Characterization of allergenic and antimicrobial properties of chitin and chitosan and formulation of chitosan-based edible film for instant food casing

A thesis submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy

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Declaration

I certify that the work described in this thesis was carried out while I was an enrolled student for the degree of Doctor of Philosophy in the School of Applied Sciences at Royal Melbourne Institute of Technology (RMIT) University. Except where due acknowledgement has been made, the work is that of the author alone. This work has also not been submitted previously in whole or in part to qualify for any other academic award.

Signature:……. (Minh Xuan Hong Nguyen)

Date:……

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List of Abbreviations

AAS	Atomic absorption spectroscopy
ACIAR	Australian Centre for International Agricultural Research
AN01 serum	Serum from patient who has allergic symptom with crustacean
AOAC	Association of Official Agricultural Chemists
BSA	Bovine serum albumin
CLSI	Clinical and Laboratory Standards Institute
CRUS	Total crustacean protein
CT	Chitin
CTS	Chitosan
DD	Degree of deacetylation
dH_2O	Deionised water
DMAc	Dimethylacetamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
g	Gram
h	Hour
HMW	High molecular weight
HRP	horseradish peroxidase
IC50	50 % inhibition value
IEC	Ion-Exchange chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
JB21 serum	Serum from patient who has allergic symptom with crustacean
kDa	kilo Dalton
LB agar	Luria Bertani agar

LB broth	Luria Bertani broth
LiCl	Lithium chloride
mAbαTM	Monoclonal antibody against tropomyosin
MBC	Minimum Bactericidal Concentration
MC	Dichloromethane
MDa	Million Dalton
ME	Mercaptoethanol
MHA	Mueller Hinton agar
MIC	Minimum inhibition concentration
min	Minute
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
NBT/BCIP	Nitro-blue tetrazolium chloride / 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
nCTS	Normal chitosan
NPN	1-N-phenylnaphthylamine
OD	Optical density
OD	Optical density
OPP	Oriented Polypropylene
pAbαCR	Polyclonal antibodies against crustacean protein
PBS-T	Phosphate buffer saline - Tween
PCA	Plate count agar
pCTS	purified chitosan
PE	Polyethylene
PVDF	Polyvinylidene diflouride
SDS	Sodium dodecyl sulphate
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
STD	Standard of derivation
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethylene-ethylenediamine
TMB	3,3'-5,5' tetramethylbenzidine
TROP	Purified tropomyosin
WVP	Water vapor permeation

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SUMMARY

Crustacean shell waste contains 20 – 30 % chitin which can be extracted to produce more valuable products such as chitosan, glucosamine and their derivatives. Due to their natural source, they are non-toxic, biodegradable and biocompatible. They have been applied in a wide range of industries, especially in the production of functional foods, pharmaceutics and cosmetics due to their binding, film-forming and antimicrobial properties. Although the safety of these products is highly regarded, some residual proteins from shrimp shell such as tropomyosin and arginine kinase could remain in these products and cause allergic reactions among consumers. Therefore, chitin and chitosan properties must be characterized in detail before applying them, especially in the food and drug industry.

At present, chitosan-based edible films are already used as a new material for food packaging. However, there is still very limited research in the application of chitosan film for instant food products due to its poor solubility in water. Therefore, it is necessary to mix chitosan with other water-soluble materials to apply it to instant food casing.

This study is aimed at investigating the allergenicity of residual shrimp proteins in chitin and chitosan samples. Antimicrobial property of normal chitosan was examined and compared with purified chitosan. The film-forming property of chitosan and some biopolymers was also characterized to formulate an edible film which is soluble and sealable to make spice sachets for instant noodles.

At the beginning, the study focuses on the production of rabbit polyclonal antibodies. These antibodies were raised by injecting antigens into rabbits to generate specific antibody containing sera. Two types of antigens were prepared. The first antigen was the purified tropomyosin from four different shrimp species. The second antigen was the mixed protein extracted from three crustacean species. The injection of antigens and collection of rabbit sera was conducted by a specialised company in Australia and not conducted at the University. These sera were analysed for titre and immunoreaction with different immunogens. The generated sera were used to investigate the immunoreactions of different shrimp extracts as well as residual shrimp proteins and allergens in chitin and chitosan preparations.

Next, the study focuses on the identification of the allergenic protein tropomyosin in different protein extracts of the two most common shrimp species sold in the Australian market: Black Tiger shrimp and Banana shrimp. Six different protein extractions from each shrimp species were prepared by different methods: raw extraction, heat-treated extraction and whole-cooked extraction. These protein extracts were then compared according to their SDS-PAGE profiles, immunoblotting reactions by Western blotting as well as cross-reaction through inhibition ELISA. The result demonstrated that the allergenic tropomyosin protein not only presents in the tail meat but also in the shell extracts of shrimp. The tropomyosin seemed to be more stable to heat processing in whole-cooked extracts. The IC50 values of rabbit sera demonstrate strong cross-reactivity of both sera to most extracts with the highest concentration of tropomyosin in cooked shrimp.

The allergenic protein tropomyosin in the shrimp shell may be present in the residual proteins in chitin and chitosan samples. Therefore the characterisation of possible allergenic residual proteins in chitin and chitosan has been conducted. Numerous methods were used in an attempt to pull the proteins out of the chitin and chitosan samples. However, due to the small amount of protein residue and the resistant property of tropomyosin, it is difficult to isolate the protein residue from chitin and chitosan using SDS-PAGE and Western blot. However, the Dot blot results demonstrated that there is a certain amount of the tropomyosin remaining in the chitin and chitosan samples. The inhibition ELISA also confirmed the immunoreactivity of the protein residue in chitin and chitosan samples to rabbit sera which are specific to crustacean protein and shrimp tropomyosin. This result could somewhat explain the reason that some people may get adverse reactions when dealing with products containing chitin and chitosan.

The next section of the study focuses on the antimicrobial properties of chitosan. Firstly, the biochemical properties of some chitin and chitosan products were characterized based on their moisture content, ash content, heavy metal content, and protein content. The results indicated that these chitin and chitosan samples have acceptable biochemical properties that are suitable for application in the food industry. The protein content of normal chitosan samples was less than 1 %, while it cannot be detected in the purified sample. Some variability in the results indicated that chitin and chitosan need to be characterized and standardized for their properties before application in the food industry. Secondly, the antimicrobial property of normal chitosan at different chitosan concentrations against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* was examined. The results showed that chitosan at 0.05 % and 0.1 % concentration can inhibit the growth of both *E. coli* and *S. aureus* after 4 hours of incubation.

After that, the antimicrobial properties between normal chitosan and purified chitosan were compared to each other by MIC, MBC and disc diffusion methods. The results demonstrated that purified chitosan still retains its antimicrobial property comparable to the normal chitosan. Finally, the application of the normal and purified chitosan in the preservation of shrimp was a confirmation of the antimicrobial property of chitosan. Both normal and purified chitosan can inhibit the outgrowth of bacteria on shrimps during 15 days of storage at 0 – 4 ^{o}C while limiting weight loss and maintaining hardness better than in the non-treated shrimps.

In the following work, the film forming capacity of chitosan has been investigated to find appropriate methods for making chitosan film. In addition, the film forming ability of some other biopolymers were also examined to find out which polymer is best to combine with chitosan to make films with desired properties. The composite edible film was investigated for its formulation as well as antimicrobial properties. The use of this edible film in spice casing for instant noodles is an attempt to apply chitosan-based films in the instant food industry. This study found that the neutralization process, the degree of deacetylation of chitosan and the chitosan concentration affected significantly the solubility of chitosan film. Among the tested biopolymers, gelatin showed the best appropriate properties to blend with chitosan in forming soluble and sealable edible film. The composition of 0.5 % chitosan and 5 % gelatin has been shown as the most appropriate formulation to make the desired edible film. This edible film showed excellent antimicrobial property to both Gram-negative and Gram-positive bacteria. Therefore, it could be used in the preservation of food products. This composite chitosan / gelatin edible film was applied in making seasoning and oil sachets for instant noodles to examine the feasibility of this film. The spice stored in these edible film sachets can still maintain quality after 6 months of storage. These sachets should be kept in another container to prevent the exposure to humidity, but overall, this edible film is suitable for application in spice casings for instant noodles.

The current study confirmed the presence of shrimp protein residue in chitin and chitosan samples and associated strong immunoreactivity with specific antibodies against the shellfish allergen tropomyosin, as well as with crustacean - sensitized patient sera. This indicated the presence of the major allergen tropomyosin and the potential risk of allergic reactions in sensitized consumers of contaminated chitosan products. The purification did not affect the antimicrobial property of chitosan. So, commercial chitosan should be purified before applying in the food industry to protect consumers from allergic reactions. In addition, a combination of chitosan and gelatine formed a water-soluble and sealable edible film which can be applied to instant food casing.

Chapter 1
INTRODUCTION

1.1 OVERVIEW OF CHITIN AND CHITOSAN

1.1.1 Source and structure of chitin and chitosan

Chitin, which was first identified in 1884 (Rinaudo, 2006), is among the most abundant natural polymers in the world, just after cellulose. It is a linear polysaccharide consisting of (1-4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (Hudson and Jenkins, 2002; Ravi Kumar, 1999) (Figure 1.1). It functions as a structural polysaccharide in animals. Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas (Nair and Madhavan, 1992)

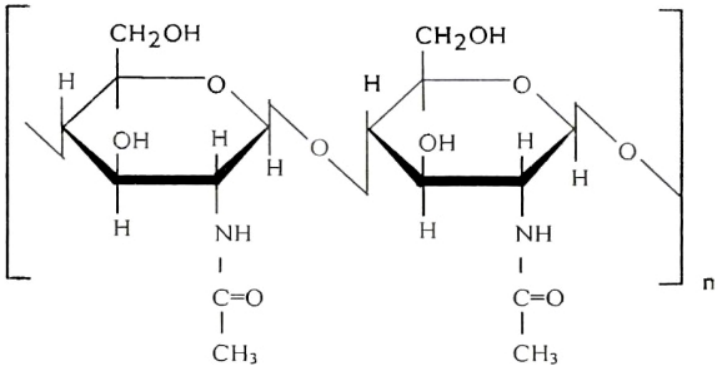


Figure 1.1: Structure of chitin

Chitin can be found in a wide variety of sources such as in the cell walls of fungi of *Zygomycetes*, in the green algae *Chlorella*, yeast and protozoa as well as insect cuticles and especially in the exoskeletons of crustaceans (Alishahi and Aïder, 2011; Rinaudo, 2006). Shells of crustaceans such as crab, shrimp, and crawfish contain approximately 15-40 % chitin (Kurita, 2006) (Table 1.1). It is produced to an amount of a hundred billion tons each year by these natural sources (Rege and Block, 1999).

Chitin is extracted from crustacean shell through two major steps. Deproteination is taken place at diluted alkaline conditions while demineralization occurs at diluted acidic conditions. Chitin is then converted into chitosan through the deacetylation at very strong alkaline conditions with high temperature (Stevens, 2000). The conversion of chitin to chitosan can be

carried out effectively at milder condition by enzymatic method (Stevens, 2005) to remove the acetyl groups from chitin structure. However, because chitin exits as a naturally partially deacetylated form depending on the source (Rinaudo, 2006), it is very difficult to clearly distinguish between chitin and chitosan. Therefore, the term chitin and chitosan can be used interchangeably. Usually, the term chitosan is used when there is more than a 50% degree of deacetylation.

Table 1.1: Contents of chitin and calcium carbonate (Kurita, 2006)

Source	Chitin (%)	$CaCO_3$ (%)
Crab cuticle	15–30	40–50
Shrimp cuticle	30–40	20–30
Krill cuticle	20–30	20–25
Squid pen	20–40	Negligible
Clam/oyster shell	3–6	85–90
Insect cuticle	5–25	Negligible
Fungi cell wall	10–25	Negligible

Chitosan, obtained from chitin by deacetylation, is a linear polysaccharide consisting of (1-4)-linked 2-amino-2-deoxy-β-D-glucopyranose (Figure 1.2). In the solution, the $–NH_2$ groups at C-2 position of chitosan structure are protonated to form the NH_3^+ cations. Due to its relatively higher polyelectrolyte nature, chitosan has more active properties than chitin such as adsorption, film-forming and antimicrobial properties which brings chitosan to wide applicability (Stevens, 1996).

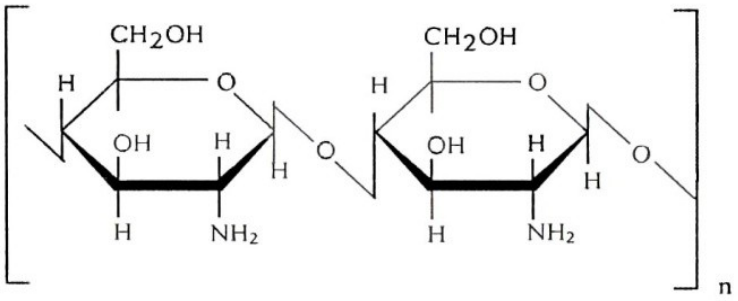


Figure 1.2: Structure of chitosan

Chitosan is a reactive polymer susceptible to many chemical modifications (Muzzarelli, 1983). Many derivatives from chitosan have been investigated for their properties and application (Table 1.2).

Table1.2: Chitosan Derivatives and Proposed Uses (Muzzarelli, 1983)

Chitosan Derivatives	Proposed Uses
N-Acyl chitosans	acetyl, propionyl, butyryl, hexanoyl, octanoyl, decanoyl, dodecanoyl, tetradecanoyl, lauroyl, myristoyl, palmitoyl, stearoyl, benzoyl, dichloroacetyl, carbamoyl (textiles, membranes)
N-Carboxyalkyl chitosans	N-carboxymethyl (metal ion collection)
N-Carboxyacyl chitosans	from anhydrides such as: maleic, itaconic, (acetylthio)-succinic, glutaric, cyclohexane-l,2-dicarboxylic, phthalic, *cis*-tetrahydrophthalic,5-norbornene-2,3-dicarboxylic, diphenic, salicyl
O-Carboxyalkyl chitosans	O-carboxymethyl, epichlorohydrin-cross-linked Ocarboxymethyl (membranes, molecular sieves)
Deoxyglycit-l-yl chitosans	1-deoxygalactit-l-yl, 1-deoxyglucit-l-yl, 1-deoxymelibiit-1-yl, 14actit-l-yl (gels, drilling muds)
Metal ions - chitosan chelates	palladium, copper, silver, iodine (catalysis, photography, health products, insecticides)
Semi-synthetic resins of chitosan	methyl methacrylate, poly(urea-urethane) poly(amide-ester), acrylamide-maleic anhydride-ehitosan copolymer)
Natural polysaccharide complex	chitosan-glucan (flocculation, metal ion chelation)
Miscellaneous	alkali chitin (intermediate), benzyl chitin (serine protease purification), hydroxybutyl chitosan (desalting), cyanoethyl chitosan (filtration, dialysis, insulating papers), glycol chitosan (dialysis, special papers), glutaraldehyde chitosan (enzyme immobilisation), linoleic acid--chitosan complex (food additive, anticholesterolaemic), uracyl chitosan, theophylline chitosan, adenine chitosan, chitosan salts of acidic polysaccharides.

1.1.2 Properties of chitin and chitosan

Chitin and chitosan have higher nitrogen content (6.89 %) than synthetically substituted cellulose (1.25 %), thus make them more attractive for many industrial applications (Rathke and Hudson, 1994). Due to their natural sources, chitin and chitosan are considered biocompatible, biodegradable and non-toxic (Muzzarelli and Muzzarelli, 2005).

Table 1.3: Specification of chitin and chitosan (technical grade) in the world market (Changrkrachang, 1996)

Parameter	Commercial chitin			Commercial chitosan		
	England	Norway	India	England	Norway	India
Source	-	Shrimp shells	-	-	-	-
Form	-	Flakes	-	Ground	-	-
Color	-	White to yellow	-	White	-	-
Moisture content (%)	≤ 8	≤ 10	< 10	≤ 10	< 10	-
Ash content (%)	≤ 2	≤ 1	< 2	≤ 1	< 1.5	< 1
Heavy metal content (ppm)	-	≤ 10		-	-	-
Deacetylation degree (%DD)	≥ 15	-	-	80 - 90	> 70	-
Protein content (%)	≤ 3-4	-	-	-	-	-
Viscosity (cps)	-	-	-	a) 20 b) 50-150 c) 200-400 d) 400-1000 e) >1000	> 200	a) 200-800 b) 800-2000 c) >2000
Insoluble				< 0.5	< 2	< 0.5

Note: - = Data is not available

While most of the naturally occurring polysaccharides such as cellulose, dextran, pectin, alginic acid, agar, agarose, carragenans are neutral or acidic in nature, chitosan is a highly basic polysaccharide (Rinaudo, 2006).

The properties of chitin and chitosan depend largely on their molecular weight and the degree of deacetylation (DD). Chitosan is characterized by either the degree of acetylation (DA), which corresponds to N-acetylamine groups or the degree of deacetylation (DD) (DD=100-DA), D-glucosamine groups. The degree of deacetylation has an influence on the physicochemical properties of chitosan (viscosity, solubility, swelling index, etc.) (Stevens, 1996).

1.1.2.1 Solubility

Due to its rigid structure, chitin is a highly insoluble material with low chemical reactivity. This is the major reason that limits the development of processing and uses of chitin. Chitin can dissolve in strong inorganic salts such as LiCNS, $Ca(CNS)_2$, CaI_2, $CaBr_2$, or strong acid and polar solvents such as trichloroacetic acid (TCA), dichloroacetic acid (DCA), lithium chloride – tertiary amide solvent systems, calcium chloride – dihydrate-saturated methanol solvent system (Pillai *et al.*, 2009).

While chitin is insoluble in water, alkaline solution and most organic solvents, chitosan is readily soluble in aqueous inorganic acids with pH below 6.0. There are some critical factors that contribute to chitosan solubility such as temperature and time of deacetylation, alkali concentration, prior treatments applied to chitin isolation, ratio of chitin to alkali solution, particle size, etc. (Pillai *et al.*, 2009). Acetic acid is a common solvent to dissolve chitosan.

1.1.2.2 Viscosity

When dissolving in aqueous organic acid, chitosan forms a very viscous solution. This is due to its high molecular weight and linear unbranched structure. Thus, chitosan can be used as a thickening agent in an acidic environment. In some acids the concentration ratio between chitosan and acid affects the viscosity of the solution (Kristl *et al.*, 1993).

1.1.2.3 Adsorption property

Chitosan acts as a cationic substance in acidic solution. Therefore it can bind to other anionic substances. Chitosan with molecular weight of 0.7×10^6 has high dye binding capacity (No *et al.*, 2007). However, in low pH conditions (pH less than 5), chitosan gradually dissolves and

therefore loses its function as an absorbent. Due to its porous polymer structure, affinity for water and ionic properties, chitosan can bind to water, protein, fat, and metal (Knorr, 1983; Shafaei *et al.*, 2007; Trung *et al.*, 2003).

1.1.2.4 Film-forming property

Chitin and chitosan belong to very interesting family of β-(1-4) linked polysaccharides. This kind of glycosidic linkage leads to relatively extended and rigid structures in the solid state, particularly in the dry state. As a consequence, these polymers, as cellulose, have a good film and fiber forming capability (Muzzarelli, 1983). The positively charged groups on chitosan interact with opposite charged groups of the solvent yield three-dimensional networks (Khoshgozaran-Abras *et al.*, 2012). Chitosan films are tough, long-lasting, flexible and very difficult to tear (Shahidi *et al.*, 1999). Chitosan films have moderate water vapour permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values (Shahidi and Abuzaytoun, 2005; Shahidi *et al.*, 1999). It exhibits an excellent barrier to oxygen permeation (Butler *et al.*, 1996).

The properties of the chitosan films depend on intrinsic characteristics of chitosan (source, molecular weight, degree of deacetylation), type and amount of solvents, plasticizers, copolymers, dispersants, and method for film preparation and storage condition (Caner *et al.*, 1998; Khoshgozaran-Abras *et al.*, 2012). The mechanical strength of the film increases with an increase in the molecular weight and the degree of deacetylation of chitosan (Chen *et al.*, 2011; Chen *et al.*, 1996; Wan *et al.*, 2003)..

1.1.2.5 Antimicrobial property

The antimicrobial property of chitosan is often examined based on its bacteriostatic and bactericidal properties (Tayel *et al.*, 2010). The antimicrobial property of chitosan is interesting for the food industry due to its natural, biocompatible and non-toxic attributes (Dutta *et al.*, 2009). Chitosan is often used in the form of a solution or coating film for food preservation. Many studies showed that chitosan can express a high inhibition rate to a wide range of microorganisms (Gram positive and Gram negative bacteria, fungi and yeast) at a low concentration, and thus assist in maintaining food safety (Kanatt *et al.*, 2008a, 2008b; Sagoo *et al.*, 2002; Zheng and Zhu, 2003). Chitosan is known as more effective against fungi and yeast compared to Gram positive and Gram negative bacteria (Rhoades and Rastall, 2003). Chitosan can also suppress viral infection (Chirkov, 2002).

Chitosan can inactivate different pathogenic bacteria including *Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Pseudomonas spp* at low concentration (MIC = 0.05 %) in four hours (Kanatt *et al.*, 2008b). In the case of Gram-negative *Salmonella typhimurium*, the growth of the bacterium was inhibited for 4 hours in the presence of 0.1 % chitosan but not for 24 hours of incubation.

In recent studies, it was discovered that chitosan extracted from fungi such as *Mucor, Zygomyces, Rhizomucor* has better antimicrobial properties than chitosan extracted from crustaceans (Tajdini *et al.*, 2010; Tayel *et al.*, 2010).

1.1.2.5.1 Mode of antimicrobial action of chitosan

The exact antibacterial mechanism of chitosan is unclear but there are many suppositions. The most supportive hypothesis is related to the solubility of chitosan in acid condition. It is presumed that positively charged NH_3^+ in glucosamine inhibit the growth of bacteria by binding to the negatively charged cell wall. Some reports demonstrated that chitosan-treated cell walls of bacteria were weakened and frayed, which may lead to leakage of intracellular materials such as electrolytes, proteins, nucleic acids, through changes in the membrane permeability (Fernández-Saiz and Lagaron, 2011; Tang *et al.*, 2010; Tayel *et al.*, 2010). The antimicrobial activity can be due to the metal binding capacity of chitosan which can inhibit various enzymes in the cell that lead to the death of the microorganism (Darmadji and Izumimoto, 1994; Dutta *et al.*, 2009; Tayel *et al.*, 2010). Chitosan also has the capacity to absorb nutrients and thereby inhibit the growth of microorganisms (Knorr, 1983; Tayel *et al.*, 2010)

Helander *et al.* (2001) studied about the effect of chitosan on Gram-negative bacteria by measuring the uptake of the hydrophobic probe 1-N-phenylnaphthylamine (NPN). The increase of NPN uptake is equivalent to the decrease of barrier function of the outer membrane. The study demonstrated that chitosan binds to the outer membrane and destroys its barrier function (Helander *et al.*, 2001).

Chitosan has different actions towards Gram-negative and Gram-positive bacteria. Chitosan prevents the transfer of nutrients into Gram-positive cells by covering its outer membrane; however, chitosan with low molecular weight can enter the cell and inhibit DNA synthesis (Dutta *et al.*, 2009). In another study, the greater effect of chitosan on Gram-positive compared to Gram-negative bacteria was explained by the difference in the cell wall structure

of these bacteria (Fernández-Saiz and Lagaron, 2011). The main components of Gram-positive cell walls, peptidoglycan (50 – 90 %) and the anionic teichoic acid, bind with the NH_3^+ group of chitosan.

The antifungal property of chitosan was also confirmed by rupturing of the outer membrane of the fungus and was not related to the sequestration of microbial nutrients (Tajdini *et al.*, 2010).

1.1.2.5.2 Factors affected the antimicrobial of chitosan

The antimicrobial activity of chitosan is affected by its intrinsic characteristics (degree of deacetylation, molecular weight, concentration, viscosity), by the environment (pH, temperature), microbial species, status of the microorganism or the nutrient composition. The type of acid used in the preparation of chitosan solution influences antimicrobial activity. Chitosan prepared with acetic acid exhibits more immediate antifungal effects as compared with chitosan prepared with lactic acid (Cuero et al., 1990; 1991).

1.1.2.5.2.1 Degree of deacetylation (DD)

The DD index affects not only the physical and mechanical properties of chitosan film but also its antimicrobial properties. A higher DD will increase the number of NH_3^+ groups, thus raising the solubility of chitosan in acid environments and the binding with negative components on the cell wall (Aider, 2010).

The study of Tayel (2010) on the antimicrobial activity of fungal chitosan with different DD against eleven bacterial strains showed that chitosan with the highest DD (95 %) had the lowest minimal inhibitory concentration indicating the highest antimicrobial effect (Table 1.4).

1.1.2.5.2.2 Molecular weight (MW)

The molecular weight of chitosan has different effects on Gram-negative and Gram-positive chitosan. The study of Zheng and Zhu (2003) showed that chitosan with lower MW has greater effects on Gram-negative *E. coli* (Chitosan with MW < 5 kDa can inhibit 100 % *E. coli* at 0.25 % concentration) (Table 1.5). In contrast, the antimicrobial activity of chitosan against Gram-positive *S. aureus* increases as MW increases (Table 1.6).

Table 1.4: Minimal inhibitory concentrations (MIC) (mg/ml) of different chitosan types against determined bacterial strains (means of triplicates) (Tayel *et al.*, 2010)

Bacterial strains	MIC (mg/ml)			
	CTS1 (84.9 % DD)	CTS2 (83 % DD)	CTS3 (95 % DD)	CTS4 (84.9 % DD)
Escherichia coli1	2.0	2.0	1.25	1.75
Staphylococcus aureus 1	0.75	1.0	0.75	0.5
Samonella typhimurium	2.5	2.0	2.0	2.0
Pseudomonas fluorescens	2.25	3.25	1.75	2.25
Psedomonas aeruginosa	2.0	2.0	1.5	1.75
Bacillus subtilis	1.5	1.25	0.75	1.0
Escherichia coli O157:H7	2.25	1.75	1.75	1.5
Sarcina lutea	1.0	1.75	1.0	0.75
Sarratia marcescens	1.75	1.75	1.25	2.0
Staphylococcus aureus 2	1.75	1.5	1.0	1.25
Escherichia coli 2	2.5	1.75	1.5	2.25

Table 1.5: The antimicrobial effect of chitosan on *E. coli* (Zheng and Zhu, 2003)

MW (kDa)	Inhibition rate η (%)			
	Chitosan concentration			
	0.25 %	0.5 %	0.75 %	1.0 %
< 5	50	60	90	100
48.5	30	80	90	100
72.4	5	10	50	100
129	0	5	90	100
166	0	40	80	100
305	0	40	50	100

Table 1.6: The antimicrobial effect of chitosan on *S. aureus* (Zheng and Zhu, 2003)

MW (kDa)	Inhibition rate η (%)			
	Chitosan concentration			
	0.25 %	0.5 %	0.75 %	1.0 %
< 5	0	0	0	0
48.5	0	95	99	100
72.4	0	96	99	100
129	40	100	99	100
166	95	100	100	100
305	99	100	100	100

Chitosan only has its antimicrobial activity within a certain MW range. Chitosan with MW below 1.4 kDa or above 400 kDa showed very weak antimicrobial effect (Zheng and Zhu, 2003).

Similarly, chitosan oligomers cannot inhibit the growth of bacteria in sausages (Jo *et al.*, 2001) while N-acetylated chitosan cannot inhibit *E. coli*, *S. aureus* and *Candida albicans* (Qin *et al.*, 2006).

1.1.2.5.2.3 pH

Many studies showed that the antimicrobial activity of chitosan increases in low pH conditions. This is because more cationic NH_3^+ groups are formed (Tang *et al.*, 2010) and also the effect of acidic solutions in preventing the growth of bacteria.

Chitosan inhibited *Salmonella typhimurium* and *Serratia marcescens* more at pH 4 than pH 4.5 (Tayel *et al.*, 2010). A higher number of *S. aureus* were killed at pH 6.2 (3 log cfu/ml) than at pH 7.4 (1.5 log cfu/ml) (Fernández-Saiz and Lagaron, 2011). Chitosan with 0.005 % concentration at pH 4 exhibited 100 % inhibition rate to *Candidan lambica* while lengthened the lag phase and had no inhibition at pH 6.

1.1.2.5.2.4 Other factors

The incubation temperature when testing the microorganism or when testing the preservation of food also affects the antimicrobial of chitosan (Tayel *et al.*, 2010).

The age of bacteria also has an effect on the antimicrobial properties of chitosan. *Staphylococcus aureus* is more sensitive to chitosan at its mid log phase (Fernández-Saiz and Lagaron, 2011).

The ingredients of food such as starch, whey protein, sodium chloride and essential oil were recognised to enhance the antimicrobial activity of chitosan.

1.1.2.6 Health and safety factors

Chitin and chitosan have very low toxicity. Therefore, they can be used as a dietary fiber for osteoarthrosis treatment, body weight control and lowering of blood plasma cholesterol level (Hudson and Jenkins, 2002).

The toxicity of chitosan examined by Knorr (1984) indicated that only concentrations above 18 g of free chitosan /kg of body weight/day were harmful to mice.

Chitosan was also confirmed for its non-toxicity for use in excipients (Paul, 2010).

1.1.3 Application of chitin and chitosan in food industry

Due to its excellent properties such as adsorption, film-forming and antimicrobial properties, chitosan has a wide range of application in many industrial fields such as food, biotechnology, paper manufacture, cosmetics, agriculture, environment and medical fields (Fernández-Saiz and Lagaron, 2011; Knorr, 1983; Kristl *et al.*, 1993; Muzzarelli and Muzzarelli, 2005; Muzzarelli, 1983; Nair and Madhavan, 1992; Ravi Kumar, 1999; Stevens, 2005; Synowiecki and Al-Khateeb, 2003; Trung *et al.*, 2003)

In the food industry, chitosan is used in edible films, immobilization of enzymes, antimicrobial activity, additives (color stabilization agent, emulsifying agents, antioxidant agents, and gelling agents), dietary fiber (fat entrapment, glucose dialysis, and water holding capacity) (Agulló *et al.*, 2003; Aider, 2010; Alishahi and Aïder, 2011; Kanatt *et al.*, 2008b; No *et al.*, 2007). Some important applications of chitosan in the food industry are presented in Table 1.7.

Table 1.7: Food applications of chitin, chitosan and their derivatives in the food industry (Shahidi *et al.*, 1999)

Area of application	Examples
Antimicrobial agent	Bactericidal Fungicidal Measure of mould contamination in agricultural commodities
Edible film industry	Controlled moisture transfer between food and surrounding environment Controlled release of antimicrobial substances Controlled release of antioxidants Controlled release of nutrients, flavours and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control Controlled enzymatic browning in fruits Reverse osmosis membranes
Additive	Clarification and deacidification of fruits and beverages Natural flavour extender Texture controlling agent Emulsifying agent Food mimetic Thickening and stabilizing agent Colour stabilization
Nutritional quality	Dietary fibre Hypocholesterolemic effect Livestock and fish feed additive Reduction of lipid absorption Production of single cell protein Antigastritis agent Infant feed ingredient
Recovery of solid materials from food processing wastes	Affinity flocculation Fractionation of agar

Purification of water	Recovery of metal ions, pesticides, phenols and PCB's Removal of dyes
Other applications	Enzyme immobilization Encapsulation of nutraceuticals Chromatography Analytical reagents

Chitosan in solution was applied to preserve many types of food such as meat balls and sausages to increase their shelf-life (Sagoo *et al.*, 2002). Chitosan solution can inhibit some pathogenic food bacteria in the preservation of fresh pork as well as retaining their sensory value during storage (Darmadji and Izumimoto, 1994). In the preservation of fruits, chitosan has been used as a coating and antifungal agent, resulting in increased quality and storability. Chitosan markedly reduced the growth of *Botrytis cinerea* and *Rhizopus stolonifer* on strawberries with a greater effect at higher concentrations (Shahidi *et al.*, 1999).

Chitosan was also combined with other natural substances to enhance its antimicrobial activity. The blending of chitosan – glucose can increase the shelf-life of lamb by two weeks (Kanatt *et al.*, 2008b). The mixture of chitosan and mint also has good effect on the inhibition of *Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Pseudomonas spp.* (Kanatt *et al.*, 2008a).

Chitosan film was used to preserve nutrient-rich and perishable food such as raw meat, fish, egg, fruit and vegetables, and green tea (Devlieghere *et al.*, 2004; Park and Chinnan, 1995; Siripatrawan and Harte, 2010)

1.2 OVERVIEW OF EDIBLE FILM FOR FOOD PACKAGING

1.2.1 Introduction to edible film

Edible film is defined as a thin layer of edible materials formed on food as a coating or a self-supporting thin layer placed on or in between the food components, and in both cases consumed along with the food (Gontard *et al.*, 2011). Edible films have recently become one of the most effective methods in maintaining the quality of food. Its functions are to extend the shelf life of food and maintain its quality by inhibiting migration of moisture, oxygen, carbon dioxide, aromas, lipid, and solute as well as to solve environment waste problems.

Edible films have potential in a number of different areas. They can coat food surfaces, separate different compounds, act as casings, pouches or wraps. They also can preserve product quality by forming oxygen, aroma, oil or moisture barriers, carrying functional ingredients, such as antioxidants, antimicrobials, and improving appearance, structure and handling. Recent have shown that edible films and edible coatings also are able to reduce oil uptake of some fried food products (Butler *et al.*, 1996).

1.2.2 Materials for edible film

The material for making films should meet Food and Drug Administration (FDA) requirements, since they are applied to foods. The material must be generally regarded as safe (GRAS), hence will be accepted in the wide market. The commercial production of edible films has been already undertaken by some companies. They sell specific edible films with explanation of usage including their properties.

Many researches now focus intensively on biomaterials from natural sources such as gelatine, casein, pectin, starch, carageenan, agar and carboxymethyl cellulose. Those biomaterials can form homogenous films or be mixed together to support and enhance their film properties. The combination of two or more biomaterials will form a blended film or a composite film.

1.2.3 Roles of edible film in food application

1.2.3.1 Barrier

The properties of films act as a barrier to either gas, oil, or more often water. Moisture levels in food are critical for maintaining freshness, controlling microbial growth, and providing mouth-feel and texture. Edible films can control water activity preventing either moisture loss or uptake (Pérez-Gago and Krochta, 2005).

1.2.3.2 Binding

Edible coating is applied to the surface of snack food and crackers to serve as a foundation or adhesive for seasoning (Bravin *et al.*, 2006).

1.2.3.3 Glaze

Edible film can also act as glazes to enhance the appearance of baked good (Petersen *et al.*, 1999). For instance, a wheat-gluten replaces the traditional eggs-based coating. In the food industry, zein is commonly used as a confectioner's glaze to provide a shine of product such as jellybeans. The alginate coating gives a glossy appearance to the product coated.

1.2.3.4 Encapsulating agent

It is used for various applications in the food industry and also the pharmaceutical industry. For example zein is common used as a tablet coating and in sustained release applications. (Gontard *et al.*, 2011)

1.2.3.5 Secondary packaging

Edible film helps maintain the quality of foods after the packaging is opened by protecting against moisture change, oxygen uptake, and aroma loss. Edible films formed or placed between food components can also improve the quality of multicomponent food.(Gómez-Guillén *et al.*, 2011; Gontard *et al.*, 2011; Kołodziejska and Piotrowska, 2007; Miller and Krochta, 1997)

1.2.3.6 Protecting from mechanical damage

Edible films with adequate mechanical properties conceivably serve as edible packaging for select foods (e.g., pouches for dried soup which would become part of the prepared soup). The sanitary condition of the edible package would need to be maintained during storage, transportation, and marketing. (Debeaufort *et al.*, 1998; Janjarasskul and Krochta, 2010; Phan *et al.*, 2005)

1.2.3.7 Protection from mechanical disintegration

Application of whey protein in freeze-dried chicken dice is one example. Application of carragenan-based coating in frozen fish is also to protect mechanical disintegration.(Petersen *et al.*, 1999)

1.2.3.8 Bioactive carrier agents

Active ingredient and seasoning can be incorporated into edible films and coatings. They may carry antioxidants, antimicrobial agents, colorants, flavours, fortified nutrients, and spices. (Langer and Peppas, 1983)

1.2.4 Current studies on chitosan-based film

Chitosan can make homogenous films as well as composite film when mixing with other substances such as polymer Low density polyethylene (LDPE), metal zinc, hydroxypropyl methylcellulose, pectin laminate, whey, pectin, methylcellulose (Aider, 2010; Li *et al.*, 2011). The aim of blending chitosan with other substances is to enhance its biochemical, physical, mechanical as well as antimicrobial properties.

Numerous studies on chitosan-based films have been conducted. Among those, the combination of chitosan with other biopolymers to make composite edible film is of interest to achieve attributes of biodegradability and biocompatibility with the environment.

Composite film made of chitosan (90 % DD) and corn starch (25 % amylose) has higher tensile strength when the ratio of chitosan and corn starch increases to 1:1. However, when the ratio rises up to 1:2, the tensile strength of the composite film decreases. The increase of the tensile strength was explained by the increase of binding between $-NH_3^+$ groups of chitosan to $–OH^-$ groups of corn starch. However, when all the $-NH_3^+$ groups are already bound, the excess $–OH^-$ groups will dissociate, resulting in separation between chitosan and starch and decreasing the tensile strength. The deformation value of the mixed film also increases proportionally to the ratio of corn starch while its water vapour permeation decreases (Xu *et al.*, 2005).

The blending of chitosan film with active substances, in addition to increasing the antimicrobial properties of the film, is to slowly release these substances, therefore lengthen the action of these active substances to food. Ouattara *et al.* (2000) examined the release of acids (acetic, propionic, lauric) and cinnamaldehyde on several types of meat such as bologna, cooked ham, and pastrami. After a period of time, those foods were tested for the residual amount of acid or cinnamaldehyde on chitosan film. It was found that propionic acid was released totally after 48 hours; acetic acid remained 2-22 % after 168 hours. The addition of lauric acid lengthened the retention of acetic acid significantly.

In another study, Pranoto blended active substances such as garlic oil, potassium sorbate and nisin into chitosan film to increase its antimicrobial properties. This type of film has good appearance, physical, mechanical properties and has antimicrobial properties against *Bacillus cereus, Staphylococcus aureus*, *Salmonella typhimurium* (Pranoto et al., 2005).

One of the unfavourable properties of natural polymer films, especially protein films is the fragility of the material. For this reason, such films have to be plasticized. Hydrophilic plasticizers are most often used to improve flexibility of the films (Arvanitoyannis *et al.*, 1997; Arvanitoyannis *et al.*, 1998; Butler *et al.*, 1996; Caner *et al.*, 1998). Incorporation of plasticizers into natural polymer films eliminates the fragility of the films and improves their elongation.

1.3 OVERVIEW OF FOOD ALLERGY

1.3.1 Introduction to food allergy

Food allergy is an immunological reaction to a component (the allergen) of a food. The resultant symptoms may affect the gastrointestinal tract, respiratory tract, skin, a combination of any of these, or the whole body (Carins, 2010).

In recent times, people have more concerns about allergic reaction because food allergy affects up to 4% of adults and 8% of children. About 90% of allergic reactions is due to the contact within eight allergenic food groups including fish and crustaceans (Lopata, 2007).

To protect the customers from the exposure to allergens, food producers are required by law to declare the major food allergens present in their products, or even to remove unnecessary major allergens from their products. (Carins, 2010)

Food allergy reaction is related to the binding between the antigen and the IgE antibody, a class of serum immunoglobulins (Eaton *et al.*, 1998). This type of reaction is usually named "Type I – IgE mediated hypersensitive reaction" to distinguish with other adverse reactions to food (Lehrer *et al.*, 2002). The mechanism of food allergic reactions is presented in Figure 1.3.

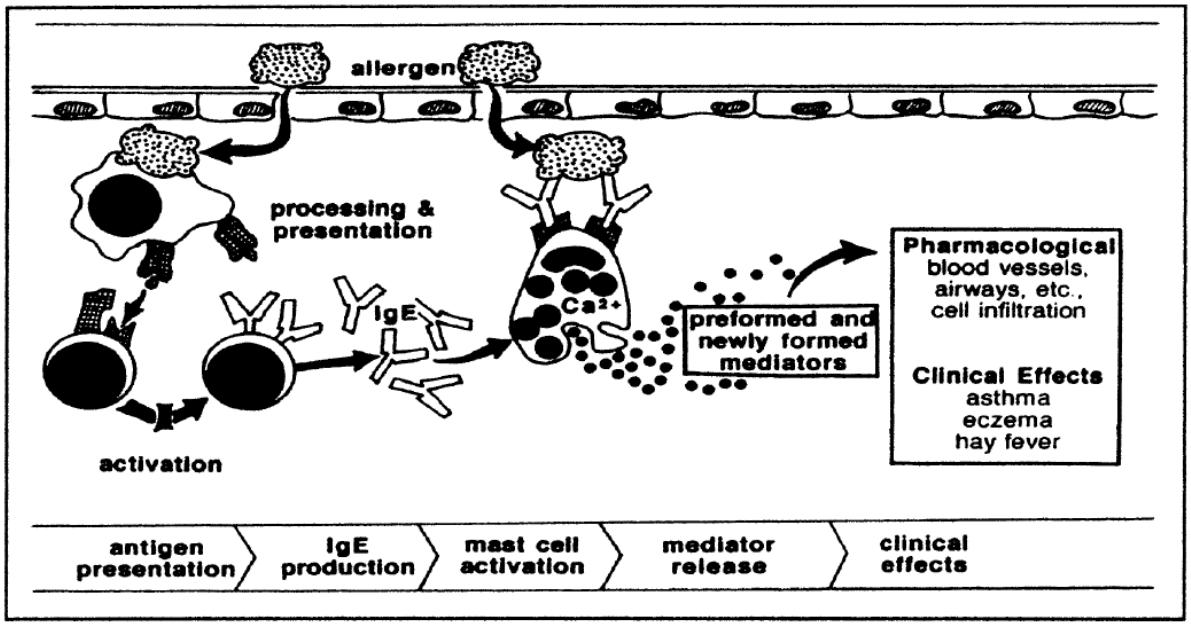


Figure 1.3: Induction and provocation of an allergic reaction

(Lehrer *et al.*, 2003)

When an antigen passes through the mucosal membrane, it is processed by macrophages and presented to T and B lymphocytes, resulting in the production of antigen-specific IgE antibodies. These antibodies can bind to the surfaces of mast cells and basophils. These cells are therefore sensitized to the allergen and the first exposure of the allergen to the body is called the sensitizing dose. When the antigens enter the cell the second time, the reaction between IgE and antigen can cross-link two or more cell-membrane-bound IgE antibodies, which release the preformed mediators and synthesis and release newly formed mediators. These mediators cause significant changes in cell migration, mobility, and dilation of blood vessels as well as smooth muscle contraction, which result in the typical symptoms of an allergic reaction.

1.3.2 Seafood allergy

Seafood allergy often develops in adulthood. The prevalence of seafood allergy in adults has been estimated to be between 1.3 % and 2.8 % (Sicherer and Sampson, 2006). It occurs more frequently in areas which have more fish in the diet (Lopata and Lehrer, 2009) (Table 1.8).

Table 1.8: Prevalence of sensitization to shelfish in various countries among individuals (adults and children) with food allergy (Lopata and Lehrer, 2010)

Country	Number of individuals investigated	Prevalence (%)
Philippines	38	58
South Africa	105	55
Singapore	227	39
France	580	34
Indonesia	600	24
Thailand	202	22
Taiwan	392	21
Singapore	334	15
Spain	355	6.8
United States*	14,948	2.0

Note: A survey among the general population is indicated by an asterisk.*
Sensitization established by SPT and/or quantification of specific IgE antibody.

Table 1.9: Classification of seafood groups causing allergies (Lopata, 2007)

Phylum	Class	Common Name	Allergens
Molluscs	Gaastropods	Abalone, snail	Tropomyosin ?
	Bivalves	Clams, oyster, mussel	Tropomyosin ?
	Cephalopods	Quid (cuttlefish), octopus	Tropomyosin ?
Arthropods	Crustacean	Crab, rock lobster, prawn, shrimp	Tropomyosin Arginine kinase
Chordates	Osteichtyes (bony fish)	Salmon, hake, tuna, herring, sardine , mackerel, carp	Parvalbumin Skin collagen? Roe?

Note: ? = indicates allergens not well characterised.

Among the allergens listed in Table, tropomyosin is considered the major allergenic protein in seafood (Lopata and Lehrer, 2010; Reese *et al.*, 1999).

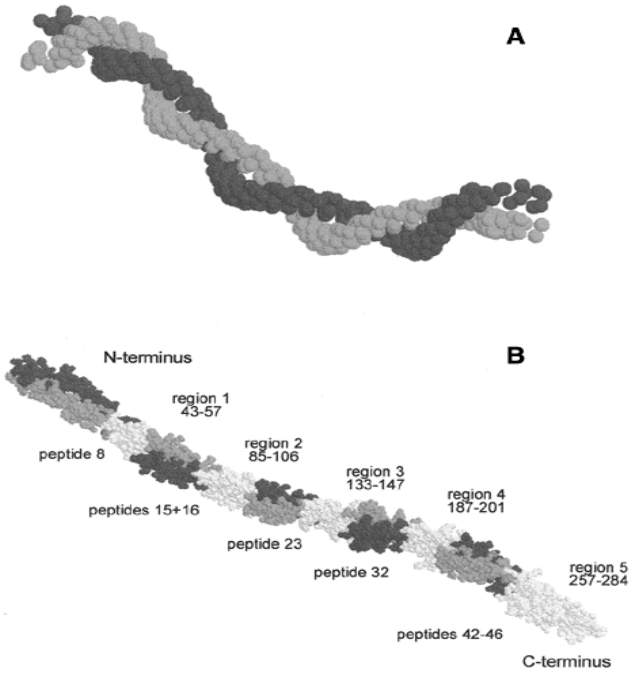


Figure 1.4: Structure of tropomyosin

(Lehrer *et al.*, 2002)

Tropomyosin is a heat – stable muscle protein which has a very conserved structure (Figure 1.4) (Lehrer *et al.*, 2002). It is a coiled-coil dimer consist of two parallel alpha-helical tropomyosin molecules wound around each other (Lehrer *et al.*, 2003; Reese *et al.*, 1999). Its average molecular weight is approximately 34 – 38 kDa with pI at 4.5 (Reese *et al.*, 1999).

Tropomyosin has also been identified as an important allergen in other invertebrates including house dust mites and cockroaches. This molecular and clinical cross-reactivity of tropomyosin between crustaceans, molluscs, house dust mites and insects has proposed it to be an invertebrate pan allergen (Lehrer *et al.*, 2003; Lopata *et al.*, 2010; Reese *et al.*, 1999). Five different major IgE-binding regions in shrimp tropomyosin have been identified as containing 8 epitopes (Lehrer *et al.*, 2003). Mutations of these shrimp allergenic epitopes can reduce seafood allergenicity; methods utilizing such mutations will provide safer vaccines for more effective treatment of seafood-allergic patients, and in the future less-allergenic seafood products for consumption (Lehrer *et al.*, 2003).

Recently, other cross-reactive allergens have also been identified and characterized. The allergen Pen m 2, identified in the Black tiger shrimp (*Penaeus mondon)* has high similarity to the enzyme arginine kinase. This 40 kDa protein may represent a new class of crustacean pan allergen (García-Orozco *et al.*, 2007; Yu *et al.*, 2003). Also in the Black tiger shrimp, a 20 kDa protein, which has been identified as Sarcoplasmic calcium binding protein (SCP), has been found to be a cross-reactive allergen to lobsters but not to crabs (Shiomi *et al.*, 2008). Another new major shrimp allergen, Lit v 3.0101, a 20-kDa myosin light chain protein extracted from the white leg Pacific shrimp (*L. vannamei*) has been successfully characterized and cloned (Ayuso *et al.*, 2008). Other IgE binding proteins which have molecular size ranging from 8-166 kDa have also been found but not completely characterized.

1.3.3 Current studies on the allergenicity of chitin and chitosan

Although chitin and chitosan is considered safe and non-toxic, there are still some reports related to the hypersensitivity to the products which contain chitin and chitosan.

The first case related to a moisturizing cream which is an oil / water emulsion containing chitin derivative 0.3 %, glucosamine, gluconic acid, pyrrolidone carboxylic acid, specific reconstituted sebum 3%, preservative, aroma composition, and purified water. Chitosan gluconate is the active ingredient of this product. The patient was tested with crustacean by cutaneous contact and eating but there were no symptoms of Type I allergy. However,

chitosan is still an unknown allergen, so this case is only a reference for the dermatologist to find the reason for skin allergy (Cleenewerck *et al.*, 1994).

The second case was an immediate-type allergy for a chitosan – containing health food. The patient had systemic urticaria and difficulty in breathing after oral ingestion of chitosan. The skin test with another commercial chitosan was positive. After avoiding using that product, the patient no longer had the condition. Therefore chitosan was assumed to have function as a food allergen due to its molecular weight and general properties (Kato Y, 2005).

The third case related to a cream containing chitin and a Carbitol. The patient was sensitized not only to chitosan gluconate / chlorhydrate glucosamine but also to ethyl diglycol Carbitol (Pereira *et al.*, 1998).

Overall, there has been no detailed research to confirm the allergenicity of chitin and chitosan. Therefore, it is necessary to undertake these studies to have the appropriate recommendations for the safety of the consumers.

1.4 AIMS AND OBJECTIVES OF CURRENT STUDY

1.4.1 Aims

This study aimed at investigating the presence of the allergenic protein tropomyosin in residual proteins of chitin and chitosan samples. After that, the antimicrobial properties of normal chitosan and purified chitosan were compared to examine if the purification process changed the properties of chitin and chitosan. The purified chitosan was then applied in food technology by the formulation of a chitosan-based edible film which is sealable and soluble in hot water and feasibility for application in instant food casing was investigated.

1.4.2 Objectives

There were five objectives for this study:

Objective 1: Production of polyclonal antibodies against shrimp tropomyosin and crustacean proteins

The aim of this objective was to produce two polyclonal antibodies from rabbits to investigate the presence of the shellfish allergen tropomyosin and/or other crustacean proteins in the shrimp tissues as well as chitin and chitosan preparations.

Objective 2: Investigation of the allergenicity of shrimp proteins

The aim of this objective was to examine the immunoreactivity of the allergenic tropomyosin protein in shrimp products due to variations in the shrimp species, parts of shrimp and the processing methods.

Objective 3: Investigation of the allergenicity of residual proteins in chitin and chitosan

The aim of this objective was to examine the presence of tropomyosin in residual proteins of chitin and chitosan samples.

Objective 4: Investigation of the biochemical and antimicrobial properties of chitin and chitosan

The aim of this objective was to characterize some biochemical properties of chitin and chitosan samples and to compare the antimicrobial property between normal and purified chitosan.

Objective 5: Formulation of chitosan-based edible film for application in instant food casing

The aim of this objective was to formulate a chitosan-based edible film which is sealable and soluble in hot water and then to examine its potential application in instant food casing.

Chapter 2
GENERAL MATERIALS AND METHODS

This chapter describes the general methods used in this project. Specific methods are described in each chapter. All materials, chemicals, and equipments used in this project are listed in this chapter.

2.1 GENERAL PROCEDURES

All chemicals used were of analytical, chromatography and electrophoresis reagent grade. Solutions were prepared in deionised water (dH_2O) delivered from a Millipore Milli-Q® water system, unless otherwise specified. Solutions were dispensed using Finnpipette micropipettes (Pathtech Pty Ltd., Australia) with the following volume ranges: 1 mL to 5 mL, 200 µL to 1 mL, 20 µL to 200 µL, 5 µL to 50 µL, 0.5 µL to 10 µL and 0.1 µL to 2 µL.

Glassware was washed using Pyroneg detergent, rinsed in tap water, with a final rinse in dH_2O. All glassware, micropipette tips, plastic ware, media and solutions used for bacterial work was sterilised by autoclaving at 121°C for 20 min, unless stated otherwise.

2.2 MATERIALS

2.2.1 General equipment

Equipment	Supplier
Amicon ultrafilter	Millipore, USA
Balance:	
Analytical balance (0.0001 – 220 g)	Sartorius Gottingen, Germany
Balance (0.01 – 500 g)	U-Lab, Australia
Cellulose acetate membrane	Sartorius Gottingen, Germany
Centrifuge:	
Eppendorf centrifuge	Eppendorf Geratebau, Germany
Bench top centrifuge	Thermal Scientific, Australia
Bench top centrifuge	Beckman Allegra, USA

Centrifuge tubes:	
1.5 mL centrifuge tubes	Sarstedt, Germany
10 mL centrifuge tubes	Greiner Labortechnik, Germany
50 mL centrifuge tubes	Greiner Labortechnik, Germany
Chromatography (Biologic LP system)	Bio-Rad, USA
Colony counter	Stuart Scientific, United Kingdom
Desiccator	
Dry block heater	Ratek, Australia
ECL Hyperfilm	GE Healthcare, USA
Electrophoresis Units	BioRad, USA
Filter	Sartorius Gottingen, Germany
Gel Doc image system	Bio-Rad, USA
Homogeniser	ART, Germany
Micropipette plastic tips (Blue and Yellow)	Greiner Bio-One, Germany
Micropipette plastic tips (5 mL and 10 mL)	Pathtech Pty Ltd., Australia
Microplate reader	Thermal Scientific, Australia
Panme ruler	Mitutoyo, Japan
Petri dish	Nunc, Denmark
pH meter	Metrohm, Germany
PVDF membrane	Bio-Rad, USA
Shaker	Ratek Instruments, Australia
Sonicator	Branson Sonic Power Co., USA
Spectrophotometer-Dynatek MR7000	Baxter Dagnostics, Australia
Syringe (1 mL, 5 mL, 10 mL, 20 mL, 50 mL)	Terumo Pty, Ltd., Australia
Texture machine	Zwick/Roell, Germany
Vortex mixer	Ratek Instruments, Australia
Water bath	Ratek, U-lab, Australia
Whatman paper	Whatman, England

2.2.2. General chemicals

Reagents	Supplier
10X PBS Buffer	Oxoid, England
Acetic Acid, glacial	BDH Chemicals, Australia
Acetone	BDH Chemicals, Australia
Acrylamide (electrophoresis grade)	Sigma-Aldrich, USA
Bis-Acrylamide	Sigma-Aldrich, USA
Ammonium acetate	Ajax Chemicals Ltd, Australia
Ammonium persulfate	Sigma-Aldrich, USA
Ammonium sulfate	Ajax Chemicals Ltd, Australia
Ampicillin disc	Oxoid, England
Bacteriological agar (No. 1)	Oxoid, Australia
Bacteriological peptone	Oxoid, Australia
Bovine serum albumin (BSA)	Promega, USA
Bromophenol blue	Sigma-Aldrich, USA
Chloramphenicol disc	Oxoid, England
Calcium chloride	Merck, USA
Chloroform	Merck, USA
Coomassie blue R -250	Sigma-Aldrich, USA
Dithiothreitol (DTT)	Bio-Rad, USA
Dimethylsulfoxide (DMSO)	BDH Chemicals, Australia
Ethylenediamine tetra acetic acid (EDTA)	BDH Chemicals, Australia
Ethanol	BDH Chemicals, Australia
D-Glucose	BDH Chemicals, Australia
Glycerol	BDH Chemicals, Australia
Glycine	BDH Chemicals, Australia
Hydrochloric acid (32%)	Ajax Chemicals Ltd., Australia
Mercaptoethanol	Sigma-Aldrich, USA
Methanol	Merck, USA
NBT/BCIP substrate solution	Roche Diagnostics, Germany
Potassium acetate	BDH Chemicals, Australia
Potassium chloride	BDH Chemicals, Australia
Potassium dihydrogen orthophosphate	BDH Chemicals, Australia
Precision Plus Dual stain protein marker	Bio-Rad, USA

Skim milk	Diploma. Australia
Sodium acetate	Amresco, USA
Sodium chloride	BDH Chemicals, Australia
Sodium dodecyl sulphate (SDS)	BDH Chemicals, Australia
Sodium hydroxide (pellets)	BDH Chemicals, Australia
Sodium phosphate	Mallinckrodt Inc., USA
Sorbitol	BDH Chemicals, Australia
Sulfuric acid	BDH Chemicals, Australia
TEMED (N, N, N', N'-tetramethylene-ethylenediamine)	Sigma-Aldrich, USA
Tris-Base	Roche Diagnostics, Germany
Tris-HCl	Roche Diagnostics, Germany
Tween 20	Sigma-Aldrich, USA
Yeast Extract	Oxoid, Australia

2.2.3. General media

Luria Bertani agar (LB agar): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride (NaCl), 1% (w/v) bacteriological agar

Luria Bertani broth (LB broth): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride (NaCl)

Mueller Hinton Agar (Oxoid): 3.8% medium mix. Autoclave.

Mueller Hinton Broth (Oxoid): 2.1% medium mix. Autoclave.

Plate count agar (Merck): 2.25 % medium mix. Autoclave.

2.3 METHODS

2.3.1 Methods of analysing basic properties of samples

2.3.1.1 Moisture content: drying method to stable weight

The crucible was dried at 105 ^{o}C for 3 hours and let cool in the desiccator before being recorded its weight (W_0). One gram of sample was added into each crucible. The initial weight of both crucible and sample was recorded (W_i). The crucible and sample was weighed (W_1) after drying at 105 ^{o}C for 8 hours. Then it was dried and weighed for each 2 hours until the difference between two weights was not bigger than 5 %. The last weight was recorded as W_d.

$$\text{The moisture content of sample (\%)} = (W_i - W_d) / (W_i - W_0) \times 100$$

2.3.1.2 Ash content: standard AOAC method (AOAC, 2000)

The crucible was heated at 550 ^{o}C overnight and let cool in desiccators before being recorded its weight (W_0). About 1 g of sample was put into each crucible. The initial weight of crucible with sample was recorded (W_i). The crucible with sample was heated at 550 ^{o}C overnight and weighed (W_d) after cooling down in the dessicator.

$$\text{The ash content of sample (\%)} = (W_i - W_d) / (W_i - W_0) \times 100$$

2.3.1.3 Heavy metal content

Atomic absorption spectroscopy (AAS) method was used to test the heavy metal content in chitin and chitosan samples following procedure of ACIAR (Australian Centre for International Agricultural Research, 2007).

This test was conducted by the Institute of Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam.

2.3.1.4 Protein concentration quantification

The Quick Start Bradford Protein Assay Kit (Bio-Rad, USA) which followed the Bradford method (Bradford, 1976) was used in this study. This technique was based on the binding between the protein in the sample solution and the Coomassie Brilliant blue G-250 dye in the Bradford reagent. The protonated cationic dye which predominantly exhibited a dark red colour in acidic conditions converted to a stable unprotonated blue form which exhibits a maximum absorbance at 595 nm when binding to a protein.

In this research, the microplate assay technique was used for the protein concentration measurement using the Thermo Scientifics' Multiskan Ascent microplate reader. A standard curve of the absorbance reading at 595 nm against bovine serum albumin (BSA) in the concentration range of 0.01 - 2 mg/mL was generated, and the protein concentration of the extracts were estimated based on this standard curve. The blank, standard and the protein extract readings were performed in duplicate to increase the accuracy of the assay.

2.3.2 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) of protein

The method, described by Bollag, Rozycki and Edelste (Bollag *et al.*, 1996), was used to create protein profiles of protein extracts. This technique could help to separate proteins from a mixture based on its molecular size under the influence of an electrical charge (Walker, 2002).

In this study, the Mini Protean III gel electrophoresis system (Bio-Rad, USA) was used for SDS-PAGE. The gels, which had the thickness of 0.75 mm, had two parts: the stacking gel (5 % polyacrylamide) and the separating gel (12 % polyacrylamide). This kind of gel had the separating resolution range between 15 and 60 kDa (Hames and Rickwood, 1990). The purpose of the stacking gel was to concentrate proteins before passing through the separating gel, therefore resulting in shaper protein bands.

2.3.2.1 Preparing the working solutions

- Solution A (Acrylamide stock solution), 100 mL: 29.2 g acrylamide, 0.8 g bis-acrylamide, and water to make 100 mL total volume
- Solution B (4 x Separating gel buffer), 100 mL: 75 mL 2M Tris-HCl (pH 8.8), 4 mL 10 % SDS, and 21ml water
- Solution C (4 x Stacking Gel Buffer), 100mL: 50 mL 1M Tris-HCl (pH 6.8), 4 mL 10 % SDS, and 46 mL water
- 10% ammonium persulfate, 5 mL: 0.5 g ammonium persulfate, and water to make 5 mL total volume
- Electrophoresis Buffer, 1000 mL: 3g Tris, 14.4g glycine, 1g SDS, and water to make 1liter total volume
- 5x Sample Buffer, 10mL: 0.6 mL 1M Tris-HCl (pH 6.8), 5 mL 50 % glycerol, 2 mL 10 % SDS, 0.5 mL 2-mercaptoethanol, 1 mL 1 % bromophenol blue, and 0.9 mL water

- Stain solution, 100 mL: 0.1 g coomassie blue, 45 mL methanol, 10 mL glacial acetic acid, and 45 mL water
- Destain solution, 100 mL: 10 mL methanol, 10 mL glacial acetic acid and 80 mL water

2.3.2.2 Preparing the gel

The formulation of gels used in this study was described in Table 2.1.

Table 2.1: Amounts of reagents for making 2 gels

Gels / Reagents	12 % separating gel	5 % stacking gel
Solution A	4 mL	2.3 mL
Solution B	2.5 mL	0.67 mL
Solution C	-	1 mL
Milli-Q water	3.5 mL	4.8 mL
10% ammonium persulfate	50 µL	30 µL
TEMED	5 µL	5 µL

Mixed separating gel solution was degassed and poured into the glass plate assembly. Then, they were overlaid with water saturated butanol to remove any bubbles and linearise the level. The gels were allowed to polymerize for 1 hour. After that, water saturated butanol was poured off before pouring staking gel. Mixed stacking gel solution was also degassed and poured into the glass plate assembly. 10 or 15-well combs were then immediately inserted and the gels were allowed to polymerize for 20 minutes.

Both gels were fit to electrode assembly before being inserted into electrophoresis chamber. Electrophoresis buffer was added to inner and outer reservoir to make the gels immersed in buffer. The combs were then carefully removed from the stacking gels.

2.3.2.3 Preparing samples and operation of SDS-PAGE

The prepared protein extracts were diluted to make a final concentration of 1 mg/mL, and then mixed with the loading dye. This solution was then heated at 100˚C for 10 minutes in a water bath. After cooling down to room temperature, the mixtures were centrifuged at 13,000 rpm for 30 seconds to settle any precipitated particles prior being loaded into the wells.

An amount of 15 or 20 µg of each protein mixture was introduced into the wells. The Precision plus dual stain protein marker 10 – 250 kDa (Bio-Rad, USA) was also added to one of the wells as a reference to estimate the molecular weight of the separated protein bands.

The apparatus was run into two stages: stage 1 with 80 voltages in 20 minutes to have the proteins settled in the stacking gel, and stage 2 with 180 voltages in 50 minutes to separating the protein solution.

After the run was completed, the gels were removed from the glass plate assembly and put in the Coomassie blue stain solution for 10 minutes with shaking and then washed in the destain solution for 3 hours till the protein bands were visible as dark blue bands against a transparent background. The gels were then scanned their images and saved in computer for later analysis.

2.3.3 Immunoblotting techniques

2.3.3.1 Western Blotting

This method was used to detect possible allergenic protein in our samples. This study followed semi-dry blot technique, where the gel and membrane are fitted between buffer-wetted filter papers that are in direct contact with two closely spaced solid-plate electrodes (Gravel, 2002).

The proteins separated in the SDS-PAGE were transferred onto polyvinylidene diflouride (PVDF) membrane, which have a strong affinity to proteins. The membrane was then probed with a primary antibody which specifically bound to target protein. Next, a secondary antibody which was conjugated with an enzyme was added to bind with the primary antibody. Finally, a substrate which reacts with the enzyme was added to the membrane to form a colour reaction or a chemiluminescent reaction. This method can detect as little as 10 pg protein (Gershoni and Palade, 1982).

2.3.3.1.1Equipment

- Trans-Blot Semi Dry transfer cell (Bio-Rad, USA)
- Power supply (capacity 200V, 500mA)
- Polyvinylidene diflouride (PVDF) membrane (Bio-Rad, USA)
- Extra thick filter pads (Whatman)
- X-ray film (Amersham hyperfilm ECL film, GE healthcare, UK)
- Micro-pipettes
- Rotary shaker (Ratek)
- Other glassware (beakers, cylinders, flasks,...)

2.3.3.1.2 Reagents

Primary antibodies:

- Monoclonal antibody (mAbαTM): Commercial rat anti-tropomyosin IgG antibody, MAC-141 (Abcam). This antibody was raised against troponin-tropomyosin complex extracted from an insect muscle (*Lethocerus indicus*).
- Polyclonal antibodies against tropomyosin (pAbαTM): This is rabbit serum containing antibodies which was raised against the shrimp tropomyosin.
- Polyclonal antibodies against crustacean protein (pAbαCR): This is rabbit serum containing antibodies which was raised against the whole-cooked crustacean protein.

Secondary antibody:

- Rabbit anti-rat-IgG, alkaline phosphatase conjugated (Sigma-Aldrich)
- Goat anti-mouse IgG (H+L), horse radish peroxidase conjugated (Promega)

Substrate:

- NBT-BCIP substrate: solution of 18.8 mg/ml nitro-blue tetrazolium chloride; 9.4 mg/ml 5-bromo-4-chloro-3-indolylphosphate toluidine salt in 67% Dimethyl sulfoxide. (Sigma-Aldrich, USA)
- Novex® ECL Chemiluminescent Substrate Reagent Kit (Life Technologies): a two-part reagent consisting of Reagent A (luminol) and Reagent B (an enhancer) used in equal volumes to attain the most intense light emission.

Other reagents:

- Tris-HCl (Roche Diagnostics, Germany)
- Glycine (BDH Chemicals, Australia)

- Methanol (Merck, USA)
- NaCl (BDH Chemicals, Australia)
- Skim milk (Diploma, Australia)
- Tween 20 (Sigma-Aldrich, USA)
- Transfer buffer: 25mM Tris, 192 mM Glycine, 20% methanol
- Tris Buffered Saline (TBS): 20 mM Tris-HCl, 150 mM NaCl (pH 7.5)
- Blocking solution: 5 % skim milk in TBS
- TBS-T: 0.05 % Tween 20 in TBS

2.3.3.1.3 Operation of Western blotting system

The gel sandwich was setup to facilitate the transfer of the separated proteins from the polyacrylamide gel to the PVDF membrane, without mixing of any protein lanes. An extra thick blot paper was soaked in freshly prepared transfer buffer and placed on the positive-electrode base plate. Then a PVDF membrane was placed on the blot paper after being activated in 100 % methanol for 10 seconds and soaked in transfer buffer until it was completely submerged. Next, the gel, after being equilibrated in transfer buffer for 2 minutes, was placed carefully on the membrane. Finally, another extra thick blot paper pre-soaked in transfer buffer was placed on the top. The negative-electrode upper lid was placed on top of the sandwich and locked in its position.

The transfer was conducted at 12 volts for 25 minutes. During the transfer, the negative charged proteins in the gel went towards the base positive electrode and binding to the PVDF membrane. After the transfer was completed, the membrane was placed in blocking solution to cover up the rest of the exposed membrane apart from the bound proteins region. After that, the membrane was incubated in the primary antibody solution with the dilution of 1:3000 in 1 % skim milk in TBS for 1 hour at room temperature or overnight at 4 ˚C. Then it was incubated for 30 minutes in the secondary antibody solution with the dilution of 1:5000. Next, it was also incubated for 30 minutes in the conjugate solution with the dilution of 1:5000. Between each step, the membrane was washed with TBS or TBS-T to get rid of unbound antibodies. The final step was to develop the blot using the substrate solutions.

If the alkaline phosphatase conjugate was used, the blot would be incubated in NBT-BCIP substrate solution for 10 minutes in the dark until visible bands appeared, and it was washed with distilled water to stop the reaction. The blot was dried and scanned its image for further analysis.

If the horse radish peroxidase conjugate was used, this step must be done in the dark room. First, the blot would be incubated in the Novex® ECL Chemiluminescent substrate for five minutes. The membrane was then covered between two transparent plastic sheets, and an X-ray film was placed over it. This setup was closed tightly in a photographic cassette for about 1 second to 1 minute to receive the clearest bands. The X-ray film was then removed from the cassette and immediately dipped into the developer solution for a minute, then rinsed with water. Next, the film was placed in the fixer solution for one minute and finally washed with water. The film was allowed to air dry before being scanned.

2.3.3.2 Dot Blot

This method was used for chitin / chitosan samples to detect possible allergenic proteins as they cannot be separated by conventional SDS-PAGE due to their high molecular weight and viscosity.

The principal and reagents of this method were similar to the Western Blot. This study used nitrocellulose membrane (Bio-Rad, USA) instead of PVDF membrane and followed the protocol from Abcam company (www.abcam.com/technical).

The nitrocellulose membrane was cut into 8 x 10 cm dimension and drawn grid by pencil to mark the blotting region. Then 2 µl of each sample was spotting slowly onto the membrane at the centre of the grid so that the spot diameter was limited in 3 - 4 mm. A serial concentration of purified tropomyosin was also added as reference.

After drying, the membrane was blocked non-specific sites by soaking in blocking solution for 1 hour at room temperature. Then it was incubated with primary antibody for 1 hour at room temperature and washed 3 times with TBS-T for 5 minutes. Next, the membrane was incubated with secondary antibody conjugated with HRP for 30 minutes at room temperature, then washed 2 times with TBS-T for 5 minutes and once with TBS for 5 minutes. Finally, it was incubated with ECL reagent for 5 minute and expose X-ray film in the dark room.

The signals from the samples were compared to that of standards and the concentration of tropomyosin in each sample was estimated.

2.3.3.3 Immunoblotting with human sera from shellfish allergic patients

This work was conducted at Monash Medical Centre, Melbourne to investigate the reaction between human serum IgE and the allergenic protein(s). Tropomyosin IgE-positive results has been shown to be a good predictor of shrimp allergy (Gámez *et al.*, 2011).

The human sera were collected from patients who showed a strong reactivity to ingested shellfish by the Alfred hospital, Melbourne and were stored at -20 ˚C at the Monash Medical Centre. The total IgE and specific IgE concentration to lobster, crab and shrimp were tested using UniCAP system (Phadia, Sweden). The patient's clinical histories were summarized in Table 2.2. Patient JB21 had a slightly higher specific IgE concentration then patient AN01. Atopy refers to the status of the patients making also specific IgE antibody to inhalant allergens such as house dust-mites and pollen.

