Effect of genipin cross-linking on properties of chitosan based bio-composite film

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ผลของการเชื่อมขวางด้วยเจนิพินต่อสมบัติของฟิล์มไบโอคอมโพสิตฐานไคโตซาน

น.ส.ภัทรารีย์ อินทมาตร์

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ฟิล์มไคโตซาน (CS) ที่เติมแต่งด้วยแอสตร้าแซนทินมีศักยภาพในการใช้เป็นบรรจุภัณฑ์เชิงแอกทีฟสำหรับอาหาร อย่างไรก็ตามฟิล์มดังกล่าวยังมีข้อจำกัดในการนำไปใช้ในอุตสาหกรรมอาหาร เนื่องจากฟิล์มมีอัตราการซึมผ่านของไอน้ำสูงและมีความแข็งแรงต่ำ งานวิจัยนี้จึงปรับปรุงสมบัติของฟิล์มโดยใช้เทคนิคการเชื่อมขวางโครงสร้างฟิล์มด้วยเจนิพินและการบ่มด้วยความร้อน ขั้นแรกเป็นการศึกษาผลของความเข้มข้นของเจนิพินซึ่งเป็นสารเชื่อมขวางที่ได้จากธรรมชาติ ต่อสมบัติของไคโตซานฟิล์มที่เติมแต่งด้วยแอสตร้าแซนทิน (CA หรือ CSA) โดยเตรียมไคโตซานในกรดแลคติก 2% (น้ำหนักต่อน้ำหนัก) เติมแอสตร้าแซนทินที่ความเข้มข้น 0 และ 1% (น้ำหนักต่อน้ำหนักของไคโตซาน) และเติมเจนิพินที่ความเข้มข้น 0 0.5 1.0 และ 1.5% (น้ำหนักต่อน้ำหนักไคโตซาน) วัดสมบัติของฟิล์ม ได้แก่ ปริมาณและโครงสร้างการเชื่อมขวาง อัตราการซึมผ่านไอน้ำ คุณสมบัติเชิงกล อัตราการไหลผ่านของออกซิเจน สี ความต้านทานความร้อน ความเป็นผลึก และลักษณะผิวหน้าของฟิล์ม ผลการทดลองแสดงให้เห็นว่าความเข้มข้นของเจนิพินที่เพิ่มขึ้นมีผลทำให้ปริมาณการเชื่อมขวางในโครงสร้างของฟิล์มเพิ่มขึ้น จากผลการวิเคราะห์ด้วย Fourier Transform Infrared Spectroscopy (FTIR) พบพันธะเชื่อมขวางระหว่างไคโตซานและเจนิพิน และพบว่าการเพิ่มความเข้มข้นของเจนิพินทำให้อัตราการซึมผ่านของไอน้ำ ความเป็นผลึกของฟิล์ม และความยืดหยุ่นของฟิล์มลดลงอย่างมีนัยสำคัญ (p<0.05) ในขณะที่ความต้านทานแรงดึง มอดูลัสของสภาพยืดหยุ่น การต้านทานความร้อน และอัตราการซึมผ่านของออกซิเจน เพิ่มขึ้น เมื่อตรวจสอบลักษณะพื้นผิวของฟิล์มด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด พบว่าฟิล์มเชื่อมขวางมีพื้นผิวหน้าที่ขรุขระกว่าฟิล์มที่ไม่ได้เชื่อมขวาง จากผลการทดลองสรุปได้ว่าฟิล์มไคโตซานเชื่อมขวางด้วยเจนิพิน 1.5% (CS1.5G) และไคโตซานที่เติมแต่งด้วยแอสตร้าแซนทินและเชื่อมขวางด้วยเจนิพิน 1% (CSA-1G หรือ CAG) มีอัตราการซึมผ่านไอน้ำ และความแข็งแรงของฟิล์ม ดีที่สุด จึงเลือกใช้ฟิล์ม CS1.5G และ CAG ในการศึกษาต่อไป

ขั้นตอนที่ 2 เป็นการศึกษาผลของอุณหภูมิการบ่มต่อโครงสร้างการเชื่อมขวางทางเคมีและสมบัติของฟิล์ม CS, CS1.5G, CSA และ CAG โดยแปรอุณหภูมิในการบ่มเป็น 25 องศาเซลเซียส (CS-25, CS1.5G-25, CSA-25, CAG-25) 80 องศาเซลเซียส (CS-80, CS1.5G-80, CSA-80, CAG-80) และ 105 องศาเซลเซียส (CS-105, CS1.5G-105, CSA-105, CAG-105) วัดอัตราการซึมผ่านไอน้ำ มุมสัมผัสของหยดน้ำ โครงสร้างทางเคมี ความต้านทานแรงดึง ความยืดหยุ่น มอดูลัสของสภาพยืดหยุ่น และความต้านทานความร้อน ผลการทดลองพบว่าฟิล์ม CS1.5G และ CAG ที่บ่มที่ 105 องศาเซลเซียส มีอัตราการซึมผ่านไอน้ำต่ำที่สุดอย่างมีนัยสำคัญ (p<0.05) ซึ่งต่ำกว่าฟิล์ม CS และ CSA 54-55 เท่า ในขณะที่องศาของมุมสัมผัสหยดน้ำเพิ่มขึ้น การบ่มทำให้ฟิล์ม CS1.5G-105 และ CAG-105 เพิ่มพันธะเคมีระหว่าง C-C double bond ของเจนิพินที่เชื่อมขวางกับสายโซ่ไคโตซาน นอกจากนี้การบ่มทำให้ฟิล์ม CS1.5G-105 และ CAG-105 มีค่าความต้านทานแรงดึงและมอดูลัสของสภาพยืดหยุ่นสูงขึ้นถึง 60-55 เท่า และ 42-50 เท่า เมื่อเทียบกับฟิล์มCS1.5G-25 และ CAG-25 ตามลำดับ และมีความต้านทานความร้อนของฟิล์มเพิ่มขึ้นเช่นกัน นอกจากนี้ยังพบว่าฟิล์มที่ได้สามารถลดการส่องผ่านของแสงได้ดีขึ้น ผลการทดลองชี้ให้เห็นว่าอุณหภูมิการบ่มด้วยความร้อนสามารถปรับปรุงคุณสมบัติของฟิล์มเชื่อมขวางฐานไคโตซานได้ และเนื่องจากเจนิพินเป็นสารเชื่อมขวางที่ได้จากธรรมชาติและไม่เป็นพิษ ฟิล์มที่พัฒนาได้จึงสามารถใช้กับอาหารได้อย่างปลอดภัย

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: Active packaging, Astaxanthin, chitosan, genipin, heat curing

Patthrare Inthamat : Effect of genipin cross-linking on properties of chitosan based bio-composite film. Advisor: Prof. UBONRATANA SIRIPATRAWAN, Ph.D. Co-advisor: Prof. Youn Suk Lee, Ph.D.

Active packaging from chitosan film incorporated with astaxanthin is limited commercial application in the food industry due to its high moisture permeability and poor tensile strength. To overcome these problems, crosslinking of the film with genipin and heat curing to improve film properties were proposed. Firstly, the effects of genipin concentration on the physical, moisture barrier and mechanical properties of chitosan films incorporated with astaxanthin were investigated. Chitosan film was prepared by mixing chitosan in 2% (w/w) lactic acid. The chitosan was incorporated with astaxanthin at 0 and 1% (w/w of chitosan) (marked as CS and CA or CSA) and crosslinked with genipin at various concentrations including 0.5, 1.0 and 1.5% (w/w of chitosan) (marked as CS0.5G, CS1G, CS1.5G, CSA-0.5G, CSA-1G or CAG and CSA-1.5G). The chemical crosslinking structure, crosslinking degree, water vapor permeability, mechanical properties, oxygen permeability, color, thermal stability, crystallinity and surface characterization of the films were investigated. The results showed that an increase in genipin concentration increased crosslinking degree, as confirmed by Fourier transform infrared spectroscopy showing that there were interactions between chitosan and genipin. Increasing the concentration of genipin significantly decreased water vapor permeability (WVP), crystallinity and elongation at break (EAB) while tensile strength (TS), Young's modulus (YM), thermal stability and oxygen permeability increased. The surface characteristic of the films was observed by scanning electron microscopy (SEM) and it was found that crosslinked film had rougher surface than non-crosslinked film. Since chitosan crosslinked with 1.5% genipin film (CS1.5G) and chitosan-astaxanthin film crosslinked with 1% genipin (CAG) showed optimal properties (WVP, TS and YM), CS-1.5G and CSA-1G films were used further to study the effect of heat curing temperature.

The second study aims to evaluate the influence of curing temperature on chemical-crosslinking and properties of the films. The CS, CS-1.5G, CSA and CAG films were cured at 25 (marked as CS-25, CS1.5G-25, CSA-25 and CAG-25, respectively), 80 (marked as CS-80, CS1.5G-80, CSA-80 and CAG-80, respectively) and 105°C (marked as CS-105, CS1.5G-105, CSA-105 and CAG-105, respectively) for 30 min. The films were analyzed for WVP, contact angle, chemical structure, TS, EAB, YM and thermal stability. The results showed that CS1.5G and CAG films cured at 105°C had the lowest (p<0.05) WVP value which by decreased by 54-55% while the contact angle increased, when compared to CS-25 and CSA-25 film. Heat curing increased the chemical interaction between C-C double bond of genipin already linked with chitosan chain. These results led to an improvement of TS and YM by 55-60% and 42-50%, respectively for CS1.5G-105 and CAG-105 films. Moreover, thermal stability of CS1.5G-105 and CAG-105 films were enhanced and the resulting films can lower light transmission through film. The results suggested heat curing can improve the properties of genipin-crosslinked chitosan-based film which has potential to be used for active food packaging applications. Moreover, since genipin is a nontoxic natural crosslinker, the developed film is considered safe to use with food.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

CS or CS-25	Pure chitosan film or pure chitosan cured at 25°C
CS-80	Pure chitosan with curing at 80°C
CS-105	Pure chitosan with curing at 105°C
CS0.5G	Chitosan crosslinked with 0.5% genipin
CS1G	Chitosan crosslinked with 1% genipin
CS1.5G or	Chitosan crosslinked with 1.5% genipin or
CS1.5G-25	Chitosan crosslinked with 1.5% genipin and cured at 25°C
CS1.5G-80	Chitosan crosslinked with 1.5% genipin and cured at 80°C
CS1.5G-105	Chitosan crosslinked with 1.5% genipin and cured at 105°C
CA or CSA or	Incorporated chitosan with astaxanthin
CSA-25	Incorporated chitosan with astaxanthin and cured at 25°C
CSA-80	Chitosan incorporated with astaxanthin and cured at 80°C
CSA-105	Chitosan incorporated with astaxanthin and cured at 105°C
CA-0.5G	Incorporated chitosan with astaxanthin and crosslinked with 0.5% genipin
CA-1G or CAG or CAG-25	Incorporated chitosan with astaxanthin and crosslinked with 1% genipin or Incorporated chitosan with astaxanthin, crosslinked with and cured at 25°C
CAG-80	Chitosan incorporated with astaxanthin, crosslinked with 1% genipin and cured at 80°C
CAG-105	Chitosan incorporated with astaxanthin, crosslinked with 1% genipin and cured at 105°C
CA-1.5G	Incorporated chitosan with astaxanthin and crosslinked with 1.5% genipin
L*	Lightness
a*	Redness
b*	Yellowness
TS	Tensile strength
EB, EAB	Elongation at break

YM	Young’s modulus
WVP	Water vapor permeability
WVTR	Water vapor transmission rate
OTR	Oxygen transmission rate
OP	Oxygen permeability
FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscopy
DSC	Differential scanning calorimetry
DPPH	1,1-Diphenyl-2-picrylhydrazyl
TEAC	Trolox equivalent antioxidant capacity
ABTS	2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
XRD	X-Ray diffraction
TGA	Thermogravimetric analyzer

CHAPTER I
INTRODUCTION

1.1 Background, motivation and linking of the study

Chitosan, natural biopolyaminosaccharide (a linear polysaccharide consisting of β-(1-4)-2-acetamido-D-glucose and β-(1-4)-2-amino-D-glucose units), is produced from natural-skeletal materials of crustaceans as shown in Figure 1.1. It is widely used in food packaging due to its non-toxicity, biodegradability and biocompatibility. Chitosan has the potential to be employed for packaging, particularly as edible films, due to its transparency film-forming capacity and good mechanical properties (Rachtanapun et al., 2021; Youssef, Abou-Yousef, El-Sayed, & Kamel, 2015). However, chitosan film has low moisture barrier and mechanical strength, limiting their use for some food products. To overcome these drawbacks, various modification methods has been used such as copolymer blending, grafting (Nunes et al., 2013) and crosslinking (Klein et al., 2016).

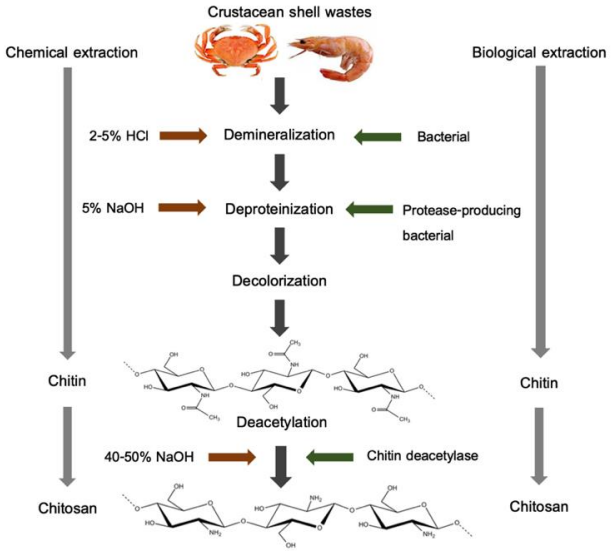

Figure 1.1 Fabrication of chitosan

Crosslinking by using synthetic chemical crosslinkers such as glutaraldehyde, sodium tripolyphosphate and formaldehyde is an effective technique to improve moisture barrier and mechanical strength of the films. However, these agents have been reported to cause cytotoxic (Klein et al., 2016). Hence, natural crosslinking

agents which are biocompatibility and non-cytotoxicity for their possible applications in food packaging field are needed. Genipin isolated from the fruits of *Gardenia jasminoides Ellis*, is accepted as an alternative crosslinking agent because of nontoxicity and thus can be applied as an effective natural crosslinking agent for biopolymers containing amino groups, especially chitosan. The crosslinking by covalent bond between chitosan and genipin related with two main processes, including (i) the formation of a heterocyclic compound caused from the nucleophilic attack of a primary amino group on olefin carbon atom (C3) of genipin-ring structure (Butler, Ng, & Pudney, 2003; Wu et al., 2018). (ii) The ester group of genipin is attacked by amino group to form a secondary amide bond, following the conjugation between genipin molecules already linked with chitosan chains with C-C double bond under oxygen induced condition, as shown in Figure 1.2 (Dimida et al., 2015; Inthamat, Lee, Boonsiriwit, & Siripatrawan, 2021). Based on these interactions, several studies reported that the crosslinked chitosan with genipin gave rise to improvement in functional properties of chitosan film (Hisham et al., 2016; Kildeeva et al., 2020).

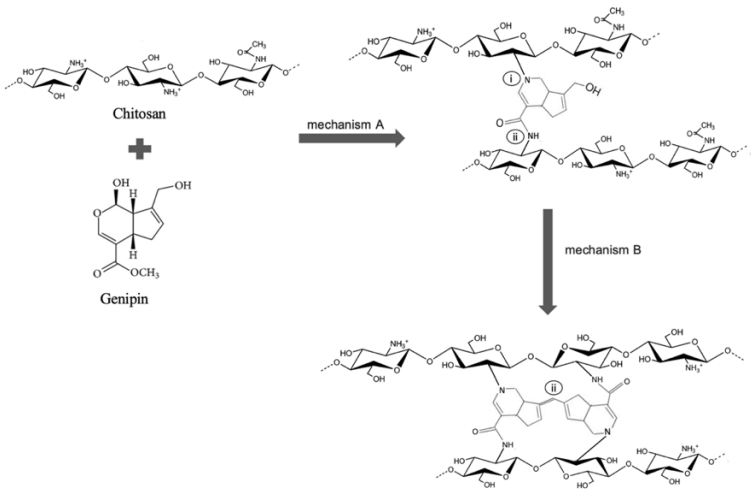

Figure 1.2 Chemical-crosslinking structure between chitosan and genipin

The curing processes, conducted through UV (Yun, Lee, Kim, & Yoon, 2017), electromagnetic wave (Biswas, Arief, Panja, & Bose, 2017), pressures (Gan, Sam, Abdullah, Omar, & Tan, 2020), or heat is widely used to improve more film properties. Heat curing is a best choice because it is green anergy, safe and inexpensive and mostly, it is popularly used together with crosslinking technique.

Several research indicated that the use of heat curing method is not only applied to improve film properties, especially, moisture barrier and mechanical properties, but it also catalyzes the crosslinking reaction including enhances a more stability of the crosslinking structure (Benbettaïeb, Gay, Karbowiak, & Debeaufort, 2016; Rubentheren et al., 2016; Zawadzki & Kaczmarek, 2010). According to the literatures, Rivero, García, and Pinotti (2011) reported the use of heat treatment on modified structure and improved moisture barrier, mechanical and stability properties of chitosan-based film. Moreover, a successful improvement of moisture barrier property in crosslinked chitosan film by genipin and cured with heat treatment above the glass transition temperature (T_g) was also reported by Dimida et al. (2015).

Therefore, an improving moisture barrier and mechanical properties of chitosan film by crosslinking with genipin as natural crosslinker and heat curing were studied. The effect of the genipin concentration and thermal curing on the chemical structure and properties of chitosan film is presented in Chapter 2, which is published in *Agriculture and Natural Resources*.

In case of food packaging, in addition to the developed chitosan film by crosslinking and heat-curing technique, another main objective of food packaging is the potential development in term of active packaging to focus on maintaining the quality and extending shelf life of products. Currently, active packaging is mostly demanded for several packaging industries, and it continues being an area of interest for researchers in the field of food packaging. Therefore, active food packaging made from chitosan incorporated with bioactive compounds, such as antioxidants is developed.

