การเตรียมและสมบัติของไคโตแซนชนิดเม็ดจากเปลือกกุ้งกุลาดำ

Penaeus monodon

นางสาว เกศสุดา เหลืองธีรภาพ

PREPARATION AND PROPERTIES OF CHITOSAN MICROSPHERES
FROM GIANT BLACK TIGER SHRIMP Penaeus monodon SHELL

Miss Kessuda Luengteerapap

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PREPARATION AND PROPERTIES OF CHITOSAN

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เกศสุดา เหลืองธีรภาพ : การเตรียมและสมบัติของไคโตแซนชนิดเม็ดจากเปลือกกุ้งกุลาดำ <u>Penaeus</u> <u>monodon</u> (PREPARATION AND PROPERTIES OF CHITOSAN MICROSPHERES FROM GIANT BLACK TIGER SHRIMP <u>Penaeus</u> <u>monodon</u> SHELL) อาจารย์ที่ปรึกษา: อ.ดร. เลอสรร ธนสุกาญจน์, xx หน้า, ISBN 974-17-2717-8.

ไคโตแซนที่เตรียมได้จากเปลือกกุ้งกุลาดำมีน้ำหนักโมเลกุล 2.67×10^5 ดาลตัน ที่ 69 to 76 เปอร์เซ็นต์ดีอะเซทิลเลชัน มีเถ้า 0.101% (SD 0.08%, n=4) ความชื้น 13.471%(SD 3.35%, n= 4) และผลผลิต 42% (SD 7.94%, n=4). จากการปั่นสารละลายไคโตแซนกับไตรเอทธานอลามีน ในพาราฟินแวกซ์ที่มีสแพน (Span®80) ไคโตแซนมิลลิสเฟียร์ที่เกิดขึ้นมีลักษณะใส ขนาดเส้นผ่าศูนย์กลางประมาณ 1.5 มิลลิเมตร จากการพิจารณาภาพถ่ายกล้องจุลทรรศน์อิเล็กตรอน ไคโตแซนมิลลิเสฟียร์มีทรงเกือบกลม (spheroid) และ ลักษณะพื้นผิวหยาบ สมบัติการขยายตัวของไคโตแซนมิลลิสเฟียร์ที่สภาวะค่าความเป็นกรดและด่าง และความแรงของไอออนต่างๆ อธิบายได้ด้วยทฤษฎีของ Tanaka ซึ่งอนุภาคของโพลิเมอร์จะถูกกระทำด้วย 3 ปัจจัย คือ 1) แรงดันออสโมติก สามารถคำนวณจากสมดุลของดอนแนน 2) polymer-polymer affinity ไม่ได้นำมาพิจารณาในงานวิจัยนี้ 3) rubber elasticity จะพิจารณาร่วมกันระหว่างผลของ "salting out" และการเกิด " polar bridge " จาก Span® จากการศึกษาสมบัติการปลดปล่อยโปรตีนทดสอบ 3 ชนิดของไคโตแซนมิลลิสเฟียร์ ได้แก่ ไคโมทริปซิน โบไวน์ซีรัมอัลบูมิน และ โปรตามีน ใช้เวลาประมาณ 1 ชั่วโมงในการปลดปล่อยและจะลดลงในกรณีโปรตามีนเท่านั้น ผลของค่าความเป็นกรดและด่างและความแรงของไอออนในสารละลายต่อการปลดปล่อยโปรตีนสามารถอธิบายในรูปของประจุบนไคโตแซน และโมเลกุลโปรตีน รวมถึงความเป็นขั้วลบของ Span®

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KEY WORD   : CHITIN/ CHITOSAN/ DEACETYLATION/ PHYSICOCHEMICAL PROPERTIES

KESSUDA LUENGTEERAPAP : PREPERATION AND PROPERTIES OF CHITOSAN MICROSPHERES FROM GIANT BLACK TIGER SHRIMP Penaeus monodon SHELL. THESIS ADVISOR : LERSON TANASUGARN, Ph.D. xx pp. ISBN 974-17-2717-8

Chitosan has been prepared from shrimp shells. The molecular weight and per cent deacetylation have been determined to be 2.67×10^{-5} kDa and 69 - 76%, respectively. The ash and moisture were 0.101%(SD 0.08%) and 13.417% (SD 3.35%) respectively (n=4). The yield was 42%(SD 7.94%, n=4). Chitosan millispheres have been prepared by mixing and stirring chitosan and triethanolamine in paraffin wax pre-dissolved with Span$^{®}$ 80. The size of the millisphere was approximately 1.5 mm in diameter. The spheroids were clear with rough surface under scanning electron microscopy. The swelling properties of chitosan millispheres (up to 20% diameter at 360 minutes incubation) under different pH's and ionic strengths have been explained by Tanaka's theory of gel polymers. According to Tanaka, pressures acting on a polymer particle come from three sources (1) osmotic pressure (calculated by Donnan equilibrium) (2) polymer-polymer affinity (not applicable) and (3) rubber elasticity (in this case a combination of polymer "salting out" and the polar bridging of positive charges on by Span$^{®}$). The release of time test proteins, chymotrypsin, bovine serum albumin and protamine took about an hour to approach the peak value and declined only in the case of protamine. The effects of pH and ionic strength of the medium on protein release were explained in terms of charges on chitosan and protein molecule as well as the negative polarity of Span$^{®}$.

Department……………Biochemistry…..……..   Student's signature…………………………..

Field of study….………Biochemistry…………   Advisor's signature…………………………..

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
μg	microgram
μl	microlitre
A	Absorbance
BSA	bovine serum albumin
g	gram
hr	hour
HCl	hydrochloric acid
kDa	kilodalton
l	liter
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide

LIST OF ABBREVIATIONS (continued)

pI   isoelectric point

W/V   weight by volume

CHAPTER I

INTRODUCTION

1.1 The Global View

Shrimp is the single most valuable seafood product that enters into world trade today. This seafood industry is an important part of the Thai economy, particular Black Tiger shrimp (Figure 1.1).   The total shrimp production in Thailand increased dramatically from 120,413 tons in 1986 to 364,796 tons in 1998. By the year 1992, Thailand became the world's leading producer and exporter of Black Tiger shrimp; and supplies 20 percent of the world trade in shrimp and prawn. [Patmasiriwat D, 1999] The frozen shrimp was the country's leading food export and third most important export in terms of overall earnings by 1995 (Figure 1.2). The pattern of shrimp exports indicates that 65 percent of the annual output is processed into frozen shrimp in the form of blocks or individually "quick frozen" (www.thaitrade.com). This leads to an inevitable increase in waste produced by the shrimp industry as 40 to 45 percent of the raw shrimps caught are largely of no use. Currently shrimp waste, which is rich in chitin, protein, lipids, pigments and flavour compounds: all of potential value, is used as a supplement in animal feed or discarded as waste. Shrimp waste, however, is an important source of chitin. It has been found that shrimp and crab processing waste contains 14 to 27% and 13 to 15% by dry weight of chitin respectively (No *et al*., 1989).

Figure 1.1 Giant black tiger shrimp (*Penaeus monodon*)

Source:http://www.beck-liner.com/images/shrimpblacktiger.htm
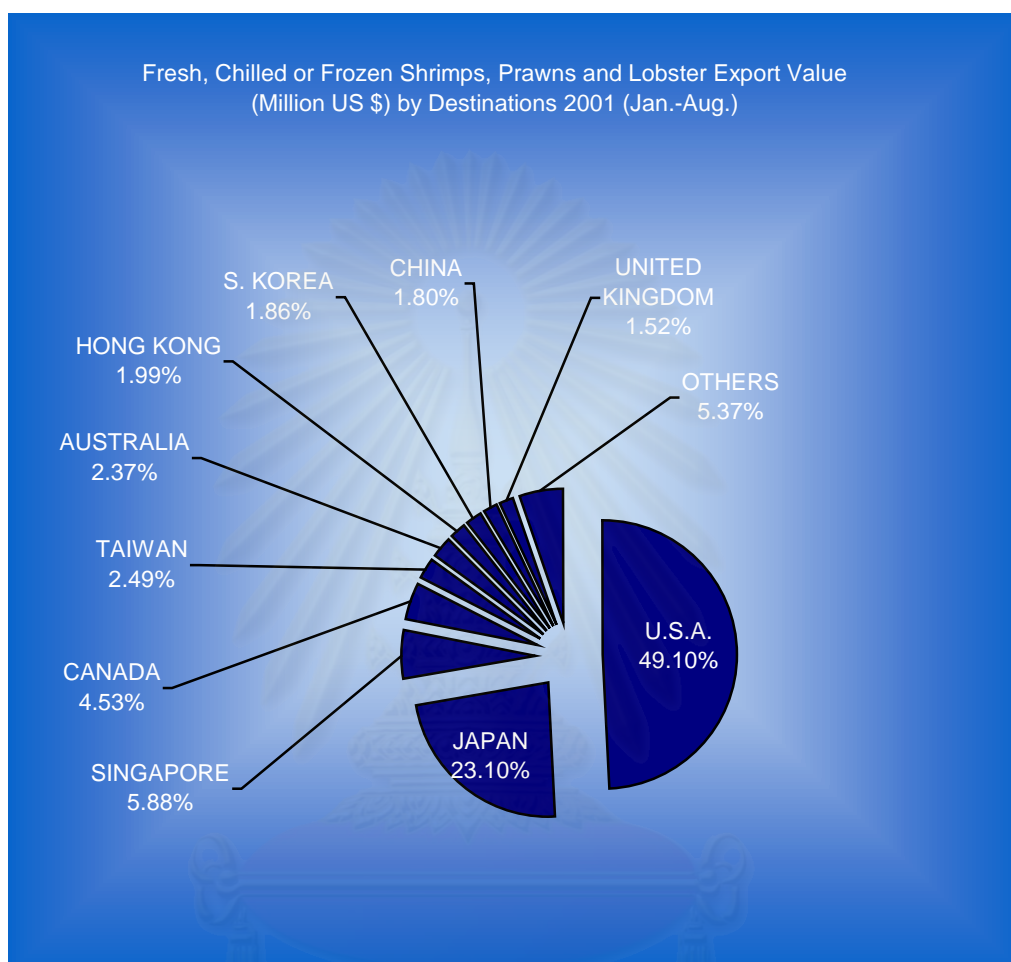
Figure 1.2 Fresh, chilled or frozen shrimps, prawns and Lobster

Export value (Million US $) by Destinations 2001 (Jan. – Aug.)

Source: http://www.thaitrade.com/en/report_doc.

Chitin (to be reviewed in section 1.2), chitosan (to be reviewed in section 1.3) and their derivatives have received much attention from scientists in different parts of the world. Many investigations have focused on its properties and various applications. Although studies on chitin and chitosan were initiated in the early nineteenth-century, most of the reports available today on its medical and pharmaceutical applications have been obtained only during the last couple of decades. This has been investigated in depth or applied to various industrial and medical applications as shown in Table 1.1

In short, the multi-industry roles of chitin and chitosan are the results of their chemical and biological properties as follow:

- Being a main, biologically reproducible component of biomass such as the shells of crab, shrimp and krill.

- Biodegradability to form environment-friendly substances

- Biocompatibility not only with animals but also with plant tissues

- Biopolymers (aminopolysaccharide) with several biological functions

- Very low toxicity (LD_{50} 16 g/kg body weight in mice)

- Changeable molecular conformations

- Ease of manufacture into gels, beads, fibers, colloids, films, etc.

- Possessing chemically modifiable amino and hydroxyl groups

Table 1.1 Applications of chitin, chitosan and its derivatives

Field	Application
Medical and Health care	Contact lens/eye bandages
	Wound-healing ointments and dressings (skin treatments)
	Orthopaedics (Temporary bioengineering material)
	Anticholesterol and fat-binding (hypercholesterolemic agent)
	Surgical sutures, Drug delivery
	Ophthalmology, Dentistry
	Transportation of cells
Food and Beverages	Anticholesterol and fat-binding
	Food stabilizer, Fruit preservation
	Flavour and tastes, Nutritional additives
	Food packaging
Agriculture	Seed treatments (coating)
	Feed ingredients (animal feed)
	(controlled release formulations)
	Nematocides and insecticides
Cosmetics and Toiletries (personal care)	Hair treatment (coating)
	skin care (moisture retention)
	Oral care
Waste and water Treatment (clarification)	Sewage effluents
	Drinking water, Pools and spas
	Recovering metals
	Treating food wastes, (food processor waste)
Product separation and recovery (bioapplications)	Membrane separations
	Chromatographic matrix
	Immobilization of enzymes/cells
	Recover valuable bioproducts (flocculate)

Source : Jaiyongka, 1993; Paul 2000

In addition to fiber, film, and gel blocks, chitosan have found practical uses in the form of beads or "spheres" [Chandrkrachang, 1999]. These chitosan "spheres" could be fabricated in different sizes, from the submicron range (nanosphere) to the micron range (microsphere) to the millimeter range.

Many of these spheres with adequately small pore sizes, are suitable as slow releases whereas larger one have found uses in cosmetics manufacturing. These chitosan spheres will be review in more detail in section 1.3
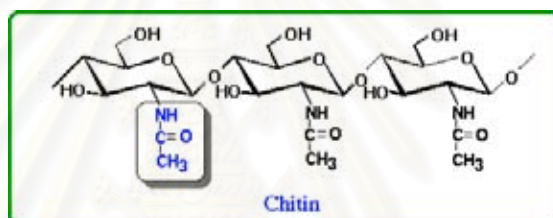
Figure 1.3 Poly [β-(1-4)-2-acetamino-2-deoxy-D-glucopyranose]

Source : www.wellable.com/product/chitosan.gif

1.2 Chitin

1.2.1 Chemical structure of chitin

Chitin *N*-acetyl-D-glucosamine and glucosamine copolymerized by β-1,4 linkages, is a major structural heteropolysaccharide found in invertebrate animals and lower plants (see Figure 1.3). It is noticeably present in the outer skeletons of arthropods, especially in crustaceans such as crabs and shrimps. In plants chitin is a cell wall constituent of most fungi, molds and yeasts [Muzzarelli, 1977].

Chitin is associated with other polysaccharides in the fungal cell walls, while in animal forms chitin is associated with proteins. It occurs naturally in three polymorphic forms, α-chitin is the most abundant, crystalline, tightly compacted and stable form in which the chains are arranged in an antiparallel fashion. In β-chitin the chains are arranged in parallel whereas in γ-chitin there are two <up> chains to every <down> chain. α-chitin is found where extra hardness proves essential whereas β and γ-chitin provide toughness, flexibility and mobility together with control electrolytes and polyanionic characteristics [Muzzarelli, 1977]. Chitin is insoluble in common organic solvents due to extensive hydrogen bonding and its highly crystalline structure. These properties strongly restrict many practical applications of chitin.

