CHARACTERIZATION OF CHITIN AND CHITOSAN FROM SQUID PENS

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Thesis Title	Characterization of Chitin and Chitosan from Squid Pens
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ชื่อวิทยานิพนธ์ การศึกษาคุณลักษณะของไคตินและไคโตแซนจากกระดองปลาหมึก

ผู้เขียน นายประสาท ศรประสิทธิ์

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บทคัดย่อ

วิทยานิพนธ์ฉบับนี้กล่าวถึงการศึกษาคุณลักษณะของเบต้า-ไคตินและไคโตแซนซึ่งเตรียมจากกระดองปลาหมึก 2 ชนิด คือ ปลาหมึกกล้วย (Splendid squid: *Loligo formosana*) และปลาหมึกหอม (Soft cuttlefish : *Loligo lessoniana*) การทดลองมุ่งชี้ให้เห็นถึงคุณสมบัติทั่วไปทางเคมี ชีวเคมีและฟิสิกส์ของไคติน และไคโตแซนที่เก็บในช่วงฤดูกาล และสภาวะการเตรียมที่ต่างกัน การศึกษาประกอบด้วย การหาปริมาณผลผลิต การวิเคราะห์หาปริมาณโลหะ การหมุนแสงราบเดี่ยว (optical activity)ระดับการกำจัดหมู่อะซีติล (degree of deacetylation) น้ำหนักโมเลกุล (viscosity average molecular weight, Mv) การดูดความชื้น การจับอิออนโลหะและความว่องไวในการย่อยสลายไคตินโดยเอนไซม์ ในบางคุณลักษณะได้ทำการศึกษาเปรียบเทียบกับไคตินและไคโตแซนที่เตรียมจากเปลือกกุ้งกุลาดำ (*Penaeus monodon*) ภายใต้สภาวะที่เหมือนกัน

จากการศึกษาพบว่ากระดองปลาหมึกอบแห้งมีปริมาณเถ้า(ash)น้อยมาก ดังนั้นขั้นตอนในการกำจัดแร่ธาตุจึงได้ข้ามไป การเตรียมไคตินทำโดยกำจัดโปรตีนด้วยโซเดียมไฮดรอกไซด์เข้มข้น 1.0 โมลาร์ ที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 5 ชั่วโมง ผลผลิตไคตินที่ได้จากกระดองปลาหมึกทั้ง 2 ชนิดมีปริมาณร้อยละ36 ส่วนไคโตแซนซึ่งเตรียมได้จากไคติน โดยกำจัดหมู่อะซีติลด้วยโซเดียมไฮดรอกไซด์เข้มข้นร้อยละ 50 (น้ำหนัก/ปริมาตร) ที่อุณหภูมิ 60 องศาเซลเซียส ภายใต้บรรยากาศไนโตรเจนเป็นเวลาประมาณ 5 ชั่วโมง ผลผลิตมีปริมาณร้อยละ 27 โดยเทียบกับน้ำหนักกระดองปลาหมึกอบแห้ง ไคตินและไคโตแซนที่ได้เป็นผงสีขาวนวลและมีธาตุโลหะปนเปื้อนน้อยมาก นอกจากนี้ไคตินและไคโตแซนที่เตรียมภายใต้สภาวะที่เหมือนกันและช่วงฤดูกาลที่เก็บต่างกันของกระดองปลาหมึก ทั้ง 2 ชนิดแสดงคุณลักษณะที่ตรงกันทั้งทางด้านเคมี ชีวเคมีและฟิสิกส์

สารละลายไคตินสามารถหมุนแสงราบเดี่ยวไปทางซ้ายและไม่เปลี่ยนแปลงการหมุนตลอดระยะเวลาการเก็บที่อุณหภูมิห้องนาน 2 สัปดาห์ ไคโตแซนที่เตรียมได้มีระดับการกำจัดหมู่อะซีติลสูงกว่า

ร้อยละ 90 และมีน้ำหนักโมเลกุล (Mv) ประมาณ 9.5 x 10^6 ดาลตัล ไคตินสามารถดูดความชื้นได้สูงถึงร้อยละ 21 เมื่อเปิดทิ้งไว้ที่อุณหภูมิห้องในขณะที่ไคโตแซนสามารถดูดความชื้นได้น้อยกว่าซึ่งอยู่ในช่วงร้อยละ 17-19 ขึ้นอยู่กับระยะเวลาที่ใช้ในการกำจัดหมู่อะซีติล

การจับอิออนโลหะของไคตินและไคโตแซนพบว่าขึ้นอยู่กับหลายปัจจัยเช่นแหล่งของวัตถุดิบที่ใช้เตรียมไคตินและไคโตแซน ระดับการกำจัดหมู่อะซีติลชนิดและความเข้มข้นของอิออนโลหะเป็นต้น พบว่าความชอบในการจับของอิออนทองแดงกับไคโตแซนต่ำกว่าอิออนตะกั่วโดยที่ไคโตแซนจับอิออนทองแดงได้ 200 มิลลิกรัม และจับอิออนตะกั่วได้ถึง 900 มิลลิกรัมต่อไคโตแซน1 กรัม ในขณะที่ไคโตแซนจากเปลือกกุ้งสามารถจับกับอิออนโลหะทั้งสองได้ในปริมาณที่ต่ำกว่าครึ่งหนึ่งของปริมาณที่จับโดยไคโตแซนจากกระดองปลาหมึก ความว่องไวในการย่อยสลายไคตินโดยไคติเนสและเบต้า-กลูโคซิเดส สามารถหาได้โดยใช้เบต้า-กลูโคซิเดสจากชีรั่มกุ้ง โดยไคตินจากกระดองปลาหมึกถูกย่อยสลายได้ง่ายกว่าไคตินจากเปลือกกุ้ง

นอกจากคุณสมบัติที่ต้องกันแล้ว จากตัวอย่างที่ศึกษาในครั้งนี้สามารถชี้และแสดงให้เห็นว่าไคตินและไคโตแซนที่เตรียมจากกระดองปลาหมึกทั้ง 2 ชนิด ภายใต้สภาวะที่เหมาะสมมีคุณภาพดีกว่าและสูงกว่าไคติน และไคโตแซนที่ได้จากแหล่งอื่น ๆ ดังนั้นมีเหตุผลที่ยืนยันได้ว่า ไคตินและไคโตแซนที่เตรียมได้นี้น่าที่จะมีความเหมาะสมในการนำมาประยุกต์ใช้ทางการแพทย์ได้ดี

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ABSTRACT

This thesis describes a study on characteristics of β-chitin and chitosan prepared from two species of squid pen, namely *Loligo lessoniana* and *Loligo formosana*. Experiments were designed to elucidate general chemical, biochemical and physical properties of the chitinous materials. These were included the percent yield for each preparation step, proximate and trace metal analyses, optical activity, degree of deacetylation, viscosity average molecular weight, moisture and metal ions adsoption, and rate of enzymatic hydrolysis. In some circumstances, the characteristics of chitin and chitosan from the prawn shell (*Penaeus monodon*) prepared under identical conditions were also compared.

In preparation of chitin from two species of the squid pens demineralization was omitted from the preparation scheme. Since both contained negligible amount of ash. Deproteinization was performed by treatment of the pen powder with 1M NaOH at 50°C for 5 hours, and this provided 36% yield. Deacetylation of the chitin with 50% (w/v) NaOH at 60°C under nitrogen atmosphere for approximately 5 hours gave chitosan of more than 27% yield. Chitin and chitosan products were white soft powder comprising a negligible amount of trace elements. Chitinous materials from two species of the squid pen prepared under identical condition exhibited consistency in chemical, biochemical and physical characteristics, regardless of season of sample collection.

The chitin solution showed levorotatory activity which did not change after standing at ambient temperature for 2 weeks.The chitosan product prepared by the use of conditions described above carried more than 90% degree of deacetylation, and

gained viscosity average molecular weight of approximately 9.5 x 10^6 daltons. Chitin from the squid pens exhibited a high hygroscopic activity when deproteinized with 1M NaOH at 50°C for 5 hours. It can adsorb moisture about 21%. For chitosans, the ability to adsorb moisture was lower than that of the chitin, and falled within a range of 17-19% due to the deacetylation period.

Chelation properties of chitin and chitosan were influenced by several factors such as source, degree of deacetylation, type and concentration of metal ion. Binding affinity of Cu (II) on chitosan was lower than Pb (II). Chitosan from squid pen adsorbed Cu (II) and Pb (II) about 200 and 900 mg/gm of the sample, respectively. In comparison, half lower amounts were found on binding with the chitosan from prawn shell. An activity of chitinolytic enzyme system on native form of chitin could be assayed by the use of β-glucosidase from prawn hemolymph. Chitins from squid pens were hydrolyzed easier than the comparable sample from prawn shell.

Apart from the consistency in properties, a number of instances in this study indicated that chitin and chitosan from both species of squid pen prepared under optimum conditions exhibited superior quality than those from other sources. It is thus quite reasonable to propose that these chitinous materials are suitable to use in medical field of application.

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I would like to express my appreciation to all my teachers, technicians, staff members of the Department of Biochemistry, and all my friends for their helpful suggestions and friendship. Finally, I am indebted and thankful to my father, mother and sister for their encouragement and attention throughout my life. Special thanks and love go to my wife, Sudchada, and my daughter, Dhirada, for her sacrifices and understanding throughout this study.

Prasart Sornprasit

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LIST OF ABBREVIATIONS

approx.	=	approximately
As	=	arsenic
Ca	=	calcium
Cd	=	cadmium
cps	=	centipoise
$CuSO_4.5H_2O$	=	cupric sulphate pentahydrate
Cu	=	copper
Cu (II)	=	cupric ions
dal	=	dalton
dl	=	decilitre
DMAc	=	dimethylacetamide
EDTA	=	ethylenediaminetetraaceticacid
Fe	=	iron
gm	=	gram
HCl	=	hydrochloric acid
Hg	=	mercury
H_2SO_4	=	sulfuric acid
Kg	=	kilogram
KOH	=	potassium hydroxide
K_2SO_4	=	potassium sulphate
$LiCl_2$	=	lithium chloride
M	=	molar
Mg	=	magnesium
mg	=	milligram
ml	=	millilitre

LIST OF ABBREVIATIONS (continue)

mm	=	millimeter
Mn	=	number average molecular weight
Mr	=	relative average molecular weight
Mv	=	viscosity average molecular weight
Mw	=	weight average molecular weight
µm	=	micrometre
µmole	=	micromole
N	=	normal
NaOH	=	sodium hydroxide
$(NH_4)_2SO_4$	=	ammonium sulphate
nm	=	nanometre
Pb	=	lead
Pb (II)	=	lead ions
$Pb(NO_3)_2$	=	lead nitrate
ppm	=	part per million
UV	=	ultraviolet
p -	=	para -
^oC	=	degree celsius
rpm	=	revolution per minute
U	=	unit
v/v	=	volume/volume
w/v	=	weight/volume
α -	=	alpha -
β -	=	beta -
γ -	=	gamma -
%	=	percent

1. INTRODUCTION

Chitin is a linear polymer of N-acetyl-D-glucosamine linked by β (1-4) glycosidic bond. It is recognized to be the second most abundant natural polysaccharides on earth, next to cellulose. It occurs primarily as a structural component in the exoskeleton of crustaceans, insects, squid pen, and also be found to a lesser extent in other animals and plants. Chemically, chitin is fairly dull molecule and insoluble in most ordinary solvents such as water, alcohols, acetone, hexane, diluted acids, diluted and concentrated alkalines. Hence, these restrict a scope of application for chitin.

Deacetylation of chitin with concentrated alkali produces poly-D-glucosamine or chitosan. Chitosan molecule carries strong positive charges under acidic aqueous media, because it possesses a high density of free amino group per unit weight. This causes the molecule more soluble in weak acidic solvent such as acetic acid or formic acid. Since the pKa of the ammonium group is about 6.2, when the pH is raised above 6.5, chitosan precipitates in gelatinous flocculation form. Under basic condition, on the other hand, chitosan carries lone pair electrons of uncharged amino groups. It thus can chelate transition metal ions with high binding capacity. By these reasons, chitosan receives more attention than its parent molecule.

Apart from chitosan, a number of chitin derivatives have been synthesized in order to use for modifying chemical and physical properties of chitin and chitosan for specific purpose. These are, for example, carboxymethyl chitin, hydroxyethyl chitin, ethyl chitin, glycol chitin, glucosylated chitin, nitrochitin, chitin sulphate, and cyanoethylated chitosan *etc.* Since chitin and its derivatives exhibit varieties of chemical and physical properties, there are widely used in several fields of application. For example, due to the high charge density giving a high potential binding capacity, they are used as the ion-exchanger, metal recovery from waste streams. Film forming property is used for ion-exchange membrane for electrodialysis. Coagulation property is used for waste water treatment. Strong binding to negative charge polymeric products is

used for paper-strenghtening additive, dye binder for textile, binding agent for nonwoven fabrics, sausage-casing component, adhesives *etc.* Also, wound healing promotion and nonthrombogenic properties are used for wound treatment and surgical adjunct. Although there are other compete synthesized products, chitin and its derivatives are used up as waste and are biodegradable. These make them more acceptable by users than those of the synthetic products.

Chitin and its derivatives have been studied extensively and many forms are commercially available, but most of them are obtained from crab and shrimp shells. Although it was noted that squid pen comprising of chitin by 41% of the dried weight, with especially for the *Loligo* species, detail information of the chitin from these sources is very limited.

Thailand exports various form of squid which is accounted for more than 60 metric tons anually, and that produces waste as squid pens for approximately 600 tons. Therefore, chitin could be commercially prepared from the waste at least 200 tons per year. It was claimed that chitin and chitosan prepared from squid pen possessed superior quality. Their prices were expected to be much higher than those prepared from prawn and crab shells. In addition, preliminary study in this laboratory indicated that chitin from squid pens consists of a very trace amount of ash. Therefore, classical demineralization step which can be rapidly depolymerized chitin, could be omitted from the preparation scheme. Thus, chitin from squid pen is not only a superior quality product but it also be able to manufactured without acid pollution and in a low production cost.

The aim of this thesis is to clarify and compare the physicochemical and biochemical properties of chitin and chitosan prepared from two species of squid pen, namely, *Loligo lessoniana* and *Loligo formosana.* The effects of duration for deacetylation reaction which is thought to be a principal factor determining properties of chitosan were also evaluated. In some circumstances, comparison was made among

chitin and chitosan from two species of squid pen and from prawn shell (*Penaeus monodon*) prepared under identical conditions.

Literature Review

1.1 Scope of Review

This review attempts to provide essential knowledge of chitin, chitosan and their derivatives. Due to the ease of accessibility, chitins from prawn and crab shells, which are α-form, have been received more attention and well documented. In contrast, a study on β–form of chitin which is principally found in squid pen has been paid very scarcely interested. Therefore, most essential information in this review represent for the α-form of chitin, unless otherwise indicated. The following topics will be covered: distribution of chitin in nature, chemical structure and complex forms in nature, biosynthetic pathway, enzymatic hydrolysis, preparation of chitin and chitosan, chemical and physical characterization, some properties, other chitin derivatives and their applications, summary and conclusion.

1.2 Distribution of Chitin in Nature

In 1811, Henri Bracannot was the first who described chitin. Because it was found in the insoluble fraction after extraction of fungi with water, alcohol and dilute alkali, he called it as "fungine" (cited by Kong, 1975 and Nicol, 1991). Since then, chitin from numerous sources in both animal and plant kingdoms have been reported as summarized in Table 1.1. It was noted that chitin content among organisms varies enormously from trace up to about 40% of the body weight.

Principally, chitin is found in the exoskeleton, gastro-intestinal linings and the respiratory tracts of arthropods, the largest phylum of the animal kingdom including insects, arachuids (spiders, scorpions, *etc.*) and crustaceans (crabs, lobsters, shrimps, Antarctic krill *etc.*). It also occurs in some other classes, such as cephalopods (squid, cuttlefish, *etc.*), gastropods (mollusks) and in some annelids (segmented worms). Furthermore, chitin is found in the cell wall of most fungi and the extracellular fibers of algae (diatoms). Its occurance in vertebrate, however, has not been reported.

Table 1.1 Chitin contents of some crustaceans, insects, molluscan organs and fungi. (Kong, 1975 and Naczk, *et al.*. 1981 cited by Knorr, 1984)

Type	Chitin content (%)
Crustacea	
Cancer (crab)	72.1[c]
Carcinus (crab)	0.4-3.3[a] ; 8.29[b] ; 64.2[c]
Paralithodes (king crab)	35[b]
Callinectes (blue crab)	14[a]
Pleuroncodes (red crab)	1.3-1.8[b]
Crangon (shrimp)	5.8[b] ; 69.1[c]28[d]
Nephrops (lobster)	69.8[c] ; 6.7[b]
Homarus (lobster)	60.8-77.0[c]
Lepas (barnacles)	58.3[c]
Insect	
Periplaneta(cockroach)	2.0[a]
Blatella (cockroach)	18.4[c]; 10[b]; 35[c]
Colcoptera (beetle)	5-15[b]; 27-35[c]
Tenebrio (beetle)	2.1[a]; 4.9[b]; 31.3[c]
May beetle	16[b]
Diptera (true fly)	54.8[c]
Pieris (sulfur butterfly)	64[c]
Grasshopper	2-4[a]; 20[c]
Bombyx (silk worm)	44.2[c]
Calleria(wax worm)	33.7[c]

Table 1.1 (continue)

Type	Chitin content (%)
Molluscan organs	
Clam shell	6.1
Oyster shell	3.6
Squid, skeletal pen	41.1
Krill, deproteinized shell	40.2±5.2
Fungi	
Aspergillus niger	42.0[e]
Penicillium notatum	18.5[e]
Penicillium chrysogenium	20.1[e]
Saccharomyces cerevisiae (bakers yeast)	2.9[e]
Mucor rouxii	44.5
Lactarius vellereus (mushroom)	19.0

The percentages are expressed on the basis of:

[a]Wet body weight
[b]Dry body weight
[c]Organic weight of cuticle
[d]Total dry weight of cuticle
[e]Dry weight of the cell wall

Chitin is recognised as the second most abundant natural occurring polysaccharides on earth, next to cellulose. Functionally, it serves as a structural supporting material which is comparable to cellulose in plants; and to collagen, chondroitin sulfate, keratin in vertebrates, urochordata, pterobranchia, enteropneusta and echinoderma.

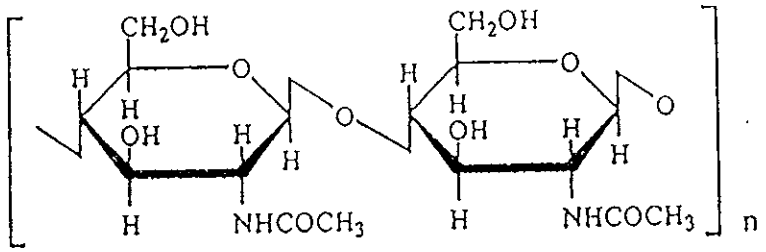
1.3 Chemical Structure and Complex Forms of Chitin and Chitosan in Nature

Ideally, chitin is a linear polymer of N-acetyl-D-glucosamine linked by β (1-4) glycosidic bond of which an official name is (1->4)-2-acetamido-2- deoxy-β-D-glucan (Figure 1.1). It may be regarded as cellulose derivative in which each C-2 hydroxyl group substitutes by acetyl amino group, -NHCOCH3. However, chitin from various sources or even within the same source, is more or less varied in degree of deacetylation, chain length, and may include saccharide units other than N-acetyl-D-glucosamine. Brine and Austin (1981) pointed out that chitin properties are not only depend upon their sources but also influence by method of preparation.

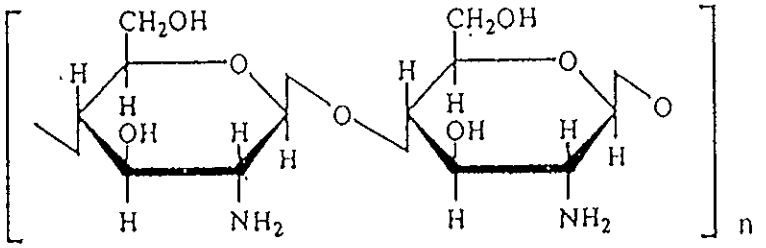
Several reports revealed that natural occuring chitin is usually form complex with other biomolecules in varying proportions with only exception for diatoms (Kong, 1975). Among the biomolecules, proteins and minerals are the principal components (Table 1.2 and Table 1.3) whereas other simple sugars and polyphenols are the minor constituents .