Table 2.2: Clinical characteristics of shellfish allergic patients

Patient Code	IgE Total (kU/l)	Specific IgE (kU/l)			Atopic	Allergic reaction after ingestion
		Lobster *(Homarus gammarus)*	Crab *(Cancer pagurus)*	Shrimp *(Penaeus monodon)*		
ANO1	242	1.65	1.17	1.32	Yes	Yes
JB21	100	Not Done	2.04	2.11	Yes	Yes

(Source: Alfred Hospital, Melbourne, 2008)

For the immunoblotting, the membrane with proteins transferred from the SDS-PAGE was incubated in the human serum which was diluted 10 times in PBS (4.7 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, 150 mM NaCl, dH_2O) containing 1% skim milk overnight at 4˚C. The next day, it was shaken on a shaker for 20 minutes before being washed three times with PBS-T for five minutes each. The membrane was then incubated for 1 hour in 5 mL of rabbit antihuman IgE antibody (Sigma-Aldrich) diluted 1000 times in PBS-T containing 1% skim milk.

After being washed with PBS-T thrice for five minutes each, it was incubated for 30 minutes in 5 mL of goat anti-rabbit polyclonal antibody - HRP conjugated (Dako, USA) diluted 1000 times in PBS-T containing 1% skim milk. It was then washed with PBS thrice for five minutes each before being developed using the enhanced chemiluminescence technique.

2.3.4 Enzyme-linked immunosorbent assay (ELISA) methods

The indirect ELISA and inhibition ELISA have been used in this study depending on the aim of each experiment.

2.3.4.1 Indirect ELISA

Indirect ELISA was used to measure the optimal antigen concentration to coat wells and to check the titre of our home-made antibodies (Crowther, 1995). In this assay, a constant amount of antigen was absorbed to wells, and then a serial dilution of rabbit serum was added to react with the antigen. The second antibody, Goat anti-rabbit IgG conjugated with HRP (Dako, Denmark), was added to bind with the serum. TMB One-Step Substrate System (TMB) containing 3,3'-5,5' tetramethylbenzidine and hydrogen peroxide was then used to create color reaction which can be detected by microplate reader with the optical density at 450 nm.

In this study 200 µL of immunizing antigen diluted in PBS pH7 (4.7 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, 150 mM NaCl, dH_2O) was first coated onto each well. The plate was sealed with cling wrap to avoid evaporation and incubate overnight at 4^oC. Then it was washed 3 times wih PBS-Tween before being added with 250 µL of blocking buffer (PBS containing 5% skim milk) to each well. The plate was washed again 3 times wih PBS-Tween. Then 200 µL of rabbit serum (serially diluted in PBS with 0.5% skim milk) was added to each well and incubated for 1 hour at 37^oC, following by shaking 30 minutes at room temperature. After being washed 3 times with PBS-Tween, 200 µL of diluted goat-anti rabbit antibody conjugated with HRP (1:10,000 in PBS with 0.5% skim milk; Dako, Denmark) was added and incubated 30 minutes at 37^oC, following by shaking 30 minutes at room temperature. The plate was then washed 6 times with PBS-Tween, then added with 100 µL of TMB substrate solution (Dako, Denmark) to each well and incubated for 20-30 min at 25^oC (wait until the first well at the second row turn very dark). Finally, 100 µL of 2 N HCl or H_2SO_4 was added to each well to stop the reaction. The absorbance at 450 nm wavelength was determined using a Multiskan plate reader (Labsystems).

2.3.4.2 Inhibition ELISA

Inhibition ELISA was used to examine the immunoreactivity of tropomyosin in different protein extracts and to estimate the quantity of tropomyosin in shrimp extracts as well as chitin and chitosan samples. This method was followed the protocol of Lopata (2005) with modifications described below (Lopata *et al.*, 2005).

The wells were first coated with 200µL antigen (0.01 µg/mL of purified tropomyosin or 0.1 µg/mL of mix crustacean protein) in PBS then incubated for 1 hour at 37 ^{o}C and for 3 hours at room temperature. After being washed 3 times with PBS-Tween, the wells were blocked with 250 µL of 5 % Skim milk in PBS and incubated for 1 hour at 37 ^{o}C, then washed again 3 times with PBS-Tween. A double strength dilution of antibody and inhibitor were prepared in 0.5 % skim milk in PBS, and then mixed together with the ratio 1:1. The wells were then coated with 200 µL of "antibody-inhibitor" mixture and incubated for 1 hour at 37 ^{o}C following by washing 3 times with PBS-Tween. After that, 200 µL of the secondary antibody (goat anti- rabbit polyclonal HRP labelled) diluted 1:10,000 in 0.5 % skim milk in PBS were added and incubated for 30 minutes at 37 ^{o}C. The wells were then washed 5 times with PBS-Tween and 1 time with PBS before adding 100 µL of TMB substrate solution (Dako, Denmark) and incubating for 20-30 minutes (or longer) at room temperature. The reaction was stopped by adding 100 µL of 2 N HCL and the absorbance of the solution in the well was read at 450nm wavelength by using a Multiskan plate reader (Labsystems).

Calculating percentage inhibition in inhibition ELISA:

The *Y* values were calculated by substract the absorbance of each sample to the absorbance of the blank (only TMB and HCl). The Y_{max} value is the absorbance of the well containing no inhibitor. The percentage inhibition (PI) was calculated as $100 \times (Y_{\max} - Y) / Y_{\max}$.

2.3.5 Ion-Exchange chromatography (IEC)

The ion-exchange chromatography was used for the purification of tropomyosin from the whole-cooked prawn extracts. This technique can separate proteins based on the intrinsic protein charge. An ion exchange column is basically a resin containing charged moieties which are capable of binding to the opposite-charged proteins through electrostatic binding. The unbound proteins are washed out of the column. The bound proteins are then eluted by increasing the salt concentration in the mobile phase which mainly contains the counter ion of the column.

2.3.5.1 The equipment and the buffer system

In this study, the Biologic LP system (Bio-Rad) was used. It has an integrated system with a buffer mixing system, sample loader, low pressure pump, UV absorbance detector, conductivity detector and a separate fraction collector. A strong anion exchange column, Bio-

Scale mini Macro-prep, High Q prepacked column (1 mL volume) was used for the purification.

The starting buffer was 30 mM Acetate buffer with a pH of 5.5. The eluting buffer was 1000 mM Sodium chloride with pH 5.5. All buffers were filtered through 0.45 μm membrane and sterilized by autoclaving before each run.

2.3.5.2 Sample preparation

A protein extract containing approximately 3 mg of protein was pipetted into a falcon tube and the volume was made up to 6 mL with the starting buffer. The tube was centrifuged at 4000 g for 10 minutes to separate any precipitated proteins due to the pH change. The supernatant was filtered through a 0.45 μm membrane, then concentrated in an Amikon tube (Millipore, USA) with a 3 kDa cut-off value by spinning at 4000 g until the volume came down to 1 mL. This retentate was added to 9 mL of the starting buffer to a final volume of 10 mL.

2.3.5.3 Purification procedure

Before starting the purification run, the column was regenerated by purging with 2 M NaCl solution for 5 column volumes, then with the eluting buffer until the conductivity reading stabilized to a certain value. It was then equilibrated with the starting buffer until the conductivity reading dropped down and stabilized at a lower value. After this step, the system was ready for a purification run.

The run was started with the starting buffer passing through the column at 2.5 mL/min. Then the prepared sample solution was injected into the system. After the initial solvent peak was visible, the starting buffer was passed through the column until the absorbance reading went down to baseline. The gradient salt concentration was then added, with the concentration of sodium chloride increased from 0 mM to 500 mM over 25 minutes, and from 500 mM to 1000 mM over 10 minutes. Finally the 100 % eluting buffer was passed through the column to remove any bound proteins, and then again equilibrated with the starting buffer for the next run.

During the run, the fractions which corresponded to the peaks containing the eluted proteins were collected and concentrated in Amikon filter tubes with a 3 kDa cut-off to a final concentration of 1 mg/mL. The fractions were stored at -80 ˚C for further analysis. After the purification runs, the column was purged with 70 % ethanol as a column sanitizer. Finally it was purged with 20 % ethanol before storage.

2.3.6 Antimicrobial susceptibility tests

The antimicrobial susceptibility tests of chitosan samples were performed employing the micro-dilution method and disc diffusion method described by the Clinical and Laboratory Standards Institute (CLSI, 2006 (a), 2006 (b)). The tests were run in triplicate.

Quality control strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used in this experiment. A well-isolated colony was inoculated into LB-broth and incubated overnight at 37^{o}C. The bacterial suspensions were prepared by adjusting the turbidity of the 16-hour-old test micro-organisms to an equivalent 0.5 McFarland standard which equals to 1x10^{8} CFU/mL with Mueller-Hinton broth. This suspension was used for the MIC test.

100 µl of the above suspension was further diluted into 2 ml of Mueller-Hinton broth, which was used as the final inoculums (approximately 5 × 10^{6} CFU/mL) for the minimum inhibition concentration and disc diffusion test.

Chitosan stock solutions were prepared in 0.2 N acetate buffer pH 4.3 at a concentration of 0.1% (equivalent to 1000 mg/L).

2.3.6.1 Minimum inhibition concentration (MIC) test

A 150 µL aliquot of each prepared bacterial suspension (approximately 5 × 10^{6} CFU/mL) was mixed with 150 µL of sterilised chitosan solutions, which were twofold serially diluted in advance with 0.85 % sterile NaCl to the designated concentration. The chitosan solutions have the concentration ranging from 3.77 to 500 mg/L. A corresponding mixture containing no test strain was used as a control. The mixture was then applied to each well of a 96-well microplate and incubated at 37 ^{o}C for each micro-organism for 48 hours. Growth of the test micro-organism was monitored by determining OD590 every 12 hours for 48 hours using the Thermo Scientifics' Multiskan Ascent microplate reader. All treatments were conducted three times. MIC was ultimately defined as the lowest concentration of sample required to inhibit bacterial growth for over 48 h beyond that of the control sample.

2.3.6.2 Minimum Bactericidal Concentration (MBC) test

The MBC was measured by inoculating the broths used for MIC determinations onto drug-free medium. The MBCs were determined by transferring 0.1 mL from each well without visible growth of the MIC microdilution plate to a petri dish containing Mueller-Hinton agar

(MHA). The plates were incubated at 37°C for 48 hours. The MBC was read as the lowest concentration of drug at which no growth was observed (Du *et al.*, 2009).

2.3.6.3 The disc diffusion test

The Muller Hinton agar medium was prepared according to the manufacturer's instructions. It was cooled and poured into Petri dishes of approximate 4 mm depth. The plates were used within seven days after preparation.

Chitosan samples were dissolved in 0.2 N acetate buffer pH 4.3 to obtain the concentration of 10 μg/μL (1 %). The prepared bacterial suspension was inoculated onto the entire surface of a Mueller-Hinton agar plate with a sterile cotton-tipped swab to form an even lawn. Sterile paper discs (6 mm in diameter, Whatman paper) were placed on the surface of each MHA plate using a sterile pair of forceps. 20 μL of diluted chitosan solutions were carefully pipetted onto the discs. The plates were then incubated aerobically at 37 ^{o}C for 24 hours. The diameter (mm) of the growth-inhibition zone including the disc diameter was measured after 24 h incubation using a ruler (CLSI, 2006 (a); Mayachiew *et al.*, 2010).

2.3.7 Total aerobic plate count method

Conventional plate count was performed for all samples as described in the FDA Bacteriological Analytical Manual (Maturin and Peeler, 2001). Standard plate count agar (AES CHEMUNEX, France) has been used. One millilitre of sample homogenate in each decimal dilution was plated in duplicate on plate count agar and incubated at 37 °C for 24-48 h.

The plates which had 25 – 250 colonies were used to calculate the number of viable cell.

$$\text{The number of viable cell } N = \Sigma C / [(1 * n_1) + (0.1 * n_2)] * (d)$$

Where: N = Number of colonies per mL (CFU/mL)

ΣC = Sum of all colonies on all plates counted

n_1 = Number of plates in first dilution counted

n_2 = Number of plates in second dilution counted

d = Dilution from which the first counts were obtained

If all plates from both dilutions yield fewer than 25 CFU each, the number of viable cell was recorded as less than 25 x 1/d when d is the dilution factor for the dilution from which the first counts were obtained.

2.3.8 Methods of analysing basic properties of edible films

2.3.8.1 Film thickness

The thickness of the film was measured using a panme ruler (Mitutoyo, Japan) with the smallest unit 0.001 mm. The data recorded was the average of 10 measurements.

2.3.8.2 Solubility in hot water

Approximately 0.5 g of edible film was soaked in 50 mL of distilled water and stirred well at 70 ^{o}C. The soluble part and insoluble part were then separated by filtering through filter paper (Whatman). The insoluble part was dried at 105 ^{o}C for 6 hours and recorded its weight.

The solubility (%) of the edible film was estimated as following equation:

$$\text{Solubility (\%)} = (M_1 - M_2) / M_1 \times 100$$

Where: M_1: initial weight of edible film (g)

M_2: weight of insoluble part of film

2.3.8.3 Mechanic properties

Mechanic properties of edible films were measured by the texture measurement machine (Zwich/Roell, Germany). Edible film was cut into each small pieces with the dimension of 12 x 2 cm (Figure 4.3).

The film was affected by stretching force at both ends at an instant speed 100 mm/min to evaluate the tensile strength (N) and the deformation (%). The data recorded was the average of 10 measurements.

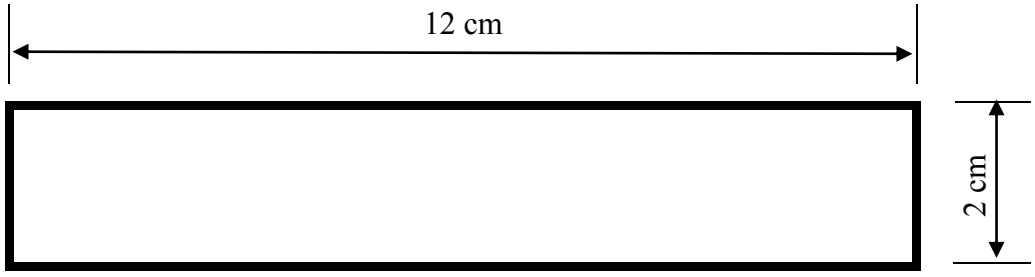


Figure 2.1: Film sample to measure mechanic properties

2.3.8.4 Seal ability

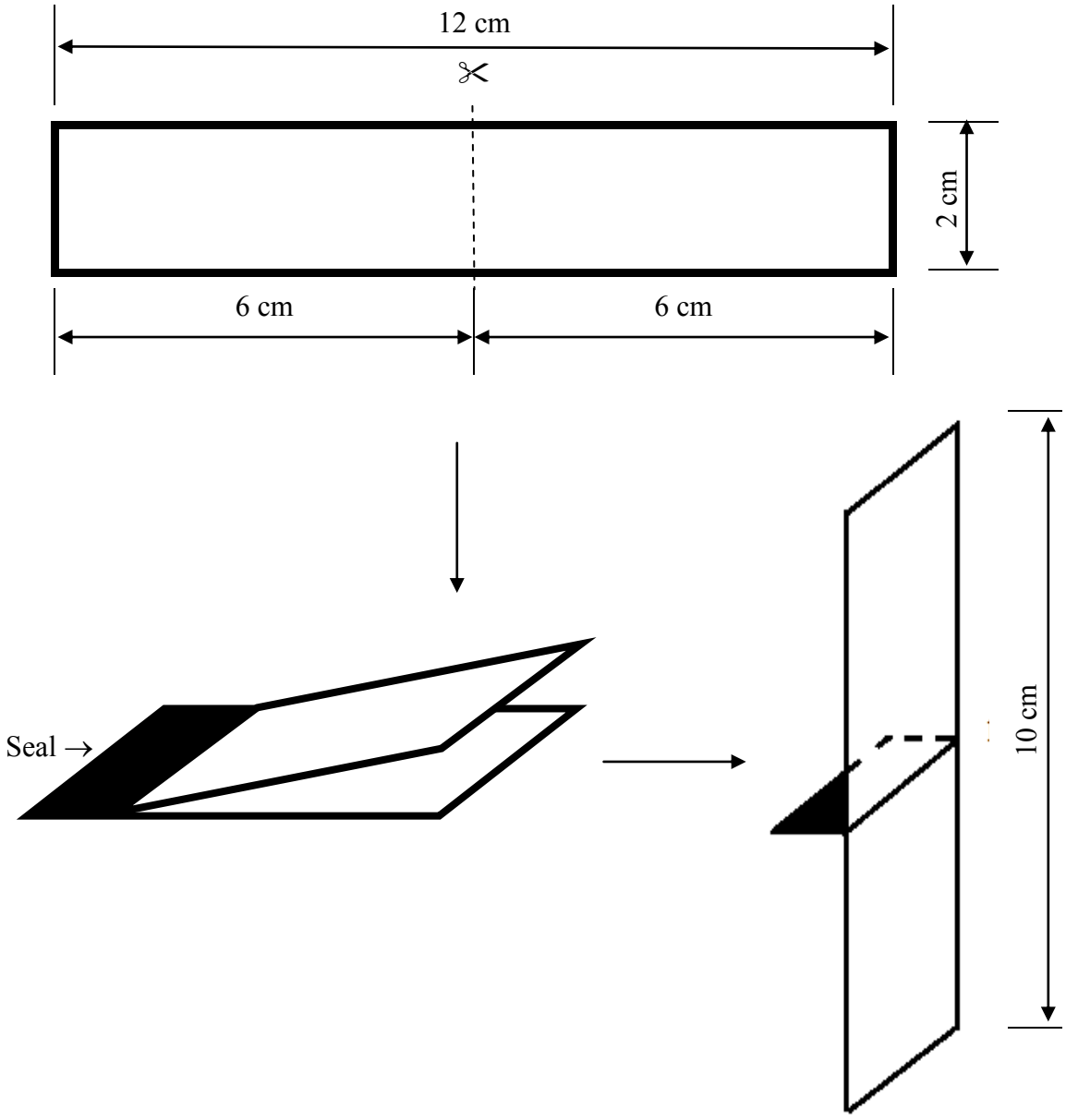


Figure 2.2: Process of sealing film

Edible film sample with the size of 2 × 12 cm is cut into 2 identical parts. They were then sealed together to make a film that have the size of 2 x 10 cm with the sealed part at the middle of the film (Figure 2.2). The film was stored in dessicators at room temperature.

The strength of the seal was measured by force that break the film seal using texture measurement machine (Zwick/Roell, Germany). The data recorded was the average of 10 measurement.

2.3.8.5 Water vapor permeation (WVP)

Water vapor permeation of edible films was estimated based on the increase of the film weight at a relative humidity differential of 84 – 22 % using a modified French standard method AFNOR NF H00-030 and ASTM Method E96-80 (Phan-The *et al.*, 2002).

Prior to measurement of the water vapour permeation, all films were stored at 25 ^{o}C in desiccators containing saturated potassium chloride which has a water activity (a_w) of 0.84 for 48 hours. Then the film was fixed between two Teflon rings on the top of a glass cell containing saturated potassium acetate which has a water activity of 0.22 at 25 ^{o}C. Finally, the test cells were put in desiccators containing saturated potassium chloride (a_w = 0.84) at 25 ^{o}C and periodically weighed until a constant weight variation rate was attained (Figure 2.3).

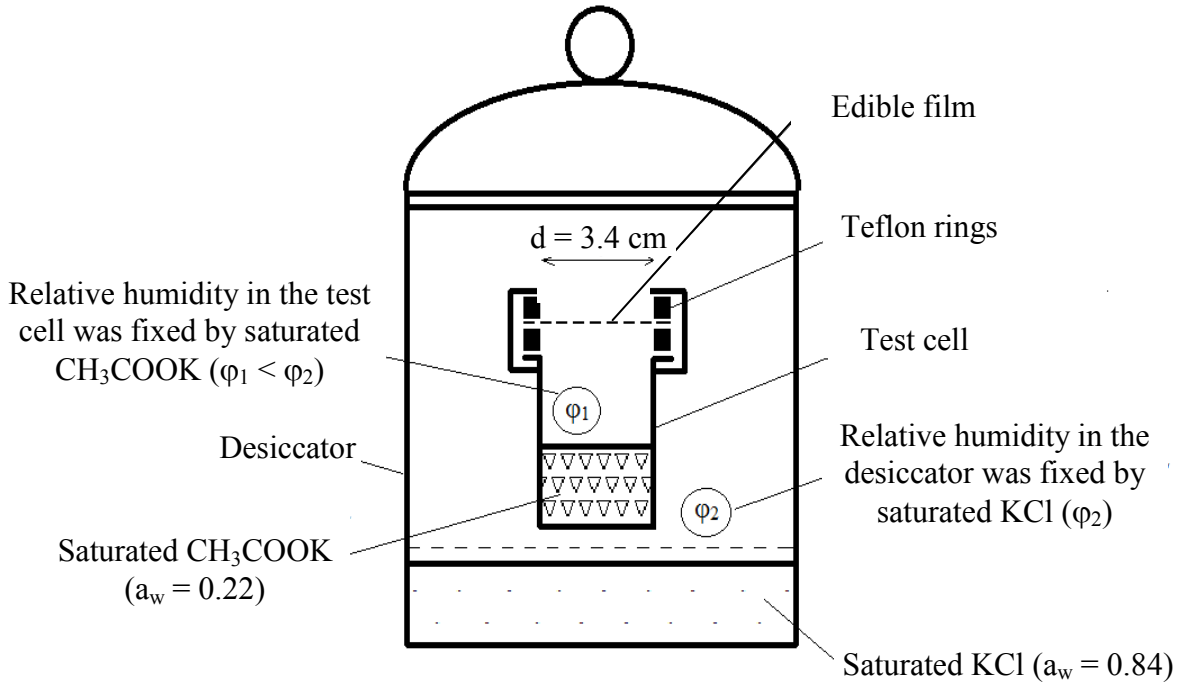


Figure 2.3: Principle outline of water vapour permeation measurement

The water vapour permeation was calculated using the equation:

$$WVP = \frac{\Delta m\, x}{S\, \Delta t\, \Delta p} \qquad (g.m^{-1}.\, s^{-1}.Pa^{-1})$$

Where:

Δm: the weight gained by the permeation cell (g)

x: the film thickness (m)

S: the exposed area of edible film during water vapour transfer (S = 8.04 x 10^{-4} m^2)

Δt: duration of water vapour transfer (s)

Δp: partial water vapor pressure differential (Pa)

$$\Delta p = a_w \text{ x } p_o = \Delta a_w \times 23{,}756 \times \frac{101300}{760} \quad \text{(Pa)}$$

2.4 DATA ANALYSIS

Most of the experiments in this project were carried out in triplicate. The obtained data were calculated using Microsoft Excel 2007. The average values with standard deviation were reported. The analysis of variance was performed to determine the significance of the differences using SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, Ill, USA) software package version 17.0.

Chapter 3
PRODUCTION OF RABBIT ANTI-CRUSTACEAN PROTEIN AND ANTI-SHRIMP TROPOMYOSIN POLYCLONAL ANTIBODIES

3.1 INTRODUCTION

Tropomyosin is a protein which is present widely in all species of vertebrates and invertebrates. However, only invertebrate tropomyosin such as crustacean, arachnids, insects, and molluscs can cause allergic reactions (Lehrer *et al.*, 2003; Lopata and Lehrer, 2009; Lopata *et al.*, 2010; Reese *et al.*, 1999). In nature, it exists in multiple isoforms which are coiled-coil dimers consisting of two parallel alpha-helical tropomosin molecules that are wound around each other (Reese *et al.*, 1999).

The antibody can bind with the antigen at one or more regions which are defined as epitopes. The monoclonal antibody is an antibody which can react with the antigen at only one epitope while the polyclonal antibody can react at two or more epitopes on the antigen structure. The cross-reactivity of tropomyosin among crustaceans (Ayuso R *et al.*, 2002; Lehrer, 1986; Lopata *et al.*, 2010; Reese *et al.*, 1999; Taylor and Steve, 2008) indicates that a monoclonal antibody can recognise the tropomyosin of different species at the same epitope (Lehrer and McCants, 1987). Therefore, the monoclonal antibody to a specific species can be used to investigate the presence of that antigen in other species. However, because different B cells can be stimulated by the tropomyosin, the blood will contain a variety of anti-tropomosin antibodies which can have different immune-reactivity (ABcam, 2012). Therefore, there is a need to use polyclonal antibodies in analysis under different experimental conditions (Newcombe and Newcombe, 2007; Seiki *et al.*, 2007; Yeung *et al.*, 1997). In the other hand, no polyclonal antibodies to crustacean proteins or crustacean tropomyosin are currently commercially available. Hence these antibodies have been made and they were described in this chapter. Without these antibodies no further analysis of chitin or chitosan products would have been possible.

The polyclonal antibodies are made by immunizing with the same antigen at intervals of several weeks to stimulate specific B cells to produce antibodies in the blood (ABcam, 2012; Stills, 2012; Yeung *et al.*, 1997). The methodologies are varied depending on the immunogen, the adjuvant and the intended purpose in producing the antibodies (Stills, 2012). The immune-sera can be used in its crude form or antibodies isolated from the immune-sera. Among laboratory animals, rabbits are used most commonly in the production of polyclonal antibodies due to their many advantages such as the adequate body size, the ready assessibility and the excellent responsiveness to a wide variety of antigens (Duranthon *et al.*, 2012; Seiki *et al.*, 2007; Stills, 2012).

The generated polyclonal antibodies are examined for immunological characteristics by immunoreacting with the antigen by Western blot and ELISA method (Crowther, 1995; Seiki *et al.*, 2007; Werner *et al.*, 2007).

The aim of this chapter is to produce two polyclonal antibodies from rabbits to investigate the presence of the shellfish allergen tropomyosin and/or other crustacean proteins in the shrimp tissues as well as chitin and chitosan preparations.

The rationale for choosing these protein preparations for antibody production is based on the knowledge that tropomyosin is the major allergen found in most crustaceans. Therefore the presence and concentration of this protein in food preparations derived from chitosan is central to manage the risk of the sensitised consumer to allergic reactions.

In addition antibodies to total protein extracts of crustacean, which in addition to tropomyosin, contain other crustacean derived proteins was also generated. One representative species from each crustacean group of shrimp, lobster and crab was chosen. It was expected that these antibodies should be able to identify if any residual crustacean proteins were present in chitosan containing products.

Summary of Chapter 3:

This chapter focuses on the production of rabbit polyclonal antibodies. These antibodies were raised by injecting antigens into rabbits to generate specific antibody containing sera. Two types of antigens were prepared. The first group of antigens were the mixed protein extracted from three crustacean species. The second group of antigens were the purified tropomyosin from four different shrimp species. The injection of antigens and collection of rabbit sera was conducted by a specialised company of the Institute of Medical and Veterinary Science (IMVS), Australia and not conducted at the University. These sera were analysed for their titters and immune reactions with the different antigens. The generated sera were used to investigate the immune reactions of different shrimp extracts as well as with residual shrimp proteins and allergens in chitin and chitosan preparation in following chapters.

3.2 MATERIALS AND METHODS

Throughtout this study, a commercial monoclonal anti-tropomyosin antibody was used for testing the immunoblotting with the samples investigated. However, this antibody was raised against the troponin – tropomyosin complex extracted from an insect. Therefore, polyclonal antibodies were generated to the total crustacean proteins and major shrimp allergen tropomyosin for use in experimental trials in comparison with the monoclonal antitropomyosin preparation.

Two types of rabbit sera were generated:

A) Rabbit serum containing polyclonal IgG antibodies against mixed crustacean protein extracted from Mud crab, Slipper lobster, and Black Tiger shrimp, referred to as "Rabbit serum pAbαCR".
B) Rabbit serum containing polyclonal IgG antibodies against purified tropomyosin extracted from four shrimp species (Black Tiger, Banana, Vannamei, and School shrimp), referred to as "Rabbit serum pAbαTM".

3.2.1 Preparation of the antigens

The two antigens used for the production of polyclonal antibodies were extracted from different crustacean species:

A) The first antigen was the total protein seperatedly extracted from each of three crustacean species: Mud crab, Slipper lobster, and Black Tiger shrimp.
B) The second antigen was the protein tropomyosin purified seperatedly from the total protein of Black Tiger, Banana, Vannamei, and School shrimp.

All crustacean samples were collected fresh or frozen from the local market and transported to the laboratory on ice. The list of these samples is described in Table 3.1.

Table 3.1: Common and scientific names of the crustacean samples

No.	Common name	Scientific name	Specimens' picture
1	Banana Shrimp	*Fenneropenaeus merguiensis*	
2	Black Tiger Shrimp	*Penaeus monodon*	
3	Vannamei Shrimp	*Litopenaeus vannamei*	
4	School Shrimp	*Metapenaeus macleayi*	
5	King Shrimp	*Melicertus latisulcatus*	 *(http:// www.austfish.com.au/king.html)*
6	Mud Crab	*Scylla serrata*	
7	Slipper lobster (Moreton Bay Bugs)	*Thenus orientalis*	 *(http://www.oceanexports.com.au/ crustaceans.htm)*

3.2.1.1 Preparation of the total protein extracts from crustacean

Protein extract from Mud crab, Slipper lobster, and Black Tiger shrimp were prepared separately by boiling whole fresh crustacean at 100 ^{o}C for 5 min in Milli-Q water. After that they were peeled to remove the shell. The tail meat part of the crustacean was cut into thin shreds using a pair of scissors. Approximately 20 g of the mass was added to 300 mL of PBS, homogenized for 10 min and then tumbled (shaker, Ratek Instruments, Australia) overnight (approximately 16 h) at 4^{o}C.

The next day, the mixture was decanted into 50 mL conical Falcon tubes (Sarstedt, Germany) and centrifuged at 4000 g for 30 minutes. The supernatant was passed through a glass fibre pre-filter (Sartorius, Germany), and then through a 0.45 μm cellulose acetate filter membrane (Sartorius, Germany).

The protein concentration of the resultant filtrate was measured by the Bradford method using Quick Start Bradford Protein Assay (Bio-Rad, USA) and adjusted to the 1 mg/mL concentration by dilution with PBS.

3.2.1.2 Preparation of purified tropomyosin from shrimp

The proteins from four shrimp species: Black Tiger, Banana, Vannamei, and School shrimp were extracted using the same procedure as described in Section 3.2.1.1.

These protein extracts were then separated by the ion-exchange chromatography (Biologic LP system, Bio-Rad) to collect the tropomyosin fractions. A strong anion exchange column, Bio-Scale mini Macro-prep, High Q prepacked column (1 mL volume) was used for the purification. The starting buffer was 30 mM Acetate buffer, pH 5.5. The eluting buffer was 1 M Sodium chloride, pH 5.5. All buffers were filtered through 0.45 μm membrane to remove all insoluble articles and sterilized by autoclaving for long storage.

Purified tropomyosin fractions were tested for their purity by SDS-PAGE. The protein concentration of each purified tropomyosin samples were measured by the Bradford method, concentrated to a level of 1 mg/mL by centrifugation at 3000 g through an Amicon Ultracel membrane (3 kDa cut-off, Millipore, USA) and then mixed together in equal volumes. Aliquots (0.5 mL) were also stored at the -80 ^{o}C freezer for use in an antibody production.

3.2.2 Procedure of antibody production

The antigens used in the production of polyclonal antibodies were 1) total crustacean protein and 2) purified tropomyosin prepared following the procedure described in Sections 3.2.1.1 and Section 3.2.1.2.

The production was conducted at a specialised company, the Polyclonal Antibody Production, Institute of Medical and Veterinary Science (IMVS), Veterinary Services Division, South Australia. Their address is at 101 Blacks Road, Gilles Plains SA 5086. Their website is www.imvs.sa.gov.au/vet. The protocol used in this production has been approved by the IMVS Animal Ethics Committee.

Three rabbits were used for the antibody production:

- Rabbit 1 and rabbit 2 were injected with the mixed purified tropomyosin to obtain the sera pAbαTM.
- Rabbit 3 were injected with the mixed crustacean protein to get the serum pAbαCR.

Four doses of antigen were injected to each rabbit using Freunds adjuvant at specific time as scheduled in Table 3.2. Each dose contained 0.5mL solution of 500µg antigen in Phosphate buffer saline pH 7.4. The first dose (primary immunisation) was emulsified in Freunds complete adjuvant and the other boost doses were emulsified in Freunds incomplete adjuvant.

Table 3.2: Immunization schedule

	Dose No	Bleed type
Week 0	1	Prebleed
Week 3	2	
Week 4		Test bleed 1
Week 5	3	
Week 6		Test bleed 2
Week 7	4	
Week 9		Bleed out

The sera were taken four times to examine their response by methods described in Section 3.2.3. The pre-bleed sera were stored frozen before injecting the immunogen and the bleed-out sera were collected under anaesthesia after which the rabbits were euthanased.

The blood was collected at 37^{o}C in sterile containers to accelerate clotting. After clots were formed, the blood was centrifuged and the serum was transferred to a sterile container and filtered (sterile 0.2 μm membrane) prior to storage at -80^{o}C.

3.2.3 Analytical methods

The rabbit immune sera were assessed for their characteristics using different methods.

The confirmation of antibody specificity was conducted by SDS-PAGE and Western Blot.

The screening of the rabbit immune sera was determined by indirect ELISA, as described in Chapter 2 – Section 2.3.4.1.

The immunoreaction of rabbit sera to the immunogens (the mixed purified tropomyosin and the mixed total crustacean protein) was investigated by the indirect ELISA method, at three dilutions: 1:5,000; 1:20,000; and 1: 30,000.

The immunoreaction of rabbit sera to different separate crustacean extracts (purified tropomyosins from different shrimp species and total protein extracts from different crustaceans) were investigated by using the indirect ELISA method, at three dilutions: 1:5,000; 1: 20,000; and 1:30,000.

In indirect ELISA, the goat-anti rabbit antibody conjugated with horse radish peroxidase (HRP) was used as the second antibody to react with the tetramethylbenzidine (TMB) substrate solution (Dako, Denmark). The absorbance at 450 nm wavelength was determined using a Multiskan plate reader (Labsystems).

All tests were conducted in triplicate. The obtained data were processed by using Microsoft Excel 2007 and ANOVA statistically analysed by SPSS 17.0 program.

3.3 RESULTS AND DISCUSSION

The production of polyclonal antibody involved two steps. The first step is the preparation of antigen and the second step is the immunisation.

3.3.1 Preparation of antigen

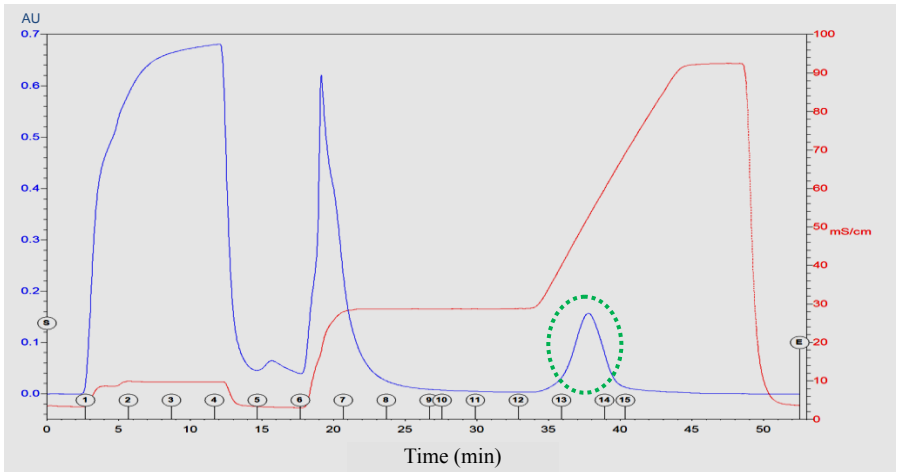
The preparation of antigen is very important because it can affect the quantity and the quality of the generated antibody. The optimal immunogen dose depends upon the specific immunogen (including factors like immunogenicity, purity, pH, contaminants, the adjuvant used, the frequency and number of immunisations, and the route of immunisation (Stills, 2012). The recommended maximum quantity of each immunogen dose at 500 μL for each rabbit was advised by the Polyclonal Antibody Production, IMVS, Veterinary Services Division, South Australia,

Two different antigens were prepared to generate immune sera containing:

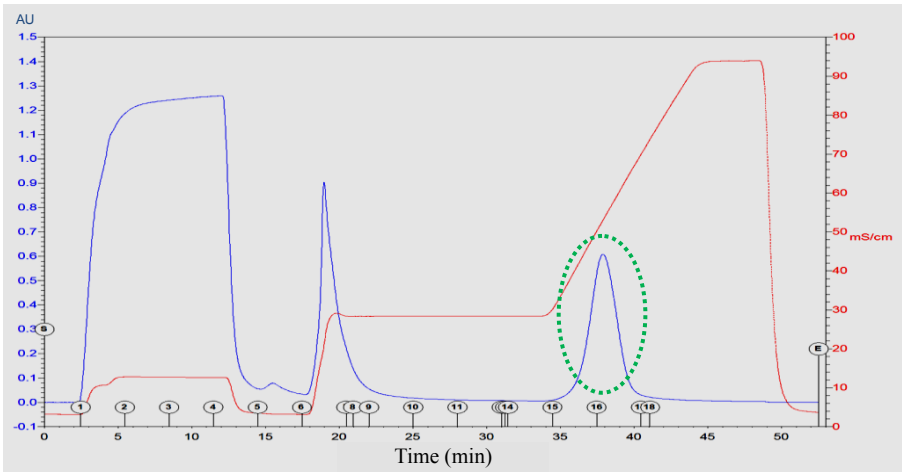
- The crustacean protein polyclonal antibody in response to mix crustacean protein.
- The tropomyosin polyclonal antibody in response to purified shrimp tropomyosin.

Four protein extracts from different shrimp species had similar chromatography (Figure 3.1). Three fractions from the protein extract were collected and examined to obtain the exact tropomyosin fraction. Fraction 1 was collected from 22 to 30 min; fraction 2 was collected from 30 to 35 min; and fraction 3 was collected from 35 to 40 min.

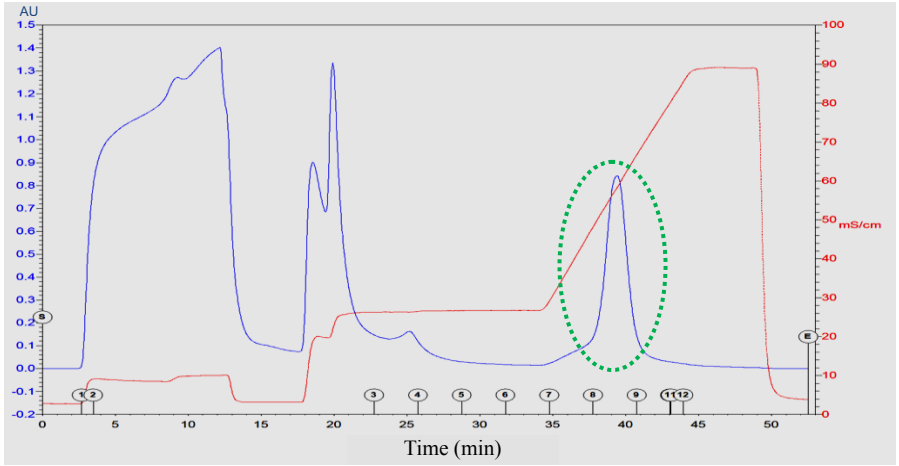
The fractions were investigated with SDS-PAGE and immunoblot with monoclonal shrimp antibody to examine specificity (Figure 3.2). The results indicated that fraction 3 contained the tropomyosin protein, which was eluted off the ion-exchange column once the salt concentration in the elution buffer reached 600 mM level. This fraction was then subjected to the ion-exchange chromatography again to collect the most purified tropomyosin sample.



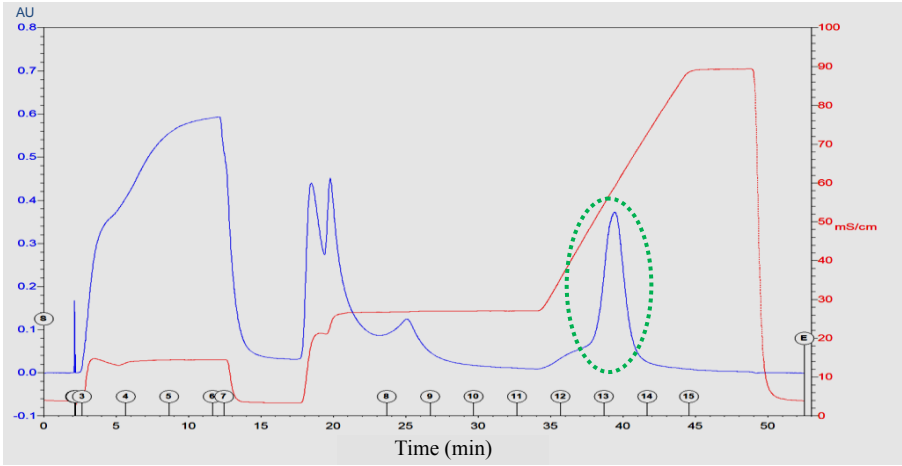
(a) Banana shrimp



(b) Black Tiger shrimp



(c) School shrimp



(d) Vannamei shrimp

: running buffer chromatography **:** protein chromatography **:** fractions which contained the tropomyosin

Figure 3.1: The ion-exchange chromatography of tropomyosin purification from different shrimp species

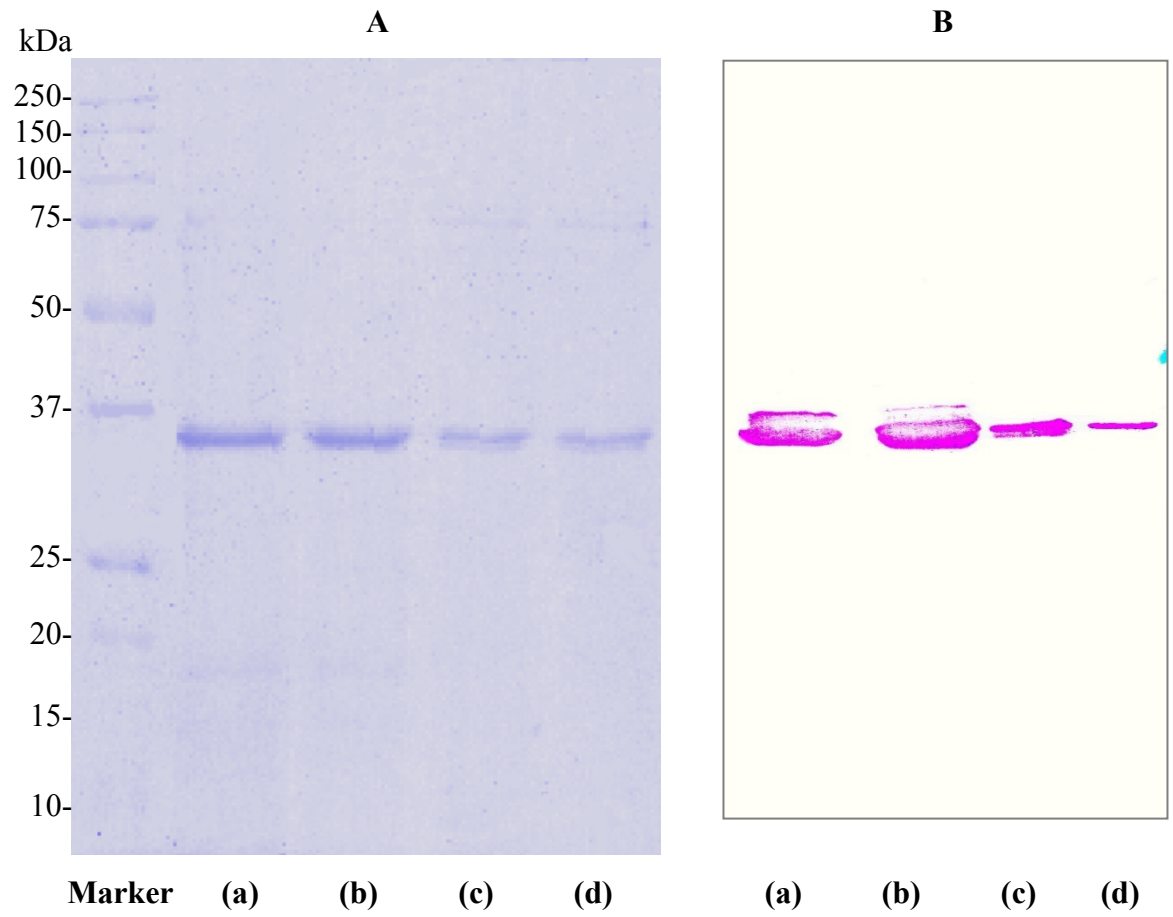


Figure 3.2: The SDS-PAGE (A) and immunobloting (B) of purified tropomyosin from (a) Banana shrimp; (b) Black Tiger shrimp; (c) School shrimp and (d) Vannamei shrimp.

Figure 3.2 presented the protein profile and immunoblotting of the final purified trpomyosin of each shrimp species. They all had highly purified tropomyosin at approximately 35 kD.

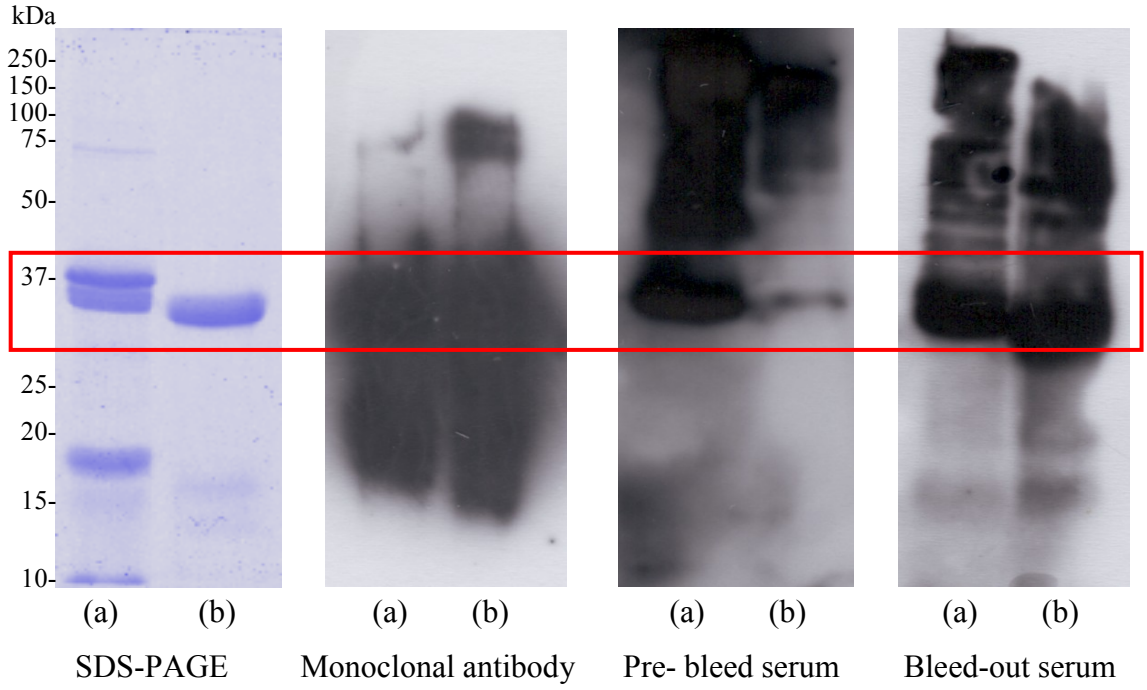
3.3.2 Immunization of rabbit

Three rabbits were used to obtain the polyclonal antibodies. The first two rabbits were injected with the purified shrimp tropomyosin to generate anti - tropomyosin sera and the third rabbit was injected with the mixed crustacean protein to get anti - crustacean protein serum. The number of rabbits was prioritised on the importance of the antibody and available project resources. Because the purified tropomyosin could cause reaction in the rabbit immune system and the important need of the anti - tropomyosin serum, two rabbits were used for this type of antibody production.

Unfortunately, rabbit 1 reacted too strongly to the antigen and died after the second dose injected. However, rabbit 2 and 3 reacted well with the antigens. They were injected with totally 4 doses of antigen as scheduled in Table 3.2. Each dose contained 0.5 mL solution of 500 µg antigen in Phosphate buffer saline pH 7.4. The Phosphate buffer saline is recommended by the IMVS Veterinary Services Division for use in the handling of the antigen to avoid high concentrations of denaturing or toxic compounds. The sera were taken four times to test their immune responses. The aim of injecting several boosts was to give time for the rabbits to generate the appropriate antibodies against the injected immunogen.

3.3.3 Confirmation of antibody specificity

This experiment was carried out to examine the affinity of the rabbit sera to the immunogens. The sera of each rabbit were tested for immunological reactivity using a Western blotting technique. The pre-bleed, test-bleed and bleed-out sera of each rabbit were immunoblotted with the purified tropomyosin and the mixed crustacean protein. The reactions of those sera were compared with the reaction of the monoclonal antibody against insect tropomyosin. The results were presented in Figures 3.3 – 3.5.



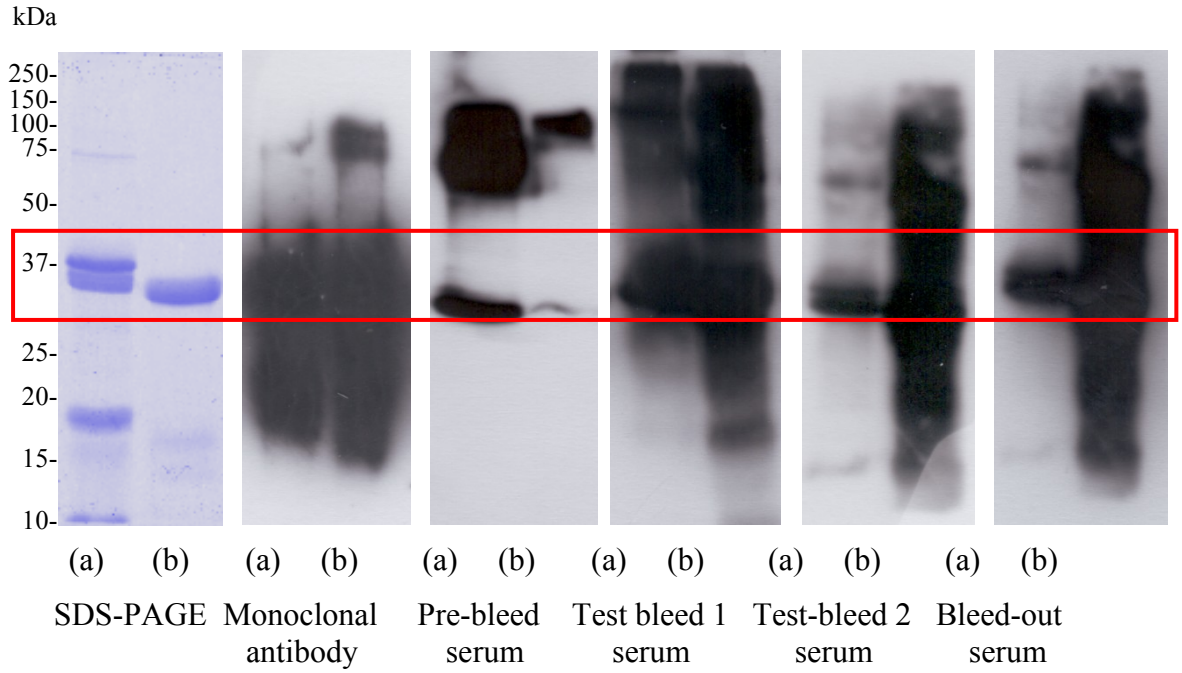
Note: The red box indicates the range of tropomyosin

Figures 3.3: Immunoblotting of (a) crustacean protein and (b) purified shrimp tropomyosin with monoclonal antibody mAbαTM and rabbit 1's sera pAbαTM.

The immunoreactivity of the rabbit 1's sera is presented in Figure 3.3. Both the crustacean protein and the purified shrimp tropomyosin reacted so strongly to the monoclonal antibody that they exhibited very thick diffuse dark bands around the 20 - 37 kDa range. The pre-bleed serum of rabbit 1 already expressed strong reaction to the purified tropomyosin. However, after the second dose was injected with the purified tropomyosin, the antibody titre in rabbit 1's blood increased sharply. The serum recognized a clear dark band of about 35 kDa, the same size as the original immunogen. This result indicated that rabbit 1 was very sentitive to tropomyosin.

The monoclonal antibody mAbαTM reacted with the crustacean protein and the purified shrimp tropomyosin at different bands. This result indicated that the presence of tropomyosin in these extracts could exist in different isoforms.

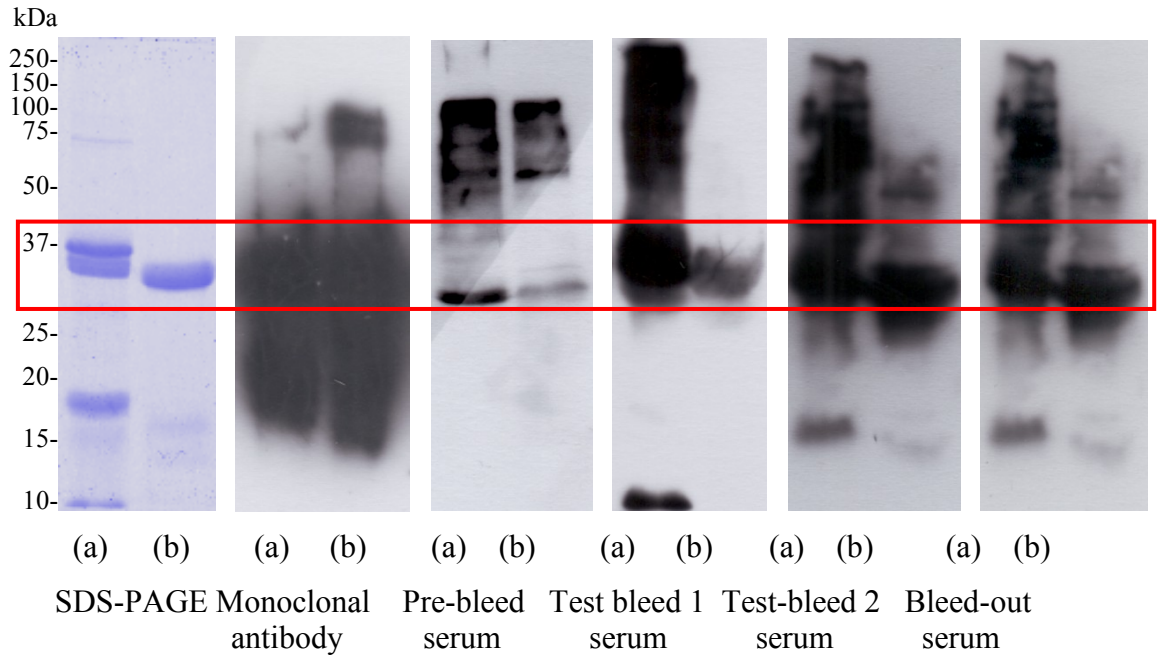
The immunoreactivity of the rabbit 2's sera is presented in Figure 3.4. The pre-bleed serum of rabbit 2 also expressed weak reaction with the mixture of purified tropomyosin. After the second dose was injected with the mixture of purified tropomyosin, the antibody titre in rabbit 2's blood increased significantly. The serum also recognized a dark band of about 35 kDa, the size of the allergic tropomyosin (Lopata *et al.*, 2010). The reaction was still very strong in the bleed-out serum.



Note: *The red box indicates the range of tropomyosin*

Figures 3.4: Immunoblotting of (a) crustacean protein and (b) purified shrimp tropomyosin with monoclonal antibody mAbαTM and sera of rabbit 2 pAbαTM.

The sera also demonstrated strong binding with tropomyosin in the mixture of crustacean protein. However, after being immunised the second time, most of the antibody in rabbit serum was produced against the tropomyosin antigen. This result indicated that the rabbit 2's immune system had very good reaction to the purified tropomyosin immunogen.



Note: The red box indicates the range of tropomyosin

Figures 3.5: Immunoblotting of (a) mix crustacean protein and (b) mix purified tropomyosin with monoclonal antibody mAbαTM and with sera from rabbit 3 pAbαCR.

The pre-bleed of rabbit 3 expressed strong reaction with tropomyosin in the mixture of crustacean protein but weak reaction with the mixture of purified tropomyosin (Figure 3.5). The reaction to the crustacean protein seemed stronger after the second dose with a very thick dark band in the 37 kDa range. The rabbit antibody could also bind with other proteins in the mixture of crustacean protein.

The test-bleed and bleed-out sera of rabbit 3 expressed strong binding with both mixture of crustacean protein and mixture of purified tropomyosin.

In general, it could be concluded that the immunization of antigen into rabbits was successful in generating the desired antibodies in the rabbit's sera.

3.3.4 Screening of antibody titers in the rabbit sera

The screening of the rabbit sera is an important step before using them in the research. This titration experiment was conducted to find out the optimum titer or dilution which gives the best result with minimum non-specific binding by using a series of dilutions of the rabbit sera.

In this experiment, a serial concentration of antigen and a serial dilution of antibody were carried out to investigate the appropriate concentration of antigen and the suitable dilution of rabbit serum which provided the highest immunoblotting reaction. The examined concentration of purified shrimp tropomyosin and crustacean protein varied from 0.001 µg/mL to 10 µg/mL while the dilution of the rabbit sera increased from 1,000 to 500,000 times.

The indirect ELISA was carried out in this experiment (Crowther, 1995). Data analysis was performed reading absorbance at 450 nm and analyzing with Microsoft Excel 2007. The results were presented in Figure 3.6 and Figure 3.7.

3.3.4.1 Checkerboard titration of rabbit serum pAbαCR

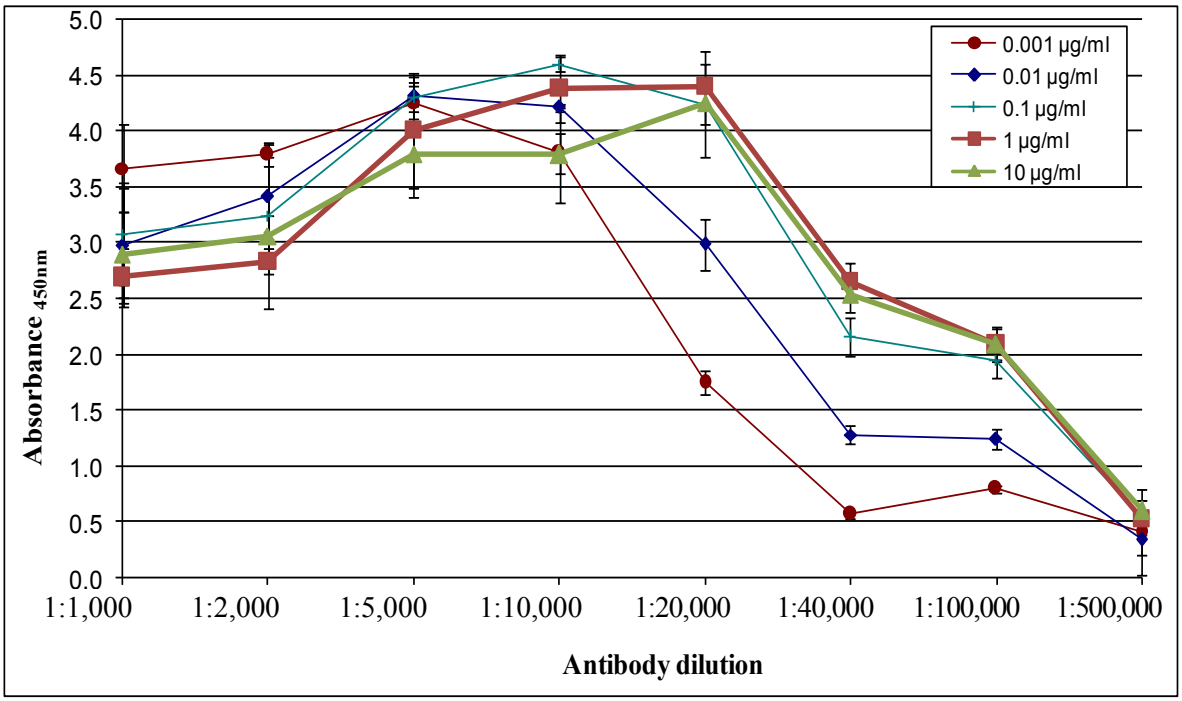


Figure 3.6: Checkerboard titration of rabbit serum pAbαCR with different concentrations of crustacean protein

Figure 3.6 demonstrated that the immunoreations between rabbit serum and crustacean protein was varied depending on the crustacean protein concentration and the dilution of the serum.

Both crustacean protein concentrations of 0.001 µg/mL and 0.01 µg/mL expressed the lowest binding reactions to the rabbit serum. The reaction reduced significantly when the rabbit serum was diluted more than 10,000 times.

The higher crustacean protein concentrations, 0.1 µg/mL, 1 µg/mL and 10 µg/mL, expressed strong immunoreactions when the rabbit serum was diluted 20,000 times. Therefore, the crustacean protein concentration of 0.1 µg/mL was chosen to use in the next experiments to get the highest immunoreactions at the lowest antigen concentration.

3.3.4.2 Checkerboard titration of rabbit serum pAbαTM

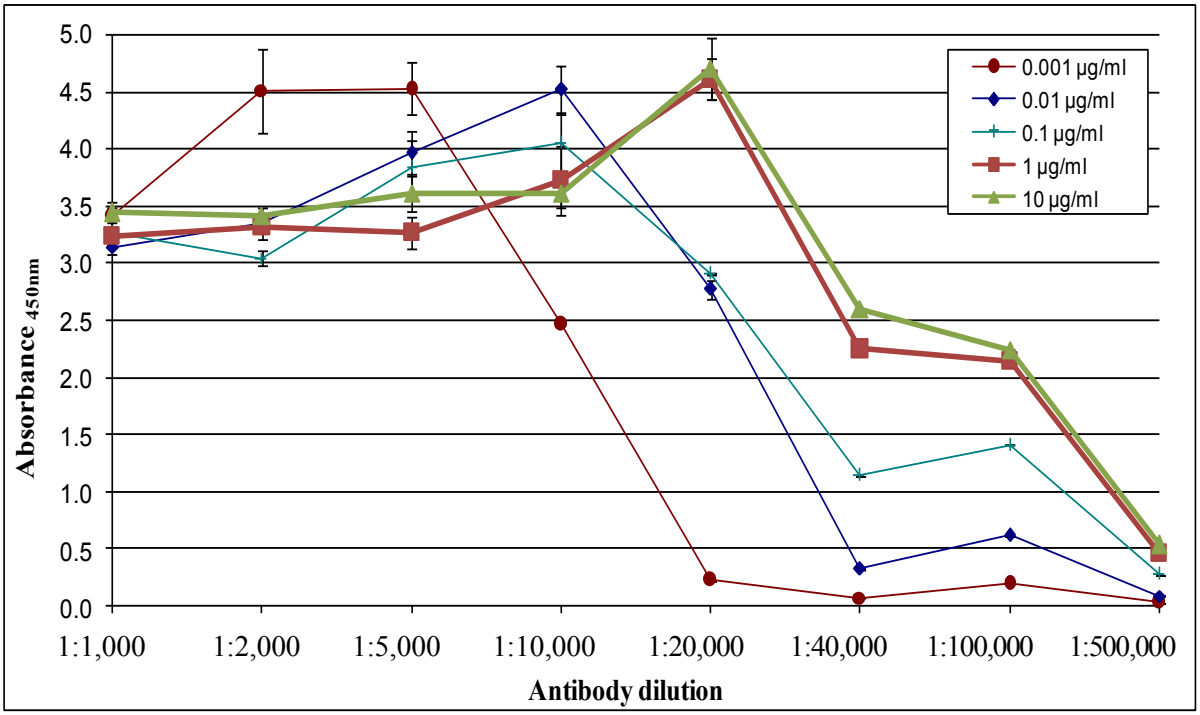


Figure 3.7: Checkerboard titration of rabbit serum pAbαTM with different concentrations of tropomyosin

Figure 3.7 also demonstrated that the immunoreations between rabbit serum and tropomyosin varied depending on the tropomyosin concentration and the dilution of the serum.

The tropomyosin concentration of 0.001 µg/mL expressed the lowest binding reaction to the rabbit serum. The reaction reduced sharply when the rabbit serum was diluted more than 5,000 times. On the other hand, the tropomyosin concentration of 1 µg/mL and 10 µg/mL expressed the highest binding reaction. The reactions were still very strong even when the rabbit serum was diluted 20,000 times.

The tropomyosin concentration of 0.01 µg/mL and 0.1 µg/mL still expressed strong immunoreactions when the rabbit serum was diluted 20,000 times. Therefore, the tropomyosin concentration of 0.01 µg/mL was chosen for use in the next experiments to obtain the highest immunoreactions at the lowest antigen concentration.

3.3.5 Immunoreactivity of different rabbit sera to the immunogens

This study was conducted to investigate immunoreactions of different rabbit sera to the purified tropomyosin and the crustacean protein.

In this experiment, the four sera (pre-bleed, test bleed 1, test bleed 2 and bleed-out) of each rabbit were incubated with the purified shrimp tropomyosin and crustacean protein to examine their immunoreactions. The examined concentration of the purified shrimp tropomyosin was 0.01 µg/mL, and the concentration of the crustacean protein was 0.1 µg/mL. The rabbit sera were investigated with three dilutions: 1:5,000; 1:20,000; and 1: 30,000.

The indirect ELISA has been conducted in this experiment. Data analysis was performed on the A450 readings using Microsoft Excel 2007. The results were presented in Figure 3.8 – 3.10.

3.3.5.1 Sera dilution 1:5,000

When the sera were diluted 5,000 times, the immunoreactions increased when more antigens was injected into the rabbits in the case of test-bleed 1. The reaction of the test-bleed 1, test-bleed 2, and bleed-out sera of the rabbit 2 were unchanged. The reaction of the test bleed 2 of the rabbit 3 continued to increase after the third boost, but the reaction of its bleed-out serum slightly decreased.

The rabbit 2's test-bleed 1 had the highest immunoreactions with both purified tropomyosin and crustacean protein. Both test-bleed 2 sera of the rabbit 2 and 3 had high bindings and the reactions continued increasing slightly in their bleed-out sera.

All sera exhibited higher immunoreactions to the crustacean protein than to the purified tropomyosin. This could be because the concentration of the crustacean protein was 10 times more than that of the purified tropomyosin.

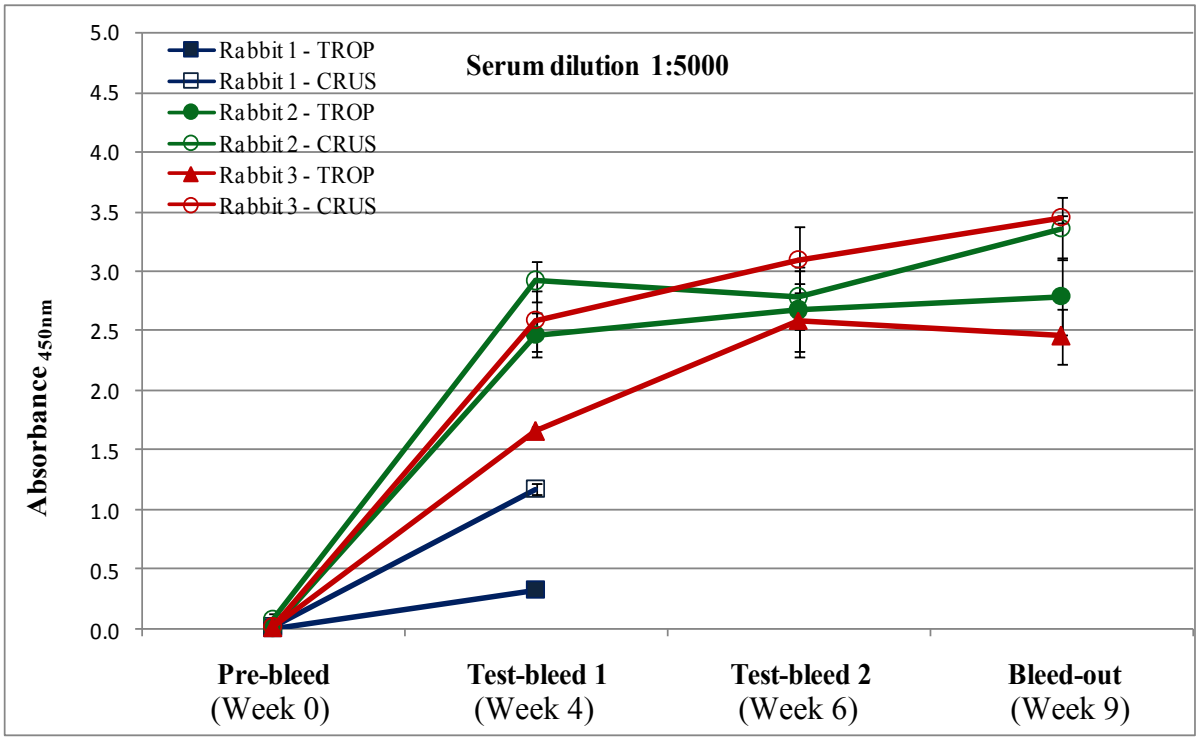


Figure 3.8: The immunoreactions of rabbit sera to purified tropomyosin and crustacean protein at sera dilution 1:5,000

Note: TROP: Purified tropomyosin; CRUS: crustacean protein

3.3.5.2 Sera dilution 1:20,000

The sera of rabbit 1 exhibited very weak reaction with both purified shrimp tropomyosin and crustacean protein. Reverselly, the sera of rabbit 2 exhibited very strong reaction with both purified shrimp tropomyosin and crustacean protein with the maximum bindings achieved in the test-bleed 2 sera.

The sera of rabbit 3 had high reaction with the crustacean protein but low reaction with the purified tropomyosin. This is because the rabbit 3 was immunised with the crustacean protein to produce anti- crustacean protein sera.

The immunoreactions of the bleed-out sera of both rabbit 2 and 3 were slightly decreased as compared to that of the test-bleed 2.

