



**Effect of Molecular Weight of Chitosan from Black Tiger Prawn
(*Penaeus monodon*) on Fat Adsorption and Antioxidative Activity**

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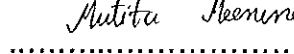
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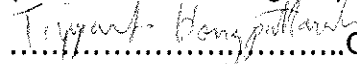
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ชื่อวิทยานิพนธ์ ผลของน้ำหนักริมโกลของโคโตแซนจากเปลือกกุ้งกุลาดำ
ต่อคุณสมบัติการดูดซับไขมัน และกิจกรรมการต้านออกซิเดชัน
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บทคัดย่อ

จากการศึกษาการเตรียมโคโตแซนจากเปลือกกุ้งกุลาดำในสภาวะที่มีโซเดียมโบโรไฮดรไรต์ ความเข้มข้นต่างๆ (0, 0.5, 1.0 และ 2.0 โมลาร์) พบว่าการเติมโซเดียมโบโรไฮดรไรต์ในขั้นตอนการกำจัดหมู่อะซิทิล มีผลให้น้ำหนักริมโกลของโคโตแซนสูงขึ้น อย่างไรก็ตามโคโตแซนที่เตรียมโดยเติมและไม่เติมโซเดียมโบโรไฮดรไรต์ มีองค์ประกอบทางเคมี (ปริมาณแก้ว ไขมัน และไนโตรเจน) และระดับการกำจัดหมู่อะซิทิล ไม่แตกต่างกัน เมื่อความเข้มข้นของโซเดียมโบโรไฮดรไรต์เพิ่มขึ้น มีผลให้ความหนืดและน้ำหนักริมโกลของโคโตแซนเพิ่มขึ้น

ความสามารถในการดูดซับไขมันของผงโคโตแซนที่เตรียมโดยไม่เติมและเติมโซเดียมโบโรไฮดรไรต์ ระดับต่างๆ (0, 0.5, 1.0 และ 2.0 โมลาร์) ไม่มีความแตกต่างกัน ($p>0.05$) กรดไขมัน และ องค์ประกอบไขมัน ของน้ำมันปาล์ม น้ำมันถั่วเหลือง และน้ำมันหมูที่ถูกดูดซับและที่ผ่านการกรองด้วยโคโตแซนผง ไม่แตกต่างจากน้ำมันเริ่มต้น($p>0.05$) สำหรับระบบอิมัลชันโคโตแซนที่เตรียมในสภาวะที่มีโซเดียมโบโรไฮดรไรต์เข้มข้น 0.5 โมลาร์ ในขั้นตอนการกำจัดหมู่อะซิทิล มีความสามารถในการดูดซับไขมันสูงสุด องค์ประกอบกรดไขมันในน้ำมันชนิดต่างๆที่สกัดจากเจลโคโตแซนไม่แตกต่างจากไขมันเริ่มต้น ในขณะที่ฟอสโฟลิปิดของน้ำมันทุกชนิดในเจลโคโตแซนเพิ่มขึ้น เมื่อเปรียบเทียบกับฟอสโฟลิปิดของน้ำมันเริ่มต้น ปริมาณไขมันในเจลโคโตแซนที่มีการเติมกรดแอสคอร์บิก (chitosan-AsA-HCl gel) สูงกว่าในเจลที่ไม่มีการเติมกรดแอสคอร์บิก(chitosan-HCl-gel) โดยกรดแอสคอร์บิกสามารถช่วยลดความหนืดของโคโตแซน ทำให้เกิดการกระจายตัวที่ดีของหยดไขมันในเจลโคโตแซน

