ฟิล์มบริโภคได้จากโปรตีนที่ละลายน้ำได้ในน้ำล้างซูริมิ

นาย เถวียน บัวตุ่ม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีทางอาหาร ภาควิชาเทคโนโลยีทางอาหาร

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2546

ISBN 974-17-3939-7

EDIBLE FILMS FROM WATER SOLUBLE PROTEINS IN SURIMI WASH WATER

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A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Food Technology

Department of Food Technology

Faculty of Science

Chulalongkorn University

Academic year 2003

ISBN 974-17-3939-7

Thesis Title	EDIBLE FILMS FROM WATER SOLUBLE PROTEINS IN SURIMI WASH WATER
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เถวียน บัวตุ่ม: ฟิล์มบริโภคได้จากโปรตีนที่ละลายน้ำได้ในน้ำล้างซูริมิ
(EDIBLE FILMS FROM WATER SOLUBLE PROTEIN IN SURIMI WASH WATER)
อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร. พันธิพา จันทวัฒน์ อาจารย์ที่ปรึกษาร่วม: ผู้ช่วยศาสตราจารย์ ดร.รมณี สงวนดีกุล, Professor Dr. Manjeet S. Chinnan, 314 หน้า. ISBN 974-17-3939-7

งานวิจัยนี้ได้พัฒนาและปรับปรุงสมบัติของฟิล์มบริโภคได้จากโปรตีนที่แยกได้จากน้ำล้างซูริมิ สามวิธีได้แก่ ทำแห้งน้ำล้างซูริมิแบบเยือกแข็ง ตกตะกอนโปรตีนโดยการปรับพีเอช และตกตะกอนโปรตีนด้วยเอทธานอล พบว่าโปรตีนละลายได้จากน้ำล้างซูริมิมีปริมาณแตกต่างกันขึ้นอยู่กับลำดับการล้าง ซึ่งน้ำล้างที่หนึ่งมีปริมาณโปรตีนมากที่สุด รองลงมาคือน้ำล้างที่สอง และสามตามลำดับ จากผลการวิเคราะห์ พบว่าโปรตีนในน้ำล้างที่หนึ่งมีน้ำหนักโมเลกุลส่วนใหญ่ในช่วง 23.2-71.6 kDa ขณะที่โปรตีนจากน้ำล้างที่สองและสามมีน้ำหนักโมเลกุลในช่วง 23.2-71.6 kDa เช่นกันแต่ปริมาณต่ำกว่าที่พบในน้ำล้างที่หนึ่ง จากการที่น้ำล้างที่หนึ่งมีปริมาณโปรตีนมากที่สุดจึงนำมาตกตะกอนสำหรับขึ้นรูปแผ่นฟิล์ม พบว่าได้ตะกอนโปรตีนสูงสุดที่พีเอช 3.5 (66.30%) และที่ความเข้มข้นของเอทธานอล 60.0 % โดยปริมาตร (64.98%) สภาวะดังกล่าวโปรตีนที่ได้มีสมบัติด้านการละลายต่ำสุด การเพิ่มอุณหภูมิขณะตกตะกอนเป็นผลให้ได้ปริมาณตะกอนเพิ่มขึ้นและตะกอนมีสมบัติด้านการละลายต่ำลง ขณะที่เวลาในการตกตะกอนไม่มีผลต่อปริมาณตะกอน จากการศึกษาผลของพีเอชและอุณหภูมิในการให้ความร้อนที่ระยะเวลาต่างๆแก่สารละลายฟิล์มที่เตรียมจากโปรตีนทั้งสามตัวอย่าง พบว่า พีเอชและอุณหภูมิมีผลต่อสมบัติของฟิล์มมากกว่าเวลาในการให้ความร้อน ตัวอย่างฟิล์มจากโปรตีนที่ทำแห้งโดยการแช่เยือกแข็งที่พีเอช 10.0 อุณหภูมิ 70 ^{o}C ฟิล์มจากโปรตีนที่ตกตะกอนด้วยการปรับพีเอช ที่พีเอช 2.0 อุณหภูมิ 80 ^{o}C และฟิล์มจากโปรตีนที่ตกตะกอนด้วยเอทธานอล ที่พีเอช 11.5 อุณหภูมิ 60 ^{o}C มีค่าความต้านทานแรงดึงขาด (tensile strength, TS) สูงสุด และค่า % การยืดตัวเมื่อขาด (elongation at break, E) สูงเช่นกัน ขณะที่ค่าการซึมผ่านไอน้ำ (water vapor permeability, WVP) ค่าการซึมผ่านก๊าซออกซิเจน (oxygen permeability, OP) ค่าการละลายของฟิล์ม (film solubility, FS) และค่าการละลายของโปรตีน (protein solubility, PS) ของแผ่นฟิล์มต่ำที่สุด การใช้พีเอชและอุณหภูมิสูงแก่สารละลายฟิล์มส่งผลให้ฟิล์มที่ได้มีสีเหลืองเข้มกว่าฟิล์มที่เตรียมโดยใช้พีเอชและอุณหภูมิต่ำ ค่าความไม่ชอบน้ำของผิวหน้า (surface hydrophobicity) และปริมาณพันธะไดซัลไฟด์ (content of disulfide bond) เพิ่มขึ้นเมื่อพีเอชและอุณหภูมิเพิ่มขึ้นขณะที่ปริมาณหมู่ซัลไฟดริล (available sulfhydryl group) ลดลง การเพิ่มปริมาณโปรตีนในสารละลายฟิล์มจากร้อยละ 1.5 ถึง 4.5 ทำให้ฟิล์มที่ได้มีค่า TS เพิ่มขึ้น ขณะที่ค่า E, WVP, OP, FS, PS ลดลง และสีเข้มขึ้น จากการศึกษาชนิดและปริมาณสารพลาสติไซเซอร์ (plasticizer) ได้แก่ ซอร์บิทอล (sorbitol) กลีเซอรอล (glycerol) และพอลิเอธิลีนไกลคอล (polyethylene glycol) ที่ความเข้มข้น 25 50 และ 75% โดยน้ำหนักโปรตีน พบว่าเมื่อปริมาณพลาสติไซเซอร์เพิ่มขึ้น แผ่นฟิล์มมีค่า TS ลดลงขณะที่ ค่า E, WVP, OP, FS, และ PS เพิ่มขึ้น พบว่าฟิล์มที่ใช้ซอร์บิทอลเป็นพลาสติไซเซอร์มีค่า TS, FS และ PS สูงที่สุดขณะที่ค่า E, WVP, OP และสีต่ำที่สุดเมื่อเปรียบเทียบกับตัวอย่างที่ใช้กลีเซอร์รอลและพอลิเอทธิลีนไกลคอล ฟิล์มจากโปรตีนที่ละลายน้ำได้ทั้งสามตัวอย่างมีสมบัติทางกลใกล้เคียงกับฟิล์มที่ผลิตจากวัสดุชีวภาพชนิดอื่น แต่ WVP สูงเมื่อเทียบกับพอลิเมอร์สังเคราะห์ ขณะที่ OP ต่ำกว่าฟิล์มจากพอลิแซคคาไรด์และพลาสติกสังเคราะห์ชนิด พอลิเอทธิลีนความหนาแน่นต่ำและสูง (low density polyethylene และ high density polyethylene) พอลิโพรพิลีน (polypropylene) และ พอลิสไตรีน (polystyrene)

ภาควิชาเทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต.....
สาขาวิชาเทคโนโลยีทางอาหาร	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2546	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

###4373817923 : MAJOR FOOD TECHNOLOGY
KEY WORD : WATER-SOLUBLE FISH PROTEINS/THREADFIN BREAM FISH/EDIBLE FILMS pH SHIFT/ORGANIC SOLVENT/TENSILE STRENGTH/ELONGATION AT BREAK/WATER VAPOR/PERMEABILITY/OXYGEN PERMEABILITY.
THAWIEN BOURTOOM: EDIBLE FILMS FROM WATER SOLUBLE PROTEIN IN SURIMI-WASH WATER. THESIS ADVISOR: ASSOC. PROF. Dr. PANTIPA JANTAWAT, THESIS CO- ADVISOR: ASSOC. PROF. Dr. ROMANEE SANGUANDEEKUL and PROF. Dr. MANJEET S. CHINNAN. 314pp. ISBN 974-17-3939-7

This research was to test the methodology involved in recovery of water-soluble fish proteins in surimi wash-water by freeze-drying, shifting the pH and ethanol and development of edible films from water-soluble proteins from different recovery methods. Water-soluble fish proteins were recovered in decreasing concentration with each successive wash. The amounts of proteins were highest for wash stage I (WS-I), followed by wash stages II (WS-II), and III (WS-III). For WS-I, most of the proteins had molecular weights (MW) ranging between 23.2 and 71.6. WS-II and-III also had proteins with MW between 23.2 and 71.6 kDa; however, the protein yield was relatively small. Maximum protein precipitation was obtained at pH 3.5 (66.30 %) and 60 % v/v of ethanol (64.98%), however, precipitated proteins had lowest solubility. There was direct correlation between percentage of precipitation and reaction temperature, which reversed with protein solubility. Reaction time for shifting the pH, and ethnol had little or no effect ($p > 0.05$) on precipitation. Water-soluble fish proteins film from three different recovering methods (freeze-drying, shifting the pH and ethanol precipitation) were developed and effects of pH, heating temperature, heating time and additive variable on various film properties were determined. The impact of pH and heating temperature was most significant, overall, on the film's properties than heating time. Tensile strength (TS) and elongation at break (E) were highest at pH about 10.0 and heating temperature, 70 ^{o}C for freeze-dried proteins and pH 2.0 and heating temperature, 80.0 ^{o}C for proteins precipitated by shifting the pH and pH 11.50 and heating temperature 60 ^{o}C for proteins precipitated by organic solvent. In contrast, at this same condition, the water vapor permeability (WVP), oxygen permeability (OP), films solubility (FS) and proteins solubility (PS) values were at their lowest. Film color was darker and more yellow with increase in pH and heating temperature of film solutions. The surface hydrophobicity and content of disulfide bond increased with increase in pH and heating temperature, while the available sulfhydryl group decreased. Increase of the protein concentration provided the film with higher TS but lowers E, WVP, OP, FS and PS and development of darker and more yellowish films. The properties of edible films from water-soluble fish proteins plasticized with different plasticizers were determined. Three plasticizers comprising sorbitol, glycerol and polyethylene glycol were studied over a range of concentration from 25 to 75%. Increasing in contents of these plasticizers resulted in decreased TS, but increased E, WVP, FS and PS. Sorbitol provided the films with the highest TS, FS and PS but lowest E, WVP, while glycerol and polyethylene glycol provided the films with high E, WVP, but low TS, FS and PS. The color of edible films changed with the plasticizer type. Mechanical properties of all three water-soluble fish protein films were pretty similar to those of various biomaterials based films. However, the films had substantially lower mechanical properties than polymeric materials. The water-soluble fish proteins films were characterized by relatively poor water vapor barrier properties, but showing lower oxygen permeability than values of other polysaccharide and common plastic films, such as low and high density polyethylene, polypropylene and polystyrene.

Department…Food Technology……..…… Student's signature……...
Field of Study…Food Technology……..… Advisor's signature……..
Academic year…….2003……...…. Co-advisor's signature……..

ACKNOWLEDGMENTS

I would like to express my respect and appreciation to all the members on my advisory committee: Assoc. Prof. Dr. Pantipa Jantawat, Asst. Prof. Dr. Romanee Sanguandeekul, of Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand and Prof. Dr. Manjeet S. Chinnan, of the Department of Food Science and Technology, University of Georgia, Griffin Campus, Georgia, USA, for their guidance and support. Special thanks are also conveyed to my examining committees, Asst. Prof. Dr. Surapongse Nawangkasatusas, Assoc. Prof. Dr. Ninnart Chinprahast and Assoc. Prof. Paiboon Thammaratwasik, for their invaluable suggestions.

I admire Assoc. Prof. Dr. Pantipa Jantawat's intellectual vitality and consider myself very fortunate that she accepted me as his graduate student. Dr. Pantipa Jantawat's guidance has roused my interest enormously in Food Science and Technology.

I am extremely thankful to Prof. Dr. Manjeet S. Chinnan, for his supporting, sharing his time, useful discussions and his assistance during the preparation of manuscript, graciously providing me the equipments used in this research during the period I was doing my experiment at Department of Food Science and Technology, University of Georgia and giving me constructive suggestions throughout the research.

I would also like to thank Ms.Vijayalakshmi Mantripragada and Mr. Glenn Farrell for their technical assistance, friendly encouragement and compassion throughout my work in the Food Engineering Lab., Department of Food Science and Technology, University of Georgia, Griffin Campus, Georgia, USA. My dear friends for their affection and care: Jintana, Nisanarth, Pattama, Piyarat, Pornpong, Pornrat, Srisuwan, Yuthana, Vassana, Worapong, Chun Fang, Dr. Cha and his family, Kasia, Rashmi, Sharon, Vandana, Lary and Dr. Saalia for his skill in statistical analysis.

I am grateful to Prince of Songkhla University for providing me with the scholarship to pursue my doctoral studies at Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The financial support from the Royal Thai Government and Chulalongkorn University Graduate School are acknowledged.

I would like to express my overwhelmingly gratitude to my dearest mom and dad including my brother and sister, for their unconditional and moral support.

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CHAPTER I

INTRODUCTION

Food packaging provides important information to the consumer and enables convenient dispensing of food. However, food packaging has become a central focus of waste reduction efforts. Over 5 billion tons of packing-related solid wastes are discarded every year, and 30 % of the wastes are plastics (Rawatt, 1993). Plastic biodegradation is a slow process, and the rate is affected by the nature of the material and its form. It takes several hundred years to degrade petroleum-based synthetic plastics, which have caused serious solid waste contamination in the world (Park *et al*., 2001). Edible and biodegradable polymer films offer alternative packaging without adverse effect to the environment although edible films are not meant to totally replace synthetic packaging and to limit moisture, aroma and lipid migration between food component where traditional packaging cannot function. For instance, edible films can be used for many food products to reduce loss of moisture, to restrict absorption of oxygen, to lessen migration of lipids, to improve mechanical handling properties, to provide physical protection, or to offer an alternative to the commercial packaging materials (Kester and Fennema, 1986; Murray *et al*., 1973; Nelson and Fennema, 1991). Components used for the preparation of edible films can be classified into three categories: hydrocolloids (such as protein, polysaccharide, alginate), lipid (such as fatty acids, acylglycerol, waxes) and composites (Donhowe and Fennema, 1993). Hydrocolloid films have good barrier properties to oxygen, carbon dioxide, and lipids but not to water vapor. Most hydrocolloid films also possess superb mechanical properties, which are quite useful for fragile food products. It has been generally accepted that the mechanical and barrier properties of protein films are superior to those of polysaccharide films (Cuq *et al*., 1995). This may be because protein was composed of 20 different amino acids and have

a specific structure which confers a wider variety of functional properties, especially high intermolecular binding potential (Guilbert and Graille, 1994). They can form bonds at different positions, with different types and energies as a function of temperature, solvation condition, pH and additive characteristic (plasticizer) compare with polysaccharides which are mostly homopolymer. Furthermore, the chemical treatment to modify functional properties can be performed more easily on protein-based materials than on polysaccharide based–materials (Osawa and Walsh, 1993).

Proteins in their native state generally exist as either fibrous or globular forms. Fibrous proteins, which are water insoluble, are fully stretched and are closely associated with each other in parallel structures. These proteins can directly be casted into edible film. On the contrary, globular proteins generally have to be structural denatured, so that the protein molecules extended. As a result, hydrogen, electrostatic, hydrophobic and covalent bondings are consequently formed between extended protein molecules. The sequence of amino acid residues and the degree of structure extension influence the protein-protein interaction, which generates film. As a result, edible films with good water vapor permeability can be prepared from various types of globular protein (McHugh *et al.*, 1993; Gennadios *et al*., 1998 and Sanchez *et al*., 1998)

In industrial surimi manufacturing process, minced flesh fish is repeatedly washed with chilled water to remove sarcoplasmic proteins to produce a tasteless and odorless product. As a result of washing, approximately 40-50 % of minced fish solids (containing primarily water-soluble proteins) are lost in the process (Pacheo-Aguilar *et al*., 1989 and Lin *et al*., 1994). This means that about 40-50% of the product is considered as waste and has the potential for recovery. In the seafood industry, solid waste from surimi processing is usually converted to animal feed or fishmeal. However,

liquid waste is generally discarded into the waste stream of the plant. Increasing concerns over the negative impact of direct wastewater discharge has led to research in proteins recovery in surimi wash-water. Recovering of water-soluble fish proteins from surimi wash-water would not only reduce the negative environmental impact and the cost of waste disposal, but also generate potential profits. Several attempts were made to recover proteins from surimi process (Huang *et al*., 1997). Ultrafiltration is the system of choice when it comes to producing a concentration with good functional properties (Horton *et al*., 1972). An early problem with ultrafiltration involved severe fouling of membranes (Morr, 1976). Jaouen and Que'men'eur (1992) noted that recovering of water-soluble fish proteins from surimi wash-water by ultrafiltration, without pretreatment was not practical. Huang *et al*. (1997) studied the effect of ohmic heating (70 ^{o}C) on proteins coagulation from surimi wash-water and found that water-soluble fish proteins could be removed, however an important consideration with heat precipitation was the possibility of proteolysis. Protease, like other enzymes, was more active at higher temperatures. Several flocculating agents such as ferric chloride and aluminium sulfate were also used in proteins preparation, but these flocculants were not suitable due to their high toxicity in humans even at low concentrations (Marti *et al*., 1994). Methods such as precipitation by shifting the pH and use of organic solvent can be employed to recover water-soluble fish proteins from surimi wash-water. The benefits of each method with reference to quality, quantity and end use of the recovered proteins need thoroughly investigation. Moreover, the recovered proteins are mostly used as animal feeds and fertilizer because the lack of techniques to use them as foodstuffs. At the moment very little research on edible films from water-soluble fish proteins in surimi wash-water has been performed, so there is a need for conducting research to understand the mechanism

of edible film formations from water-soluble fish proteins, obtain information on film characteristics, develop methods to improve the properties and identify their applications on food according to their characteristic. Therefore, this study was conducted to test the methodology involving the recovery of water-soluble fish proteins by shifting the pH and use of organic solvent. The proteins recovered were then characterized.

Later, various parameters affecting properties of films casted from the water-soluble fish proteins were studied. These parameters were pH, heating temperature, time, additive variables like proteins concentration and plasticizer type and concentration on physico-chemical of the film from different recovering methods and comparison of prepared film with those produced from polysaccharide and synthetic polymers.

1.1 Objectives

Overall goal of this research was to test the methodology involved in the recovery of water-soluble fish proteins from surimi wash-water by shifting the pH and use of organic solvent. The proteins recovered were characterized and developed into edible films. The specific objectives of this study were:

1) Investigate the methodology in recovery of water-soluble fish proteins.
2) Determine the properties of recovered protein from different methods.
3) Determine the effect of selected parameters and additive variables on the properties of edible films from water-soluble fish proteins from different recovering methods and comparing their properties with those produced from polysaccharide and synthetic polymer.

CHAPTER II

LITERATURE REVIEW

2.1 Edible Films and Coatings

Edible films are defined as thin layer of material which is edible and can provide a barrier to moisture, oxygen and solute movement for the food (Guilbert, 1986). Edible films can be formed as food coatings and free-standing films, and have potential to be used with food as gas aroma barrier (Kester and Fennema, 1986). However, the technical information is still needed in order to develop films for food application (Donhowe and Fennema, 1993). The edible films and coatings have received a consideration attention in the recent years because of their advantages over the synthetic films. The advantages of edible films over other traditional non edible polymeric packaging materials are summarized by Gennadios and Weller (1990) as below:

1. They could be consumed with the package products. This was obviously of critical importance since it represents the environmentally ideal package.
2. There was no package to dispose of even if the films were not consumed they could still contributed to the reduction of environmental pollution.
3. The films were produced exclusively from renewable, edible ingredients and therefore were anticipated to degrade more readily than polymeric materials.
4. The films could be enhanced for the organoleptic properties of packaged foods provided that various components (flavorings, colorings, sweeteners)

5. The films could be supplemented for the nutrition value of the foods. This was particular true for films made from proteins.
6. The films could be used for individual packaging of small portion of food, particularly products that currently were not individually packaged for practical reasons such as pears, beans, nuts and strawberries.
7. The films could be applied inside heterogeneous foods at the interfaces between different layers of components. They could be tailored to prevent deteriorative intercomponent moisture and solute migration in foods such as pizzas, pies and candies.
8. The films could be functioned as carriers for antimicrobial and antioxidant agents. In a similar application they also could be used at the surface of food to control the diffusion rate of preservative substances from the surface to the interior of the food.
9. The films could be very conveniently used for microencapsulation of food flavoring and leavening agents to efficiently control their additional and released into the interior of foods.
10. Another possible application for edible films could be their uses in multilayer food packaging materials together with nonedible films. In this case, the edible films would be the internal layers in direct contact with food materials.

Production of edible films causes less waste and pollution, however, their permeability and mechanical properties are generally poorer than synthetic films (Kester and Fennema, 1986). Extensive research is needed on the development of new materials,

methods of film formation, methods to improve film properties and the potential applications (Kester and Fennema, 1986).

2.2 Classification of Edible Films and Coatings

Edible films can be produced from materials with film forming ability. During manufacturing, film materials must be dispersed and dissolved in the solvent such as water, alcohol or mixture of water and alcohol or mixture of other solvents. Plasticizer, antimicrobial agent, colors or flavor can be added in this process. Adjusting pH and/or heating the solutions may be done for the specific polymer to facilitate the dispersion. Film solution is then casted and dried at desired temperature and relative humidity to obtain a free-standing films (Donhowe and Fennema, 1993). In the food application, film solutions could be applied to food by several methods such as dipping, spraying, brushing and panning followed by a drying step. Kester and Fennema (1986) classified the edible films based on the nature of material as polysaccharide, protein, lipid and composite films.

2.2.1 Polysaccharide Films

Polysaccharides used for edible films or coatings include cellulose and derivatives, starch and derivatives pectin, seaweed extracts, exudate gums, microbial fermentation gums and chitosan (Krochta and Mulder-Johnson, 1997). Polysaccharides are generally very hydrophilic resulting in poor water vapor and gas barrier properties. Although coating by polysaccharide polymers may not provide a good water vapor barrier, these coatings can act as sacrificing agent retarding moisture loss from food products (Kester and Fennema, 1986).

2.2.1.1 Cellulose and derivatives

Cellulose is composed of repeating D-glucose units linked through ß-1, 4 glycosidic bonds. In its native state, the hydroxymethyl groups of anhydroglucose residues are alternatively located above and below the plane of the polymer backbone. This results in very tight packing of polymer chains and a highly crystalline structure that resists salvation in aqueous media (Krochta and Mulder-Johnson, 1997). Water solubility could be increased by treating cellulose with alkali to swell the structure, followed by reaction with chloroacetic acid, methyl chloride or propylene oxide to yield carboxy methyl cellulose (CMC), methyl cellulose (MC), hydroxy propyl methyl cellulose (HPMC) or hydroxy propyl cellulose (HPC) (Kamper and Fennema, 1985; Kester and Fennema, 1989b; Rico-Pena and Torres, 1990). Placement of bulky substituents along the cellulose molecule, in the form of ether linkages at reactive hydroxyls, separated the polymer chains and interfere with formation of the crystalline unit cell, thereby enhancing aqueous solubility (Krumel and Lindsay, 1976). MC, HPMC, HPC and CMC film possess good film-forming characteristic; films were generally odorless and tasteless, flexible and were of moderate strength, transparent, resistance to oil and fats, water-soluble, moderate to moisture and oxygen transmission (Krochta and Mulder-Johnson, 1997). MC was the most resistant to water and it was the lowest hydrophilic cellulose derivatives (Kester and Fennema, 1986); however, the water vapor permeability of cellulose ether film was still relatively high. MC and HPMC had ability to form thermally induced gelatinous coating; they have been used to retard oil absorption in deep frying food product (Kester and Fennema, 1986; Balasubramanium *et al*., 1997). MC could be applied as coating on confectionery products as barrier to lipid migration (Nelson and Fennema, 1991). A

number of groups have investigated composite films composed of MC or HPMC and various kinds of solids, such as beeswax and fatty acids (Debeaufort *et al.*, 1993, Greener and Fennema, 1989a, Kamper and Fennema, 1984; Kester and Fennema, 1989a; Koelsch and Labuza, 1992; Park *et al.*, 1994). Many of these had water vapor permeability as low as low density polyethylene (LDPE). These composite films were all polymer-lipid bilayer formed either in one step from aqueous ethanolic solutions of cellulose ether fatty acids.

Cellulose could also be chemically modified to ether, ethyl cellulose (EC), which is biodegradable but not edible. EC films could either be cast from non-aqueous solutions or extruded. Like the other cellulose ethers. EC films were poor moisture barrier, but they have been reported to be good oil and fat barriers (Hanlon, 1992).

2.2.1.2 Pectin

Pectin was a complex group of structural polysaccharides found in the middle lamella of plant cells. It is composed mainly of (1, 4) α-D-galactopyranosyluronic acid units with varying degrees of esterification (DE). Chemical de-esterification yielded low-methoxyl pectins which when dissolved in aqueous media were capable of forming gels in the presence of calcium ions (Schultz *et al.*, 1948). The function of the ionic calcium is to bridge free carboxyl group on adjacent polymer molecules (Morris, 1986). Pectin with DE more than 50% was classified as a high-methoxyl and lower than 50%, as a low methoxyl pectin (deMan, 1990). Pectin might be used to form film alone or blended with other polymers (Coffin and Fishman, 1993). These films or coatings gave a glossy, non sticky surface, high water vapor permeability.

Although pectinate coatings were poor in moisture barrier, they could reduce the moisture loss from food product by sacrificing (Kester and Fennema, 1986).

Schultz *et al.* (1948) evaluated water vapor permeability of dried low-methoxyl pectinate films. They found that the permeability was very high, averaging approximately 300 g. H_2O . mil. m^2. day^{-1}. mm Hg^{-1} at 25 ^oC at 50% relative humidity (RH). The authors concluded that increasing of resistance to water vapor transmission would be required to broaden the range of potential applications of dried pectinate films and this could be at least partially achieved by coating the original film with lipids. Applications of pectinate coatings were reported in confectionery products and almonds as oil barrier. (Swenson *et al*., 1953, Braker and Fennema, 1993); in candied fruits products as stickiness reducer (Swenson *et al*., 1953); in cheese, sausage, ham, fish and frozen foods as moisture barrier (Schultz *et al*., 1948)

2.2.1.3 Chitin and Chitosan

Chitin is the second most abundant naturally occurring biopolymer (after cellulose) and it is found in the exoskeleton of crustaceans, in fungal cell walls and other biological materials (Andrady and Xu, 1997). It is mainly poly (ß-(1-4)-2-acetamide-D-glucose), which is structurally identical to cellulose except that secondary hydroxyl on the second carbon atom of the hexose repeat unit is replaced by an acetamide group. Chitosan is derived from chitin by deacetylation in the presence of alkali. Therefore, chitosan is a copolymer consisting of (ß-(1-4)-2-acetamido-D-glucose and (ß-(1-4)-2-acetamide-D-glucose units with the latter usually exceeding 80% (Sandford, 1989). Chitosans are described in terms of the degree of deacetylation and average molecular weight and their importance resides in their antimicrobial properties in

conjunction with their cationicity and their-forming properties (Muzzarelli, 1996). Chitosan could form semi-permeable coatings, which could modify the internal atmosphere, thereby delaying ripening and decreasing transpiration rates in fruits and vegetables. Films from aqueous chitosan were clear, tough, flexible and good oxygen barriers (Sandford, 1989; Kaplan *et al*., 1993). Carbon dioxide permeability could be improved by methylation of polymer. Butler *et al*. (1996) observed that films from chitosan were rather stable and mechanical and barrier properties changed only slightly during storage. Chitosan coatings were usually used on fruit and vegetable products such as strawberries, cucumbers, bell peppers as antimicrobial coatings (El Ghaouth *et al*., 1991a, 1991b), and on apples, pears, peaches and plums as gas barrier (Elson and Hayes, 1985; Davis *et al.*, 1989).

2.2.1.4 Starch

Starch consists of amylose and amylopectin, the ratio of amylose and amylopectin depends on the type and variety of raw material. Amylose is a linear chain of D-glucose residues linked through α-1,4 glycosidic bonds. Amylopectin is a branched molecule consisting of glucose units connected by α-1,4 and α-1,6 linkages (Whistler and Daniel, 1985). High amylose starch as corn starch is a good source for films formation. Free-standing films could be produced from aqueous solution of gelatinized amylose and drying. Normal corn starch consisted of approximately 25% amylose and 75% amylopectin. Mutant varieties of corn were produced which contained starch with up to 85% amylose (Whistler and Daniel, 1985). Wolf *et al*. (1951) produced self-supporting films by casting aqueous solutions of gelatinized amylose, followed by solvent evaporation. The films were transparent and had very low permeability to oxygen

at low RH (Rankin *et al*., 1958). Mark *et al*. (1966) reported that films produced from high a mylose corn starch (71% amylose) had no detectable oxygen permeability at RH levels less than 100%. This was true for both unplasticized and plasticized (16% glycerol) films. This result was surprising in light of the fact that addition of plasticizers and absorption of water molecules by hydrophilic polymers increased polymer chain mobility and generally led to increased gas permeability (Banker, 1966)

Partial etherification of high-amylose starch with propylene oxide, to yield the hydroxypropylated derivative, improved water solubility. As expected, films produced from hydroxypropylated starch possess virtually no resistance to the passage of water vapor: however, as with the pure amylose films, resistance to oxygen transport is substantial (Jokay *et al*., 1967). Oxygen permeation through plasticized and unplasticized films was not detectable at 25 ^{o}C and RH up to 78% (Roth and Mehltretter, 1967). At the high RH, films became distorted due to the moisture absorption and were not tested for oxygen permeability; however, it is likely that oxygen transport increased greatly as the film became hydrate. Jokay *et al*. (1967) applied hydroxylpropylated starch films on almond nutmeats, and organoleptic evaluation revealed that the film retarded development of oxidative rancidity of the products during storage.

Starch hydrolysates (dextrin) of low dextrose equivalent (DE) have been suggested for use as protective coatings. Although hydrophilic in nature, starch hydrolysates do provide a limited resistance to transport of water vapor. Allen *et al*. (1963) evaluated the relative barrier properties of edible film materials by coating them onto a cellulose acetate support. Starch films displayed minimal resistance to water transport, while films of low-DE dextrin and corn syrup were approximately 2-and 3-fold

more resistant, respectively. Murray *et al*. (1973) coated almond nutmeats with a 50% solution of a 10-DE starch hydrolysate. Sensory evaluation indicated that the coated nuts maintained a more desirable texture than uncoated controls during storage. Presumably, this was attributable to a reduction in the rate of moisture absorption by coated almonds.

Films of starch hydrolysates may exhibit some resistance to oxygen transmission. Dipping of fresh sliced apples in a 40% solution of a 15-DE hydrolysate prior to dehydration prevented browning of the tissue, probably by retarding the entrance of oxygen (Murray *et al*., 1973).

2.2.1.5 Seaweed and Gum Polymers

Alginate, carrageenan and agar are seaweed products and have good film forming characteristics. Alginate is the salt of alginic acid, a linear (1 → 4) linked polyuronic acid extracted from brown seaweed. Film formations, which may or may not involve gelation, can be achieved by evaporation, electrolyte crosslinking, or injection of a water-miscible nonsolvent for alginate (Kelco, 1976). Alginate coating possessed good oxygen and lipid barrier but poor water vapor barrier properties (Cottrell and Kovacs, 1980; Conca and Yang, 1993). Additionally, coating with alginate could improve flavor, texture and batter adhesion. Carrageenan is an extract from red seaweed which consists of a family of sulfated polysaccharides of D-galactose and 3, 6-anhydro-D-galactose. Upon cooling, a warm aqueous solution of the polymer, gelation occurs presumably by the formation of a double-helix structure to yield a three-dimensional polymer network (Glicksman, 1983). Coating with carrageenan has been used in food to incorporate antimicrobial agents, and reduce moisture loss, oxidation or disintegration (Krochta and Mulder-Johnson, 1997).

2.2.2 Lipid Films

Lipid compounds utilized as protective coating consist of acetylated monoglycerides, natural wax, and surfactants. The most effective lipid substances are paraffin wax and beeswax. The primary function of lipid coating is to block transport of moisture due to their relative low polarity. In contrast, the hydrophobic characteristic of lipid forms thicker and more brittle films. Consequently, they must be associated with film forming agents such as proteins or cellulose derivatives (Kester and Fennema, 1986). Generally, water vapor permeability decreases when the concentration of hydrophobicity phase increases. Lipid-based films are often supported on a polymer structure matrix, usually a polysaccharide, to provide mechanical strength.

2.2.2.1 Waxes and Paraffin

Paraffin wax is derived from distillate fraction of crude petroleum and consists of mixture of solid hydrocarbon resulting from ethylene catalytic polymerization. Paraffin wax is permitted for use on raw fruit and vegetable and cheese. Carnauba wax is exudates from palm tree leaves (*Copoernica cerifera*). Beeswax (white wax) is produced from honeybees. Candelilla is obtained from candelilla plant. Mineral oil consists of a mixture of liquid paraffin and naphtheric hydrocarbon (Hernandez, 1994). Waxes are used as barrier films to gas and moisture (skin on fresh fruits) and to improve the surface appearance of various foods (e.g., the sheen on sweet). Applied in a thick layer, they must be removed before consumption (certain cheese); when used in thin layers, they are considered edible. Waxes (notably paraffin, carnauba, candellila and beeswax) are the most efficient edible compounds providing a humidity barrier (Guilbert, 1986).

2.2.2.2 Acetoglyceride

Acetylation of glycerol monosterate by its reaction with acetic anhydride yielded 1-stearodiacetin. This acetylated monoglyceride displayed the unique characteristic of solidifying from the molten state into a flexible, wax-like solid (Feuge *et al*., 1953). Most lipids in the solid state could be stretched to only about 102% of their original length before fracturing. Acetylated glycerol monostearate, however, could be stretch up to 800% of its original length (Jackson and Lutton, 1952). Water vapor permeability of this film is much less than that of polysaccharide film with the exception of methyl cellulose or ethyl cellulose (Kester and Fennema, 1986). Acetylated monoglyceride coatings have been used on poultry and meat cuts to retard the moisture loss during storage (Kester and Fennema, 1986).

2.2.2.3 Resins and Rosins

2.2.2.3.1 Shellac Resins

Shellac resins are a secretion by the insect *Laccifer lacca.* It is composed of a complex mixture of aliphatic alicyclic hydroxyl acid polymers. This resin is soluble in alcohols and in alkaline solutions. Shellac is not a GRAS substance; it was only permitted as an indirect food additive in food coatings and adhesives. It was mostly used in coating for the pharmaceutical industry and a few work has been reported on foods (Hernandez, 1994). Rosins which are obtained from the oleoresins of the pine tree, is a residue left after distillation of volatile from the crude resin. Resin and it derivatives were widely used in coating for citrus and other fruits (Sward, 1972).

2.2.3.2.2 Surfactants

Coating of foods with surface-active agents (16-18 carbon fatty alcohol) have been used for coating foods to reduce superficial a_w and rate of moisture loss by evaporation. Furthermore, thin film of surface active agents (lecithin, hydroxylate lecithin or tweens) tended to inhibit undersirable light-induced greening (chlorophyll) in potato tubes (Kester and Fennema, 1986).

2.2.3 Protein Films

In their native states, proteins generally exist as either fibrous proteins, which are water insoluble and serve as the main structural materials of animal tissues, or globular proteins, which are soluble in water or aqueous solutions of acids, bases or salts and function widely in living system (Morrison and Boyd, 1959). The fibrous proteins are fully extended and associated closely with each other in parallel structures, generally through hydrogen bonding, to form fibers. The globular proteins fold into complicated spherical structures held together by a combination of hydrogen, ionic, hydrophobic and covalent (disulfide) bonds (Bushuk and Wrigley, 1974). The chemical and physical properties of these proteins depend on the relative amounts of the component amino acid residues and their placement along the protein polymer chain. Of the fibrous proteins, collagen has received the most attention in the production of edible films. Several globular proteins, including wheat gluten, corn zein, soy protein, and whey protein, have been investigated for their film properties. Protein films were generally formed from solutions or dispersions of the protein as the solvent/carrier

evaporates. The solvent/carrier was generally limited to water, ethanol or ethanol-water mixtures (Kester and Fennema, 1986).

Generally, globular proteins must be denatured by heat, acid, base, and/or solvent in order to form the more extended structures that are required for film formation. Once extended, protein chains can associate through hydrogen, ionic, hydrophobic and covalent bonding. The chain-to-chain interaction that produces a cohesive films are affected by the degree of chain extension and the nature and sequence of amino acid residues. Uniform distribution of polar, hydrophobic, and/or thiol groups along the polymer chain increase the likelihood of the respective interactions.

Increased polymer chain-to-chain interaction resulted in films that were stronger but less flexible and less permeable to gases, vapors and liquids (Kester and Fennema, 1986). Polymers containing groups that could associate through hydrogen or ionic bonding resulted in films that were excellent oxygen barrier but susceptible to moisture. Thus, protein films were expected to be good oxygen barriers at low relative humidities (Salame, 1986). Polymers containing a preponderance of hydrophobic groups are poor oxygen barriers but excellent moisture barriers. However, the fact that they were not totally hydrophobic and contain predominantly hydrophilic amino acid residues limits their moisture-barrier properties. Creation of protein-versed edible films with low water vapor permeability requires addition of lipid components. This was analogous to the situation with synthetic polymers where moisture-sensitive oxygen-barrier polymers might be either co-polymerized with a hydrophobic polymer or sandwiched between hydrophobic polymer layers to limit the ability of water to reduce barrier properties (Kester and Fennema, 1986).

Various types of protein had been used as edible films. These included collagen, casein, whey protein, corn zein, wheat gluten, soy protein, mung bean protein, and peanut protein (Gennadois *et al*. 1994).

2.2.3.1 Collagen Films

Collagen is a fibrous protein generally isolated from hides, tendon, cartilage, bone and connective tissues (Balian and Bowes, 1977). Production of films from animal hides could be accomplished using a dry or wet process with some similarity, including (a) alkaline treatment to dehair and removed collagen from carbohydrates and other proteins, (b) acid swelling and homogenization to form a ~ 4.5% moisture gel (wet process) or ~ 10% moisture gel dough (dry process), (c) extrusion into a tube and (d) neutralization of the extruded tube, washing the tube of salts, treating the tube with plasticizer and cross-linkers and drying to 12-14% moisture, with the order depending on whether the wet or dry process is used (Hood, 1987). Collagen was used to make the most commercially successful edible protein films. Collagen casing had largely replaced natural gut casing for sausage (Hood, 1987). Collagen film was eaten with the meat product after removal of the netting. Besides providing mechanical integrity to meat products, collagen film was generally seen as reducing oxygen and moisture transport (Baker *et al*., 1994).

2.2.3.2 Gelatin Films

Gelatin, a protein derived from collagen formed by thermally reversible gels when warm aqueous suspensions of the polypeptide were cooled, had good film forming properties. Gelatin films could be formed from gelatin

20-30%, plasticizer (glycerin or sorbitol) 10-30% and water 40-70% followed by drying the gelatin gel (Guilbert, 1986). Gelatin was used to encapsulate low moisture or oil phase food ingredients and pharmaceuticals. Such encapsulation provided protection against oxygen and light, as well as defining ingredient amount or drug dosage (Gennadios *et al*., 1994). In addition, gelatin films have been formed as coatings on meats to reduce oxygen, moisture and oil transport (Gennadios *et al.,* 1994). For example, gelatin coating formed on cut poultry before freezing reduced the amount of rancidity developed during storage (Klose *et al*., 1952). The effect was enhanced by adding an antioxidant to the coating.

2.2.3.3 Corn Zein Films

Corn zein is prolamin fraction (soluble in 70% ethanol) of corn gluten, making up approximately 70% of the corn gluten. Differential solubility in aqueous ethanol yielded two zein fractions. A low content of polar amino acid or high content of non-polar amino acids (leucine, alanine and proline) make corn as in anhydrous alcohol (Gennadios *et al*., 1994). Edible film could be formed by drying aqueous ethanol solution of zein (Gennadios and Weller, 1990). Formation of films was believed to involve development of hydrophobic, hydrogen and limited disulfide bonds between zein chains in the film matrix (Gennadios *et al*., 1994). The resulting films are brittle and therefore require plasticizer addition for increasing flexibility (Park, 1991). Zein films were relatively good water vapor barriers compare to other edible films (Guilbert, 1986). Water vapor barrier properties could be improved by adding fatty acids or by using a cross-linking reagent. But when cross-linking agent was used, the edibility of those films was concern (Alikonis, 1979). Food application of corn zein-acethylated monoglyceride was seen in confectionaries and nut products as oxygen and lipid barrier

(Alikonis, 1979; Alikonis and Cosler, 1961) and in intermediate moisture foods to delay the diffusion of antimicrobial chemical (sorbic acid) (Torres, 1987). Zein coating had also been used to coat vitamin-enriched rice, for protecting vitamins from loss (Mickus, 1955). Zein and zein-based coating formulations were markedly commercialized for these food-uses and related pharmaceutical applications (Andres, 1984). Zein coating was also shown an ability to reduce moisture and firmness loss and delay color change (reduce oxygen and carbon dioxide transmission) in fresh tomatoes (Park *et al*., 1994a). Zein had also been explored as a replacement for collagen in the manufacture of sausage casting (Turbak, 1972) and for the production of water-soluble pouches for dried food (Georgevits, 1967).

2.2.3.4. Wheat Gluten Films

Wheat gluten which is generally termed for water-insoluble proteins of wheat flour is composed of a mixture of polypeptide molecules, considered to be globular proteins. Cohesiveness and elasticity of gluten give integrity to wheat dough and facilitate film formation. Wheat gluten contains the prolamin and glutelin fractions of wheat flour proteins, typically referred to as gliadin and glutenin, respectively. While gliadin is soluble in 70% ethanol, glutenin is not (Gennadios and Weller, 1990). Although insoluble in natural water, wheat gluten dissolves in aqueous solutions of high or low pH at low ionic strength (Krull and Inglett, 1971). Edible films could be formed by drying aqueous ethanol solution of wheat gluten (Gennadios and Weller, 1990). Cleavage of native disulfide bonds during heating of film-forming solutions and then formation of new disulfide bonds during film drying were believed to be important to the formation of wheat gluten films structure, along with hydrogen and hydrophobic bonds

(Gennadios and Weller, 1990). Addition of plasticizer such as glycerin in gluten films was necessary to improve film flexibility (Gennadios *et al*., 1994). However, increasing film flexibility by increasing sorbitol content reduced film strength, elasticity and water vapor barrier properties (Gontard *et al*., 1992). A review on the field of gluten film was published by Gennadios and Weller (1990). A summary of research results on wheat gluten films formation was presented as follows:

Gennadios and Weller (1992) confirmed the earlier studies of Wall and Beckwith (1969) on the effect of wheat gluten purity on film's appearance and mechanical properties, i.e., a greater purity gluten resulted in a stronger and clearer films. However, cost of additional purification steps must be considered. Anker *et al*. (1972) adjusted pH for wheat gluten dispersion in alcohol-water mixture by using volatile alkali such as ammonia, to produce neutral pH films. Herald *et al*. (1995) investigated the effect of plasticizer size of wheat gluten and concluded that films prepared from spray-dried wheat gluten was stronger than films from flash-dried sample with larger size particles. When used as a coating on grade A-quality shell eggs, the egg quality could be maintained for 30 days.

Tensile strength of gluten films could be improved by using a cross-linking agent such as glutaraldehyde, or heat curing at 80 ^{o}C (Gennadios and Weller, 1992; Kolster *et al*., 1992)

2.2.3.5. Soy Protein Films

The protein content of soybeans (38-44%) is much higher than the protein content of cereal grain (8-15%). Most of protein in soybeans is insoluble in water but soluble in dilute neutral salt solutions. Thus, soy protein belongs to the

globulin classification (Kinsella, 1979). Soy protein is globular in nature and is further classified into 2S, 7S, 11S and 15S fractions according to relative sedimentation rates (Gennadios *et al*., 1994). The principal components are the 7S (conglycinin) and 11S (glycinin) fractions, both of which have a quaternary (subunit) structure (Kinsella *et al*., 1985). Soy protein is high in asparagine and glutamine residues. Both conglycinin and glycinin are tightly folded proteins. While the extent of disulfide cross-linking of conglycinin is limited due to only two to three cysteine groups per molecule, glycinin contains 20 intramolecular disulfide bonds (Kinsella, 1979). Alkali and heating both caused dissociation and subsequent unfolding of glycinin due to disulfide bond cleavage (Kinsella, 1979). Edible films based on soy protein could be produced in either of two ways: surface film formation on heated soymilk or film formation from solutions of soy protein isolate (SPI) (Gennadios and Weller, 1991). Soymilk could be produced by grinding soybeans with water followed by separation of milk from extracted soybeans. To form films from both soymilk and SPI, the film-solutions were heated to disrupt the protein structure, cleave native disulfide bonds and expose sulfhydryl groups and hydrophobic groups. Formation of new disulfide, hydrophobic and hydrogen bonds during film drying were believed to be important to the formation of soy protein film structure (Gennadios *et al*., 1994).

The use of soy protein in the formation of films or coatings on food products has been investigated (Gennadios *et al*., 1994). Soy protein concentrate and SPI were used successfully to aid batter adhesion and encase meat fibers to aid flavor retention. Soy protein-based coatings showed limited ability to reduce moisture migration in raisin and dried peas (Bolin, 1976).

2.2.3.6. **Casein Films**

Milk proteins are classified into two types comprising casein and whey protein. Casein consists of three principal components, α, ß, and κ, which together form colloidal micelles in milk containing large numbers of casein molecules and are stabilized by calcium-phosphate bridge (Kinsella, 1984). The casein molecules possess little defined secondary structure, exhibiting instead an open random-coil structure. Casein, which comprises 80% of milk protein, precipitates when skim milk was acidified to the casein isoelectric pH of approximately 4.6 (Dalgleish, 1989). Acidification would solubilize the calcium phosphate, thus releasing individual casein molecules, which were associated to form insoluble acid casein. The acid casein could be converted to functional soluble caseinates by neutralization through addition of alkali. Sodium and calcium caseinates were most common, but magnesium and potassium caseinates were also available commercially (Kinsella, 1984). Edible films based on various caseinates could be obtained by solubilization in water followed by casting and drying. Caseinates formed films from aqueous solution without heat treatment due to their random coil nature. Interactions in the film matrix involved hydrophobic, ionic, and hydrogen bonding (Avena-Bustillos and Krochta, 1993).

Glycerin-plasticized caseinate films were transparent and flexible, but had poor water barrier properties. Treatment with lactic acid or tannic acid improved water barrier properties (Guilbert, 1986). At comparable test conditions, caseinate films appeared to be similar in moisture barriers to wheat gluten films and soy protein films but somewhat poorer moisture barriers when compared with corn zein films (Avena-Bustillos and Krochta, 1993). Casein has been extensively investigated in the formation of films and coatings on food products (Gennadios *et al*., 1994). Laminated

films that include casein were reported to protect dried fruits and vegetables from moisture absorption and oxidation. Caseinate-lipid emulsion coatings were successful in reducing moisture loss from peeled carrot and zuchini (Avena-Bustillos and Krochta, 1993).

2.2.3.7. Sarcoplasmic Protein Films

Proteins that were located inside the sarcolemma and were soluble in low salt concentration (< 0.01 M KCl) were referred to as sarcoplasmic proteins. Sarcoplasmic proteins comprised about 30-35% of the total muscle proteins or about 5.5% of the weight of muscle in mature animals (Xiong, 1997). Fish sarcoplasmic proteins were more or less like those from land animals, i.e. they include myoglobin, hundred of enzymes, and other albumin (Gazzaz and Rasco, 1993). The main enzyme groups known to affect the edible qualities of fish were hydrolase, oxidoreductase and transferase (Haard, 1990). Hydrolytic enzymes of importance in post harvest fish include proteinases and peptidases, lipases and phospholipases, and glycogen hydrolases. Constitutive proteinases important in seafood included those present in: (i) muscle cells, (ii) extracellular matrix and connective tissue surrounding muscle cells and (iii) digestive and other organs (Haard, 1995). Polyphenol oxidases were particularly important in crustacean because they caused post harvest discoloration. Lipoxygenases had been identified in fish skin, gill and muscle tissue. These enzymes had been implicated in skin carotenoid bleaching (Tsukuda, 1970) and fresh fish aroma (Haard, 1995).

Several nitrogeneous compounds contributed to the color of fish and shell fish. Other than myoglobin and haemoglobin, most of these compounds were associated with epithelial tissue (Haard, 1995). Nitrogen containing pigments included the blue-green to red carotenoproteins, blue purple indigoids, brown-black

melanins and melanoproteins, variously colored ommatids, which were polycyclic, aromatic compounds from cephalopods, red-green tetrapyrroles such as bilividin, flavins associated with the skin of scaleless fishes, which provided white iridescence in leucophores of fish skin and pterins which contributed to blue iridescence in fish scales. The heme proteins were particularly important because of their ubiquity, discoloration and influence on the lipid oxidation in fish meat (Haard, 1995).

In surimi manufacturing process, fish flesh was repeatedly washed with chilled water to remove sarcoplasmic proteins (Pacheo-Aguilar, *et al.*, 1989). The sarcoplasmic proteins in surimi wash-water were regarded as a hindrance in gelation of myofibrillar proteins (Niki *et al.*, 1985). Some researchers reported that sarcoplasmic proteins in surimi wash-water did not only interfere with gel formation of myofibrillar proteins, but also formed gel, if sufficiently concentrated (Ninomiya, *et al.*, 1990). Ko and Hwang (1995) reported that adding of sarcoplasmic proteins recovered by ultrafiltration from milkfish to the thermal gelation of meat paste and myofibrillar proteins (10 mg/g) improved thermal gelation.

A review on the field of sarcoplasmic proteins from water-soluble fish proteins were conducted by some researchers. A summary of research results are presented as follows:

Iwata *et al.* (2000) formed films, using water-soluble fish proteins extracted from blue marlin flesh by deposition method. The film solutions were prepared from 3% of water-soluble fish protein and pH was adjusted at 10.0 with 1.5% glycerol as a plasticizer, followed by heating at 70 ^{o}C for 25 min and drying at 25 ^{o}C for 20 h. The result revealed that water-soluble fish protein had to be denatured somehow to unfold the protein structure, and the interaction of water-soluble fish protein molecules,

particularly through disulfide linkage was attributed to the formation of films. The films had better flexibility and low water vapor permeability compared to most other protein films.

Tanaka *et al.* (2001) studied the effects of type and concentration of plasticizers on the mechanical and water vapor permeability of edible films from water-soluble fish proteins. They found that increase of glycerol resulted in decrease of tensile strength with concomitantly increase of elongation at break and water vapor permeability.

Kerdsup *et al*. (2002) prepared film from water-soluble proteins extracted from threadfin bream. At pH 9.0 and heating time-temperature at 15 min at 70 ^{o}C for 15 min, at this same conditions, the film with highest tensile strength resulted. Sorbitol plasticized film demonstrated higher tensile strength than using glycerol and polyethylene glycol. Films with 40% sorbitol yielded the highest tensile strength of 5.11 MPa concomitantly with the lowest elongation at break and water vapor permeability.

2.2.4 Composite Films

Edible films and coatings may be heterogeneous in nature and consist of a blend of polysaccharides, protein, and/or lipids. This approach enabled one to advantageously utilize the distinctive functional characteristics of each class of film former (Kester and Fennema, 1986). The combination between polymers to form films could be from proteins and carbohydrates, proteins and lipids, carbohydrates and lipids or synthetic polymers and natural polymers. The main objective to produce composite films was to improve the permeability or mechanical properties as dictated by the need of a

specific application. These heterogeneous films were applied either in the form of an emulsion, suspension, or dispersion of the nonmiscible constituents, or in successive layers (multilayer coating or films), or in the form of a solution in a common solvent. The method of application affected the barrier properties of the films obtained (Guilbert, 1986).

Schultz *et al*. (1948) incorporated lipids into low-methoxy pectinate films to improve resistance to water vapor permeation. Kamper and Fennema (1984) introduced the emulsion films from methyl cellulose and fatty acid to improve water vapor barrier of cellulose films. Recently, many researchers have extensively explored the development of composite films based on the work of Kamper and Fennema (1984). Examples of these studies were using lipid and hydroxypropyl methyl cellulose (Hagenmaier and Shaw, 1990), methyl cellulose (MC) and lipid (Greener and Fennema, 1989a), MC and fatty acid (Sapru and Labuza, 1994), corn zein, MC and fatty acid (Park *et al*. 1996), whey isolate and lipids (McHugh and Krochta, 1994), and casein and lipids (Avena-Bistillos and Krochta, 1993). Rico-Pena and Torres (1990) used methyl cellulose-palmitic acid films as moisture barrier on sundae ice creams cones. Coated sample could store for 10 weeks without moisture loss at -23 ^{o}C , for 4 weeks at -12 ^{o}C.

Shih (1994) formed films, using soy protein isolate and carbohydrate (sodium alginate and propylene glycol alginate), by deposition method and dried at 50 ^{o}C for 15 min. The auther reported that the solubility and emulsifying activity were maintained or improved and films with alkylation process showed better film-making properties.

Holton *et al*. (1994) combined polyethylene with 6% of corn starch. The corn-starch containing polyethylene package provided protection equal to that made of polyethylene.

2.3 Formation of Protein Films

Protein films could be formed by two different methods: surface film formation (Wu and Bates, 1972) and deposition method (Jaynes and Chou, 1975). The first method has been employed by the oriental from ages for making traditional edible wrapping films from soy milk.

2.3.1 Surface Film Formation

Films were obtained by prolong heating of film-solutions and films were periodically harvested from the surface, drained and dried (Wu and Bates, 1972). Wu and Bates (1972) also prepared similar films from peanut milk. During heating of the peanut milk, the high molecular weight peanut protein was broken down into lower molecular weight moiety, conarachin and arachin fractions (Cherry *et al*., 1975; Aboagye and Standley, 1985). Interfacial forces might initiate the formation of a protein matrixes capable of trapping oil droplets and water released from the surface and facilitating the formation of protein matrixes (Fanum *et al*., 1976).

2.3.2 Deposition Method

Films obtained from this method generally are made by casting and drying film-forming solution on a non-stick surface. Jaynes and Chou (1975) used this method to produce soy protein-lipid films. The researchers used protein isolate solution at natural pH 6.6, casting on Teflon coated baking pan and drying at 100 ^{o}C. Films from deposition technique were more uniform compared to the surface forming method. The film thickness could be controlled by the amount of total solids in the film solutions which was not the case when the surface formation method was employed. Most researchers have been using the deposition technique in recent years to produce

edible films. However, casting material and casting temperature might vary depending upon the state and type of substrates. Deposition technique has been used to make protein films from wheat gluten, corn zein, casein, whey protein isolate, soy protein isolate and rice protein concentrate (Gontard *et al*., 1992; Park *et al*., 1994a; Aydt *et al*., 1991; Mahmoud and Savello, 1992; Brandenburge *et al*., 1993; Gennadiose *et al*., 1993ab; Shih, 1994 and 1996).

2.4 Factor Affecting Films Formation and Films Properties

2.4.1 Type of Material

Raw materials used in film solutions are classified, according to their solubility characteristics into two categories, hydrophilic and hydrophobic. Hydrophilic materials such as soy protein isolate, whey protein isolate and water soluble fish proteins are water soluble where as hydrophobic materials such as corn zein, wax are water-insoluble but they dissolve in non-polar solvent such as alcohol. The difference in soluble properties of these raw material influences the amount of energy needed to obtain dried films and their use on foods. Carbohydrates such as alginate, carregeenan, pectin, starch, cellulose and cellulose derivatives provided a strong matrix free standing films, but these films had poor water barrier properties because hydrophilic nature of raw materials used (Kester and Fennema, 1986). Proteins provided a good gas barrier but poor water vapor barrier properties, however, some protein films such as corn zein films exhibited better water resistance than protein films because zein contains high amount of hydrophobic side chain amino acid. Lipid films, made from hydrophobic

materials such as wax, fatty acid, showed excellent water vapor barrier but poor mechanical properties (Guilbert, 1986).

2.4.2 Polymer Chemistry

The regular structure molecule is more diffusible than irregular stereochemical structure whereas branched molecule may provide a greater cohesive strength than non-branched molecule. Lower molecular weight fraction shows a greater cohesion and a greater change in cohesion with temperature change. In highly polar polymer such as protein and cellulosic, self-adhesion by diffusion is not significant due to the minimal flexibility and fixed order of the macromolecule caused by the internal molecular forces holding the polymer chains. Cellulosics have a rigid ring structure chain back bone whereas proteins tend to form helical chain structure (Banker, 1966).

Kinsellar and Phillips (1989) summarized the desired molecular characteristic of proteins for films formation as follows: 1) high soluble molecules promoted rapid diffusion; 2) the large molecules allowed more interactions at the interface resulting in strong film; 3) amphiphatic molecules provided unbalanced distribution of charged and apolar residuals for improved interfacial interaction; 4) flexible domains facilitated phase behavior and unfolding at interface; 5) dispersion of charged groups affected protein-protein interaction in the films and charge repulsion between neighboring bubbles; 6) polar residue could provide hydratable or charged residues to keep bubbles apart, binding and retaining water; 7) retention of structure could enhance overlap and segmental interactions in film; 8) interactive regions could

affect depositions of different functional segments and facilitate secondary interactions in the air, and aqueous phases.

2.4.3 pH

pH plays an important role in protein films made from water-soluble materials, such as soy protein isolate and whey protein isolate, as solubility of these proteins depend on their isoelectric point (pI). During the dissolution of macromolecular substance, the cohesive forces between the solute macromolecules are neutralized by unions with the solvent molecules (Banker, 1966). The functionality of the polymer was related to solution properties which further influenced film characteristics. The charged groups repelled each other and produced a stretching of the polymer chain when the functional groups on a linear polymer became ionized during dissolution. The greater the degree of dissolution and more extensively the chain was charged, the greater was uncoiling of chain. The interaction between the charged polymer molecules and the molecules of the polar solvent increased with increasing charge on the chain (Banker, 1966). The maximum protein solubility was obtained at pH away from its isoelectric point (pI). But to produce an edible films at extreme pH, the sensory property was also considered along with other films properties. Gennadios *et al*. (1993a) studied the effect of pH on soy protein isolate film and found that highly acidic (pH < 1) or alkaline condition (pH > 12) inhibit soy protein isolate film formation. Kinsella and Phillips (1989) reported that films formed near the isoelectric point of major proteins are more condensed and stronger.

2.4.4 Casting Temperature

The interaction forces in protein structure are affected by temperature. Hydrophilic interactions increase, hydrogen bonds and electrostatic interaction decrease when temperature increase (Kinsella and Phillips, 1989) resulting in facilitation of adhesion between polymer films and substrate (Banker, 1966). High temperature (70-100 ^{o}C) affected the forming of rigid structure in protein solutions because of protein denaturation (Chefel *et al*., 1986). The excessive heat or excessive solvent evaporation rate during process might produce non-cohesive films (Guilbert *et al*., 1986). Water soluble proteins such as soy protein, whey protein needed a higher temperature and longer time for films formation than films from alcohol-soluble protein such as corn zein or wheat gluten. The higher drying temperature of water-soluble based-films might limit films use. However, low relative humidity could also be employed for film formation at low temperature as reported by McHugh and Krochta (1994b) in whey protein film formation (40%RH, 23 ^{o}C) and McHugh *et al*. (1996) in fruit puree film formation (40% RH, 24^{o}C).

2.4.5 Concentration

Concentration of film solutions affected the self adhesion of high polymers and rate of matrix forming in film preparations. At low concentration or high concentration, self-diffusion was promoted. At optimum concentration of film solutions, an intermediate viscosity could be obtained which resulted in the highest cohesive strength (Banker, 1966; Guilbert, 1986).

2.4.6 Film Additives

Various materials can be incorporated into edible films to influence mechanical, protective, sensory, or nutritional properties. Plasticizer was a major component of edible films. Generally two types of plasticizers were distinguished. Internal plasticization was a result of modifications to the chemical structure of the polymer, for example, by copolymerization or selected hydrogenation or transesterification in the case of edible fats or similar; external plastification is obtained by adding an agent which modifies the structure and energy within the three-dimensional arrangement of the film polymer (Banker, 1966). It was the second method which, on the basis of the type of material and the technology, was mainly used for edible packaging and coatings. A plasticizer may be defined as a compound, when added to another materials and under given conditions, modifies certain physical and mechanical properties of the material. The addition of a plasticizer to films produced films, which were less likely to break and more flexible and stronger. The reduction of the intermolecular bonds between the polymer chains, and thus the overall cohesion, facilitated elongation of the films and reduced its glass transition temperature. This is manifested by a reduction in the barrier properties to gases, vapors, and film solutes (Banker, 1966; Kumins, 1965).

A plasticizing agent must be compatible with the film-forming polymer and be permanently presented within the solvent–polymer system and under the conditions used. To be compatible, it must be miscible with the polymer, which implied the use of molecular reactions of similar nature. It was important to remember that the formulation of the whole films system (polymer, solvent, plasticizer, and other additives) had a direct effect on the nature and characteristics of the films

produced. As a result, the polymer and the plasticizer must not only be compatible, but must also have similar solubility in the solvent used. A soluble plasticizer will be generally be sought for the development of soluble coating and an insoluble plasticizer (or dispersible) for an insoluble coatings or for a slow solubilization (Guilbert, 1986)

The permanence of a plasticizer was also of prime importance since this influence the physical and mechanical stability of the films. The plasticizer should not be volatile (or only very slightly volatile) and its degree of retention by the films should be high. Other properties, such as its chemical stability, hygroscopicity, color, and flavor and so on, were also more or less important depending on the type of films under consideration. In addition, the concentration of a plasticizer necessarily varies from 10-60 % (dry base) according to the nature and type of films and the method of application (Guilbert and Biquet, 1996).

The plasticizers that were most often used in the field of edible coatings and films are

- mono-, di-, and oligosaccharides (generally glucose syrups or glucose-fructose honey)

- polyols (principlly glycerol and its derivatives, polyethylene glycols, sorbitol)

- lipids and its derivatives (fatty acids, monoglycerides and their esters, phospholipids and other emulsifiers).

Plasticizing of hydrophilic polymer-based films would generally be achieved by the addition of a compound belonging to one of the first two groups and that of a wax or fat-based film by a compound from the third group. The efficiency, stability, compatibility and permanence of a plasticizing agent could be

evaluated by various semi-empirical tests. The final method of plasticization consisted of adding to the films system relatively inert solids (fillers which reduce the molecular reactions and cohesion of the final films). The size of these particles and their dispersion were of prime importance. Microcrystalline cellulose, various protein isolates, and cocoa were used as plasticizers, particularly, in fat–based films (Guilbert and Biquet, 1996).

Glycerol and polyethylene glycol were found to be the most effective plasticizers for MC (Donhowe and Fennema, 1993) and HPMC, films (Aulton *et al*., 1981). Park *et al*. (1993) studied the effect of three plasticizer comprising polyethylene glycol (PEG), propylene glycol (PG), glycerin (G)-at 4 level concentrations (0.17, 0.33, 0.50, 0.66 ml/ g of cellulose on cellulose based films. They found decrease in tensile strength (TS) and increase in elongation (E) when plasticizer content increase. PEG was found to be most effective to improve flexibility among glycerin and polyethylene glycol. However, PEG did not affect film's permeability properties. Glycerin did not have effect on oxygen permeability (OP) of cellulose films but affected water vapor permeability (WVP), WVP increased with increase of glycerin from 0-0.33 G/g but decreased beyond 0.33ml/g cellulose. Water vapor and oxygen permeability of cellulose films increased with an increase of PG. Chinnan and Park (1995) reported that increasing PEG from 0 to 0.33 ml/g cellulose increased the WVP of hydroxy propyl cellulose films. Whereas, in MC films, WVP decreased when PEG was increased from 0 to 0.11 ml/g cellulose but WVP increased when PEG was increased from 0.11 to 0.33 ml/g cellulose.

In whey protein films, Mahmoud and Savello (1992) reported change in water vapor transmission rate at glycerin level of 0.125 to 2%. McHugh and Krochta (1994b) determined the effect of sorbitol and glycerol on whey

protein films and concluded that oxygen permeability was affected by glycerol more than that by sorbitol. Films with sorbitol showed lower oxygen permeability than films with glycerol at equal tensile strength. Tensile strength decreased and elongation increased with an increase of plasticizer.

In pectin films, Coffin and Fishman (1993) found glycerin performed better than urea and PEG. In their study, mechanical properties (elongation and tenacity) improved with increasing glycerin (9 to 19% (w/w)).

Guo (1994) investigated the effect of PEG-600 on sucrose permeability of cellulose acetate films and reported that permeability of sucrose showed decrease with increasing plasticizer concentration, however when the concentration of plasticizer increased above 30% dramatic increase in sucrose permeability was resulted.

Butler *et al*. (1996) investigated the effect of glycerin on properties of chitosan films at 0.25 and 0.50 ml/g chitosan. As they expected, the barrier properties and elongation at break increased but tensile strength decreased with increasing glycerin. In order to decrease water vapor permeability on whey protein isolate films by using glycerol or sorbitol as plasticizer, Fairley *et al*. (1996) used sodium dodecyl sulfate (SDS) as plasticizer. SDS could not be used as plasticizer by itself, however, when used as co-plasticizer with sorbitol at mass ratio of SDS to whey protein isolate (WPI) of 1:2, films improved in flexibility and solubility without water vapor permeability change. When SDS was used with glycerol at the same ratio of SDS to WPI, a less flexibility and solubility with slight increase in water vapor permeability were shown.

2.5 Enhancement of Properties of Edible Protein Films

Many approaches were employed to improve the barrier properties of edible protein films. These included by modifying properties of protein by chemical and enzymatic methods, combining with hydrophobic material, combining with some polymers, and using a physical method.

2.5.1 Modification of Protein by Chemical Methods

Chemical treatments with acid, alkali or crosslinking agent have been extensively used to improve films properties. However, this approach might restrict films edibility (Gennadios *et al*., 1994). Hydrolyzed protein results in greater solubility at high pH and high temperature (Bain *et al*., 1961). Guilbert (1986) reported that denatured protein formed less flexible and transparent, but more moisture resistant films. Theoretically, the more protein interaction from chemical treatment such as alkaline or acid modification, the less permeability but higher tensile strength would be obtained. However, Brandenburg *et al*. (1993) found that alkaline treatment on soy protein isolate did not affect water vapor permeability, oxygen permeability and tensile strength but alkaline treatment improved film's appearance (clearer, more uniform, less air bubble) and elongation at break.

2.5.2 Modification of Protein by Enzymatic Treatments

Mahmoud and Savello (1992) investigated the production of whey protein films using transglutaminase as the catalytic crosslinking enzyme. Transglutaminase could catalyze the covalent polymerization of whey protein. However, the effect of using transglutaminase on film's permeability was not available. Stuchell

and Krochta (1994) studied enzymatic treatments of edible soy protein films. They reported that treatment with horseradish peroxidase provided no further improvement in water vapor permeability, but increase tensile strength and decrease elongation. Yildirim *et al*. (1996) prepared biopolymer from crosslinking whey protein isolate and soybean by transglutaminase. The biopolymer showed an excellent stability. They then postulated that the polymers should be able to form a better water moisture barrier films.

2.5.3 Combination with Hydrophobic Materials or Other Polymers

Ukai *et al*. (1976) patented the use of protein-lipid emulsion (caseinate-based emulsion) for coating agricultural products. Kamper and Fennema (1984) used emulsion technique to produce bilayer films from lipid (bee wax, paraffin, hydrogenated palm oil or steric acid) and hydroxypropyl methyl cellulose with water vapor permeability (25^{o}C) lower than that of low density polyethylene. Guilbert (1986) developed bilayer emulsion protein based films using casein or gelatin, with stearic-palmitic acid and canauba wax. These films showed good water barrier properties, but poor mechanical properties and residual waxy taste. McHugh and Krochta (1994a) developed whey protein-lipid emulsion films and found that the water vapor permeability of films was reduced through lipid incorporation,while fatty acid and beeswax emulsion films exhibited very low water vapor permeability. Gontard *et al*. (1994) reported that beeswax was the most effective lipid to improve moisture barrier of films prepared from wheat gluten. Combining wheat gluten protein with diacetyl tartaric ester monoglycerides reduced water vapor permeability, increased tensile strength and maintained transparency. Park *et al*. (1994b) reduced water vapor permeability of corn zein by lamination with methylcellulose and zein-fatty acid comprising lauric acid,

plamitic or blends of stearic acid and palmitic acid. Shih (1994) found that alkylated complexes, protein-propylene glycol alginate, showed better film making properties and good stability in water but the non-edibility of specific reducing agent, sodium cyanoborohydride, may limit its use on food.

2.5.4 Combination with Synthetic Polymers

Park *et al*. (1993) employed the extrusion process to make films from a mixture of corn zein and low density polyethylene. They reported reduction in tensile strength and elongation, and increase in water vapor permeability with increase of corn zein content. Ghorpade *et al*. (1995) combined soy protein isolate with polyethylene oxide, and reported that no improvement in tensile strength and water vapor barrier properties was observed. This method focused only on environmental friendly, non-edible films.