1.2.2 Preparation of chitin

Chitin is the second most abundant natural polymer after cellulose. It was first isolated in 1811 by Henry Broconnot, chemist and botanist [Muzzarelli, 1997]. The amount of chitin with respect to total dry weight is the highest in crustaceans, mainly decapods. Crab and shrimp shells contain approximately 15 – 20 and 15 – 30 % chitin on a dry weight basis, respectively. This observation may explain the use of crustacean shells as the main source of chitin to support any commercial chitin/chitosan industry. Within the vast amounts of shellfish waste now available, applications requiring large amounts of chitin and chitosan are now possible. Crustacean shell waste mainly consists

of protein (30-40%), calcium carbonate (30-50%), and chitin (20-30%) [No and Meyers, 1997]. These proportions vary with species and with the season (Table 1.2).

Table 1.2 Chemical composition of various crustacean wastes.

Source	Chemical composition(%)				
	Moisture	Protein	Ash	Lipid	chitin
Lobster*(Linuparus trigonus)*					
	13.5	17.0	54.7	-	-
Crab					
1) *Callinectes sapidus*					
	-	30-35	50.0	-	13-15
	4.5	24.0[a]	56.0	2.0	12.9
2) *Chinonecetes opilio*					
	-	29.19	40.60	1.35	26.65
3) *Portunus trituberculatus*					
	12.9	10.3	57.9	0.3	17.1
Shrimp					
1) *Penaeus monodon*					
(Carapace)	9.1	26.8	29.3	0.5	34.9[b]
(Shell)	9.7	42.8	20.8	1.2	36.5[b]
2) *Pandalus borealis*					
	-	23.5	33.9	14.7	30.0
3) *Penaeus* sp.					
(Head)	3.2	55.8	22.6	8.9	11.9[c]
(Hull)	4.0	45.0	31.7	0.4	27.2[c]
Prawn	9.24	61.6*	26.67	1.40*	30.00
Crawfish *(Procambarus clarkii)*					
	-	16.9	63.6	0.6	23.5
	5.7	28.1[a]	44.0	4.4	12.5
Krill *(Euphausia superba)*					
	-	41.0	23.0	11.6	24.0
	-	44.6[a]	24.7	1.8	19.9

*dry basis   [a]corrected for chitin.   [b]based of 6.9% N present in chitin.
[c]expressed as fiber.

Source: No and Meyers, 1997

Preparation of chitin from crustacean shell waste consists of three basic steps: (1) Protein separation–deproteinization, (2) calcium carbonate (and calcium phosphate) separation – demineralization, and (3) removal of pigments – decoloration. The former two steps also can be conducted in a reverse of order, i.e., demineralization, followed by deproteinization. However, if protein recovery is an objective, its extraction before demineralization is preferred so as to maximize protein yield and its quality [No and Meyers, 1997].

1.2.2.1 Deproteinization

Crustacean shell waste usually is ground and treated with dilute (1-10%) sodium hydroxide solution at elevated temperature (65-100°C) to dissolve the protein present. For example, Bough, *et al.* (1978) extracted protein from shrimp hulls with 3% NaOH at 100°C for 1 hour. No, *et al.* (1989) treated crawfish shell waste with 3.5% NaOH at 65°C for 30 hours. Brzeski, (1982) deproteinized with 3.5% NaOH at 25°C for 30 hr. Optimal deproteinization also can be accomplished by treatment with dilute potassium hydroxide solution reaction time usually ranges from 0.5 to 6 hours depending on preparation methods, although Hackman, (1954) and Whistler and BeMiller, (1962) attempted to extract protein for several days. In a study of extraction time of proteins from crab and shrimp shell wastes it has been found that a minimum period of 1 hour was needed to extract over 90% of the proteins at 35°C in 4% NaOH.

[No *et al.,* 1997]. In recent studies, more complete deproteinization of shrimp waste using two proteolytic enzymes [Gagne and Simpson, 1993] has been used.

1.2.2.2 Demineralization

Demineralization is conventionally accomplished by extraction with dilute hydrochloric acid at room temperature to dissolve calcium carbonate (Hackman, 1954, Anderson *et al.*,1978 and No *et al.*, 1989). On the other hand, Whistler and BeMiller, performed the demineralization step in concentrate in (37%) HCl at 20°C. Horowitz *et al.* (1975) accomplished demineralization at room temperature using 90% formic acid instead of hydrochloric acid. Some of other methods involve drastic treatments that may cause depolymerization and deacetylation of the native chitin. To avoid this, certain methods using mild acids have been developed to minimize degradation. For example, Hackman used ethylene diaminetetraacetic acid (EDTA) for decalcification (Hackman, 1957). Demineralization usually can be achieved in 2 to 3 hours with proper agitation. However, reaction time varied with preparation method from 30 minutes (No *et al*, 1989) to over 2 days ((Hackman, 1954). Prolonged demineralization time, even to 24 hours, results in only a very slight drop in the ash content but can cause polymer degradation (Brzeski, 1982). For demineralization, it is also important that the amount of acid be stoichiometrically equal to, or greater than, all minerals present in shells to ensure complete reaction.

1.2.2.3 Decoloration

Acid and alkali treatments alone produce a colored chitin product. When bleached chitin is desired, pigments can be removed with reagents as alcohol, acetone, ether and permanganate solution (Waiprib, 1991). Nevertheless, this decoloration step may be omitted since the deproteinization and demineralization reactions often result in almost complete decoloration.

1.2.3 Characterization of chitin

1.2.3.1 Protein

Experimental evidence of covalent bonds between chitin and protein has been obtained by many authors. *N*-acetylglucosamine and chitin can react with α-aminoacids, especially tyrosine, peptides and cuticular proteins, to give stable complexes, dissociable, however, upon pH changes. The proportion of protein in these complexes varies according to the isolation method. Chitin, as it occurs naturally in association with proteins, might be expected to have properties slightly different from those of chitin when isolated as a powder: for instance, the enzymic digestion of chitin preparations is far from complete, while the chitin in the endocuticle at the time of molting is digested by enzymes (Muzzarelli, 1977).

1.2.3.2 Ash

Ash content was indicated mineral present in crustacean shell wastes which, varies with species and the season. Calcium carbonate as calcium chloride extraction,

demineralization, accomplished with dilutes hydrochloric acid at room temperature.

Usually can be achieved in 2 to 3 hours. However, reaction time varied with preparation

methods from 30 minutes to over 2 days and vary with sources to meet compositional

differences (No *et al.,* 1989). Prolonged demineralization time, even to 24 hours, results

in only a very slight drop in the ash content but can cause polymer degradation

(Brzeski, 1982).

1.2.3.3 Infrared spectroscopy

Many authors on both naturally occurring chitins and purified or modified

chitins and chitosan have published the infrared spectra of chitin and chitosan. The

most significant parts of the spectra are those showing the amide bands at 1665, 1555

and 1313 cm^{-1}, all of which show perpendicular dichroism and which are, respectively,

assigned to the C=O stretching, to the N-H deformation in the CONH plane and to CN

bond and CH_2 wagging. The chitosan spectrum differs from that of chitin in that the new

band at 1590 cm^{-1} predominates over the one at 1665 cm^{-1} and the band at 1555 cm^{-1} is

absent. This is the most important feature for practical purposes (Muzzarelli, 1977).

1.3 Chitosan
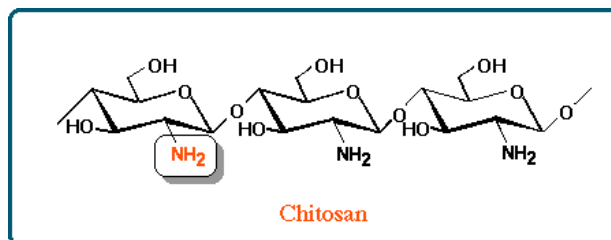
Figure 1.4   Poly [β-(1-4)-2-amino-2-deoxy-D-glucopyranose]

Source: www.wellable.com/product/chitosan.gif

1.3.1 Chemical structure of chitosan

Chitosan (Figure 1.4), is β-1,4 linked linear biopolymer of 2-amino-2-deoxy-β-D-glucose, and thus it possesses an amino group instead of a hydroxyl group on the C-2 position of the glucose residue of cellulose. Chitosan, discovered by Rouget in 1859, composes the cell wall of *Mucor rouxii* and is easily obtained by alkaline deacetylation of chitin. In general, commercially available chitin and chitosan are 10-20% deacetylated and 15-25% *N*-acetylated, respectively [Jaiyongka, 1993].

Chitosan, a cationic polyelectrolyte, is soluble in organic acids such as 0.2-100 % (v/v) formic acid, diluted solutions of hydrochloric acid, nitric acid and phosphoric acid.

Chitosan is readily soluble in acidic water as a result of salt formation by the C-2 amino group of its glucosamine residue with various inorganic and organic acids

(Shigemasa, 1998).   On the other hand, chitosan is insoluble in alkaline or neutral solvent (pH>6) such as common organic solvent [Rinaudo, 1997].

Chitosan is solution a viscous, non-newtonian, clear solution and plastic like flexibility.   The solution can be cast into many form such as beads, gels, membranes, fiber and coating agent [Chandrkrachang, 1999].

1.3.2 Preparation of chitosan

The physicochemical characteristics of chitin and chitosan differ with crustacean species and preparation methods.   Thus, the relationship between process conditions and characteristics of chitin/chitosan products must be constantly monitored to achieve uniformity and proper product quality control [No and Meyers, 1997].

1.3.2.1 Deacetylation

Conversion of chitin to chitosan is generally achieved by treatment with concentrated (40-50%) sodium or potassium hydroxide solution usually at $100^{\circ}C$ or higher to remove some or all of the acetyl groups from the polymer.   During deacetylation, conditions are necessary that will, in a reasonable time, sufficiently deacetylate the chitin to yield a chitosan product soluble in dilute acetic acid, but one which is not significantly degraded.   Factors affecting the deacetylation of chitin to produce chitosan are (1) Temperature, (2) Duration and alkali concentration, (3) Atmosphere, (4) Ratio of chitin to alkali solution, (5) Particle size of chitin

First, higher temperature increases the percentage of deacetylation but reduces molecular size. The alkali treatment should be carried out at temperature as low as possible to avoid degradation of the molecular chain [Lusena and Rose, 1953] needed for deacetylation chitin.

Second, the reaction time depends on the degree of deacetylation of chitosan product required, the alkali concentration and the reaction temperature. Under strong condition (50% NaOH at 100°C) the time required to obtain 80% deacetylation chitosan is about 5 hours [Wu and Bough, 1978].   Under this condition, reaction time longer than 5 hours dose not deacetylated significantly and only degrades the molecular chain.

Third, deacetylation in an atmosphere of nitrogen yielded chitosans of higher viscosity and molecular weight distribution than deacetylation in air because oxygen is catalyst in hydrolysis degradation of molecular chain [Lusena and Rose, 1953].

Fourth, during deacetylation, proper agitation is needed to obtain uniformity in reaction.   The reported solids to solution ratios ranged from 1:10 to 1:100. Increasing the chitin to alkali solution ratio above 1:10 (to1:15 and 1:20) did not show any effect on the deacetylation efficiency of chitin [Benjakul and Wisitwuttikul, 1994].

Finally, the effect of particle size of the initial raw material on the final quality of chitosan products has been investigated [Bough et al., 1978].   Results indicated that although the compositions of nitrogen and ash were similar, smaller particle size (1 mm diameter) gave chitosan products of both higher viscosity and molecular weight than those with larger particle sizes (2 or 4 mm diameter). Lusena and Rose (1953)

concluded that the size of chitin particle within the range 20-40, 40-60, and 60-80 mesh had no effect on deacetylation and viscosity.

1.3.3 Characterization of chitosan

The main characteristics of a chitosan sample are the molecular weight and degree of deacetylation.

1.3.3.1 Molecular weight

The molecular weight of chitosan is perhaps the most difficult parameter to obtain with precision. In order to evaluate the average molecular weight of polymeric chains, various methods can be used. The more popular ones are viscometric and gel permeation chromatographic techniques, which are relatively fast and easy to perform.

1.3.3.1.1 Viscosity

Viscometric techniques given correlation between intrinsic viscosity and molecular weight as expressed in the equation $[\eta] = KM^a$ where K and a are functions of the solvent as well as of the polymer type. For chitosan in 0.5 M acetic acid 0.2 M sodium acetate the K and a values are 3.5×10^{-4} and 0.76 and are independent on the degree of acetylation [Terbojevich et al., 1997].

1.3.3.1.2 Gel permeation chromatography

The technique is applicable to chitosan at different degrees of acetylation in salt solutions. In gel permeation or size exclusion chromatography (SEC)

molecules are separated according to their sizes, yielding a molecular weight distribution [Terbojevich *et al.,* 1997].

1.3.3.1.3 Light scattering

This method measures, the intensity of light scattered from a polymer solution, as a function of scattering angle (θ) and concentration. Hackman and Goldberg, (1974) reported that chitin are random coil structures, polydisperse in molecular weight with a polydispersity parameter of one. Muzzarelli *et al.*, (1972) determined the molecular weight of chitosan by light scattering in 8.5% formic acid and 0.5 M sodium formate, with the value dn/dc = 0.174 ml x g^{-1}

1.3.3.2 Degree of deacetylation

Chitosan is a partial or complete deacetylation of chitin. For this reason, the degree of deacetylation needs to be determined. A simple and reliable method for the determination of this parameter has been sought over many decades.

1.3.3.2.1 Colloidal titration (Amino residue analysis)

Chitosan hydrochloride is prepared by adding an excess of concentrated hydrochloric acid. Titration of ammonium cation is then performed by using negative colloid solution, such as potassium salt of polyvinyl sulfate [Terayama, 1952], or sodium hydroxide or silver nitrate [Hayes, 1978]. The end point is detected by indicator, such as phenolphthalein.

1.3.3.2.2 Infrared spectroscopy

The ratio of the absorbance of the 1655 cm^{-1} band to that of the CH stretching band at 2867 cm^{-1} can be used to estimate CONH content of chitosan with excellent accuracy. This method will be limited to the samples deacetylated by over 90% per glucosamine residue [Jaiyongka, 1993].

1.3.3.2.3 First derivative ultraviolet spectroscopy

This method is, for detecting *N*-acetylglucosamine in chitosan, simple, fast and accurate. Sensitive and precise measurements can be carried out in the simplest way by using commonly available spectrophotometers [Muzzarelli. 1997].

1.3.3.2.4 ^{1}H-NMR spectroscopy

High-resolution NMR spectroscopy is one of the most useful non-invasive methods for studies of chemical as well as steric structures of carbohydrates [Inoue, 1997]. The characterization by NMR is a novel method to determine the degree of deacetylation proposed by Hirai, Odani, and Nakajima (1991).

1.3.3.2.5 Gas chromatography

This method is based on the measurement of the retention time of methanol in a chitin/chitosan column that is proportional to the degree of acetylation [Jaiyongka, 1993].

1.3.3.2.6 Thermal analysis

The determination of the percentage of acetyl groups is performed by using thermogravimetric analysis with the empirical calibration technique.

Alonso, Peniche-Covas and Nieto (1983) proposed this method. They stated that this method compared satisfactory with the one that makes use of IR spectroscopy and was more rapid and simple than the chemical methods.

1.3.3.2.7 Elemental analysis

This method determines carbon/nitrogen ratio. Since N-acetylglucosamine and glucosamine have C/N rations, the C/N ratio can based on an indicator for per cent deacetylation [Hayes, 1978].