จากการศึกษากิจกรรมการต้านออกซิเดชันของผงโคโคแซนที่มีน้ำหนักโมเลกุลต่างกัน พบว่ากิจกรรมการต้านออกซิเดชันสูงขึ้นเมื่อปริมาณของโคโคแซนเพิ่มขึ้น คุณสมบัติในการกำจัดอนุมูลไฮดรอกซิลของโคโคแซนเพิ่มขึ้นตามปริมาณของโคโคแซนที่เพิ่มขึ้น นอกจากนี้โคโคแซนสามารถกำจัดไอออนโลหะ โดยประสิทธิภาพเพิ่มขึ้นเมื่อปริมาณที่ใช้เพิ่มขึ้น อย่างไรก็ตามน้ำหนักโมเลกุลของโคโคแซนไม่มีผลต่อคุณสมบัติการต้านออกซิเดชัน การเติมโคโคแซน (50 -200 มิลลิกรัม/กิโลกรัมเนื้อหมูปอด) สามารถยับยั้งการเพิ่มขึ้นของค่าเปอร์ออกไซด์ ปริมาณคอนจูเกตไทไดอิน ปริมาณของเหล็กอิสระ และค่า TBARS ในเนื้อหมูปอด ที่ผ่านการให้ความร้อนระหว่างการเก็บรักษาที่อุณหภูมิ 4 องศาเซลเซียส เป็นระยะเวลา 15 วัน การเติมโคโคแซนที่ระดับความเข้มข้นที่เพิ่มขึ้น มีผลให้ประสิทธิภาพการยับยั้งปฏิกิริยาออกซิเดชันของเนื้อหมูปอดเพิ่มขึ้น($p < 0.05$) โคโคแซนที่มีน้ำหนักโมเลกุลต่างกันมีกิจกรรมการต้านออกซิเดชัน ไม่แตกต่างกันในเนื้อหมูปอด($p < 0.05$)

Thesis Title	Effect of Molecular Weight of Chitosan from Black Tiger Prawn (<i>Penaeus monodon</i>) on Fat Adsorption and Antioxidative Activity
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Abstract

Effect of sodium borohydride at different concentrations (0, 0.5, 1.0 and 2.0 M) on chitosan properties was investigated. Higher molecular weight chitosan was obtained in presence of sodium borohydride. Nevertheless, no significant differences in chemical compositions (ash, fat and nitrogen content) and degree of deacetylation (DD) were observed among chitosans prepared without and with sodium borohydride at different concentrations. The viscosity of chitosan increased with a concomitant increased molecular weight as the concentration of sodium borohydride increased.

In chitosan powder system, no differences in oil adsorption capacity were observed among chitosan prepared by deacetylation in presence or absence of sodium borohydride. No differences in fatty acid profiles and lipid compositions between percolated, adsorbed fractions and original oil for all samples including palm oil, soybean oil and lard were found. However, in emulsion system, chitosan prepared by deacetylation in presence of 0.5 M sodium borohydride showed the highest oil adsorption capacity. Fatty acid compositions in oil extracted from chitosan gel were not different, compared with the original oil. Conversely, phospholipid content of all oils in chitosan gel was greater, compared with that found in the original oil. With addition of ascorbic acid, (AsA), high oil content was adsorbed in chitosan-AsA-HCl gel, compared to that in chitosan-HCl-gel. Ascorbic acid

was shown to reduce the viscosity of chitosan, leading to uniformly dispersed oil droplets in chitosan gel. As a result, the improvement of oil dispersion in chitosan gel was obtained with a concomitant enhanced oil holding capacity of chitosan.

Antioxidant activity of chitosan powder with different molecular weights increased with increasing amount of the chitosan used. Chitosan powder showed the pronounced activities as a radical scavenger toward hydroxyl radical in a concentration-dependent manner. Therefore, chitosan possibly worked as primary antioxidant, which potentially reacts with free radicals. Chitosan powder also possessed Fe^{2+} ion chelating activity. The efficiency increased as the amount of chitosan powder increased. Chitosan powder (50-200 ppm) retarded the increase in peroxide, conjugated diene, nonheme and TBARS values in cooked ground pork during storage at 4°C for 15 days. Higher amounts of chitosan added resulted in greater efficacy in retardation of lipid oxidation in cooked ground pork ($p < 0.05$). No differences in antioxidant activity of chitosan powder with different molecular weights were observed in cooked ground pork ($p < 0.05$).