2.5.5 Modification of Films Properties by Physical Methods

Stuchell and Krochta (1994) improved appearance and water vapor permeability of soy protein isolate film by thermal treatment of the forming solution at 85 ^{o}C, and then cooled quickly to room temperature before casting. Gennadios *et al*. (1996) improved water barrier and tensile strength of soy protein film by heating at a desired temperature after peeling off from the casting surface. Banerjee *et al*. (1996) improved some mechanical properties of milk protein-based films by using ultrasound. However, this practice did not have any effect on the water vapor permeability of the films.

2.6 Application of Protein Films

Several researchers have studied the application of protein films in food uses and an excellent review was given by Gennadios *et al*. (1994). In this reviewed, the applications of several edible protein-based films, such as corn zein on nut and fruit product, casein emulsion film on fruit, whey protein films on fruit product were cited but the application of edible films from water-soluble fish protein in surimi wash-water was not mentioned.

One of the potential uses of protein films is to reduce lipid oxidation due to the excellent barrier properties of the film. Herald *et al*. (1996) used corn zein with an antioxidant and emulsifier to maintain the quality of cooked turkey. They found that dipping in corn zein resulted in a very dry products for the sensory panelists. However, corn zein with an antioxidant and emulsifier reduced hexanol after 3 days compared with PVDC films. Stuchell and Krochta (1995) used whey protein isolate and acetylated monoglyceride to maintain quality of frozen king salmon and found a delay in the lipid oxidation onset and a reduction in moisture loss rate.

2.7 Protein Precipitation

The solubility of a protein molecule in an aqueous solvent is determined by the distribution of charged hydrophilic and hydrophobic groups on its surface. The charged groups on the surface will interact with ionic groups in the solution. Protein precipitates are formed by aggregation of the protein molecules, induced by changing pH or ionic strength, or by addition of organic miscible solvent or other inert solutes or polymers. Temperature will also affect the degree of aggregation achieved. Precipitates can be recovered by filtration or centrifugation, washed and redisolved in an appropriate

buffer, if required (Harris and Angal, 1989). Numerous methods have been reported for protein precipitation.

2.7.1 Precipitation by Shifting the pH

One of the easiest methods in precipitating a protein and achieving a degree of purification is by adjusting the pH of the solution to close or equal to the isoelectric point (pI) of the protein. The surface of protein molecules is generally covered by both negatively and positively charged groups. Above pI the surface is predominantly negatively charged, and therefore like-charged molecules will repelled from one another; conversely below pI the overall charge will be positive and again like-charged molecules repel one another. However, at the pI of the protein the negative and positive charges on surface of the molecule cancel one another out, electrostatic repulsion between individual molecules no longer occurs and electrostatic attraction between molecules may occur, resulting in formation of a precipitate (Harris and Angal, 1989). The temperature was reported to be important, in a more extreme pH conditions i.e temperature around 0-10 ^{o}C rather than 50-60 ^{o}C may cause protein denaturation. In view of the great difference that a few degrees make, pH precipitation should be carried out at a carefully specified temperature (Scope, 1994). The pH adjustment should be carried out with an appropriate acid or base. Strong acids and bases should be avoided unless necessary. Tris and acetic acid could be used for adjusting pH values in the range 4.5-8.5; stronger acids or bases were needed only to go outside this range. Lactic acid was suitable down to about pH 3.5, after which phosphoric or even sulfuric acid might be needed. If the enzyme was stable at pH 2, then brief exposure to a drop of strong acid was less likely to cause harm than the same exposure of a protein that denatured at about

pH 5. For high pH, diethanolamine (to pH 9) or sodium carbonate (to pH 10.5) could be used; sodium or potassium hydroxide would be needed to get above pH 11. In these extremes of pH it was likely that a substantial proportion of denatured protein would remain in solution, even if the salt concentration was moderate. But on readjusting to neutrality, denatured proteins would normally precipitate out. It was advisable, after neutralization, to incubate proteins before centrifuging off the precipitate (Scope, 1994).

2.7.2 Precipitation by Decreasing the Ionic Strength

Some proteins could be precipitated by lowering the ionic strength. This could rarely be achieved with crude extracts, since the ionic strength could only be lowered by addition of water, which also lead to a decrease in the concentration and hence an increased solubility (a notable exception is the serum globulin). However, this form of precipitation could often occur at later stages of purification, such as, in salts removal by diafiltration, dialysis or gel filtration. Precipitation at low ionic strength was more likely to occur at or close to the pI of the protein (Harris and Angal, 1989).

2.7.3 Precipitation by Increasing the Ionic Strength (Salting Out)

Precipitation by addition of neutral salts is probably the most commonly used method for fractionating proteins by precipitation. The precipitated proteins were usually not denatured and activity was recovered upon redissolving the pellet. In addition these salts could stabilize proteins against denaturation, proteolysis or bacterial contamination. Thus, a salting-out step was an ideal step at which to store an extract overnight, either before or after centrifugation. The cause of precipitation was different from that for isoelectric precipitation, and therefore the two were often used

sequentially to obtain differential purification (Harris and Angal, 1989). Salting-out was dependent on hydrophobic nature of the protein. Hydrophobic groups predominate in the interior of the proteins, but some are located at the surface, often in patches. Water is forced into contact with these groups, and in so doing becomes ordered. When salts were added to the system, water solvated the salt ions and as the salt concentration increased water was removed from around the protein, eventually exposing the hydrophobic patches. Hydrophobic patches on one protein molecule can interact with those on another, resulting in aggregation. Thus, proteins with larger or more hydrophobic patches will aggregate and precipitate before those with smaller patches (Glatz, 1990). The aggregates formed are a mixture of several of proteins, and like isoelectric precipitation, the nature of the raw material affected the concentration of salt required to precipitate the protein of interest. In contrast to isoelectric precipitation, increasing the temperature increased the amount of precipitation; however, salting out was usually performed at 4 ^{o}C to decrease the risk of inactivation (by, e.g. proteases) (Harris and Angal, 1989).

2.7.4 Precipitation by Organic Solvents

Many proteins could be precipitated by addition of water-miscible organic solvents, such as ethyl alcohol. The factor which influenced the protein precipitation behavior of proteins was similar to those involved in isoelectric precipitation and different from those involved in salting-out; thus, this method could be used as an alternative to isoelectric precipitation. Addition of organic solvent lowered the dielectric constant of the solution, and hence its solvating power. Thus, the solubility of a protein was decreased and aggregation through electrostatic attraction could occur.

Precipitation occured more readily when the pH was close to the pI of the protein. The size of the protein also influenced its precipitation behavior; thus a larger protein would precipitate in low concentrations of organic solvent than smaller protein with otherwise similar properties (Harris and Angal, 1989). However, some hydrophobic proteins, particularly those, which were located in the cellular membranes, were not precipitated by organic solvents, and in fact could be solubilized from the membranes by addition of organic solvents. With these proteins the organic solvent would displace the water molecules from around the hydrophobicity patches of the protein, resulting in an increased solubility (Findlay, 1989).

To minimize the denaturation, precipitation with organic solvents should be carried out at or below 0 ^{o}C. At higher temperature the protein conformation rapidly changed, thus enabling the organic solvent to gain access to interior of proteins, where they could disrupt the hydrophobic interactions and caused denaturartion. Acetone and ethanol were the most commonly used solvents; others which have been used included methanol, propan-1-ol, and propan-2-ol. Safety aspects should be considered, particularly when working on a large scale; thus the solvent should be relatively non-toxic and have a relative high flashing point, above 20 ^{o}C. The longer chain alcohols, such as butanol, caused a high degree of denaturation than ethanol. In many cases acetone was preferable, since lower concentrations were required, and therefore less denaturation occurred (Harris and Angal, 1989).

2.7.5 Precipitation by Organic Polymers

Polyethylene glycol was the most commonly used organic polymer (Ingham *et al.*, 1984). The mechanism of precipitation was similar to that of

precipitation by organic solvents; however, lower concentrations were required, usually below 20%. Higher concentration resulted in viscous solutions, making recovery of the precipitate difficult. The molecular weight of the polymer should be greater than 4000. The most commonly used molecular weight was 6000 to 20000. Polyethylene glycol was removed by ultrafiltration, provided its molecular weight differed significantly from that of the protein of interest (Harris and Angal, 1989).

2.7.6 Precipitation by Denaturation

Precipitation by denaturation could be used as a purification step if the protein of interest was not denatured by the treatment, whilst many of the contaminant protein were. This method could also be used to concentrate the proteins in a solution prior to analysis. Denaturation could occur by the changes in temperature, pH or addition of organic solvents. The tertiary structure of proteins was disrupted during denaturation, resulting in the formation of random coil structures. In solution these random coils became entangle with one another, thus forming aggregates. Aggregate formation was influenced by pH and ionic strength, occurring more readily close to the pI of the protein, and at lower ionic strength (Harris and Angal, 1989).

CHAPTER III

METHODOLOGY

3.1 Recovery and Characterization of Water-Soluble Fish Proteins Precipitated from Surimi Wash-Water

3.1.1 Preparation of Raw Material

Fresh whole threadfin bream fish (*Nemipterus hexodon*) was purchased from a local fish dealer and transported on ice to Chulalongkorn University in Bangkok where they were hand-skinned and filleted on the day received. The fillets were then placed immediately in polyethylene bags and kept frozen at -20 ^{o}C until used.

Surimi was produced in the laboratory using the commercial method described by Toyoda *et al*. (1990) (Fig. 3.1). Moisture, fat, protein (total N x 6.25-non protein nitrogen) and ash of threadfin bream fish (*Nemipterus hexodon*) were determined according to the procedure outlined by Association of Official Analytical Chemists (AOAC) (1995) followed by total volatile base nitrogen (TVB) determined by the method of Hasegawa (1986). The freshness of fish was analyzed for TVB and samples within the range of 10-20 mg / 100 g sample was used in this study. Surimi wash-water was collected from WS-I, II and III and analyzed for their water soluble proteins content by Lowry's method (Lowry *et al.*, 1951). The molecular weight distribution of the protein was also determined by using the sodium dodecyl sulfate polyacrylamide Gel (SDS-PAGE) according to the methods described by Laemmli (1970).

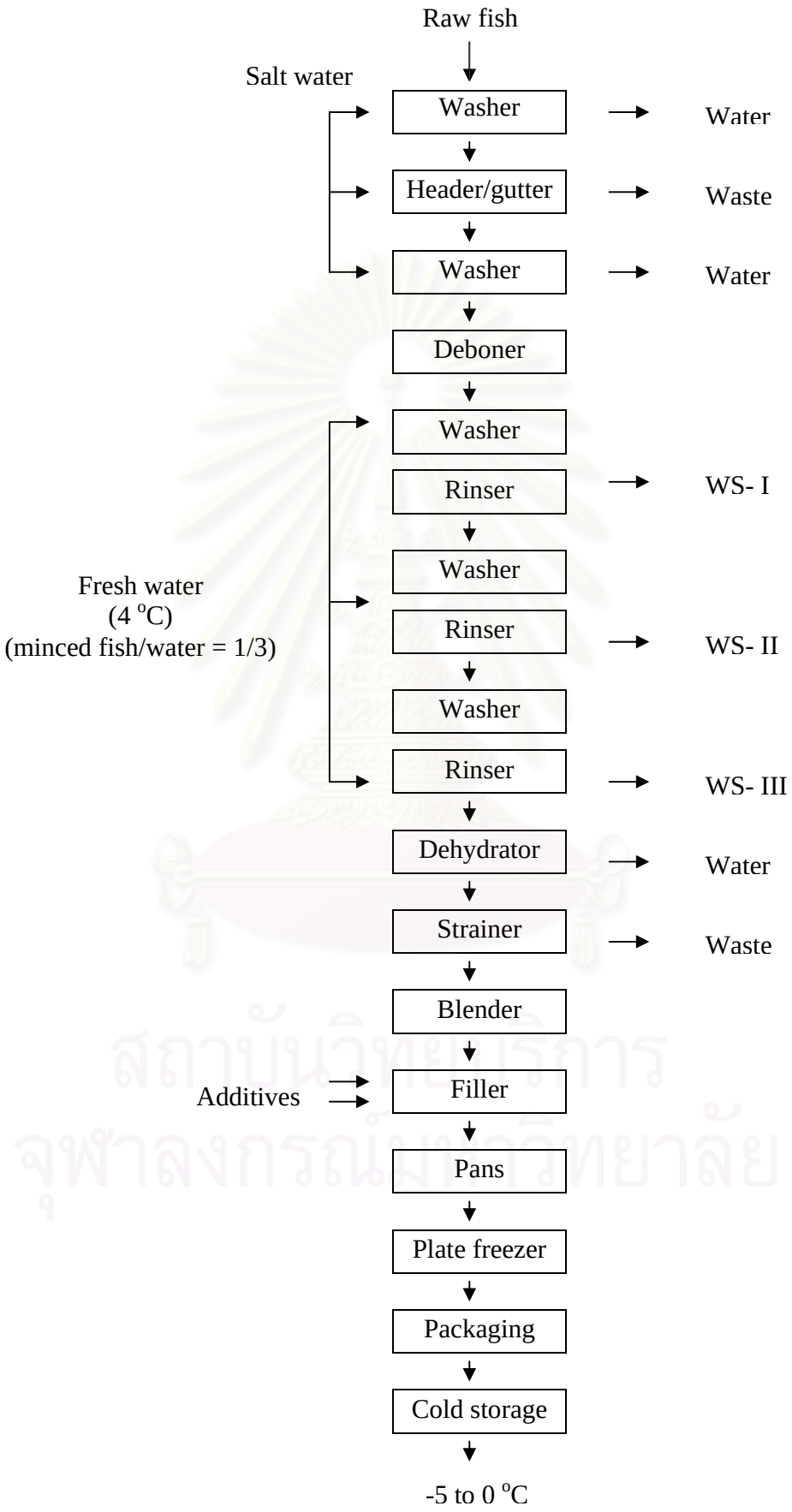


Figure 3.1 Commercial surimi manufacturing process

3.1.2 Precipitation of Water-Soluble Fish Proteins

Two methods were explored for precipitating water-soluble proteins in surimi wash-water: a) by shifting the pH, and b) by using ethanol (95%).

3.1.2.1 Shifting the pH

Precipitation by shifting the pH was achieved by using 0.1 M sodium phosphate buffers at various pH (3.0-6.0), time (5, 10 and 15 min) and temperature (4, 17 and 30 ^{o}C) conditions. Sample solutions (150 ml) of surimi wash-water (pH 6.7-7.0) were mixed with buffer solutions to a final volume of 300 ml and evaluated on the effect of pH, reaction time and reaction temperature related to proteins precipitation. The percentage of proteins precipitated was assessed by determining the concentration of water-soluble fish proteins in the supernatant.

3.1.2.2 Ethanol

Ethanol (95%) was used to precipitate proteins from the surimi wash-water. Sample solution (150 ml) of surimi wash-water was mixed with ethanol and evaluated for concentration (10, 20, 30, 40, 50 and 60 % v/v) and time (10, 15, 20 and 25 min) related to proteins precipitation. The percentage of proteins precipitated was assessed by determining the concentration of water-soluble fish proteins in the supernatant.

3.1.3 Determination of Water-Soluble Proteins (Lowry *et al.*, 1951)

A 0.5 ml of test solution was placed in a test tube. A 2.5 ml of the mixture of 0.2 M sodium hydroxide and 4% sodium carbonate was pipetted into each sample and vortexed to mix thoroughly and stand for 10 min. Then 0.25 ml folin ciocalture & phenol reagent was added into each sample and vortexted to mix thoroughly

and stand for 30 min at room temperature. Absorbance at 750 nm was determined by diode array spectrophotometer (Hewlett Packard Model 6541A, Avondale, PA). A standard curve was developed using bovine serum albumin.

3.1.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970). The precipitated supernatant proteins were boiled for 120 s in 10 % SDS-8 M Urea and 0.5 M Tris-HCl (pH 6.8) in the presence of 2-mercaptoethanol (2ME). Fifteen micrograms of proteins applied on 4 % stacking and 12.5 % running polyacrylamide gel. The molecular weight standard mixture (stock No. SDS-7, Sigma Chemical Company, St. Louis, MO) made up of albumin, bovine (66.0 kDa), albumin, egg (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase rabbit muscle (36.0 kDa), carbonic anhydrase, bovine (29.0 kDa), trypsinogen, bovine (29.0 kDa), trypsinogen, bovine pancrease (24.0 kDa), trypsin inhibittor, soybean (20.1 kDa) and α-lactalbumin, bovine milk (14.2 kDa) was used. Protein bands were stained with 0.125 % of Coomassie blue and destained in 40 % methanol and 7 % acetic acid solution followed by 5 % methanol and 7 % acetic acid solution.

3.1.5 Protein Solubility

Protein solubility was determined by the method of Lee *et al*. (1992) with slight modification. The recovered proteins were diluted to 1% (w/v) with distilled water and centrifuged for 20 min at 10,000 x *g* in a centrifuge Model J2-21M (Beckman Instruments Inc., Palo Alto, CA) to sediment insoluble proteins. Protein concentrations in the total solution and supernatant fractions were determined by the Lowry method and the protein solubility was computed as:

$$\text{Protein solubility} = \% \frac{\text{protein in supernatant}}{\text{\% total protein}} \times 100$$

3.1.6 Statistical Analysis

Analysis of variance and Duncan's New Multiple Range Test of Statistical Analysis (SAS Program for windows version 6.08, Cary, NC) were employed ($\alpha = 0.05$) for statistical analysis of data.

3.2 Effect of pH, Heating Temperature, Heating Time, Protein Concentration and Plasticizer on the Properties of Edible Films from Water–Soluble Fish Proteins in Surimi Wash-Water

3.2.1 Preparation of Raw Materials

Water-soluble fish proteins from threadfin bream fish (*Nemipterus hexodon*) recovered from first stage of surimi wash-water was produced in the laboratory. These protein samples were recovered by shifting the pH using 0.1 M sodium phosphate buffer at pH 3.5 and 17 ^{o}C for 5 min or using 60% ethanol at 4 ^{o}C for 25 min. Both recovered proteins from surimi wash-water were freeze dried for 24 hr (Dura-Top^{TM} l TD2DOTS002, FTS Systems, Inc). The controlled sample was prepared by freeze-drying surimi wash-water for 24 hr. All samples were stored in plastic bag at –20 ^{o}C until used.

3.2.2 Preparation of Water-Soluble Fish Protein Films

Freeze-dried water-soluble fish proteins were dissolved in distilled water (3% w/v) to prepare film-solutions. The pH was adjusted by 0.5 M sodium hydroxide prior to adding plasticizer (sorbitol), the ratio of protein: plasticizer was applied at 2:1. The film-solutions were heated (60, 70 and 80 ^{o} C) on a hot plate with magnetic stirrer for the given time (10, 20 and 30 min)

3.2.3 Film Casting and Drying

The film solutions were filtered through polyester screen (mesh no. 140) to remove small lumps, cooled to room temperature (23±2 ^{o}C) and a vacuum was applied to remove dissolved air and poured into leveled non-stick plates (Teflon plate, 25 cm x 37 cm) to set overnight at 30 ^{o}C by using hot air oven. The films were peeled off the plates after cooling and were conditioning by keeping in the plastic bag in the desiccator at 50 %RH and 25 ^{o}C for further testing.

3.2.4 Film Testing

3.2.4.1 Conditioning

All films were conditioned prior to permeability and mechanical tests according to Standard method, D618-61 (ASTM, 1993a). Films used for testing water vapor permeability (WVP), oxygen permeability (OP), tensile strength (TS) and elongation (E) were conditioned at 50% RH and 23±2 ^{o}C by placing them in a desiccator over a saturated solution of Mg $(NO_3)_2$.$6H_2O$ for 48 h or more. For other tests, film were transferred to plastic bags after peeling and placed in the desiccator.

3.2.4.2 Moisture and Protein Contents

Moisture content was determined by drying samples under vacuum at 70 ^{o}C (3.4 KPa) for 24 h (Jangchud and Chinnan, 1999). Nitrogen content was determined by using a Leco FP2000 combustion oven apparatus (Model FP-2000, Leco Corporation, Warrendale, PA). A protein conversion factor of 6.25 was used to calculate protein content.

3.2.4.2 Film Thickness

Thickness of the films were measured with a digital micrometer (Digitrix-Mark II, Cole-Palmer Instrument Company, and Niles, IL) to the nearest 0.0001 in at five random locations around the film. Precision of the thickness measurements was $\pm$5%. Mean thickness for each sample was calculated and used in water vapor permeability (WVP), oxygen permeability (OP) and tensile strength (TS) calculation.

3.2.4.3 Film Solubility

Method modified from Stuchell and Krochta (1994) was used to measure film solubility. Film pieces 20 mm x 20 mm were dried at 70 ^{o}C in a vacuum oven (3.4 kPa) for 24 h, and then weighed to the nearest 0.0001 g for the initial dry weight. Film was immersed into 20 ml of distilled water in 50 ml screw centrifuge tube containing 0.01 % potassium sorbate. The tubes were capped and placed in shaking water bath for 24 h at 25 ^{o}C. The solution was removed and set aside for later testing of protein solubility as described later. The remaining solution and small film piece was pour onto (Whatman #1) qualitative filter paper, rinsed with 10 ml distilled water, and dried at 70 ^{o}C in a vacuum oven for 24 h, the dried weight of film was determined. Triple measurements were done for each treatment. Total soluble matter was calculated from the initial gross weight and final dry weight using the following equation:

$$\% \text{ Film solubility (db)} = \frac{\text{(film weight before test – film weight after test)}}{\text{Film weight before test}} \times 100$$

3.2.4.5 Protein Solubility

Solution set aside from films solubility was analyzed for protein content by the Lowry method (Lowry *et al.* 1950). A 0.5 ml of test

solution was placed in a test tube. A 2.5 ml of the mixture of 0.2-M sodium hydroxide and 4% sodium carbonate was pipetted into each sample and vortexed to mix thoroughly and left for 10 min. Then 0.25 ml folin ciocalture & phenol reagent was added into each sample and vortexted to mix thoroughly and stand for 30 min at room temperature. Absorbance at 750 nm was determined by diode array spectrophotometer (Hewlett Packard Model 6541A, Avondale, PA). A standard curve was developed using bovine serum albumin. The protein solubility (% PS) was calculated as followed:

$$\% \text{ Protein solubility} = \frac{\text{Weight of protein in 20 ml solution x 100}}{\text{Initial weight of film x (\%protein in film) x (\%dry matter of film)}}$$

3.2.4.6 Film Color

A portable colorimeter (MiniScan XE, Associate Laboratory, Inc., Reston, Verginia) was used to determined film L*, a* and b* color value (L* = 0 (black) to 100 (white); a* = -60 (green) to +60 (red); and b* = -60 (blue) to +60 (yellow). Yellow standard plate (calibration plate CR-A47, L*= 85.45, a*= -0.15 and b*= 54.55) was used as standard. Color (means of five measurements at different locations on each specimen) was measured on 10 cm X 10 cm. Film specimens were placed on a black plate when measurements were performed. Total color difference (ΔE_{ab}*), hue angle (H) and chroma (C) were calculated as the following equation:

$\Delta L^* = L^*_{sample} - L^*_{standard}$, $\Delta a^* = a^*_{sample} - a^*_{standard}$, $\Delta b^* = b^*_{sample} - b^*_{standard}$,

$\Delta E_{ab}^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$, $C = [(a^*)^2 + (b^*)^2]^{0.5}$ and

$H = \tan^{-1}(b^*/a^*)$ when $a^* > 0$ and $b^* > 0$

$H = 180^o + \tan^{-1}(b^*/a^*)$ when $a^* < 0$

$H = 360^o + \tan^{-1}(b^*/a^*)$ when $a^* > 0$ and $b^* < 0$

Prior to color measurement, film specimens were conditioned at 50% RH and 23±2 °C for 3 days.

3.2.4.7 Water Vapor Permeability

3.2.4.7.1 Infrared Sensor Technique

Water vapor permeability was determined by water transmission rate instrument (Permatran-W1A, Modern Controls, Inc. Minneapolis, MN). Testing method as described by ASTM F1249-90 Standard Method was used (ASTM, 1993b). Film samples were double masked by aluminium foil mask with effective film test area of 5 cm^2. Testing was performed at 25± 2 °C and 50% RH. Water vapor permeability was calculated by multiplying water vapor transmission rate (WVTR) with the thickness and dividing by water vapor pressure gradient across the exposed films.

3.2.4.7.2 Gravimetric Technique

The gravimetric Modified Cup Method based on ASTM E96-92 (McHugh *et al.,* 1993) was used to determine water vapor permeability. The test cups were filled with 20 g of Drierite (W.A. Hommond Drierite Co.) (desiccant) to produce a 0% RH below the film. An edible films produced from water-soluble fish proteins in surimi wash-water were in between the cup and the ring cover of each cup coated with silicone sealant (high vacuum grease, Dow Corning Midland, Mich., USA) and held with 4 screws around the cup circumference. The air gap at approximately 0.6 cm between films surface and desiccant. The water vapor transmission rates (WVTR) of films were measure 50± 5 % RH and 25±2 °C. After taking initial weights of the test cup, the cup were placed into an environmental chamber

with an air velocity rate of 450 ft/min (Model NQ2 Incubator with TC2A microcontroller, EGC Corp, Chagrin Falls, Ohio). Weight gain measurements were taken by weighing the test cup to the nearest 0.001g with an electronic scale (Sartorious Corp.) every 2 h for 16 h. A plot of weight gained versus time was used to determine the WVTR. The slope of the linear portion of this plot represents the steady state amount of water vapor diffusing through the film per unit time (g/h). WVTR was expressed in unit of grams per meter square per day. Steady state over time (slope) yielded a regression coefficient of 0.99 or greater. Eight samples were used at each treatment. The water vapor permeability of films were calculated by multiplying the steady WVTR by the films thickness and dividing by the water vapor pressure difference across the films.

Water vapor permeability of edible films produced from sections 4.2.1, 4.3.1 and 4.4.1 were analyzed by Infrared sensor technique (Permatran –W1A), while, edible films produced from sections 4.2.2, 4.2.3, 4.3.2, 4.3.3, 4.4.2 and 4.4.3 were analyzed by the gravimetric Modified Cup Method.

3.2.4.8 Oxygen Permeability

Oxygen permeability was determined with a MOCON unit (Ox-Tran 100A, Modern Control, Inc., Minneapolis, MN) according to ASTM D3985-81 Standard Method (ASTM, 1993c). Film samples were masked by aluminium foil mask with effect film test area of 5 cm^2. Testing was performed at 25$\pm$2 ^oC and 50% RH. Oxygen permeability was calculated by multiplying oxygen gas transmission rate (OGTR) with the thickness and dividing by partial pressure difference of oxygen across the films surface.

3.2.4.9 Tensile Strength and Elongation at Break

Tensile strength was performed with an Instron universal testing instrument (Model 1122, Instron Corp., Canton, MA) as per ASTM D882-91 Standard Method (ASTM, 1995). Fifteen samples, 3 cm x 10 cm, were cut from each film. Initial grip separation and cross head speed were set at 50 mm and 50 mm/min, respectively. Tensile strength was calculated by dividing the maximum force at break by initial specimen cross-sectional area, and percent elongation at break was calculated as follows;

$$E = 100 \text{ X } (d_{\text{after}} - d_{\text{before}})/d_{\text{before}}$$

Where d was the distance between grips holding the specimen before or after the break of the specimen.

3.2.4.10 Determination of Surface Hydrophobicity

Surface hydrophobicity of film-solutions were determined by using hydrophobic fluoresence probe, 1-anilino-8-naphthalene sulfonate (ANS). Measurement was performed according to the method of Kato and Nakai (1980) with slight modification. Film-solutions were serially diluted with 0.1 M phosphate buffer (pH 7.0) containing water, 0.2 M H_3BO_4; 0.05 M $H_3C_6H_5O_7$. H_2O and 0.1 M $Na_3PO_4.12H_2O$ to obtain protein concentration ranging from 0.005% to 0.05 %. Then 40 µl ANS (8.0 mM in 0.1 phosphate buffer pH 7.0) solution was added to 40 ml of sample. After keeping at room temperature for 15 min, fluoresence intensity (FI) of ANS was measured with an spectrofluorometer (Model RF-5301PC; Shimadzu Co., Kyoto, Japan) using excitation wave length at 390 nm and emission wave length at 470 nm. The initial slopes (S_o) of the fluoresence intensity (FI) versus protein concentration (%) were

plotted, calculated by linear regression analysis, was used as an index of protein hydrophobicity.

3.2.4.11 Determination of Available SH Group and Content of SS bond.

Each of film-solution was analyzed for available SH group (free and reactive) by the method of Habeeb (1972) and Beveridge *et al.* (1974) with slight modification using Ellman's reagent (5,5', dithio-bis-2-nitrobenzoic acid). The available SH group of film forming solution was determined by mixing 5.0 ml distilled water, 2.0 ml TRIS-Glycine buffer (pH 8.0) containing 0.12% (w/v) EDTA, 20 μl sample of known amount of protein, and 20 μl of Ellman's reagent in a test tube. After 2 min, the absorbance at 412 nm was read concurrently with the determination of reagent blank.

The content of SS bond of the same film-solutions were determined by mixing a 1.0 ml sample of known protein content and 20 μl 2-mercaptoethanol (ME). After 1 h, 10 ml of 12% (w/v) trichloroacetic acid was added and mixed. After an additional hour, samples were centrifuged at 5000 X *g* for 10 min to remove ME. The precipitated was dissolved in 10 mL 8M urea in TRIS-Gly and 40 μl Ellman's reagent and the solution was read at 412 nm.

3.2.5 Experimental Design

General Response Surface Methodology (GRSM) was used to determine the optimum combinations of pH, heating temperature and heating time. GRSM is given in terms of coded variable, x_i (Cochran and Cox, 1957; Cox, 1958; Myers, 1971; Thompson, 1982). They are used in specific applications after

transformations of the uncoded (actual) variables, ξ_i. In this study a three level, three-factor design was adopted (Box and Behnken, 1960). It fulfills the requirements for multiple factor response surface designs and considers the overall error (variance or sampling error and bias error).

Selection of levels for independent variables was based on results from preliminary tests and observation of the work done by Iwata *et al.* (2000). For edible film from freeze-dried water-soluble fish proteins, when adjusting pH of film-solutions from 3.0 to 9.0 the film could not be formed well. In contrast, the viscosities of the film-solutions with pH below 2.5 or above 11.0 were too high to develop into films. Therefore, edible films from freeze-dried water-soluble fish proteins were studied at pH range from 9.5 to 10.5 (Table 3.1). For edible films produced from shifting the pH of water-soluble fish proteins, adjusting the pH of film-solutions from 7.0 to 12.0 and 3.0 and 3.5, the film could not be formed well. Therefore, edible films from protein precipitated by shifting the pH were studied at pH range from 1.5 to 2.3 (Table 3.2). For edible films produced from ethanol of the water-soluble fish proteins, adjusting the pH of film-solutions from 1.5 to 5.5 and 9.0 to 10.5, the film could not be developed, however, adjusting the pH of film-solutions higher than 12.0, the film could be formed but there were brittle. Therefore, edible films from protein precipitated by ethanol were studied at pH range from 11.0 to 12.0 (Table 3.3).

The levels of input variables in coded (x_i) and uncoded (ξ_i) forms of are given in Table 3.1, 3.2 and 3.3. The complete design consisted of 15 experimental points (each sample), which included three replications of the center point. The films were prepared in random order. Each of the fifteen dependent Y variables (responses) was assumed to be affected by the three independent variables. Responses under observation were: tensile strength (X_1), elongation at break (X_2), water vapor

permeability (X_3), Oxygen permeability (X_4), film solubility (X_5), protein solubility (X_6), L* value (X_7), a value (X_8), b* value (X_9), ΔE^*_{ab} (X_{10}), chroma (X_{11}), hue (X_{12}), hydrophobicity (X_{13}), available sulfhydryl group (X_{14}) and disulfide bond (X_{15}). Each value represented the mean of three replications. The product thus obtained was analyzed and experimental values were compared with model predictions.

Table 3.1 Fractional factorial design for freeze-dried water-soluble fish proteins in surimi wash-water

Independent variables	Coded	Uncoded	Coded	Uncoded
pH	x_1	pH	1	10.5
			0	10.0
			-1	9.5
Temperature, ^oC	x_2	T	1	80.0
			0	70.0
			-1	60.0
Time, min	x_3	t	1	30.0
			0	20.0
			-1	10.0

Table 3.2 Fractional factorial design for protein precipitated by shifting the pH in surimi wash-water

Independent variables	Coded	Uncoded	Coded	Uncoded
pH	x_1	pH	1	2.5
			0	2.0
			-1	1.5
Temperature, ^oC	x_2	T	1	80.0
			0	70.0
			-1	60.0
Time, min	x_3	t	1	30.0
			0	20.0
			-1	10.0

Table 3.3 Fractional factorial design for protein precipitated by ethanol in surimi wash-water

Independent variables	Coded	Uncoded	Coded	Uncoded
pH	x_1	pH	1	12.0
			0	11.5
			-1	11.0
Temperature, ^oC	x_2	T	1	80.0
			0	70.0
			-1	60.0
Time, min	x_3	t	1	30.0
			0	20.0
			-1	10.0

3.2.6 Statistical Analysis

The PROC RSREG (response surface regression) procedure of SAS Institute, Inc (1996) was used to determine the effects of independent variable (pH, heating temperature and heating time) on physical, barrier and chemical properties of edible films from water-soluble fish proteins in surimi wash-water. Tensile strength (TS), % Elongation (%E), water vapor permeability (WVP), oxygen permeability (OP), films solubility (FS), protein solubility (PS), L* value, a* value, b* value, ΔE^*_{ab} , chroma , hue, color of prepared films were analyzed, additionally hydrophobicity (HQ), available SH-group and disulfide (SS) bond values of film

solutions were also analyzed. RSREG is based on a second order polynomial equation to perform regression:

$$Y = \beta_{ko} + \sum_{i=1}^{3} \beta_{ki} x_i + \sum_{i=1}^{3} \beta_{kii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{kij} x_i x_j \quad \text{..... (3.1)}$$

Where: β_{ko}, β_{ki}, β_{kij} are constant coefficients and X_is are the coded independent variables. If the lack of fit was not significant, regression coefficients (β_{ko}….β_{k9}) were used to generate contour plots for response variable. If the regression model from RSREG showed a significant lack of fit, simple mathematical transformations were performed on independent and response variables to improve the fit (Box and Draper, 1987) before response surfaces were generated. Response surface and contour plot of responses for these models were also be drawn using the Statistica for Windows Version 5.0 by plotting the responses as a function of two variables, while keeping other variable at the constant value. The optimum conditions of the selected parameters on the properties of edible films from water-soluble fish proteins in surimi wash-water can be determined by superimposing the contour plots an acceptably high tensile strength, high elongation at break, low water vapor permeability and oxygen permeability. After data analysis, the experiments were performed to verify the response models at the optimum conditions using the same film-forming procedure.

3.3 Effect of Protein Concentration on the Properties of Edible Films from Water–Soluble Fish Proteins in Surimi Wash-Water

Freeze-dried water-soluble fish proteins were dissolved in distilled water at varying protein concentrations of 1.5, 3.0 and 4.5% w/v to prepare film-solutions. The optimum pH (selected from 3.2) was adjusted by 0.5 M sodium hydroxide prior to adding plasticizer (sorbitol), the ratio of protein: plasticizer was applied at 2:1. The film-

solutions were heated at optimum heating temperature on a hot plate with magnetic stirrer at optimum heating time obtained from 3.2.

3.4 Effect of Plasticizer Type and Concentration on the Properties of Edible Films from Water–Soluble Fish Proteins in Surimi Wash-Water

Freeze-dried water-soluble fish proteins were dissolved in distilled water at optimum protein concentration selected from 3.3 to prepare film-solutions. The optimum pH (selected from 3.2) was adjusted by 0.5 M sodium hydroxide prior to adding the different type of plasticizer (sorbitol, glycerol and polyethylene glycol) at various concentrations (25, 50 and 75%). The film-solutions were heated at optimum heating temperature on a hot plate with magnetic stirrer for the optimum heating time obtained from 3.2.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Recovery and Characterization of Proteins Precipitated from Surimi Wash-Water

4.1.1 Compositional Profile of Threadfin Bream Fish

The proximate composition of threadfin bream fish was found to be 84.84, 4.02 and 6.26 % dry basis of crude protein, crude fat, and ash, respectively and total volatile base nitrogen was 8.20-11.90 mg/ 100 g sample.

4.1.2 Characteristics of Surimi Wash-Water

Water-soluble fish proteins extracted from WS-I contained the highest level of proteins (1.23$\pm$0.08 mg/ml) followed by those from WS-II and WS-III with protein content of 0.64$\pm$0.06 and 0.54$\pm$0.05 mg/ml, respectively (Fig. 4.1), which was closed to amount of water-soluble proteins from surimi-wash water (0.85% and 1.50%) reported by Huang and Morrissey (1998). Water-soluble fish proteins in WS-I had molecular weights primarily ranging between 23.2 and 71.6 kDa and some with traces of less than 23.2 kDa (Fig. 4.2). Water-soluble fish proteins in WS-II and WS-III also had molecular weights between 23.2 and 71.6 kDa, as could be seen from some bands; however, the amounts were meager. Surimi wash-water from WS-I had the maximum water soluble-soluble fish proteins, hence surimi WS-I was selected for next study.

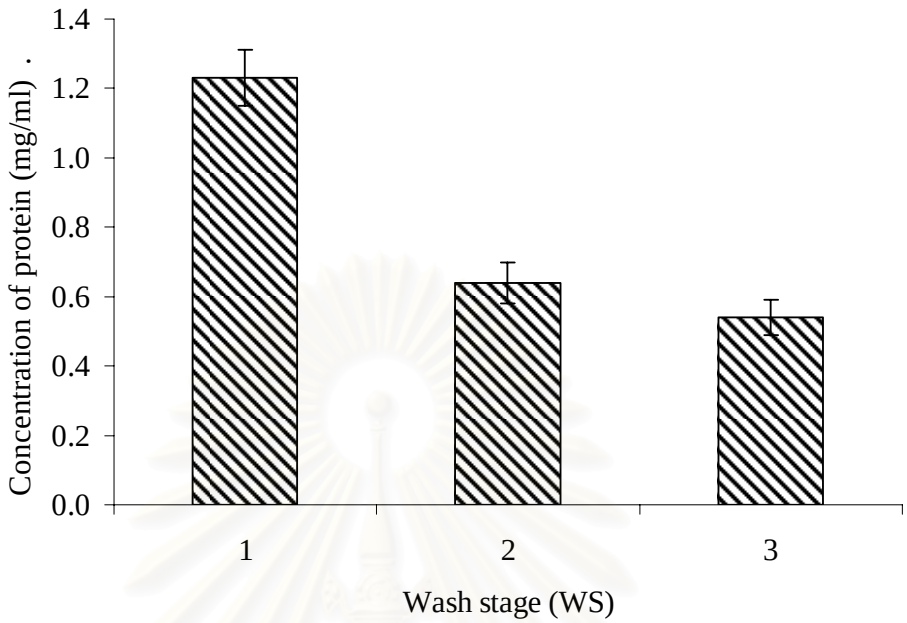


Fig. 4.1 Concentration of water-soluble fish proteins in surimi wash-water at different wash stages.

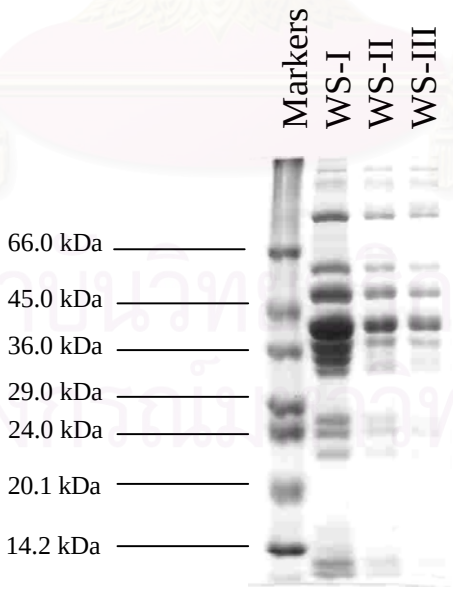


Fig. 4.2 SDS–PAGE patterns of water-soluble fish proteins from surimi wash-water at different wash stages.

4.1.3 Precipitation by Shifting the pH

The proteins precipitation from surimi-wash water at various levels of pH, temperature and time are shown in Figure 4.3. Increasing rate at proteins precipitation with each decreasing level of pH was observed, with maximum precipitation (66.30 %) attained at pH 3.5. The proteins precipitation was found to vary considerably with shifting the pH. This was because at pH 3.5 which is also isoelectric point (pI) of the protein, the negative and positive charges on the surface of molecule cancel one another out. The electrostatic repulsion between individual molecules no longer occurs, instead electrostatic attraction between molecules sets in, resulting in the formation of precipitation. Above and below the isoelectric point, the surface charge of protein is negative and positive, respectively, and therefore like-charged molecules take on a positive charge and tend to repel one another (Harris and Angal 1989) resulting in overall low precipitation. Percentage of precipitation of proteins was directly related with temperature. The proteins precipitated at 4 ^{o}C (pH 3.5) were in the range of 52.80-54.06 %. However, increased temperatures to 17 and 30 ^{o}C resulted in higher precipitation (63.56- 66.30 %). Nishioka and Shimizu (1983) reported similar results for the effect of temperature on the recovery of washing of minced fish meat, for obtaining the maximum recovery at 10 ^{o}C for 60 min was needed, but at 25 ^{o}C for 20 min was enough. Scope (1994) also showed that the temperature was important for protein denaturation; higher temperatures created more denaturation than lower temperatures resulted in higher proteins precipitation. Additionally, in extreme pH conditions the temperatures of around 0-10 ^{o}C rather than 50-60 ^{o}C may be more conductive to denature of proteins. Reaction time of pH shift had little or no significant effect ($p > 0.05$) on the percentage of precipitation.

Figure 4.4 shows SDS-PAGE patterns of supernatant water-soluble fish proteins from surimi wash-water subjected to protein precipitate at different levels of pH, temperature and time. The patterns in lane 2-4, which were obtained from the surimi wash-water, subjected to pH 3.0-4.0 indicate that most of the proteins were absent in the running gel. This confirms that the proteins precipitation increased with decreasing pH and because pH 3.5 was the isoelectric point, only faint bands were noticeable in the supernatant.

The protein solubility was considered one of the functional properties of proteins, hence the loss of solubility was taken as a criterion for protein denaturation (Wu and Inglett 1974). The solubility profiles of recovered proteins at various levels of pH are shown in Figure 4.5. A decrease in the solubility was observed of recovered proteins with decreasing pH. Extreme pH (pH 3.0-4.5) caused more denaturation because the sensitive areas of the protein molecule acquired more like charges, resulting in internal repulsion or perhaps loss of charges which were previously forces of attraction holding the protein together (Scope, 1994). Results confirm that the minimum solubility (pH 3.5) were in the range of 10.5-23.6 %, while, higher recovered proteins solubility was observed at pH value 5.0-6.0. Percentage of solubility of recovered proteins was directly related to temperature. The solubility of recovered proteins at 4 ^{o}C (pH 3.5) was in the range of 20.9-23.6 %. However, increased temperatures to 17 and 30 ^{o}C resulted in decreased solubility (10.5-21.5 %). This tendency seems to be due to the fact that lower pH and higher temperature leading to excessive denaturation and thus decrease in solubility of recovered proteins (Whitaker 1996). Reaction time for pH shift had little or no significant effect ($p > 0.05$) on solubility.

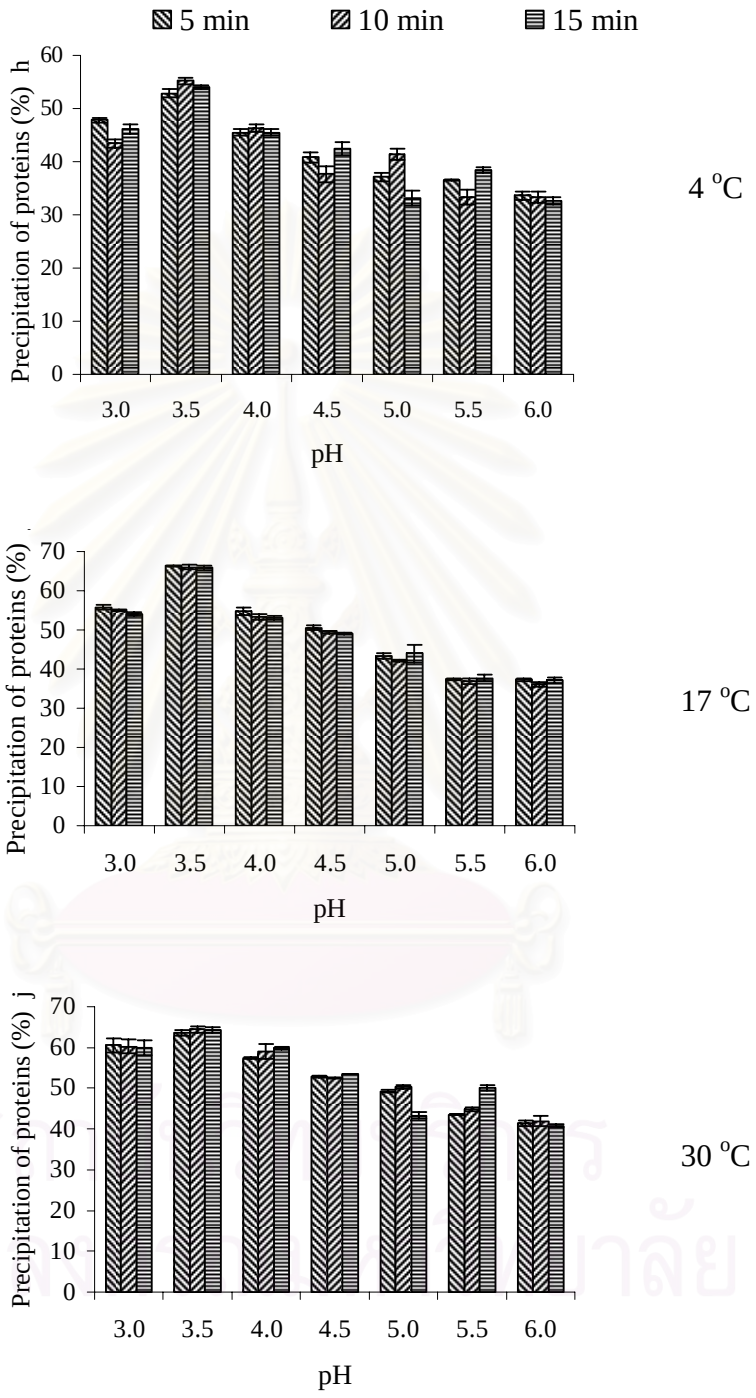


Fig. 4.3 Effect of pH, temperature and reaction time on precipitation of water-soluble fish proteins from surimi wash-water.

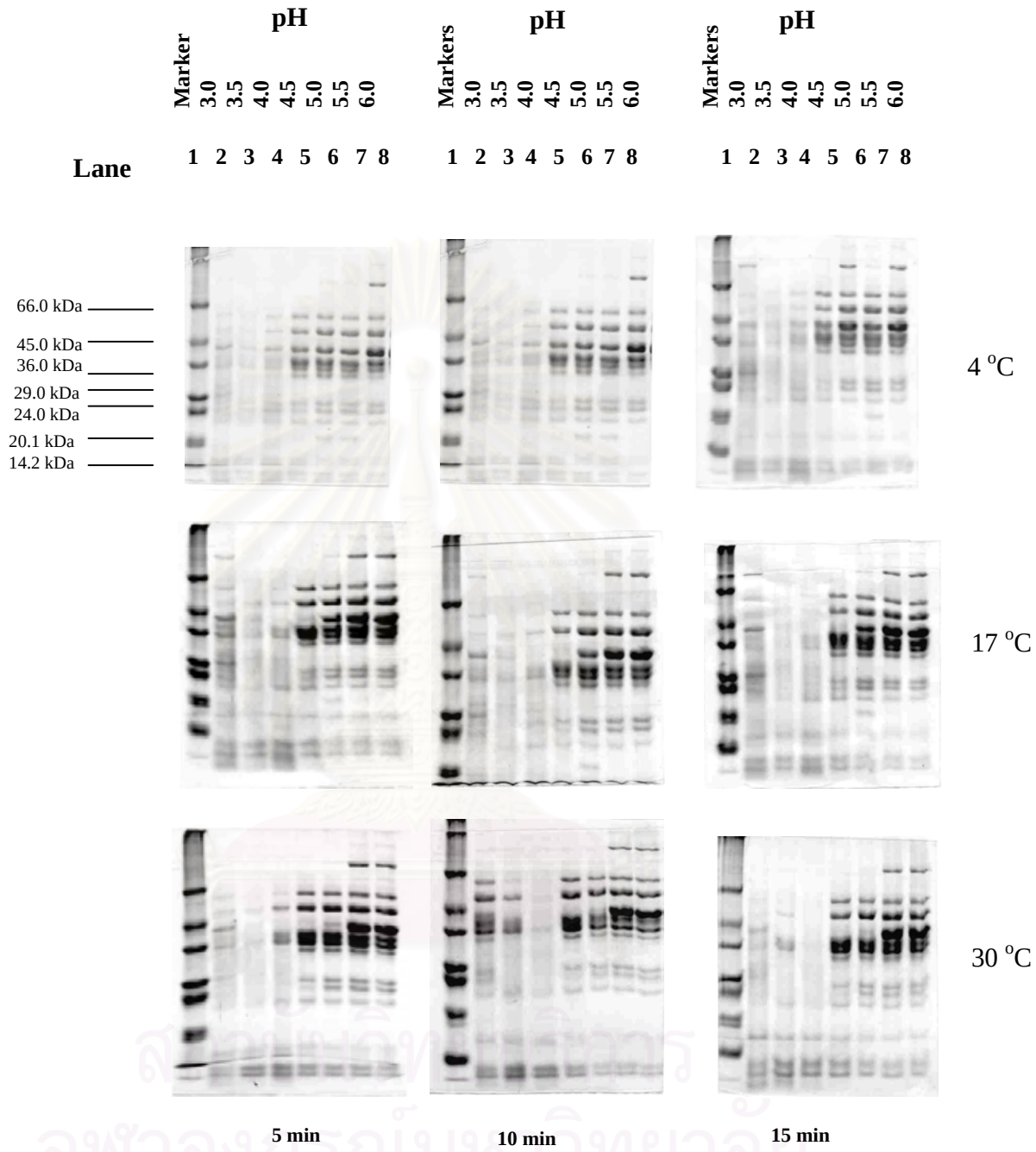


Fig. 4.4 Effect of pH, temperature and reaction time on SDS-PAGE patterns of supernatant of water-soluble fish proteins from surimi wash-water.

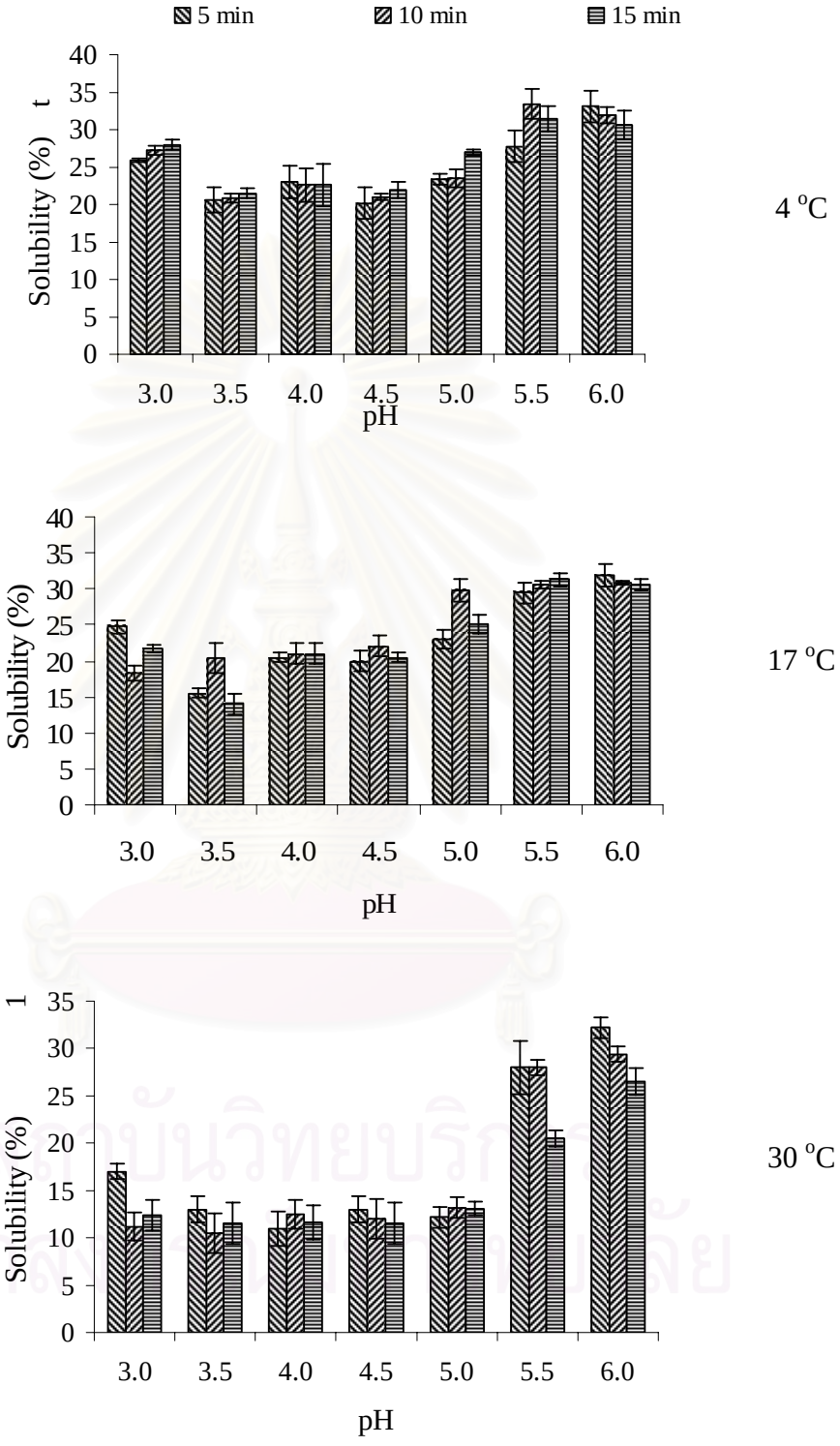


Fig. 4.5 Effect of pH, temperature and reaction time of precipitation on solubility of recovered proteins from surimi wash-water.

4.1.4 Precipitation by Ethanol

Figure 4.6 shows the effect of various levels of ethanol concentration on proteins precipitation at different exposure times. Addition of ethanol to surimi wash-water containing proteins has a variety of effects which lead to protein precipitation. The principle effect is the reduction in water activity. Increased proteins precipitation with increasing ethanol concentration was observed for the simple reason that the solvating power of water for a charged, hydrophilic protein molecule decreased as the concentration of organic solvent increased. This can be described in terms of the reduction of the dielectric constant of the solvent, or simply in terms of a bulk displacement of water, plus the partial immobilization of water molecules through hydration of the organic solvent (Scope, 1994). The maximum precipitation (64.98%) at 60% w/w of ethanol was observed. Reaction time (10, 15, 20 and 25 min) of ethanol on proteins precipitation had little or no significant effect ($p > 0.05$) on the percentage of precipitation, because most miscible organic solvents have lower densities than water, the sedimentation of aggregated protein can be very rapid (Scope, 1994)

The SDS-PAGE patterns of supernatant water-soluble proteins in surimi wash-water subjected to the different levels of ethanol concentration and time were compared (Fig. 4.7). Lanes 3-5 obtained from ethanol concentrations 10, 20 and 30 % w/w indicate that most of the proteins were similar to that of untreated surimi wash-water (lane 2). Most of the proteins were not present from the running gel in lanes 6-8 (40, 50 and 60 % w/w of ethanol concentration, respectively). This indicates that the proteins precipitation increased with increasing ethanol concentration whereby, at highest ethanol concentration (60 % w/w), only one band was noticeable in the supernatant.

Figure 4.8 shows the proteins solubility profiles of recovered proteins at various levels of ethanol concentration. When the concentration of ethanol was increased, the solubility of recovered proteins tended to decrease. Lower solubility of recovered proteins, in the range of 26.70-34.30 % occurred when 40-50 % of ethanol was used. A higher solubility of recovered proteins was observed at ethanol concentration of 10-30 % (24.40-41.80 %) because lower concentration of organic solvent created less denaturation than higher concentration (Harris and Angal 1989). Reaction time of ethanol had little or no significant effect ($p > 0.05$) on the percentage of solubility.

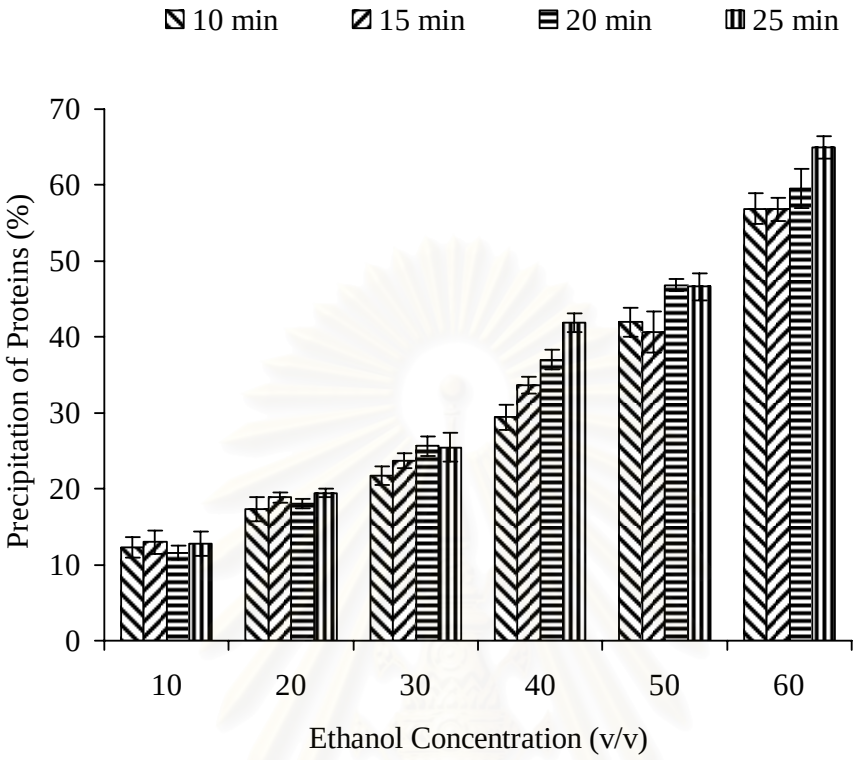


Fig. 4.6 Effect of ethanol concentration and reaction time on precipitation of water-soluble fish proteins from surimi wash-water.

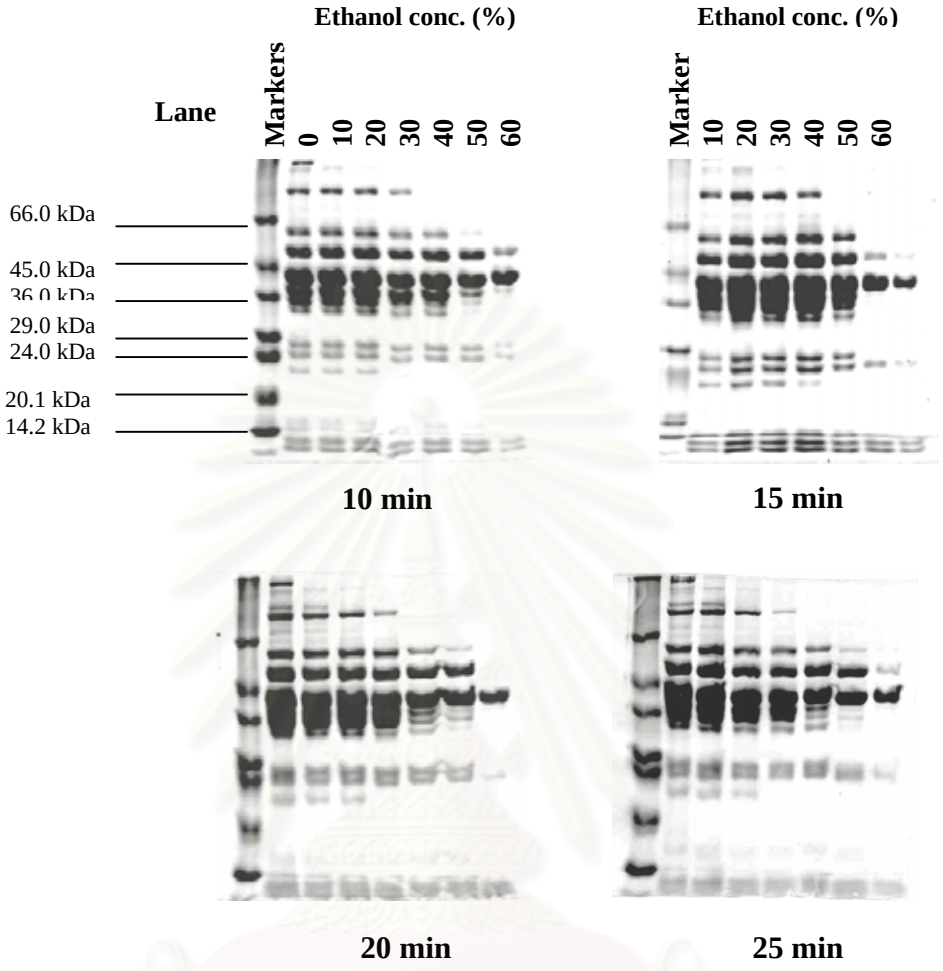


Fig. 4.7 Effect of ethanol concentration and reaction time on SDS– PAGE patterns of supernatant water-soluble protein from surimi wash-water.

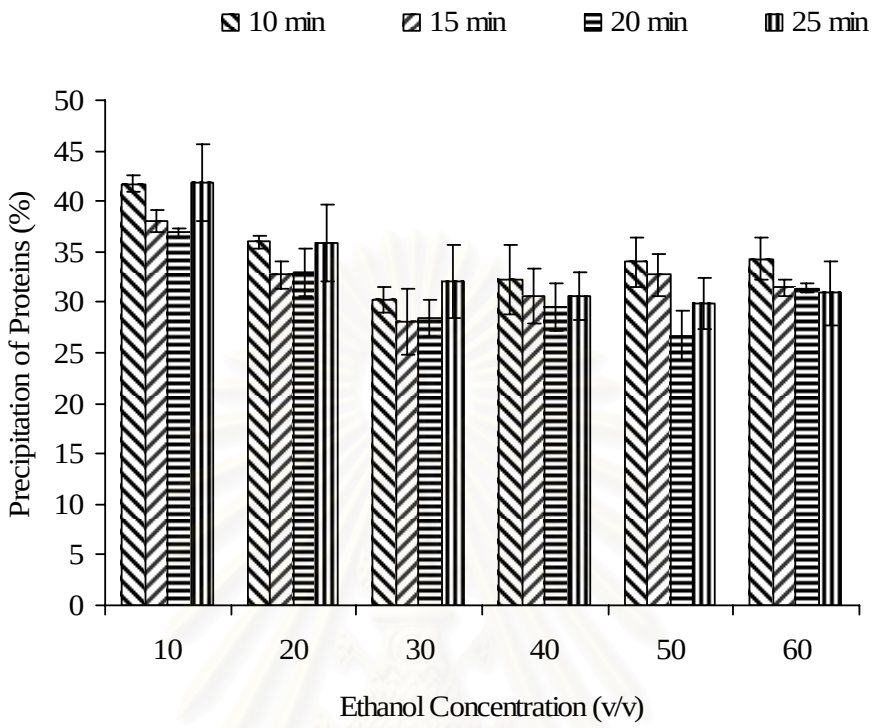


Fig. 4.8 Effect of ethanol concentration and reaction time on solubility of recovered proteins from surimi wash-water.

Water-soluble fish proteins were recovered from WS-I of surimi wash-water; which recovery by freeze-drying of surimi wash-water, shifting the pH and organic solvent, that was achieved by using 0.1 M sodium phosphate buffer at pH 3.5 and 17 ^{o}C for 5 min and that was achieved by using 60% ethanol at 4 ^{o}C for 25 min, respectively (optimum condition from 4.1). All recovered proteins from surimi wash-water were freeze-dried and stored in plastic bag at –20 ^{o}C until used. Freeze-dried water-soluble fish proteins were studied on the effects of some process parameters such as pH, heating temperature and heating time; additive variables like proteins concentration and plasticizer type and concentration on mechanical and barrier properties

of edible films from water-soluble fish proteins from different recovery methods. The results of these studies were presented in section 4.2, 4.3 and 4.4.

4.2 Effect of pH, Heating Temperature, Heating Time, Protein Concentration and Plasticizer on the Properties of Edible Films from Water–Soluble Fish Proteins from Surimi Wash-Water

4.2.1 Effect of pH, Heating Temperature and Heating Time on the Properties of Edible Films from Water–Soluble Fish Proteins from Surimi Wash-Water

4.2.1.1 Model Fitting

The RSREG procedure of Statistical Analysis System (SAS, 1996) was used to fit the second order polynomial equation (3.1) to the film properties data shown in Table 1 (Appendix A). The regression coefficients (β_{ki}) obtained thereof, were presented in Table 2 (Appendix A). The analysis of variance for the response variables (Table 3, Appendix A).) indicated that the model developed for the tensile strength (TS), elongation at break (E), oxygen permeability (OP), protein solubility (PS), color (L*, a*, b*, ΔE^*_{ab}, Hue angle, and Chroma), hydrophobicity (HQ), content of SS bond (SS) and available SH group (ASH) were adequate, and had no significant lack of fit. However, regarding water vapor permeability (WVP) and film solubility (FS), values showed highly significant lack of fit, suggesting that the chosen model did not represent the system appropriately (Thompson, 1982). In such a case it was desired that some kind of mathematical transformation be performed on the

dependent or independent variables, to obtain an acceptable model with non-significant lack of fit. Several such transformations of the experimental data were tried. The model obtained by the logarithmic transformation of water vapor permeability and film solubility data yielded the best results and are given below:

$$A = \ln(WVP) = 4.74+0.014X_1-0.06\ X_2+0.001\ X_3+0.235\ X_1\ X_1 +0.022X_1\ X_2+0.208\ X_2\ X_2-0.035\ X_1\ X_3-0.013\ X_2\ X_3 +0.115\ X_3\ X_3 \quad (4.1)$$

$$B = \ln(FS) = 4.047+0.073X_1-0.024\ X_2-0.014\ X_3+0.111\ X_1\ X_1 -0.003X_1\ X_2+0.084\ X_2\ X_2+0.012\ X_1\ X_3-0.031X_3\ X_3 \quad (4.2)$$

Where Y = response variable; X_1, X_2 and X_3 = independent variables (pH, heating temperature and heating time, respectively)

Equation 4.1 and 4.2 were the most appropriate for calculating water vapor permeability and film solubility, giving a statistically non-significant lack of fit and explaining 88.08 and 83.22% of the variability, respectively. Further statistical analysis (Table 4, Appendix A) was then performed. Results revealed that pH, heating temperature and heating time had a significant ($p \leq 0.05$) overall effect on all responses. The pH and heating temperature of film solutions significantly ($p \leq 0.05$) affected tensile strength, water vapor permeability and a* value, while highly significant effects ($p \leq 0.01$) were observed on elongation at break and oxygen permeability. Meanwhile, heating time affected only elongation at break, oxygen permeability. Films solubility, proteins solubility, hydrophobicity and available SH

groups were most affected by pH. However, pH, heating temperature and heating time did not showed significant ($p > 0.05$) effect on L* value and content of SS bond.

4.2.1.2 Tensile Strength and Elongation at Break

An edible film must withstand the normal stress encountered during its application, subsequent shipping and handling of the food, to maintain its integrity and also barrier properties. High tensile strength is generally required but deformation values must be adjusted according to the intended application of the films, whether it should be undeformable material to provide structural integrity to reinforce structure of the food (Gontard *et al*., 1992). Tensile strength is the maximum tensile stress sustained by the sample during tension test. If maximum tensile stress occurs at either the yield point or the breaking point, it is designated tensile strength at yield or at break, respectively (ASTM, 1991). Elongation at break is an indication of films flexibility and stretchability (extensibility), which is determined at point when the film breaks under tensile testing and is expressed as the percentage of change of the original length of the specimen between the grips of a film to stretch (extend). The main factors that influenced the film's properties were pH and heating temperature of film-solutions, while heating time had the lowest effect (Table 4, Appendix A).