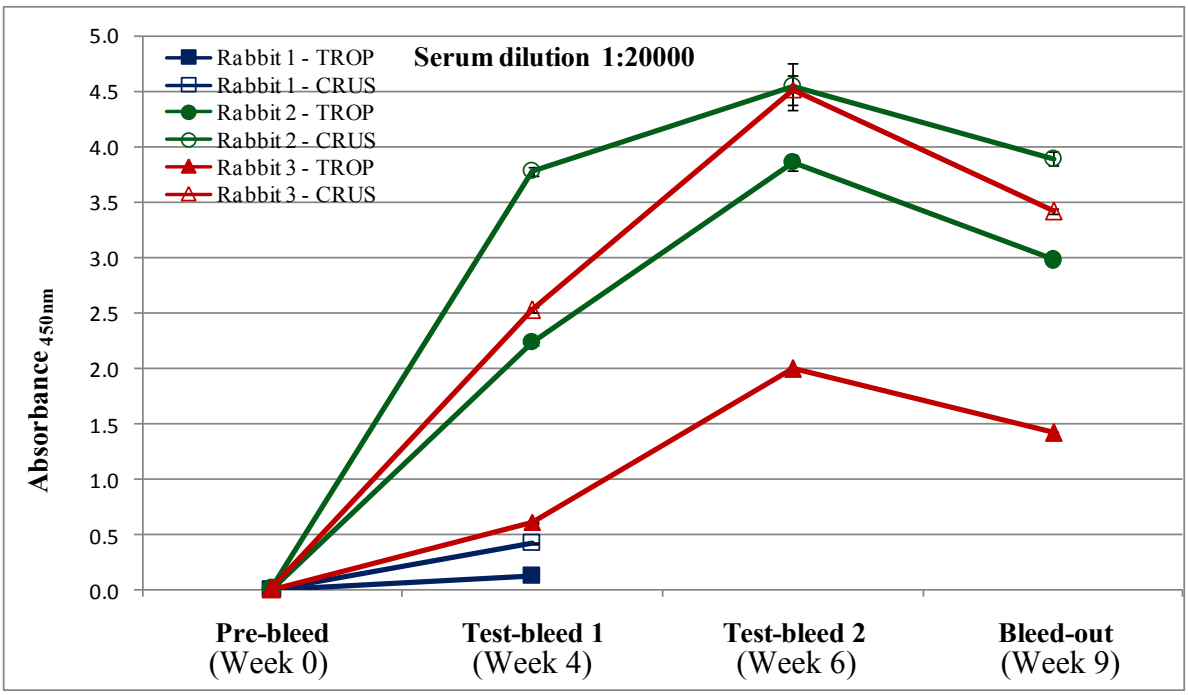


Figure 3.9: The immunoreactions of rabbit sera to purified tropomyosin and crustacean protein at serum dilution 1:20,000

Note: TROP: Purified tropomyosin; CRUS: crustacean protein

3.3.5.3 Sera dilution 1:30,000

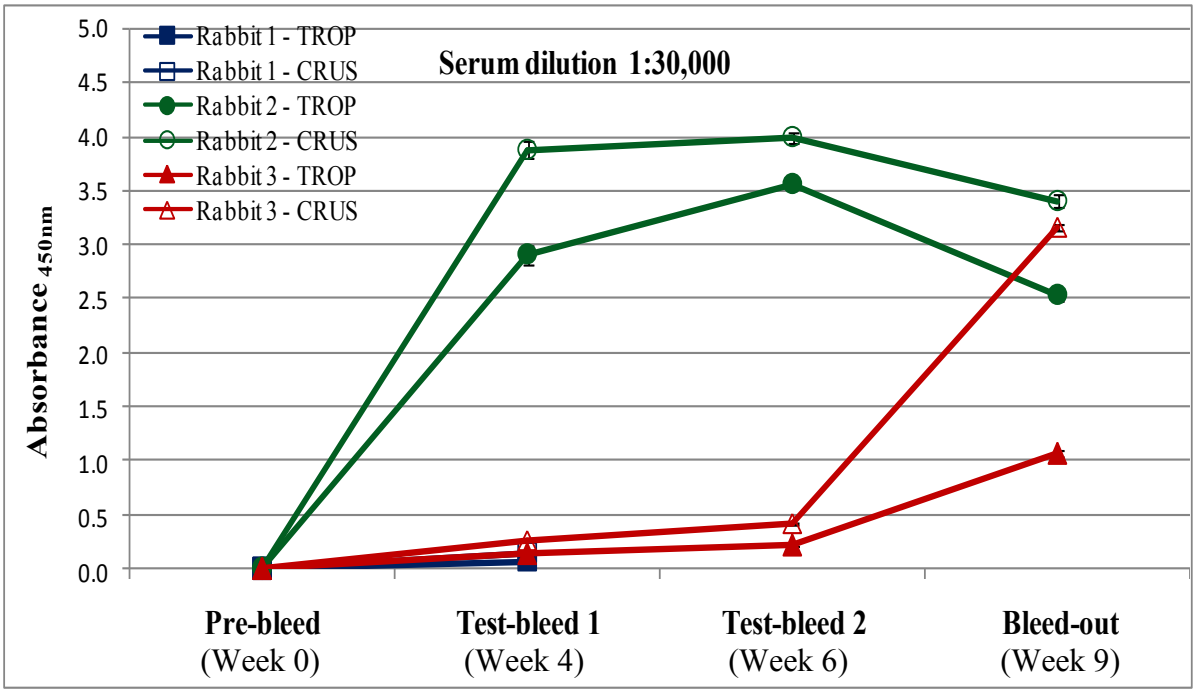


Figure 3.10: The immunoreactions of rabbit sera to purified tropomyosin and crustacean protein at serum dilution 1:30,000

Note: TROP: Purified tropomyosin; CRUS: crustacean protein

The sera might be too diluted in this case. The immunoreactions of rabbit 1 and 3 were very low. Only the rabbit 2's sera can exhibite the high bindings with the antigens.

In general, the immunoreactions of different rabbit sera changed with the change of the serum dilution. The 20,000 times diluted sera had the highest reaction, and the 5,000 times diluted sera had the lowest reaction. The immunoreactions were also different among different sera in the same dilution.

3.3.6 Immunoreactivity of rabbit sera to specific crustacean protein extracts

This experiment was aimed to compare the immunoreactions of rabbit serum pAbαTM and rabbit serum pAbαCR to different purified tropomyosin and protein extracts.

In this experiment, the rabbit sera were incubated with purified tropomyosin from different shrimp species and with different crustacean protein extracts. The purified tropomyosin was used at the 0.01 μg/mL concentration and the crustacean protein was used at the 0.1 μg/mL concentration. The immunoreactions were examined with three dilutions of rabbit serum: 1:5,000; 1: 20,000; and 1:30,000. The results are presented in Figure 3.11.

The immunoreactions of the rabbit sera changed with the change of the serum dilution, in which the 5,000 times diluted sera had the lowest reaction. The immunoreactions were also different between the two rabbit sera and among different protein extracts in the same dilution.

With the dilution 1:5,000, the rabbit serum pAbαTM exhibited stronger reaction to purified tropomyosin than crustacean extracts. The immunoreactions of this serum to the purified tropomyosin from Black Tiger, Banana, Vannamei and School shrimp were even higher than the mixed purified tropomyosin. The reaction of the rabbit serum pAbαCR to the purified tropomyosin extracts was similar to the crustacean extracts.

The 20,000 and 30,000 times dilution of rabbit sera exhibited similar immunoreactions. They both had high binding to the crustacean protein extracts than the purified tropomyosin. This might be due to the appropriate dilution of the sera that expressed the maximum binding reactions.

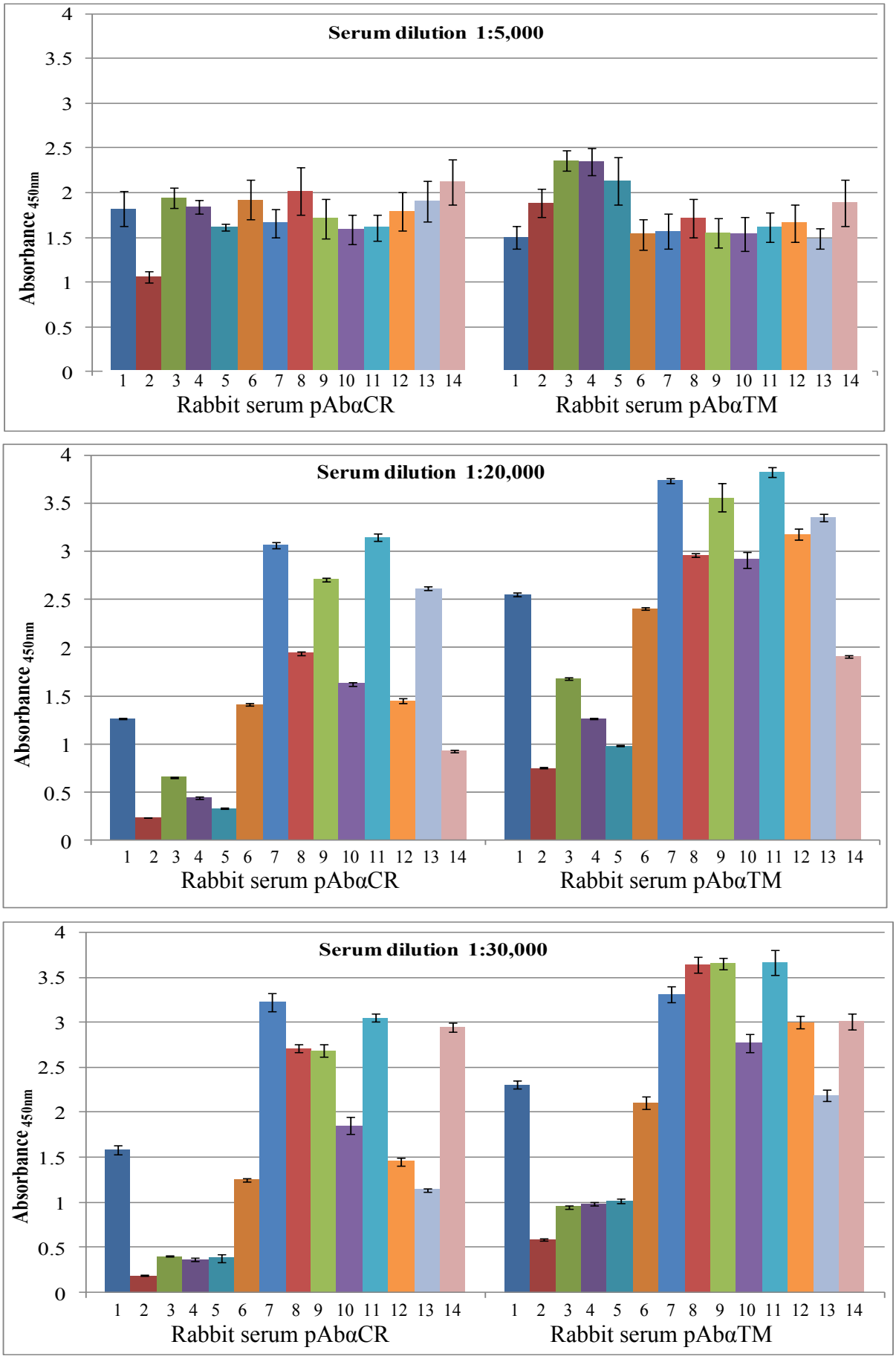


Figure 3.11: The immunoreactions of rabbit sera to different protein extracts

(1) *Mix purified tropomyosin*

(2) *Black Tiger shrimp – Tropomyosin*

(3) *Banana shrimp – Tropomyosin*

(4) *Vannamei shrimp – Tropomyosin*

(5) *School shrimp – Tropomyosin*

(6) *King shrimp – Tropomyosin*

(7) *Mix crustacean protein*

(8) *Mud crab protein*

(9) *Slipper lobster protein*

(10) *Black Tiger shrimp protein*

(11) *Banana shrimp protein*

(12) *Vannamei shrimp protein*

(13) *School shrimp protein*

(14) *King shrimp protein*

Overall, the dilution 1:30,000 of rabbit sera was chosen to use for the next experiments due to their high binding immunoreactions to the crustacean protein extracts.

3.4 GENERAL DISCUSSION AND CONCLUSION

Two rabbit polyclonal antibodies were successfully generated. These antibodies were raised against the total crustacean proteins from Mud crab, Slipper lobster, and Black Tiger shrimp and against the purified tropomyosin from four species of shrimp. This research showed that rabbit is a suitable laboratory animal for antibody production in this study. Other researchers were also successful in using rabbit for antibody production in their studies (Duranthon *et al.*, 2012; Hashimura *et al.*, 1982; Ridder *et al.*, 1995).

All antigen preparations in this chapter had very compact bands arounds 37 kDa range in SDS-PAGE and Western Blot with anti-tropomyosin monoclonal antibody. It indicated the presence of tropomyosin there. This result was confirmed by other researchers in various shellfish allergens (Ayuso R *et al.*, 2002; Lopata and Lehrer, 2009; Lopata *et al.*, 2010).

Both rabbit sera had very thick dark bands at the 37 kDa range, which indicated that the rabbit sera are very sensitive for the detection of the presence of tropomyosin. In addition, the rabbit serum against higher molecular weight crustacean proteins which are most likely polymers of tropomyosin. The rabbit sera not only recognise the immunogen but also proteins from other species such as King shrimp. This strong reaction between the rabbit sera against proteins extracted from other species of shrimp as well as other crustacean species can confirm the cross reactivity of the allergenic tropomyosin which was discovered by other scientists (Hoffman *et al.*, 1981; Lopata and Lehrer, 2010; Lopata *et al.*, 2010; Mills and Breiteneder, 2005; Patrick *et al.*, 1998; Shanti *et al.*, 1993; Wild and Lehrer, 2005).

The rabbit sera showed high affinity and specificity to their antigen in Western blotting. The antibody titration was carried out using the indirect ELISA. The immunoreactivity of the rabbit sera were still very strong when the antibody was diluted 30,000 times with very low concentrations of the purified tropomyosin and the crustacean protein, with 0.01 µg/mL and 0.1 µg/mL respectively. These results demonstrated that the obtained polyclonal antibodies from rabbits are suitable for the analysis of chitin and chitosan in the next chapters.

Chapter 4
INVESTIGATION OF THE ALLERGENICITY OF SHRIMP PROTEIN

4.1 INTRODUCTION

Consumers are getting more and more concerned about their health. Therefore, there is an increasing demand for healthy food to support and strengthen the quality of lives (Alishahi and Aïder, 2011). Most of the nutritious food products which provide functional substances such as eicosapentaenoic acid, docosahexaenoic acid and omega-3 can be found in aquacultural products. As a result, the aquaculture industry has become one of the fastest developed industries over recent years (Kandra *et al.*, 2012). South East Asia countries such as Thailand, Vietnam, Indonesia and Philippins are among the world's leader producer of cultured shrimp (FAO, 2003).

On the other hand, shellfish allergy is one of the most prevalent food allergies, especially in adults (Asero *et al.*, 2012; Hill *et al.*, 1997; Lopata and Lehrer, 2010; Mills and Breiteneder, 2005; Ng *et al.*, 2011; Sicherer *et al.*, 2004; Sicherer and Sampson, 2006; Taylor and Steve, 2008; Wild and Lehrer, 2005). It is estimated that about 1% of the world population is allergic to shrimp (Leung *et al.*, 1998; Lopata and Potter, 2000; Sicherer *et al.*, 2004).

The shrimp muscle protein tropomyosin has been shown to be the major heat-stable allergen causing sensitisation in consumers (Ayuso *et al.*, 2002; Hoffman *et al.*, 1981; Leung *et al.*, 1998; Liu *et al.*, 2010; Lopata, 2010; Nagpal *et al.*, 1989). The molecular weight of tropomyosin varies from 34 to 38 kDa (Reese *et al.*, 1999; Shanti *et al.*, 1993). It has a coiled-coil dimer structure of two paralled alpha-helical tropomyosin molecules (Reese *et al.*, 1999). The processing of shrimp may affect the IgE binding properties of tropomyosin (Liu *et al.*, 2010). Generally, shrimp is exported in frozen form without exoskeleton (Kandra *et al.*, 2012). About 48 – 56 % by weight of shrimp raw material is discarded as waste depending on species (Sachindra *et al.*, 2007).

Therefore, this research is aimed at investigating the allergenic tropomyosin protein in shrimp products due to variations in the shrimp species, body parts of shrimp and the processing methods.

Summary of Chapter 4:

This chapter focuses on the identification of the allergenic protein tropomyosin in different protein extracts of the two most common shrimp species sold in the Australian market: Black Tiger shrimp and Banana shrimp.

Six different protein extractions from each shrimp species were prepared by different methods: raw extraction, heat-treated extraction and whole shrimp-cooked extraction. These protein extracts were then compared together according to their SDS-PAGE profiles, immunoblotting reactions by Western blotting as well as their immunological cross-reactivities through inhibition ELISA.

The result demonstrated that the allergenic tropomyosin protein is not only present in the tail meat but also in the extracted shell waste of shrimp. The tropomyosin seemed to be more stable to heat processing when extracted after heating the whole shrimp.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of protein extracts

Two shrimp species were used for this study, the Australian Banana shrimp (*Fenneropenaeus merguiensis*) and the Black Tiger shrimp (*Penaeus monodon*). A general procedure of the three methods of protein extraction from shrimp is shown in Figure 4.1.

Raw specimens were bought from the local market and stored in ice during the transit. They were peeled to separate the shell (including shell, head - carapace and rostrum, and legs - pereopods and pleopods) and the tail meat (which is termed "tail" for the remainder of the thesis) of the shrimp to prepare two different protein extracts, the shell protein extract and the tail protein extract. Those parts were then cut into thin shreds using a pair of scissors and approximately 20 g was added homogenized in PBS as described in Section 3.2.1.1 and refererred to as "Mixture A".

The "**raw protein extract**" was aliquots from mixture A, filtered and frozen at -80 ^{o}C freezer (Section 3.2.1.1).

The "**heat-treated protein extract"** was prepared from mixture A by heating at 70 ^{o}C for 5 min, clarifying by centrifugation, filtered sterilising, aliquoting and storing at -80 ^{o}C (Section 3.2.1.1).

The "**whole-cooked protein extract"** was prepared from whole raw shrimp cooked at 100 ^{o}C for 5 min in Milli-Q water and then treated as for other preparations as described in Section 3.2.1.1.

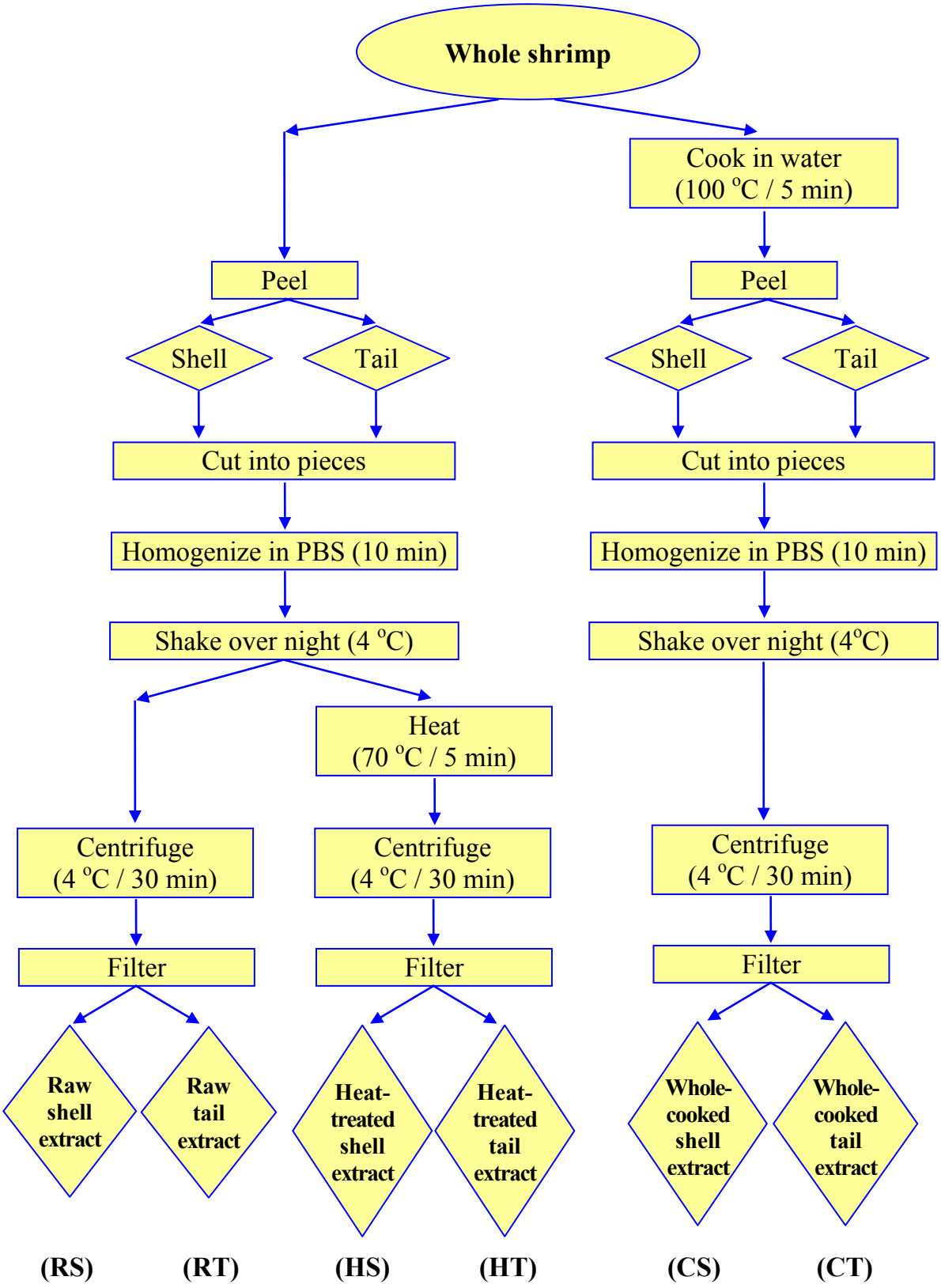


Figure 4.1: A general layout of the protein extraction procedure from shrimp

4.2.2 Analytical requirements

4.2.2.1 Protein concentration of shrimp protein

The protein concentration of each extract was quantified by Bradford method using the Quick Start Bradford Protein Assay (Bio-Rad) (Chapter 2 – Section 2.3.1.4). The protein concentration of each extract was calculated based on the initial amount of shrimp tissue used for extraction and expressed as % (w/w).

4.2.2.2 Protein profile of different shrimp protein extracts

The 12% SDS polyacrylamide gel electrophoresis was used to separate the proteins of the different extracts by molecular weight (Chapter 2 – Section 2.3.2). The protein profiles of different protein extracts were discussed and compared together.

4.2.2.3 Immunoblotting of different shrimp protein extracts

The presence of tropomyosin was examined by Western blotting (Chapter 2 – Section 2.3.3.1). The immunoblotting was conducted with the monoclonal antibody and sera of two patients with clinical symptoms to crustacean:

- Monoclonal antibody mAbαTM: Commercial rat anti-tropomyosin IgG antibody, MAC-141 (Abcam).
- Human serum AN01: Serum from patient who has allergic symptoms with specific IgE against lobster, crab and shrimp.
- Human serum JB21: Serum from patient who has allergic symptoms with specific IgE against crab and shrimp.

4.2.2.4 Immunoreactivity of different shrimp protein extracts

The immunoreactivity of tropomyosin in shrimp extracts was examined by the indirect ELISA as described in Chapter 2 – Section 2.3.4.1. In this assay, two rabbit sera were used to detect the presence of tropomyosin: the rabbit serum pAbαTM containing antibodies which was raised against the shrimp tropomyosin and the rabbit serum pAbαCR containing antibodies which was raised against the whole-cooked crustacean protein. Protein extracts from Black Tiger and Banana shrimp was first coated onto the wells. The rabbit sera pAbαTM and pAbαCR diluted 30,000 times were then added. The goat-anti rabbit antibody conjugated with HRP was used as the second antibody to react with the TMB substrate solution (Dako, Denmark). The absorbance at 450 nm wavelength was determined using a Multiskan plate reader (Labsystems).

Serial concentrations of the purified tropomyosin from 0.0001 µg/mL to 0.1 µg/mL were also loaded as antigens to create a standard curve for the estimation of tropomyosin concentration in the shrimp protein extracts.

To determine the cross-reactivities of each antibody to different protein extracts, inhibition ELISA was performed (Chapter 2 – Section 2.3.4.2). A high percentage of inhibition indicates a high degree of cross-reactivity of the antibody to the antigen. In this assay, the wells were first coated with antigen (0.01 µg/mL of purified tropomyosin or 0.1 µg/mL of crustacean protein). The inhibitors were the different protein extracts from Black Tiger and Banana shrimp. The inhibitor was mixed with rabbit serum pAbαTM or pAbαCR (depending on the coated antigen) at different concentrations before coating onto the wells. The goat anti-rabbit polyclonal antibody labelled with HRP was used as the second antibody. After adding TMB One-Step Substrate System, the reaction was stopped by HCl and the OD was read at 450 µm.

The percentage inhibition of each shrimp protein extract was calculated following the below formula:

$$\text{Percentage inhibition (\%)} = 100 \times (Y_{max} - Y) / Y_{max}$$

Where Y_{max}: the absorbance of the well containing no inhibitor

Y: the absorbance of the well containing inhibitior

The 50 % inhibition value of different protein extracts were recorded and compared together.

4.2.3 Data analysis

The experiments were conducted in triplicate. The data were analysed by Microsoft Excel 2007 program and by SPSS 17.0 programs for the analysis of variances.

4.3 RESULTS AND DISCUSSION

4.3.1 Evaluation of protein concentration in shrimp

The protein concentration of different extracts from Black Tiger shrimp and Banana shrimp were determined by Bradford method (Bradford, 1976) using the Quick Start Bradford Protein Assay kit from Bio-Rad (USA) (Figure 4.2). The protein concentrations were different significantly at $p < 0.05$ (Appendix – Chapter 4, Table A.4.2).

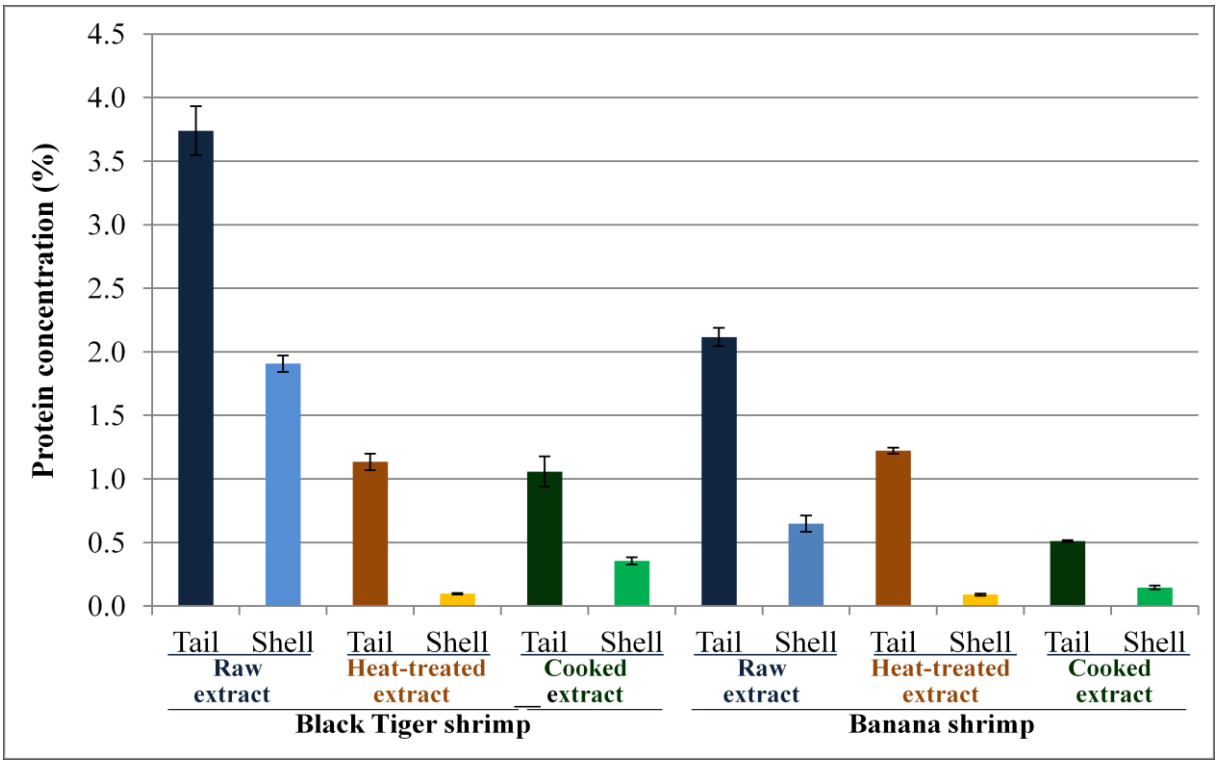


Figure 4.2: The protein concentration (%) of different shrimp protein extracts

4.3.1. 1 Comparison of the protein concentration of different shrimp species

The amount of protein extracted from the Black Tiger shrimp was double the amount from the Banana shrimp in the raw and cooked extracts. However, the concentrations of the heat-treated protein extracts were mostly similar between these two species.

4.3.1.2 Comparison of the protein concentration of different extraction methods

The protein concentration in the heat-treated and cooked extracts had much lower protein concentrations as compared to the raw extracts in both Black Tiger shrimp and Banana shrimp. This could be because some proteins have been hydrolysed during the heating process (An *et al.*, 1988). Some shrimp proteins might be denatured or come out to the solution during heating process of the cooked extracts.

As for the tail meat, the raw shell extracts had the highest protein concentration amongst different extraction methods of raw, heat-treated and cooked.

The protein content in the heat-treated tail extract was double the amount in the cooked tail extract in Banana shrimp while it remained the same in the Black Tiger shrimp.

4.3.1.3 Comparison of the protein concentration of different parts of shrimp

The results also proved that there is a certain amount of protein in the shell extracts. As the proteins exist mostly in the muscle of the shrimp, the shell extracts contained smaller amount of protein than the tail extract. However, the protein concentration in the raw shell extract from Black Tiger was even higher than the protein concentration in the heat-treated tail extracts. These proteins may remain in the derived products from the shrimp shell. Therefore, it is very important to investigate the protein content in products derived from shrimp shell such as chitin and chitosan.

4.3.2 SDS Gel electrophoresis profile of different shrimp protein extracts

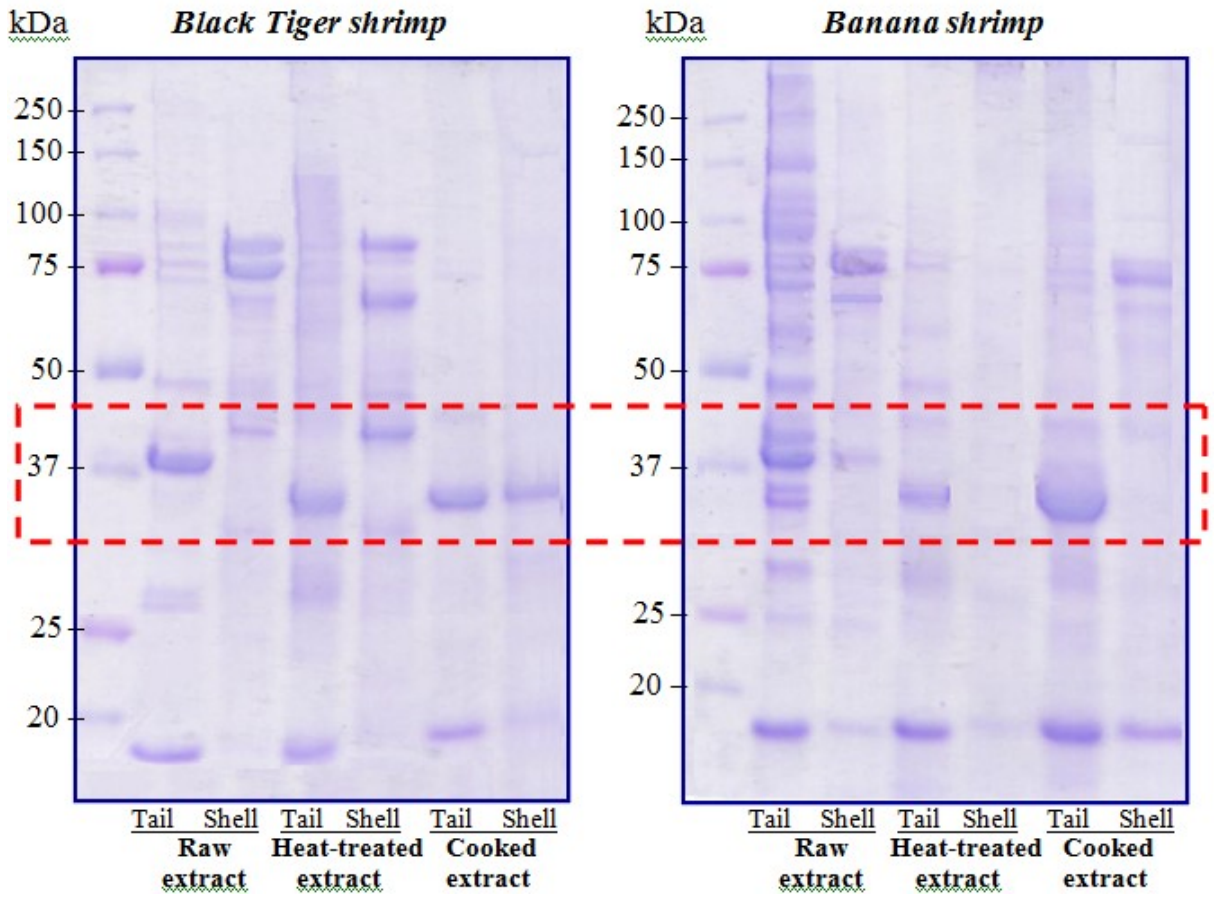


Figure 4.3: SDS Gel electrophoresis profile of different shrimp tail and shell protein extracts.
Note: The red box indicates the position of the major allergen tropomyosin.

Equal amounts (20μg) of the protein extract were loaded onto each well to compare SDS-PAGE profiles. The results were presented in Figure 4.3. Several proteins bands were detected after electrophoresis with molecular weight between 15-250 kDa. The most visible bands could be observed near the 75, 37 and 20 kDa regions.

4.3.2.1 Comparison of the protein profile of different shrimp species

The raw tail extract from Banana shrimp seemed to have more types of protein than from Black Tiger shrimp. The raw shell, heat-treated tail and cooked tail extracts from these two species had similar protein profiles. However, the heat-treated shell extract from Black Tiger shrimp had very strong bands at 75 and 37 kDa regions while it could not be seen in this extract from Banana shrimp. This could be because most of the proteins that remained in the shell of the Banana shrimp were hydrolysed during the heating process. Besides that, the cooked shell extract from Black Tiger shrimp had only one strong band at 37 kDa range, while there were visible bands at 75 and 20 kDa ranges in the protein profile of cooked shell extract from the Banana shrimp.

4.3.2.2 Comparison of the protein profile of different extraction methods

The raw and the heat-treated tail extracts had more protein bands than the cooked tail extracts. The raw and heat-treated shell extracts from the Black Tiger shrimp had similar protein profile with 3 major bands at 75 and 37 kDa ranges which could not be detected in the Banana shrimp.

4.3.2.3 Comparison of the protein profile of different parts of shrimp

Most proteins in the raw shell extracts are in at the 75 kDa range compared to the protein profile of the raw tail extracts. The shell extracts seemed to have fewer protein bands than the tail extracts, which demonstrated a large variety of proteins.

4.3.3 Immunoblotting of different shrimp protein extracts (Western blotting)

The mouse monoclonal anti-tropomyosin antibody and the crustacean allergic patient serum were used for the detection of the allergen tropomyosin in the shrimp species (Figure 4.4 – 4.5). Most of the protein extracts expressed clear dark bands at the 37 kDa range, indicating the presence of tropomyosin in these extracts.

The immunoblotting with the monoclonal antibody (Figure 4.4) demonstrated much stronger bands of at around 37 kDa range in the cooked extracts as compared to the others.

The treated tail extracts of these two species expressed two dark bands when reacted with the monoclonal antibody, while the raw extracts only had one band. This could be due to the changes in the structure of tropomyosin during the heat treatment.

All the shell extracts from the Black Tiger shrimp had the binding reactions with the monoclonal antibody at 37 kDa range, while the cooked shell extract and the raw shell extract from the Banana shrimp expressed reactions at 37 kDa and at lower band, respectively.

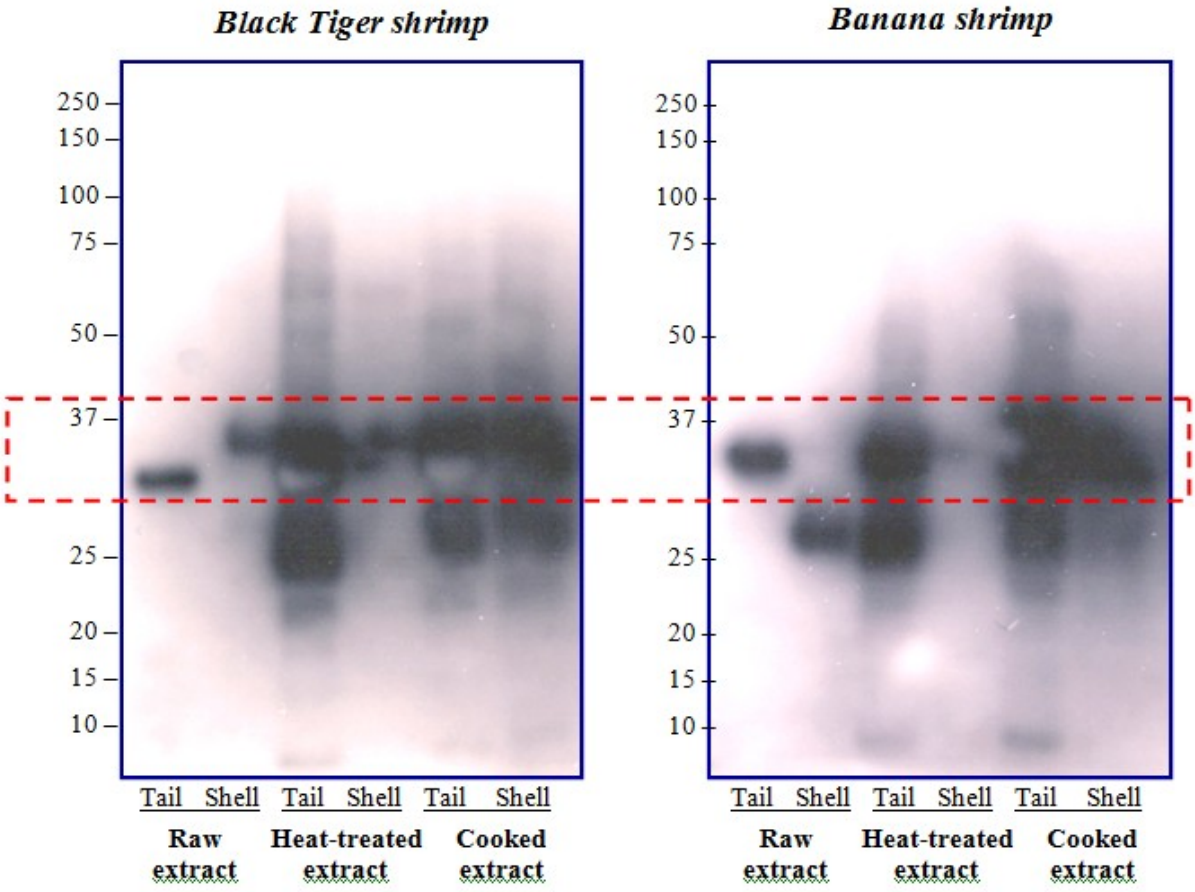


Figure 4.4: Immunoblotting of different shrimp tail and shell protein extracts with monoclonal antibody

Note: The red box indicates the position of the major allergen tropomyosin.

The immunoreactions were confirmed when probing protein extracts with the human patient sera (Figure 4.5).

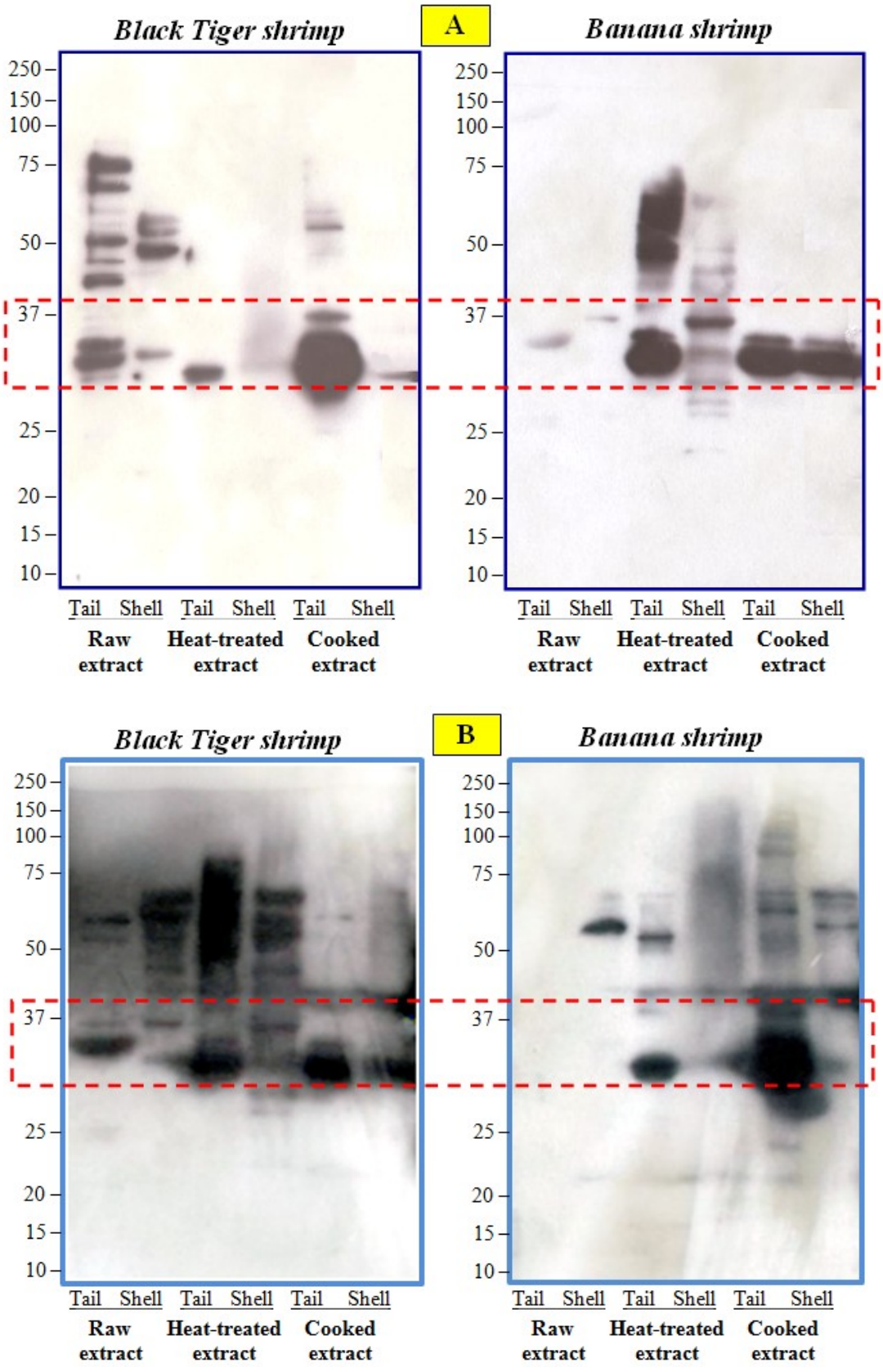


Figure 4.5: Immunoblotting of different shrimp protein extracts with crustacean allergic patient serum AN01 (A) and JB21 (B)

Note: The red box indicates the position of the major allergen tropomyosin.

Most protein extracts expressed visible bands at the 37 kDa range, indicated the IgE bindings to the tropomyosin in these extracts. The JB21 serum has more specific IgE to Black Tiger shrimp than the AN01 serum. Hence, it can bind to more proteins in the Black Tiger shrimp extracts, especially the treated ones. The treated protein extracts of both Black Tiger shrimp and Banana shrimp had stronger reactions to both human sera than the raw extracts while similar to the monoantibody blots. The whole-cooked shrimp shell extract shows very dark bands after immunoblotting with the patient sera at around 37 kDa. This indicated the presence of high amounts of tropomyosin in these extracts as well as possible isoforms at about 33 – 37 kDa and dimers at about 65 – 75 kDa.

4.3.4 Immunoreactivity of different shrimp protein extracts to rabbit sera

In this experiment, the indirect ELISA was designed to examine the binding reaction of tropomyosin and shrimp protein extracts to the two in-house produced polyclonal antibodies, the pAbαTM and the pAbαCR. The tropomyosin concentrations in these extracts were also estimated based on their reaction with the pAbαTM and the standard curve from purified tropomyosin.

The inhibition ELISA was conducted to determine the cross-reactivity of rabbit sera pAbαTM and pAbαCR to different protein extracts.

4.3.4.1 Immunoreactivity of shrimp protein extracts to rabbit serum pAbαCR

4.3.4.1.1 The bindings of shrimp protein extracts to rabbit serum pAbαCR

The bindings of different shrimp protein extracts to rabbit serum pAbαCR were demonstrated through the absorbance value obtained from the indirect ELISA (Figure 4.6). The absorbance values of different shrimp protein extracts to rabbit serum pAbαCR were different significantly at $p < 0.05$ (Appendix – Chapter 4, Table A.4.4).

Most tail extracts expressed high binding with the rabbit serum pAbαCR, except the heat-treated tail extract from Black Tiger shrimp. It can be assumed that the heat-treated process can break down or denature most of the proteins in Black Tiger shrimp.

Most protein extracts from the shrimp shell had lower immunoreactions which can be compared as the reaction of the 0.001 μg/mL crustacean protein concentration. However, the cooked shell extract from Black Tiger shrimp showed very high immunoreactions to pAbαCR.

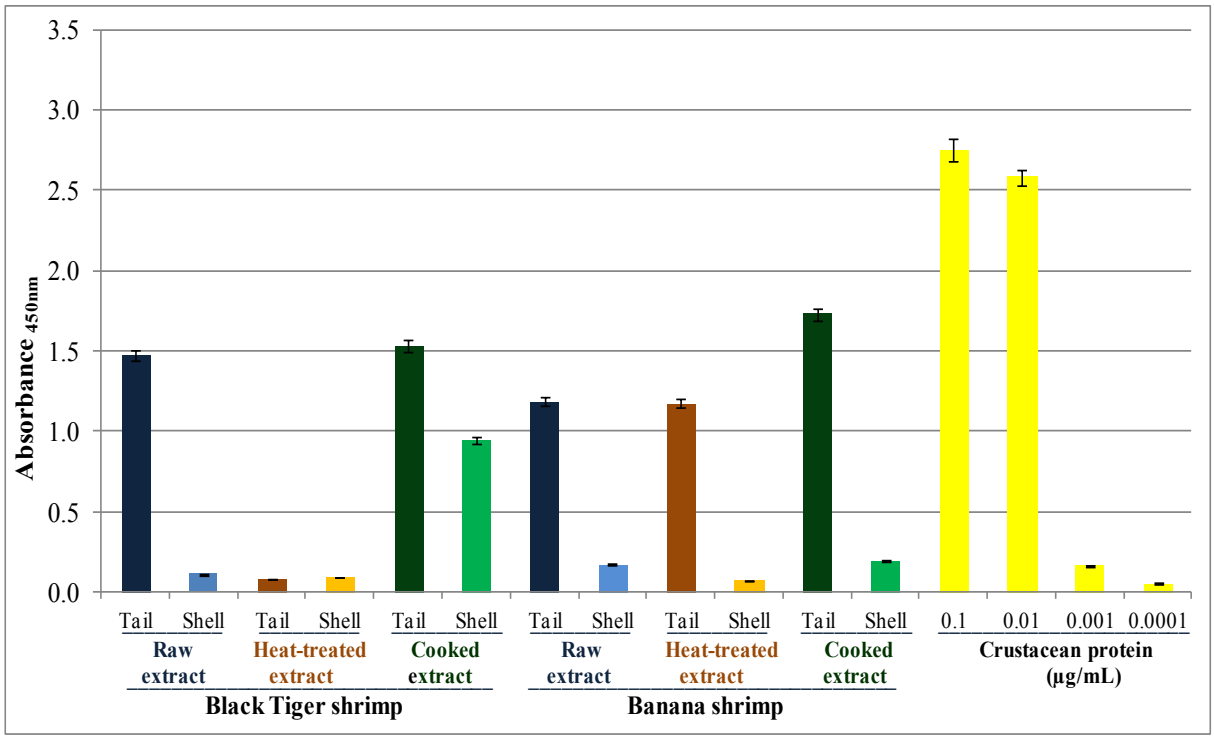


Figure 4.6: Absorbance values of the bindings of different shrimp protein extracts to rabbit serum pAbαCR

4.3.4.1.2 Percentage inhibition of shrimp protein extracts to rabbit serum pAbαCR

To compare the similarity of tropomyosin between the different extracts and the concentration of antigen, the inhibition ELISA was performed. An amount of 0.01 µg/mL of purified tropomyosin was coated onto the wells before adding the inhibitor and rabbit serum mixture. The percentage inhibitions of shrimp protein extracts at different concentrations were calculated. A high degree of inhibition demonstrates a high concentration of tropomyosin. The results were presented in Figure 4.7 and 4.8.

The percentage inhibition of different concentration of protein extracts from both Black Tiger shrimp and Banana shrimp to rabbit serum pAbαCR increased with the increasing concentration of the inhibitors.

All the tail protein extracts from Black Tiger shrimp as well as Banana shrimp had significantly higher percentage inhibition than the shell extracts with $p < 0.05$ (Appendix – Chapter 4, Table A.4.6 and Table A.4.8). The cooked tail extracts from both shrimp had the highest percentage inhibition (> 90 % at 10 µg/mL inhibitor concentration). The lowest percentage inhibition occurred at the heat-treated shell extracts from both Black Tiger and Banana shrimp.

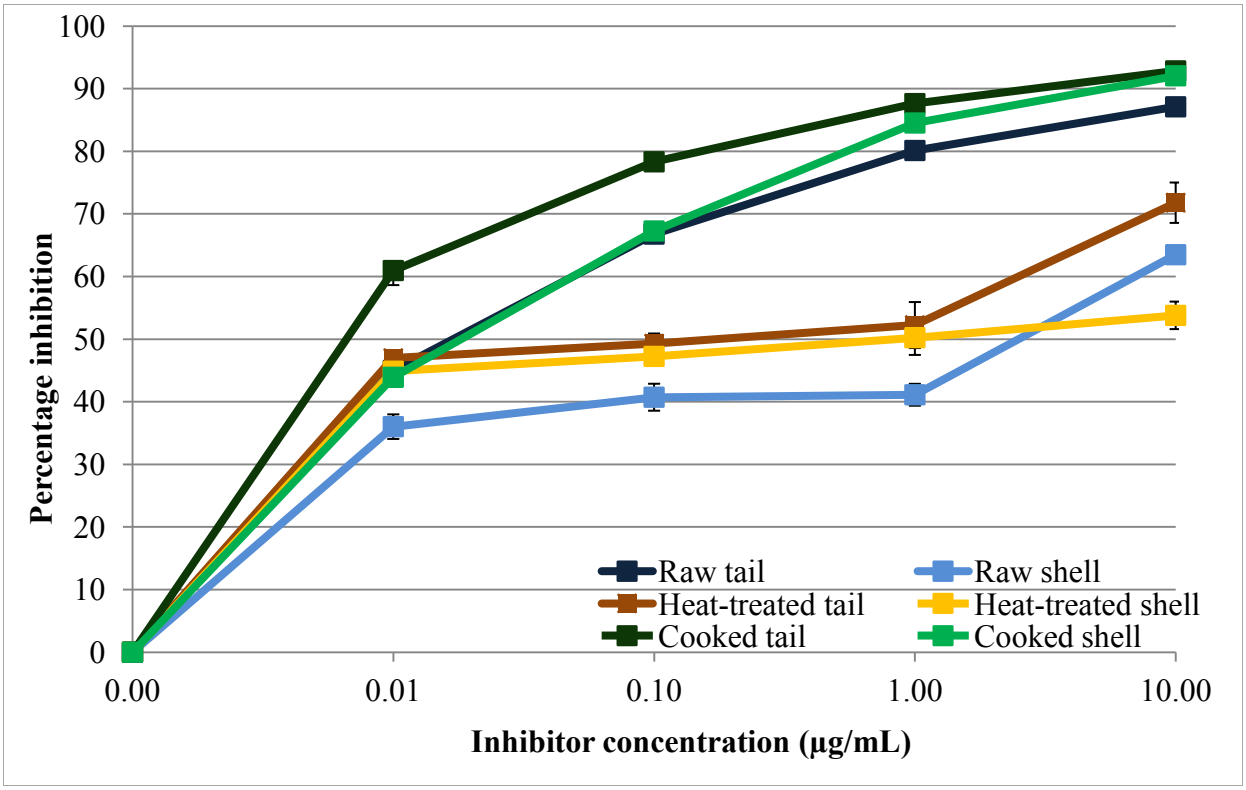


Figure 4.7: Percentage inhibition to rabbit serum pAbαCR of different protein extracts from Black Tiger shrimp

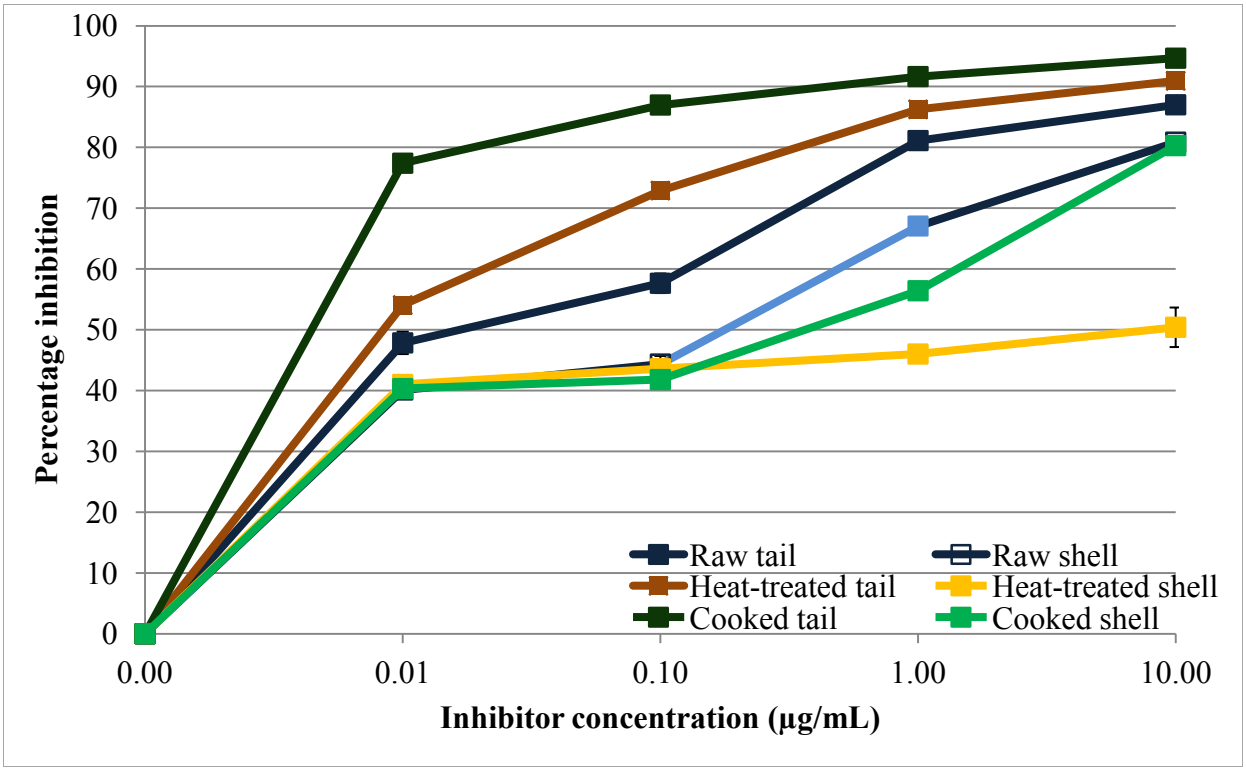


Figure 4.8: Percentage inhibition to rabbit serum pAbαCR of different protein extracts from Banana shrimp

For the Black Tiger shrimp, the cooked shell extract had the highest percentage inhibition with the 92 % inhibition at the 10 µg/mL inhibitor concentration. The percentage inhibition of heat-treated and raw shell extracts increased slightly when the inhibitor concentration increased, and it was just more than 50 % at the 10 µg/mL inhibitor concentration. This result indicated that the cooked shrimp shell extract contained a higher amount tropomyosin than other shrimp shell extracts.

For the Banana shrimp, the shell extracts of all processing methods had similar percentage inhibition at low inhibitor concentration (0.01 – 0.1 µg/mL). However, the percentage inhibition of the raw and cooked shell extracts increased steadily at higher inhibitor concentration, while the percentage inhibition of the heat-treated shell extracts just slightly increased.

In general, the tail protein extracts from both Black Tiger shrimp and Banana shrimp had higher percentage inhibition to both rabbit sera than the shell protein extracts. The cooked shell extracts expressed the significantly higher percentage inhibition than other shell extracts.

4.3.4.2 Immunoreactivity of shrimp protein extracts to rabbit serum pAbαTM

4.3.4.2.1 The bindings of shrimp protein extracts to rabbit serum pAbαTM

The bindings of different shrimp protein extracts to rabbit serum pAbαTM were similar to the rabbit serum pAbαCR but at higher bindings (Figure 4.9A). The absorbance values of different shrimp protein extracts to rabbit serum pAbαTM are different significantly at $p < 0.05$ (Appendix – Chapter 4, Table A.4.10).

Most tail extracts expressed high binding with the rabbit serum pAbαTM, except the heat-treated tail extract from Black Tiger shrimp. Their immunoreactions were as strong as the 0.01 and 0.1 µg/mL tropomyosin reaction. Most protein extracts from the shrimp shell had lower immunoreactions which can be compared as the reaction of the 0.0001 µg/mL tropomyosin concentration. However, the cooked shell extract from Black Tiger shrimp showed as high immunoreactions as the tail extracts.

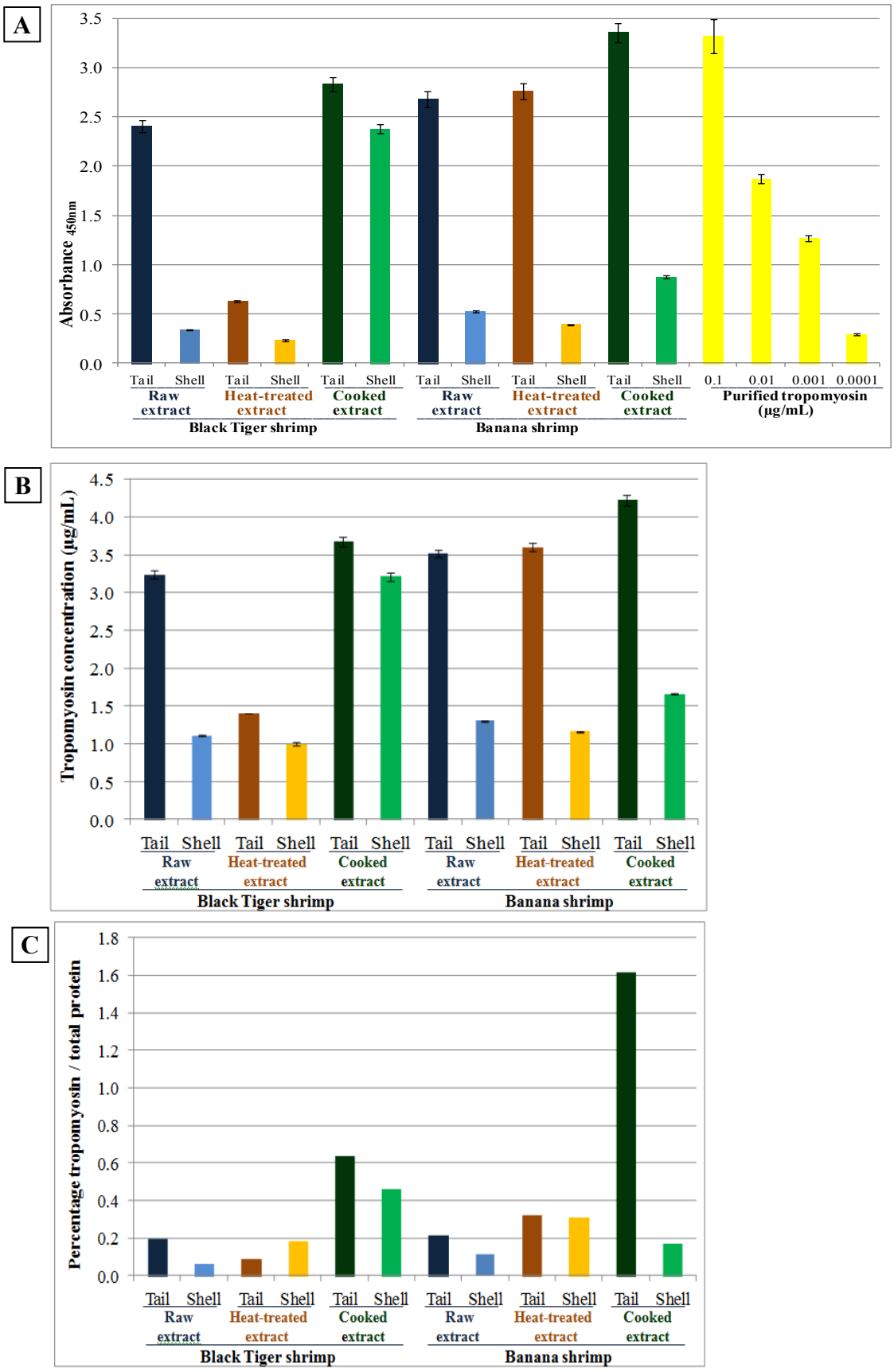


Figure 4.9: Immunoreactivity of shrimp protein extracts to rabbit serum pAbαTM
A - Absorbance values of the bindings of different shrimp protein extracts to pAbαTM
B - Tropomyosin concentration (µg/mL) of shrimp protein extracts
C - Percentage of tropomyosin compared to total protein extracted

4.3.4.2.2 Evaluation of tropomyosin content in shrimp protein extracts

Based on the binding of known concentrations from purified tropomyosin (Figure 4.9A), the tropomyosin concentration in each shrimp protein extracts was also estimated. The absorbance values of known concentrations from purified tropomyosin were used as a standard curve for the conversion from absorbance values to tropomyosin concentration values. The result was presented in Figure 4.9B. There were significant differences between these values at $p < 0.05$ (Appendix – Chapter 4, Table A.4.12).

All the tail protein extracts had higher concentration of tropomyosin than the shell extracts. Among them, the heat-treated extract from the Black Tiger shrimp had the lowest tropomyosin concentration (1.4 μg/mL). This result reflected the binding of these extracts in Figure 4.9A.

The tropomyosin concentrations in the shell extracts from Banana shrimp were rather similar at approximately 1.3 μg/mL. On the other hand, the tropomyosin concentration in the cooked shell extracts from Black Tiger shrimp was very high and comparable to the tropomyosin concentration in the tail extracts.

The percentage of tropomyosin concentration over the total protein of each extract has been calculated in order to compare the level of tropomyosin among these extracts (Figure 4.9C).

The results presented in Figure 4.9C demonstrated that the percentage of tropomyosin to total shrimp protein in each extract was in general very low with about 0.2 – 0.6 %. Most extracts had a tropomyosin percentage less than 0.5 %. Amongst all shrimp protein extracts, the cooked tail extract from Banana shrimp had the highest percentage at 1.6 %. As even very small amounts of allergens can cause allergic reactions (Lopata and Lehrer, 2010), these allergen concentrations can be considered high.

4.3.4.2.3 Percentage inhibition of shrimp protein extracts to rabbit serum pAbαTM

The percentage inhibition of shrimp protein extracts to rabbit serum pAbαTM was carried out using inhibition ELISA. The results were presented in Figure 4.10 and 4.11.

The percentage inhibition to rabbit serum pAbαTM of different protein extracts from Black Tiger shrimp increased with the increasing concentration of the inhibitors (Figure 4.10). The differences were significant at $p < 0.05$ (Appendix – Chapter 4, Table A.4.15).

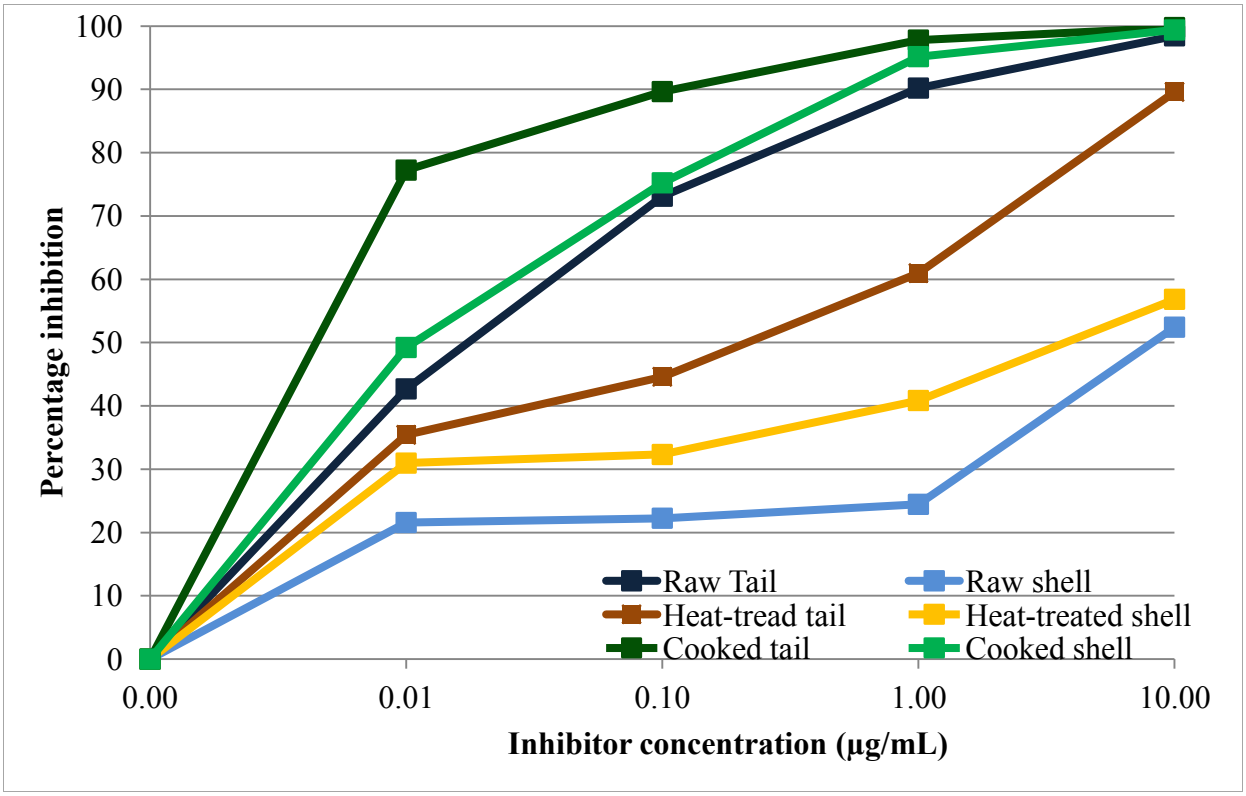


Figure 4.10: Percentage inhibition to rabbit serum pAbαTM of different protein extracts from Black Tiger shrimp

The cooked protein extracts from Black Tiger shrimp expressed the highest percentage inhibition. It seemed to reach the maximum percentage inhibition (100 %) at the 10 µg/mL inhibitor concentration. The results of the inhibition ELISA confirm the data of the indirect ELISA in Figure 4.8, that cooked tail and shell extracts have the highest concentrations of tropomyosin and the raw shell extracts have the lowest values.

All the tail protein extracts from Black Tiger shrimp had higher percentage inhibition than the shell extracts. This was consistent with the tropomyosin concentrations (µg/mL) in the tail extracts being higher than the shell extracts.

Amongst the shell protein extracts from Black Tiger shrimp, the cooked shell extract had the highest percentage inhibition with the maximum percentage inhibition (100 %) at the 10 µg/mL inhibitor concentration. The percentage inhibitions of other shell extracts were just more than 50 % at the 10 µg/mL inhibitor concentration. This result was consistent with the cooked shrimp shell containing a high amount tropomyosin. It is similar to the result obtained from the above indirect ELISA.

The percentage inhibition of different concentration of protein extracts from Banana shrimp to rabbit serum pAbαTM also increased with the increasing concentration of the inhibitors (Figure 4.11). The differences were significant at $p < 0.05$ (Appendix – Chapter 4, Table A.4.17).