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Chapter 1

INTRODUCTION

Chitosan is a natural substance derived from chitin, a polysaccharide found in the exoskeleton of shellfish, such as shrimp or crabs (Shahidi *et al.*, 1999). This polymer is known to be nontoxic, odorless, biocompatible in animal tissues, and enzymatically biodegradable (Zong *et al.*, 2000). The characteristics of chitosan depend on the deacetylation, the distribution of acetyl groups, chain length, and molecular mass distribution (Schmuhl and Keizer, 2001).

Chitosan is known to interact with hydrophobic compounds such as cholesterol, triglycerides, fatty acid and bile acid (Rockway, 2000). As a consequence, it can reduce their absorption or reentry into the mucosal cells of animals and man. It is claimed that the cationic nature of chitosan makes it particularly suitable for sequestering bile acid and fatty acids (Muzzarelli *et al.*, 2000). Also, it is believed that chitosan is dissolved in an acidic medium of stomach, and mixed well, leading to the formation of fat – chitosan emulsification. Chitosan exists in the aqueous phase at the surface of the fat droplets as a surfactant. This mixture is transferred to the intestines. In the intestinal stage, rough emulsion of fat with chitosan immediately changes into the insoluble gel with the entrapped fat droplets, which could not be attacked by pancreatic or intestinal enzymes (Kanauchi *et al.*, 1995).

Lipid peroxidation is a major cause of food deterioration, leading to loss of functional properties and nutritional value (Yen *et al.*, 1999). Transition metals, particularly those with two or more valency states and a suitable oxidation-reduction potential between them (e.g., cobalt, copper, iron, manganese, and nickel) are effective pro-oxidants (Nawar, 1996).

Synthetic antioxidants and chelating agent may be added to food products in order to prevent lipid oxidation. However, the growing consumer demand for food without of synthetic antioxidants has focused efforts on the discovery of new natural preservatives (Madsen and Bertelsen, 1995). Several sources of natural antioxidants are known (Shahidi, 1997), and some of them, such as those of rosemary and sage, are currently used in a variety of food products. Chui *et al.* (1996) confirms that the amino group of chitosan is the major effective binding sites for metal ions, forming stable complexes by coordination. The nitrogen eletrons present in the amino and N-acetylamino groups can establish bonds with transition metal ions. However, fundamental studies on chitosan as a natural antioxidative agent in fish and seafood are lacking.

Therefore, the objective of this study was to examine the effect of chitosan with different molecular weights on fat adsorption and antioxidative activity.

Literature review

Chitin

Chitin, namely, poly[(1 → 4)-*N*-acetyl-β-D-glucosamine], the natural polymer second in abundance to cellulose, has become a subject of considerable interest (Neugebauer and Brzezinski, 1989). Chitin is the major structural component of the exoskeleton of invertebrates and the cell walls of fungi (Tan *et al.*, 1996; Cairns *et al.*, 1992). Depending on the sources, this polymer has been found in three polymorphic forms: α-, β- and γ-chitin, which differ, depending upon the arrangement of the chains within the crystalline regions, implying different networks of hydrogen-bonds (Duarte *et al.*, 2002). Chitin can be degraded by chitinase (Wang and Chio, 1998). Its immunogenicity is exceptionally low, in spite of the presence of nitrogen. It is a highly insoluble material resembling cellulose in its solubility and low chemical reactivity. It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group (Figure 1) (Ravikumar, 2000).