Astaxanthin (3,3′-dihydroxy-β, β′-carotene-4, 4′-dione) is a natural antioxidant with higher singlet oxygen quenching activity than β-carotene, vitamin E and α-tocopherol (Xu, Wei, Jia, & Song, 2020). Astaxanthin is a kind of carotenoid pigment found in various marine plants and animals such as algae, rainbow trout, shrimp and lobster as well as crustacean aquaculture, and some types of microorganisms (Liu, Zhang, McClements, Wang, & Xu, 2019). Astaxanthin molecule presents a liner polyene chain of 11 conjugated double bonds, determining the pink or red color of astaxanthin, linked by two oxygenated ionone ring systems as

shown in Figure 1.3. The interest in astaxanthin is supported by Stachowiak and Szulc (2021) who noted that the presence of the polar and non-polar phase in the structure of astaxanthin can fit both hydrophilic (two β rings) and hydrophobic phase (a liner polyene chain). Consequently, astaxanthin displays 10- and 100-times greater anti-oxidative activity in lipid systems than that of β-carotene and vitamin E, respectively when compared with other carotenoids (Stachowiak & Szulc, 2021).

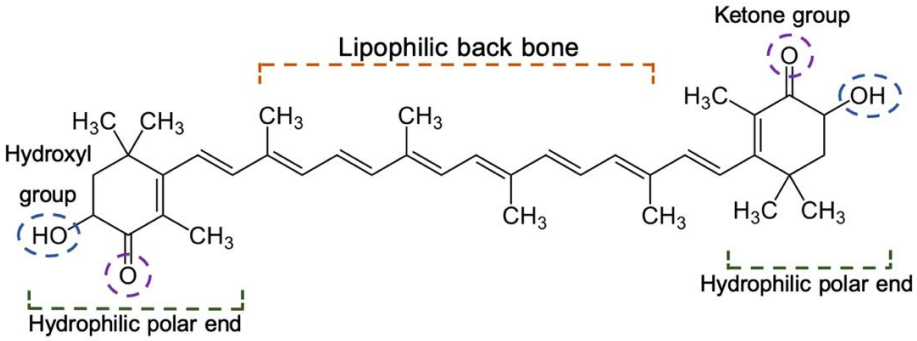

Figure 1.3 Chemical structure of astaxanthin

In this work, astaxanthin as a natural antioxidant was incorporated in chitosan film and studied the effect of genipin crosslinking at various concentrations on film properties. The study of this part of the research is described in Chapter 3, which is published in *Food Processing and Preservation*.

Furthermore, an improving the properties of active film from genipin-crosslinked chitosan/astaxanthin film by heat curing was also investigated. This research is presented in Chapter 3, which is published in *International Journal of Food Science and Technology*.

1.2 Objectives of research

1. To determine the optimal genipin crosslinker concentration to improve properties of chitosan and chitosan/astaxanthin films.

2. To study the effect of heat curing on the functional properties of the developed chitosan-based films.

1.3 Scope of the study

This research contains two main parts.

1. The effect of different concentration of genipin (0, 0.5, 1.0 and 1.5% (w/w of chitosan)) on the properties of chitosan film with/without astaxanthin (0 and 1%

(w/w)) were obtain by measuring the tensile strength, Young's modulus, elongation at break, water vapor and oxygen permeability, crystallinity and thermal stability of the films

2. The effects of heat curing (25, 80 and 105°C, for 30 min) on the properties of the developed film, including chitosan film (CS), chitosan crosslinked with 1.5% (w/w of chitosan) genipin film (CS1.5G), chitosan incorporated with 1% (w/w) astaxanthin (CSA) and chitosan-astaxanthin crosslinked with 1% (w/w of chitosan) genipin (CSA-1G) were determined.

1.4 Benefits of this study

The success of this research can lead to the potential development of active chitosan-based film which has improved moisture barrier and strength. The developed film can be applied as food packaging to maintain the quality and extend shelf life of meat products.

CHAPTER II
MANUSCRIPT I

Thermal curing to improve properties of genipin-crosslinking chitosan film

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Abstract

Importance of the work: Chitosan film as a natural biodegradable packaging material has limited food applications due to its high moisture permeability and poor mechanical strength. Crosslinking with genipin, a natural crosslinker, and thermal curing were applied to eliminate these drawbacks.

Objective: To study the effect of the genipin concentration and thermal curing on the chemical structure and properties of chitosan film.

Materials & Methods: Chitosan (CS, 1.5 g) solution were prepared crosslinked with genipin at 0.5%, 1.0% or 1.5 (all weight per weight, w/w of chitosan) % and cured at 25 °C, 80 °C or 105 °C. Film properties were investigated: water vapor permeability (WVP), tensile strength (TS), elongation at break (EAB), Young's modulus (YM), thermal stability, crystallinity and contact angle.

Results: Increasing the genipin concentration significantly increased the degree of crosslinking, TS and YM of the films, with the best film obtained at 1.5% w/w genipin (CS1.5G). The CS1.5G film with curing at 105°C (CS1.5G-105) had the highest ($p < 0.05$) TS (36.45 MPa), YM (315.99 N/m^2) and thermal stability due to a high conjugation of C double bonding between the genipin molecules in the chitosan chains. However, CS1.5G-105 decreased the crystallinity, EAB and WVP, while the contact angle value increased.

Main finding: Improving properties, such as the mechanical strength and moisture barrier of the genipin-crosslinked film, could be achieved by thermal curing, thus increasing the potential of the film for use as food packaging in the food industry.

Keywords:
Chitosan,
Chemical structure,
Functional properties,
Genipin,
Thermal curing

2.1 Introduction

Chitosan, a natural polysaccharide formed between β-(1-4)-linked 2 acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose, generally deacetylated from the chitin of crustaceans, has been used for numerous applications in the food industry, particularly as a packaging material due to its available film-forming, biocompatible and biodegradable properties (Varun et al., 2017; Venkatesan, Ramachandran, & Ramachandran, 2017). Chitosan with its functional properties of being bacteriostatic and fungistatic, has potential to be applied as active packaging (Dutta, Tripathi, Mehrotra, & Dutta, 2009; Martins, Cerqueira, & Vicente, 2012) to extend the shelf life of food. Chitosan film has a good oxygen barrier property (Azeredo, Britto, & Assis, 2011). However, its poor mechanical properties limit its practical applications for food packaging. Modifying the chitosan matrix through a crosslinking structure is applied to overcome these drawbacks by enhancing the mechanical strength through forming chemical bridges at amine groups of the chitosan (Khouri, Penlidis, & Moresoli, 2019). The most common synthetic chemicals used as crosslinking reagents are aldehyde substances, such as glyoxal (Yang, Dou, Liang, & Shen, 2005), glutaraldehyde (Li, Ren, Qiu, Mou, & Liu, 2013) and formaldehyde (Xue, Li, Wu, Wang, & Ma, 2011). However, these substances are reported to have a certain degree of toxicity due to the presence of both unreacted molecules of such crosslinkers and by-products formed during the reactions (Dimida et al., 2015). Therefore, an alternative natural crosslinker, such as genipin, has attracted substantial interest.

Genipin is a natural crosslinking agent isolated from the fruit of *Gardenia jasminoides* Ellis which can be used to replace the synthetic-chemical crosslinker because it is safe and nontoxic and therefore it is primarily used as scaffolds and hydrogel in tissue engineering and pharmaceutical applications (Dimida et al., 2017; Jin, Song, & Hourston, 2004; Xu, Strandman, Zhu, Barralet, & Cerruti, 2015). The crosslinking reaction between genipin and the amino groups of chitosan can be formed with a covalent bond, resulting in improved mechanical properties, as well as thermal stability of the resulting chitosan film (Butler, Ng, & Pudney, 2003; Liu et al., 2019; Nunes et al., 2013; Wu et al., 2018). However, the reaction is sometimes incomplete and not rapid enough for some applications. To finalize the crosslinking

process, curing processes have been studied, using ultraviolet light (Yun, Lee, Kim, & Yoon, 2017), electromagnetic waves (Biswas, Arief, Panja, & Bose, 2017), pressure (Gan, Sam, Abdullah, Omar, & Tan, 2020) or heat (Dimida et al., 2015).

Many investigations have focused on the use of heat curing method because it is easy, safe and inexpensive and has been reported to increase the kinetic rate of polymerization and to stabilize the biopolymer network (Dimida et al., 2015; Rubentheren et al., 2016). In addition, some authors reported that heat curing improved the mechanical, thermal stability and moisture barrier properties of the polymer (Falamarzpour, Behzad, & Zamani, 2017; Sandra Rivero, García, & Pinotti, 2011). Although genipin has been reported as a crosslinking agent in chitosan films, few studies have been conducted on the chemical-crosslinking structure and the properties of genipin-crosslinking chitosan film under thermal treatment. Thus, this study evaluated the effect of the genipin concentration and thermal curing on the morphology, moisture barrier, mechanical properties, crystallization and thermal stability of the film.

2.2 Materials and Methods

2.2.1 Materials

Chitosan (MW: 50-190 KDa) was purchased from Sigma-Aldrich, USA. Genipin (purity = 99.1%) was ordered from Tokyo Chemical Industry Co., Ltd, Japan.

2.2.2 Film preparation

The genipin was evaluated as a natural crosslinking agent. In this study the optimal genipin concentration was determined by adding genipin at different concentrations into chitosan film (CS) solution and observing the properties of the resulting films. An amount of chitosan (1.5 g) as melted in 100 mL aqueous solution containing lactic acid at 2% (weight per weight, w/w) and then homogenized using a magnetic stirrer (Corning PC-420D; Korea) at 500 revolutions per minute and 25 °C until the solution was clear. Genipin concentrations at 0% (CS), 0.5% (CS0.5G), 1.0% (CS1G) or 1.5 (CS1.5G)%, (all w/w of chitosan) were mixed in chitosan solution for 45 min. The mixture solution was added with 15% (w/w of chitosan) of glycerol, stirred at 50 °C for 15 min and the gas was removed using an ultrasonicator (Sonicator bath 500 series; Powersonic Hwashin Co. Ltd.; Korea) for 30 min. A sample volume of film solution (100 mL) was poured onto a polytetrafluoroethylene

tray (125 mm × 125 mm × 20 mm) and then dried in a hot air oven (400D-003, TESTONE Co., Ltd.; Korea) at 35 °C for 48 h. The films were maintained under controlled conditions of 50% relative humidity (RH) at 25 °C for 24 h. Characterization of film samples was carried out of the crosslinking degree and functional properties: vapor permeability (WVP), tensile strength (TS), elongation at break (EAB) and Young's modulus (YM).

2.2.3 Thermal curing

The influence of thermal curing on the chemical-crosslinking structure and properties of CS and the crosslinked film (obtained from the optimal crosslinking condition: CS1.5G) were investigated by curing the films (CS and CS1.5G) at 25 °C, 80 °C or 105 °C for 30 min, according to the method of Falamarzpour et al. (2017) with slight modifications. All films were stored under controlled conditions at 25 °C, 50% RH for 24 h before measurement.

2.2.4 Measurement of film properties

Chitosan and the chitosan film crosslinked with genipin with and without thermal curing were characterized as follows.

Thickness

Each sample (50 mm × 50 mm) was measured for thickness at five random positions using a digital micrometer (M-547 Thickness Gage Series; Mitutoyo; Japan). Ten films were measured for each treatment, with the mean values calculated.

Crosslinking degree

The crosslinking degree was carried out following the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay method (Kanoujia, Parashar, Singh, Tripathi, & Saraf, 2018). The mixture solution between 0.5% (weight per volume, w/v) TNBS and sodium hydrogen carbonate (4% w/v) solution was prepared at a 1:1 ratio; then, 1 mL of the mixed solution was added in a sample (6 mg) tube. The sample was cured at 40 °C for 2 h. To stop the reaction, 6 M of hydrochloric acid (3 mL) was added in solution and continuously cured at 60 °C for 90 min. The absorbance was measured at 345 nm using a spectrophotometer (Optizen 2120 UV; Mecasys Co. Ltd.; Korea). The percentage of crosslinking was calculated using Equation 1:

$$\% \text{ Crosslinking} = \left(\frac{\mathcal{X}NH2 - \mathcal{X}NH2cross}{\mathcal{X}NH2}\right) \times 100 \quad (1)$$

where $\mathcal{X}NH2$ is he absorbance value at 345 nm of free amines in CS and $\mathcal{X}NH2cross$ is the absorbance value at 345 nm for the crosslinked film (CS0.5G, CS1G or CS1.5G).

Fourier-transform infrared spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy was performed to note the chemical-crosslinking structure of chitosan and genipin. The FTIR spectra of films were recorded from 4000 cm^{-1} to 400 cm^{-1} with 4 cm^{-1} resolution and an accumulation testing at 32 scans using an FTIR spectrometer (Spectrum 65; Co., Ltd.; Korea), according to (Dimida et al., 2017).

Moisture permeability

The water vapor permeability (WVP) was determined on the basis of a modified ASTM method E96-80 (ASTM., 1992) as described by Siripatrawan and Vitchayakitti (2016). The WVP of each sample was investigated for four replications. An aluminum cup was determined as 0% RH using silica gel (20 g) and then covering with film at a radius of 30.06 mm. All samples were stored at 25 °C in desiccator (controlled at 75% RH with sodium chloride solution) and weighed intermittently to a steady state. The WVP (measure in grams millimeters per hour per aquare centimeter per pascal) was calculated according to Equation 2:

$$\text{WVP}=\frac{\Delta m}{\Delta t \text{A}}\times\frac{x}{\Delta\rho} \quad (2)$$

where $\frac{\Delta m}{\Delta t}$ is the slope from moisture weight gain per time unit (measured un grams per hour), A is the transferred area of film (in square millimeters), x is the thickness of film (in millimeters), and $\Delta\rho$ is the difference in water vapor pressure (in pascals) between the inner and outer surface of the film.

Contact angle measurement

The wettability on the surface sample was measured using an optical tensiometer (SDS-TEZD10014; FEMTOFAB Co., Ltd.; Korea). Each sample (20 mm × 40 mm) was placed on a glass plate and fixed with tape adhesive. Then 2 μL of water were dropped on the film surface. The angle (°) was measured to within 5 s to calculate the mean values of the test on both side of water droplet (Wu et al., 2018).

Mechanical properties

TS, EAB and YM were measured using a universal testing machine (MC Tester version 12.5.0; Quality & Measurement Systems; USA) according to the ASTM method D882-88 (ASTM., 1992). Each sample (20 mm × 90 mm) was fixed in a specific grip. An initial separation distance was determined at 50 mm and the test speed was 50 mm/min. Fifteen films were investigated for each treatment.

Surface morphology

Film morphology was assessed using scanning electron microscopy (SEM; JSM-IT500HR; JEOL Ltd.; Japan) with an accelerating voltage at 5 kV. Each sample was coated with 1 nm of gold to improve conductivity (Dimida et al., 2017).

X-ray diffraction

The crystalline characteristics of the films were investigated using an X-ray diffractometer (D8 Discover; Bruker Co., Ltd.; Germany) according to the method of Sandra Rivero et al. (2011). Cu radiation, operated at 25 °C, was generated at 40 kV. The relative intensity was recorded for a scanning range of (2θ) 3–60º, with a step size $2\theta = 0.02°$.

Thermal stability

The thermal stability of the films was measured using a thermogravimetric analyzer-TGA (Pyris series-TGA4000, PerkinElmer Co., Ltd., Korea). Sample (10–11mg) was heated from 30 °C up to 800 °C at the rate of 20 °C min^{-1} under nitrogen atmosphere. The changes in sample weight with increasing temperature were recorded on the excel graph (Klein et al., 2016).

2.2.5 Statistical analysis

Statistical analyses were carried out using the IBM SPSS Statistics for Windows (Version 20.0; IBM Corp.; USA). To assess the effects of genipin concentrations on the film properties, data on thickness, crosslinking degree, TS, EAB and YM of control (CS) and crosslinked films were analyzed using one-way analysis of variance. Then, all data were subjected to two-way analysis of variance to test the effects of genipin-crosslinking, curing temperatures and their interaction. Subsequently, differences between treatments were analyzed using Duncan's multiple

range test. Mean and standard deviations were analyzed at a significance level of $p < 0.05$.

2.3 Results and Discussion

2.3.1 Optimal genipin-crosslinking film

Film thickness is shown in Table 2.1. The addition of genipin concentrations did not affect the film thickness. The higher the genipin concentration, the darker the film color. CS1.5G had the darkest-blue color compared to the other film samples, probably due to the crosslinking reaction between genipin and chitosan being related with oxygen radical-induced polymerization, as similarly reported by Dimida et al. (2015) and Lee, Lim, Bhoo, Paik, and Hahn (2003).

The crosslinking degree percentages of CS0.5G, CS1G and CS1.5G film were 26.74%, 55.81% and 74.42%, respectively, as shown in Table 2.1. The percentage degree of crosslinking increased as the crosslinker concentration increased, probably because of the reduction of the free amines within chitosan. This suggested that genipin as a crosslinking agent could form intermolecular crosslinking with chitosan (Kanoujia et al., 2018).

TS, EAB and YM are three important parameters to evaluate the strength and flexibility of biodegradable film (Jakubowska, Gierszewska, Nowaczyk, & Olewnik-Kruszkowska, 2020). In the current study, the values for TS, EAB and YM of non-crosslinked and crosslinked chitosan films are shown in Table 2.1. Increasing the genipin concentration significantly increased TS and YM, while EAB slightly decreased. The values for TS and YM of CS0.5G film increased by 28% and 22%, respectively, and increased by 37% and 88%, respectively for CS1G film, compared to those of the CS film. Moreover, CS1.5G had the highest values for TS and YM with increases of 47% and 91%, respectively, compared to the CS film. However, the EAB of films significantly decreased when the genipin was added, which was probably related to the crosslinking between chitosan and genipin by covalent bonding (C-N), as reported by Inthamat, Boonsiriwit, Lee, and Siripatrawan (2021), resulting in the films being more rigid.