Table 1.2 Characteristic of percent compositions of chitin from different sources and other biomolecules (Knorr, 1984)

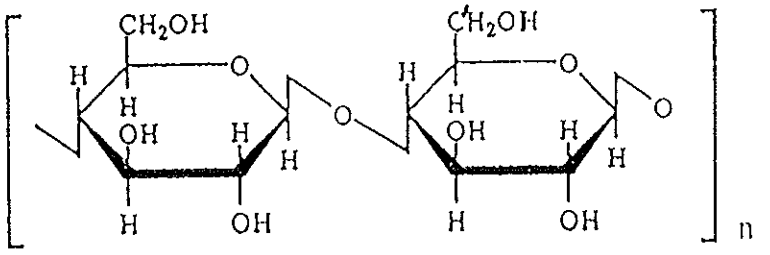
Source	Dry-weight composition (%)		
	Inorganic	Protein/Fats	Chitin
Shellfish	25-50	25-50	14-35
Krill	24	61	7
Clams/Oysters	85-90	negligible	3-6
Squid	negligible	76-95	1-2
Fungi	negligible	25-50	10-15
Insects	negligible	60-80	0-8



chitin



chitosan



cellulose

Figure 1.1 Chemical structures of chitin, chitosan and cellulose (adapted from Brine, 1984)

Table 1.3 Percentages of chitin and the protein content of dry shell (Austin, *et al.* 1981).

Organism	Chitin	Total protein	Co-valently bound protein*	Ratio of chitin to bound protein
Blue crab	14.9	16.4	5.3	2.8 to 1
Stone crab	18.1	15.4	5.7	3.2 to 1
Red crab	27.6	12.3	3.1	9.0 to 1
Brine shrimp	27.2	34.9	16.0	1.7 to 1
Horseshoe crab	26.4	73.4	27.9	0.9 to 1

*Total covalently bound protein as percent of total dry shell.

Austin, *et al.* (1981) demonstrated that the association between protein and chitin may be covalently linkage as well as weak interactions with varying proportions depend upon animal species (Table 1.4). Muzzarelli and Jeuniaux (1976) stated that the structural organizations as above described reflect properties of chitinous materials *i.e.* exhibit high tensile strength with essentially pliable and flexible, allowing movements and limit expansion. After sclerotization of proteins through quinone by the action of diphenol oxidase, the resulting chitin-tanned protein complex gains considerable stability and confers to the structural hardness, rigidity and resistance to enzymatic hydrolysis. Recently, detail studies on the nature of covalently linkage between chitin and protein using infrared spectroscopy indicated that amide type bonding through N-glycosidic linkages to aspartic acid predominates in all investigated samples from various species (Brine, 1982).

Table 1.4 Fractionation of chitin-protein complexes (Austin, *et. al.* 1981)

Organism	Total protein (%)[a]	Physical association (%)[b]		Covalently bound protein (%)[b]			
		EDTA 20 °C 12 hr	7M urea 20 °C 48 hr	0.1 N NaOH 20 °C 5 hr	1N NaOH 50 °C 6 hr	1N NaOH 100 °C 48 hr	Residual in chitin
Blue crab	16.4	31.4	36.9	9.1	22.1	0.2	0.3
Stone crab	15.4	22.4	40.6	1.7	33.1	1.8	0.4
Red crab	12.3	58.4	16.6	2.0	16.0	6.9	0.1
Brine shrimp	34.9	30.0	24.1	12.5	28.2	4.7	0.5
Horseshoe crab	73.4	15.6[c]	46.4	2.8	32.7	2.4	0.1

[a] Total protein in dry shell was determined independently by extraction with 1 N NaOH for 48 hours.

[b] Percent of total shell protein.

[c] Water extraction (50 °C, 6 hours) 15.0 percent then 0.16 N Na_2SO_4 (20 °C, 72 hours) 0.6 percent, prior to urea treatment.

In addition to proteins and minerals, chitin may associate with pigments such as melanin, carotenoid and astraxanthine.

Chitosan is deacetylated product of chitin which ideally is recognized as a long straight chain of (1->4)-linked 2-amino-2-deoxy-β-D-glucan (Figure1.1). Commonly, the deacetylation reaction could be carried out by treatment chitin with strong alkali such as sodium hydroxide. Since the behaviours of chitosan depend on nature of the starting chitin

as well as processing conditions, it may not be regarded as a well-defined chemical. On the other word, structure and properties of chitosan varies markedly according to source of starting material, degree of deacetylation and molecular weight.

1.4 Biosynthesis of Chitin and Chitosan

As shown in Figure 1.2, the biosynthesis of chitin represents the first case in which substantial evidence is presented for the formation of a polysaccharide from a sugar nucleotide, namely, uridine diphosphate-N-acetylglucosamine. Its chain polymerization accomplished by chitin synthetase (uridine diphosphate-2-acetamido-2-deoxy-D-glucose: chitin-4-β-acetamidodeoxyglucosyl transferase: E.C. 2.4.1.16). The newly synthesized enzyme behaves like zymogen whose proteolytic enzyme is required for the conversion to its active form. Detail studies on biosynthetic pathways of chitin were reviewed by Muzzarelli (1977).

Although there is rarely, natural occuring chitosan also found in cell wall of filamentous and yeast-like forms of *Mucor rouxii* grown under air and under carbon dioxide (Bartricki-Garcia, 1973). Muzzarelli and Jeuniaux (1976) explained that it is synthesized from chitin which the post deacetylation reaction is catalyzed by deacetylase.

1.5 Enzymatic Hydrolysis of Chitin and Chitosan

Although chitin is regarded as a stable compound, it is readily hydrolyzed by chitinolytic enzyme system which generally contributes by successive action of two hydrolases; namely, chitinase (chitin glucanohydrolase, E.C. 3.2.1.14) and chitobiase (chitobiose acetylaminodeoxyglucohydrolase, E.C. 3.2.1.29). Ohtakara (1964, cited by Muzzarelli, 1976) concluded that chitinase cleaves the endo-β-glucosaminic bonds in the chitin chain, forming chitooligosaccharides which are further hydrolyzed by chitobiase to produce N-acetylglucosamine. These enzyme systems play several important roles in nature.

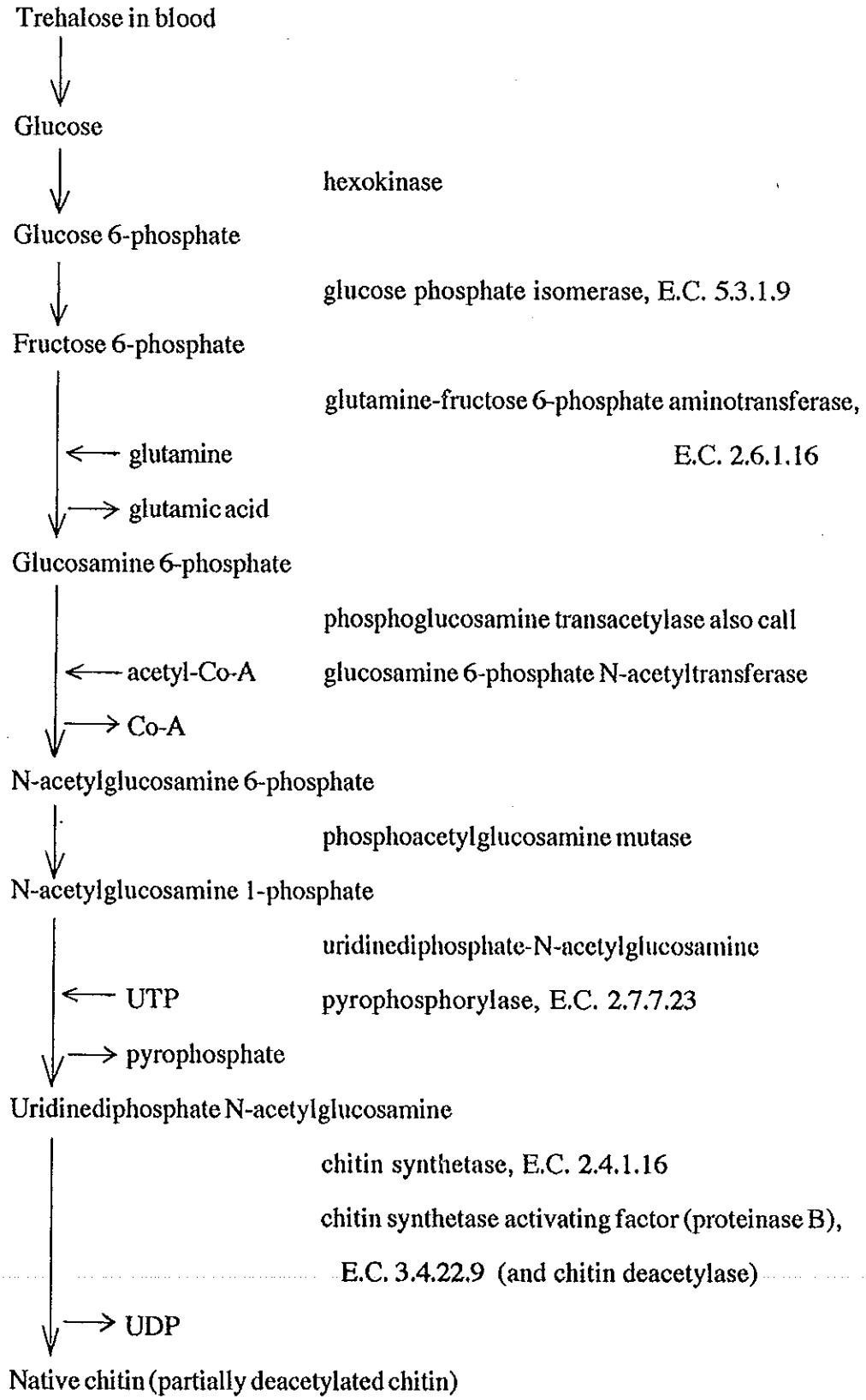


Figure 1.2 Pathway of chitin synthesis (Muzzarelli and Jeuniaux, 1976)

In marine ecosytems, it was estimated that several million tons of chitin are annually produced principally by various species of planktonic and benthic crustaceans. It appears, however, that a very small amount of chitin is accumulated in marine sediments. This suggests that the biopolymer is actively decomposed in marine environments. Since the rates of chitin degradation in marine ecosytems are greatly significant as it enters into both carbon and nitrogen biogeochemical cycles, mechanisms of these processes have been studied by a number of workers. Results from these experiments are consistently to each other which indicated that most of the decomposition processes are accomplished by bacterial activities as a series of work carried out by Seki and Taka (Seki, 1965a, b; Seki and Taga, 1963a, b, c,d; 1965a, b) and some others (Campbell, and Williams, 1951; Hillman, *et al.* 1989; Gooday, *et al.* 1991).

In invertebrate, Stevenson (1985) demonstrated that epithelial cells of prawn secrete chitinase to digest chitin from its old cuticle at pre-ecdysis stage in order to facilitate loosening of the exuvia for moulting, and to resorb the digested products. In vertebrates, the secretion of chitinolytic enzymes is not a matter of systematics, but is related to the nature of the usual diet for each species. Jeuniaux and Cornelius (1978) demonstrated that the gastric chitinases were at least occasionally found in some bird species that were more or less insectivorous. No chitinase was found in two strictly grain-eating birds, the pigeon and the parrot. In fish, Okutani (1978) showed that chitin from food seems to be decomposed simultaneously by both microflora and *in situ* chitinolytic enzymes. Similar relation was also observed for mammals and omnivorous, while the more specialized species for both carnivorous and herbivorous did not. The occurrence of chitinase in plants such as in sugar maple and okes was explained by Wago (1975, cited by Muzzarelli, 1976) in term of protection against invasion by microorganisms.

Enzymatic hydrolysis of chitosan accomplished by chitosanase (E.C. 3.2.1.99) was reported in many microorganisms. Monaghan, *et al.* (1973; cited by Muzzarelli, 1976) studied chitosanase in 200 strains of microorganisms and reported that 25 fungal and 15 bacterial strains synthesized the enzyme.

Beside the chitinolytic system and chitosanase as described, lysozyme (endo-β-N-acetylmuraminidases; E.C. 3.2.1) is able to hydrolyze chitin and chitosan to the oligomers of N-acetyl-D-glucosamine and glucosamine, respectively. The enzyme is widely distributed in mammalian tissues, insects, plants, birds and microorganisms. The activity of chitinolytic enzymes could be determined by three principles. Firstly, determination for initial rates of the liberated product, N-acetyl-D-glucosamine which is usually carried out by colorimetric techniques (Jeuniaux, 1966; Imoto and Yakishita, 1971). Secondly, determination for the rates of substrate degradation which is usually carried out by viscometric method (Jeuniaux, 1966). Thirdly, initial rates of the liberated product are monitored by the use of radioisotope labelled substrate (Malano, *et. al.* 1979). Although the last is the most reliable and sensitive, it is not practical in general laboratories. The first method seems to be the most simple and gain considerable ranges of precision, therefore, it is commonly accepted for the assay of chitinolytic activity and was used in this thesis.

1.6 Preparation of Chitin and Chitosan

1.6.1 Preparation of chitin

As described in Section 1.3, natural occuring chitin usually associate with minerals and proteins. Therefore, preparation of chitin commonly involves two processes in either order, namely, demineralization and deproteinization. Generally, the former process is conducted by treatment the chitinous material with diluted acid whereas the latter is carried out by treatment with diluted alkali solution. As glycosidic bond is readily hydrolyzed in both acidic and basidic conditions, variations of the treatment conditions determine the quality and performance of the prepared chitin. Thus, many attempts have been studied on the two processes in order to obtain appropriate products which were extensively reviewed by Muzzarelli (1977) and Ashford, *et al.* (1977) as some essential works described below.

In some instances, pigment free chitin is required for specific purpose especially with medical field of applications. These could be achieved by treatment with 0.02% potassium permanganate at 60 °C, or bleaching with sodium metabisulfite, hydrogen peroxide, sulfuric acid *etc.* (Muzzarelli, 1977).

1.6.1.1 Demineralization step

Crustacean shell generally contains 30-35% of minerals depend on species, and most of which is calcium carbonate. The common practice to separate minerals carries out by treatment the chitinous material with dilute hydrochloric acid. Since glycosidic bond is readily hydrolyzed in acid solution, this is the most critical step determine to a great extent the characteristics of the product. Hence, the effects of acid concentration, temperature, and duration of the treatment on properties of chitin from different sources have been evaluated.

Rutherford and Austin (1978) demonstrated that ash content in chitin from the shell of brown shrimp (*Penaeus aztecus*) remained at 0.6% after demineralization with 1.5% (w/v; 0.5 N) HCl at room temperature. Bough, *et al.* (1978) decalcified prawn shell with 1N (3.0%; w/v) HCl at room temperature for 30 minutes with continuous stirring using solid to acid ratio 1: 10 (w/v), and reported 0.62% of ash content in the chitin product. Benjakul (1991) isolated minerals from deproteinized prawn shell (*Penaeus indicus*) with 1.25 N (0.4%, w/v) HCl at ambient temperature for an hour using the ratio of reactant to acid solution 1: 10 (w/v), and reported 0.18% of ash content in the chitin product.

No, *et al.* (1989) showed that the optimum condition for demineralization of the dried ground crawfish shell could be done by treatment the sample with 1N (3.0 %, w/v) HCl with constant stirring at ambient temperature for 30 minutes using a solid to acid ratio of 1: 15 (w/v). The ash content in the chitin product was 0.1 %. Broussignac (1968) reported that ash content in chitin from crab shell powder (approx. 1-6 mm) remained at 0.4 - 0.5% after decalcification with 5% (w/v ; 1.7 N) HCl at room temperature for 24 hours.

Rutherford and Austin (1978) demineralized the blue crab shell (*Callinectes sapidus*), that has been deproteinized, with 5% acetic acid at room temperature for 5 hours using a ratio of sample to acid 6.8 : 1 (w/v). The ash contents in the chitin product remained 4.0% which were higher than those treated traditionally with HCl.

Anderson, *et al.* (1978) demineralized krill offals with 0.6 N HCl (1.8%, w/v) at room temperature for 2 hours using the ratio of sample to acid solution 1: 22 (w/v), and reported 0.4% ash contents remained in the product. Similar results were obtained when demineralized the chitinous material with 1.2 N (3.6%, w/v) HCl at room tempertaure for 1.5 hours, using the ratio of sample to acid 1 : 4 (Bruzeski, 1982). Synowiecki, *et al.* (1981) reported a very low ash content (0.06%) in chitin from the same chitinous source demineralized with 22% (w/v; 7.3 N) HCl at room temperature for 2 hours, using the ratio of solid to acid 1 : 10 (w/v).

1.6.1.2 Deproteinization step

Due to the differences in nature of chemical bonding and percent composition of chitin-protein complex from various sources, many protein separation conditions have been proposed to minimize cleavage of glycosidic bond and deacetylation of the native chitin. The effects of alkali concentration, temperature and duration of treatment on properties of the chitin prepared from various sources have been reported by a number of investigators.

Benjakul (1991) treated prawn shells (*Penaeus indicus*) with 2% (w/v; 0.5 M) NaOH at 100 °C for an hour by the ratio of sample to alkali as 1: 10 (w/v). Bough, *et al.* (1978) separated protein from prawn shell which was ground to a powder of 1 mm particle size, with 3% (w/v; 0.75 M) NaOH at 100 °C for an hour using a solid to alkali solution 1 : 10 (w/v).

No, *et al.* (1989) suggested that the optimum condition for deproteinization of crawfish shell could be achieved using 3.5% (w/v; 0.9M) NaOH at

65 °C for 2 hours with constant stirring, and with a ratio of solid to alkali solution was 1 : 10 (w/v).

Anderson, *et al.* (1978) isolated protein from krill offals with 3.5% (w/v; 0.9 M) NaOH at 90-95 °C for 2 hours, using a solid to solvent ratio of 1 : 10 (w/v). Synowiecki, *et al.* (1982) deproteinized the same chitinous source with 28% (w/v; 7.0M) NaOH by heating at 90°C with stirring, using a solid to solvent ratio of 1 : 10 (w/v).

Kandaswamy (1978) prepared squid (*Loligo indica*) chitin by deproteinization the pens with 1M (4%, w/v) NaOH at 100 °C for 16-18 hours. In addition, Kurita, *et al.* (1993) separated protein from the pens of *Ommastrephes bartrami* by soaking the powder in 2M (8%, w/v) NaOH at room temperature overnight. The residue was washed with water to a neutral pH, and further heating in 2M (8%, w/v) NaOH at 100 °C for 4 hours.

In an attempt to minimize the side reactions, other procedures for isolation of protein were also proposed. Muzzarelli (1977) separated protein from shell powder (approx. 850 μm.) by treatment with formalin solution (in neutral phosphate buffer) and saturated with disodium EDTA at room temperature for 12 hours. Austin, *et al.* (1981) showed that this treatment was also effective in simultaneously removing physically admixed and salt-bound protein.

Shimahara, *et al.* (1982) showed that deproteinization of decalcified prawn shells (*Penaeus japonicus*) could be achieved by the use of growing cells of proteolytic bacteria. The optimized conditions carried out by incubating the deproteinized shell powder (200 mesh) with bacteria in medium at 30 °C, pH 7.0. Among 224 of the tested strains, *Pseudomonas maltophilia* (LC 102) exhibited the highest proteolytic activity. Analysis for the protein contents in the prepared chitin showed that there were decreased from initial 19.9% to 1.1% after 3 days of incubation. Comparative studies were also made between this procedure and the use of classical caustic alkali treatment. Results indicated that degree of deacetylation of the chitin

products from bacterial proteolysis was 10.8% which was much lower than that of 20.0% for alkali treatment. In addition, the solubility in N,N-dimethylacetamide - 5% litium chloride as well as crystallinity determined by X-ray diffraction were higher for the chitin deproteinized by the use of the former procedure than that of the latter one.

Brine and Austin (1981) studied the effects of some deproteinization conditions on properties of chitin from horseshoe crab, *Limulus polyphemus* which was naturally uncalcified but well hardened with sclerotized protein. The compared conditions were made between the conventionally treatment using 1N NaOH (4%, w/v) at 50 °C for 6 hours with constant stirring, and the treatment by other methods *i.e.* the use of dimethylforamide which was the milder conditions and ethylenediamine tetraacetate (EDTA) which was the mildest condition. Results indicated that the acetyl values for all prepared chitins were significantly lower than the theoretical value of 21.2% by weight and the experimental value of 20.7% determined from pure N-acetylglucosamine. These suggested that partial deacetylation occurred with all studied conditions. Data from molecular weight analysis for the chitin products confirmed that using the more drastic condition the more degree of deacetylation, and the more depolymerization was taken place. These conclusions were consistent with the experiments revealed by Brine and Austin (1981). The authors stated that although the use of an exhaustive mild alkali treatment, the significant amounts of protein still remained in the chitins prepared from stone crab (*Callinectes sapidus* and *Menippe mercenaria*), red crab (*Geryon quirquedons*), horseshoe crab (*Limulus polyplemus*) and brine shrimp (*Artemia salina*). Therefore, it is likely that the residual proteins associate with chitin by covalently linkage. Analysis for amino acid profile and contents of the remaining protein showed considerably species dependence, but aspartic acid, serine and glycine appeared to be the most common (Muzzarelli, 1985 and No, *et al.* 1989).

Green and Kramer (1979) suggested that if proteins in soluble portion are required, there could be recovered in precipitated form by adjusting pH of the solution to their isoelectric point which is generally is 4.0.