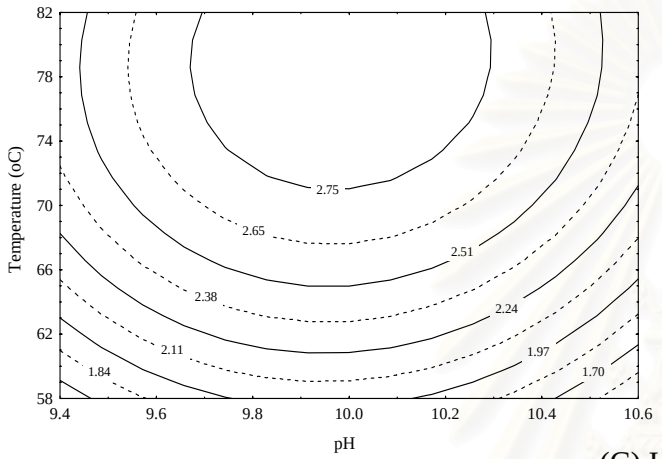
Contour plots of tensile strength and elongation at break as affected by pH and heating temperature were given in Figure 4.9 and 4.10. Depending upon the film conditions, tensile strength and elongation at break showed a high variation between 1.70-3.02 MPa and 8.50-14.72 %, respectively (Fig. 4.9). Comparing within the same heating temperature of film-solutions, the results demonstrated that, tensile strength increased as pH of film-solutions increased. This result implied that higher pH of film

solutions induced formation of resistant films. Banker (1966) reported that pH played an important role in protein films made from water-soluble materials. At alkaline pH away from the isoelectric point of 3.5 (Bourtoom *et al*., 2002), denaturation of proteins was promoted and resulted in unfolding and solubilizing of the proteins. During solubilization, the cohesive forces between the protein macromolecules were neutralized by complexing with the solvent molecules (Banker, 1966). In general, functions of polymers were related to solution properties which further influenced the film characteristics. The same charged groups repelled each other and produced a stretching of the polymer chain when functional groups on a linear polymer became ionize during dissolution. This phenomenon, facilitated molecule orientation and fine-stranded network (Banker, 1966). The resulting interaction between polymers may have been responsible for this result. Anker *et al*. (2000) reported that, when the pH of the film-solutions from ß-lactoglobulin was increased above 8, SH/S-S interchange reactions or thiol/thiol (SH/SH) oxidations could occur upon heating and intermolecular disulfide (S-S) bonds formed. The highest tensile strength value was obtained at pH about 10.0 (Fig. 4.9). However, increasing pH of film-solutions higher than 10.0 resulted in decrease of tensile strength, by the reason that strongly repulsive force occurred between negative (extreme pH) charges along the protein chains could have decreased the occurrence of molecular associations within the protein matrix (Rhim *et al*., 2002). Gennadios *et al*. (1993a) studied the effect of pH on soy protein isolate film and found that highly alkaline condition (pH > 12) inhibited soy protein isolate film formations. The weakest film was obtained at the lowest pH of the film-solution. The very low tensile strength (1.70 MPa) was observed at pH 9.5, most likely due to less protein-protein interaction.

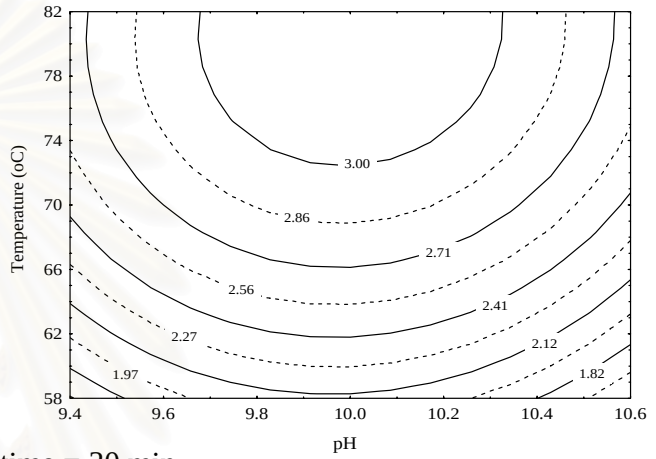
The tensile strength was enhanced as heating temperature of film-solutions increased from 60-80 ^{o}C. This result demonstrated that, tensile strength increased from 1.70 MPa to almost 3.0 MPa when heating temperatures of film solutions increased from 60 to 80 ^{o}C. This might be due to the fact that higher heating temperature induced protein denaturation and resulted in increase in the number and/or a better localization of bonds between protein chains. The weakest film was obtained at the lowest heating temperature and a very low tensile strength (1.70 MPa) was observed at heating temperature of film solutions around 60 ^{o}C. The contour plots (Fig. 4.9) indicated an interaction between the effect of pH and heating temperature on tensile strengths of the resulting films. It was observed that, the lowest tensile strength could be expected with low pH and relatively low heating temperature of film solutions. According to the contour plots, the experimental condition involving higher of both pH (10.0) and heating temperature resulted in higher film formations and high tensile strength of the formed films. Heating time seemed to have less effect on tensile strength of the film.

The elongation at break value was also affected by pH and heating temperature of film-solutions. All linear, quadratic and interaction terms for pH, heating temperature and heating time were significant (Table 3, Appendix A). The contour plots of elongation at break (Fig. 4.10) showed a high variation between 8.50 and 14.72 % and showed the highest elongation at break when lower pH and higher heating temperature of film-solutions were employed. An increase in elongation at break of heat-induced edible films was suggested to be due to an increased number of intermolecular disulfide (SS bond) bonds (Shimada and Cheftel, 1988). Prolonged heating time, however, resulted in increase in elongation at break.

(A) Heating time = 10 min



(B) Heating time = 20 min



(C) Heating time = 30 min

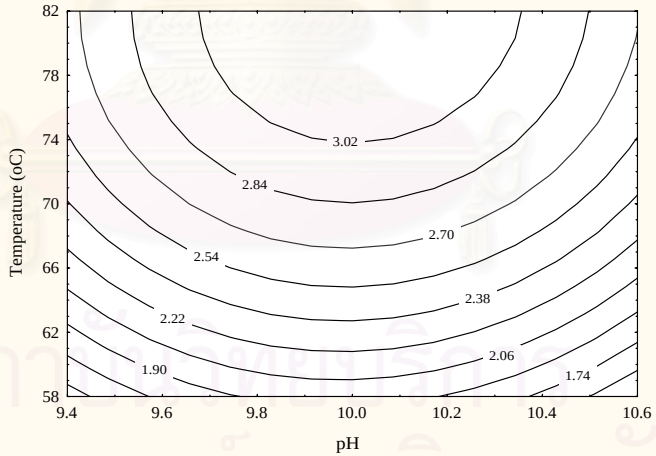


Fig. 4.9 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent tensile strength (KPa) of films at given heating.

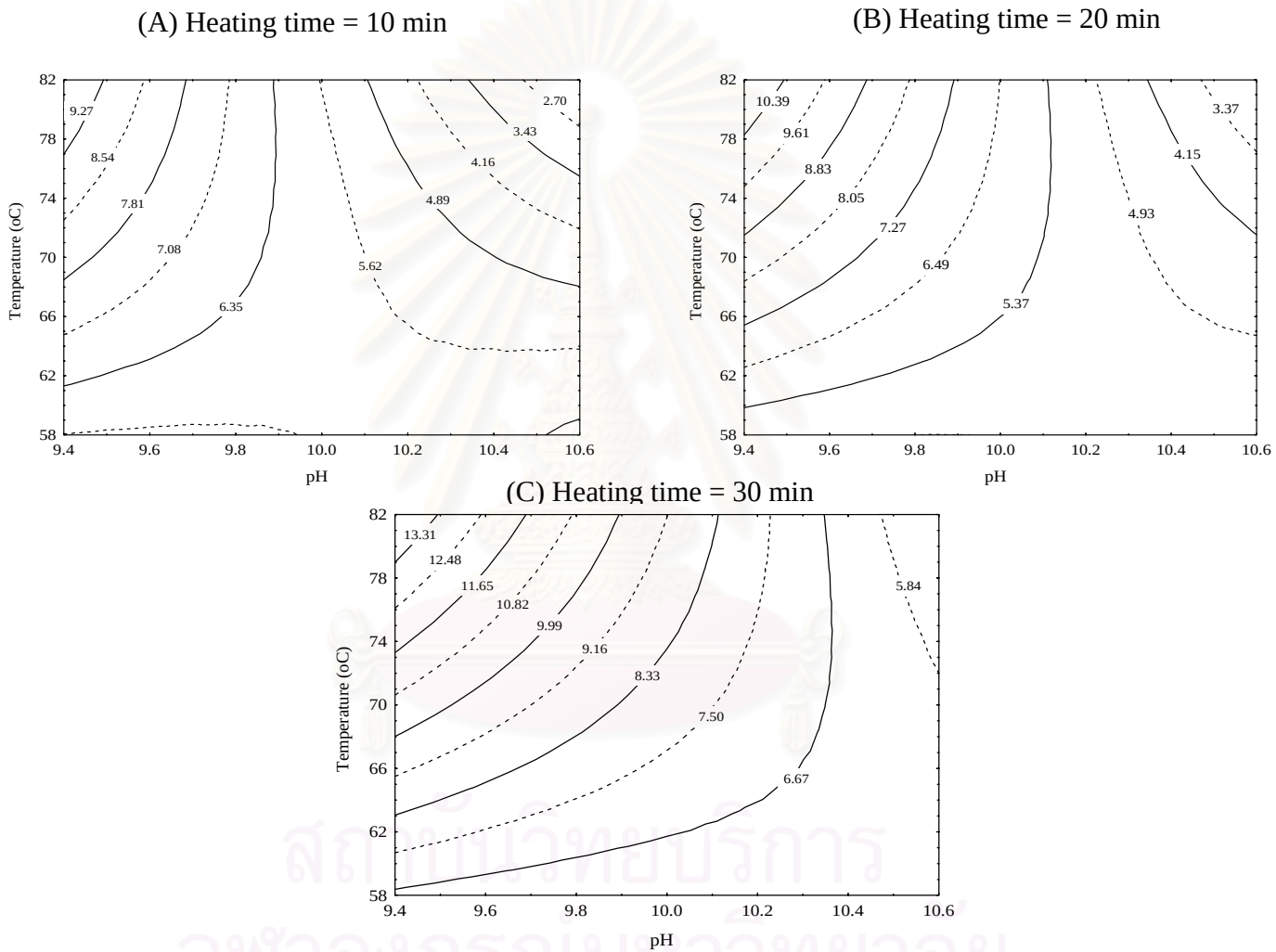


Fig. 4.10 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent elongation at break (%) of films at given heating time.

4.2.1.3 Water Vapor Permeability

Constant water vapor permeabilities as affected by the changing of pH, heating temperature and time suggested an independence of the water vapor pressure gradient applied across the films. However for hydrophilic (edible or nonedible) materials, such as protein films, the derivation from this ideal behavior might be due to interactions of permeating water molecules with polar groups in the films structure (Hagenmaier and Shaw, 1990). Deviation from the ideal behavior could also be induced by temperature effects on materials (Myers *et al*., 1962). Since the main function of edible films or coatings are often to impede moisture transfer between food and the surrounding atmosphere, or between two components of a heterogeneous food products, water vapor permeability should therefore be as low as possible. The main factor influencing water vapor permeability of water-soluble fish protein films were pH and heating temperature (Table 4, Appendix A). The contour plots (Fig 4.11) were characteristics of the effects of these variables and showed that the water vapor permeability value was the highest at pH around 9.5 (117.92-138.38 g.mm/m^2.d.kPa) and tended to decline when pH of film-solutions reached to 10.0 (58.55-65.96 g.mm/m^2.d.kPa). However, the water vapor permeability increased again when the pH was adjusted to 10.5 (117.92-129.28 g.mm/m^2.d.kPa). These results could arise from the fact that, at higher pH protein can denature, unfolds and solubilize. This phenomenon facilitated favorable molecule orientation and formation of intermolecular disulfide bond by thiol-disulfide interchange and thiol oxidation reactions. The function of disulfide bonds on protein coagulation during drying of soymilk was studied by Fukushima and Van Buren (1970). Thiol-disulfide interchanged via thiol oxidation also implicated in whey protein gelation (Donovan and Mulvihill, 1970; Shimada and Cheftel, 1988).

Extreme pH (pH > 10.0) of film-solutions as in this study might inhibit the water-soluble fish protein film formation. Most likely, strong repulsive forces between highly negative charges prevented protein molecules form associating and forming the films. The highest water vapor permeability was observed at the lowest and the highest pH of this study. The water vapor permeability of edible films was affected by heating temperature of the film-solution as well. Basically, proteins must be denatured in order to form a more extended structures that are required for film formations. Once extended, protein chains can associate through hydrogen, ionic, hydrophobic and covalent bondings. The chain-to-chain interaction that produces cohesive films is affected by the degree of chain extension and the nature and sequence of amino acid residues. Uniform distribution of polar, hydrophobic, and/or thiol groups along the polymer chain increased the likelihood of the respective interactions. (Kester and Fennema, 1986). The result of this experiment showed that increasing of heating temperature of film solutions (60-70 ^{o}C) resulted in lower water vapor permeability (Fig 4.11). The thermal energy might promote a greater cross-link between protein-protein chains resulting in a tight and compact protein network and structure. Shimada and Matsushita (1980) reported that the first step of ovalbumin aggregation involved the formation of SS bonds and the exposure of hydrophobic groups, and that, during further heating, ovalbumin was then polymerized and the intermolecular sulfhydryl/disulfide (SH/SS) exchanged to form a higher protein net work structure. However, extremely heating temperature (> 70 ^{o}C) of film-solutions provided an increase in water vapor permeability, most likely due to increase in protein denaturation and the protein precipitation that obstructed the film formation. The highest water vapor permeability of edible films was found at lowest and highest heating

temperature of film-solutions. The effect of heating time of film-solutions on water vapor permeability of edible film showed similar trend with that of the heating temperature.

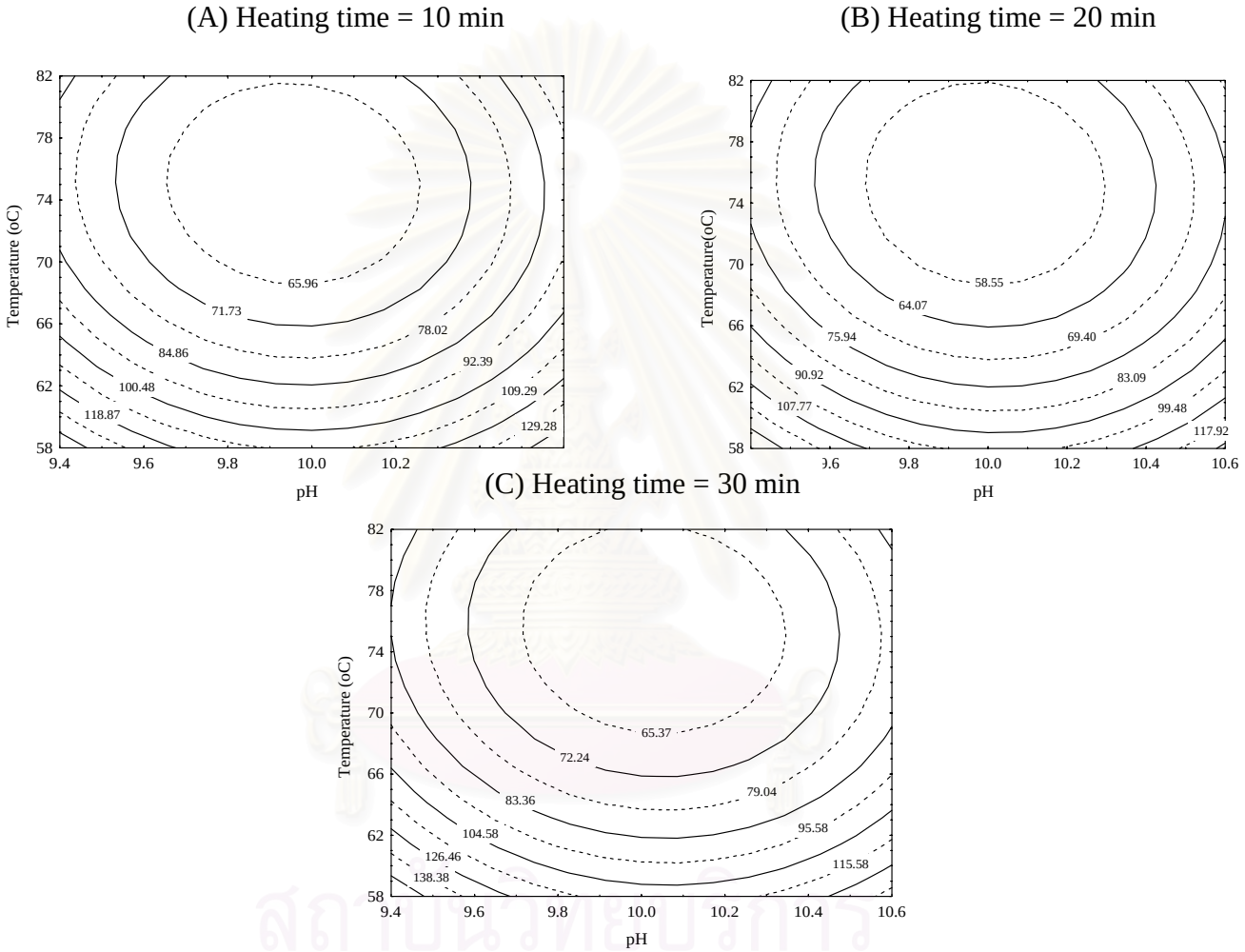


Fig. 4.11 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent water vapor permeability (g.mm/m^2.d.kPa) of film at given heating time.

4.2.1.4 Oxygen Permeability

Within the same heating time, it was observed that at lower pH (9.5) and heating temperature (60 ^{o}C) the oxygen permeabilities of the film were high (1153.94-1640.25 $cm^3.\mu m/m^2.d.kPa$). As heating temperature and pH increased, the oxygen permeability decreased and reached the minimum values (351.33-624.18 $cm^3.\mu m/m^2.d.kPa$) at pH around 10 and heating temperature at around 70-74 ^{o}C (Fig. 4.12).

Further increasing of the pH and heating temperature provided the films with higher oxygen permeability. The initial decrease of oxygen permeability as a function of pH and heating temperature might be due to the formation of the protein-protein interactions at pH of around 10 and the occurrence greater of cross linkings among the protein molecules as a result of the increase thermal energy. However as the pH and heating temperature were adjusted to higher than 10 and more than 70-74 ^{o}C, respectively the intensity of the protein denaturation was increased to the critical level that the protein films could not properly formed and the higher oxygen permeability resulted.

Oxygen permeability was also affected by heating time, the lowest oxygen permeability was obtained at 20 min of heating time. At lower and higher time of heating, the extent of the thermal energy might be too low and too high for the appropriate intensity of the protein denaturation and the compact protein net work and structure. Thus to maintain relatively low oxygen permeability, the pH around 10.0, heating temperature about 70 ^{o}C and 20 min heating time were required. As discussed previously, the higher pH provided protein denatures, unfolds, solubilizes and facilitating

favorable molecule orientation, while extreme pH (pH >10.0) inhibited edible film formations. Most likely strong repulsive forces between highly negative charges prevented protein molecules form associating and forming films. Moreover, at highest heating temperature (80 ^{o}C), oxygen permeability was increased, most likely due to the fact that increase in proteins denaturation could induced proteins precipitation and improper formation of films.

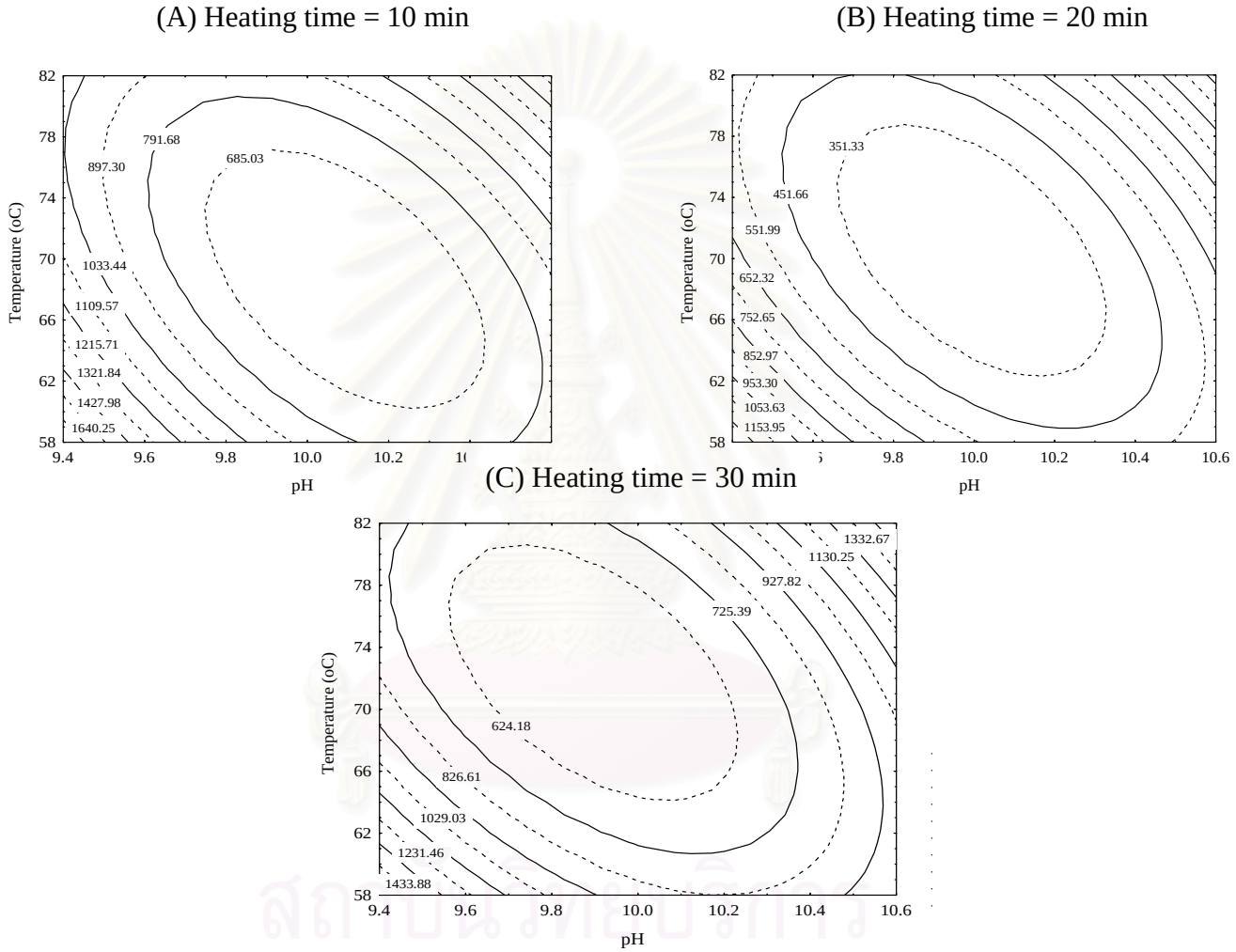


Fig. 4.12 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent oxygen permeability ($cm^3.\mu m/m^2.d.kPa$) of film at given heating time.

4.2.1.5 Film and Protein Solubilities

Water resistance is an important property of edible films for applications as food protection where water activity is high, or when the film must be in contact with water during processing of the coated food e.g. to avoid exudation of fresh or frozen products (Gontard *et al.* 1992). Generally, higher solubility indicates lower water resistance. However, a high solubility may have an advantage for some applications. High solubility is advantageous in case that the films are to be consumed with a product and may also be important factor that determines biodegradability of films when used as packaging wrap. Water-soluble fish protein films maintain their integrity (i.e., did not dissolve or break apart) even after 24 hr of incubation at 25 ^{o}C with gentle motion. This indicated that the protein polymer network remained intact and those only monomers, small peptides and non-protein material might be soluble (Stuchell and Krochta, 1994).

Only pH of film-solutions significantly ($p \leq 0.05$) affected the film and protein solubilities. The values of film and protein solubilities significantly ($p \leq 0.05$) increased when pH of film-solutions increased (Fig.4.13 and 4.14) and the higher solubility were observed when pH of the film-solution was higher than 10.0. Increase of soluble matters might due to increase of protein solubility. Higher pH of film-solutions (pH >10.0) facilitated dispersion of the protein in water and loosening of the film structure, causing dissolution of the non-protein materials (Gnanasambandam *et al*., 1997). It was observed that both film and protein solubilities were the lowest at pH around 10.0, most likely due to better films formation as mentioned before.

The contour plots of the effect of heating temperature and time of film-solutions on films solubility and proteins solubility are shown in Figure 4.13

and 4.14. Increasing of heating temperature and time affected less to film and protein solubilities comparing to the pH. Roy *et al* (1999) reported that wheat gluten film solubility and protein solubility decreased ($p \leq 0.05$) as heating temperature of film-solutions increased. This was attributed to more pronounced heat-induced protein denaturation at higher temperatures. Heat induced protein denaturation (unfolds), resulted in exposing previously "buried" groups such as hydrophobic and sulfhydryl (SH) which producing a strong films resulted in lower both films solubility and proteins solubility (Fukushima and Van Buren, 1970; Farnum *et al*., 1976; Schofield *et al*., 1983 and Mine *et al*., 1990). However, the effect of the pH changes as designed in this experiment might contribute more to these two properties, when studied concomitantly with those of the heating temperature and time.

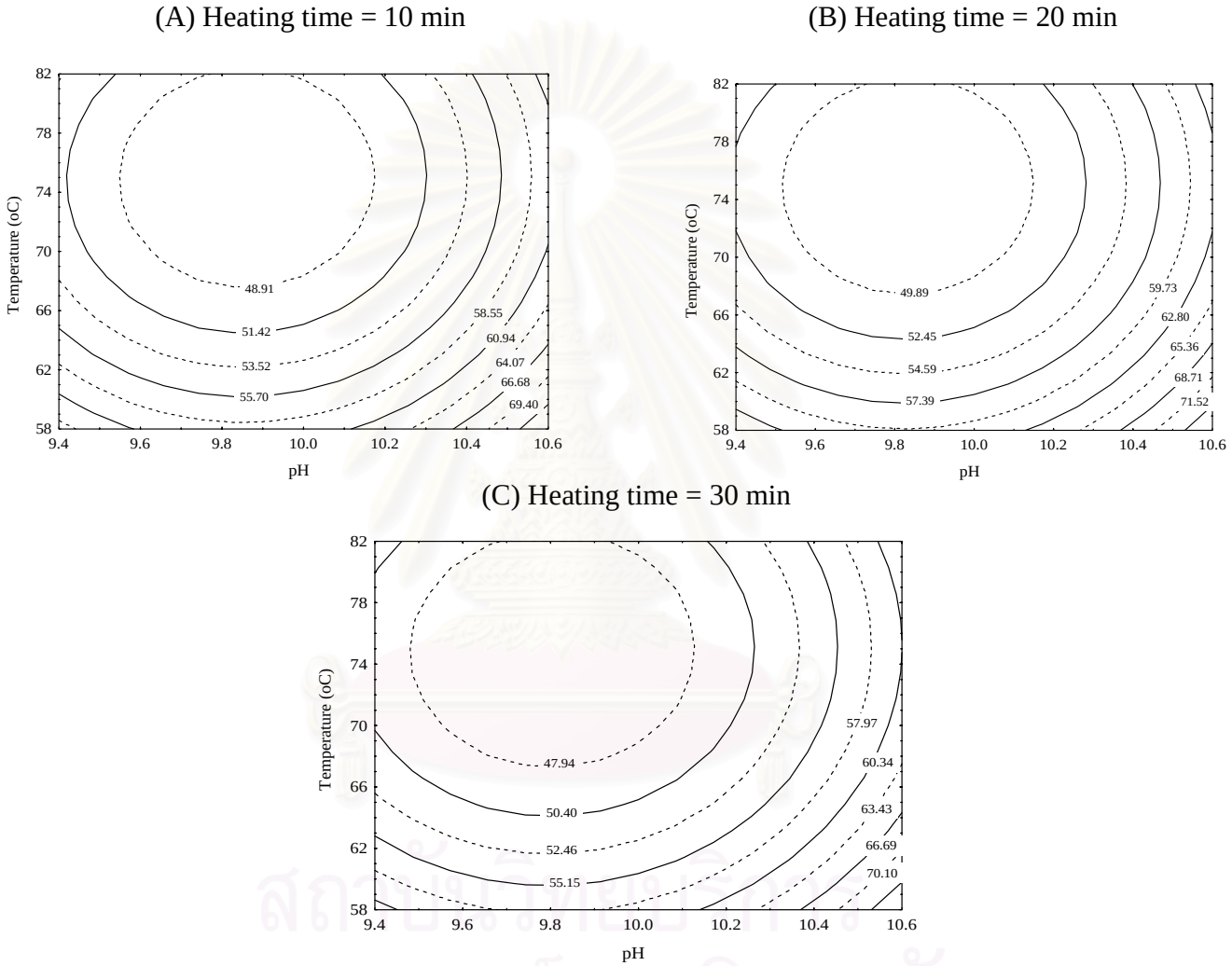


Fig. 4.13 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent film solubility (%) of film at given heating time.

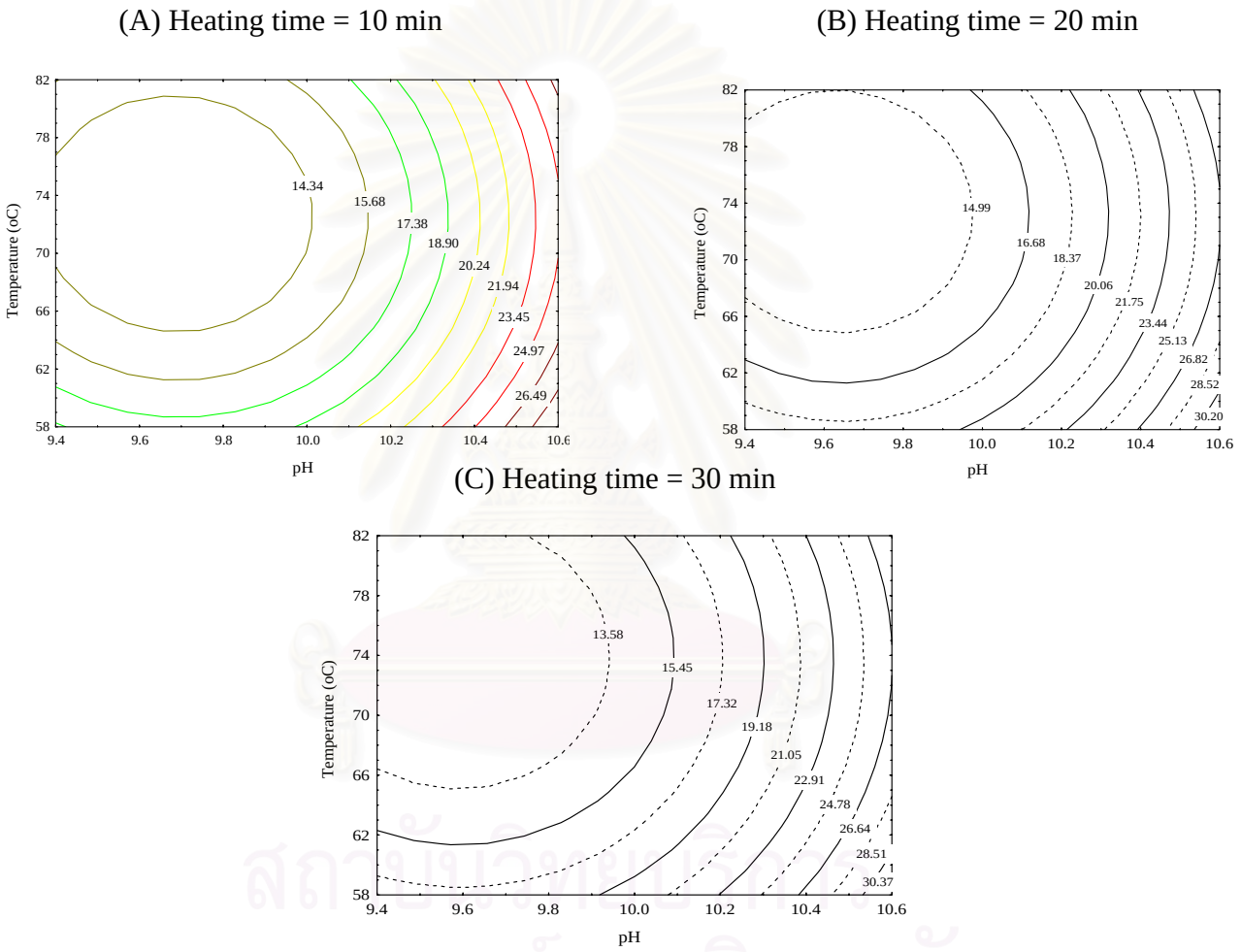


Fig. 4.14 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent protein solubility (%) of film at given heating time.

4.2.1.6 Film Color

The results of the color measurements performed on the films were expressed in accordance with the CIELAB system, and the rectangular coordinates (L*, a* and b*) were defined. The film colors were most affected by pH of film solutions, while heating temperature and heating time affected less (Table 4, Appendix A). Films formed at lower pH and heating temperature were lighter yellow than those formed at high pH and heating temperature. Instrumental color parameters L* was not significantly affected by heating temperature and heating time of the film solutions (Fig. 4.15 and 4.16), however, value b* dramatically increased with increasing in the pH of film-solutions (Fig. 4.17), and the films appeared more yellowish. At alkali pH, proteins were able to form complex substances with polyphenolic compounds. Such complexes might have contributed to discoloration of films prepared at higher pH (Gnanasambandam *et al.*, 1997). The value a* increased as pH of film solutions increased, which reversed with change in heating temperature (Fig. 4.16). As a result, the films became reddish yellow at higher pH.

The main factor influencing ΔE^*_{ab} of edible films were pH, heating temperature and heating time of film solutions, while hue angle and chroma values, only pH of the film-solutions were the most important factor (Table 4.4, Appendix A). According to the model, ΔE^*_{ab} was plotted against pH and heating temperature of film solutions at each heating times (Fig. 4.18), as can be seen, the pH and heating temperature of film solutions were greater affected this variate than heating time (Fig 4.18-4.20).

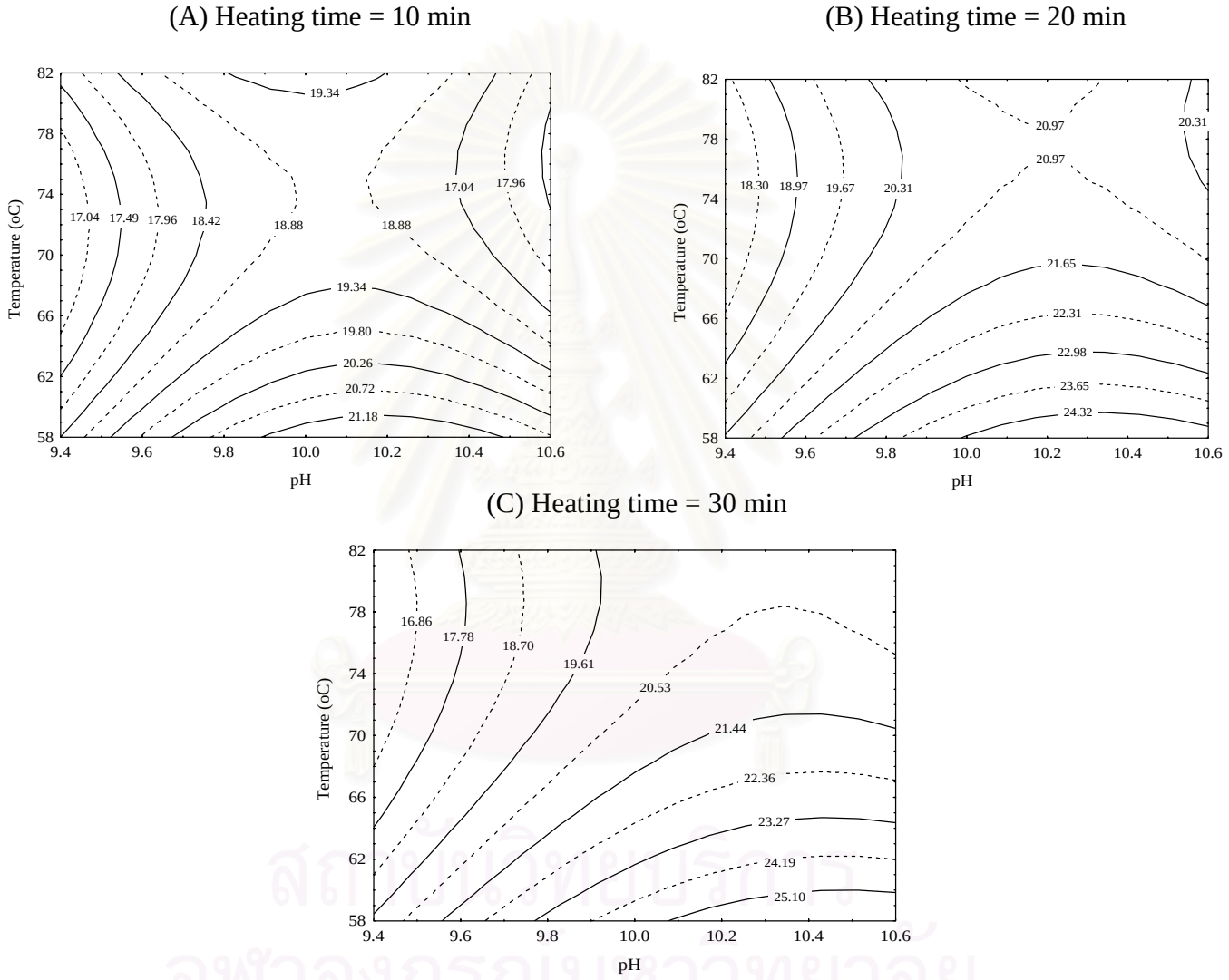


Fig. 4.15 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent L* value of film at given heating time.

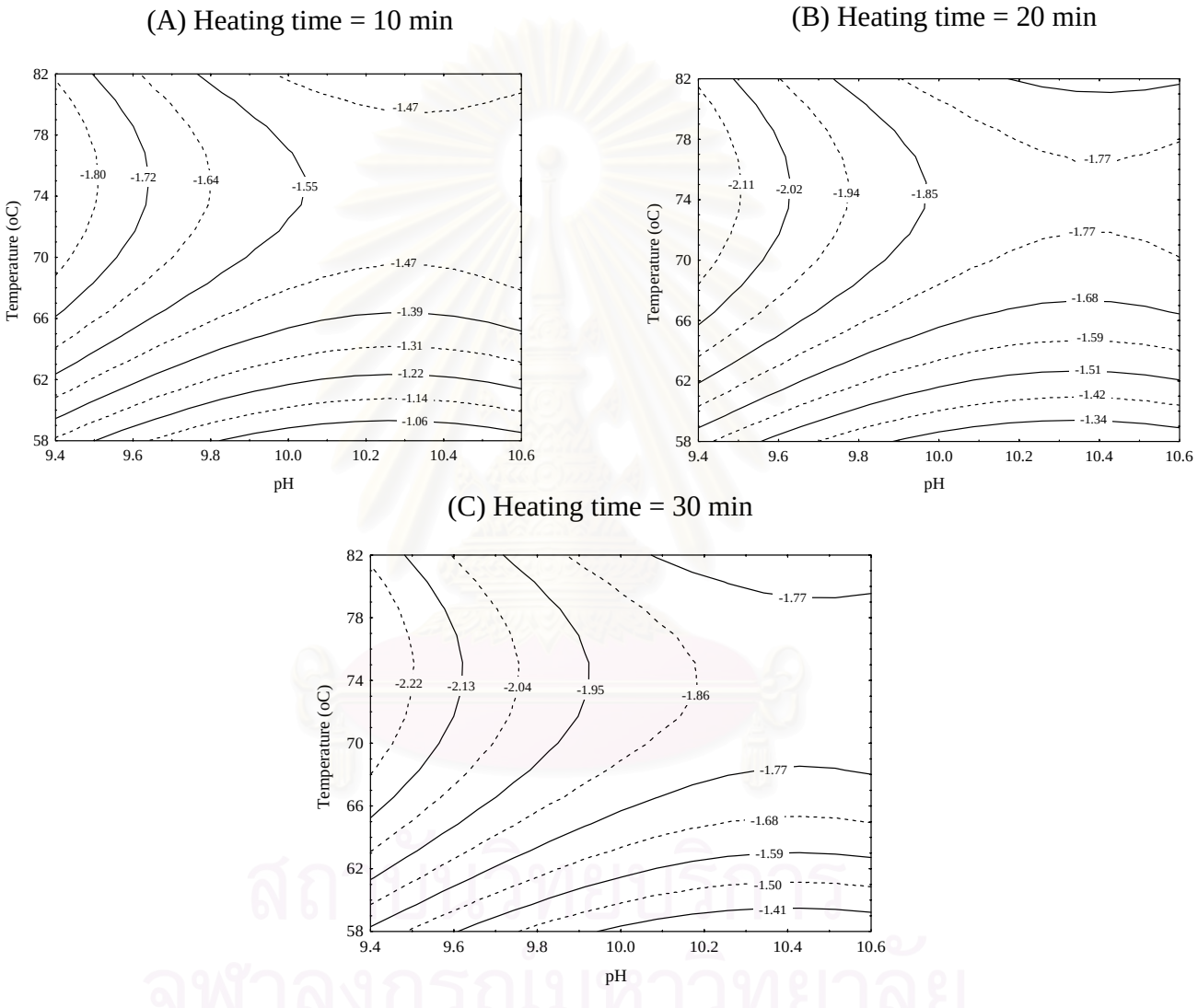


Fig. 4.16 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent a* value of film at given heating time.

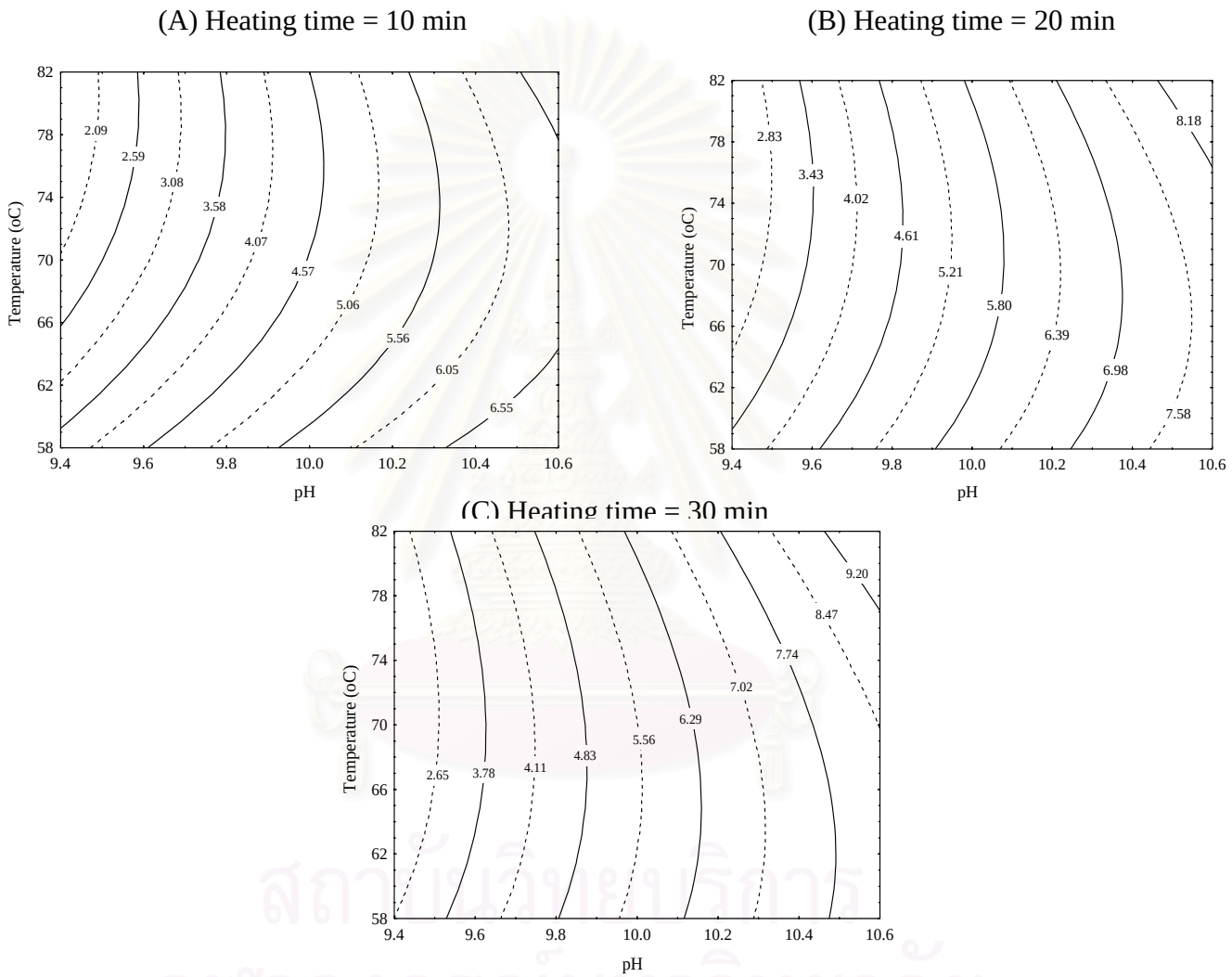
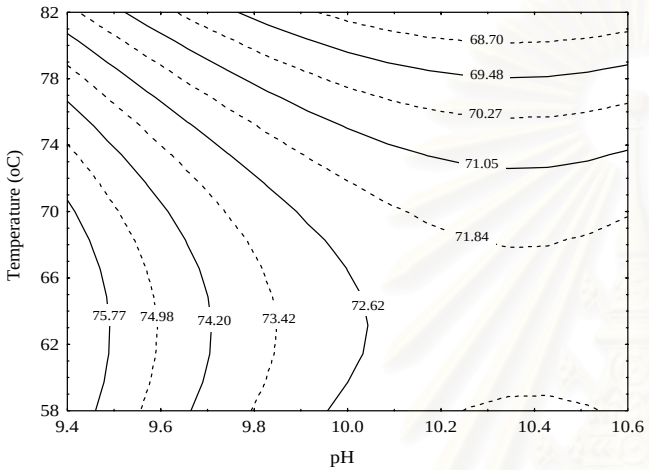
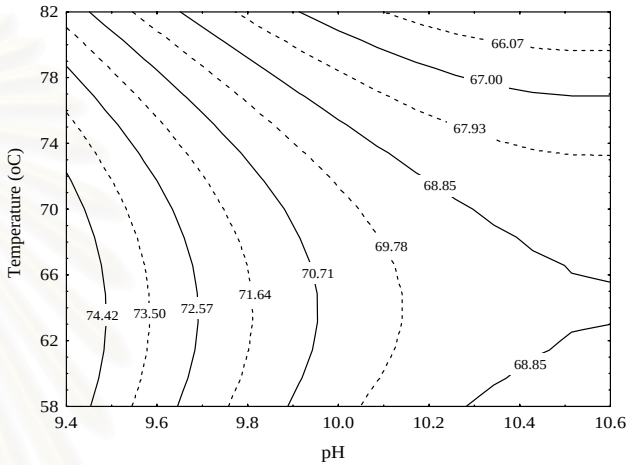


Fig. 4.17 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent b* value of film at given heating time.

(A) Heating time = 10 min



(B) Heating time = 20 min



(C) Heating time = 30 min

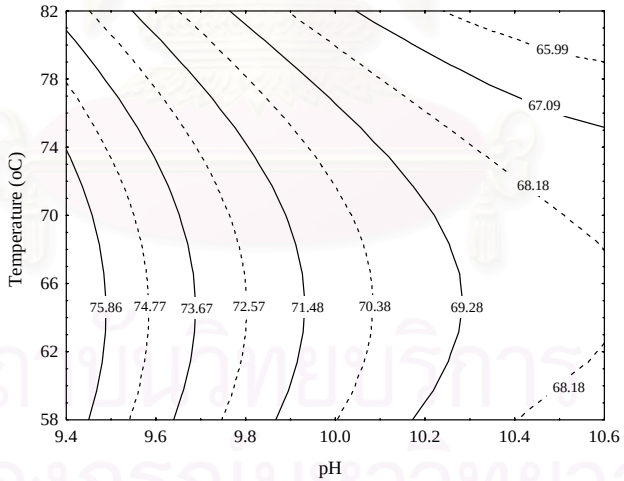


Fig. 4.18 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent ΔE^*_{ab} value of film at given heating time.

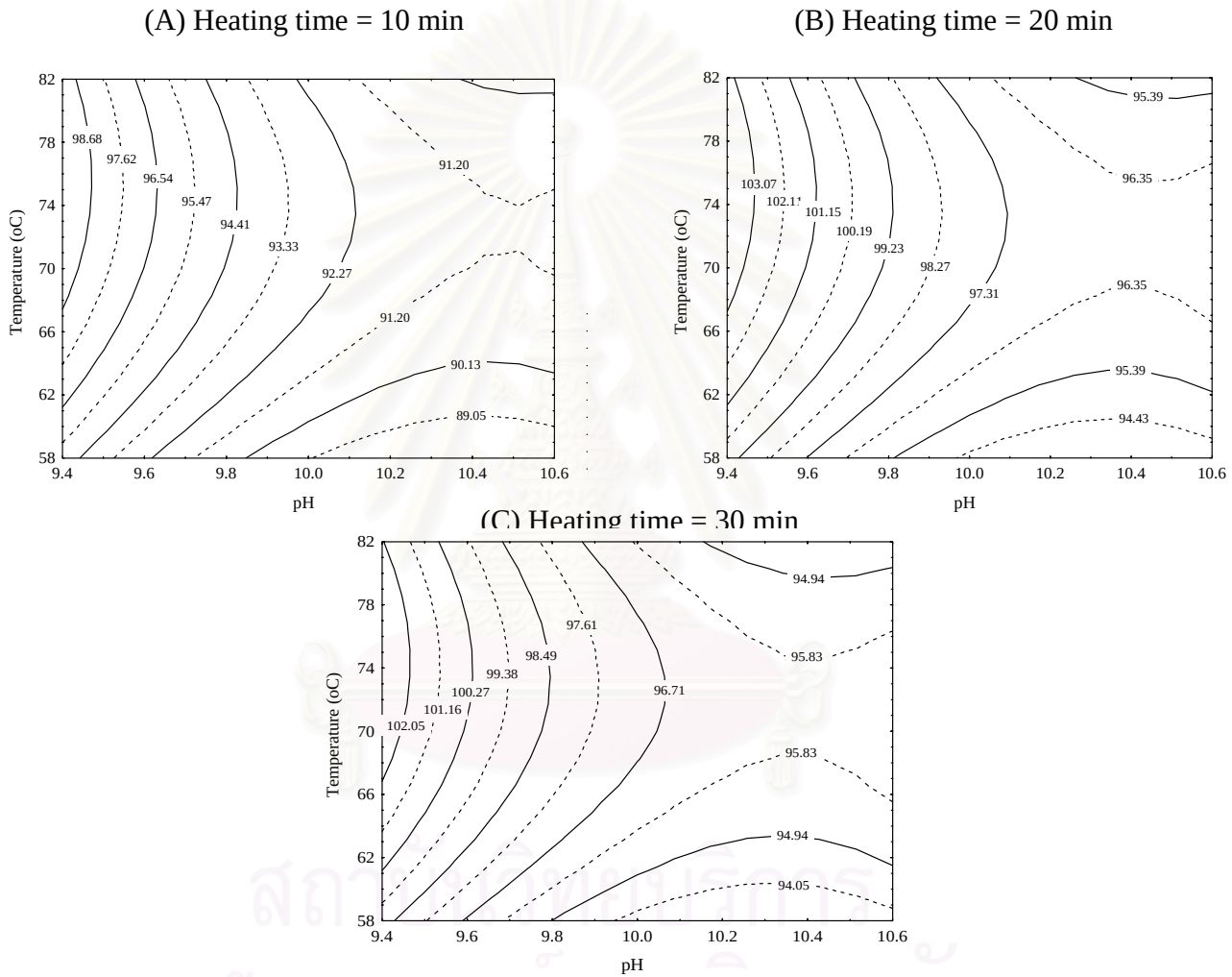


Fig. 4.19 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent hue angle value of film at given heating time.

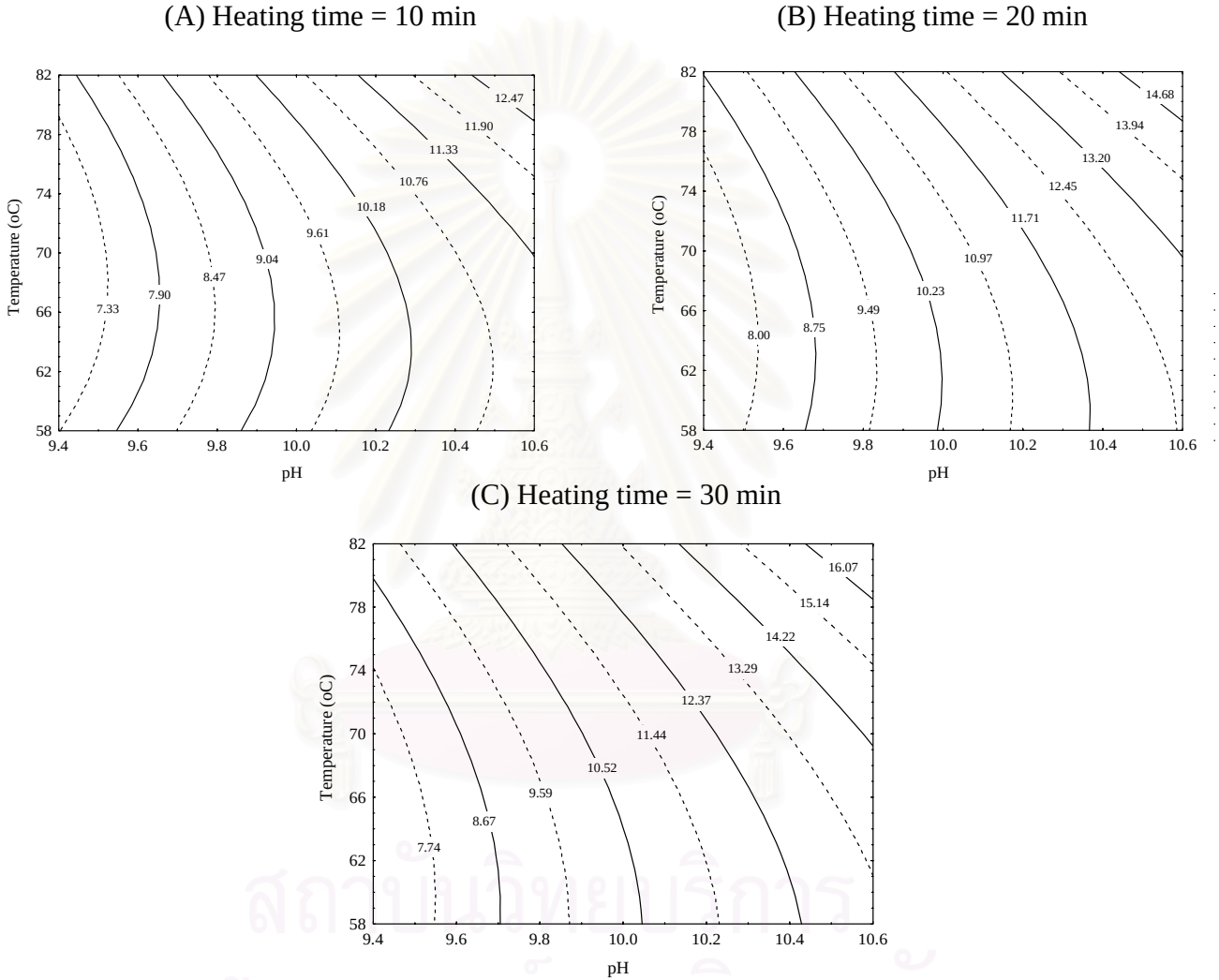


Fig. 4.20 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent chroma value of film at given heating time.

4.2.1.7 Surface Hydrophobicity, Available SH Group and Content of SS Bond

Surface hydrophobicity of protein changed in proportion to conformation change (Kato *et al.*, 1981). The main factors influencing surface hydrophobicity, available SH group and content of SS bond were pH followed by heating temperature and heating time (Table 4, Appendix A). According to the result of this experiment, pH was the most important factor determining the surface hydrophobicity, available SH group and content of SS bond.

Figure 4.21 demonstrated the contour plots of surface hydrophobicity of film-solutions as affected by pH, heating temperature and heating time. It was observed that the surface hydrophobicity increased markedly with increasing pH of film-solutions from 9.4 to 10.3-10.4 and thereafter decreased again. Increase of the surface hydrophobicity could be due to the increase of unfolded protein molecules resulting in increase exposure of hydrophobic groups (Iwata *et al.*, 2000). Heating temperature and time in the ranges of this studiy provided a minor effect comparing to that of the pH on surface hydrophobicity.

Changes in available SH groups of the film-solutions as affected by the pH, heating temperature and time were shown in Figure 4.22. The highest amount of available SH groups were found at the highest pH and heating temperature. This tendency was quite similar to that of surface hydrophobicity shown in Figure 4.21, which suggests that protein molecules in film-solutions are unfolded by increasing pH and heating temperature. Beveridge and Arnfield, (1979) reported that available SH groups in film-solutions of egg white protein increased with increasing pH. Extensive alkaline hydrolysis of SS bonds (with subsequent formation of SH groups) in egg white

protein has been reported (Beveridge and Arnfield, 1979). Increasing of pH and heating temperature of film-solutions obtained greater concentration of available SH groups. This was expected since alkaline condition and heating denature (unfold) water-soluble fish proteins and other protein chains, exposing previously "buried" SH group (Schofield *et al.,* 1983; Mine *et al.*, 1990) resulted in higher available SH group.

Changes in content of SS bonds of the film-solutions as affected by the pH, heating temperature and time were shown in Figure 4.23. The content of SS bonds decreased as pH of film-solutions increased, but increased with increasing heating temperature and time. Heat treatment allowed the formation of intermolecular SS bond by thiol-disulfide (SH/SS) interchange and thiol oxidation reaction (McHugh *et al.*, 1994). Arntifield *et al*. (1991***)*** investigated the role of SS bonds in heat-induced net works from hen ovalbumins and vicilin, and the results indicated that SS bonds may contribute to the elasticity and strength of the net work. They suggested that SH/SS interchange reactions mediated by the free SH groups of the ovalbumins were very important in the primary stage of gelation or the formation of soluble SS-link aggregate. SH/SS interchange and thiol oxidation reactions was also been previously implicated in whey protein gelation studies (Donovan and Mulvihill, 1970; Shimada and Cheftel, 1988). Therefore, covalent bonds, such as SS bonds, were considered to be important in the formation and /or maintenance of ovalbumin gel structure. Similar films formation mechanisms involving cross linking by SS bonds were postulated for casted films from other sulfur-containing proteins such as soy protein (Gennadios and Weller, 1991).

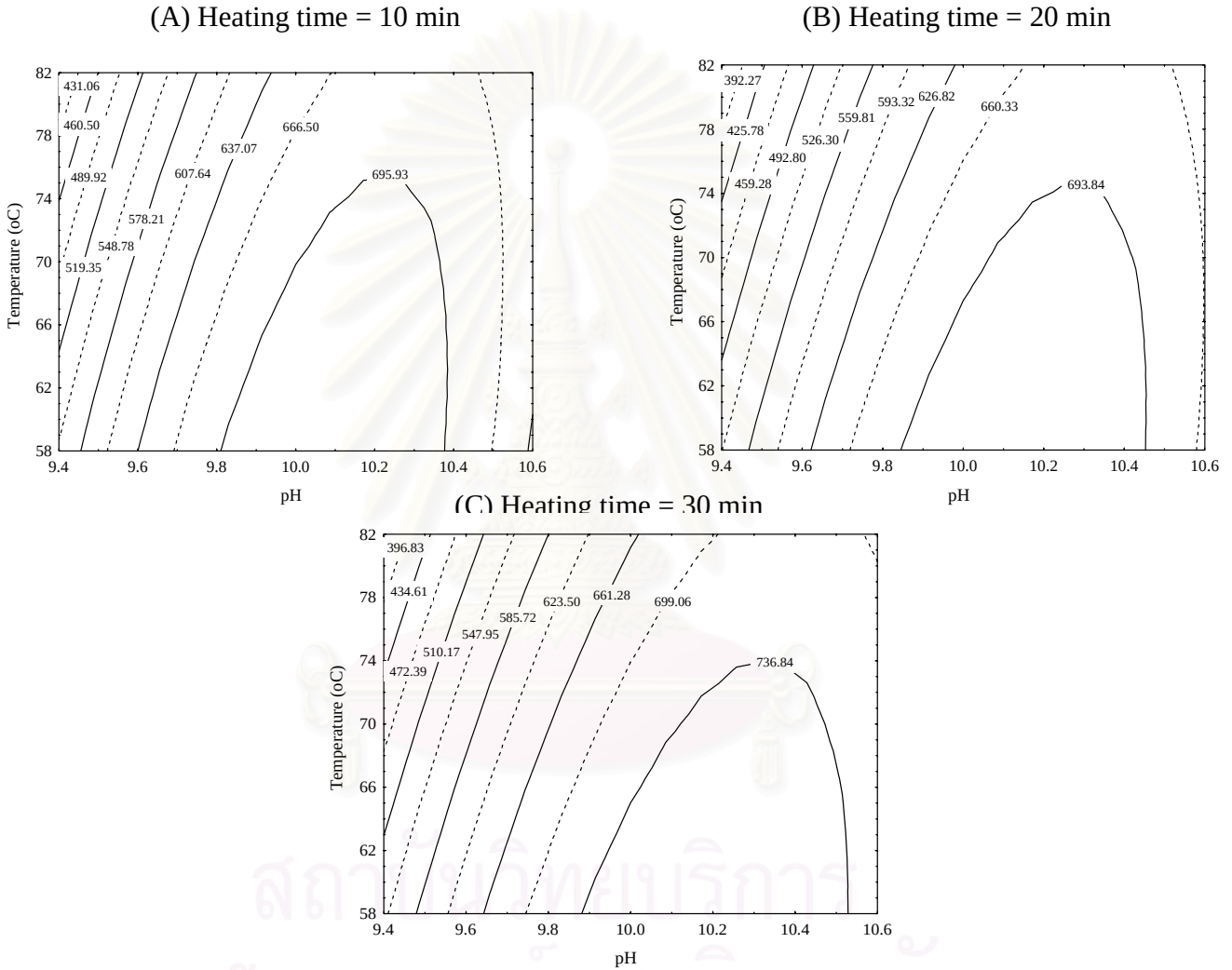


Fig. 4.21 Contour plots showing response behavior of pH and heating temperature under constant reaction time. The numbers inside the contours represent hydrophobicity of film-solutions at given heating time.

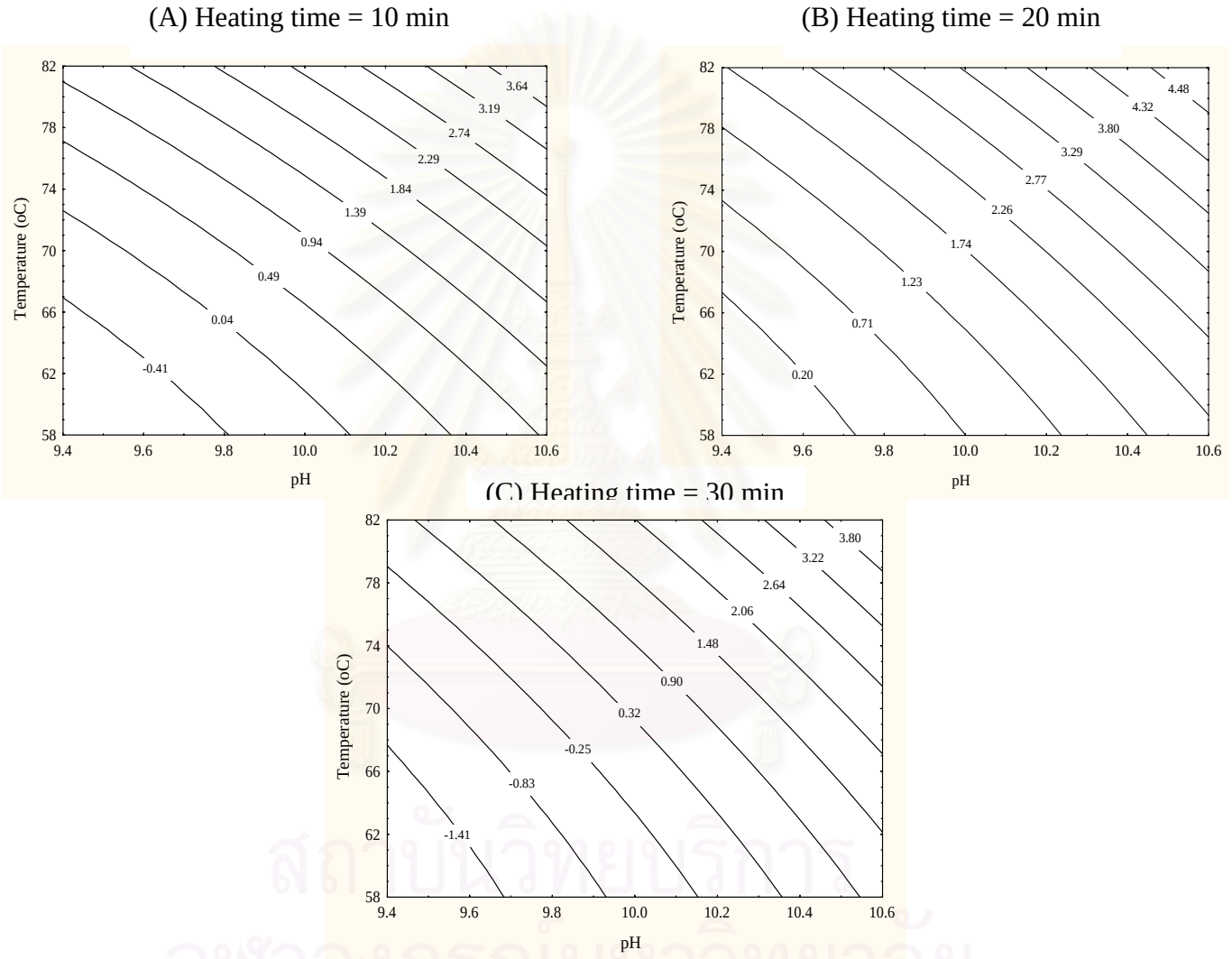


Fig. 4.22 Contour plots showing response behavior of pH and heating temperature under constant reaction time. The numbers inside the contours represent available SH of film-solutions at given heating time.

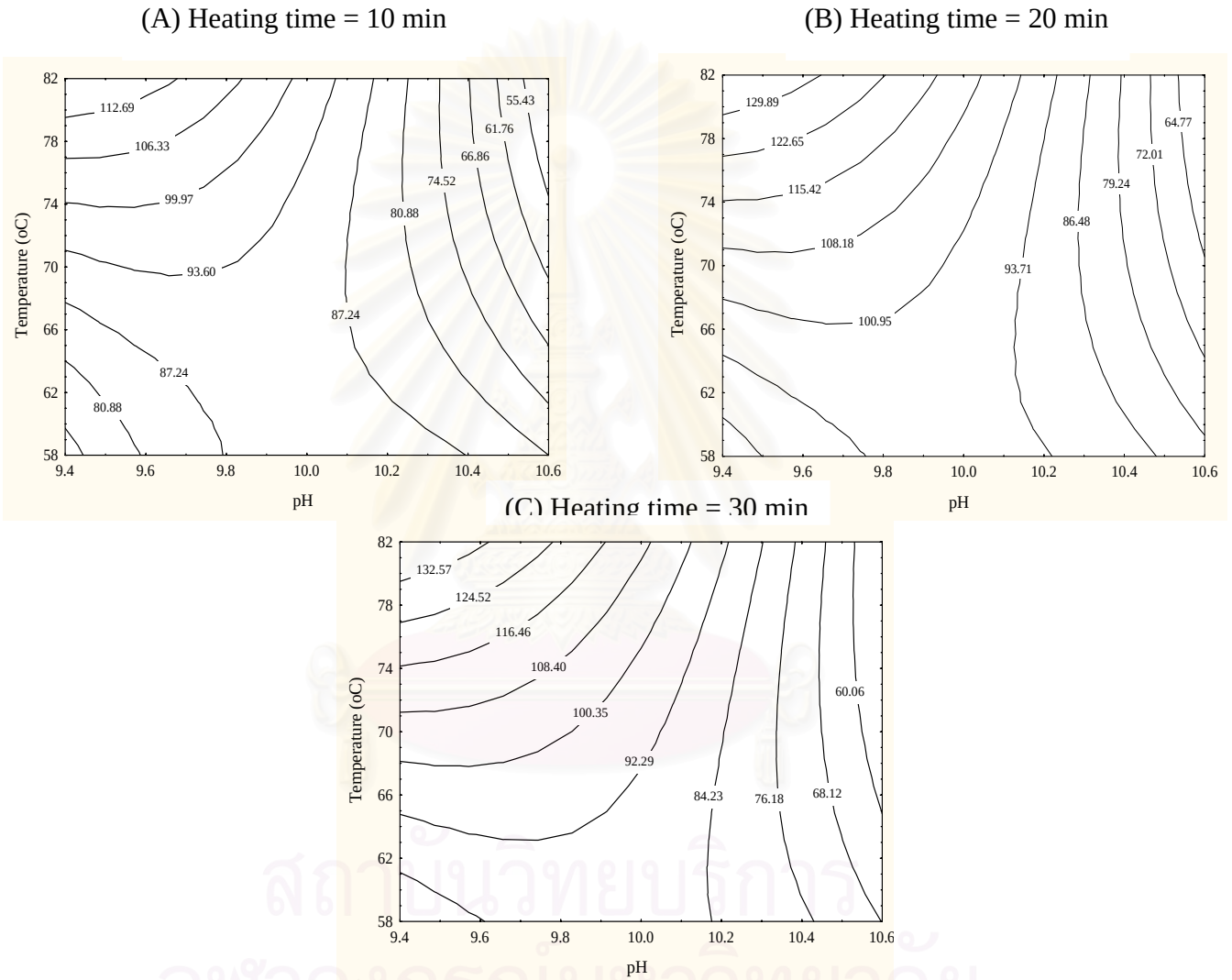


Fig. 4.23 Contour plots showing response behavior of pH and heating temperature under constant reaction time. The numbers inside the contours represent content of SS bond of film-solutions at given heating time.