The current results indicated that chitosan crosslinked with 1.5% genipin provided optimal film properties (TS and YM). Therefore CS1.5G was used further to study the effect of heat curing.

Table 2.1 Thickness, crosslinking degree, tensile strength (TS), elongation at break (EAB) and Youn's modulus (YM) of film crosslinked at genipin concentrations

Sample	Thickness (mm)	Crosslinking degree (%)	TS (MPa)	EAB (%)	YM (N/m^2)
CS	0.17 ± 0.03^{a}	ND	7.61 ± 0.94^{c}	122.66 ± 11.02^{a}	8.81 ± 0.61^{c}
CS0.5G	0.17 ± 0.04^{a}	26.74^{c}	10.67 ± 1.31^{b}	106.22 ± 2.01^{b}	11.33 ± 2.42^{c}
CS1G	0.17 ± 0.04^{a}	55.81^{b}	12.09 ± 1.53^{b}	50.04 ± 8.63^{c}	77.20 ± 4.82^{b}
CS1.5G	0.17 ± 0.03^{a}	74.42^{a}	14.21 ± 1.37^{a}	44.25 ± 7.98^{c}	105.65 ± 4.19^{a}

CS = pure chitosan film; CS0.5G = chitosan crosslinked with 0.5% genipin; CS1G = chitosan crosslinked with 1% genipin; CS1.5G = chitosan crosslinked with 1.5% genipin

Mean (± SD) values within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different; ND = not detected in studied samples

2.3.2 Effect of thermal curing on chitosan and crosslinked chitosan film

The CS and CS1.5G films were cured at 25 °C (CS-25 or CS1.5G-25), 80 °C (CS-80 or CS1.5G-80) or 105 °C (CS-105 or CS1.5G-105) and then were then analyzed for chemical structure, WVP, contact angle, TS, EAB, YM, crystallinity and thermal stability. Analysis of variance showed that thermal curing was the main effect causing a decrease in WVP and an increase in TS, EAB and YM for both the CS and CS1.5G films. There were significant interactions between thermal curing and genipin-crosslinking for TS, EAB and YM values of the films.

Fourier-transform infrared spectroscopy of thermal curing films

Figure 2.1 displays the FTIR spectra of film cured at 25 °C, 80 °C and 105 °C. The signals observed at 1719 cm^{-1} and 1568 cm^{-1} were assigned to amide I (C=O stretching) and amide II (N–H stretching), respectively. Generally, the crosslinking between the amino groups at amide I and amide II of chitosan and the carbon atom at the C–3 position on the olefinic ring of genipin was formed as shown in the CS1.5G-25 film. The characteristic signals of CS1.5G-25 at 1723 cm^{-1} and 1571 cm^{-1} were shifted to 1725 cm^{-1} and 1574 cm^{-1} for CS1.5G-80. Similarly, these peaks were moved to 1728 cm^{-1} and 1577 cm^{-1} for CS1.5G-105. A peak at 1410 cm^{-1} of CS1.5G-80 and CS1.5G-105 was detected when those films had higher density due to heat curing, probably attributed to the C–C binding between the genipin molecules already

linked to the amino groups of the chitosan chain (Inthamat, Lee, Boonsiriwit, & Siripatrawan, 2021).

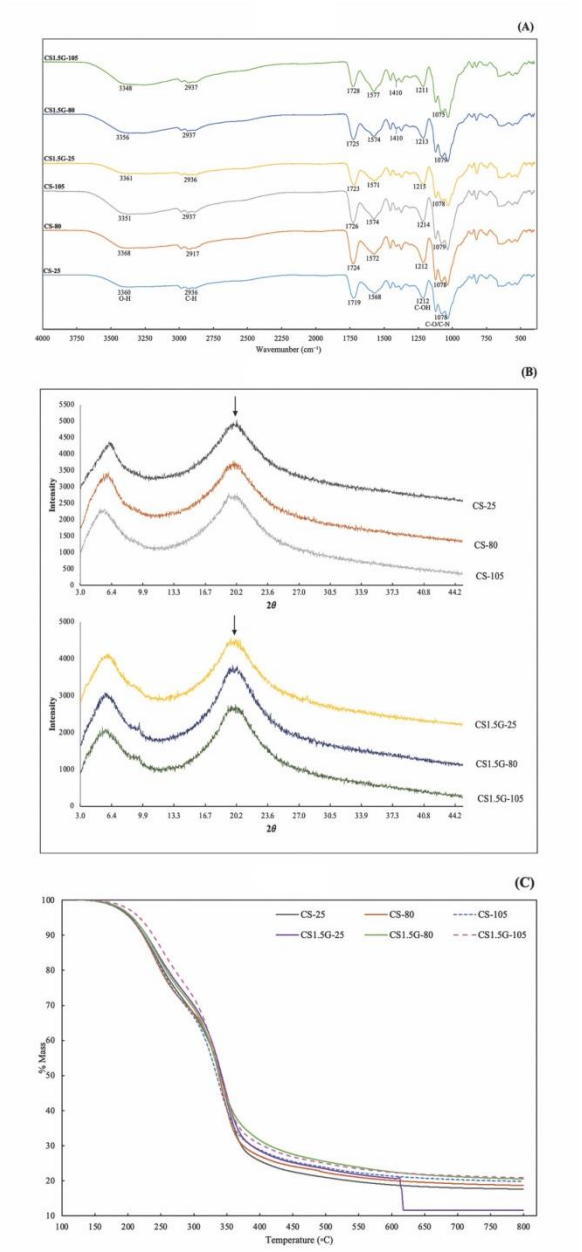

Figure 2.1 Chitosan (CS) and CS with 1.5% w/w genipin (CS1.5G) films with curing temperatures at 25 °C, 80 °C or 105 °C: (A) Fourier-transform infrared spectra; (B) X-ray diffractometry; (C) thermogravimetric analyzer curves

Water vapor permeability of thermal curing films

The CS-25 film had an WVP value of 2.79 ± 0.19 $\times 10^{-4}$ g mm/h/mm^2/Pa when cured at 80 °C and 105 °C; however, the WVP values of CS-80 and CS-105 were

significantly reduced to 2.65 ± 0.09×10^{-4} g mm/h/mm^2/Pa and 1.95 ± 0.13×10^{-4} g mm/h/mm^2/Pa, respectively. Films crosslinked with genipin had lower WVP values than non-crosslinked films. For example, the WVP of CS-25 (2.79 ± 0.19×10^{-4} g mm/h/mm^2/Pa decreased to 1.77 ± 0.11×10^{-4} g mm/h/mm^2/Pa after crosslinking with genipin (CS1.5G-25). The WVP of CS1.5G-80 significantly decreased from 1.77 ± 0.11×10^{-4} g mm/h/mm^2/Pa (CS1.5G-25) to 1.37 ± 0.08×10^{-4} g mm/h/mm^2/Pa when cured at 80°C. A similar trend was observed for the CS1.5G-105 film with curing at 105 °C, producing the lowest WVP value of 0.81 ± 0.01×10^{-4} g mm/h/mm^2/Pa compared with that of CS1.5G-25. These results agreed well with those obtained by Inthamat, Lee, et al. (2021) and Sandra Rivero et al. (2011) who reported that the molecular alterations in film structure due to curing at high temperatures, probably because the carboxylic acid could react with the amine group to form an amide resulting in water being eliminated (De Castro, Campos, Bruno, & Reges, 2013).

Contact angle of heat curing films

A contact angle image of cured films is shown in Figure 2.2; the contact angles increased gradually with increasing curing temperature. The CS-25 film had a value of 55.8° that the contact angles of crosslinked-chitosan film with increasing heat curing, with a value of 43.1° for CS1.5G-25, increasing to 54.9° for CS1.5G-80 and to 60.1° for CS1.5G-105, respectively. The cured films had the highest contact angle values, indicating that thermal curing played an important role in decreasing the hydrophilic nature of the film surface. Similar effect had been observed in the works of S. Rivero, Garc®™a, and Pinotti (2013) and Czibulya et al. (2021).

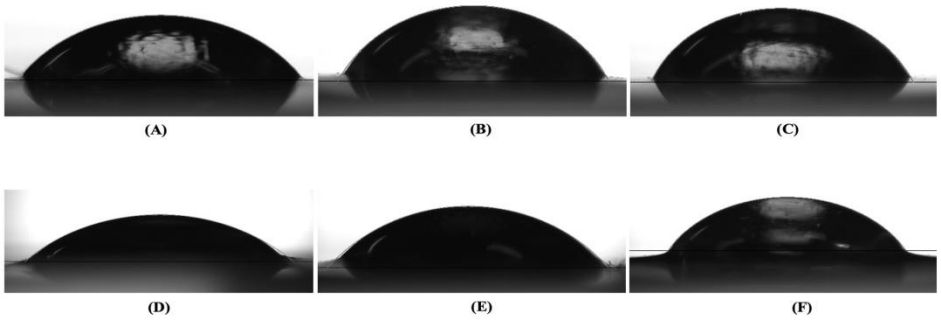

Figure 2.2 Contact angles of chitosan (CS) and CS with 1.5% w/w genipin (CS1.5G) films with curing temperature at 25 °C, 80 °C and 105 °C: (A) CS-25; (B) CS-80; (C) CS-105; and for pure chitosan with curing at 25 °C, 80 °C and 105 °C: (D) CS1.5G-25; (E) CS1.5G-80; (F) CS1.5G-105, where chitosan is crosslinked with 1.5% genipin and cured at 25 °C, 80 °C and 105 °C, respectively.

Mechanical properties of thermal curing films

The values for TS, EAB and YM of CS and CS-1.5G film with curing temperature at 25 °C, 80 °C and 105 °C are shown in Table 2.2. The results indicated that curing the films at high temperature significantly increased TS and YM while EAB slightly decreased. TS increased from 7.61 MPa (CS-25) to 13.73 MPa (CS-80) and 15.22 MPa (CS-105), while YM increased from 8.81 N/m^2 (CS-25) to 162.19 N/m^2 (CS-80) and 179.24 N/m^2 (CS-105). Similarly, with increasing curing temperature, TS of the crosslinking films increased from 14.26 MPa (CS1.5G-25) to 23.02 MPa (CS1.5G-80), while CS1.5G-105 had a maximum TS of 36.45 MPa, which was a 60% increase compared to CS-1.5G-25. YM significantly increased from 105.65 N/m^2 (CS1.5G-25) to 270.24 N/m^2 and 315.96 N/m^2 for CS1.5G-80 and CS1.5G-105, respectively. This fact could indicate that heat curing was a reinforcement of both the CS-25 and CS1.5G-25 networks. The improvement in TS and YM of the chitosan-crosslinked films after heat curing was also observed by S. Rivero et al. (2013), which suggested that the thermal treatment affected structural stabilization. Conventionally, when mechanical strength increases, polymer elasticity decreases. The elongation values decreased on average by 87–88% for the cured films compared to their corresponding controls (CS-25 and CS1.5G-25). A similar observation was reported by Sandra Rivero et al. (2011), where heat curing markedly

reduced the flexibility of films. In the same way, Inthamat, Lee, et al. (2021) reported that a declination in the segmental movement of polymer chains due to the development of the C–C double bond between genipin molecules resulted in a decrease in the elongation at break.

Table 2.2 Tensile strength (TS), elongation at break (EAB) and Young's modulus (YM) of CS and CS1.5G film with curing temperatures at 25 °C, 80 °C and 105 °C

Sample	TS (MPa)	EAB (%)	YM (N/m^2)
CS-25	7.61 ± 0.94^{d}	122.66 ± 11.02^{a}	8.81 ± 0.61^{f}
CS-80	13.73 ± 0.71^{c}	42.85 ± 6.12^{b}	162.19 ± 4.15^{d}
CS-105	15.22 ± 2.08^{c}	14.04 ± 1.78^{c}	170.24 ± 9.35^{c}
CS1.5G-25	14.26 ± 1.37^{c}	44.25 ± 7.98^{b}	105.65 ± 4.19^{e}
CS1.5G-80	23.02 ± 3.23^{b}	6.07 ± 2.10^{d}	270.24 ± 6.76^{b}
CS1.5G-105	36.45 ± 0.64^{a}	5.36 ± 0.80^{d}	315.96 ± 1.57^{a}

CS-25, CS-80, CS-105 = pure chitosan with curing at 25 °C, 80 °C and 105 °C, respectively; CS1.5G-25, CS1.5G-80 and CS1.5G-105 = chitosan crosslinked with 1.5% genipin and cured at 25 °C, 80 °C and 105 °C, respectively

Mean (± SD) values within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Morphology of thermal curing films

The surface morphologies of the CS and CS1.5G films at 25 °C, 80 °C and 105 °C are shown in Figure 2.3. The film surfaces of the CS-25, CS-80 and CS-105 films (Figure 2.3A–2.3C, respectively) were smoother than that of the CS1.5G-25 film (Figure 2.3E). Increasing curing temperature increased the roughness of the CS1.5G-80 (Figure 2.3F) and CS1.5G-105 (Figure 2.3G) films, probably due to a rapid release of moisture on the film surface during the heat curing process (Gan et al., 2020). As shown in Figure 2.3D and 2.3H, the cross-sectional surface of the CS-105 film was smooth. However, the CS1.5G-105 film had a dense-layered structure on the cross-sectional surface of the film, leading to improved mechanical and barrier properties of the genipin-crosslinked film.

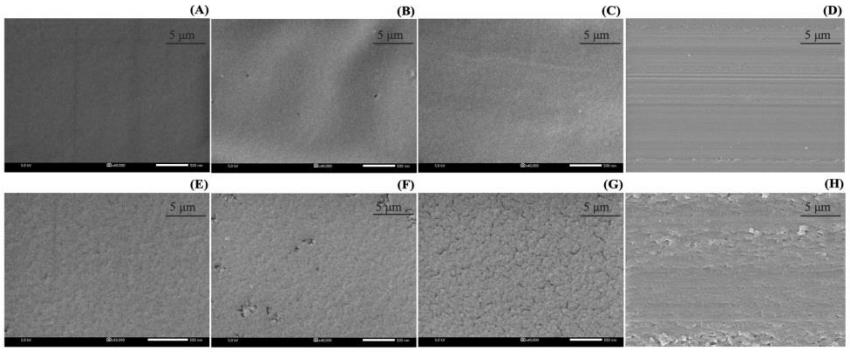

Figure 2.3 Film morphology of CS and CS1.5G film surfaces with curing temperature at 25 °C, 80 °C and 105 °C: (A) CS-25; (B) CS-80; (C) CS-105; and of pure chitosan with curing at 25 °C, 80 °C and 105 °C: (E) CS1.5G-25; (F) CS1.5G-80; (G) CS1.5G-105, where chitosan is crosslinked with 1.5% genipin and cured at 25 °C, 80 °C and 105 °C, respectively; and cross-section scanning electron microspore images of: (D) CS-105; (H) CS1.5G-105

X-ray diffractometry analysis of thermal curing films

The main objective of the XRD analysis was to observe the influence of curing temperature on the crystallinity of the CS and CS1.5G films, as shown in Figure 2.1B. The X-ray diffractograms of the CS-25 film displayed two main peaks at 2θ = 8–9° and 2θ = 20–21° that were assigned to the typical crystalline forms I and II, respectively. The heat-treated CS-80 and CS-105 films had a flattening peak at 20–21°. This result further showed that heat curing affected the molecular structure of chitosan, probably relating to an increment in the number of hydrogen bonds in the chitosan matrix (Liu et al., 2019). Furthermore, the diffractograms of the CS1.5G-80 and CS1.5G-105 films cured at different temperatures showed a broader state at the peak of 2θ = 20–21° because heat curing caused further crosslinking of the film matrix, as described by Sandra Rivero et al. (2011) and Rubentheren et al. (2016), reducing the formation of a crystalline structure.

Thermal stability of thermal curing films

Figure 2.1C shows the TGA curves of the CS and CS1.5G films after curing at 25 °C, 80 °C and 105 °C. The observed first stage at 180–280 °C corresponded to an elimination of water molecules in both free and associated states in the chitosan-based

film structure. The loss in the second stage at 300–380 °C was related to complex processes, such as the dehydration of the polysaccharide rings and the decomposition of units of acetylated and deacetylated chitosan (Rivero et al., 2020). However, the third stage at 571 °C, attributed to crosslinking degradation, was observed in the CS1.5G-25 film. The thermal stability levels of CS-80 and CS-105 film with curing at 80 °C and 105 °C were higher than that of the CS-25 film as shown by their slightly increasing curves. Furthermore, the curves of cured films had the highest percentage remaining mass and decomposition temperature, indicating the development of a more stable structure. In addition, this result could indicate that heat curing encouraged crosslinking reactions between chitosan and genipin.

2.4 Conclusions

An increase in the genipin concentration increased the crosslinking degree, TS and YM of the films After curing, the structure of crosslinking between chitosan and genipin was enhanced. Chitosan film crosslinked with 1.5% genipin and cured at 105 °C (CS1.5G-105) had the lowest WVP value and the most reduced hydrophilic surface. The highest TS, YM and thermal stability values were measured in the CS1.5G-105 film. Therefore, thermal curing of genipin-crosslinked chitosan improved the film properties, increasing the potential for these films to be used in food packaging applications.

2.5 Conflict of Interest

The authors declare that there are no conflicts of interest.

2.6 Acknowledgments

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CHAPTER III
MANUSCRIPT II

Effects of genipin as natural crosslinker on barrier and mechanical properties of chitosan-astaxanthin film

Running title: Functional properties of crosslinked active film

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Abstract

The aim of this research was to study the effect of genipin crosslinking (0.5, 1.0 and 1.5% (w/w of chitosan)) on moisture barrier property and mechanical strength of chitosan-astaxanthin film. The film functional properties including transparency, tensile strength (TS), elongation at break (EB), water transmission rate (WVTR) and oxygen transmission rate (OTR) were investigated. The results showed that genipin crosslinking was significant decrease the film transparency and %EB while OTR slightly increased. Fourier transform infrared observation on the chemical-crosslinking structure showed the shifted peak at 1720 and 1569 cm^{-1}, indicating the forming covalent linkage between chitosan and genipin. The morphology characterization of film was evaluated by scanning electron microscopy (SEM) and found that the crosslinked film was rougher surface than non-crosslinked film. Chitosan-astaxanthin film with crosslinking of 1% (w/w) genipin significantly improved WVTR and TS compared to other samples.