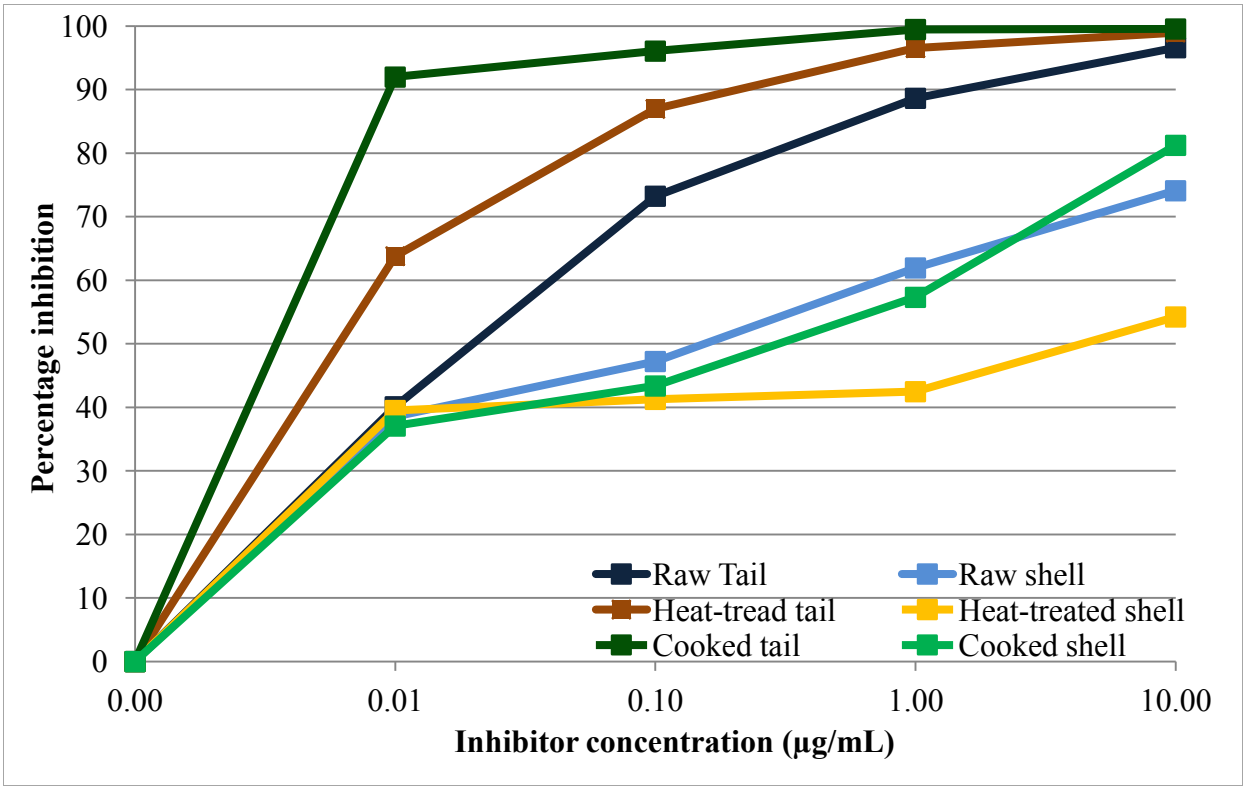


Figure 4.11: Percentage inhibition to rabbit serum pAbαTM of different protein extracts from Banana shrimp

All the tail protein extracts from Banana shrimp had higher percentage inhibition than the shell extracts due to their higher tropomyosin concentration.

All the tail extracts reached the maximum percentage inhibition (100 %) at the 10 µg/mL inhibitor concentration. At lower inhibitor concentration, the cooked tail extract had the highest percentage inhibition with more than 90 % at the 0.01 µg/mL inhibitor concentration. The heat-treated tail extract and the raw tail extract had lower percentage inhibition with approximately 65 % and 40 % at the 10 µg/mL inhibitor concentration, respectively.

Among the shell protein extracts from Banana shrimp, the heat-treated shell extract had the lowest percentage inhibition with 40 - 45 percentage inhibition when the inhibitor concentration increased from 0.01 to 10 µg/mL. This result indicated that the heat-treated shrimp shell contained the least amount tropomyosin than others.

4.3.4.3 The 50 % inhibition value (IC50) of shrimp protein extracts to rabbit sera

The IC50 is the median concentration that causes 50 % inhibition. From the percentage inhibition results of Section 4.3.4.1 and Section 4.3.4.2, the IC50 of different shrimp protein extracts to the two rabbit sera were calculated (Table 4.1).

Table 4.1: IC50 (µg/mL) value of shrimp protein extracts to rabbit sera pAbαCR and pAbαTM

Shrimp protein extracts	pAbαCR	pAbαTM
Black Tiger **raw shell extract**	**4**	**9**
Black Tiger heat-treated shell extract	1	5.5
Black Tiger heat-treated tail extract	0.3	0.3
Black Tiger cooked shell extract	0.025	0.01
Black Tiger **cooked tail extract**	0.008	0.0065
Black Tiger **raw tail extract**	0.022	**0.0025**
Banana **heat-treated shell extract**	9.8	**6.5**
Banana cooked shell extract	0.55	0.5
Banana raw shell extract	0.24	0.2
Banana raw tail extract	0.02	0.03
Banana **cooked tail extract**	**0.0065**	0.0055
Banana **heat-treated tail extract**	0.0092	**0.008**

For the rabbit anti-crustacean protein antibody pAbαCR, the highest IC50 were observed at the heat-treated shell extract of the Banana shrimp (9.8 µg/mL), then the raw shell and the heat-treated shell extracts of the Black Tiger shrimp (4 µg/mL and 1 µg/mL, respectively). Other extracts had very low IC50 (less than 0.5 µg/mL).

For the rabbit anti-shrimp tropomyosin antibody pAbαTM, the highest IC50 were observed at the raw shell extract of the Black Tiger shrimp (9 µg/mL), then the heat-treated shell extracts of Banana and Black Tiger shrimp (6.5 µg/mL and 5.5 µg/mL, respectively). Other extracts had very low IC50 (less than 0.5 µg/mL).

4.4 GENERAL DISCUSSION AND CONCLUSION

The protein concentration of the protein extracts from Black Tiger and Banana shrimp varied depending on the shrimp species, body part of shrimp and the processing method. There was still a remarked amount of protein in the shell extracts although the shrimp was pre-treated by boiling at high temperature. Most proteins in the shrimp extracts concentrated near the 75, 37 and 20 kDa regions of the SDS-PAGE. Other studies also showed that thermal processing affected sharply the amount of allergenic proteins from seafood (Liu *et al.*, 2010; Lopata, 2010). However, because tropomyosin is a heat-stable allergen, it can still exist in the processed food (Hoffman *et al.*, 1981; Lopata and Lehrer, 2010; Nagpal *et al.*, 1989; Reese *et al.*, 1999). The 75 kDa protein is most likely a dimer of tropomyosin, whereas the 20 kDa is a large fragment of tropomyosin (Kamath, 2012).

The immunobloting of these extracts with monoclonal antibody as well as crustacean sensitive patients has confirmed the presence of tropomyosin in these extracts. The tropomosin concentrations in the tail extracts and shell extracts were approximately at 3.5 µg/mL and 1.0 µg/mL, respectively. In another study, Rahman et al. (2010) analysed the allergenic proteins in Black Tiger shrimp using peptide mass finger printing and peptide fragment fingerprinting methods. Their study found the presence of tropomyosin, myosin light chain, and arginine kinase at 33 kDa, 20 kDa and 40 kDa, respectively (Rahman *et al.*, 2010). Due to thermal process, the quantities of proteins in heat-treated and whole-cooked samples were less than the raw samples; however, their percentage amount of tropomyosin compared to total proteins were significantly higher.

Most of the IC50 values for shrimp protein extracts were obtained at very low concentration of inhibitor (less than 0.5 µg/mL). These results indicated that the affinity between the tropomyosin and the sera was very strong. Therefore, special care should be given to the processing of shrimp derived products to prevent contamination with tropomyosin, which could cause negative health effects in crustacean sensitized people.

Chapter 5
INVESTIGATION OF THE ALLERGENICITY OF RESIDUAL PROTEINS IN CHITIN AND CHITOSAN

5.1 INTRODUCTION

Chitin, an animal polysaccharide, is a major structural component of the exoskeleton of invertebrates (Shahidi *et al.*, 1999). It is composed of poly (β-(1-4)-N-acetyl-D-glucosamine). It can be extracted from a variety of sources such as marine animal, insect and fungi (Mathur and Narang, 1990; Stevens, 2005). The major source of chitin from marine animal is from crustacean shell waste (Rinaudo, 2006) which contain 20 – 30 % chitin (Agulló *et al.*, 2003).

Chitosan, derived from chitin through a deacetylation process, is composed of poly (β-(1-4)-N-acetyl-D-glucosamine). Therefore, it has more active properties than chitin due to its solubility and cationic property in queous acidic conditions (Hudson and Jenkins, 2002; Rinaudo, 2006). Due to the natural source of these subtances, chitin, chitosan and their derivative compounds are promising materials with non-toxic, biodegrable and biocompatible properties (Arai *et al.*, 1968; Carreño-Gómez and Duncan, 1997; Kean and Thanou, 2010; Rao and Sharma, 1997; Richardson *et al.*, 1999; VandeVord *et al.*, 2002; Zhang *et al.*, 2002). They have been applied in many industrial fields, especially in the production of functional foods, pharmaceutics and cosmetics due to their binding property, film-forming property and antimicrobial properties (Ilium, 1998; Muzzarelli and Muzzarelli, 2005; Ravi Kumar, 2000; Rinaudo, 2006; Shahidi and Abuzaytoun, 2005).

Although the safety of these products is highly appreciated (Paul, 2010; Rao and Sharma, 1997; Waibel *et al.*, 2011), some residual proteins from shrimp shell such as tropomyosin and arginine kinase could remain in these products and cause allergic reactions among consumers. Until now there is no obvious evidence which indicates chitin and chitosan could cause adverse reactions. However, some cases of anaphylaxis related to chitin and chitosan have been reported (Cleenewerck *et al.*, 1994; Kato Y, 2005; Pereira *et al.*, 1998). Therefore, this study attempts to evaluate the presence of possible allergenic protein(s) residues in chitin and chitosan preparations.

Summary of Chapter 5:

This study focuses on the characterisation of possible residual allergenic proteins in chitin and chitosan. Various methods were utlized to extract the proteins from chitin and chitosan samples. However, due to the small amount of protein residue and the resistant property of tropomyosin, it is difficult to isolate the protein residue from chitin and chitosan using SDS-PAGE and Western blot. However, the Dot blot results demonstrated that there are considerable quantities of the allergenic tropomyosin remaining in the chitin and chitosan samples. The inhibition ELISA also confirmed the immunoreaction of the protein residue in chitin and chitosan sample to rabbit sera which are specific to crustacean protein and shrimp tropomyosin. This result is consistent with the observed allergenic responses that experience when exposed to products containing chitin and chitosan.

5.2 MATERIALS AND METHODS

5.2.1 Extraction of proteins from chitin

5.2.1.1 Materials

A chitin sample produced in Vietnam was used to examine its protein residue. It was donated by Prof. Willem F. Stevens, Mahidol University, Thailand. This is technical chitin obtained from shrimp waste by decalcification (using HCl) and deproteination (using 1 N NaOH). It has a degree of deacetylation between 5 and 15 %.

5.2.1.2 Extraction methods

5.2.1.2.1 Extraction method 1: Using a combination of chemicals

1 g of chitin (dry basis) was treated with 20 ml of 0.2 M NaOH, 0.001 M EDTA, 0.001 M mercapto-ethanol at 70 ^{o}C for 4 hours with 5 times sonication, each time 5 seconds every 15 minutes. Then, 5 mL of 5 M NaCl was added and incubated for 10 minutes at room temperature following by sonication as previous step. The mixed solution was centrifuged at 3600 g for 30 min at 4 ^{o}C. The supernatant I was collected and filtered through 0.45 µm cellulose acetate membrane. This sample was named "Chitin – supernatant I".

The pellet was suspended in 20 mL of 0.2 M phosphoric acid at room temperature for 1 hour with 5 times sonication, each time 5 seconds for every 15 minutes. Then, 5 mL of 5 M NaCl was added and followed the procedure as previous step. The supernatant II was collected after centrifugation and filtration process. It was then run through 3 kDa ultra-filtration until reach 3 mL residual volume. Then 5 mL of 0.1 M phosphate buffer pH 7 was added and applied ultra-filtration for 2 times. The collected supernatant after ultra-filtration was named "Chitin – supernatant II". Both supernatants were aliquoted in 1 mL quantities and kept at -20 ^{o}C for further analysis.

5.2.1.2.2 Extraction method 2: Using phosphoric acid

1 g (dry basis) chitin was soaked in 20 mL of 0.2 M phosphoric acid at room temperature for 1 h with 5 times sonication for every 15 min, each time 5 sec. Next, 5 mL of 5 M NaCl was added and incubated for 10 min at room temperature following by 5 times sonication for every 15 min, each time 5 sec. The mixed solution was centrifuged at 3600 g for 30 min at 4 ^{o}C. The supernatant I was collected filtered through 0.45 µm cellulose acetate membrane and estimated the protein concentration. After that, the supernatant was concentrated by running through the 3 kDa ultra-filtration until reach 3 mL residual volume. Then 5 mL of 0.1 M phosphate buffer pH 7 was added and applied ultra-filtration for 2 times. The concentrated supernatant after ultra-filtration was named "Chitin – phosphoric".

5.2.1.3 Analytical requirements

The protein extracts from chitin sampples were estimated for their protein concentration and calculated the percentage of protein loss. After that they were separated by SDS-PAGE and blotted onto Western blot to examine the presence of the allergenic protein tropomyosin.

5.2.2 Extraction of proteins from chitosan

5.2.2.1 Materials

A chitosan sample produced in Vietnam was used to examine its protein residue. It was donated by Prof. Willem F. Stevens, Mahidol University, Thailand. This is technical chitosan obtained from shrimp waste by decalcification (using HCl), deproteination (using 1 N NaOH) and deacetylation (using 12.5 N NaOH at 70 ^{o}C). It has the degree of deacetylation of about 85 %.

5.2.2.2 Extraction methods

Table 5.1: Protein extraction from chitosan using different buffers

Sample	Extraction methods
A1	1 g chitosan was soaked in 50 mL Phosphate buffer saline pH 7.4. It was ultra-sonicated for 5 min before shaking overnight at 4 ^{o}C.
A2	1 g chitosan was soaked in 50 mL Ammonium sulphate 50 %. It was ultra-sonicated for 5 min before shaking overnight at 4 ^{o}C.
A3	1 g chitosan was soaked in 50 mL 0.2 M Phosphate buffer pH 9. It was ultra-sonicated for 5 min before shaking overnight at 4 ^{o}C.
A4	1 g chitosan was dissolved in 100 mL acetic acid 1 % by shaking overnight at 4 ^{o}C.
A5	Extraction A4 was adjusted to pH 10 by NaOH.
A6	Extraction A5 was neutralised to pH 7 by HCl.
A7	1 g chitosan was dissolved in 100 mL acetate buffer pH 4 by shaking overnight at 4 ^{o}C
A8	Extraction A7 was adjusted to pH 10 by NaOH.
A9	Extraction A8 was neutralised to pH 7 by HCl.
A10	Extraction A4 was adjusted to pH 8 by NaOH before adding 1 % (w/v) SDS. Next, it was heated at 90 ^{o}C for 10 min and adjusted to pH 10 by NaOH.
A11	Chitosan solution (A4) was adjusted to pH 8 by NaOH before adding 1 % (w/v) SDS and 0.2 % (w/v) DTT. Next, it was heated at 90 ^{o}C for 10 min and adjusted to pH 10 by NaOH.

Similar to chitin, several methods have been employed in an effort to extract the proteins from the chitosan sample. The methods of extraction protein from chitosan sample were described in Table 5.1.

All the extracted protein solutions were filtered through cellulose acetate membrane 0.45 μm to remove all insoluble materials or precipitates. The filtrate were collected and kept at -20 ^{o}C for further analysis.

5.2.2.3 Analytical requirements

The protein extracts from chitin and chitosan sampples were evaluated by their protein content. They were separated by SDS-PAGE to examine their protein profiles. The immunoblottings of these extracts to different antibodies by Western blot, Dot blot and ELISA was aimed at finding the presence of the allergenic protein tropomyosin.

The protein concentration of each extract was quantified by Bradford method using Quick Start Bradford Protein Assay (Bio-Rad) (Chapter 2 – Section 2.3.1.4).

The 12% SDS polyacrylamide gel electrophoresis was used to separate the proteins in the protein extracts by molecular weight (Chapter 2 – Section 2.3.2).

The presence of tropomyosin was examined by Western blot teachnique (Chapter 2 – Section 2.3.3.1) and Dot blot technique (Chapter 2 – Section 2.3.3.2). The immunoblotting was conducted with the monoclonal antibody and sera of two patients with clinical symptoms to crustacean:

- Monoclonal antibody mAbαTM: Commercial rat anti-tropomyosin insect IgG antibody, MAC-141 (Abcam).
- Rabbit serum pAbαCR: Rabbit serum containing antibodies which was raised against the whole-cooked crustacean protein
- Rabbit serum pAbαTM: Rabbit serum containing antibodies which was raised against the shrimp tropomyosin
- Human serum AN01: Serum from patient who has allergic symptom with specific IgE against lobster, crab and shrimp.
- Human serum JB21: Serum from patient who has allergic symptom with specific IgE against crab and shrimp.

The percentage inhibition (PI) of protein extracts from chitin and chitosan samples was examined by inhibition ELISA (Chapter 2 – Section 2.3.4.2). In this assay, the wells were first coated with antigen (0.01 μg/mL of purified tropomyosin for rabbit serum pAbαTM or 0.1 μg/mL of crustacean protein for rabbit serum pAbαTM). The inhibitors were the different protein extracts from chitin and chitosan preparation. The inhibitor was mixed with rabbit serum pAbαTM or pAbαCR (depending on the coated antigen) at different concentrations before coating onto the wells. The goat anti-rabbit polyclonal antibody labelled with HRP was used as the secondary antibody. After adding TMB One-Step Substrate System, the reaction was stopped by HCl and the OD read at 450 μm.

The percentage inhibition of each protein extract was calculated following the below formula:

$$\text{Percentage inhibition (\%)} = 100 \times (Y_{max} - Y) / Y_{max}$$

Where Y_{max}: the absorbance of the well containing no inhibitor

Y: the absorbance of the well containing inhibitior

The 50 % inhibition value of different protein extracts were recorded based on the percentage inhibition.

5.2.3 Data analysis

The experiments were conducted in triplicate. The data were analysed by Microsoft Excel 2007 program and the analysis of variances were obtained by SPSS 17.0 programs.

5.3 RESULTS AND DISCUSSION

Commercial chitin/chitosan products still contain a certain amount of proteins which are always stated by the manufacturer. The chitin and chitosan samples used in this study were extracted from shrimp in which the most dominant allergenic protein is tropomyosin. Therefore, this study focusses on evaluation of tropomyosin in chitin and chitosan products.

Several methods have been used in order to separate the protein from the chitin and chitosan samples. However, the collected protein amount was too low to express distinct bands on SDS-PAGE and Western blots.

5.3.1 Extraction of protein from chitin sample

Chitin is a biopolymer which is hardly dissolved in normal chemical solution. On the other hand, the protein residue in chitin sample often less than 1 %. Therefore, it is difficult to extract protein out of the chitin sample. Some methods have been employed in order to extract protein out of chitin sample to investigate its allergenicity.

5.3.1.1 Extraction method 1

In this method, a combination of chemical agents has been employed in order to detach proteins out of chitin sample. At high alkaline condition (pH >10), chitin will be more or less neutral while protein will be negative. The OH- of the solvent will also weaken the binding of the dissociated carboxylate groups of the protein and possible cationic sites on chitin. Mercapto-ethanol (ME) was used to open the disulfide bridges in the protein structure. Ethylenediaminetetraacetic acid (EDTA) can bind metal ions and so withdraws metal ions that might play a role in ligand formation between chitin and protein. Sonic treatment can assist in providing a more effective chemical treatment. Moderate amounts of salt can enhance the dielectric property of the solvent and weaken electrostatic forces between chitin and protein. Ultra-filtration was used to remove all low molecular weight material, including the salt prior applying in SDS-PAGE.

After the extraction process as described in Section 5.2.1.2.1, the protein fractions were measured for volume and protein concentration. The results were presented in Table 5.2 and Table 5.3.

Table 5.2: Protein fractions extracted from chitin

	Total volume of added buffer	Volume before ultra-filtration	Volume after ultra-filtration
Supernatant I:			
Replicate 1	25 mL	15 mL	1 mL
Replicate 2	25 mL	15.5 mL	1.6 mL
Supernatant II:			
Replicate 1	25 mL	21 mL	2.4 mL
Replicate 2	25 mL	21 mL	3.1 mL

Table 5.3: Protein fraction concentration (mg/mL)

	Before ultra-filtration	After ultra-filtration	Protein loss (%)
Supernatant I:			
Replicate 1	0.1646	1.6259	34.15
Replicate 2	0.1776	1.1953	30.52
Supernatant II:			
Replicate 1	Not detected	0.0324	Not available
Replicate 2	Not detected	0.0138	Not available

The result in Table 5.3 showed that the protein concentration in the supernatant I is relatively high while the protein concentration in the supernatant II cannot be detected before ultra-filtration and very low after ultra-filtration. This indicated that the extraction procedure of supernatant I could extract most of proteins in chitin sample.

The protein loss after ultra-filtration was relatively high (> 30 %). This might be because the small molecular-weight proteins can pass through the membrane during filtration under high centrifugation speed.

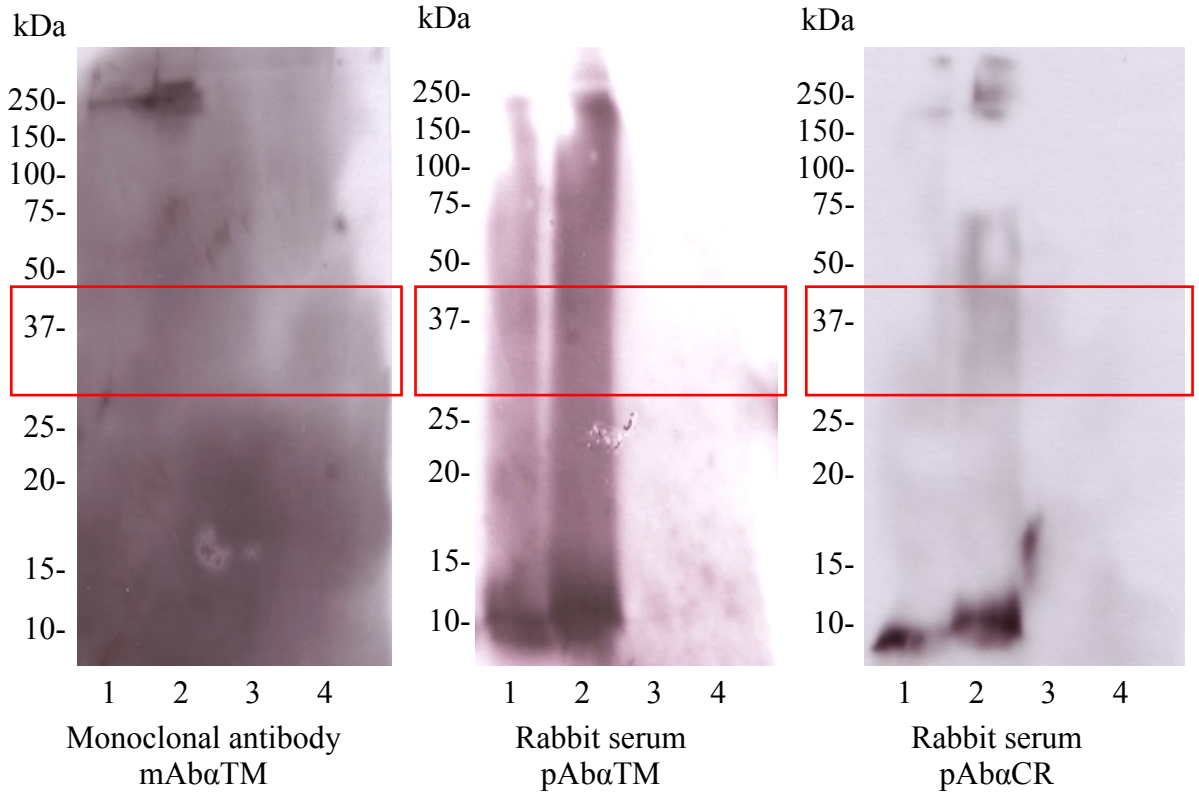
From the result obtained, the total protein content in chitin sample can be roughly estimated by taking the sum of protein amount of both supernatant I and II.

Total protein in 1g chitin = (Protein concentration before ultra-filtration x Total volume of added buffer) of supernatant I + (Protein concentration after ultra-filtration x Total collected volume after ultra-filtration) of supernatant II.

- ⇨ The amount of total protein in Replication 1: 4.1932 mg/g chitin (0.42 % (w/w))
- ⇨ The amount of total protein in Replication 2: 4.4824 mg/g chitin (0.45 % (w/w))

The extracted protein fractions were separated by SDS-PAGE. However, no band appeared on the gel. This could be because most of the protein residue in chitin had the molecular weight less than 10 kDa due to the deproteinization process.

The gel was immunoblotted with anti-tropomyosin monoclonal antibody, rabbit anti-shrimp tropomyosin polyclonal antibody and rabbit anti-crustacean protein polyclonal antibody. The results were expressed in Figure 5.1.



Note: 1 and 2 are two replicates of Chitin - supernatant I.
3 and 4 are 2 replicates of Chitin - supernatant II.
The red line indicated suspected area where the allergenic protein tropomyosin can be.
The molecular weight of tropomyosin is 32-37 kDa. However, it can exist as a dimer or a fragment, so it can be somewhere in the range 20-75kDa.

Figure 5.1: Immunoblottings of proteins extracted from chitin by method 1

The Chitin – supernatant I (extraction with NaOH, EDTA and ME) showed significantly dark bands in the low molecular range of about 10 kDa compared to the Chitin – supernatant II (extraction with phosphoric acid).

The polyclonal antibodies bind strongly to proteins at 10 kDa. At other ranges, the extracted protein solutions showed some bindings with polyclonal antibodies, but still unclear. In contrast, the monoclonal antibody did not show any binding.

5.3.1.2 Extraction method 2

In the previous method, the supernatant II which had proteins extracted in phosphoric acid still contained a small amount of proteins. However, the protein quantity was too small to run on the SDS-PAGE. Therefore, this second method tried to extract proteins in phosphoric acid to examine whether more proteins can be extracted.

Table 5.4: Protein fractions extracted from chitin by method 2

	Total volume of added buffer	Volume before ultra-filtration	Volume after ultra-filtration
Chitin - Phosphoric:			
Replication 1	25 mL	17 mL	1.94 mL
Replication 2	25 mL	17.5 mL	1.62 mL

Table 5.5: Protein fraction concentration (mg/ml) method 2

Sample	Protein concentration (mg/mL)	
	Replication 1	Replication 2
Chitin – Phosphoric - before ultra-filtration	Not be detected	
Chitin – Phosphoric - after ultra-filtration	0.0446	0.0592
Chitin – Phosphoric < 3 kDa	Not be detected	

This method aimed to weaken the electrostatic forces between the chitin and the proteins by changes in the pH. Chitin is a weak cationic compound so acidic condition (pH < 5) will make it positively charged. However, the protein in general will be cationic below their isoelectric point. Tropomyosin is an acidic protein which has the pI at 4.5 (Reese *et al.*, 1999).

Therefore, acidic condition might assist to repel the protein from the chitin. In this method, the polyvalent phosphate ions will be beneficial for protein extraction, especially at lower pH.

The sample Chitin-Phosphoric < 3 kDa is the supernatant which go through the 3 kDa ultra-filtration membrane. The protein concentration was meaasured to check the protein loss. However, the protein concentration in the supernatant before ultra-filtration as well as in the supernatant < 3 kDa cannot be detected, so the protein loss cannot be measured.

From the obtained result, the total extracted protein content from chitin sample by method 2 can be roughly estimated as the following:

Replication 1:

0.0446 mg/mL x 1.94 mL = 0.0865 mg protein / 1g chitin (0.0087 %)

Replication 2:

0.0592 mg/mL x 1.62 mL = 0.0959 mg protein / 1g chitin (0.0096 %)

The concentration of protein extracted in phosphoric acid only (< 0.01 %) was much lower than in the first extraction method (in NaOH + EDTA + ME) (> 0.4 %). Therefore, it can be assumed that the NaOH + EDTA + ME combination method was more effective than the phosphoric acid method in the extraction of protein from chitin sample.

The concentrated protein extracted in phosphoric acid was separated by SDS-PAGE and compared with other extractions from method 1. However, no band appeared on the gel from these extractions. This could be because most of the protein residue in chitin had the molecular weight less than 10 kDa due to the deproteinization process.

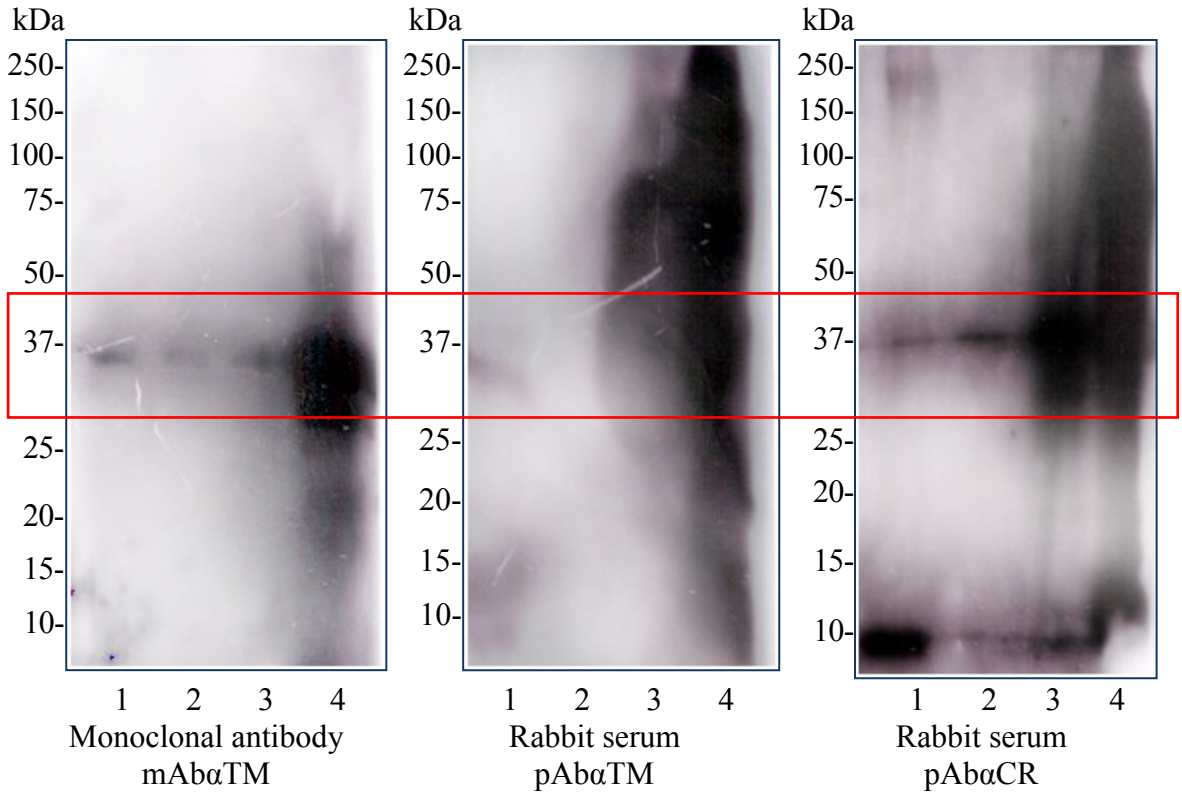
The gel was also immunoblotted with monoclonal antibody mAbαTM and two rabbit sera pAbαTM and pAbαCR. The results are expressed in Figure 5.2.

All samples showed bands at 37 kDa when blotting with monoclonal antibody. That means there is tropomyosin present in these chitin extracts. Although the protein concentration in Chitin - phosphoric sample was much less than that in Chitin - supernatant I sample, they both showed stronger bands than Chitin - supernatant II sample.

The samples Chitin - supernatant I and Chitin - phosphoric also showed bindings with the anti-tropomyosin rabbit serum at 37 kDa. Moreover, there were some bands at lower

molecular weight (20 - 25 kDa) at Chitin - supernatant I sample. Therefore, these bands might contain fragments of tropomyosin.

All samples showed band at 37 kDa when blotting with the anti-crustacean protein rabbit serum at 37 kDa. The Chitin - phosphoric sample showed stronger band than other samples at 37 kDa. The Chitin - supernatant I sample had strong bands at 10 kDa and 250 kDa while other samples also had faint bands at 10 kDa.



Note: *1. Protein extracted in NaOH+EDTA+ME (Chitin - supernatant I sample)*
2. Protein extracted in phosphoric acid (Chitin - supernatant II sample)
3. Protein extracted in phosphoric acid only (Chitin - phosphoric sample)
4. Protein extracted from cooked Black Tiger shrimp shell

The red line indicated suspected area where the allergenic protein tropomyosin can be.

Figure 5.2: Immunoblottings of proteins extracted from chitin sample

In summary, these extraction methods demonstrated the presence of tropomyosin in chitin samples. The extraction of protein from chitin sample can be conducted in a combination of NaOH, EDTA and ME or with phosphoric acid.

5.3.2 Extraction of protein from chitosan sample

The extraction of proteins from chitosan sample was much more difficult than from chitin sample because the protein amount in chitosan samples is even less than that in chitin. Most deproteination methods might also lead to the destruction of the protein of interest, although protein allergens might be extra resistant.

Several methods have been employed in an effort to extract the proteins from chitosan sample (Table 5.1). The deproteination has been tried on the solid chitosan as well as on dissolved chitosan in acetic acid. The deproteination on the solid chitosan has the advantage that chitosan can be removed easily out of the solution by filtration. On the other hand, the deproteination on dissolved chitosan in acetic acid can affect proteins located in the interior of the chitosan particle.

The chitosan No. 1 has been used for this experiment. The protein concentration in each extraction has been measured to examine which method was more effective (Table 5.6).

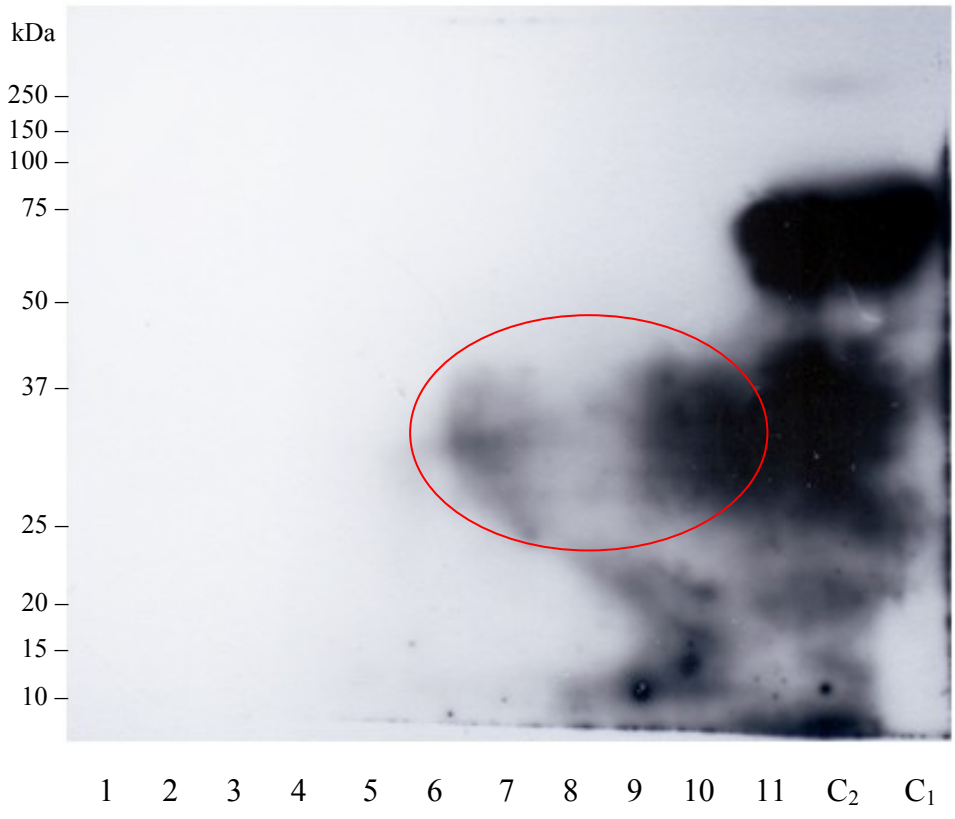
Table 5.6: Protein concentration (%, w/w) of extractions from chitosan sample

Sample	Original extractions	Concentrated extractions
A1	0.0624 ± 0.0103	0.0029
A2	0.1311 ± 0.0178	0.0258
A3	0.1102 ± 0.0150	0.0125
A4	0.6736 ± 0.0692	Not available
A5	0.3081 ± 0.0476	0.0355
A6	0.2454 ± 0.0450	0.0167
A7	1.0222 ± 0.0940	Not available
A8	0.2719 ± 0.0267	0.0413
A9	0.2287 ± 0.0428	0.0327
A10	0.0516 ± 0.0045	Not available
A11	0.1228 ± 0.0321	Not available

The results in Table 5.6 showed that samples A4, A5, A7 and A8 got higher amount of proteins than other samples. However, the samples A2, A4 and A7 cannot be separated by SDS-PAGE due to high amount of salt or acidic condition. All samples showed no band after applying on SDS-PAGE.

After those samples were concentrated by ultra-filtration with 3 kDa molecular weight cut-off, the protein concentration was droped even more to 5-20 times lower. That could be because most of the proteins were degraded and break down into small fragments. Due to little amount of proteins, these samples also showed no bands on the SDS gel.

The protein extractions were immunoblotted with the monoclonal antibody mAbαTM to see if there is any presence of tropomyosin in those extracts (Figure 5.3).



Note: C_1: *Control – Black Tiger Shrimp Tail* protein extract
C_2: *Control – Black Tiger Shrimp Shell* protein extract
1→ 11: Protein extracts A1 →A11 from chitosan (Table 5.1)

Figure 5.3: Immunoblotting of different protein extracts from chitosan with the monoclonal antibody mAbαTM

There were some smears on the blot from the extractions A8, A10 and A11 at the position of 25 - 37 kDa. Therefore, it can be assumed that there could be the presence of tropomyosin in these extractions. The sodium hydroxide seemed to be effective in isolating protein and chitosan (sample A8). The result of samples A10 and A11 showed that the combination of sodium hydroxide, SDS and DTT might be able to extract protein from chitosan sample to examine its allergenicity.

5.3.3 Immunoblottings of proteins extracted from chitin and chitosan

Table 5.7: Methods of extraction protein from chitin and chitosan samples

No.	Extraction methods description
1	1 g chitosan was dissolved in 100 mL acetate buffer pH 4 by shaking overnight at 4 ^{o}C.
2	1 g chitosan was dissolved in 100 mL acetate buffer pH 4 by shaking overnight at 4 ^{o}C. It was adjusted to pH 8 by NaOH before adding 1 % (w/v) SDS and 0.2 % (w/v) DTT. Next, it was heated at 90 ^{o}C for 10 minutes and adjusted to pH 10 by NaOH.
3	1 g of chitin / chitosan was treated with 20 mL of 0.2 M NaOH, 0.001 M EDTA, 0.001 M mercapto-ethanol at 70 ^{o}C for 4 hours with 5 times sonication, each time 5 seconds every 15 minutes. Then, 5 mL of 5 M NaCl was added and incubated for 10 minutes at room temperature following by sonication as previous step. The mixed solution was centrifuged at 3600 g for 30 minutes at 4 ^{o}C. The supernatant was collected and filtered through 0.45 µm cellulose acetate membrane. It was then run through 3 kDa ultra-filtration for concentration and changed to phosphate buffer pH 7.
4	1 g chitosan was treated with 20 mL of 0.2 M phosphate buffer pH 9, 0.001 M EDTA, 0.001 M mercapto-ethanol at 70 ^{o}C for 4 hours with 5 times sonication, each time 5 seconds every 15 minutes. Then, 5 mL of 5 M NaCl was added and incubated for 10 minutes at room temperature following by sonication as previous step. The mixed solution was centrifuged at 3600 g for 30 minutes at 4 ^{o}C. The supernatant was collected and filtered through 0.45 µm cellulose acetate membrane. It was then run through 3 kDa ultra-filtration for concentration and changed to phosphate buffer pH 7.

No.	Extraction methods description
5	20 mL of 0.2 M NaOH, 0.001 M EDTA, 0.001 M mercapto-ethanol at 70 ^{o}C for 4 hours with 5 times sonication, each time 5 seconds every 15 minutes. Then, 5 mL of 5 M NaCl was added and incubated for 10 minutes at room temperature following by sonication as previous step. The mixed solution was centrifuged at 3600 g for 30 minutes at 4 ^{o}C. The pellet was suspended in 20 mL of 0.2 M phosphoric acid at room temperature for 1 hour with 5 times sonication. Then, 5 mL of 5 M NaCl was added and incubated for 10 minutes at room temperature following by sonication. The mixed solution was centrifuged at 3600 g for 30 minutes at 4 ^{o}C. The collected supernatant was then run through 3 kDa ultra-filtration for concentration and changed to phosphate buffer pH 7.
6	1 g chitin was soaked in 20 mL of 0.2 M phosphoric acid at room temperature for 1 hour with 5 times sonication, each time for 5 seconds, for every 15 minutes. Next, 5 mL of 5 M NaCl was added and incubated for 10 minutes at room temperature following by sonication. The mixed solution was centrifuged at 3600 g for 30 minutes at 4 ^{o}C. The supernatant was collected and filtered through 0.45 µm cellulose acetate membrane. It was then run through 3 kDa ultra-filtration for concentration and changed to phosphate buffer pH 7.

From the prior trial extractions, the chitin and chitosan samples were extracted again using the most effective methods from above with modification. The detail extraction methods were described in Table 5.7.

Two types of immunoblotting methods have been carried out, the Western blot and the Dot blot. The PVDF membrane was used in Western blot while the nitro-cellulose membrane was used in Dot blot. The advantage of the Dot blot is that viscous samples such as chitosan solution can be applied easily onto the membrane.

Due to the high viscosity and high acidic condition, the chitosan solutions cannot be applied in SDS-PAGE. The fish (Baramundi) sample was used as a negative control in Dot blot**.**

Table 5.8: List of sample preparation for immunoblotting

No	Sample code	Extraction method
1	CTS1-Acetate	Chitosan No. 1, extraction method 1
2	CTS1-SDS-DTT	Chitosan No. 1, extraction method 2
3	CTS1-NaOH	Chitosan No. 1, extraction method 3
4	CTS1-Phosphate	Chitosan No. 1, extraction method 4
5	CTS2- Acetate	Chitosan No. 2, extraction method 1
6	CTS2-SDS-DTT	Chitosan No. 2, extraction method 2
7	CTS2-NaOH	Chitosan No. 2, extraction method 3
8	CTS2-Phosphate	Chitosan No. 2, extraction method 4
9	CT-Supernatant I	Chitin No. 1, extraction method 3
10	CT-Supernatant II	Chitin No. 1, extraction method 5
11	CT-Phosphoric	Chitin No. 1, extraction method 6
12	TROP	Mixed purified tropomyosin from 4 shrimp species: Banana, School, Vannamei and Black Tiger
13	CRUS	Mixed proteins extracted from Black Tiger shrimp, Mud crab, and Morton Bay Bug

Different types of antibodies were used in this experiment. They were:

- Monoclonal antibody mAbαTM: Commercial rat anti-tropomyosin IgG antibody, MAC-141 (Abcam).
- Rabbit serum pAbαTM: Rabbit serum pAbαTM containing antibodies which was raised against the shrimp tropomyosin
- Rabbit serum pAbαCR: rabbit serum pAbαCR containing antibodies which was raised against the whole-cooked crustacean protein
- Human serum AN01: Serum from patient who has allergic symptoms with specific IgE against lobster, crab and shrimp.
- Human serum JB21: Serum from patient who has allergic symptoms with specific IgE against crab and shrimp.

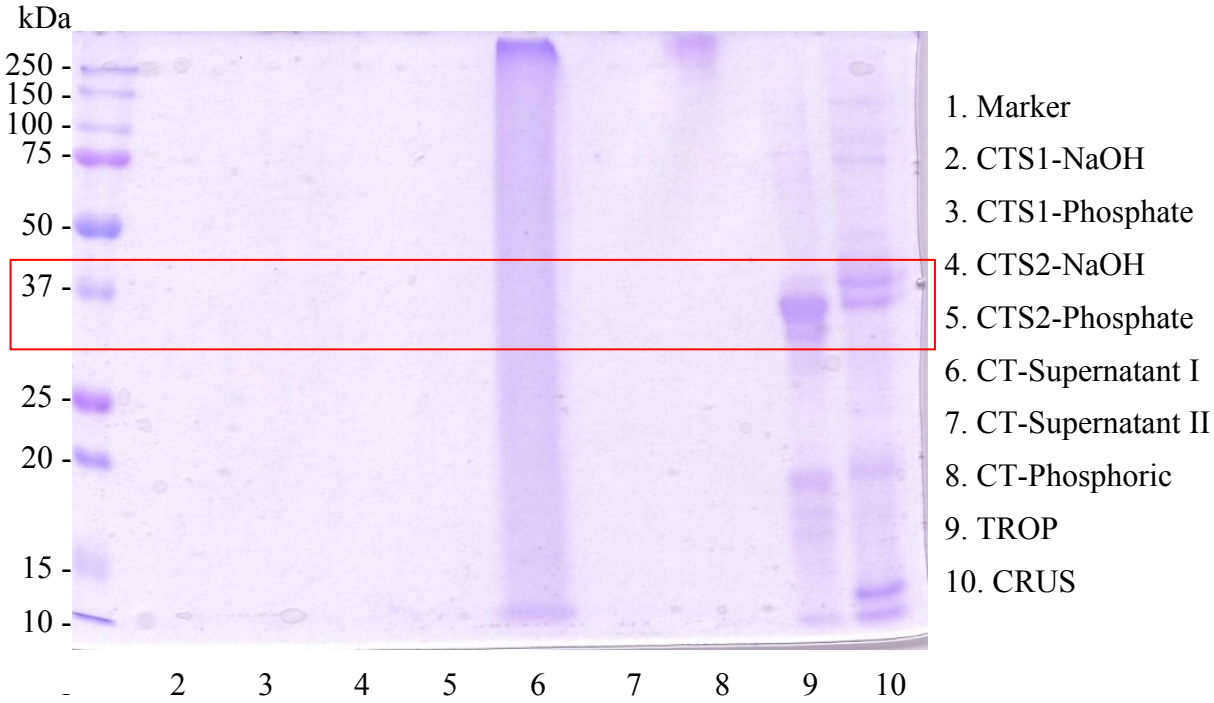
Table 5.9: SDS-PAGE and Western Blot design for immunoblottings of proteins extracted from chitin and chitosan

No	Sample
1	Marker
2	CTS1-NaOH (Double loaded)
3	CTS1-Phosphate (Double loaded)
4	CTS2-NaOH (Double loaded)
5	CTS2-Phosphate (Double loaded)
6	CT-Supernatant I (Double loaded)
7	CT-Supernatant II (Double loaded)
8	CT-Phosphoric (Double loaded)
9	TROP (2.5 µg/well)
10	CRUS (2.5 µg/well)

Table 5.10: Dot blot design for immunoblottings of proteins extracted from chitin and chitosan

Sample	Triplicates			Triplicates			Triplicates		
	1	2	3	4	5	6	7	8	9
A	TROP 1 µg/ml			CTS1- Acetate			CTS2- Acetate		
B	TROP 10 µg/ml			CTS1-SDS-DTT			CTS2-SDS-DTT		
C	TROP 100 µg/ml			CTS1-NaOH			CTS2-NaOH		
D	CRUS 1 µg/ml			CTS1-Phosphate			CTS2-Phosphate		
E	CRUS 10 µg/ml			CT-Supernatant I			CT-Phosphoric		
F	CRUS 100 µg/ml			CT-Supernatant II			Fish (Baramundi)		

5.3.3.1 SDS-PAGE of protein extractions from chitin and chitosan



Note: *The **red** area indicates tropomyosin range*

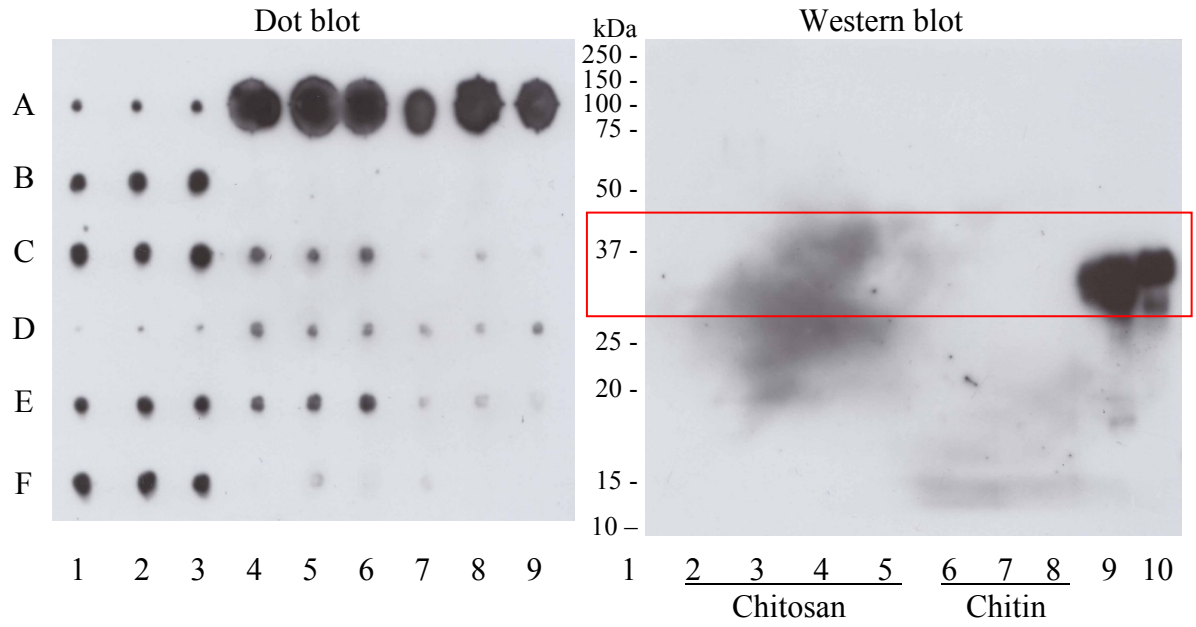
Figure 5.4: The SDS-PAGE of protein extractions from chitin and chitosan

Four extractions from chitosan samples and three extractions from chitin sample have been run through the SDS-PAGE (Figure 5.4). Due to their low protein concentration, these samples were double loaded (40 µL/well) into the wells in order to have more proteins for the SDS-PAGE.

The resulted gel showed that no visible band could be seen from chitosan extractions. This could be because the protein concentrations in these extractions were too low to be detected on SDS-PAGE.

Lane 6, which was the position of chitin supernatant I sample, presented a smear column from the top to the end of the gel without any distinct band. Lane 8, which was the position of chitin supernatant I sample, presented a smear around 250 kDa. The results proved that chitin sample still contain certain amount of protein.

5.3.3.2 Immunoblottings of protein extractions from chitin and chitosan with monoclonal antibody mAbαTM



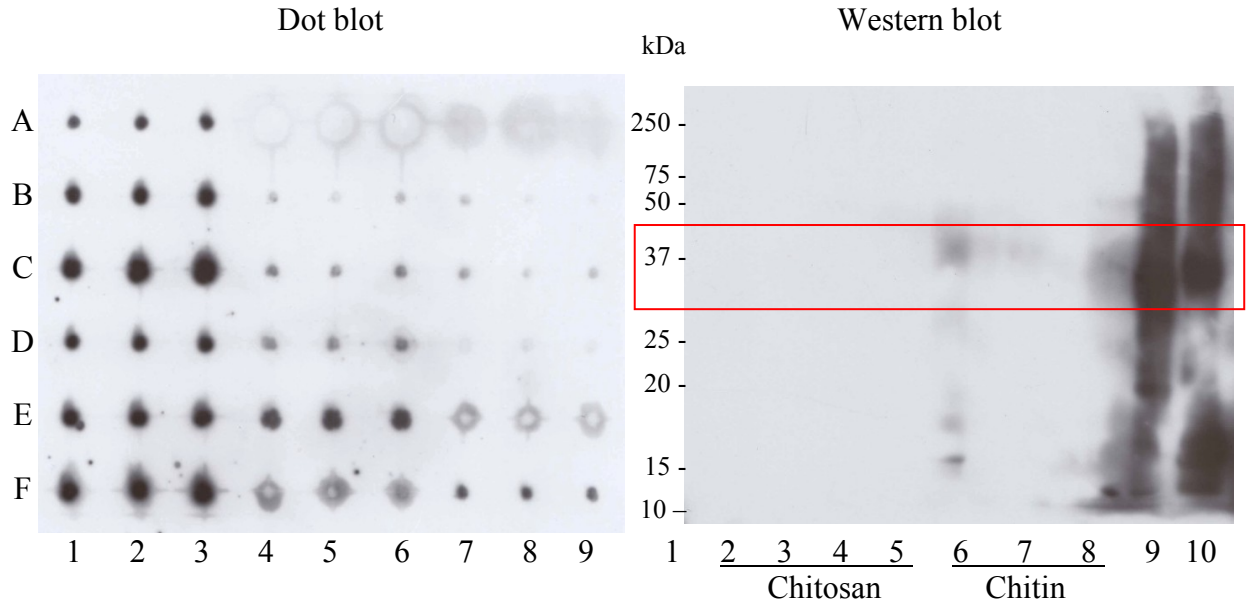
Note: *The **red** area indicates tropomyosin range.*

Figure 5.5: The immunoblottings of protein extracts from chitin and chitosan with monoclonal antibody **mAbαTM**

In the Western blot, there were very dark bands at 37 kDa of purified tropomyosin and whole cooked crustacean protein. It indicated the strong bindings between tropomyosin in the shrimp extracts with the monoclonal antibody mAbαTM. More important, there was the presence of a cloud at the position of chitosan samples in the range of 20 - 37 kDa. This could be because of the binding of protein in chitosan samples with the monoclonal antibody. There were also faint bands at the position of chitin samples but at lower molecular weight (10 – 20 kDa).

The result from the Western blot was strongly confirmed by Dot blot. There were very strong reactions with the monoclonal antibody mAbαTM from both chitosan samples which were dissolved in Acetate buffer pH 4. The intensity of the dots from these samples was even more than the 100 µg/ml tropomyosin or crustacean protein. There was also positive response from chitosan samples with extraction method 3 and 4. The chitin-supernatant I preparation also showed strong binding to the monoclonal antibody The dot intensity was equivalent to the 10 µg/ml tropomyosin or crustacean protein.

5.3.3.3 Immunoblottings of protein extracts from chitin and chitosan with rabbit serum pAbαCR



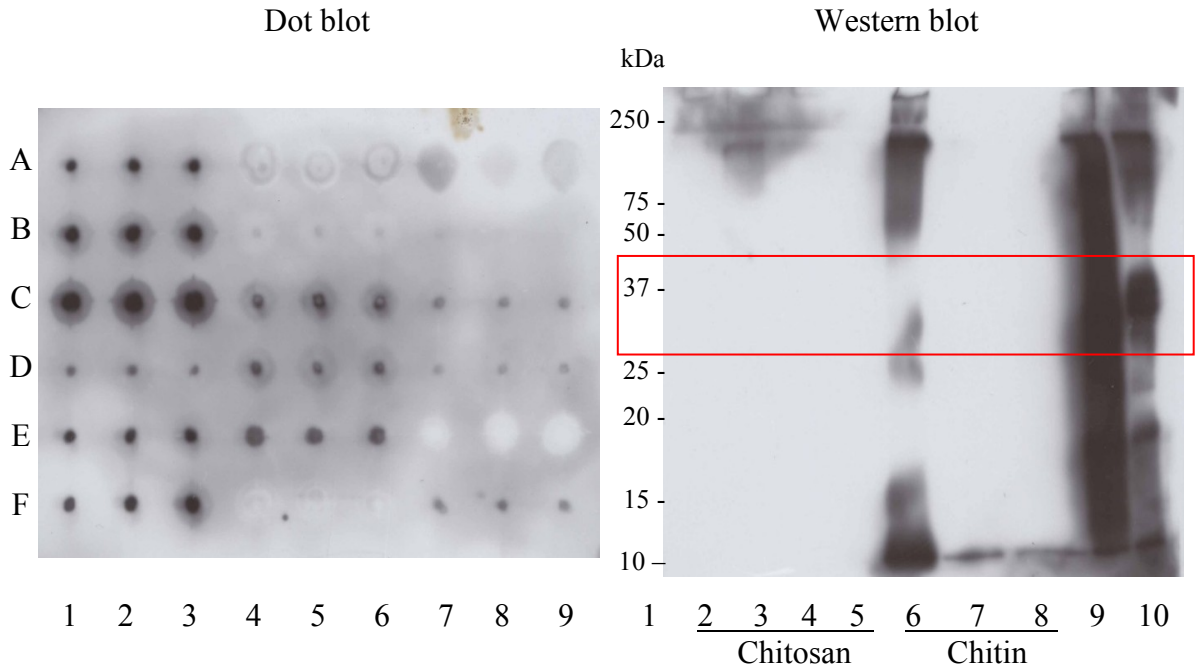
Note: *The **red** area indicates tropomyosin range.*

Figure 5.6: The immunoblottings of protein extracts from chitin and chitosan with rabbit serum **pAbαCR**

In the Western blot, the rabbit serum pAbαCR did not show much response to chitosan preparations. However, it expressed strong bindings with protein extracts from chitin samples (lane 6). Although the bands were not very distinct, there were visible bands at the range of 37 kDa which may indicate the presence of tropomyosin.

In the Dot blot, there were very faint intensity dots with chitosan No.1 – NaOH and phosphate extractions. However, there was strong reaction between the pAbαCR and the chitin – supernatant I with high intensity dots which equivalent to about 10 µg/ml tropomyosin or crustacean protein.

5.3.3.4 Immunoblottings of protein extracts from chitin and chitosan with rabbit serum pAbαTM



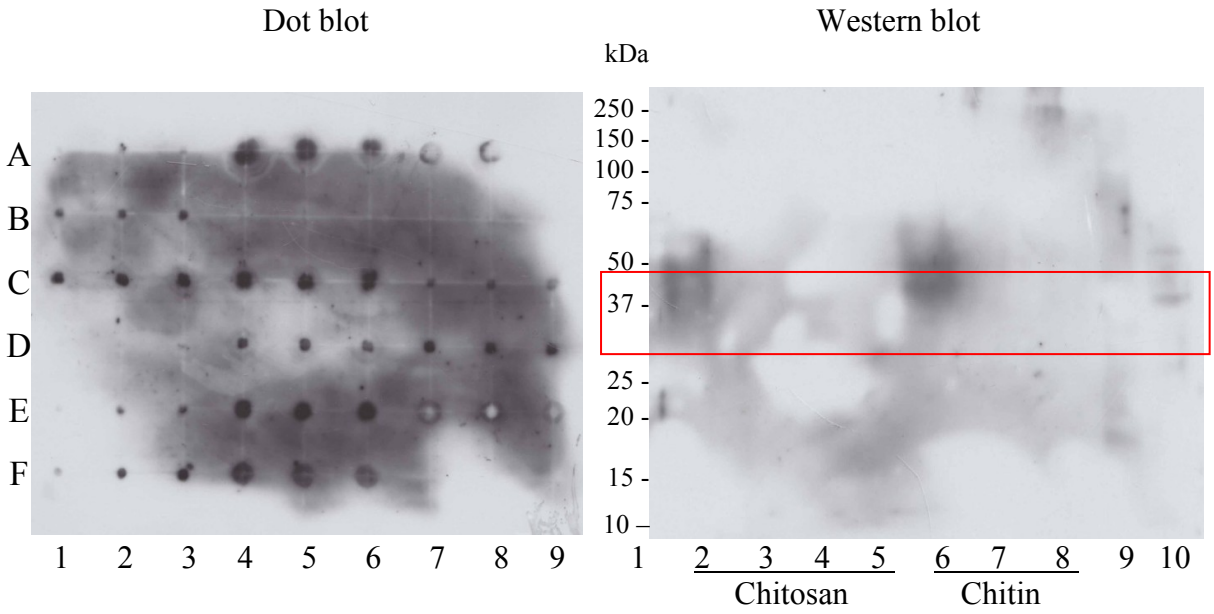
Note: *The **red** area indicates tropomyosin range.*

Figure 5.7: The immunoblottings of protein extracts from chitin and chitosan with rabbit serum **pAbαTM**

In the Western blot, there was not much response from chitosan samples. However, the chitin-supernatant I showed a dark column with some distinct bands at 250 and 10 kDa positions. There were also bands at 10 kDa of the chitin preparations (lane 6 – 8) which might contain fragments of tropomyosin.

In the Dot blot, the rabbit anti - purified shrimp tropomyosin serum only recognised the chitosan – NaOH and chitosan - phosphate preparations with low intensive dots (less than 1 µg/ml tropomyosin or crustacean protein). The chitin-supernatant I preparation also showed strong reaction to the rabbit serum. The dot intensity was equivalent to about 10 µg/ml tropomyosin or crustacean protein.

5.3.3.5 Immunoblottings of protein extracts from chitin and chitosan with human serum AN01



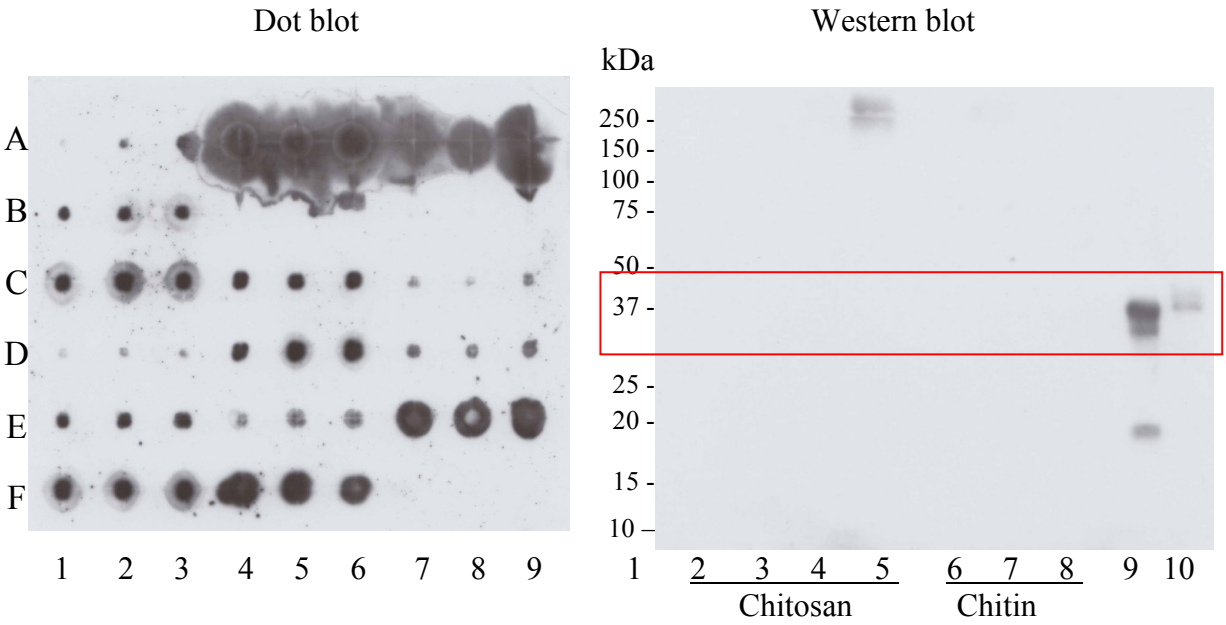
Note: *The **red** area indicates tropomyosin range.*

Figure 5.8: The immunoblottings of protein extractions from chitin and chitosan with human serum **AN01**

Human serum AN01 was donated from a patient who has urticarious allergic reaction to lobster, crab and shrimp. The results in Figure 5.8 showed that the serum had positive reaction to both chitosan and chitin sample (lane 2, 6), especially chitosan No. 1.

In the dot blot, sample CTS – acetate, CTS – NaOH, CTS – phosphate, CT – supernatant II and CT – phosphoric showed intensive black dots compared to the purified tropomyosin and crustacean protein. The result indicated that human serum AN01 was very sensitive to protein in the chitin and chitosan samples. This also might be due to the presence of tropomyosin in these samples.

5.3.3.6 Immunoblottings of protein extractions from chitin and chitosan with human serum JB21



Note: *The **red** area indicates tropomyosin range.*

Figure 5.9: The immunoblottings of protein extractions from chitin and chitosan with human serum JB21

Human serum JB21 was donated from a patient who has anaphylaxis allergic reaction to crab and shrimp. The immunoblotting of this serum with chitin and chitosan samples did not show many bindings, except sample CTS2 – phosphate which showed some faint bands at around 250 kDa (lane 5).

However, very large and intensive dots could be seen from the Dot blot of both chitin and chitosan samples. This result indicated that there were very strong reactions between human serum JB21 and protein in chitin and chitosan samples.

5.3.3.7 Summary of immunoblottings between protein extractions from chitin and chitosan with different antibodies by Western blot and Dot blot

The antibodies were coded as the followings:

- A : Monoclonal antibody mAbαTM
- B : Rabbit serum pAbαCR
- C : Rabbit serum pAbαTM
- D : Human serum AN01
- E : Human serum JB21

In the Western blot, the immunoblotting reaction between protein extractions from chitin and chitosan with different antibodies was recorded as positive (+) or negative (-).

Table 5.11: The immunoblotting reaction between protein extractions from chitin and chitosan with different antibodies by Western blot

Antibodies / Samples	A	B	C	D	E
CTS1 – Acetate	NA	NA	NA	NA	NA
CTS1 – SDS – DTT	NA	NA	NA	NA	NA
CTS1 – NaOH	+	-	-	+	-
CTS1 – Phosphate	+	-	+	-	-
CTS2 – Acetate	NA	NA	NA	NA	NA
CTS2 – SDS – DTT	NA	NA	NA	NA	NA
CTS2 – NaOH	+	-	+	-	-
CTS2 – Phosphate	+	-	-	-	+
CT – Supernatant I	+	+	+	+	-
CT – Supernatant II	+	+	+	-	-
CT - Phosphoric	+	+	+	+	-

Note: NA: not available ***+: possitive reaction*** *-: negative reaction*

In the Dot blot, the immunoblotting reaction between protein extractions from chitin and chitosan with different antibodies was compared to the reaction of TROP and CRUS standards and symbolised as the following scale:

0: no reaction

1: the reaction was less than the reaction of 1 µg/mL TROP or CRUS

2: the reaction was equivalent to the reaction of 1 µg/mL TROP or CRUS

3: the reaction was equivalent to the reaction of 10 µg/mL TROP or CRUS

4: the reaction was equivalent to the reaction of 100 µg/mL TROP or CRUS

5: the reaction was more than the reaction of 100 µg/mL TROP or CRUS

Table 5.12: The immunoblotting reaction between protein extractions from chitin and chitosan with different antibodies by Dot blot

Antibodies / Samples	Compared to TROP					Compared to CRUS				
	A	B	C	D	E	A	B	C	D	E
CTS1 – Acetate	5	0	0	4	5	5	0	0	5	4
CTS1 – SDS – DTT	0	0	1	0	0	0	0	1	1	0
CTS1 – NaOH	2	1	1	4	4	2	2	1	4	4
CTS1 – Phosphate	1	1	1	3	4	2	2	1	4	4
CTS2 – Acetate	5	0	0	0	4	5	0	0	1	3
CTS2 – SDS – DTT	0	0	1	0	0	0	0	1	1	0
CTS2 – NaOH	1	1	1	2	2	1	1	1	3	2
CTS2 – Phosphate	1	1	0	3	2	2	1	0	4	2
CT – Supernatant I	2	2	3	5	2	3	2	3	5	2
CT – Supernatant II	0	0	0	5	5	0	0	0	5	5
CT - Phosphoric	1	0	0	0	5	1	0	0	0	5

Overall, the data indicated that tropomyosin in addition to other proteins can be detected in the chitin and chitosan preparation by a combination of antibodies with different specificity.

In the Western blot, positive bindings could be seen in both chitin and chitosan samples. Chitin samples had reactions with almost all antibodies, except the human serum JB21. The

reactions of chitosan samples were weak and probably due to the presense of less IgE antibody in this patients serum.

However, importantly the dot-blots were very positive. The two chitosan preparations of CTS1 - Acetate and CTS2 - Acetate were very strong in the monoclonal antibody dot blots and both patient sera. The Chitin – Supernatant I preparation came strong up with all five antibodies.

The monoclonal antibody is specific to tropomyosin from insects. However, the dot-blots and Western blots confirmed that the tropomyosin of crustacean was also recognised, based on their molecular similarity.

The rabbit anti-shrimp tropomyosin and anti-crustacean protein detected both tropomyosin as well as other proteins in crustaceans. The specific recognition was shown in the two controls, TROP and CRUS, as well as the concentration - dependent increase of reactivity in the dot-blot. Both antibodies recognised proteins in chitin and chitosan.

In the patient serum, IgE antibody reactivity was examined. Both patients detect tropomyosin in the control extracts. These antibody responses are always not very strong as a very small quantity of this specific antibody is produced in allergic patients. Importantly very strong reactivity was demonstrated in the dot-blots of both chitin and chitosan samples.

The advantage of the Dot blot to the Western blot is that even small antibody binding proteins can be detected, as the SDS gel does not separate well below 10 kDa. Secondly, probably more important, is that the different pH and salt conditions have a negative effect on the separation of proteins eluted from the different preparations. An argument for this would be that there were actually no proteins seen on the SDS-gel. Also, tropomyosin can form dimers or polymers and it is very possible that smaller fragments bind antibody. It has been shown that tropomyosin of brown shrimp has about 8 IgE antibody binding sites which are only 10-20 amino acid long (Leung *et al.*, 1994). This would mean that tropomyosin -fragment of 1000-2000 kDa could still bind antibodies, but can not be detected using conventional protein separation by SDS-gel electrophoresis. However, these smaller tropomyosin protein fragments are still detectable by dot-blot and therefore the preferred method.