Chitosan

Chitosan is the *N*-deacetylated derivative of chitin, although this *N*-deacetylation is never complete (Ravikumar, 2000). Structurally, chitosan is a polysaccharide, derived from the deacetylation (removal of a carbonyl and methyl group) of chitin. Chemically, chitosan is a (1 → 4) 2 -amino - 2 - deoxy - β-D-glucosamine residue chain. In other words, chitosan is a 1 → 4 linked polymer of D- glucosamine (Figure 1) (Savant and Torres, 2000). Due to the presence of an amino group at C-2 of its cellulose like backbone, chitosan is a cationic polyelectrolyte and soluble in acidic media (Chen *et al.*, 2002).

Chitosan are recommended as suitable functional materials, because these natural polymers have excellent properties such as hydrophilicity, biocompatibility, biodegradability, non toxicity and adsorption properties (Ito *et al.*, 1999; Ravikumar, 2000).

This biopolymer is polycationic, i.e. positively charged at $\text{pH} < 6$. Due to the presence of protonated amino groups (the amino group in chitosan has a $\text{p}K_a$ of approx. 6.5), chitosan in dilute acid aqueous solution exhibits a polyelectrolyte character (Nystrom *et al.*, 1998 ; Ormrod *et al.*, 1998).

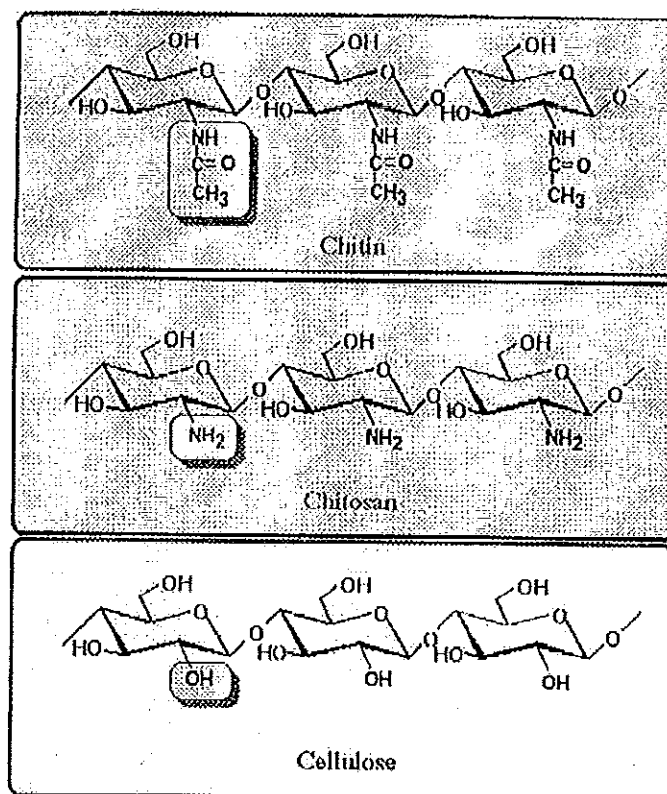


Figure 1 Structure of chitin, chitosan and cellulose

Source : Okuyama *et al.* (2000)

1. Sources of chitin and chitosan

Chitin occurs in wide variety of species, from fungi to the lower animals. Arthropod shells (exoskeltons) are the most easily accessible sources of chitin (Table 1). These shells contain 20-50% chitin on a dry weight basis. From a practical viewpoint, shells of crustaceans such as crabs and shrimps are conveniently available as wastes from seafood processing industries and are used for the commercial production of chitin. Other potential sources for chitin production include krill, crawfish, insects, clams, oysters, jellyfish, algae, and fungi (Knorr, 1984).

Squid pens also contain chitin that is classified as β -chitin (Tolaimate *et al.*, 2000). This material is distinguished from the ordinary α -chitin in crustacean shells according to the difference in crystalline structure. β -Chitin has weaker intermolecular forces and is quite attractive as another form of chitin having some characteristics considerably different from those of α -chitin. The chemistry of β -chitin is rapidly advancing, although this starting material is less abundant and is not yet produced commercially. The cell walls of some fungi (Zygomycetes) contain chitosan as well as chitin and these may be used as sources of chitosan (Tan *et al.*, 1996).