1.6.2 Preparation of chitosan

Conversion of chitin to chitosan is conventionally carried out by heating chitin powder in approximately concentration 40-55% (10.0-12.5 M) sodium hydroxide (Green and Kramer, 1979). This reaction removes acetyl groups from C-2 position of chitin, leaving free amino groups ($-NH_2$) behind. It is well documented in literature that increasing temperature of treatment or alkali concentration resulting in the more deacetylation and the more chain depolymerization and the more breaking of glycosidic bond which effect the product properties. It also appears that if the deacetylation reaction carries out in the presence of oxygen and water, it may give rise to considerable polymer chain breakage by oxidative reaction, yielding a mixture of chain length products. Hence, Wu, *et al.* (1978) recommended to carry out the reaction under nitrogen atmosphere. Several works proposed for the preparation condition providing a wide range in molecular forms and their applications.

Bough, *et al.* (1978) deacetylated prawn chitin by refluxing with 50% (w/v; 12.5M) NaOH at 145-150 ^{o}C in oxygen free atmosphere for 5-15 minutes, in which the ratio of sample to alkali was 1: 5 (w/v). The authors also showed that grinding of the dry shell to 1 mm prior to any treatment resulted in a chitosan product of higher viscosity and higher molecular weight than those ground to either 2 or 6.2 mm. Benjakul (1991) studied several conditions for deacetylation of the chitin from prawn shell (*Penaeus indicus*). The results suggested that the optimum condition could be performed by treatment the chitin with 50% (w/v; 12.5M) NaOH at 100 ^{o}C under vacuum for 30 minutes, in which the ratio of chitin to alkali solution was 1: 15 (w/v).

Anderson, *et al.* (1978) prepared chitosan from krill offals chitin (ground to 60 mesh) by continuous refluxing with the mixture of 53.5gm KOH and 34.5gm ethylene glycol in 48.5 ml of 95% ethanol for 20 hours. The chitosan product contained 7.9% nitrogen and showed viscosity as a 1% solution in 5% acetic acid of 60 cps.

Kurita, *et al.* (1993) prepared chitosan from squid pen chitin (*Ommastrephes bartrami*) by treatment with 40% (w/v; 10.0M) NaOH at 80 ^{o}C under

a nitrogen stream with stirring for 3 hours using a ratio of chitin to alkali solution 1: 20 (w/v). The degree of deacetylation of the chitosan product was 0.80. Results also suggested that the longer alkali treatment the higher degree of deacetylation was obtained, and that could be increased to 0.97 after 6 hours.

Since deacetylation process usually performs under strong alkali with drastic conditions, most proteins and amimo acids are solubilized. Chitosan is practically considered to be free of these contaminants. It is known that the ash content of chitosan could be kept below 0.5%. Hence, little attention has been paid to the presence, the origin and the consequences of inorganic contaminants in chitosan. Muzzarelli (1985) pointed out that most of the trace metals in commercial chitosan did not come from the animals, but principally from foreign matters, equipment and water. Accordingly, chitosan suitable to use in medical applications should be prepared from the clean shell and by using demineralized water. With these procedures, the author demonstrated that the levels of some trace metals detected in chitosan from crab shells were : V, 0.12; Cr, 0.04; Mn, 0.09; Ni, 1.3; Cu,1.03; Ag, 0.02; Cd , 0.22; Hg, 0.025; Pb, 0.15 μg/ gm of chitosan.

1.7 Characteristics of Chitin and Chitosan

1.7.1 Degree of deacetylation

Chitin and chitosan molecules with higher degree of deacetylation provide the more free amino group or positive charges at neutral pH. It is, therefore, an important parameter reflect chemical and physical properties of the polymers. Several methods were proposed to determine the degree of deacetylation of chitin and chitosan such as the use of UV spectroscopy (Castle, *et al.* 1984 and Muzzarelli and Rocchetti, 1985), IR spectroscopy (Sannan, *et al.* 1978), mass spectrometry (Hayes and Devies, 1978), conductometric titration (Kurita, *et al.* 1993), colorimetric methods (Neugebauer and Brzezinski, 1989; Curotto and Aros, 1993; Clarke and Knowles, 1994) and enzymatic method (Nanjo, *et al.* 1991).

In 1985, Muzzarelli developed the UV spectrophotometric technique by reading an absorbance of the sample in acetic acid solution from the first derivative spectra at wavelength 202 nm where the disturbance reading from N-glucosamine is negligible. At this wavelength, the reading absorbances are not influenced by any concentrations of the acetic acid within a range usually encountered in the dilute solution. The author claimed that this was the most precise, accurate and non-destructive method, and was used in this thesis.

The degree of deacetylation were usually approximately 10% in chitin, 60% in chitosan, between 90-100% in fully deacetylated chitosan (Muzzarelli, 1985) and 70-90% in commercial chitosans (Anonymous, 1989 cited by Benjakul, 1991).

1.7.2 Molecular weight

The chemical and physical properties of chitin and chitosan are influenced to a great extent by their molecular weight. Since the prepared polymer could be regarded as a polydisperse molecule, an expression of the molecular weight can be defined in a number of ways. The most common encountered are number-average molecular weight (Mn), weight-average molecular weight (Mw) and viscosity-average molecular weight (Mv). As appear in general text books of physical chemistry, however, the values of Mv and Mw are the most close to each other and might be the same in many circumstances (Chang, 1980).

There are several methods employed to determine molecular weight of chitin and chitosan, for example, light scattering (Hackman and Goldberg, 1974), membrane osmometry, viscometry and high-performance liquid chromatography (Wu, *et al.*; 1976; Domard and Rinaudo, 1983). The viscometry is a simple and rapid method for determination of viscosity average molecular weight, and it was thus determined the molecular weight of chitosan in this thesis.

As earlier described, molecular weight of the prepared chitin and chitosan depended on both sources of chitinous material and preparation conditions. It appears,

however, that the smaller molecular weight would be expected if demineralization performed with stronger acid, longer treatment and higher temperature. These is also true for deacetylation with stronger base, longer treatment, higher temperature of treatment and in the presence of oxygen.

Hackman and Goldberg (1974) reported that chitin from crab shell (*Scylla serrata*) 150 mesh powder decalcified using cold 1M (3.0%, w/v) HCl and deproteinized twice with 1M (4%, w/v) NaOH at 100 °C for 24 hours, has the number-average molecular weight of 1.306×10^6 daltons. Lee (1974; cited by Muzzarelli, 1976) investigated the effect of preparation conditions on molecular weight of β-chitin and chitosan in order to compare with that of the α- form. Pen from Loligo species was deproteinized with pronase and three washed with 5M (20%, w/v) NaOH. A portion of chitin was further deacetylated in 45% (w/v; 11.3M) NaOH under nitrogen atmosphere for three intervals minutes at 140 °C. Results indicated that the number-average molecular weights of chitin and chitosan deacetylated for 40, 60 and 80 minutes were 2.5×10^6, 7.25×10^5, 4.92×10^5 and 2.35×10^5 daltons, respectively.

Brine and Austin (1981) compared the molecular weight of chitin prepared under identical condition from different sources (blue crab, *Callinectes sapidus*; horseshoe crab, *Limulus polyphemus*; stone crab, *Menippe mercenaria* and red crab, *Geryon quirquendons*). The effects of preparation condition were also investigated in this thesis. Results were clearly indicated that decalcification was the most critical step determining the weight-average molecular weight of the product. The greatest depolymerization occurred when hydrochloric acid was used, followed by acetic acid and sulfurous acid, with minimal degradation occurred when EDTA was used.

Muzzarelli (1985) reviewed that molecular weight of native chitin, commercial chitin and commercial chitosan were 1×10^6, $1\text{-}5 \times 10^5$ and $1\text{-}5 \times 10^5$ daltons, respectively. Nowaday, commercial chitosan produces notably a wide range in molecular weight which are generally between 10,000 - 1,000,000 daltons (Anonymous, 1989 cited

by Benjakul, 1991). Fluka company is manufacturing and selling chitin of molecular weight 400,000 daltons, and is selling chitosan according to the molecular weight, *i.e.* 70,000, 750,000 and 2,000,000 daltons for low, medium and high molecular weight, respectively.

1.7.3 Physical forms of chitin

Natural isolated chitin is a white amorphous solid, generally possessing some degree of crystallinity with a flaky or fibrous nature. Due to the differences in stereoisomer, intermolecular and intramolecular hydrogen bonding, a few crystalline polymorphic forms, designated α, β and γ have been reported (Muzzarelli, 1977). Each of these has a characteristic X-ray diffraction pattern. Alpha chitin has orthorhombic unit cell and forms the most tightly compacted crystalline where the chains are arranged in an anti-parallel fashion; β-chitin has a monoclinic unit cell where the chains are parallel, while γ-chitin is the form where two chains are "up" to everyone "down". By far, the most abundant is α-chitin which widely distributes in artropod exoskeletons and in certain fungi. Beta chitin is found principally in the squid pen of *Loligo* species, whereas the γ-chitin is not common, it has been reported only in the cocoons of the beetles *Ptinus tectus* and *Rhynchaenus fungi*. However, the three forms may distribute in different parts of the same organism, for instance, in the squid *Loligo* species. whose beak contains α-chitin, whose pen contains β-chitin and whose stomach linings contains γ-chitin. This indicates that each form contributes for the different functions rather than animal grouping.

Kandaswamy (1978) demonstrated that the conversion of β-chitin from squid pen (*Loligo indica*) and polychaete annelids (*Nereis diversicolor*) to α-chitin was accomplished by treatment with fuming nitric acid or 6 M hydrochloric acid. After the treatment, however, their acetyl contents were reduced from 9.62 and 9.57, to 7.4 and 7.6 %, respectively, and the X-ray patterns showed α-form. In contrast, the treatment of α- chitin from crab (*Neptunes sanguinolentus*) with those conditions, did not change

both acetyl content and form.

1.7.4 Chiroptical activity of chitin.

Monomeric unit of chitin, N-acetylglucosamine comprises a number of asymmetric carbon atoms, therefore, it exhibits a chiroptical activity. Austin, *et. al.* (1981) showed that native form of chitin which could be prepared from horseshoe crab using a mild alkaline condition was lavorotatory whereas the others prepared by harsher treatments were dextrorotatory. It was latterly found, however, that the specific optical rotation of a certain chitin solution gradually changed with time (Table 1.5). These evidences indicated that its chiroptical activity arise not only from the asymmetric carbon atom but also its helical conformation. Unfortunately, no report has been found for the optical activity of the β-chitin.

Table 1.5 Optical rotation of chitins from various sources. Samples were dissolved in DMAc- 5% $LiCl_2$ (Adapted from Austin, *et al.* 1981).

Chitin sources	Rotation, $[\alpha]_D$	
	Initial	After 2 weeks
Horseshoe crab (*Limulus polyphemus*)	-56°	-56°
Blue crab (*Callinectes sapidus*)	+33°	-52°
Japanese crab (*Chionects opilio*)	+23°	-22°
Pink shrimp (*Pendalis borealis*)	+75°	-54°
Brown shrimp (*Penaeus aztecus*)	-36°	-36°

1.8 Some Properties of Chitin and Chitosan

1.8.1 Solubility

As above mentioned, chitin does not soluble in all ordinary solvents such as water, dilute acids, dilute and concentrated alkali as well as simple organic solvents. Although it appears to soluble in concentrated acid, for instance, methanesulfonic, sulfuric acid and anhydrous formic acids, partially hydrolysis of glycosidic bond and removing of the acetyl groups always take place (Kong, 1975; Tokura, *et al.* 1983). These may due to its rigid crystalline structure through intramolecular and intermolecular hydrogen bonds (Pearson, *et al.* 1960 cited by Tokura, *et al.* 1983).

Chitin swell in cold alkali suspension, with partially deacetylation reaction occurs. Ashford, *et al.* (1977) suggested that the swelling mechanism is presumably due to some free amino groups associate with the cations which render a spreading of the interchain structure. Repeat freezing in alkali suspension causes dissolution, but the regenerated chitin is still insoluble in water. Chitin may also be swollen in dimethylsulfoxide (DMSO), yielding a good reactant for subsequent treatments to produce other water soluble derivatives such as carboxymethyl or hydroxyethyl chitin. Rutherford and Austin (1978) revealed that chitin readily dissolved in the mixture solution of N,N-dimethylacetamide (DMAc)-5% lithium chloride and N-methyl-2-pyrrolidone (NMP)-5% lithium chloride which did not hydrolyse the polymer.

In comparison, β-chitin gives higher solubility in organic solvents than that of α-chitin. Kurita, *et al.* (1993) demonstrated that β-chitin prepared from squid pen (*Ommastrephes batrami*) was soluble in dichloroacetic acid and dimethylacetamide containing 5% lithium chloride in addition to formic acid to give a clear viscous solutions. Furthermore, it was almost soluble in m-cresol and highly swelled in polar organic solvents such as dimethyl sulfoxide, dimethylformamide, hexamethylphosphoramide, and pyridine.

Although chitosan is insoluble in water, it readily dissolves in weak acid solvents such as 1% of acetic, formic, malic, malonic, propionic acids and 5% succinic

acid (Muzzarelli and Jeuniaux, 1976). It is also soluble in some other acids at the higher concentration (approx. 10%), for instances, oxalic, citric, pyruvic or tartaric acids. However, its solubility saturation in each of the acid solvent is different markedly. In inorganic acids, chitosan dissolves in the solvent of concentration approximately 1% after prolong stirring and warming, with an exception for sulfuric and phosphoric acids. It has been noted that the solubility increased as chain length decreased.

1.8.2 Hygroscopic activity

Muzzarelli and Jeuniaux (1976) stated that hygroscopicity of chitin was close to that of mercerized cellulose fibers and considerably greater than that of ramie fibers. However, the chitin surface was less active and permeable to water than cellulose fibers. Water uptake of chitin, microcrystalline chitin and chitosan was found to be significantly higher than that of microcrystalline cellulose (Knorr, 1983). Possible explanation for the differences in water uptake between the cellulose and chitinous polymers is the differences in the crystallinity and protein contents within the materials. Filar and Wirick (1978) reported that the equilibrium of moisture content for chitosan was a function of relative humidity, but it was not affected appreciably by sample molecular weight.

Kurita, *et al.* (1993) compared the hydrophilic nature between β-form of chitin and chitosan isolated from squid pen (*Ommastrephes bartrami*) and that of the α-form from prawn shell (*P. japonicus*). It was found that chitinous materials from squid pen exhibited much higher hygroscopicity and retention of absorbed water than those obtained from the shrimp samples. These may due to the looser arrangement with relatively low crystallinity of the β-form of chitin.

1.9 Other Chitin Derivatives and Their Properties

Since chitin and chitosan do not dissolve in water and in most ordinary solvent, these strictly limit their ranges of applicaltion. According to molecular structure, however,

they comprise of primary and secondary hydroxyl groups as well as amino group on which several reactions could be carried out. Thus, numerous derivatives were synthesised in order to modify their properties for appropriated purposes. Some of the known well derivatives are included : carboxymethyl chitin (CM-chitin), chitin and chitosan sulfates, ethyl and hydroxyethyl chitin, ethyl and hydroxyethyl chitin, *etc.* Details of preparation procedures and properties of them were extensively reviewed by Muzzarelli and Jeuniaux (1976) and Ashford, *et al.* (1977) as summarized below.

1.9.1 Carboxymethyl chitin (CM-chitin)

Carboxymethyl chitin or poly [N-acetyl-6-O-(carboxymethyl)-D-glucosamine] is generally synthesized by swelling chitin powder in dimethylsulfoxide (DMSO), then treated the swollen mixture with 2-propanol, NaOH and chloroacetic acid, successively. The solid residue of CM-chitin is further treated with methanol, then is neutralized with acid. The product dissolves in water at 5%, and is degradable by lysozyme (Ashford, *et al.* 1977). With this procedure, carboxyethyl chitosan could also be synthesized.

Tokura, *et al.* (1983) suggested the more simple procedure for the synthesis of CM-chitin. They swelled chitin from queen crab shells in 60% NaOH containing 0.2% sodium dodecylsulfate, treated the alkali-chitin with isopropyl alcohol, then neutralized with monochloroacetic acid. The product was filtered, washed and extracted with ethanol and water, respectively. The soluble CM-chitin sodium salt was precipitated with acetone, centrifuged and washed the solid residue with acetone several times. Salt free CM-chitin could be obtained by redissolving the residue in water, adjusted pH to 2.0 by the addition of 2N HCl.

1.9.2 Chitin and chitosan sulfates

Ashford, *et al.* (1977) reviewed that sulfonation of chitosan resulted in chitosan sulfate, poly [6-O-sulfo-D-glucosamine]. These can be conducted by treatment of chitosan with chlorosulfonic acid in pyridine to yield an amorphous sodium salt containing both N- and O-sulfate groups. Chitin sulfate can also be prepared by

sulfonation with chlorosulfonic acid, yielding the chitin disulfate half-ester which is water soluble and has about quarter the anticoagulant activity of heparin.

1.9.3 Hydroxyethyl and ethyl chitin

Hydroxyethyl chitin, poly [N - acetyl - 6 - O -(2 - hydroxyethyl) - D-glucosamine], can be prepared by reacting ethylene oxide in an autoclave with chitin swollen in alkali and then dimethyl sulfoxide (DMSO). It is water soluble at 5% and is degradable by lysozyme (Ashford, *et al.* 1977).

Ethyl chitin, poly [N - acetyl - 6 -O- (ethyl) - D - glucosamine], can be synthesized by a procedure similar to that of hydroxyethyl chitin except for the substitution of ethyl chloride in spite of ethylene oxide and an increase in the reaction time or temperature. Variations of reaction condition influence the degree of substitution of which determines the product solubility. This derivative is water soluble if the degree of substitution is not greater than about 1, and is soluble in a wide range of organic solvents if the value is greater than 1, such as benzene, xylene, toluene, methyl or ethyl ketone, chloroform and alcohols. Ethyl chitin, like hydroxyethyl chitin, is degradable by lysozyme (Ashford, *et al.* 1977).

1.10 Applications of Chitin and Its Derivatives

A number of chitin derivatives could be chemically modified, each of which displays a characteristic chemical and physical properties, several fields of application including biomedical, food technology, cosmetic, paper and other industries have been reported. Many forms are commercially available as some important applications given below.

1.10.1 Biomedical applications

1.10.1.1 Bacteriocidal and fungicidal agents

The bacteriostatic and fungicidal activities have been noted for chitin, chitosan and their derivatives. Allan, *et al.* (1984) found that the growth inhibitory characteristics of chitosan from crab shell against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the bacteria often encountered in burns, were effective at 1% in acetic acid, whereas *Staphylococcus epidermis*, the common skin bacterium was completely inhibited over the range of 0.1-1% in the acid solution. Moreover, Muzzarelli, *et al.* (1990) demonstrated that N-carboxybutyl chitosan, a modified chitin of crustacean origin, displayed inhibitory, bacteriocidal, and candidacidal activities when tested against several pathogenic bacteria.

1.10.1.2 Wound healing accelerator

It has been well documented that a powder extracted bone cartilage as well as chitin was traditionally used as an accelerator for the natural fusion and healing of incisions and other wounds for long time ago. Balassa and Prudden (1978) proposed that the mechanism of action primarily attributed by the presence of hexosamine, and more specifically, to the presence of N-acetyl-D-glucosamine. However, the authors demonstrated that acceleration rates using chitin and chitosan showed much higher activity than that of the bone cartilage. For chitin and chitosan when free from naturally-associated protein, they are non-antigenic in human and may be inserted under skin, in mucous-wetted cavities or in contact with body fluids, without any side effects. In such a location, it will be gradually hydrolyzed by lysozymes and absorbed. Hence, several interesting spectra of surgical applications has been studied (Prudden, *et al.* 1970 cited by Ashford, *et al.* 1977).

Allan, *et al.* (1984) pointed out that chitosan is an attractive candidate for wound-healing treatment because of several advantages. Firstly, it forms a tough biocompatible film that is conducted directly on the burn by application of aqueous solution of chitosan. Secondly, the film is oxygen permeable that prevents

oxygen deprivation from tissue. Thirdly, the film could absorb water up to 50% by weight. Fourthly, since it is slowly degraded by lysozyme, the chitosan film need not be periodically removed from the wound.

Numerous products used for wound healing accelerator made from chitin and chitosan are commercially available. Nicol (1991) reviewed that between 1968 and 1975 the American pharmaceutical company (Lescarden of Goshen, New York) patented five products manufactured in various forms such as chitin mats, fibres, sponges, sutures and films. The company claimed that the efficiency of using these products were much better than standard cartilage-based ones. The review also mentioned that Katakurachikkarin Company, Hokkaido, Japan, has manufactured chitosan film to make an artificial skin which effectively enhanced recovery from surgical wounds or burns.