5.3.3.8 Inhibition ELISA of protein extractions from chitin and chitosan with rabbit sera

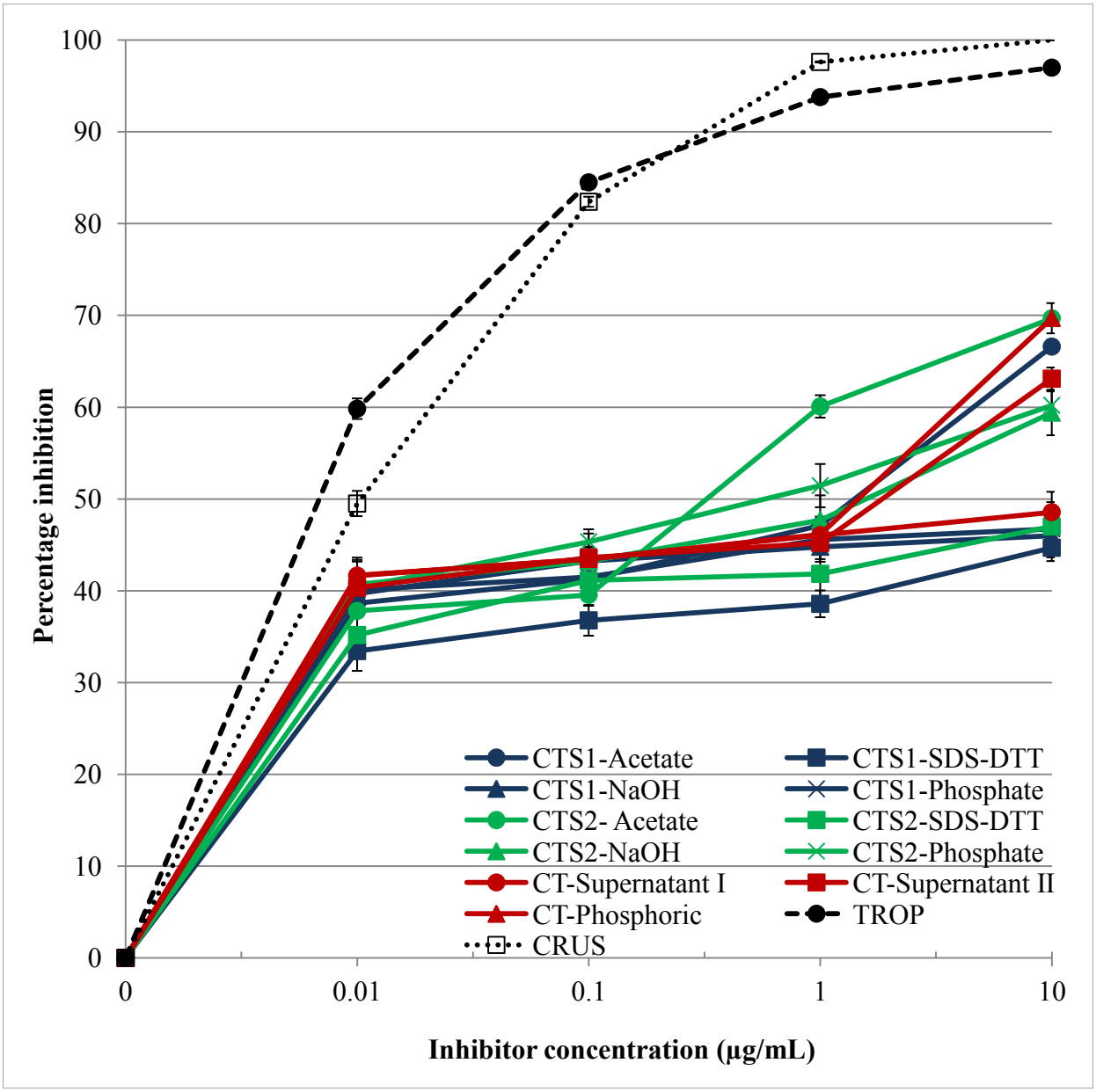


Figure 5.10: Percentage inhibition of protein extracts from chitin and chitosan samples to rabbit serum pAbαCR

The results in Figure 5.10 and 5.11 showed that the percentage inhibition of chitin and chitosan samples increased with the increase of the inhibition concentration. The percentage inhibition values of these samples were different significantly at $p < 0.05$ (Appendix – Chapter 5, Table A.5.2 and Table A.5.4).

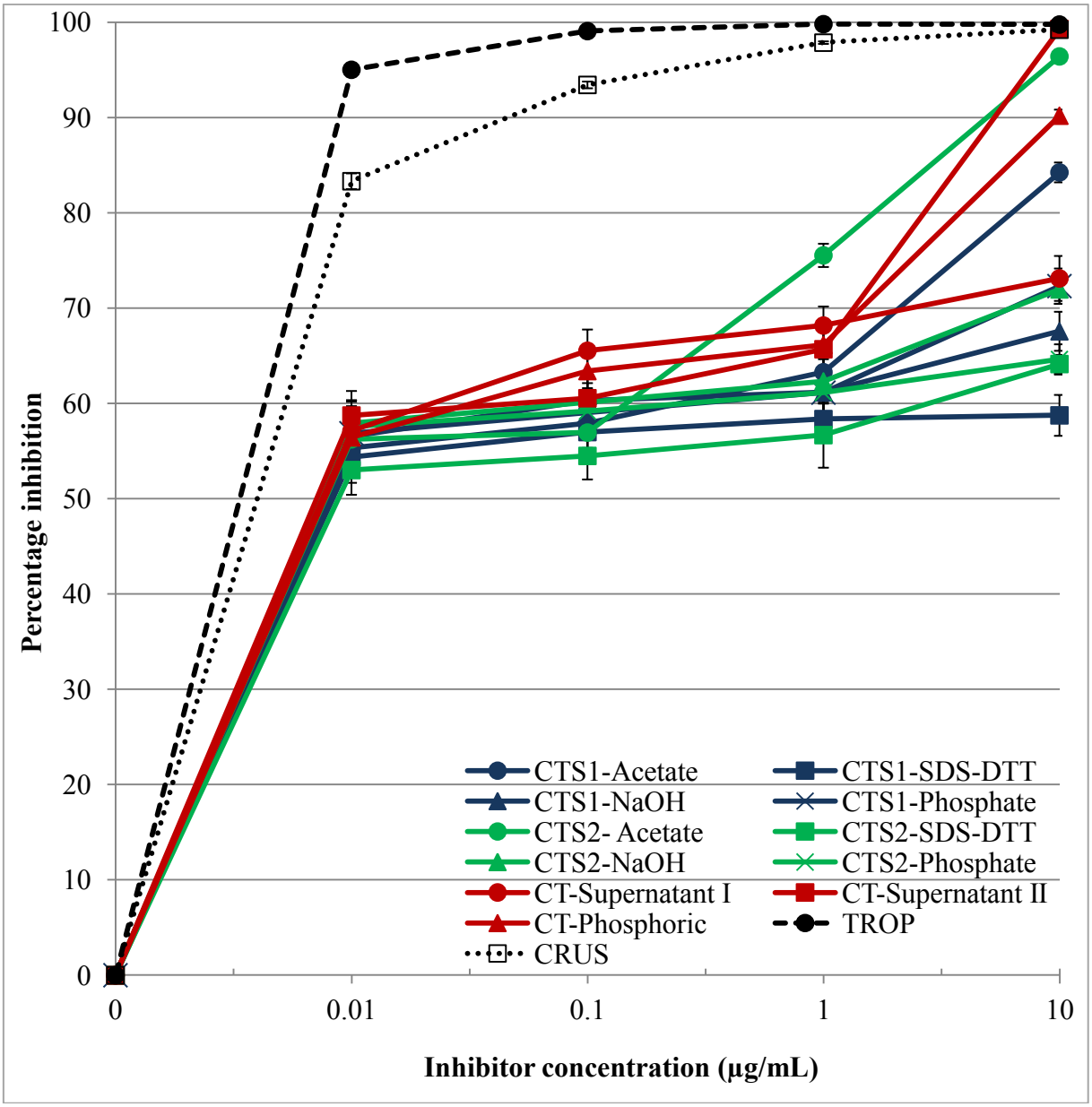


Figure 5.11: Percentage inhibition of protein extracts from chitin and chitosan samples to rabbit serum pAbαTM

The percentage inhibition of the rabbit serum pAbαTM was higher than the rabbit serum pAbαCR. When comparing the 50 % inhibition values of the rabbit serum pAbαTM one can clearly see that much more tropomyosin was present in most of the extracts, inhibition over 50 % of the binding already at 0.01 µg/mL. In contrast the 50% inhibition values of the rabbit serum pAbαCR was in the range from 0.5 to 3 µg/mL of several extracts only. This indicated that the different extraction preparations contain about 10-fold different amounts of crustacean proteins.

5.4 GENERAL DISCUSSION AND CONCLUSION

Many methods have been tried to isolate and identify the residual proteins in chitin and chitosan samples. However, they were not successful. There are many possibilities that make it hard to separate proteins from chitin and chitosan sample. Firstly, the residual proteins must be combined tightly in the structure of the chitin and chitosan powder, so they can survive after treatment with high acidic and alkaline solutions during their extraction. The second reason could be related to the solubility of chitin and chitosan. Chitin cannot dissolve in normal solution (Pillai *et al.*, 2009; Sannan *et al.*, 1975); if strong chemicals were used to dissolve chitin, harsh environment will break down the residual proteins. On the other hand, chitosan can dissolve in light acidic condition; however this solution is too viscous to run through the filter to separate proteins. Chitosan solutions are also very sticky that they cannot be separated by SDS-PAGE. Another reason could be because most of the proteins remaining in the chitin and chitosan were degraded and broken down into small fragments during extraction from the shrimp shell, so they cannot be separated by SDS-PAGE and Western blot.

Although this study cannot isolate the residual proteins from the chitin and chitosan, it can demonstrate the presence of tropomyosin in these samples. The immunoreactivities of the chitin and chitosan protein preparation were very strong when analysing by Dot blot. It is because antibodies can interact with residual proteins while they were still in combination with chitosan as well as they can recognise small fragments of proteins.

The IC50 values of these extracts, ussing the rabbit serum pAbαTM, were also obtained at very low concentration of inhibitor (approximately 0.01 %). This result demonstrated that small fragments of residual protein in chitin and chitosan products may act as an antigen. When these allergens bind to the high-molecular-weight chitin and chitosan molecules, they can possible cause allergic reaction in sensitized patients.

Overall, the data in this chapter demonstrated the presence of tropomyosin protein in chitin and chitosan extracted from shrimp shell. Therefore, special care should be taken when using chitin and chitosan in food or medical preparations. Warning statements should state clearly the presence of tropomyosin in products derived from chitin or chitosan, especially when the consumers are sensitised to crustaceans.

Chapter 6
CHARACTERIZATION OF THE BIOCHEMICAL AND ANTIBACTERIAL PROPERTIES OF CHITIN AND CHITOSAN

6.1 INTRODUCTION

Crustacean shell waste contains 20 - 30% of chitin which can be extracted to be a more valuable product (Kandra *et al.*, 2012; Kurita, 2006; Stevens, 2005). The amount of chitin in marine sources worldwide is currently about 1560 million tons (Agulló *et al.*, 2003), of which over 50 thousand tons will be commercially produced by 2012 (eMediaWire, 2008). The world market for chitin is estimated at USD 1 billion per year (Archer and Russell, 2008). Shrimp shell products such as chitin, chitosan and glucosamine from the seafood industry can be used in the production of functional foods, pharmaceutics and cosmetics due to their excellent characteristics such as biochemical, absorption, film-forming and anti-microbial properties (Agulló *et al.*, 2003; Alishahi and Aïder, 2011; Coma *et al.*, 2003; Dutta *et al.*, 2009; Helander *et al.*, 2001; Hudson and Jenkins, 2002; Kurita, 2006; Muzzarelli and Muzzarelli, 2005; Muzzarelli, 1983; No *et al.*, 2007; Rabea *et al.*, 2003; Ravi Kumar, 2000; Rinaudo, 2006; Stevens, 2005; Zheng and Zhu, 2003).

The anti-microbial property of chitosan is based on the binding between cation groups on chitosan molecules to the negative group on the outer membrane of the bacteria. This reaction changes the permeability of the cells as well as inhibits the production of toxin and microbial growth (Cuero *et al.*, 1991; Rabea *et al.*, 2003). This property depends on the molecular weight, degree of deacetylation of chitosan and the pH of chitosan solution (No *et al.*, 2002; Rabea *et al.*, 2003). There have been many studies on the application of anti-microbial properties of chitosan in the preservation of food (Darmadji and Izumimoto, 1994; Devlieghere *et al.*, 2004; Friedman and Juneja, 2010; Kanatt *et al.*, 2008a, 2008b; Simpson *et al.*, 1997).

The aim of work described in this chapter is to examine some properties of chitin and chitosan samples and compare the anti-microbial properties of normal and purified chitosan for application in preservation of food.

Summary of Chapter 6:

This chapter contains four main parts:

The first part focuses on the characterization of the biochemical properties of some commercial chitin and chitosan products. All samples were examined the following properties: moisture content, ash content, heavy metal content, and protein content. The results indicated that these chitin and chitosan samples have acceptable biochemical properties that are suitable for application in food industry.

The second part examined the antimicrobial property of normal chitosan at different chitosan concentrations against two types of bacteria, Gram negative (*Escherichia coli*) and Gram positive (*Staphylococcus aureus*) bacteria. The results showed that chitosan at 0.05 % and 0.1 % concentration can inhibit totally the development of both *E. coli* and *S. aureus* after 4 hours of incubation.

The third part compared the antimicrobial properties between normal chitosan and purified chitosan by MIC, MBC and Disc diffusion methods. The results demonstrated that purified chitosan retains its antimicrobial property compared to the normal chitosan.

The last part compared the antimicrobial property between normal chitosan and purified chitosan by application in the preservation of shrimp. Both normal and purified chitosan can reduce the development of bacteria on shrimps during 15 days of storage at 0 – 4 ^{o}C while controlling their weight loss and hardness better than the non-treated shrimps.

6.2 MATERIALS AND METHODS

6.2.1 Characterization of biochemical properties of some chitin and chitosan products

6.2.1.1 Chitin and chitosan samples

All the samples were obtained from Prof. Willem F. Stevens, Mahidol University, Thailand.

Table 6.1: Chitin and chitosan samples

No.	Sample	Product description
1	Chitin (CT1)	This is technical chitin which was produced in a research institution in Viet Nam. It is obtained from shrimp waste by decalcification (using HCl) and deproteination (using 1 N NaOH). It has the degree of deacetylation between 5 and 15 %.
2	Chitin (CT2)	This is produced in Mahidol University, Thailand. It has the degree of deacetylation between 5 and 15 %.
3	Chitin (CT3)	This is produced in Mahidol University, Thailand. It has the degree of deacetylation between 5 and 15 %.
4	Chitosan (CTS1)	This is technical chitosan made in Viet Nam obtained from technical chitin using 12.5 N NaOH at 70 ^{o}C.
5	Chitosan (CTS2)	This is high quality technical chitosan made in India. Its degree of deacetylation is about 85 %.
6	Chitosan (CTS3)	This is purified chitosan made from Mahidol University, Thailand. Its degree of deacetylation is about 85%. This protein-free chitosan was made by dissolution of the chitosan in diluted acetic acid, followed by precipitation using NaOH, neutralization and drying.

6.2.1.2 Analytical requirements

Chitin samples were examined their moisture content, ash content, and heavy metal content. Chitosan samples were examined the following properties: moisture content, ash content, heavy metal content, and protein content.

6.2.1.2.1 Moisture content

The moisture content of the chitin and chitosan samples were examined using the drying method as described in Chapter 2 – Section 2.3.1.1

6.2.1.2.2 Ash content

The standard AOAC method (AOAC, 2000) described in Chapter 2 – Section 2.3.1.2 was performed to measure the ash content of the chitin and chitosan samples.

6.2.1.2.3 Heavy metal content

The heavy metal contentof the chitin and chitosan samples was measured by the atomic absorption spectroscopy (AAS) method following the ACIAR procedure (Australian Centre for International Agricultural Research, 2007). This test was conducted by the Institute of Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam.

6.2.1.2.4 Protein content: Bradford assay (as described in Chapter 2 – Section 2.3.1.4)

6.2.2 Characterization of the antimicrobial property of normal chitosan

The aim of this experiment was to determine the concentration of chitosan at which it can inhibit the growth of bacteria.

The antimicrobial property of normal chitosan was investigated at different chitosan concentrations and on two types of bacteria, Gram negative (*Escherichia coli*) and Gram positive (*Staphylococcus aureus*) bacteria.

Two methods have been used in this experiment:

- Method 1: Evaluation of antimicrobial ability of chitosan by spectrophotometric methods. This method was performed to monitor the growth kinetics of each bacterium in different chitosan solution concentrations.

- Method 2: Evaluation of antimicrobial ability of chitosan by the plate count method. This method was performed to examine the viable cell count of each bacterium in different chitosan solution concentrations.

6.2.2.1 Materials and chemicals

Chitosan: 85 % DD; 1.08 MDa, made in Vietnam.

1 % acid acetic

5 N NaOH

Sterilised 0.85 % NaCl

Microbial cultures: *Escherichia coliandStaphylococcus aureus* obtained from Medical University, Ho Chi Minh city, Vietnam. They were kept in nutrient agar slants at 4 ^{0}C.

Nutrient broth (NB) media (HiMedia Laboratories, India)

Mueller Hinton agar (MHA) (HiMedia Laboratories, India)

6.2.2.2 Methodology

Preparation of chitosan solutions: Determined amounts of chitosan powder were dissolved into 1 % acetic acid solution to make 100 mL of chitosan solutions. These solutions were then filtered to remove insoluble particles, adjusted to pH 5.5 by 5 N NaOH, and autoclaved at 121 ^{o}C for 15 minutes.

Preparation of the bacterial enrichment broth: A loop of bacteria was transferred from a slant to 10 mL of Nutrient Broth and incubated at 37 ^{o}C for 18 hours.

The experiment was carried out using the modified protocol from previous studies (Darmadji and Izumimoto, 1994; Stubbings *et al.*, 2004). 100 mL of chitosan solutions at 0 %, 0.01 %, 0.05 % and 0.1 % concentration were prepared as the above description. After that, 0.1 % of the enriched bacterial cultures (*Escherichia coli* and *Staphylococcus aureus*) were added and incubated at 37 ^{o}C.

The optical density of the mixed solution at 660 nm after 0, 4 and 24 hours was recorded to monitor the growth of each bacterium.

At the same time, the viable cell count of each bacterium in different chitosan solution was also determined by the aerobic plate count method (Maturin and Peeler, 2001) which is described in Chapter 2 – Section 2.3.7.

From the viable cell count results, the inhibition rate of chitosan solution was determined as the following formula:

$$\eta = \frac{N_1 - N_2}{N_1} \text{ x } 100\ \%$$

Where η: inhibition rate of chitosan solution (%)

N_1: number of colonies of the control sample (without chitosan)

N_2 : number of colonies of the sample (with chitosan)

6.2.3 Comparison of the antimicrobial property between normal chitosan and purified chitosan

6.2.3.1 Materials and chemicals

- Normal chitosan (85 % DD, HMW) and purified chitosan (85 % DD, HMW) were provided by Prof. Willem F. Stevens, Mahidol University, Thailand.
- Test micro-organisms (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923) were provided by the Microbiology lab, School of Applied Sciences, RMIT University.
- Mueller-Hinton broth and agar
- Glacial acetic acid

6.2.3.2 Methodology

The comparison of the antimicrobial property between normal chitosan and purified chitosan was conducted using the antimicrobial susceptibility tests. These tests were performed following the micro-dilution method and disc diffusion method described by the National Committee of Clinical Laboratory Standards (CLSI, 2006a). The tests were run in triplicate.

The normal chitosan and purified chitosan samples were examined and compared to each other based on the minimum inhibition concentration **(**MIC), minimum bactericidal concentration (MBC) and disc diffusion test results to two standard test micro-organisms: the Gram negative *Escherichia coli* ATCC 25922 and the Gram positive *Staphylococcus aureus* ATCC 25923. These methods were described in Chapter 2 – Section 2.3.6.

6.2.4 Comparison of the antimicrobial activity of normal and purified chitosan in the preservation of raw shrimp tissue

6.2.4.1 Materials

Black Tiger shrimps (*Penaeus monodon*) with the size of 30-40 items / kg were bought at Vietnamese market as fresh. They were kept on ice (< 4 ^{o}C) during transportation and processing.

Fresh Black Tiger shrimp Shrimp kept in ice (0-4^{o}C)

Figure 6.1: Black Tiger shrimps (*Penaeus monodon*)

Normal and purified chitosan were used in this experiment. An amount of 0.5 g chitosan (dry basis) was dissolved in 100 mL of 1.5 % acetic acid and its solution was filtered to remove all insoluble particles. Their pH was adjusted to 5.5 prior conducting the experiment.

6.2.4.2 Methodology

Pre-treatment: The fresh shrimpswere peeled with tail-on and then washed with sterilised NaCl 0.85 %.

Figure 6.2: Pre-treated shrimp

Four treatments were examined in this experiment:

- Shrimps were soaked into normal chitosan solution (0.5 % Chitosan in 1.5 % acetic acid solution) (nCTS)
- Shrimps were soaked into purified chitosan solution (0.5 % Chitosan in 1.5 % acetic acid solution) (pCTS)
- Shrimps were soaked into 1.5 % acetic acid solution (Acetic)
- Non-treated shrimps (Control)

The soaking time was 5 minutes for each treatment. After that, all treated shrimps were kept dry in the freezer 5 minutes before being put on trays, wrapped and stored in the fridge (0 – 4 ^{o}C) (Figure 6.3). Three trays of shrimp were taken out for analysis after 5, 10 and 15 days of storage.

6.2.4.3 Analytical requirements

The shrimp treatments were examined for their weight loss, texture and total plate counts before storage and after 5, 10 and 15 days of storage.

6.2.4.3.1 Weight loss

The weight of shrimp in each tray was obtained before storage and after 5, 10 and 15 days of storage. Data were used to calculate the percentage of weight loss during storage.

$$\text{The weight loss (\%)} = (W_i - W_t) / W_i \times 100\%$$

Where W_i: the initial weight of the shrimp before storage

W_t: the weight of the shrimp at the time of analysis

6.2.4.3.2 Texture

The texture of shrimp was expressed by the hardness index which was evaluated by a texture machine (Zwick/Roell, Germany) using the TestXpert II V3.1 program. The hardness of the shrimp was conducted by a penetration test and was defined as the maximum force (N) of the load application.

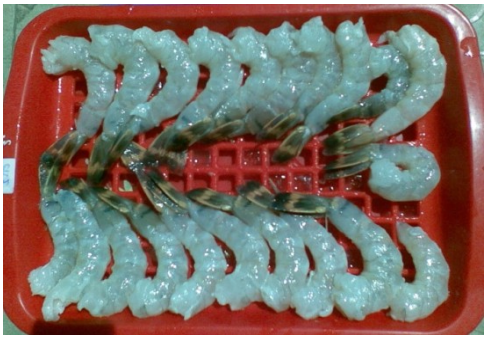
6.2.4.3.3 Total plate counts

The total plate count of shrimp treatments during storage was measured following the method in Chapter 2 – Section 2.3.7.

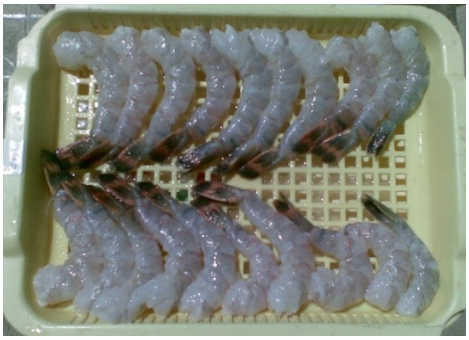
Shrimp soaked in acetic acid

Shrimp soaked in chitosan solution

Shrimp treated in normal chitosan

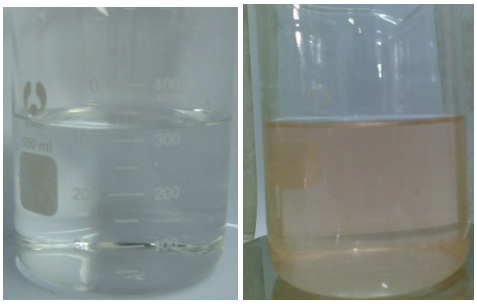


Shrimp treated in purifed chitosan



Shrimp treated with acetic acid

Shrimp without treatment



(a) (b)

Acetic acid (a) before and (b) after treament

Shrimp stored in the fridge at 0 – 4 ^{o}C

Figure 6.3: Treatment of shrimp

6.2.5 Data analysis

The experiments were conducted in triplicate. The data were analysed by Microsoft Excel 2007 program and the analysis of variances were conducted using SPSS 17.0 program.

6.3 RESULTS AND DISCUSSION

6.3.1 Characterization of some properties of chitin and chitosan products

6.3.1.1 Moisture content

The moisture content of chitin and chitosan samples varied between 4 % and 11 %. The data were expressed in Figure 6.4.

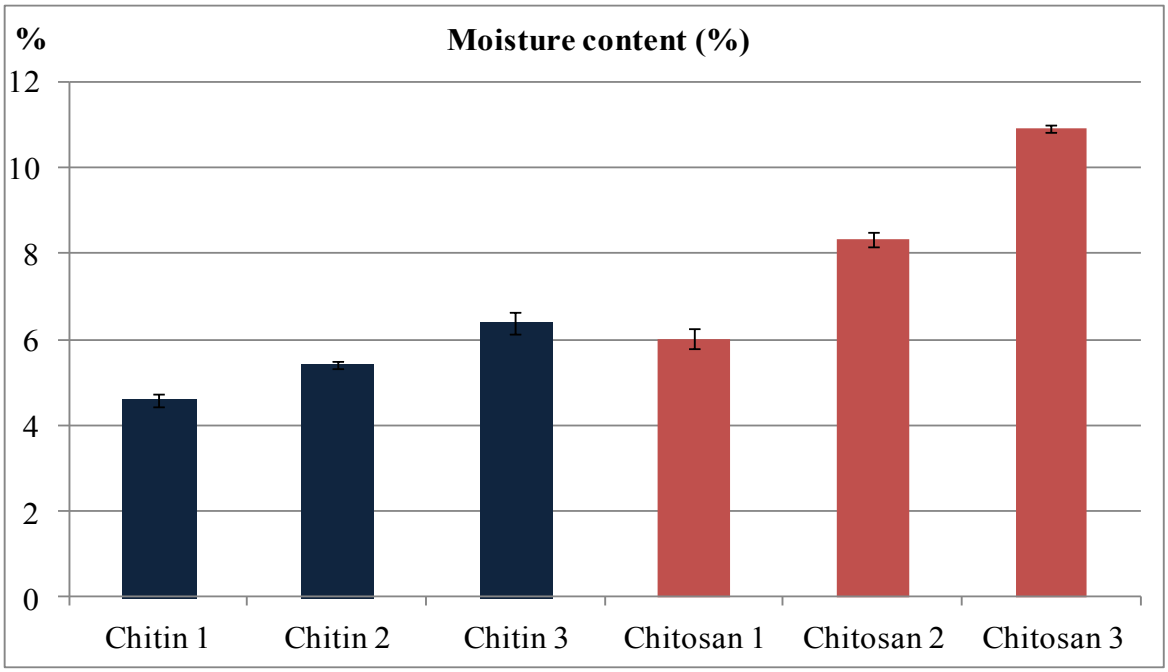


Figure 6.4: Moisture content of chitin and chitosan samples

The chitin samples seemed to generally have less moisture content than the chitosan samples. This could be because the chitosan samples were not properly dried after deacetylation process. Among them, chitosan 3, the purified one, had the highest moisture content (10.9278 %).

The difference in moisture content could also be seen among chitin samples and among chitosan samples. This indicated that the quality of chitin and chitosan samples from different sources were not similar.

Statistical analysis results demonstrated that the differences in moisture content between these chitin and chitosan samples were significant at 95 % of confidence (Appendix – Table A.6.2). Therefore, it is important to measure the moisture content of chitin and chitosan before using to determine the exact quantity as required in an industrial application.

6.3.1.2 Ash content

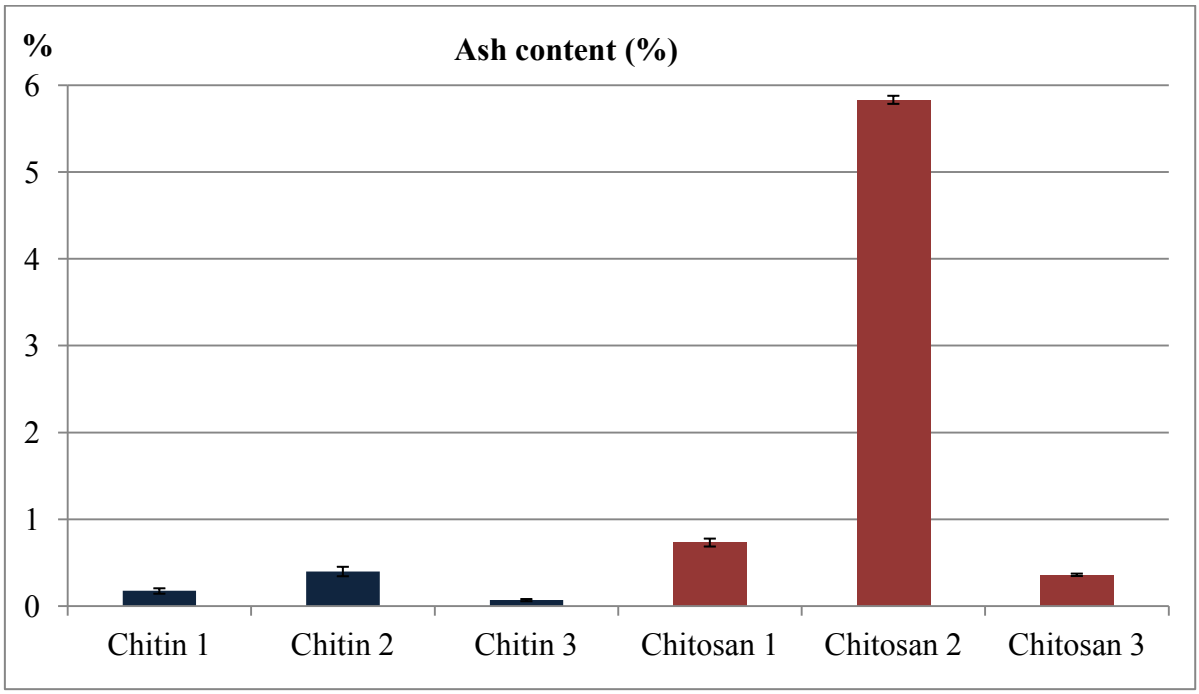


Figure 6.5: Ash content of chitin and chitosan samples

The results in Figure 6.5 demonstrated that the ash content in chitin and chitosan samples was very low, less than 1%, except the chitosan 2. This product had the highest value in ash content (5.8325 %). This could be because this product might not been deionised thoroughly during the extraction process.

The differences in ash content between chitin and chitosan samples were significant at $p < 0.05$ (Appendix – Table A.6.4).

6.3.1.3 Heavy metal content

The heavy metal content of these chitin and chitosan samples was tested by the Institute of Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam. The results are presented in Table 6.2.

Table 6.2: The heavy metal content of chitin and chitosan samples

Sample	Pb (ppm)	Cd (ppm)	As (ppb)	Hg (ppb)
Chitin 1	< 0.01	<0.002	<0.2	<0.2
Chitin 2	< 0.01	<0.002	<0.2	<0.2
Chitin 3	< 0.01	**<0.004**	<0.2	<0.2
Chitosan 1	< 0.01	<0.002	<0.2	<0.2
Chitosan 2	< 0.01	**<0.004**	<0.2	<0.2
Chitosan 3	< 0.01	<0.002	<0.2	<0.2
Methods	ACIAR-AAS 015-2007	ACIAR-AAS 004-2007	ACIAR-AAS 001-2007	ACIAR-AAS 009-2007
Detection limits	0.01	0.002	0.2	0.2
Trace detection limits	0.02	0.004	0.4	0.4
Australian MPC (Anon, 1987) (*)	1.5 ppm	0.2 ppm	1.0 ppm	0.5 ppm

Note: (*) MPC (maximum permitted concentration) of heavy metal in crustacean (Australian food standards – 1987)

This test was focused on four heavy metals which might affect to the health of the consumers: lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg). Most of the samples did not have heavy metal content above the detection limit of the machine using ACIAR-AAS methods. Among these samples, only chitin 3 and chitosan 2 showed the presence of cadmium at trace level. This implicated that both chitin and chitosan samples can contain a certain amount of heavy metal.

However, the concentration of these selected heavy metals in chitin and chitosan samples are very low compared to the Australian food standards for maximum permitted concentration of heavy metal in crustaceans (Anon, 1987). This indicated that these chitin and chitosan samples are safe for usage in food industry.

6.3.1.4 Protein content

Chitin samples could not have their protein concentration determined because of their poor solubility (Hudson and Jenkins, 2002; Kurita, 2006; Muzzarelli, 1983). Chitosan samples were examined the protein content by dissolving in 0.1 N acetate buffer pH 4 (Shepherd *et al.*, 1997). The results were presented in Table 6.3.

Table 6.3: The protein content of chitosan samples

Sample	Protein content (%)
Chitosan 1	0.6353 ± 0.0233
Chitosan 2	0.4436 ± 0.1807
Chitosan 3	Too low to be detected

Table 6.3 showed that there was certain amount of protein in chitosan samples although they had to pass a deproteinization process with sodium hydroxide (Hudson and Jenkins, 2002; Nguyen Van *et al.*, 2006). The protein concentration varied between samples. This could be because these chitosan samples were produced under different conditions. The processing procedure such as reaction temperature and processing time significantly affects the chitosan properties (Rege and Block, 1999).

The protein concentration in chitosan 3 cannot be estimated because this is a purified chitosan. The normal chitosan has been dissolved in acetic acid, filtered and precipitated by sodium hydroxide to collect the purified chitosan. This process removes protein. Therefore, the protein amount in this sample is too low to be detected.

Overall, the properties of these chitin and chitosan samples varied due to their different source. Another study about the differences between the labelled value and the value determined by the researchers on the degree of deacetylation and degree of the polymerization of some commercial chitosans demonstrated that the quality control procedure of chitin and chitosan should be improved (Rege and Block, 1999). Therefore, chitin and chitosan samples should be examined their properties before each application, especially in food and drug industry, for the safety of the consumers as well as the accuracy of processing technology.

6.3.2 Antimicrobial properties of normal chitosan

This experiment was aimed at studying the antimicrobial property of the normal chitosan. The growth of microbiology and the number of viable cells in cultures added with different concentrations of chitosan were investigated by spectrophotometric method and viable cell count method.

6.3.2.1 Spectrophotometric method

The growth of bacteria increases the mass density, hence prevents the transmission of the light. Therefore, the optical density (OD) index can be used to indicate the turbidity of the culture which is indicative of the growth of the bacteria. The optical density of *E. coli* and *S. aureus* cultures were expressed by absorbance values at 600 nm wavelength.

6.3.2.1.1 The growth of *E. coli* in media with different chitosan concentrations

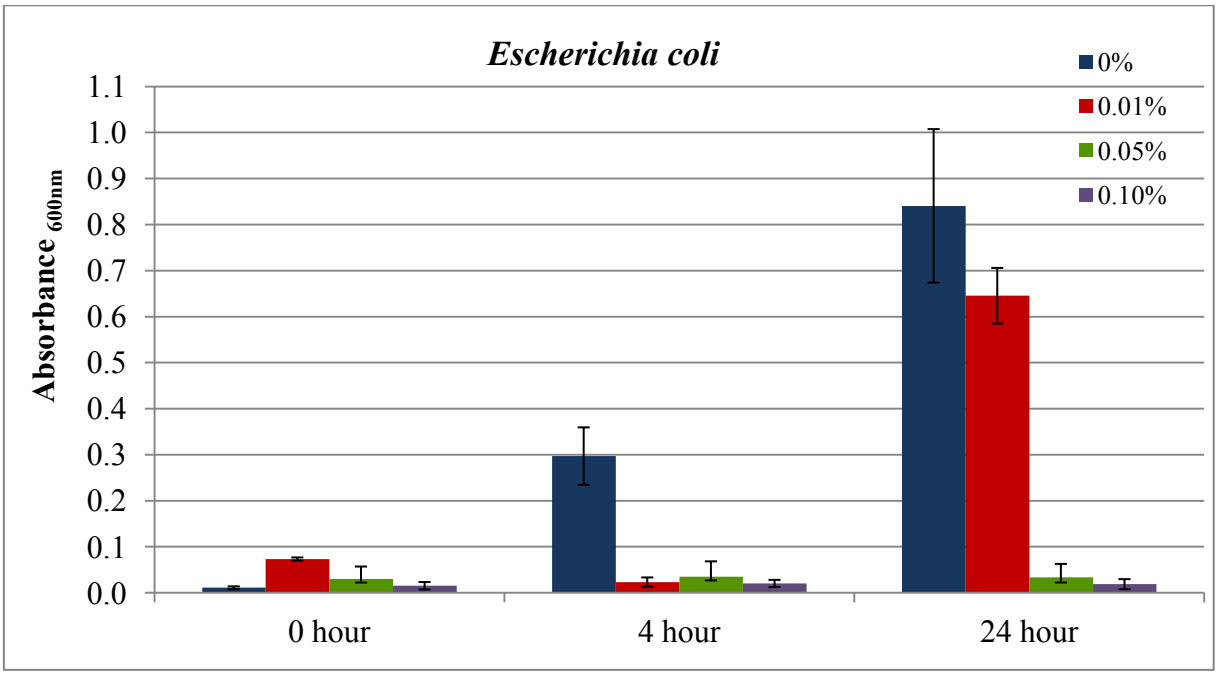


Figure 6.6: The optical density of *E. coli* after incubation in nutrient broth with different chitosan concentrations

The observed OD data in Figure 6.6 and statistical analysis showed that the growth of *E. coli* in nutrient broth medium with different chitosan concentrations were different significantly at $p < 0.05$ (Appendix – Chapter 6, Table A.6.6).

The OD of the control sample and the 0.01% chitosan sample increased rapidly, from 0.011 to 0.841 and from 0.073 to 0.645 respectively, after 24 hours of incubation. The turbidity of the broths of these samples indicated that the growth of *E. coli* was very good (Figure 6.7).

In contrast, the OD of *E. coli* in nutrient broth medium with 0.05 % and 0.1 % chitosan concentration was still very low after 4 hours and 24 hours of incubation. The broths with 0.05% and 0.1% chitosan still remained their clear solution. This demonstrated that chitosan at 0.05 % and 0.1 % concentrations can completely inhibit the growth of *E. coli*, a Gram-negative bacterium.

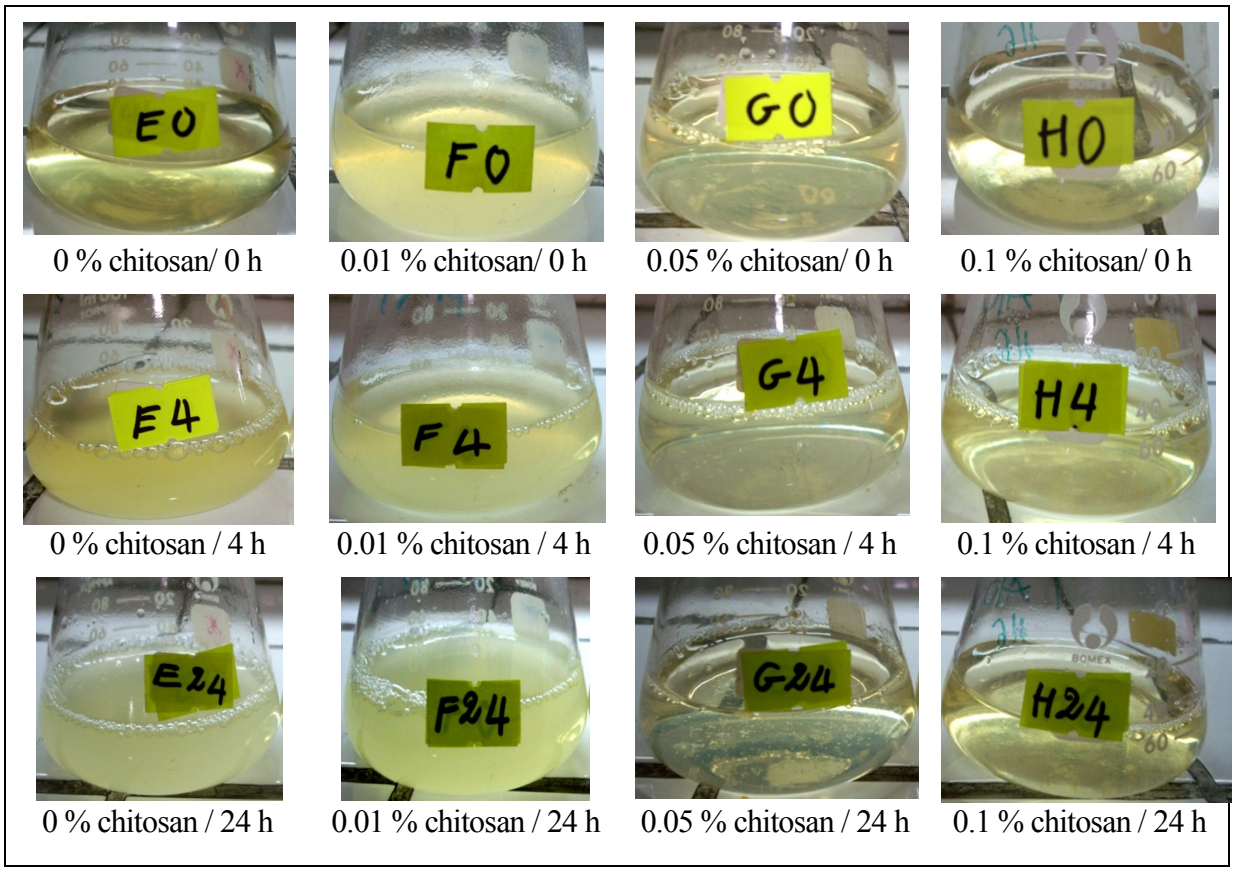


Figure 6.7: The development of *E. coli* in nutrient broth with different chitosan concentrations

In Figure 6.7, the treatment F0 (0.01 % chitosan / 0 hour) looked a bit cloudy. This might due to the precipitation of chitosan when changing to the new medium with higher pH than its pKa. In this case, the concentration of chitosan used to mix with the culture was too low, and hence it could be affected easily by the new medium.

6.3.2.1.2 The growth of *S. aureus* in media with different chitosan concentrations

Similar to *E. coli*, the development of *S. aureus* in nutrient broths without chitosan was significantly different from the broths with 0.05 % and 0.01 % chitosan at $p < 0.05$ (Appendix – Chapter 6, Table A.6.7). The OD of the broths with 0.01 % chitosan and without chitosan increased sharply to more than 0.8 after 24 hours of incubation, while the OD of the broths with 0.05 % and 0.01 % chitosan were still less than 0.03 (Figures 6.8 and 6.9).

This result demonstrated that chitosan at 0.05 % and 0.1 % concentration also effectively prevents the growth of *S. aureus*, a Gram-positive bacterium.

In general, chitosan at 0.05 % and 0.1 % concentration can inhibit the development of both *Escherichia coli*, a Gram-negative bacterium, and *Staphylococcus aureus*, a Gram-positive bacterium.

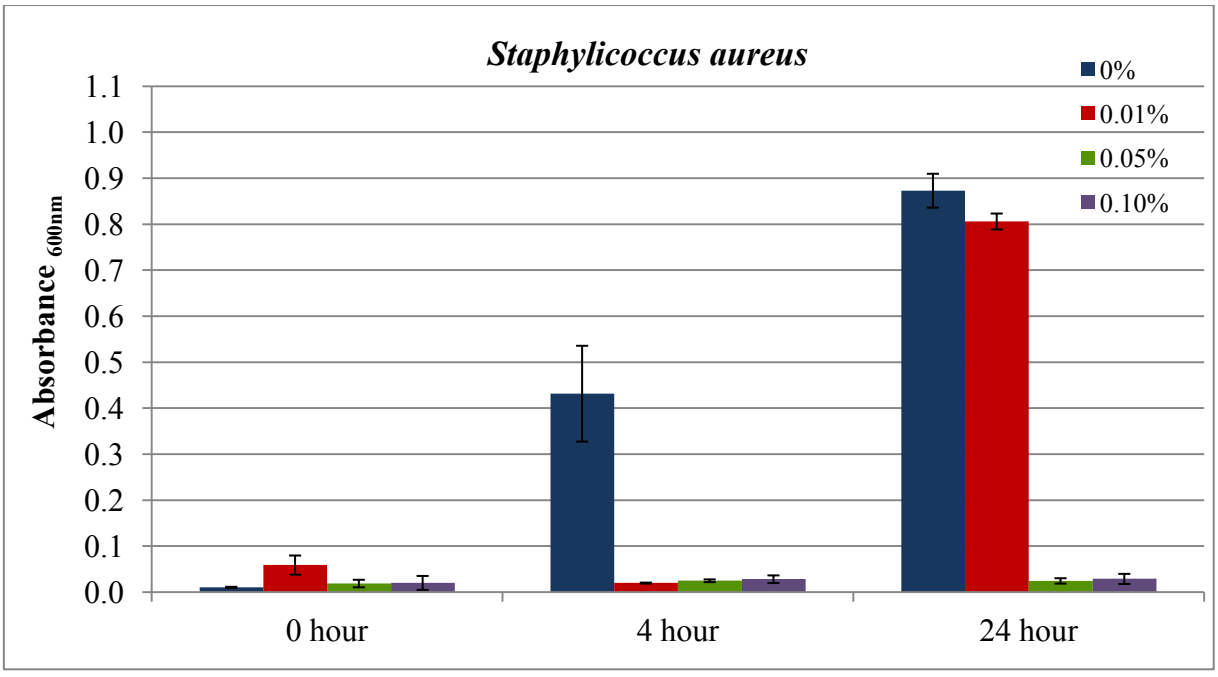


Figure 6.8: The optical density of *Staphylococcus aureus* after incubation in nutrient broth with different chitosan concentration

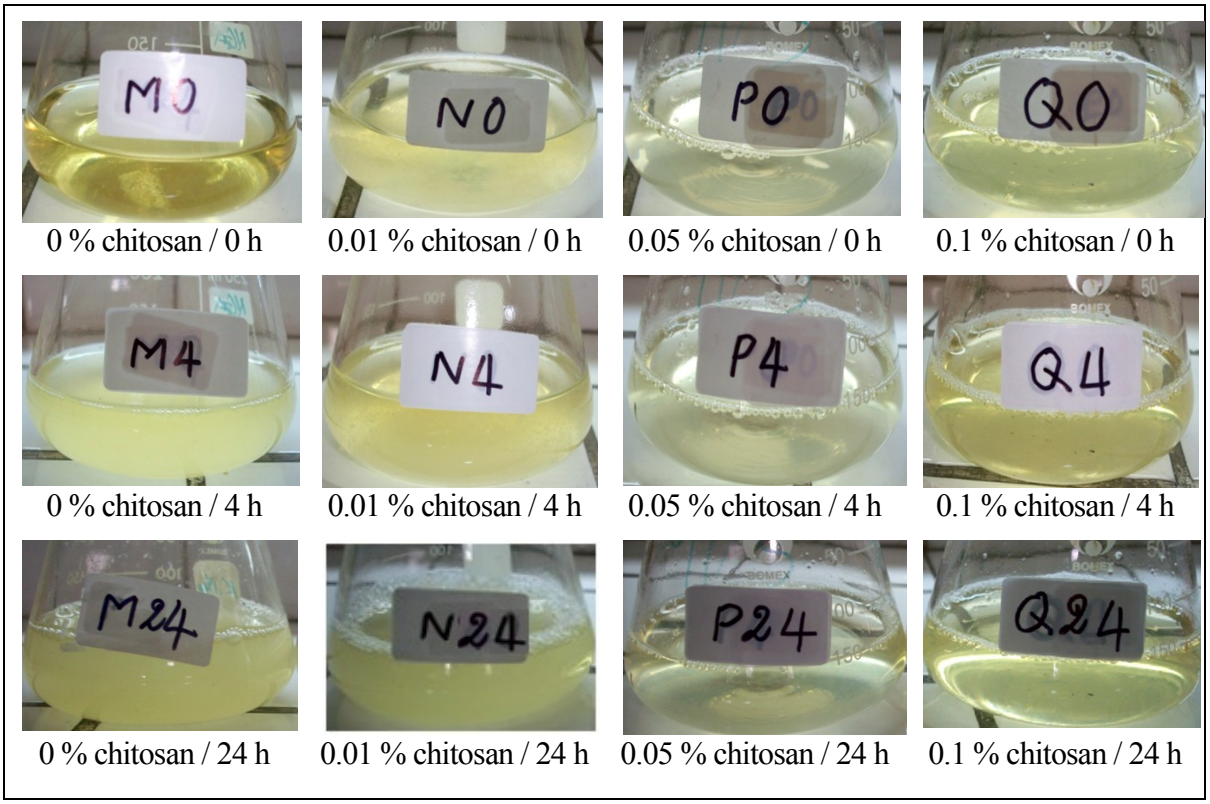


Figure 6.9: The development of *Staphylococcus aureus* in nutrient broth with different chitosan concentrations

6.3.2.2 Plate count method

The spectrophotometric method is a quick method to detect the development of bacteria. However, this method is affected by many factors such as the broth composition or substances released during metabolized process of the bacteria which cause the change in the optical density of the solution. Therefore, viable cell plate count method was used to determine the exact quantity of bacteria in a solution.

6.3.2.2.1 The viable *E. coli* count

The development of *E. coli* after incubation in nutrient broth with different chitosan concentrations was described in Table 6.4 and Figure 6.10.

Table 6.4: Number of *E. coli* (CFU/mL) after incubation in nutrient broth with different chitosan concentrations

Time / Chitosan concentrations	0 hour	4 hours	24 hours	Inhibition rate η (%) after 4 h	Inhibition rate η (%) after 24 h
0%	1.70E+09 ± 1.00E+08	2.40E+11 ± 5.29E+10	1.87E+12 ± 1.53E+11	-	-
0.01%	1.73E+09 ± 2.52E+08	1.90E+11 ± 1.00E+10	1.60E+12 ± 1.00E+11	-	-
0.05%	1.87E+09 ± 2.31E+08	1.57E+04 ± 1.15E+03	2.03E+04 ± 4.93E+03	100	100
0.1%	1.73E+09 ± 2.31E+08	1.60E+04 ± 1.00E+03	1.63E+04 ± 4.04E+03	100	100

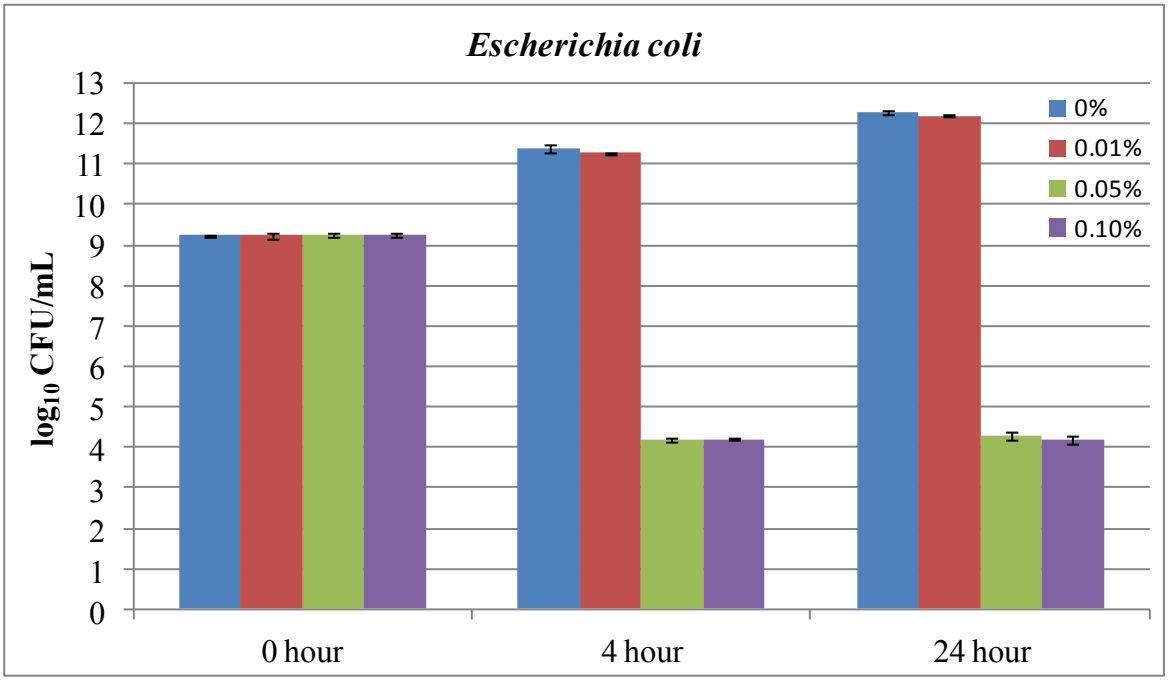


Figure 6.10: Number of *E. coli* ($\log_{10}$ CFU/mL) after incubation in different chitosan concentrations

The result showed that chitosan had a high antimicrobial effect to *E.coli*. In the control as well as the 0.01% chitosan treatment, the number of *E.coli* increased steadily to 24 hours of incubation, from approximately 9 to 12 $\log_{10}$ CFU/mL. In contrast, the number of *E.coli* decreased sharply after 4 hours of incubation in nutrient broth containing 0.5 % or 1 % chitosan, from approximately 9 to 4 $\log_{10}$ CFU/mL. This result indicated that the 0.01 % chitosan concentration was too low to inhibit the development of *E.coli*while the 0.05 and 0.1 % chitosan concentrations had an effective inhibition to *E.coli*just after 4 hours of incubation.

Statistical analysis also showed that the numbers of *E.coli* developed in different chitosan concentration as well as after different incubation times were different significantly at $p < 0.05$ (Appendix – Chapter 6, Table A.6.9).

6.3.2.2.2 The viable *S. aureus* count

The development of *S. aureus*after incubation in nutrient broth with different chitosan concentrations was described in Table 6.5 and Figure 6.11.

Table 6.5: Number of *S. aureus*(CFU/mL) after incubation in nutrient broth with different chitosan concentrations

Time / Chitosan concentrations	0 hour	4 hours	24 hours	Inhibition rate η (%) after 4 h	Inhibition rate η (%) after 24 h
0%	1.17E+09 ± 1.53E+08	1.63E+10 ± 3.51E+09	2.80E+10 ± 1.00E+09	-	-
0.01%	1.37E+09 ± 2.89E+08	1.70E+10 ± 2.65E+09	2.20E+10 ± 3.46E+09	-	-
0.05%	1.17E+09 ± 2.08E+08	2.50E+02 ± 0.00E+00	2.50E+02 ± 0.00E+00	100	100
0.1%	1.16E+09 ± 2.16E+08	2.50E+02 ± 0.00E+00	1.60E+03 ± 2.34E+03	100	100

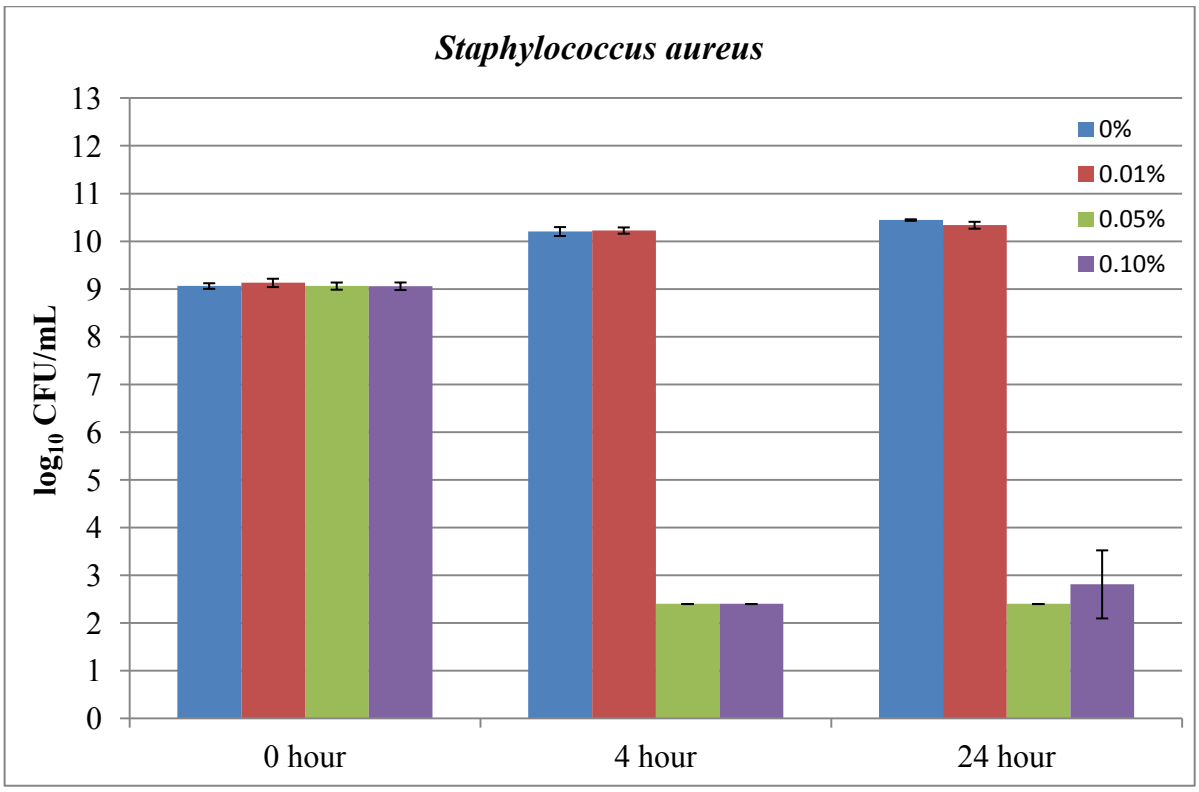


Figure 6.11: Number of *S. aureus* (log_{10} CFU/mL) after incubation in nutrient broth with different chitosan concentrations

Similar to *E. coli*, the result showed that the 0.05% and 0.1% chitosan solution can inhibit *S. aureus* effectively with a 100 % inhibition rate after 4 hours and 24 hours of incubation while the 0.01% chitosan concentration cannot inhibit the development of *S. aureus*. The difference of the growth of *S. aureus* in different chitosan concentrations as well as after different incubation time was significant at $p < 0.05$ (Appendix – Chapter 6, Table A.6.10).

In general, the plate count method gave similar results to the spectrophotometric method in the development of both *E. coli*and *S. aureus* in nutrient broth with different chitosan concentrations, except the 0.01 % chitosan treatment. In the 0.01 % chitosan treatment, the growth of *E. coli* and *S. aureus* after 4 hours of incubation could not be detected by the spectrophotometric method while it was recognised as increasing by viable cell method. This could be due to the decrease of pH of the medium after 4 hours that make the chitosan dissolved, hence the OD looked unchanged (Table 6.6).

Table 6.6: The pH of the culture media in the control treament

Time / Bacteria	0 hour	4 hours
Escherichia coli	6.8567 ± 0.2511	6.5133 ± 0.2804
Staphylococcus aureus	7.0267 ± 0.4373	6.7533 ± 0.6424

The pH recorded at the beginning and after 4 hours of incubation (Table 6.6) showed that the pH of bacteria culture was higher than the pKa of chitosan which is normally at 6.2 (Rabea *et al.*, 2003).

The result of this experiment was similar to that observed in a previous study (Domínguez *et al.*, 2001). Similar to the other research, the growth of these bacteria could be almost 100 % inhibited when incubated with 0.05 % - 0.1 % chitosan in the medium (Kanatt *et al.*, 2008a).

In another study, Darmadji and Izumimoto showed that 0.01 % chitosan can inhibit the growth of both *E. coli*and *S. aureus* in yeast extract peptone glucose broth (Darmadji and Izumimoto, 1994).This dissimilar finding maydue to the different culture and chitosan used for the research because the antimicrobial property of chitosan depends on many factors such as the pH, the age of cell, the molecular weight, and degree of deacetylation of chitosan (No *et al.*, 2002; Tsai and Su, 1999; Zheng and Zhu, 2003).

In our study, the 0.01 % chitosan concentration cannot inhibit the development of *E. coli*and *S. aureus* might be because of the low chitosan concentration or the high pH of the culture media (Table 6.6). Chitosan can inhibite the growth of bacteria better at lower pH values (No *et al.*, 2002; Tsai and Su, 1999). When the pH of the culture media is higher than the pKa of the chitosan, there are less cation groups on the structure of chitosan, therefore, it doesn't react as strongly with the negative-charged outer membrane of the bacteria (Rabea *et al.*, 2003).

6.3.3 Comparison the antimicrobial property between normal chitosan and purified chitosan

In order to test whether the purified chitosan can still keep its original property, the susceptibility tests were performed to compare between a normal chitosan and a purified one based on guidance of the National Committee for Clinical Laboratory Standards.

The minimum inhibitory concentration technique (MIC) (CLSI, 2006a), minimal bactericide concentration technique (MBC) (Fernandez-Saiz *et al.*, 2009; No *et al.*, 2002; Wiegand *et al.*, 2008), and disc diffusion technique (CLSI, 2006b) have been used in this experiment.

6.3.3.1 Minimum inhibitory concentration (MIC)

The chitosan stock solution (1000 mg/L) was serially diluted from 1 to 1/128 in 0.85 % sterile NaCl. When being mixed with the bacterial suspension at the same volume, the final concentration of chitosan in incubation culture would be in the range from 3.77 to 500 mg/L.

The MIC is defined as lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism (CLSI, 2006a). The more resistant an organism is, then the higher will be the MIC.

Table 6.7: The MICs of chitosan samples to *E. coli* and *S. aureus*

Microorganism	Replication	nCTS	pCTS	Acetate
Escherichia coli	1	31.25 ppm	31.25 ppm	62.5 ppm
	2	31.25 ppm	31.25 ppm	125 ppm
	3	31.25 ppm	31.25 ppm	125 ppm
Staphylococcus aureus	1	31.25 ppm	31.25 ppm	62.5 ppm
	2	31.25 ppm	31.25 ppm	125 ppm
	3	31.25 ppm	31.25 ppm	125 ppm

Note: nCTS: normal chitosan
pCTS: purified chitosan
Acetate: 0.2 N acetate buffer pH 4.3 (control)

The MIC of chitosan to *E. coli* and *S. aureus* is presented in Table 6.7. The MIC results demonstrated that chitosan can inhibit the growth of both Gram negative and Gram positive bacteria. From Table 3.8, it could be concluded that the MIC of the normal chitosan and the purified chitosan was 31.25ppm to both *E. coli* and *S. aureus*, while MIC of acetate buffer was 125ppm.

This result was close to the results of another research (Seo *et al.*, 1992) , which showed that the MIC of chitosan to both *E. coli* and *S. aureus* was 20 ppm. This experiment showed that the purified chitosan can retain its antimicrobial property at the same level of the normal chitosan.

6.3.3.2 Minimal bactericideconcentration (MBC)

For each well that didn't show the visible growth of bacteria after 24h of the MIC test, the number of bacteria was counted by the plate count method on Muller-Hinton agar. The concentration of chitosan that resulted in no colony on the plate count was obtained as the MBC. The MBC of chitosan to *E. coli* and *S. aureus* is presented in Table 6.8.

Table 6.8: The MBC of chitosan to *E. coli* and *S. aureus*

Microorganism	Replication	nCTS	pCTS	Acetate
Escherichia coli	1	62.5ppm	62.5ppm	250ppm
	2	31.25ppm	31.25ppm	250ppm
	3	31.25ppm	31.25ppm	500ppm
Staphylococcus aureus	1	62.5ppm	31.25ppm	250ppm
	2	62.5ppm	62.5ppm	500ppm
	3	125ppm	125ppm	500ppm

Note: *nCTS: normal chitosan*
pCTS: purified chitosan
Acetate: 0.2 N acetate buffer pH 4.3 (control)

From Table 6.8, it could be concluded that the MBC result of normal chitosan and purified chitosan is 31.25 ppm for *E. coli* and 62.5 ppm for *S. aureus* while MBC of Acetate buffer was 250 ppm for *E. coli* and 500 ppm for *S. aureus.* Tsai *et al.* also showed that the MBC of shrimp chitosan to *E. coli* and *S. aureus* was about 100 ppm (Tsai *et al.*, 2002).

There was no difference between purified chitosan and the normal chitosan in the term of MBC value.

6.3.3.3 Disc diffusion

The inhibitory activity of chitosan was evaluated based on the clear zone surrounding a circular disc. If there is no clear zone, it is assumed that there is no inhibition of bacterial growth.

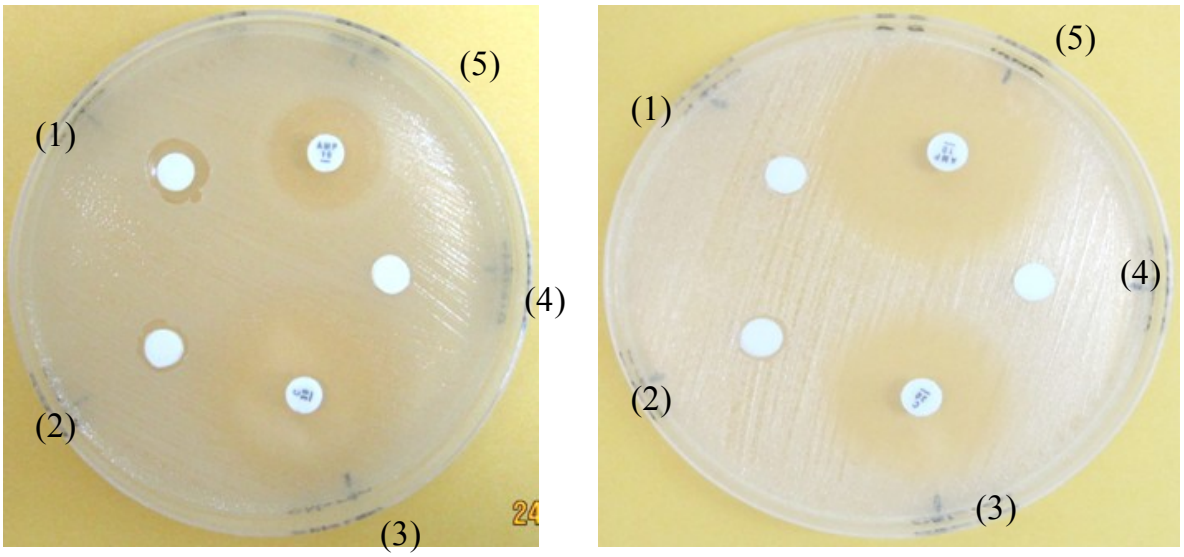
An amount of 200 µg of each type of chitosan was dropped into separate discs. The 30-µg chloramphenicol disc and 10-µg ampicilin discs were used as positive control, while the acetate buffer was used as negative control. The diameter (mm) of the growth-inhibition zone including the disc diameter was measured after 24 h incubation using a ruler (CLSI, 2006b; Mayachiew *et al.*, 2010).

Table 6.9 expressed antimicrobial activity of chitosan by the disc diffusion method. The antibiotic action of chitosan was very low compared to chloramphenicol and ampicillin. This is because chitosan is not a real antibiotic but only a biopolymer which has the antimicrobial property.

Table 6.9: The antibiotic circle diameter (mm)

No.	Chitosan /Antibiotics	*Escherichia coli*	*Staphylococcus aureus*
1	Normal chitosan	9.17 ± 0.76	7.33 ± 0.58
2	Purified chitosan	9.17 ± 0.76	7.17 ± 0.76
3	Acetate buffer	6.50 ± 0.00	6.00 ± 0.00
4	Chloramphenicol	24.00 ± 0.00	24.67 ± 1.15
5	Ampicillin	18.83 ± 0.00	33.33 ± 1.15

Chitosan seems to inhibit Gram-negative bacteria better than Gram-positive bacteria, while chloramphenicol and ampicillin showed higher antibiotic property to Gram-positive bacteria (Figure 6.12). Normal chitosan and purified chitosan had the same effects on bacterial inhibition to both *E. coli* and *S. aureus*. There was no significant difference at $p < 0.05$ (Appendix – Chapter 6, Table A.6.13).



(A) **(B)**

Figure 6.12: Zone inhibition of discs containing (1) normal chitosan, (2) purified chitosan, (3) chloramphenicol, (4) acetate buffer, and (5) ampicillin in petri dishes with culture of (A) *E. coli* and (B) *S. aureus.*

In general, through the MIC, MBC and disc diffusion tests, chitosan showed its antimicrobial property which still remains after the purification process. Although the antibiotic activity of chitosan is rather low, it can be applied in food preservation. The following experiment was a trial to confirm this property of chitosan.

6.3.4 Comparison of the antimicrobial activity of normal and purified chitosan in preservation of raw shrimp

6.3.4.1 Weight loss

The weight of shrimp decreases during the storage at cool temperature. The weight loss (%) of shrimp in different preservation methods was presented in Figure 6.13.

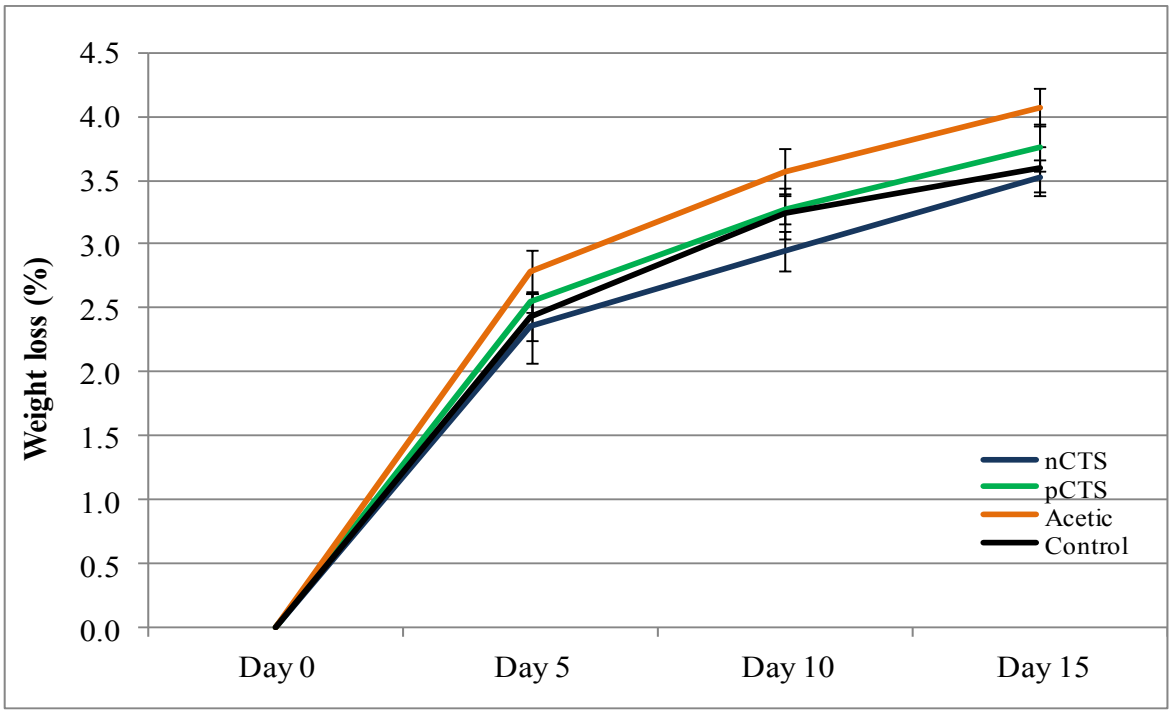


Figure 6.13: Weight loss (%) of shrimp during storage

The results in Figure 6.13 and statistical analysis in Appendix – Chapter 6, Table A.6.15 demonstrated that the weight loss (%) of shrimp in all treatments increased gradually over time during storage ($p < 0.05$). Among them, shrimps soaked in acetic acid solution showed the highest weight loss which was significantly different to other treatments at $p < 0.05$.