4.2.1.8 Localization of Optimum Conditions

To determine the optimum conditions of the selected parameters on the properties of edible films from water-soluble fish proteins, the graphical method used in RSM was employed. The variables tensile strength, water vapor permeability and oxygen permeability were considered the most important of the 15 responses followed by elongation at break. The contour plots in Figure 4.25 were obtained from the predictive model of tensile strength, elongation at break, water vapor permeability and oxygen permeability at 20 min of heating time. A plot of Figure 4.25 was superimposed over those of Figure 4.24(A), 4.24(B), 4.24(C) and 4.24(D) to locate regions of the highest of tensile strength, elongation at break and lowest water vapor permeability and oxygen permeability. The shaded area in Figure 4.25 satisfied the specified constraints. As shown, the optimum condition for edible films forming from water-soluble fish proteins at shaded area are: pH of film-solutions of 9.72 and heating temperature of 77.42 ^{o}C for 20 min of heating time. At this combination, values of 2.86 MPa, 11.57 %, 81.45 g.mm/m^2.d.kPa and 344.13 cm^3. μm/m^2.d.kPa for tensile strength, elongation at break, water vapor permeability and oxygen permeability, respectively, were resulted. Besides, film solubility, protein solubility, hydrophobicity, available SH group and content SS bonds were found to be 49.90%, 19.19%, 567.04, 3.98 and 117.77 μM SH/g protein, respectively.

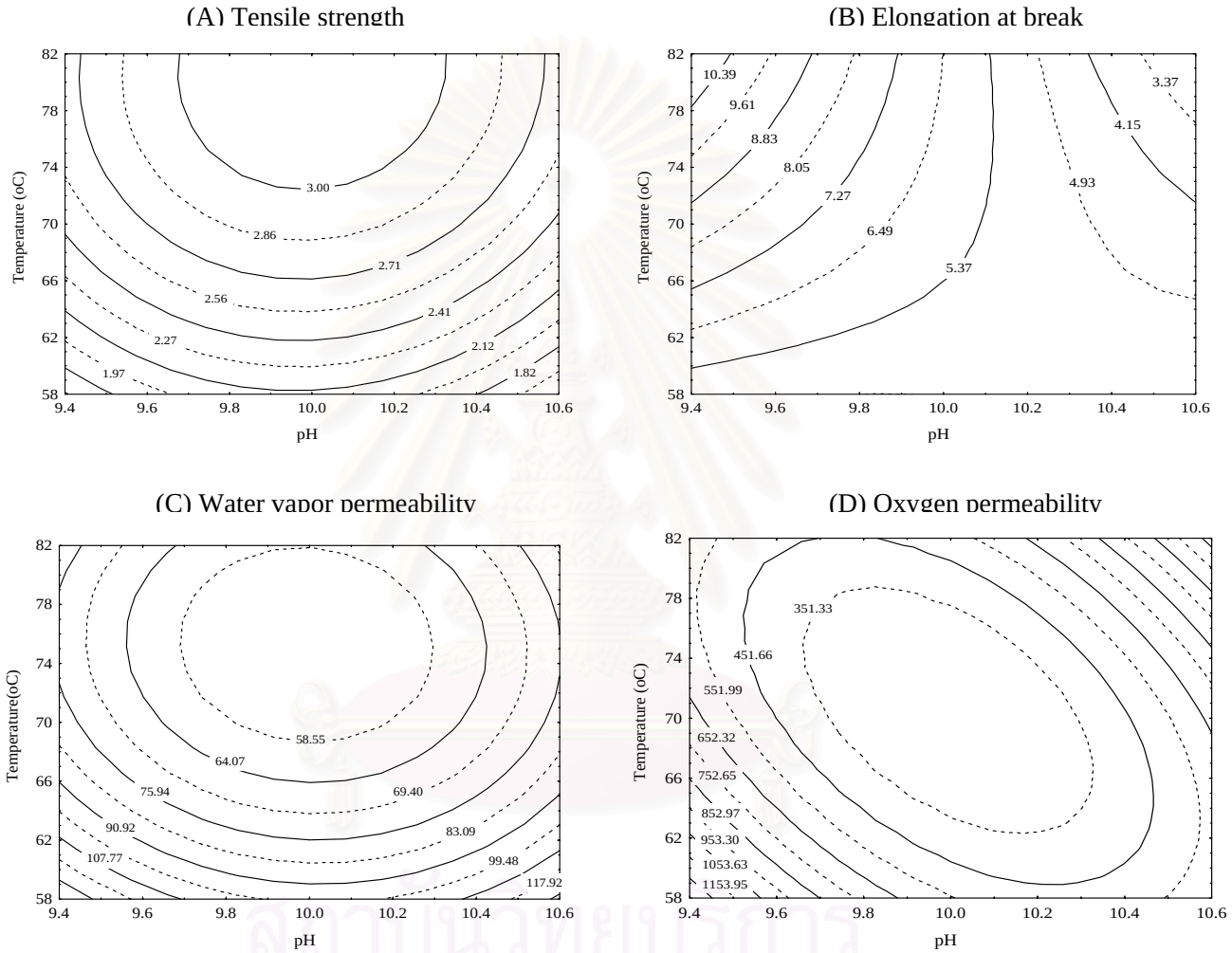


Fig. 4.24 Contour plots showing response behavior of pH and heating temperature of film solutions heated for 20 min on the; tensile strength (MPa), elongation at break (%), water vapor permeability (g.mm/m^2.d.kPa) and oxygen permeability (cm^3.μm/m^2.d.kPa) before superimposition.

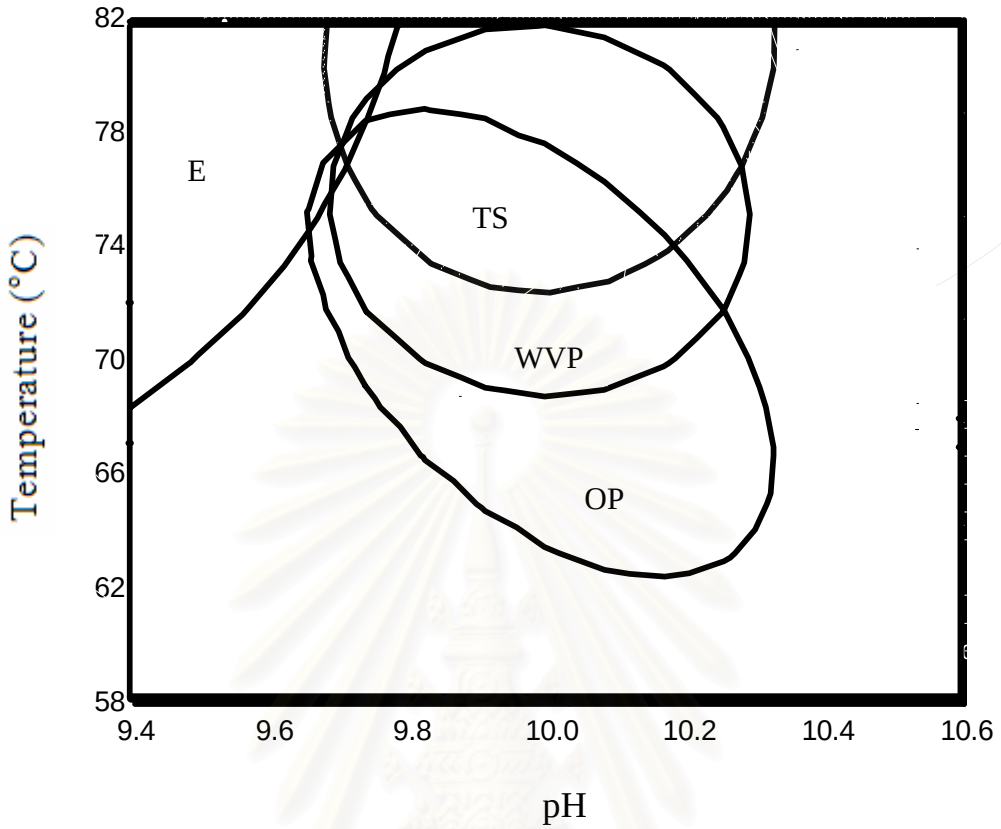


Fig. 4.25 Optimum film solution condition as function of the independent variables after superimposition of contour plots over those of 4.24(A), 4.24(B), 4.24(C) and 4.24 (D). Shaded area indicates region of the highest tensile strength (TS), elongation at break (E) and lowest water vapor permeability (WVP) and oxygen permeability (OP).

Validation tests were performed to determine the adequacy of the single order polynomial (SOP) model (Floros and Chinnan, 1988; Mudahar *et al*., 1990). This was performed because a fractional factorial design was used as the experimental design. A model is deemed adequate if the predicted values (of the model) are close to the experimental values observed during the validation tests. Table 4.1

shows the predicted and observed values for the responses at optimum condition for the selected parameters on the properties of edible film from water-soluble fish proteins. The experimental values were averages of three replicates and were very close to the predicted values indicating that the SOP models generated were acceptable. The high CV values for some models were due to their lesser reproducibility (Montgomery, 1984) that may have contributed to the statistical insignificance of some of these models. Despite the lesser effect of these responses to the optimum conditions, predictions were within fairly acceptable limits.

Table 4.1 Predicted and observed values for the independent variables after superimposition conditions

Response variable	Predicted value	Actual value ± SD
Tensile strength (MPa)	2.86	2.88 ± 0.05 (1.79%)
Elongation at break (%)	11.57	11.00 ± 0.32 (2.91%)
Water vapor permeability ($g.mm/m^2.d.kPa$)	81.45	72.16 ± 5.07 (7.02%)
Oxygen permeability ($cm^3.\mu m/m^2.d.kPa$)	344.13	322.92 ± 33.6 (10.40%)

Numbers in parentheses are coefficients of variation (CV).

4.2.2 Effect of Protein Concentrations on the Properties of Edible Films from Water-Soluble Fish Proteins from Surimi Wash-Water

The effect of water-soluble fish proteins concentrations on the tensile strength and elongation at break of edible films were presented in Figure 4.26. Water-soluble fish proteins solutions with varying protein concentrations of 1.5, 3.0 and

4.5% at fixed pH of 9.7, heating temperature of 77.4 and 20 min heating time were studied. When 4.5 % of protein concentration was used, the formation of films was inhibited due to the precipitation of proteins after heating the film solutions. Hence, only the effect of water-soluble fish proteins concentration at 1.5 and 3.0 % w/w were compared.

Varying the concentration of water-soluble fish proteins elucidated the influence on the tensile strength and elongation at break (Fig.4.26). The tensile strength and elongation at break were significantly ($p \leq 0.05$) higher at the 3.0% of water-soluble fish proteins (Fig. 4.26), this implied that, the higher content of proteins (3.0% w/w) induced favorable structure regarding the ability of the films to form and stretch.

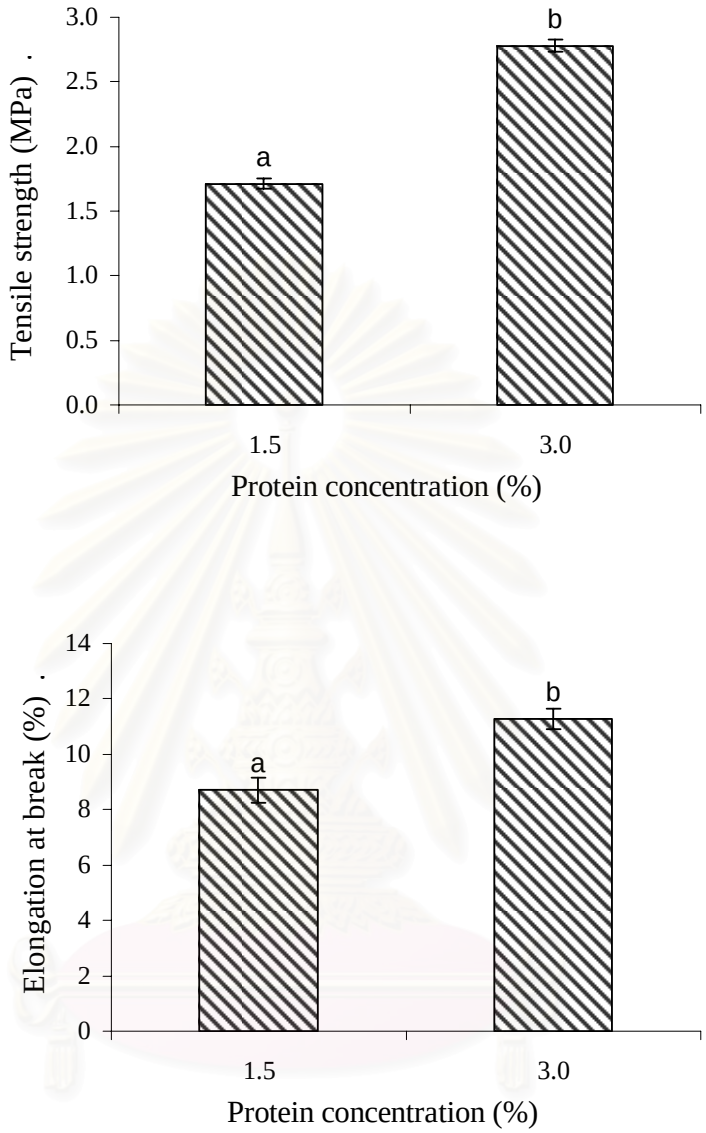


Fig. 4.26 Effect of protein concentration on the tensile strength (MPa) and elongation at break (%) of edible films from water-soluble fish proteins. Standard error bars are shown.

a, b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

Water vapor permeability and oxygen permeability of water-soluble fish proteins films at 1.5 and 3.0% w/w of water-soluble fish proteins were observed (Fig. 4.27). The results showed that the protein concentration had significantly ($p \leq 0.05$) effect on the water vapor permeability and oxygen permeability (Fig. 4.27). It was observed that higher protein concentration (3.0% w/w) provided lower both water vapor permeability and oxygen permeability. This result might be due to the fact that, increasing of the protein concentration, a more aggregated structure with a denser protein structure network formed (McHugh and Krochta, 1994a). As a result, decrease in water vapor and oxygen permeabilities concomitantly with decrease in film and protein solubilitis obtained (Fig. 4.28). However, Anker *et al.* (2000) reported that varying of whey protein isolated (WPI) concentration over and under the critical gel concentration (Cg) elucidated the influence of the polymer network on the film properties. The strain at break showed a maximum at the Cg and this implied that the most favorable structure regarding the ability of the film to stretch was formed at this concentration. Reduced mechanical properties for films formed below and above Cg indicated that different protein network was formed. However effect of varying the concentration of WPI influencing the barrier property was studied by McHugh *et al.* (1994a). This result showed that 12% (w/w) WPI film had higher water vapor permeability value than 8% (w/w) WPI film but lower oxygen permeability. The reason for the increased water vapor permeability was probably the large pores formed at high concentration, compared to the smaller pores formed at low concentration. When the pore size increased the water and plasticizer and, since the water molecules were hydrophillic, their easiest way through the film matrix was through the hydrophillic water (Anker *et al.*, 2000). Similar results were reported by McHugh *et al.* (1994), who showed that when the concentration

increased from 8-12 % (w/w) WPI, the water vapor permeability increased for the WPI films. However, at a high protein concentration, a denser protein structure was formed and, consequently, the oxygen permeability was lowered due to the increased obstacle to the oxygen molecules passing through the more closely packed protein network (Anker *et al.*, 2000). This is in agreement with Pascat (1986), who stated that a higher density decreases the permeability. Miller and Krochta (1997) further confirmed that the permeability was highly affected by how closely packed the polymer chains were. Although this showed how large an effect of the protein concentration was on the barrier propeties, several other factors were known to affect the permeability: the microstructure, the plasticizer, the density, the orientation, cross-linking, and the molecular weight of the polymer chains, the nature of the permeant etc (McHugh *et al.*, 1994).

The color of water-soluble fish protein based-edible films was affected by protein concentrations, higher protein concentration showed significantly ($p \leq 0.05$) higher in b* and chroma value, but lower in a* (Fig. 4.29-4.31), hence, the films color was darker and more yellow than that found at lower content of protein. However, the lightness (L*) of films was not significantly ($p \leq 0.05$) different (Fig. 4.29). It was well known that protein could undergo browning reactions during processing, causing yellowing and loss of nutritional value of product (Coultate, 1988). Yellowing was attributed to the reaction of protein lysine group with reducing sugars such as lactose and glucose. Labuza and Saltmarch (1981) found that the rate of browning pigment formation in whey powder increased as storage temperature and water activity increased from 25-45 ^{o}C and 0.33 to 0.65, respectively. Heat curing of whey protein isolated film at 60, 70 and 80 ^{o}C for up to 48 h qualitatively increased film yellowing (Miller *et al.* 1997).

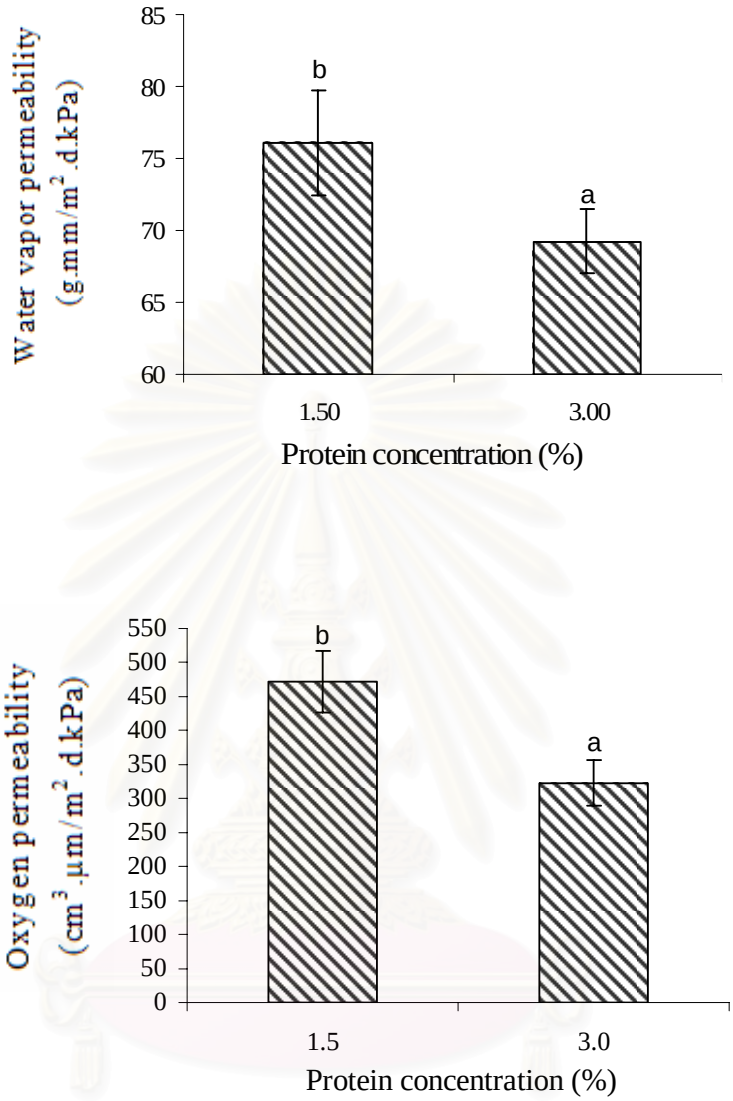


Fig. 4.27 Effect of protein concentration on the water vapor permeability (g.mm/m^2.d.kPa) and oxygen permeability (cm^3.µm/m^2.d.kPa) of edible films from water-soluble fish proteins. Standard error bars are shown.

a, b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

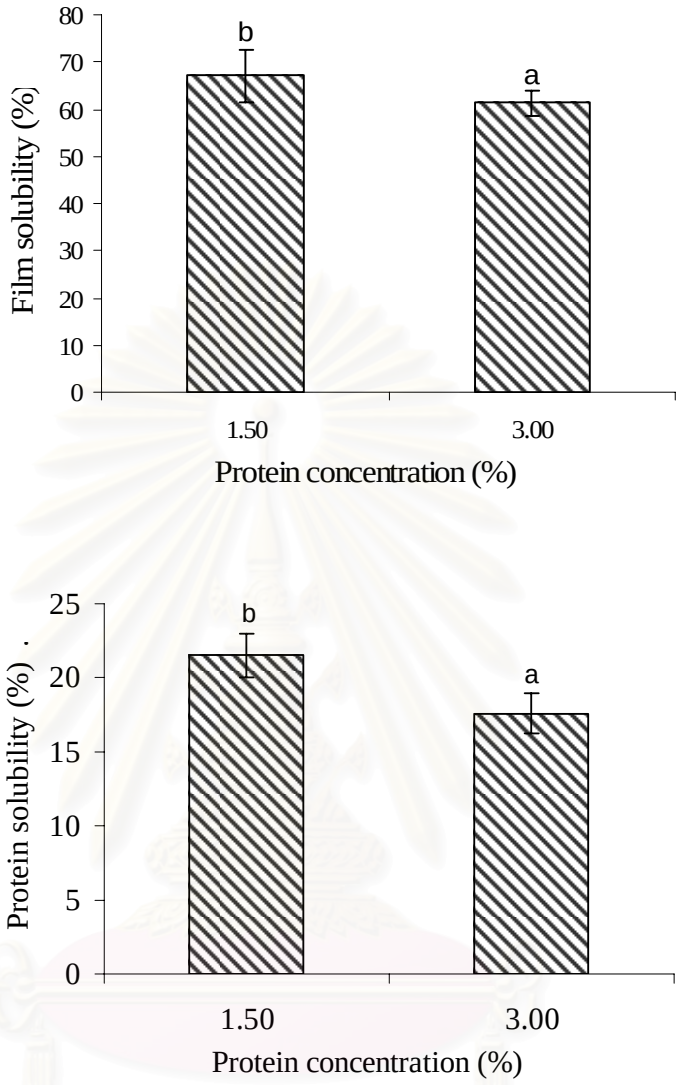


Fig. 4.28 Effect of protein concentration on the film solubility (%) and protein solubility (%) of edible films from water-soluble fish proteins. Standard error bars are shown.

a, b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

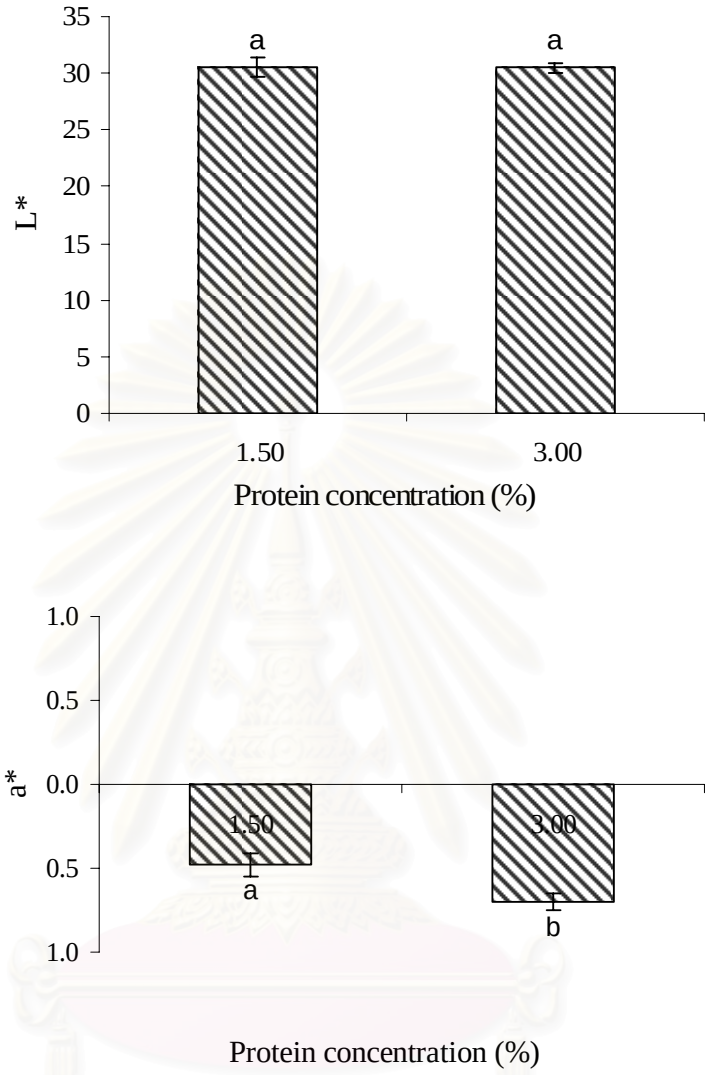


Fig 4.29 Effect of protein concentration on the L* and a* values of edible films from water-soluble fish proteins. Standard error bars are shown.

a, b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

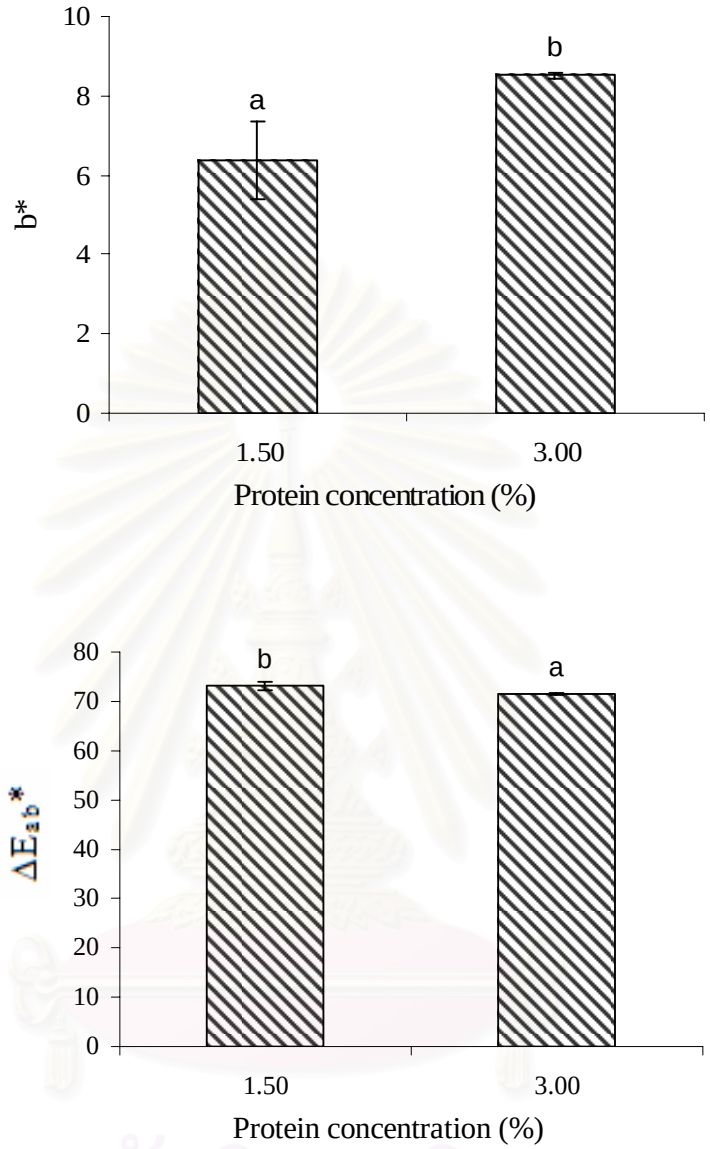


Fig. 4.30 Effect of protein concentration on the b* and ΔE_{ab}* values of edible films from water-soluble fish proteins. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

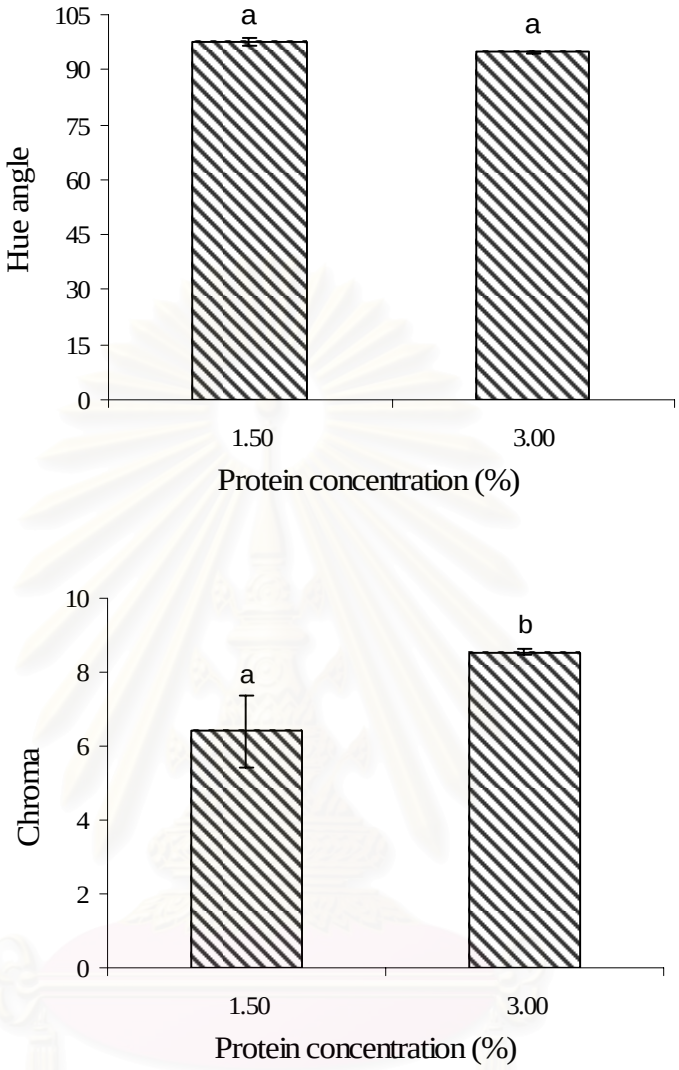


Fig. 4.31 Effect of protein concentration on the hue angle and chroma values of edible films from water-soluble fish proteins. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

4.2.3 Effect of Plasticizer Type and Concentration on Properties of Edible Films from Water-Soluble Fish Proteins from Surimi Wash-Water

4.2.3.1 Tensile Strength and Elongation at Break

Preliminary work demonstrated that edible films from water-soluble fish proteins formed without plasticizer were relatively brittle and broke easily when peeled off. Hence desirable mechanical properties of edible films were improved by using three types of plasticizer (sorbitol, glycerol and polyethylene glycol) at different concentrations (25, 50 and 75 %). The mechanical properties of films plasticized by sorbitol, glycerol or polyethylene glycol, at different concentration were assessed by measuring their tensile strength and elongation at break. The results are shown in Figure 4.32. It was observed that an increase in content of these plasticizers resulted in decrease in mechanical resistance (decrease in tensile strength) and increase in extensibility (increase in percentage of elongation). Tensile strength decreased from 3.14 to 0.82, 2.13 to 0.82 and 1.8 to 0.62 MPa when the sorbitol, glycerol and polyethylene glycol concentration increased from 25 to 75 % w/w., while, elongation at break increased from 5.76 to 15, 26.24 to 61.01 and 23.49 to 52.21%, respectively (Fig. 4.32). Sorbitol, glycerol and polyethylene glycol are low molecular weight hydrophilic molecules that could easily fit into protein chains and establish hydrogen bonding with reactive groups of proteins. Bringing together plasticizers and proteins induced formation protein-plasticizer interactions to the detriment of protein-protein interactions. As a consequence, the density of intermolecular interaction in material decreased and the free volume between polymer chains increased (Cuq *et al.*, 1997). The changes in mechanical properties as affected by hydrophilic plasticizers were observed for various

hydrocolloid-based films (Park and Chinnan, 1990; Gontard *et al.*, 1993). The mechanical property changed due to decrease in density and reversibility of intermolecular and molecular interaction occurring in the edible films from water-soluble fish proteins network that formed films.

The mechanical properties of sorbitol, glycerol and polyethylene glycol plasticized films at an equal concentration were compared (Fig.4.28). The sorbitol plasticized films had significantly ($p \leq 0.05$) higher tensile strength and lower elongation at break than glycerol and polyethylene glycol plasticized films at both 25 and 50 % w/w. However, no significant difference ($p > 0.05$) in tensile strength was observed when 75% w/w was used. This could be attributed to the ring molecular conformation of sorbitol molecules, which may sterically hinder insertion between the protein chains resulted in less effective in disrupting the protein-protein interruptions. McHugh and Krochta (1994) studied whey protein isolated/sorbitol = 1 and whey protein isolated/glycerol = 2.3 films and presented similar tensile strength values. They concluded that a higher amount of sorbitol than glycerol was needed to obtain similar tensile strength properties and suggested that the smaller size of glycerol molecule enable it to influence the films properties more readily than the sorbitol molecule.

The glycerol plasticized films were more mechanical resistance and stretchable (at 75% w/w) than the polyethylene glycol plasticized films (Fig. 4.32), suggesting that glycerol could be a more effective plasticizer in edible films from water-soluble proteins films than polyethylene glycol. The effectiveness of glycerol in the edible films from water-soluble fish proteins films are most likely due to its small size which allows it to be more readily inserted between the polymer chains, and consequently exert more influence on the mechanical properties than the larger

polyethylene glycol molecule. Donhowe and Fennema (1993) found that plasticizer with low molecular weights such as glycerol was more effective than those with high molecular weights (polyethylene glycol) in methylcellulose-based films. Similarly, McHugh and Krochta (1994) suggested that smaller size plasticizer was more effective than larger size plasticize in whey protein films. In addition, at an equal percentage concentration, the total number of glycerol molecules in the film-solution was greater than that of the higher molecular weight polyethylene glycol, therefore glycerol had more functional groups (-OH) than polyethylene glycol, which should promote the plasticizer-polymers interactions in the films (Donhowe and Fennema, 1993; McHugh and Krochta, 1994).

Gennadios *et al.* (1993) reported that, the polar group (-OH) along plasticizer chains are believed to developed polymer-plasticizer hydrogen bonds replacing the polymer-polymer interaction in the biopolymer films. Molecular size, configuration and total number of functional hydroxide groups of the plasticizer as well as its compatibility with the polymer could affect the interactions between the plasticizer and the polymer (Yang and Paulson, 2000)

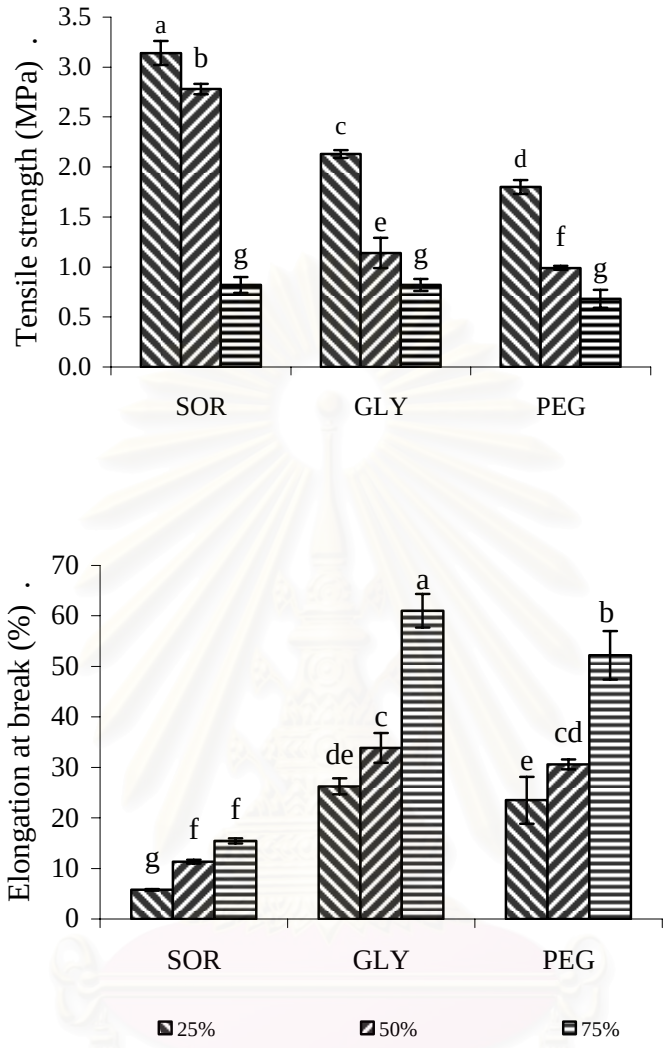


Fig 4.32 Effect of plasticizer type and concentration on the tensile strength (MPa) and elongation at break (%) of edible films from water-soluble fish proteins. Standard error bars are shown.

a-g; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

4.2.3.2 Water Vapor Permeability

Water vapor permeability of edible films from water-soluble proteins with different type and concentration of plasticizer were examined (Figure 4.33). The water vapor permeability increased with increasing of plasticizer concentration. The water vapor permeability increased from 30.41 to 79.76, 125.80 to 234.67 and 89.52 to 225.45 g.mm/m^2.d.kPa respectively, when the concentration of sorbitol, glycerol and polyethylene glycol increased from 25 to 75 % w/w (Fig. 4.33). This tendency could be explained by structural modifications of the protein network. The incorporation of plasticizers modified the molecular organization of the protein network, with an increase in free volume. The network becames less dense and as a consequence more permeable (Banker *et al.*, 1966; Ashley, 1985). Permeability increased with plasticizer content could be related to hydrophillicity of plasticizer molecules. Introducing hydrophillic plasticizers, favorable to adsorption and desorption of water molecules, was reported to enhance the water vapor permeability of hydrocolloid-based films (Gontard *et al*., 1993; McHugh *et al.* 1994).

Comparing of the successive values of the water vapor permeability for each plasticized film was shown in Figure 4.33. Films plasticized with sorbitol had lower water vapor permeability than those with glycerol and polyethylene glycol at each plasticizer concentration due to the fact that sorbitol had ability to bind less water than glycerol and polyethylene glycol, thereby, provided a lower water vapor permeability (McHugh *et al.*, 1994). Chick and Ustanol (1998) reported that casein-based films plasticized with glycerol had higher water vapor permeability values than films plasticized with sorbitol when the same amounts of plasticizers were used. The high hydrophillicity of glycerol and polyethylene glycol molecules, which is favorable to

the adsorption of water molecules, could also be contribute to the increase in the films water vapor permeability (Gennadios *et al.*, 1993b). The increase in water vapor permeability with increasing hydrophillicity plasticizer concentration was also common in edible films (McHugh *et al*., 1994; Cuq *et al.*, 1997). Sorbal *et al.* (2001) reported that hydrophilicity of the plasticizers will increase the water content of the films, consequently increasing the mobility of the molecules. In addition, increasing water content could also affect permeate solubility in the film.

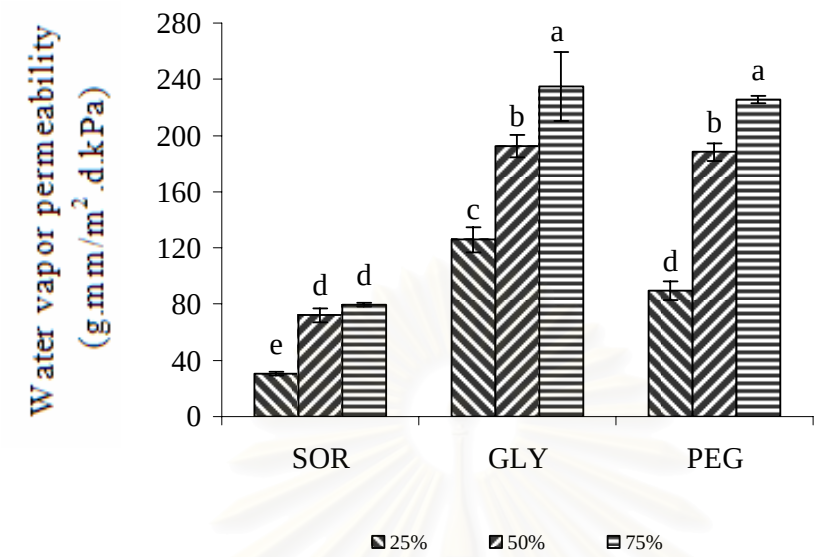


Fig. 4.33 Effect of plasticizer type and concentration on the water vapor permeability (g.mm/m^2.d.kPa) of edible films from water-soluble fish proteins. Standard error bars are shown.

a-e; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

4.2.3.3 Film and Protein solubilities

From visual observations and irrespective of plasticizer type and content, the edible films from water-soluble fish proteins clearly did not lose integrity after a 24 h immersion in water. Irrespective of the type, an increase in plasticizer content leads to an increase in films and proteins solubilities (Fig. 4.34). It could be hastily concluded that hydrophilic plasticizers enhanced films solubility in water. Low molecular weight protein chains (i.e. monomers and small peptides) formed during storage of film solutions and entrapped in the network (Cuq *et al.*, 1995) could then constitute the protein-based materials that solubilize in water. The dry matter

solubilized in water was likely to be composed mainly of the plasticizer. The protein network was then not likely to solubilize or disperse in water. High interaction density and more certainly, the presence of intermolecular covalent bonds or “physical knots” (i.e. chain entailments) are responsible for partial insolubility of these films. This water solubility behavior could not be generalized, and understanding the films solubility remains a complex subject. Plasticizer solubilization in water was already observed for films based on wheat gluten or treated soy proteins or transglutaminase catalytic cross-linking whey protein (Gontard *et al.*, 1992; Stuchell and Krochta, 1994). Stuchell and Krochta (1994) pointed out that increase in the content of protein solubilized in water was obtained when the hydrophilic content of treated whey protein-and soy protein-based films increased. A decrease in the polymer network interaction density due to the presence of plasticizer was thus associated with this increase in solubility property. The lowest films and proteins solubility of water-soluble fish proteins films plasticized by 25% w/w of these plasticizers were noticed, while increasing the amount of plasticizer content showed higher films solubility and proteins solubility (Fig. 4.34). It could be explained that, at higher content of plasticizer, more molecules of plasticizer were untrapped in the protein cross linked network and able to escape into solution, while, lower content of plasticizer gave lowered plasticizer molecules untrapped in the crosslinked network and less ability to escape into solution. The film and protein solubilities were higher for the sorbitol plasticized film comparing with those plasticized with glycerol and polyethylene glycol. The sorbitol had a ring molecular conformation which may sterically hinder insertion between the protein chains (Yang and Paulson, 2000) thus facilitated its escape into solution, while glycerol and polyethylene glycol have straight chains, which promote the insertion between protein-protein chains.

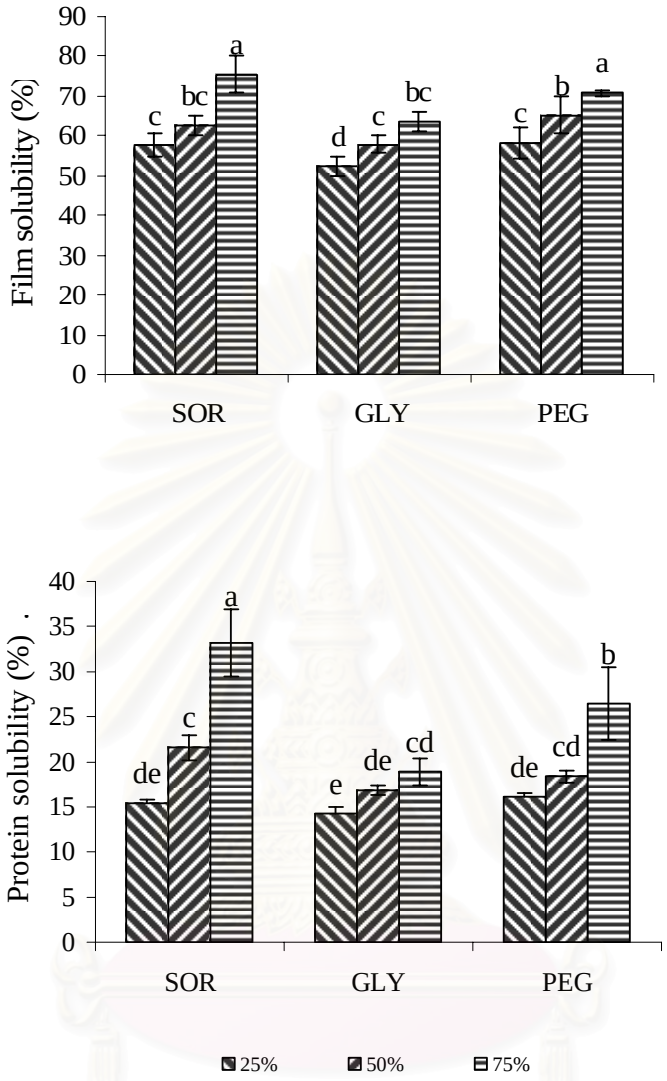


Fig. 4.34 Effect of plasticizer type and concentration on the films solubility (%) and proteins solubility (%) of edible films from water-soluble fish proteins. Standard error bars are shown.

a-e; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

4.2.3.4 Film Color

The results of the measurements performed on the films color were expressed in accordance with the CIELAB system, and the rectangular coordinates (L*, a* and b*) were defined. The color of films was more affected by the nature of the plasticizer rather than by concentration. L* values of water-soluble fish protein films plasticized by sorbitol, glycerol and polyethylene glycol were not significantly different ($p > 0.05$) (Fig. 4.35). In contrast, increased yellowness (greater + b* and chroma) occurred when glycerol and polyethylene glycol were used (Fig. 4.36 and 4.37). This was somewhat expected since color change depend on the type of plasticizer.

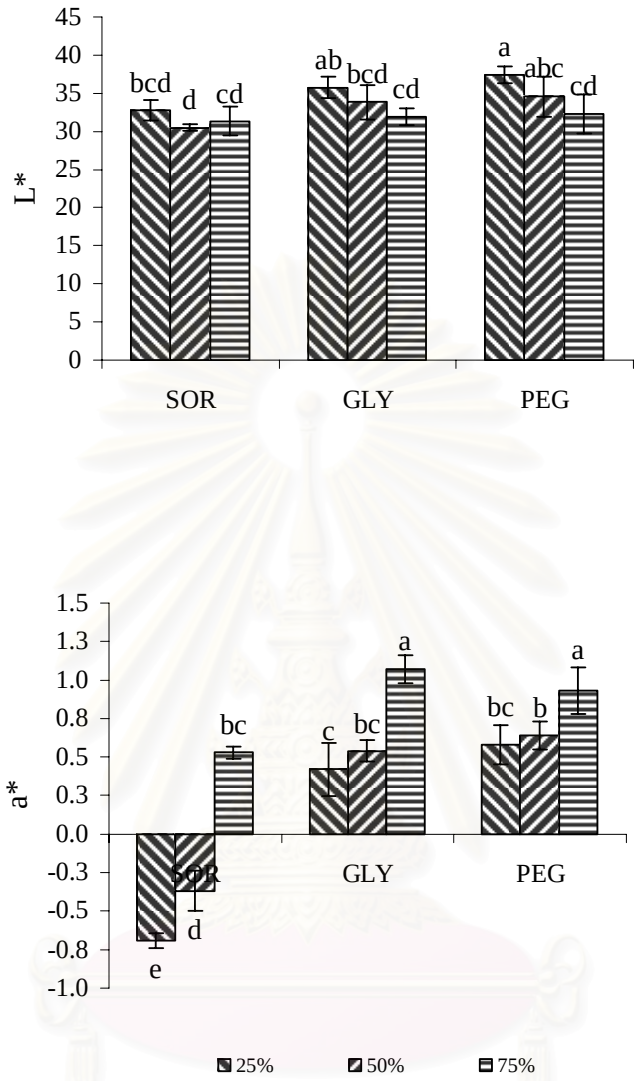


Fig 4.35 Effect of plasticizer type and concentration on L* value and a* values of edible films from water-soluble fish proteins. Standard error bars are shown.

a-e; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

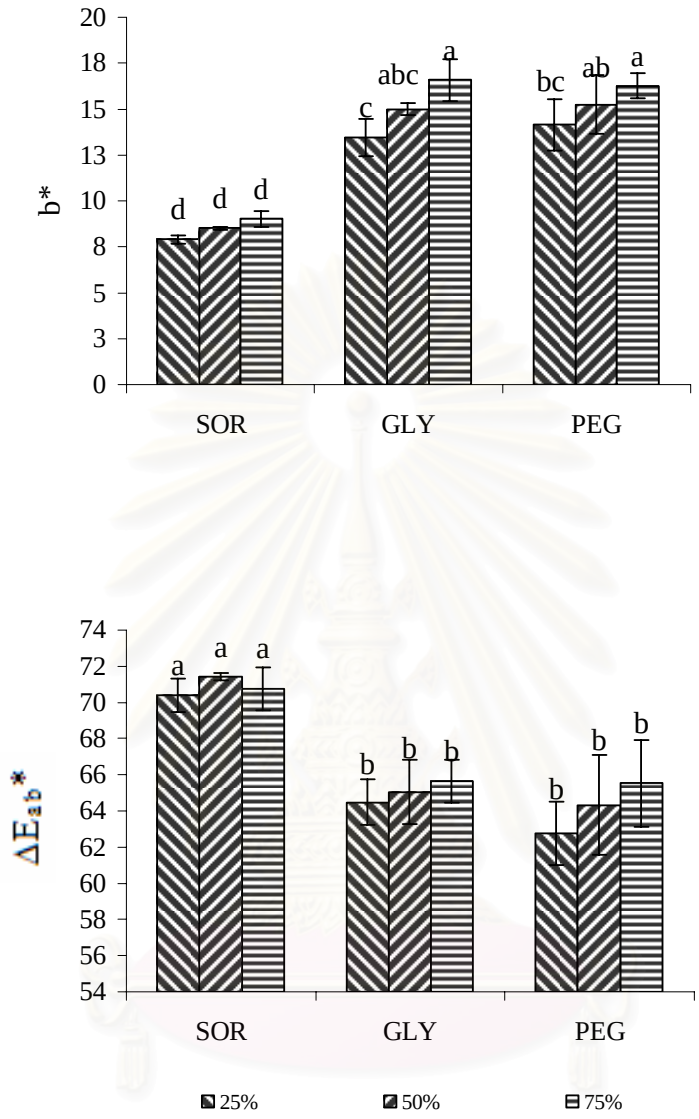


Fig. 4.36 Effect of plasticizer type and concentration on b* and ΔE_{ab}* values of edible films from water-soluble fish proteins. Standard error bars are shown.

a-d; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

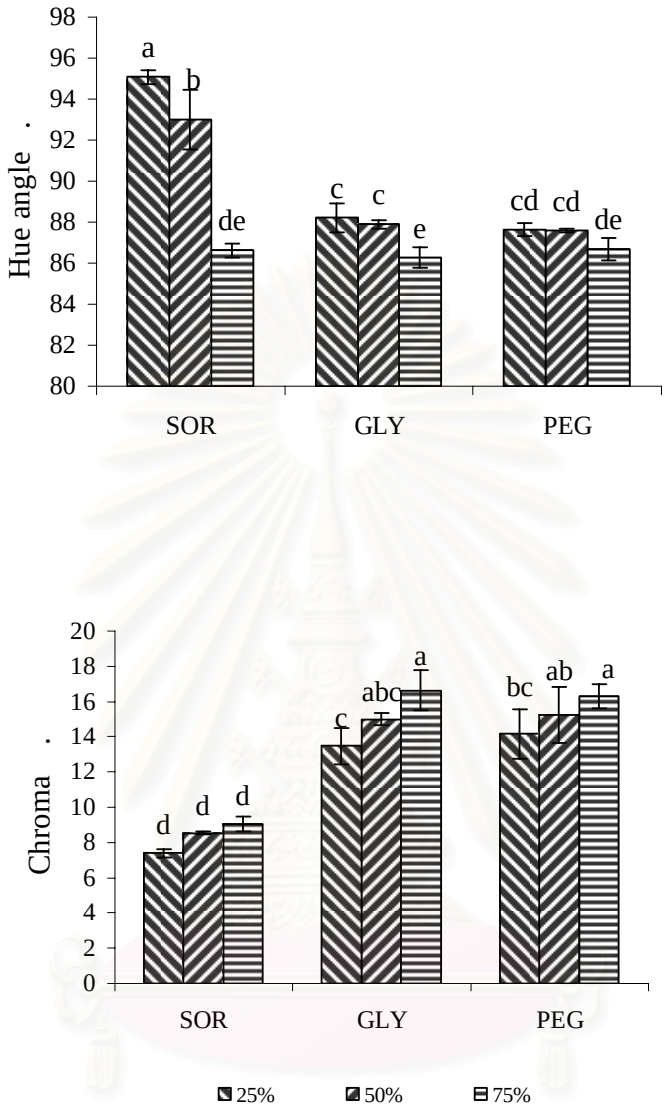


Fig. 4.37 Effect of plasticizer type and concentration on hue angle and chroma values of edible films from water- soluble fish proteins. Standard error bars are shown.

a,e; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

4.3 Effect of pH, Heating Temperature, Heating Time, Protein Concentration and Plasticizer on the Properties of Edible Films from Proteins Precipitated by Shifting the pH in Surimi Wash-Water

4.3.1 Effect of pH, Heating Temperature and Heating Time on the Properties of Edible Films from Proteins Precipitated by Shifting the pH in Surimi Wash-Water

4.3.1.1 Model Fitting

The RSREG procedure of Statistical Analysis System (SAS, 1996) was used to fit the second order polynomial equation (1) to the films properties data shown in Table 5 (Appendix A). The regression coefficients (β_{ki}) obtained thereof, were presented in Table 6 (Appendix A). The analysis of variance for the response variables (Table 7, Appendix A) indicated that the model developed for the tensile strength (TS), elongation at break (E), water vapor permeability (WVP), oxygen permeability (OP), film solubility (FS), protein solubility (PS), color (L*, a* b*, ΔE^*_{ab} , Hue angle, and Chroma values), hydrophobicity (HQ), available SH group (ASH) and content of SS bond (SS) were adequate, and had no significant lack of fit.

Further statistical analysis (Table 4.8**,** Appendix A**)** was then performed. Results revealed that pH, heating temperature and heating time had a significant overall effect on the all responses. The pH had the most significant ($p \leq 0.05$) effect on tensile strength, elongation at break, L*, a*, ΔE^*_{ab} values and available SH group, while heating temperature and heating time was the lowest effect on these responses. The b* hue angle and chroma values were the most affected by pH and heating temperature. Water vapor permeability, oxygen permeability content of SS bond were most affected by pH, heating

temperature and heating time. However, pH, heating temperature and heating time did not show significant ($p > 0.05$) effect on film and protein solubilities.

4.3.1.2 Tensile Strength and Elongation at Break

Tensile strength is an important mechanical property that expressed the maximum stress developed in films during tensile testing (Gennadios *et al*., 1993a), while elongation at break is an indication of films flexibility and stretchability (extensibility). The main factors that had impacts the film's properties were pH of film-solutions, while heating temperature and heating time had lower effects (Table 8, Appendix A). Contour plots of tensile strength and elongation at break as affected by pH and heating temperature were given in Figure 4.38 and 4.39. Depending upon the film conditions tensile strength and elongation at break demonstrated a high variation between 1.83-5.21 MPa and 1.87-72.14, respectively (Figure 4.38 and 4.39).

Comparing within the same heating temperature of film-solutions, the results showed that tensile strength increased as pH of film-solutions decreased. This result implied that lower pH of film-solutions induced formation of resistant films. At pH away from the isoelectric point of 3.5 (Bourtoom *et al*., 2002), unfolding and solubilizing of proteins were occurred resulted in facilitating molecule orientation and fine-stranded network (Banker, 1966). The resulting interaction between polymers may have been responsible for this result. The highest tensile strength value was obtained at pH of film solutions about 2.0 (Figure 4.38). However, decreasing pH of the film-solution lower than 2.0 resulted in decrease of tensile strength due to the fact that strongly repulsive force occurred between the positive (extreme pH) charges along protein chain could have decreased the occurrence of molecular associations within the protein matrix and formation film (Rhim *et al*., 2002). Contrarily, increasing of pH of the

film-solution higher than 2.0 resulted in decrease in tensile strength, most likely due to less unfolding and solubilizing of proteins were promoted and resulted in less protein-protein interaction. The weak films were obtained at the lowest and highest pH of film-solutions: The very low tensile strength (2.79 and 3.15 MPa, respectively) was observed.

The tensile strength was enhanced as heating temperature of film-solutions increased from 60-80 ^{o}C. This result demonstrated, the tensile strength increased from 2.36 MPa to almost 5.21 MPa, when heating temperature of film-solutions increased from 60 to 80 ^{o}C. This may be due to the fact that higher heating temperature induced protein denaturation and resulted in increase in the number and/or a better localization of bonds between protein chains. Hayakawa and Nakai (1985) reported that heating of film-solutions induced surface SH group which was converted to SS bonds resulted in contributing gel network formation between protein-protein chains which provided higher interaction between protein polymers. The weakest film was obtained at the lowest heating temperature and a very low tensile strength (2.36 MPa) was observed at heating temperature of film-solutions around 60 ^{o}C. The contour plot (Fig. 4.38) indicated an interaction between the effect of pH and heating temperature on tensile strength of the resulting films. It was observed that the lowest tensile strength could be expected with low pH and relative low heating temperature of film-solutions. According to the contour plots, the experimental condition involving lower pH (2.0) and higher heating temperature resulted in higher film formations and high tensile strength of the formed films. Heating time of film-solutions seemed to have less effect on tensile strength of the film.

Elongation at break was mostly affected by pH of film-solutions. The linear and quadratic terms for pH, heating temperature and heating time

were significant (Table 7, Appendix A). The contour plots of elongation at break (Fig 4.39) showed a high variation between 1.87 and 72.14 % and showed the highest elongation at break when lower pH (2.0) and higher heating temperature of film solution were employed. An increased in elongation at break with decrease in pH (2.5 to 2.0) could be explained by adjusting of pH away from the isoelectric point (3.5) was assumed to be induced a high solubility, facilitating favorable molecule orientation and protein-protein interaction was promoted, resulted in increase in elongation at break. Heating temperature and heating time had less effect on elongation at break of the film.

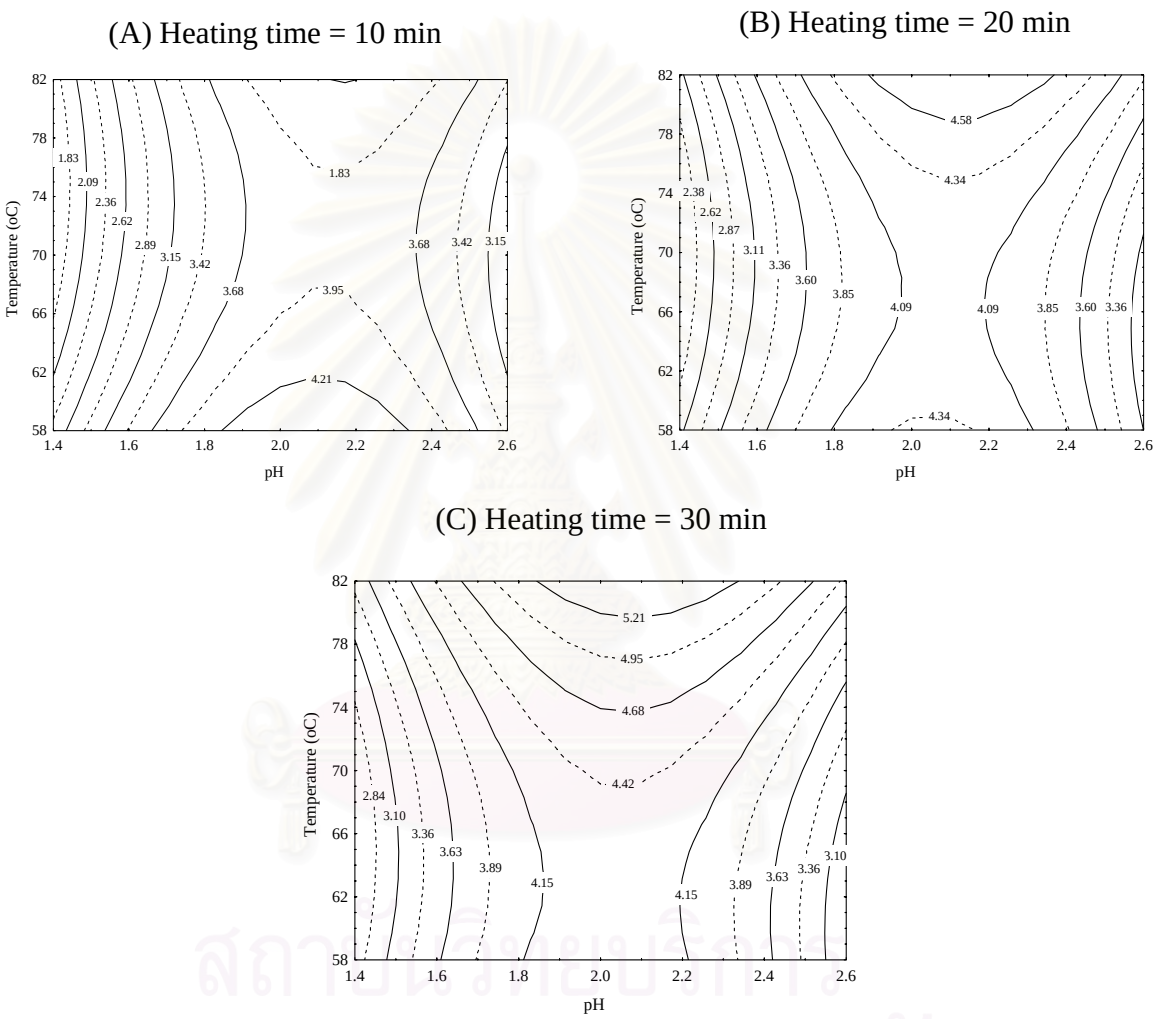


Fig. 4.38 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent tensile strength (KPa) of films at given heating time.

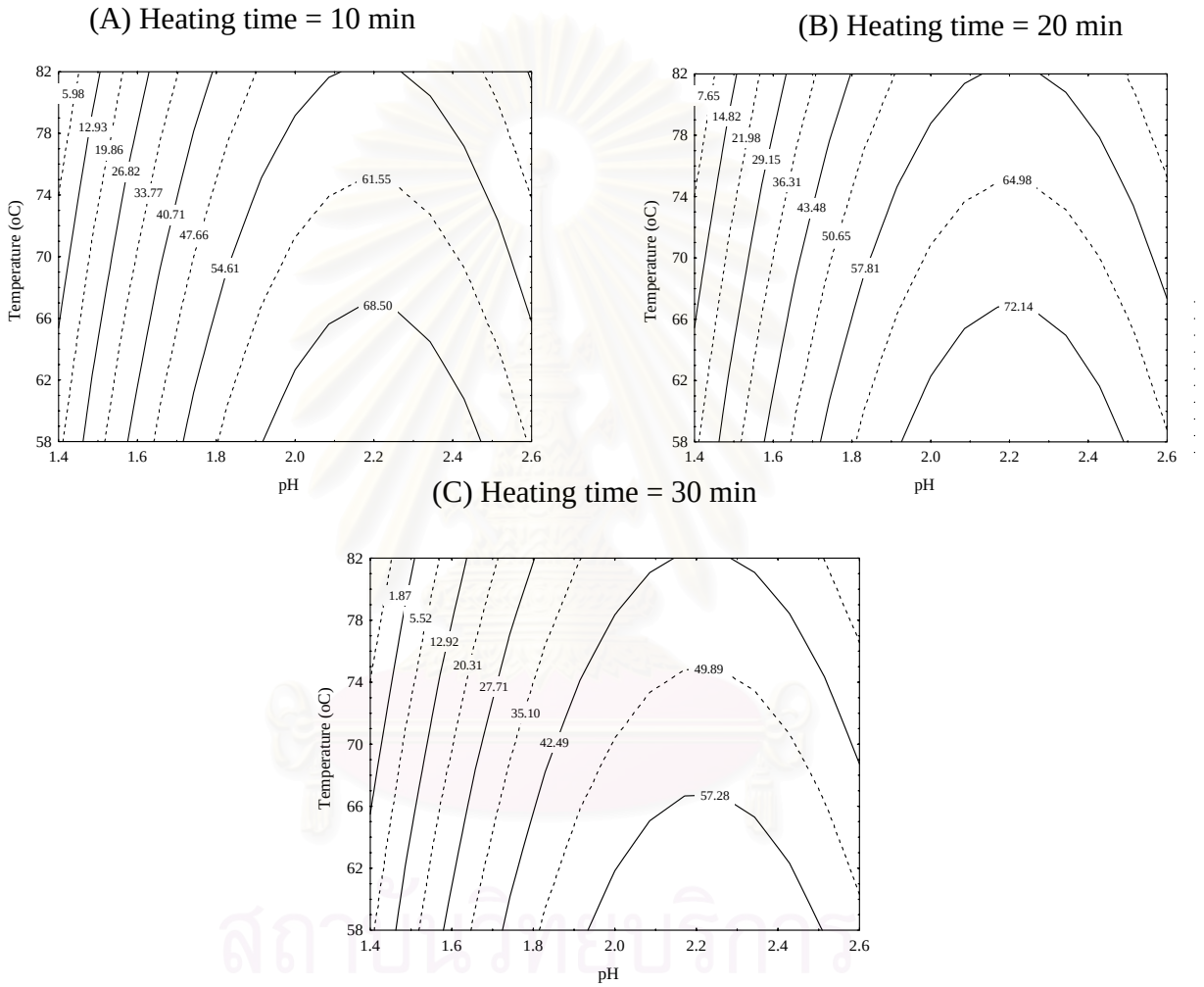


Fig. 4.39 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent elongation at break (%) of film at given heating time.

4.3.1.3 Water Vapor Permeability

Water vapor permeability is an important property that greatly utilized in food systems (Gnanasambandam *et al.*, 1997). Since a main function of an edible films or coatings are often to impede moisture transfer between food and the surrounding atmosphere, or between two components of a heterogeneous food products, water vapor permeability and oxygen permeability should therefore be as low as possible. The main factor influencing water vapor permeability of edible films from proteins precipitated by shift the pH from surimi wash-water were pH, heating temperature and heating time (Table 8, Appendix A). The contour plots (Fig 4.40) were characteristic of the effects of these variables and showed that the water vapor permeability was high at pH around 1.5 (14.39-16.22 g.mm/m^2.d.kPa) and tended to decline when pH of film-solutions reached 2.0 (5.19-5.61 g.mm/m^2.d.kPa). However, the water vapor permeability increased again when pH was adjusted to 2.5 (11.58-12.86 g.mm/m^2.d.kPa). These results could arise from the fact that, at lower pH (2.0) protein can unfold and solubilize. This phenomenon facilitated favorable molecule orientation and formation of SS bond by SH/SS interchange and thiol oxidation reactions and this could have promoted higher protein-protein net work (Donovan and Mulvihill, 1970; Shimada and Cheftel, 1988). Extreme pH (pH 1.5) of film-solutions as in this study might inhibit the film formations. Most likely, strong repulsive force between highly positive charges prevented protein molecules form associating and formation of films. Meanwhile, when pH of film-solutions was adjusted to 2.5 higher water vapor permeability could be observed as well. At the pH of film-solution closer to the isoelectric point of pH 3.5 (Bourtoom *et al.*, 2002), insufficient denaturation of proteins could prevail and resulted in less unfolding

and solubilizing of protein molecules. This phenomenon facilitated less molecule orientation and fine-stranded network (Banker *et al.*, 2000) provided higher water vapor permeability. The highest water vapor permeability was at the lowest pH and heating temperature of film-solutions of this study. The water vapor permeability was affected by heating temperature of the film-solution as well. The result of this study showed that increasing of heating temperature of film-solutions (60 to 80 ^{o}C) resulted in lower water vapor permeability (Fig. 4.40). The thermal energy might promote greater cross-link of protein- protein chains resulting in a tight and compact protein network and structure. Water vapor permeability was also affected by heating time of film-solutions. The result of this experiment demonstrated that increasing of heating time of film-solutions resulted in lower water vapor permeability (Fig. 4.40).