1.10.1.3 Hemostatics and blood anticoagulants.

Chitosan itself is an hemostatic substance that can be applied to open wounds in surgery and prosthetics, however, a few derivatives are blood anticoagulants (Malette, *et al.* 1983). Muzzarelli (1985) reviewed that the sulfation product of N-carboxymethyl chitosan prepared from *Euphausia superba* showed the anticoagulant property similar to that of heparin. The author proposed the mechanism of action that it occured by association with thrombin to form antithrombin as well as the inhibition of factor-X which prevent the conversion of prothrombin to thrombin. It appeared also that no appreciable hemolysis was produced, and no adverse effects were observed on lymphocytes or erythrocytes (Muzzarelli, 1983).

1.10.1.4 Hypocholesterolemic and hypolipidemic activities

Furda (1980) patented the use of chitosan as a lipid-binding food additive or pharmaceutical preparation. Knorr (1982) reported the fat binding capacity of chitin, chitosan and microcrystalline chitin in a ranging from 170 to 215 % (w/v). It was found that chitosan having the lowest and chitin having the highest binding capacity. Microcystalline chitin showed good emulsifying properties whereas

chitin and chitosan did not form emulsion under the test conditions. In addition, changing in concentration of chitin or chitosan did not affect the emulsion capacity. The hypocholesterolemic activity of chitosan in rats has been demonstrated by Sugano, *et al.* (1978; cited by Muzzarelli, 1985). The review stated that levels of plasma cholesterol in male rats fed on a high-cholesterol diet (0.5%, w/w) incorporated with 2 to 5 % chitosan for 3 weeks, were reduced significantly (25-30%) without influencing food intake and growth. Plasma cholesterol lowering by chitosan was similar to that by cholestyramine, whereas the liver cholesterol reduction was less extensive, although it still showed a reduction to one-half. The hypocholesterolemic activity of chitosan was also demonstrated with chickens, hens and broilers by oral administration (Hirano, *et al.* 1990). For the water soluble form, chitosan acetate exhibited superior to pectin as a general hypolipidemic agent (Nagyvary, *et al.* 1979 cited by Muzzarelli, 1985).

Nauss, *et al.* (1983 ; cited by Muzzarelli , 1985) demonstrated that chitosan is a powerful bile salt sequestrant. Under optimal conditions, its binding capacity with bile salt might be 4-12 times of its own weight, and that no other natural or synthetic products have been showed this property. Since the binding capacity was strongly influenced by pH, the sequestrant mechanism was presumably due to ionic interactions. Muzzarelli (1985) proposed that the hypocholesterolemic and hypolipidemic activity of chitosan is probably due to the inhibition of micelle formation. At pH 6.0-6.5, chitosan begins to precipitate (depending on its origin and quality) and, as the chains of the polysaccharide aggregate, they can entrap the whole micelles. The entrapped cholesterol, fatty acids and monoglycerides thus escape adsorption. It is important to note, however, that the hypocholesterolemic activity of chitosan is likely due primarily to lowering lipid absorption. Hence, further investigation on the effects of interaction between chitosan and individual lipids which would reflect other physiological changing are required.

1.10.2 Food and Feed Industries

Food processing industries use polysaccharides to alter or control functional properties of food. Many are incorporated into foods as thickener to control foam, improve cling, moisture uptake; as gelling agents and agents to control ice crystal formation in frozen foods, change texture, add bulk, biomass recovery, remove undesirable substances, *etc.* (Ashford, *et al.* 1977).

Dye binding properties of chitin and chitosan have been investigated (Knorr, 1983) by using FD&C Red No.40. The results showed the significant correlations between dye concentration (ranging from 0.2 to 1.6 mg.dye/gm of chitin or chitosan) and dye binding capacity of chitin or chitosan. Between pH 2 - 7, dye uptake was constant at approximately 0.8 mg dye/gm chitin. The same results were found for chitosan between pH 7.0 - 5.5, at a lower pH chitosan forms gels. The potential of chitin as a nonabsorbable carrier for food dyes and found in in vitro experiments that dyed chitin powder did not release dye within a pH range of 2.0 to 6.0 and released 2.1, 2.9 and 5.6 % of the total amount of dye bound at pH 7.0, 1.0 and 8.0, respectively (Knorr, 1984).

Dunn and Farr (1974) showed that microcrystalline chitin produced dispersions which were superior to microcrystalline cellulose under room-temperature storage conditions, repeated retort sterilization and repeated freeze thaw cycles. The use of microcrystalline chitin as a stabilizer-thickener in foods was proposed. The developing of a solvent system for chitin and subsequent precipitation of chitin that renatures it in highly ordered crystalline form closely similar to native chitin was developed and indicated that filaments and films had a good degree of order crystallinity and could be cooled down to more than double their original length to enhance their properties. Proposed application of these films and filaments such as oven and other food wraps based on its (chitin) edibility and temperature stability. Work has been done regarding the film-forming properties of chitosan. Mechanical properties of chitosan

films such as breaking stress, tensile strength and percent elongation have been investigated (Kienzle-Sterzer, *et al.* 1982).

The effects of microcrystalline chitin addition on specific bread volume of wheatflour breads and protein fortified breads (8% replacement level of the flour by potato-protein) at different levels of water resulted in increase specific loaf volume. It should be noted that a specific loaf volume about 6 cm^3/gm could be obtained even for the protein-fortified samples although the bread formula contained no emulsifiers and shortenings. This indicates surfactant properties of chitin which can overcome the loaf volume depression that occurs when potato proteins (and others) are added to wheat-bread formulations (Knorr and Betschart, 1978).

Experimental evidence regarding the toxicity of chitosan has been documented by Aria, *et al.* (1968) who demonstrated that only concentrations above 18 gm of free chitosan /kg body weight/day were harmful to mice. Landes and Bough (1976) reported that rats receiving up to 5 % of free dietary chitosan had no differences in growth rates, inner organs and blood serum compositions when compared to control animals. The authors also showed that protein efficiency ratio (PER) values of rats fed on casein, whey solids, and whey solid coagulate incorporated with 2.15% chitosan, were not significant difference. These indicated that incorporation of chitosan in food at these levels had no adverse effects on the nutritional value of protein.

Hirano, *et al.* (1990) demonstrated that no abnormal symptom was observed with rabbits, broilers and hens which had been orally administered chitosan and chitin at a dosage 0.7-0.8 gm/kg body weight/day for up to 239 days. Apparently, digestibility by rabbits was 28-38 % for chitin and 39-79 % for chitosan, and that both polymers were digested almost completely by hens and broilers. The authors also reported that no abnormal symptom was observed with rabbits when low molecular weight-chitosan (M.W. 3,000) or chitosan oligosaccharides (M.W. 304-1,162) were injected intravenously at a dosage 4.5 mg/kg body weight/day for 7-11 days.

1.10.3 Waste water treatment

Polyelectrolytic compounds such as polyacryamide have been commonly used for coagulation and separation of colloidal and dispersed particles from food processing wastes. The mechanism of action is due primarily to charge to charge interactions between the particle and the polyelectrolytic polymer. Although the synthetic polymer with particular for polyacryamide is claim to be a safety material, their degradability as well as toxicity of the degraded products remain doubtful. Hence, chitosan, biodegradable polyelectrolytic compound receives more attention during the past decade (Green and Kramer, 1979).

Bough (1976) and Knorr (1984) demonstrated an effectiveness of chitosan for the treatment of water from various food processing wastes including poultry, egg, meat, shrimp, cheese and vegetable plants. It was also effective for dewatering of activated sludge suspensions. The authors showed that the use of chitosan in conjunction with other cationic polymers or multivalent inorganic salts such as aluminium sulfate or ferric sulfate, was more effective coagulant than those of the synthetic one.

It appears that coagulation efficiency of chitosan is a function of its charge, structure and molecular weight. Therefore, proper preparation conditions are required for these applications. Bough, *et al.* (1978) demonstrated that chitosan made by deproteinization with 3% NaOH at 100 °C for 1 hour, demineralization with 1N HCl at ambient temperature, and deacetylation with 50% NaOH at 145-150 °C under nitrogen atmosphere gave the best performance for the activated sludge. Apart from coagulation properties, it has been well documented on chelating ability of chitin and chitosan. Much of this work compiled with an excellent and comprehensive summary of the characteristic and properties of chitin and related material has been reviewed (Muzzarelli, 1976 ; Muzzarelli and Rocchetti, 1985). The authors stated that complex formation between the polymer with metals were contributed by both its free amino

groups and hydroxyl groups. Therefore, chitosan exerted the higher chelating capacity than that of the parent molecule, chitin.

Yaku and Koshijima (1978) demonstrated that chitosan has the characteristics selectively to combine particular metal ions, with particular for the transition elements. These tendencies were most effective for Cu (II), Hg (II), Fe (II), Ag (II), Cd (II), Ni (II); whereas the weakest binding were Mn (II), Co (II) and Cr (III). The authors also showed that aqueous solutions containing alkali earth metals such as Sr (II), Mg (II), Ca (II) and Ba (II) did not influence the binding capacity of Cu (II)-chitosan complex. The selective of binding was also observed in the presence of alkali metals such as Na, K, Ca and Mg which were the dominant constituents in seawater. Accordingly, several workers have been reported the potential use of the biopolymers for removal of undesirable metals from industrial wastes as well as in seawater such as Hg, Pb, Zn, Cu, Cr, Pt and U (Muzzarelli and Weckx, 1972; Marsi, *et al.* 1974; Muzzarelli, 1977; Silver, 1978; Eiden, *et al.* 1980; Galun, *et al.* 1983).These could be performed by either bulk adsorption in suspension or often more efficiently in a packed column of chitosan.

Ramachandran-Nair and Madhavan (1982) studied the influence of time for mixing, and pH on the binding characteristics of chitosans from crab (*Scyll serrata*), prawn (*Penaeus indicus*), squilla (*Oratosquillanepa*) and squid (*Loligo sp.*) on Fe, Co, Ni, Hg and Cu ions. Results indicated that adsorption behaviour of chitosan prepared from the four sources did not differ markedly, with only exception for Hg ion whose adsorption rate by chitosan from squilla was faster and the adsorption capacity was more than those from the others. The differences in nitrogen content of chitosan samples showed no correlation with adsorption rate. An adsorption was more or less completely by one hour and pH of the solutions did not influence significantly either the rate of adsorption or quantity of metal adsorbed. In all cases, the quantity of the different metals adsorped varied considerably irrespective of the sources of chitosan. The maximum and minimum adsorption capacities were Hg and Co ions, respectively.

Apart from the removal of biomass and metal ions from waste water, Mckay, *et al.* (1982 and 1984) showed the potential application of chitin and chitosan to adsorb substantial quantities of dyestuffs from dye processing effluents. These could be performed by both batch and continuous fixed bed adsorption columns. In addition, a significant uptake of pesticides by chitin and chitosan has been demonstrated (Muzzarelli,1977; Kemp and Wightman, 1981).

1.10.4 Enzyme immobilization and chromatographic applications

Owing to chitin and chitosan comprising of a high density of both hydroxyl and amino groups, one would expect a considerable number of fortuitous affinities between the polymers and macromolecules. Immobilization of enzymes and whole cells on the polymer was investigated by many authors. The most commonly preferred technique is based on the use of glutaraldehyde as a cross-linking agent to form aldimine bonds between chitosan and protein. However, certain enzymes lend themselves to direct immobilization because they contain specific chitin binding sites, the occupation of which does not detract from the catalytic activity of the protein. Examples of the reported enzymes immobilized on chitin base are lactase (Stanley, *et al.* 1975), glucoamylase (Stanley, *et al.* 1978), α-amylase (Flor and Hayashida, 1982), invertase (Illanes, *et al.* 1986), diastase and amyloglucosidase (Synowiecki, *et al.* 1987), glucose oxidase and catalase (Liu, 1982), urease (Iyengar and Prabhakara-Rao, 1982). Immobilization of enzymes with chitosan was also studied such as acid phosphatase (Muzzarelli, *et al.* 1976), β-galactosidase (Sicsic, *et al.* 1986) and penicillin G acylase (Braun, *et al.* 1989).

Purification of wheat-germ agglutinin using regenerated chitin and reported binding capacity about 10 mg of agglutinin/gm of the chitin has been examined by Bloch and Burger (1974). The authors suggested that due to simplicity, speed and economy of the proposed chitin affinity chromatography for the purification of wheat-germ agglutinin, these should make it useful for a large-scale preparation. Nagasawa, *et*

al. (1970) showed that suspension of chitosan mixed with microcrystalline cellulose and dried as a thin layer on glass plates was useful for the preparation of nucleic acid constituents .

Tobacco mosaic virus (TMV), a protein-encapsulated organism suspended in a solution of 0.01 M phosphate buffer at pH 6.8 can be adsorbed on chitosan and eluted to its active form by potassium hydrogen phosphate (K_2HPO_4) in the pH range between 7.2 and 7.7 (Ashford, *et al.* 1977).

1.10.5 Cosmetic ingredient

Nicol (1991) stated that chitin was used in cosmetics since 1969. The German cosmetics giant " Wella " has been researching chitosan as a hair treatment for more than 10 years. More recently, Japanese and German companies had developed a water soluble form of chitosan and used it for skin and hair cosmetic base. They used these derivatives as a replacement for hyaluronic acid, a common component thickening effects in creams, lotion and conditioners. It has experimented with the firm-forming properties of chitosan on hair sprays and nail varnishes providing. Chito-Bios of Ancona in Italy sells N-carboxybutyl chitosan, under the trade name Evalsan R for shampoos, bath foams, liquid soaps, toothpaste, personal-hygiene detergent and face creams.

1.10.6 Paper industries

The greatest amount of chitin is perhap supplied for paper manufacturing industries (Nicol, 1991). Adding only one percent by weight of chitin to pulp increases the strength of paper. The quantity of fibres retained and speeds up the rate at which water drains from the pulp when making sheets of paper. These save up to 90% of the energy for drying the pulp. In addition, chitin makes the paper easier to print on and greatly improves its wet strength which are advantage for manufacturing of disposable nappies, shopping bags and paper towels *etc.*

1.10.7 Film and membrane formation

As cellulose and their derivatives, chitin and chitosan could be made to a form of membrane, film or fiber using similar processes. Since chitosan is soluble in a wider range of solvent, it is easier to use as starting material than chitin. However, production of chitin membrane is possible by either dissolving chitin in hexafluoroisopropanol or hexafluoroacetone (Capozza, 1975; cited by Muzzarelli, 1976) or reacetylating chitosan membrane using mixture of methanol and acetic anhydride. Rutherford and Dunson (1984) studied the characteristics of chitin film prepared by dissolving the material in N,N-dimethylacetamide (DMAc)-5% $LiCl_2$. The authors reported that the chitin film is clear and flexible; water flux was about 34,000 $\pm$ 5,942 $\mu mole \cdot cm^{-2} \cdot h^{-1}$ and several solutes flux was 100-300 times less than the water flux. Chitin fiber could be obtained by extruding chitin solubilized in anhydrous tricarboxylic acid solvent system, into potassium hydroxide and 2-propanol (Ashford, *et al.* 1977).

The most simple procedure to prepare chitosan membranes could be performed by dissolving the material in weak acid, spreading the solution on a glass plate, neutralizing the dried acetate form of membrane in ammonia or other alkali, and followed by washing with water. In addition, chitosan films may be cast on metallic surface from acidic solutions of chitosan and dried by heating. The chitosan membrane and film is also clear and flexible; and it is insoluble in water, in alkali solutions and resists dissolution down to a pH about 4 (Muzzarelli, 1976; Averbach and Clark, 1978; Yang and Zall, 1984). The outstanding properties of chitosan membrane and film are its ability to prevent passage of oxygen, carbon dioxide, nitrogen and permeability to water. Therefore, these could be used for reverse osmosis membrane instead of the traditional one, cellulose acetate.

Ashford, *et al.* (1977) reviewed the physical examination of chitosan membranes and suggested that the polymer interchain spacing was not rigid that various compounds can be inserted and accomodated in the film. From these standpoint,

various characteristics of chitin and chitosan membranes such as flexibility, permeability, conductivity as well as mechanical strength, could be manipulated. Muzzarelli (1976) stated that the membrane that made from chitosan and polyhexamethyleneadipamide of 12-18 mm thick showed osmotic flow between 100-250 mm^3 x 100 cm^{-2} x h^{-1}. In addition to chitin and chitosan base membranes, their derivatives could also be modified to used in varieties of specific purpose such as sulfoethylchitosan and sulfonatechitosan for medical application (Muzzarelli, 1976).

Since chitin and chitosan comprising of amino groups with regularly spacing along a polymer chain, it exhibits a strong binding affinity to both metallic and non-metallic materials. This supports a number of potential applications in adhesives, coatings, binders, *etc.*, for cotton, wood and paper.

1.11 Summary of Review

Chitin is a polymer of N-acetyl-D-glucosamine which is regarded as the second most abundant polysaccharide on earth next to cellulose. It is widely distributed in both animal and plant kingdoms, and plays important role principally as structural supporting material in living organisms. Natural occuring chitin usually associated with proteins and minerals, and found in three polymorphic forms designated as α, β and γ. Therefore, isolation of the polymer from chitinous source with particular from crustacean shell involves two processes *i.e.* demineralization and deproteinization . The former process could be accomplished by treatment the chitinous material with strong diluted acid whereas the process is conducted using strong diluted alkali. However, variations of reaction condition influence properties of product to a great extent. Although chitin is regarded as an innert molecule, it is readily hydrolysed by chitinolytic enzyme system as well as lysozymes. Chitin is thus more attractive for application than those of the other synthetic polymers.

Since chitin is insoluble in water and all ordinary solvents, its application are restricted. Fortunately, deacetylation of chitin using concentrated strong alkali yields

chitosan which is soluble in most weak acids and some other solvents. Variations of the deacetylation condition provide products with different properties, and wide ranges of application. In conclusion, properties of chitin and chitosan depend on their average molecular weight, degree of deacetylation as well as physical form of crystallinity. General characteristics of commercially available chitin and chitosan were shown in Appendix 1. Apart from chitosan, several forms of chitin derivative have been synthesized such as carboxymethyl chitin, carboxyethyl chitin, chitin sulfate, ethyl- and hydroxyethyl chitin *etc.*

Since chitin and its derivatives exhibit varieties of chemical and physical properties, therefore, they could be used in several fields of application.

Objectives

1. To establish the optimal conditions for preparation of chitin and chitosan from two species of the squid pen, namely *Loligo lessoniana* and *Loligo formosana*.

2. To clarify the chemical, physical and biochemical properties of the products prepared under different conditions.

3. In some instances, comparisons of the characters will be made between the chitin and chitosan from prawn shell prepared under identical conditions and those from squid pens.

2. MATERIALS AND METHODS

Materials

Chemicals : All chemicals used were analytical grade or otherwise indicated.

Chemical name	Manufacture
Ammonium metavanadate	Ajax, Australia
Potassium tetraborate	Ajax, Australia
Lead nitrate	BDH, Germany
Acetone	E. Merck, Germany
Hydrochloric acid (37 %)	E. Merck, Germany
Methanol	E. Merck, Germany
Methyl red	E. Merck, Germany
Nitric acid (65 %)	E.Merck, Germany
Perchloric acid	E. Merck, Germany
Potassium hydroxide	E. Merck, Germany
Sodium hydroxide	E. Merck, Germany
Sulfuric acid (98 %)	E. Merck, Germany
Ammonium sulphate	Fluka, Switzerland
Chitosan, Mr 75,000	Fluka, Switzerland
Chitosan, Mr 2,000,000	Fluka, Switzerland
Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA)	Fluka, Switzerland
Hexamethylenetetramine	Fluka, Switzerland
Concentrated ammonia solution (25%)	Ridel-deHaen, Switzerland

Chemicals: (continue)

Chemical name	Manufacture
Cupric sulphate pentahydrate	Ridel-deHaen, Switzerland
Lithium chloride	Ridel-deHaen, Switzerland
Cupric sulphate	Ridel-deHaen, Switzerland
N- acetylglucosamine	Sigma, U.S.A.
Chitinase, 0.5 U/ mg (*Streptomyces griscus*)	Sigma, U.S.A.
N,N- dimethylacetamide (99+%)	Sigma, U.S.A.
p-dimethylaminobenzaldehyde (DMAB)	Sigma, U.S.A.
Fast sulphon black F	Sigma, U.S.A.
β-glucosidase, 27 U/mg (Almonds)	Sigma, U.S.A.
Xylenol indicator	Sigma, U.S.A.

Equipments:

Equipment	Manufacturer
Analytical balance, H 45	Mettler, Switzerland
Analytical balance, BP 110 S	Mettler, Switzerland
Analytical balance, AE 240	Mettler, Switzerland
Atomic Absorption spectroscopy (AAS)	G.B.C., Australia
Cutting mill, SR-2	Retsch, West-Germany
Digestion unit	Labcenco, U.S.A.
Furnace muffle, Type 6000	Thermolyne, U.S.A.
Inductively Coupled Plasma Emission Spectroscopy (ICPS), Plasma 1000	Perkin-Elmer, U.S.A.
Polarimeter, Polax-D	Atago, Japan
Spectrophotometer, Spectronic 21	Baush & Lomb, U.S.A.
Sinter glass, ASTM No. 40-60, C	Pyrex,U.S.A.
Steam distillation unit	Exelo, England
Test sieve ASTME 11	Endecotts Ltd., England
UV-visible spectrophotometer, UV-160	Shimadzu, Japan
Viscometer Ubbelohde No. 200, M.548	Cannon, U.S.A.