Table 1 Chitin content of some crustaceans, insects, mollusc and fungi.

Type	Chitin content (%)
Crustaceans	11
Shrimp heads	27
Shrimp shells	24
Shrimp hulls	35
Shrimp (Black tiger)-carapace	37
Shrimp (Black tiger)-shell	12-18
Commercial shrimp waste	24
Snow crab-claws	32
Snow crab-legs	30-35
Squid pens (beta-chitin)	34-49
Krill-crude de-proteinized shell	25
Krill-dried concentrated waste	
Insect	2.0 ^a
<i>Periplaneta</i> (cockroach)	18.4 ^c ; 10 ^b ; 35 ^c
<i>Blatella</i> (cockroach)	5-15 ^b ; 27-35 ^c
<i>Colcoptera</i> (beetle)	2.1 ^a ; 4.9 ^b ; 31.3 ^c
<i>Tenebrio</i> (beetle)	16 ^b
May beetle	54.8 ^c
<i>Diptera</i> (true fly)	64 ^c
<i>Piesis</i> (sulur butterfly)	2-4 ^a ; 20 ^c
Grasshopper	44.2 ^c
<i>Bombyx</i> (silk worm)	33.7 ^c
<i>Calleria</i> (wax worm)	

Table (continued)

Type	Chitin content (%)
Mollusc	6.1
Clam shell	36.6
Oyster shell	41.1
Squid, skeletal pen	40.2 ± 5.2
Krill, deproteinized shell	
Fungi	
<i>Aspergillus niger</i>	42.0 ^d
<i>Penicillium notatum</i>	18.5 ^d
<i>Penicillium chrysogenum</i>	20.1 ^d
<i>Saccharomyces cerevisiae</i> (bakers yeast)	2.9 ^d
<i>Mucor rouxii</i>	44.5
<i>Lactarius vellereus</i> (mushroom)	19.0

The percentages are expressed on the basis of :

^aWet body weight

^cOrganic weight of cuticle

^bDry body weight

^dDry weight of the cell wall

Sources: Kong, 1975 and Naczek *et al.*, 1981 cited by Knorr (1984);
Subasinghe (1999)

2. Production of chitin

2.1 Deproteinization

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 the cleavage of glycosidic bond and deacetylation of the native chitin (Okuyama *et al.*, 2000; Austin *et al.*, 1981). The effects of alkali concentration, temperature and duration of treatment on properties of the chitin from various sources have been reported by a number of investigators (Muzzarelli, 1977).

No *et al.* (1989) suggested that the optimum condition for deproteinization of crawfish shell could be achieved using 3.5% (w/v) NaOH at 65°C for 2 hours with constant stirring and with a ratio of solid to alkali solution of 1:10 (w/v). Shahidi and Synowiecki (1991) showed that deproteinization involved treatment with 1% KOH solution for shrimp (*Pandalus borealis*) and 2% KOH for crab (*Chionoecetes opilio*) shells at 90°C for 2 hours with a shells to alkali solution ratio of 1 : 20 (w/v). Mima *et al.* (1983) isolated protein from shells of the crab with 1 N NaOH solution for 3 h at about 80 °C. Chang *et al.* (1997) deproteinized pink shrimp (*Solenocera melanthero*) shell waste with 2.5 N NaOH (12.5 mL per gram of shrimp shell powder) at 75°C for 6 h. Rodriguez *et al.* (2002) separated protein from shrimp (*Pleoticus mulleri*) waste by treatment with 9% (w/w) NaOH at 65°C for 90 min.