1.3.3.2.8 Mass spectroscopy

Mass spectra provide information of degradation temperature and polymer identification and permit the determination of the degree of acetylation in a polymer and possibly the $-NH_2$/-NHCOCH$_3$ ratio [Hayes, 1978].

1.3.3.3 Solubility

The term "chitosan" may be consider as referring to a family of polymers derived from chitin that has been deacetylated to provide sufficient free amino groups to render the polymer soluble is not readily determined, and it undoubtedly varied with such factors as polymer molecular weight, temperature, and concentration and nature of the acid species. In general, solubilization begins at about 60%, usually about 75% deacetylation depending on the molecular weight of chitosan formed. Chitosan samples 75% or more deacetylation dissolve readily in dilute organic acids to give clear, homogeneous and viscous solutions.

For practical purposes, chitosan is insoluble in sulfuric acid and phosphoric acid, while a certain solubility exists for other mineral acids like hydrochloric acid, nitric acid and perchloric acid. Compared with the more common organic acids, the solubility in inorganic acids seems more limited concerning the concentration ratio chitosan/acid. The solubility of chitosan in some organic acid is up to 50%(w/v) such as acetic acid, lactic acid, formic acid, and propionic acid (The standard solvent commonly used for solution property measurement is acetic acid).

1.3.4 Application of chitosan

Chitosan is useful as a cationic precipitant for waste treatment. In addition, Table 1.1 lists other uses in the fields of biotechnology, personal care products and medicine.

1.4 Chitosan millispheres

1.4.1 Application of microparticles/microspheres

Use of chitosan microsphere as a drug delivery system

Chitosans and their derivatives have been employed in the area of drug delivery system for the preparation of drug loaded microparticles [Yao, 1995]. The use of chitosan in controlled drug delivery systems aims to prepare microparticulate or macromolecular systems kinetically controlling drug release in order to make the release more dependent on the pharmaceutical formulation than the physico-chemical characteristics of the drug [Zecchi *et al.,* 1997].

Use of chitosan microsphere as support material for enzyme immobilization

Chitosan microspheres have been used as support material for enzyme immobilization. For example in one preparation for sorption immobilization of acid phosphatase, chitosan was cross-linked with different dosages of glutaraldehyde and glyoxal (100-80000 mg/l). The amount of sorption of solutes and the immobilization capacities of enzymes onto the swollen chitosan beads were significantly affected by the degrees of cross-linking. Activities and lifetimes of the immobilized enzymes were measured to evaluate the potential of practical applications [Juang *et al*., 2002].

Use of chitosan microparticle as waste water treatment

Since chitosan has high density of amino groups on the polymer chain, it has been used as an excellent coagulant or flocculent as well as a strong metal uptaker. A novel method of synthesis of cross linked *N*-methylthiobenzylated chitosan beads give

a high selectivity in adsorption of metal (Pd^{2+}, Au^{3+}, and Hg^{2+}) ions at pH 4 of the test solution [Han, 1998].

1.4.2 Covalently cross-linked microparticles

The earliest work in chitosan microstructures predominantly involved chemical cross-linking reactions to the polymer chain. The chitosan microspheres for drug delivery applications. Using a water-in-oil (W/O) emulsion method followed by glutaraldehyde cross-linking of the chitosan amino groups. The group produced microspheres which contained 5-fluorouracil (5-FU), an anti-cancer drug [Ohya *et al.*, 1994]. The chitosan microspheres developed by Thanoo *et al.,* (1991) microspheres were prepared by the glutaraldehyde cross-linking of an aqueous acetic acid dispersion of chitosan in paraffin oil using dioctylsulfosuccinate as the stabilizing agent. For (theophylline, aspirin, or griseofulvin), the incorporation efficiencies exceed 80% could be achieved.

1.4.3 Ionically cross-linked microparticles

The cationic nature of chitosan has been conveniently exploited for the development of particulate drug delivery systems. Aside from its complexation with negatively charged polymers, an interesting property of chitosan is its ability to gel on contact with specific polyanions. This gelation process is due to the formation of inter- and intramolecular cross-linkages mediated by these polyanions. First reported was the

ionotropic gelation of chitosan with tripolyphosphate (TPP) for drug encapsulation [Bodmeier *et al.,* 1989]. However, the approach was aimed at designing chitosan-TPP beads rather than nanoparticles. More recently, the developed chitosan microspheres based upon the same principle was investigated by compared TPP between other anions, sulfate and citrate [Shu *et al.*, 2002].

1.4.4 Desolvated microparticles

The use of desolvating agents for the synthesis of chitosan particles originally emerged from the field of microencapsulation. Sodium sulfate was first proposed as a precipitating agent to form chitosan particles. They were able to adsorb significant amounts (up to 30 % loading) of the hydrophilic, anionic corticosteroid, prednisolone sodium phosphate, to the particle surface [Berthold *et al.*, 1996]. A variation of this technique was later employed for the controlled release of antineoplastic proteoglycans for immunostimulation [Tian *et al.*, 1999].

1.4.5 Millispherical

Microspheres and microcapsules made from chitosan or cross-linked chitosan in the size range of 50 nm to 2 mm have been reported in literature. (see review in Yao *et al.*, 1995) The term "millisphere" is used here merely to emphasize the millimeter size range of the chitosan particles.

1.5 Volume changes in polymergels

While working with acrylamide-acrylic acid copolymer gels; Tanaka postulated three factors that control the expansion and contraction of the polymer. The first factor is called "rubber elasticity" which tends to contract expanding gels and expand contracting gels. The second factor is called "polymer-polymer affinity" which corresponds to van der Waals interaction among polymer chains. The third factor is the osmotic pressure of the gel, which is related to the ionization of the polymer network [Tanaka, 1981].

1.6 Objectives

The objectives of this study of chitosan from shrimp shells are:

1. To prepare chitosan from shrimp shells

2. To prepare chitosan millispheres.

3. To investigate the characteristics of chitosan millispheres.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autopipette : Pipetman, Gilson, France

Centrifuge, benchtop centrifuge : Model H-11n, Kokusan, Japan

Centrifuge, small bench centrifuge : Model 1010 series Labquip, U.K.

Centrifuge, Hettich Zentrifugen : Model MIKRO 12-24, Switzerland

Electric blender & mill : Model SMB-001, Srithai Marketing Co.,Ltd., Thailand

Electron microscope : Model JSM-5800LV, JEOL, Japan

Hot air oven : Isotemp$^{®}$ oven Model 517G, Fisher Scientific, U.S.A.

Infrared spectrometer : Model 1760x Perkin Elmer, U.S.A.

Magnetic stirrer and heater : Model IKAMA$^{®}$GRH, Janke & Kunkel Gmbh & Co. KG, Japan

Objective micrometer (stage micrometer) : 0.01 mm, Olympus,Japan.

Occular micrometer : Olympus, Japan.

pH meter : PHM 83 Autocal pH meter, Radiometer, Denmark

Spectrophotometer : Spectronic$^{®}$ genesys^{TM}8, Spectronic Instrument Inc, England

Stereo microscope : Stereo Zoom$^{®}$4, Bausch & lomb, U.S.A.

Thermolyne : Furnatrol Π Model F-A1830 , Sybron corporation, U.S.A.

Viscometer : U-tube viscometer(A, B, F, G, μ1) Cannon instrument Co.,Ltd., U.S.A.

Vortex: Model K-550-GE, Scientific Industrial, U.S.A.

Water bath shaker : Model G 76D New Brunswick Scientific Co., Inc. Edison, N.J. U.S.A.

2.2 Chemicals

- Chloroform (67-66-3), and Hydrochloric acid (7647-01-0) was obtained from Merck, U.S.A.

- α-Chymotrypsin (9004-07-3), benzoyl - L- tyrosine ethyl ester (BTEE), Bovine serum albumin (BSA), Protamine sulfate (53597-25-4), Coomassie brilliant blue G-250, and Glycine (56-40-6) were obtained from Sigma, U.S.A.

- Paraffin wax (8002-74-2), Span®80 (1338-43-8) were obtained from Fluka, Switzerland.

- Phenolphthalein (77-09-8) was obtained from BDH, England

- Sodium hydroxide (1310-73-2) was obtained from Carlo, Italy

- Triethanolamine (102-71-6) was obtained from Unilab, Australia.

Other common chemicals were obtained from Fluka, Sigma, Carlo or BDH. All chemicals are of reagent grade unless otherwise specified. All water used was distilled once on an automatic water distiller.

Other materials

Filter paper No.1: Whatman International LTD., England.

2.2.1 Preparation of chitin and chitosan

Chitin was extracted from giant black tiger shrimp shells (*Penaeus monodon*) which was obtained from T.C. Union frozen foods Co., Ltd., Thailand. The shrimp shells, without carapace, were used as the wet starting material. The shell was kept at -20°C prior to chitin extraction.

2.2.1.1 Chitin extraction

Frozen shrimp shells were deproteinization with 1 M NaOH in the ratio 1 : 10 (w/v) at 35°C for 24 hours. The deproteinized shells were washed with deionized water until the filtrate was neutral. The deproteinized shells were then demineralized with 1 M HCl solution in the ration 1 : 15 (w/v) at 35°C for 12 hours. Crude chitin was obtained after washing until no chloride ions were detected by reacting with a 0.1% silver nitrate solution. Unwanted color of chitin was bleached by sunlight after washing with distilled water.

2.2.1.2 Chemical characterization of chitin
2.2.1.2.1 Protein determination

The protein concentration was determined according to the method of Chandrkrachang (1999) as follow :

About 50 g of chitin was soak overnight in 30 ml of 1 M NaOH at room temperature and then heat in water bath for 30 minutes. Then filter the solution through

Whatman No. 1 paper. Collect the filtrate and make up the volume to 100 ml. Assay for

proteins in this filtrate by the Biuret method [Gornall *et al.,* 1949].

Further treat the residue with 30 ml of 1 M HCl for 30 minutes in water

bath. Filter, collect the filtrate and make up the volume to 50 ml. Assay for protein in

this second filtrate by the Bradford method [Bradford *et al*., 1976].

The total protein contents were calculated as the sum of the alkaline-

soluble protein contents from the Biuret method and the acid-soluble protein contents

from the Bradford method.   The protein concentrations were expressed as the total

protein contents per dry weight of chitin.

2.2.1.2.2 Ash determination

The ash content was determined according to the method of Association of

Official Analytical Chemists (AOAC) 7.009 as follow : weigh accurately about 2 g of the

sample into a porcelain crucible and place the container in a temperature controlled

furnace preheated to 600°C. Hold at this temperature for 2-3 hours or until free from

carbon. When ashing was complete, allow the crucible to cool in a desiccator and the

weigh it to the nearest 0.1 mg. Then calculate the ash content as percent of ash weight

to weight of sample.

2.2.1.2.3 Moisture determination

The moisture content was determined according to the method of

Association of Official Analytical Chemists (AOAC) 7.007 as follows : weigh accurately

about 2 g of the sample into a porcelain crucible and place in a temperature controlled

oven at 135°C. Hold at this temperature 2 hours and allow the crucible to cool in a desiccator. Weigh it to the nearest 0.1 mg: The moisture content was calculated as follows:

$$\text{Moisture content (\%wt)} = \frac{(W_0 - W_1)}{W_0} \times 100$$

where $W_0 =$ Weight of sample before dry (g)

$W_1 =$ Weight of sample after dry (g).

2.2.1.3 Chitosan preparation

Chitin was deacetylated with 50%(w/v) NaOH in the ratio 1 : 10 (w/v) at 50°C for 30 hours. After reaching the reaction time chitosan was washed with deionized water until the filtrate was neutral and dried in a 60°C oven.

2.2.2 Physicochemical characterization

2.2.2.1 Ash determination

The procedure was essentially the same as the one in section 2.2.1.2.2.

2.2.2.2 Moisture determination

The procedure was essentially the same as the one in section 2.2.1.2.3.

2.2.2.3 Solubility determination

About 1 g of sample was dissolved in 100 ml of 0.5%(w/v) acetic acid and incubate at 40°C on a rotary shaker at 100 rpm for 12 hours. Undissolved substances were filtered through Whatman No. 1 paper. The undissolved substances were dried in a 50°C oven until constant weight was obtained : The dissolution was calculated as follows :

$$\text{Dissolution (\%wt)} = \frac{(W_s - W_p)}{W_s} \times 100$$

where W_s =   Weight of sample (g)

W_p =   Weight of undissolved substance (g).

2.2.2.4 Degree of deacetylation of chitosans determination

2.2.2.4.1 Colloidal titration

The degree of deacetylation of chitosan was determined by the colloidal titration [Hayes and Davies, 1977].

Preparation of chitosan hydrochloride

About 1 g of chitosan was weighed and dissolved in 100 ml of 10%(w/v) acetic acid and incubate at 40°C on a rotary shaker at 100 rpm for 12 hours. Undissolved particles were removed by suction filtration using polyester cloth (< 100 mesh) as a filter. Concentrated hydrochloric acid was added slowly to the chitosan

solution with rapidly stirring until no further precipitation of chitosan hydrochlorides was obtained. The precipitate was filtered through a sintered glass (No. 1). The solid was made into a slurry with 60 ml of methanol and refiltered. This operation was repeated until the filtrate was free of chloride ions. (test with 0.1% silver nitrate solution). The chitosan hydrochloride precipitate was dried in a hot air oven at 50°C until constant weight was obtained.

Titration of chitosan hydrochloride

Accurate weight about 1 g of dried chitosan hydrochloride was dissolved in 100 ml of distilled water. 50 ml of solution was transferred to a 125-ml Erlenmeyer flask. Next, the solution was titrated with 0.1 M NaOH solution using phenolphthalein as an indicator. The volume of sodium hydroxide solution used was recorded as ml and percentage of degree of deacetylation of chitosan sample was calculated.

2.2.2.4.2 Infrared spectroscopy

The degree of deacetylation of chitosan was determined by infrared spectroscopy method [Robert, 1977]. The degree of deacetylation was calculated using the equation

$$\text{Degree of deacetylation (\%)} = 100 - [(A_{1655}/A_{3450}) \times 115]$$

2.2.2.5 Infrared spectroscopy

Infrared spectra were examined by using a Fourier transform infrared spectrophotometer (Perkin Elmer, model 1760x, USA) and KBr disc.

2.2.2.6 Average molecular weight determination

The average molecular weight (M_v) of the chitosan was determined according to the method of Robert (1982). The intrinsic viscosity $[\eta]$ of chitosan was determine by using a U-tube viscometer. Solution of 0.1 M acetic acid-0.2 M sodium chloride was used as standard. The molecular weight of chitosan was estimated using Mark-Houwink equation

$$[\eta] = K\, M_v^{\,a}$$

where $[\eta]$ is intrinsic viscosity expressed in ml/g, K $= 1.81 \times 10^{-3}$ and a $= 0.93$.