Methods

2.1 Preparation of Chitin and Chitosan

2.1.1 Collection and preparation of squid pen samples

Squid pen of two species, namely *Loligo lessoniana* and *Loligo formosana* were freshly collected from the Pitak Frozen Co. Ltd., Pattani. They were washed several times with tap water and dried at 60 °C overnight in force air oven. The dried samples were pulverized with Waring blender, then ground to a powder through 0.75 mm sieve with cutting mill. In order to investigate the effects of season on properties of the chitin and chitosan products, samples were collected two times during summer and rainy. They were studied separately, and results were combined where statistical analyses showed no significant differences.

The sample of prawn shell (*P. monodon*) was freshly collected from Kiang Huat Sea Gull Trading Frozen Food Co., Ltd., Songkhla. Only abdominal section was used in order to minimize lipid and protein contamination. After thoroughly washing with tap water, the sample was dried at 80 °C overnight in force air oven. The dried sample was pulverized and ground to a powder through 0.75 mm sieve with cutting mill.

2.1.2 Preparation of chitin from squid pen

Preliminary study in this laboratory suggested that squid pen from both species compose of very trace amount of ash (approx. 0.03 %), demineralization was, therefore, omitted from the preparation scheme of chitin. Deproteinization was carried out by slowly adding the sample powder into 1.0 M NaOH solution to obtain a ratio of solid to alkali solution 1: 13 (w/v). Temperature of the reaction mixture was maintained at 50 °C with constant stirring for 5 hours. The residue was filtered through 4 layers of gauze using vacuum pump, washed with deionized distilled water until pH became neutral. The chitin product was dehydrated 2 times with methanol, followed a time

with acetone, transferred to glass tray, then dried overnight at 50 °C in force air oven. In order to determine percent yield accurately, weighing was performed immediately after the sample was cooled in desiccator. The dried chitin was tightly kept in polyethylene bottle for preparation of chitosan and for further studies. To evaluate the differences between species as well as seasons of collecting sample, each sample was separately prepared by duplication.

2.1.3 Preparation of chitosan from squid pen

Preliminary study on preparation of chitosan was carried by slowly adding the dried chitin powder into three-neck head boiling flask containing a solution of 50% NaOH (w/v) to obtain a ratio of solid to alkali solution 1: 15 (w/v). The temperature of reaction mixture was maintained at 100 °C and refluxed under nitrogen atmosphere (oxygen free). Results suggested that color of product changed from off-white to light brown after 2 hours of treatment. Moreover, the chitosan powder aggregated and became cluster after 4 hours of deacetylation. Hence, preparation of chitosan under the lower temperature, 60 °C was selected for deacetylation of squid pen chitin throughout the studies.

To investigate the effects of deacetylated duration on properties of chitosan, a portion of sample was taken from the reaction mixture at 2, 4, 6 and 8 hours successive intervals. At each interval, the reaction mixture was filtered and a portion of residue was taken while the remaining was further refluxed with approximately the same proportion of 50% NaOH and the same condition as described above. The taken sample was washed with deionized distilled water and filtered through a sinter glass several times until a neutral pH was obtained. The chitosan product was dehydrated 2 times with methanol, followed a time with acetone, transferred to glass tray, then dried overnight at 50 °C in force air oven. Weighing was immediately determined after the powder was cooled in desiccator. To evaluate the differences between species as well as seasons of collecting sample, each sample was separately prepared by duplication.

2.1.4 Preparation of chitin and chitosan from prawn shell

The shell powder was demineralized with 1M (3.0 %, w/v) HCl at room temperature with constant stirring for 1.5 hours, using a ratio of solid to acid solution 15 : 190 (w/v) (modified from Bough, *et. al.* 1978). The decalcified product was washed and filtered several times with deionized distilled water until a neutral pH was obtained, followed by methanol, acetone and then dried overnight at 50 °C in force air oven. The decalcified product was, then, deproteinized and deacetylated under identical conditions as described in the preparative schemes for the chitin and chitosan from the squid pens. These was performed in order to obtain comparable properties of samples prepared under identical conditions.

2.2 Determination of Nitrogen Content

Nitrogen contents in chitin and chitosan were determined using standard semi-micro Kjeldahl method (A.O.A.C., 1984). Accurated weight of approximately 0.5 gm sample and 7.0 gm catalyst mixture (499.0 gm K_2SO_4 and 1.3 gm $CuSO_4.5H_2O$) wrapped with ashless filter paper and put in digestion tube. It was digested with 10 ml concentrated H_2SO_4 in heating block until a clear solution was obtained. The cooled solution was diluted to 50 ml with distilled water. Aliquot of 10 ml of the diluted solution was taken to react with 10 ml of 45% NaOH in steam distillation unit. The liberated ammonia was trapped with standardized of approximately 0.2 N HCl containing a few drops of methyl red indicator. The remaining HCl was evaluated by titration with standardized of approximately 0.2 N NaOH, and percent nitrogen was calculated as follow:

$$\% \text{ Nitrogen} = \frac{[(\text{N. acid})(\text{ml. acid}) - (\text{ml. NaOH})(\text{N.NaOH})] \times (1400.67 \times \text{dilution factor})}{\text{mg sample}}$$

Prior to analysis, accuracy and precision of procedure was evaluated using standard $(NH_4)_2SO_4$ solution. Results indicated that standard error of estimation (SE) for these

procedure was ± 0.0016 for 0.14% Nitrogen. Triplication were carried out for each sample.

2.3 Ash Contents Analyses

Ash contents in samples were analysed using standard procedure that has been described in A.O.A.C. (1984). An accurated weight of approximately 3 gm sample in a known dried weight crucible was heated on bunsen flame until a white smoke disappeared. The burned sample was then placed in furnace and heated at 850 °C for 8 hours, transferred to desiccator, and weighed immediately after cooling using the microanalytical balance reading to 5 decimal places. Four replications were determined for each sample.

2.4 Trace Elements Analyses

Samples were digested according to the procedure recommened in the Handbook from Kerven, G. An accurated weight of approximately 0.5 gm sample was digested in digestion tube with 15 ml digestion mixture prepared by mixing 625 ml concentrated nitric acid and 125 ml concentrated perchloric acid, containing 30 mg ammonium metavanadate previously dissolved in 5 ml warm deionized distilled water. To avoid a vigorous reaction with evolution of brown NO_2 fume, initial temperature of sand bath was maintained at 100-120 °C for 2 hours. Temperature was brought up to 155-165 °C while no more brown fume evolved and the reactions subsided. The digestion was maintained at this temperature until completion of digestion obtained that could be observed by the appearance of a clear solution with white perchloric acid fume. After cooling, the digested sample was quantitatively transferred and made the volume up to 25 ml with deionized distilled water. The diluted sample was sealy kept in scientillation vial until analysis. To substract background contaminated in reagents and glasswares, a blank was carried out by the same procedure. Concentrations of cadmium (Cd), copper (Cu), iron (Fe) and lead (Pb) were analysed by Atomic Absorption Spectroscopy

(AAS), while arsenic (As), calcium (Ca), magnesium (Mg) and mercury (Hg) were analysed by Inductively Coupled Plasma Emission Spectroscopy (ICPS). Four replications were determined for each sample.

2.5 Molecular Weight Determination

The viscosity average molecular weight (Mv) of chitosan from the two species of squid pen was determined and compared using viscometric method (modified from Bronswijk, 1975). Chitosan sample of 0.05 gm was dissolved in 1% (v/v) glacial acetic acid 100 ml. It appeared that all chitosan samples prepared at the different deacetylated intervals were completely dissolved within 1 hour by low speed stirring. The solution was filtered through a sinter glass (ASTM No. 40-60, C), various concentrations were prepared from this stock solution by diluting with 1% acetic acid. Viscosity of each chitosan solution and solvent were determined at 25 ^{o}C with Ubbelohde viscometer, and these values were used in molecular weight determination of the samples. Triplicate readings were performed for each concentration. The values of relative viscosity (η_{rel}), specific viscosity (η_{sp}), specific viscosity per concentration (η_{sp}/C), ln.relative viscosity (ln η_{rel}) and ln.relative viscosity per concentration (ln η_{rel}/C) were calculated. The values of η_{sp}/C were plotted against concentration (C) and the values of $\ln\eta_{rel}/C$ were plotted against concentration (C). The intrinsic viscosity [η] was estimated by extrapolating η_{sp}/C to a zero concentration, then the viscosity average molecular weight of chitosan was calculated from Standinger equation as follows:

$$\log [\eta] = \log K + a \log Mv$$

Where, K and a are the constant values; 8.93×10^{-4} and 0.71, respectively.

Mv = viscosity average-molecular weight

[η] = intrinsic viscosity

Relative viscosity (η_{rel}) definded as: $\eta_{rel} = t / t_o$

Where, t = flow time of solution and t_o = flow time of solvent.

Specific viscosity (η_{sp}) definded as: $\eta_{sp} = \eta_{rel} - 1 = (t - t_o)/t_o$

The specific viscosity per concentration (η_{sp}/C) or reduced specific viscosity (η_{red}) defind as:

$$\eta_{sp}/C = \frac{(t - t_o)}{t_o}/C$$

where, C = concentration of solution (gm/100 ml)

And ln relative viscosity per concentraion ($\ln \eta_{rel}/C$) or inherent viscosity (η_{inh}) defined as:

$$\ln \eta_{rel}/C = \ln (t / t_o)/C$$

The stability of chitosan in 1% acetic acid solution was investigated by measuring viscosity of the solutions of various concentrations as described above. This was determined dialy until the constant level of intrinsic viscosity per unit time [η/t] for each sample was reached. Triplications were conducted for each sample.

2.6 Determination for Degree of Deacetylation of Chitosan

Several attempts have been evaluated in order to establish a reliable process to determine the degree of deacetylation of chitosan. Finally, it was recognized that the parameter could be determined by the use of spectroscopic method recommended by Muzzarelli and Rocchetti (1985). It was the most simple with excellent accuracy and precision method.

Preliminary study performed by evaluation for the crossing point of the first derivative absorption spectra of acetic acid at concentration of 0.01, 0.02 and 0.03 M. The spectra were read against water blank. This was carried out in order to determine the wavelength where disturbance absorption at any concentration of acetic acid solvent could be set as zero. As shown in Figure 2.1, the crossing point was found at 205 nm, and was closed to the maximum signal height of the first derivative absorption spectrum of N-acetylglucosamine whose the signal height was proportion with concentration.

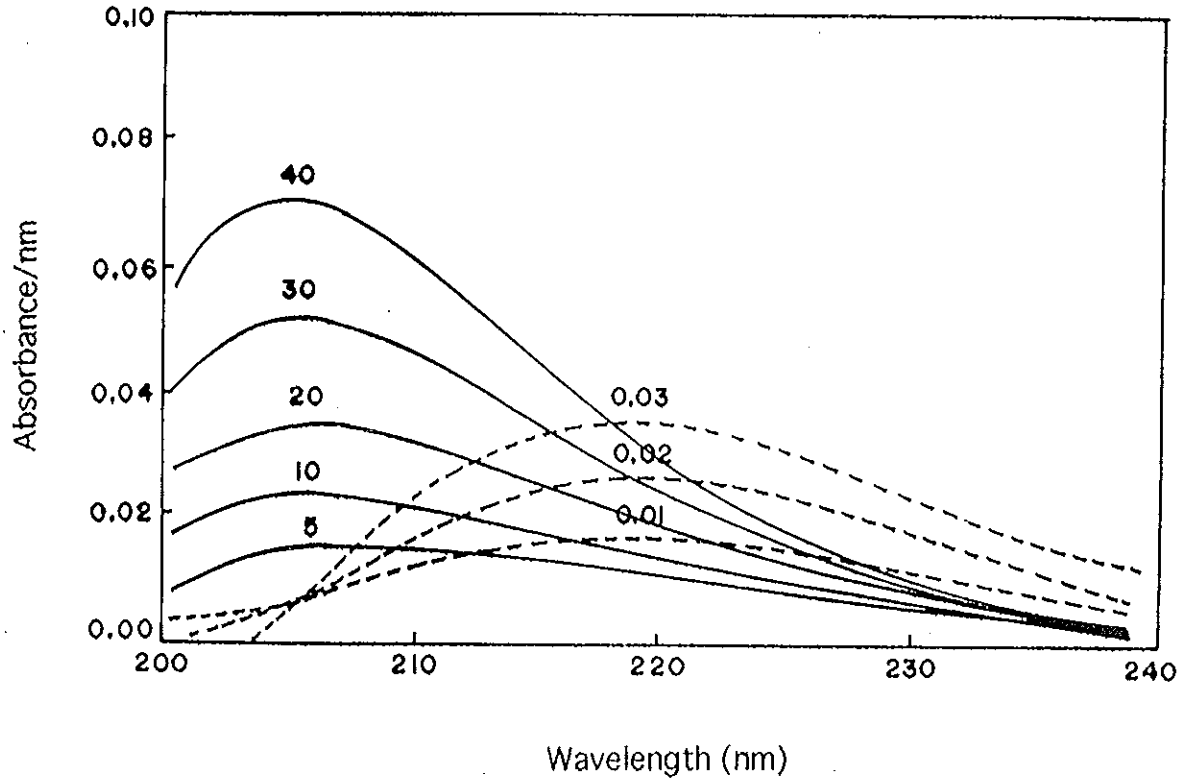


Figure 2.1 First derivative spectra of 0.01, 0.02, 0.03 M acetic acid solution (three lower curves crossing at 205 nm), and of N-acetylglucosamine at various concentrations (mg/l) in 0.01M acetic acid (- - - - - and —— figure to the first derivative spectra of acetic acid and N-acetylglucosamine solutions, respectively). Actual recording with Shimadzu UV-160 spectrophotometer.

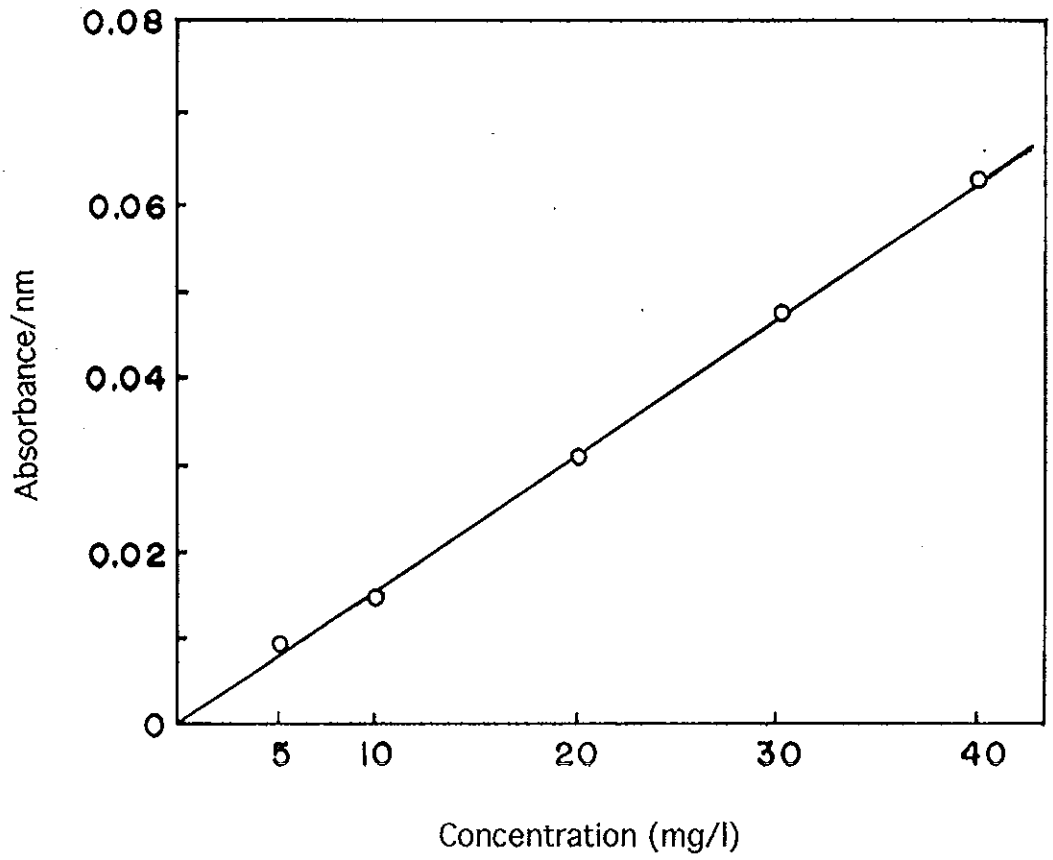


Figure 2.2 Calibration curve of the first derivative spectra of N-acetylglucosamine (5-40 mg/l) at 205 nm.

Accordingly, five concentrations (5-40 mg/l) of N-acetylglucosamine in 0.01 M acetic acid were prepared, an absorbance of the first derivative spectrum for each concentration was read. Then, calibration curve was achieved from linear correlation between the concentrations and the absorbance values (Figure 2.2).

To determine the degree of deacetylation of chitosan, 10 mg of dried sample was dissolved in 10 ml of 0.1 M acetic acid and diluted to 100 ml with distilled water. An absorbance of the first derivative spectrum was read. Then, the concentration of N-acetylglucosamine unit in the sample was determined from the calibration curve. Five replications were determined for each sample.

2.7 Optical Activity of Chitin

The procedure used to investigate an optical activity of chitin in this study was adapted from Austin, *et al.* (1981). An accurated weight of 0.1 gm of the sample was dissolved with slowly stirring in 100 ml dimethylacetamide (DMAc) containing 5% lithium chloride. Generally, the chitin from both species of squid pen were completely dissolved within 6 hours, but a little amount of particle appeared in the solution of chitin from prawn shell, and it was removed by filtration through sinter glass. An optical activity of the solution in a polarimetric tube of 1 decimetre path length was determined with polarimeter against the solvent blank. In order to investigate changing of the property, an optical activity was read after standing the solution at room temperature (29±1 °C) for nine intervals within 14 days. Four replications were determined for each sample. An optical activity was expressed in a term of specific angle of rotation which could be calculated as follow:

$$[\alpha]_D^T = \frac{\alpha \times 100}{L \times C}$$

Where, $[\alpha]_D^T$ = Specific angle of rotation

α = Angle of optical rotation of solution (degree)

L = Path length of polarimetric tube (decimetre)

C = Concentration of solution (gm/100 ml)

T = Temperature (°C)

D = Light source (D-line, 589 nm)

2.8 Determination of Hygroscopic Activity of Chitin and Chitosan

To investigate moisture reabsorption properties of chitin and chitosan from the two species of squid pens, an accurated weight of approximately 3 gm samples were transferred to a dried and known weight of 100 ml beaker. Beakers containing the samples were dried at 100-105 ^{o}C in air force oven until a constant weight was obtained which was taken approximately 48 hours. The samples were allowed to stand at room temperature in desiccator, and their initial weights were determined immediately after cooling by using analytical balance.

Beakers containing the dried sample were covered with gauze and stood in open air at room temperature. Weights were daily determined until constant values were reached, percent moisture reabsorption of each sample was then calculated with respect to its initial dried weight. Four replications were determined for each sample.

2.9 Determination for Adsorption Capacity for Metals

In this study, adsorption capacity for metal ions on chitin and chitosan from both species of the squid pens as well as the prawn shell prepared under identical conditions was compared. Samples were sieved through 150 μm screen in order to standardize the surface area. Two metal ions namely, Cu (II) in a form of $CuSO_4.5H_2O$ and Pb (II) in a form of $Pb(NO_3)_2$ were selected for investigation. Solutions of these compounds were dissolved in deionized distilled water and the pH was adjusted to 4.0 with nitric acid.

Prior to analysis with other samples, an appropriate metal concentration and incubation period providing maximum adsorption on the chitosan which were expected to have the highest binding capacity, were preliminary determined. So, the chitosan from the squid pens which had been deacetylated for 8 hours, and were presumed to carry the maximum density of amino group were selected to meet the purpose.

An appropriated concentration for each metal ion adsorbed by chitosan was determined by weighing 1.0 gm of the chitosan sample in conical flask, slowly added

5.0 ml of six concentrations for Cu (II) ranging from 2.5-30.0 mg/ml, and for Pb (II) ranging from 10-160 mg/ml. The mixture was equilibrated at room temperature with shaking using orbital shaker at 150 rpm for 60 minutes, then filtered (Whatman, No. 1). The non-adsorbed portion for each ion dissolved in filtrate was determined by complexometric titration with standardized EDTA solution (0.01 M) as procedures described by Vogel (1961) (Appendix 4 and 5). The adsorbed portion was then calculated. From these results the maximum adsoption capacity on 1 gm chitosan for each metal ion was evaluated and was regarded as an appropriated concentration for further examination.