There were no significant difference between chitosan-treated shrimp and the control at $p < 0.05$. This result demonstrated that chitosan has no effects on preventing the loss of weight in shrimp during storage at 0 – 4 ^{o}C. However, the weight loss of shrimp treated with normal chitosan and purified chitosan different significantly at $p < 0.05$. The normal chitosan can help shrimp remains its moisture content slightly better than the purified one with the mean difference just around 0.25 % (Appendix – Chapter 6, Table A.6.15).

6.3.4.2 Texture

The texture of shrimp in this experiment was expressed as the hardness index. It was measured by texture equipment from Zwick/Roell company (Germany). The hardness is defined as the maximum force at load application and measured in Newton. The result was presented in Figure 6.14.

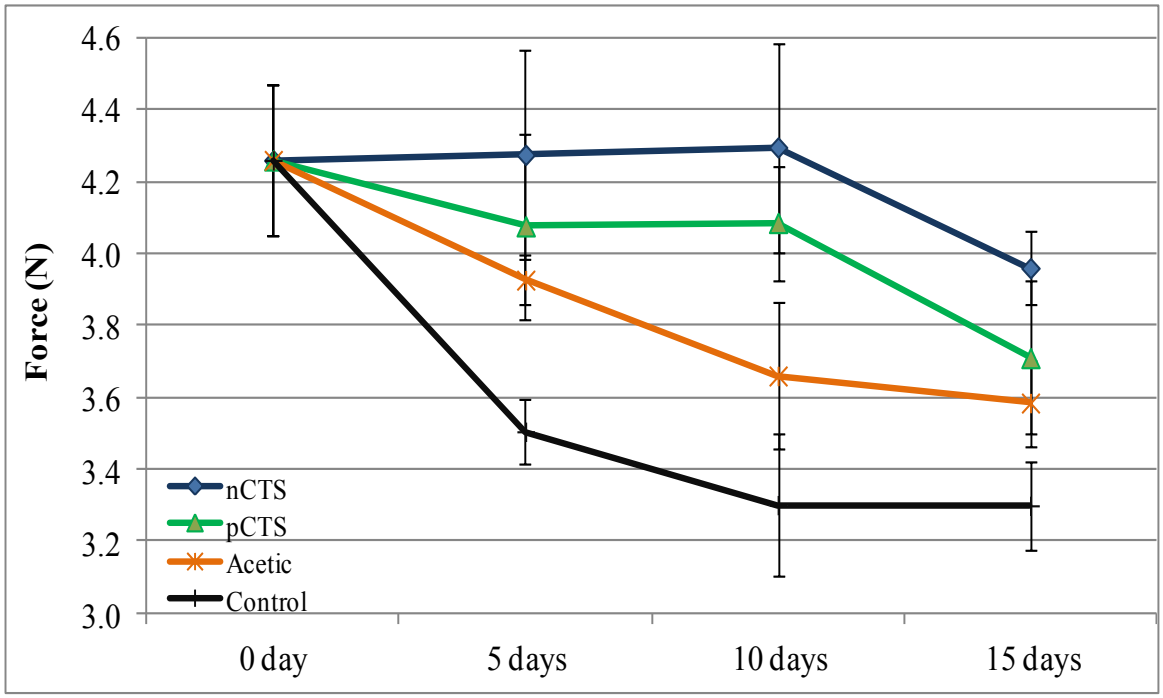


Figure 6.14: Hardness of shrimp (force - N) during storage

The texture of shrimp changed with different treatments and decreased during storage. All treatments resulted in better texture of shrimp when compared to the control. The differences among treatments were significant at $p < 0.05$ (Appendix – Chapter 6, Table A.6.17). Although there was a slight difference in the texture of shrimp between normal chitosan and purified chitosan treatments ($p = 0.048$), both treatments showed higher hardness value than others.

However, there was no significant difference in the hardness of shrimps between 5 days and 10 days of storage, especially with the chitosan-treated shrimps ($p < 0.05$). After 15 days of storage, the hardness of shrimps treated with chitosan decreased while other treatments seemed unchanged.

6.3.4.3 Total plate counts

The micribiological index of shrimp during storage was measured by the total plate counts. The results were presented in Figure 6.15.

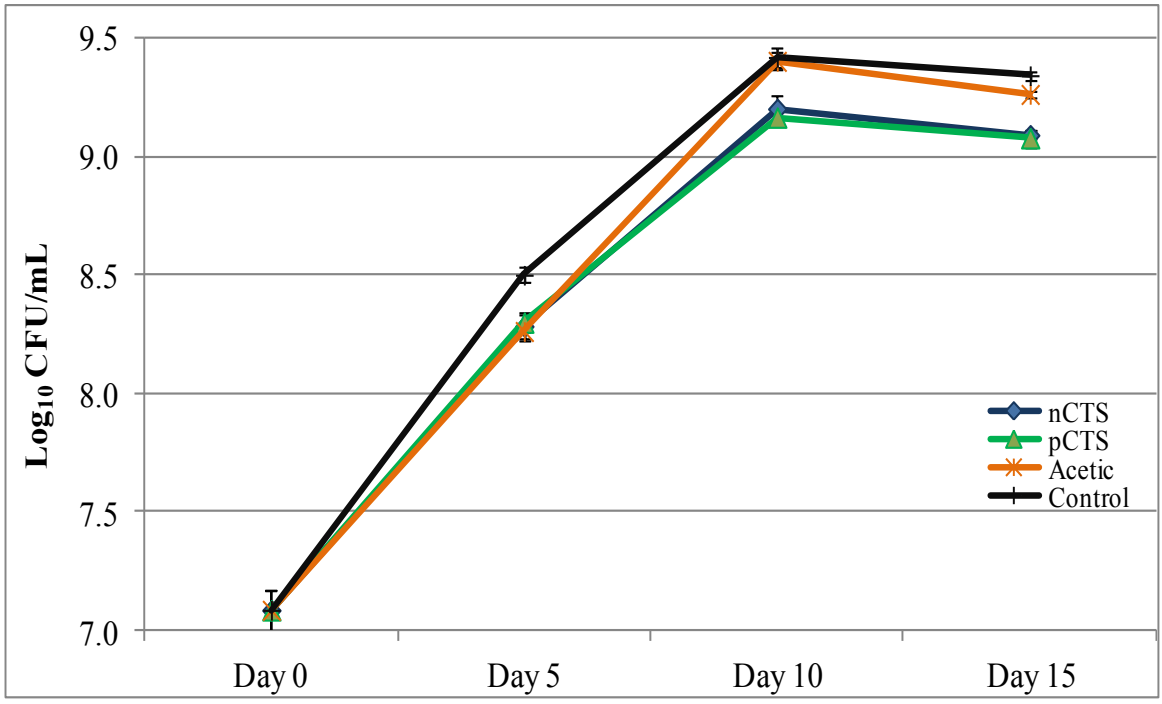


Figure 6.15: Total plate counts (log_{10} CFU/mL) of shrimp during storage

The total plate counts (log_{10} CFU/mL) of shrimp increased sharply after 10 days of storage at 0 – 4 ^{o}C and slightly decreased after 15 days. This could be because the spoilage of shrimp occurred during storage time. After 10 days, the growth of bacteria started to decrease. This might be due to the accumulation of toxic waste that they excreted during their metabolism. This result was similar to the research of Simpson *et al.* on preservation of *Pandalus borealis* shrimp (Simpson *et al.*, 1997). According to their findings, *Bacillus cereus* required chitosan concentrations of 0.02 % (for bactericidal effect), while *Escherichia coli* and *Proteus vulgaris* showed minimal growth at 0.005 %, and complete inhibition at ≥ 0.0075 %.

Figure 6.15 showed that the total plate counts in shrimps were reduced significantly ($p < 0.05$) by chitosan treatments as compared with the control up to 15 days of storage. This result indicated that chitosan has good effect on the inhibition of bacterial growth on shrimp. There was no significant difference in total plate counts between shrimps treated with normal chitosan and purified chitosan (Appendix – Chapter 6, Table A.6.20). This result demonstrated that purified chitosan can still keep its antimicrobial property as the normal one.

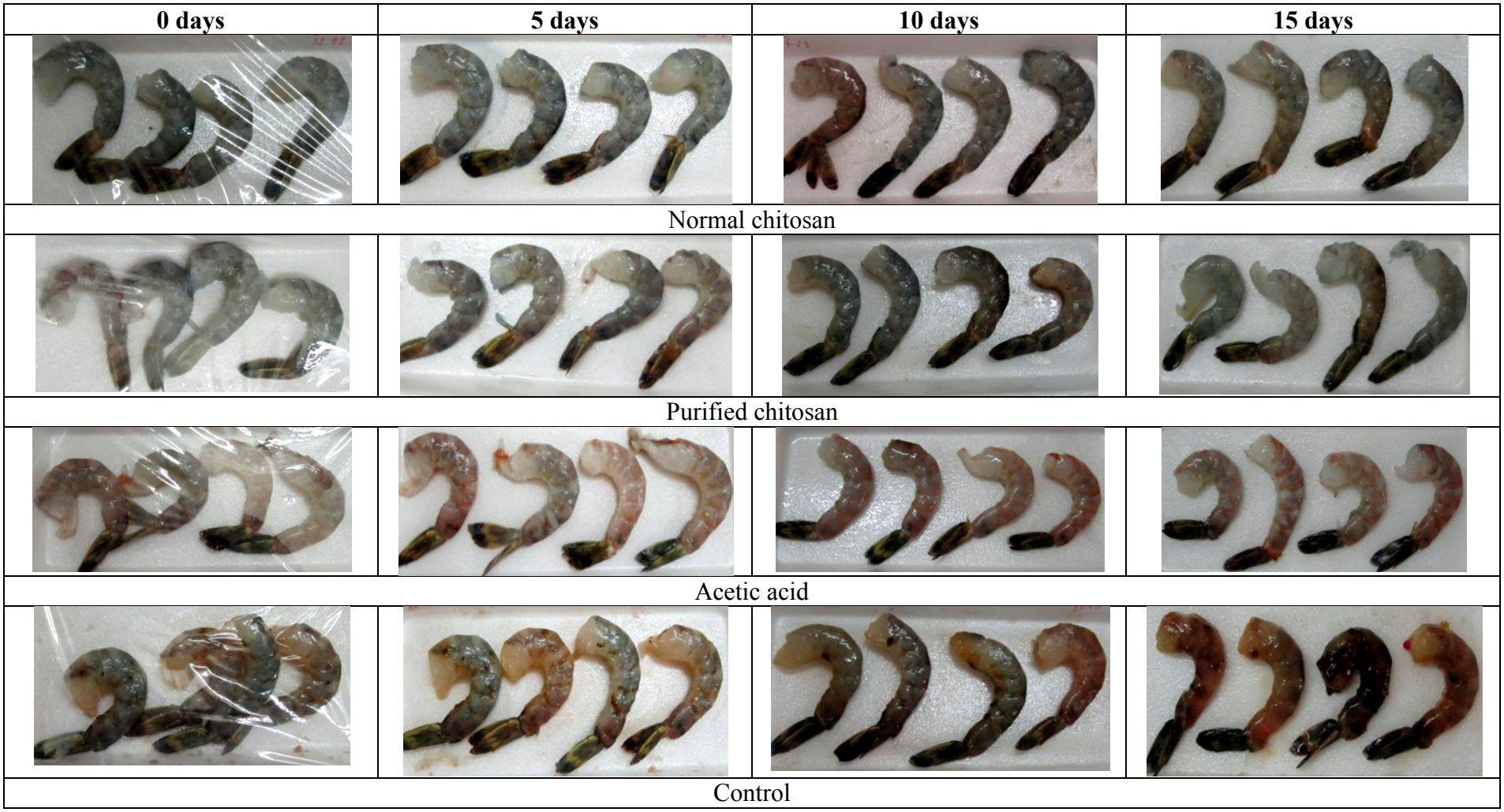


Figure 6.16: Shrimp images after 0, 5, 10 and 15 days of storage at 0 – 4 ^{o}C

Figure 6.16 presented the change of shrimp colour during preservation. The shrimp turned pink after treated in acetic acid. This could be due to the acidic condition. Although chitosan was also dissolved in acetic acid, its solution had the pH raised up to 5.5 before treatment of shrimp. Therefore, the acetic acid did not affect the colour of the shrimp. The increasing pH was aimed to prevent the shrimp from being affected by the smell and taste of the acetic acid.

The images in Figure 6.16 demonstrated that treated shrimp still retain their original colour after 5 days of storage at 0 – 4 ^{o}C. Their colour started to change after 10 days of storage with some small black spot on the skin of the shrimp. However, their appearance still looked fresh even after 15 days of storage.

In contrast, the colour of shrimp in the control treatment changed significantly during 15 days of storage. The black spots appeared just after 5 days of storage. The black spots are composed of melanin pigments formed by the oxidation of phenolic compound with the activation of polyphenol oxidases (Simpson *et al.*, 1997). The shrimp start to have bad smell with darker skin after 10 days of storage. It was completely spoiled after 15 days of storage with the darkening on the whole shrimp. The shrimp had very bad smell and soft texture at this stage.

6.4 GENERAL DISCUSSION AND CONCLUSION

The biochemical properties of selected chitin and chitosan samples varied due to their different sources. Most chitin and chitosan samples have moisture content less than 10 %. Their ash contents were less than 1 %, except for chitosan 2. The heavy metal content of these samples were very low compared to the Australian food standards for maximum permitted concentration of heavy metal in crustacean (Anon, 1987). It appears that each of the tested samples would be safe product for use in the food industry. The protein content of normal chitosan samples was less than 1 %, while it cannot be detected in the purified sample. The results indicated that chitin and chitosan need to be characterized and standardized their properties before application in food industry.

Chitosan at 0.05 % and 0.1 % concentration can inhibit the development of *Escherichia coli*, a Gram-negative bacterium, and *Staphylococcus aureus*, a Gram-positive bacterium. It can absolutely inhibit the growth of the *E. coli* and *S. aureus* after 4 hours of incubation. This result was confirmed by other researches (Devlieghere *et al.*, 2004; Rabea *et al.*, 2003; Zheng and Zhu, 2003).

The purified chitosan can still retain its antimicrobial property as the normal chitosan through MIC, MBC and disc diffusion results. Their MICs to *E. coli* and *S. aureus* were 31.25 ppm while their MBCs were 31.25 ppm to *E. coli* and approximately 62.5 ppm to *S. aureus*. These chitosan samples inhibit the Gram-negative bacteria better than Gram-positive bacteria although the antibiotic activity of chitosan is rather low compared to chloramphenicol and ampicilin. Due to its antimicrobial property, chitosan is a promising material in the preservation of food products.

The application of chitosan in shrimp preservation has confirmed its antimicrobial property. Both purified and normal chitosan can reduce the development of bacteria on shrimps during 15 days of storage at 0 – 4 ^{o}C. Moreover, it can help the shrimp to lower the weight loss and keep their hardness higher than the non-treated ones during storage.

Chapter 7
FORMULATION OF CHITOSAN-BASED EDIBLE FILM FOR APPLICATION IN INSTANT FOOD CASING

7.1 INTRODUCTION

During recent years, the food industry is developing very fast to fulfill the requirements of the consumers. The food producers always want to make new food products to supply for the higher demands of the consumers as well as try to develop new materials that are friendly to the environment. On the other hand, due to the development of the industrial society, people's lives are getting busier. Therefore, fast foods become more dominant in the market because of their ease (Buckley *et al.*, 2007; de Boer *et al.*, 2004; Glanz *et al.*, 1998).

Instant noodles are among the instant foods that are used very frequently due to their cheap price, nutritious value and ease of preparation (Fu, 2008; Tan *et al.*, 2009). However, the consumers must open their spice sachets by knife, scissors or by hands that can cause contamination or loss of spice. These spice sachets are usually made of plastics which are not degradable and can affect the environment and human health. Many researchers are trying to make self-degradable packaging to solve this problem (Duncan, 2011; Farris *et al.*, 2009; Siró, 2012; Tharanathan, 2003). However, there are still no studies on making edible films for spice casing in instant food.

Vietnam is a tropical country which has the coastal length of more than 3,000 kilometers. Therefore, the seafood industry is very developed and gives out large amount of seashell which is considered as waste. Only 5% of shrimp waste which contains head and body carapace (48-56 % of the raw shrimp) (Sachindra *et al.*, 2007) is used for animal feed (Kandra *et al.*, 2012). The shrimp waste composed mainly of protein (40%), minerals (35%) and chitin (14–30%) (Synowiecki and Al-Khateeb, 2003). Nowadays, people can isolate chitin from the shell of crustacean such as shrimp and crab.

Chitin and its derivatives such as chitosan and glucosamine are being applied widely in many fields (Dutta *et al.*, 2009; Friedman and Juneja, 2010; Kurita, 2006; Muzzarelli and

Muzzarelli, 2005; Ravi Kumar, 2000; Rinaudo, 2006; Stevens, 2005). In the food industry, chitosan can be used as a protective membrane or film (Alishahi and Aïder, 2011; Fernández-Saiz and Lagaron, 2011; Mohammed, 2010; Rathke and Hudson, 1994). Therefore, the use of chitosan film in food production should be more developed.

This research aimed to use chitosan as a biomaterial to make spice sachets which can be used in instant food products. They should have the following properties: edible, soluble in hot water, and sealable. However, chitosan film has very poor solubility in neutral or alkaline solution. Therefore, it is necessary to mix chitosan with other water-soluble materials to apply it in instant food casing.

Summary of Chapter 7:

In work described in this chapter, the film forming capacity of chitosan has been investigated to find appropriate methods for making chitosan film. Besides that, the film forming ability of some other biopolymers were also examined to find out which polymer to combine with chitosan to make films with desired properties. The composite edible film was investigated for its formulation as well as antimicrobial properties. The use of this edible film in spice casing for instant noodles is an attempt to apply chitosan-based films in the instant food industry.

This study found that the neutralization process, the degree of deacetylation of chitosan and the chitosan concentration affected significantly the solubility of chitosan film. Among the tested biopolymers, gelatin showed the best appropriate properties to blend with chitosan in forming soluble and sealable edible film. The composition of 0.5 % chitosan and 5 % gelatin has been shown as the most appropriate formulation to make the desired edible film. This edible film showed excellent antimicrobial property to both gram negative and positive bacteria. Therefore, it could be used in the preservation of food products. This composite chitosan/gelatin edible film was applied in making seasoning and oil sachets for instant noodles to examine the feasibility of this film. The spice stored in these edible film sachets can still keep their quality after 6 months of storage. These sachets should be kept in another container to prevent the exposure to humidity. Overall, this edible film is suitable for application in spice casings for instant noodles.

7.2 MATERAILS AND METHODS

7.2.1 Materials and equipments

7.2.1.1 Materials

- Agar: Food grade (Viet Nam)
- Carrageenan: Food grade (Denmark)
- Carboxymethyl cellulose: Food grade (Japan)
- Chitosan: Food grade (Viet Nam)

Two types of chitosan which have 94 % and 76 % degrees of deacetylation were used in this study. They both have less than 1 % ash content, 1 % protein content, 1.5 NTU turbidity, and 450 cps viscosity. They were purified before using in this study.

- Gelatin: Food grade (Japan)
- Pectin: Food grade (USA)
- Corn starch: Food grade (France)

7.2.1.2 Chemicals

- Plasticizer: glycerol ($C_3H_8O_3$) 98 % purity
- Glacial acid acetic (CH_3COOH, Merck)
- Saturated potassium cloride (KCl, Merck)
- Saturated potassium acetate (CH_3COOK, Merck)
- Seasoning powder (Knorr, Viet Nam)
- Vegetable oil (Crisco, Australia)
- Other chemicals for analysis

7.2.1.3 Equipments

- Film casting tray
- Drying machine
- Analytical balance (0.0001 – 220 g) (Sartorius Gottingen, Germany)
- Panme ruler (Mitutoyo, Japan)
- Texture machine (Zwick/Roell, Germany)

7.2.2 Method of making edible films

7.2.2.1 Method of making homogeneous edible films

The homogeneous edible film was cast following the diagram in Figure 7.1.

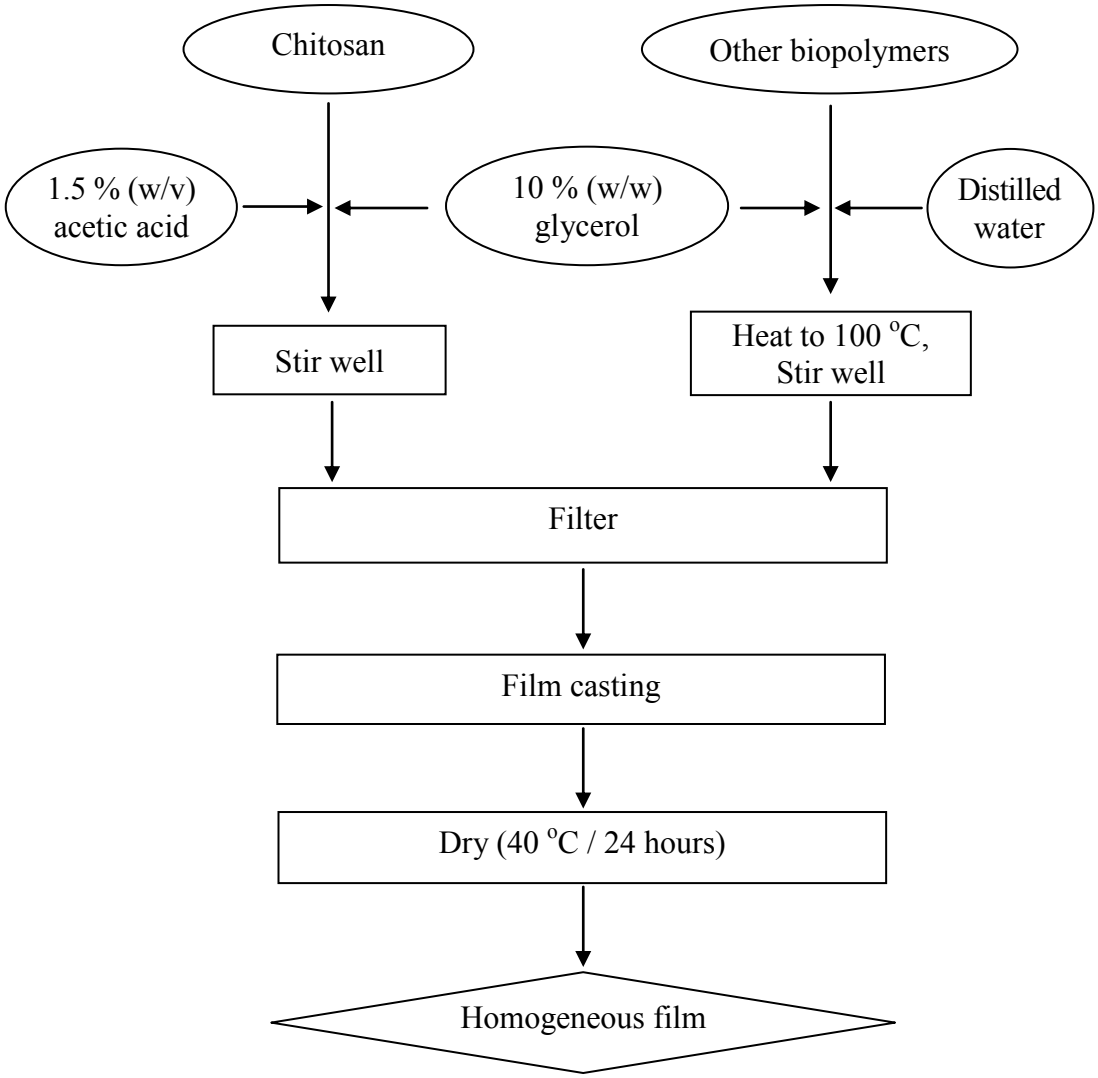


Figure 7.1: Process of casting homogeneous edible film

Chitosan was dissolved in 1.5 % acetic acid containing 10 % glycerol (w/w, dry basis) as a plasticizer by stirring overnight using a magnetic stirrer. Other biomaterials were dissolved in distilled water containing 10 % glycerol (w/w, dry basis) as a plasticizer by stirring overnight using a magnetic stirrer at 100 ^{o}C. All solutions were filtered before casting to remove insoluble particles. A volume of 50 mL of each solution was poured onto a casting tray with a dimension of 20 x 20 cm. It was dried at 40 ^{o}C for 24 hours in a drying machine. The forming film was taken out of the tray and stored in desiccators prior to analysis.

7.2.2.2 Method of making composite edible film

The casting of composite edible films was conducted following the diagram in Figure 7.2.

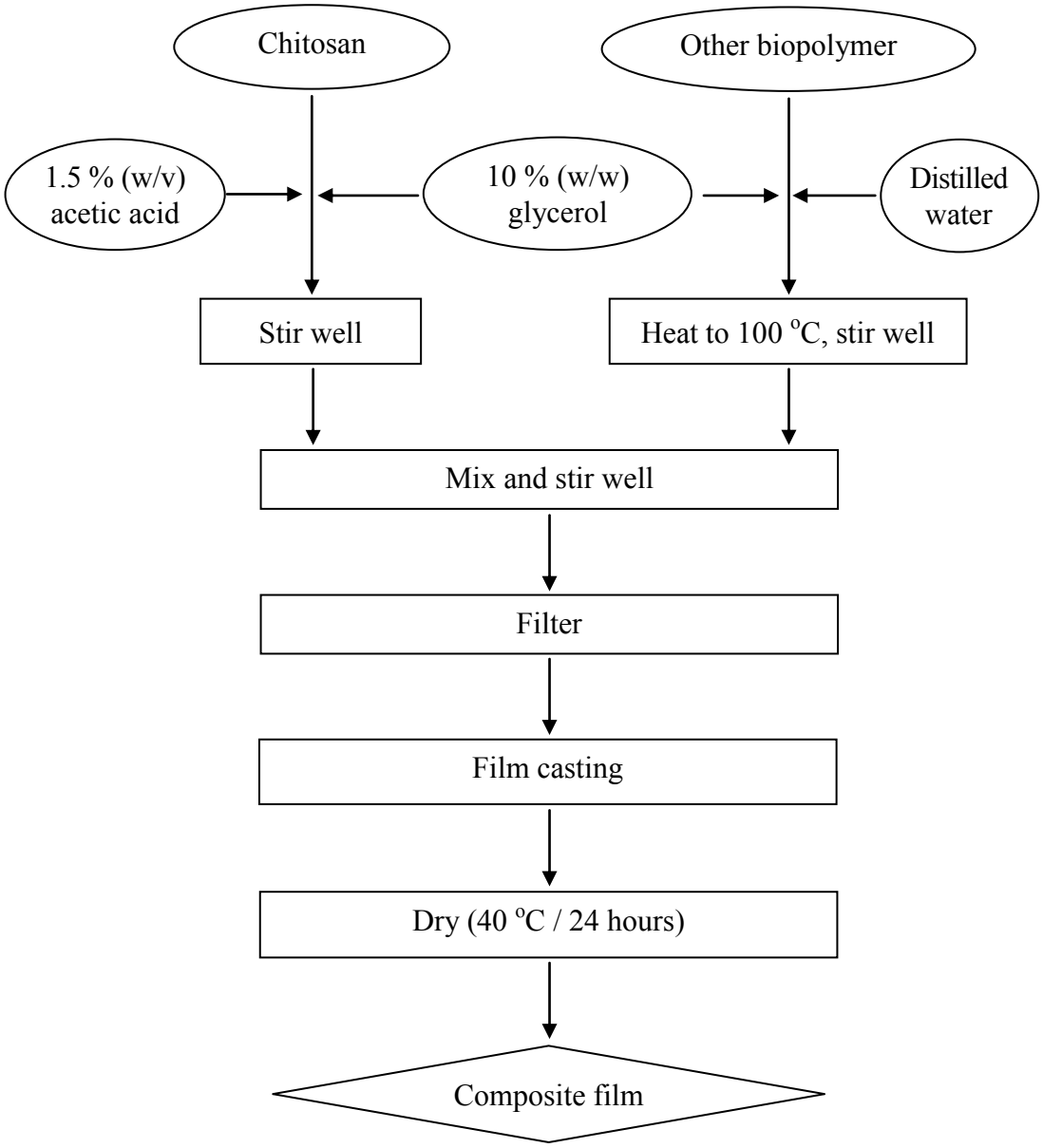


Figure 7.2: Process of casting composite edible film

Chitosan was dissolved in 1.5 % acetic acid containing 10 % glycerol (w/w, dry basis) as a plasticizer by stirring overnight using magnetic stirrer. Other biomaterials were dissolved in distilled water containing 10 % glycerol as plasticizer by stirring overnight using magnetic stirrer at 100 °C. Chitosan solution was mixed with other biopolymer solution(s) with a determined ratio. The combined solution was then filtered after mixing thoroughly by stirring to remove insoluble particles.

A 50 mL volume of the mixed solution was poured onto a casting tray with a dimension of 20 x 20 cm. It was dried at 40 ^{o}C for 24 hours in a drying machine. The forming film was taken out of the tray and stored in desiccators prior to analysis.

7.2.3 Investigation of film forming capacity of chitosan

7.2.3.1 Evaluation of the effect of the neutralization to the solubility of chitosan films

This experiment was aimed at finding the appropriate process for making chitosan film which has better solubility in hot water.

The chitosan film was made following the procedure in Section 6.2.2.1. Chitosan with 94 % degree of deacetylation was used in this assay. One gram chitosan was dissolved in 100 mL of 1.5 % acetic acid. The solution was filtered to remove all insoluble substances before pouring into the casting tray. The chitosan films were formed after drying at 40 °C for 24 hours.

The chitosan film was cut into two parts:

- The first part was neutralized by soaking in 1 N NaOH solution for 5 minutes, then washed thoroughly by distilled water. The neutralized film was dried again before storage in a desiccator.
- The second part was stored in a desiccator without neutralization.

The solubility of each type of chitosan film was measured following the method described in Chapter 2 – Section 2.3.8.4. The chitosan films were conditioned in desiccators for at least 24 hours before performing the test. The experiment was performed in triplicate. The film treatment which resulted in better solubility was chosen to conduct in the next experiments.

7.2.3.2 Evaluation of the effect of the degree of deacetylation of chitosan and the concentration of chitosan solution on the properties of chitosan film

This experiment was aimed at finding the appropriate degree of deacetylation of chitosan and the concentration of chitosan solution to make chitosan film which has better solubility in hot water.

The chitosan film was made following the procedure in Session 7.2.2.1. Two variants were examined in this experiment:

- Types of chitosan: Chitosan 1 (94 % DD) and chitosan 2 (76 % DD)
- Concentration of chitosan (w/v): 0.5 %, 1 % and 1.5 %

The formed chitosan films were evaluated for their thickness, solubility and mechanical properties. The films were conditioned in desiccators for at least 24 hours before performing the tests. The film which has better solubility and mechanical properties was chosen to conduct the next experiments.

7.2.4 Improvement of the solubility and seal-ability of chitosan films

The chitosan films had poor seal-ability and low solubility in water. Therefore, this study attempted to find another biomaterial to mix with chitosan to improve these properties for application in food casing.

7. 2.4.1 Evaluate the film forming capacity of some biopolymers

The aim of this experiment was to determine the biopolymer which can make sealable and soluble films.

Different concentrations of agar, carrageenan, carboxyl methyl cellulose (CMC), chitosan, gelatin, pectin, and corn starch were examined to make homogeneous biofims which have the desired properties. The chosen concentrations were different depending on the polymer:

- Agar: 1 %, 2 %, and 3 %
- Carrageenan: 2 %, 3 %, and 4 %
- CMC: 1 %, 1.5 %, and 2 %
- Gelatin: 3 %, 4 %, and 5 %
- Pectin: 1 %, 1.5 %, and 2 %
- Corn starch: 1 %, 2 %, and 3 %

The forming films were measured for the following criteria:

- Film thickness
- Solubility in hot water
- Mechanical properties
- Water vapor permeation
- Sealability

The films were conditioned in desiccators for at least 24 hours before performing the tests. The biopolymer which had better sealable and soluble properties would be chosen to use in the next experiments.

7. 2.4.2 Evaluate the effect of blending ratio to the forming film properties

This experiment was aimed at finding the appropriate concentration of chitosan solution and another biopolymer to make composite film which is sealable and soluble in hot water.

The composite film was made following the procedure in Session 6.2.2.2. From the results of the previous experiments, the blending ratio of chitosan and other biopolymer was chosen based on the best concentrations of chitosan and other biopolymer in making films with desired properties. Two variants were examined in this experiment:

- Chitosan ratio: 100 %, 75 %, and 50 % of the best chitosan concentration obtained from Section 7.3.1.2.
- Other biopolymer ratio: 100 %, 80 %, and 60 % of the best concentration of other biopolymer obtained from Section 7.3.2.1.

The formed composite edible films were evaluated their thickness, solubility, mechanical and antimicrobial properties. The composite films were conditioned in desiccators for at least 24 hours before performing the tests. The experiment was carried out in triplicates. The film which had better properties, especially the solubility and sealability was chosen to conduct the next experiments.

7. 2.5 Characterization of the microbiological properties of composite film

The composite film was prepared using the best results from the previous experiments. After that, it was examined for its microbiological properties before application in instant food casing. In this study, the bacterial growth and the viable cell count methods have been used.

For the microbial property characterization, the composite film was cut into 50 mm diameter circular pieces and placed onto the petri dish. After that, the plate count agar medium was poured onto the whole film and allow to settle on top of the film. The plate was incubated at 37 ^{o}C for 24 hours. The number of bacterial colonies formed on top of the film was then counted (Thomas *et al.*, 2009).

For the antimicrobial activity characterization, the viable cell count method was used (Thomas *et al.*, 2009; Zivanovic *et al.*, 2007). Test films of 50-mm diameter were soaked into 9 mL of culture tubes containing approximately 10^6 CFU of the bacterium species *E. coli* (ATCC 25922) or *S. aureus* (ATCC 29213). The tubes were vortexed to make the films contact thoroughly with the media. A tube containing only bacteria culture (without film) was used as the positive control. A tube containing only 0.85 % NaCl was used as the negative control. All tubes were then incubated at 37 ^{o}C for 24 hours. After 2 h, 4 h and 24 h of incubation, a volume of 1 mL of the innoculum was spread onto a PCA petri dish and incubated at 37 ^{o}C overnight. The number of surviving bacteria was determined by counting the colonies formed after incubation.

The percentage reduction of tested bacteria was determined following the below formula (Abou-Zeid *et al.*, 2011):

$$\text{Percentage reduction (\%)} = (C - A) / C \times 100$$

where C: the colonies counted from the plate of the control sample

A: the colonies counted from the plate of the treated sample.

7. 2.6 Application of composite edible films in spice casing for instant noodles

This experiment was aimed at examination of the feasibility of application of the composite edible film in food casing.

The composite edible films with the dimension of 50 x 120 mm were folded and sealed to make sachets with the size 40 x 50 mm. Approximately 1 g of seasoning or oil was put into the sachets and sealed tightly. These sachets were stored at two different conditions to examine their stability: in oriented polypropylene (OPP) wrap and in ambient environment. The OPP wrap is a common material for instant noodles packaging. The PE sachets was used to store the spice instead of composite sachets as a control.

The experiment was carried out within six months. The sachets were evaluated their sensory quality by observation of their appearance. The oil and seasoning powder in each sachet was measured for weight loss and total plate counts after each month of storage. The measurement was conducted in triplicates.

The weight loss of spice sachet during storage was calculated using the following formula:

$$\text{The weight loss (\%)} = (W_i - W_t) / W_i \times 100\%$$

Where W_i: the initial weight of the spice sachet before storage

W_t: the weight of the spice sachet at the time of analysis

The total plate counts was determined as described in Chapter 2 - Session 2.3.7.

7.2.7 Data analysis

The data were analysed by Microsoft Excel 2007 and presented as mean with standard derivation (STD). The SPSS 17.0 programs were used to examine the difference between treatments.

7.3 RESULTS AND DISCUSSION

7.3.1 Film forming capacity of chitosan

7.3.1.1 Effect of the neutralization to the solubility of chitosan films

The solubility of the neutralised and non-neutralised chitosan films were examined to determine which method is suitable for this study. The chitosan films were conditioned in desiccators for at least 24 hours before performing the test. The results were presented in Figure 7.3.

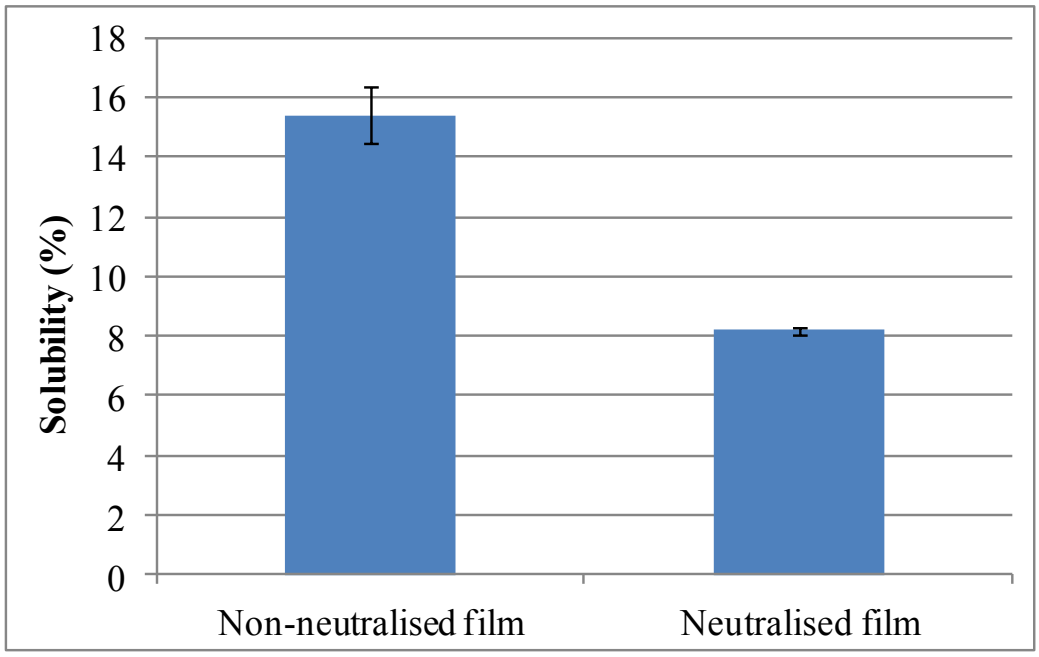


Figure 7.3: The solubility of neutralised and non-neutralised chitosan films

It could be clearly observed from the figure that the solubility of the non-neutralised chitosan (15.42 %) was almost double the solubility of the neutralized one (8.18 %). The difference in the solubility of these films was significant at $p < 0.05$ (Appendix – Chapter 7, Table A.7.2).

The chitosan powder had to be dissolved completely in diluted acetic acid prior casting into chitosan film. In the acidic solution, the $–NH_2$ groups in the chitosan structure were ionized to NH_3^+ groups (Kurita, 2006; Rinaudo, 2006). After casting into film, these cations are still present and easily combine with water. This makes the non-neutralised chitosan more soblule in water.

When the film was neutralized with sodium hydroxide, the NH_3^+ groups were reversed into $-NH_2$ groups. These groups are less active when the chitosan film is soaked in water. This makes the neutralized chitosan film have lower solubility in water than the non-neutralised one.

The aim of this study is to make a film which can dissolve easily in hot water. Therefore, the non-neutralised film was chosen to be processed in the next experiments.

7.3.1.2 Effect of the degree of deacetylation of chitosan and the concentration of chitosan solution on the properties of chitosan film

7.3.1.2.1 Thickness

Six types of chitosan films have been prepared with differences in the degree of the acetylation and the chitosan concentration. The thickness of these films was measured by the Panme rular (Mitutoyo, Japan). The results were presented in Figure 7.4.

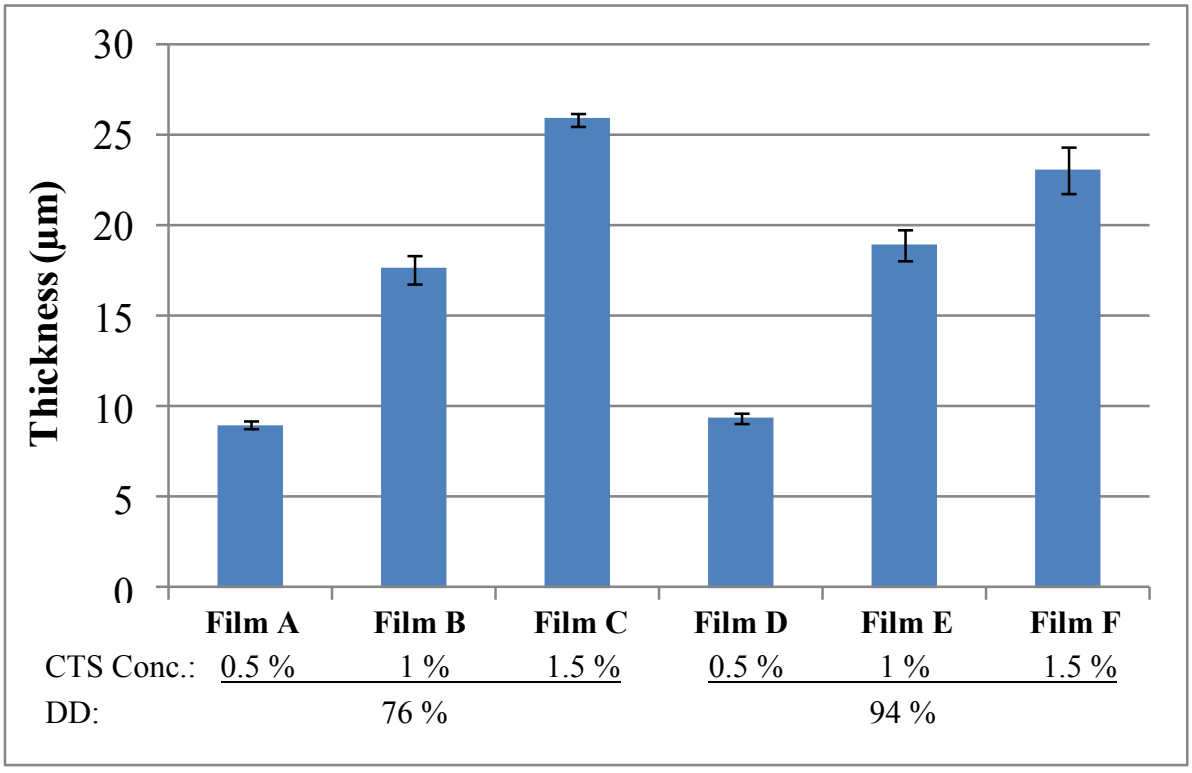


Figure 7.4: The thickness of chitosan films

Note: CTS Conc.: Chitosan concentration

DD: Degree of deacetylation

The results demonstrated that the thickness of chitosan films changed depending on the chitosan concentration. Their thickness increased together with the increase of the chitosan concentration. This result is due to the increase of the chitosan mass in the film. The difference in the thickness of chitosan films with different chitosan concentration was significant at $p < 0.05$ (Appendix - Chapter 7, Table A.7.4).

On the other hand, the degree of deacetylation did not have any effect on the thickness of chitosan films. There was no significant difference at $p < 0.05$ in the thickness of chitosan films made from chitosan with different degree of deacetylation (Appendix - Chapter 7, Table A.7.4).

Therefore, the thickness of chitosan film can be controlled by adjusting the chitosan concentration.

7.3.1.2.2 Solubility

The solubility of chitosan films was presented in Figure 7.5.

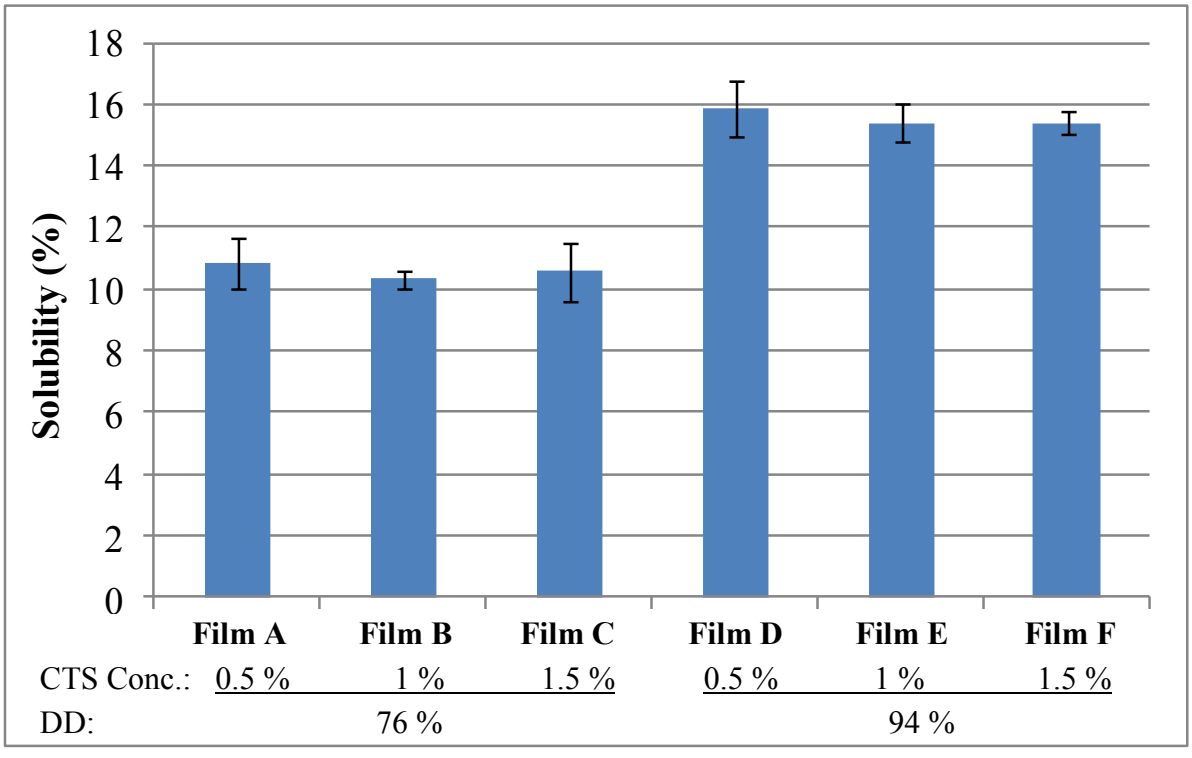


Figure 7.5: The solubility of chitosan films

Note: CTS Conc.: Chitosan concentration

DD: Degree of deacetylation

The solubility of chitosan films was relatively low. The films did not dissolve totally in distilled water. The films mostly absorbed water and swelled.

The obtained data demonstrated that the chitosan concentration did not affect the solubility of chitosan films. There was no significant difference between the films with different concentrations ($p > 0.05$).

Conversely, the degree of deacetylation had a significant effect on the solubility of chitosan films ($p < 0.05$). Chitosan which has higher degree of deacetylation will lead to films which have higher solubility. The results were related to the amino ($-NH_2$) groups on the polymer chain of chitosan (Rinaudo, 2006). Chitosan with a higher degree of deacetylation has more amino groups. When chitosan film is soaked into water, the amino groups will be protonated into $-NH_3^+$ groups. These groups will make hydrogen bonds with the OH^- groups of the water molecules. Therefore, the films can dissolve in water. However, because just a few amino groups was ionised at pH higher than 6, only part of chitosan film is dissolved in water (Fernández-Saiz and Lagaron, 2011; Kurita, 2006).

7.3.1.2.3 Mechanical properties

The tensile strength and the deformation of chitosan films were tested to examine their mechanical properties. The tests were followed the standard DIN EN 527-1 (CEN, 1996) using the Zwick/Roell texture testing machine. The results were presented in Figures 7.6 and 7.7.

The degree of deacetylation of chitosan and the chitosan concentration did not have a large effect on the tensile strength of chitosan films. The tensile strength of chitosan film is approximately 40 MPa. The difference between the films was not significant at $p > 0.05$. This result is close to the study of Butler (1996) which confirmed that the mechanical properties of chitosan film were as strong as films of other commercial polymers such as high density polyethylene (HDPE) and low density polyethylene (LDPE) (Butler *et al.*, 1996; Rabea *et al.*, 2003).

On the other hand, the deformation of chitosan films was affected by the concentration of chitosan. The 0.5 % chitosan concentration films had lowest percentage of deformation (approximately 17 %) which was significantly different to other films at $p < 0.05$. The percentage of deformation of the 1 % and 1.5 % chitosan concentration films were not different at $p < 0.05$. The degree of deacetylation also did not have any effects on the deformation of chitosan films. (Appendix – Chapter 7, Table A.7.8)

The results demonstrated that the 1 % and 1.5 % chitosan concentration films can be stretched longer than 0.5 % chitosan concentration films without changing the tensile strength.

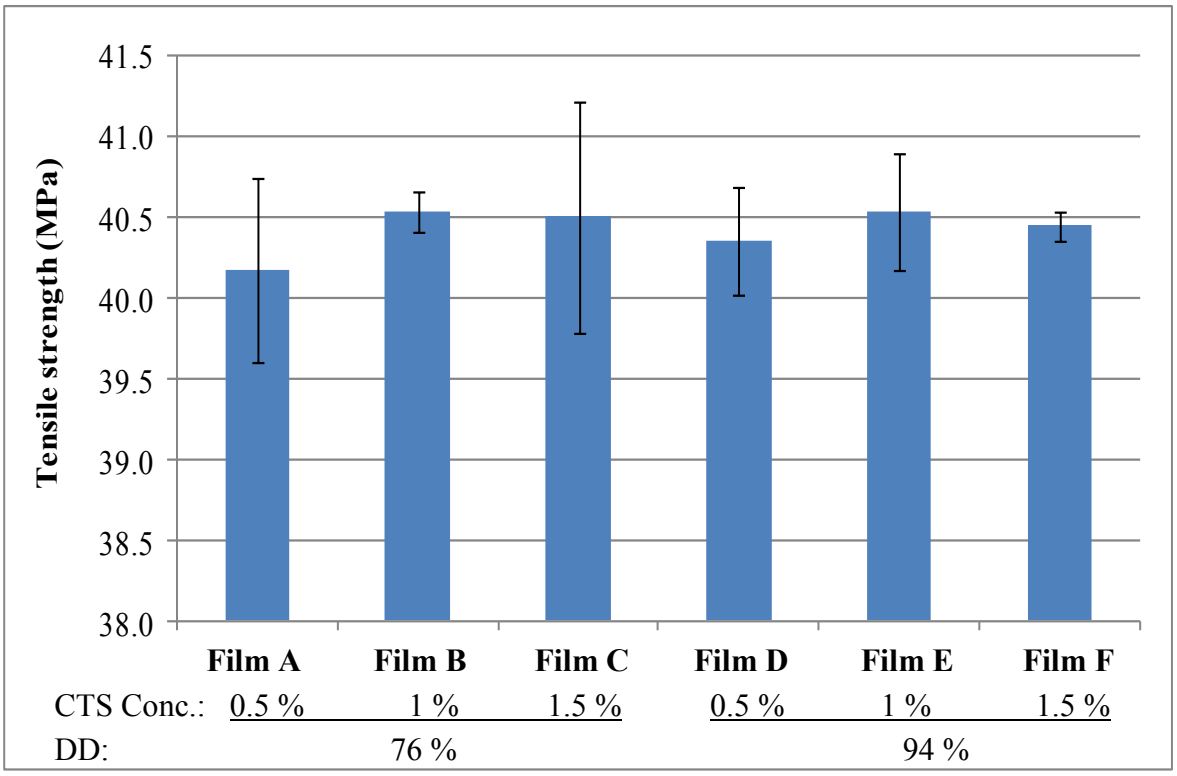


Figure 7.6: The tensile strength of chitosan films

Note: CTS Conc.: Chitosan concentration

DD: Degree of deacetylation

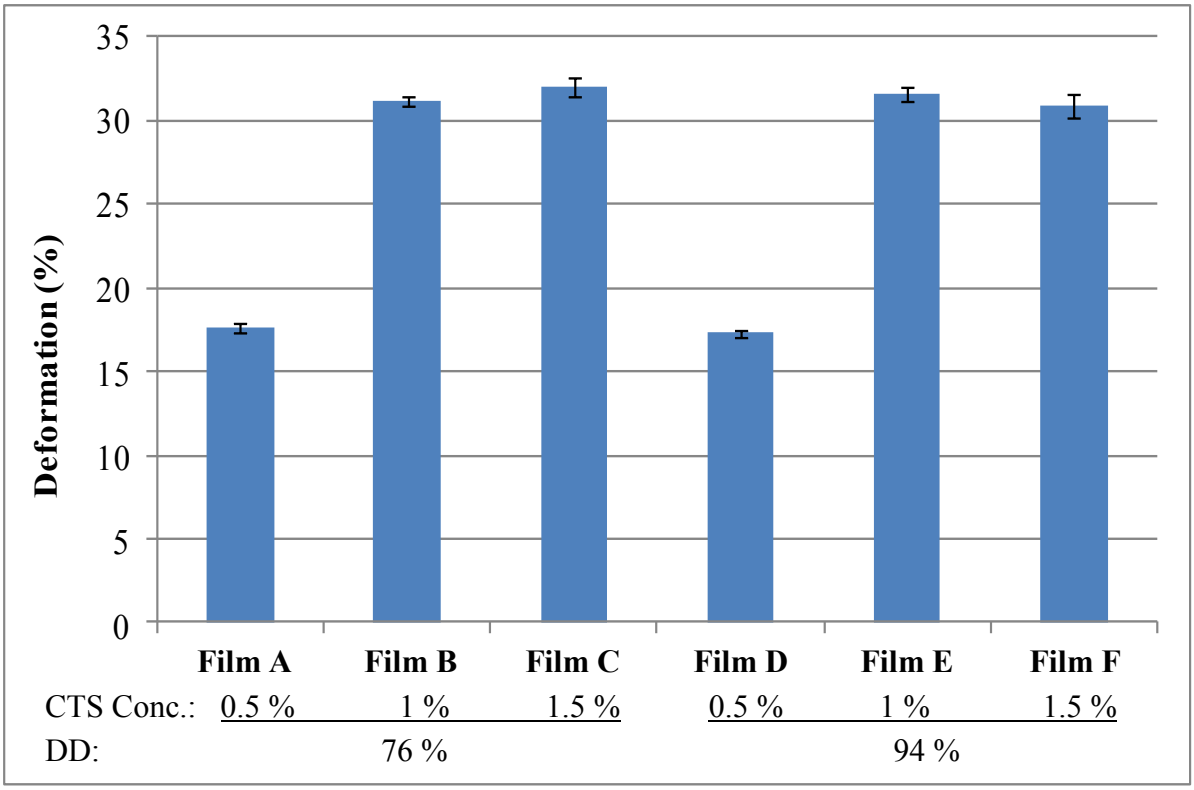


Figure 7.7: The deformation of chitosan films

Note: CTS Conc.: Chitosan concentration

DD: Degree of deacetylation

Overall, the data obtained from this experiment showed that there were no differences between the films made from chitosan with 76 % DD and 94 % DD in terms of thickness, tensile strength and deformation. However, the 94 % DD chitosan film had higher solubility than the 76 % DD chitosan film. This is an important requirement to apply chitosan for packaging in instant food product. Therefore, the 94 % DD chitosan was chosen for usage in the next experiments.

The concentration of chitosan did not have a large effect on the solubility or the tensile strength of chitosan film. However, the 0.5 % chitosan concentration film has very low thickness and deformation. Between the 1 % and 1.5 % chitosan concentration films, the 1 % chitosan concentration film consumed less chitosan but still has similar properties of the 1.5 % chitosan concentration film. Therefore, the 1 % chitosan concentration film was chosen to conduct the next experiments.

7.3.2 Improvement of the solubility and seal-ability of chitosan films

Chitosan is a very promising materials for apllication in the food industry due to its biodegradable and non-toxic properties. However, the main dissadvantage of chitoasn film is its low solubility. Therefore, chitosan needs to be formulated with other biopolymers to enhance its solubility as well as sealability to make it feasible for application in instant food casing.

Some biopolymers were investigated for film forming properties. The biomaterials which can make the films with high solubility and sealability will be chosen to mix with chitosan. The concentration of each biopolymer chosen for examination varied depending on its intrinsic film-forming properties and the results of previous studies (Chen *et al.*, 1996; Cheng *et al.*, 2003; De Yao *et al.*, 1996; Hambleton *et al.*, 2009; Jo *et al.*, 2005; Phan *et al.*, 2005; Tharanathan, 2003).

7.3.2.1 Evaluate the film forming capacity of some biopolymers

7.3.2.1.1 Thickness

The thickness of some homogenous edible films was presented in Figure 6.8. For each type of edible film, ten measurements have been conducted. The measurements were repeated three times. The data were presented as the mean and the standard deviation of the three replications (Appendix - Chapter 7, Table A.7.9).

The results in Figure 6.8 indicated that the thickness of edible films made from a biopolymer with different concentration was different significantly at $p < 0.05$ (Appendix A.7.10). The thickness of edible film increased when the concentration of biopolymer increased. Among seven biololymers, gelatin film had the highest thickness. This is obvious because of the high gelatin concentration used to cast the film. Comparing with other edible films with the same concentration, the thickness of chitosan film was similar to the pectin and CMC films and much lower than corn starch and agar films.

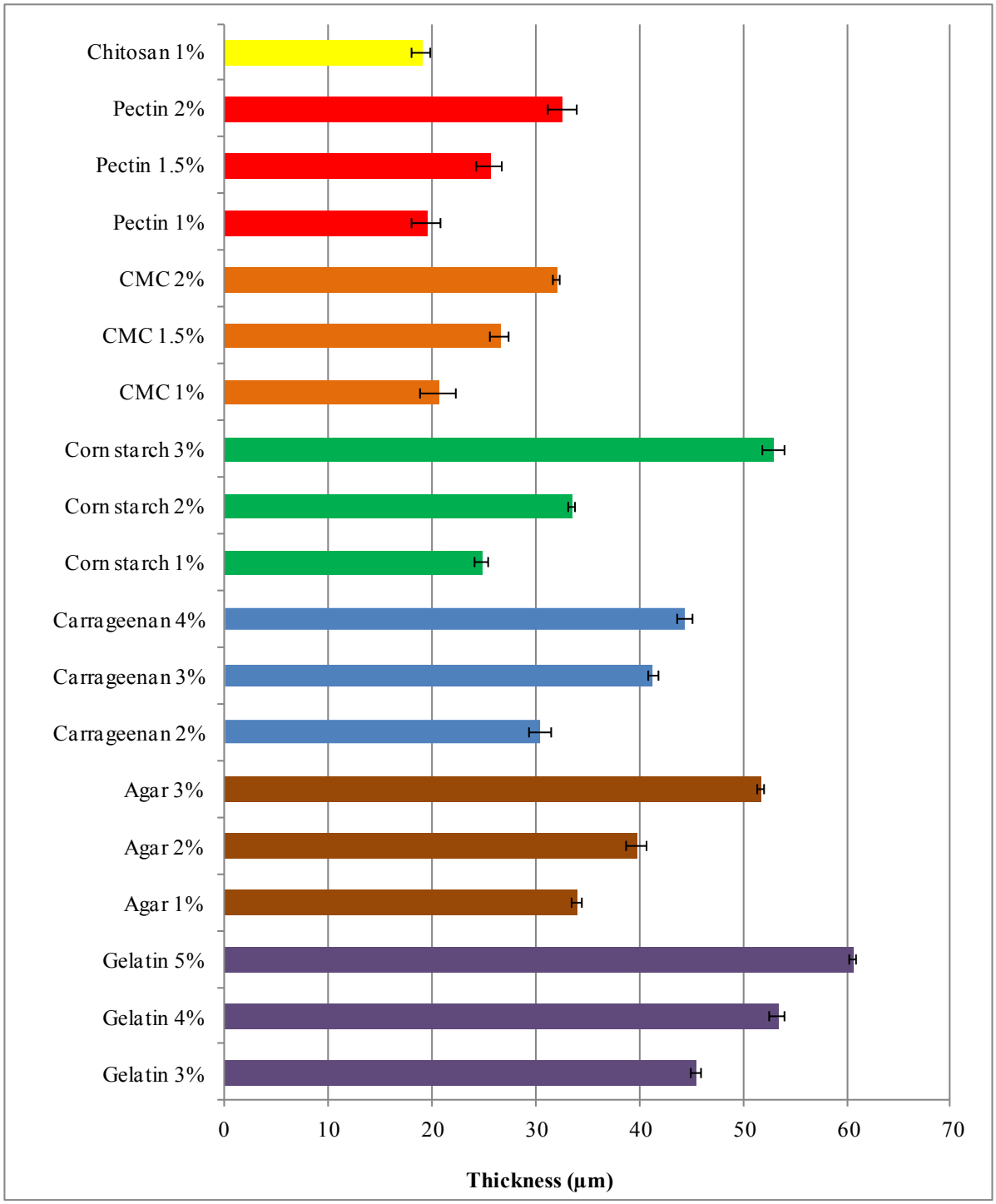


Figure 7.8: The thickness of homogenous edible films

7.3.2.1.2 Solubility

The solubility of homogeneous edible films was measured in triplicate. The results were presented in Figure 7.9. The solubility of selected edible films was significantly higher than that of the chitosan film.

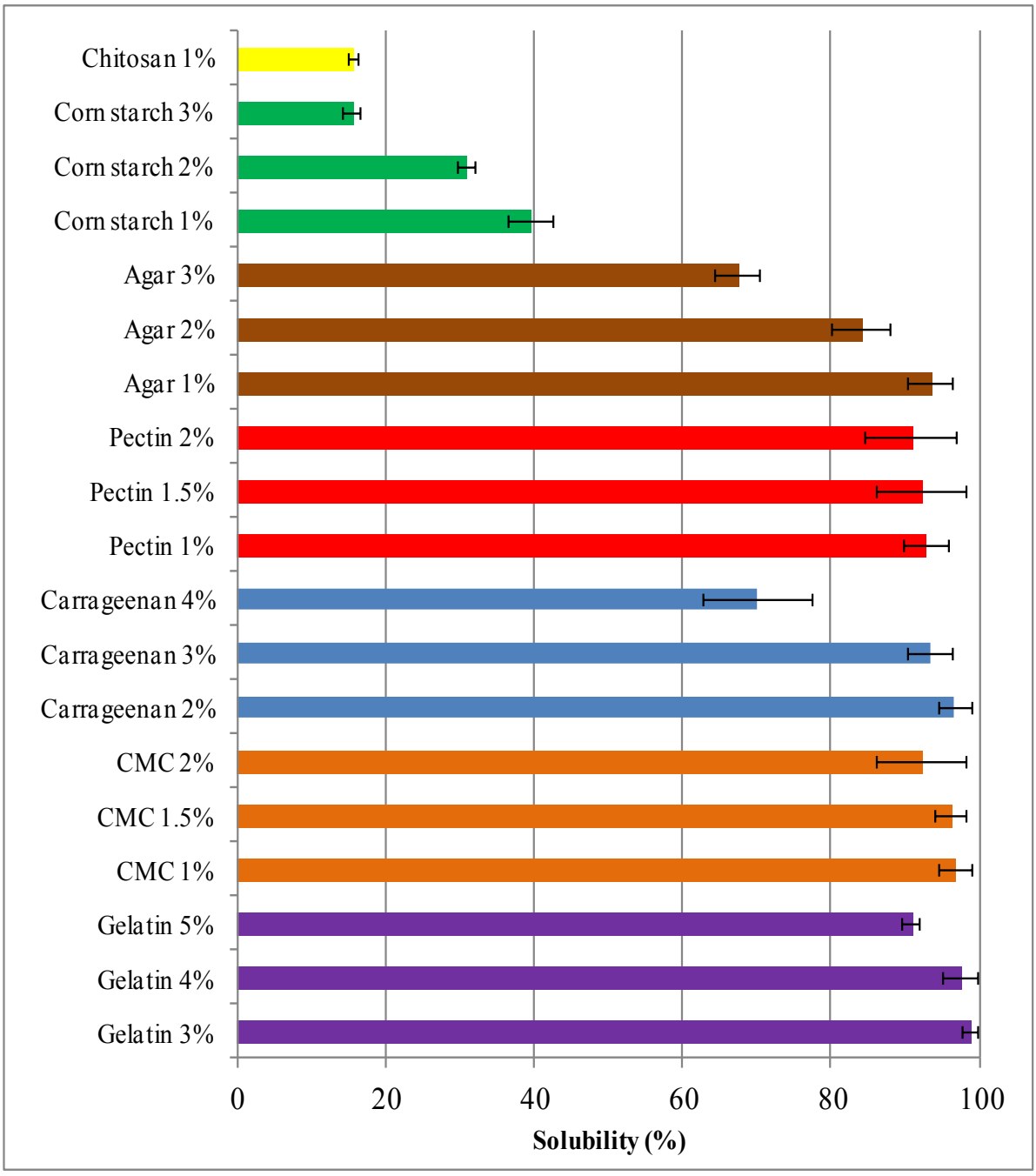


Figure 7.9: The solubility of homogenous edible films

The results showed that the solubility of chitosan and corn starch was very low (< 40%) compared to other biopolymers. The difference was significant at $p < 0.05$ (Appendix - Chapter 7, Table A.7.12). Most of the corn starch film was not dissolved in hot water but only

absorbed water and swelled to make gel. The film which had higher starch concentration had lower solubility which was significantly different at $p < 0.05$.

Agar, pectin, carrageenan, CMC and gelatin had high solubility in the hot water. They dissolved more than 70 % in water at 70 ^{o}C for 5 minutes. This may be due to their hydrophylic properties. As the melting point of gelatin is just at the body temperature (Achet and He, 1995), it can dissolve easily in hot water.

Overall, their solubility decreased in reverse proportion to their concentrations. The solubility of agar, carrageenan and gelatin films was higher at lower polymer concentration. The difference was significant at $p < 0.05$.

However, the solubility of pectin and CMC films at different biopolymer concentrations was not different at $p < 0.05$. They can dissolve more than 90 % in hot water.

7.3.2.1.3 Mechanical properties

The tensile strength and deformation properties of edible films was tested by a texture machine (Zwich/Roell, Germany) to evaluate their mechanical propeties. The speed of pulling force was 100 mm.min^{-1}. The results wre presented in Figure 7.10 and Figure 7.11.

7.3.2.1.3.1 Tensile strength

In this experiment, the tensile strength of edible films fluctuated depending on the concentration of each biopolymer.

The tensile strength of corn starch film increased when the concentration increased from 1 % to 2 %. However, when the concentration of corn starch was raised to 3 %, the tensile strength slightly decreased. Conversely, the tensile strength of carrageenan film and CMC film decreased and then increased when the concentration of these biopolymers increased.

The tensile strength of pectin and agar film increased proportional to their concentration while the tensile strength of gelatin film seemed unchanged with the change of gelatin concentration. This could be because there was the presence of the plasticizer glycerol which enhances the mechanical property of the edible film. Chitosan film had rather high tensile strength compared to other films. The difference was significant at $p < 0.05$ (Appendix - Chapter 7, Table A.7.14).

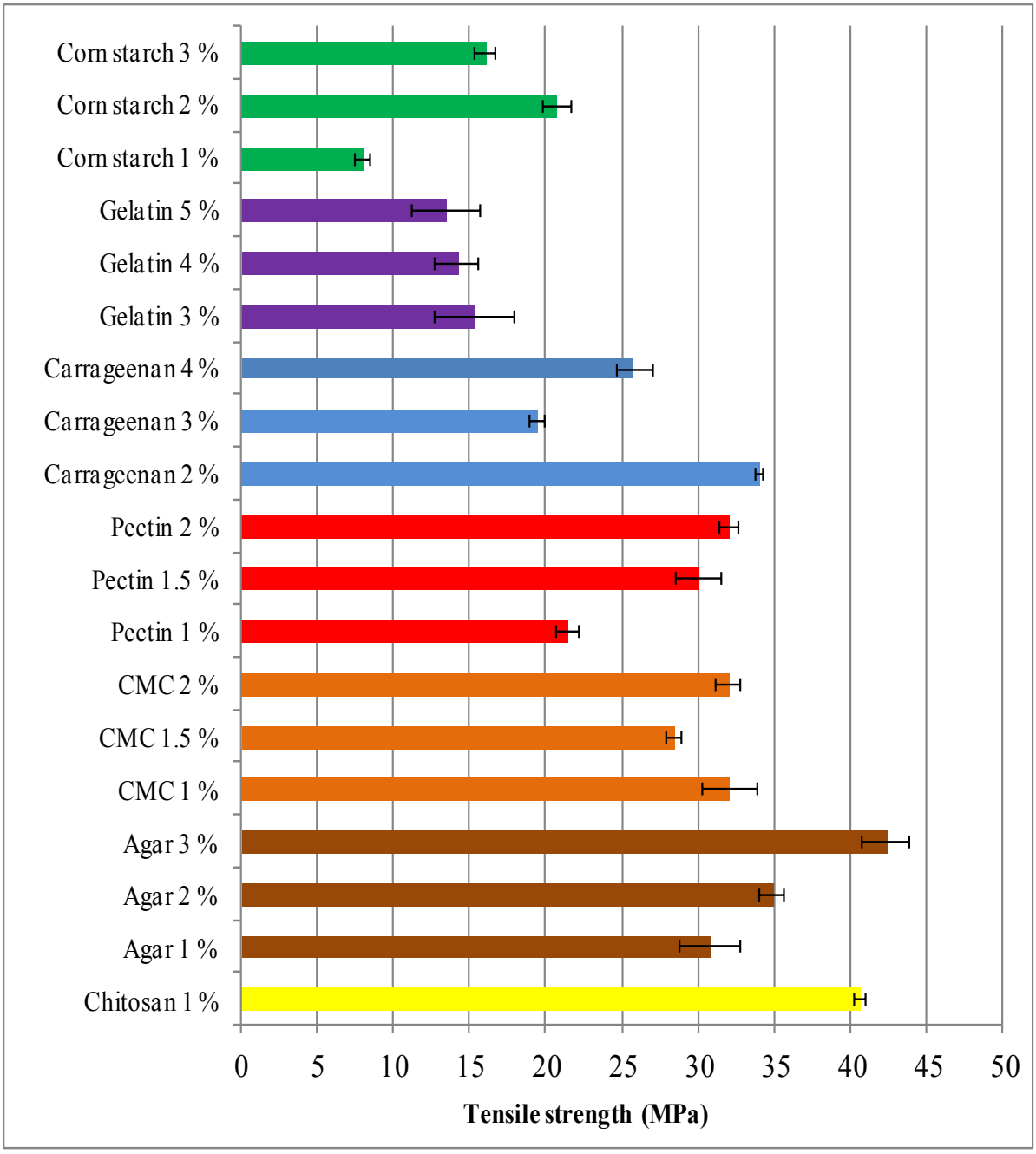


Figure 7.10: The tensile strength of homogenous edible films

7.3.2.1.3.2 Deformation

The deformation of edible films was presented in Figure 7.11. Overall, the deformation of edible films increased with the increase of their concentrations, except pectin and CMC films. The percentages of deformation of edible films with different concentrations were significantly different at $p < 0.05$ (Appendix - Chapter 7, Table A.7.14).