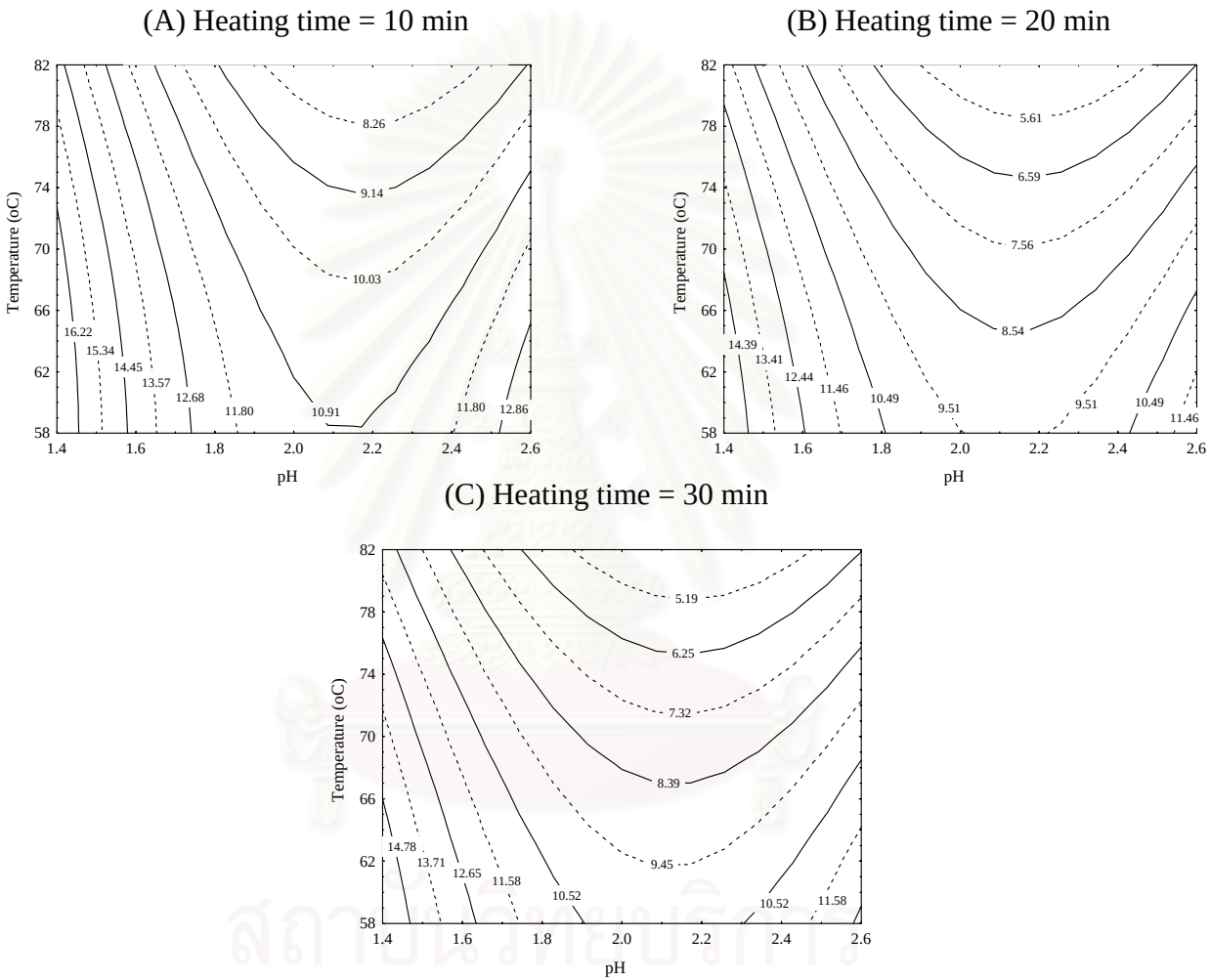


Fig. 4.40 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent water vapor permeability (g.mm/m^2.d.kPa) of film at given heating time.

4.3.1.4 Oxygen Permeability

The main factors impacting oxygen permeability of edible films from proteins precipitated by shifting the pH in surimi wash-water were pH, heating temperature and heating time (Table 8, Appendix, A). Within the same heating time, the result demonstrated that at the lowest pH (1.5) and heating temperature (60^{o}C) the oxygen permeabilities of the film were high (34.36-71.45 $cm^3.\mu m/m^2.d.kPa$). As heating temperature and pH increased, the oxygen permeability decreased and reached the minimum value (13.27-22.43 $cm^3.\mu m/m^2.d.kPa$) at pH around 2.2 and heating temperature around 74-80 ^{o}C (Fig. 4.41). The initial decrease in oxygen permeability with increase in pH and heating temperature might be due to the fact that the highest formation of protein-protein interactions was occurred at pH around 2.2 and the occurrence greater of cross linking among the protein molecules as a result of the increase thermal energy. However as the pH was adjusted to higher than 2.2 higher oxygen permeability was resulted. At the pH of film-solution closer to the isoelectric point of pH 3.5 (Bourtoom *et al.,* 2002), insufficient of unfolding and solubilizing of protein molecules might prevail. This phenomenon, facilitated less molecule orientation and fine-stranded network (Banker *et al.,* 2000) provided higher oxygen permeability, while extreme pH (pH 1.5) inhibited film formations, most likely strong repulsive forces between highly positive charges prevented protein molecules form associating and forming films.

Oxygen permeability was also affected by heating time, the lowest oxygen permeability was obtained at 20 min of heating time. At lower and higher time of heating, the extent of thermal energy might be too low and too high for the appropriate intensity of the protein unfolding solubilizing and the tight and compact

protein network and structure. Thus to maintain relatively low oxygen permeability, the pH around 2.2, heating temperature about 74-80 ^{o}C and the 20 min heating time were required.

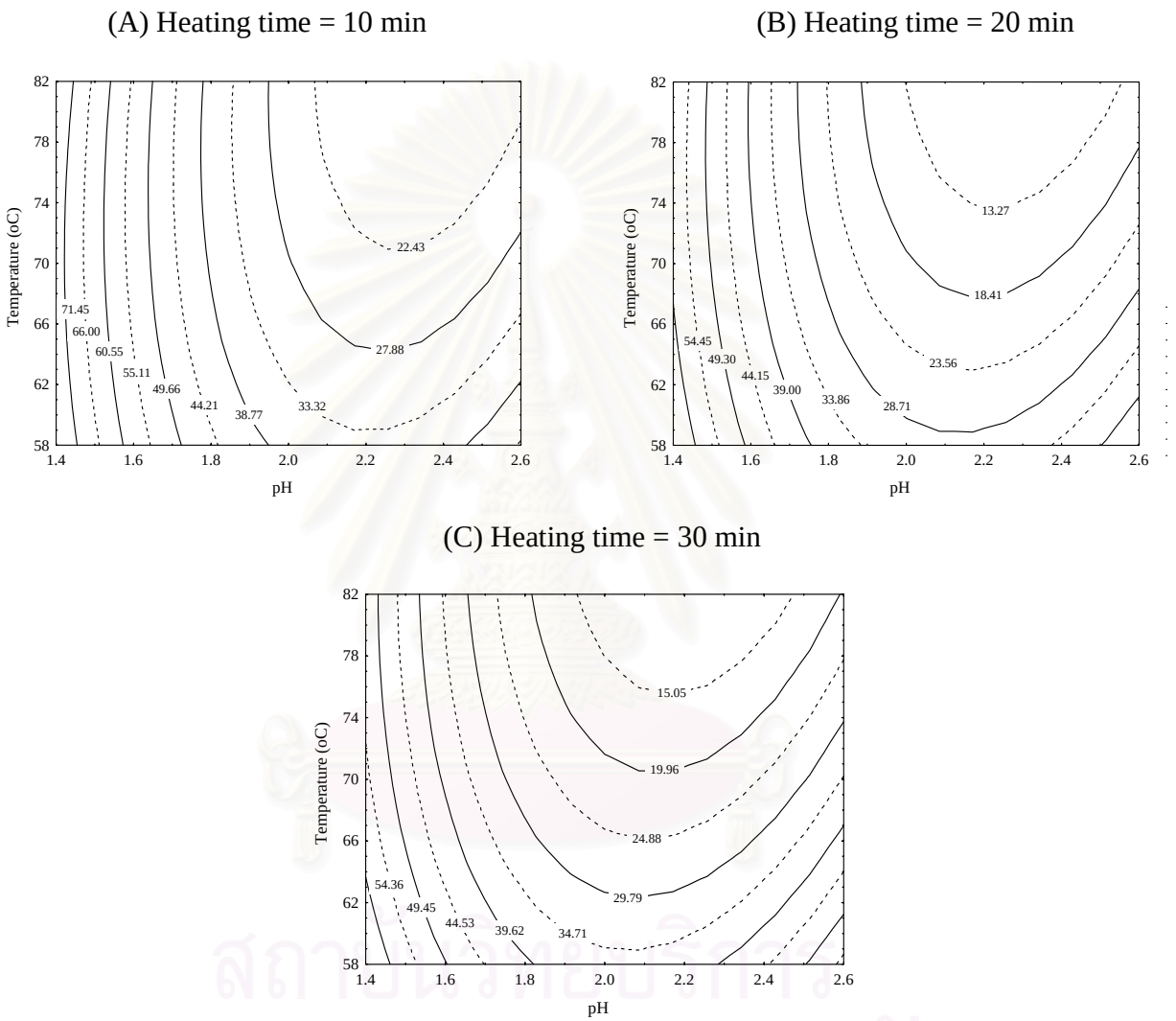


Fig. 4.41 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent oxygen permeability ($cm^3.\mu m/m^2.d.kPa$) of films at given heating time.

4.3.1.5 Film and Protein Solubilities

The solubility of edible films indicates their integrity in an aqueous environment. Generally, higher solubility would indicate lower water resistance. However, a high solubility may be an advantage for some applications. Films solubility is advantageous in situations when the films are to be consumed with a product that is heated prior to consumption and may also be an important factor that determines biodegradability of film when used as packaging wrap.

The values of film and protein solubilities increased dramatically when pH of film-solutions increased (Fig. 4.42 and 4.43) and the higher solubility were observed when pH of the film-solution was higher and lower than 2.0. Lower pH of film-solutions (< 2.0) facilitated dispersion in water and loosing of the film structure, causing dissolution of the non-protein materials (Gnanasambandam *et al*., 1997). Besides, higher pH of film-solutions (> 2.0) provided higher both film and protein solubilities, as discussed previously, adjusting of pH of film-solution closer to the isoelectric point of pH 3.5 (Bourtoom *et al.,* 2002), insufficient of unfolding and solubilizing of protein molecules was occurred, facilitated in lesser molecule orientation and fine-stranded network (Banker *et al.,* 2000) resulted in higher film and protein solubilities, however, when extreme pH condition was adjusted (pH 1.5) resulted in inhibition of film formations, most likely strong repulsive forces between highly positive charges prevented protein molecules from associating and film formation.

The contour plots of the effect of heating temperature and heating time of film-solutions on the films solubility and proteins solubility were shown in Figure 4.37 and 4.38. Increasing of heating temperature and time affected less to film and protein solubilities comparing to the pH. Irrespective to pH of film-solutions,

increasing of heating temperature and time resulted in decreases in film and protein solubilities. This was attributed to more pronounced heat-induced protein denaturation at higher temperature (Fukushima and Van Buren, 1970).

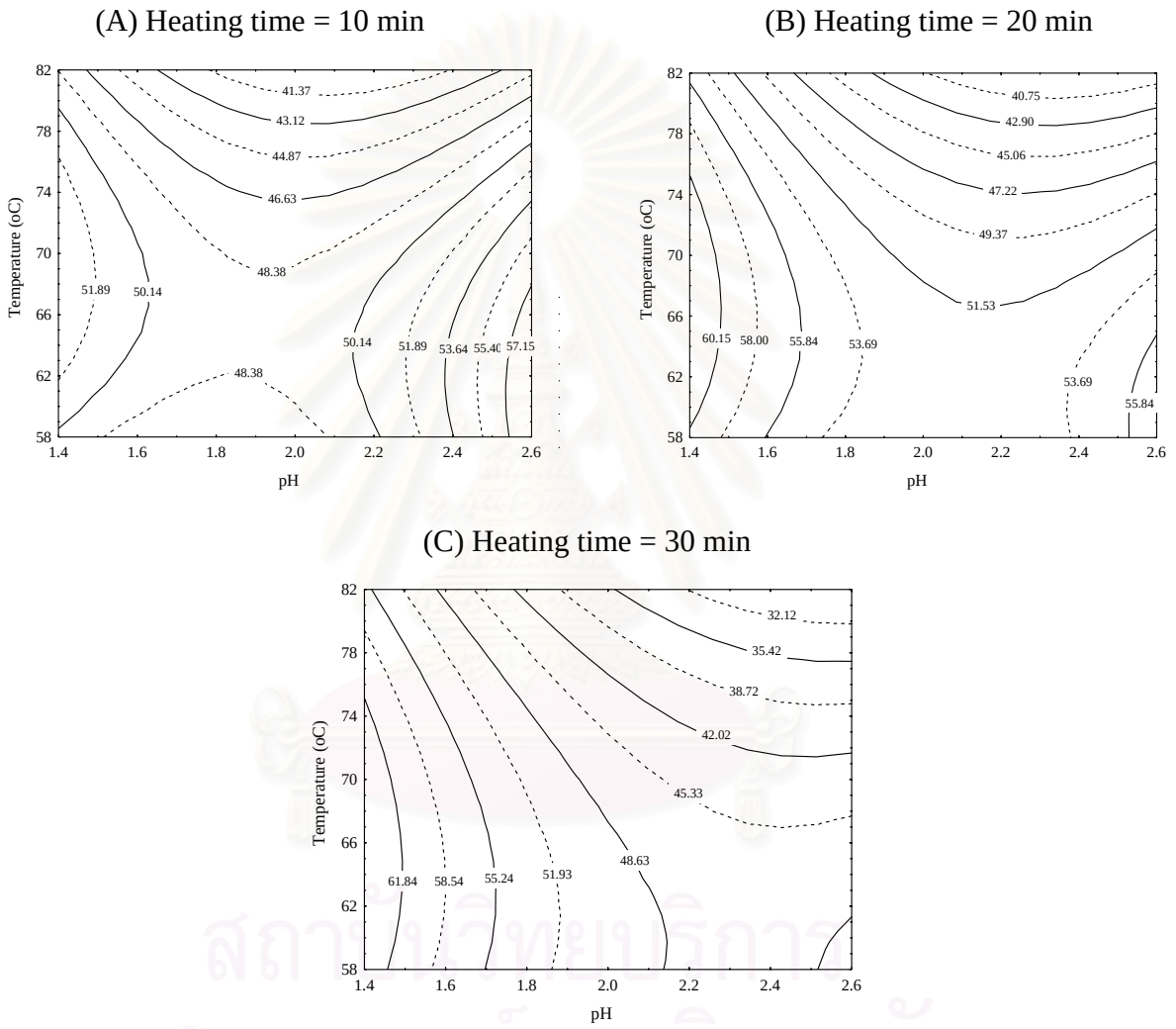


Fig. 4.42 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent film solubility (%) of films at given heating time.

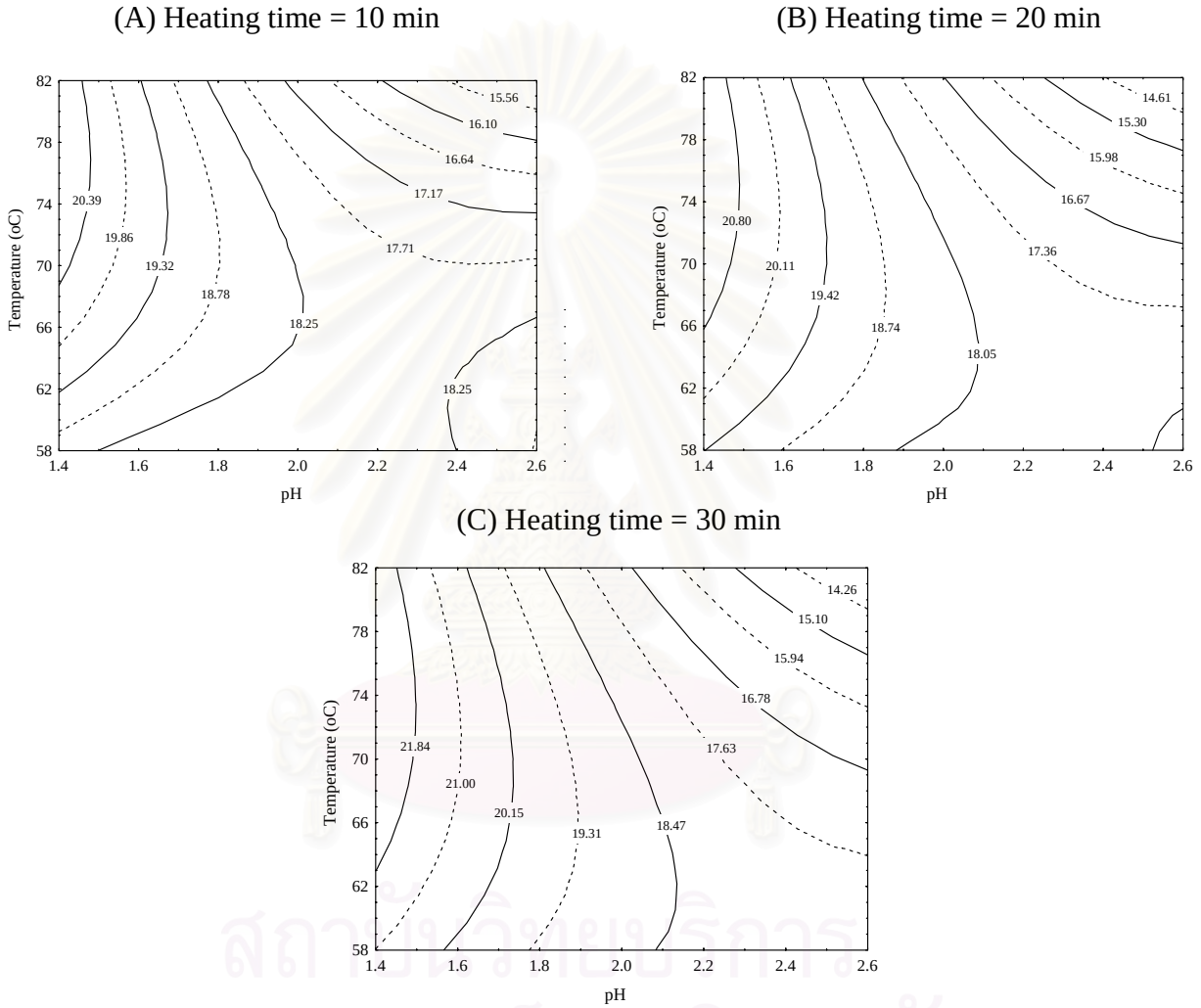


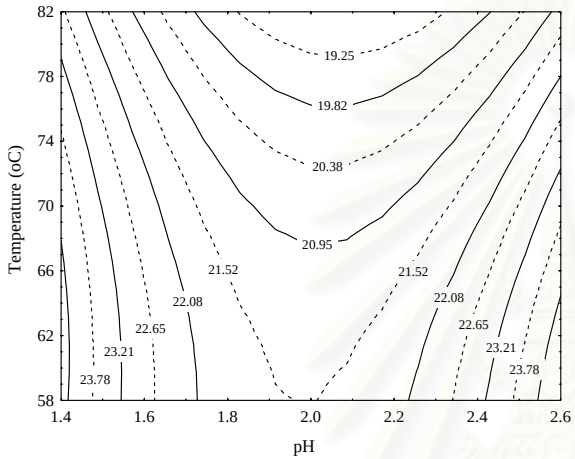
Fig. 4.43 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent protein solubility (%) of film at given heating time.

4.3.1.6 Film Color

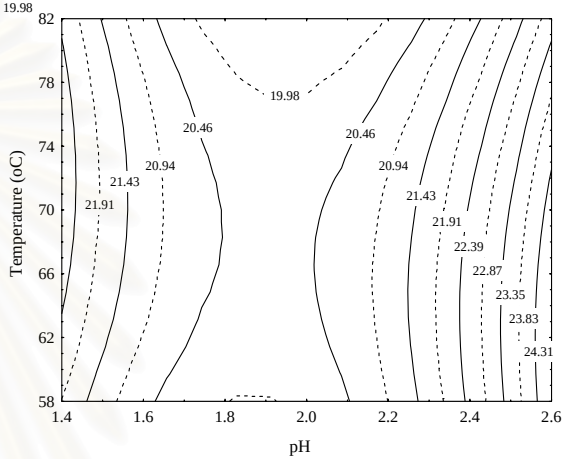
The results of the measurements performed on the films were expressed in accordance with the CIELAB system, and the rectangular coordinates (L*, a* and b*) were defined. The color of films was most affected by pH of film-solutions, while heating temperature and heating time affected less (Table 8, Appendix A). Films formed at higher pH and lower temperature were lighter yellow than films formed at lower pH and higher heating temperature. Instrumental color parameter L* value had a little increase with decreasing pH and increasing heating temperature of the film-solution (Fig. 4.44), however, value b* markedly increased with decreasing pH and increasing heating temperature of the film-solution (Fig. 4.46), and this made the film appear more yellowish. The value a* decreased as pH and heating temperature of the film-solutions decreased (Fig. 4.45).

The main factor influencing ΔE^*_{ab} of edible film from proteins precipitated by shifting the pH in surimi wash-water were pH, while hue angle and chroma, the pH and heating temperature of the film solutions was the most important factor (Table 4.8, Appendix A) . According to the model, ΔE^*_{ab} was plotted against pH and heating temperature at each heating times (Fig. 4.47), as can be seen, the pH and heating time of film-solutions were greater affected this variate than heating time. There appeared to be positive correlation between hue angle and pH of film-solutions but reversed with change in heating temperature (Fig. 4.48). Increasing in pH of film-solutions, concomitant with decrease heating temperature resulted in increased in chroma value (Fig. 4.49).

(A) Heating time = 10 min



(B) Heating time = 20 min



(C) Heating time = 30 min

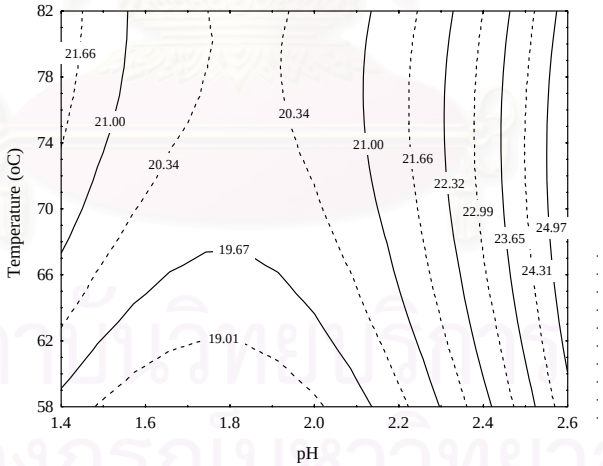


Fig. 4.44 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent L* value of films at given heating time.

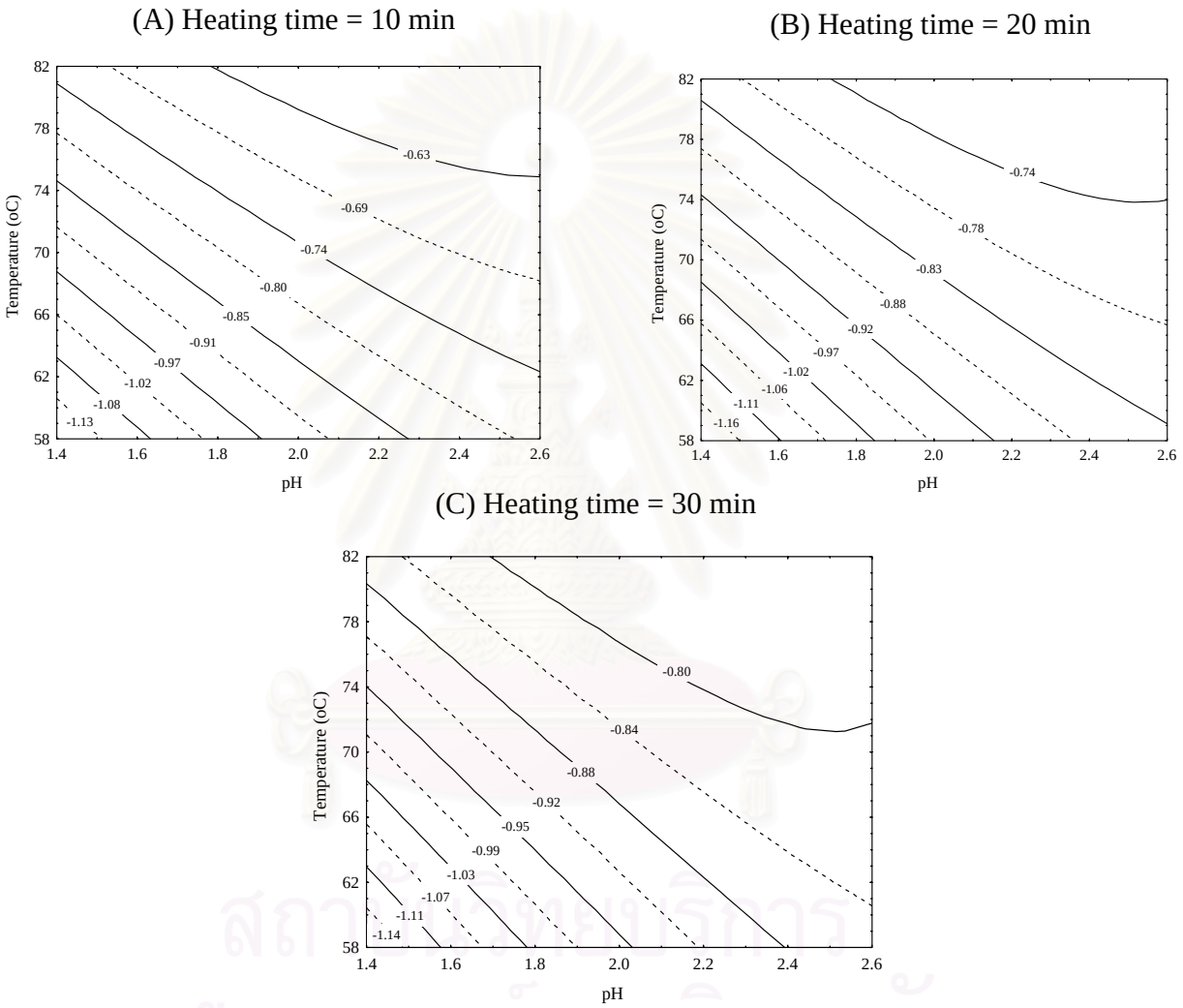


Fig. 4.45 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent a* value of films at given heating time.

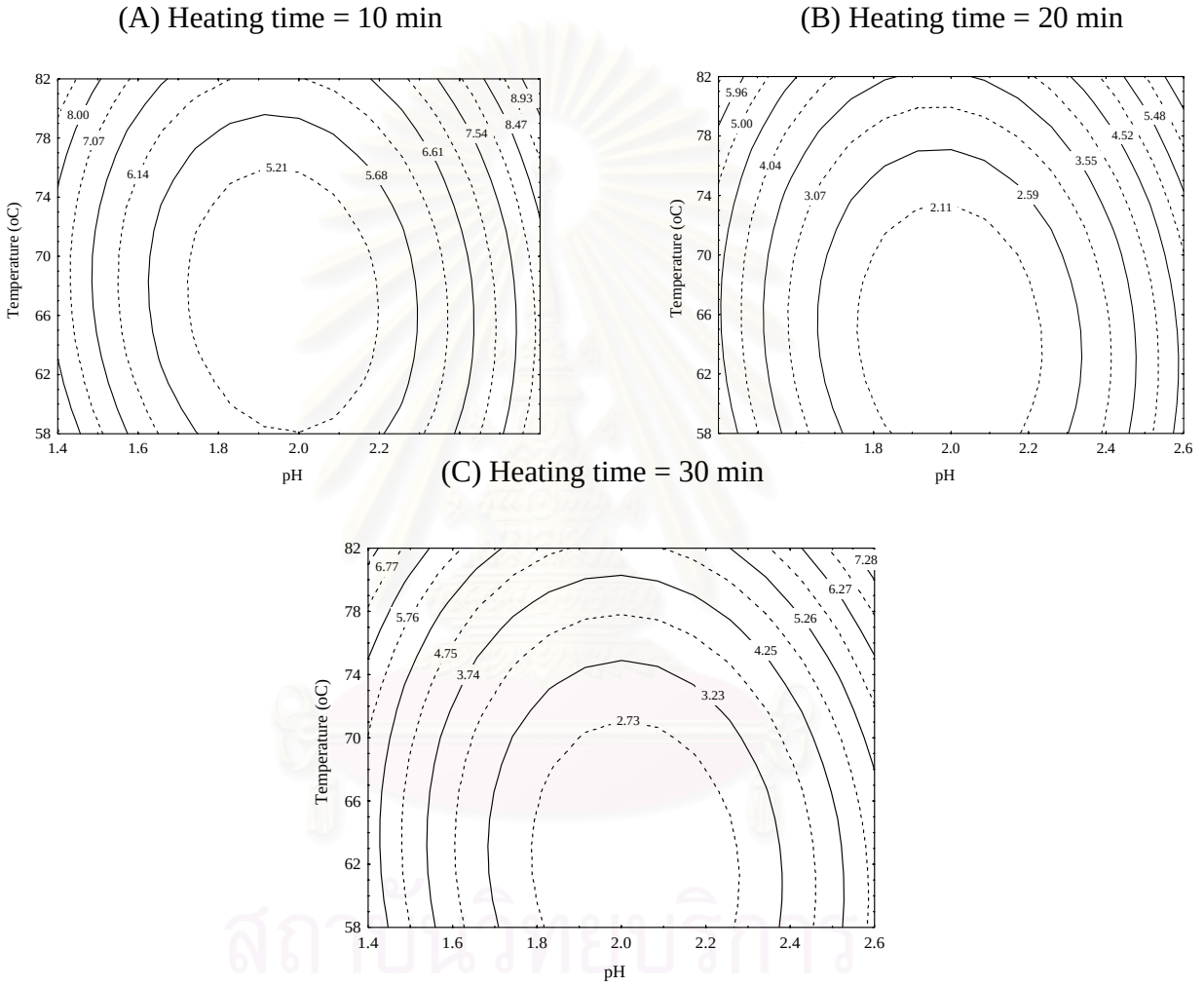


Fig. 4.46 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent b* value of films at given heating time.

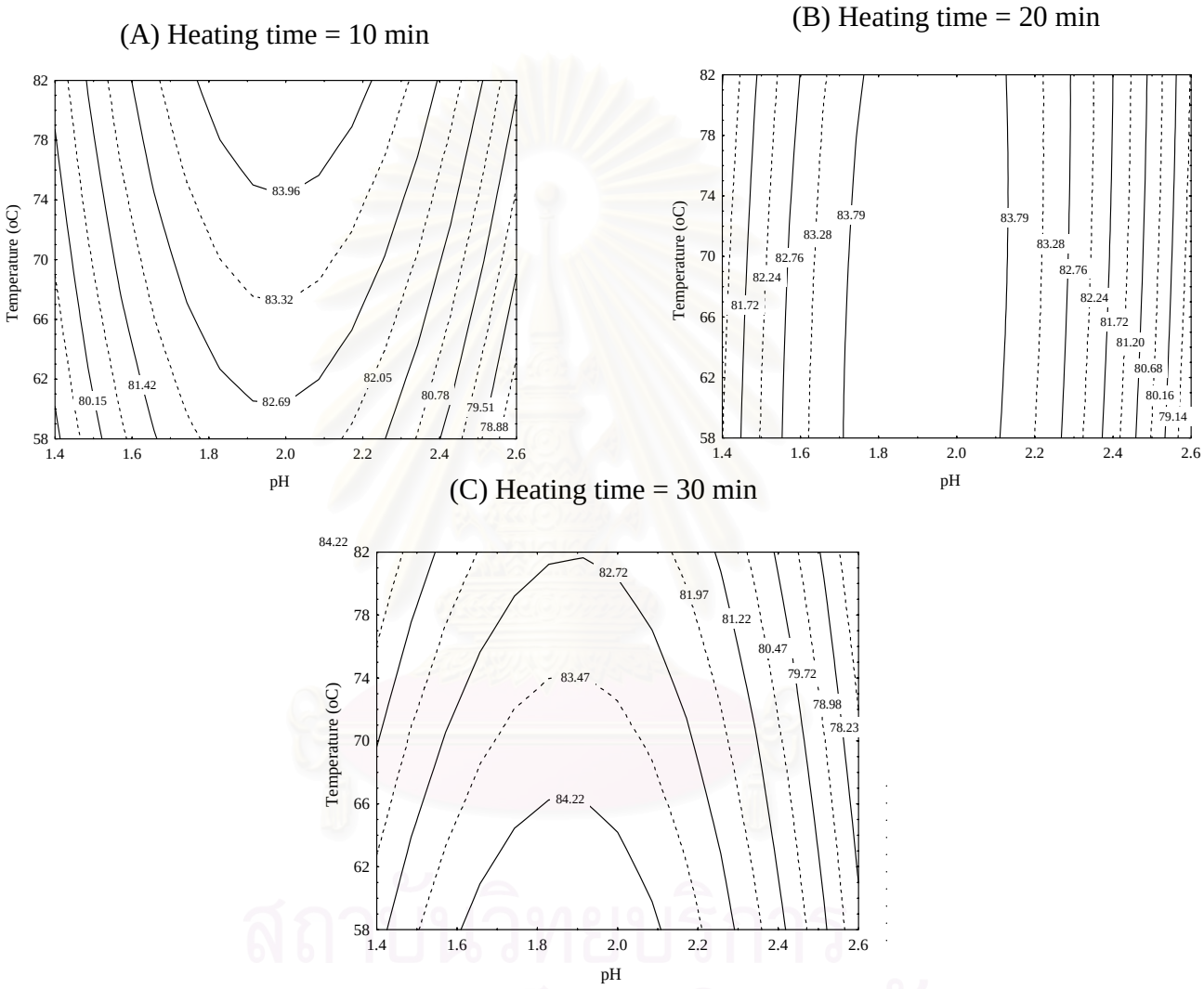


Fig. 4.47 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent ΔE^*_{ab} value of films at given heating time.

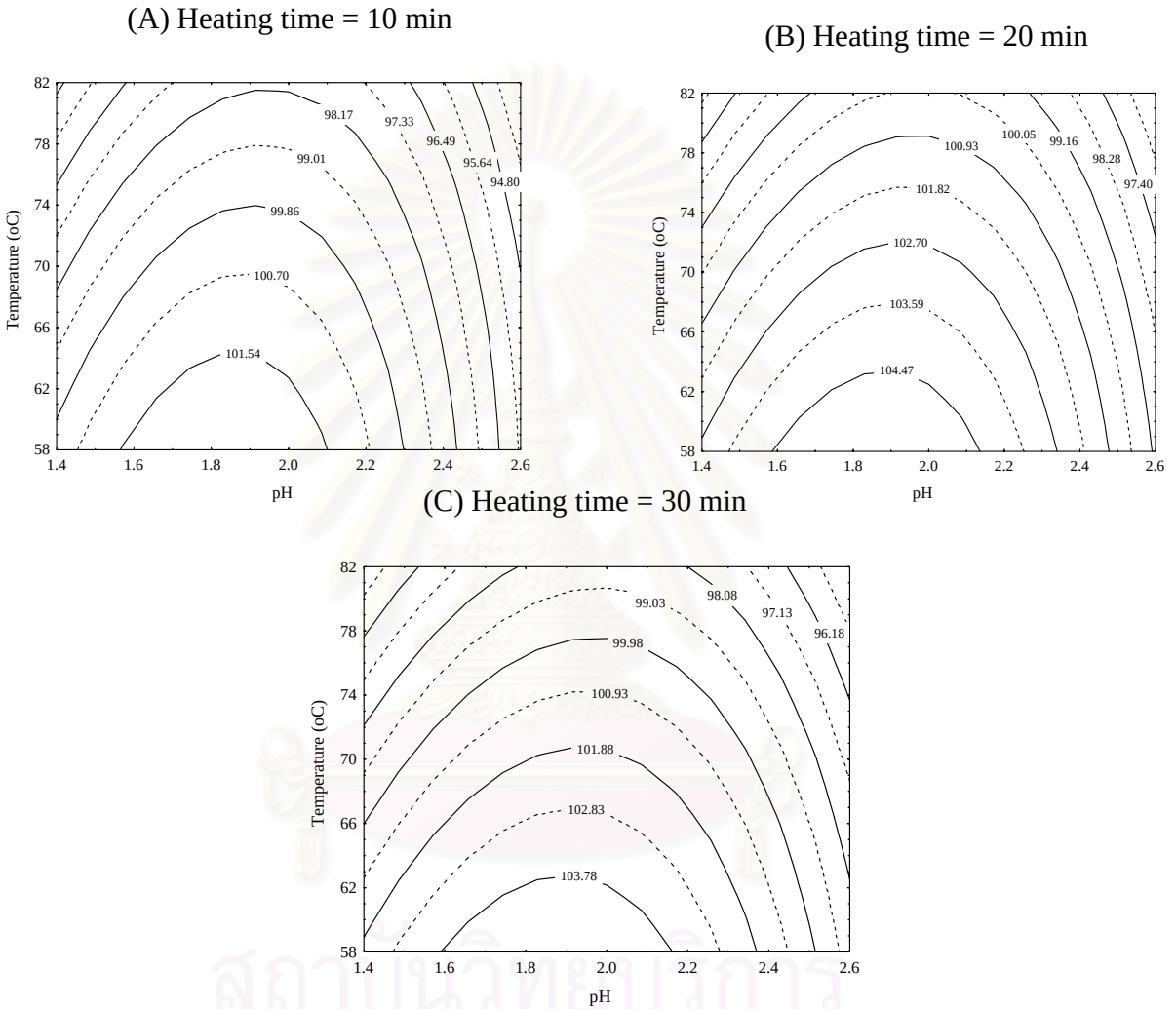


Fig. 4.48 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent hue angle value of film at given heating time.

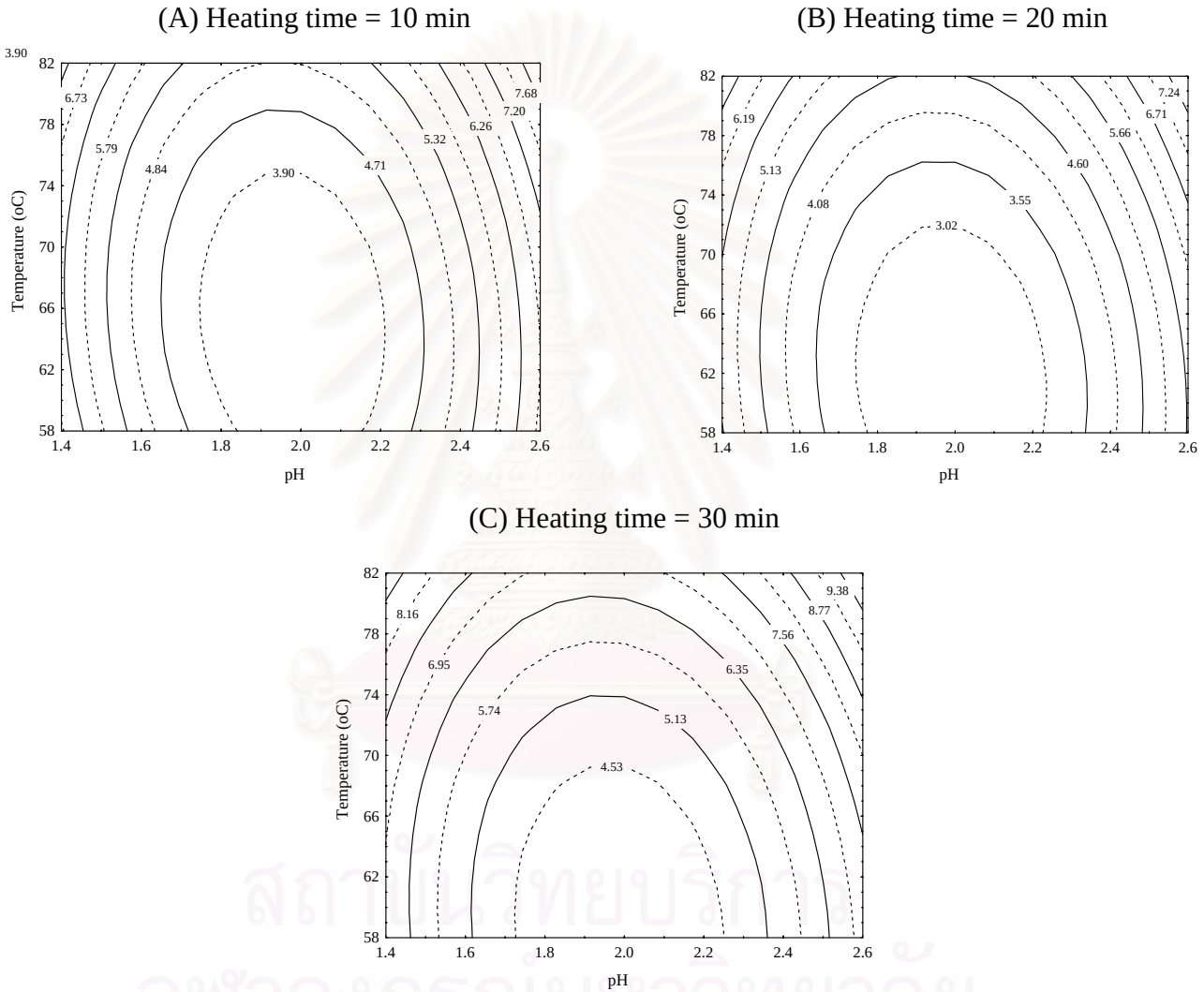


Fig. 4.49 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent chroma value of films at given heating time.

4.3.1.7 Surface Hydrophobicity, Available SH Group and Content of SS Bond

The main factors influencing content of surface hydrophobicity, available SH group and content SS bond were pH and heating temperature of the film-solution, while heating time was the lowest (Table 8, Appendix A). According to the result of this study, pH was the most important determining affects on the surface hydrophobicity, available SH group and content SS bond.

Figure 4.50 showed the contour plots of surface hydrophobicity of film-solutions as affected by pH, heating temperature and heating time. It was demonstrated that the surface hydrophobicity increased markedly with decreasing pH of film-solution from 2.5 to 1.5. Increase of the surface hydrophobicity could be due to increase of unfolded protein molecules resulting in increase exposure of hydrophobic groups (Iwata *et al.*, 2000). Irrespective to the pH of film-solutions, increasing heating temperature and time of film solutions resulted in increase in surface hydrophobicity. This was attributed to more pronounced heat-induced protein unfolding at higher temperature.

Changes of available SH groups of film-solutions as affected by pH, heating temperature and heating time were given in Figure 4.51. The highest amount of available SH groups were found at the lowest pH (1.5) and highest heating temperature. This tendency was quite similar to that of surface hydrophobicity shown in Figure 4.51, which suggested that protein molecules in film-solutions are unfolded by decreasing pH and heating temperature.

Changes of content of SS bonds of film-solutions were given in Figure 4.52. The content of SS bonds showed the lowest value as pH of film-

solutions was about 2.0 and tended to increase when pH of film solutions reached to 1.5 and 2.5 and it was noticed that heating temperature of film-solutions demonstrated similar trend with change of pH of film-solutions. This tendency suggested that protein molecules in film-solutions are unfolded by changing in pH and heating temperature.

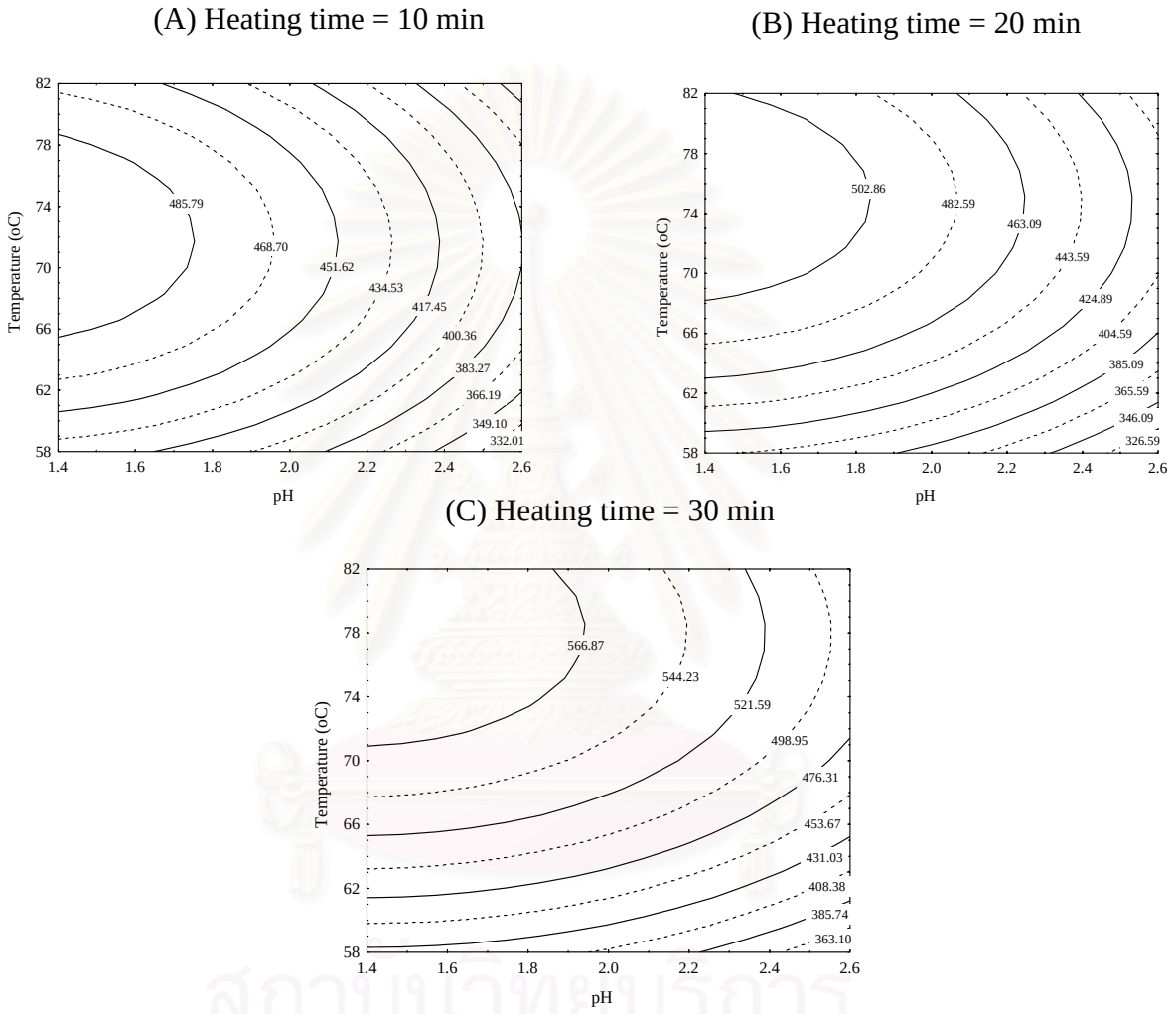


Fig. 4.50 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent hydrophobicity of film-solutions at given heating time.

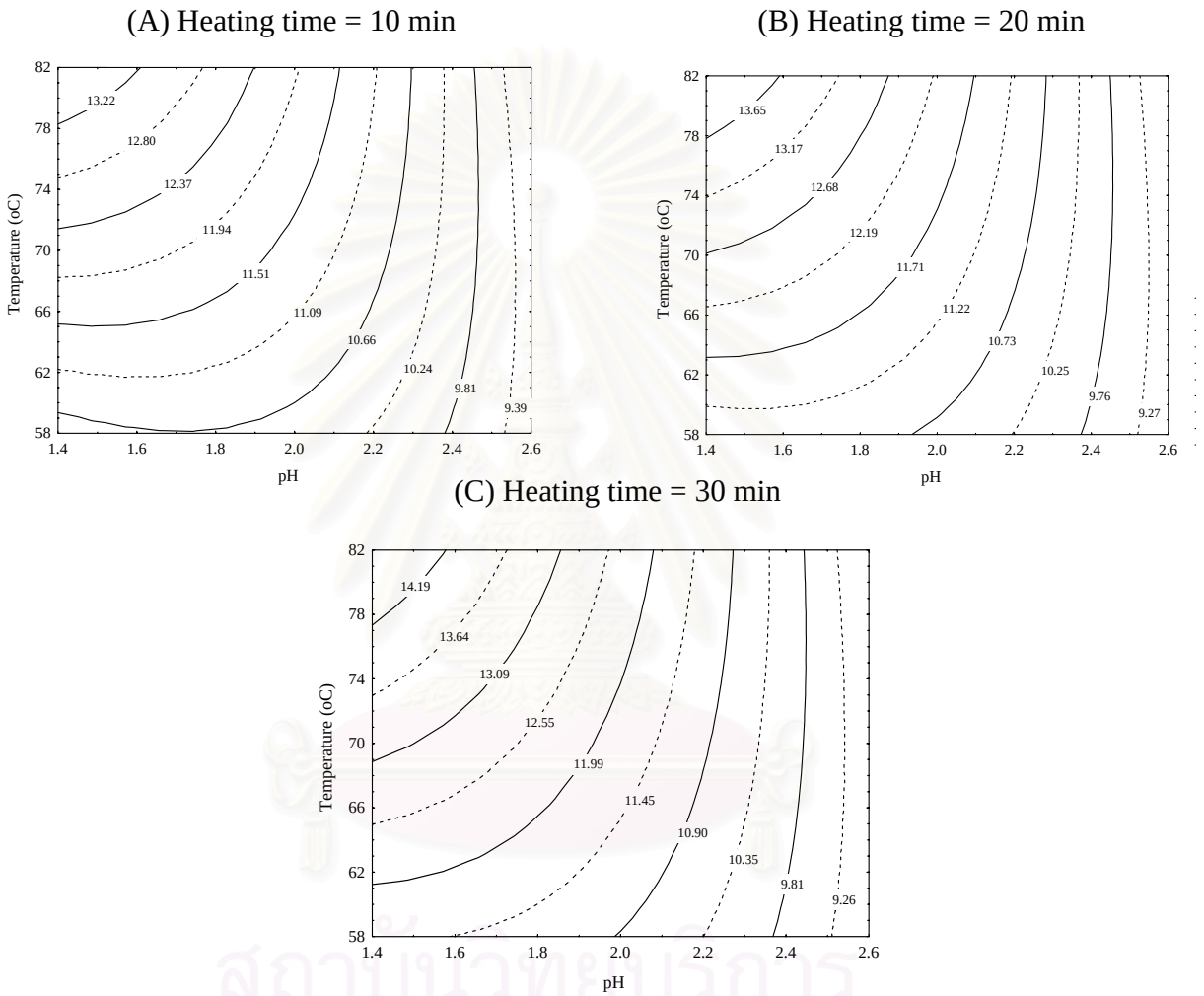


Fig. 4.51 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent available SH group of film-solutions at given heating time.

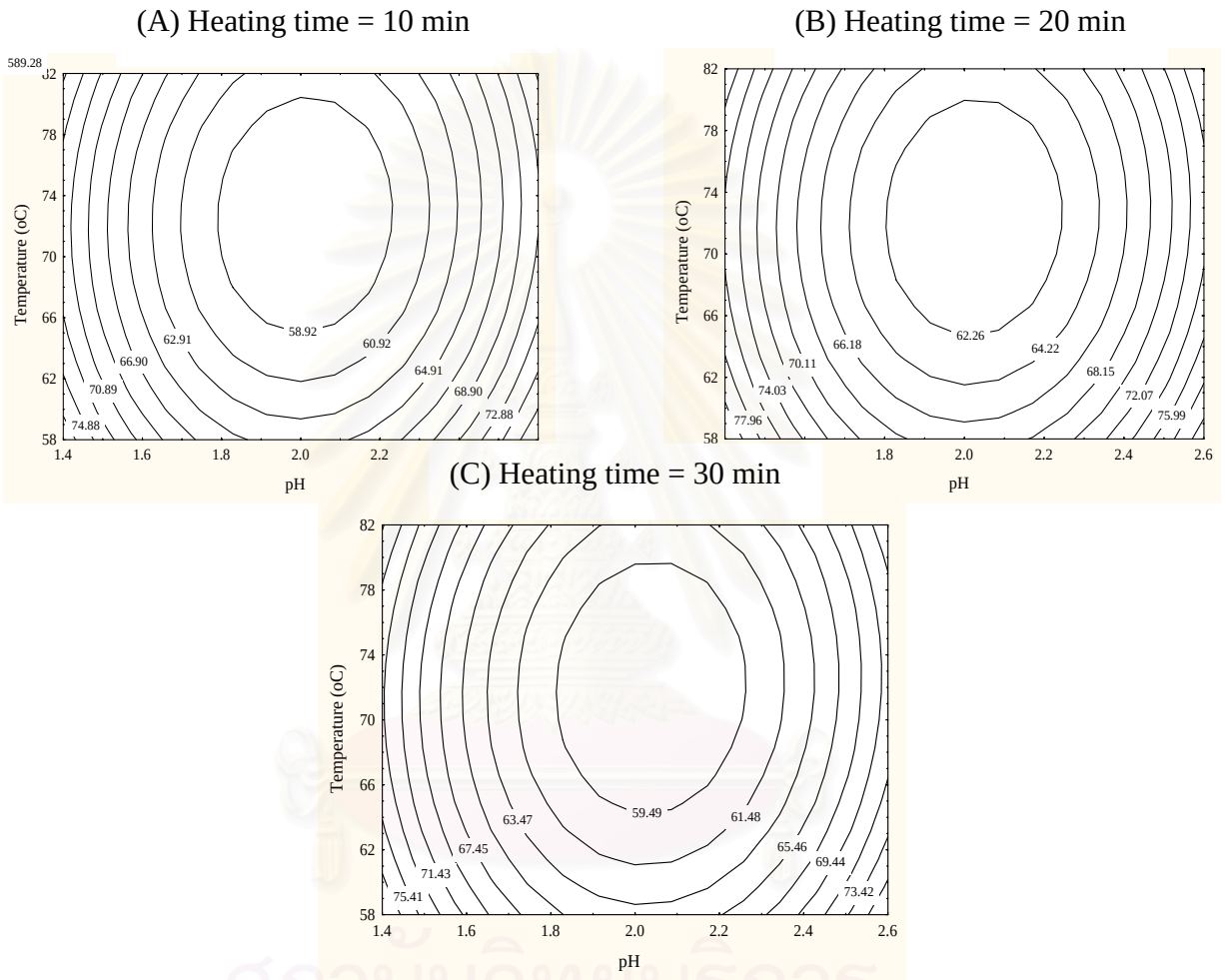


Fig. 4.52 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent content of SS bond of film-solutions at given heating time.

4.3.1.8 Localization of Optimum Conditions

To determine the optimum conditions of the selected parameters on the properties of edible films from proteins precipitated by shifting the pH from surimi wash-water, the graphical method used in RSM was employed. The variable tensile strength, water vapor permeability and oxygen permeability were considered the most important of the 15 responses followed by elongation at break. The contour plots in Figure 4.53 were obtained from the predictive model of tensile strength, elongation at break, water vapor permeability and oxygen permeability at 20 min of heating time, respectively. Plot of Figure 4.54 were superimposed over those of Figure 4.53 (A), 4.53 (B), 4.53 (C) and 4.53 (D) to locate regions of the highest of tensile strength, elongation at break and the lowest water vapor permeability and oxygen permeability. The shaded area in Figure 4.54 satisfied the specified constraints. As shown, the optimum conditions for edible films from proteins precipitated by pH shift in surimi wash-water at shaded area are: pH of film-forming solution of 2.15 and heating temperature of 80.86 ^{o}C for 20 min of heating time. At this combination, 4.83 MPa, 61.25 %, 6.95 g.mm/m^2.d.kPa, 11.20 cm^3.μm/m^2.d.kPa of tensile strength, elongation at break, water vapor permeability and oxygen permeability, respectively were resulted. Meanwhile, film solubility, protein solubility, hydrophobicity, available SH group and content of SS bonds were found to be 40.75%, 16.06%, 460.12, 11.44 and 60.61 μM SH/g protein, respectively.

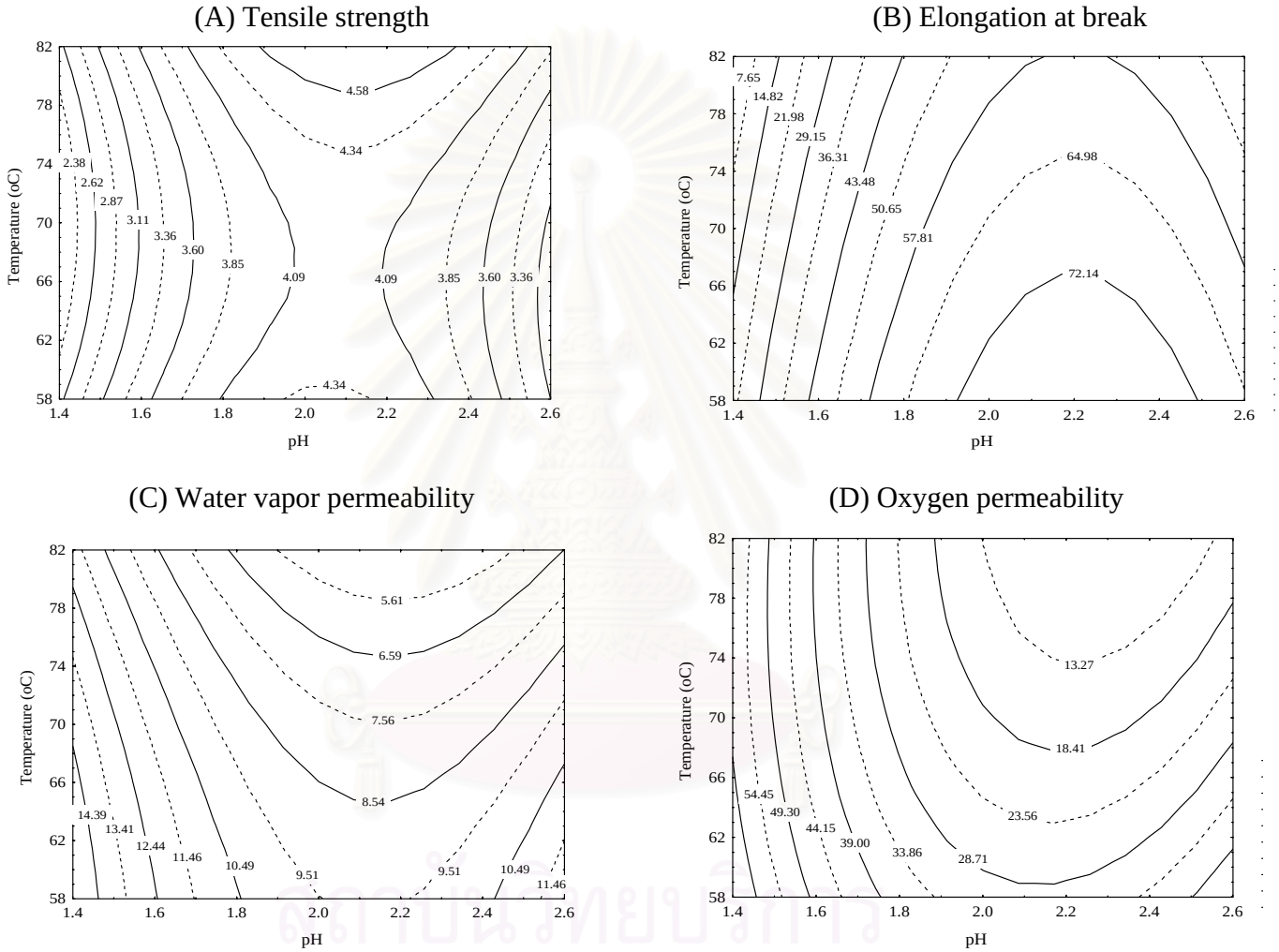


Fig. 4.53 Contour plots showing response behavior of pH and heating temperature of film-solutions heated for 20 min on the; tensile strength (MPa), elongation at break (%), water vapor permeability (g.mm/m^2.d.kPa) and oxygen permeability (cm^3.μm/m^2.d.kPa) before superimposition.

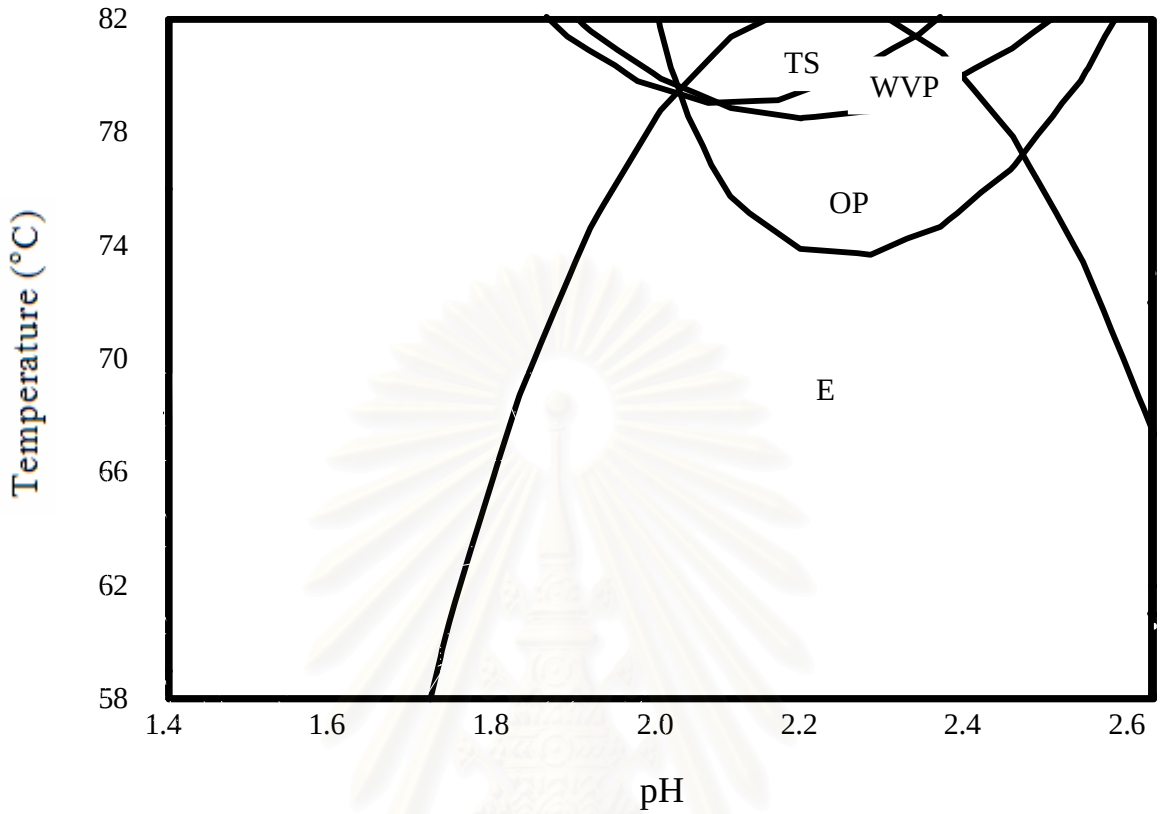


Fig. 4.54 Optimum film condition as a function of the independent variables after superimposition of contour plots over those of 4.53(A),4.53(B), 4.53(C) and 4.53(D). Shaded area indicates regions the highest of tensile strength (TS), elongation at break (E) and the lowest water vapor permeability(WVP) and oxygen permeability (OP).

Validation tests were performed to determine the adequacy of the single order polynomial (SOP) model (Floros and Chinnan, 1988; Mudahar *et al*., 1990). This was performed because a fractional factorial design was used as the experimental design. A model is deemed adequate if the predicted values (of the model) are close to the experimental values observed during the validation tests. Table 4.2 shows the predicted and observed values for the responses at optimum condition for the

selected parameters on the properties of edible films from water-soluble fish proteins precipitated by pH shift. The experimental values were averages of three replicates and were very close to the predicted values indicating that the SOP models generated were acceptable. The high CV values for some models were due to their lesser reproducibility (Montgomery, 1984) that may have contributed to the statistical insignificance of some of these models. Despite the lesser effect of these responses to the optimum conditions, predictions were within fairly acceptable limits.

Table 4.2 Predicted and observed values for the independent variables after superimposition conditions

Response variable	Predicted value	Actual value $\pm$ SD
Tensile strength (MPa)	4.83	5.24 $\pm$ 0.42 (8.01%)
Elongation at break (%)	61.25	56.50 $\pm$ 5.62 (9.94%)
Water vapor permeability ($g.mm/m^2.d.kPa$)	6.95	7.18 $\pm$ 0.64 (8.91%)
Oxygen permeability ($cm^3.\mu m/m^2.d.kPa$)	11.20	12.01 $\pm$ 0.72 (6.00%)

Number in parentheses are coefficients of variance (CV)

4.3.2 Effect of Protein Concentrations on the Properties of Edible Films from Proteins Precipitated by Shifting the pH from Surimi Wash-Water

The effect of protein concentrations on the tensile strength and elongation at break of edible films from water-soluble fish protein precipitated by shifting the pH were presented in Figure 4.55. Edible films with varying protein concentrations of 1.5, 3.0 and 4.5% at fixed pH 2.2, heating temperature of 80.9 ^oC and

20 min heating time were investigated. Varying the proteins concentration influenced of the tensile strength (Fig. 4.55). Tensile strength was significantly ($p \leq 0.05$) higher at the 4.5% of water soluble fish proteins (Fig. 4.55), this implied that higher protein content induced favorable structure regarding the ability of the films to form. However, there was no significantly ($p > 0.05$) different of tensile strength when 1.5 and 3.0% w/w of protein concentrations were employed. Changes in elongation at break of the film from water-soluble fish protein precipitated by shifting the pH as affected by content of proteins was given in Figure 4.55. The proteins concentration had significantly ($p \leq 0.05$) effect on elongation at break. Films prepared at 3.0 % w/w provided higher elongation at break. Reduces in elongation at break of the film formed at the lowest (1.5% w/w) and the highest protein concentration (4.5%) were observed indicating that different protein net work was formed. The lowest protein concentration provided less protein-protein interaction, while the highest protein concentration (4.5% w/w) yielded less order of the protein network due to the enhanced and fast gelling that occur at high protein concentrations (Anker *et al.*, 1999).

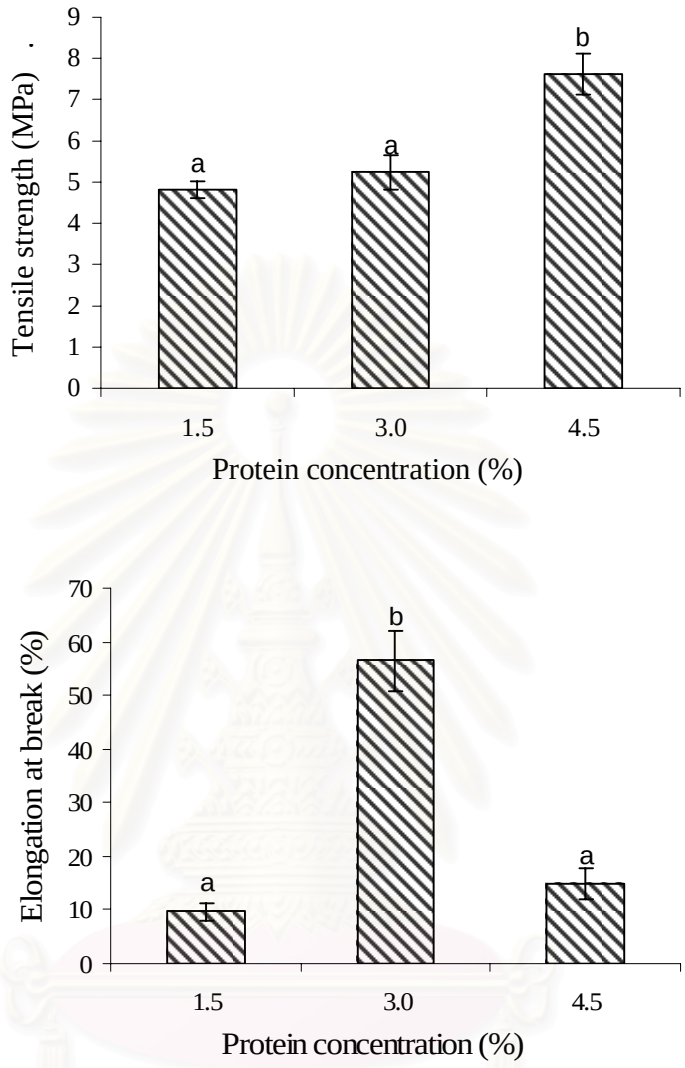


Fig. 4.55 Effect of protein concentration on the tensile strength (MPa) and elongation at break (%) of edible films from water-soluble fish proteins precipitated by shift the pH. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

Water vapor permeability and oxygen permeability of the film from proteins precipitated by shifting the pH in surimi wash-water at 1.5, 3.0 and

4.5% w/w of precipitated protein were investigated (Fig. 4.56). The result shows that the protein concentrations had significantly ($p \leq 0.05$) effect on water vapor permeability and oxygen permeability (Fig. 4.56). It was observed that higher protein concentration (3.0 and 4.5% w/w) provided both lower water vapor permeability and oxygen permeability. This result might be due to the fact that, increasing of the protein concentration, a more aggregated structure was formed, with a denser protein structure (McHugh and Krochta, 1994) resulting in increased obstacle to the water and oxygen molecule passing through the more closely packed protein network. This was in agreement with Pascat (1986) who reported that a higher density network decreased the permeability. Miller and Krochta (1997) further confirmed that the permeability was highly affected by how closely packed the polymer chains were. However, lower protein concentration provided lesser aggregated structure, with a loser protein structure, resulting in an increase in water vapor permeability and oxygen permeability. The result showed no significant ($p > 0.05$) different in water vapor permeability and oxygen permeability of the film when 3.0 and 4.5% w/w of proteins concentration were used.

Film solubility and protein solubility of edible films from proteins precipitated by shifting the pH in surimi wash-water were affected by protein concentration (Fig 4.57). Lower and above 3.0% w/w of protein concentration demonstrated significantly higher film and protein solubilities. It could be the result from less aggregated structure was formed, with a loser protein structure, provided an increased in film and protein solubilities when 1.5% w/w of protein concentration was used. While, using of protein concentration at 4.5% w/w, there was insufficient time for the unfolded protein molecules to rearrange in the most favored structure prior to

aggregation and led to poor protein structure network resulting in higher film and protein solubilities.

The color of edible film from protein precipitated by shifting the pH in surimi wash-water was affected by protein concentration, higher protein concentration resulted in significantly higher b* and chroma values, but lower in a* (Fig. 4.59 and 4.60), hence, the films color was darker and more yellow than lower content of protein. The lightness (L*) of films at various protein concentration was not significant different ($p \leq 0.05$) (Fig. 4.58).