An optimum adsorption period was determined by the use of similar analogy to evaluate an appropriated adsorption concentration as described above. However, variation of binding period for five intervals within 120 minutes was performed while the concentration for each metal ion was fixed at the appropriated levels as previously evaluated.

To compare adsorption capacity of chitin and chitosan from different sources as well as preparation conditions, the solution of the metal ions at the appropriated concentrations and optimum adsorption period for each ion were used as a standard condition. The comparison was made between chitin and chitosan from the two species of squid pen. Moreover, the adsoption capacity for the metal ions on the chitosan samples prepared with different deacetylated times was studied as well. In addition, this property of chitin and chitosan from prawn shell prepared under identical condition with those from the squid pen were also conducted. Nine replications were determined for each sample from squid pen and six replications were determined for the sample from prawn shell.

2.10 Chitinase and Chitobiase Activities on Chitin from the two Species of Squid Pen and from Prawn Shell

To evaluate a potential use of chitin from the different sources in biomedical field of applications, the rates of chitinolytic hydrolysis on the substrate from the two species of squid pen and prawn shell were compared. These were performed by the procedure adapted from Jeuniaux (1966) as follow.

In order to obtain reliable initial rate of the enzymatic hydrolysis, the optimum assay conditions were preliminary studied by variation of particle sizes of substrate, enzyme concentrations and reaction periods. Results suggested that particle size of chitin substrates should be standardized by passing through 150 μm sieve. To a glass vial, 1 ml chitin suspensed in water (containing approx. 5.0 mg), 1.0 ml buffer solution (0.6 M citric acid and 1.2 M disodium hydrogen phosphate which was adjusted pH to 5.1), 0.2 ml chitinase (*Streptomyces griseus*) solution (1U/ml) and 0.5 ml β-glucosidase (Almonds) solution (0.6 U/ml) were added, respectively. The volume of reaction mixture was made up to 3.3 ml with distilled water. It was then incubated at 37 ^{o}C with gently shaking. Liberated products from the enzymatic hydrolysis were determined at 0, 1, 3, 5, 7 and 10 hours intervals. To each interval, the reaction was stopped by adding 0.7 ml KOH (10 %, w/v) and boiled for exactly 10 minutes, centrifuged at 3,000 rpm for 10 minutes after cooling. The supernatant of 0.5 ml was taken into test tube, 0.1 ml 0.8 M potassium tetraborate was added and boiled for 10 minutes. Color was developed by adding 3.0 ml of p-dimethylaminobenzaldehyde (DMAB) which was freshly prepared by adding 1.0 gm DMAB in 100 ml glacial acetic acid containing 2.4% (v/v) 10 N HCl, incubated at 37 ^{o}C for 15 minutes after thoroughly mixing. Absorbance at 585 nm was read within 10 minutes using Spectronic 21 spectrophotometer. Then, concentrations of liberated N- acetyl-glucosamine were determined from the calibration curve.

It was noted that considerable amount of chitobiase which was an expensive enzyme, was required for each determination. In the reference method, Jeuniaux (1966)

suggested the use of lobster hemolymph as an alternative source of chitobiase. Due to its ease of accesibility, prawn hemolymph was thought to be an alternative source and was, therefore, included in this study.

Prawn hemolymph was withdrawn from pericardium using 1 ml disposable syringe containing 0.2 ml cold distilled water. The sample was centrifuged at 2500 rpm for 15 minutes, serum was collected and protein concentration was determined using Lowry method (Thorne, 1978).

Units. The activity was expressed as µmole N-acetylglucosamine liberated per hour (µmole/hr) and specific activity was expressed as µmole per hour per miligram protein (µmole-hr^{-1}- mg^{-1}protein).

3. RESULTS AND DISCUSSION

3.1 Appearance and Percent Yield of Chitin and Chitosan from Squid Pens

Figure 3.1 shows a general shape and size of the two species of squid pen used in this thesis namely, *Loligo lessoniana* and *Loligo formosana.* , A white and soft fluffy fibrous of chitin and chitosan prepared from squid pen powder were shown in Figure 3.2. A similar appearance (did not shown) was observed for *L. formosana*.

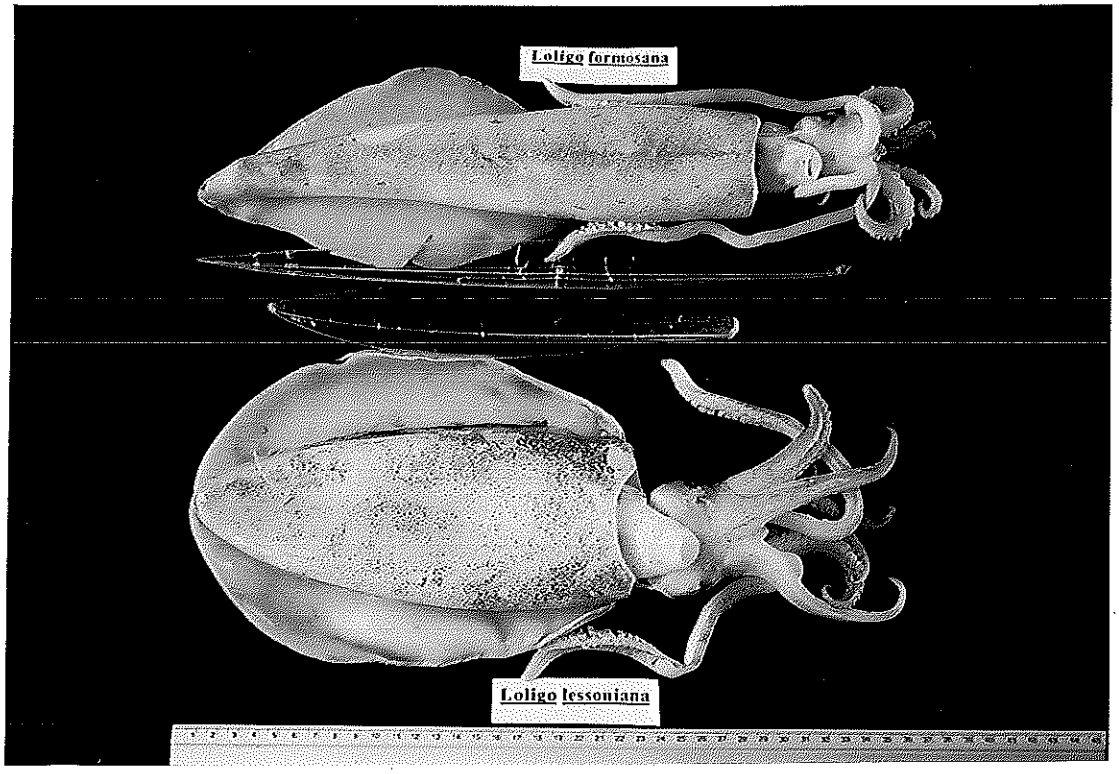


Figure 3.1 Shape and size of two species of squid *L. lessoniana* and *L. formosana* and their pens.

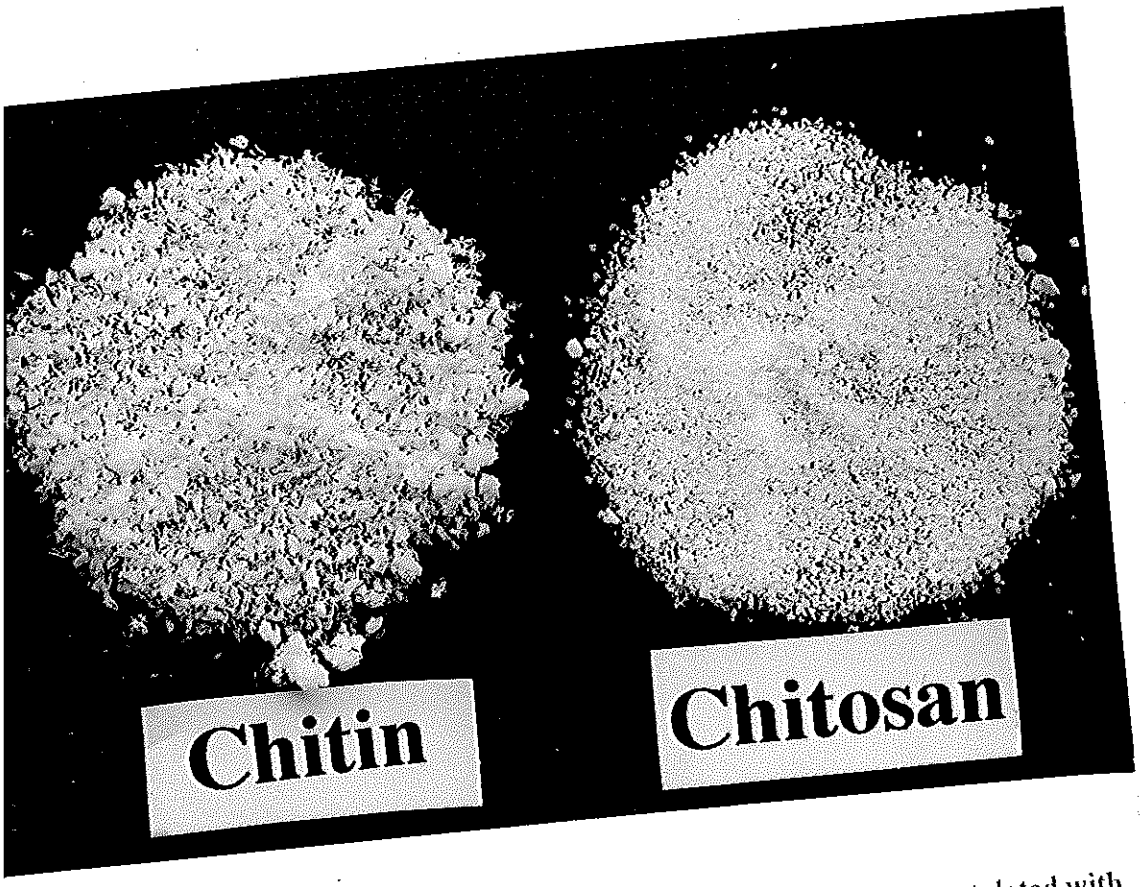


Figure 3.2 Appearance of chitin and chitosan powders (0.75 mm) deacetylated with 50% NaOH at 60 °C for 6 hours under nitrogen atmosphere from squid pen, *L. lessoniana*.

Within the same species, there were no considerable differences in percent yield of chitin prepared from samples collected during summer and rainy, therefore, data of the same species were combined (Table 3.1). In comparison between the two species, there was no significant difference in percent yield, approx.36 %yield (Appendix 2). These are comparable to other species (*Ommastrephes bartrami*) studied by Kurita, *et al.* (1993) who reported 35-40% percent yield. Although a slightly lower in percent yield was found in the present study, this variation may contribute by the difference of weighing procedure which was not described in the published data.

Table 3.1 Percent yield of chitin and chitosan from *L. lessoniana* and from *L. formosana*. Values are calculated on the basis of dry weight and are mean of duplicate preparations.

Species	Percent yield		
	Chitin	Chitosan[1]	Chitosan[2]
L. lessoniana	36.06	27.59	77.55
L. formosana	36.55	28.21	77.21

Chitosan[1], calculated on the basis of the dried squid pen powders

Chitosan[2], calculated on the basis of the dried chitin

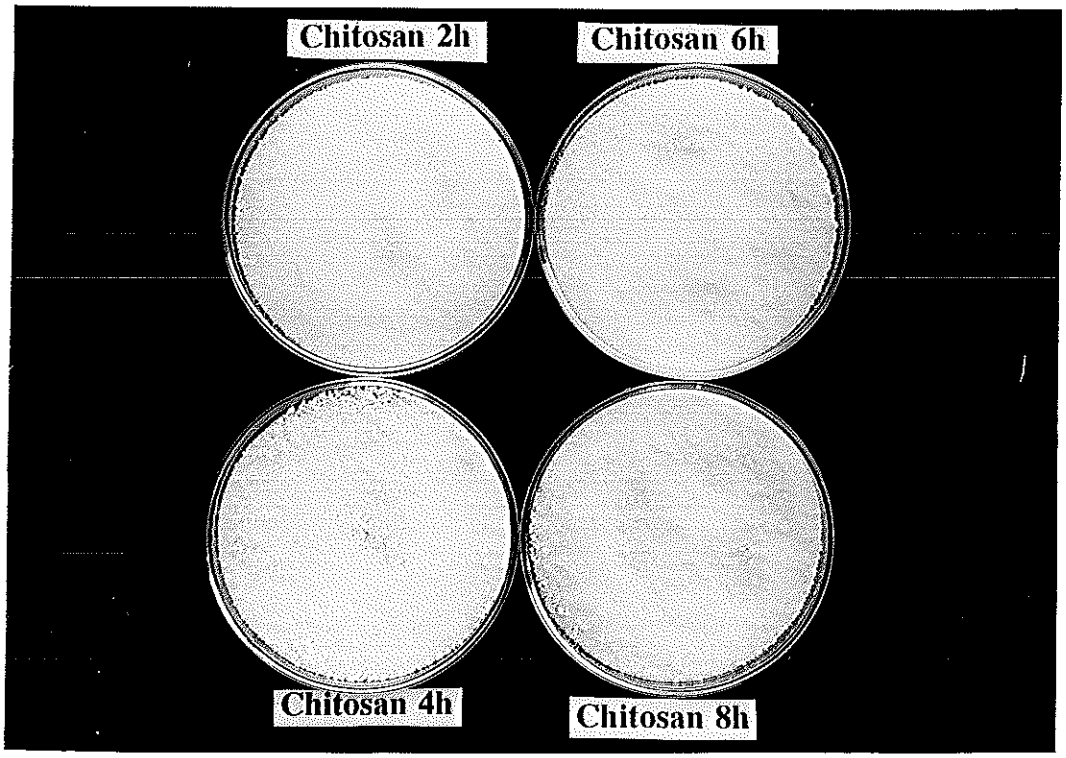


Figure 3.3 Appearance of chitosan prepared from a powder (0.75 mm) of squid pen (*L. lessoniana*), deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for various intervals 2, 4, 6 and 8 hours.

For chitosan, percent yield calculated with respect to the weight of dried pen from *L. lessoniana* and *L. formosana* again indicated no significant differences, which were 27.6% and 28.2%, respectively. Appearances of sample prepared under the different conditions, whereas the compact powder was increased accordingly to period of deacetylation, were shown in Figure 3.3.

3.2 Nitrogen Contents in Squid Pen, Chitin and Chitosan

Statistical analyses indicated that there were no significant differences of nitrogen content ($P>0.05$) in the same species of squid pen collected during summer and rainy seasons. Similar results were noted for the nitrogen content in their isolated chitins. Therefore, data of the same species was pooled, and showed in Table 3.2.

For chitosan, analysis of varian (ANOVA) indicated that there were no significant differences ($p>0.05$) between the two spceies as well as the two collection seasons (Appendix 3). Although significant interactions ($P<0.05$) among the treatment conditions and the collection periods were noted, the differences were obviously seen during the first four hours of deacetylation. Therefore, data of the same species and deacetylated interval was pooled and showed in Table 3.2. For *L. lessoniana*, the nitrogen content were increased from 6.37% in chitin to 7.73% in chitosan after 2 hours of deacetylation, then maintained at the level of approximately 7.88% thereafter. Similar variation in pattern of nitrogen contents was also observed for *L. formosana*. The most likely was due to the progressingly lost of acetyl group from the squid pen chitin.

Nitrogen contents in the pen of *L. lessoniana* and *L. formosana* were 11.56% and 11.80%; and contents in the isolated chitin samples were 6.37 and 6.23%, respectively. Statistical analysis from the pooled data also showed no significant difference ($P>0.05$) of nitrogen content between samples from the two species of squid pen. These were slightly lower than theoretical value of 6.9% (cited by Kandaswamy, 1978) for pure chitin.

Table 3.2 Nitrogen contents in squid pen, chitin and chitosan from *L. lessoniana* and *L. formosana*. Chitosan samples were prepared by deacetylation with 50% NaOH at 60 °C under nitrogen atmosphere for 2,4,6 and 8 hours. Values are calculated on a dried weight basis, and are mean ± S.E. of six replicate for squid pens and twelve replicate determinations for chitin and chitosan.

Sample	Nitrogen content (%)	
	L. lessoniana	*L. formosana*
Squid pens	11.56 ± 0.03	11.80 ± 0.01
Chitin	6.37 ± 0.06	6.23 ± 0.04
Chitosan (2h)	7.73 ± 0.02	7.63 ± 0.01
Chitosan (4h)	7.86 ± 0.02	7.82 ± 0.02
Chitosan (6h)	7.88 ± 0.02	7.90 ± 0.02
Chitosan (8h)	7.88 ± 0.02	7.98 ± 0.02

Similar has been noted by Kandaswamy (1978) who reported that chitin from *Loligo indica* which was deproteinized with 1 M NaOH at 100 °C for 16-18 hours comprised of 6.42-6.58% nitrogen.

3.3 Ash and Trace Element Contents

Table 3.3 indicated that both species of squid pen comprising very small amount of ash within the range of 0.03-0.04% (30-40 ppm), while the content could not be detected in their chitin and chitosan products. This confirms that it is not necessary to include the demineralization step in preparation scheme of chitin from squid pens.

Table 3.3 Ash contents in pens, chitin and chitosan deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours from *L. lessoniana* and *L. formosana* . Values are mean ± S.E. of four replications.

Sample	Ash (%)	
	L. lessoniana	*L. formosana*
Squid pen	0.025±0.005	0.042±0.003
Chitin	nd	nd
Chitosan (2h)	nd	nd
Chitosan (4h)	nd	nd
Chitosan (6h)	nd	nd
Chitosan (8h)	nd	nd

nd, non detected

Analysis for trace elements in the pen of *L. lessoniana* indicated that Ca was the predominant composition (17.73 ppm), followed by Fe (7.74 ppm), Mg (3.30 ppm) and Cu (0.93 ppm); whereas As, Cd, Hg and Pb fallen below detection limit (Table 3.4). Although the higher levels of these elements were detected in the pen of *L. formosana* (Table 3.5), these were presumably due to the contamination from foreign matters rather than its inherent composition as pointed out by Muzzarelli (1985).

Kurita, *et al.* (1993) demonstrated that the contents of Ca, Na, Mg and Fe in squid pen (*Ommastrephes bartrami*) were 344, 170, 121 and 4 ppm, respectively. Although the authors included demineralization process in preparation of chitin, the metal contents were still remained in the product with considerably higher levels than those found in this study.

Table 3.4 The contents of some typical elements in pens, chitin and chitosan deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours from *L. lessoniana*. Values are mean ± S.E. of four replications for squid pen and eight replications for chitin and chitosan samples.

Sample	Element content (ppm)							
	Ca	Mg	Cu	Fe	As	Cd	Hg	Pb
Squid pen	17.73±3.32	3.30±0.80	0.93±0.28	7.74±0.50	nd	nd	nd	nd
Chitin	3.25±1.57	2.43±0.34	nd	3.14±1.13	nd	nd	nd	nd
Chitosan								
(2h)	3.19±2.03	1.54±0.73	nd	2.59±1.11	nd	nd	nd	nd
(4h)	2.79±1.43	1.21±0.57	nd	1.69±0.95	nd	nd	nd	nd
(6h)	3.74±0.35	1.06±0.36	nd	nd	nd	nd	nd	nd
(8h)	3.39±1.50	0.67±0.32	nd	nd	nd	nd	nd	nd

nd; non detected or below detection limit

Detection limit (ppm); Cd, 0.0004; Cu, 0.001; Fe, 0.005; Pb, 0.01 for AAS

As, 0.02; Ca, 0.001; Hg, 0.02; Mg, 0.001 for ICPS

Table 3.5 The contents of some typical elements in pens, chitin and chitosan deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours from *L. formosana*. Values are mean ± S.E. of four replications for squid pen and eight replications for chitin and chitosan samples.

Sample	Element content (ppm)							
	Ca	Mg	Cu	Fe	As	Cd	Hg	Pb
Squid pen	24.19±5.82	5.53±1.03	16.19±5.69	17.16±2.82	nd	8.14±0.23	nd	nd
Chitin	6.60±3.52	2.50±0.32	8.49±2.01	4.68±0.35	nd	2.83±0.15	nd	nd
Chitosan								
(2h)	3.18±2.06	2.12±0.71	6.89±2.44	2.38±1.10	nd	2.92±0.16	nd	nd
(4h)	3.22±1.61	1.83±0.80	5.31±1.28	2.30±0.92	nd	2.34±0.14	nd	nd
(6h)	2.53±1.62	1.48±0.58	3.22±0.62	1.67±1.08	nd	1.12±0.24	nd	nd
(8h)	1.56±1.27	1.22±0.47	2.77±0.44	nd	nd	0.28±0.12	nd	nd

nd; non detected or below detection limit

Detection limit (ppm); Cd, 0.0004; Cu, 0.001; Fe, 0.005; Pb, 0.01 for AAS

As, 0.02; Ca, 0.001; Hg, 0.02; Mg, 0.001 for ICPS

3.4 Average Molecular Weight of Chitosan

Intrinsic viscosity [η] of chitosan dissolved in 1% acetic acid was evaluated by either extrapolating η_{sp}/C or $\ln \eta_{rel}/C$ to zero concentration as example shown in Figure 3.4. The viscosity average molecular weight (M_V) was then calculated as described in Section 2.5. On the basis of this procedure, it was found that the calculated viscosity average molecular weight of standard chitosan (M_r = 750,000; Fluka) showed 810,535

daltons. However, when the standard chitosan of $M_r = 2 \times 10^6$ daltons was examined, much higher value (4.33×10^6 daltons) was obtained. These suggested that the reliable value of the viscosity average molecular weight would be obtained within the range of 10^6 daltons.