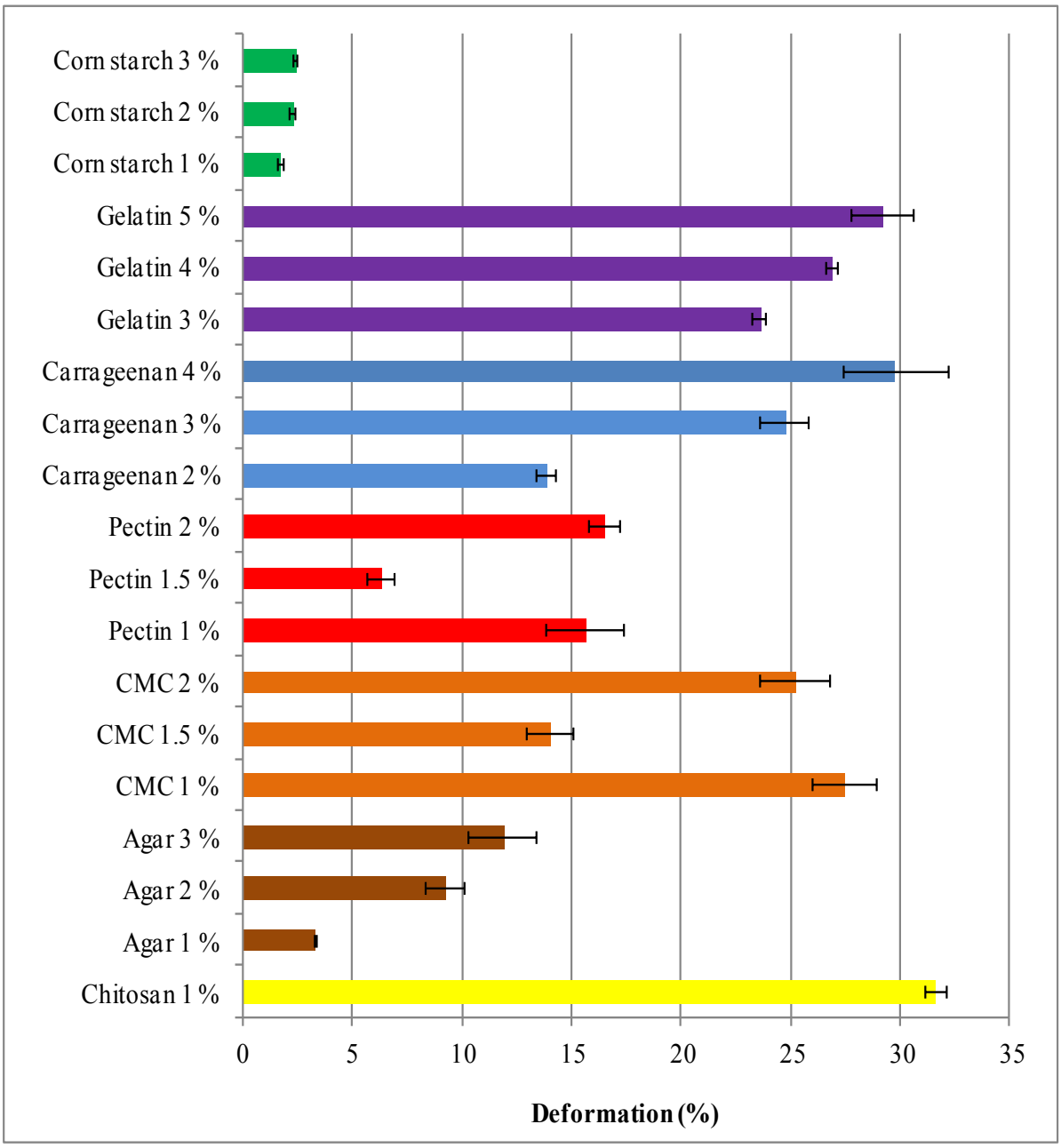


Figure 7.11: The deformation of homogenous edible films

The deformation of chitosan films (31.58 %) was rather high compared to other edible films while the corn starch film had the lowest percentage of deformation (1.64 % – 2.33 %). Not only the corn starch but the cassava starch, rice starch and sticky rice starch also make films which have very low percentage of deformation (2.64 %, 2.07 %, and 0.72 %, respectively) (Phan *et al.*, 2005). Most of the films made from starch are very brittle.

7.3.2.1.4 Water vapor permeation (WVP)

The WVP of homogenous edible films was measured at 25 ^{o}C in an environment with the water activity changed from 0.22 to 0.84. The low density polyethylene (LDPE) film was measured as the control. The experiment was conducted in triplicate. The average results were presented in Figure 7.12.

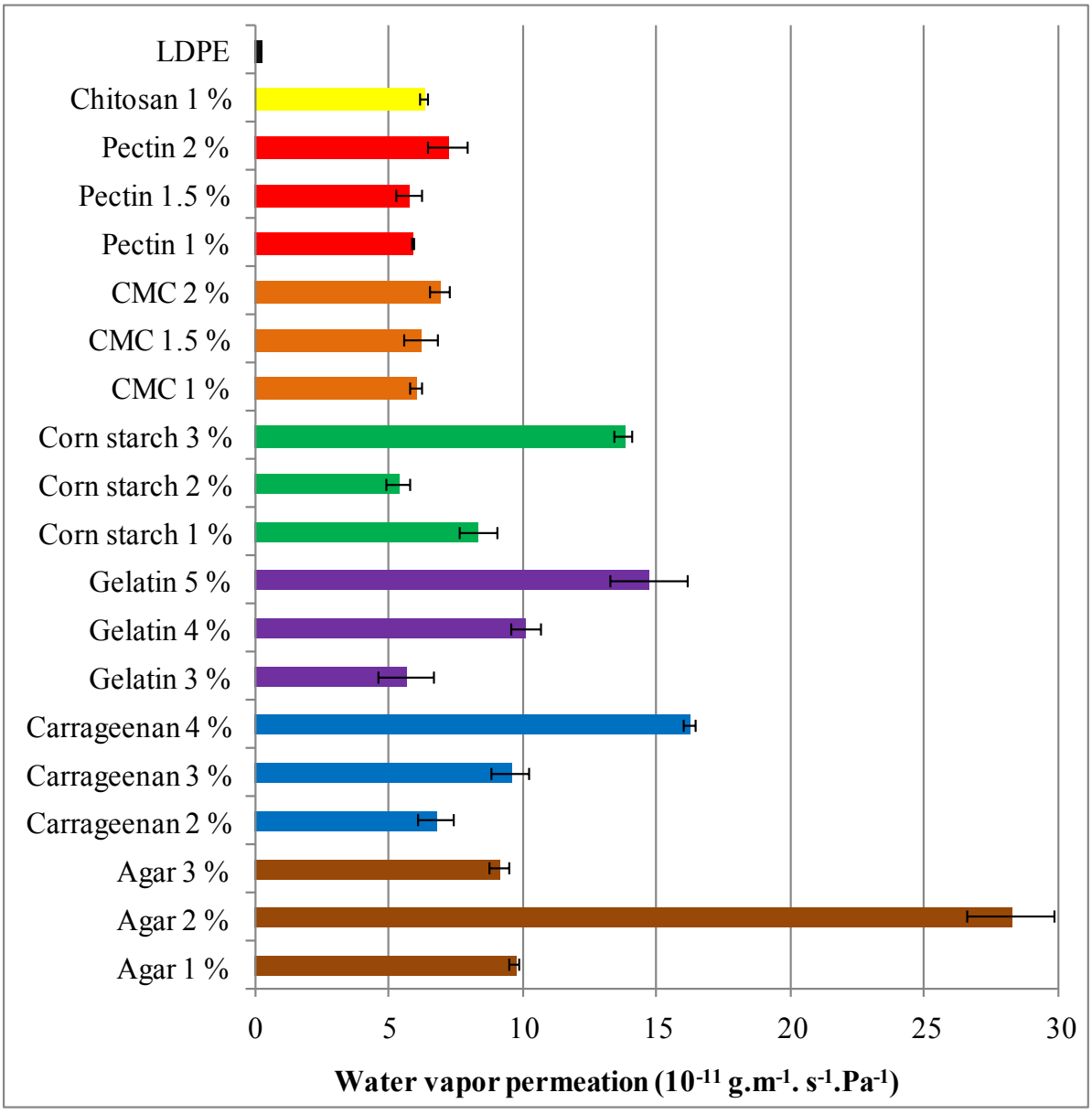


Figure 7.12: The water vapor permeation of homogenous edible films

The concentration of biomaterials had marked effect to the WVP of edible films. The films which had higher biomass concentration had higher WVP.

The 2 % agar film had high water vapor permeation (28.2 x 10^{-11}g.m^{-1}. s^{-1}.Pa^{-1}) which was significantly different to other concentrations at $p < 0.05$ (Appendix - Chapter 7, Table A.7.16). The 3 % corn starch film, 5 % gelatin film and 4 % carrageenan film had medium WVP (around 15 x 10^{-11}g.m^{-1}. s^{-1}.Pa^{-1}) while the other films had lower WVP (< 10 x 10^{-11}g.m^{-1}. s^{-1}.Pa^{-1}).

Chitosan film is among the films which has low WVP. This property of chitosan film make it feasible for application in food packaging to protect the food from the outer environment.

The WVP of all examined biofims were higher than the LDPE film. This result showed that these edible films can transfer a certain amount of water through the film after a period of time. This is due to the hydrophylic property of biomaterials. Especially gelatin is a strong polar protein, therefore it can transfer water which is also a polar subsstance (Miller and Krochta, 1997).

On the other hand, the WVP of edible films also increase when the concentration of plasticizer increases (Bertuzzi *et al.*, 2007; Park and Chinnan, 1995). The incorporation of plasticizer into a polymer matrix can enhance the flexibility of the polymeric chain, therefore the edible film can transfer moisture easier (Arvanitoyannis and Biliaderis, 1998, 1999; Trommsdorff and Tomka, 1995).

7.3.2.1.5 Sealability

The film was sealed at 100 ^{o}C for 15 seconds. The testing samples were conditioned for 24 hours at room temperature before measuring the tensile strength of the seal. The sealability was estimated based on the tensile strength of the seal that makes the film separated or broken.

The results showed that carrageenan, chitosan, and corn starch films cannot be sealed. The agar, CMC and pectin films can be sealed, but the seals were too weak to be measured because they were separated before the force reached the threshold value (F_{Min} = 0,1 N).

Among the investigated edible films, only the gelatin films can be sealable. The film melted very quickly at 100 ^{o}C. The films were very dry and britlle at the seal position of 3 % and 4 % gelatin films. So, they were torn at the side of the seal before the force reached the threshold value.

Only the seal of the 5 % gelatin film can be measured for its mechanical properties by the texture machine (Zwich/Roell, Germany). It had the tensile strength of 1.61 MPa with 10.55 % deformation (Appendix - Chapter 7, Table A.7.17). Most seals were not separated but torn at the side of the seal due to the changes of the thickness of the film during the sealing process.

In general, the results from this experiment showed that only the 5% gelatin can fullfil the requirements for application in food casing of instant noodles:
- The sealablity: The film can be made into small sachets which are tight, stable and can protect an amount of seasoning or oil.
- The solubility: The film can dissolve in hot water. Therefore, the spice sachet can dissolve by itself within 3-5 minutes.

Therefore, the 5 % gelatin was chosen for combination with chitosan to make spice sachets for instant noodles.

7.3.2.2 The effect of blending ratio to the forming film properties

From the results of previous experiments, gelatin was chosen to mix with chitosan to enhance the sealbility and solubility of edible films for application in food casing of instant noodles. Different ratio of gelatin solution (at 100 %, 80 % and 60 % of the 5 % gelatin solution) and chitosan solution (at 100 %, 75 % and 50 % of the 1 % chitosan solution) were blended together to examine which ratio was appropriate to make composite film with the required properties. The ratio of gelatin and chitosan were converted to the concentration of gelatin and chitosan in the film-forming solution. The blending concentration of gelatin and chitosan were described in Table 7.1.

The experiment was designed randomly with 2 factors and 3 replications. Chitosan solutions were mixed thoroughly with gelatin solutions by a homogenizer. A volume of 50 mL of the blending solution was casted onto a flat glass tray to make edible film. After drying at 40 ^{o}C for 24 hours, these films were taken off the trays and stored in desiccators for at least 24 hours before analysis of their properties.

Table 7.1 The concentration of gelatin and chitosan in film-forming solution for composite edible films

	Concentration (%, w/v) of gelatin	Concentration (%, w/v) of chitosan
Film 1	3	0.50
Film 2	3	0.75
Film 3	3	1.00
Film 4	4	0.50
Film 5	4	0.75
Film 6	4	1.00
Film 7	5	0.50
Film 8	5	0.75
Film 9	5	1.00

7.3.2.2.1 Thickness

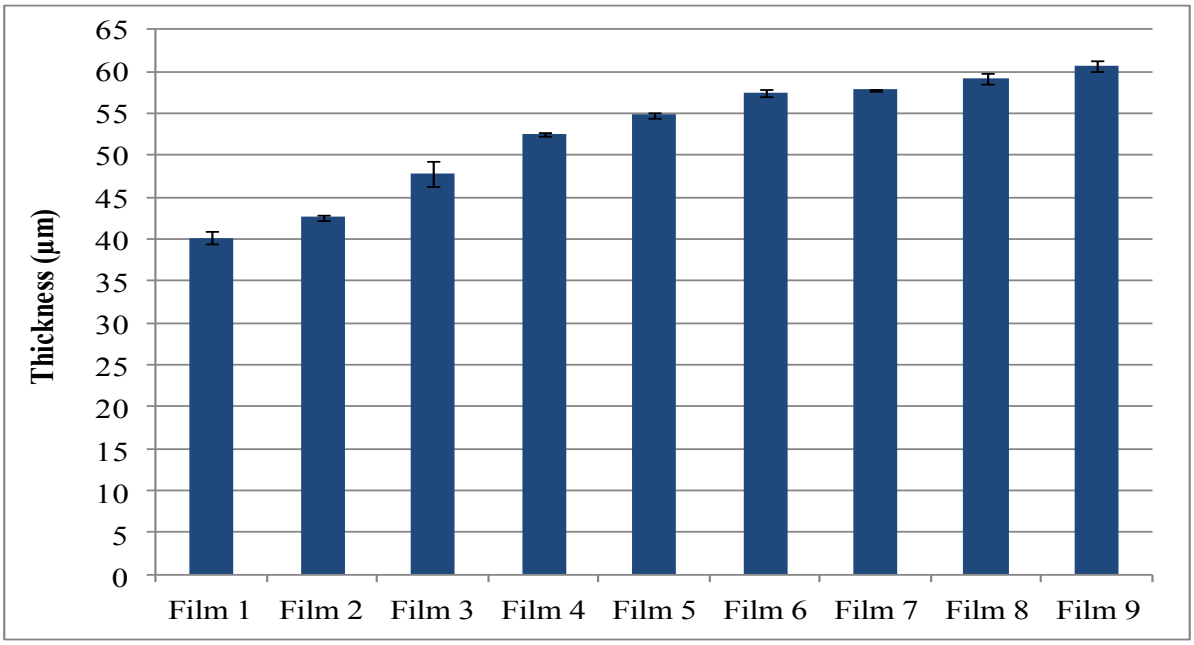


Figure 7.13: The thickness of chitosan and gelatin composite films

The thickness of the composite film was presented in Figure 7.13. Each film was measured 10 times for each replication. Three replications were conducted.

The thickness of edible films increased proportionally to the biomass of the film-forming solution. Film 1 (0.5 % chitosan, 3 % gelatin) had the lowest thickness (40.18 µm) due to its lowest biomass content while film 9 (1 % chitosan, 5 % gelatin) had the highest thickness (60.70 µm) due to its highest biomass content. The difference of the thickness of these films was significant at $p < 0.05$ (Appendix - Chapter 7, Table A.7.19).

The statistical analysis showed that both chitosan concentration and gelatin concentration had obvious effects on the thickness of edible films (Appendix - Chapter 7, Table A.7.19). At the same chitosan concentration, the thickness of the composite film increased when the gelatin concentration increased. Similarly, at the same gelatin concentration, the thickness of the composite film increased when the chitosan concentration increased.

7.3.2.2.2 Solubility

The solubility of each composite film was measured three times. The average and its standard derivation were presented in Figure 7.14.

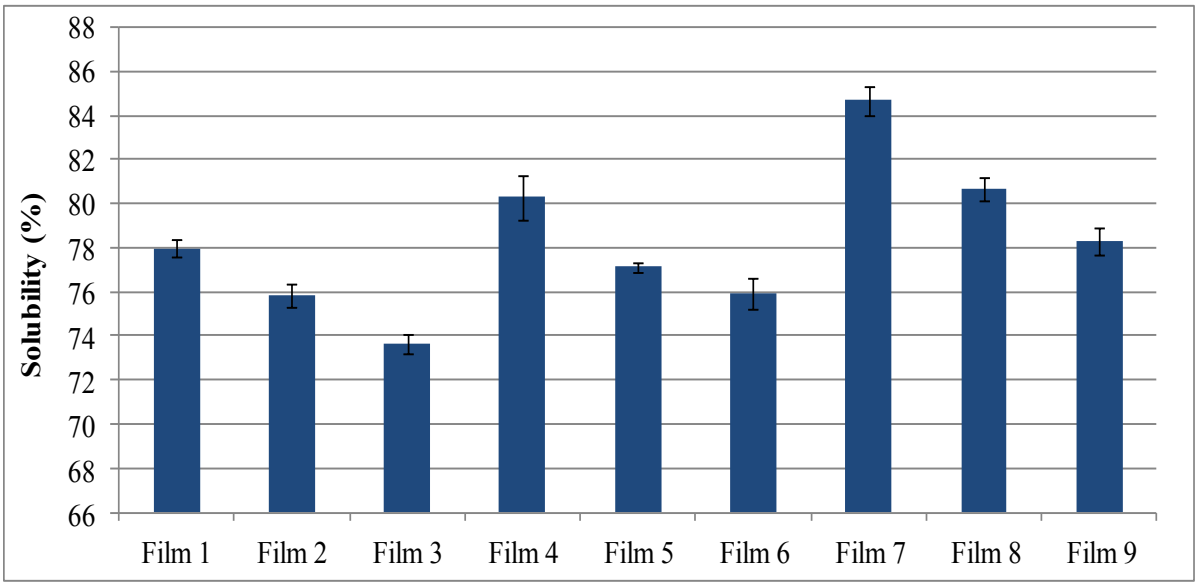


Figure 7.14: The solubility of chitosan and gelatin composite films

The result showed that the solubility of composite film changed with the change of concentration of gelatin and chitosan in the film forming solution.

The film which had a higher gelatin concentration had better solubility. Film 7 had higher solubility (84.69 %) than film 4 (80.29 %) and film 1 (77.98 %). The difference was significant at $p < 0.05$ (Appendix - Chapter 7, Table A.7.21)

Conversely, the film which had a higher chitosan concentration had lower solubility. Film 3 had lower solubility (73.67 %) than film 2 (75.86 %) and film 1 (77.98 %). The difference was significant at $p < 0.05$ (Appendix - Chapter 7, Table A.7.21).

Comparing the solubility between composite film and homogeneous chitosan film, the composite film had distinctly higher solubility than the chitosan film (15.41 %). This result demonstrated that the combination of gelatin has successfully increased the solubility of the edible film.

7.3.2.2.3 Mechanical properties

7.3.2.2.3.1 Tensile strength

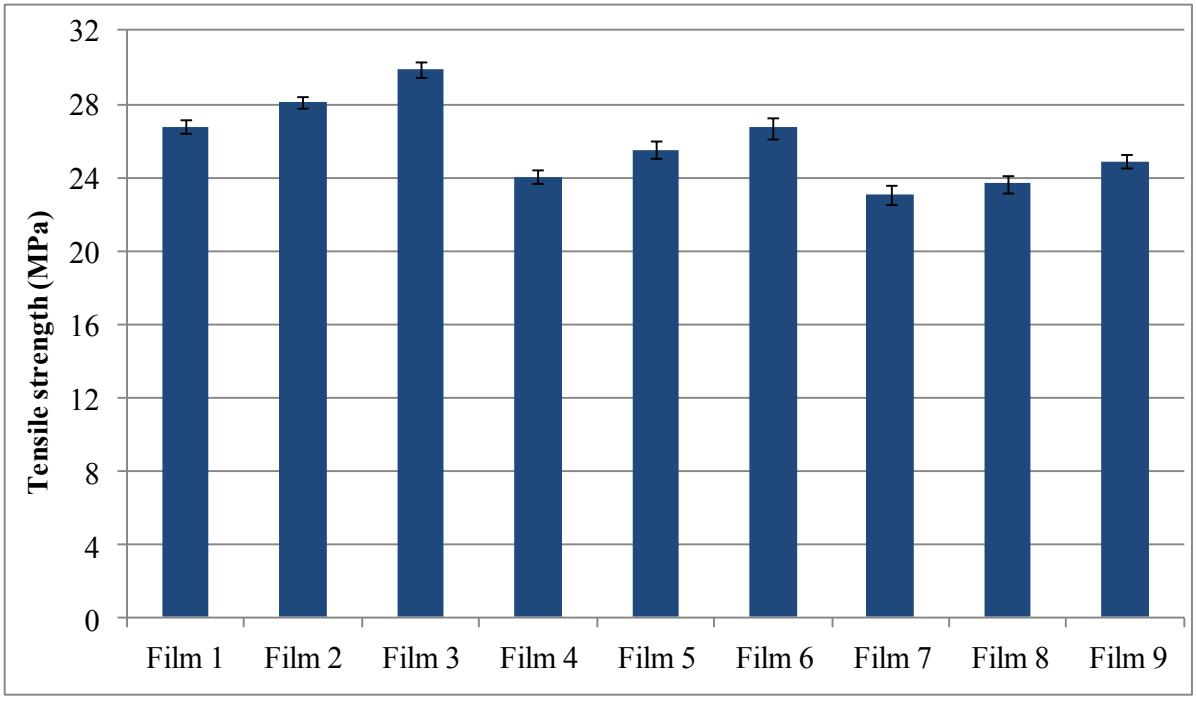


Figure 7.15: The tensile strength of chitosan and gelatin composite films

The results in Figure 7.15 showed that the concentration of chitosan and gelatin affected significantly the tensile strength of the composite film at $p < 0.05$ (Appendix - Chapter 7, Table A.7.23).

At the same chitosan concentration, the increase of gelatin concentration will lead to a film with lower tensile strength. For example, the tensile strength of composite film decreased respectively from 26.78 MPa (film1) to 24.05 MPa (film 4) and 23.03 (film 7).

On the other hand, at the same gelatin concentration, the increase of chitosan concentration will lead to a film with higher tensile strength. For example, the tensile strength of composite film increased respectively from 26.78 MPa (film1) to 28.11 MPa (film 2) and 29.88 MPa (film 7).

The tensile strength of the composite film (29.88 MPa) was higher than the homogeneous gelatin film (15.25 MPa) but lower than the chitosan film (40.54 MPa). The results could be due to the intrinsic properties of gelatin and chitosan. Gelatin is a protein which can easily absorb water, while chitosan, the polysaccharide, is more resistent to water. The combination of these two polymers produces a film which has the average tensile strength. Moreover, the addition of plasticizer glycerol also affected the tensile strength of the edible film (Butler *et al.*, 1996). The tensile strength of edible film can raise up to 50 % when added 30 % of plasticizer (Arvanitoyannis *et al.*, 1998).

7.3.2.2.3.2 Deformation

The deformation of the composite films was presented in Figure 7.16.

The results showed that the concentration of chitosan and gelatin also affected significantly the deformation of the composite film at $p < 0.05$ (Appendix - Chapter 7, Table A.7.23). The deformation increased with the increasing concentration of gelatin but it decreased with the increasing concentration of chitosan.

Among examined films, film 4, film 7, film 8 and film 9 had the highest percentage of deformation.

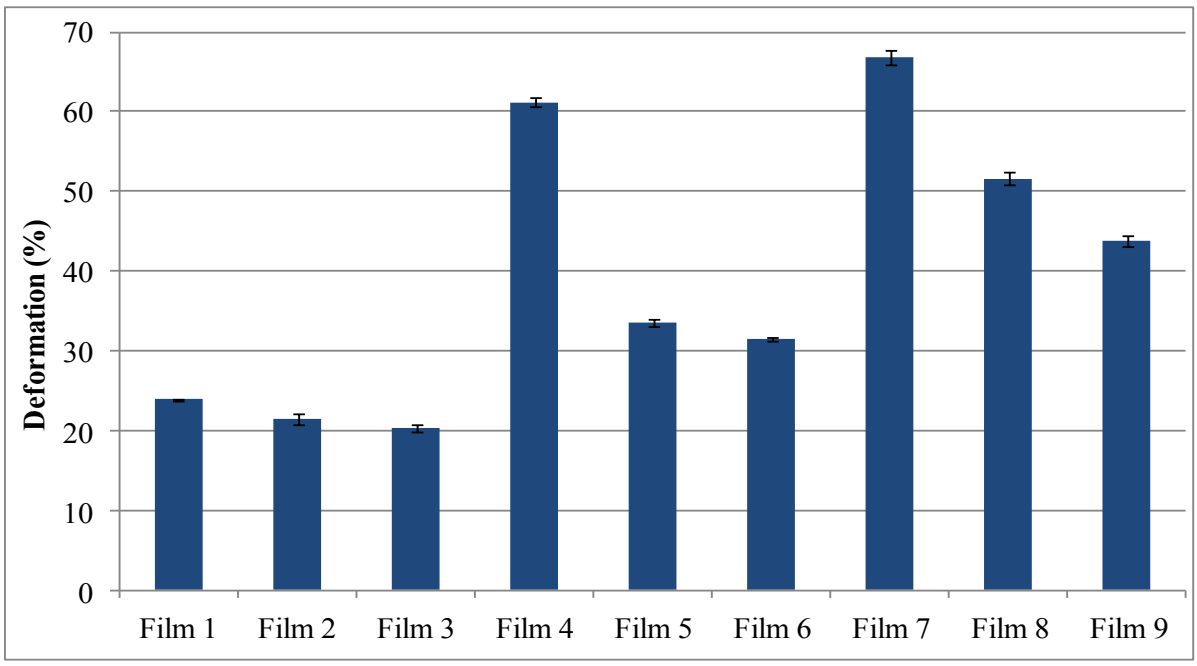


Figure 7.16: The deformation of chitosan and gelatin composite films

7.3.2.2.4 Water vapor permeation (WVP)

The WVP of composite edible films was measured at 25 ^{o}C in an environment with the water activity changed from 0.22 to 0.84. The LDPE film was measured as the control. The experiment was conducted in triplicate. The average results were presented in Figure 7.17.

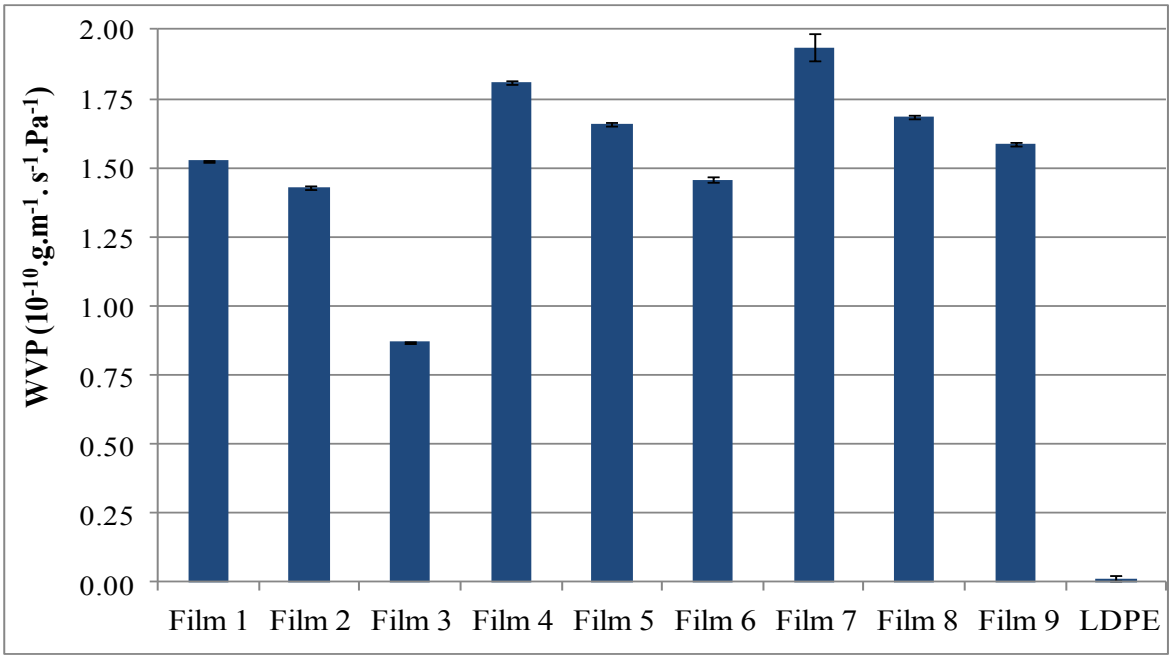


Figure 7.17: The water vapor permeation of composite edible films

The WVP of the composite edible films was higher than the homogeneous edible films and the LDPE film. It increased proportionally to the ratio of gelatin and inversely proportionally to the ratio of chitosan. The difference was significant at $p < 0.05$ (Appendix - Chapter 7, Table A.7.25). Film 7 (4 % gelatin and 0.5 % chitosan) had the highest WVP among the examined films.

This result is due to the hydrophylic properties of gelatin film (Gómez-Guillén *et al.*, 2011). Moreover, the presence of glycerol as a plasticizer increases the transportation of water molecules (Park *et al.*, 1994).

7.3.2.2.5 Sealability

In this experiment, the sealing temperature and time were reduced to enhance the tensile strength of the seal. The composited films were sealed at 90^{o}C for 10 seconds. The testing samples were conditioned for 24 hours at room temperature before measuring the tensile strength of the seal. The sealability was estimated based on the tensile strength of the seal. The results were presented in Figure 7.18.

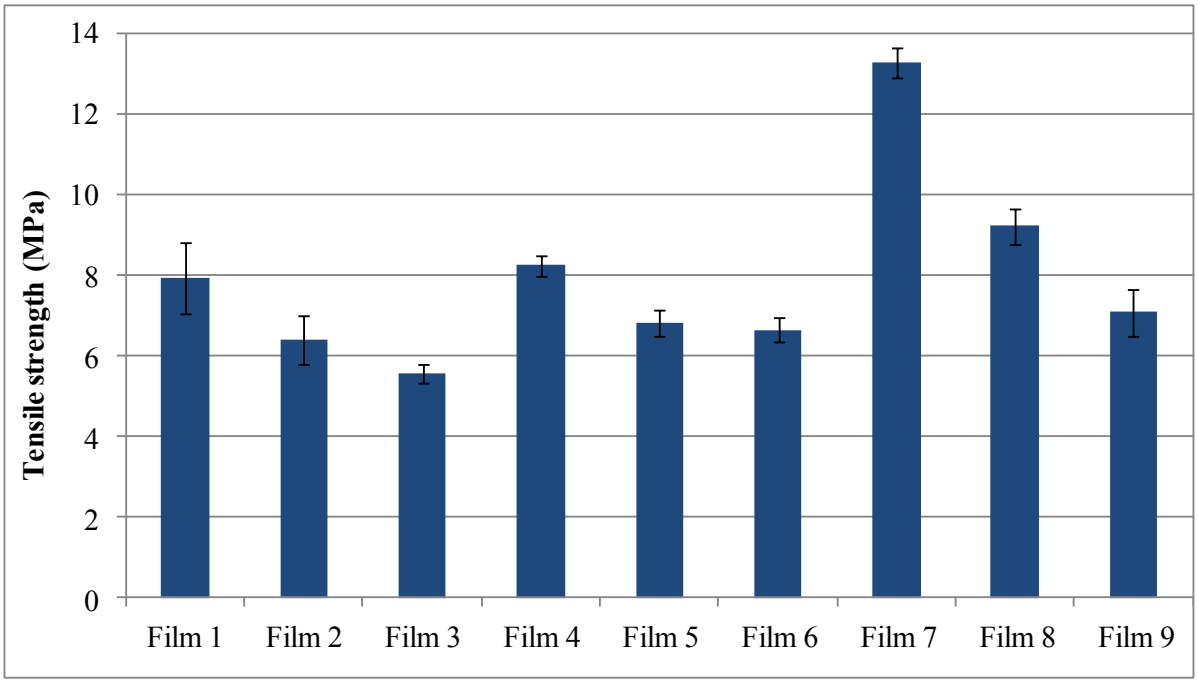


Figure 7.18: The sealability of composite edible films

The results showed that the sealability of the composite films improved markedly over the homogeneous films. The tensile strength increased from 1.61 MPa (gelatin film) to more than

6 MPa (composite film). Most seals were not separated but torn at the side of the seal due to the changes of the film thickness during the sealing process.

At lower blending ratio of gelatin (60 % and 80 %), there was no significant difference among the tensile strength of the composite films at $p < 0.05$ (Appendix - Chapter 7, Table A.7.27). However, the tensile strength increased sharply when the film was formed by 100 % gelatin and 50 % chitosan (film 7). The tensile strength of the seal decreased when the blending ratio of chitosan increased.

Throughout this experiment, the results showed that the properties of the composite edible films have been enhanced markedly compare to the homogeneous edible films.

Among the examined films, film 7 has better properties than other composite films:

- The thickness is around 58 μm. This film thickness is enough to make a small bag to contain the seasoning and oil of the instant food product.

- The solubility is the highest (approximately 85 %). This is one of the most important criteria to choose the film for application in instant noodles food casing because it need to dissolve in hot water as soon as possible.

- The tensile strength (23 MPa) is not so high comparing to other composite films. However, it is better than the gelatin homogenous film and strong enough to store the food inside its bag. Moreover, its high percentage of deformation (67 %) is an advantage for this film to dissolve in hot water.

- The water vapor permeation of this film is the highest (1.94×10^{-10} $g.m^{-1}.s^{-1}.Pa^{-1}$). This property is really a disadvantage of this edible film in the application in food casing because it cannot prevent the transfer of moisture between the food and the outer environment. However, in this study, edible film is aimed to make small bags for the storage of the spice (seasoning and oil) of instant noodles. These small bags are covered by an OPP bag which has better moisture resistance. Therefore, this disadvantageous property of the film is not too much of a concern.

- The sealability of this film is very good. The seal has the highest tensile strength (13.27 MPa) comparing to other composite films. This property is an advantage for the edible film application in food casing.

Due to its excellent properties, film 7 was chosen for application in making spice sachets for instant noodles. This film is formed by a combination of 5 % (w/v) gelatin concentration and 0.5 % (w/v) chitosan concentration in the film-forming solution.

7.3.3 Characterization of properties of composite edible film

The best results from previous experiments were chosen to formulate an edible film which can dissolve in hot water and has good sealablity for application to instant food casing. The film was prepared and examined for its physical-chemical properties and microbiological properties before application in instant noodle spice casing.

7.3.3.1 Preparation of the composite edible film

The composite edible film used in this study was formed with the following formulation:

- Chitosan concentration: 0.5 % (w/v)
- Gelatin concentration: 5 % (w/v)
- Glycerol concentration: 10 % (w/w, dry matter)

To prepare the composite edible film, chitosan solution and gelatin solution were prepared separately before mixing them together. An amount of 0.25 g of chitosan was dissolved in 25 mL of 1.5 % acetic acid solution by shaking overnight. The pH of the chitosan solution was 3.8. An amount of 2.5 g of gelatin was dissolved in 25 mL of distilled water by heating to 100 ^{o}C for 1 minute with stirring. The pH of the gelatin solution was 5.5. Chitosan solution and gelatin solution was mixed together and 0.275 g glycerol added as a plasticizer.

The blending solution was homogenized for 5 minutes to mix all the components thoroughly. It was then filtered through a Whatman filter paper to remove all insoluble particles. The solution was degassed by heating to 100 ^{o}C on a hot-plate magnetic-stirrer device with gentle stirring to form a clear solution. The resulting film forming solution had the pH of 4.1 which was then raised to pH 6.0 by adding 1 N NaOH solution.

The film forming solution was poured carefully onto a casting tray with the dimension of 20 x 20 cm so that no bubble was formed. It was settled flatly in the drying machine. It was dried at 40 ^{o}C for 24 hours. The forming film was peeled off the tray and stored in a PE bag in desiccators for at least 24 hours prior analysis.

The physical-chemical properties of the composite edible film are summarized in Table 7.2. The formed composite film had high solubility. Its mechanical properties was strong enough to make edible bags for storage of a small amount of food. The high sealablity of this film makes it feasible for application in food casing.

Table 7.2: The physical-chemical properties of the composite edible film

No.	Parameters	Value
1	Thickness	60 μm
2	Moisture content	10 %
3	Solubility	85 %
4	Tensile strength	23 MPa
5	Percentage of deformation	67 %
6	Water vapor permeation	1.94×10^{-10} $g.m^{-1}. s^{-1}.Pa^{-1}$
7	Tensile strength of the seal	13.27 MPa

7.3.3.2 Microbiological properties of the composite edible film

The microbiological properties of composite chitosan/gelatin film were examined followed modified method from previous studies (Abou-Zeid *et al.*, 2011; Li *et al.*, 2010; Thomas *et al.*, 2009; Zivanovic *et al.*, 2007).

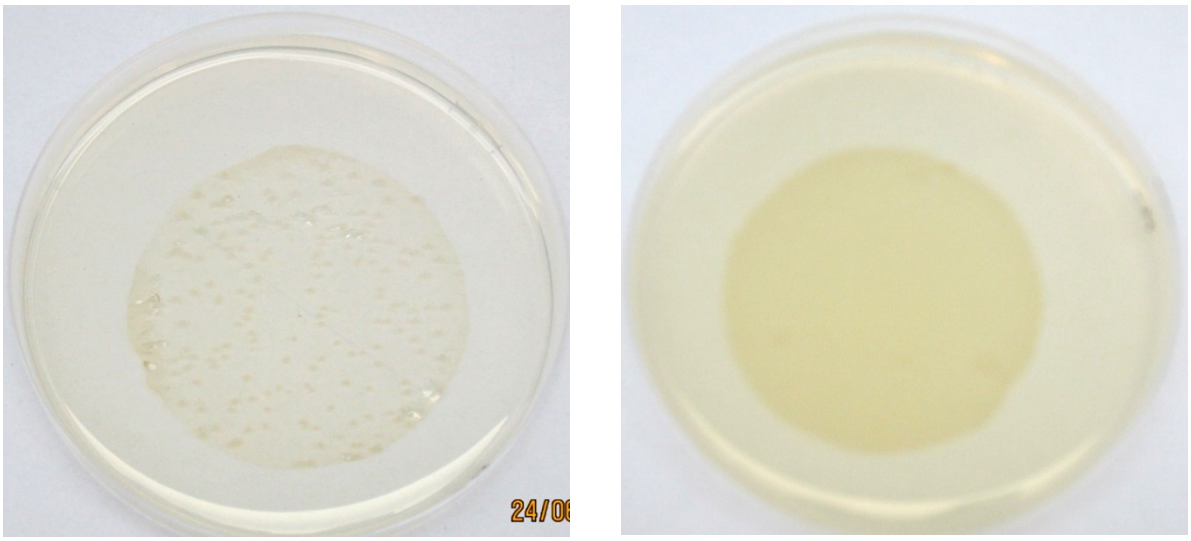
The bacterial growth on the composite edible film was examined 3 months after casting. The 1 % chitosan film was used as the control. The results are presented in Table 7.3 and Figure 7.19. The obtained data showed that the composite film had better microbial properties than the homogeneous chitosan film. There were no bacteria on the petri dish containing the composite chitosan/gelatin film, while there are bacteria on the chitosan film.

When casting into a membrane, chitosan may lose its effectiveness that it has in its solution form because it can only exhibit its antibacterial activity in acidic conditions (Fernández-Saiz and Lagaron, 2011; Rabea *et al.*, 2003). Possitive charges on the chitosan molecule are lost when the pH of the film forming solution is increased to pH 6. Moreover, some charges are involved in gel forming when casted into film. This is the reason why chitosan film loses its

antimicrobial activity. This result indicated that the combination of gelatin and chitosan can enhance the microbial inhibition ability of the chitosan-based edible film.

Table 7.3: The microbial activity of the composite edible film

Sample	Replication	CFU
Chitosan film	1	185
	2	174
	3	92
Composite film	1	0
	2	0
	3	0



(a) chitosan film (b) composite film

Figure 7.19: Bacterial growth in PCA petri dishes containing edible films

The antimicrobial activity of the composite edible film was determined by the viable cell count method. The films were soaked in bacteial suspensions of *E. coli* (ATCC 25922) or *S. aureus* (ATCC 29213). The viable cells were enumerated by incubating 1 mL of the suspension on the plate count agar petri dishes after 2 hours, 4 hours and 24 hours of immersion for 24 hours at 37 ^{o}C. The results were presented in Table 7.4 and Table 7.5.

Table 7.4: Effect of the edible films on the survival of *E. coli* (ATCC 25922) (CFU)

	Replication	0 h	2 h	4 h	24 h
Chitosan film	1	1.1×10^7	73	22	20
	2	1.1×10^7	71	20	0
	3	1.1×10^7	67	30	0
Composite film	1	1.1×10^7	0	0	0
	2	1.1×10^7	0	0	0
	3	1.1×10^7	0	0	0
Positive control (without film)		1.1×10^7	1.6×10^7	1.1×10^8	1.8×10^7

Table 7.5: The effect of the edible films on the survival *S. aureus* (ATCC 29213) (CFU)

	Replication	0 h	2 h	4 h	24 h
Chitosan film	1	2.3×10^6	1465	180	20
	2	2.3×10^6	0	0	0
	3	2.3×10^6	0	0	0
Composite film	1	2.3×10^6	0	0	0
	2	2.3×10^6	393	245	14
	3	2.3×10^6	416	335	8
Positive control (without film)		2.3×10^6	2.5×10^6	1.1×10^7	1.0×10^7

The percentage reduction (%) of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 was calculated based on the viable cell counts. The results were presented in Table 7.6.

Table 7.6: Effect of the edible films on the percentage reduction (%) of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213

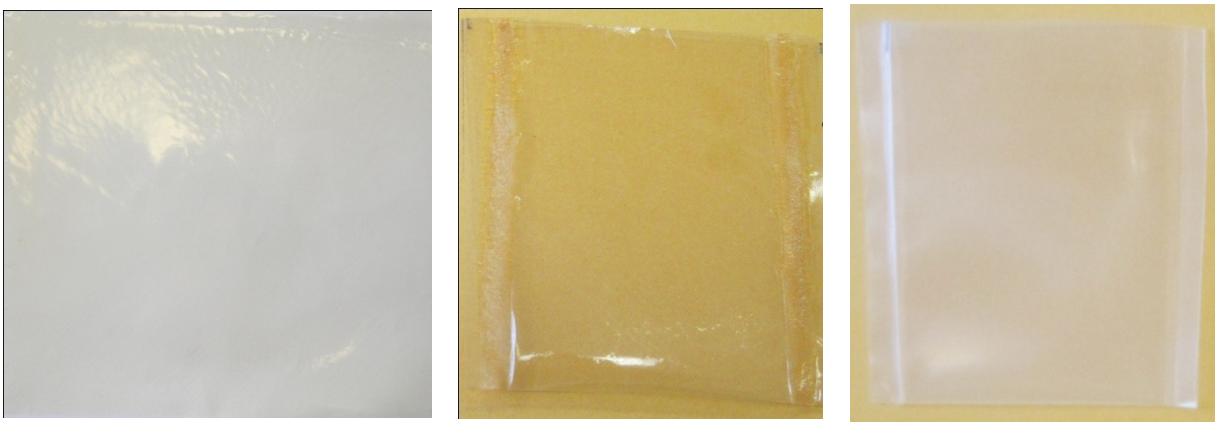
	Sample	2 h	4 h	24 h
E. coli ATCC 25922	Chitosan film	99.9994 ± 0.0000	99.9998 ± 0.0000	99.9999 ± 0.0001
	Composite film	100.0000 ± 0.0000	100.0000 ± 0.0000	100.0000 ± 0.0000
	Positive control	-46.5021	-895.8848	-69.5473
S. aureus ATCC 29213	Chitosan film	99.9968 ± 0.0000	99.9989 ± 0.0000	99.9999 ± 0.0000
	Composite film	100.0000 ± 0.0000	100.0000 ± 0.0000	100.0000 ± 0.0000
	Positive control	-5.9387	54.5977	57.4713

The results showed that most bacteria were killed only after 2 hours of incubation with the edible films. The composite film exhibited 100 % of its effectiveness on the inhibition of the bacterial growth to both bacteria while the chitosan film seemed to have more effect on the Gram-negative bacterium.

Other researches also found that when chitosan is blended with other substances such as polyethylene oxide, cellulose acetate or nanosilver, the antibacterial activity of the film increased significantly (Abou-Zeid *et al.*, 2011; Thomas *et al.*, 2009; Zivanovic *et al.*, 2007).

7.3.4 Application of composite edible films in spice casing for instant noodles

The composite edible films were applied to spice casing for instant noodles to examine their effectiveness for practical application. Small sachets with the dimension of approximately 40 x 50 mm were prepared for the containing of seasoning powder and vegetable oil (Figure 7.20).



Composite edible film | Edible film sachet | PE sachet

Figure 7.20: Composite edible film and small sachets ready for spice storage

Approximately 1 g of seasoning powder or 1 mL of vegetable oil was put into each sachet. The sachets were sealed tightly at 90 ^{o}C for 10 seconds to prevent the loss of spice. The PE sachets were used as the control to determine the stability of the edible film sachets.

The spice sachets were stored 6 months at ambient environment and in OPP wrap to investigate the effect of the storage condition to the stability of the edible film sachets. The oil and seasoning in each bag were evaluated for their sensory properties and had their weight loss and total plate counts measured after each month.

7.3.4.1 Sensory evaluation

The spice sachets were evaluated for their sensory properties before and during 6 months of storage. The evaluation was carried out by observation the status of the sachets and the inside seasoning powder and vegetable oil. The observation was recorded in Table 7.7 and Table 7.8.

Table 7.7: Sensory evaluation of seasoning sachets during 6 months of storage

Sample	Status of sachets	Status of seasoning
Before storage		
A, B, C, D	Dry, tight	Dry, yellowish, good smell
After 1 month of storage		
A, B, C, D	Dry, tight	Dry, yellowish, good smell
After 2 months of storage		
A, B, C, D	Dry, tight	Dry, yellowish, good smell
After 3 months of storage		
A, B	Dry, tight	Dry, faint yellowish, good smell
C, D	Dry, tight	Dry, yellowish, good smell
After 4 months of storage		
A	Dry, tight, slightly soft	Dry, white, good smell
B	Dry, tight	Dry, white, good smell
C, D	Dry, tight	Dry, yellowish, good smell
After 5 months of storage		
A	Dry, tight, slightly soft	Slighly wet, white, good smell
B	Dry, tight	Dry, white, good smell
C, D	Dry, tight	Dry, yellowish, good smell
After 6 months of storage		
A	Dry, tight, soft, elastic	Wet, white, good smell
B	Dry, tight	Slighly wet, white, good smell
C	Dry, tight, slightly soft	Dry, yellowish, good smell
D	Dry, tight	Dry, yellowish, good smell

Note: A: Edible film sachets - Ambient environment C: Edible film sachets - OPP wrapped
B: PE sachets - Ambient environment D: PE sachets - OPP wrapped

Table 7.8: Sensory evaluation of oil sachets during 6 months of storage

Sample	Status of sachets	Status of oil
Before storage		
A, B, C, D	Dry, tight	Clear, good smell
After 1 month of storage		
A, B, C, D	Dry, tight	Clear, good smell
After 2 months of storage		
A, C	Little leakage at the seal	Clear, good smell
B, D	Dry, tight	Clear, good smell
After 3 months of storage		
A, C	Little leakage at the seal	Clear, good smell
B, D	Dry, tight	Clear, good smell
After 4 months of storage		
A, C	Little leakage at the seal	Clear, good smell
B, D	Dry, tight	Clear, good smell
After 5 months of storage		
A	Dry, tight, slightly soft and elastic, little leakage at the seal	Clear, good smell
B, C	Dry, tight, little leakage at the seal	Clear, good smell
D	Dry, tight	Clear, good smell
After 6 months of storage		
A	Dry, tight, slightly soft and elastic, little leakage at the seal	Clear, good smell
B, C	Dry, tight, little leakage at the seal	Clear, good smell
D	Dry, tight	Clear, good smell

Note: A: Edible film sachets - Ambient environment *C: Edible film sachets - OPP wrapped*
B: PE sachets - Ambient environment *D: PE sachets - OPP wrapped*

The results showed that during 6 months of storage, the composite edible film sachets still retained their original shape no matter whether they were being stored in another packaging such as OPP or in contact directly with the ambient environment.

The seasoning sachets showed no leakage during storage. The seal was still in a good position without any detachment. However, the edible film sachets which were stored in the ambient environment started to get wet after 5 months of storage. This could be because this composite edible film can absorb water from the environment. The edible film changed its properties remarkably after 6 months of storage. It was wet, soft and elastic that can not be torn by hands.

There was no change in the appearance of the seasoning powder in both PE bags and edible film sachets which were covered by OPP wraps. However, a significant change in the color of the seasoning powder which was let in the ambient environment was recognized after 3 months of storage. The yellow color of the seasoning powder faded gradually over time. After 4 months, the yellow color was disappeared totally and the seasoning powder had a white color. This could be due to the oxidation of active substances in the seasoning powder such as iodine.

For the preservation of oil, the oil samples still retained their quality during 6 months of storage without any changes in its color and smell. However, there was leakage of oil in the edible film sachets at the sealing lines starting from the second month of storage although there was no detachment at the seal of the sachets. This may due to the high sealing temperature that caused the films to be thinner at the sealing lines and allowed the oil to pass through.

After 5 months of storage, there was a significant difference in the sensory properties between oil sachets under different storage conditions. Only the PE sachets kept in OPP wrap did not show the leakage of oil. The edible film sachets exposed to ambient environment turned soft and elastic, so they can not be torn by hand.

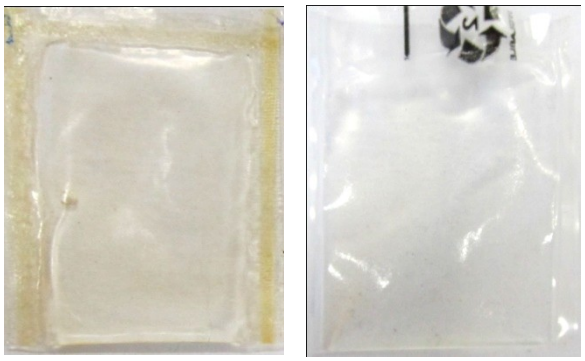
Spice sachets before storage

Seasoning bio-bag | Seasoning PE bag | Oil bio-bag | Oil PE bag

Spice sachets after 6 months of storage

Ambient environment

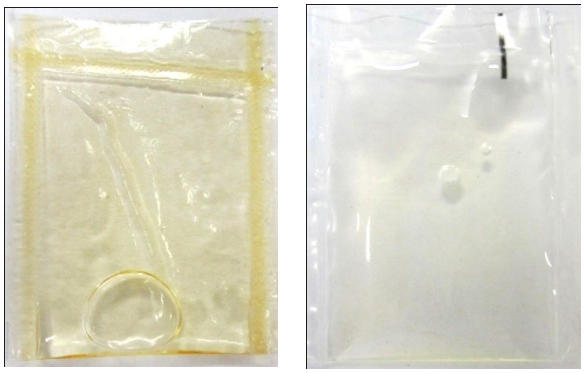
Seasoning bio-bag | Seasoning PE bag



Oil bio-bag | Oil PE bag

OPP wrapped

Seasoning bio-bag | Seasoning PE bag



Oil bio-bag | Oil PE bag

Figure 7.21: Seasoning and oil sachets before and after 6 months of storage

7.3.4.2 Weight loss

The weight of seasoning sachets and oil sachets were recorded before storage and at each testing time. From these data, the percentage weight loss of seasoning sachets and oil sachets were calculated. The results were presented in Figure 7.22 and Figure 7.23.

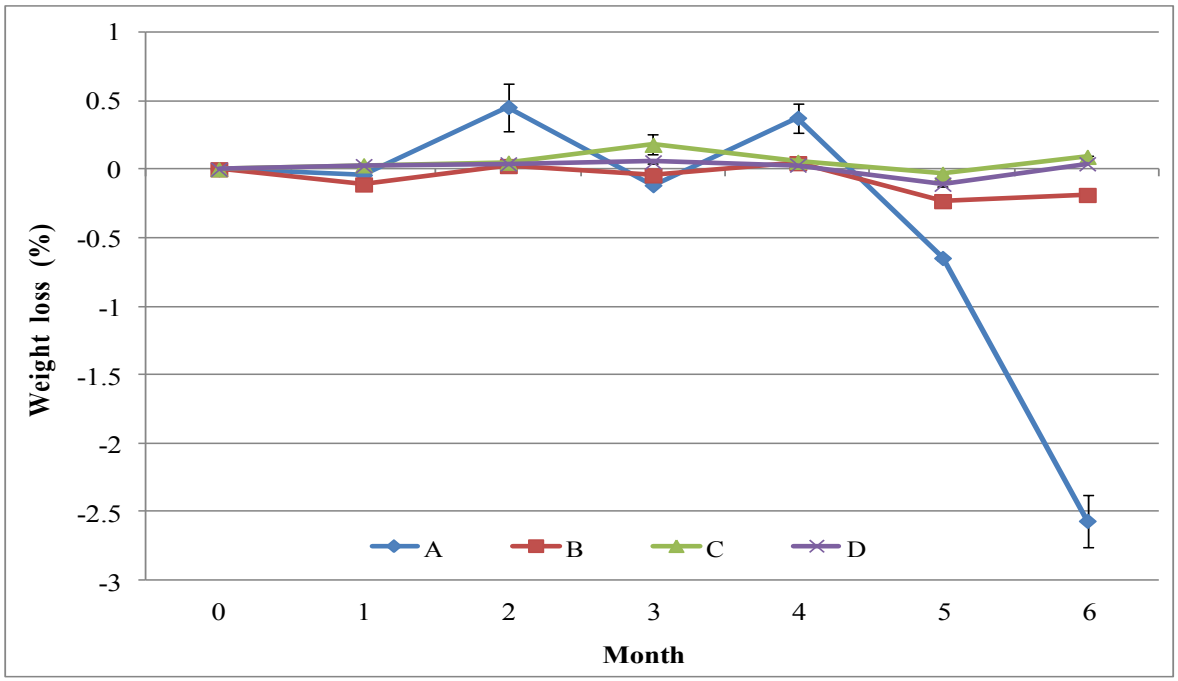


Figure 7.22: The weight loss (%) of the seasoning sachets during 6 months of storage

Note: *A: Edible film sachets - Ambient environment* *C: Edible film sachets - OPP wrapped*
B: PE sachets - Ambient environment *D: PE sachets - OPP wrapped*

The percentage weight loss of the seasoning sachets fluctuated during 6 months of storage. However, the changes in the weight of these sachets, except the edible film sachets at ambient environment, were negligible. This could be due to the change of their weights was too low to be differentiated by a 4-decimal digit scale. The result showed that these sachets were stable at the storage conditions.

The weight loss of the edible film sachets stored at ambient environment fluctuated at higher amplitude. Its weight rose up to 0.65 % and 2.5 % after 5 and 6 months of storage, respectively. It implied that these edible film sachets were not stable in the ambient environment. It can absorb water from the humidity of the environment, especially after 5 months of storage. This result was compatible with the observation of its appearance in the previous section.

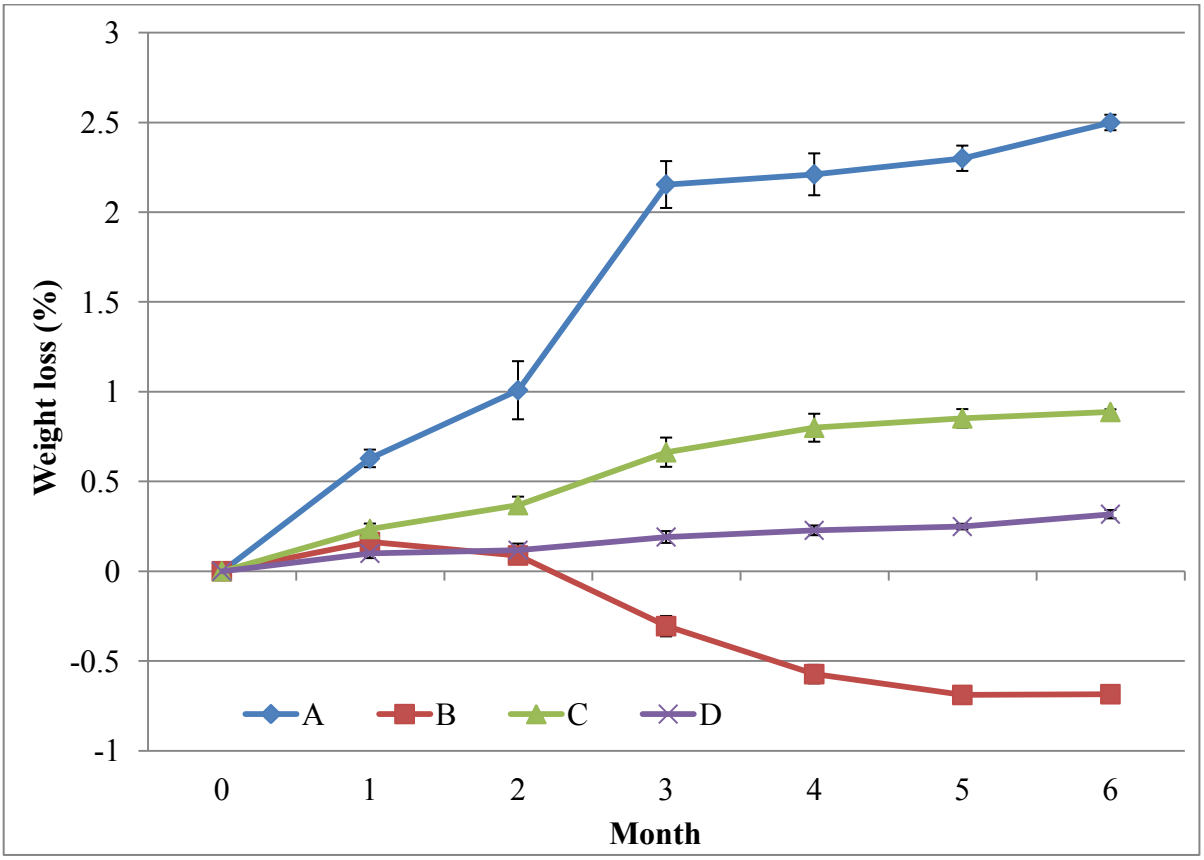


Figure 7.23: The weight loss (%) of the oil sachets during 6 months of storage

Note: A: Edible film sachets - Ambient environment *C: Edible film sachets - OPP wrapped*
B: PE sachets - Ambient environment *D: PE sachets - OPP wrapped*

The percentage weight loss of the oil sachets increased over 6 months of storage, except the PE sachets stored at ambient environment. The edible film sachets at the ambient environment lost weight sharply at the first 3 months of storage, then slowly till the sixth month. This could be due to the leakage of oil which was observed after 2 months of storage. The PE sachets stored at ambient environment seemed stable within the first 2 months, and then slightly increase its weight. The oil sachets stored in OPP wrap were stable with very low percentage of weight loss (< 1 %) during 6 months of storage.

In general, the composite edible film sachets will be more stable with a very slight change in their weight if they are stored in a tight container such as OPP wrap. This kind of wrap prevents the contact between the edible film sachets with the outer environment, and thus inhibits the transmission of water through the edible film.

7.3.4.3 Total plate counts

The seasoning powder and the vegetable oil stored in composite edible film sachets and PE sachets under different storage conditions were examined for their anti-microbiological properties by total plate count method (Table 7.9).

Table 7.9: Total plate counts (CFU/g or CFU/mL) of seasoning powder and the vegetable oil during 6 months of storage

Sample	0 month	1 month	2 months	3 months	4 months	5 months	6 months
Seasoning powder							
A	< 10	< 250	< 10	< 10	< 250	< 10	< 10
B	< 10	< 10	< 10	< 10	< 10	< 10	< 10
C	< 10	< 250	< 10	< 250	< 250	< 10	< 10
D	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Vegetable oil							
A	< 10	< 250	< 10	< 10	< 10	< 10	< 10
B	< 10	< 10	< 10	< 250	< 10	< 10	< 10
C	< 10	< 250	< 10	< 10	< 10	< 10	< 10
D	< 10	< 250	< 10	< 10	< 10	< 10	< 10

Note: A: Edible film sachets - Ambient environment C: Edible film sachets - OPP wrapped
B: PE sachets - Ambient environment D: PE sachets - OPP wrapped

The results in Table 7.9 demonstrated that there is very limited growth of bacteria in these samples during 6 months of storage. This could be due to the high level of salt in the seasoning powder and the hydrophobic property of vegetable oil. The antibacterial property of the composite edible film could also take part in this inhibition activity. Therefore, although the edible film sachets were wet after 6 months of storage, the seasoning powder and the vegetable oil can still keep out bacteria. The moisture content of seasoning powder and vegetable oil was 0.5 % and 1 %, respectively. This low moisture content can also help the spice avoid the growth of bacteria.

7.4 GENERAL DISCUSSION AND CONCLUSION

Throughout this work, a formulation of edible film for application in spice casing for instant noodles has been developed. This edible film was a combination of chitosan and gelatin with the support of glycerol as a plasticizer.

The film forming solution is composed of 0.5 % (w/v) of chitosan which has 94 % DD, 5 % (w/v) of gelatin and 10 % (w/w dry matter) of glycerol. The film was formed after the solution was cast and dried at 40 ^{o}C for 24 hours. This kind of film had high solubility and sealability. Its mechanical properties were as strong as other polymers. It could inhibit the growth of bacteria better than the homogeneous chitosan film. Therefore, this film can be applied in instant food casing.

The attempt to use this composite chitosan/gelatin edible film in making seasoning and oil sachets for instant noodles have confirmed the usability of this film. The spice stored in these edible film sachets can still keep their sensory quality and can avoid the presence of bacteria after 6 months of storage. These sachets retain their quality better if they are kept in another container to prevent contact with the outer environment. Therefore, this edible film is suitable for application in spice casing for instant noodles.

Chapter 8
CONCLUSION AND FUTURE DIRECTIONS

8.1 CONCLUSION

Two rabbit polyclonal antibodies were successfully generated and are suitable for further studies. These antibodies were raised against the purified tropomyosin from four species of shrimp and against the total crustacean proteins from Mud crab, Slipper lobster, and Black Tiger shrimp. The rabbit sera showed high affinity and specificity to their antigen in Western blotting. The antibody titration was carried out using the indirect ELISA. The immunoreactivity of the rabbit sera were still very strong when the antibody was diluted 30,000 times at very low concentrations of the purified tropomyosin and the crustacean protein, with 0.01 μg/mL and 0.1 μg/mL respectively. This indicated that the rabbit sera are very sensitive for the detection of the presence of tropomyosin. In addition, the rabbit serum against total crustacean proteins also recognised other proteins at higher mass. The strong reaction between the rabbit serum against proteins extracted from other species of shrimp as well as other crustacean species confirms the cross reactivity of the allergenic tropomyosin.

The protein concentration of the extracts from Black Tiger and Banana shrimp varied and depended on shrimp species, part of the shrimp and the processing method. There was still a marked amount of protein in the shell extracts although the shrimp was treated by boiling. Most proteins in the shrimp extracts concentrated near the 75, 37 and 20 kDa regions in SDS-PAGE. The immunobloting of these extracts with monoclonal antibody as well as crustacean sensitive patients has confirmed the presence of tropomyosin in these extracts. The tropomosin concentrations in the tail extracts and shell extracts were approximately at 3.5 μg/mL and 1.0 μg/mL, respectively. Most the IC50 values of shrimp protein extracts to rabbit sera demonstrate strong cross-reactivity of both sera to most extracts and the highest concentration of tropomyosin in cooked shrimp. Therefore, special care should be given to the processing of shrimp derived products to prevent contamination with tropomyosin, which could cause allergic reactions in crustacean sensitized people.

Most of the proteins remaining in the chitin and chitosan could be degraded and broken down into small fragments during extraction, reflected in the fact that no proteins could not be seen on the SDS-PAGE and Western blot. However, the immunoreactivities of the chitin and chitosan preparation were very strong in the Dot blot. The IC50s of these extracts to the rabbit serum pAbαTM were also obtained at very low concentration of inhibitor (approximately 0.01 %). This result demonstrated that the small fragments of residual proteins in chitin and chitosan products may act as an allergen which can cause allergic reaction to sensitized patients. When they bind to the high-molecular-weight chitin and chitosan molecules, they can cause allergic reaction to sensitized patients.

The biochemical properties of selected chitin and chitosan samples varied due to their different sources. Most samples have their moisture content less than 10 %. Their ash contents were less than 1 %, except for chitosan 2. The heavy metal content of these samples was very low compared to the Australian food standards for maximum permitted concentration of heavy metal in crustacean (Anon, 1987). It appears that each of the tested samples would be a safe product for use in the food industry. The protein content of normal chitosan samples was less than 1 %, while it cannot be detected in the purified sample. The results indicated that chitin and chitosan need to be characterized and standardized their properties before application in food industry.

Chitosan at 0.05 % and 0.1 % concentration can inhibit the development of *Escherichia coli*, a Gram-negative bacterium, and *Staphylococcus aureus*, a Gram-positive bacterium. It can completely inhibit the growth of *E. coli* and *S. aureus* after 4 hours of incubation. The purified chitosan can still retain its antimicrobial property comparable to the normal chitosan as assayed through MIC, MBC and disc diffusion results. Their MICs to *E. coli* and *S. aureus* were 31.25 ppm while their MBCs were 31.25 ppm to *E. coli* and approximately 62.5ppm to *S. aureus*. These chitosan samples inhibit the Gram-negative bacteria better than Gram-positive bacteria although the antibiotic activity of chitosan is rather low compared to chloramphenicol and ampicillin. However, due to its modest antimicrobial property, chitosan may be a promising material in the preservation of food products.

The application of chitosan in shrimp preservation has confirmed its antimicrobial property. Both purified and normal chitosan can reduce the development of bacteria on shrimps during 15 days of storage at 0 – 4 ^{o}C. Moreover, it can help the shrimp to lower the weight loss and keep their hardness higher than the non-treated ones during storage.

Throughout this work, a formulation of edible film for application in spice casing for instant noodles has been developed. This edible film is a combination of chitosan and gelatin with the support of glycerol as a plasticizer. The film forming solution was composed of 0.5 % (w/v) of chitosan which has 94 % DD, 5 % (w/v) of gelatin and 10 % (w/w dry matter) of glycerol. The film was formed after the solution was cast and dried at 40 ^{o}C for 24 hours. This kind of film has high solubility and sealability. Its mechanical properties were as strong as other polymers. It can inhibit the growth of bacteria better than the homogeneous chitosan film. Therefore, this film can be applied to instant food casing. The attempt to use this composite chitosan / gelatin edible film in making seasoning and oil sachets for instant noodles has confirmed the usability of this film. The spice stored in these edible film sachets can still keep their sensory quality and can avoid the presence of bacteria after 6 months of storage. These sachets retain their quality better if they are kept in another container to prevent contact with the outer environment. Therefore, this edible film is suitable for application in spice casing for instant noodles.

Overall, the presence of the major allergen tropomyosin in chitin and chitosan could cause potential risk to crustacean sensitized consumers. So, commercial chitosan should be purified before applying in the food industry to protect consumers from allergic reactions. In addition chitosan can be combined with gelatine to form a water-soluble and sealable edible film which can be used in instant food casing.

8.2 DIRECTIONS FOR FURTHER STUDY

Followings are some suggestions for further study related to this topic:

- This study only detected the presence of the allergenic protein tropomyosin in the residual proteins in chitin and chitosan samples. Therefore, the next study should focus on the comparison of the immunoreactivity between normal and purified chitosan to antibodies against tropomyosin to confirm that tropomyosin has been removed completely out of chitin and chitosan.

- In addition to tropomyosin, there are some other proteins in crustaceans such as arginine kinase or myosin light chain which could cause allergic reactions. So, there is also a need to examine the presence of these proteins in chitin and chitosan.

- In this study, raw shrimp was preserved at cool temperature (0 – 4 ^{o}C). However, most shrimp in the market are preserved in the frozen state. Therefore, it is necessary to examine the antimicrobial property of normal and purified chitosan on shrimp preservation at frozen temperature.

- The composite edible film still has some disadvantages such as it is not 100 % soluble, and there was some leakage of oil at the seal. Therefore, further studies to improve the quality of composite edible film are needed.