2.2.3 Preparation of chitosan millispheres

A 1.6% (w/v) chitosan solution was prepared in 1%(v/v) acetic acid. The solution was slowly added under mechanical stirring to liquid paraffin solution containing 0.5% (w/v) Span® 80 in the ratio 1:10 (v/v). The dispersion was mechanically stirred at 150 rpm at 25°C for 30 minutes after the addition of triethanolamine (1:1 v/v ratio). The formed spherical beads were washed with chloroform several times and vacuum dried for 24 hours.

In protein-loaded millispheres preparations, the 1.6% (w/v) chitosan in 1%(v/v) acetic acid was replaced with 1 mg/ml protein (either chymotrypsin, BSA, or protamine) in the 1.6% (w/v) chitosan in 1%(v/v) acetic acid. The remaining steps were the same as those of the unloaded chitosan millispheres preparation.

2.2.4 Characterization of chitosan millispheres

2.2.4.1 Physical characterization of chitosan millispheres

The shape and surface morphology of the millispheres were examined by light microscopy and scanning electron microscopy (model JSM-5800LV, JEOL, Japan). Sample were mounted on metal stubs, using double-side adhesive tape, vacuum coated with gold and then scanned for their shape, surface morphological structures.

2.2.4.2 Swelling of chitosan millispheres

The conditions for swelling of chitosan millispheres were investigated by varying the pH and ionic strength of unstirred buffer solutions. The pH was set at 5.0 (20 mM acetate buffer), 7.0 (20 mM phosphate buffer), and 9.1 (20 mM glycine-NaOH buffer). Sodium chloride was added between 75 and 500 mM. The volume ratio of millispheres to buffer was approximately 1 : 50. The diameter of the millispheres was measured under an optical microscope that has been calibrated using a reticule and a stage micrometer. The swelling ratio in the media was estimated by measuring the bead size at three different positions and expressed as :

$$\text{Swelling ratio} \quad = \quad \frac{(D_t - D_o) \times 100}{D_o}$$

where D_t the bead diameter at time t and

D_o the initial bead diameter

2.2.4.3 Release of protein from chitosan millispheres

A certain amount of millispheres containing approximately 1.0 mg/ml model proteins were suspended in 25 ml Erlenmeyer flask containing 5 ml release media and incubate at 25°C on a rotary shaker at 100 rpm. Release media at different ionic strengths were buffered in the same manner as the ones described in the proceeding section. At appropriate intervals, 1 ml samples were withdrawn and protein content in the medium determined by the Bradford method [Bradford *et al.*, 1976]. An equal volume of the same release medium was added back to maintain a constant volume. Concentrations of species present in the reaction mixture were calculated to compensate for this change in reaction volume.

2.2.4.4 Protein determination

Protein concentrations were determined by the Coomassie blue micro method according to Bradford (1976), using bovine serum albumin as standard. (Appendix C)

A 100 μl sample was mixed with 1 ml of Bradford reagent and incubated at room temperature for 5 minutes before the absorbance at 595 nm was measured.

The Coomassie blue reagent was prepared by mixing together 100 mg Coomassie blue G-250, 50 ml of 95% ethanol and 100 ml of 85% H_3PO_4. Double distilled water was then added to give a one liter solution.

Chitosan up to 1 mg/ml was mixed with a test protein (Bovine serum albumin in the range of 0 to 10 mg/ml). No interference with protein determination was detected. (Data not shown.)

Fresh shrimp shells

--Deproteinization

with 1 M NaOH at 35°C for 24 hours.

Solid : solvent, 1 : 10

Deproteinized shells

--Demineralization

with 1 M HCl at 35°C, for 12 hours.

Solid : solvent, 1 : 15

Chitin

- Protein determination

- Ash and Moisture determination

--Deacetylation

with 50%(w/v) NaOH at 50°C, for 30 hours.

Solid : solvent, 1 : 10

Chitosan

- Ash and moisture determination

- Solubility determination

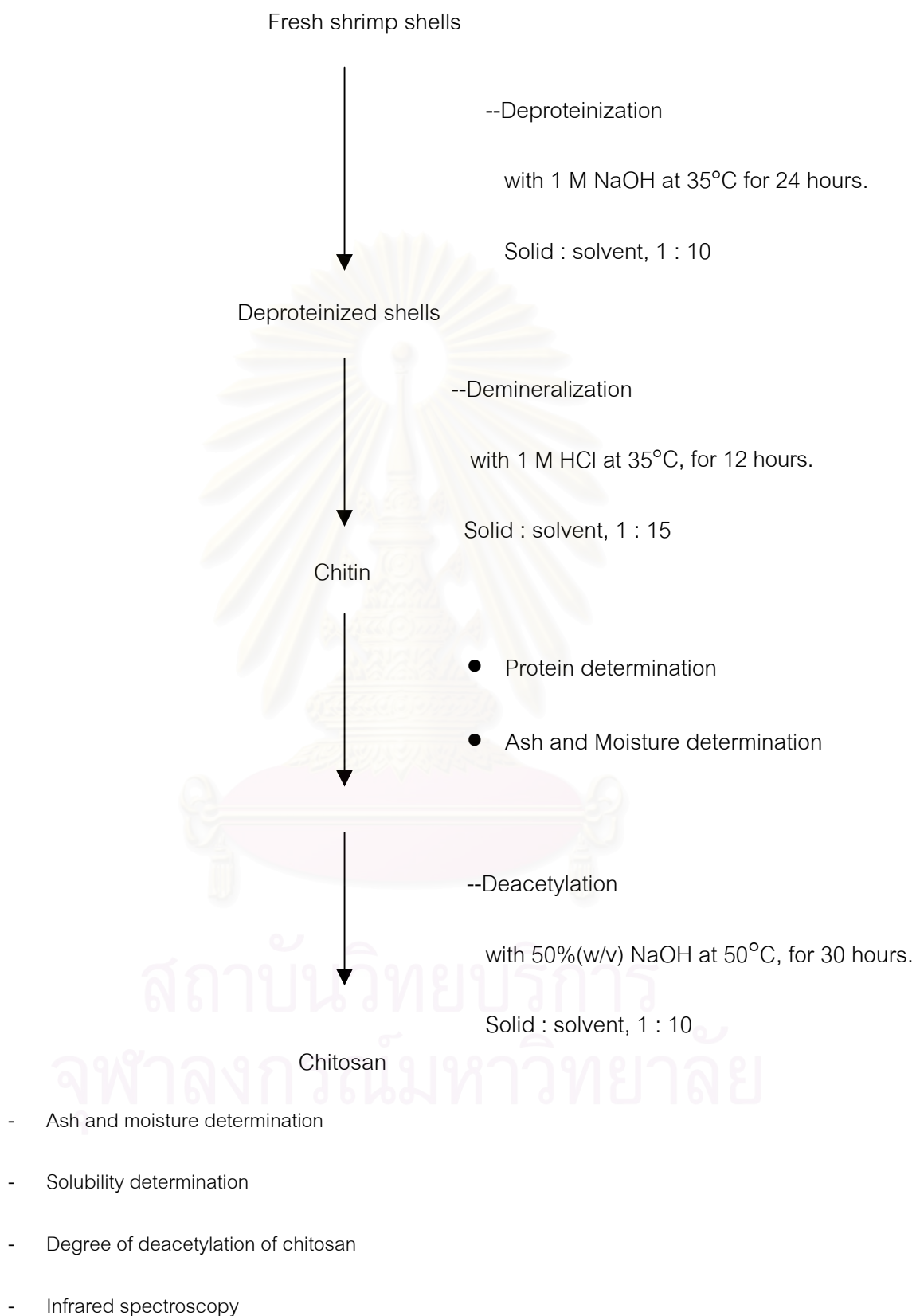- Degree of deacetylation of chitosan

- Infrared spectroscopy

Figure 2.1 Flow chart for chitin and chitosan preparation

CHAPTER III

RESULTS

3.1 Chitin extraction

Chitin was extracted according to the methods outlined in section 2.2.1.1. After

deproteinization with 1 M NaOH at 35°C for 24 hours, the shrimp shell was orange in

color. After the calcium carbonate was removed, the extracted solid turned into white,

lightweight flakes.

3.1.1 Yield

Chitin was extracted from four batches of shrimp shells for further chitosan

preparation. The conversion of shrimp shells to chitin was about 45.89% (w/w) of chitin

to the dry weight of shrimp shells (moisture included). Table 3.1 compares the four

batches of chitin preparation.

3.1.2 Protein content

The concentrations of proteins remaining in the deproteinized shrimp shells were

determined according to the method outlined in section 2.2.1.2.1. The protein

concentrations of the deproteinized shrimp shells was found to be about

0.8 – 1.0%(w/w) (Table 3.1). The protein content of the extracted chitin is similar to that

of commercial chitins (No and Meyer, 1997).

3.1.3 Ash content

The ash, which is the main composition in shrimp shell, was removed with 1 M

HCl at 35°C for 12 hours as described in section 2.2.1.2.2. The ash contents were in

the range of 0.07 to 0.17 percent dry weight (Table 3.1). The resulting ash content was

comparable if not better than commercial chitins (<1%).

3.1.4 Moisture content

The moisture content of the extracted chitin as measured by the method outlined

in section 2.2.1.2.3. It was presented as percentage of moisture with respect to the dry

weight of chitin. The moisture contents were about 20.35 to 36.83 percent dry weight

(Table 3.1).

Table 3.1   The chemical composition of chitin from fresh shrimp shells

Chitin extraction was performed using the method outlined in section 2.2.1.1

Physical content of chitin % (dry weight)	Batch 1	Batch 2	Batch 3	Batch 4	Average
Yield	40.55	35.51	51.60	55.90	45.89
Protein	0.82	0.78	0.95	0.99	0.89
Ash	0.173	0.146	0.085	0.074	0.24
Moisture	20.35	28.46	21.40	36.83	26.76

3.1.4 Infrared spectroscopy

Infrared spectroscopy was employed to determine the absorption band of carbonyl (C=O) and amide (-NH) interaction of acetamido groups in chitin products. Each spectrum shows a peak at about 1650 cm^{-1} for carbonyl group and absorption band of N-H bending at about 1550 cm^{-1}. The IR spectra of two batches of chitin was shown in Figure 3.1
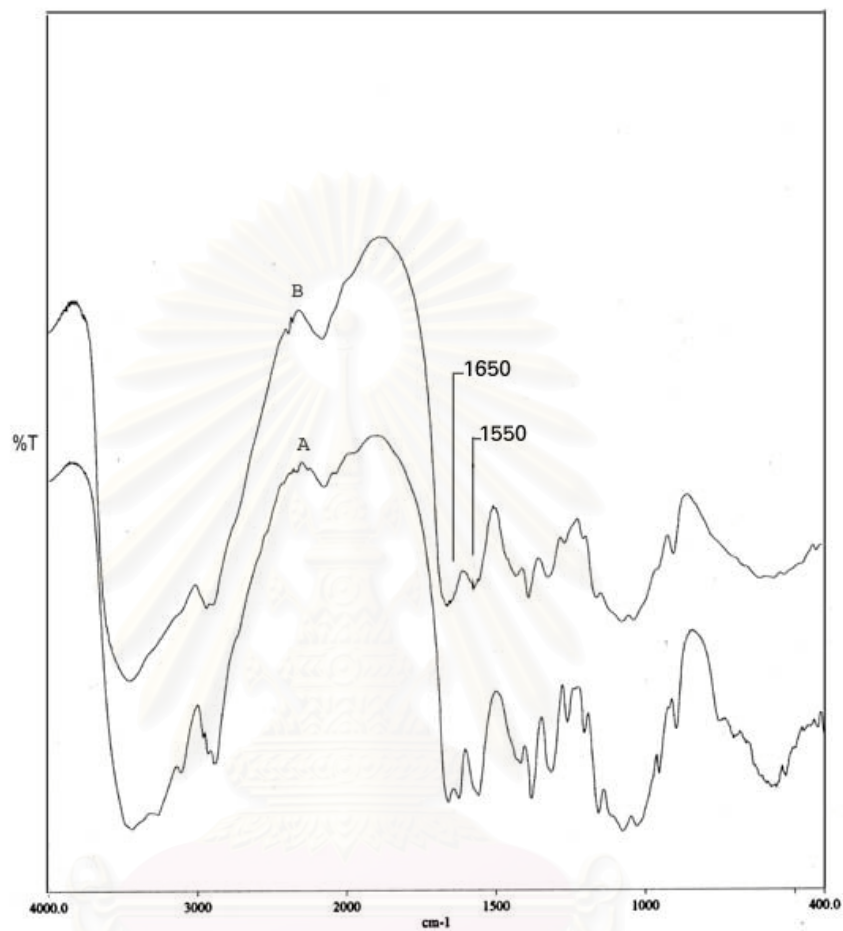
Figure. 3.1 Infrared spectra of chitin
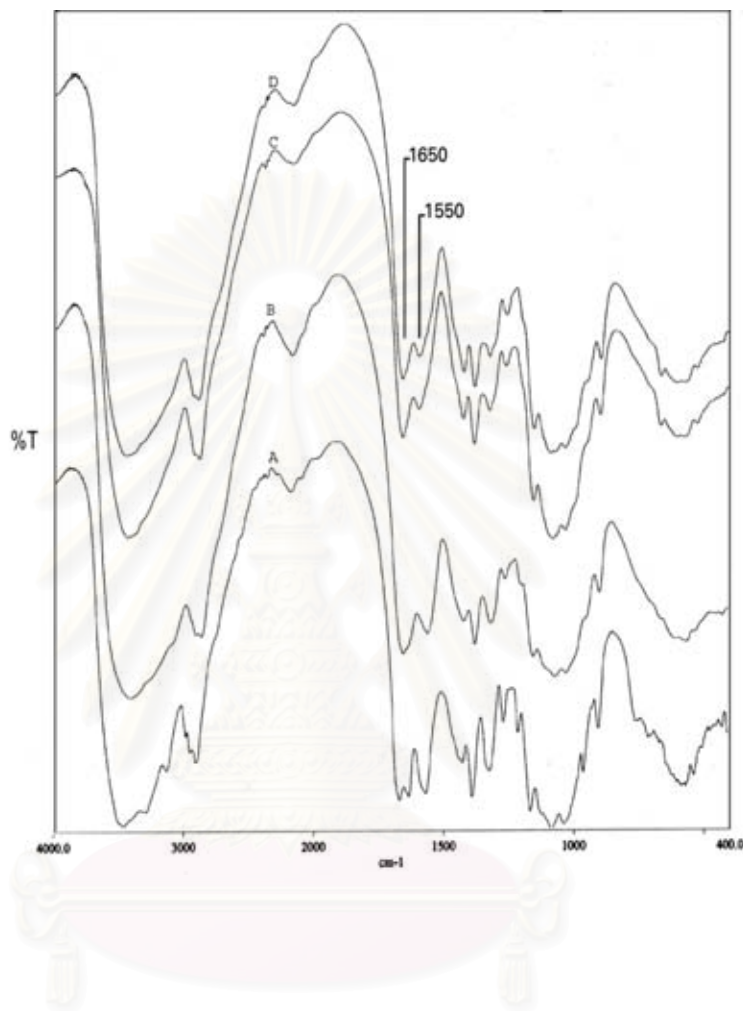
A = Chitin 1st batch

B = Chitin 2nd batch

Figure. 3.2 Infrared spectra of chitosan

A = Chitosan 1st batch

B = Chitosan 2nd batch

C = Chitosan 3rd batch

D = Chitosan 4th batch

3.2 Chitosan preparation

Four batches of chitosan were produced from chitin according to the methods outlined in section 2.2.1.3. The chitin was deacetylated in 50% (w/v) NaOH solution at 50°C for 30 hours.