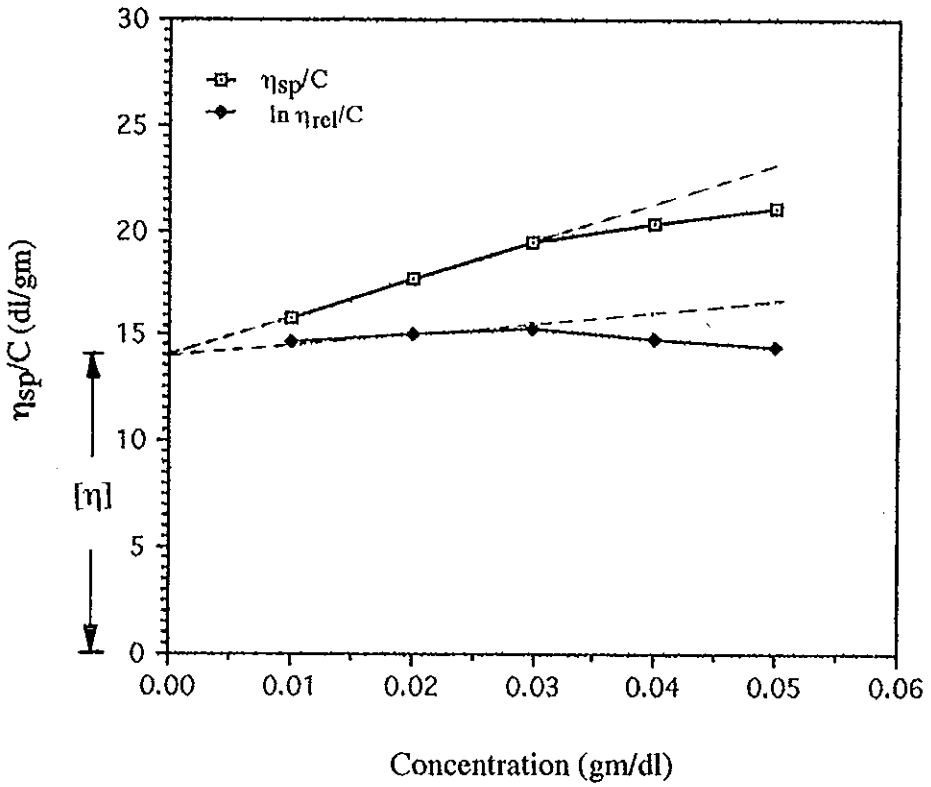


Figure 3.4 The values of η_{sp}/C and $\ln\eta_{rel}/C$ from the standard solution of chitosan (M_r = 750,000 daltons) were plotted against concentration (gm/decilitre). Intrinsic viscosity, [η], was determined by extrapolating of η_{sp}/C and $\ln\eta_{rel}/C$ to zero concentration.

Analysis of variance (Table 3.6) showed that the molecular weight of chitosans from *Loligo lessoniana* and *Loligo formosana* prepared under identical condition were not significant differences ($P>0.05$). It appeared, however, that the longer deacetylation time yielded the lower molecular weight of products, significantly ($P<0.01$). Although

the analysis indicated a significant effect of collection periods on the molecular weight, this was presumably due primarily to the variation of reaction temperature rather than genuine characteristic of raw materials. Therefore, data of the same species was combined as presented in Table 3.7.

Table 3.6 Analysis of variance for the viscosity average molecular weight (M_V) of chitosan prepared by deacetylation chitin from *L. lessoniana* and *L. formosana* with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours. Values of separated analysis for summer and rainy collecting period are shown, and are mean of duplications for each sample.

Species	Deacetylation time (hour)	M_V (dal x 10^6)		ANOVA		
		summer	rainy	T	C	Sp
L. lessoniana						
	2	9.86	10.62	**	**	ns
	4	9.65	10.03			
	6	9.32	9.69			
	8	8.78	9.44			
L. formosana						
	2	10.28	10.20	**	**	ns
	4	10.07	9.94			
	6	9.86	8.98			
	8	8.90	8.66			

T, deacetylation time (hour)
C, collection period (summer and rainy)
Sp., species

**, significant at P< 0.01
ns, non-significant

Table 3.7 Intrinsic viscosity, [η], and viscosity average molecular weight (M_V) of chitosan prepared by deacetylation chitin with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours from *L. lessoniana* and *L. formosana*. Values are mean±S.E. of four replications.

Species	Deacetylation time (hour)	Intrinsic viscosity (dl/gm)	M_V (dal x10^6)
L. lessoniana			
	2	84.75±1.30	10.24±0.22
	4	82.62±0.94	9.84±0.14
	6	80.38±0.75	9.50±0.12
	8	78.00±1.15	9.11±0.19
L. formosana			
	2	84.75±0.25	10.24±0.04
	4	83.38±0.31	10.00±0.05
	6	81.50±0.61	9.69±0.10
	8	76.00±0.54	8.78±0.09

The viscosity averge molecular weight of chitosan from the two species of squid pen was similar, 10.24 x 10^6 daltons after 2 hours of deactylation, and was gradually decreased to 8.78 - 9.11 x 10^6 daltons after 8 hours. In comparison with chitosans from the same source deacetylated in this laboratory using 50% NaOH at 100 °C for 4, 6, and 8 hours, molecular weight of the products were 3.80 x 10^6, 3.10 x 10^6 and 2.20 x 10^6, respectively (data did not published). Similar results were also reported by Lee (1974; cited by Muzzarelli, 1976) who investigated the effect of deacetylation conditions on

molecular weight of β-chitin from the pen of *Loligo* species. These confirm the significant influence of deacetylation temperature on cleavage of the polymer chain.

Unfortunately, no other published data on the viscosity average molecular weight of chitosan from the squid pen is available to compare. It should be noted that the molecular weights of chitosans in these studies were markedly higher than those reported from other sources. However, the actual values may be lower than those present in Table 3.7. These were presumably due to an inaccuracy in nature of viscoscopic method as earlier demonstrated for the standard high molecular weight chitosan.

Intrinsic viscosity of chitosan dissolved in 1.0% acetic acid decreased notably during the first 10 days of standing at ambient temperature, then, slowly declined throughout 35 days of investigation (Figure 3.5). Similar pattern was observed for chitosan from both species of the squid pen.

3.5 Degree of Deacetylation

According to data from Kurita, *et al.* (1993) , chitin polymer from squid pen (*Ommastrephes bartrami*) comprising of 8% deacetylation. Results in this study implied that chitin from *L. lessoniana* and *L. formosana* were extensively deacetylated during the first two hours of the alkali treatment which were increased from 8% to 75.1 and 69.3%, respectively (Table 3.8). Deacetylation were progressively continued for two hours furthermore, then it was noteably slow down to the end of the experiment. Deacetylation behaviors of chitin to chitosan in this study agreed well with that of the chitin from *Ommastrephes bartrami* treated with 40% NaOH at 80 ^{o}C for 3 hours (Kurita, *et al.*, 1993).

In an attempt to elucidate the effects of collecting seasons and species of the pens, it appeared that degree of deacetylation in the products at comparable period of treatment varied during the first four hours (Table 3.8). However, it was maintained at about 92% after 8 hours of the alkali treatment regardless of season and species.

Unfortunately, chitosan from prawn shell (*P. monodon*) prepared under identical conditions with those from the squid pens could not be compared because of the former did not soluble in 1.0% acetic acid solvent. These suggested, however, that the more drastic alkali treatment was required for deacetylation of the chitin from prawn shell. Further study on the chitin from prawn shell indicated that treatment of the sample with 50% NaOH at 100 °C for 10 hours yielded the chitosan product soluble in 1.0% acetic acid, and showed 89.11% degree of deacetylation (data not shown).

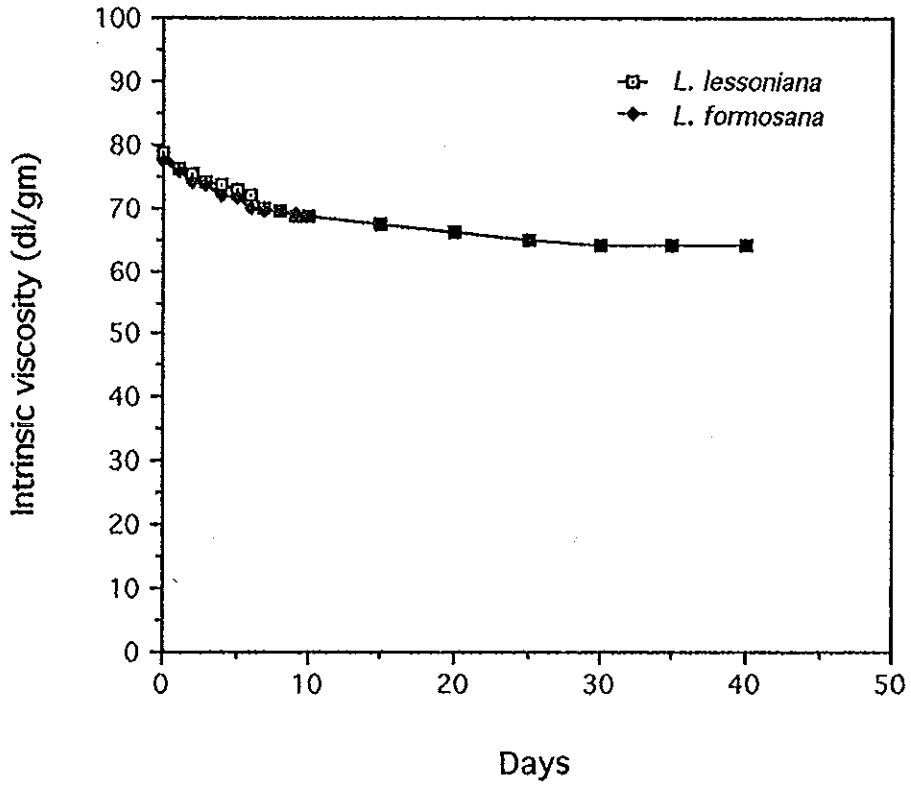


Figure 3.5 Changing of intrinsic viscosity of chitosan dissolved in 1% acetic acid solution after standing at ambient temperature for 35 days. The samples were from *L. lessoniana* and *L. formosana* deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 8 hours. Values are mean of duplications.

Table 3.8 Degree of deacetylation of chitosan deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours from *L. lessoniana* and *L. formosana*. Values of separated analysis in the samples for summer and rainy collecting periods are shown, and are mean ± S.E. of five replications.

Species	Deacetylation Time (h)	Degree of deacetylation (%)		Average
		summer	rainy	
L. lessoniana	2	73.76±0.19	76.38±0.09	75.07
	4	85.80±0.22	88.75±0.05	87.28
	6	90.90±0.32	90.76±0.06	90.83
	8	92.57±0.09	92.26±0.10	92.42
L. formosana	2	69.34±0.12	69.26±0.20	69.30
	4	84.57±0.29	80.21±0.08	82.39
	6	90.86±0.49	89.44±0.12	90.15
	8	92.60±0.12	92.26±0.26	92.43

3.6 Characteristic of Optical Activity

Determination on the optical activity of chitin dissolved in dimethylacetamide -5% $LiCl_2$ from both species of the squid pen indicated that they were levorotatory (Table 3.9). To investigate the effects of collecting seasons and species of the pens, results showed that there were no significant difference between the two collection seasons as well as between the two species. In addition, the specific angle of rotation remained at -96.57 degrees cm^2 g^{-1} throughout 14 days of observation. The optical activity characteristic of chitin from the squid pens are the same as the chitin from horseshoe crab reported by Austin, *et al.* (1981).

Table 3.9 Characteristic of the optical activity of chitins dissolved in dimethylacetamide -5% $LiCl_2$ at initial and after standing at room temperature for 14 days from *L. lessoniana*, *L. formosana* and *P. monodon*.

Species	$[\alpha]_D^{25}$	
	Initial	14 days
L. lessoniana	-96.57	-96.57
L. formosana	-96.58	-96.58
P. monodon	+98.82	-98.82

In contrast, chitin from the prawn shell showed dextrorotatory with specific angle of rotation +98.82 degrees $cm^2\ g^{-1}$ during the first 4 days. Then, it changed to levorotatory with specific angle of rotation -98.82 degrees $cm^2\ g^{-1}$ after 7 days, and stable at this value thereafter. Austin, *et al.* (1981) demonstrated the similar optical activity behavior of chitin from the pink shrimp.

3.7 Hygroscopic Activity

Moisture was dramatically adsorbed by dried squid pen powders, chitin and chitosan from a pen of *L. lessoniana* during the first day of exposing in open air at ambient temperature (Table 3.10). The adsorption was further continued with slow rate, and reached to the equilibrium after 6 days of observation, then it was slightly declined (see details in Appendix 6.1 and 6.2). The decrement was presumably due to an effect of relative humidity during the day of investigation.

At equilibrium, pen powder from *L. lessoniana* adsorbed moisture with minimum content, and the reverse was true for its chitin (Table 3.10). The saturation levels of pen powder and its chitin product were about 15% and 21%, respectively. For chitosans, the

contents varied from 17-20% depend upon deacetylated period, and that the longer alkali treatment the lower content of moisture was uptaken. The variations of hygroscopicity may be attributable by the difference in degree of compact crystalline structure of the chitosan products. In comparison, the moisture was uptaken by chitosan with lower content than its parent chitin. The same conclusions coluld also be applied for the samples from *L. formosana* (Table 3.10). In addition, the correspond results were observed by Kurita, *et al.* (1993) who studied the samples from other species of squid pen (*Ommastrephes bartrami*).

Table 3.10 Moisture adsorption at ambient conditions of the dried squid pen, chitin and chitosan from *L. lessoniana* and from *L. formosana* deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours. Values are mean ± S.E of four replicate determinations.

Sample	Moisture adsorption (%)			
	1st day		Sat. adsorption (6 days)	
	L. lessoniana	*L. formosana*	*L. lessoniana*	*L. formosana*
Squid pen	11.02±0.36	12.09±0.10	15.16±0.26	15.64±0.14
Chitin	15.68±0.67	15.83±0.56	21.46±0.14	21.27±0.09
Chitosan (2h)	15.10±0.43	15.28±0.63	19.79±0.03	19.62±0.41
Chitosan (4h)	14.53±0.84	14.32±0.68	19.04±0.26	18.86±0.28
Chitosan (6h)	13.89±0.94	13.53±0.43	17.70±0.23	17.64±0.35
Chitosan (8h)	13.31±0.45	13.21±0.45	17.49±0.15	17.31±0.33

3.8 Determination of the Metal Adsorption on Chitin and Chitosan

Preliminary study suggested that adsorption characteristics of the chitosan from squid pen depend on both concentration and type of metal ion (Table 3.11).

Table 3.11 Effects of metal ion concentrations on adsorption capacity of squid pen chitosan preapared by deacetylation its parent chitin with 50% NaOH at 60 °C for 8 hours. Values are mean ± S.E of six replications.

Metal ions	Concentration (mg/ml)	mg of metal adsorbed/gm chitosan
Cu (II)*	2.5	117.42 ± 0.43
	5.0	152.63 ± 2.09
	10.0	185.86 ± 2.71
	15.0	201.21 ± 1.32
	20.0	204.74 ± 2.46
	30.0	195.92 ± 5.30
Pb (II)**	10.0	223.40 ± 1.45
	20.0	394.99 ± 2.90
	40.0	660.48 ± 2.73
	80.0	969.14 ± 7.29
	120.0	971.13 ± 5.79
	160.0	954.03 ± 7.96

* used chitosan from *L. lessoniana*

** used chitosan from *L. formosana*

The adsorbed quantities of Cu (II) and Pb (II) markedly increased when concentration of the former ion was varied from 2.5-15.0 mg/ml, and from 10-80 mg/ml for the latter. Saturation of binding was observed when their concentrations were increased beyond the levels. Thereby, the concentrations of Cu (II) and Pb (II) solutions at 20.0 and 120.0 mg/ml were selected and used in studying for the adsorption rates of the metal ions on other chitin and chitosan samples.

Rates of metal ions uptaken by chitosan samples were shown in Table 3.12. The results clearly indicated that uptaking of Pb (II) on the chitosan sample could be saturated within few minutes whereas Cu (II) required much longer equilibrating period of more than 30 minutes. This indicated that the binding affinity of Cu (ll) on chitosan was lower than Pb (ll).

Table 3.12 Adsorption rates of metal ions on chitosan preapared by deacetylation its parent chitin with 50% NaOH at 60 °C for 8 hours. Values are mean ± S.E of six replications for Pb (ll) and mean ± S.E. of nine replications for Cu (ll).

Time (min)	mg of metal adsorbed/gm chitosan	
	Cu (ll)*	Pb (ll)**
2	108.55 ± 2.65	902.23 ± 7.96
6	142.96 ± 3.97	915.18 ± 5.46
30	188.86 ± 1.76	988.56 ± 4.32
60	204.74 ± 2.46	971.13 ± 5.79
120	200.33 ± 1.76	965.26 ± 5.38

* used chitosan from *L. lessoniana* ; ** used chitosan from *L. formosana*

Similar observation has been demonstrated for the chitosan from prawn shell (Madhavan and Ramachandran-Nair, 1978; Ramachandran-Nair and Madhavan, 1982). From the above conclusions, therefore, the concentrations of Cu (II) and Pb (II) solutions used for studying an adsorption capacity on other chitin and chitosan samples were selected at 20 and 120 mg/ml, respectively. To ensure their saturation of binding, an equilibration period for 60 min was used throughout further studies.

In comparison between chitinous products from the two species of squid pen, there were no remarkable differences in adsorption capacity for Cu (II) on chitin as well as chitosan prepared under identical condition (Table 3.13).

Table 3.13 Comparison for adsorption capacity of Cu (II) on chitin and chitosan from the pens of *L. lessoniana*, *L.formosana* and from the prawn shells, *P. monodon*. Chitosan samples were prepared by different times of deacetylation. Values are mean ± S.E of nine replications for samples from *L.lessoniana* and *L. formosana*, and mean ± S.E. of six replications for samples from *P. monodon* .

Sample	mg Cu (II) adsorbed/gm sample		
	L. lessoniana	*L. formosana*	*P. monodon*
Chitin	27.36 ± 3.53	23.83 ± 1.87	9.27 ± 1.32
Chitosan (2h)	162.38 ± 1.40	168.56 ± 2.21	92.66 ± 1.67
Chitosan (4h)	195.03 ± 1.40	202.09 ± 1.92	127.08 ± 0.00
Chitosan (6h)	200.33 ± 1.76	210.04 ± 1.92	135.02 ± 0.00
Chitosan (8h)	204.74 ± 2.46	217.10 ± 1.32	137.67 ± 1.67

However, an adsorption capacity for the ion on the chitin and chitosan from squid pens was noteably higher than that obtained from the prawn shell. These may due primarily to the difference in physical forms between the two chitinous materials.In contrast, Ramachandran-Nair and Madhavan (1982) demonstrated that there were no significant differences in binding capacity of Cu (II) as well as some other ions on chitosan from squid pen and from prawn shell. Unfortunately, the authors did not show details of sample preparation which is likely to be the principal factor determined the result.

Table 3.13 indicated that chitosan from squid pens adsorbed Cu (II) more than their parent chitin 7 times where the difference of more than 10 times were observed for samples from prawn shell. These characteristics were also true for the adsorption of Pb (II) on all studied samples, with only exception for the samples from prawn shell which the difference between chitin and chitosan was only 5 times (Table 3.14). The higher binding capacity of metal ions on chitosan than its parent chitin confirms that chelating mechanism of chitinous materials contributes primarily by amino group.

Chitins from squid pen adsorbed Cu (II) within a range of 23.8-27.4 mg/gm chitin (0.10-0.11 mmole/gm chitin) and the sample from prawn shell adsorbed the ion 9.27 mg/gm (0.04 mmole/gm chitin). It was noted that chitosan prepared by deacetylation of chitin from all studied sources for 2 hours with 50% NaOH at 60 ^{o}C under nitrogen atmosphere showed slightly lower adsorption capacity than those of the other conditions. However, no significantly increase in adsorption capacity was observed for samples deacetylated longer than 4 hours. These conclusion could also be applied for an adsorption behavior of Pb (II) (Table 3.14).