Kamil *et al.* (2002) treated snow crab with 4% (w/v) NaOH at 60°C for 3 hours with a sample to alkali ratio of 1:20 (w/v) and the alkali treatment was repeated twice. These workers also observed that an increase in shell to KOH solution ratio above 1: 4 (w/w), a minimum ratio, had only a minor effect on the deproteinization efficiency of crustacean shells. However, relatively high

ratios of solids to alkali solution, 1:10 or above, usually were used to obtain uniformity in reaction with proper agitation.

Traditionally, chitin is isolated from crustacean shells by deproteinization in a hot base solution. It causes changes in molecular weight and degree of deacetylation of the product and degradation of nutritionally valuable proteins. Thus, enzymatic procedures for deproteinization of the shells or mold mycelia were investigated. Synowiecki and Khateeb (2003) deproteinization of shrimp shells by Alcalase, *Pseudomonas aeruginosa* K-187 (Wang and Chio, 1998 ; Oh *et al.*, 2000) and *Bacillus subtilis* (Yang *et al.*, 2000).

2.2 Demineralization

Crustacean shell generally contains 30-35% of minerals, depending on species and most of them is calcium carbonate (Johnson and Peniston, 1982). The common practice to separate minerals is carried 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 determining the characteristics of the product. Hence, the effects of acid concentration, temperature and duration of the treatment on properties of chitin from different sources were evaluated (Muzzarelli, 1977).

The efficiency of demineralization is generally monitored by measuring the residual ash content of the isolated chitin. The ash content for deproteinized shell wastes from shrimp and crab on a dry basis was 41.96 ± 0.52 and $45.77 \pm 0.53\%$, respectively. After 30 min of demineralization at 20° C by a sufficient amount of acid [2.5% (w/v) HCl solution at a ratio of shell to acid of 1:20 (w/v)], the ash content in chitin from shrimp and crab decreased to 0.1 ± 0.04 and $0.25 \pm 0.05\%$, respectively. Addition of more HCl solution or longer time for demineralization showed no beneficial effects (Shahidi and

Synowiecki, 1991). According to No *et al.* (1989), similar conditions were necessary for demineralization of the dried ground crawfish shell.

Demineralization could be done by treatment the sample with 1 N HCl with constant stirring at ambient temperature for 30 min using a solid to acid ratio of 1:15 (w/v). Wu *et al.* (2000) isolated minerals from deproteinized fishery wastes (shrimp, lobster and crab shell) with 10% HCl for 18 h. Benjakul and Sophanodora (1993) showed that the optimal condition for demineralization were 1.25 N and 0.75 N HCl for 0.5 h with a solid to solvent ratio of 1:12 (w/v) for carapace and shell, respectively. Kamil *et al.* (2002) demineralized the snow crab shell, that has been deproteinized, with 10 volumes of 10% (w/v) HCl at 25°C for 2 h and the acid treatment was repeated twice.

Chang *et al.* (1997) demineralization pink shrimp (*Solenocera melanthro*) shell waste with 1.7N HCl (9 mL per gram of shrimp shell powder) at ambient temperature for 6 h. The ash and calcium contents in chitin were 1.4% and 0.0004%, respectively. Rutherford and Austin (1978) demineralized the deproteinized blue crab shell (*Callinectes sapidus*) with 5% acetic acid at room temperature for 5 h using a sample to acid ratio of 6.8 : 1 (w/v). The ash content in the chitin product remained 4.0% which were higher than those treated traditionally with HCl. The demineralization squid pens, from *Loligo vulgaris* species, was carried out by washing twice at room temperature with 0.55 M HCl for 2 h each time (Tolaimate *et al.*, 2000).

2.3 Decoloration

The structures of some carotenoids found in shellfish are shown in Figure 2. Acid and alkali treatments alone produce a colored chitin product. When a bleached chitinous product is desired, pigments can be removed with reagents (Table 2). No *et al.* (1989) prepared a white colored crawfish chitin by extracting with acetone, followed by bleaching with 0.315% sodium hypochlorite solution. The color of chitin products varied from white to extremely pink. Kamil *et al.* (2002) washed chitin residue, firmly complexed with the carotenoid pigments with 20 volumes of acetone and dried for 2 h at ambient temperature, followed by bleaching with a 0.32% (v/v) sodium hypochlorite (containing 5.25% available chlorine) for 5 min with a solid to solvent ratio of 1 : 10 (w/v).