Keywords: chitosan, astaxanthin, genipin, crosslinking, moisture barrier and mechanical property

Novelty Impact Statement

Active biodegradable packaging made from chitosan, such as chitosan film incorporated with natural antioxidants, is preferred but lack the water barrier and strong mechanical properties necessary for high moisture food application. In this study, genipin-crosslinking is not only a promising technique to improve the performance and applicability of our developed chitosan-astaxanthin film, but genipin is also a natural crosslinker and an alternative to most chemical crosslinkers which cause undesirable changes and cytotoxicity.

3.1 Introduction

Active packaging is produced under the aim of maintaining and extending product shelf life. In current it has increasingly been required for several packaging industries, and it continues being an area of interest for researchers in the field of food packaging. The development of innovative biodegradable active packaging has been underway for many years due to the major environmental threats from plastic wastes.

In particular, bioactive packaging based on chitosan is widely used as a potential food preservative, due to its antimicrobial activity against a wide range of microorganisms. Although antimicrobial activities of chitosan have been widely reported (Malinowska-Pańczyk, Staroszczyk, Gottfried, Kołodziejska, & Wojtasz-Pajak, 2015; Zarzor, Dahham, & Thalij, 2019), other functions of chitosan as active food packaging can also be improved to maintain qualities, and prolong shelf life of foods by the addition of natural bioactive compounds, for example, essential oils, polyphenols, and vitamin, as well as astaxanthin.

Astaxanthin, found in various marine plants and animals e.g. algae, rainbow trout, shrimp and lobster, is a kind of carotenoid pigments (Liu et al., 2019). Astaxanthin has been reported to have higher powerful antioxidant capacity than vitamin E and beta-carotene and have been confirmed their potential uses in the pharmaceutical, medical and food fields. A wide number of studies demonstrated that an incorporation of astaxanthin into chitosan can improve biological activities as active material (Arancibia et al., 2014; Xu, Wei, Jia, & Song, 2020). However, chitosan films have low moisture barrier properties and mechanical strength (Liang et al., 2019), limiting their use in food products. To overcome these problems, the modify polymer matrix by crosslinking is employed.

Crosslinking is a structural modification technique of biopolymer with the aim to improve structural and functional properties of polymer to be suitable for various applications. Improving moisture barrier and tensile strength of crosslinked films has been described in literature (Hisham et al., 2016; Kanoujia, Parashar, Singh, Tripathi, & Saraf, 2018). A common technique for crosslinking of chitosan films is based on the formation of a Schiff base between the amino groups of the chitosan chain and the aldehyde groups from cross-linking agents. Although these agents can efficiently produce a high degree of crosslinking, the application is limited because they cause cytotoxicity after interaction between chitosan and aldehyde (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002; Kildeeva et al., 2020). Therefore, in recent years the attention has been focused on the green and natural crosslinking agents, such as ferulic, tannic acid, citric acid, and genipin.

Genipin is isolated from *Gardenia* fruits, primarily used as a medicine (Jin, Song, & Hourston, 2004), suggesting it is a safe and nontoxic natural cross-linking agent. Moreover, it has been regarded as a new bifunctional agent to replace conventional chemical crosslinkers, since lesser cytotoxicity was observed in genipin crosslinked films than in those with chemical crosslinking (Bi et al., 2011; Klein et al., 2016; Liu et al., 2019). Although the crosslinking between genipin and chitosan has been reported to improve moisture barrier and mechanical properties of chitosan-based film (Kildeeva et al., 2020), the genipin crosslinking of chitosan-astaxanthin composited film has never been documented. Therefore, the objective of this research was to study the effect of genipin crosslinking on functional properties, including water and oxygen transmission rate, tensile strength, and elongation at break of chitosan-astaxanthin film.

3.2 Materials and Methods

3.2.1 Materials

Commercial chitosan powder (Mw: 50-190 KDa, 76% DD) and astaxanthin (purity>90%) were purchased from Sigma-Aldrich, USA and Nanjing Zelang Medical Technology Co., Ltd, China, respectively. Analytical grade genipin (purity >98%) was ordered from Tokyo Chemical Industry Co., Ltd (TCI), Japan.

3.2.2 Preparation of the crosslinked chitosan-astaxanthin film

Chitosan film containing astaxanthin was used as based film (control film). The crosslinking film was prepared using the solution casting method as described by Li, Ren, Qiu, Mou, and Liu (2013) and Zeng et al. (2015). The chitosan-based film was carried out by dissolving of 1.5% (w/v) chitosan and 1% (w/w) astaxanthin in 2% (w/w) lactic acid. Briefly, genipin as crosslinking agent was added at 0, 0.5, 1.0 and 1.5% (w/w of chitosan) into the film solution. Glycerol 15% (w/w) was added and continuously stirred using magnetic stirrer (Corning PC-420D, Korea) for 15 min. The film solution (100 ml) was casted on a polytetrafluoroethylene (PTFE) tray (12.5 x 12.5 cm) and dried at 35°C for 48 hr. The obtained films were stored under 50% relative humidity for 24 hr before characterization.

3.2.3 Thickness and transparency

Thickness was measured at five different positions on the film (5 x 5 cm) using a handheld micrometer (Mitutoyo gage, Japan) with a precision of 0.001 mm. Four samples were used for calculation the average of film thickness.

The light transmittance of the films was measured using a UV/VIS spectrophotometer (Optizen 2120UV, Mecasys co., ltd., Korea) following the method of Lin, Wang, and Weng (2020). The samples (1 cm x 4 cm) were tested at 600 nm. The transparency was calculated using equation (1).

$$\text{Transparency} = \frac{-\log T}{x} \quad (1)$$

where T is the transmittance at 600 nm and x is the film thickness (mm)

3.2.4 Degree of crosslinking

The degree of crosslinking was determined by using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay (Kanoujia et al., 2018). The weighed sample (6 mg) was mixed with 1 ml of TNBS (0.5% w/v) solution and 1 ml of 4% (w/v) sodium hydrogen carbonate. The solution was heated at 40°C for 2 hr, and then 3 ml of 6 M HCl was added to stop the reaction. The sample was heated again at 60°C for 90 min. The optical absorbance of sample was determined at 345 nm using UV-Vis spectrophotometer. The degree of crosslinking was calculated using equation (2).

$$\%\text{Degree of crosslinking} = \left[\frac{(XNH2 - XNHcross)}{XNH2} \times 100\right] \quad (2)$$

where XNH2 is the absorbance of free amines in non-crosslinked sample and XNHcross is the absorbance of crosslinked sample.

3.2.5 Mechanical properties

Tensile strength (TS) and elongation at break (EB) were measured with an Instron Universal testing machine (MC_Tester version 12.5.0, Quality & Measurement System, USA) following the ASTM Standard Test method D 882-91 (ASTM., 1992). The film (size of 2 cm x 5 cm) was determined with a testing condition at load of 20 kgf and pulling up at a head speed of 50 mm/min.

3.2.6 Water vapor transmission rate (WVTR)

WVTR was determined an according to ASTM Standard Test method E95 (ASTM., 1992). Sample was cut with the diameter size of 60.12 mm and then placed on the top of test cup contained 20 g silica gel as determined 0% RH. The cup was stored in controlled condition of 75% RH (sodium chloride solution) at 25°C and weighed for 8 hr. The WVP was calculated as the following formular (3).

$$\text{WVTR} = \frac{\Delta m}{\Delta t \text{A}} \quad (3)$$

where $\frac{\Delta m}{\Delta t}$ is the weight of moisture gain per unit of time (g/hour) and A is an area of the exposed film surface (m^2).

3.2.7 Oxygen transmission rate (OTR)

OTR of the films was analyzed by using an oxygen permeability analyzer (OTR-8001, Illinois Instruments Inc., Korea) according to the method of Siripatrawan and Vitchayakitti (2016). The sample (size of 133.28 m^2) was measured the amount of oxygen passing through films with the setting condition at 25°C, 50% RH and carrier gas pressure > 0.28 MPa with flowing of gas at OTR > 1,000 cc/m²/day. The condition of bypass time and purge level were determined at 10 min and 100 ppm, respectively. The OTR (Eq (4)) was recoded as the quantity (q) of oxygen molecules passing through a film surface area (A) during time (Δt) at steady state.

$$\text{OTR} = \frac{q}{A\Delta t} \quad (4)$$

3.2.8 Fourier transform infrared (FTIR) analysis

The chemical-crosslinking structure between chitosan and genipin of the films was evaluated by a FTIR spectrometer (Spectrum 65, PerkinElmer (precisely) CO., LTD, Korea). The absorbance range was determined from 400 cm^{-1} to 4000 cm^{-1} with 32 scans (Dimida et al., 2015).

3.2.9 Morphology characterization

The surface microcosmic morphology of the film was evaluated by scanning electron microscopy (SEM) (JSM-IT500HR, JEOL Ltd., Japan) following the method

of Dimida et al. (2017). A small piece of films was placed on a double-sided adhesive tape and then coated with a layer of gold. The sample was examined at a voltage of 5.0 kv with magnification at 40,000X.

3.2.10 Differential scanning calorimetry (DSC)

Crystallization of the film was analyzed using a Differential Scanning Calorimetry (DSC) analyzer (Series Q20, TA Instrument, Korea). Five mg of the film sample was put into a small aluminum cup. The sample cup and pure cup (without sample) were heated at the temperature ranging from 35°C to 350°C with an increasing heat rate at 10°C min^{-1} (Aldana, González, Strumia, & Martinelli, 2012). The heat of melting (ΔHm) and the post-crystallization (ΔHc) were analyzed by integrating the areas (J/g) under the peak by using Universal Analysis 2000 program. The percent crystallinity was determined using the following equation (5);

$$\%\text{Crystallinity} = \frac{\Delta Hm - \Delta Hc}{\Delta Hm^{\circ}} \times 100 \quad (5)$$

Where ΔHm° is a reference value of melting entralpy for 100% crystallinity of polymer.

3.2.11 Statistical analysis

All experiments were performed with three replications (n=3). The statistical analysis was performed by using SPSS software (SPSS statistics software version 20.0; IBM Crop., USA). All of data was reported as the mean ± standard deviation using Duncan's multiple range test to identify differences between each treatment under the level of significance set at $P<0.05$.

3.3 Results and discussions

3.3.1 Thickness and transparency

The chitosan-astaxanthin (CA) as control film and those crosslinked with 0.5, 1.0 and 1.5% (w/w) genipin (CA-0.5G, CA-1G and CA-1.5G, respectively) are determined. The film thickness was in the range of 0.17±0.04 to 0.16±0.04 mm as reported in Table 3.1. The thickness of crosslinked film tended to be lower, but showed no significant difference ($P\geq0.05$), when compared with the control film.

Transparency of the films is presented in Table 3.1. The transparency of the crosslinked films decreased with the genipin concentration, probably due to the degree of the dihydropyran ring from the crosslinking agent dispersing in the chitosan matrix (Liang et al., 2019). In fact, this behavior can be attributed to the crosslinking of the chitosan-based films by genipin leading to the formation of stronger bonds resulting in less spaces between the polymer chains, which reduces the light that passes through the films and consequently decreases transparency (Costa et al., 2018).

TABLE 3.1 Thickness and transparency value of crosslinked film at different concentrations of genipin.

	Thickness (mm)	Transparency value
CA	0.17 ± 0.04^{a}	13.50 ± 0.21^{a}
CA-0.5G	0.17 ± 0.05^{a}	13.29 ± 0.21^{a}
CA-1G	0.16 ± 0.05^{a}	12.98 ± 1.13^{ab}
CA-1.5G	0.17 ± 0.05^{a}	12.67 ± 0.27^{b}

Abbreviations: CA, incorporated chitosan with astaxanthin; CA-0.5G, incorporated chitosan with astaxanthin and crosslinked with 0.5% genipin; CA-1G, incorporated chitosan with astaxanthin and crosslinked with 1% genipin; CA-1.5G, incorporated chitosan with astaxanthin and crosslinked with 1.5% genipin. Mean values within a row for each sample with different letters (a-b) were significantly different ($p < 0.05$, Duncan).

3.3.2 Degree of crosslinking

The crosslinking degree of chitosan-astaxanthin crosslinked with genipin concentrations is shown in Table 3.2. The crosslinking degree of chitosan-astaxanthin film crosslinked with genipin (0.5, 1 and 1.5% (w/w)) was found to be 22.73, 45.45 and 46.59%, respectively. The result suggested that the percentage of crosslinking degree increased with genipin concentrations, due to the content of free amino group within chitosan film decreased correspondingly with an increase of crosslinking (Kanoujia et al., 2018).

TABLE 3.2 Degree of crosslinking and % crystallinity of crosslinked film at different concentration of genipin.

	Degree of crosslinking (%)	Crystallinity (%)
CA	-	42.52
CA-0.5G	22.73	35.73
CA-1G	45.46	33.31
CA-1.5G	46.59	24.29

Abbreviations: CA, incorporated chitosan with astaxanthin; CA-0.5G, incorporated chitosan with astaxanthin and crosslinked with 0.5% genipin; CA-1G, incorporated chitosan with astaxanthin and crosslinked with 1% genipin; CA-1.5G, incorporated chitosan with astaxanthin and crosslinked with 1.5% genipin.

3.3.3 Mechanical properties

TS (MPa) and EB (%) of chitosan-astaxanthin film crosslinked with different concentrations of genipin were investigated, and the results are shown in Table 3.3. TS and EB of CA was 10.17±0.34 MPa and 65.69±2.46%, respectively. TS of the crosslinked film significantly increased (P<0.05), that was 11.26±0.63 MPa of CA-0.5G, 16.77±0.65 MPa of CA-1G and 17.82±0.70 MPa of CA-1.5G while %EB decreased significantly (P<0.05) when the concentration of genipin increased. These results suggest that the addition of genipin as crosslinking agent could effectively strengthen the chitosan-astaxanthin composited films. However, a decrease in %EB with increasing genipin concentration is probably attributed to producing a three-dimensional network structure between genipin and chitosan can inhibit the sliding of the molecular chain (Liu et al., 2019; Wu et al., 2018).

TABLE 3.3 TS and EB of crosslinked film at different concentrations of genipin.

	TS (MPa)	EB (%)
CA	10.17±0.34^{d}	65.69±2.46^{a}
CA-0.5G	11.26±0.63^{c}	29.95±5.47^{b}
CA-1G	16.77±0.65^{b}	26.64±3.54^{b}
CA-1.5G	17.82±0.70^{a}	11.18±2.27^{c}

Abbreviations: CA, incorporated chitosan with astaxanthin; CA-0.5G, incorporated chitosan with astaxanthin and crosslinked with 0.5% genipin; CA-1G, incorporated

chitosan with astaxanthin and crosslinked with 1% genipin; CA-1.5G, incorporated chitosan with astaxanthin and crosslinked with 1.5% genipin. TS, tensile strength; EB, elongation at break. Mean values within a row for each sample with different letters (a-d) were significantly different ($p < 0.05$, Duncan).

3.3.4 Water vapor transmission rate

WVTR is the most extensively studied property of edible films mainly because of the importance of water in deteriorative reactions in food. The effect of genipin concentration on the WVTR of the films is shown in Table 3.4. A significant decrease in WVTR was observed in CA-0.5G ($4.55 \pm 0.12 \times 10^{-6}$ g/h.mm^2) and CA-1G ($4.47 \pm 0.14 \times 10^{-6}$ g/h.mm^2) when compared with CA ($4.83 \pm 0.05 \times 10^{-6}$ g/h.mm^2). However, WVP increased when the genipin concentration was increased to 1.5% in CA-1.5G ($5.76 \pm 0.25 \times 10^{-6}$ g/h.mm^2). This is probably due to the imbalanced ratio of hydrophilic/hydrophobic constituents which made the polymer matrix more open to the transport of water molecules.

TABLE 3.4 WVTR and OTR of crosslinked film at different concentration of genipin.

	WVTR (g/h.mm² (x10^{-6}))	OTR (cc/m²/day)
CA	4.83±0.05^{b}	38.44±0.21^{d}
CA-0.5G	4.55±0.12^{c}	40.30±0.10^{b}
CA-1G	4.47±0.14^{c}	40.00±0.10^{c}
CA-1.5G	5.76±0.25^{a}	41.13±0.23^{a}

Abbreviations: CA, incorporated chitosan with astaxanthin; CA-0.5G, incorporated chitosan with astaxanthin and crosslinked with 0.5% genipin; CA-1G, incorporated chitosan with astaxanthin and crosslinked with 1% genipin; CA-1.5G, incorporated chitosan with astaxanthin and crosslinked with 1.5% genipin. WVTR, water vapor transmission rate; OTR, oxygen transmission rate. Mean values within a row for each sample with different letters (a-d) were significantly different ($p < 0.05$, Duncan).

3.3.5 Oxygen transmission rate

OTR of CA and the chitosan-astaxanthin crosslinked with genipin at 25ºC and 75% RH is presented in Table 3.4. The CA film had OTR of 38.44±0.21 cc/m²/day, respectively, while the addition of genipin slightly increased the OTR value. This is probably because the covalent bond between chitosan and genipin enhanced the

hydrophobicity of the film surface, resulting an increase in the adsorption of non-polar O_2 molecules on the film surface. The similar results were observed in Keratin/polyvinyl alcohol/Tris (hydroxymethyl) aminomethane blend film crosslinked with transglutaminase, $CaCl_2$ and genipin (Wu et al., 2018).