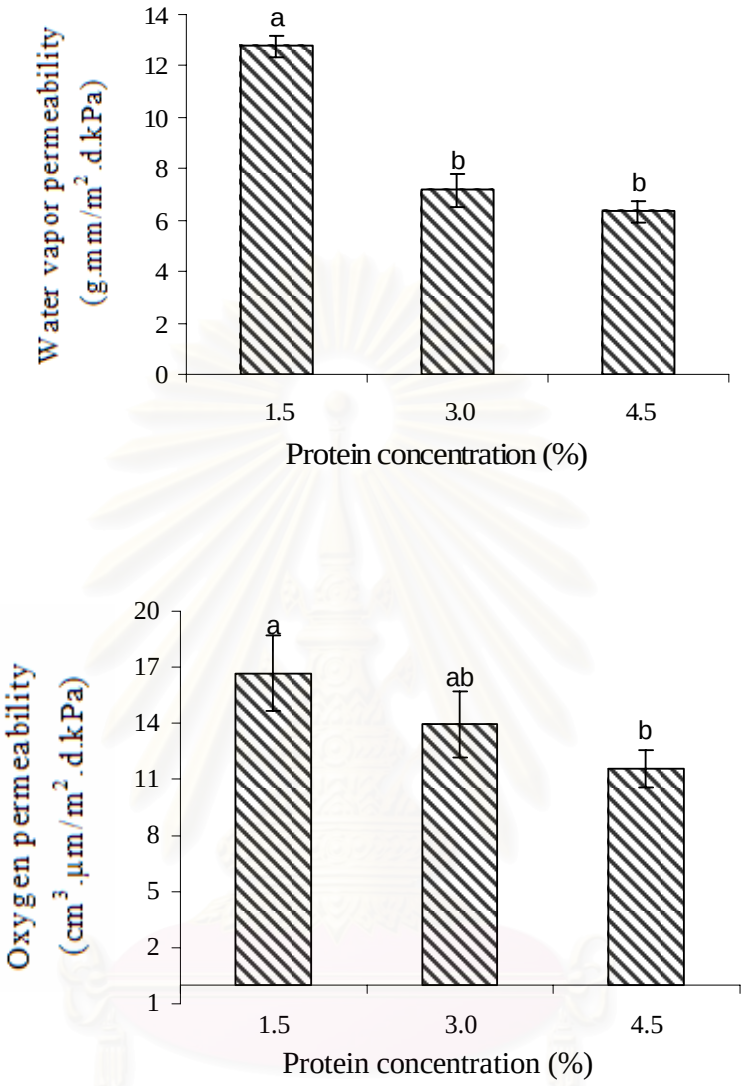


Fig. 4.56 Effect of protein concentration on the water vapor permeability (g.mm/m^2.d.kPa) and oxygen permeability (cm^3.µm/m^2.d.kPa) of edible films from water-soluble fish proteins precipitated by shifting the pH. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

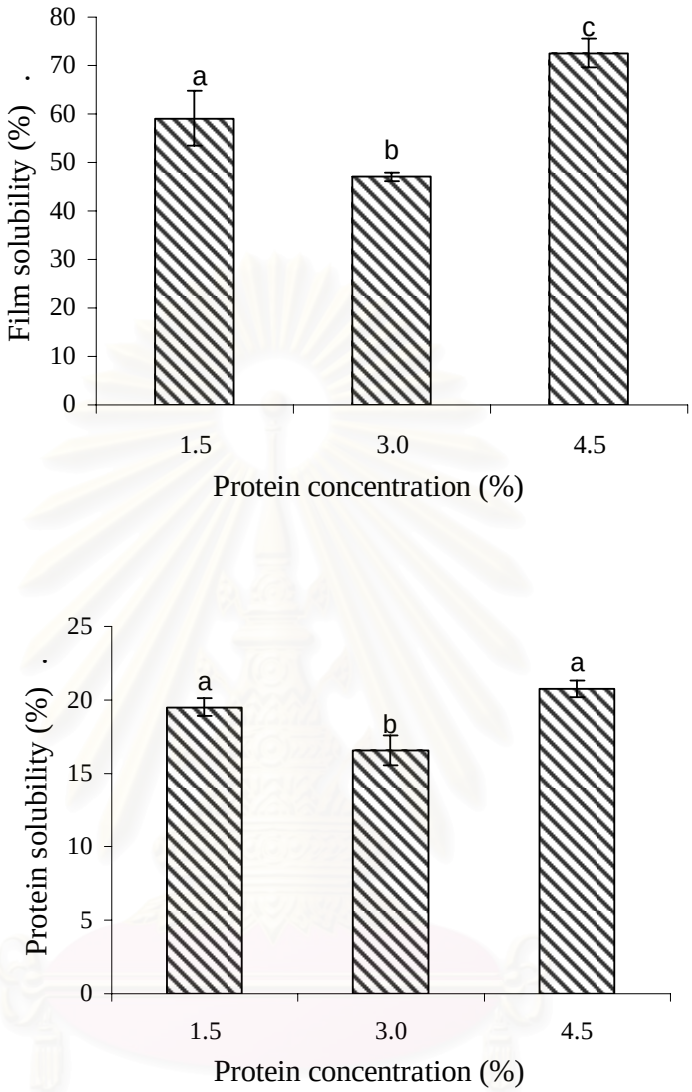


Fig. 4.57 Effect of protein concentration on the film solubility (%) and protein solubility (%) of edible films from water-soluble fish protein precipitated by shifting the pH. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

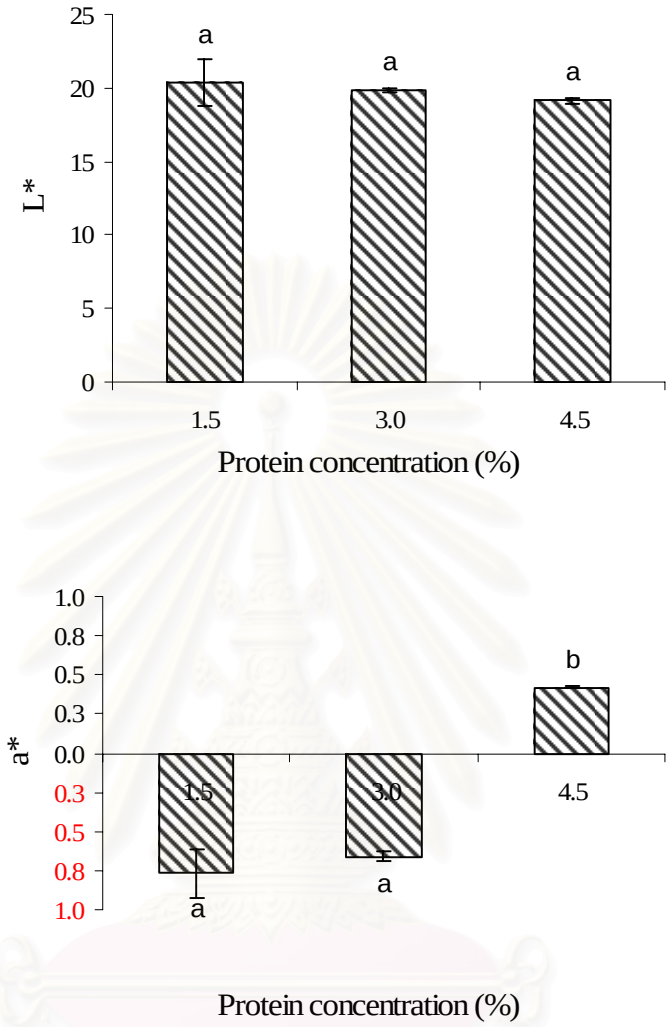


Fig. 4.58 Effect of protein concentration on the L* and a* values of edible films from water-soluble fish protein precipitated by shifting the pH. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

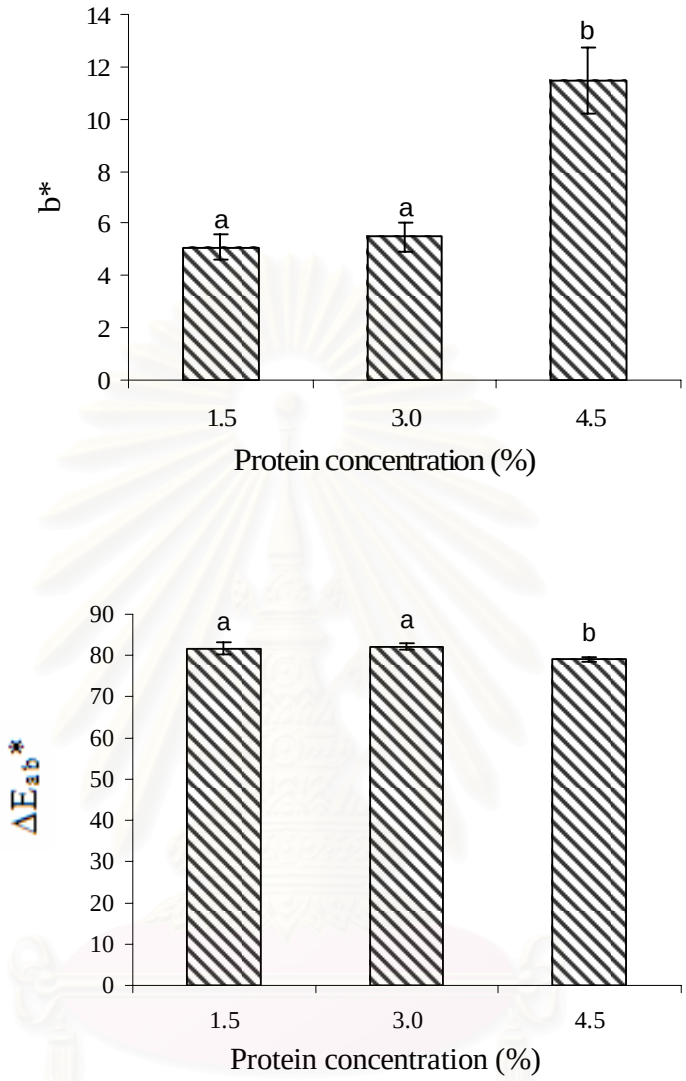


Fig. 4.59 Effect of protein concentration on the b* and ΔE_{ab}*values of edible films from water-soluble fish protein precipitated by shifting the pH. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

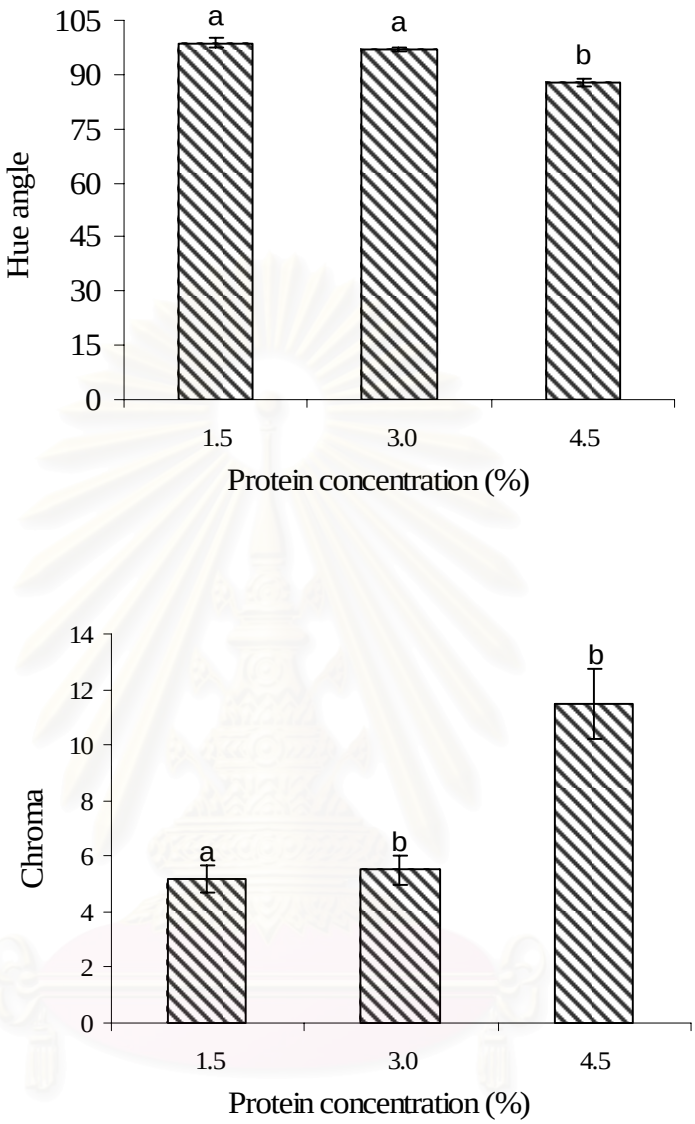


Fig. 4.60 Effect of protein concentration on the hue angle and chroma values of edible films from water-soluble fish protein precipitated by shifting the pH. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

4.3.3 Effect of Plasticizer Type and Concentration on the Properties of Edible Films from Proteins Precipitated by Shifting the pH from Surimi Wash-Water

The plasticizers which were selected in this study had been used to plasticize in protein and polysaccharides film based on compatibility (no phase separation). Our work found that the edible film from proteins precipitated by pH shift in surimi wash-water was compatible with all plasticizers in this study. The plasticizers represent different chemical compositions, sizes and shapes, thus providing the opportunity to explore the effects of these factors on film mechanical properties. Preliminary work demonstrated that the edible film from proteins precipitated by shifting the pH in surimi wash-water formed without plasticizer were relatively brittle and broken easily when peeled off.

4.3.3.1 Tensile Strength and Elongation at Break

The mechanical properties of edible films plasticized by sorbitol, glycerol or polyethylene glycol were assessed by measuring their tensile strength and elongation at break for three types of plasticizer (sorbitol, glycerol and polyethylene glycol) at different concentrations (25, 50 and 75 %). The results were shown in Figure 4.61. It was observed that an increase in content of these plasticizers resulted in decrease in tensile strength and increase in elongation at break. Tensile strength decreased dramatically from 7.07 to 4.40, 5.39 to 2.10 and 4.27 to 1.92 MPa when the sorbitol, glycerol and polyethylene glycol concentration increased from 25 to 75 % w/w, while, elongation at break increased from 20.30 to 66.19, 62.24 to 173.68 and 74.30 to 153.21%, respectively (Fig. 4.61). Gontard *et al.* (1993) observed a linear reduction of the mechanical resistance (puncture force) in gluten film, from 1.9 N to 0.3 N when glycerol

increased from 19 to 49 %. Cuq *et al*. (1997) also observed a linear reduction of the puncture force of edible films based on myofibrillar proteins of Atlantic sardine from 5.1 to 2.6 N, when glycerol increased from 0 to 40 g of glycerol/100 g of protein. The changes in mechanical properties as affected by hydrophilic plasticizers were previous observed for various hydrocolloid-based films (Park and Chinnan, 1990; Gontard *et al*., 1993). The mechanical property changes characterize decrease in density and reversibility of intermolecular molecular interaction occurring in the edible films from water-soluble proteins network. Sorbitol, glycerol and polyethylene glycol were low molecular weight hydrophilic molecules that could easily fit into protein chains and establish hydrogen bondings with reactive groups of proteins. Bringing together plasticizers and proteins induced formation of protein-plasticizer interactions to the detriment of protein-protein interactions. As a consequence, the density of intermolecular interaction decrease in material and the free volume between polymer chains increases (Cuq *et al*., 1997).

The mechanical properties of sorbitol, glycerol and polyethylene glycol plasticized films at an equal concentration were compared (Fig.4.61). The sorbitol plasticized films had significantly ($p \leq 0.05$) higher tensile strength and lower elongation at break. This could be attributed to the ring molecular conformation of sorbitol molecules, which may sterically hinder insertion between the proteins chains resulted in less effect in disrupting the protein-protein interruptions.

The glycerol plasticized films were more mechanical resistance and stretchable than the polyethylene glycol plasticized films (Fig. 4.61), suggesting that glycerol could be a more effective plasticizer in edible films from proteins precipitated by shifting the pH from surimi wash-water than polyethylene glycol.

Donhowe and Fennema (1993) reported that plasticizer with low molecular weights such as glycerol was more effective than those with high molecular weights (polyethylene glycol) in methylcellulose-based films. The effectiveness of glycerol in edible films from proteins precipitated by pH shift from surimi wash-water was most likely due to its small size which allows it to be more readily inserted between the polymer chains, and consequently exert more influence on the mechanical properties than the larger polyethylene glycol molecule. Similarly, McHugh and Krochta (1994a) suggested that, due to its small size of plasticizer was more effective than larger size of plasticizer in whey proteins films.

In addition, at an equal percentage concentration, the total number of glycerol molecules in the film-forming solution was greater than that of the higher molecular weight polyethylene glycol and therefore glycerol had more functional group (-OH) than polyethylene glycol which should promote the plasticizer-polymer interactions in the films (Donhowe and Fennema, 1993). Polar group (-OH) along plasticizer chains were believed to develop polymer-plasticizer hydrogen bonds replacing the polymer-polymer interaction in the biopolymer films (Gennadios *et al.*, 1993b). Molecular size, configuration and total number of functional hydroxide groups of the plasticizer as well as its compatibility with the polymer could affect the interactions between the plasticizer and the polymer (Yang and Paulson, 2000).

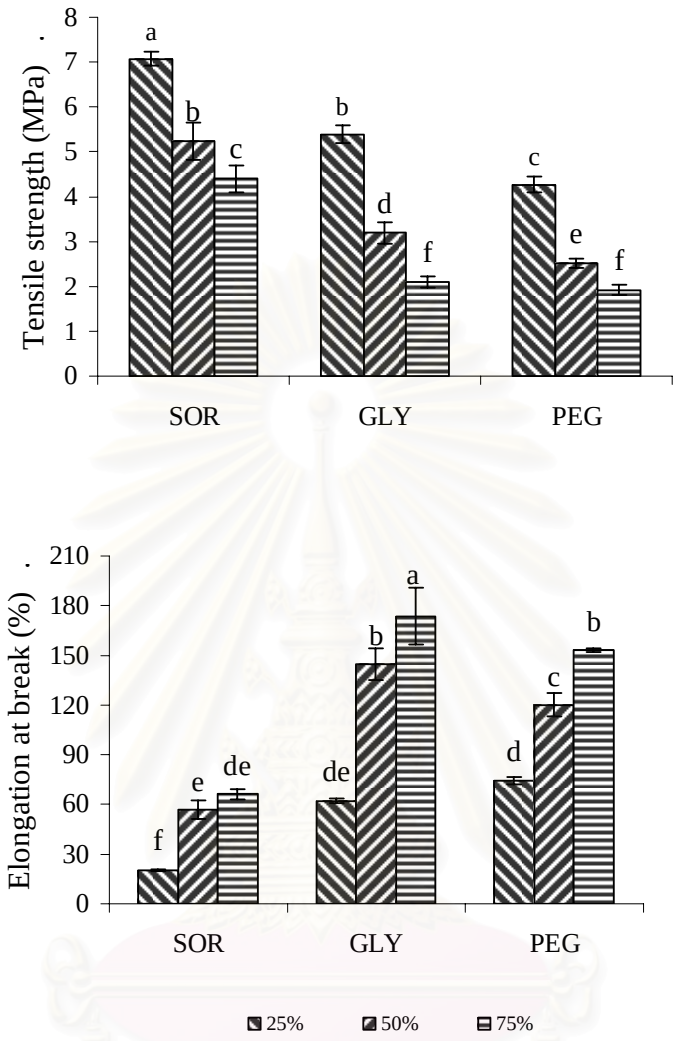


Fig. 4.61 Effect of plasticizer type and concentration on the tensile strength (MPa) and elongation at break (%) of edible films from water-soluble fish proteins precipitated by shifting the pH. Standard error bars are shown. a-f; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol.

4.3.3.2 Water Vapor Permeability and Oxygen Permeability

Water vapor permeability and oxygen permeability of edible films from proteins precipitated by shifting the pH from surimi wash-water with different type and concentration of plasticizer were examined (Fig. 4.62). The results demonstrated that water vapor permeability and oxygen permeability increased with increase of plasticizer concentration. The water vapor permeability and oxygen permeability of the film increased from 4.16 to 14.40, 37.44 to 156.70 and 25.30 to 120.30 g.mm/m^2.d.kPa and 10.37 to 41.30, 23.39 to 179.45 and 54.52 to 200.56 cm^3.µm./m^2.d.kPa, respectively, when the sorbitol, glycerol and polyethylene glycol concentration increased from 25 to 75 %w/w (Fig 4.62). An increase in plasticizer content enhanced the mobility of the polymer matrix thereby facilitating the water vapor or gas diffusion and permeation. Plasticization of the polymer leads to widening of the interchain hydrogen bonds, thus, facilitating permeation (Park *et al*., 1994). Banker (1966) reported that the plasticizer could retard or enhance moisture transmission depending on its concentration. Mahmoud and Savello (1992) also reported that plasticizer content influenced the water vapor permeability of whey protein isolate films. This tendency could be explained by structural modifications of the proteins network. The incorporation of plasticizers modifies the molecular organization of the protein network resulted in increase in free volume of protein network. Permeability increased with plasticizer content could be related to hydrophilicity of plasticizer molecules. Introducing hydrophilic plasticizers, favorable to adsorption and desorption of water molecules, was known to enhance the water vapor permeability of hydrocolloid-based films (Gontard *et al*., 1993; McHugh *et al.*, 1994a).

Comparison of the successive values of the water vapor permeability and oxygen permeability for each plasticized edible films were shown in Figure 4.62. Films plasticized with sorbitol had the lower water vapor permeability and oxygen permeability than glycerol and polyethylene glycol plasticized films due to the fact that sorbitol had ability to bind less water than glycerol and polyethylene glycol, thereby, provided a lower water vapor permeability and oxygen permeability. (McHugh *et al.,* 1994a). Chick and Ustanol (1998) showed that casein-based films plasticized with glycerol had higher water vapor permeability values than films plasticized with sorbitol when the same amounts of plasticizer were used. The high hydrophilicity of glycerol and polyethylene glycol molecules, which is favorable to the adsorption of water molecules, could also be contributed to the increase in the film water vapor permeability (Gennadios *et al*., 1993). The increase in water vapor permeability with increasing hydrophilicity plasticizer concentration is also common in edible films (McHugh *et al*., 1994a; Cuq *et al.*, 1997). Sorbal *et al.* (2001) reported that hydrophilicity of the plasticizers will increase the water content of the films, consequently increasing the mobility of the molecules. In addition, increasing water content could also affect permeant solubility in the films. Effect of edible films plasticized by glycerol and polyethylene glycol on water vapor permeability and oxygen permeability were compared (Fig. 4.62). Water vapor permeability of edible films plasticized by glycerol was significantly ($p \leq 0.05$) higher than those of edible films plasticized by polyethylene glycol which reversed with oxygen permeability (Fig. 4.62). It could be explained by the effect of the small size of glycerol which allows it to be more readily inserted between the polymer chains, and consequently exert more influence on oxygen permeability properties than the larger polyethylene glycol molecule. Moreover, comparing at an equal percentage concentration, the total

number of glycerol molecules in the film solutions is greater than that of the higher molecular weight polyethylene glycol and therefore glycerol has more hydrophilic group than polyethylene glycol which should promote the solubility and diffusivity of water through film structure resulted in higher water vapor permeability.

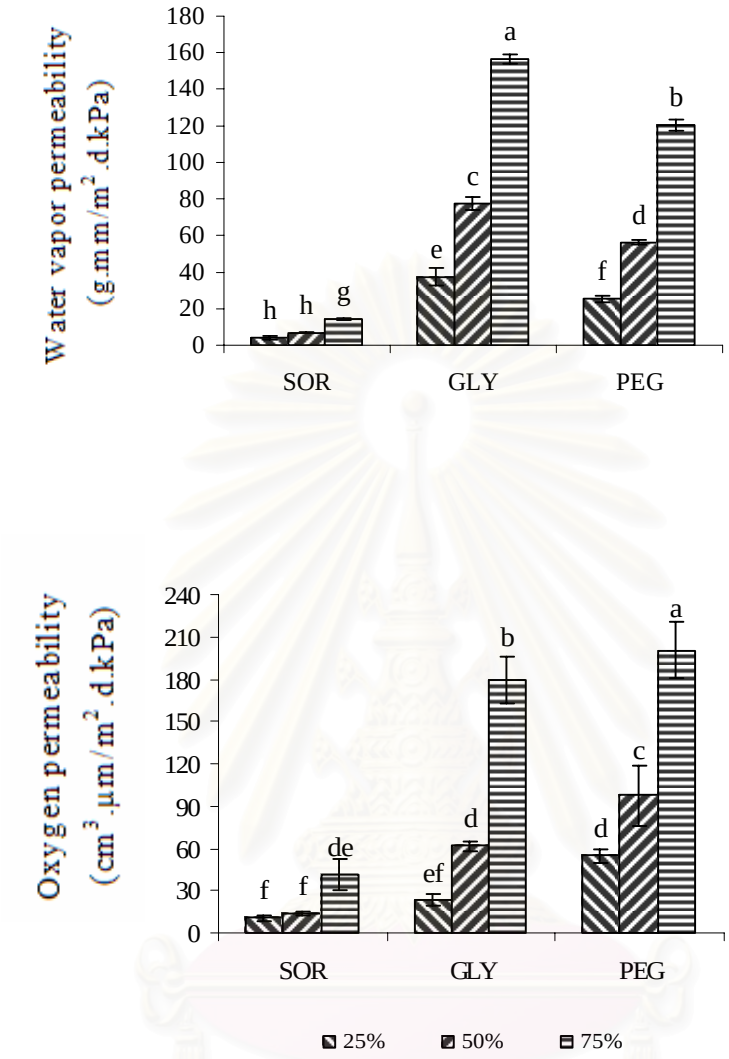


Fig. 4.62 Effect of plasticizer type and concentration on the water vapor permeability (g.mm/m^2.d.kPa) and oxygen permeability (cm^3.µm/m^2.d.kPa) of edible films from water-soluble fish proteins precipitated by shifting the pH. Standard error bars are shown. a-f ; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol.

4.3.3.3 Film and Protein Solubilities

Irrespective of type, an increase in plasticizer content led to an increase in film solubility and protein solubility (Fig. 4.63). It could be hastily concluded that hydrophilic plasticizers enhance films solubility in water. Low molecular weight protein chains (i.e. monomers and small peptides) formed during storage of film-solutions and entrapped in the network (Cuq *et al*., 1995) could thus constitute the protein-based materials that solubilize in water. The dry matter solubilized in water was likely to be constituted mainly by the plasticizer. Plasticizer solubilization in water was already observed for film based on wheat gluten or treated soy proteins or produced by transglutaminase catalytic cross-linking of whey protein (Gontard *et al.,* 1992; Stuchell and Krochta, 1994). Stuchell and Krochta (1994) had pointed out increase in the content of protein solubilized in water when the hydrophilic content increased for treated whey protein-and soy protein-based films. A decrease in the polymer network interaction density due to the presence of plasticizer was thus associated with this increase in solubility properties. The lowest film solubility and protein solubility of edible films plasticized by 25% w/w of these plasticizer were noticed, while increasing the amount of plasticizer resulted in higher film solubility and protein solubility (Fig. 4.63). It could be explained by the fact that at the higher content of plasticizer, there were more molecules of plasticizer untrapped in the cross linked network and able to escape into solution, while, lower content of plasticizer provided lowered plasticizer molecules untrapped in the cross linked network resulted in lesser ability to escape into solution. The film solubility and protein solubility of films plasticized by sorbitol and polyethylene glycol were significantly higher than those of glycerol. The sorbitol had a ring molecular conformation which may sterically hinder insertion between the protein chains (Yang and

Paulson, 2000) resulted in an easy to escape into solution, while polyethylene glycol had a larger molecular size than glycerol, which promoted less insertion between protein-protein chains.

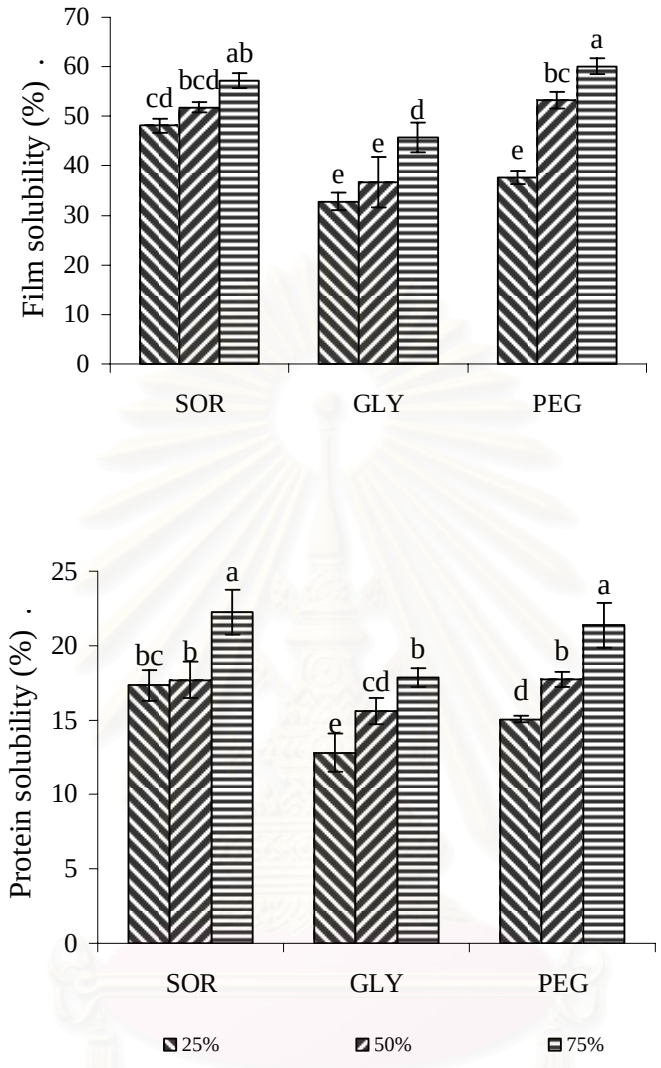


Fig. 4.63 Effect of plasticizer type and concentration on the film solubility (%) and protein solubility (%) of edible film from water-soluble fish proteins precipitated by shifting the pH. Standard error bars are shown.

a-e; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

4.3.3.4 Film Color

The results of the measurements performed on the films color were expressed in accordance with the CIELAB system, and the rectangular coordinates (L*, a* and b*) were defined. The color of films was more affected by the nature of the plasticizer than by its concentration. L* value of edible film from proteins precipitated by pH shift in surimi wash-water plasticized by sorbitol, glycerol and polyethylene glycol were not significantly different ($p > 0.05$) (Fig. 4.64). It was observed that a*, b* and chroma values of glycerol and polyethylene glycol plasticized films showed significantly ($p \leq 0.05$) higher than glycerol plasticized films (Fig. 4.65 and 4.66); hence, the films color was more yellow than sorbitol plasticzed films. It was somewhat expected since color change depend on the type of plasticizer.

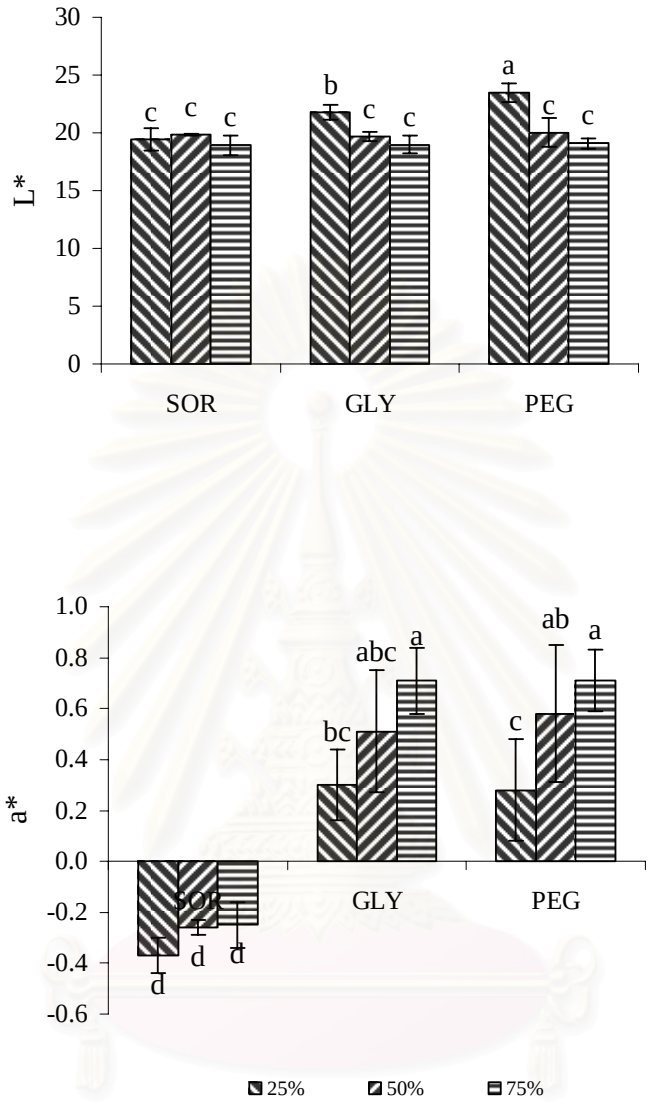


Fig. 4.64 Effect of plasticizer type and concentration on L* value and a* values of edible films from water-soluble fish proteins precipitated by shifting the pH in surimi wash-water. Standard error bars are shown.

a-d; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

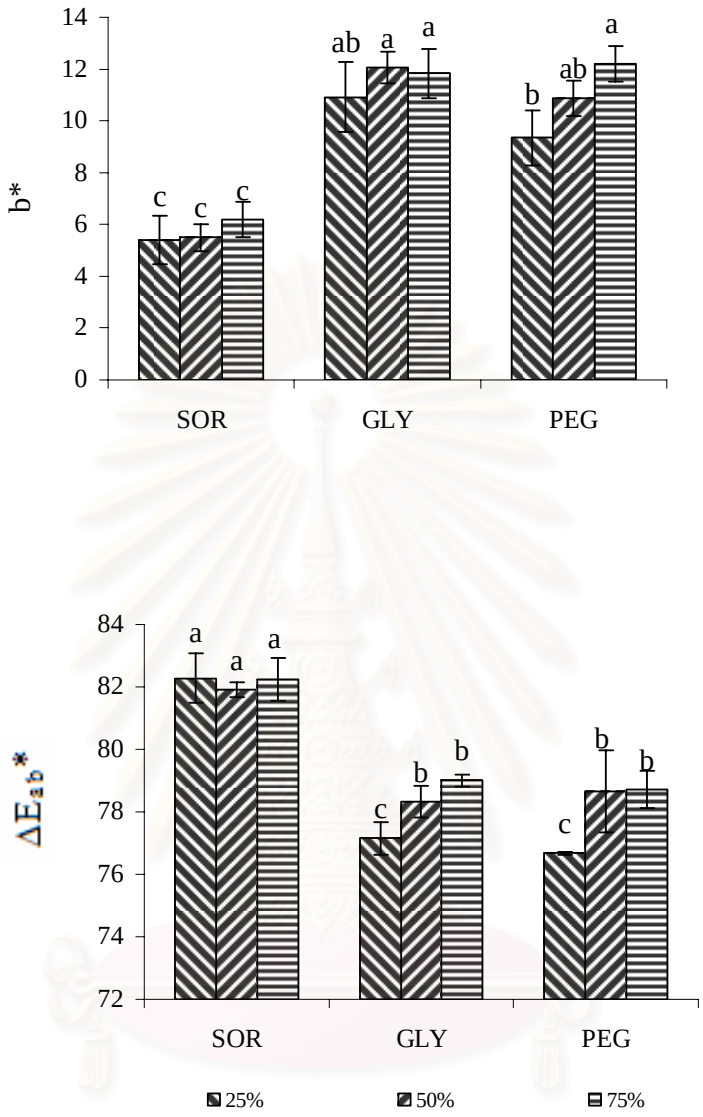


Fig. 4.65 Effect of plasticizer type and concentration on b* and ΔE_{ab}* values of edible films from water-soluble fish proteins precipitated by shifting the pH in surimi wash-water. Standard error bars are shown.

a-c; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

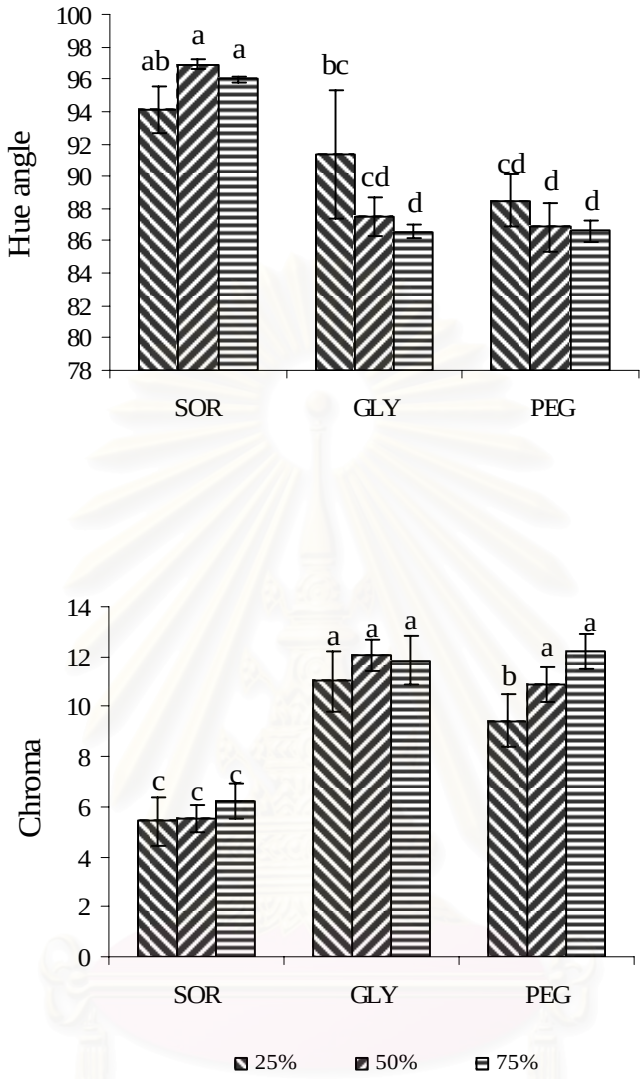


Fig. 4.66 Effect of plasticizer type and concentration on hue angle and chroma values of edible films from water-soluble fish proteins precipitated by shifting the pH in surimi wash-water. Standard error bars are shown. a-d; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

4.4 Effect of pH, Heating Temperature, Heating Time, Protein Concentration and Plasticizer on the Properties of Edible Films from Proteins Precipitated by Ethanol from Surimi Wash-Water

4.4.1 Effect of pH, Heating temperature and Heating Time on the Properties of Edible Films from Proteins Precipitated by Ethanol from Surimi Wash-Water

4.4.1.1 Model Fitting

The RSREG procedure of Statistical Analysis System (SAS, 1996) was used to fit the second order polynomial equation (3.1) to the films properties and the data were shown in Table 9 (Appendix A). The regression coefficients (β_{ki}) obtained thereof, are presented in Table 10 (Appendix A). The analysis of variance for the response variables (Table 11, Appendix A) indicated that the model developed for the tensile strength (TS), elongation at break (E), film solubility FS), protein solubility (PS), color (L*, a*, b*, Hue angle, ΔE^*_{ab} and Chroma), hydrophobicity (HQ), available SH group (ASH) and content of SS bond (SS) were adequate, and had no significant lack of fit. However, regarding water vapor permeability (WVP) and oxygen permeability (OP), values showed highly significant lack of fit suggesting that the chosen model does not represent the system appropriately (Thompson, 1982). In such a case it was desired that some kind of mathematical transformation be performed on the dependent or independent variables, to obtain an acceptable model with non-significant lack of fit. Several such transformations of the experimental data were tried. The model obtained by the logarithmic transformation of water vapor permeability and oxygen permeability data yielded the best results and are given below:

$$A = \ln(WVP) = 2.433+0.100X_1+0.098\ X_2+0.029\ X_3+0.206\ X_1\ X_1 +0.060X_1\ X_2+0.007\ X_2\ X_2-0.002\ X_1\ X_3-0.020\ X_2\ X_3 -0.087\ X_3\ X_3 \quad \text{4.3}$$

$$B = \ln(OP) = 5.423-0.063X_1-0.213\ X_2-0.120\ X_3+0.421X_1\ X_1+0.198X_1\ X_2 +0.161\ X_2\ X_2+0.045\ X_1\ X_3+0.105X_2X_3+0.101X_3\ X_3 \quad \text{4.4}$$

Where Y = response variable; X_1, X_2, and X_3 = independent variables (pH, heating temperature and heating time, respectively)

Equation 4.3 and 4.4 were the most appropriated for calculating water vapor permeability and oxygen permeability, giving a statistically non-significant lack of fit and explaining 92.98 and 85.19 of the variability, respectively. Further statistical analysis (Table 12, Appendix A) was then performed. Results revealed that pH, heating temperature and heating time of film-solutions had a significantly ($p \leq 0.05$) overall effect almost on all responses. The pH and heating temperature of film-solution significantly ($p \leq 0.05$) affected tensile strength, proteins solubility, while heating time was the lowest effect on these responses. Meanwhile, pH, heating temperature and heating time affected film solubility and surface hydrophobicity. Elongation at break, water vapor permeability, oxygen permeability, content of SS bond available SH group, b* , ΔE_{ab}*, hue angle and chroma values were most affected by pH, However, pH, heating temperature and heating time did not show significant ($p > 0.05$) effect on L* and a* values.

4.4.1.2 Tensile Strength and Elongation at Break

Tensile strength is the maximum tensile stress a films could sustain, is a measure of film strength, while elongation at break is an indication of films flexibility and stretchability (extensibility). The main factors influencing the film's properties were pH and heating temperature of the film-solutions, while, heating time had the lowest effect (Table 12, Appendix A). Contour plots of tensile strength and elongation at break as affected by pH, heating temperature and heating time were given in Figure 4.67 and 4.68. Depending upon the film conditions, tensile strength and elongation at break showed a high variation between 1.86-4.19 MPa and 4.88-33.49 %, respectively (Fig. 4.67 and 4.68).

Irrespective of heating temperature and heating time of film-solutions, the results showed that, tensile strength increased as pH of film-solutions increased. This result implied that higher pH of film-solutions induced formation of resistant films. Banker (1966) reported that pH played an important role in protein films made from water-soluble materials. At alkaline pH away from the isoelectric point of 3.5 (Bourtoom *et al*., 2002), denaturation of proteins was promoted and resulted in unfolding and solubilizing of the proteins. During solubilization, the cohesive forces between the protein macromolecules were neutralized by complexing with the solvent molecules (Banker, 1966). In general, functions of polymers were related to solution properties which further influenced the film characteristics. The charged groups repelled each other and produced a stretching of the polymer chain when functional groups on a linear polymer became ionized during dissolution. This phenomenon, facilitated molecule orientation and fine-stranded network (Banker, 1966). The resulting interaction between polymers may have been responsible for this result. Anker *et al*. (2000) reported that,

when the pH of the film- solutions from ß-lactoglobulin was increased above 8, SH/S-S interchange reactions or thiol/thiol (SH/SH) oxidations could occur upon heating and intermolecular disulfide (S-S) bonds formed. The highest tensile strength value was obtained at pH about 11.5 (Fig. 4.67). However, increasing pH of film-solutions higher than 11.5 resulted in decrease of tensile strength, by the reason that strongly repulsive force occurred between negative (extreme pH) charges along the protein chains could have decreased the occurrence of molecular associations within the protein matrix (Rhim *et al.*, 2002). Gennadios *et al*. (1993a) studied the effect of pH on soy protein isolate film and found that highly alkaline condition (pH > 12) inhibited soy protein isolate film formations. The weakest film was obtained at the lowest pH of the film-solution. The very low tensile strength (1.86-2.01 MPa) was observed at pH lower than 11.0, most likely due to less protein-protein interaction.

Tensile strength was decreased as heating temperature of film-solutions increased from 60-80 ^{o}C. All linear and quadratic terms for pH, heating temperature and heating time were significant (Table 4.11, Appendix A). This result demonstrated that, tensile strength decreased from 5.04 MPa to almost 2.19 MPa when heating temperature of film-solutions. This might due to the fact that higher heating temperature may cause a high protein denaturation and resulted in precipitation of the protein. According to the contour plots (Fig. 4.67) indicated the effect of pH and heating temperature of film-solution on the film's properties. It was observed that, the lowest tensile strength could be expected with the lowest and the highest pH and relative high heating temperature of film-solutions. The results showed that, the experimental condition involving lowest in heating temperature (60 ^{o}C) and medium pH (11.5) of film-

solutions provided highest tensile strength. Heating time seemed to have less effect on tensile strength of the film.

Elongation at break also most affected by pH of film solutions. The contour plots of elongation at break (Fig. 4.68) showed a high variation between 4.88 and 33.49 % and showed the highest elongation at break when higher pH (11.5) and higher heating temperature (70-80 ^{o}C) were used. An increased in elongation at break with increase in pH of film-solutions around 11.5 was assumed to be induced higher protein-protein interaction, resulted in increase in elongation at break. Heating temperature and heating time at the range of this studied did not significantly ($p > 0.05$) affect elongation at break.

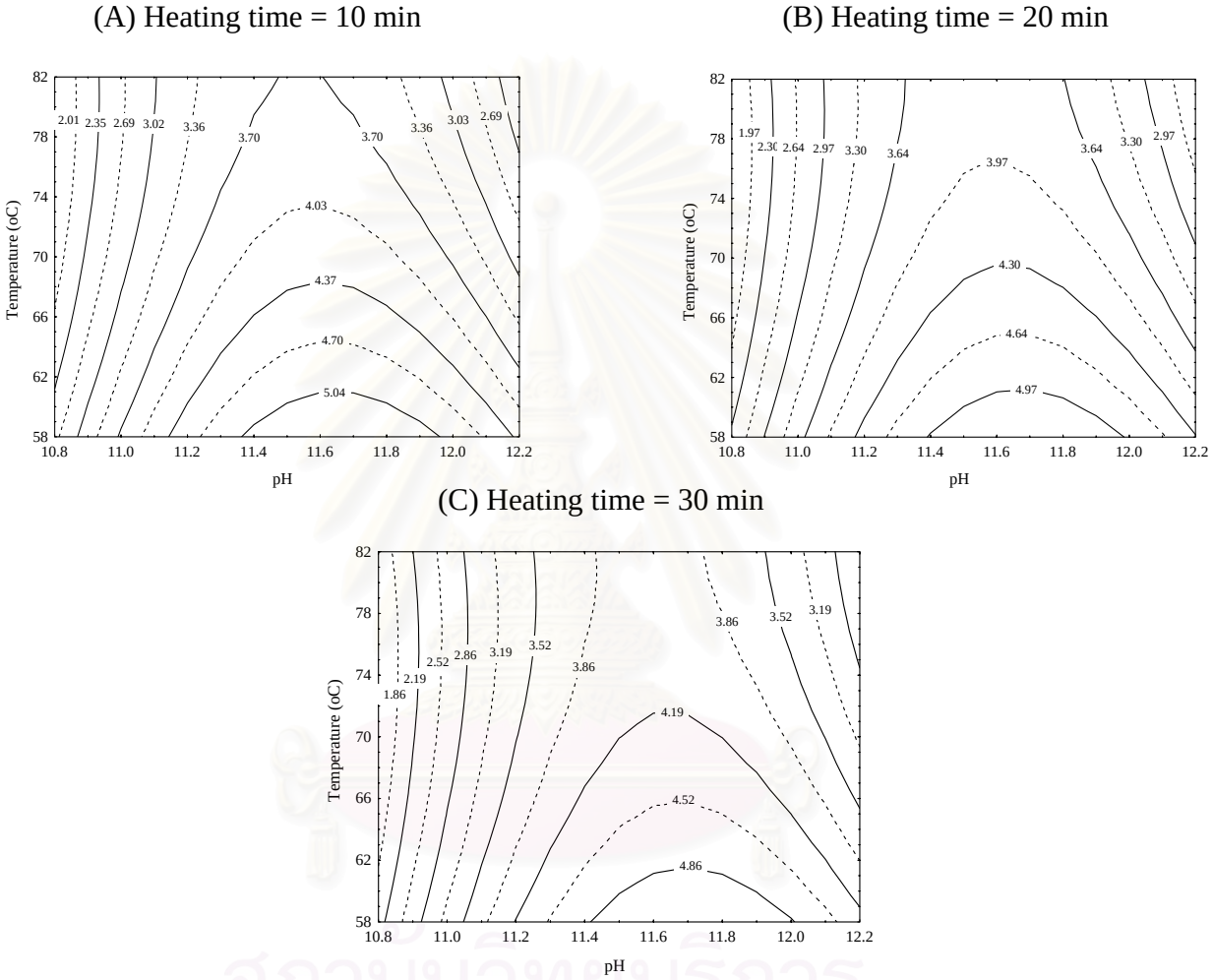
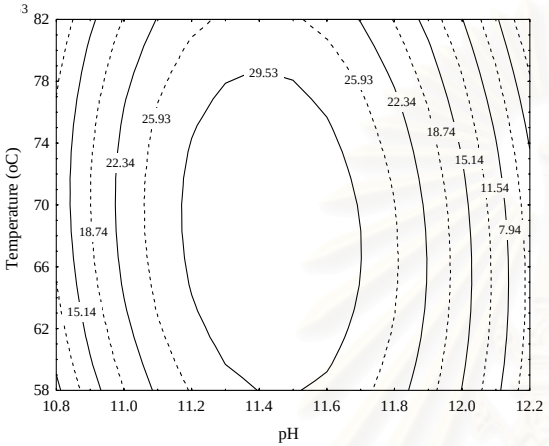
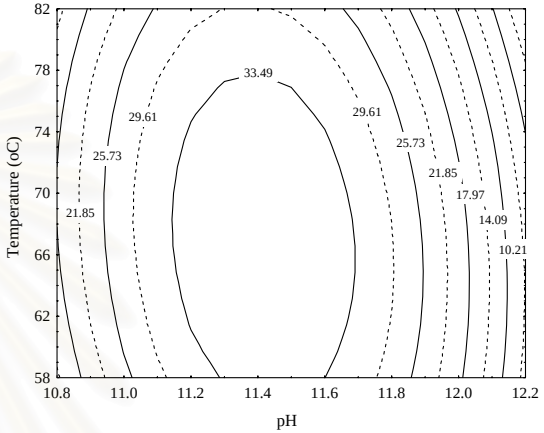


Fig. 4.67 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent tensile strength (KPa) of film at given heating time.

(A) Heating time = 10 min



(B) Heating time = 20 min



(C) Heating time = 30 min

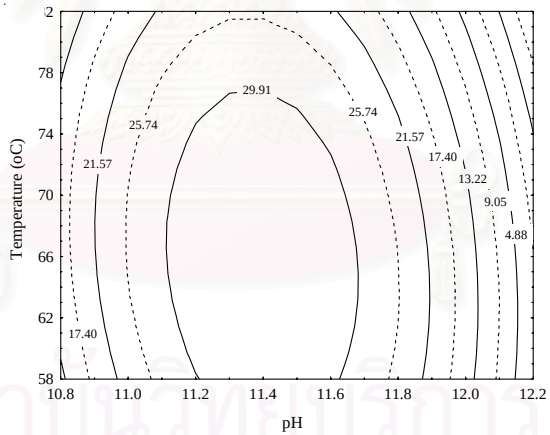


Fig. 4.68 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent elongation at break (%) of film at given heating time.

4.4.1.3 Water Vapor Permeability

The main factor influencing water vapor permeability of the film from water-soluble fish proteins precipitated by ethanol was pH (Table 12, Appendix A). The contour plots (Fig 4.69) were characteristics of the effect of these variables and showed that the water vapor permeability value was the highest at pH around 11.0 (11.70-12.30 g.mm/m^2.d.kPa) and tended to decline when pH of film-solutions closed to 11.5 (8.41-9.47 g.mm/m^2.d.kPa). However, the water vapor permeability increased again when pH was reached to 12.0 (12.68-14.30 g.mm/m^2.d.kPa). These results could be due to the fact that, at higher pH protein could denature, unfold and solubilize. This phenomenon induced favorable molecule orientation and formation of intermolecular disulfide bond by thiol–disulfide interchange and thiol oxidation reactions resulted in higher protein-protein net work. Extreme pH (pH >11.5) of film-solutions as in this experiment might inhibit the film formations. Most likely, strong repulsive forces between highly negative charges prevented protein molecules from associating and formation of films. The highest water vapor permeability was observed at the lowest pH and highest pH of this study. The water vapor permeability increased as heating temperature of film-solutions increased from 60 to 80 ^oC. It was believed that increase in water vapor permeability was due to increased heating temperature of film-solutions and might cause higher denaturation of proteins from water-soluble fish proteins precipitated by organic solvent and resulted in precipitation. The lowest water vapor permeability was obtained at the lowest heating temperature. The contour plots from Figure 4.69 showed the effects of pH and heating temperature of film-solutions on water vapor permeability of the water-soluble fish proteins film. The results demonstrated that, the highest water vapor permeability value

could be expected with the lowest and the highest pH and relative high heating temperature of film-solutions. However, regarding the contour plots, the experimental condition involving low heating temperature and medium pH of film-solutions gave the lowest water vapor permeability. Heating time at the range of this study did not affect on water vapor permeability of the film from water-soluble fish proteins precipitated by ethanol.

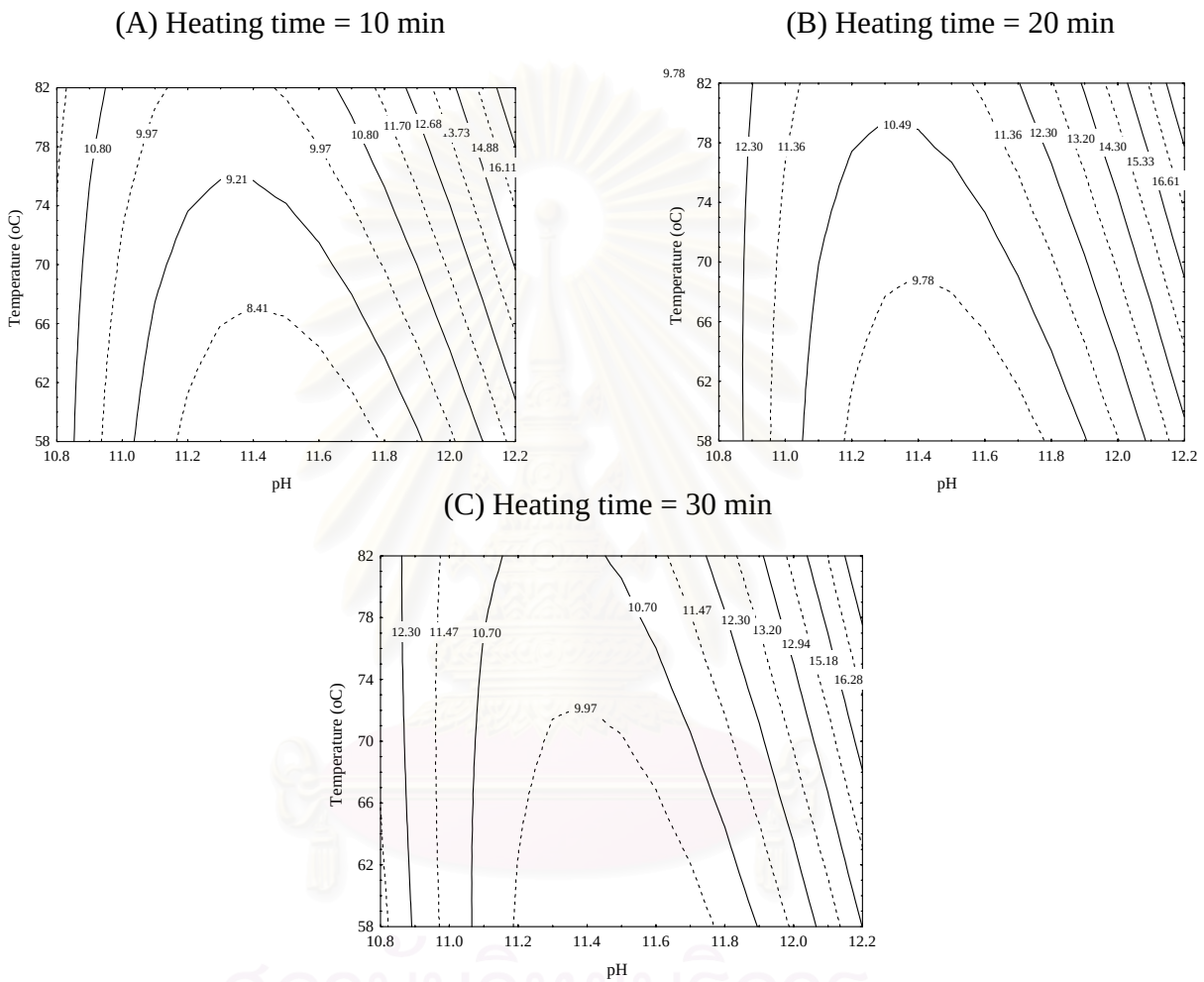


Fig. 4.69 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent water vapor permeability ($g.mm/m^2.d.kPa$) of film at given heating time.

4.4.1.4 Oxygen Permeability

The pH of film-solutions was the most important factor determining the oxygen permeability, while heating temperature and heating time were lowest (Table 12, Appendix A). Within the same heating temperature and heating time, it was observed that at lower pH (11.0) the oxygen permeability of the film were high (163.6-272.44 $cm^3.\mu m/m^2.d.kPa$). As pH increased, the oxygen permeability decreased and reached to the minimum value **(**95.34-104.91 $cm^3.\mu m/m^2.d.kPa$) at pH around 11.5. Further increasing of the pH provided the film with higher oxygen permeability (Fig. 4.70).

The initial decrease of oxygen permeability as a function of pH might be due to the formation of the protein-protein interactions at the pH of around 11.5 and occurrence greater of cross linkings among the protein molecules. However as the pH was adjusted higher than 11.5 an increases in oxygen permeability was provided. The decrease in oxygen permeability with increase in pH (11.5) could be explained by higher protein-protein interaction, inversely, further increasing of pH of film-solutions reached to 12.0 yielded higher oxygen permeability by the reason that highly negative (extreme pH) changes along protein chain could have decreased the occurrence of molecular associations within the protein matrix and formation films (Rhim *et al.*, 2002). Comparing with same pH of film-solutions, the oxygen permeability was increased as heating temperature of film-solutions increased from 60 to 80 ^oC (Fig. 4.70), the intensity of the protein denaturation was increased to the critical level that the protein film could not properly formed and the high oxygen permeability resulted.

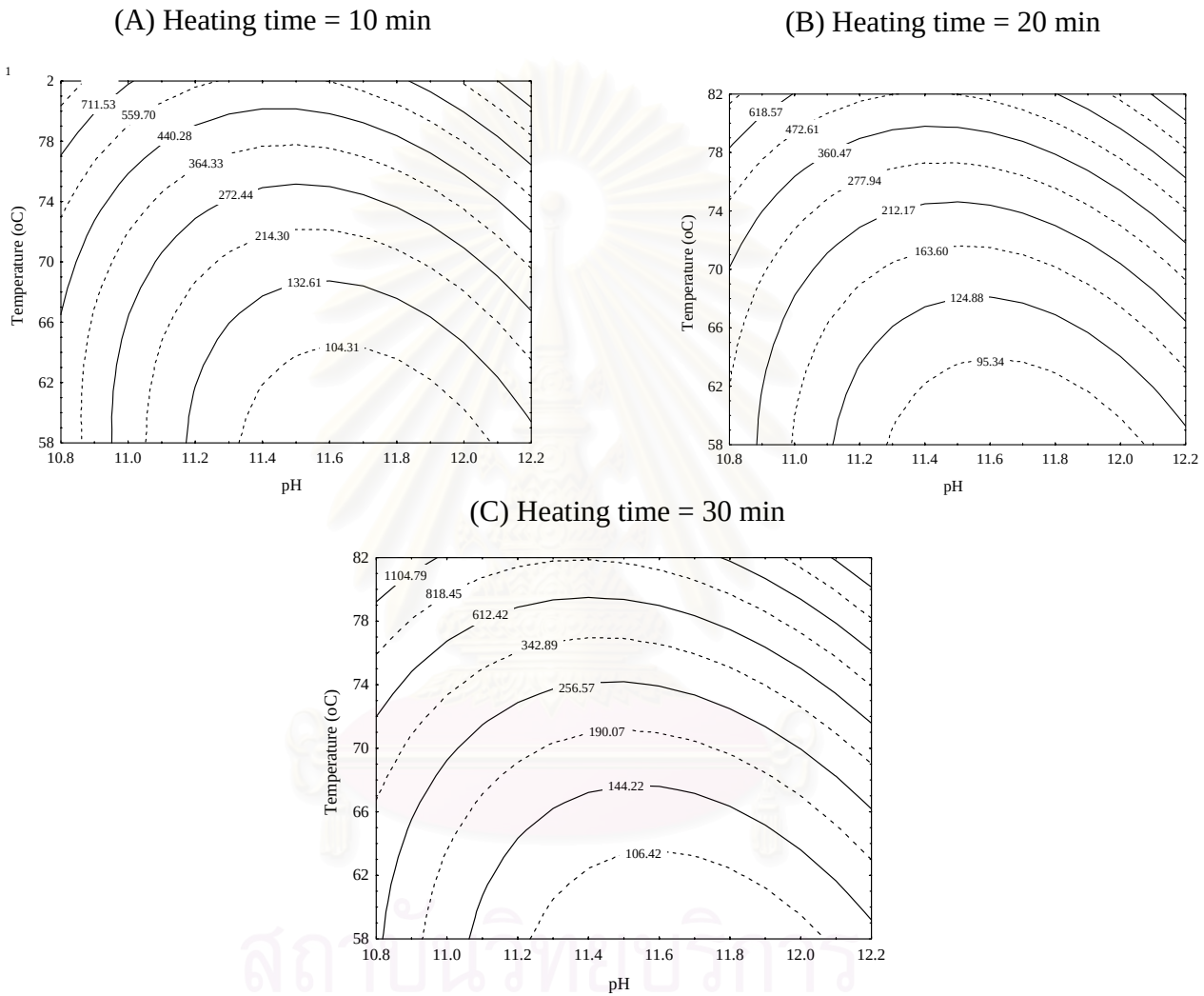


Fig. 4.70 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent oxygen permeability ($cm^3.\mu m/m^2.d.kPa$) of films at given heating time.

4.4.1.5 Film and Protein Solubilities

The main factors influencing the film solubility and protein solubility were pH, heating temperature and heating time of film-solutions, (Table 12, Appendix A). The value of film and protein solubility significantly ($p \leq 0.05$) increased when pH of film-solutions increased (Fig. 4.71 and 4.72) and the higher solubility were observed when pH of the film-solution increased. Increased in film solubility and protein solubility might be due to increase of protein solubility. Higher pH of film solutions facilitated dispersion of protein in water and loosening the film structure, causing dissolution of the non-protein materials (Gnanasambandam *et al.*, 1997). It was observed that film solubility and protein solubility showed the lowest values at pH around 11.5, most likely due to better film formation as mentioned before.

The contour plots of the effect of heating temperature of film-solutions on film solubility and protein solubility were shown in Figure 4.71 and 4.72. Comparing with the same pH of film-solutions, it was observed that, an increase in heating temperature of film-solutions from 60 to 70 ^{o}C resulted in increase in film and protein solubilities. This was attributed to more pronounced heat-induced protein denaturation at higher temperatures. Heating time at the range used in this study did not affect the oxygen permeability of edible films from proteins precipitated by ethanol in surimi wash-water.

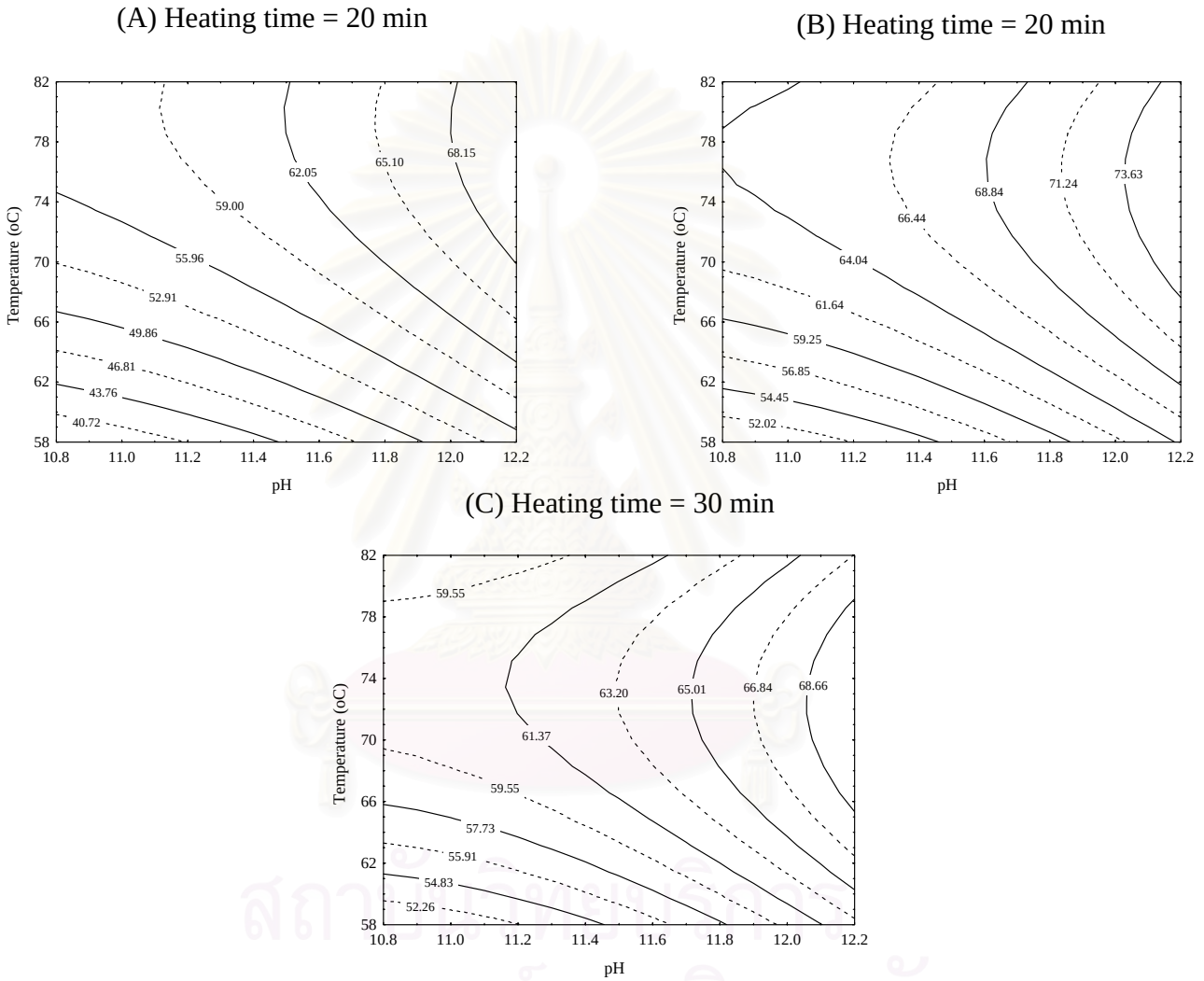


Fig. 4.71 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent film solubility (%) of films at given heating time.

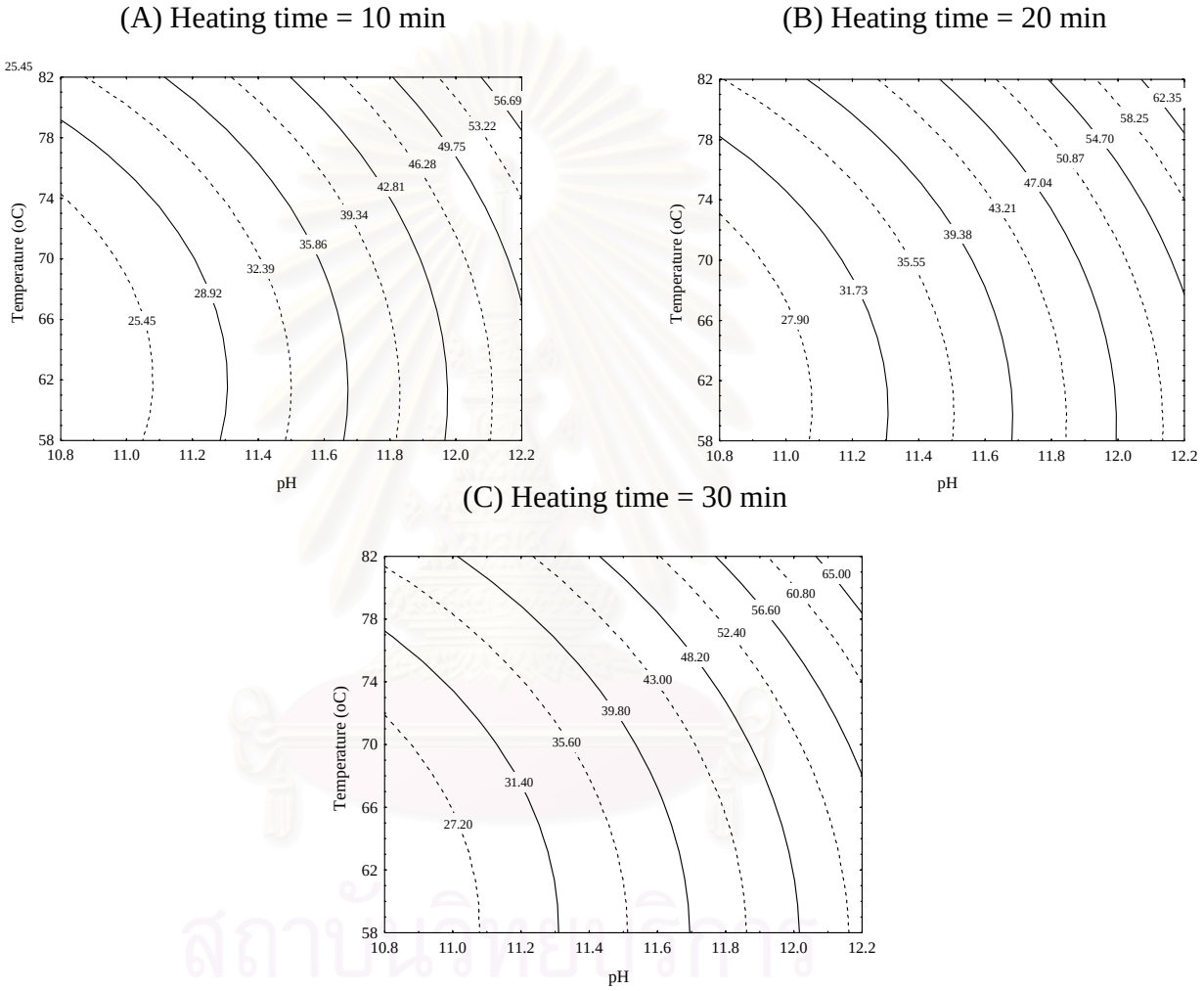


Fig. 4.72 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent protein solubility (%) of film at given heating time.