3.2.1 Infrared spectroscopy

Infrared spectra of chitosan are demonstrated in Figure 3.2. Each spectrum shows a peak at about 1650 cm^{-1} for C=O stretching and 1550 cm^{-1} for NH bending. It was seen that spectra B, C and D appeared to be similar. From this observation, The absorption peak at about 1650 cm^{-1} in each chitosan associated with the degree of deacetylation measured by colloidal titration. The lower the 1650 cm^{-1} peak (The lower the amount of carbonyl groups), the higher the degree of deacetylation.

3.2.2 Yield

The conversion of chitin to chitosan produced about 42.28% (w/w) of chitosan to the dry weight of shrimp shells (moisture included). The chemical composition of chitosan are shown in Table 3.2

3.2.3 Ash content

The ash was determined from chitosan according to the method of Association

of Official Analytical Chemists 7.009 (section 2.2.1.2.2). The ash of chitosan about

0.10% (w/w) (Table 3.2)

3.2.4 Solubility

The solubility of chitosan was evaluated according to the method outlined in

section 2.2.2.3. The solubility of chitosan in the first and second batches was not

measured due to lack of solubility in 1% (v/v) acetic acid. The solubility of chitosan in

the last two batches (3rd and 4th batches) was 100 % (Table 3.2).

3.2.5 Moisture content

The moisture contents of chitosan preparation are presented as percentage of

moisture with respect to the dry weight of chitosan. The moisture contents were about

11.51 to 18.43 percent dry weight (Table 3.2).

3.2.6 Degree of deacetylation

3.2.6.1 Colloidal titration

The degree of deacetylation of the prepared chitosan was determined

according to the methods outlined in section 2.2.2.4.1. The calculation was performed

according to Appendix A. The degrees of deacetylation of chitosan are shown in Table 3.2.

Note that the first and second batches were not measured because chitosan from the

first two batches was not soluble in acetic acid. Chitosan from the third and fourth batches had approximately 70% degree of deacetylation.

3.2.6.2 Infrared spectroscopy

Degree of deacetylation of chitosan was determined according to the method outlined section 2.2.2.4.2. The degrees of deacetylation of chitosan from the four batches were 52.50, 54.50, 60.43 and 60.43 percent respectively.

3.2.7 Average molecular weight

The average molecular weight (M_v) of chitosan in the fourth batch was determined according to the method outlined in section 2.2.2.6. The intrinsic viscosity [η] of a chitosan solution was 201.36 ml/g and the average molecular weight was 2.67×10^5 kDa.

Table 3.2 The chemical composition of chitosan from fresh shrimp shells

Physical content of chitin % (dry weight)	Batch 1	Batch 2	Batch 3	Batch 4	Average
Yield	39.095	34.140	43.000	52.880	42.279
Ash	0.222	0.063	0.040	0.079	0.101
Solubility	-	-	100	100	100
Moisture	18.432	12.00	11.510	11.725	13.417
Degree of deacetylation					
- Colloidal titration	-	-	76.770	69.825	73.298
- Infrared spectrometry	52.50	54.50	60.43	60.43	56.965
Average molecular weight	*	*	*	2.67×10^5	2.67×10^5

- The solubility and degree of deacetylation could not be determined

   due to poor solubility in 1% acetic acid.

* Average molecular weights were not measured.

3.3 Chitosan millispheres

The chitosan millispheres were prepared according to the method outlined in section 2.2.3. The millispheres were transparent as shown below in Figure 3.3. The average size of millispheres was 1.0 to 1.5 mm (in diameter).
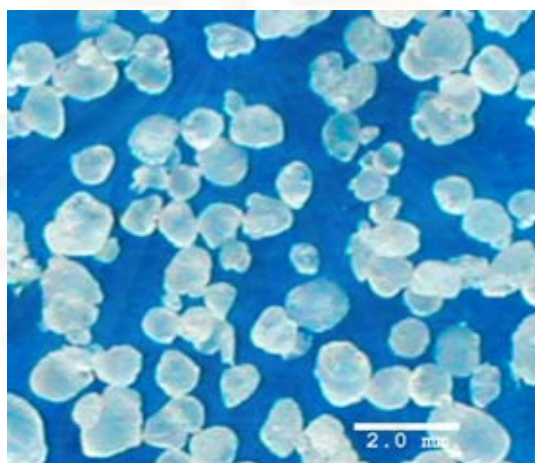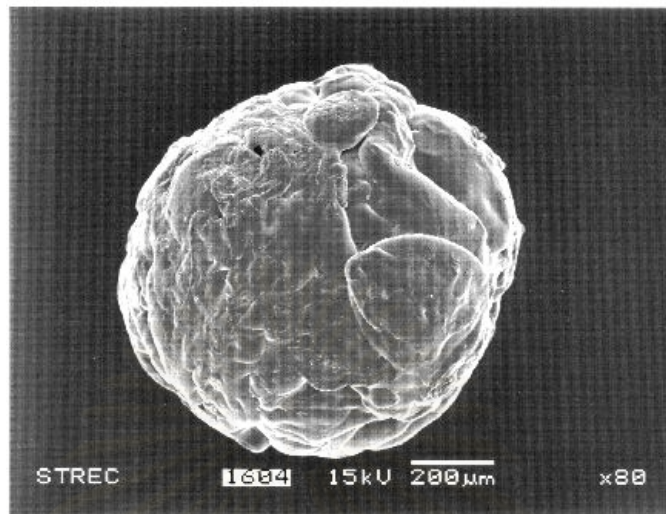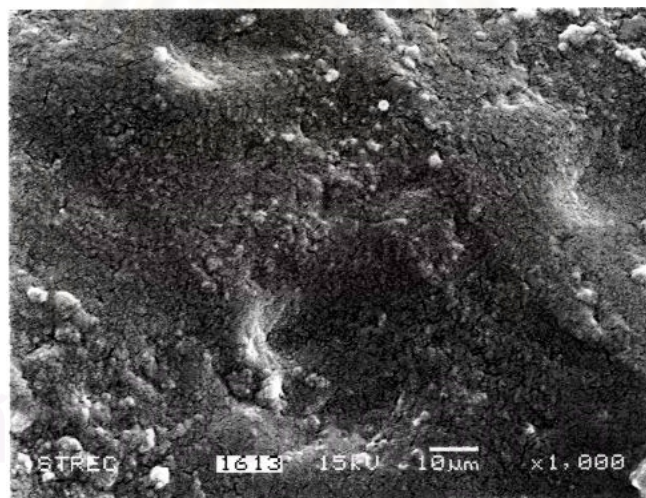

Figure 3.3 Photographs of Chitosan millispheres

(magnification 3x)

3.3.1 Physical characterization of chitosan millispheres

Scanning electron micrographs of chitosan millispheres and their surface morphology are shown in Figure 3.4. The chitosan millispheres were not spherical in shape and had a rough surface with large wrinkles.

(a)

(b)

Figure 3.4 Scanning electron micrographs of chitosan millispheres (a)

and surface morphology (b)

3.4 Swelling of chitosan millispheres

The swelling of chitosan millispheres was determined according to the method outlined in section 2.2.4.2. The swelling of chitosan millispheres in a buffer without NaCl was labelled "control" in Figure 3.5. It was found that in pH 5.0 acetate buffer, the swelling ratio decreased with the increase in NaCl, while concentration 75 mM NaCl was an exception (Figure 3.5a). As demonstrated in Figure 3.5b, swelling of millispheres in pH 7.0 phosphate buffers with 150 mM NaCl was not different from control. At 75, 500 mM NaCl gave the increase of swelling rate. The observed swelling rate in pH 9.1 glycine buffer increased with the increase NaCl concentrations (Figure 3.5c).
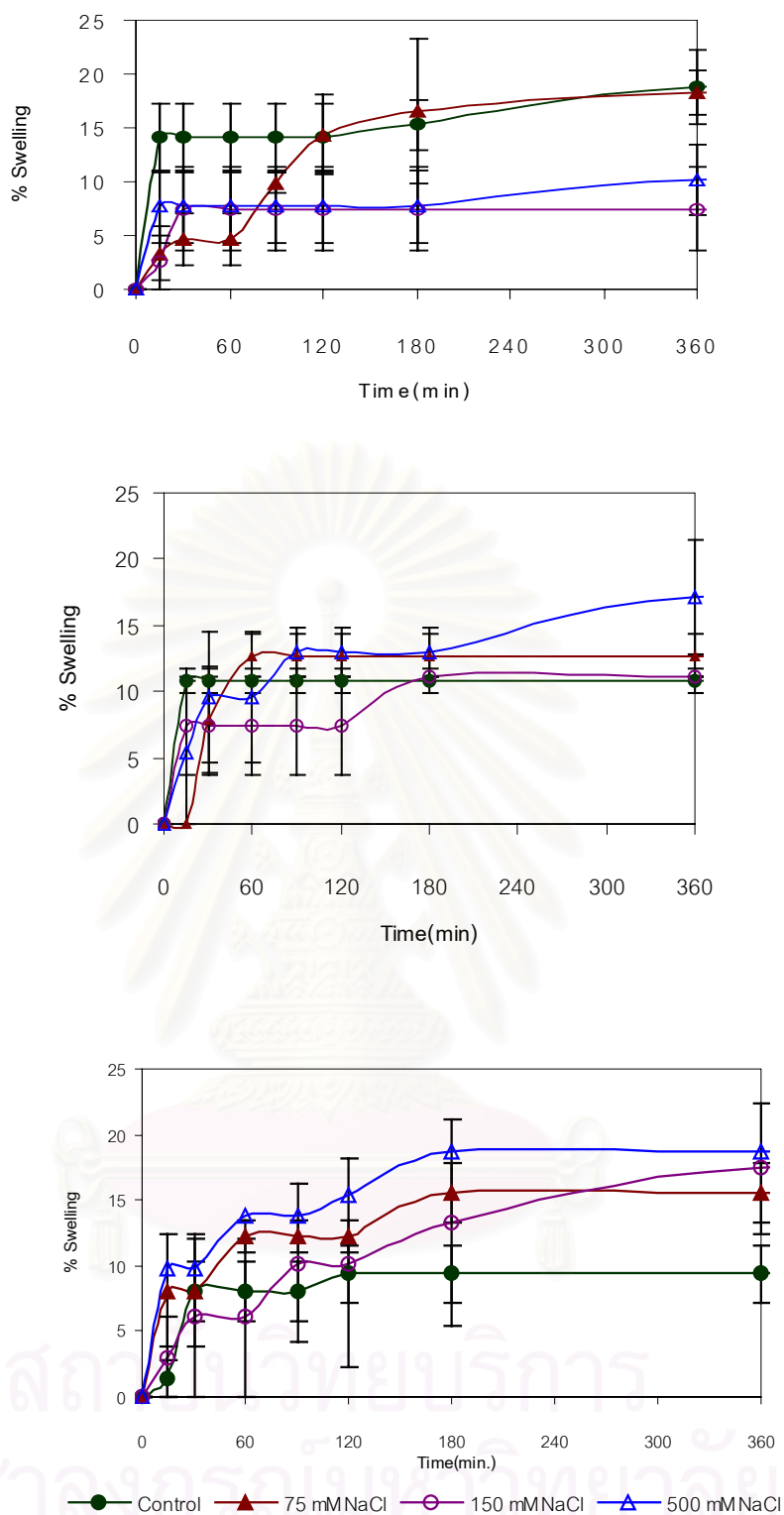
Figure 3.5 The swelling curves of chitosan millispheres in 20 mM buffer and different

NaCl concentration : (a) Acetate buffer pH 5: (b) Phosphate buffer pH 7.0: (c)

Glycine buffer pH 9.1. Actual experiments ran for 24 hours, with minimal

changes in swelling after 6 hours.

(The error bar is expressed in standard error of the mean, n=3)

3.5 Release of protein from chitosan millispheres

The release of model protein from chitosan millispheres was determined according to the method outlined in section 2.2.4.3.

3.5.1 α-Chymotrypsin

The release of α-chymotrypsin from chitosan millispheres in a buffer without NaCl was labelled "control" in Figure 3.6. This "control" sample gave the lowest release profile (concentration of protein recovered in the surrounding medium) regardless of pH or ionic strength. Figure 3.6 shows that at 75, 150 and 500 mM NaCl in acetate buffers, chitosan millispheres exhibited similar "saturation" profiles. Except at pH 7 where obvious declining profiles were found at 75 and 150 mM NaCl. The protein released from millisphere in 500 mM NaCl buffer solutions consistently gave the highest concentration at 180 minutes of releasing time.

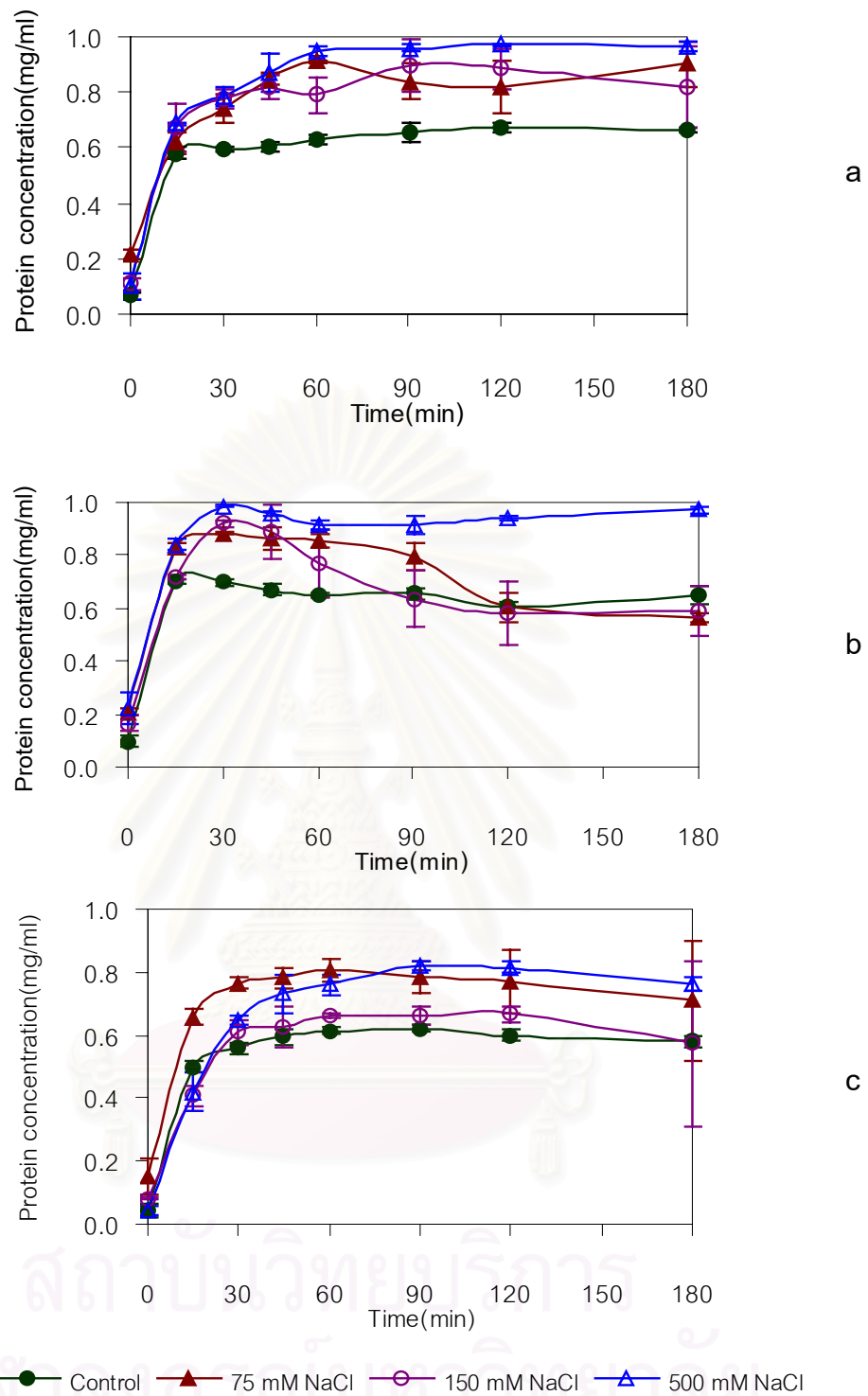**Figure 3.6** The release curves of α-chymotrypsin in 20 mM buffer and different

NaCl concentration: (a) Acetate buffer pH 5; (b) Phosphate buffer

pH 7; (c) Glycine buffer pH 9.1.