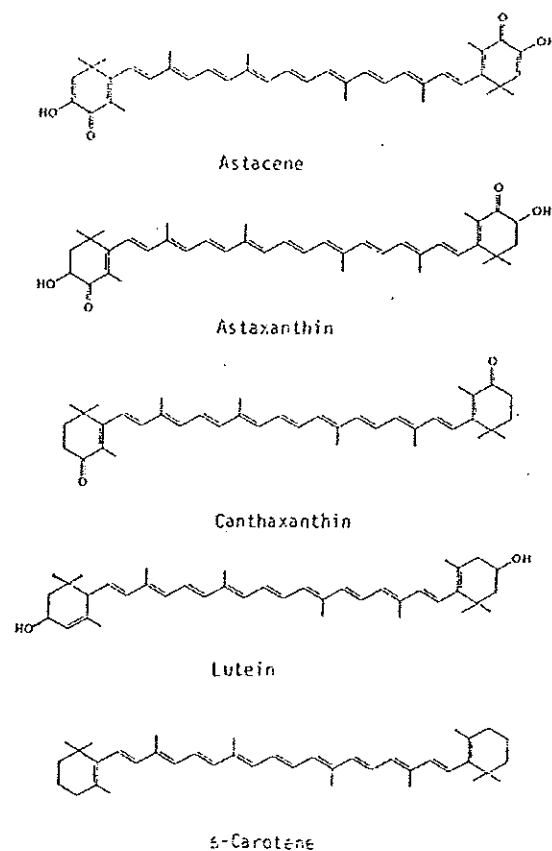


Figure 2 Structures of some carotenoids in shellfish

Source : Simpson (1978)

Table 2 Condition used for decoloration of chitins

Source	Decoloration conditions			
	Reagent	Temp. (°C)	Time (min)	Ratio (w/v)
Lobster	ethanol + ether	ambient	washing	-
	NaOCl	cold	-	-
	ethanol+acetone+ether	ambient	washing	-
Krill	chloroform	-	-	-
	ethyl acetate	30	30	1:4
Shrimp	acetone	-	-	-
Crawfish	acetone & 0.315% NaOCl	ambient	5	1:10
	diethyl ether	ambient	180	-
Crab	acetone	reflux	-	-
	acetone	-	-	-
	3% H ₂ O ₂	-	-	-
	acid H ₂ O ₂	cold	360	1:20
Prawn	acetone	reflux	45	1:15-20
	0.5% H ₂ O ₂	ambient	overnight	-
	NaOCl	cold	30	-

Source : No and Meyers (1995)

3. Preparation of chitosan

Conversion of chitin to chitosan is achieved by treatment with concentrated sodium or potassium hydroxide solution (40 – 50%) generally at 100°C or higher to remove some or all of the acetyl groups from the polymer (No and Meys, 1995). *N*-acetyl group can not be removed by acidic reagents without hydrolysis of the polysaccharide, thus alkaline methods must be employed for *N*-deacetylation (Muzzarelli, 1987).

Deacetylation is carried out by heating a suspension of chitin flakes (or powders) in strong aqueous bases such as sodium and potassium hydroxide at 100 – 160°C to give chitosan with degree of deacetylation between 0.70 and 0.90. The extent of deacetylation does not increase much after a few hours. To attain complete deacetylation, it is necessary to isolate chitosan and treat it with alkali in the same manner repeatedly (Mima *et al.*, 1983).