3.3.6 FTIR analysis

Figure 3.1 shows FTIR spectra of chitosan-astaxanthin films without and with crosslinking at different concentration of genipin. The adsorption was observed to relatively increase at 1716 cm^{-1} while it decreased at 1573 cm^{-1} when the concentration of genipin increased. The characteristic peaks shifted from crosslinking were noticed on the absorption peak in the range of 1717-1712 cm^{-1}, assigned to C=O stretching vibrations in primary amine, while the peak at 1569-1573 cm^{-1} was related to N-H stretching in secondary amine and the C-N stretching band of covalent bond (Aldana et al., 2012; Dimida et al., 2015). The characteristic band of CA at 1569 cm^{-1} shifted to higher wavenumbers of 1573, 1571 and 1570 cm^{-1} in CA-0.5G, CA-1G and CA-1.5G, respectively. These indicated the crosslinking process form between amino group of chitosan and the olefinic carbon atom at C3 of genipin, followed by the opening of the dihydropyran ring to form the heterocyclic amine as shown in Figure 3.2. Moreover, the C=O stretching vibration at 1722 cm^{-1} and 1717 cm^{-1} of CH and CA, respectively, was shifted to 1716 cm^{-1} of CA-0.5G, 1719 cm^{-1} of CA-1G and 1720 cm^{-1} of CA-1.5G; this is attributed to the hydrogen bonds between C=O and NH_2 within chitosan being broken by genipin to release free C=O (Kawadkar & Chauhan, 2012).

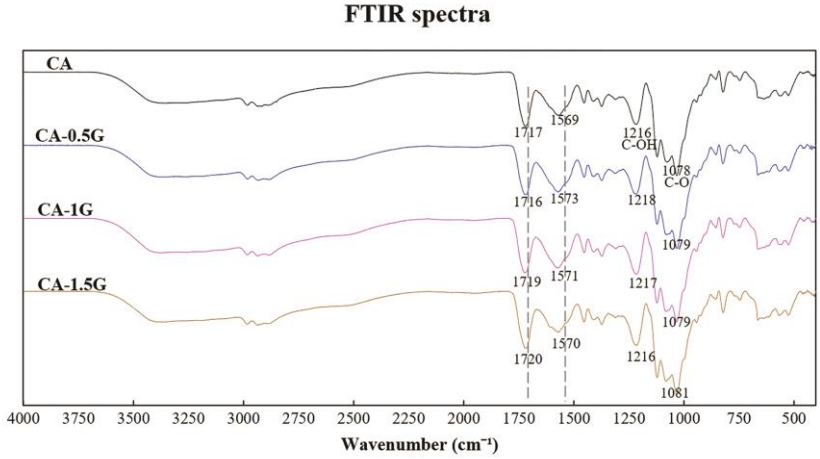

Figure 3.1 FTIR spectra of non-crosslinked and crosslinked films. CA: Incorporated chitosan with astaxanthin; CA-0.5G: Incorporated chitosan with astaxanthin and crosslinked with 0.5% genipin; CA-1G; Incorporated chitosan with astaxanthin and crosslinked with 1% genipin; CA-1.5G; Incorporated chitosan with astaxanthin and crosslinked with 1.5% genipin.

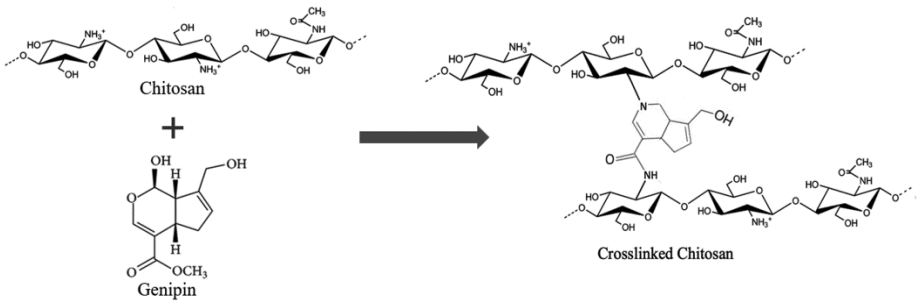

Figure 3.2 Crosslinked interaction between chitosan and genipin.

3.3.7 Morphology characterization

The effect of genipin as crosslinking agent on the morphology of the films were examined by SEM. Figure 3.3 (a)-(d) shows the surface of CA film with crosslinking at 0.5, 1.0 and 1.5% (w/w of chitosan), respectively. The fracture surface morphology of CA film appeared more homogeneous than the crosslinked films, which indicated the occurrence of chemical interaction between genipin and chitosan film as similar result was also observed by Hisham et al. (2016) and Yeamsuksawat and Liang (2019).

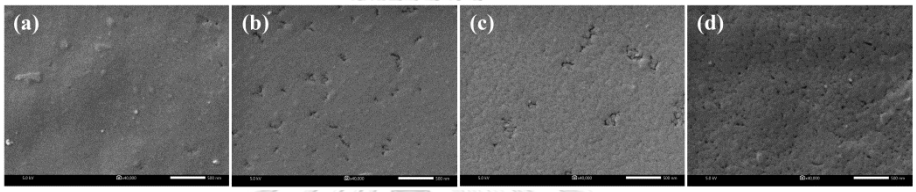

Figure 3.3 SEM micrographs of film surface. CA: Incorporated chitosan with astaxanthin; CA-0.5G: Incorporated chitosan with astaxanthin and crosslinked with 0.5% genipin; CA-1G; Incorporated chitosan with astaxanthin and crosslinked with 1% genipin; CA-1.5G; Incorporated chitosan with astaxanthin and crosslinked with 1.5% genipin.

3.3.8 Differential scanning calorimetry

The percent crystallinity is summarized in Table 4.2. The results showed that the decrease of ΔHm with the addition of genipin was associated with the decrease of the film crystallinity. It is known that the chitosan film is semi-crystalline which genipin crosslinker reduces the relative number of crystalline structures in the film, leading to a more amorphous structure due to the interruption of the crystalline structure formation in the chitosan matrix (Dimida et al., 2015; Guerrero, Muxika, Zarandona, & de la Caba, 2019).

3.4 Conclusions

This research demonstrates a successful improvement of the functional properties of chitosan-astaxanthin based film. In fact, the genipin crosslinking decreased the film transparency and increased roughness of the film. The addition of genipin decreased the film crystalline while degree of crosslinking of the film was

increased as similarly observed by FTIR analysis with the shifted peak at 1717 and 1569 cm^{-1} which can be associated with an increase of OTR. Moreover, this crosslinking structure led to significantly decrease WVTR and increased tensile strength of the film. As a result, genipin crosslinking can be an alternative choice in the field of active film.

3.5 Acknowledgments

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CHAPTER IV
MANUSCRIPT III

Improving moisture barrier and functional properties of active film from genipin-crosslinked chitosan/ astaxanthin film by heat curing

Running title: Improving active film properties by heat curing

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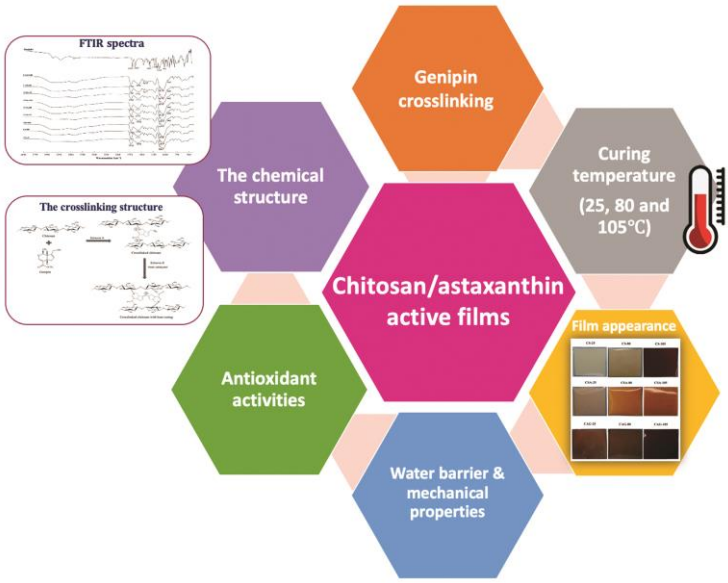

Figure 4.1 Graphical abstract

Summary

This research aimed to improve moisture barrier and functional properties of genipin-crosslinked chitosan/astaxanthin film by heat curing. The effect of heat curing (25, 80 and 105°C) on film properties was investigated by measuring water vapor permeability (WVP), contact angle, tensile strength (TS), elongation at break (EAB), Young's modulus (YM), opacity, chemical structure and antioxidant activity. Results showed that genipin-crosslinked chitosan/astaxanthin film and heat cured at 105°C (CAG-105) have the lowest WVP value which decreased by 55% ($p<0.05$) while a contact angle increased from 50.2° to 74.6°, when compared to CAG-25 film. Heat curing affected the chemical-crosslinked interaction between chitosan and genipin with the establishment of new chemical bonds, leading to the improvement of TS and YM by 55% and 42%, respectively, for CAG-105 film. However, EAB and antioxidant activity of the CAG-105 slightly decreased. Moreover, heat curing changed the color of genipin-crosslinked chitosan/astaxanthin film from light brown to reddish-brown and increased the film opacity which can lower light transmission through the film. The developed film not only had better water barrier, TS and YM but, due to its opacity, could provide light protection, and thus would be appropriate for packaging material of foods that degrade when exposed to moisture or light.

Keywords: Astaxanthin, chitosan, genipin, heat-curing, moisture barrier

4.1 Introduction

Active packaging has been one of the most innovative developments in food packaging to maintain the quality and extend the shelf-life of food products. Biodegradable polymers incorporated with bioactive compounds, especially antioxidants (Liu, Zhang, McClements, Wang, & Xu, 2019) have been developed as active food packaging. Among them, the antioxidant film which can protect food products from oxidation reactions and thus improve food shelf-life have gained interest. Astaxanthin is a kind of carotenoid pigment found in various marine plants and animals, such as algae, shrimp and lobster. Astaxanthin has more powerful antioxidant activity than other carotenoids due to the presence of two oxygenated groups, hydroxyl (OH) and carbonyl (C=O), in each of its ionone rings (Xu, Wei, Jia, & Song, 2020). These bonds play an important role in preventing the production of reactive oxygen species (ROS), causing tissue damage (Gao, Zhu, & Xu, 2021). Consequently, astaxanthin has been widely used as an ingredient in food and pharmaceutical industry (Xu et al., 2020). However, its use as a natural antioxidant incorporated into chitosan film to be used as active packaging is scarce (Sanches-Silva et al., 2010; Sendon et al., 2012).

Chitosan, natural biopolyaminosaccharide, has high potential to be used for the development of active packaging due to its non-toxicity, biodegradability and biocompatibility. The addition of astaxanthin into chitosan film has been reported to enhance functional film properties such as oxygen permeability and intrinsic antimicrobial activity (Xu et al., 2020); however, it has poor moisture barrier properties. To overcome these drawbacks, various modification methods are used such as copolymer blending, grafting (Nunes et al., 2013) and chemical crosslinking (Klein et al., 2016). Chemical crosslinking is a structure modifying technique to form covalent bonds between two polymer chains by using synthetic chemical agents. Although synthetic chemical crosslinkers such as glutaraldehyde, sodium tripolyphosphate and formaldehyde are considered effective, they have been reported to cause cytotoxicity (Klein et al., 2016). Hence, natural crosslinking agents which are biocompatibility and non-cytotoxicity for their possible applications in food packaging are needed. Genipin isolated from *Gardenia* is accepted as an alternative crosslinking agent owing to nontoxicity and thus can be applied as an effective natural

crosslinking agent for biopolymers containing amino groups, especially chitosan. Several studies have reported that genipin as crosslinker can improve functional properties of chitosan film (Hisham et al., 2016; Kildeeva et al., 2020). Our previous study has reported that genipin crosslinker can improve physical, moisture barrier and mechanical properties of chitosan-astaxanthin film to a moderate extent (Inthamat, Boonsiriwit, Lee, & Siripatrawan, 2021a) but the certain functional properties, especially, high moisture barrier and strong mechanical properties necessary for high moisture food application have not been conclusively established and thus required further investigation.

In general, heat curing is applied to stabilize the synthetic polymer network and to improve film properties, especially, moisture barrier and mechanical properties. Sandra Rivero, García, and Pinotti (2011) reported the use of heat treatment to modify structure and improve moisture barrier, mechanical and stability properties of chitosan-based film. Moreover, a successful improvement of moisture barrier property of genipin-crosslinked chitosan film by heat curing above the glass transition temperature (T_g) was reported by Dimida et al. (2015). Although improving properties of chitosan films with genipin crosslinker or heat curing has been reported in the literature, to the best of our knowledge the use of genipin crosslinker together with heat curing to improve film properties has never been reported on chitosan film incorporated with astaxanthin.

Therefore, this research aimed to study the effect of heat curing at different temperature on the functional properties including water vapor permeability (WVP), mechanical properties (tensile strength (TS), elongation at break (EAB) and Young's modulus (YM)), contact angle, color, opacity, chemical structure and antioxidant activity of the chitosan (CS) film, CS incorporated with astaxanthin (CSA) film, and CSA crosslinked with genipin (CAG) film.

4.2. Materials and methods

4.2.1 Materials

Chitosan powder (Mw: 50-190 KDa, 76% DD) and astaxanthin (purity>90%) were purchased from Sigma-Aldrich, USA and Nanjing Zelang Medical Technology Co., Ltd., China, respectively. Genipin (purity = 99.1%) was purchased from Tokyo Chemical Industry Co., Ltd. (TCI), Japan.

4.2.2 Preparation of genipin-crosslinked chitosan/astaxanthin film

Film was prepared by incorporating of astaxanthin into chitosan following the method suggested by J. Li, Ren, Qiu, Mou, and Liu (2013) with slight modifications using the optimal conditions obtained from the preliminary study. Briefly, chitosan (1.5 g) was dissolved in 100 mL of 2% (w/v) lactic acid and stirred (Corning PC-420D, Korea) with a rotation speed of 500 rpm at 30°C. Astaxanthin (1% w/w) was mixed into chitosan solution. The contained chitosan/astaxanthin film was used as control film (CSA). Glycerol 15% (w/w) was added and then stirred at 30°C for 15 min. Film solution (100 mL) was cast on a polytetrafluoroethylene tray (125 × 125 × 20 mm) and dried at 25°C for 48 h (Klein et al., 2016). The dried films were stored at relative humidity (RH) 50% for 24 h. The chitosan/astaxanthin (CSA) film was used as control.

In this study, genipin was used as a natural crosslinker. Our previous study (Inthamat et al., 2021) have suggested that chitosan-astaxanthin crosslinked with genipin at 1% (w/w of chitosan) gave optimal film properties and thus used as CAG film in this current study.

4.2.3 Heat curing of composite films

The prepared film (CS, CSA, or CAG) was enclosed in aluminum foil and then cured at 80°C (CS-80, CSA-80, or CAG-80) and 105°C (CS-105, CSA-105, or CAG-105) for 30 min using a drying oven (400D-003, TESTONE Co., Ltd., Korea). CS, CSA, or CAG films cured at 25°C (CS-25, CSA-25, or CAG-25) was determined as control. All samples were then stored at 50% RH, 25°C for 48 h before all measurements (Falamarzpour, Behzad, & Zamani, 2017).

4.2.4 Film thickness

Thickness was measured at five different positions on the film (5 cm x 5 cm) using a handheld micrometer (M-547 Thickness Gage Series, Mitutoyo, Japan). Ten samples were used for calculating the average of film thickness.

4.2.5 Water vapor permeability (WVP)

WVP was examined according to the ASTM Standard Test method E96 (ASTM., 1992) with slight modification following the method described by Siripatrawan and Vitchayakitti (2016). The circular test cups which contained silica gel (0% RH) were covered with the testing films. All samples were stored in the controlled RH of 75% (sodium chloride solution) at 25°C. WVP was determined from the equilibrium weight gain of the permeation cell, using equation (1):

$$\text{WVP} = \frac{\Delta m}{\Delta t \text{A}} . \frac{x}{\Delta \rho} \quad (1)$$

where $\frac{\Delta m}{\Delta t}$ is the slope of the weight of moisture gained per unit of time (g/h), A is the exposed area of film surface exposed (mm^2), x is the film thickness (mm), and $\Delta\rho$ is the difference in partial pressure (Pa).

4.2.6 Mechanical properties

Tensile strength (TS), elongation at break (EAB) and Young's modulus (YM) were analyzed using an Instron Universal testing machine (MC Tester version 12.5.0, Quality & Measurement System, USA) following the ASTM Standard Test Method D882-91 (ASTM., 1992). The film (20 mm × 50 mm) was gripped at an initial separation of 50 mm and tested at load of 20 kg_f with pulling up at a head speed of 50 mm/min under the condition of 25°C and 50% RH (Nunes et al., 2013).

4.2.7 Contact angle

The contact angle of the developed film (2 cm × 4 cm) was measured by using a contact angle machine (SDS-TEZD10014, FEMTOFAB CO., Ltd., Korea). Water (7 µL) was dropped onto the film surface using a microsyringe. The angle (°) was recorded within 5 s (Palakattukunnel, Thomas, Sreekumar, & Bandyopadhyay, 2011).

4.2.8 Color and opacity

The lightness (L*), redness (a*) and yellowness (b*) were recorded using an automatic color reader (CR-10, Konica Minolta Sensing, Inc., Japan). A white reflector standard plate was used as background.

The opacity of films was measured using UV/Vis spectrophotometer (Optizen 2120 UV, Mecasys Co., Ltd., Korea). A rectangular piece of film was placed directly onto the internal side of the spectrophotometer cell. The film was measured at an absorbance of 600 nm by determination of the blank condition with air (Yuan, Lv, Zhang, Sun, & Chen, 2016), and the opacity was then the data were calculated by using equation (2):

$$\text{Opacity} = \frac{Abs}{d} \quad (2)$$

where *Abs* is the absorbance at 600 nm and d is the film thickness (mm).

4.2.9 Fourier transform infrared (FTIR) analysis

The chemical-structural interactions between chitosan and genipin of the films were evaluated by a FTIR spectrometer (Spectrum 65, PerkinElmer Co., Ltd., Korea), according to the method of Martins, Cerqueira, and Vicente (2012). The measurement was scanned over the absorbance range of 400 to 4,000 cm^{-1} at a resolution of 4 cm^{-1} with an accumulation setting at 32 scans.