The saturation level for an adsorption of Cu (II) on the chitosan from squid pens was approximately 210 mg/gm chitosan (0.84 mmole/gm) and the chitosan from prawn shell was approximately 135 mg/gm chitosan (0.54 mmole/gm). These values were much higher than that studied by Ramachandran-Nair and Madhavan (1982) who reported the binding capacity of Cu (II) on chitosan from squid pen and prawn shell as

42.3 and 45.5 mg/gm chitosan, respectively. These may be contributable by the difference of preparation conditions.

Table 3.14 Comparison for adsorption capacity of Pb (II) on chitin and chitosan from the pens of *L. lessoniana*, *L. formosana* and from the prawn shells, *P. monodon*. Chitosan samples were prepared by different times of deacetylation. Values are mean ± S.E of nine replications for samples from *L. lessoniana* and *L. formosana*, and mean ± S.E.of six replications for samples from *P. monodon* .

Sample	mg Pb (II) adsorbed/gm sample		
	L. lessoniana	*L. formosana*	*P. monodon*
Chitin	123.75 ± 3.81	126.63 ± 5.19	60.44 ± 5.46
Chitosan (2h)	771.28 ± 8.39	742.50 ± 4.32	341.03 ± 5.79
Chitosan (4h)	877.76 ± 8.21	874.89 ± 5.76	470.54 ± 5.79
Chitosan (6h)	906.54 ± 8.39	923.81 ± 6.10	526.66 ± 5.46
Chitosan (8h)	961.12 ± 8.01	990.00 ± 4.81	543.93 ± 5.46

It was noted in all cases that chitinous materials adsorbed Pb (II) with higher capacity than Cu (II), approximately 5 times (Table 3.14). The saturation level for an adsorption of Pb (II) on the chitosan from squid pens was approximately 950 mg/gm chitosan (2.87 mmole/gm) and the chitosan from prawn shell was approximately 530 mg/gm chitosan (1.60 mmole/gm). Unfortunately, no report has been found for the binding capacity of Pb (II) on chitosan from other squid pens, but similar value was demonstrated by Hauer (1978) for the chitosan from prawn shell.

3.9 Determination of Chitinolytic Activities on Chitin from Different Sources

A kinetic study of chitinolytic enzymes on the native chitin prepared from both species of squid pen (*L. lessoniana* and *L. formosana*) and from the prawn shell (*P. monodon*) was carried out by the procedure described in Section 2.10. Amounts of end product, N-acetyl-D-glucosamine liberated were linearly detected during the first two hours, and that initial rates could be determined (Figure 3.6).

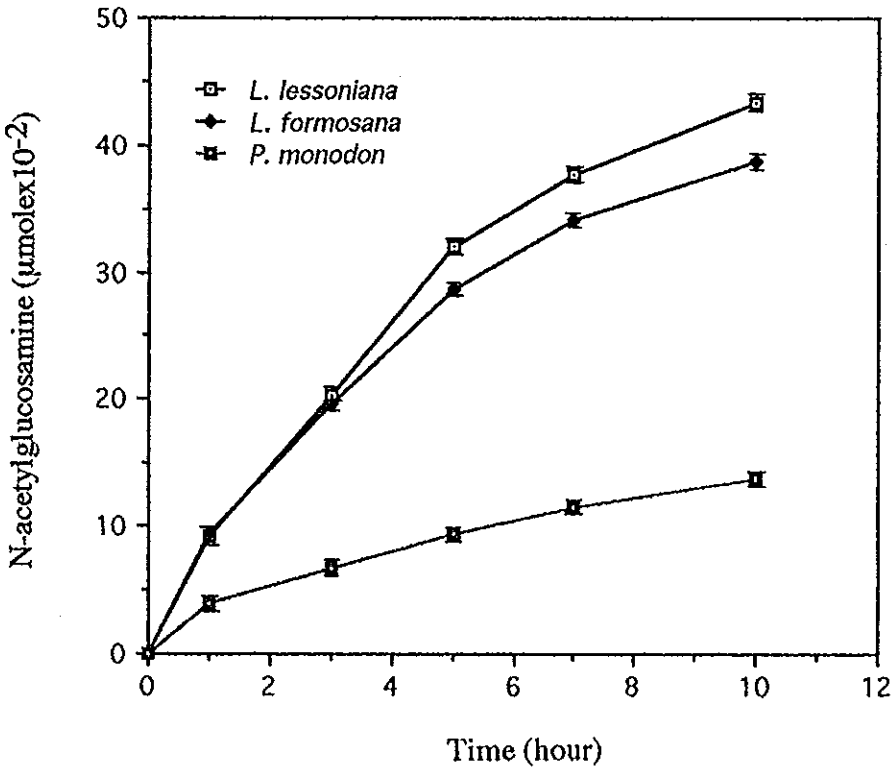


Figure 3.6 Comparison for rates of N-acetyl-D-glucosamine liberated on the hydrolysis of chitins from the pen of *L. lessoniana*, *L. formosana* and the shell of *P. monodon* by chitinase and β-glucosidase (Sigma). Values are mean ± S.E. of five replications for each sample.

According to the procedure described in Section 2.10, it was found that 0.1 ml of the prawn serum that had been diluted with distilled water to 1 : 10 could be replaced the commercial β- glucosidase (Figure 3.7).

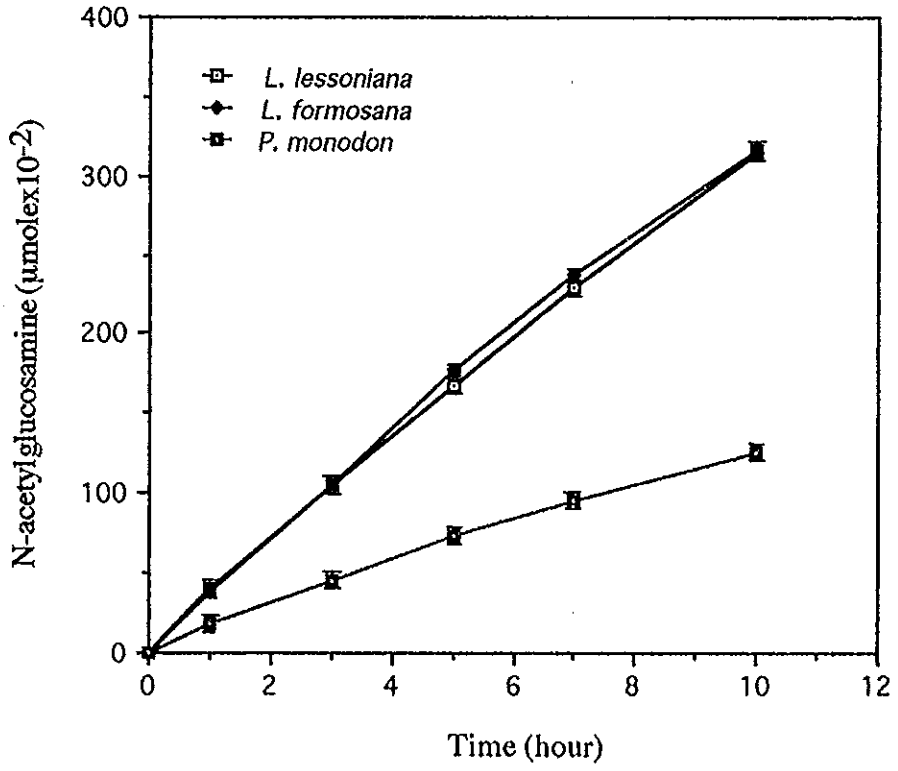


Figure 3.7 Comparison for rates of N-acetyl-D-glucosamine liberated on the hydrolysis of chitins from the pen of *L. lessoniana*, *L. formosana* and the shell of *P. monodon* by chitinase and β- glucosidase (prawn hemolymph). Values are mean ± S.E. of five replications for each sample.

As shown in Figure 3.6, although rates of the enzymatic hydrolysis on the chitin from *L. lessoniana* appeared slightly higher than *L. formosana*, statistical analysis showed no significant differences ($P>0.05$). It is worth noting, however, that the hydrolytic rates on the chitin from prawn shell were significantly lower than those from both species of squid pens.This data indicated that chitin from squid pen proved to show higher activity for enzymatic hydrolysis than that of the prawn chitin isolated by similar

condition. These presumably due primarily to the polymer chains from squid pens, β-form, which are loosely arranged and thus enhance accessibility of enzymes to hydrolyze substrate in comparison with the compact crystalline structure of the α-form of prawn chitin. Accordingly, chitin from the squid pen could be used as substrate for the assay of chitinolytic activities instead of using the other forms which are not represent the intrinsic behavior of the enzymes.

Hydrolysis of chitins from both species of the squid pen and prawn shell by β-glucosidase from prawn (*P. monodon*) hemolymph and from almonds (Sigma product) showed comparable patterns (Figure 3.6 and Figure 3.7). However, the former source of enzyme (diluted with distilled water 1 : 10) provided approximately 5 times higher hydrolytic rate than the latter one. These results suggested that prawn hemolymph should contain a considerable amounts of β-glucosidase.

It is important to note that as far as the mechanism has been documented in artropods, β-glucosidase plays two important roles, namely digestion and moulting. However, it is unlikely that the enzyme in prawn hemolymph would involve in these two mechanisms. The controversy is because of two reasons. Firstly, the enzyme should be secreted directly into the digestive tract if it plays digestive function as demonstrated by Koga, *et al* (1996). Secondly, it should be secreted into moulting fluid at pre-ecdysis stage of the animal by epithelial cells as described by Stevenson (1985). Therefore, further elucidation on the roles of β-glucosidase in prawn hemolymph is required.

4. SUMMARY

Squid pens from *Loligo lessoniana* and *Loligo formosana* comprise of ash less than 0.03%, therefore, isolation of chitin from their pens could be achieved by omitting demineralization step. Deproteinization process carried out in 1.0M NaOH at 50 °C for 5 hours by using a ratio of solid to alkali as 1:15 (w/v). A white soft chitin was resulted with 36% yield. Chitin products constituted a negligible amount of ash and contained about 6.3% nitrogen. Chitins dissolved in dimethylacetamide-5% $LiCl_2$ showed levorotatory property, and this was remained throughout 14 days of observation. It was noted in general that there were no remarkably different in properties between two species of the squid pen as well as between the two samples collecting in summer and rainy seasons.

Optimum conditions to prepare chitosan from both species of the squid pen could be performed by deacetylation of the chitin with 50% NaOH at 60 °C for 5 hours under nitrogen atmosphere using solid to alkali solution ratio of 1: 15 (w/v). The product was a white soft powder and yielded about 27% calculated with respect to raw material of dried squid pen powder. These chitosans carried more than 90% degree of deacetylation and exhibited relatively high viscosity average molecular weight of approximately 9.5×10^6 daltons.

The chitin from squid pens displayed a high hygroscopic activity which was able to adsorb moisture about 21%. Moisture adsorptivity of the chitosan was 17-19% which was slightly lower than its parent chitin. This also depended on an extension of deacetylation period. In contrast, this behavior was opposite with those from the prawn shell. These properties therefore consider to be useful as the high hydrophilic biopolymers.

Chelation properties of the chitin and chitosan were influenced by several factors such as source, degree of deacetylation, type and concentration of metal ions. The binding affinity of Cu (II) on chitosan was lower than Pb (II), but the reverse was true for the binding capacity. Chitosan from the squid pen adsorbed Cu (II) and Pb (II) about 200 and 900 mg/gm of the sample, respectively. In comparison, the half lower amounts were found on binding with the chitosan from prawn shell.

The native form of chitin was hydrolyzed by chitinolytic enzyme system with higher rate than the prawn chitin. Therefore, it might be use as a good substrate to assay the enzyme activity instead of other chitin derivatives which may provide dubious results. On the assay procedure, β-glucosidase from prawn hemolymph could be perfectly used to replace other expensive enzyme from commercial sources.

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APPENDICES

Appendix 1 Specifications for chitin and chitosan (adapted from Muzzarelli, 1985).

Specification	Description
Moisture	From 2-10% under normal laboratory conditions.
Nitrogen	Usually from 6-7% in chitins, from 7.0-8.4% in chitosans.
Degree of deacetylation	Usually estimate 10% in chitins, 60% in current chitosan, and between 90 and 100% in fully deacetylated chitosans.
Ashes at 900°C	Usually lower than 1.0%.
Viscosity of 1% acid (for chitosans only)	From 200-3,000 cps: degraded chitosans solution in 1% acetic acid have lower viscosities.
Molecular weight	Native chitins, > $1x10^6$; commercial chitins and chitosans $1\text{-}5x10^5$; polydispersity is rarely indicated.
Titrations	With potassium polyvinylsulfonate and by alkalimetry; moisture content and pH of the waters form which chitosans were isolated should be taken into account.
Dissociation constant, Ka	Between 6.0 and 7.0; most often 6.3
X-ray diffraction data	Typical peaks at 8° 58'- 10° 26' and 19° 58'- 20° 00'

Appendix 1 (continue)

Specification	Description
carotenoids;	Carotenoids Chitins and chitosans may contain otherwise, it should be indicated whether they have been extracted or bleached.
Amino acids	Glycine, serine and aspartic acid may be present.
Transition metals	With the exception of iron, normally below a total of 5.0 ug/g.; typically, for crab chitosan: V, 0.12; Cr, 0.04; Mn,0.09; Ni,1.3; Cu,1.03; Ag,0.02; Cd,0.22; Hg,0.025; Pb,0.15 μg/gm

Appendix 2 Statistical analysis for the percent yield of chitin prepared from the pen of *Loligo lessoniana* and *Loligo formosana*. Chitin samples were prepared by deproteinization with 1M NaOH at 50°C for 5 hours. Values are calculated on a dried weight basis, and are mean of duplications for each sample collection.

Species	Percent yield		ANOVA	
	summer	rainy	C	Sp
L. lessoniana	36.54	35.59	*	ns
L. formosana	35.98	37.12		

C, collection period (summer and rainy) *, significant at (P<0.05)

Sp, species ns, non-significant

Appendix 3 Statistical analysis for the nitrogen contents of chitosan from *Loligo lessoniana* and from *Loligo formosana*. Chitosan samples were prepared by deacetylation with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours. Values are calculated on a dried weight basis, and are mean ± S.E. of six replicated determinations for each sample collection.

Species	Deacetylation time (hour)	Nitrogen content (mg/gm) summer	Nitrogen content (mg/gm) rainy	ANOVA T	ANOVA C	ANOVA Sp.
L. lessoniana						
	2	77.45±0.21	77.15±0.47	**	**	ns
	4	77.95±0.16	79.30±0.11			
	6	78.30±0.10	79.34±0.08			
	8	78.42±0.09	79.25±0.16			
L. formosana						
	2	76.60±0.08	75.96±0.14	**	**	ns
	4	78.73±0.16	77.60±0.15			
	6	79.81±0.13	78.25±0.07			
	8	80.47±0.09	79.22±0.01			

T, treatment or deacetylation time (hour) * *, significant at P< 0.01

C, collection period (summer and rainy) ns, non-significant

Sp., species

Appendix 4 Titration for Cu (II)

The procedure for obtaining the Cu adsorption on samples was carried on as follow: 0.1 ml of filtrate was diluted with 5.9 ml deionized distilled water and transferred into a conical flask, 10 drops of concentrated ammonia solution and 2 drops of fast sulphon black F indicator (0.5 gm in 100 ml deionized distilled water) was added. After mixed gently, the mixture was titrated with standard EDTA solution (0.01 M) until the dark green color was obtained. The Cu adsorption was calculated from the millilitres of standard EDTA solution which was used for titration, in which 1 ml. standard EDTA solution (0.01 M) will be reacted completely with 0.6354 mg Cu.

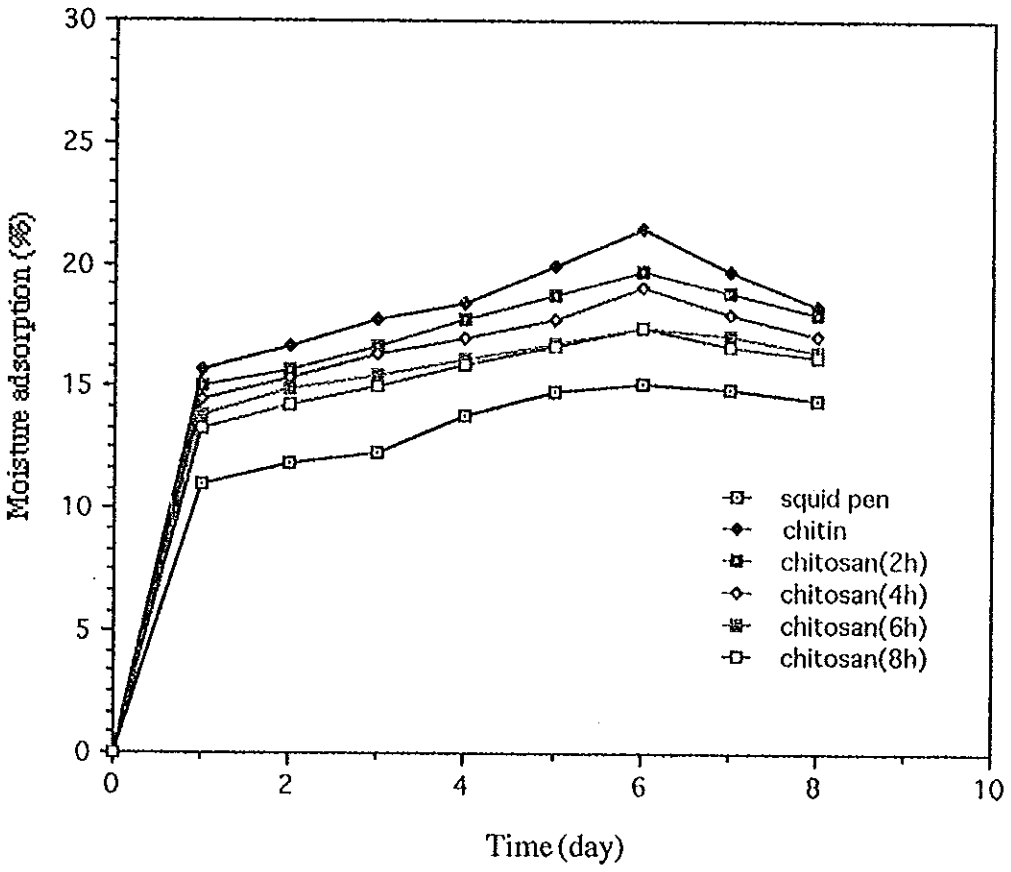
Appendix 5 Titration for Pb (II)

The procedure for obtaining the Pb adsorption on samples was carried on as follow: 0.2 ml of filtrate was diluted with 3.8 ml deionized distilled water and transferred into a conical flask, a drop of xylenol orange indicator (0.5 gm in 100 ml deionized distilled water) was added and the diluted nitric acid (2 %, v/v) was applied drop by drop until a yellow color of mixture was obtained, An approximately 0.2-0.3 gm hexamethylenetetramine powder was added and mixed gently. The mixture was titrated with standard EDTA solution (0.01 M) until the lemon yellow color was obtained. The Pb adsorption was calculated from the millilitres of standard EDTA solution which was used for titration, in which 1 ml.of standard EDTA solution (0.01 M) will be reacted completely with 2.0721 mg Pb.

Appendix 6.1 Moisture adsorption at ambient conditions of the dried squid pen, chitin and chitosan from *L. lessoniana* and deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours (similar results were found from *L. formosana*). Values are mean ± S.E of four replicate determinations.

Time (day)	Moisture adsorption (%) Samples					
	squid pen	chitin	chitosan(2h)	chitosan(4h)	chitosan(6h)	chitosan(8h)
1	11.02±0.35	15.68±0.67	15.10±0.43	14.52±0.84	13.31±0.45	13.90±0.94
2	11.89±0.47	16.74±0.28	15.71±0.49	15.34±0.68	14.90±0.80	14.27±0.42
3	12.38±0.52	17.73±0.25	16.71±0.49	16.31±0.54	15.46±0.74	15.04±0.56
4	13.88±0.39	18.49±0.25	17.80±0.26	17.01±0.29	16.13±0.59	15.89±0.47
5	14.79±0.26	19.92±0.11	18.75±0.17	17.75±0.38	16.80±0.44	16.64±0.46
6	15.16±0.26	21.46±0.14	19.79±0.03	19.04±0.26	17.45±0.26	17.49±0.15
7	14.92±0.19	19.74±0.23	18.91±0.23	18.04±0.45	17.13±0.42	16.73±0.26
8	14.46±0.14	18.37±0.28	17.96±0.44	17.12±0.38	16.47±0.35	16.22±0.30

Appendix 6.2 Moisture adsorption at ambient conditions of the dried squid pen, chitin and chitosan from *L. lessoniana* and deacetylated with 50% NaOH at 60 °C under nitrogen atmosphere for 2, 4, 6 and 8 hours (similar results were found from *L. formosana*). Values are mean of four replicate determinations.



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