4.4.1.6 Film Color

The colors of films were most affected by pH of film-solutions, while heating temperature and heating time were least affected (Table 12, Appendix, A). The films prepared at lower pH and lower heating temperature of film-solutions showed lighter yellow than films formed at higher pH and heating temperature. Instrumental color parameters L* and b* markedly increased with increasing pH (Fig.4.73 and 4.75), and this made the films appear more yellowish. The value a* increased as pH and heating temperature of film solutions increased (Fig. 4.74). At alkali pH, proteins are able to form complex substances with polyphenolic compounds. Such complexes might have contributed to discoloration of films prepared at higher pH (Gnanasambandam *et al.,* 1997). The heating temperature and heating time had the lowest effect on L*, a* and b* values.

The main factor influencing ΔE^*_{ab} , hue angle and chroma of the films were pH of film-solutions, while, heating temperature and heating time were less affected. According to the model, ΔE^*_{ab} was plotted against pH and heating temperature at each heating times (Fig. 4.76), as can be seen, the pH and heating temperature were greater affected this variate than heating time. Increased in pH showed decrease in ΔE^*_{ab}, which reversed which the change in heating temperature of film-solutions. Hue angle decreased when pH of film-solutions increased and showed a little increase when heating temperature of film-solutions was increased (Fig. 4.77). The chroma value showed increase as pH and heating temperature of film-solutions increase (Fig 4.78) and this made films appeared yellowish.

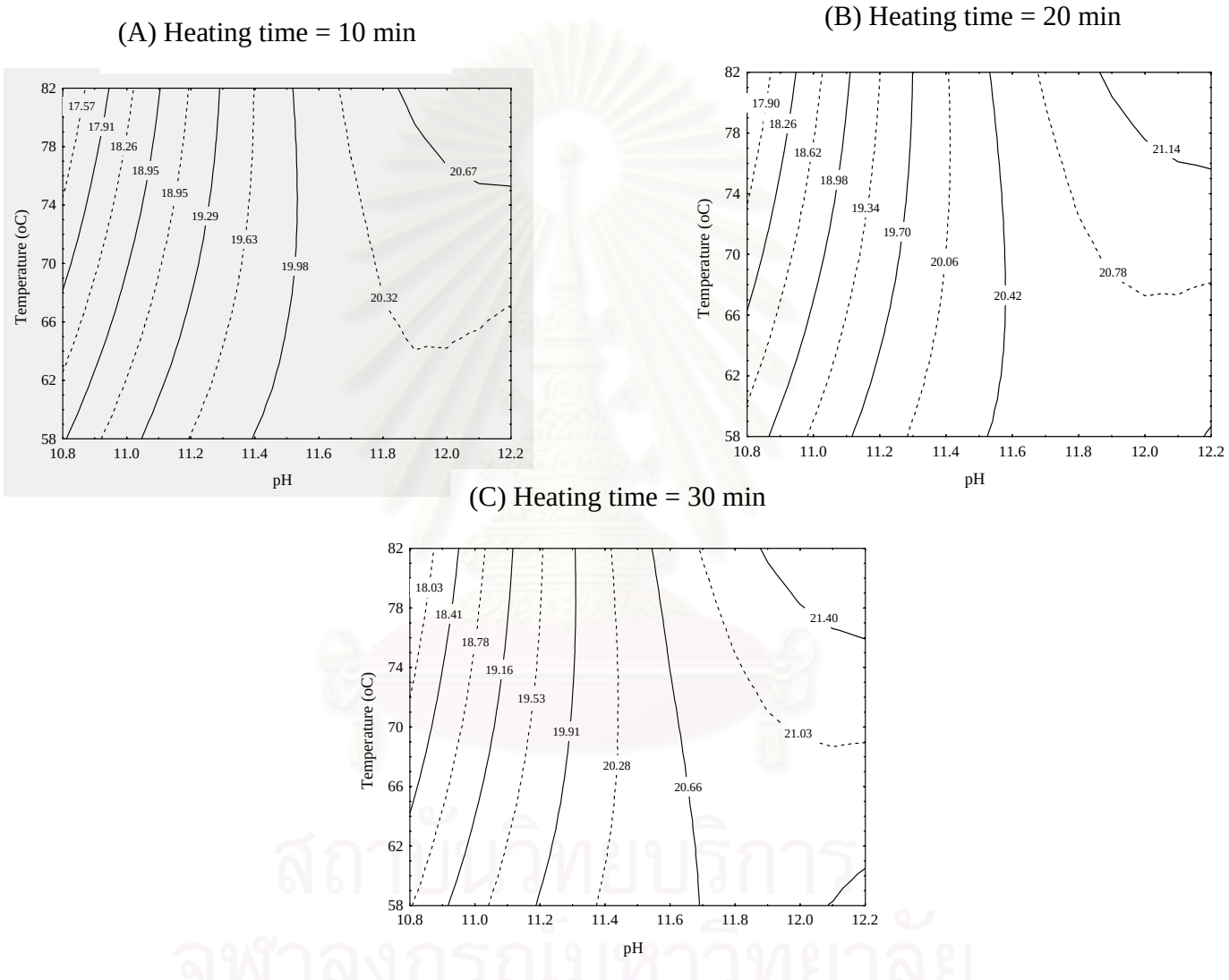


Fig. 4.73 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent L* value of film at given heating time.

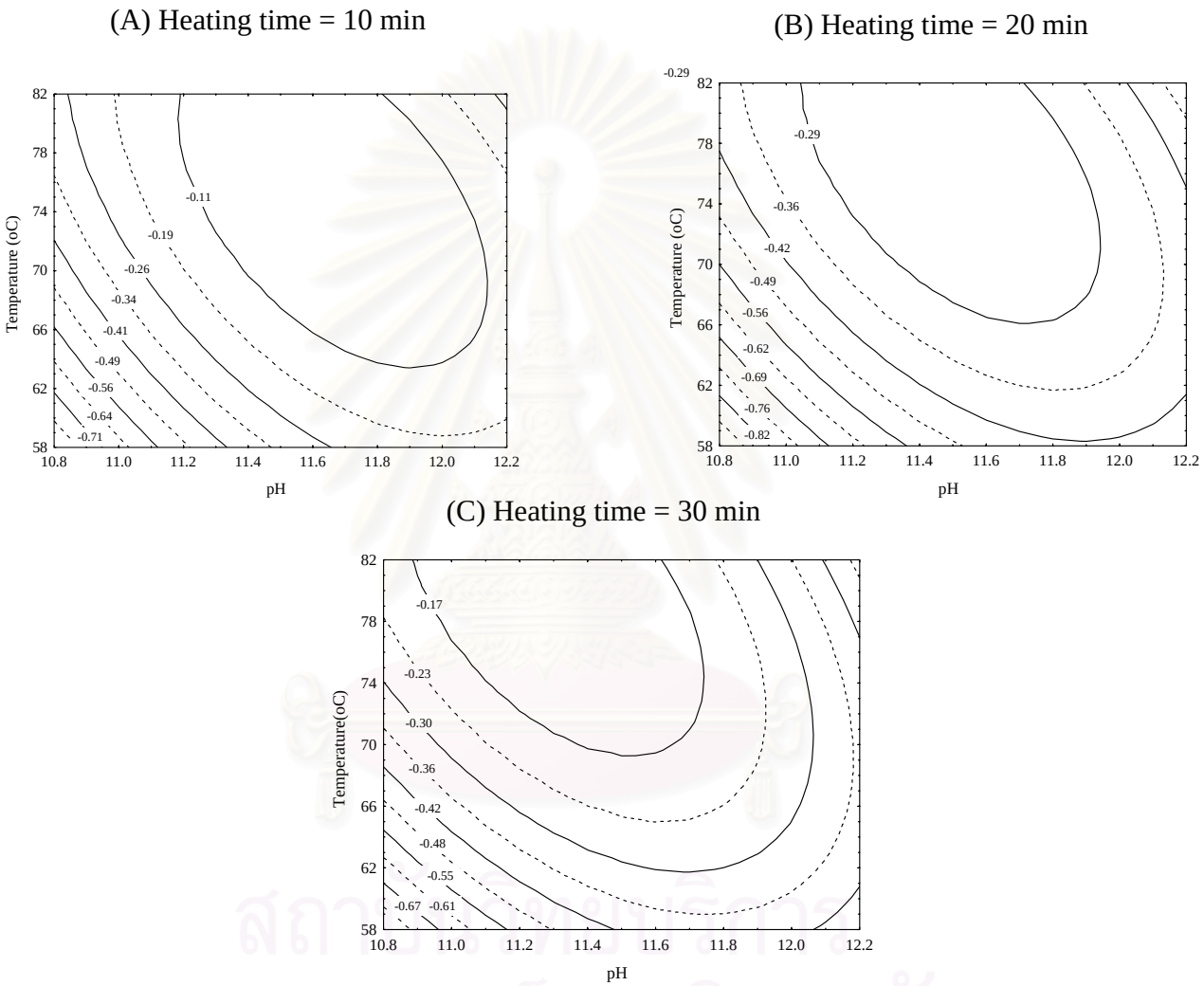


Fig. 4.74 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent a* value of film at given heating time.

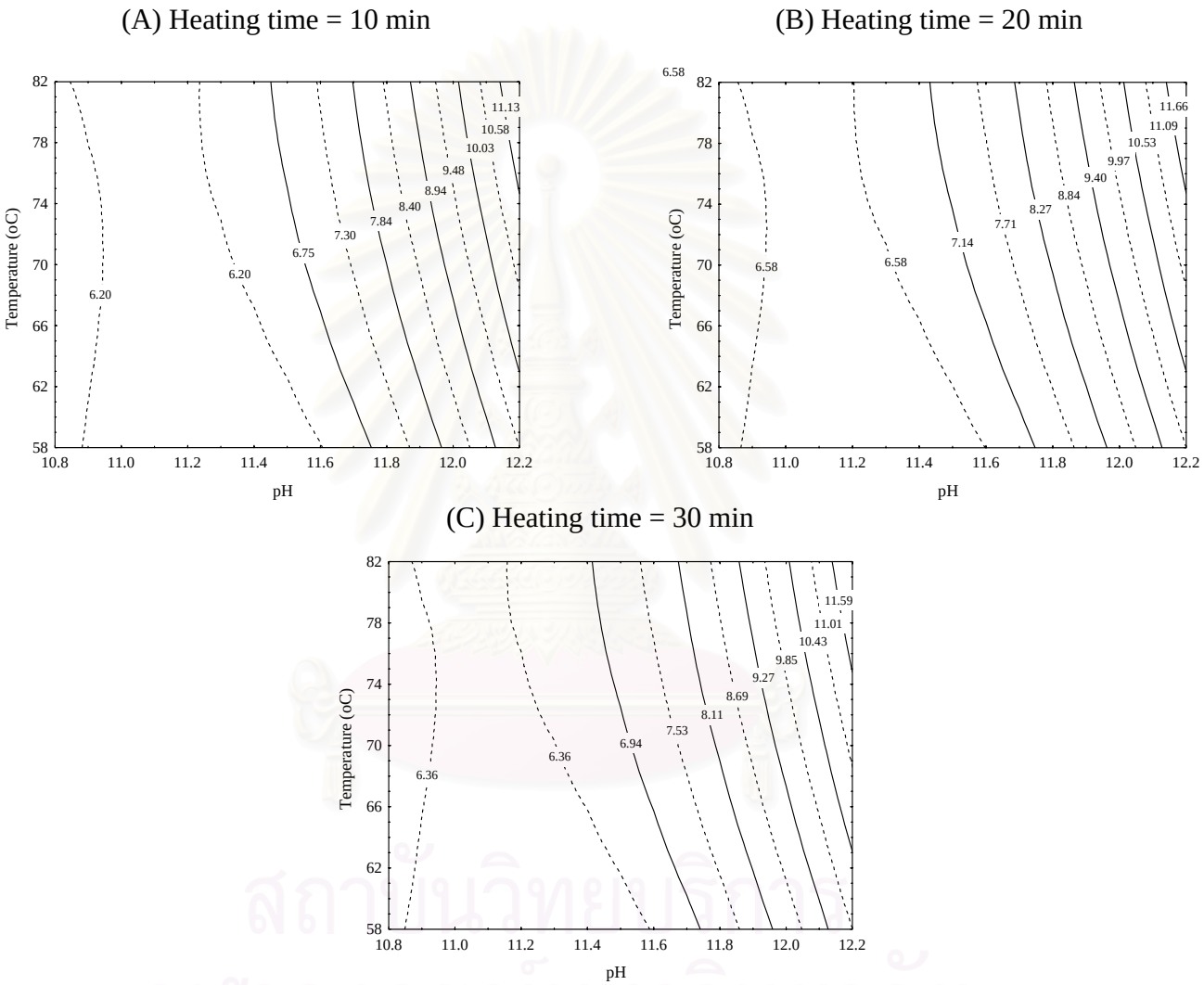


Fig. 4.75 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent b* value of film at given heating time.

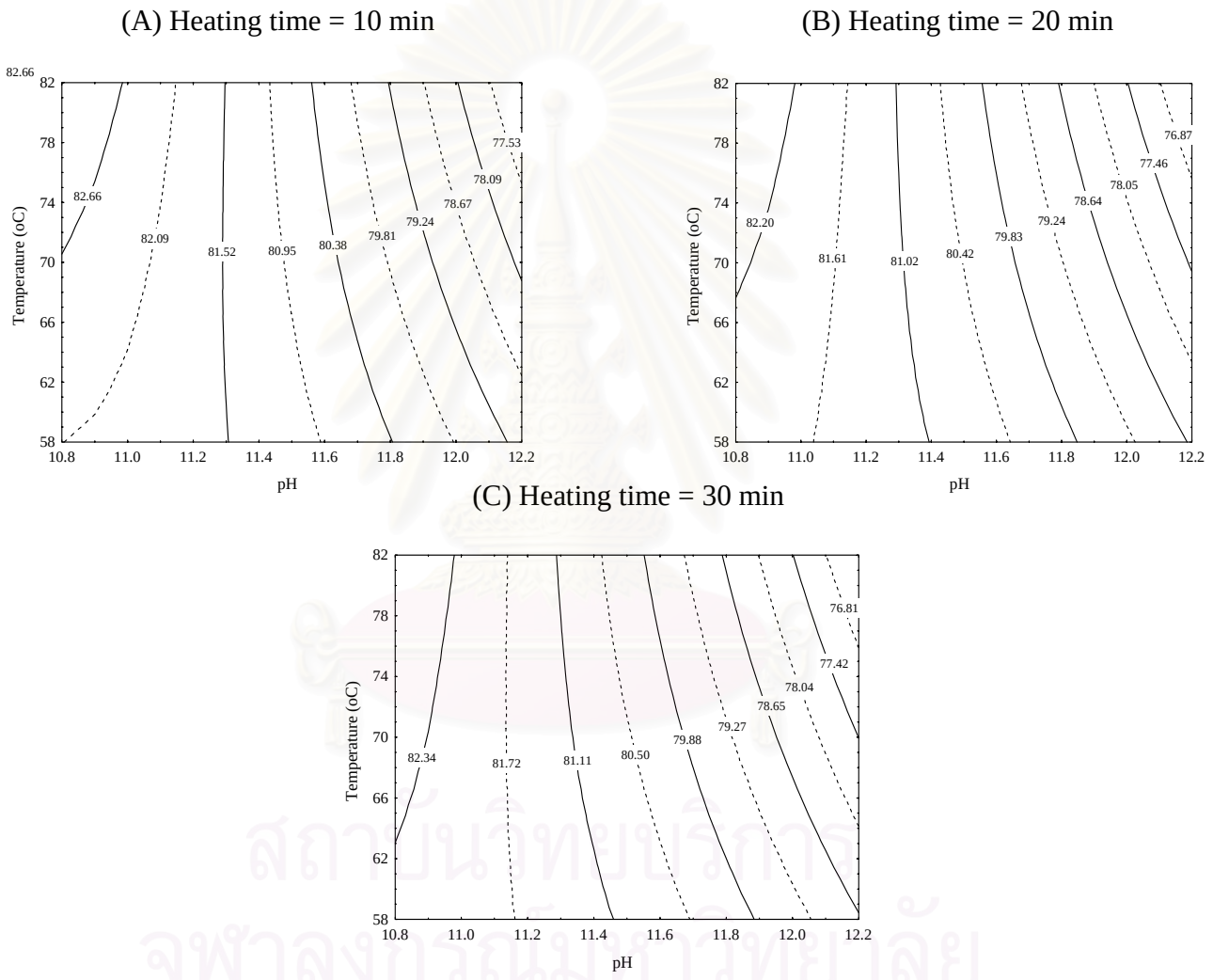


Fig. 4.76 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent ΔE^*_{ab} value of film at given heating time.

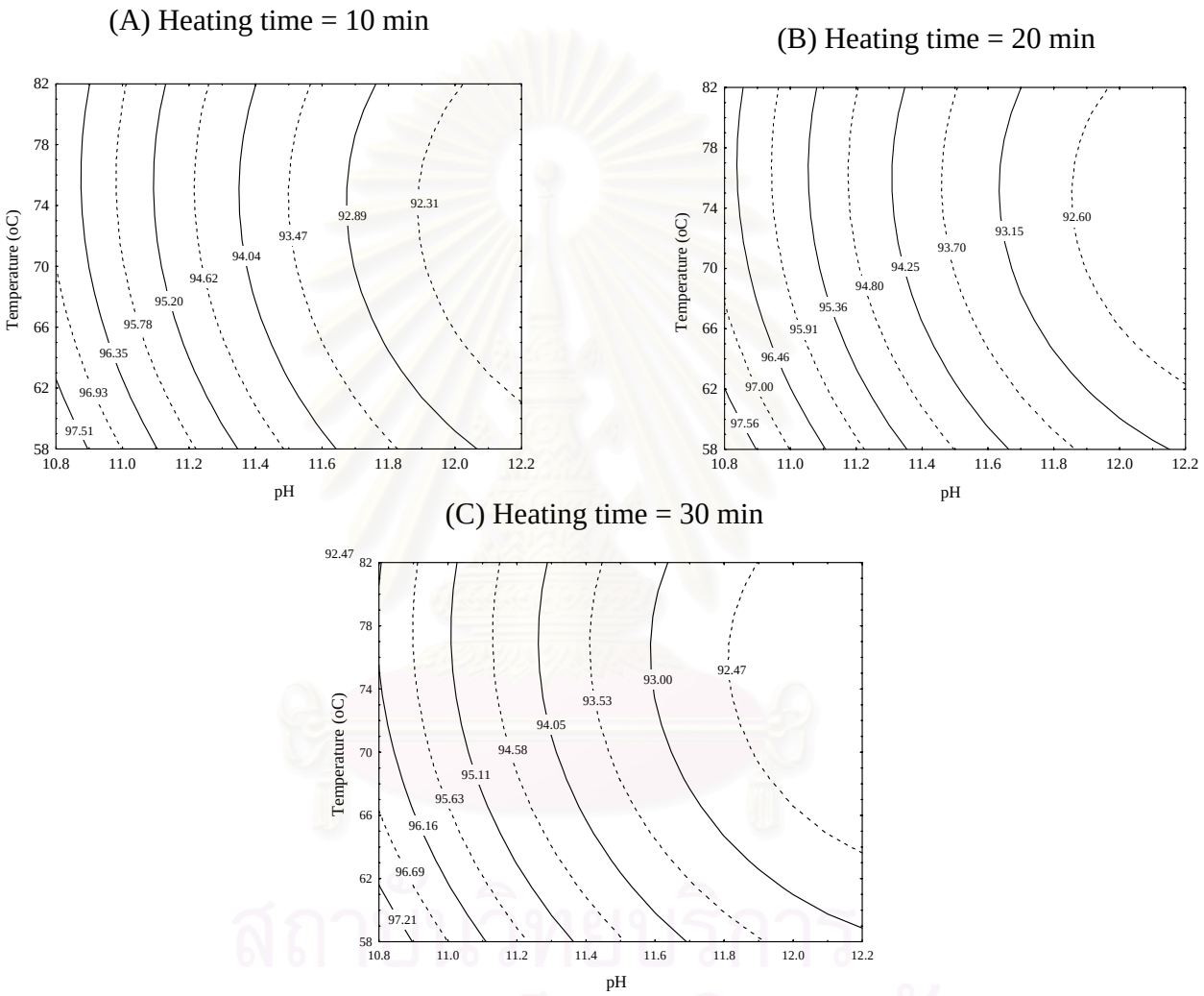
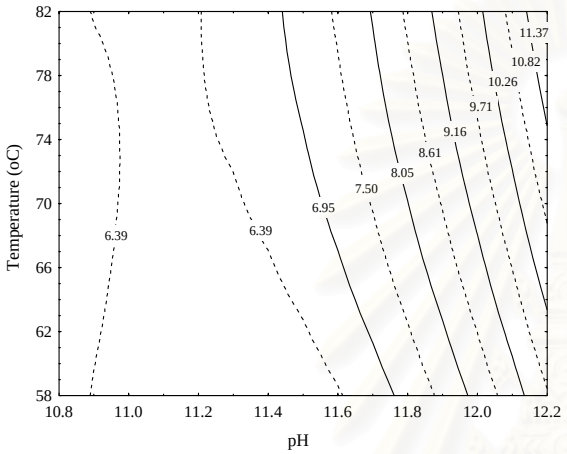
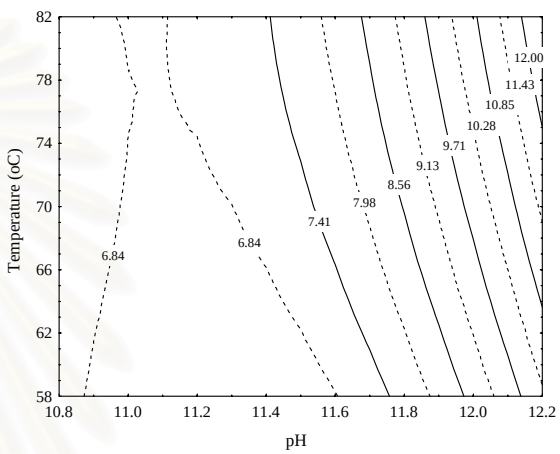


Fig. 4.77 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent hue angle value of film at given heating time.

(A) Heating time = 10 min



(B) Heating time = 20 min



(C) Heating time = 30 min

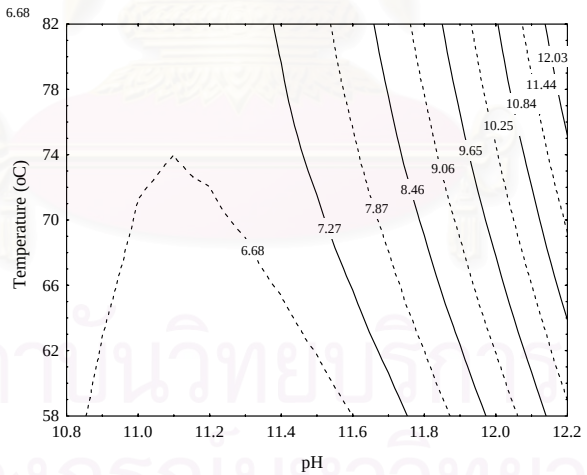


Fig. 4.78 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent chroma value of film at given heating time.

4.4.1.7 Surface Hydrophobicity, Available SH Group and Content of SS Bond

The main factors influencing available SH group and content of SS bond were pH of film-solutions, while heating temperature and heating time had the highest influenced on surface hydrophobicity (Table 12, Appendix A).

Figure 4.79 showed the contour plots of surface hydrophobicity of film-solutions as affected by pH, heating temperature and heating time. It was observed that surface hydrophobicity increased with increasing pH of film-solutions from 11.0-11.6 and thereafter decreased again. Increase of the surface hydrophobicity could be due to the increase of unfold protein molecules resulting in increase exposure of hydrophobic groups (Iwata *et al.*, 2000). Heating temperature and heating time of film-solutions resulted in increase in surface hydrophobicity. Increasing of surface hydrophobicity might be due to the fact that increased flexibility of denatured protein molecules resulting in increased exposure of hydrophobic group.

Changes of available SH groups of film-solutions as affected by pH, heating temperature and heating time were shown in Figure 4.80. The highest amount of available SH groups were found at the highest of pH. This tendency was quite similar to that of surface hydrophobicity shown in Figure 4.79, which suggests that protein molecules in film-solutions were unfolded by increasing pH. Handa *et al.* (1999) reported that available SH groups in film-solutions of egg white protein increased with increasing pH. Extensive alkaline hydrolysis of SS bonds (with subsequent formation of SH groups) in egg white protein has been reported (Beveridge and Arnfield, 1979).

Changes in content of SS bonds of the film-solution as affected by the pH, heating temperature and heating time were given in Figure 4.81. The content of SS bonds decreased when pH of film-solutions increased from 11.0 to 11.5 but increased again when pH of film solutions was higher than 11.5.

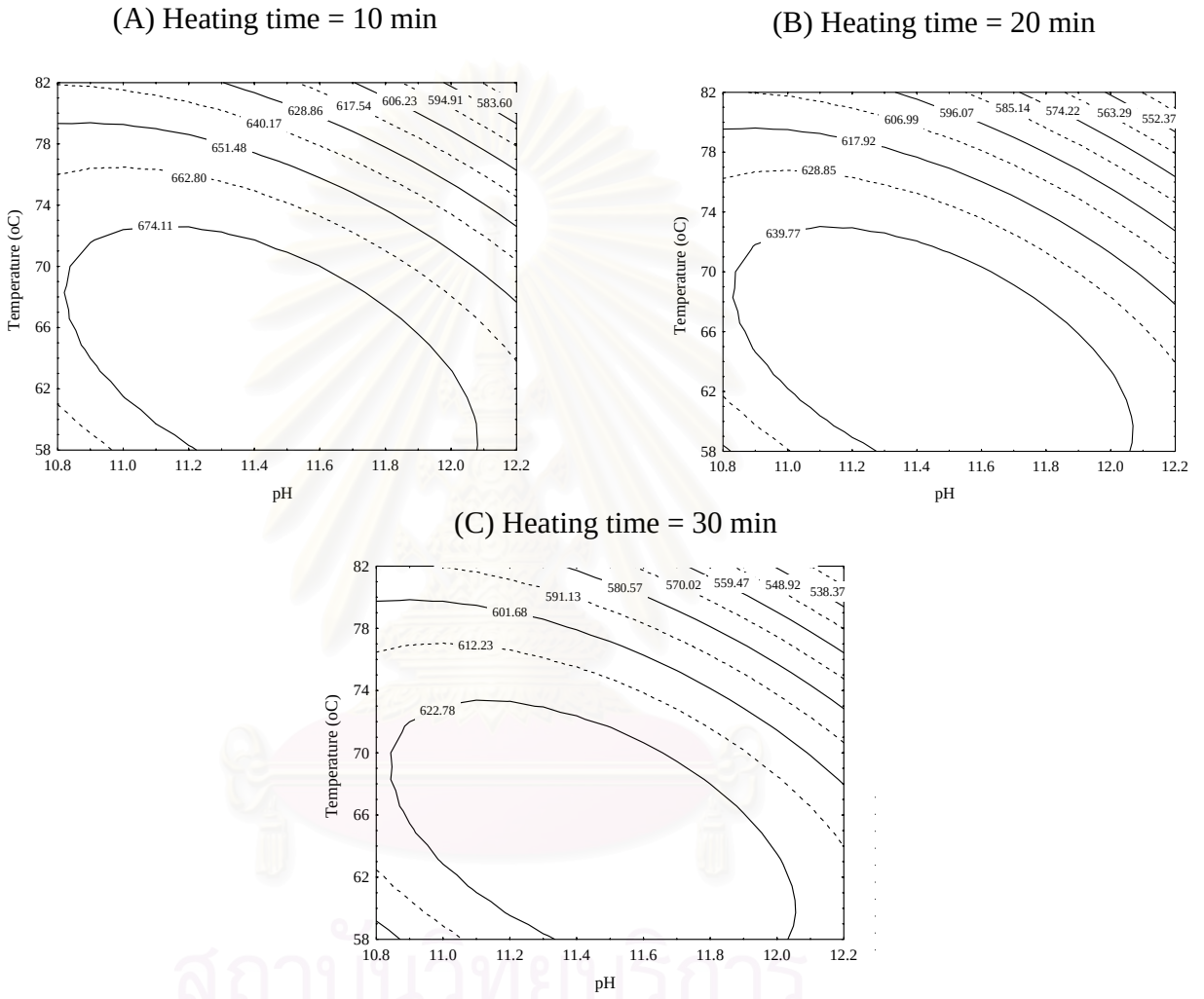
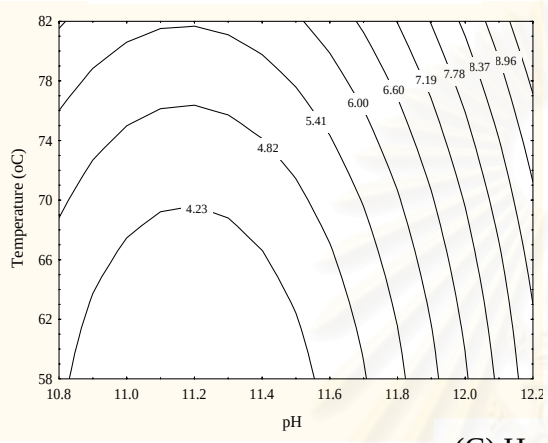
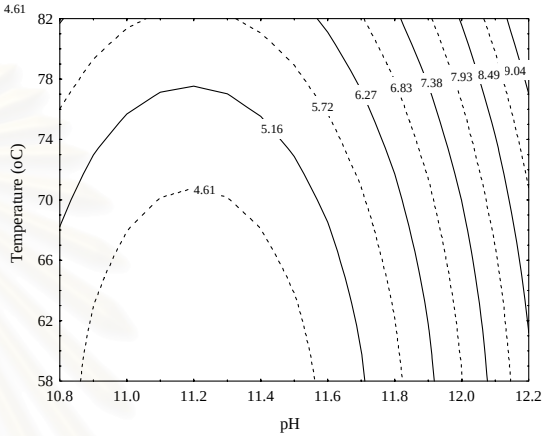


Fig. 4.79 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent hydrophobicity of film-forming solution at given heating time.

(A) Heating time = 10 min



(B) Heating time = 20 min



(C) Heating time = 30 min

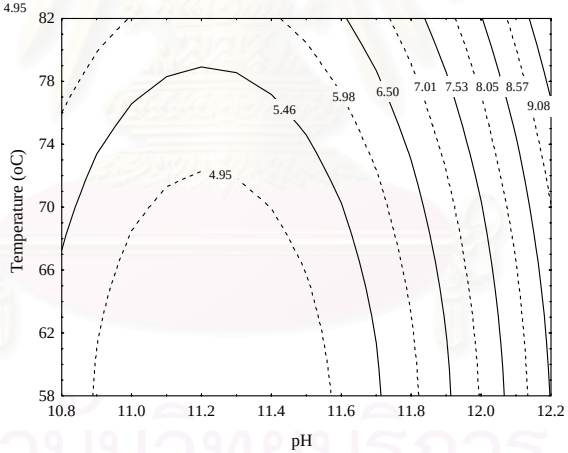


Fig. 4.80 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent available SH group of film-solutions at given heating time.

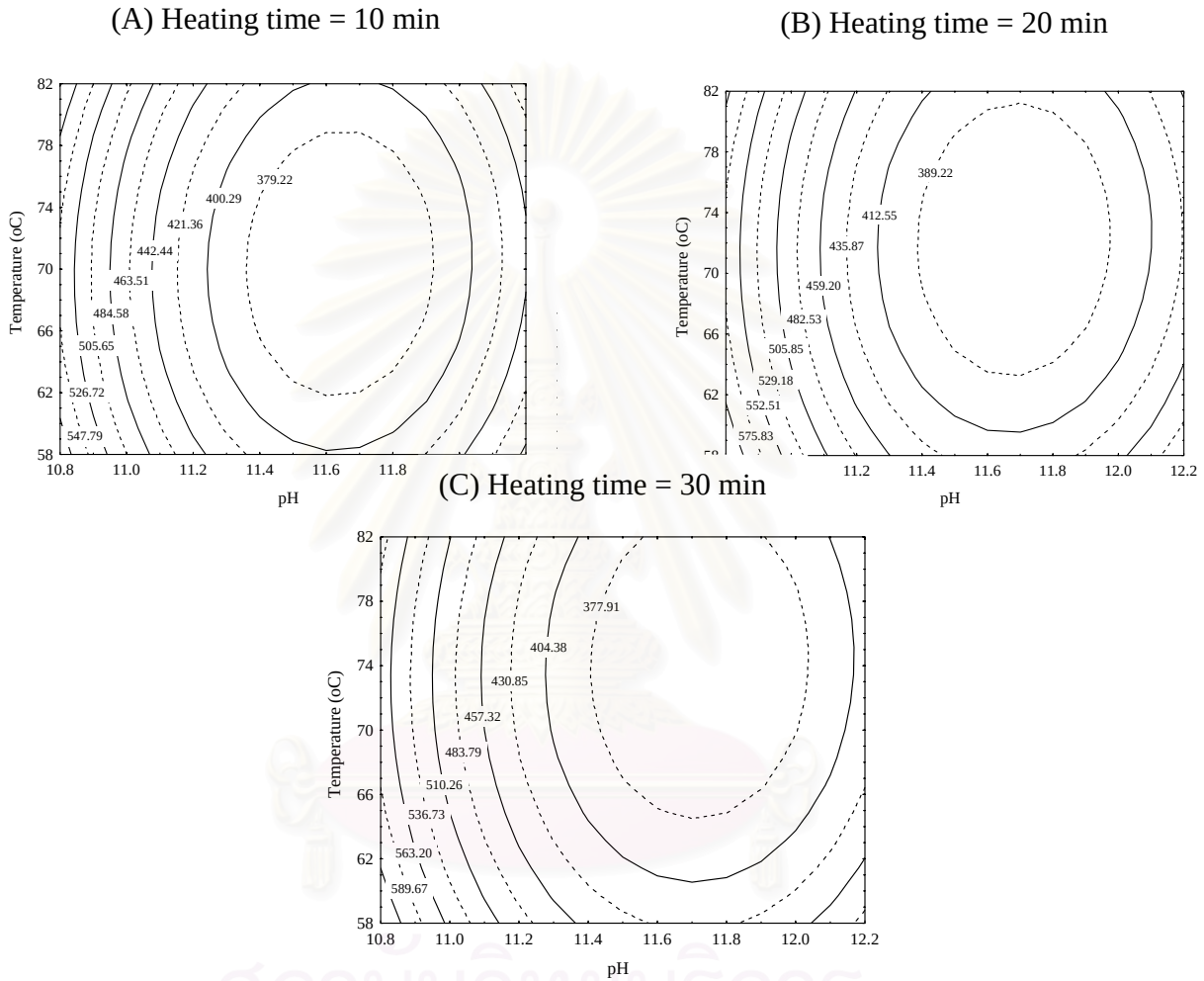


Fig. 4.81 Contour plots showing response behavior of pH and heating temperature of film-solutions under constant heating time. The numbers inside the contours represent content of SS bond of film-solutions at given heating time.

4.4.1.7 Localization of Optimum Conditions

To determine the optimum conditions of the selected parameters on the properties of edible film from proteins precipitated by ethanol from surimi wash-water, the graphical method used in RSM was employed. The variable tensile strength, water vapor permeability and oxygen permeability were considered the most important among the 15 responses followed by elongation at break. The contour plots in Figure 4.82 were obtained from the predictive model of tensile strength, elongation at break, water vapor permeability and oxygen permeability at 10 min of heating time. Plot of Figure 4.83 were superimposed over those of Figure 4.82(A), 4.82 (B), 4.82 (C) and 4.82(D) to locate regions of the highest of tensile strength, elongation at break and the lowest water vapor permeability and oxygen permeability. The shaded area in Figure 4.83 satisfies the specified constraints. As shown, the optimum condition for edible film forming from water-soluble fish proteins precipitated by ethanol at shaded area are: pH of film-solutions of 11.51 and heating temperature of 59.50 ^{o}C for 10 min of heating time. At this combination, 4.96 MPa, 21.03 %, 8.00 g.mm/m^2.d.kPa and 108.4 cm^{3}μm/m^2.d.kPa of tensile strength, elongation at break, water vapor permeability and oxygen permeability, respectively, were resulted. Besides, film solubility, protein solubility, hydrophobicity, available SH group and content of SS bonds were found to be 46.55%, 32.68%, 720.81, 3.12 and 39.49 μM SH/g protein, respectively.

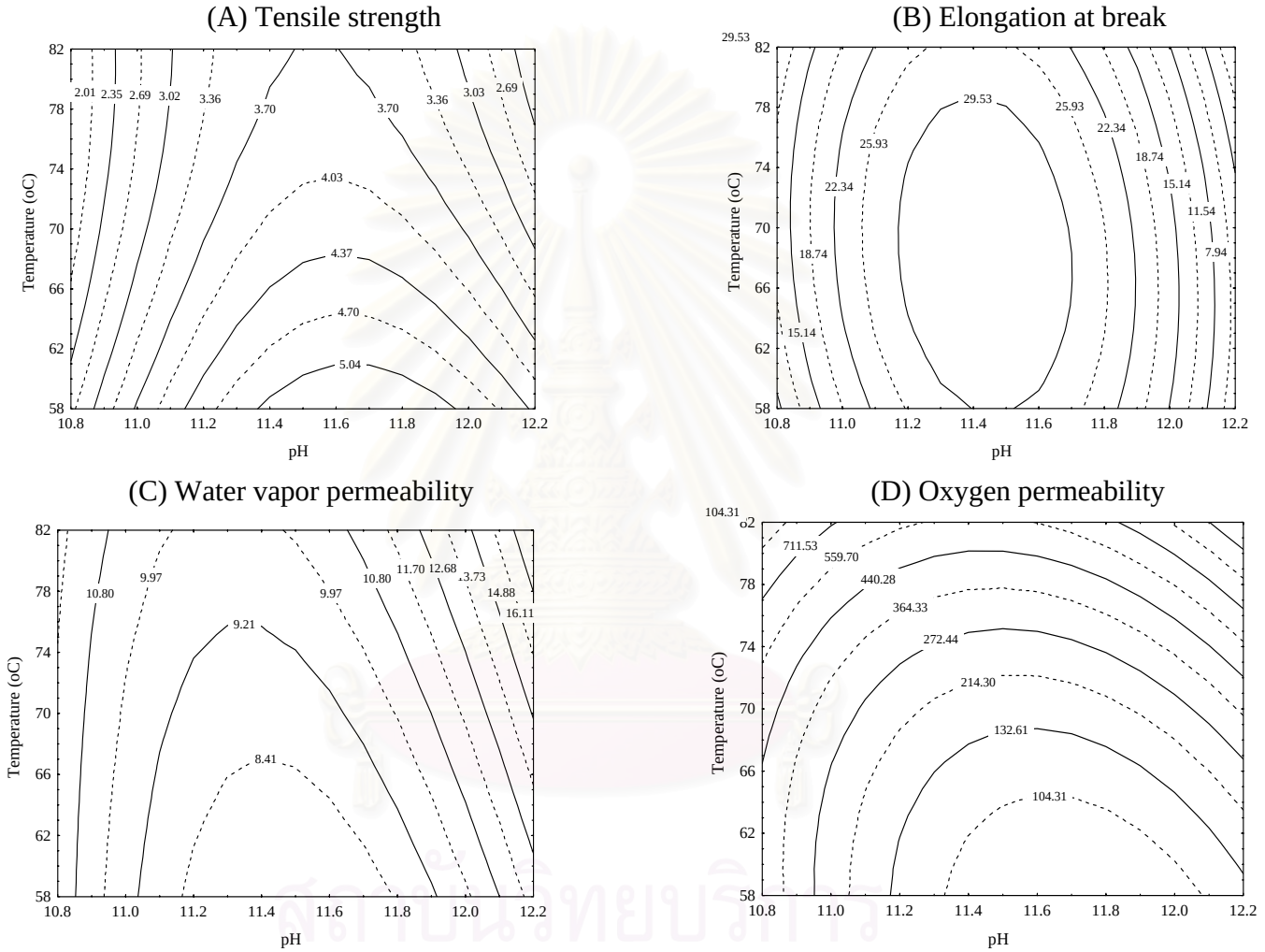


Fig. 4.82 Contour plots showing response behavior of pH and heating temperature of film-solutions heated for 10 min on the; tensile strength (MPa),elongation at break (%), water vapor permeability ($g.mm/m^2.d.kPa$) and oxygen permeability ($cm^3.\mu m/m^2.d.kPa$) before superimposition.

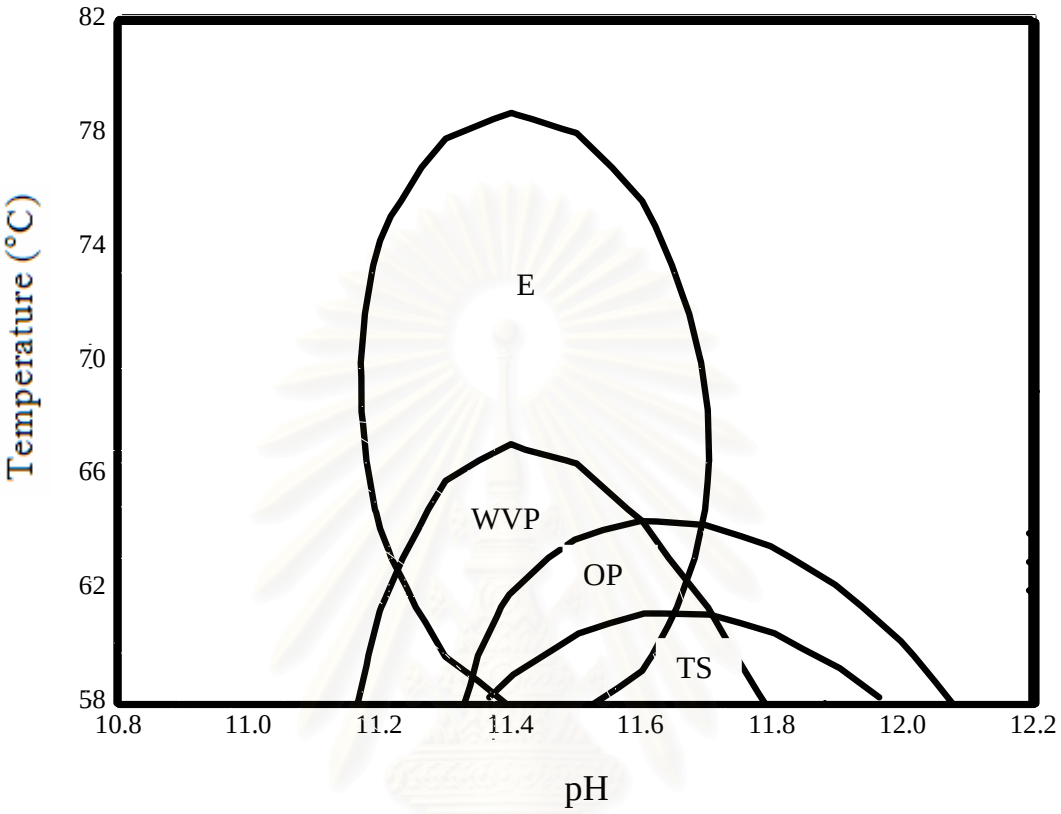


Fig. 4.83 Optimum film soltions condition as a function of the independent variables after superimposition of contour plots over those of 4.82(A), 4.82(B), 4.82(C) and 4.82(D). Shaded area indicates regions the highest of tensile strength (TS), elongation at break (E) and lowest water vapor permeability (WVP) and oxygen permeability (OP).

Validation tests were performed to determine the adequacy of the single order polynomial (SOP) model (Flores and Chinnan, 1988; Mudahar *et al.*, 1990). This was performed because a fractional factorial design was used as the experimental design. A model is deemed adequate if the predicted values (of the model) are close to the experimental values observed during the validation tests. Table 4.3

shows the predicted and observed values for the responses at optimum condition for the selected parameters on the properties of edible film from proteins precipitated by organic solvent in surimi wash-water. The experimental values were averages of three replicates and were very close to the predicted values indicating that the SOP models generated were acceptable. The high CV values for some models were due to their lesser reproducibility (Montgomery, 1984) that may have contributed to the statistical insignificance of some of these models. Despite the lesser effect of these responses to the optimum conditions, predictions were within fairly acceptable limits.

Table 4.3 Predicted and observed values for the independent variables after superimposition conditions

Response variable	Predicted value	Actual value $\pm$ SD
Tensile strength (MPa)	4.96	5.30 $\pm$ 0.49 (9.24%)
Elongation at break (%)	21.03	21.77 $\pm$ 1.94 (8.91%)
Water vapor permeability ($g.mm/m^2.d.kPa$)	8.00	7.67 $\pm$ 0.48 (6.25%)
Oxygen permeability ($cm^3.\mu m/m^2.d.kPa$)	108.41	116.37 $\pm$ 8.38 (7.20%)

Number in parentheses are coefficients of variation (CV).

4.4.2 Effect of Protein Concentration on the Properties of Edible Films from Protein Precipitated by Ethanol from Surimi Wash-Water

The effect of protein precipitated by ethanol concentration on the tensile strength and elongation at break of edible films were presented in Figure 4.84. Edible films with varying protein concentrations of 1.5, 3.0 and 4.5% at fixed pH 11.51, heating temperature of 59.50 and 10 min of heating time were investigated.

Varying the concentration of fish proteins elucidated the influence of the tensile strength and elongation at break (Fig. 4.84). Tensile strength was significantly ($p \leq 0.05$) higher at the 4.5% of water soluble fish proteins (Fig. 4.84), this implied that higher protein content induced favorable structure regarding the ability of the films to form. However, there was no significantly ($p > 0.05$) different of tensile strength when 3.0 and 4.4% w/w of protein concentrations were employed.

The effect of protein concentration on elongation at break of the film was given in Figure 4.84. The results demonstrated that, the protein concentration had significantly ($p \leq 0.05$) effect on elongation at break. Increasing of proteins concentration from 1.5 to 3.0 % w/w provided higher elongation at break; however, when the concentration of proteins was increased to 4.5% w/w lower elongation at break was resulted. Reducing in elongation at break of the film formed at the lowest and highest protein concentration might be due to the fact that the different of protein net work was formed. The lowest protein concentration probably caused less protein-protein interaction, while the highest protein concentration caused less order alignment, due to the fact that fast gelling occurred at high protein concentration concentration (Anker *et al.*, 1999).

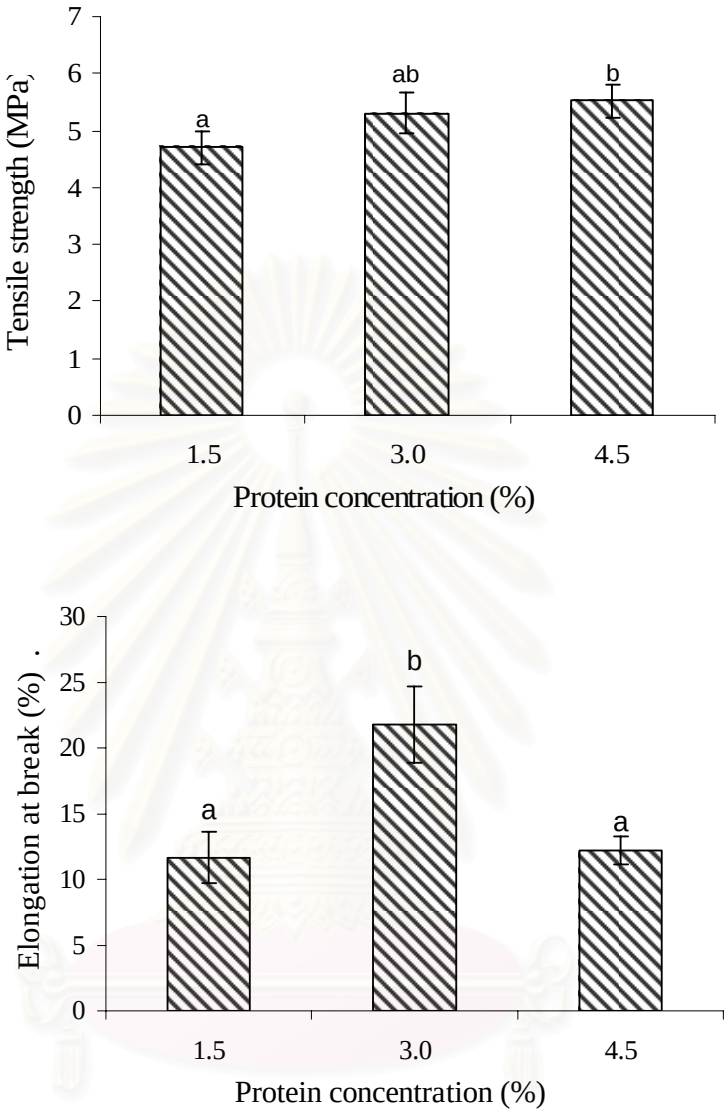


Fig. 4.84 Effect of protein concentration on the tensile strength (MPa) and elongation at break (%) of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

Water vapor permeability and oxygen permeability of edible films from water-soluble fish proteins precipitated by ethaol at 1.5, 3.0 and 4.5% w/w were investigated (Fig. 4.85). The results showed that the protein concentration had significant ($p \leq 0.05$) effect on the water vapor permeability and oxygen permeability (Fig. 4.85). It was observed that the highest protein concentration (4.5% w/w) provided lower both water vapor permeability and oxygen permeability. This result might be due to the fact that when increasing protein concentration, a more aggregated structure with a denser protein network was formed (McHugh and Krochta, 1994a). While at lower protein concentration, a less aggregated structure was formed, with a loser protein structures provided in increase in water vapor permeability and oxygen permeability. However, at 3.0 and 4.5% w/w of protein concentration did not significantly different in water vapor permeability.

Film and protein solubilities of edible films were affected by protein concentration (Fig 4.86). Lower and above 3.0% w/w of protein concentration tended to increase film and protein solubilities. This result might be due to the fact that less aggregated structure was formed, with a loser protein structure, provided an increase in films and protein solubility when 1.5% w/w of protein concentration was used. While, above 3.0% w/w of protein concentration, there was insufficient time for unfolded protein molecules to rearrange in the most favored structure prior to aggregation and led to poor protein-protein network facilitated in dissolution.

The color of edible films from proteins precipitated by ethanol in surimi wash-water was affected by protein concentration, higher protein concentration showed significantly higher in b*, a* and chroma value, but lower in L*

(Fig. 4.87, 4.88 and 4.89), hence, the films color was darker and more yellow than lower content of protein.

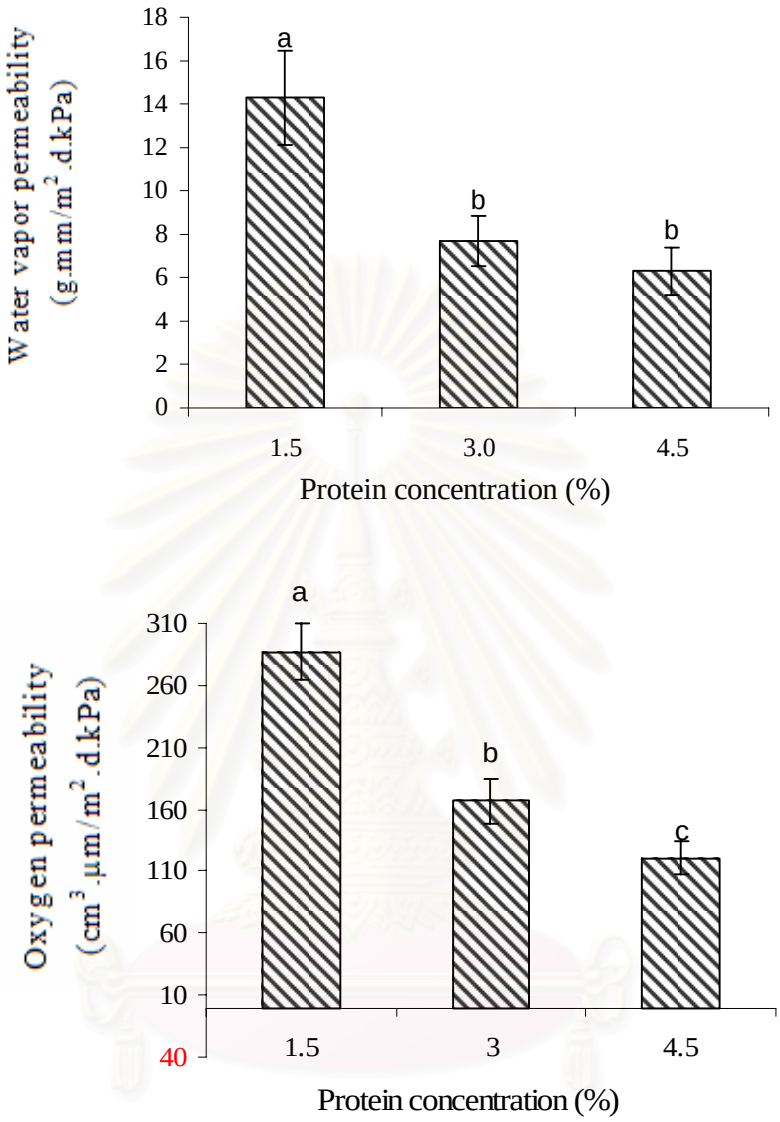


Fig. 4.85 Effect of protein concentration on water vapor permeability (g.mm/m^2.d.kPa) and oxygen permeability (cm^3.µm/m^2.d.kPa) of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

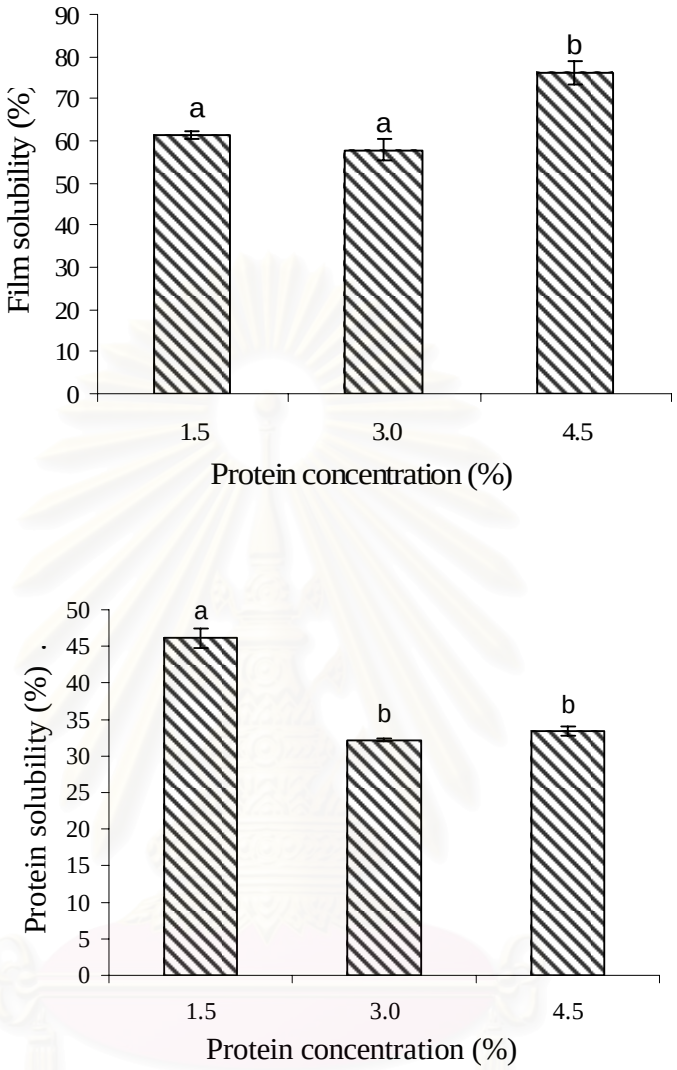


Fig. 4.86 Effect of protein concentration on the films solubility (%) and proteins solubility (%) of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

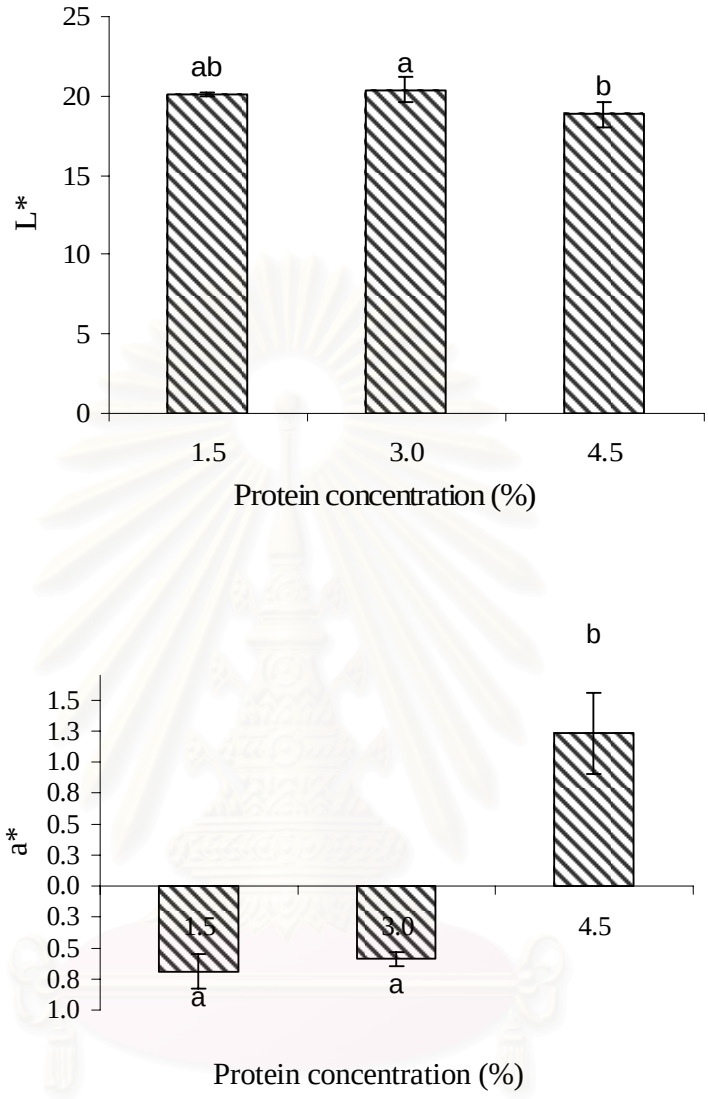


Fig. 4.87 Effect of protein concentration on the L* and a* values of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

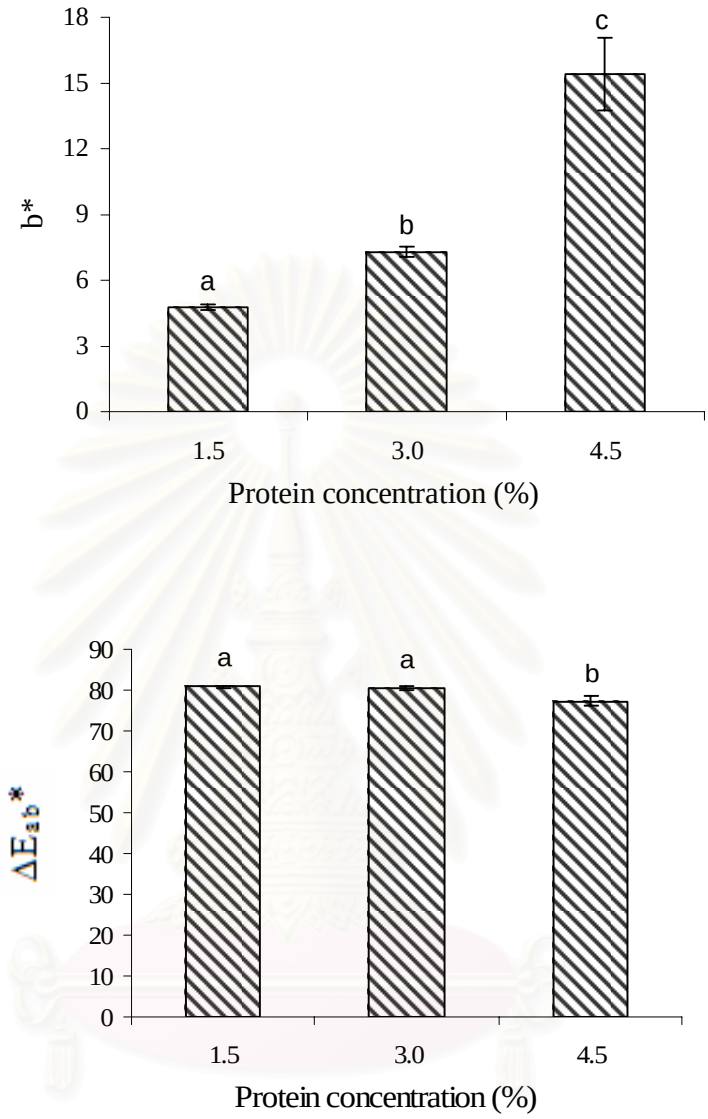


Fig. 4.88 Effect of protein concentration on the b* and ΔE_{ab}*values of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a,c; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

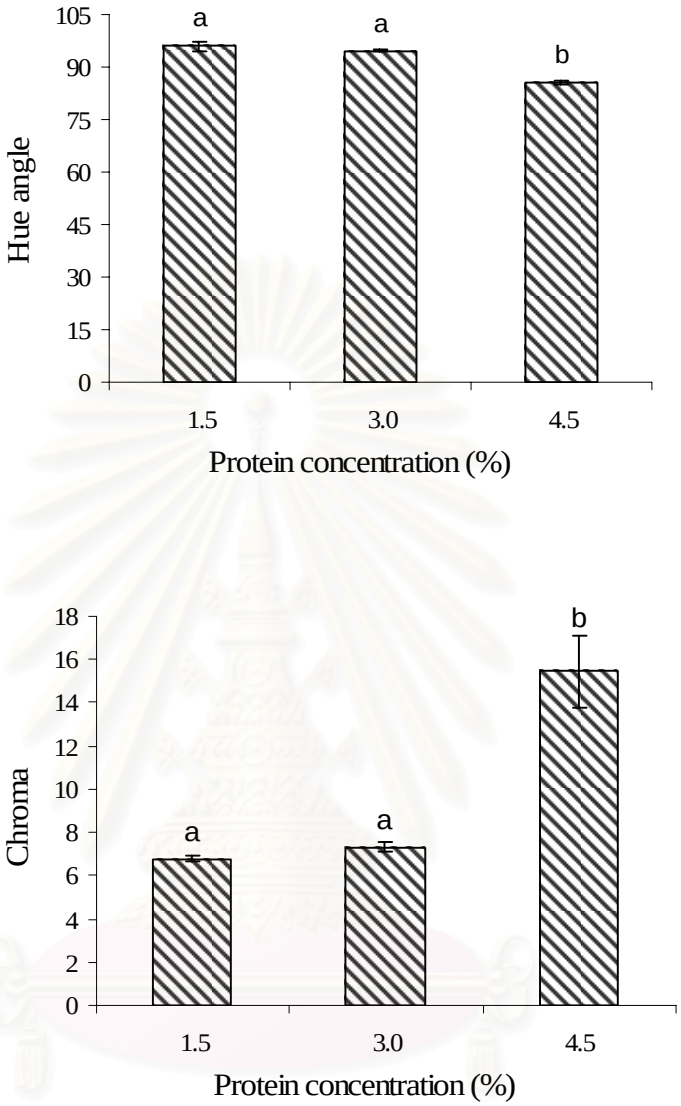


Fig. 4.89 Effect of protein concentration on the hue angle and chroma values of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a,b; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test.

4.4.3 Effect of Plasticizer Type and Concentration on the Properties of Edible Films from Proteins Precipitated by Ethanol from Surimi Wash-Water

4.4.3.1 Tensile Strength and Elongation at Break

Preliminary work demonstrated that edible films from water-soluble fish proteins precipitated by ethanol in surimi wash-water formed without plasticizer were relatively brittle and broken easily when peeled off. Hence, edible films were prepared by using three type of plasticizer (sorbitol, glycerol and polyethylene glycol) at different concentration (25, 50 and 75 %). The results were given in Figure 4.90. The results demonstrated that an increase in content of plasticizers resulted in decrease in mechanical resistance (low tensile strength), an increase in extensibility (high percentage of elongation). Tensile strength decreased from 6.10 to 0.82, 4.27 to 1.92 and 3.76 to 1.68 MPa when the sorbitol, glycerol and polyethylene glycol concentration increased from 25 to 75 % w/w, which inversely resulted in increase in elongation at break from 15.03 to 31.15, 62.20 to 173.58 and 75.30 to 153.21%, respectively (Fig. 4.90). The changes in mechanical properties as affected by hydrophilic plasticizers were previously observed in various hydrocolloid-based films (Park and Chinnan, 1990; Gontard *et al*., 1993). The mechanical property changes characterize decreases in density and reversibility of intermolecular interaction occurring in the edible films from water-soluble proteins network that forms films. Sorbitol, glycerol and polyethylene glycol are low molecular weight hydrophilic molecules that could easily fit into protein chains and establish hydrogen bonding with reactive groups of proteins. Bringing together plasticizers and proteins induces formation protein-plasticizer interactions to the detriment of protein-protein interactions. As a sequence, the density of intermolecular

interaction decrease in material and the free volume between polymer chains increases (Cuq *et al*., 1997).

The mechanical properties of sorbitol, glycerol and polyethylene glycol plasticized films at an equal concentration were compared (Fig.4.90). The sorbitol plasticized films had significantly ($p \leq 0.05$) higher tensile strength and lower elongation at break than glycerol and polyethylene glycol plasticized films at all plasticizers concentration. This could be attributed to the ring molecular conformation of sorbitol molecules, which may sterically hinder insertion between the proteins chains resulted in less effective disrupting the protein-protein interruptions. McHugh and Krochta (1994a) reported that a higher amount of sorbitol than glycerol was needed to obtain similar tensile strength properties in whey protein isolate film and suggested that the smaller size of glycerol molecule enable it to influence the film properties more readily than the sorbitol molecule.

The glycerol plasticized films were more mechanical resistant and stretchable than the polyethylene glycol plasticizer films (Fig. 4.90), suggesting that glycerol could be a more effective plasticizer than polyethylene glycol. The effectiveness of glycerol was most likely due to its small size which allows it to be more readily inserted between the polymer chains, and consequently exert more influence on the mechanical properties than the larger polyethylene glycol molecule. In addition, at an equal percentage concentration, the total number of glycerol molecules in the film-forming solution is greater than that of the higher molecular weight polyethylene glycol and therefore glycerol has more functional groups (-OH) than polyethylene glycol, which should promote the plasticizer-polymers interactions in the films (Donhowe and Fennema, 1993; McHugh and Krochta, 1994a). Donhowe and Fennema (1993) found

that plasticizer with low molecular weights such as glycerol (92.09) was more effective than those with high molecular weights polyethylene glycol (400) in methylcellulose-based films. Similarly, McHugh and Krochta (1994a) suggested that, due to its small size of plasticizer was more effective than larger size of plasticizer in whey protein films.