4.2.10 Antioxidant activity

The antioxidant activities of the films were conducted using two different methods. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay is based on the measurement of free radical scavenging capacity of antioxidant compounds. DPPH analysis was carried out according to the method as described by Ruengdech and Siripatrawan (2021) with slight modifications. The film sample (100 mg) was cut into small pieces and mixed with 5 mL of absolute methanol for 12 h. Ascorbic acid was dissolved in methanol and used as a standard solution. Fifty microliters of sample or ascorbic acid solution were mixed with 1950 µL of DPPH. The absorbance at 517 nm of the residual DPPH solution was determined using a UV/Vis spectrophotometer.

Trolox equivalent antioxidant capacity (TEAC) assay based on $ABTS^{+}$ radical scavenging activity was determined by adaptation of the method of decolorization of

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation (Kittikaiwan, Powthongsook, Pavasant, & Shotipruk, 2007). $ABTS^+$ solution was prepared by mixing 1 mL of potassium persulfate (2.45 mM) and 1 mL of ABTS (7 mM) and then kept in the dark at 25°C for 16 h. Trolox (1.5 mM) was used as a standard solution. Twenty microliters of sample or Trolox solution was mixed with 2000 µL of $ABTS^+$, left in the dark for 10 min, and the sample absorbance was measured at 734 nm using a UV/Vis spectrophotometer.

4.2.11 Statistical analysis

All experiments were performed with three replications (n=3) and reported as the mean ± standard deviation. Duncan's multiple range test was determined to identify differences between each treatment under the level of significance set at $p<0.05$. The statistical analysis was performed using SPSS software (SPSS statistic software version 20.0; IBM Crop., USA).

4.3 Results and discussion

4.3.1 Thickness

The thickness of films cured at 25, 80 and 105°C is presented in Table 1. The film thickness was in the range from 0.16 ± 0.04 to 0.18 ± 0.04 mm which showed no significant difference ($p>0.05$).

3.3.2 WVP

WVP of the all film samples is shown in Table 4.1. WVP value of CS-25 was $2.79 \pm 0.19 \times 10^{-4}$ g.mm/h.mm^2.Pa and significantly decreased ($p<0.05$) to $2.06 \pm 0.20 \times 10^{-4}$ g.mm/h.mm^2.Pa when incorporated with astaxanthin (CSA-25), and to $1.87 \pm 0.06 \times 10^{-4}$ g.mm/h.mm^2.Pa when crosslinked with genipin (CAG-25). Increasing curing temperature decreased WVP of the films. CS-80 and CS-105 had WVP decreased by 5% and 30%, respectively, when compared to CS-25, whereas WVP of CSA-80 and CSA-105 decreased by 9% and 31%, respectively, when compared to that of CSA-25. The lowest WVP value was observed in CAG-105 which decreased by 55% when compared to that of CAG-25. Heat curing increased the degree of crosslinking between chitosan and genipin, causing a decrease in the number of residual H^+ ions in amino group of chitosan which led to a lower capacity to absorb water molecule (Dimida et al., 2015).

Table 4.1 Thickness, WVP and contact angle of film with curing at 25, 80 and 105°C.

	Thickness (mm)	WVP ((g.mm/h.mm².Pa (x10^{-4}))	Contact angle (°)
CS-25	0.17±0.03^{a}	2.79±0.19^{a}	55.8
CS-80	0.18±0.04^{a}	2.65±0.09^{a}	65.1
CS-105	0.17±0.02^{a}	1.95±0.13^{b}	66.8
CSA-25	0.17±0.04^{a}	2.06±0.20^{b}	46.9
CSA-80	0.17±0.04^{a}	1.82±0.09^{b}	51.7
CSA-105	0.17±0.02^{a}	1.42±0.03^{c}	58.4
CAG-25	0.17±0.02^{a}	1.87±0.06^{b}	50.2
CAG-80	0.17±0.02^{a}	1.41±0.02^{c}	50.9
CAG-105	0.17±0.02^{a}	0.84±0.05^{d}	74.6

Abbreviations: CS-25, CS-80 and CS-105, pure chitosan cured at 25, 80 and 105°C, respectively; CSA-25, CSA-80 and CSA-105, chitosan incorporated with astaxanthin and cured at 25, 80 and 105°C, respectively; CAG-25, CAG-80 and CAG-105, chitosan incorporated with astaxanthin, crosslinked with 1% genipin, and cured at 25, 80 and 105°C, respectively. WVP, water vapor permeability. Mean values within columns for each treatment with different letters ($^{a–c}$) are significantly different ($p<0.05$, Duncan).

4.3.3 Mechanical properties

The effect of heat curing temperature on the mechanical properties of the films is shown in Table 4.2. The addition of astaxanthin and crosslinking with genipin improved TS and YM of the chitosan-based film. When compared to CS-25, CSA-25 and CAG-25 had TS and YM significantly increased ($p<0.05$) from 7.61 to 10.17 and 13.12 MPa, respectively, and YM significantly increased ($p<0.05$) from 8.81 to 38.42 N/m^2 and 128.30, respectively. The results also suggested that increasing curing temperature improved TS and YM of the films. TS and YM of CS-105 film were 15.22 MPa and 170.24 N/m^2, respectively, higher than that of CS-80 film (13.73 MPa and 162.19 N/m^2, respectively). Similarly, CSA-105 film exhibited an increase to 22.75 MPa and 185.87 N/m^2 in TS and YM, respectively, when compared CSA-80 film (19.08 MPa and 167.92 N/m^2, respectively). The highest improvement of TS and YM was observed in CAG-105, which was 33.63 MPa and 219.71 N/m^2, respectively. These results indicated that heat curing decreased the ability of chitosan chain for

slippage, resulting in an improvement in tensile strength and modulus. However, heat curing slightly decreased EAB of CS, CSA and CAG films.

Table 4.2 TS, EAB and YM of film with curing at 25, 80 and 105°C.

	TS (MPa)	EAB (%)	YM N/m²
CS-25	7.61±0.94^{g}	122.66±11.02^{a}	8.81±0.61^{f}
CS-80	13.73±0.71^{e}	42.85±6.12^{c}	162.19±4.15^{e}
CS-105	15.22±2.08^{de}	14.04±1.78^{e}	170.24±9.35^{bc}
CSA-25	10.17±0.34^{f}	65.69±2.46^{b}	38.42±1.81^{e}
CSA-80	19.08±2.21^{cd}	12.26±1.56^{e}	167.92±3.50^{c}
CSA-105	22.75±1.65^{c}	10.05±0.79^{ef}	185.87±7.76^{b}
CAG-25	13.12±1.09^{e}	26.64±3.54^{d}	128.30±4.43^{d}
CAG-80	27.58±1.53^{b}	9.64±0.40^{f}	177.09±2.90^{b}
CAG-105	33.63±1.53^{a}	7.36±1.03^{g}	219.71±9.86^{a}

Abbreviations: CS-25, CS-80 and CS-105, pure chitosan cured at 25, 80 and 105°C, respectively; CSA-25, CSA-80 and CSA-105, chitosan incorporated with astaxanthin and cured at 25, 80 and 105°C, respectively; CAG-25, CAG-80 and CAG-105, chitosan incorporated with astaxanthin, crosslinked with 1% genipin, and cured at 25, 80 and 105°C, respectively. TS, tensile strength; EAB, elongation at break, YM; Young's modulus. Mean values within columns for each treatment with different letters ($^{a–g}$) are significantly different ($p<0.05$, Duncan).

3.3.4 Contact angle

Contact angle, as a basic wetting property, is a good indicator for the degree of film surface hydrophobicity or hydrophilicity. Table 4.1 shows the contact angles of all film samples (CS, CSA, and CAG). The lower the contact angle, the lower the film surface hydrophobicity. The incorporation of astaxanthin and crosslinking with genipin lowered the contact angle of the chitosan-based films. These results indicated the presence of hydrophilic compounds, probably phenolic compounds from astaxanthin and the remaining hydroxyl groups of genipin, on the film surface which facilitated the interaction with the water droplets. The results also suggested that increasing curing temperature increased the contact angle. This result could be explained that the crosslinking produced after the heat curing played a significate role in reducing the hydroxyl groups on the film surface (S. Rivero, A. GarcÌa, & Pinotti, 2020).

4.3.5 Color and opacity

The color parameters of L* (lightness/darkness), a* (redness/greenness) and b* (yellowness/blueness) of all film samples are shown in Table 4.3. The results indicated that heat curing affected film color as displayed in Figure 4.2. The original chitosan film (CS-25) had yellowish color while film incorporated with astaxanthin (CSA-25) and crosslinked with genipin (CAG-25) was reddish and brownish, respectively. After curing at 80 and 105°C, CS-80 and CS-105 film turned to reddish-brown with decreasing in L* value and increasing in a* and b* value. Additionally, CSA and CAG films cured at 80 and 105°C also showed lower L* and higher a* and b* values than films cured at 25°C. The results also suggested that the higher the curing temperature, the lower the L* value and the higher the a* and b* values of the CS, CSA and CAG films. This may be because during heat curing lactic acid used for the preparation of chitosan film forming solution accelerated the browning color formation (Park Chun & Um In, 2018).

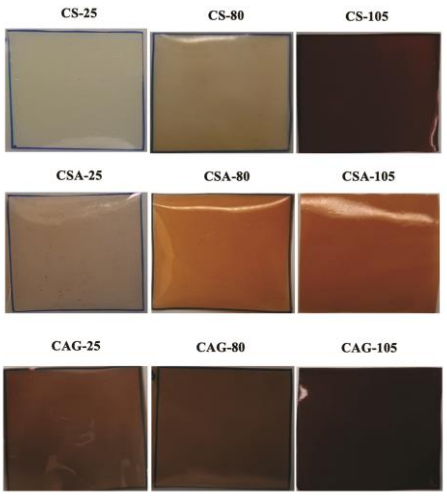

Figure 4.2 Film appearance. CS-25, CS-80 and CS-105, pure chitosan cured at 25, 80 and 105°C, respectively; CSA-25, CSA-80 and CSA-105, chitosan incorporated with astaxanthin and cured at 25, 80 and 105°C, respectively; CAG-25, CAG-80 and CAG-105, chitosan incorporated with astaxanthin, crosslinked with 1% genipin, and cured at 25, 80 and 105°C, respectively.

As shown in Table 4.3, a significant increase of opacity values ($p<0.05$) was observed in chitosan incorporated with astaxanthin and crosslinked with genipin, as well as after heat curing. This is probably due to the expansion of the molecular

chains and the presence of astaxanthin in the film matrices prevented the passage of the light (K. Li, Zhu, Guan, & Wu, 2019).

Table 4.3 Color (L* (lightness), a* (redness) and b* (yellowness)) and opacity of film with curing at 25, 80 and 105°C.

	Color			Opacity
	L*	a*	b*	(A mm^{-1})
CS-25	45.53 ± 0.41^{a}	9.78 ± 0.07^{f}	25.96 ± 0.55^{f}	0.91 ± 0.14^{c}
CS-80	43.77 ± 0.65^{b}	13.23 ± 0.61^{e}	31.87 ± 1.48^{e}	0.94 ± 0.22^{c}
CS-105	34.53 ± 0.83^{d}	24.53 ± 0.87^{b}	53.80 ± 1.32^{a}	0.95 ± 0.19^{c}
CSA-25	40.25 ± 1.86^{c}	15.30 ± 1.61^{e}	30.79 ± 2.77^{e}	1.34 ± 0.05^{b}
CSA-80	33.97 ± 1.55^{d}	24.47 ± 1.55^{bc}	52.33 ± 0.90^{a}	1.38 ± 0.06^{b}
CSA-105	30.03 ± 0.25^{e}	28.67 ± 0.32^{a}	51.10 ± 0.27^{b}	1.46 ± 0.38^{b}
CAG-25	27.78 ± 0.66^{f}	20.10 ± 0.41^{d}	32.40 ± 0.41^{e}	2.43 ± 0.04^{a}
CAG-80	26.90 ± 0.87^{f}	22.47 ± 0.97^{c}	38.07 ± 1.54^{c}	2.54 ± 0.30^{b}
CAG-105	24.47 ± 5.86^{g}	23.43 ± 1.50^{bc}	35.00 ± 1.08^{d}	2.60 ± 0.41^{a}

Abbreviations: CS-25, CS-80 and CS-105, pure chitosan cured at 25, 80 and 105°C, respectively; CSA-25, CSA-80 and CSA-105, chitosan incorporated with astaxanthin and cured at 25, 80 and 105°C, respectively; CAG-25, CAG-80 and CAG-105, chitosan incorporated with astaxanthin, crosslinked with 1% genipin, and cured at 25, 80 and 105°C, respectively. Mean values within columns for each sample with different letters ($^{a-g}$) are significantly different ($p<0.05$, Duncan).

4.3.6 FTIR analysis

FTIR was used to investigate the interaction between functional groups in the films with curing at different temperatures as shown in Figure 4.3. The main characteristic peaks of CS-25 at 1716 and 1570 cm^{-1} were assigned to C=O stretching in amide I and N–H stretching in amide II, respectively. A new peak at 941 cm^{-1}, relating with the C-O vibrations of alkoxyl group (C-O-R) was found in film incorporated with astaxanthin (CSA-25). When compared to CSA-25 film, the amine I and II band of genipin-crosslinked film (CAG-25) were shifted to 1722 cm^{-1} and 1573 cm^{-1}, respectively, as previously reported by Inthamat et al. (2021a). Both shifts were probably caused by the crosslinking between the amino groups of chitosan and the olefinic carbon atom at C-3 of genipin (Dimida et al., 2015; Pomari et al., 2019). Moreover, a band located at 1442 cm^{-1} in genipin associated with carboxymethyl groups in genipin had disappeared in the crosslinked film, suggesting there were

crosslinks between amino groups in chitosan and carboxymethyl groups in genipin (Hisham et al., 2016), which was shown in Figure 4.3 ((b): mechanism A).

The shifted vibration of the absorption bands at 1716 cm^{-1} and 1568 cm^{-1} of CS-25 and 1718 cm^{-1} and 1570 cm^{-1} of CSA-25 was observed when curing temperature increased. Moreover, the appearance of a new peak at 1410 cm^{-1} was detected for CAG after cured at 80 and 105°C, which probably indicated C-C bridges between genipin and genipin (Vlăsceanu, Crica, Pandele, & Ionita, 2020) as shown in Figure 4.3((b): mechanism B). As a result, heat curing could lead to the development of the crosslinking reaction between chitosan and genipin leading to an improvement in the strength of the film.

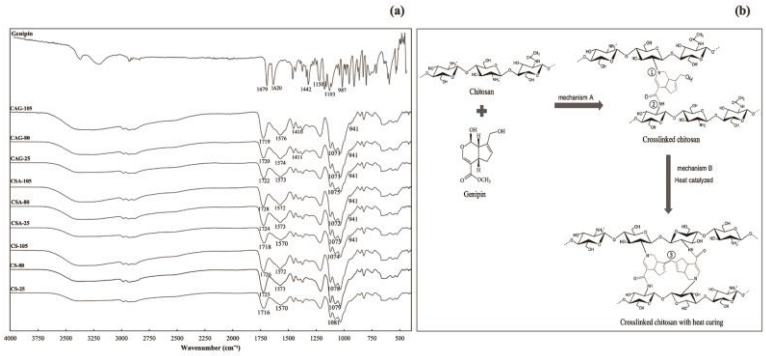

Figure 4.3 (a), FTIR spectra of cured films at different temperatures and crosslinking interaction between chitosan and genipin with/without heat curing. CS-25, CS-80 and CS-105, pure chitosan cured at 25, 80 and 105°C, respectively; CSA-25, CSA-80 and CSA-105, chitosan incorporated with astaxanthin and cured at 25, 80 and 105°C, respectively; CAG-25, CAG-80 and CAG-105, chitosan incorporated with astaxanthin, crosslinked with 1% genipin, and cured at 25, 80 and 105°C, respectively. (b), Mechanism of crosslinking between chitosan and genipin.

4.3.7 Antioxidant activity

The antioxidant activity of film was analyzed using DPPH and TEAC assay as shown in Table 4.4. Incorporation of CS film with astaxanthin and crosslinking with genipin significantly ($p<0.05$) affected the antioxidant activity of the film. CSA-25 and CAG-25 had DPPH values of 28.83 ± 1.56 and 29.80±0.45 µmol/g, respectively, which were higher than CS-25 (6.63±0.15 µmol/g). The reduction of DPPH was observed in CSA film from 18.19±1.25 (CSA-80) to 10.03±0.86 (CSA-105) µmol/g

and in CAG film from 34.47±1.12 (CAG-80) to 29.80±0.45 (CAG-105) μmol/g when the curing temperature increased from 80°C to 105°C.

The TEAC value (Table 4.4) of the films showed a similar trend as DPPH value. Film incorporated with astaxanthin (CSA-25) or crosslinked with genipin (CAG-25) had significantly higher ($p<0.05$) TEAC value than CS-25. Our results indicated that heat curing process decreased antioxidant activity of the film. The TEAC value of CAG-25 film was decreased from 47.97±0.60 μmol/g to 41.22±0.60 μmol/g (CAG-80) and to 34.10±1.24 μmol/g (CAG-105) after cured at 80 and 105°C, respectively. Moreover, the results in Table 4.4 also showed that films crosslinked with genipin had higher TEAC value than those of non-crosslinked films.

Table 4.4 Antioxidant activities of film with curing at 25, 80 and 105°C.