(The error bar is expressed in standard error of the mean, n=3)

3.5.2 Bovine serum albumin (BSA)

The release of BSA from chitosan millispheres in a buffer without NaCl was labelled "control" in Figure 3.7. These controls gave the highest release profile in all three pH buffers. As more NaCl was added to 75 mM, the release profile was shifted downward. At 150 mM NaCl, the release profile was shifted upward from the one at 75 mM except at pH 9.1 where the shift was not significant. Finally, as NaCl was increased further to 500 mM, the release profile seemed to move downward at pH 5. In the pH 7 buffer, no shift was detected until after 90 minutes, where the profile shifted slightly upward. In the pH 9.1 buffer, the shift was slightly upward until after 60 minutes, where no shift was detected. At 180 minutes, the shift was slightly downward.

3.5.3 Protamine

The release profile of protamine from chitosan millispheres in a buffer without NaCl was labelled "control" in Figure 3.8. The profile of these controls showed a peak of 0.6-0.8 mg/ml protein at around 30-60 minutes. These profile level off at around 0.4 mg/ml protein except in pH 5, where the protein concentration dropped to 0.1 mg/ml at 180 minutes. As the concentration of NaCl was raised to 75 mM, the profile tend to move downward in pH 7 and 9.1 buffers. The shifts are move complicated in pH 5 buffer, where the only downward shift occurred at 90 minutes. At 150 mM NaCl, the profile tend to move downward in pH 5 and 7 buffers, and slightly upward in pH 9.1 buffer. At 500 mM NaCl, the profiles are the smallest of all. It is interesting to point out that most of the protamine profiles exhibit a broad peak around 30-90 minutes.
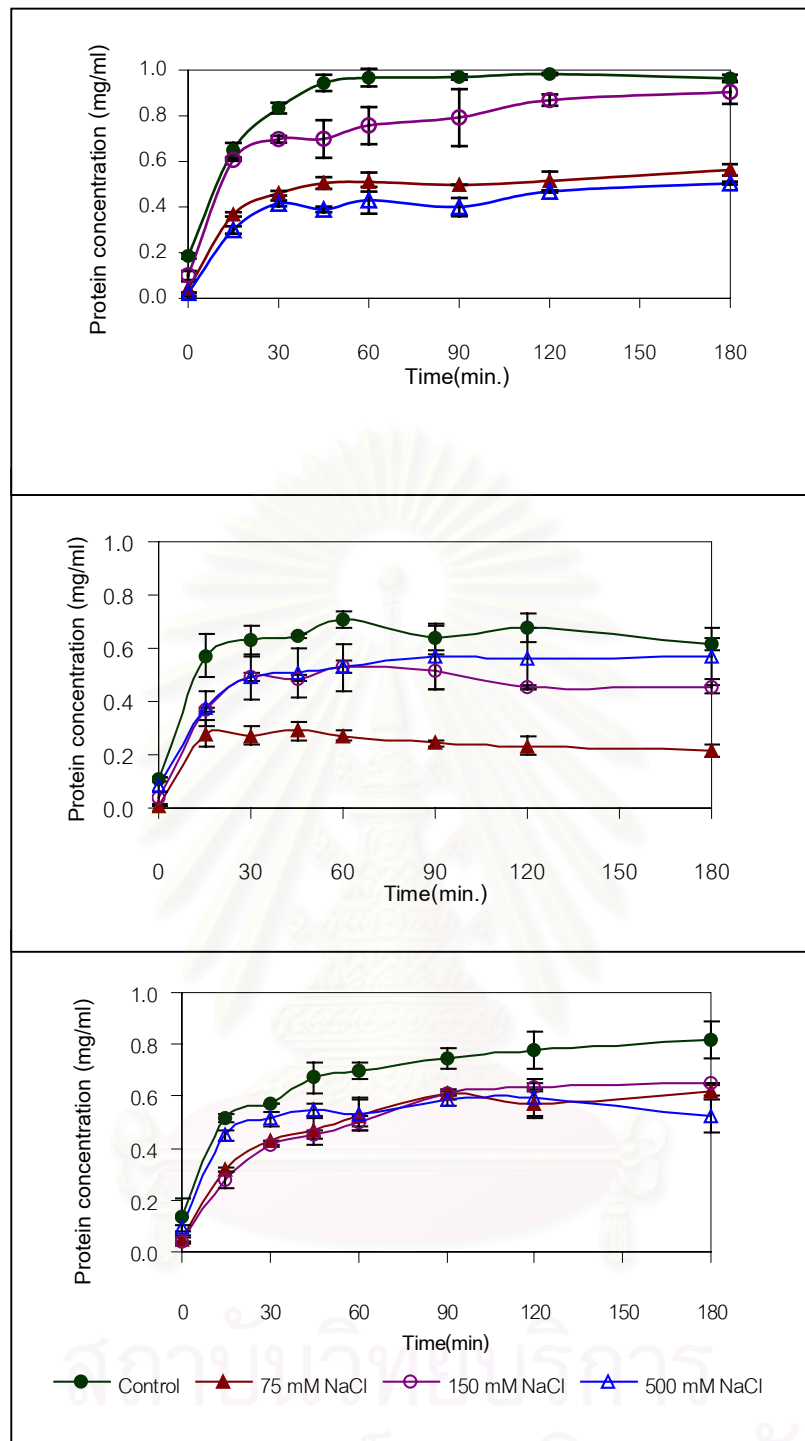
Figure 3.7 The release curves of BSA in 20 mM buffer and different NaCl

concentration : (a) Acetate buffer pH 5; (b) Phosphate buffer pH 7;

(c) Glycine buffer pH 9.1.

(The error bar is expressed in standard error of the mean, n=3)
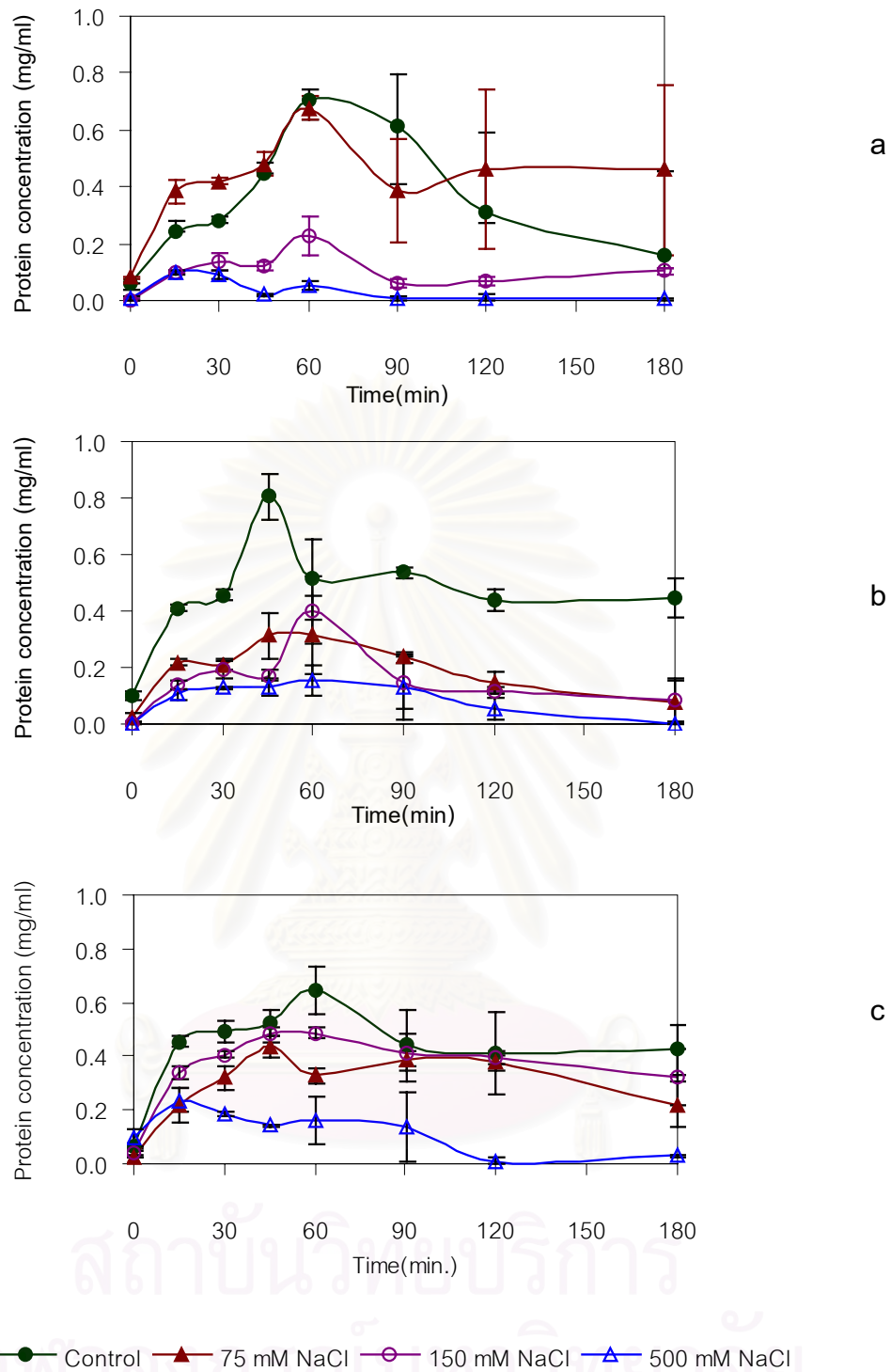
Figure 3.8 The release curves of protamine sulfate in 20 mM buffer and different

NaCl concentration: (a) Acetate buffer pH 5: (b) Phosphate buffer

pH 7: (c) Glycine buffer pH 9.1.

(The error bar is expressed in standard error of the mean, n=3)

CHAPTER IV

Discussion

This research consists of three parts: chitosan preparation, swelling of chitosan millispheres in pH buffers at various ionic strengths, and release of proteins from chitosan millispheres. For chitosan preparation, the chitosan prepared from shrimp shells was a high-molecular weight type, with degree of deacetylation comparable to commercially produced chitosan. For the swelling phenomena, Tanaka's swelling model was used to explain the swelling behavior of millispheres, with the osmotic pressure component of the model estimated from Donnan equilibrium. Finally, the release of test proteins from chitosan millispheres could be explained in terms of ionic interactions and the salting-out behavior of polyelectrolytes in aqueous environments.

4.1 Chitosan preparation

Commercially produced chitosan of relatively low molecular weights utilize high temperature (e.g. 100° C) briefly (1-3 h) in the deacetylation step. It was reported that alkali treatment should be carried out at low temperature if the degradation of the molecular chain is to be avoided [Lusena and Rose, 1953]. The preparation procedure used in this thesis was based on an established method [Chandrkrachang, 1999]. Such method was suitable for preparing high molecular weight chitosan because of its simplicity and milder condition. The procedure to extract chitin from shrimp shells

started with deproteinization by incubating chitin in 1 M NaOH, for 24 hours, at 35°C. The decalcification step was then accomplished with 1 M HCl, at 35°C for 12 hours. Chitin was deacetylated with 50%(w/v) NaOH at 50°C for 30 hours.

As reviewed earlier, the molecular weight of chitosan can be determined by various methods such as viscometry and gel permeation chromatography [Terbojevich and Cosani, 1997]. In this study, viscometry was chosen because of its simplicity. The molecular weight of chitosan was also confirmed by gel permeation chromatography.

Viscometric measurements showed that the prepared chitosan had an average molecular weight of 2.67 x 10^{5}. In comparison, commercial chitosans have molecular weights around below 100,000 (low molecular weight), 500,000-600,000 (medium molecular weight) and above 1,000,000 dalton (high molecular weight) [Berthold *et al,* 1996]. The medium molecular weight chitosan obtained in the present experiment is presumably the consequence of the low deacetylation temperature, which favors longer deacetylation chain chitosan, and the longer deacetylation time, which favors more shorter-chain chitosans. These results in agreement with those from the study by Wu and Bough (1978) that increasing the deacetylation time reduced the molecular sizes.

The degree of deacetylation in chitosan was determined by colloidal titration and infrared spectroscopy. Colloidal titration is a classical method for measuring the per cent deacetylation of chitosan. It is, however, more complicated and time-consuming than the IR method. Furthermore, colloidal titration requires an acidic solution of chitosan, thus precluding chitosan with low percent deacetylation. Infrared spectroscopy was a rapid method to determine the residual CONH groups in chitosan products. It could be used to confirm the value attained from the titration method. The lower the 1650 cm^{-1} peak, the lower amount of carbonyl groups and the higher the degree of deacetylation. Results indicate a positive correlation between deacetylation percentages from colloidal titration and IR techniques.

4.2 Chitosan millispheres

Chitosan millispheres can be prepared using different techniques: emulsion or multiple emulsion cross-linking, simple or complex coacervation, emulsion/solvent evaporation [Genta, 1997]. The choice of technique is dependent on the nature of the starting materials and on the desired compositional and morphological characteristics of the resultant millispheres. Preliminary results indicated that (1) The size of chitosan microspheres decreased by increasing the stirring rate. (2) Millispheres did not form from chitosan solutions that had been kept for over approximately 2 months. The inability to form particulate structures seemed to correlate with the decrease in viscosity of an aging chitosan solution. (3) The average size of the particles decreased with an

increasing concentration of emulsifier (Span[®]80) over the range of 1 to 3 percent (w/v). This can be explained by noting the decrease in interfacial tension between the aqueous droplets and the organic suspension medium caused by increasing amounts of the emulsifier. The term "millispheres" is used here merely to emphasize that the sizes of chitosan particles are in the millimeter range.