The polar group (-OH) along plasticizer chains are believed to develop polymer-plasticizer hydrogen bonds replacing the polymer-polymer interaction in the biopolymer films (Gennadios *et al.*, 1993). Molecular size, configuration and total number of functional hydroxide groups of the plasticizer as well as its compatibility with the polymer could affect the interactions between the plasticizer and the polymer (Yang and Paulson, 2000)

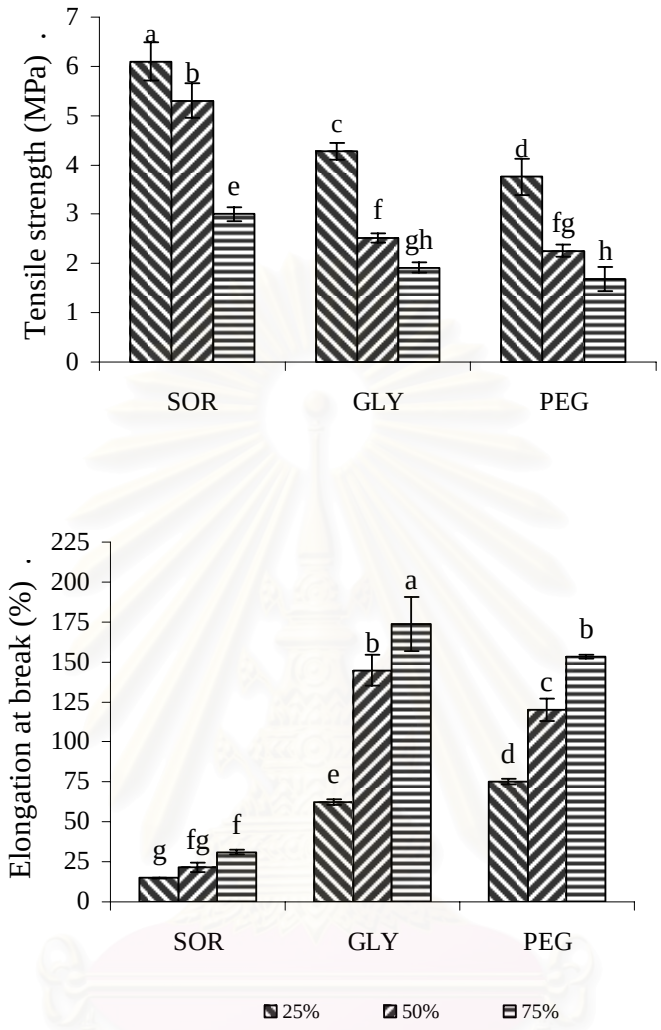


Fig. 4.90 Effect of plasticizer type and concentration on the tensile strength (MPa) and elongation at break (%) of edible film from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown. a-h; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol.

4.4.3.2 Water Vapor Permeability and Oxygen Permeability

Water vapor permeability of edible films from proteins precipitated by ethanol with different type and concentration of plasticizer were investigated (Figure 4.91). The water vapor permeability and oxygen permeability increased with increasing amount of plasticizer. The water vapor permeability and oxygen permeability increased from 4.61 to 28.94, 51.62 to 183.75 and 32.11 to 159.21 g.mm/m^2.d.kPa and 93.54 to 229.10, 134.84 to 274.94 and 170.00 to 308.16 cm^3.µm./m^2.d.kPa, respectively when the concentration of sorbitol, glycerol and polyethylene glycol increased from 25 to 75 % w/w (Figure 4.91). This tendency could be explained by structural modifications of the protein network. The incorporation of plasticizers modifies the molecular organization of the proteins network by increasing in the free volume. The network becomes less dense and as a consequence more permeable (Banker, 1966; Ashley, 1985). Permeability increase with plasticizer content could be related to hydrophilicity of plasticizer molecules. Introducing hydrophilic plasticizers, favorable to adsorption and desorption of water molecules, was reported to enhance the water vapor permeability and oxygen permeability of hydrocolloid-based films (Gontard *et al.*, 1993; McHugh *et al*., 1994).

Comparison of the successive values of the water vapor permeability and oxygen permeability for each plasticized films was shown in Figure 4.91. Films plasticized with sorbitol had the lower water vapor permeability and oxygen permeability than glycerol and polyethylene glycol at each plasticizer concentration due to the fact that sorbitol had ability to bind less water than glycerol and polyethylene glycol, thereby, provided a lower water vapor permeability (McHugh *et al*., 1994a). Chick and Ustanol (1998) showed that casein-based films plasticized with glycerol had

higher water vapor permeability and oxygen permeability values than films plasticized with sorbitol when the same amounts of plasticzer were used. The high hydrophilicity of glycerol and polyethylene glycol molecules, which is favorable to the adsorption of water molecules, could also be contributed to the decrease in barrier properties (Gennadios *et al.*, 1993). The increase in water vapor permeability with increasing hydrophilicity plasticizer concentration is also common in edible films (McHugh *et al*., 1994a; Cuq *et al*., 1997). Sorbal *et al*. (2001) reported that hydrophilicity of the plasticizers will increase the water content of the films, consequently increasing the mobility of the molecules. In addition, increasing water content could also affect permeate solubility in the films.

Water vapor permeability of edible films plasticized by glycerol was significantly ($p \leq 0.05$) higher than polyethylene glycol plasticized films (Fig. 4.91). It could be due to the fact that the small size of glycerol which allows it to be more readily inserted between the polymer chains, and consequently exert more influence water vapor permeability and oxygen permeability properties than the larger polyethylene glycol molecule. However, comparing at an equal percentage concentration, the total number of glycerol molecules in the film solutions is greater than that of the higher molecular weight polyethylene glycol and therefore glycerol had more hydrophilic group than polyethylene glycol which should enhance the solubility and diffusivity of water through films structure resulted in higher water vapor permeability.

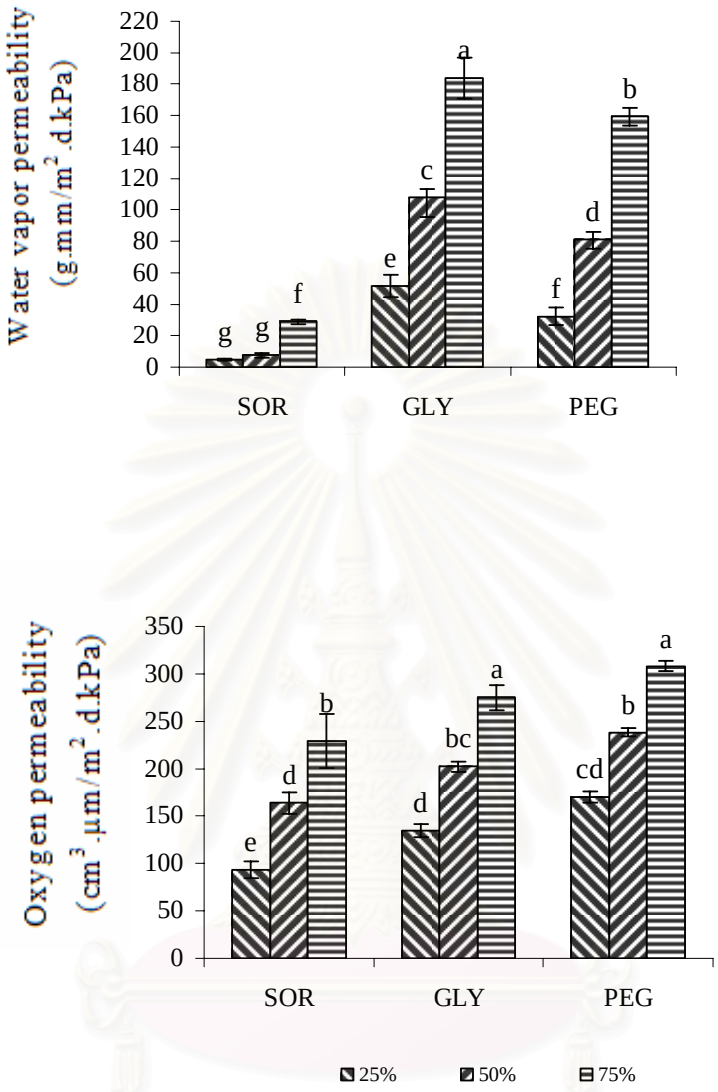


Fig. 4.91 Effect of plasticizer type and concentration on the water vapor permeability (g.mm/m^2.d.kPa) and oxygen permeability (cm^3.µm/m^2.d.kPa) of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a-g; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol.

4.4.3.3 Film and Protein Solubilities

Irrespective of plasticizer type, an increase in plasticizer content led to an increase in film and protein solubilities (Fig. 4.92). It could be concluded that hydrophilic plasticizers enhance films solubility in water. Low molecular weight protein chains (i.e. monomers and small peptides) formed during storage of film solutions and entrapped in the network (Cuq *et al.*, 1995) could thus constitute the protein-based materials that solubilize in water. The dry matter solubilized in water was likely to be constituted mainly by the plasticizer. Plasticizer solubilization in water was already observed for film based on wheat gluten or treated soy proteins or produced by transglutaminase catalytic cross-linking of whey protein (Gontard *et al*., 1992; Stuchell and Krochta, 1994). Stuchell and Krochta (1994) had pointed out that increase in the content of protein solubilized in water was obtained when the hydrophilic content of treated whey protein-and soy protein-based films increased. A decrease in the polymer network interaction density due to the presence of plasticizer was thus associated with this increase in solubility properties. The lowest film and protein solubilities of the edible films plasticized by 25% w/w of these plasticizer were observed, while increasing the amount of plasticizer content showed higher film solubility and protein solubility (Fig. 4.92). It could be explained that, at higher content of plasticizer, there were more molecules of plasticizer untrapped in the cross linked protein network and able to escape into solution, while, lower content of plasticizer provided lowered plasticizer molecules untrapped in the crosslinked network resulted in less ability to escape into solution. The film and protein solubilities were higher for the edible films plasticized with sorbitol than those of glycerol and polyethylene glycol. The sorbitol had a ring molecular conformation which may sterically hinder insertion between the protein chains (Yang and

Paulson, 2000) thus facilitated its escaped into solution, while glycerol and polyethylene glycol have a straight chains, which promote the insertion between protein chains.

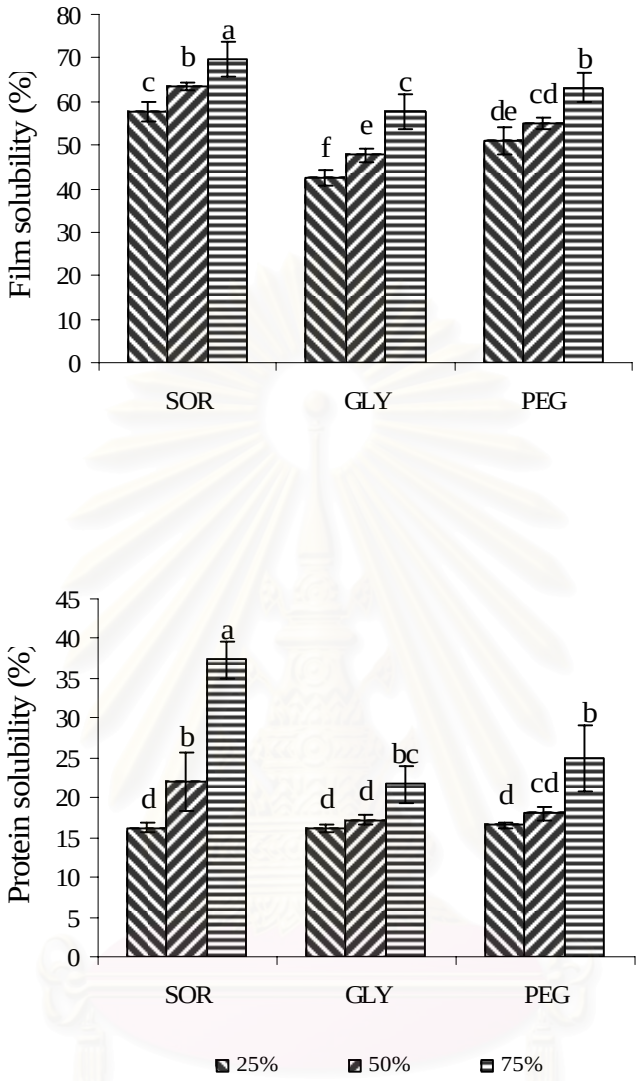


Fig. 4.92 Effect of plasticizer type and concentration on the film solubility (%) and protein solubility (%) of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a-f; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol.

4.4.3.4 Film Color

The results of the measurements performed on the films color were expressed in accordance with the CIELAB system, and the rectangular coordinates (L*, a* and b*) were defined. The color of films was more affected by the nature of the plasticizer than by its concentration. L* value of edible films plasticized by sorbitol, glycerol and polyethylene glycol were not significantly different ($p > 0.05$) (Fig. 4.93). In contrast, increase in the yellowness (greater + b*) and a* value occurred when glycerol and polyethylene glycol were used (Fig. 4.94 and 4.95). This was somewhat expected since color change depend on the type of plasticizer.

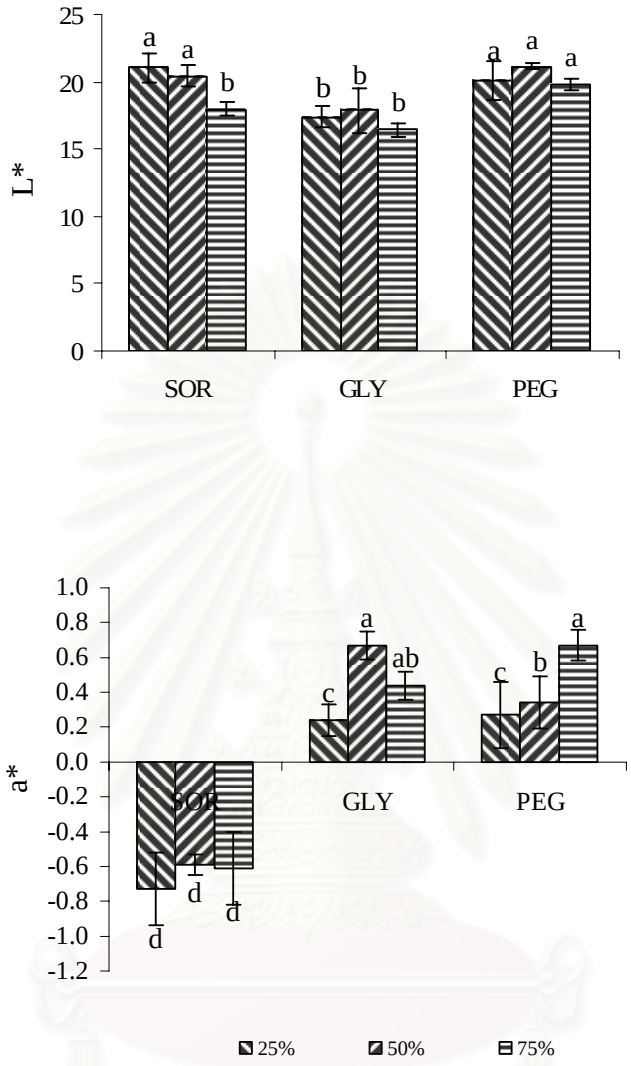


Fig. 4.93 Effect of plasticizer type and concentration on L* value and a* values of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a-d; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol.

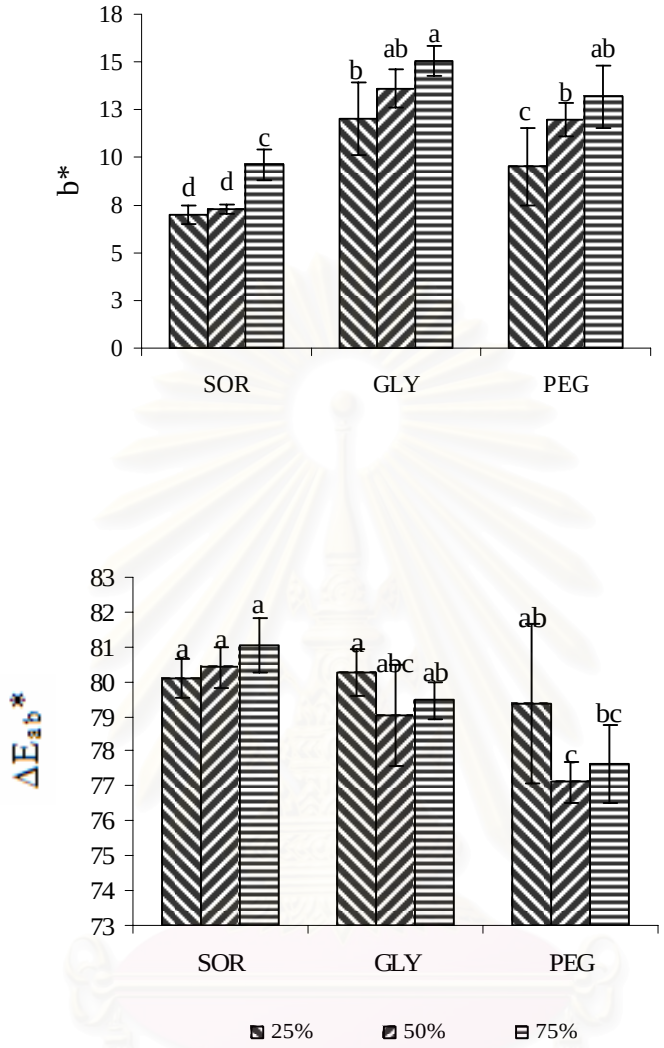


Fig. 4.94 Effect of plasticizer type and concentration on b* and ΔE_{ab}* values of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a-d; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

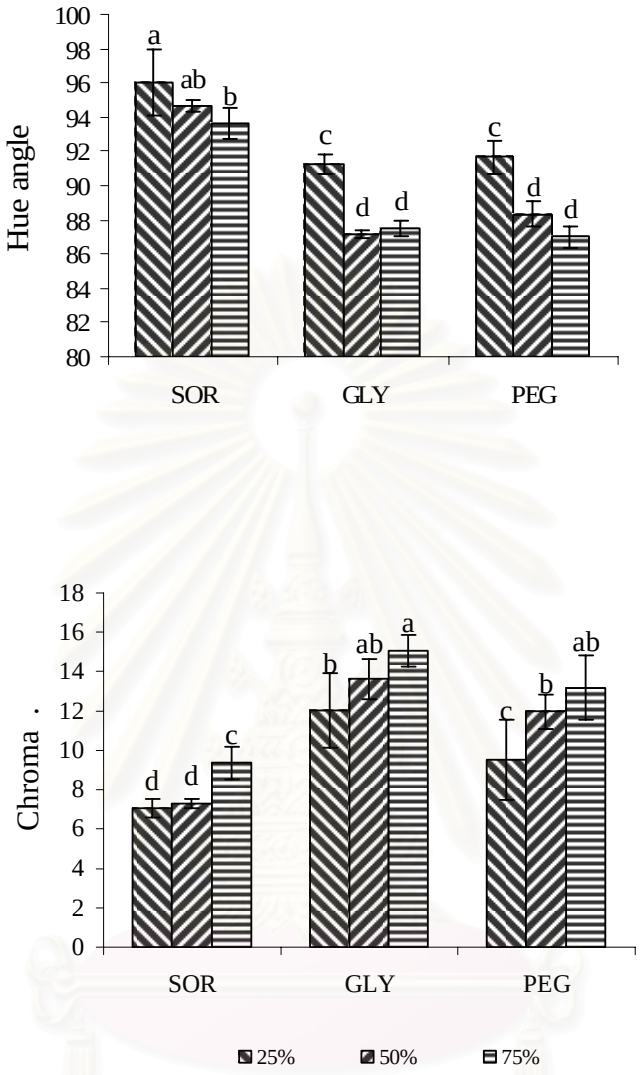


Fig. 4.95 Effect of plasticizer type and concentration on hue angle and chroma values of edible films from water-soluble fish proteins precipitated by ethanol. Standard error bars are shown.

a-d; means with different letters represent significantly different value at $p \leq 0.05$ using Duncan's Multiple Range Test, where, SOR = sorbitol, GLY = glycerol and PEG = poly ethylene glycol

4.5 Comparison of Water–Soluble Fish Proteins Films with Selected Biopolymer and Synthetic Polymer Films

The mechanical properties (tensile strength and elongation at break) of water-soluble fish proteins based-edible films prepared from different recovering method and of various films were compared (Table 4.4). Water-soluble fish proteins based-edible films had mechanical properties similar to those other protein sources. Tensile strength of edible films from water-soluble fish proteins precipitated by shifting the pH and ethanol were slightly above (films were more mechanically resistant) but elongation was slightly below (film were less deformable) than casein, soy protein isolate, wheat gluten and the water-soluble fish proteins reported by Iwata *et al.* (2000) (Table 4.4). Edible films from water-soluble fish proteins had substantially lower both tensile strength and elongation at break than synthetic polymer (low density polyethylene, high density polyethylene, polyvinyl chloride, cellulose acetate and polyester) (Table 4.4). However, edible films from water-soluble fish proteins precipitated by shifting the pH had higher percentage of elongation at break (77.14%) than polyvinylidine chloride and cellulose acetate films, meanwhile elongation at break of edible films from water-soluble fish proteins precipitated by ethanol had almost similar to polyvinylidine chloride and cellulose acetate films (Table 4.4).

Water vapor permeability of edible films from water-soluble fish proteins and various films were presented in Table 4.5. Water vapor permeability of edible films from water-soluble fish proteins were characterized by relative low water vapor barrier properties than other protein sources (soy protein isolate, whey protein isolate and milk proteins) especially the edible films from water-soluble fish proteins from proteins precipitated by shifting the pH and ethanol. Water vapor permeability

determined in this study of edible films from water-soluble fish proteins were higher than lower density polyethylene and high density polyethylene, however edible films from water-soluble fish proteins precipitated by shifting the pH and ethanol had lower water vapor permeability than cellophane (Table 4.5). Resistance of protein-based edible films to water vapor transmission is limited due to the inherent hydrophilicity of proteins. Transmission of water vapor through protein-based edible film is also facilitated by the presence of, a hydrophilic plasticizer, which favors adsorption of water molecules (Cuq *et al.,* 1995).

Oxygen permeability values of the three edible films from water-soluble fish proteins and of various films were compared (Table 4.5). The edible films from water-soluble fish proteins precipitated by shifting the pH had lower oxygen permeability than whey protein isolate film. However, edible films from water-soluble fish protein from freeze-drying and precipitated by ethanol had oxygen permeability higher than whey protein isolate, peanut protein, amylose and amylopectin films. Oxygen permeability of edible films from water-soluble fish proteins were lower than those of common plastic films (low density polyethylene, high density polyethylene, and polypropylene) especially edible films from water-soluble fish proteins precipitated by shifting the pH. Even polyamide, 6, a packaging materials regarded as a good oxygen barrier (Billing, 1989), had very close permeable to oxygen with edible films from water-soluble fish proteins precipitated by shifting the pH.

Table 4.4 Tensile strength (TS) and elongation at break (E) of various films

Film Type (polymer: plasticizer)	Test condition	Tensile strength (MPa)	Elongation at break (%)	Reference
Water-soluble fish proteins film				
Sample 1[a]: Sorbitol (2: 1)	23 °C, 50% RH	3.02	13.31	Current study
Sample 2 [b]: Sorbitol (2: 1)	23 °C, 50% RH	5.21	72.14	Current study
Sample 3 [c]: Sorbitol`(2: 1)	23 °C, 50% RH	5.04	33.49	Current study
Casein: Glycerol (49: 1)	25 °C, 50% RH	4.1	38.00	Motoki *et al.* (1987)
Soy protein isolate: Glycerol (5:3)	25 °C, 50% RH	3.6	139.00	Gennadiose *et al.* (1993a)
Wheat gluten: Glycerol (12: 4.4)	25 °C, 50% RH	4.4	170.00	Gennadiose *et al.* (1993a)
Milk protein: Glycerol (25:7.5)	25 °C, 65% RH	8.6	22.10	Maynes and Krochta (1994)
Wheat gluten: Glycerol (3: 1)	23 °C, 55% RH	2.12	-	Herald *et al.* (1995)

[a] Freeze-dried proteins; [b] Proteins precipitated by shift the pH; [c] Proteins precipitated by ethanol

Table 4.4 Tensile strength (TS) and elongation at break (E) of various films (continued)

Film Type (polymer: plasticizer)	Test condition	Tensile strength (MPa)	Elongation at break (%)	Reference
Peanut protein: Glycerol (1: 2)	25 °C, 50% RH	4.35	105.00	Jangchud and Chinnan (1999)
Water-soluble fish proteins: Glycerol (2:1)	23 °C, 50% RH	3.0-5.5	40-70	Iwata *et al.* (1999)
Water-soluble fish proteins: Glycerol (2:1)		1.84	48.72	Kerdsup *et al.* (2002)
Low density polyethylene	38 °C, 90% RH	7.60-17.30	500	Briston (1988)
High density polyethylene	38 °C, 90% RH	17.3-34.6	300	Briston (1988)
Polyvinylidine chloride	38 °C, 90% RH	48.4-138.0	20-40	Briston (1988)
Cellulose acetate	38 °C, 90% RH	48.5-82.7	15-45	Briston (1988)
Polyester	38 °C, 90% RH	178.0	70.0-100.0	Briston (1988)

Table 4.5 Water vapor permeability (WVP) and oxygen permeability (OP) of various films

Film Type (polymer: plasticizer)	Test condition [d]	Water vapor permeability (gram.mm/m^2.day.kPa)	Oxygen permeability (cm^3.µm/m^2.day.kPa)	Reference
Water-soluble fish proteins				
Sample 1[a]: Sorbitol (2: 1)	23 ^oC, 50/0% RH	58.55	351.33	Current study
Sample 2 [b]: Sorbitol (2: 1)	23 ^oC, 50/0% RH	5.19	15.05	Current study
Sample 3 [c]: Sorbitol (2: 1)	23 ^oC, 50/0% RH	8.41	95.34	Current study
Soy protein isolate: Glycerol (5:3)	25 ^oC, 50/0% RH	284	-	Gennadiose *et al.* (1993)
Whey protein isolate: Glycerol (1.6:1)	25 ^oC, 0/65% RH	119.8	-	McHugh *et al.* (1994)
Whey protein isolate: Sorbitol (1.6:1)	25 ^oC, 0/79% RH	62.0	-	McHugh *et al.* (1994)
Whey protein isolate: Glycerol (2.3:1)	25 ^oC, 50/0% RH	-	76.1	McHugh and Krochta (1994)
Whey protein isolate: Glycerol (3.5:1)	25 ^oC, 70/0% RH	-	43.3	McHugh and Krochta (1994)
Non fat dried milk : Glycerol (4:1)	25 ^oC, 0/65% RH	70.3	-	Maynes and Krochta (1994)

[a] Freeze-dried proteins; [b]Proteins precipitated by pH shift; [c]Proteins precipitated by organic solvent

[d] Relative humidity (RH) were those on out side and inside of the test cup

Table 4.5 Water vapor permeability (WVP) and oxygen permeability (OP) of various films (continued)

Film Type (polymer: plasticizer)	Test condition [d]	Water vapor permeability (gram.mm/m^2.day.kPa)	Oxygen permeability (cm^3.µm/m^2.day.kPa)	Reference
Wheat gluten: glycerol (3:1)	23 ^oC, 55/0% RH	66.37	-	Herald *et al.* (1995)
Water soluble fish proteins: Glycerol (2:1)	30 ^oC, 100/0% RH	10.08	-	Iwata *et al.* (1999)
Peanut protein: Glycerol (3:5)	37.8 ^oC, 55/0% RH	10.35	2.13	Jangchud and Chinnan (1999)
Amylose: Glycerol (7:3)	20 ^oC, 50/0% RH	-	15.0	Forssell *et al.* (2002)
Amylopectin: Glycerol (7:3)	20 ^oC, 50/0% RH	-	10.89	Forssell *et al.* (2002)
Low density polyethylene	38^oC 90/0% RH	0.079	-	Smith (1986)
High density polyethylene	38^oC 90/0% RH	0.02	-	Smith (1986)
Cellophane	38^oC 90/0% RH	7.27	-	Taylor (1986)

[d] Relative humidity (RH) were those on out side and inside of the test cup

Table 4.5 Water vapor permeability (WVP) and oxygen permeability (OP) of various films (continued)

Film Type (polymer: plasticizer)	Test condition [d]	Water vapor permeability (gram.mm/m^2.day.kPa)	Oxygen permeability (cm^3.µm/m^2.day.kPa)	Reference
Low density polyethylene	23°C 50/0% RH	-	1870	Salame (1986)
High density polyethylene	23°C 50/0% RH	-	427	Salame (1986)
Cellophane	23°C 50/0% RH	-	16	Taylor (1986)
Polypropylene	23°C 50/0% RH	-	741	Billing (1989)
Polystyrene	23°C 50/0% RH	-	1235.7	Billing (1989)
Polyvinyl chloride	23°C 50/0% RH	-	29.1	Billing (1989)

[d] Relative humidity (RH) were those on out side and inside of the test cup

4.6 Comment on Properties and Film Forming Ability of Water-Soluble Fish Proteins in Surimi Wash-Water from Different Recovery Methods

Difference in film-forming condition and films properties were obtained when edible film prepared from different recovery water-soluble fish proteins method. Edible film from freeze-dried water-soluble fish proteins and proteins precipitated by ethanol were formed under alkali conditions, while edible film from protein precipitated by shifting the pH could not be formed well. Contrarily, the edible film from protein precipitated by shifting the pH was formed well under acid condition. The difference of film-forming conditions resulted from the different in degree of denaturation of recovered proteins. Addition of ethanol lowered the dielectric constant of the solution, and hence, its solving power. Thus, the solubility of a protein was decreased and denatured through electrostatic attraction could occur. While, recovery of proteins by shifting the pH resulted from the negative and positive charges on surface on the molecule cancel one another out, electrostatic repulsion between individual molecules no longer occurs and electrostatic attraction between molecules may occur, resulting of a precipitation. The temperature was also reported to be important on these recoveries, higher temperature may cause higher protein denaturation. Tensile strength and elongation at break were highest at pH about 10.0 and heating temperature , 70 ^{o}C for freeze-dried water-soluble fish proteins and pH 2.0 and heating temperature, 80 ^{o}C for proteins precipitated by shifting the pH and pH 11.5 and heating temperature, 60 ^{o}C for proteins precipitated by ethanol. In contrast, at this same condition, the water vapor permeability oxygen permeability, film solubility and protein solubility values were at their lowest. The mechanical properties (tensile strength and elongation at break) and barrier properties (water vapor permeability and oxygen permeability) of edible protein

films produced from various recovery methods were different. Tensile strength and elongation at break (5.21 MPa and 72.14%, respectively) of edible film produced from proteins precipitated by shifting the pH showed higher than edible film produced from proteins precipitated by ethanol (4.19 MPa and 33.49%, respectively) and freeze-dried (3.02 MPa and 14.72%, respectively). In contrast, at this same condition, the water vapor permeability, oxygen permeability, film solubility and protein solubility values (5.19 $g.mm/m^2.d.kPa$, 13.27 $cm^3.\mu m/m^2.d.kPa$, 48.63% and 17.71%, respectively) of edible film produced from proteins precipitated by shifting the pH were lower than edible film produced from proteins precipitated by ethanol (8.41 $g.mm/m^2.d.kPa$, 95.34 $cm^3.\mu m/m^2.d.kPa$, 49.86% and 35.85%, respectively) and freeze-dried water-soluble fish proteins (58.55 $g.mm/m^2.d.kPa$, 351.33, $cm^3.\mu m/m^2.d.kPa$, 47.94% and 14.34%, respectively). The surface hydrophobicity and content of disulfide bond increased with increase in pH and heating temperature, while the available sulfhydryl group decreased. Increase of the protein concentration provided the film with higher tensile strength but lowers elongation at break, water vapor permeability, oxygen permeability, film solubility and protein solubility and development of darker and more yellowish films. Increasing in contents of plasticizers resulted in decreased tensile strength, but increased elongation at break, water vapor permeability, film solubility and protein solubility. Sorbitol provided the films with the highest tensile strength, film solubility and protein solubility but lowest elongation at break and water vapor permeability, while glycerol and polyethylene glycol provided the films with high elongation at break, water vapor permeability, but low tensile strength, film solubility and protein solubility. The color of edible films changed with the plasticizer type. Mechanical properties of all three water-soluble fish protein films were pretty similar to those of various biomaterials based films.

However, the films had substantially lower mechanical properties than polymeric materials. The edible film from water-soluble fish proteins were characterized by relatively poor water vapor barrier properties, but showing lower oxygen permeability than values of other polysaccharide and common plastic films, such as low and high density polyethylene, polypropylene and polystyrene.

CHAPTER V

CONCLUSIONS

5.1 Recovery and Characterization of Water-Soluble Fish Proteins Precipitated from Surimi Wash-Water

- Most of the valuable water-soluble fish proteins, which would be otherwise lost in surimi processing, could be successfully recovered in the very first wash stage as demonstrated in this study.
- Percentage of precipitation of proteins was directly related to temperature increase; maximum precipitation (52.8-66.3%) occurred around pH 3.5, however precipitated proteins had the lowest solubility.
- Increasing organic solvent concentration yielded greater precipitation; maximum precipitation (64%) was for 60% wt/wt of ethanol.
- There was direct correlation between percentage of precipitation and reaction temperature, which reversed with protein solubility.
- Reaction time for pH shift and organic solvent had little or no significant effect ($p > 0.05$) on percentage of precipitation.

5.2 Effect of pH, Heating Temperature, Heating Time, Protein Concentration and Plasticizer on the Properties of Edible Films from Water–Soluble Fish Proteins in Surimi Wash-Water

- The pH and heating temperature of film-solutions had the greatest impact on the physico-chemical and permeability properties of edible films from water-soluble fish proteins.
- The films produced at pH ~10.0 at 70 ^{o}C exhibited the highest tensile strength and elongation at break, while water vapor permeability and oxygen permeability were at there lowest.
- There was a direct correlation between the film solubility and protein solubility and heating temperature, which reversed with change in pH.
- Color of films turned darker and more yellow with increase in the pH.
- The surface hydrophobicity and available SH group increased with increase in pH.
- The content of SS bond increased as increasing of heating temperature.
- Increasing the protein concentration provided the films with a higher tensile strength but lowered of elongation at break, water vapor permeability, oxygen permeability, film solubility and protein solubility and showed a darker and more yellowish films.
- Sorbitol resulted in greatest mechanical resistance, but poorest in film flexibility.

- Glycerol and polyethylene glycol was found to yield the most flexible structure, however, the mechanical strength was low, which inverse with water vapor permeability.
- Increase in plasticizers concentration resulted in decrease of tensile strength with concomitant increase of elongation at break and water vapor permeability.
- Increasing plasticizer concentration resulted in higher solubility.
- Sorbitol plasticized films showed higher solubility than glycerol and polyethylene glycol plasticized films.
- The change in color of edible film depended on the plasticizer type.

5.3 Effect of pH, Heating Temperature, Heating Time, Protein Concentration and Plasticizer on the Properties of Edible Films from Proteins Precipitated by pH Shift in Surimi Wash-Water

- The pH and temperature of film-solutions had the greatest impact on the physico-chemical and permeability properties of edible films from proteins precipitated by pH shift in surimi wash-water.
- The films produced at pH ~2.0 at 80 ^{o}C exhibited high tensile strength and elongation at break, while water vapor permeability and oxygen permeability were at their lowest.
- Increasing heating temperature of film solutions from 60 to 80 ^{o}C, resulted in increase in tensile strength but decrease in water vapor

permeability, oxygen permeability, film solubility and protein solubility.

- Positive correlation was observed between both film solubility and protein solubility with change in pH of film-solutions.
- Excessive pH and heating temperature of film-solutions resulted in darker and more yellowish color of edible films.
- The surface hydrophobicity, available SH group and content of SS bond increased with decrease in pH of film-solutions concomitant with increase in heating temperature.
- Increasing the protein concentration provided the films with higher tensile strength but lowered of elongation at break, water vapor permeability and oxygen permeability and provided a darker and more yellowish film.
- The effects of type and concentration of these plasticizers on physico-chemical properties of the films showed a similar trend to edible films produced from freeze dried water-soluble fish proteins in surimi wash-water.

5.4 Effect of pH, Heating Temperature, Heating Time, Protein Concentration and Plasticizer on the Properties of Edible Films from Proteins Precipitated by Organic Solvent in Surimi Wash-Water

- The pH and temperature of film-solutions had the greatest impact on the physico-chemical and permeability properties of edible films from proteins precipitated by organic solvent in surimi wash-water.
- The films produced at pH ~11.5 at 60 ^{o}C demonstrated high tensile strength and elongation at break, while water vapor permeability and oxygen permeability were at their lowest.
- Increasing heating temperature of film-solutions from 60-80 ^{o}C resulted in decreased in tensile strength but increased in water vapor permeability, oxygen permeability, film solubility and protein solubility.
- Increase in protein concentration provided the films with a higher tensile strength but lower elongation at break, water vapor permeability, oxygen permeability, film solubility and protein solubility, additionally the films showed darker and more yellowish color.
- The effects of type and concentration of these plasticizers on physico-chemical properties of the films showed a similar trend to edible films prepared from freeze dried water-soluble fish protein and proteins precipitated by shifting the pH in surimi wash-water.

5.5 Comparison of Water–Soluble Fish Proteins Films with Selected Biopolymer and Synthetic Polymer Films

- Edible films from water-soluble fish proteins films had mechanical properties similar to those other protein sources.
- Water-soluble fish proteins had substantially lower tensile strength and elongation at break than synthetic polymer.
- Edible films from water-soluble fish proteins were characterized by relative low water vapor barrier properties than other protein sources (soy protein isolate, whey protein isolate and milk proteins) especially the edible film produced from proteins precipitated by shifting the pH and ethanol.
- Edible films from water-soluble fish protein prepared from proteins precipitated by shifting the pH and ethanol had higher water vapor permeability than lower density polyethylene and high density polyethylene but lower water vapor permeability than cellophane.
- Edible films from water-soluble fish proteins produced from proteins precipitated by shifting the pH and ethanol had lower oxygen permeability than low density polyethylene, high density polyethylene and polypropylene.

CHAPTER VI

SUGGESTIONS AND POTENTIAL APPLICATIONS

6.1 Water-soluble fish protein films when apply to food products must provide the product with satisfactory appearance, aroma, flavour and mouth feel. Hence, selective of application to appropriate food and good control of environmental condition are necessary to ensure the consumer acceptability.

6.2 Edible films are usually considerably more expensive than conventional synthetic polymers. Improvement in production practice, economic of scale and increasing source of by products could all be necessary to produce a more favourable economic situation for biodegradable polymer.

6.3 Rather than only determining the optimum condition for all responses, it would be preferable to choose particular film formation combinations based on the emphasis required of specific use of the film; application techniques or other considerations. For example, if the film to be used on a superficial coating for handled products, the prime properties to optimize would be film mechanical properties and appearance.

6.4 Development of the function of edible films from water-soluble fish proteins as carrier for other food additive such as antioxidants and/or antimicrobial that will migrate into the packed food and prolong its shelf life.

6.5 Improving water-soluble properties of edible films from water-soluble fish proteins in order to enhance it application in food area by combining the protein with functional polysaccharide such as pollulan.

6.6 All protein chains are random in solution state. Rapid drying will tend to freeze the protein chains in the random state, while slow drying allow the molecules to realign themselves into a more order state. Thus, the study of the effect of drying rate on properties of edible films from water-soluble fish proteins needs to be investigated.

6.7 The sorption isotherms of edible protein films vary, depending on their water vapor permeability property and hydrophilic nature. In addition, the sorption isotherm, which is a necessary parameter to predict the properties of films at different environments pertinent to their application, thus it is important to study sorption isotherm of edible film from water-soluble fish proteins.

6.8 Although edible films from water-soluble fish proteins are poor water vapour barrier, they are found to be effective oxygen barrier. It may worth trying to be used as oxygen barrier layer in multilayer packaging materials.

6.9 Protective edible films from water-soluble fish proteins could also be used on certain food products, such as meat pies and high-moisture low sugar cake, that require films with highly permeable to water vapour.

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Appendices

APPENDIX A

Table 1 Experimental data for the three-factor, three level response surface analysis [a]

	pH	Temp	Time	Tensile strength (Mpa)	Elongation at break (%)	Water vapor permeability ($g.mm/m^2.d.kPa$)	Oxygen permeability ($cm^3.\mu m/ m^2.d.kPa$)	Films solubility	Proteins solubility
Treatment	x_1	x_2	x_3	TS	%E	WVP	OP	FS	PS
1	1	1	0	1.173	8.70	161.56	995.66	77.32	29.74
2	1	-1	0	0.891	8.50	179.46	546.04	79.22	33.13
3	-1	1	0	1.095	14.72	174.32	456.75	61.26	15.30
4	-1	-1	0	0.915	8.41	207.22	1036.12	62.30	19.12
5	1	0	1	1.036	10.04	175.49	827.25	65.32	26.95
6	1	0	-1	1.015	8.94	179.16	859.04	62.30	22.11
7	-1	0	1	1.225	13.78	157.14	712.86	60.26	18.47
8	-1	0	-1	1.280	11.76	139.92	1032.86	60.21	16.72
9	0	1	1	1.444	13.47	143.42	770.09	55.51	18.41
10	0	1	-1	1.186	10.23	153.86	752.81	60.62	21.07
11	0	-1	1	1.184	9.77	165.13	808.75	60.32	19.17
12	0	-1	-1	1.207	9.23	168.24	692.25	65.48	20.51
13	0	0	0	1.643	9.72	114.47	272.00	57.69	15.85
14	0	0	0	1.543	10.25	109.81	220.11	58.23	20.67
15	0	0	0	1.595	9.98	119.11	257.52	55.61	18.64

[a] The experimental runs were performed in a random order

Table 1 Experimental data for the three-factor, three level response surface analysis [a] (continued)

	pH	Temp	Time	Color					
Treatment	x_1	x_2	x_3	L	a	b	ΔE^*_{ab}	Hue Angle	Chroma
1	1	1	0	33.13	-0.46	12.35	67.24	92.17	12.36
2	1	-1	0	30.92	-0.36	11.35	69.36	91.80	11.69
3	-1	1	0	32.43	-0.88	5.63	72.14	98.91	5.70
4	-1	-1	0	28.69	-0.72	6.12	74.62	96.74	6.16
5	1	0	1	31.76	-0.31	11.73	68.67	91.55	11.73
6	1	0	-1	29.56	-0.58	8.08	72.69	94.14	8.10
7	-1	0	1	26.15	-0.82	7.09	76.03	96.74	7.02
8	-1	0	-1	27.10	-0.97	5.37	76.32	100.27	5.46
9	0	1	1	32.26	-0.42	9.56	69.67	92.55	9.57
10	0	1	-1	32.15	-0.33	9.32	69.83	92.04	9.45
11	0	-1	1	31.26	-0.40	7.30	71.90	93.17	7.31
12	0	-1	-1	28.74	-0.26	8.68	72.94	91.75	8.68
13	0	0	0	30.26	-0.75	9.31	71.36	94.64	9.34
14	0	0	0	31.15	-0.68	8.12	71.45	94.82	8.15
15	0	0	0	32.86	-0.70	8.64	69.81	94.66	8.67

[a] The experimental runs were performed in a random order

Table 1 Experimental data for the three-factor, three level response surface analysis [a] (continued)

	pH	Temp	Time	Hydrophobicity	SS bond (mole / g protein)	AvailableSH group (mole / g protein)
Treatment	x_1	x_2	x_3	HQ	SS	Available SH
1	1	1	0	687.79	880.12	1.76
2	1	-1	0	725.99	933.36	3.46
3	-1	1	0	395.77	1249.81	4.04
4	-1	-1	0	544.81	757.15	4.92
5	1	0	1	697.49	538.48	1.56
6	1	0	-1	645.92	549.11	2.15
7	-1	0	1	546.69	1189.07	5.27
8	-1	0	-1	574.65	1049.12	4.85
9	0	1	1	682.78	1083.89	2.65
10	0	1	-1	665.38	902.05	2.80
11	0	-1	1	748.37	947.09	4.13
12	0	-1	-1	703.81	897.78	4.13
13	0	0	0	684.53	1078.00	3.66
14	0	0	0	698.00	1016.09	4.26
15	0	0	0	671.06	972.40	3.65

[a] The experimental runs were perfomed in a random order

Table 2 Regression Coefficient of the second order polynomial for fifteen response variables

Coefficient	Tensile Strength (TS) k = 1	%Elongation (%E) k = 2	Water vapor permeability (WVP) k = 3	Oxygen permeability (OP) k = 4	Film solubility (FS) k = 5
β_{ko}	1.59	9.98	4.74	249.87	4.05
β_{k1}	-0.05	-1.56	0.01	-6.32	0.07
β_{k2}	0.09	1.40	-0.06	-18.48	-0.02
β_{k3}	0.03	0.86	0.001	-27.25	-0.01
β_{k11}	-0.35	0.28	0.23	300.39	0.11
β_{k22}	-0.23	-0.18	0.21	198.37	0.08
β_{k33}	-0.11	0.87	0.12	307.73	-0.03
β_{k12}	0.03	-1.53	0.02	247.25	-0.002
β_{k13}	0.02	-0.23	-0.04	72.05	0.01
β_{k23}	0.07	0.68	-0.01	-24.81	0.00

Table 2 Regression Coefficient of the second order polynomial for fifteen response variables (continued)

Coefficient	Protein solubility (PS) k = 6	L* k = 7	a* k = 8	b* k = 9	ΔE^*_{ab} k = 10	Hue Angle k = 11	Chroma k = 12
β_{ko}	18.39	31.42	-0.71	8.69	70.87	94.70	8.72
β_{k1}	5.29	1.38	0.21	2.41	-2.64	-2.87	2.44
β_{k2}	-0.93	1.30	-0.04	0.43	-1.24	0.28	0.41
β_{k3}	0.32	0.49	0.02	0.53	-0.69	-0.52	0.49
β_{k11}	3.60	-1.30	-0.11	-0.24	1.15	1.75	-0.21
β_{k22}	2.33	1.16	0.21	0.41	-1.19	-1.55	0.47
β_{k33}	-0.93	-1.49	0.15	-0.39	1.40	-0.78	-0.43
β_{k12}	0.11	-0.38	0.02	0.37	0.09	-0.45	0.28
β_{k13}	0.77	0.79	0.03	0.48	-0.93	0.24	0.52
β_{k23}	-0.33	-0.60	0.01	0.41	0.22	-0.23	0.37

Table 2 Regression Coefficient of the second order polynomial for fifteen response variables (continued)

Coefficient	Hydrophobicity (HQ) k = 13	SS bond (SS) k = 14	Available SH group (ASH) k = 15
β_{ko}	684.53	1022.16	3.86
β_{k1}	86.98	-168.01	-1.27
β_{k2}	-36.41	72.56	-0.67
β_{k3}	10.70	45.06	-0.04
β_{k11}	-89.92	-96.65	-0.14
β_{k22}	-6.02	29.61	-0.17
β_{k33}	27.58	-94.06	-0.26
β_{k12}	27.71	-136.47	-0.21
β_{k13}	19.88	-37.64	-0.25
β_{k23}	-6.79	33.13	-0.04

Table 3 ANOVA and model fitting from the response variables

Source	df	Sum of squares								
		Tensile strength (TS)	%Elongation (E)	Water vapor permeability (WVP)	Oxygen permeability (OP)	Films solubility	Proteins solubility	L*	a*	b*
Model	9	0.72*	55.74**	8645.53	1000440.00	557.61	304.91	55.21	0.67**	53.87
Linear	3	0.09	41.16**	856.46	8993.54	227.66	231.57*	30.42*	0.37**	50.25
Quadratic	3	0.61**	3.21*	7566.54*	723694.00**	327.56	70.47	20.26	0.30**	1.48
Cross product	3	0.02	11.37**	222.53	267753.00**	2.39	2.87	4.52	0.01	2.14
Residual	5	0.05	0.64	1475.79	36080.00	115.13	58.45	7.33	0.06	9.37
Lack of fit	3	0.05	0.51	1432.55*	34646.00	111.30*	46.73	3.48	0.06	8.66
Pure error	2	0.01	0.14	43.24	1433.92	3.83	11.71	3.50	0.01	0.71
%variability explained (R^2)		93.22	98.84	85.45	96.52	82.89	83.92	88.28	91.68	85.19

* Significant at 5% level

** Significant at 1% level

Table 3 ANOVA and model fitting from the response variables (continued)

Source	df	Sum of squares					
		ΔE^*_{ab}	Hue angle	Chroma	Hydrophobicity (HQ)	Content of SS bond (SS)	Available SH group (ASH)
Model	9	94.08**	94.33*	54.71	109655.00*	438086.00	17.33*
Linear	3	72.06**	68.93**	50.98*	71944.00**	284186.00	16.52**
Quadratic	3	18.31	24.17	1.79	32874.00**	69344.00	0.37
Cross product	3	3.70	1.24	1.94	4837.05	84556.00	0.43
Residual	5	3.06	9.17	9.82	9295.70	132557.00	1.14
Lack of fit	3	1.36	9.15	9.11	8932.82	126926.00	0.90
Pure error	2	1.70	0.02	0.71	362.88	5631.00	0.24
%variability explained (R^2)		96.85	91.14	84.79	92.19	76.77	93.82

* Significant at 5% level

** Significant at 1% level

Table 4 ANOVA: overall effect of independent variables on response variable

		Sum of squares								
Independent variable	df	Tensile strength (TS)	%Elongation (E)	Water vapor permeability (WVP)	Oxygen permeability (OP)	Film solubility (FS)	Protein solubility (PS)	ΔE^*_{ab}	Hue	Chroma
pH	4	0.47*	29.33**	0.21*	598799.00**	0.09*	274.27*	64.35**	78.44*	49.28*
Temperature	4	0.28*	26.98**	0.20*	395012.00**	0.03	27.42	17.79*	10.49	2.99
Time	4	0.07	10.78**	0.05	378820.00**	0.01	6.48	14.70*	4.87	4.26

Table 4 ANOVA: overall effect of independent variables on response variable (continued)

Independent variable	df	Sum of squares					
		L	a	b	Hydrophobicity (HQ)	content of SS bond (SS)	Available SH group (ASH)
pH	4	24.39	0.40**	48.26**	94931.00*	340483.00	13.37**
Temperature	4	20.46	0.18*	3.28	13994.00	124242.00	3.91
Time	4	13.96	0.09	4.37	58973.00	58973.00	0.52

Table 5 Experimental data for the three-factor, three level response surface analysis [a]

	PH	Temp	Time	Tensile strength (Mpa)	Elongation at break (%)	water vapor permeability ($g.mm/m^2.d.kPa$)	Oxygen permeability ($cm^3.\mu m/ m^2.d.kPa$)	Film solubility (%)	Protein solubility (%)
Treatment	X1	X2	X3	(TS)	%E	WVP	OP	FS	PS
1	1	1	0	4.43	61.21	8.03	12.98	48.10	17.36
2	1	-1	0	4.22	69.95	11.80	32.88	54.15	18.37
3	-1	1	0	3.65	18.14	12.40	49.82	55.16	21.76
4	-1	-1	0	3.99	25.91	14.82	53.32	52.29	18.51
5	1	0	1	4.41	45.18	10.18	29.83	39.45	17.51
6	1	0	-1	4.22	54.93	13.03	27.07	52.68	17.30
7	-1	0	1	3.65	12.64	13.41	44.97	61.79	23.75
8	-1	0	-1	2.79	26.79	16.80	61.92	55.59	20.92
9	0	1	1	6.53	37.57	7.79	12.36	35.52	16.58
10	0	1	-1	5.28	51.47	9.56	22.52	37.54	18.79
11	0	-1	1	4.42	63.25	11.63	35.38	54.46	18.99
12	0	-1	-1	4.37	75.85	11.36	36.08	51.20	20.25
13	0	0	0	5.27	66.09	8.97	16.37	53.00	18.80
14	0	0	0	4.57	71.23	8.85	20.31	51.28	19.58
15	0	0	0	4.64	62.77	9.88	19.46	48.44	18.41

[a] The experimental runs were performed in a random order

Table 5 Experimental data for the three-factor, three level response surface analysis [a] (continued)

	PH	Temp	Time	Color					
Treatment	X1	X2	X3	L	a	b	ΔE^*_{ab}	Hue angle	Chroma
1	1	1	0	18.76	-0.97	9.64	80.41	95.78	9.69
2	1	-1	0	23.07	-0.99	6.56	78.41	98.64	6.63
3	-1	1	0	18.43	-1.03	7.86	81.69	97.47	7.93
4	-1	-1	0	21.34	-1.24	5.56	80.69	102.56	5.70
5	1	0	1	22.91	-1.00	7.27	77.86	97.00	8.24
6	1	0	-1	21.11	-0.88	7.22	79.88	96.95	7.27
7	-1	0	1	18.81	-1.14	8.61	80.95	97.55	8.68
8	-1	0	-1	20.10	-1.08	7.56	80.50	98.17	7.63
9	0	1	1	19.57	-1.01	8.20	80.56	97.05	8.27
10	0	1	-1	17.31	-0.86	7.19	82.99	97.64	7.25
11	0	-1	1	17.48	-1.08	4.58	84.37	103.31	4.70
12	0	-1	-1	18.88	-1.05	4.94	83.03	102.06	5.05
13	0	0	0	19.30	-0.95	4.37	83.03	102.35	4.47
14	0	0	0	18.84	-0.96	4.75	83.18	101.46	4.84
15	0	0	0	17.81	-1.11	4.90	84.00	101.68	5.00

[a] The experimental runs were performed in a random order

Table 5 Experimental data for the three-factor, three level response surface analysis [a] (continued)

	pH	Temp	Time	Hydrophobicity	content of SS bond	Available SH
Treatment	X1	X2	X3	(HQ)	(SS)	(ASH)
1	1	1	0	402.30	711.07	8.38
2	1	-1	0	372.21	761.31	8.10
3	-1	1	0	507.75	729.98	13.32
4	-1	-1	0	470.86	761.47	11.68
5	1	0	1	449.03	668.30	10.22
6	1	0	-1	409.66	660.50	10.76
7	-1	0	1	544.70	703.64	11.86
8	-1	0	-1	498.74	673.89	11.74
9	0	1	1	585.69	599.70	12.32
10	0	1	-1	486.45	602.75	11.74
11	0	-1	1	396.87	612.93	11.67
12	0	-1	-1	353.73	626.59	10.90
13	0	0	0	471.11	600.98	11.84
14	0	0	0	483.78	605.45	11.10
15	0	0	0	468.70	616.28	11.44

[a] The experimental runs were performed in a random order

Table 6 Regression Coefficient of the second order polynomial for all response variables

Coefficient	Tensile Strength (TS) k = 1	%Elongation (%E) k = 2	Water vapor permeability (WVP) k = 3	Oxygen permeability (OP) k = 4	Films solubility k = 5	Proteins solubility k = 6	L* k = 7	a* k = 8	b* k = 9
β_{ko}	4.83	66.70	9.23	18.71	50.91	18.93	18.62	-1.01	4.67
β_{k1}	0.40	18.47	-1.80	-13.41	-3.81	-1.80	0.77	0.08	0.14
β_{k2}	0.36	-8.32	-1.48	-7.50	-4.47	-0.20	-0.84	0.06	1.41
β_{k3}	0.30	-6.30	-0.97	-3.13	-0.72	-0.05	0.05	-0.05	0.22
β_{k11}	-1.07	-22.52	2.90	16.45	4.61	0.64	2.23	-0.04	2.08
β_{k22}	0.31	-0.37	-0.37	2.09	-3.09	-0.57	-0.44	-0.01	0.65
β_{k33}	0.01	-9.29	1.22	5.78	-3.14	0.30	0.14	0.02	0.91
β_{k12}	0.14	-0.24	-0.34	-4.10	-2.23	-1.07	-0.35	-0.05	0.20
β_{k13}	-0.17	1.10	0.14	4.93	-4.86	-0.66	1.02	-0.02	-0.25
β_{k23}	0.30	-0.33	-0.51	-2.37	-1.32	-0.24	0.92	-0.03	0.34

Table 6 Regression Coefficient of the second order polynomial for all response variables (continued)

Coefficient	ΔE^*_{ab} k = 10	Hue angle k = 11	Chroma k = 12	Hydrophobicity (HQ) k = 13	Content of SS bond (SS) k = 14	Available SH group (ASH) k = 15
β_{ko}	83.40	101.83	4.77	476.58	607.57	11.45
β_{k1}	-0.87	-0.92	0.24	-43.92	-8.48	-1.55
β_{k2}	-0.14	-2.33	1.38	37.13	-14.85	0.61
β_{k3}	-0.33	0.01	0.34	36.82	2.61	0.25
β_{k11}	-2.98	-2.91	2.18	-15.06	99.74	-0.48
β_{k22}	-0.04	-0.31	0.54	-38.87	33.65	-0.09
β_{k33}	-0.62	-1.51	1.00	20.68	-30.73	0.06
β_{k12}	0.18	0.56	0.21	-2.36	-4.69	-0.60
β_{k13}	-0.62	0.17	-0.02	5.28	-5.49	-0.30
β_{k23}	-0.94	-0.46	0.34	25.01	2.65	0.01

Table 7 ANOVA and model fitting from the response variables

		Sum of squares								
Source	df	Tensile strength (TS)	%Elongation (E)	Water vapor permeability (WVP)	Oxygen permeability (OP)	Films solubility	Proteins solubility	L*	a*	b*
Model	9	8.37	5705.70**	88.84**	3228.85**	563.14	36.04	38.14	0.12	36.04*
Linear	3	3.01	3601.70**	50.86**	1966.50**	280.12	26.27	10.39	0.10*	6.35*
Quadratic	3	4.81	2098.50**	34.40**	1075.63**	161.78	3.28	19.74	0.01	18.82*
Cross product	3	0.55	5.50	1.57	186.73*	121.24	6.48	8.02	0.01	0.87
Residual	5	1.72	256.20	3.50	38.37	177.60	14.21	9.58	0.02	2.69
Lack of fit	3	1.42	228.86	2.87	29.74	166.99	13.05	8.24	0.00	2.54
Pure error	2	0.30	36.34	0.63	8.63	10.61	0.71	1.34	0.02	0.15
%variability explained (R^2)		82.97	95.56	96.21	98.83	76.02	71.72	79.93	88.07	93.05

* Significant at 5% level

** Significant at 1% level

Table 7 ANOVA and model fitting from the response variables (continued)

		Sum of squares					
Source	df	ΔE^*_{ab}	Hue angle	Chroma	Hydrophobicity (HQ)	Content of SS bond (SS)	Available SH group (ASH
Model	9	45.94	89.71**	37.78**	48310.00	47916.00**	25.15
Linear	3	7.12	50.20**	16.64**	37307.00	2393.07	22.54*
Quadratic	3	33.62*	37.31**	20.49**	8367.73	45286.00**	0.88
Cross product	3	5.20	2.20	0.64	2635.90	236.48	1.72
Residual	5	5.96	3.40	1.93	12920.00	851.08	4.61
Lack of fit	3	5.41	2.96	1.78	10453.00	727.30	4.27
Pure error	2	0.55	0.43	0.15	2467.24	123.78	0.34
%variability explained (R^2) 84.50		88.51	96.35	95.15	78.90	98.25	

* Significant at 5% level

** Significant at 1% level

Table 8 ANOVA: overall effect of independent variables on response variable

		Sum of squares								
Independent variable	df	Tensile strength (TS)	%Elongation (E)	Water vapor permeability (WVP)	Oxygen permeability (OP)	Films solubility	Proteins solubility	L*	a*	b*
pH	4	5.68*	4608.21**	57.45**	2601.61**	308.57	33.70	27.76*	0.07*	16.59*
Temperature	4	1.84	555.11	19.50*	555.37**	222.13	6.31	10.17	0.04	17.98*
Time	4	1.16	641.41	14.11*	321.41**	141.87	2.29	7.62	0.02	4.14

Table 8 ANOVA: overall effect of independent variables on response variable (continued)

Independent variable	df	Sum of squares					
		ΔE^*_{ab}	Hue angle	Chroma	Hydrophobicity (HQ)	Content of SS bond (SS)	Available SH group (ASH)
pH	4	40.60*	39.37**	18.13**	16404.00	37513.00**	21.66*
Temperature	4	3.84	45.82**	17.00*	19133.00	6060.78*	4.38
Time	4	7.39	9.32	5.12	15037.00	3688.80*	0.82

Table 9 Experimental data for the three factor, three level response surface analysis [a]

	PH	Temp	Time	Tensile strength (Mpa)	Elongation at break (%)	Water vapor permeability (g.mm/m^2.d.kPa)	Oxygen permeability (cm^3.µm/ m^2.d.kPa)	Films solubility (%)	Proteins solubility (%)
Treatment		X1	X2	X3 (TS)	%E	WVP	OP	FS	PS
1		1	1	0 3.99	8.74	18.95	523.09	68.34	59.69
2		1	-1	0 4.49	11.80	14.27	312.88	62.42	50.37
3		-1	1	0 3.27	25.36	12.33	355.16	57.96	33.21
4		-1	-1	0 3.44	22.45	13.90	461.75	49.53	25.29
5		1	0	1 4.24	12.32	12.94	375.17	61.46	50.21
6		1	0	-1 4.06	15.22	12.77	301.10	60.79	42.54
7		-1	0	1 3.32	20.20	11.80	440.96	56.64	32.14
8		-1	0	-1 3.52	18.56	11.78	422.24	52.60	27.48
9		0	1	1 4.48	18.44	11.77	562.72	57.61	48.36
10		0	1	-1 4.35	21.68	9.77	320.07	56.78	41.22
11		0	-1	1 5.31	32.40	9.02	219.26	54.01	34.91
12		0	-1	-1 5.58	31.90	11.69	189.67	43.22	31.75
13		0	0	0 4.56	31.46	11.34	245.28	63.39	38.68
14		0	0	0 4.68	32.96	11.05	219.56	61.40	35.90
15		0	0	0 4.66	35.50	10.86	217.77	62.47	39.87

[a] The experimental runs were performed in a random order

Table 9 Experimental data for the three-factor, three level response surface analysis [a] (continued)

	PH	Temp	Time	Color					
Treatment	X1	X2	X3	L	a	b	ΔE^*_{ab}	Hue angle	Chroma
1	1	1	0	21.90	-0.33	10.20	77.49	92.35	10.21
2	1	-1	0	21.78	-0.35	8.48	78.59	93.34	8.49
3	-1	1	0	18.49	-0.68	6.13	82.63	96.36	6.17
4	-1	-1	0	19.76	-0.71	5.84	81.79	98.70	5.90
5	1	0	1	21.34	-0.48	9.10	78.58	93.05	9.11
6	1	0	-1	20.56	-0.75	8.81	79.39	93.11	8.82
7	-1	0	1	19.88	-0.69	6.01	81.59	95.26	6.04
8	-1	0	-1	19.35	-0.61	5.94	82.05	95.90	5.97
9	0	1	1	21.39	-0.29	6.58	80.03	94.03	6.60
10	0	1	-1	21.13	-0.44	6.22	80.50	94.24	6.17
11	0	-1	1	20.28	-0.52	6.31	81.09	94.56	6.33
12	0	-1	-1	20.25	-0.50	6.09	81.23	94.45	6.11
13	0	0	0	21.29	-0.49	6.54	80.13	94.37	6.56
14	0	0	0	21.00	-0.34	6.74	80.25	94.08	6.76
15	0	0	0	20.21	-0.52	6.90	80.79	93.93	6.92

[a] The experimental runs were performed in a random order

Table 9 Experimental data for the three-factor, three level response surface analysis [a] (continued)

	pH	Temp	Time	Hydrophobicity	content of SS bond	Available SH
Treatment	X1	X2	X3	(HQ)	(SS)	(ASH)
1	1	1	0	577.50	391.47	6.50
2	1	-1	0	648.42	441.35	6.12
3	-1	1	0	609.15	505.44	4.06
4	-1	-1	0	623.44	542.94	3.85
5	1	0	1	595.70	383.47	6.50
6	1	0	-1	649.71	397.42	6.38
7	-1	0	1	632.74	486.24	3.66
8	-1	0	-1	689.31	458.63	3.19
9	0	1	1	586.62	383.35	4.31
10	0	1	-1	627.25	401.91	4.96
11	0	-1	1	635.29	407.41	3.30
12	0	-1	-1	683.08	383.76	3.44
13	0	0	0	636.09	368.27	3.78
14	0	0	0	646.17	377.88	3.64
15	0	0	0	646.87	379.26	4.11

[a] The experimental runs were performed in a random order

Table 10 Regression Coefficient of the second order polynomial for all response variables

Coefficient	Tensile Strength (TS) k = 1	%Elongation (%E) k = 2	Water vapor permeability (WVP) k = 3	Oxygen permeability (OP) k = 4	Film solubility k = 5	Protein solubility k = 6	L* k = 7	a* k = 8	b* k = 9
β_{ko}	4.63	33.31	2.43	5.42	62.42	38.15	20.83	-0.45	6.73
β_{k1}	0.46	-4.81	0.10	-0.06	4.58	10.58	1.01	0.10	1.58
β_{k2}	-0.40	-3.04	0.10	0.21	3.98	5.02	0.11	0.04	0.30
β_{k3}	-0.02	-0.50	0.03	0.12	2.04	2.83	0.20	0.04	0.12
β_{k11}	-0.93	-12.87	0.21	0.42	1.10	1.51	-0.42	-1.31	1.05
β_{k22}	0.21	-3.34	0.01	0.16	-3.87	2.48	0.06	0.06	-0.11
β_{k33}	0.09	-3.86	-0.09	0.10	-5.64	-1.57	-0.14	-0.05	-0.31
β_{k12}	-0.20	-1.49	0.06	0.20	-0.54	0.35	0.35	0.00	0.36
β_{k13}	0.10	-1.14	-0.00	0.05	-0.84	0.75	0.06	0.09	0.06
β_{k23}	0.10	-0.94	-0.02	0.11	-2.49	0.99	0.06	0.04	0.04

Table 10 Regression Coefficient of the second order polynomial for all response variables (continued)

Coefficient	ΔE^*_{ab} k = 10	Hue angle k = 11	Chroma k = 12	Hydrophobicity (HQ) k = 13	Content of SS bond (SS) k = 14	Available SH group (ASH) k = 15
β_{ko}	80.39	94.13	6.75	643.04	375.14	3.84
β_{k1}	-1.75	-1.67	1.57	-10.41	-47.44	1.34
β_{k2}	-0.25	-0.38	0.29	-23.71	-11.66	0.39
β_{k3}	-0.24	-0.10	0.13	-24.87	2.34	-0.03
β_{k11}	-0.29	0.41	1.06	-9.81	66.24	1.11
β_{k22}	0.22	0.40	-0.12	-18.61	28.92	0.18
β_{k33}	0.30	-0.21	-0.33	8.63	-9.94	-0.02
β_{k12}	-0.49	0.09	0.36	-14.16	-3.10	0.04
β_{k13}	-0.09	0.15	0.06	0.64	-10.39	-0.09
β_{k23}	-0.08	-0.08	0.05	1.79	-10.55	-0.13

Table 11 ANOVA and model fitting from the response variables

Source	df	Sum of squares								
		Tensile strength (TS)	%Elongation (E)	Water vapor permeability (WVP)	Oxygen permeability (OP)	Films solubility	Proteins solubility	L*	a*	b*
Model	9	6.78**	938.53*	65.93*	167379.00	530.27**	1210.88**	9.83	0.23	26.18**
Linear	3	2.96**	261.18	29.41*	61864.00	328.10**	1162.17**	8.61	0.10	20.90**
Quadratic	3	3.59**	659.79*	32.06*	68310.00	173.36**	41.99	0.72	0.09	4.75**
Cross product	3	0.23	17.56	4.46	37205.00	28.79	6.72	0.51	0.03	0.53
Residual	5	0.19	112.99	6.14	26938.00	22.80	55.31	2.94	0.08	0.47
Lack of fit	3	0.18	104.65	5.99*	26464.00*	20.81	47.00	2.31	0.06	0.40
Pure error	2	0.01	8.34	0.15	473.84	1.98	8.30	0.62	0.02	0.07
%variability explained (R^2)		97.24	89.25	91.48	86.14	95.88	95.63	77.00	75.06	98.28

* Significant at 5% level

** Significant at 1% level

Table 11 ANOVA and model fitting from the response variables (continued)

		Sum of squares					
Source	df	ΔE^*_{ab}	Hue angle	Chroma	Hydrophobicity (HQ)	Content of SS bond (SS)	Available SH group (ASH)
Model	9	27.19**	25.13*	25.94**	13096.00*	39412.00**	20.36**
Linear	3	25.50**	23.60**	20.49**	10316.00**	19138.00**	15.64**
Quadratic	3	0.69	1.39	4.91**	1963.26	19359.00**	4.63*
Cross product	3	1.00	0.14	0.55	816.19	915.55	0.11
Residual	5	1.48	1.88	0.48	792.64	1243.09	1.04
Lack of fit	3	1.24	1.78	0.42	719.87	1171.41	0.93
Pure error	2	0.25	0.10	0.07	72.77	71.68	0.11
%variability explained (R^2)		94.82	93.04	98.17	94.29	96.94	95.13

* Significant at 5% level

** Significant at 1% level

Table 12 ANOVA: overall effect of independent variables on response variable

	Sum of squares								
Independent variable	df	Tensile strength (TS)	%Elongation (E)	Water vapor permeability (WVP)	Oxygen permeability (OP)	Films solubility	Proteins solubility	L*	a*	b*
pH	4	5.10**	811.27*	0.25*	0.85*	176.27*	907.74**	9.34	0.17	4.66**
Temperature	4	1.62*	127.70	0.09	0.65	208.15*	228.74*	0.60	0.04	1.29
Time	4	0.11	65.58	0.04	0.24	178.74*	79.33	0.42	0.06	0.49

Table 12 ANOVA: overall effect of independent variables on response variable (continued)

		Sum of squares					
Independent variable	df	ΔE^*_{ab}	Hue angle	Chroma	Hydrophobicity (HQ)	Content of SS bond (SS)	Available SH group (ASH)
pH	4	25.81**	23.08**	24.41**	2025.95	34681.00**	19.00**
Temperature	4	1.49	1.83	1.26	6592.11*	4658.76	1.41
Time	4	0.83	0.35	0.54	5239.39*	1286.32	0.10

APPENDIX B

1. Permatran-W1A (Modern Controls, Inc.)

2. Ox-Tran 100A (Modern Control, Inc.)

3. Instron Universal Testing Instrument (Model 1122)

4. Protein analyzer (Leco FP2000 combustion oven, Model FP-2000)

5. Acrylic cups

VITAE

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