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APPENDIX - CHAPTER 3

A.3.1 Screening of the rabbit sera

Table A.3.1: Checkerboard of rabbit sera pAbαCR

Antibody dilution	Log antibody dilution	Crustacean protein concentration				
		0.001 µg/ml	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml
1,000	3.00E+00	3.6718 ± 0.3946	2.9762 ± 0.5116	3.0813 ± 0.4616	3.2342 ± 0.2631	2.6948 ± 0.3792
2,000	3.30E+00	3.7950 ± 0.1095	3.4167 ± 0.4723	3.2463 ± 0.5259	2.8273 ± 0.4150	3.0632 ± 0.0299
5,000	3.70E+00	4.2450 ± 0.2012	4.3153 ± 0.0917	4.3035 ± 0.1870	4.0057 ± 0.5205	3.7953 ± 0.3891
10,000	4.00E+00	3.8077 ± 0.1771	4.2145 ± 0.0273	4.6012 ± 0.0669	4.3835 ± 0.2969	3.7882 ± 0.4225
20,000	4.30E+00	1.7477 ± 0.1025	2.9877 ± 0.2336	4.2428 ± 0.4723	4.4082 ± 0.1899	4.2602 ± 0.1905
40,000	4.60E+00	0.5640 ± 0.0347	1.2805 ± 0.0776	2.1548 ± 0.1674	2.6562 ± 0.1714	2.5385 ± 0.1595
100,000	5.00E+00	0.7965 ± 0.0304	1.9485 ± 0.0948	1.9485 ± 0.1616	2.0847 ± 0.1475	2.0943 ± 0.1457
500,000	5.70E+00	0.4045 ± 0.3868	0.3373 ± 0.1373	0.5347 ± 0.1544	0.5293 ± 0.0553	0.5957 ± 0.1007

Table A.3.2: Checkerboard of rabbit sera pAbαTM

Antibody dilution	Log antibody dilution	Shrimp tropomyosin concentration				
		0.001 µg/ml	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml
1,000	3.00E+00	3.4125 ± 0.1252	3.1398 ± 0.0588	3.2613 ± 0.0964	3.2342 ± 0.0703	3.4408 ± 0.0453
2,000	3.30E+00	4.5135 ± 0.3664	3.3550 ± 0.0665	3.0453 ± 0.0695	3.3170 ± 0.1140	3.4128 ± 0.0730
5,000	3.70E+00	4.5235 ± 0.2281	3.9648 ± 0.1883	3.8352 ± 0.2407	3.2687 ± 0.1428	3.6108 ± 0.1500
10,000	4.00E+00	2.4695 ± 0.0038	4.5185 ± 0.2131	4.0422 ± 0.2814	3.7228 ± 0.3085	3.6117 ± 0.1200
20,000	4.30E+00	0.2288 ± 0.0010	2.7702 ± 0.0764	2.9075 ± 0.0146	4.6118 ± 0.1743	4.7048 ± 0.2657
40,000	4.60E+00	0.0753 ± 0.0003	0.3215 ± 0.0000	1.1422 ± 0.0012	2.2512 ± 0.0008	2.5997 ± 0.0035
100,000	5.00E+00	0.1983 ± 0.0003	0.6183 ± 0.0003	1.4085 ± 0.0005	2.1413 ± 0.0030	2.2432 ± 0.0015
500,000	5.70E+00	0.0320 ± 0.0005	0.0915 ± 0.0005	0.2753 ± 0.0003	0.4640 ± 0.0005	0.5363 ± 0.0003

A.3.2 Immunoreactivity of different rabbit sera to the immunogens

Table A.3.3: Sera dilution 1:5,000

	Rabbit serum			
	Pre-bleed	Test-bleed1	Test-bleed 2	Bleed-out
Rabbit 1 – Anti-shrimp tropomyosin serum (pAbαTM)				
Shrimp tropomyosin	0.0020 ± 0.0010	0.3197 ± 0.0240		
Crustacean protein	0.0150 ± 0.0108	1.1713 ± 0.0450		
Rabbit 2 – Anti-shrimp tropomyosin serum (pAbαTM)				
Shrimp tropomyosin	0.0012 ± 0.0010	2.4588 ± 0.1861	2.6655 ± 0.3357	2.7802 ± 0.3235
Crustacean protein	0.0705 ± 0.0503	2.9140 ± 0.1692	2.7755 ± 0.2587	3.3503 ± 0.2620
Rabbit 3 – Anti-crustacean protein serum (pAbαCR)				
Shrimp tropomyosin	0.0050 ± 0.0022	1.6535 ± 0.0398	2.5830 ± 0.3058	2.4517 ± 0.2276
Crustacean protein	0.0097 ± 0.0010	2.5820 ± 0.2493	3.0913 ± 0.2787	3.4390 ± 0.0320

Table A.3.4: Sera dilution 1:20,000

	Rabbit serum			
	Pre-bleed	Test-bleed1	Test-bleed 2	Bleed-out
Rabbit 1 – Anti-shrimp tropomyosin serum (pAbαTM)				
Shrimp tropomyosin	0.0005 ± 0.0005	0.1258 ± 0.0013		
Crustacean protein	0.0030 ± 0.0005	0.4162 ± 0.0025		
Rabbit 2 – Anti-shrimp tropomyosin serum (pAbαTM)				
Shrimp tropomyosin	0.0015 ± 0.0010	2.2333 ± 0.0187	3.8602 ± 0.0739	2.9775 ± 0.0219
Crustacean protein	0.0120 ± 0.0005	3.7780 ± 0.0331	4.5453 ± 0.2128	3.8958 ± 0.0560
Rabbit 3 – Anti-crustacean protein serum (pAbαCR)				
Shrimp tropomyosin	0.0030 ± 0.0005	0.6113 ± 0.0070	1.9902 ± 0.0111	1.4153 ± 0.0133
Crustacean protein	0.0085 ± 0.0000	2.5258 ± 0.0273	4.5192 ± 0.1337	3.4145 ± 0.0236

Table A.3.5: Sera dilution 1:30,000

	Rabbit serum			
	Pre-bleed	Test-bleed1	Test-bleed 2	Bleed-out
Rabbit 1 – Anti-shrimp tropomyosin serum (pAbαTM)				
Shrimp tropomyosin	0.0013 ± 0.0003	0.0720 ± 0.0013		
Crustacean protein	0.0027 ± 0.0003	0.1563 ± 0.0016		
Rabbit 2 – Anti-shrimp tropomyosin serum (pAbαTM)				
Shrimp tropomyosin	0.0015 ± 0.0015	2.9050 ± 0.0864	3.5563 ± 0.0268	2.5377 ± 0.0643
Crustacean protein	0.0108 ± 0.0008	3.8753 ± 0.0796	3.9917 ± 0.0473	3.4040 ± 0.0545
Rabbit 3 – Anti-crustacean protein serum (pAbαCR)				
Shrimp tropomyosin	0.0018 ± 0.0019	0.1432 ± 0.0014	0.2173 ± 0.0053	1.0653 ± 0.0324
Crustacean protein	0.0012 ± 0.0008	0.2660 ± 0.0022	0.4258 ± 0.0038	3.1582 ± 0.0359

A.3.3 Immunoreactivity of rabbit sera to specific crustacean protein extract

Table A.3.6: Sera dilution 1:5,000

Antigen	Rabbit serum pAbαCR	Rabbit serum pAbαTM
Mix purified tropomyosin	1.8177 ± 0.1974	1.4950 ± 0.1243
Black Tiger shrimp - Tropomyosin	1.0543 ± 0.0673	1.8793 ± 0.0624
Banana shrimp - Tropomyosin	1.9393 ± 0.1161	2.3560 ± 0.1125
Vannamei shrimp - Tropomyosin	1.8353 ± 0.0723	2.3410 ± 0.1489
School shrimp - Tropomyosin	1.6088 ± 0.0370	2.1290 ± 0.2683
King shrimp - Tropomyosin	1.9175 ± 0.2208	1.5322 ± 0.1720
Mix crustacean protein	1.6548 ± 0.1576	1.5642 ± 0.1917
Black Tiger shrimp protein	2.0152 ± 0.2652	1.7102 ± 0.2156
Banana shrimp protein	1.7070 ± 0.2202	1.5498 ± 0.1661
Vannamei shrimp protein	1.5827 ± 0.1683	1.5372 ± 0.1928
School shrimp protein	1.6052 ± 0.1437	1.6102 ± 0.1667
King shrimp protein	1.7917 ± 0.2142	1.6582 ± 0.2112
Mud crab protein	1.8980 ± 0.2275	1.4823 ± 0.1096
Slipper lobster protein	2.1153 ± 0.2523	1.8873 ± 0.2600

Table A.3.7: Sera dilution 1:20,000

Antigen	Rabbit serum pAbαCR	Rabbit serum pAbαTM
Mix purified tropomyosin	1.2582 ± 0.0055	2.5467 ± 0.0198
Black Tiger shrimp - Tropomyosin	0.2360 ± 0.0020	0.7483 ± 0.0060
Banana shrimp - Tropomyosin	0.6490 ± 0.0039	1.6755 ± 0.0140
Vannamei shrimp - Tropomyosin	0.4378 ± 0.0102	1.2573 ± 0.0077
School shrimp - Tropomyosin	0.3260 ± 0.0025	0.9793 ± 0.0068
King shrimp - Tropomyosin	1.4087 ± 0.0077	2.4058 ± 0.0163
Mix crustacean protein	3.0643 ± 0.0321	3.7307 ± 0.0262
Black Tiger shrimp protein	1.9397 ± 0.0157	2.9607 ± 0.0237
Banana shrimp protein	2.7072 ± 0.0234	3.5615 ± 0.1471
Vannamei shrimp protein	1.6213 ± 0.0153	2.9113 ± 0.0872
School shrimp protein	3.1420 ± 0.0401	3.8235 ± 0.0490
King shrimp protein	1.4503 ± 0.0264	3.1778 ± 0.0563
Mud crab protein	2.6130 ± 0.0205	3.3458 ± 0.0380
Slipper lobster protein	0.9223 ± 0.0083	1.9070 ± 0.0148

Table A.3.8: Sera dilution 1:30,000

Antigen	Rabbit serum pAbαCR	Rabbit serum pAbαTM
Mix purified tropomyosin	1.5818 ± 0.0475	2.3063 ± 0.0471
Black Tiger shrimp - Tropomyosin	0.1885 ± 0.0064	0.5845 ± 0.0126
Banana shrimp - Tropomyosin	0.4055 ± 0.0092	0.9482 ± 0.0177
Vannamei shrimp - Tropomyosin	0.3668 ± 0.0141	0.9812 ± 0.0193
School shrimp - Tropomyosin	0.3808 ± 0.0391	1.0135 ± 0.0231
King shrimp - Tropomyosin	1.2545 ± 0.0188	2.1038 ± 0.0687
Mix crustacean protein	3.2213 ± 0.0990	3.3150 ± 0.0882
Black Tiger shrimp protein	2.7062 ± 0.0445	3.6382 ± 0.0913
Banana shrimp protein	2.6853 ± 0.0746	3.6535 ± 0.0642
Vannamei shrimp protein	1.8495 ± 0.0941	2.7722 ± 0.1005
School shrimp protein	3.0505 ± 0.0493	3.6595 ± 0.1379
King shrimp protein	1.4558 ± 0.0436	3.0000 ± 0.0661
Mud crab protein	1.1365 ± 0.0215	2.1860 ± 0.0608
Slipper lobster protein	2.9437 ± 0.0540	3.0058 ± 0.0848

APPENDIX - CHAPTER 4

A.4.1 Protein concentration of shrimp protein extracts

Table A.4.1: Protein concentration in shrimp extracts (% w/w)

Shrimp protein extracts	Mean	STD
Black Tiger raw tail extract	3.7388	0.1926
Black Tiger raw shell extract	1.9070	0.0651
Black Tiger heat-treated tail extract	1.1343	0.0656
Black Tiger heat-treated shell extract	0.0975	0.0063
Black Tiger cooked tail extract	1.0580	0.1193
Black Tiger cooked shell extract	0.3550	0.0282
Banana raw tail extract	2.1163	0.0723
Banana raw tail extract	0.6486	0.0641
Banana heat-treated tail extract	1.2224	0.0248
Banana heat-treated shell extract	0.0899	0.0081
Banana cooked tail extract	0.5125	0.0070
Banana cooked shell extract	0.1460	0.0147

Table A.4.2: Statistical analysis of protein concentration (%) of shrimp protein extracts

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	38.106[a]	11	3.464	585.634	.000
Intercept	42.420	1	42.420	7171.305	.000
Shrimp	3.159	1	3.159	534.095	.000
Part	10.687	1	10.687	1806.755	.000
Status	18.706	2	9.353	1581.152	.000
Shrimp * Part	.091	1	.091	15.462	.001
Shrimp * Status	3.497	2	1.749	295.613	.000
Part * Status	1.865	2	.932	157.636	.000
Shrimp * Part * Status	.100	2	.050	8.430	.002
Error	.142	24	.006		
Total	80.669	36			
Corrected Total	38.248	35			

[a]. R Squared = .996 (Adjusted R Squared = .995)

A.4.2 Immunoreactivity of different shrimp protein extracts to rabbit sera

A.4.2.1 Immunoreactivity of shrimp protein extracts to rabbit serum pAbαCR

Table A.4.3: The absorbance values of the bindings of different shrimp protein extracts to rabbit serum pAbαCR

Shrimp protein extracts	Mean	STD
Black Tiger raw tail extract	1.4736	0.0348
Black Tiger raw tail extract	0.1069	0.0040
Black Tiger heat-treated tail extract	0.0807	0.0023
Black Tiger heat-treated shell extract	0.0888	0.0022
Black Tiger cooked tail extract	1.5303	0.0346
Black Tiger cooked shell extract	0.9399	0.0227
Banana raw tail extract	1.1826	0.0280
Banana raw tail extract	0.1715	0.0035
Banana heat-treated tail extract	1.1750	0.0286
Banana heat-treated shell extract	0.0676	0.0034
Banana cooked tail extract	1.7280	0.0400
Banana cooked shell extract	0.1896	0.0070
Crustacean protein 0.1 μg/mL	2.7492	0.0693
Crustacean protein 0.01 μg/mL	2.5803	0.0515
Crustacean protein 0.001 μg/mL	0.1640	0.0046
Crustacean protein 0.0001 μg/mL	0.0522	0.0019

Table A.4.4: Statistical analysis of the absorbance values of the bindings of different shrimp protein extracts to rabbit serum pAbαCR

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	14.701[a]	11	1.336	2578.525	.000
Intercept	19.072	1	19.072	36798.503	.000
Shrimp	.022	1	.022	41.693	.000
Part	7.857	1	7.857	15159.142	.000
Status	3.321	2	1.661	3204.120	.000
Shrimp * Part	.729	1	.729	1407.162	.000
Shrimp * Status	1.110	2	.555	1070.451	.000
Part * Status	.689	2	.345	664.772	.000
Shrimp * Part * Status	.973	2	.486	938.546	.000
Error	.012	24	.001		
Total	33.785	36			
Corrected Total	14.713	35			

[a]. R Squared = .999 (Adjusted R Squared = .999)

Table A.4.5: Percentage inhibition of different Black Tiger shrimp protein extracts to rabbit serum pAbαCR

Inhibitor	Inhibitor concentration				
	0 µg/mL	0.01 µg/mL	0.1 µg/mL	1 µg/mL	10 µg/mL
Raw tail extract	0.00 ± 0.0000	45.01 ± 1.4033	66.77 ± 1.0513	80.12 ± 0.6253	87.10 ± 0.4250
Raw tail extract	0.00 ± 0.0000	36.02 ± 1.9642	40.72 ± 2.1524	41.11 ± 1.7490	63.48 ± 1.1445
Heat-treated tail extract	0.00 ± 0.0000	46.97 ± 0.8925	49.28 ± 1.6170	52.24 ± 3.6743	71.78 ± 3.2256
Heat-treated shell extract	0.00 ± 0.0000	44.88 ± 0.7377	47.24 ± 0.7731	50.23 ± 2.7616	53.79 ± 2.1953
Cooked tail extract	0.00 ± 0.0000	60.95 ± 1.1398	78.31 ± 0.6252	87.61 ± 0.3405	92.83 ± 0.1500
Cooked shell extract	0.00 ± 0.0000	43.90 ± 2.3239	67.27 ± 1.0823	84.51 ± 0.3917	92.05 ± 0.1926
Mix crustacean protein	0.00 ± 0.0000	61.04 ± 1.0883	83.06 ± 0.4771	97.17 ± 0.0901	100.06 ± 0.0203

Table A.4.6: Statistical analysis of the percentage inhibition of different Black Tiger shrimp protein extracts to rabbit serum pAbαCR

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	76800.302[a]	29	2648.286	408.507	.000
Intercept	218341.997	1	218341.997	33679.980	.000
Part	2167.569	1	2167.569	334.355	.000
Status	5571.421	2	2785.710	429.705	.000
Concentration	62952.684	4	15738.171	2427.665	.000
Part * Status	1062.891	2	531.446	81.977	.000
Part * Concentration	609.414	4	152.354	23.501	.000
Status * Concentration	2829.793	8	353.724	54.563	.000
Part * Status * Concentration	1606.529	8	200.816	30.977	.000
Error	388.971	60	6.483		
Total	295531.269	90			
Corrected Total	77189.272	89			

[a.] R Squared = .995 (Adjusted R Squared = .993)

Table A.4.7: Percentage inhibition of different Banana shrimp protein extracts to rabbit serum pAbαCR

Inhibitor	Inhibitor concentration				
	0 µg/mL	0.01 µg/mL	0.1 µg/mL	1 µg/mL	10 µg/mL
Raw tail extract	0.00 ± 0.0000	47.84 ± 1.8388	57.64 ± 1.6100	81.12 ± 1.1078	87.01 ± 0.3029
Raw tail extract	0.00 ± 0.0000	40.10 ± 1.3966	44.36 ± 1.3191	67.05 ± 0.7634	80.85 ± 0.4205
Heat-treated tail extract	0.00 ± 0.0000	54.07 ± 1.0921	72.89 ± 0.6115	86.25 ± 0.3192	90.94 ± 0.2230
Heat-treated shell extract	0.00 ± 0.0000	41.00 ± 0.8512	43.58 ± 1.5168	46.01 ± 1.2460	50.41 ± 3.2512
Cooked tail extract	0.00 ± 0.0000	77.39 ± 0.5054	86.97 ± 0.3192	91.64 ± 0.2143	94.67 ± 0.1458
Cooked shell extract	0.00 ± 0.0000	40.32 ± 1.3911	41.81 ± 1.3944	56.42 ± 0.9919	80.29 ± 0.4853
Mix crustacean protein	0.00 ± 0.0000	73.95 ± 0.6036	89.70 ± 0.2672	97.94 ± 0.0758	99.70 ± 0.0181

Table A.4.8: Statistical analysis of the percentage inhibition of different Banana shrimp protein extracts to rabbit serum pAbαCR

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	87852.851[a]	29	3029.409	2661.716	.000
Intercept	243550.357	1	243550.357	213989.597	.000
Part	8773.641	1	8773.641	7708.747	.000
Status	1158.023	2	579.011	508.734	.000
Concentration	70961.469	4	17740.367	15587.142	.000
Part * Status	1498.852	2	749.426	658.465	.000
Part * Concentration	2621.835	4	655.459	575.903	.000
Status * Concentration	1297.922	8	162.240	142.548	.000
Part * Status * Concentration	1541.108	8	192.639	169.257	.000
Error	68.288	60	1.138		
Total	331471.497	90			
Corrected Total	87921.140	89			

[a.] R Squared = .999 (Adjusted R Squared = .999)

A.4.2.2 Immunoreactivity of shrimp protein extracts to rabbit serum pAbαTM

Table A.4.9: The absorbance values of the bindings of different shrimp protein extracts to rabbit serum pAbαTM

Shrimp protein extracts	Mean	STD
Black Tiger raw tail extract	2.4056	0.0620
Black Tiger raw tail extract	0.3387	0.0073
Black Tiger heat-treated tail extract	0.6274	0.0114
Black Tiger heat-treated shell extract	0.2308	0.0064
Black Tiger cooked tail extract	2.8278	0.0725
Black Tiger cooked shell extract	2.3788	0.0498
Banana raw tail extract	2.6768	0.0805
Banana raw tail extract	0.5263	0.0099
Banana heat-treated tail extract	2.7570	0.0807
Banana heat-treated shell extract	0.3890	0.0084
Banana cooked tail extract	3.3566	0.0988
Banana cooked shell extract	0.8713	0.0143
Purified tropomyosin 0.1 μg/mL	3.3209	0.1712
Purified tropomyosin 0.01 μg/mL	1.8711	0.0440
Purified tropomyosin 0.001 μg/mL	1.2689	0.0300
Purified tropomyosin 0.0001 μg/mL	0.2872	0.0104

Table A.4.10: Statistical analysis of the absorbance values of the bindings of different shrimp protein extracts to rabbit serum pAbαTM

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	47.716[a]	11	4.338	1490.380	.000
Intercept	93.954	1	93.954	32280.526	.000
Shrimp	.781	1	.781	268.490	.000
Part	24.583	1	24.583	8446.109	.000
Status	11.356	2	5.678	1950.785	.000
Shrimp * Part	4.184	1	4.184	1437.629	.000
Shrimp * Status	4.020	2	2.010	690.635	.000
Part * Status	.946	2	.473	162.571	.000
Shrimp * Part * Status	1.845	2	.923	316.985	.000
Error	.070	24	.003		
Total	141.740	36			
Corrected Total	47.786	35			

[a]. R Squared = .999 (Adjusted R Squared = .998)

Table A.4.11: The tropomyosin concentration (μg/mL) of shrimp protein extracts

Shrimp protein extracts	Mean	STD
Black Tiger raw tail extract	3.242	0.0483
Black Tiger raw shell extract	1.1099	0.0129
Black Tiger heat-treated tail extract	1.4079	0.0016
Black Tiger heat-treated shell extract	0.9986	0.018
Black Tiger cooked tail extract	3.6776	0.0619
Black Tiger cooked shell extract	3.2147	0.0559
Banana raw tail extract	3.5214	0.0509
Banana raw shell extract	1.3036	0.0054
Banana heat-treated tail extract	3.6042	0.0495
Banana heat-treated shell extract	1.1619	0.0114
Banana cooked tail extract	4.2227	0.0742
Banana cooked shell extract	1.6596	0.0088

Table A.4.12: Statistical analysis of the tropomyosin concentration (µg/mL) of different shrimp protein extracts

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	58.708[a]	11	5.337	3813.868	.000
Intercept	45.909	1	45.909	32806.453	.000
Shrimp	3.032	1	3.032	2166.853	.000
Part	34.260	1	34.260	24481.981	.000
Status	12.215	2	6.108	4364.466	.000
Shrimp * Part	.780	1	.780	557.558	.000
Shrimp * Status	.843	2	.421	301.097	.000
Part * Status	6.606	2	3.303	2360.402	.000
Shrimp * Part * Status	.971	2	.486	347.112	.000
Error	.034	24	.001		
Total	104.652	36			
Corrected Total	58.742	35			

a. R Squared = .999 (Adjusted R Squared = .999)

Table A.4.13: Percentage of tropomyosin compared to total protein extracted from Black Tiger shrimp and Banana shrimp

Shrimp protein extracts	Total protein (mg/mL)	% tropomyosin / total protein
Black Tiger raw tail extract	1.6863	0.1923
Black Tiger raw shell extract	1.7452	0.0636
Black Tiger heat-treated tail extract	1.5741	0.0894
Black Tiger heat-treated shell extract	0.5446	0.1834
Black Tiger cooked tail extract	0.5801	0.6339
Black Tiger cooked shell extract	0.6979	0.4606
Banana raw tail extract	1.6306	0.2160
Banana raw shell extract	1.1328	0.1151
Banana heat-treated tail extract	1.1296	0.3191
Banana heat-treated shell extract	0.3768	0.3084
Banana cooked tail extract	0.2614	1.6153
Banana cooked shell extract	0.9852	0.1685

Table A.4.14: Percentage inhibition of different Black Tiger shrimp protein extracts to rabbit serum pAbαTM

Inhibitor	Inhibitor concentration				
	0 µg/mL	0.01 µg/mL	0.1 µg/mL	1 µg/mL	10 µg/mL
Raw tail extract	0.00 ± 0.0000	0.01 ± 0.3820	0.10 ± 0.1503	1.00 ± 0.0331	10.00 ± 0.0306
Raw tail extract	0.00 ± 0.0000	42.68 ± 0.7772	73.11 ± 0.4612	90.19 ± 0.6068	98.45 ± 0.2882
Heat-treated tail extract	0.00 ± 0.0000	21.57 ± 0.9106	22.25 ± 0.4061	24.45 ± 0.1811	52.43 ± 0.0353
Heat-treated shell extract	0.00 ± 0.0000	35.42 ± 0.8062	44.59 ± 0.4810	60.97 ± 0.5020	89.58 ± 0.2199
Cooked tail extract	0.00 ± 0.0000	30.96 ± 0.3105	32.32 ± 0.0925	40.85 ± 0.0441	56.83 ± 0.0704
Cooked shell extract	0.00 ± 0.0000	77.24 ± 0.5519	89.64 ± 0.2121	97.77 ± 0.0814	99.76 ± 0.0585
Mix purified tropomyosin	0.00 ± 0.0000	49.22 ± 0.0665	75.23 ± 0.0447	95.16 ± 0.0669	99.38 ± 0.0595

Table A.4.15: Statistical analysis of the percentage inhibition of different Black Tiger shrimp protein extracts to rabbit serum pAbαTM

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	110124.945[a]	29	3797.412	27702.608	.000
Intercept	225012.000	1	225012.000	1641491.450	.000
Part	8925.754	1	8925.754	65114.520	.000
Status	15302.965	2	7651.482	55818.548	.000
Concentration	71883.603	4	17970.901	131100.030	.000
Part * Status	3274.455	2	1637.228	11943.785	.000
Part * Concentration	2563.119	4	640.780	4674.571	.000
Status * Concentration	4756.613	8	594.577	4337.513	.000
Part * Status * Concentration	3418.435	8	427.304	3117.241	.000
Error	8.225	60	.137		
Total	335145.169	90			
Corrected Total	110133.169	89			

[a.] R Squared = 1.000 (Adjusted R Squared = 1.000)

Table A.4.16: Percentage inhibition of different Banana shrimp protein extracts to rabbit serum pAbαTM

Inhibitor	Inhibitor concentration				
	0 µg/mL	0.01 µg/mL	0.1 µg/mL	1 µg/mL	10 µg/mL
Raw tail extract	0.00 ± 0.0000	40.09 ± 0.3732	73.22 ± 0.3296	88.63 ± 0.0667	96.59 ± 0.0305
Raw tail extract	0.00 ± 0.0000	38.55 ± 0.1725	47.21 ± 0.5013	61.93 ± 0.3736	74.08 ± 0.5296
Heat-treated tail extract	0.00 ± 0.0000	63.79 ± 0.3776	86.96 ± 0.1489	96.51 ± 0.0192	98.97 ± 0.0333
Heat-treated shell extract	0.00 ± 0.0000	39.51 ± 1.0815	41.26 ± 0.9269	42.47 ± 0.5231	54.22 ± 0.5089
Cooked tail extract	0.00 ± 0.0000	91.97 ± 0.1057	96.05 ± 0.0305	99.46 ± 0.0342	99.54 ± 0.0457
Cooked shell extract	0.00 ± 0.0000	37.06 ± 0.8669	43.36 ± 0.8818	57.31 ± 0.5246	81.23 ± 0.1377
Mix purified tropomyosin	0.00 ± 0.0000	95.57 ± 0.0319	98.94 ± 0.0600	99.08 ± 0.0601	99.03 ± 0.5792

Table A.4.17: Statistical analysis of the percentage inhibition of different Banana shrimp protein extracts to rabbit serum pAbαTM

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	107442.768[a]	29	3704.923	20150.905	.000
Intercept	272252.200	1	272252.200	1480767.134	.000
Part	17107.047	1	17107.047	93044.441	.000
Status	1412.103	2	706.051	3840.181	.000
Concentration	78331.548	4	19582.887	106510.417	.000
Part * Status	1680.510	2	840.255	4570.107	.000
Part * Concentration	5103.321	4	1275.830	6939.182	.000
Status * Concentration	1593.539	8	199.192	1083.398	.000
Part * Status * Concentration	2214.700	8	276.837	1505.706	.000
Error	11.032	60	.184		
Total	379705.999	90			
Corrected Total	107453.799	89			

[a.] R Squared = 1.000 (Adjusted R Squared = 1.000)

APPENDIX - CHAPTER 5

Table A.5.1: Percentage inhibition of protein extracts from chitin and chitosan samples to rabbit serum pAbαCR

Concentration / Protein extract	0 µg/mL	0.01 g/mL	0.1 µg/mL	1 µg/mL	10 µg/mL
CTS1-Acetate	0.0000 ± 0.0000	38.6551 ± 1.1483	41.2658 ± 1.3619	47.1047 ± 0.9661	66.6019 ± 0.7314
CTS1-SDS-DTT	0.0000 ± 0.0000	33.4224 ± 2.1572	36.7709 ± 1.6732	38.5722 ± 1.4612	44.6817 ± 1.0458
CTS1-NaOH	0.0000 ± 0.0000	39.6983 ± 1.5575	43.2487 ± 1.4704	44.7898 ± 1.6340	46.0113 ± 0.7264
CTS1-Phosphate	0.0000 ± 0.0000	40.0788 ± 1.2885	41.4976 ± 0.8870	45.5760 ± 0.8410	46.7418 ± 0.5571
CTS2- Acetate	0.0000 ± 0.0000	37.8007 ± 2.5792	39.5447 ± 1.1869	60.0807 ± 1.2237	69.6900 ± 1.6464
CTS2-SDS-DTT	0.0000 ± 0.0000	35.1707 ± 2.4870	41.1220 ± 1.3967	41.8433 ± 3.1663	47.0047 ± 3.7736
CTS2-NaOH	0.0000 ± 0.0000	40.6863 ± 2.9213	43.2103 ± 3.0213	47.7007 ± 2.6944	59.3970 ± 2.4577
CTS2-Phosphate	0.0000 ± 0.0000	40.3614 ± 2.8108	45.3411 ± 1.3545	51.4519 ± 2.3556	60.2050 ± 1.5162
CT-Supernatant I	0.0000 ± 0.0000	41.6494 ± 1.7082	43.4502 ± 0.9782	46.0542 ± 0.9643	48.5580 ± 1.0994
CT-Supernatant II	0.0000 ± 0.0000	40.3337 ± 1.5897	43.6099 ± 1.1956	45.2073 ± 1.7617	63.1045 ± 1.2187
CT-Phosphoric	0.0000 ± 0.0000	41.6793 ± 0.1931	43.4644 ± 0.1069	46.1113 ± 1.7330	69.7166 ± 0.1202
TROP	0.0000 ± 0.0000	59.8351 ± 1.1216	84.4939 ± 0.4346	93.7848 ± 0.0970	96.9942 ± 0.0322
CRUS	0.0000 ± 0.0000	49.4999 ± 1.3756	82.3815 ± 0.5477	97.6154 ± 0.0607	100.0197 ± 0.0466

Table A.5.2: Statistical analysis of the percentage inhibition of protein extracts from chitin and chitosan samples to rabbit serum pAbαCR

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	66199.541[a]	54	1225.917	490.671	.000
Intercept	224392.568	1	224392.568	89812.742	.000
Preparation	1634.242	10	163.424	65.410	.000
Concentration	61878.240	4	15469.560	6191.665	.000
Preparation * Concentration	2687.059	40	67.176	26.887	.000
Error	274.829	110	2.498		
Total	290866.938	165			
Corrected Total	66474.371	164			

a. R Squared = .996 (Adjusted R Squared = .994)

Table A.5.3: Percentage inhibition of protein extracts from chitin and chitosan samples to rabbit serum pAbαTM

Concentration / Protein extract	0 µg/mL	0.01 µg/mL	0.1 µg/mL	1 µg/mL	10 µg/mL
CTS1-Acetate	0.0000 ± 0.0000	55.3314 ± 3.0174	57.9086 ± 2.5726	63.2828 ± 2.6968	84.2494 ± 1.0406
CTS1-SDS-DTT	0.0000 ± 0.0000	54.3759 ± 2.7005	57.0022 ± 2.2230	58.3662 ± 2.2697	58.7536 ± 2.1467
CTS1-NaOH	0.0000 ± 0.0000	56.5252 ± 3.6675	60.3084 ± 2.6431	61.1595 ± 2.4906	67.5772 ± 2.0490
CTS1-Phosphate	0.0000 ± 0.0000	56.8770 ± 2.8236	59.0245 ± 2.5862	61.1375 ± 2.5552	72.3191 ± 1.8482
CTS2- Acetate	0.0000 ± 0.0000	56.2395 ± 2.3678	56.9502 ± 2.2287	75.5447 ± 1.2209	96.4231 ± 0.2024
CTS2-SDS-DTT	0.0000 ± 0.0000	53.0140 ± 2.5914	54.4964 ± 2.4670	56.6806 ± 3.4169	64.1391 ± 0.9640
CTS2-NaOH	0.0000 ± 0.0000	57.9202 ± 1.7871	60.1240 ± 2.5016	62.3171 ± 2.3319	71.9716 ± 1.5165
CTS2-Phosphate	0.0000 ± 0.0000	57.5728 ± 2.7651	59.1929 ± 2.9465	61.1514 ± 2.4352	64.6248 ± 1.5874
CT-Supernatant I	0.0000 ± 0.0000	57.1367 ± 3.1882	65.5325 ± 2.2291	68.1793 ± 1.9992	73.1259 ± 2.3585
CT-Supernatant II	0.0000 ± 0.0000	58.7385 ± 2.5757	60.5532 ± 2.4150	65.6914 ± 2.1737	99.3050 ± 0.0559
CT-Phosphoric	0.0000 ± 0.0000	56.4411 ± 2.5873	63.3985 ± 2.2861	66.1491 ± 2.2210	90.2027 ± 0.6362
TROP	0.0000 ± 0.0000	95.0196 ± 0.2538	99.0794 ± 0.0699	99.8061 ± 0.0451	99.7697 ± 0.0458
CRUS	0.0000 ± 0.0000	83.3230 ± 0.8228	93.4321 ± 0.3548	97.8683 ± 0.1321	99.2243 ± 0.0703

Table A.5.4: Statistical analysis of the percentage inhibition of protein extracts from chitin and chitosan samples to rabbit serum pAbαTM

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	119434.570[a]	54	2211.751	68.036	.000
Intercept	431890.398	1	431890.398	13285.395	.000
Preparation	1605.323	10	160.532	4.938	.000
Concentration	115574.821	4	28893.705	888.800	.000
Preparation * Concentration	2254.426	40	56.361	1.734	.013
Error	3575.953	110	32.509		
Total	554900.921	165			
Corrected Total	123010.523	164			

[a.] R Squared = .971 (Adjusted R Squared = .957)

APPENDIX – CHAPTER 6

A.6.1 Characterization of the properties of some commercial chitin/chitosan products

Table A.6.1: Moisture content of chitin and chitosan samples

Sample	Moisture content (%)	Standard derivation
Chitin 1	4.5885	0.1367
Chitin 2	5.3973	0.0907
Chitin 3	6.3663	0.2568
Chitosan 1	6.0210	0.2392
Chitosan 2	8.3412	0.1715
Chitosan 3	10.9278	0.0766

Table A.6.2: Statistical analysis of moisture content of chitin and chitosan samples

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	80.847[a]	5	16.169	523.375	.000
Intercept	867.034	1	867.034	28064.223	.000
Type	80.847	5	16.169	523.375	.000
Error	.371	12	.031		
Total	948.252	18			
Corrected Total	81.218	17			

[a]. R Squared = .995 (Adjusted R Squared = .994)

Table A.6.3: Ash content of chitin and chitosan samples

Sample	Ash content (%)	Standard derivation
Chitin 1	0.1755	0.0312
Chitin 2	0.3998	0.0543
Chitin 3	0.0702	0.0127
Chitosan 1	0.7333	0.0457
Chitosan 2	5.8325	0.0464
Chitosan 3	0.3619	0.0139

Table A.6.4: Statistical analysis of ash content of chitin and chitosan samples

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	75.970[a]	5	15.194	10697.626	.000
Intercept	28.677	1	28.677	20190.342	.000
Type	75.970	5	15.194	10697.626	.000
Error	.017	12	.001		
Total	104.664	18			
Corrected Total	75.987	17			

[a]. R Squared = 1.000 (Adjusted R Squared = 1.000)

A.6.2 Characterization of the antimicrobial properties of normal chitosan

A.6.2.1 Spectrophotometer method

Table A.6.5: The optical density (OD) of bacteria over time in solutions of different chitosan concentration

Bacteria	Chitosan concentration	0 hour	4 hours	24 hours
Escherichia coli	0%	0.011 ± 0.003	0.297 ± 0.062	0.841 ± 0.167
	0.01%	0.073 ± 0.004	0.023 ± 0.010	0.645 ± 0.061
	0.05%	0.030 ± 0.027	0.035 ± 0.034	0.033 ± 0.030
	0.1%	0.015 ± 0.008	0.020 ± 0.008	0.019 ± 0.011
Staphylococcus aureus	0%	0.011 ± 0.001	0.432 ± 0.104	0.873 ± 0.037
	0.01%	0.059 ± 0.021	0.020 ± 0.001	0.806 ± 0.017
	0.05%	0.019 ± 0.008	0.025 ± 0.003	0.025 ± 0.006
	0.1%	0.020 ± 0.015	0.028 ± 0.008	0.029 ± 0.011

Table A.6.6: Statistical analysis of *Escherichia coli* over time in solutions of different chitosan concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.621[a]	11	.238	74.323	.000
Intercept	1.044	1	1.044	325.567	.000
Concentration	.839	3	.280	87.210	.000
Incubation	.849	2	.424	132.343	.000
Concentration * Incubation	.934	6	.156	48.540	.000
Error	.077	24	.003		
Total	3.742	36			
Corrected Total	2.698	35			

[a]. R Squared = .971 (Adjusted R Squared = .958)

Table A.6.7: Statistical analysis of *Staphylococcus aureus* over time in solutions of different chitosan concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.441[a]	11	.313	278.649	.000
Intercept	1.377	1	1.377	1226.499	.000
Concentration	1.147	3	.382	340.725	.000
Incubation	1.075	2	.538	478.884	.000
Concentration * Incubation	1.218	6	.203	180.866	.000
Error	.027	24	.001		
Total	4.844	36			
Corrected Total	3.467	35			

[a]. R Squared = .992 (Adjusted R Squared = .989)

A.6.2.2 Viable cell counts method

Table A.6.8: The viable cell counts of bacteria (log_{10} CFU/mL) over time in solutions of different chitosan concentration

Bacteria	Chitosan concentration	0 hour	4 hours	24 hours
Escherichia coli	0%	9.23 ± 0.03	11.37 ± 0.09	12.27 ± 0.04
	0.01%	9.24 ± 0.06	11.28 ± 0.02	12.20 ± 0.03
	0.05%	9.27 ± 0.06	4.19 ± 0.03	4.30 ± 0.10
	0.1%	9.24 ± 0.06	4.20 ± 0.03	4.20 ± 0.10
Staphylococcus aureus	0%	9.06 ± 0.06	10.21 ± 0.09	10.45 ± 0.02
	0.01%	9.13 ± 0.09	10.23 ± 0.07	10.34 ± 0.07
	0.05%	9.06 ± 0.08	2.40 ± 0.00	2.40 ± 0.00
	0.1%	9.06 ± 0.08	2.40 ± 0.00	2.81 ± 0.71

Table A.6.9: Statistical analysis of viable cell counts of *Escherichia coli* (log_{10} CFU/mL) over time in solutions of different chitosan concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.460E25	11	1.328E24	439.716	.000
Intercept	3.810E24	1	3.810E24	1261.722	.000
Concentration	3.846E24	3	1.282E24	424.558	.000
Incubation	5.342E24	2	2.671E24	884.650	.000
Concentration * Incubation	5.417E24	6	9.028E23	298.984	.000
Error	7.247E22	24	3.019E21		
Total	1.849E25	36			
Corrected Total	1.468E25	35			

[a]. R Squared = .995 (Adjusted R Squared = .993)

Table A.6.10: Statistical analysis of viable cell counts of *Staphylococcus aureus* (log_{10} CFU/mL) over time in solutions of different chitosan concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.545E21	11	3.222E20	118.870	.000
Intercept	1.945E21	1	1.945E21	717.310	.000
Concentration	1.758E21	3	5.860E20	216.158	.000
Incubation	7.815E20	2	3.908E20	144.149	.000
Concentration * Incubation	1.005E21	6	1.675E20	61.800	.000
Error	6.506E19	24	2.711E18		
Total	5.554E21	36			
Corrected Total	3.610E21	35			

[a]. R Squared = .982 (Adjusted R Squared = .974)

A.6.3 Comparison the antimicrobial property between normal chitosan and purified chitosan

Table A.6.11: Statistical analysis of MIC of chitosan samples

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	21267.361[a]	5	4253.472	9.800	.001
Intercept	55555.556	1	55555.556	128.000	.000
Bacteria	.000	1	.000	.000	1.000
Type	21267.361	2	10633.681	24.500	.000
Bacteria * Type	.000	2	.000	.000	1.000
Error	5208.333	12	434.028		
Total	82031.250	18			
Corrected Total	26475.694	17			

[a]. R Squared = .803 (Adjusted R Squared = .721)

Table A.6.12: Statistical analysis of MBC of chitosan samples

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	411729.601[a]	5	82345.920	10.765	.000
Intercept	489637.587	1	489637.587	64.007	.000
Bacteria	12207.031	1	12207.031	1.596	.230
Type	397243.924	2	198621.962	25.965	.000
Bacteria * Type	2278.646	2	1139.323	.149	.863
Error	91796.875	12	7649.740		
Total	993164.063	18			
Corrected Total	503526.476	17			

[a]. R Squared = .818 (Adjusted R Squared = .742)

Table A.6.13: Statistical analysis of inhibition diameter of chitosan samples

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2595.675[a]	9	288.408	596.707	.000
Intercept	6409.408	1	6409.408	13260.845	.000
Bacteria	35.208	1	35.208	72.845	.000
Type	2268.217	4	567.054	1173.216	.000
Bacteria * Type	292.250	4	73.063	151.164	.000
Error	9.667	20	.483		
Total	9014.750	30			
Corrected Total	2605.342	29			

[a]. R Squared = .996 (Adjusted R Squared = .995)

A.6.4 Comparison of the antimicrobial activity of normal and purified chitosan in the preservation of raw shrimp tissue

Table A.6.14: Weight loss of shrimp (%) during storage

Treatments	Day 5	Day 10	Day 15
nCTS	2.36 ± 0.28	2.94 ± 0.15	3.53 ± 0.14
pCTS	2.55 ± 0.07	3.28 ± 0.12	3.76 ± 0.19
Acetic	2.79 ± 0.17	3.57 ± 0.19	4.07 ± 0.15
Control	2.43 ± 0.19	3.24 ± 0.21	3.59 ± 0.18

Table A.6.15: Statistical analysis of weight loss of shrimp (%) during storage

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10.273[a]	11	.934	29.892	.000
Intercept	363.060	1	363.060	11620.600	.000
Treatment	1.372	3	.457	14.640	.000
Time	8.834	2	4.417	141.370	.000
Treatment * Time	.067	6	.011	.359	.898
Error	.750	24	.031		
Total	374.083	36			
Corrected Total	11.023	35			

[a]. R Squared = .932 (Adjusted R Squared = .901)

Table A.6.16: Hardness of shrimp (force - N) during storage

	0 day	5 days	10 days	15 days
nCTS	4.26 ± 0.21	4.28 ± 0.29	4.30 ± 0.29	3.96 ± 0.10
pCTS	4.26 ± 0.21	4.08 ± 0.26	4.09 ± 0.16	3.71 ± 0.21
Acetic	4.26 ± 0.21	3.93 ± 0.07	3.66 ± 0.20	3.59 ± 0.12
Control	4.26 ± 0.21	3.51 ± 0.09	3.30 ± 0.20	3.30 ± 0.12

Table A.6.17: Statistical analysis of the hardness of shrimp (%) during storage

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.850[a]	15	.390	10.164	.000
Intercept	737.822	1	737.822	19228.647	.000
Treatment	2.425	3	.808	21.068	.000
Time	2.433	3	.811	21.132	.000
Treatment * Time	.992	9	.110	2.874	.013
Error	1.228	32	.038		
Total	744.900	48			
Corrected Total	7.078	47			

[a]. R Squared = .827 (Adjusted R Squared = .745)

Table A.6.18: Total plate count (CFU/mL) of shrimp during storage

	Day 0	Day 5	Day 10	Day 15
nCTS	1.23E+07 ± 2.52E+06	1.93E+08 ± 2.52E+07	1.60E+09 ± 2.00E+08	1.23E+09 ± 5.77E+07
pCTS	1.23E+07 ± 2.52E+06	2.00E+08 ± 1.73E+07	1.47E+09 ± 1.15E+08	1.20E+09 ± 1.00E+08
Acetic	1.23E+07 ± 2.52E+06	1.83E+08 ± 1.53E+07	2.53E+09 ± 2.08E+08	1.83E+09 ± 5.77E+07
Control	1.23E+07 ± 2.52E+06	3.17E+08 ± 2.31E+07	2.63E+09 ± 2.52E+08	2.20E+09 ± 1.00E+08

Table A.6.19: Total plate count ($\log_{10}$ CFU/mL) of shrimp during storage

	Day 0	Day 5	Day 10	Day 15
nCTS	7.09 ± 0.09	8.28 ± 0.06	9.20 ± 0.05	9.09 ± 0.02
pCTS	7.09 ± 0.09	8.30 ± 0.04	9.17 ± 0.03	9.08 ± 0.04
Acetic	7.09 ± 0.09	8.26 ± 0.04	9.40 ± 0.04	9.26 ± 0.01
Control	7.09 ± 0.09	8.50 ± 0.03	9.42 ± 0.04	9.34 ± 0.02

Table A.6.20: Statistical analysis of total plate count of shrimp during storage

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.243E19 [a]	15	2.829E18	240.250	.000
Intercept	4.588E19	1	4.588E19	3896.551	.000
Treatment	2.862E18	3	9.539E17	81.011	.000
Time	3.692E19	3	1.231E19	1045.326	.000
Treatment * Time	2.646E18	9	2.940E17	24.971	.000
Error	3.768E17	32	1.177E16		
Total	8.869E19	48			
Corrected Total	4.281E19	47			

[a]. R Squared = .991 (Adjusted R Squared = .987)

APPENDIX – CHAPTER 7

A.7.1 Film forming capacity of chitosan

A.7.1.1 Effect of the neutralization to the solubility of chitosan films

Table A.7.1: The solubility (%) of neutralised and non-neutralised chitosan films

Replication	Non-neutralised film	Neutralised film
1	14.72	8.07
2	15.03	8.33
3	16.50	8.14
Average	15.42	8.18
STD	0.95	0.13

Table A.7.2: Statistical analysis of the solubility of neutralised and non-neutralised chitosan films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	78.554[a]	1	78.554	170.338	.000
Intercept	835.204	1	835.204	1811.068	.000
Film	78.554	1	78.554	170.338	.000
Error	1.845	4	.461		
Total	915.603	6			
Corrected Total	80.399	5			

a. R Squared = .977 (Adjusted R Squared = .971)

A.7.1.2 Effect of the degree of deacetylation of chitosan and the concentration of chitosan solution on the properties of chitosan film

Table A.7.3: The thickness (μm) of homogeneous chitosan films at different degree of deacetylation and concentrations

Replication	DD = 76 %			DD = 94 %		
	Chitosan concentration			Chitosan concentration		
	0.5 %	1 %	1.5 %	0.5 %	1 %	1.5 %
	Film A	Film B	Film C	Film D	Film E	Film F
1	8.75	17.60	25.45	9.50	18.00	23.33
2	9.00	18.35	26.00	9.00	19.75	21.67
3	9.25	16.8	26.05	9.50	19.00	24.25
Average	9.00	17.58	25.83	9.33	18.92	23.08
STD	0.25	0.78	0.33	0.29	0.88	1.31

Table A.7.4: Statistical analysis of the thickness (μm) of homogeneous chitosan films at different degree of deacetylation and concentrations

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	723.948[a]	5	144.790	260.252	.000
Intercept	5382.031	1	5382.031	9673.919	.000
DD	.587	1	.587	1.055	.325
Concentration	709.771	2	354.885	637.888	.000
DD * Concentration	13.590	2	6.795	12.214	.001
Error	6.676	12	.556		
Total	6112.655	18			
Corrected Total	730.624	17			

Table A.7.5: The solubility (%) of homogeneous chitosan films at different degree of deacetylation and concentrations

Replication	DD = 76 %			DD = 94 %		
	Chitosan concentration			Chitosan concentration		
	0.5 %	1 %	1.5 %	0.5 %	1 %	1.5 %
	Film A	Film B	Film C	Film D	Film E	Film F
1	10.46	10.58	10.00	16.86	14.67	14.95
2	11.81	10.31	11.62	15.45	15.68	15.59
3	10.35	10.04	10.05	15.24	15.87	15.59
Average	10.87	10.31	10.56	15.85	15.41	15.38
STD	0.81	0.27	0.92	0.88	0.65	0.37

Table A.7.6: Statistical analysis of the solubility (%) of homogeneous chitosan films at different degree of deacetylation and concentrations

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	111.806[a]	5	22.361	46.086	.000
Intercept	3071.190	1	3071.190	6329.667	.000
DD	110.906	1	110.906	228.575	.000
Concentration	.842	2	.421	.868	.445
DD * Concentration	.058	2	.029	.060	.943
Error	5.822	12	.485		
Total	3188.818	18			
Corrected Total	117.628	17			

Table A.7.7: The mechanical properties of homogeneous chitosan films at different degree of deacetylation and concentrations

Replication	DD = 76 %			DD = 94 %		
	Chitosan concentration			Chitosan concentration		
	0.5 %	1 %	1.5 %	0.5 %	1 %	1.5 %
	Film A	Film B	Film C	Film D	Film E	Film F
Tensile strength (MPa)						
1	40.59	40.42	41.17	40.07	40.88	40.39
2	40.42	40.54	40.58	40.27	40.16	40.56
3	39.53	40.66	39.76	40.72	40.58	40.41
Average	40.18	40.54	40.50	40.35	40.54	40.45
STD	0.57	0.12	0.71	0.34	0.36	0.09
Deformation (%)						
1	17.43	31.40	31.45	17.24	32.14	31.47
2	18.00	31.18	32.09	17.55	31.34	30.12
3	17.44	30.92	32.56	17.03	31.25	31.18
Average	17.63	31.16	32.03	17.27	31.58	30.93
STD	0.33	0.24	0.56	0.26	0.49	0.71

Table A.7.8: Statistical analysis of the mechanic properties of homogeneous chitosan films at different degree of deacetylation and concentrations

Dependent Variable: Tensile strength

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.295[a]	5	.059	.325	.888
Intercept	29420.102	1	29420.102	161915.809	.000
DD	.008	1	.008	.042	.841
Concentration	.247	2	.123	.679	.526
DD * Concentration	.041	2	.021	.113	.894
Error	2.180	12	.182		
Total	29422.578	18			
Corrected Total	2.476	17			

Dependent Variable: Deformation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	783.707[a]	5	156.741	730.105	.000
Intercept	12895.645	1	12895.645	60068.215	.000
DD	.551	1	.551	2.568	.135
Concentration	781.423	2	390.711	1819.943	.000
DD * Concentration	1.733	2	.866	4.036	.046
Error	2.576	12	.215		
Total	13681.928	18			
Corrected Total	786.283	17			

A.7.2 Improvement of the solubility and seal-ability of chitosan films

A.7.2.1 Film forming capacity of some biopolymers

Table A.7.9: The thickness (μm) of homogeneous edible films

Biopolymer	**Code**	**Average**	**STD**
Chitosan 1 %	CHI1	18.92	0.88
Agar 1 %	A1	33.85	0.48
Agar 2 %	A2	39.66	1.02
Agar 3 %	A3	51.57	0.35
Carrageenan 2 %	CAR2	30.38	1.01
Carrageenan 3 %	CAR3	41.33	0.49
Carrageenan 4 %	CAR4	44.33	0.74
CMC 1 %	CMC1	20.56	1.71
CMC 1.5 %	CMC1.5	26.42	0.88
CMC 2 %	CMC2	31.97	0.34
Corn starch 1 %	S1	24.71	0.73
Corn starch 2 %	S2	33.41	0.38
Corn starch 3 %	S3	52.85	1.12
Gelatin 3 %	G3	45.38	0.53
Gelatin 4 %	G4	53.24	0.75
Gelatin 5 %	G5	60.49	0.30
Pectin 1 %	P1	19.38	1.35
Pectin 1.5 %	P1.5	25.47	1.28
Pectin 2 %	P2	32.47	1.35

Table A.7.10: Statistical analysis of the thickness (μm) of homogeneous edible films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8152.444[a]	18	452.914	542.616	.000
Intercept	75181.674	1	75181.674	90071.808	.000
Biomaterial	8152.444	18	452.914	542.616	.000
Error	31.718	38	.835		
Total	83365.836	57			
Corrected Total	8184.162	56			

a. R Squared = .996 (Adjusted R Squared = .994)

Table A.7.11: The solubility (%) of homogeneous edible films

Biopolymer	**Code**	**Average**	**STD**
Agar 1 %	A1	93.33	3.06
Agar 2 %	A2	84.00	4.00
Agar 3 %	A3	67.33	3.06
Carrageenan 2 %	CAR2	96.67	2.31
Carrageenan 3 %	CAR3	93.33	3.06
Carrageenan 4 %	CAR4	70.00	7.21
CMC 1 %	CMC1	96.67	2.31
CMC 1.5 %	CMC1.5	96.00	2.00
CMC 2 %	CMC2	92.00	6.00
Corn starch 1 %	S1	39.33	3.06
Corn starch 2 %	S2	30.67	1.15
Corn starch 3 %	S3	15.33	1.15
Gelatin 3 %	G3	98.67	1.15
Gelatin 4 %	G4	97.33	2.31
Gelatin 5 %	G5	90.67	1.15
Pectin 1 %	P1	92.67	3.06
Pectin 1.5 %	P1.5	92.00	6.00
Pectin 2 %	P2	90.67	6.11
Chitosan 1 %	CHI1	15.41	0.65

Table A.7.12: Statistical analysis of the solubility (%) of homogeneous edible films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	39976.702[a]	18	2220.928	168.342	.000
Intercept	339957.965	1	339957.965	25768.090	.000
Biomaterial	39976.702	18	2220.928	168.342	.000
Error	501.333	38	13.193		
Total	380436.000	57			
Corrected Total	40478.035	56			

a. R Squared = .988 (Adjusted R Squared = .982)

Table A.7.13: The mechanic properties of homogeneous edible films

Biopolymer	Tensile strength (MPa)	Deformation (%)
Agar 1 %	30.74 ± 1.95	3.27 ± 0.05
Agar 2 %	34.83 ± 0.81	9.19 ± 0.91
Agar 3 %	42.28 ± 1.60	11.81 ± 1.59
Carrageenan 2 %	33.94 ± 0.23	13.81 ± 0.45
Carrageenan 3 %	19.39 ± 0.55	24.70 ± 1.10
Carrageenan 4 %	25.80 ± 1.19	29.77 ± 2.40
CMC 1 %	32.03 ± 1.84	27.42 ± 1.46
CMC 1.5 %	28.33 ± 0.51	13.96 ± 1.07
CMC 2 %	31.96 ± 0.80	25.16 ± 1.61
Corn starch 1 %	7.88 ± 0.49	1.64 ± 0.13
Corn starch 2 %	20.66 ± 0.94	2.22 ± 0.14
Corn starch 3 %	15.96 ± 0.67	2.33 ± 0.11
Gelatin 3 %	15.25 ± 2.58	23.55 ± 0.29
Gelatin 4 %	14.10 ± 1.48	26.87 ± 0.27
Gelatin 5 %	13.37 ± 2.21	29.18 ± 1.42
Pectin 1 %	21.39 ± 0.78	15.58 ± 1.81
Pectin 1.5 %	29.99 ± 1.44	6.24 ± 0.60
Pectin 2 %	32.00 ± 0.60	16.44 ± 0.72
Chitosan 1 %	40.54 ± 0.36	31.58 ± 0.49

Table A.7.14: Statistical analysis of the mechanic properties of homogeneous edible films

Dependent Variable:Tensile

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4548.130[a]	18	252.674	143.815	.000
Intercept	34674.624	1	34674.624	19735.900	.000
Biomaterial	4548.130	18	252.674	143.815	.000
Error	66.763	38	1.757		
Total	39289.517	57			
Corrected Total	4614.893	56			

a. R Squared = .986 (Adjusted R Squared = .979)

Dependent Variable:Deformation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5241.396[a]	18	291.189	62.673	.000
Intercept	13528.902	1	13528.902	2911.837	.000
Biomaterial	5241.396	18	291.189	62.673	.000
Error	176.555	38	4.646		
Total	18946.853	57			
Corrected Total	5417.951	56			

a. R Squared = .967 (Adjusted R Squared = .952)

Table A.7.15: The water vapor permeation (WVP) ($g.m^{-1}. s^{-1}.Pa^{-1}$) of homogeneous edible films

Biopolymer	Water vapor permeation (WVP)	STD
Agar 1 %	9.66E-11	2.01E-12
Agar 2 %	2.82E-10	1.64E-11
Agar 3 %	9.08E-11	3.78E-12
Carrageenan 2 %	6.76E-11	6.61E-12
Carrageenan 3 %	9.52E-11	7.25E-12
Carrageenan 4 %	1.62E-10	2.31E-12
Gelatin 3 %	5.62E-11	1.02E-11
Gelatin 4 %	1.01E-10	5.86E-12
Gelatin 5 %	1.47E-10	1.47E-11
Corn starch 1 %	8.30E-11	7.06E-12
Corn starch 2 %	5.35E-11	4.42E-12
Corn starch 3 %	1.37E-10	3.12E-12
CMC 1 %	5.98E-11	2.12E-12
CMC 1.5 %	6.18E-11	6.40E-12
CMC 2 %	6.88E-11	3.73E-12
Pectin 1 %	5.87E-11	2.32E-13
Pectin 1.5 %	5.73E-11	4.71E-12
Pectin 2 %	7.16E-11	7.38E-12
Chitosan 1 %	6.27E-11	1.53E-12
LDPE	2.32E-12	3.81E-25

Table A.7.16: Statistical analysis of the water vapor permeation (WVP) of homogeneous edible films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.907E-19[a]	19	1.004E-20	193.200	.000
Intercept	.000	1	.000	.000	1.000
Biomaterial	.000	19	.000	.000	1.000
Error	2.078E-21	40	5.195E-23		
Total	6.881E-19	60			
Corrected Total	1.928E-19	59			

a. R Squared = .989 (Adjusted R Squared = .984)

Table A.7.17: The mechanical properties of the seal of 5 % gelatine films

Replication	Tensile strength (MPa)	Deformation (%)
1	1.71	11.44
2	1.41	10.82
3	1.72	9.40
Average	1.61	10.55
STD	0.18	1.05

A.7.2.2 The effect of blending ratio to the forming film properties

Table A.7.18: The thickness (μm) of composite edible films

	% Gelatin	% CTS	Average	STD
Film 1	3	0.50	40.18	0.75
Film 2	3	0.75	42.60	0.30
Film 3	3	1.00	47.83	1.43
Film 4	4	0.50	52.52	0.28
Film 5	4	0.75	54.83	0.35
Film 6	4	1.00	57.47	0.37
Film 7	5	0.50	57.78	0.16
Film 8	5	0.75	59.20	0.69
Film 9	5	1.00	60.70	0.58

Table A.7.19: Statistical analysis of the thickness of composite edible films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	125.624[a]	8	15.703	63.912	.000
Intercept	1686.777	1	1686.777	6865.249	.000
Gelatin	53.021	2	26.510	107.899	.000
Chitosan	54.043	2	27.022	109.979	.000
Gelatin * Chitosan	18.560	4	4.640	18.885	.000
Error	4.423	18	.246		
Total	1816.824	27			
Corrected Total	130.047	26			

a. R Squared = .966 (Adjusted R Squared = .951)

Table A.7.20: The solubility (%) of composite edible films

	% Gelatin	% CTS	Average	STD
Film 1	3	0.50	77.98	0.38
Film 2	3	0.75	75.86	0.51
Film 3	3	1.00	73.67	0.44
Film 4	4	0.50	80.29	1.02
Film 5	4	0.75	77.14	0.21
Film 6	4	1.00	75.94	0.71
Film 7	5	0.50	84.69	0.68
Film 8	5	0.75	80.68	0.56
Film 9	5	1.00	78.32	0.60

Table A.7.21: Statistical analysis of the solubility (%) of composite edible films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	254.508[a]	8	31.813	86.571	.000
Intercept	165472.962	1	165472.962	450284.714	.000
Gelatin	134.035	2	67.017	182.368	.000
Chitosan	115.113	2	57.556	156.622	.000
Gelatin * Chitosan	5.360	4	1.340	3.646	.024
Error	6.615	18	.367		
Total	165734.084	27			
Corrected Total	261.122	26			

a. R Squared = .975 (Adjusted R Squared = .963)

Table A.7.22: The mechanical properties of composite edible films

	% Gelatin	% CTS	Tensile strength (MPa)	Deformation (%)
Film 1	3	0.50	26.78 ± 0.38	23.84 ± 0.21
Film 2	3	0.75	28.11 ± 0.28	21.50 ± 0.73
Film 3	3	1.00	29.88 ± 0.41	20.33 ± 0.42
Film 4	4	0.50	24.05 ± 0.37	61.21 ± 0.57
Film 5	4	0.75	25.53 ± 0.45	33.50 ± 0.54
Film 6	4	1.00	26.70 ± 0.57	31.51 ± 0.17
Film 7	5	0.50	23.03 ± 0.55	66.81 ± 0.95
Film 8	5	0.75	23.65 ± 0.43	51.62 ± 0.83
Film 9	5	1.00	24.90 ± 0.37	43.87 ± 0.68

Table A.7.23: Statistical analysis of the mechanic properties of composite edible films

Dependent Variable:Tensile

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	119.665[a]	8	14.958	80.351	.000
Intercept	18037.406	1	18037.406	96892.534	.000
Gelatin	89.205	2	44.602	239.593	.000
Chitosan	29.046	2	14.523	78.013	.000
Gelatin * Chitosan	1.414	4	.354	1.899	.154
Error	3.351	18	.186		
Total	18160.422	27			
Corrected Total	123.016	26			

a. R Squared = .973 (Adjusted R Squared = .961)

Dependent Variable:Deformation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7259.397[a]	8	907.425	2380.552	.000
Intercept	41813.310	1	41813.310	109693.704	.000
Gelatin	4769.723	2	2384.861	6256.483	.000
Chitosan	1772.677	2	886.338	2325.234	.000
Gelatin * Chitosan	716.998	4	179.249	470.246	.000
Error	6.861	18	.381		
Total	49079.569	27			
Corrected Total	7266.259	26			

a. R Squared = .999 (Adjusted R Squared = .999)

Table A.7.24: The water vapor permeation (WVP) ($g.m^{-1}.\ s^{-1}.Pa^{-1}$) of composite edible films

	% Gelatin	% CTS	Water vapor permeation ($10^{-10}.g.m^{-1}.\ s^{-1}.Pa^{-1}$)	STD ($10^{-10}.g.m^{-1}.\ s^{-1}.Pa^{-1}$)
Film 1	3	0.50	1.5246	0.0060
Film 2	3	0.75	1.4287	0.0069
Film 3	3	1.00	0.8687	0.0006
Film 4	4	0.50	1.8125	0.0068
Film 5	4	0.75	1.6590	0.0050
Film 6	4	1.00	1.4571	0.0092
Film 7	5	0.50	1.9375	0.0502
Film 8	5	0.75	1.6863	0.0084
Film 9	5	1.00	1.5875	0.0073
LDPE	-	-	0.0145	0.0090

Table A.7.25: Statistical analysis of the water vapor permeation (WVP) of composite edible films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.217[a]	8	.277	865.278	.000
Intercept	64.979	1	64.979	202859.700	.000
Gelatin	1.078	2	.539	1683.296	.000
Chitosan	.948	2	.474	1479.859	.000
Gelatin * Chitosan	.191	4	.048	148.980	.000
Error	.006	18	.000		
Total	67.202	27			
Corrected Total	2.223	26			

a. R Squared = .997 (Adjusted R Squared = .996)

Table A.7.26: The tensile strength (MPa) of the seal of composite edible films

	% Gelatin	% Chitosan	Tensile strength (MPa)	STD
Film 1	3	0.50	7.93	0.90
Film 2	3	0.75	6.39	0.61
Film 3	3	1.00	5.55	0.24
Film 4	4	0.50	8.23	0.27
Film 5	4	0.75	6.83	0.33
Film 6	4	1.00	6.65	0.30
Film 7	5	0.50	13.27	0.39
Film 8	5	0.75	9.21	0.44
Film 9	5	1.00	7.08	0.59

Table A.7.27: Statistical analysis of the mechanic properties of the seal of composite edible films

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	125.624[a]	8	15.703	63.912	.000
Intercept	1686.777	1	1686.777	6865.249	.000
Gelatin	53.021	2	26.510	107.899	.000
Chitosan	54.043	2	27.022	109.979	.000
Gelatin * Chitosan	18.560	4	4.640	18.885	.000
Error	4.423	18	.246		
Total	1816.824	27			
Corrected Total	130.047	26			

a. R Squared = .966 (Adjusted R Squared = .951)

A.7.3 Application of composite edible films in spice casing for instant noodles

Table A.7.28: The weight loss (%) of the seasoning bags during 6 months of storage

Sample	1 month	2 months	3 months	4 months	5 months	6 months
A	-0.0412 ± 0.0029	0.4539 ± 0.1739	-0.1160 ± 0.0113	0.3756 ± 0.1063	-0.6474 ± 0.0116	-2.5660 ± 0.1908
B	-0.1109 ± 0.0419	0.0235 ± 0.0117	-0.0444 ± 0.0120	0.0445 ± 0.0061	-0.2327 ± 0.0028	-0.1875 ± 0.0447
C	0.0290 ± 0.0111	0.0482 ± 0.0095	0.1786 ± 0.0717	0.0542 ± 0.0107	-0.0332 ± 0.0053	0.0905 ± 0.0130
D	0.0261 ± 0.0094	0.0390 ± 0.0095	0.0536 ± 0.0110	0.0305 ± 0.0078	-0.1088 ± 0.0153	0.0402 ± 0.0033

Note: A: Mixed bag - Ambient environment
B: PE bag - Ambient environment
C: Mixed bag - OPP wrapped
D: PE bag - OPP wrapped

Table A.7.29: Statistical analysis of the weight loss (%) of the seasoning bags

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	21.894[a]	27	.811	256.277	.000
Intercept	.723	1	.723	228.493	.000
Sample	2.216	3	.739	233.496	.000
Month	5.654	6	.942	297.832	.000
Sample * Month	14.023	18	.779	246.223	.000
Error	.177	56	.003		
Total	22.794	84			
Corrected Total	22.071	83			

a. R Squared = .992 (Adjusted R Squared = .988)

Table A.7.30: The weight loss (%) of the oil bags during 6 months of storage

Sample	1 month	2 months	3 months	4 months	5 months	6 months
A	0.6285 ± 0.0489	1.0082 ± 0.1619	2.1541 ± 0.1305	2.2111 ± 0.1167	2.3004 ± 0.0705	2.4997 ± 0.0430
B	0.1628 ± 0.0097	0.0877 ± 0.0046	-0.3059 ± 0.0568	-0.5732 ± 0.0515	-0.6891 ± 0.0181	-0.6850 ± 0.0149
C	0.2356 ± 0.0299	0.3684 ± 0.0470	0.6630 ± 0.0815	0.7994 ± 0.0778	0.8518 ± 0.0515	0.8872 ± 0.0146
D	0.0992 ± 0.0261	0.1169 ± 0.0371	0.1907 ± 0.0329	0.2278 ± 0.0272	0.2488 ± 0.0157	0.3179 ± 0.0233

Note: *A: Mixed bag - Ambient environment* *C: Mixed bag - OPP wrapped*
B: PE bag - Ambient environment *D: PE bag - OPP wrapped*

Table A.7.31: Statistical analysis of the weight loss (%) of the oil bags

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	60.394[a]	27	2.237	652.826	.000
Intercept	20.422	1	20.422	5960.338	.000
Sample	38.129	3	12.710	3709.372	.000
Month	5.562	6	.927	270.527	.000
Sample * Month	16.703	18	.928	270.834	.000
Error	.192	56	.003		
Total	81.008	84			
Corrected Total	60.586	83			

a. R Squared = .997 (Adjusted R Squared = .995)

Table A.7.32: The moisture content (%) of the seasoning during storage

Sample	0 month	3 months	4 months	5 months	6 months
A	0.4644 ± 0.0841	0.3927 ± 0.0585	0.3476 ± 0.0700	0.3517 ± 0.0160	1.1204 ± 0.0418
B	0.4644 ± 0.0841	0.2223 ± 0.0388	0.1532 ± 0.0386	-2.0293 ± 2.2340	0.2404 ± 0.0288
C	0.4644 ± 0.0841	0.3622 ± 0.0219	0.1978 ± 0.0306	-1.8354 ± 0.0601	0.2585 ± 0.0239
D	0.4644 ± 0.0841	0.2515 ± 0.0383	0.0885 ± 0.0836	-2.4161 ± 1.0644	0.2894 ± 0.0257

Note: A: Mixed bag - Ambient environment *C: Mixed bag - OPP wrapped*

B: PE bag - Ambient environment *D: PE bag - OPP wrapped*

Table A.7.33: The moisture content (%) of the oil during storage

Sample	0 month	3 months	4 months	5 months	6 months
A	0.9866 ± 0.1546	5.6391 ± 1.0737	0.0000 ± 0.0000	-0.8848 ± 0.3427	-0.3116 ± 0.1514
B	0.9866 ± 0.1546	0.2055 ± 0.4294	1.0278 ± 0.9749	-5.1944 ± 6.2372	-1.4716 ± 0.2925
C	0.9866 ± 0.1546	-0.5144 ± 0.4279	0.1684 ± 0.2917	0.7384 ± 0.1286	0.2623 ± 0.0911
D	0.9866 ± 0.1546	0.0087 ± 0.3056	0.2252 ± 0.1972	0.4450 ± 0.0333	0.3545 ± 0.2461

Note: A: Mixed bag - Ambient environment *C: Mixed bag - OPP wrapped*

B: PE bag - Ambient environment *D: PE bag - OPP wrapped*