	Antioxidant activity (μmol/g)	
	DPPH	TEAC
CS-25	6.63±0.15^{e}	10.78±0.21^{g}
CS-80	6.42±0.05^{f}	10.63±0.14^{g}
CS-105	6.11±0.10^{g}	10.24±0.14^{h}
CSA-25	28.83±1.56^{b}	38.44±1.13^{c}
CSA-80	18.19±1.25^{c}	27.84±0.15^{e}
CSA-105	10.03±0.86^{d}	22.48±2.56^{f}
CAG-25	35.62±1.17^{a}	47.97±0.60^{a}
CAG-80	34.47±1.12^{a}	41.22±0.60^{b}
CAG-105	29.80±0.45^{b}	34.10±1.24^{d}

Abbreviations: CS-25, CS-80 and CS-105, pure chitosan cured at 25, 80 and 105°C, respectively; CSA-25, CSA-80 and CSA-105, chitosan incorporated with astaxanthin and cured at 25, 80 and 105°C, respectively; CAG-25, CAG-80 and CAG-105, chitosan incorporated with astaxanthin, crosslinked with 1% genipin, and cured at 25, 80 and 105°C, respectively. DPPH, 1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay; TEAC, Trolox equivalent antioxidant capacity assay. Mean values within columns for each treatment with different letters ($^{a–h}$) are significantly different ($p<0.05$, Duncan).

4.4 Conclusions

Heat curing was successfully improved the functional properties of chitosan-based film, imparting a high moisture barrier and mechanical properties. Curing at

105°C affected the interactions between chitosan and genipin of the film by initiating new chemical bonds which led to the highest improvement of water barrier, TS and YM. The contact angle of CAG film increased after heat curing at 105°C, suggesting an improve in film surface hydrophobicity. A slight reduction of antioxidant activity was observed in CAG-105 film, while heat curing increased the film's opacity which can lower the light transmission though films. The developed film not only had better water barrier, TS and YM but, due to its opacity, could provide light protection, and thus may be applied as active film for foods that degrade when exposed to moisture or light.

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4.6 Conflict of interest statement

The authors declare no conflict of interest.

4.7 Data availability statement

Research data are not shared.

4.8 Ethics approval statement

Ethics approval was not required of this research.

4.9 Annotated references

DIMIDA, S., DEMITRI, C., DE BENEDICTIS, V. M., SCALERA, F., GERVASO, F. & SANNINO, A. 2015. Genipin-cross-linked chitosan-based hydrogels: Reaction kinetics and structure-related characteristics. *Journal of Applied Polymer Science*, 132.

This paper reported the fabrication and characterization of cross-linked chitosan-based material. They decribed the crosslinking interaction between chitosan and genipin crosslinker.

FALAMARZPOUR, P., BEHZAD, T. & ZAMANI, A. 2017. Preparation of nanocellulose reinforced chitosan films, cross-linked by adipic acid. *International Journal of Molecular Science,* 18.

This article studied the chemical crosslinking of nanocellulose-reinforced chitosan films using adipic acid crosslinker and heat curing at different temperatures. Their information about the effects of heat curing and time on film properties are useful for our current research.

INTHAMAT, P., BOONSIRIWIT, A., LEE, Y. S. & SIRIPATRAWAN, U. 2021. Effects of genipin as natural crosslinker on barrier and mechanical properties of chitosan-astaxanthin film. *Journal of Food Processing and Preservation,* 00**,** e15707.

Our current research was extended from this article. This paper investigated the effect of genipin at different concententations on chitosan-based film properties. The result reported the effects of different crosslinker concententations on properties of chitosan based film and the optimal concentration of genipin corsslinker was investigated.

RIVERO, S., A. GARCÌA, M. & PINOTTI, A. 2020. Physical and chemical treatments on chitosan matrix to modify film properties and kinetics of biodegradation. *Journal of Materials Physics and Chemistry,* 1**,** 51-57.

This article investigated the properties chitosan crosslinked with tannic acid. Their results agree well with our current research and they provided useful information about crosslinked chitosan film and its surface hydrophilicity.

CHAPTER V
CONCLUSIONS AND FUTURE WORKS

5.1 Conclusions

The study of thermal curing to improve properties of genipin-crosslinking chitosan film revealed that an increase in the genipin concentration increased the crosslinking degree, tensile strength (TS) and Young's modulus (YM) of the films. After curing with heat, the structure of crosslinking between chitosan and genipin was enhanced. Chitosan film crosslinked with 1.5% genipin and cured at 105 °C (CS1.5G-105) had the lowest water vapor permeability (WVP) value and the most reduced hydrophilic surface. The highest TS, YM and thermal stability values were measured in the CS1.5G-105 film. Therefore, thermal curing of genipin-crosslinked chitosan improved the film properties, increasing the potential for these films to be used in food packaging applications.

The investigation of the effects of genipin as a natural crosslinker on barrier and mechanical properties of chitosan-astaxanthin film demonstrated a successful improvement of the functional properties of chitosan-astaxanthin based film. In fact, the genipin crosslinking decreased the film transparency and increased roughness of the film. The addition of genipin decreased the film crystalline while degree of crosslinking of the film was increased as similarly observed by FTIR analysis with the shifted peak at 1717 and 1569 cm^{-1} which can be associated with an increase of oxygen transmission rate (OTR). Moreover, this crosslinking structure led to significantly decrease WVTR and increased TS of the film. As a result, genipin crosslinking can be an alternative choice in the field of active film.

The study of improving moisture barrier and functional properties of active film from genipin-crosslinked chitosan/ astaxanthin film by heat curing suggested that heat curing was successfully improved the functional properties of chitosan-based film, imparting a high moisture barrier and mechanical properties. Curing at 105°C affected the interactions between chitosan and genipin of the film by initiating new chemical bonds which led to the highest improvement of water barrier, TS and YM. The contact angle of CAG film increased after heat curing at 105°C, suggesting an

improve in film surface hydrophobicity. A slight reduction of antioxidant activity was observed in CAG-105 film, while heat curing increased the film's opacity which can lower the light transmission though films. The developed film not only had better water barrier, TS and YM but, due to its opacity, could provide light protection, and thus may be applied as active film for foods that degrade when exposed to moisture or light.

5.2 Future works

The developed chitosan/astaxanthin films been improved in their moisture barrier and physical properties by crosslinking with genipin and heat curing. The films have high potential to be used as active film food packaging. Therefore, the film can be tested further for its feasibility to maintain quality and extend shelf life of various foods such as beef cut, pork chop and chicken breast etc. Its stability and heat sealability may also be further investigated.

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APPENDIX

Appendix A: Determination of genipin concentration

The calculation of percent crosslinking degree between chitosan and genipin was calculated by determining chitosan concentration at 1.5% (w/v) and genipin concentration at 0.5, 1.0 and 1.5% (w/w of chitosan). Chitosan (Mw, 50-190 kDa) was produced the purity at >/= 90% or 0.914 while genipin was produced the purity at 98% or 0.98. mol of active chitosan monomer between NH_3 and genipin and mol of genipin genipin was calculated using Equation 1 and Equation 2, respectively. Therefore, percent of crosslinking degree between chitosan and genipin was calculated according to Equation 3.

Mol of active chitosan monomer (between NH_3 and genipin) = $purity\ of\ chitosan \times purity\ of\ genipin$ (1)

Mol of genipin = $\frac{genipin\ concentration \times Volue\ of\ chitosan \times purity\ of\ genipin}{Molecular\ weight\ of\ genipin}$ (2)

%Crosslinking = $\frac{genipin\ concentration}{mol\ of\ active\ chitosan\ monomer\ (between\ NH3\ and\ genipin)}$ (3)

Table A1 Percent of crosslinking between chitosan and genipin at different genipin concentration

Genipin concentration (%)	Crosslinking between chitosan and genipin (%)
0.5	36.81
1.0	73.61
1.5	99.09

Appendix B: The optimal properties of chitosan and chitosan/astaxanthin crosslinked film

Table B1 showed that chitosan crosslinked with 1.5% (w/w of chitosan) genipin and chitosan-astaxanthin crosslinked with genipin at 1% (w/w of chitosan) gave optimal film properties based on results in WVP, TS and YM. Therefore CS1.5G and CA-1G were used further to study the effect of heat curing.

Table B1 Thickness, water vapor permeability (WVP), tensile strength (TS), elongation at break (EAB) and Young's modulus (YM) of chitosan and chitosan/astaxanthin crosslinked films.

Sample	Thickness (mm)	WVP $\times 10^{-4}$ g mm/h/mm²/Pa	Mechanical properties		
			TS (MPa)	EAB (%)	YM (N/m²)
CS	0.17±0.03^{a}	2.79±0.19^{a}	7.61±0.94^{e}	122.66±11.02^{a}	8.81±0.61^{f}
CS0.5G	0.17±0.04^{a}	2.64±0.20^{b}	10.67±1.31^{cd}	106.22±2.01^{b}	11.33±2.42^{f}
CS1G	0.17±0.04^{a}	2.48±0.17^{b}	12.09±0.83^{bc}	50.04±8.63^{d}	77.20±4.82^{d}
CS1.5G	0.17±0.03^{a}	1.77±0.11^{d}	14.26±0.37^{a}	44.25±7.98^{d}	105.65±4.19^{c}
CA	0.17±0.04^{a}	2.06±0.20^{c}	10.17±0.34^{d}	65.69±2.46^{c}	38.42±1.81^{e}
CA-0.5G	0.17±0.05^{a}	1.90±0.08^{cd}	11.26±0.63^{cd}	29.95±5.47^{e}	128.30±4.43^{b}
CA-1G	0.16±0.04^{a}	1.87±0.06^{cd}	13.12±1.09^{ab}	26.64±3.54^{e}	141.35±4.66^{b}
CA-1.5G	0.17±0.05^{a}	2.50±0.09^{b}	14.55±0.96^{a}	11.18±2.27^{f}	155.52±4.41^{a}

CS = pure chitosan film; CS0.5G = chitosan crosslinked with 0.5% genipin; CS1G = chitosan crosslinked with 1% genipin; CS1.5G = chitosan crosslinked with 1.5% genipin; CA = chitosan incorporated with astaxanthin; CA-0.5G = chitosan incorporated with astaxanthin and crosslinked with 0.5% genipin; CA-1G = chitosan incorporated with astaxanthin and crosslinked with 1% genipin; CA-1.5G = chitosan incorporated with astaxanthin and crosslinked with 1.5% genipin

Mean (± SD) values within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different

Appendix C: Determination of heat curing

An increasing heat curing temperature significantly decreased water vapor permeability of the film as shown in Figure C1. The WVP value of the film cured at 70-80°C significantly decreased, and probably led to the lowest WVP value of film when curing temperature was increase. According to the results in Figure D1, heat curing could be determined at 80 and 105°C.

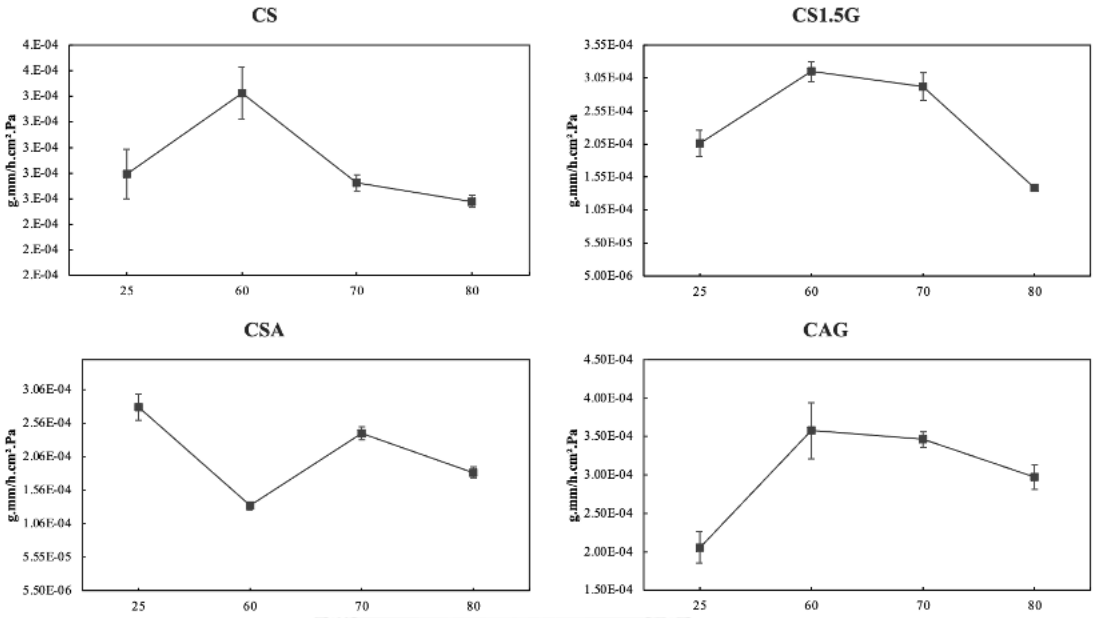

Figure C1 Water vapor permeability of chitosan (CS), CS is crosslinked with 1.5% genipin (CS1.5G), CS is incorporated with astaxanthin (CSA) and CSA is crosslinked with 1% genipin (CAG) films at different temperature

Appendix D: Kinetics reaction for determining heat curing time

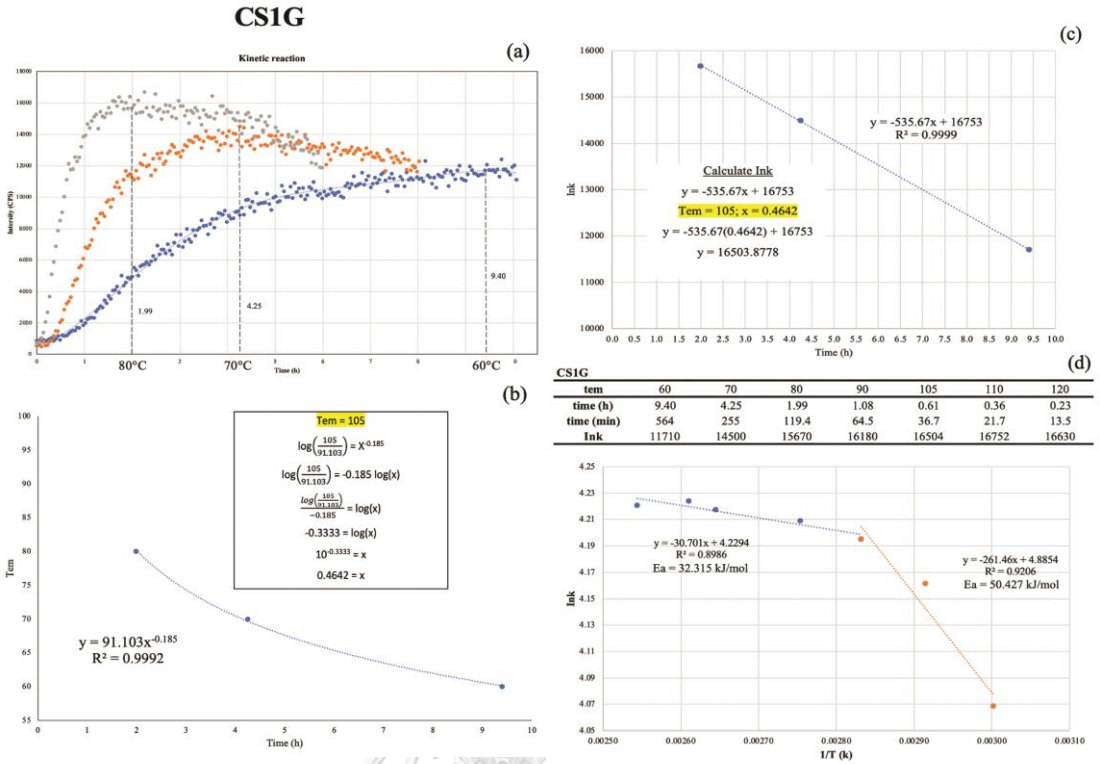

Figure D1 Crosslinking reaction of chitosan crosslinked with 1% genipin (CS1G) at different temperature: (a) Maximum time of crosslinking reaction time; (b) Calculation of maximum time at 105°C; (c) Calculation of intensity kinetic (Ink) at 105°C; (d) Time of complete-crosslinking reaction at different temperature.

Maximum time of the crosslinking reaction of CS1G was showed in Figure D1 (a). For prediction the crosslinking reaction time at 105°C, time and temperature of crosslinking reaction was plotted the line graph, and then calculated based on linear equation for various temperature as shown in Figure D1 (b) and D1 (c), respectively. The results showed that the crosslinking reaction time of CS1G cured at 80°C and 105°C was 119.4 and 36.7 min as displayed in Figure D1 (d).

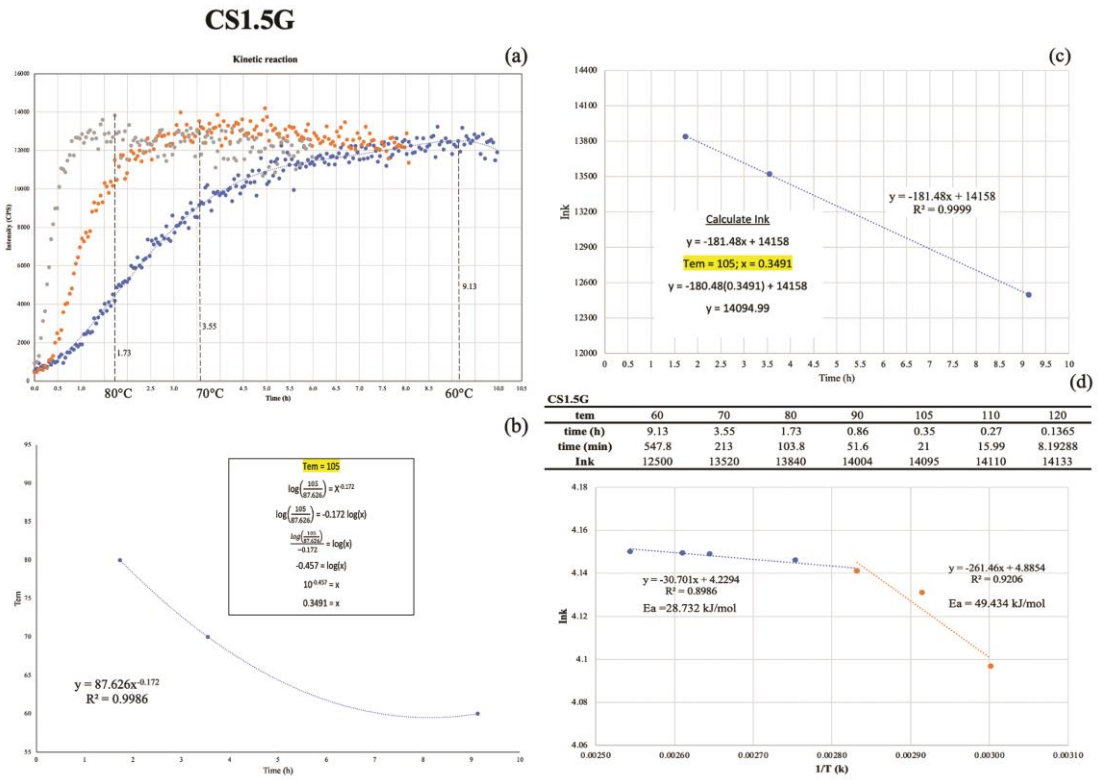

Figure D2 Crosslinking reaction of chitosan crosslinked with 1.5% genipin (CS1.5G) at different temperature: (a) Maximum time of crosslinking reaction time; (b) Calculation of maximum time at 105°C; (c) Calculation of intensity kinetic (Ink) at 105°C; (d) Time of complete-crosslinking reaction at different temperature.