4.3 Swelling of chitosan millispheres

Generally, the swelling process of chitosan spheres (covalently cross-linked or otherwise) at pH < 6 involves protonation of amino/imine groups in the beads, and mechanical relaxation of coiled polymeric chains. Although protonation takes place in a very short time, the overall process is much more complex and mainly completed in two stages [Gupta and Kumar, 2000].

In the first stage, amino/imine groups are protonated at the sample surface, leading to breaking of the hydrogen bonding between amino/imine groups and other groups, which results in penetrants invading the polymer from the surface and forming a sharp boundary or moving front separating the unsolvated polymer region ahead of the front from the swollen bead phase behind it.

In the second stage, proton and counterions diffuse into the bead, inward amino/imine groups are protonated, and the hydrogen bonding further dissociates causing the complex structure of the beads to collapse with further inward moving of the front. The process repeats itself and drives the front to move forward until the beads are

completely solvated. Finally, mechanical relaxation of the strained macromolecular chains of the beads takes place, and redistribution of mobile ions occurs in the interior and the external regions of the beads [Gupta and Kumar, 2000].

The swelling behavior of the chitosan spheres was reported in a number of publications, books and reviews. For example, Shu and Zhu, 2002 studied ionically cross-linked chitosan beads in 0-3.6% (w/v) NaCl solution. Ionic strength and pH seriously influenced the properties of these beads, which related to the strength of electrostatic interaction between anions and chitosan. In studying the swelling behavior of chitosan beads in solutions of pH 2.0 and 7.4 at 37°C, Gupta and Kumar found that the degree of swelling was much higher in pH 2.0 than in pH 7.4. The findings were explained on the basis of the inherent hydrophobicity of the chitosan beads dominating at high pH values, which prevents swelling in alkaline media.

In a landmark study of intelligent gels by Tanaka (reviewed in Tanaka, 1981). The size of polymer gel was suggested to depend on 3 factors: osmotic pressure, rubber elasticity, and polymer-polymer affinity. The first factor is related to ion concentrations and is always zero or positive. The second factor originated from covalent linkages among the polymer strands and can be negative for expanding gels or positive for contracting gels. The third factor, always zero or negative in sign, represents van der Waals interaction and is effective only between polymer stands that are very close together or touching one another.

Mathematically, the positive osmotic pressure in our chitosan millispheres was the difference between the internal osmotic pressure of the chitosan gel and the external osmotic pressure of the buffer (with or without concentrations of salt). The internal osmotic pressure was exerted by the sum of all ions within the gel. This value can be calculated from Donnan equilibrium consideration.

Figure 4.1 shows a simulation of a simple Donnan equilibrium of chitosan gel. It is interesting to note that as the outside salt concentration increases, the osmotic pressure difference decreases.

Although Figure 4.1 is a simulation at pH 5.0, similar simulations were also performed at other pH's, i.e. 7.0 and 9.1. (Data not shown) All curves followed the same shapes as those in Figure 4.1 except the Molarity or osmotic pressure values were smaller due to decreased ionization of chitosan at higher pH's.
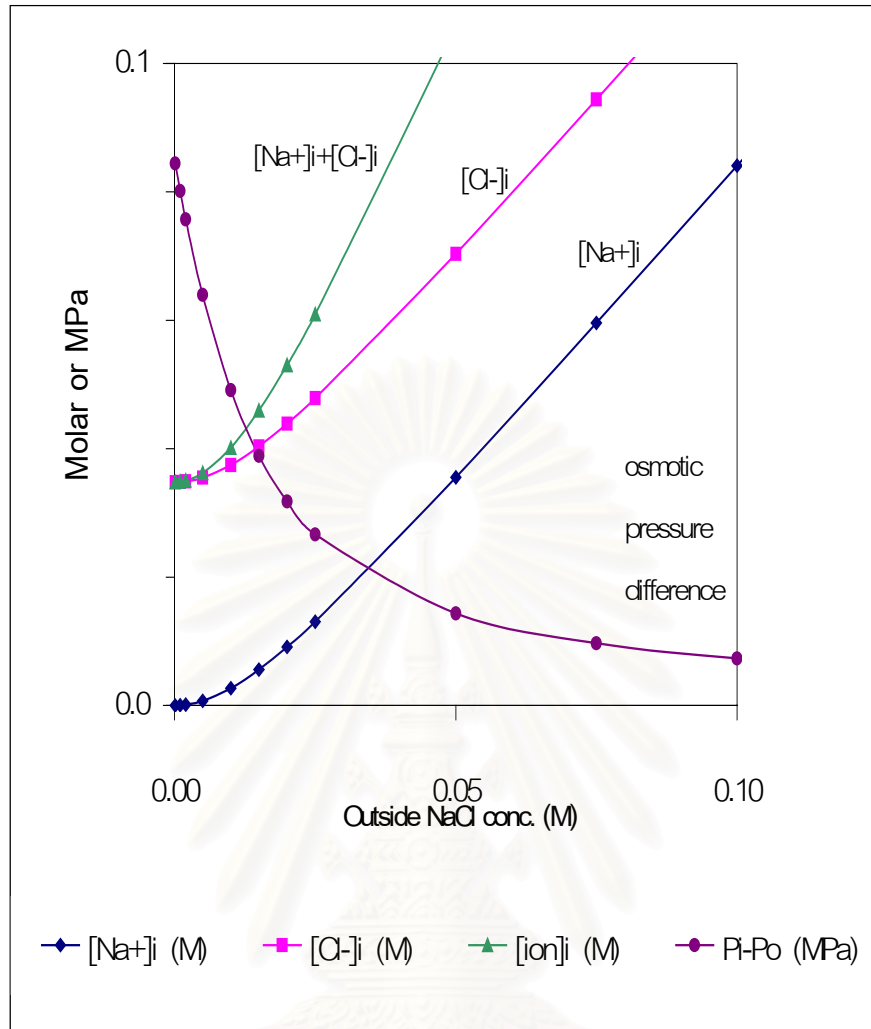
Figure 4.1 Simulation of Donnan equilibrium

The graph simulates a chitosan gel of 73% deacetylation (pK=6.5)

at pH 5.0 The upper curve ( ) is the osmolarity resulting from internal

concentrations of sodium and chloride ions. This value is directly

proportional to the osmotic pressure inside the gel. The inside osmotic

pressure minus the outside osmotic pressure equals the osmotic pressure

difference (bottom curve, ●)

In Figures 3.5 the percentages of swelling did not change appreciably after 360 minutes. The initial swelling rates in these Figures under most conditions were too fast to analyze. Therefore, only values taken at 360 minutes were extracted and plotted in Figure 4.2. The concentrations of the pH buffer in use, as well as those of NaCl, were taken into account when computing the ionic strength values.

In Figure 4.2a, at ionic strength 0.02, the swelling of the chitosan millispheres follows the expected direction, i.e. decreased swelling at increased pH. At pH 5, the swelling of the chitosan millispheres seems to follow the osmotic pressure according to Donnan equilibrium (curve ● in Figure 4.2b), i.e. as the ionic strength increases, the swelling decreases (although in an S shape curve). This corresponds to the left 4 points aligned vertically at pH 5 in Figure 4.2a. Nevertheless, the high salt graphs in Figure 4.2a and the high pH graph in Figure 4.2b cannot be explained by osmotic pressure alone.
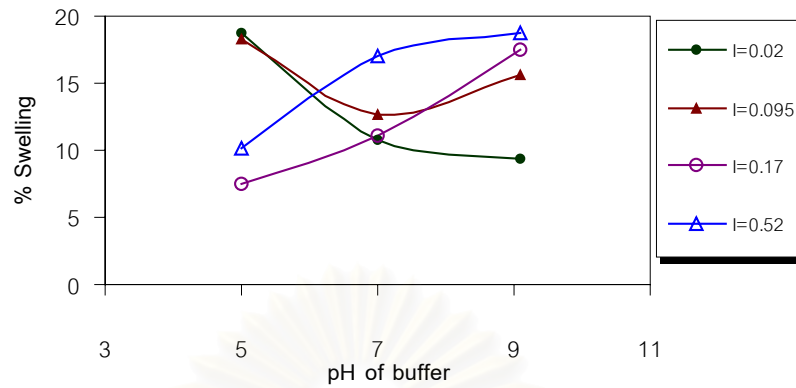
a
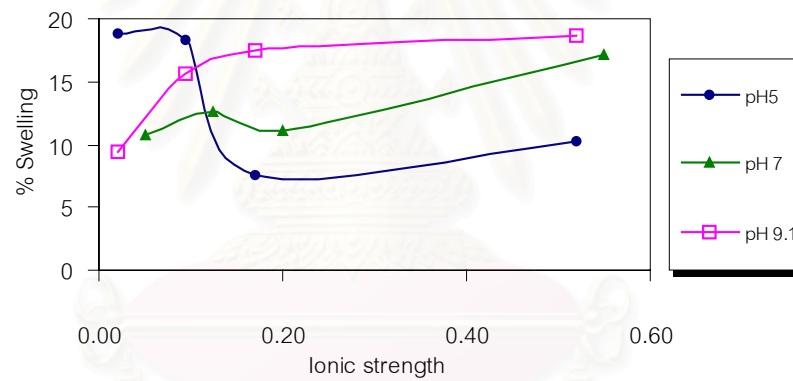
b

Figure 4.2 The swelling ratio of chitosan millispheres after incubation in different ionic strength solution for 6 hours

(a) Swelling is plotted as a function of pH at various ionic strengths

(b) Swelling is plotted as a function of ionic strength at various pH's.

In the I=0.52 curve of Figure 4.2a, as pH is increased, the ionization of chitosan (pK~6.5) is decreased, leading (according to Donnan equilibrium) to decreased osmotic pressure. The swelling of the millisphere, however, increased under this condition. In the pH 9.1 curve of Figure 4.2b, as the ionic strength is increased, the osmotic pressure is decreased according to Donnan equilibrium (similar to the bottom curve of Figure 4.1). The swelling of the millisphere, however, increased under this condition.

These seemingly anomalous results can be explained by considering Tanaka's model of polymer gels that has been mentioned earlier in Chapter I. According to this model, the size of the gel is determined by three factors: osmotic pressure, rubber elasticity, and polymer-polymer affinity. The anomaly can be explained if we assume that under this condition (high pH, high salt), the rubber elasticity of the chitosan gel is appreciably weakened to the point that even a decrease in osmotic pressure could produce greater swelling.

Such weakened rubber elasticity of chitosan gel cannot be explained by considering only chitosan in high pH-high salt (pH 9.1, 0.5 M NaCl) since such condition should diminish the positive charges on the chitosan molecule (salting out the polymer) possibly resulting in a stronger structure instead of a weaker one.

A more plausible explanation of the weakened rubber elasticity may have to do with the presence of Span[®]80 during the formation of the spheres. Span[®] works as an emulsifier by virtue of its hydrophobic tail on one side of the molecule and a markedly

negative polar group on the other side. This negative polar group could conceivably

serve in place of anionic bridges between the positive amino groups to strengthen the

gel structure of chitosan. A combination of high pH and high ionic strength diminishes

these "bridges" and weakens the overall structure, leading to more swelling under
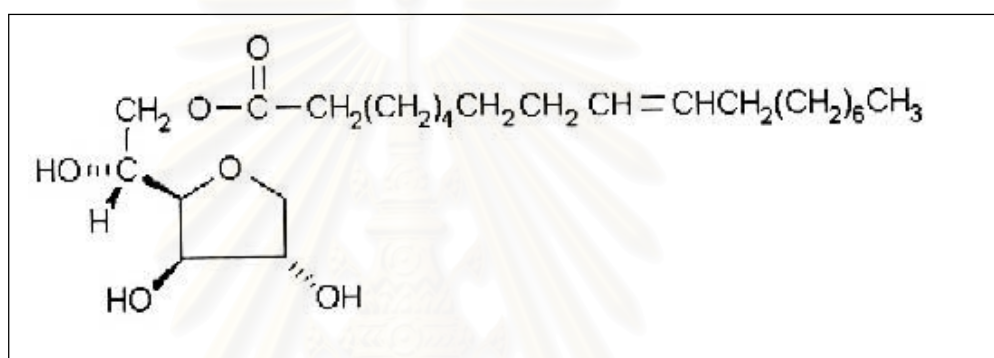
relatively mild osmotic pressure.

Figure 4.3 Structure of Span $^\circledR$80

Source: The merck index 11th edition.

4.4 Protein release

The protein concentrations released into the media at 180 minutes for α-

chymotrypsin, BSA and protamine from Figures 3.6, 3.7 and 3.8 have been plotted in

Figures 4.4, 4.5 and 4.6 respectively. The nature of the three test proteins are as follows:

• Chymotrypsin is a roughly globular 21.6 kDa protein with pI = 9.1 (Laskowski, 1955).

  It carries 22 amino groups and 11 carboxyl groups.

- BSA is a globular 68 kDa protein with pI = 4.8 [Florkin and Stotz, 1963]. It carries 83 amino groups and 99 carboxyl groups.

  In addition, BSA has non-polar lipid-binding domains.

- Protamine is a basic protein with pI = 12.1 [Florkin and Stotz, 1963]. Its molecular weight is around 4 to 4.5 kDa. It carries 22 amino groups and one carboxyl group. Due to the small number of residues and a concentration of amino groups, its conformations at the three-test pH's are assumed to be random coils.

Starting from chymotrypsin in Figures 3.6 and 4.4 as the ionic strength was increased, the concentrations of released protein did not change appreciably. At pH 9.1, which is the pI of chymotrypsin, the protein carries no net charge. At this pH, chitosan is protonated only 0.25 per cent of the maximum value, in other words, the charge interaction was small without any added salt. This condition (pH 9.1, no salt) gives a reference interaction level of approximately 0.6 mg/ml protein.

In order to explain the results further, it is postulated here that two mechanisms are responsible for binding proteins to chitosan in these millispheres (1) ionic interaction between the protein and chitosan and (2) bridges formed between the positive charges on the protein and chitosan through the negative polarity of span®80.

In Figure 3.6a and b, the no-salt condition gave somewhat stronger attachment forces (0.6 and 0.6 to 0.8 mg/ml as around 0.5 mg/ml in Figure 3.6c). The fact that there was not much chymotrypsin release as pH was varied from 5 to 7 to 9.1 may suggest that different charge clusters on the protein may interact independently. As the NaCl

concentrations were raised, the interaction between polar bridge and positive charges on the protein and chitosan were weakened, resulting in more release of about 0.9 to 0.8 mg/ml at pH 5.0 and pH 7.0 respectively. Comparison at pH 5 between the chymotrypsin-containing millispheres in Figure 3.6 and 4.4 and protamine-containing millisphere in Figure 3.8 and 4.6 indicates that the average released protamine concentrations were about the same value as that obtained with chymotrypsin but peaked and then decreased with time. This can be explained by the higher number of net charges (about 0.5/residue) in protamine at neutral pH compared to that of chymotrypsin and the ability of protamine to interact with counterions. This may have made protamine a more easily precipitated at least at pH 5 (given time), than chymotrypsin. Comparison between release curves of BSA (especially at pH 9.1 in Figures 3.7 and 4.5) with those of chymotrypsin (Figures 3.6 and 4.4) shows that the release protein concentrations is curve of BSA were slightly higher than the corresponding release concentrations for chymotrypsin. This can be explained by the partial competition between the negative polarity of the span[®] head groups and BSA for chitosan, as opposed to the interaction between charged domains on the surface of chymotrypsin and chitosan.