An increasing genipin concentration (CS1.5G) affected to reduce time of the crosslinking reaction when compared to CS1G. Figure D2 (d) showed that an increasing curing temperature induced the crosslinking reaction between chitosan and genipin. CS1.5G cured at 80°C and 105°C had time of the crosslinking reaction at 103.8 and 21 min, respectively. Heat curing at high temperature could catalyze the crosslinking reaction in both CS1G and CS1.5G to be faster completability than low temperature.

However, the crosslinking reaction is probably produced during drying the film at 30°C for 24 h. Therefore, the time for heat curing was determined at 30 min based on maximum time of heat curing at 105°C.

Appendix E: Film properties of chitosan and chitosan/astaxanthin cured at high temperature

Table E1 Thickness, water vapor permeability (WVP), tensile strength (TS), elongation at break (EAB) and Young's modulus (YM) of the cured films.

Sample	Thickness (mm)	WVP $\times 10^{-4}$ g mm/h/mm²/Pa	Mechanical properties		
			TS (MPa)	EAB (%)	YM (N/m²)
CS-25	0.17±0.03^{a}	2.79±0.19^{a}	7.61±0.94^{h}	122.66±11.02^{a}	8.81±0.61^{k}
CS-80	0.18±0.04^{a}	2.64±0.09^{a}	13.73±0.71^{f}	42.85±6.12^{c}	162.19±4.15^{g}
CS-105	0.17±0.02^{a}	1.95±0.13^{bc}	15.22±2.08^{f}	14.04±1.78^{e}	170.24±9.35^{ef}
CS1.5G-25	0.17±0.03^{a}	1.77±0.11^{c}	14.26±1.37^{f}	44.25±7.98^{c}	105.65±4.19^{j}
CS1.5G-80	0.17±0.04^{a}	1.37±0.08^{d}	23.02±3.23^{d}	6.07±2.10^{f}	270.24±6.76^{b}
CS1.5G-105	0.17±0.02^{a}	0.81±0.01^{e}	36.45±0.64^{a}	5.36±0.80^{f}	315.96±1.57^{a}
CSA-25	0.17±0.04^{a}	2.06±0.20^{b}	10.17±0.34^{g}	65.69±2.46^{b}	38.42±1.81^{j}
CSA-80	0.17±0.04^{a}	1.82±0.09^{c}	19.08±2.21^{e}	12.26±1.56^{ef}	167.92±3.50^{fg}
CSA-105	0.17±0.02^{a}	1.42±0.03^{d}	22.75±1.65^{d}	10.05±0.79^{ef}	185.87±7.76^{d}
CAG-25	0.17±0.02^{a}	2.50±0.09^{bc}	13.12±1.09^{f}	26.64±3.54^{d}	128.30±4.43^{h}
CAG-80	0.17±0.02^{a}	1.41±0.02^{d}	27.58±1.53^{c}	9.64±0.40^{ef}	177.09±2.90^{e}
CAG-105	0.17±0.02^{a}	0.84±0.05^{e}	33.63±1.53^{b}	7.36±1.03^{ef}	219.71±9.86^{c}

CS-25, CS-80, CS-105 = pure chitosan with curing at 25°C, 80°C and 105°C, respectively; CS1.5G-25, CS1.5G-80 and CS1.5G-105 = chitosan crosslinked with 1.5% genipin and cured at 25°C, 80°C and 105°C, respectively; CSA-25, CSA-80, CSA-105 = chitosan is incorporated with astaxanthin and cured at 25°C, 80°C and 105°C, respectively; and CAG-25, CAG-80, CAG-105 = chitosan is incorporated with astaxanthin, crosslinked with 1% genipin and cured at 25°C, 80°C and 105°C, respectively

Mean (± SD) values within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

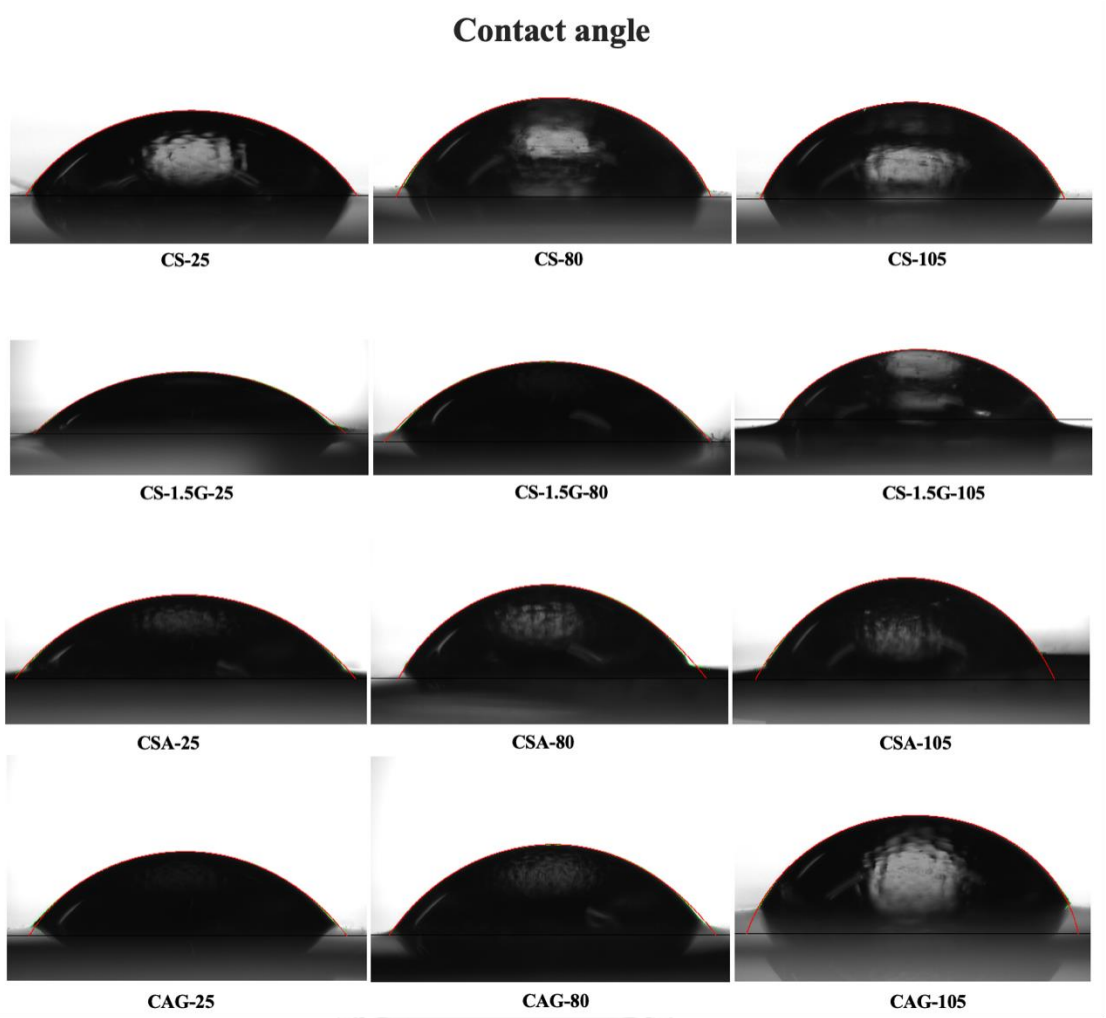

Figure E1 Contact angle of chitosan (CS) film: CS-25, CS-80 and CS-105, where CS is cured at 25°C, 80°C and 105°C, respectively; CS crosslinked with 1.5% genipin: CS1.5G-25, CS1.5G-80; CS1.5G-105, respectively; CS incorporated with astaxanthin (CSA): CSA-25, CSA-80, CSA-105, where CSA is cured at 25°C, 80°C and 105°C, respectively; CSA crosslinked with 1% genipin: CAG-25, CAG-80, CAG-105, where CAG is cured at 25°C, 80°C and 105°C, respectively

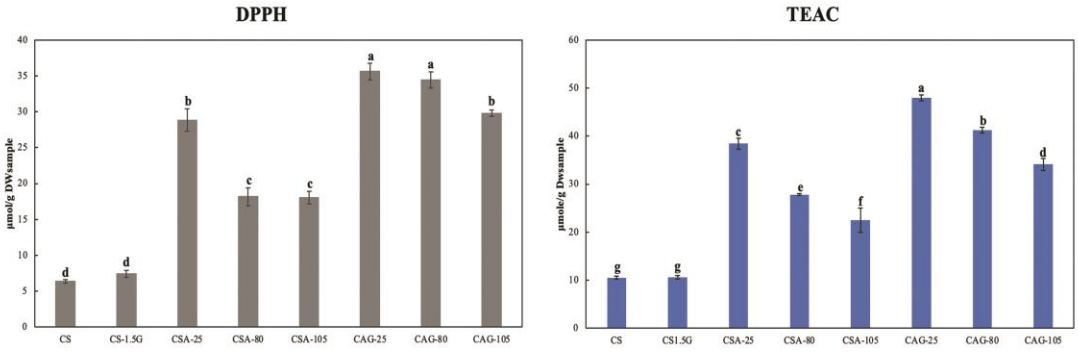

Figure E2 Antioxidant activities of chitosan (CS) film; CS crosslinked with 1.5% genipin (CS1.5G); CS incorporated with astaxanthin (CSA): CSA-25, CSA-80, CSA-105, where CSA is cured at 25°C, 80°C and 105°C, respectively; CSA crosslinked with 1% genipin: CAG-25, CAG-80, CAG-105; where CAG is cured at 25°C, 80°C and 105°C 80°C and 105 °C, respectively; DPPH: 1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay; TEAC: Trolox equivalent antioxidant capacity assay

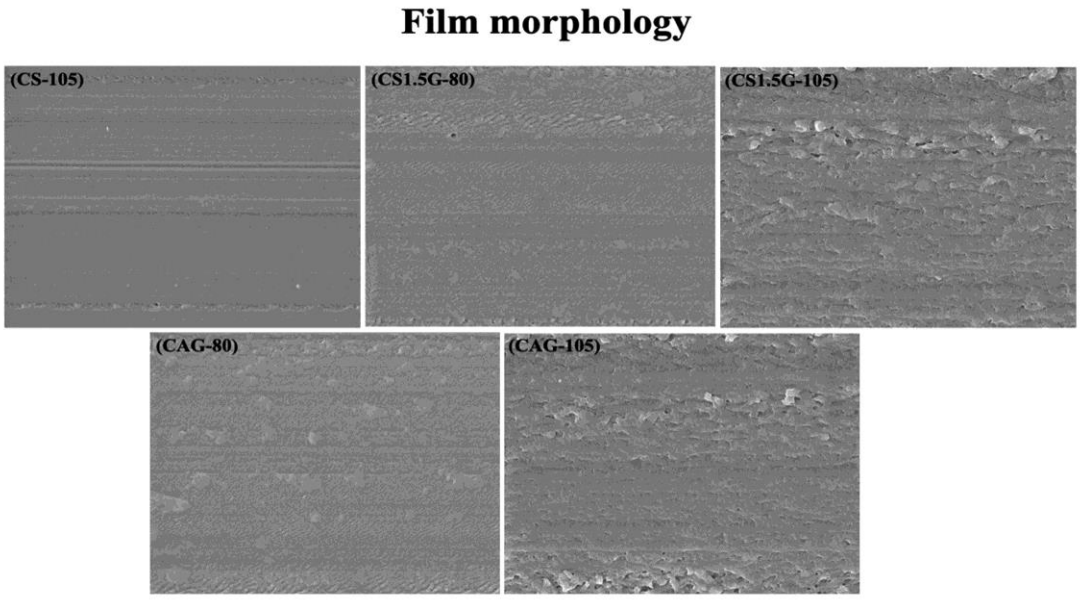

Figure E3 Cross-section scanning electron microspore images of CS-105, where pure chitosan is cured at 105°C; and CS1.5G-80; CS1.5G-105, where chitosan is crosslinked with 1.5% genipin and cured at 80°C and 105°C, respectively; and CAG-80; CAG-105, where chitosan/astaxanthin is crosslinked with 1% genipin and cured at 80°C and 105°C, respectively

Table E2 Crystallinity and thermal stability of the cured films.

Sample	Crystallinity (%)		Melting point (°C)	
	Amorphous	Crystalline	P1	P2
CS-25	25.26	74.74	110.51	190.43
CS-80	29.40	70.60	115.03	194.68
CS-105	29.61	70.38	115.62	194.84
CS1.5G-25	32.17	67.84	112.59	194.33
CS1.5G-80	32.16	67.80	116.14	194.92
CS1.5G-105	34.60	65.40	116.89	195.83
CSA-25	29.45	70.55	108.68	192.63
CSA-80	31.38	68.62	114.70	194.89
CSA-105	31.04	68.96	115.31	195.31
CAG-25	30.55	69.45	111.86	192.45
CAG-80	31.37	68.63	114.72	192.57
CAG-105	32.63	67.37	116.13	195.59

CS-25, CS-80, CS-105 = pure chitosan with curing at 25°C, 80°C and 105°C, respectively; CS1.5G-25, CS1.5G-80 and CS1.5G-105 = chitosan crosslinked with 1.5% genipin and cured at 25°C, 80°C and 105°C, respectively; CSA-25, CSA-80, CSA-105 = chitosan is incorporated with astaxanthin and cured at 25°C, 80°C and 105°C, respectively; and CAG-25, CAG-80, CAG-105 = chitosan is incorporated with astaxanthin, crosslinked with 1% genipin and cured at 25°C, 80°C and 105°C, respectively

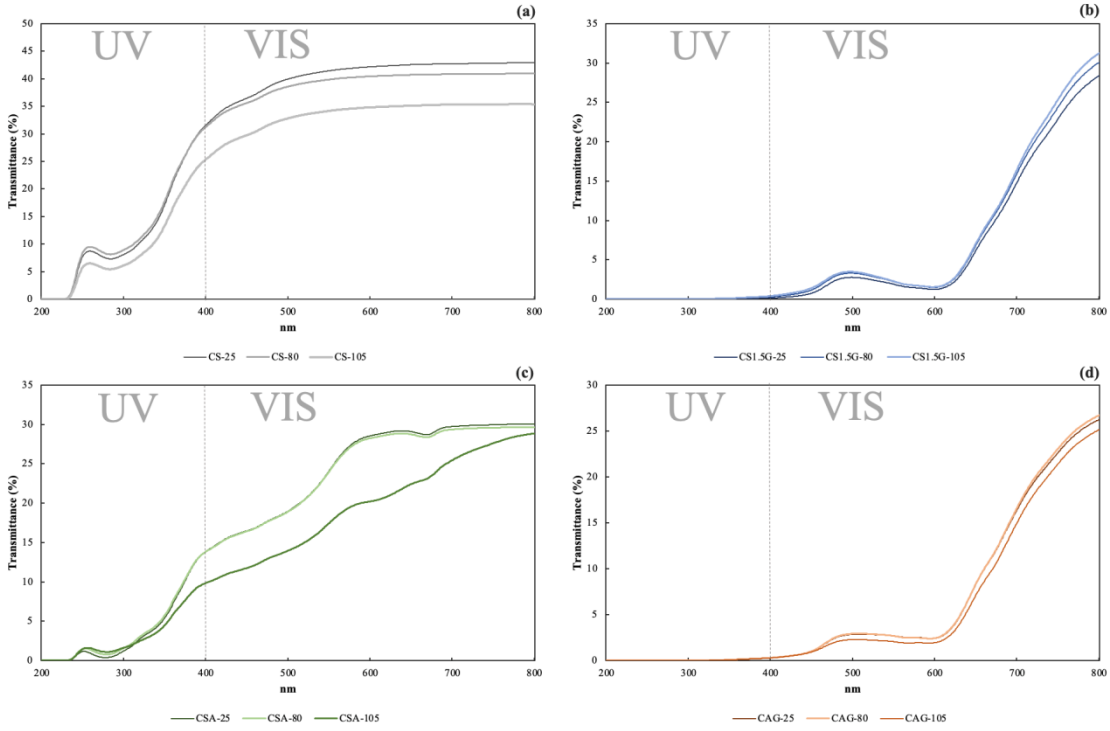

Figure E4 UV-blocking property of chitosan (CS) and chitosan incorporated with astaxanthin (CSA): (a) CS-25, CS-80 and CS-105, where CS is cured at 25°C, 80°C and 105°C, respectively; (b) CS1.5G-25, CS1.5G-80; CS1.5G-105, where CS is crosslinked with 1.5% genipin and cured at 25°C, 80°C and 105°C, respectively; (c) CSA-25, CSA-80, CSA-105, where CSA is cured at 25°C, 80°C and 105°C, respectively; (d) CAG-25, CAG-80 and CAG-105, where CSA is crosslinked with 1% genipin and cured at 25°C, 80°C and 105°C, respectively

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VITA

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