At pH 9.1 without any additional salt, chitosan carries only 0.25 per cent of its maximum value while BSA is fully charged. Although Span[®] may be present, its contribution was likely to be negligible compared to the charges on BSA. At pH 5, the situation is opposite. BSA is slightly charged while chitosan carries almost fully positive

charges. In this condition, the effect of Span[®] was not negligible due to the small

charges on BSA. The competition from Span[®] decreases the attraction between BSA

and chitosan. As a result, the pH 5, no-salt curve for BSA-chitosan millispheres reaches

the plateau at round 1.0 mg/ml protein, which is closer to the 1.5 mg/ml reference level

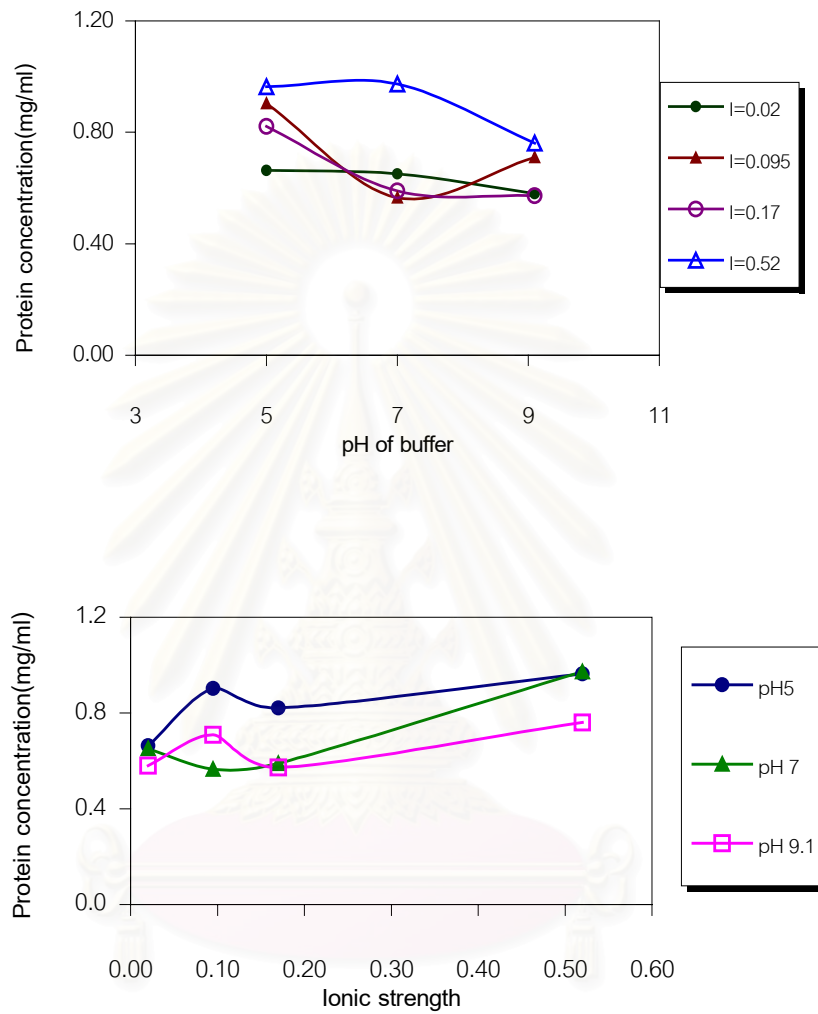in case of chymotrypsin-chitosan at pH 9.1.

Figure 4.4 The release ratio of α-chymotrypsin from chitosan millispheres after incubation in different pH and ionic strength solution for 3 hours.
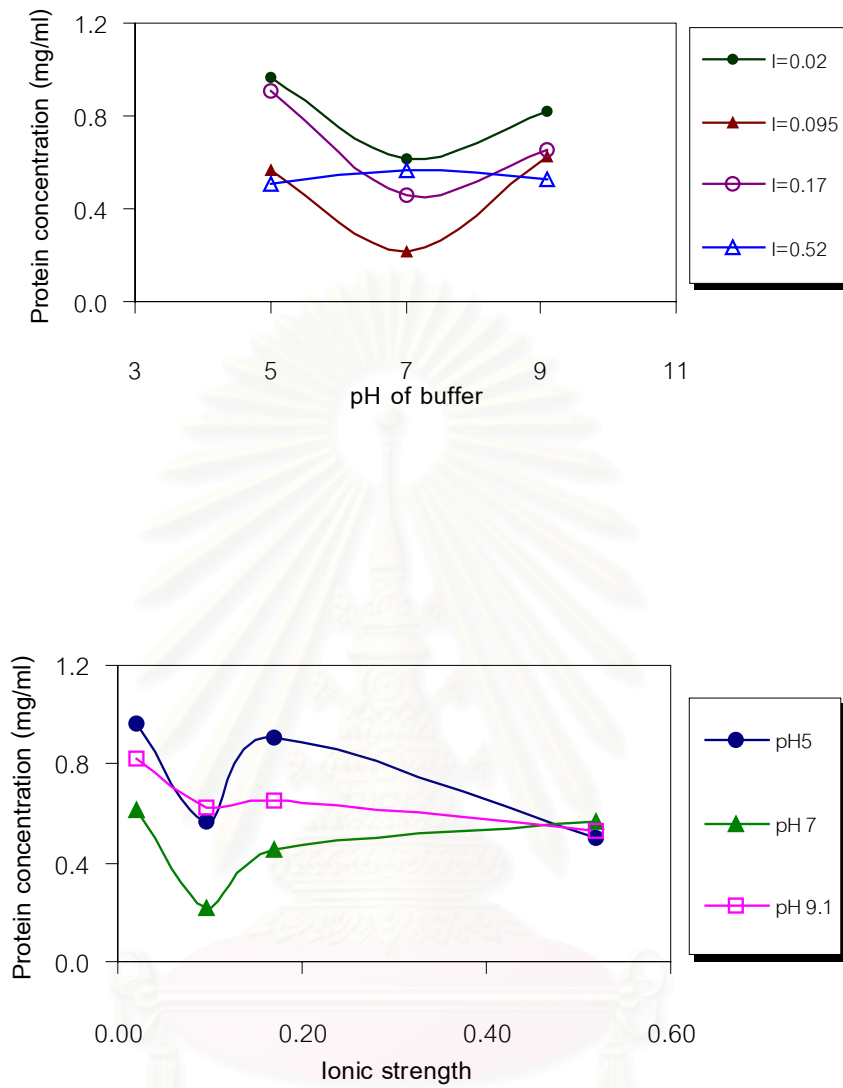
Figure 4.5 The release ratio of BSA from chitosan millispheres after incubation

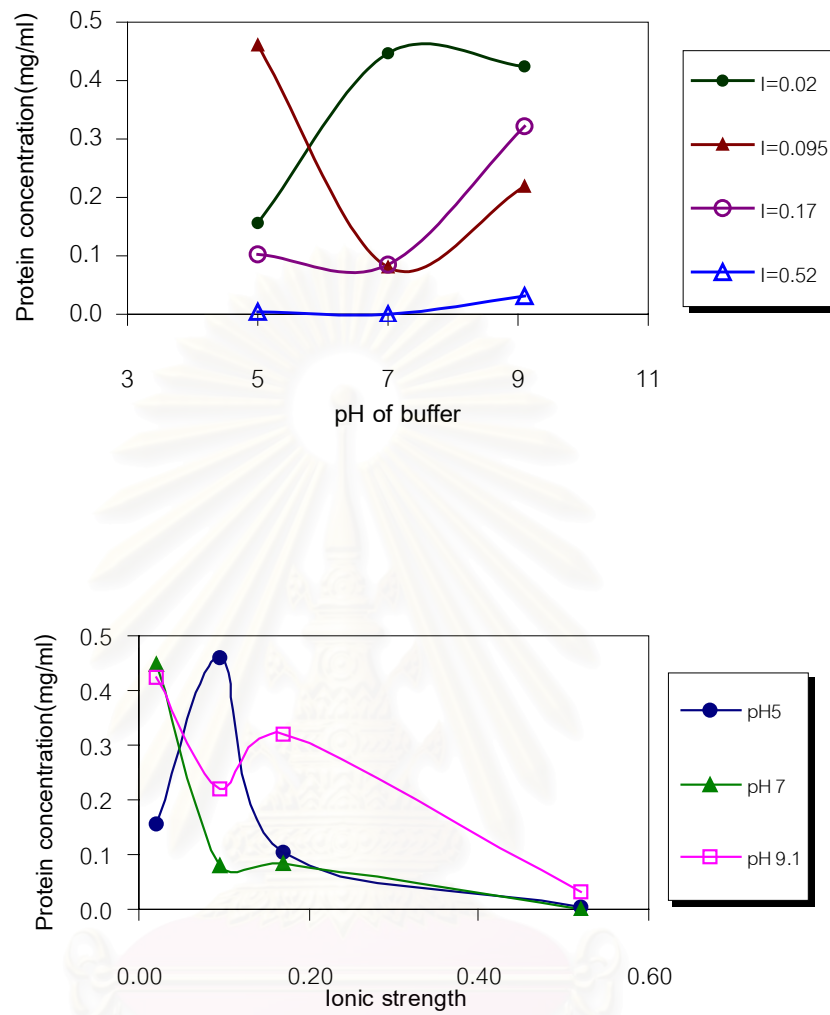in different pH and ionic strength solution for 3 hours

Figure 4.6 The release ratio of protamine from chitosan millispheres after

incubation in different pH and ionic strength solution for 3 hours

CHAPTER V

CONCLUSION

1. Chitosan has been prepared from shrimp shells, the molecular weight and per cent deacetylation have been determined to be 2.67 x 10^5 and 69 to 76 %, respectively. The ash and moisture were 0.10% and 13.42%, respectively. The yield was 42.28%.

2. Chitosan millispheres have been prepared by mixing and stirring chitosan-triethanolamine-Span$^®$ in paraffin wax. The size of the millisphere was approximately 1.5 mm in diameter. The spheres were clear and not round with rough surface under scanning electron microscopy.

3. The swelling properties of chitosan millispheres under different pH's and ionic strengths have been explained by Tanaka's theory of gel polymers. The osmotic pressure was considered according to Donnan equilibrium while rubber elasticity was considered a combination of polymer "salting out" and the polar bridging of positive charges by Span$^®$

4. The releases of three test proteins have been investigated. The results have been explained using similar models to the ones for the swelling property.

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APPENDICES

APPENDIX A

Determination of Degree of Deacetylation of Chitosan

<u>Method of calculation</u>

Sample

Weight of sample (Chitosan hydrochloride)	0.9291	g
Dissolved in water to	100	ml

Titration

Chitosan hydrochloride solution used	50	ml
Volume of standard NaOH (0.098 M) used	18.3	ml

Calculation

Chitosan hydrochloride 1 mol (197.5 g) $\equiv$ NaOH 1 mol.

The amount of monomer having NH_2 group in sample 0.9291 g

$$= 3.58 \times 10^{-3} \quad mol$$

$$= 0.7071 \quad g$$

The amount of monomer having $NHCOCH_3$ group in sample 0.9291 g

$$= 0.9291 - 0.7071 \quad g$$

$$= 0.222 \ g$$

(monomer having $-NHCOCH_3$ 1 mol = 203.19 g) $= 1.09 \times 10^{-3} \quad mol$

The total amount of monomer in sample 0.9291 g $= 3.58 \times 10^{-3} + 1.09 \times 10^{-3}$ mol

$$= 4.76 \times 10^{-3} \quad mol$$

The degree of deacetylation $= (3.58 \times 10^{-3} / 4.76 \times 10^{-3}) \times 100\%$

$$= 76.87 \ \%$$

APPENDIX B

Preparation for buffer solution

- 0.02 M Sodium acetate buffer pH 5.0

Sodium acetate	1.64	g
Glacial acetic acid	1.1	ml

Adjust to pH 5.0 by 1 M acetic acid and adjust volume to 1 litre with distilled water

- 0.02 M Potassium phosphate buffer pH 7.0

KH_2PO_4	2.72	g
K_2HPO_4	3.48	g

Adjust to pH 7.0 by 1 M HCl and adjust volume to 1 litre with distilled water

- 0.02 M Glycine-NaOH buffer pH 9.1

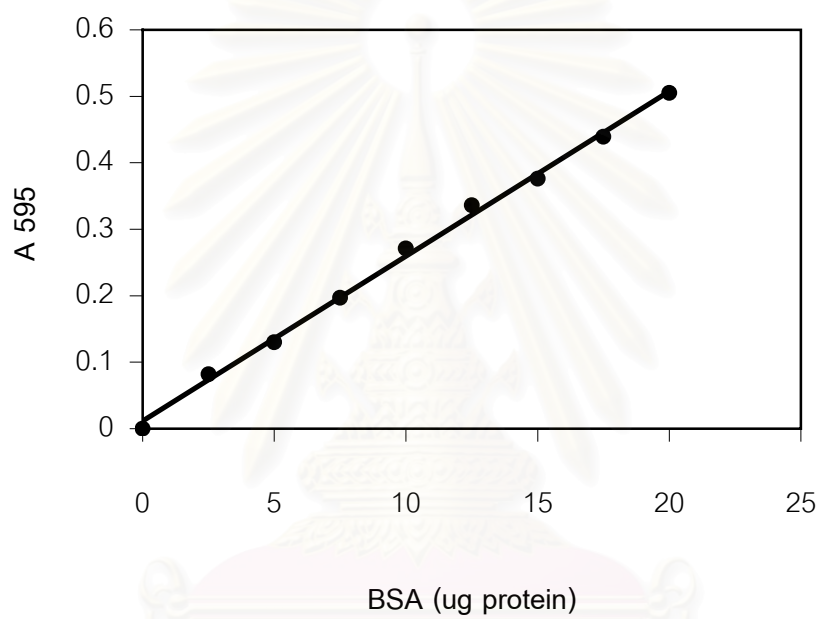|		
Glycine	1.50	g
NaOH	0.80	g

Adjust to pH 9.1 by 1 M NaOH and adjust volume to 1 litre with distilled water

APPENDIX C

Standard curve for protein determination by Bradford's method

BIOGRAPHY

Miss Kessuda Luengteerapap was born on June 30, 1975. She finished High school at Mahapruttaram Girl's School, Bangkok and enrolled in the Faculty of Science, Silpakorn University. She graduated with the B.Sc. In Biology in 1997 and continued studying for M.Sc. in Biochemistry Program, Faculty of Science, Chulalongkorn University in 1999.