This reaction removes acetyl groups from C – 2 position of chitin, leaving free amino groups (-NH₂). It is well documented in literature that increasing temperature of treatment or alkali concentration result in the more chain depolymerization and the more breaking of glycosidic bond which affect the product properties. It also appears that if the deacetylation reaction takes place 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. Different preparation conditions provide a wide range in molecular forms and their applications.

No and Meyers (1989) prepared chitosan from crawfish chitin with 50% NaOH (w/w) solution at 100°C for 30 min in air using a solid to solvent ratio of 1:10 (w/v). Deacetylation was effectively achieved by treatment of chitin under elevated temperature and pressure with 45% NaOH for 30 min using solids/solvent ratio of 1:15. Treated chitosan showed similar nitrogen content (7.42%), degree of deacetylation (90.4%) and molecular mass (1560 kDa) but

significantly higher viscosity values (2025 cP) compared with those (7.40%, 87.6%, 1304 kDa and 143 cP, respectively) of a commercial chitosan. Reduction of the solid/solvent ratio from 1:15 to 1:10 did not affect degree of deacetylation, viscosity and molecular mass of chitosan.

Kurita *et al.* (1993) prepared chitosan from squid pen chitin (*Ommastrephes bastrami*) by treatment with 40% (w/v) NaOH at 80 °C under a nitrogen stream, stirring for 3 h using a chitin to alkali solution ratio of 1:20 (w/v). The degree of deacetylation of the chitosan product was 0.80. The longer alkali treatment, the higher degree of deacetylation was obtained and that could be increased to 0.97 after 6 h.

Benjakul and Sophanodora (1993) reported that chitin from black tiger prawn carapace and shell were satisfactorily deacetylated twice with 50% NaOH (w/v) under vacuum for 30 min each time with a solid to solvent ratio of 1:15 (w/v). Chitosan products from both carapace and shell showed moderate deacetylation with 57.0 and 45.6% degree of *N* – acetylation, respectively. Tsai and Su (1999) prepared chitosan from shrimp chitin (*Solenocera meloncho*) by treatment with 50% NaOH (1 g of chitosan powder per 13 mL of NaOH) and heated at 140 °C in an oil bath for 3 h to obtain chitosan with 98% degree of deacetylation. Kamil *et al.* (2002) reported that chitosan was prepared by alkali treatment of chitin using 10 volumes of 50% (w/v) NaOH at 100 °C up to 20 h in a nickel crucible under a nitrogen atmosphere. The reactants were immediately filtered under vacuum after alkali treatment, washed with hot-deionized water to neutral pH and lyophilized for 75 h at -49 °C and 62×10^{-3} mbar.

Effective procedures for obtaining highly deacetylated chitosan from carapace and shell have been developed. The optimal deacetylation attained by intermittently washing the intermediate product in water two times during the alkali treatment involved reacting chitin with 50% NaOH (w/v) under

vacuum for 45 min each time with a solids to solvent ratio of 1:15 (w/v) at 100°C. Chitosan products from both carapace and shell showed 31.89 and 43.21% *N* – acetylation, respectively and contained 7.60 – 7.63% nitrogen with low fat and ash content (Benjakul and Wisitwuttikul, 1994). Domard and Rinaudo (1983) proposed a new method for preparation of fully deacetylated chitosan without excessive decrease in molecular weight. Such effective deacetylation was attained by use of thiophenol during two or three successive alkali treatment for 1 h at 100°C.

4. Properties of chitosan

4.1 Solubility

Chitosan is insoluble in either water or organic solvents. It is a polyamine and is soluble in aqueous dilute acids. It dissolves in hydrochloric acid and aqueous organic acids such as formic, acetic, oxalic and lactic acids (Kurita, 2001). The required amount of acid to solubilize chitosan depends on the characteristics of the acid used. Compared with the more common organic acids, the solubility in inorganic acids seems more limited with regards to the concentration, and chitosan/acid ratio (Onsoyen and Skaugrud, 1990). The extent of solubility depends on the concentration and on the type of acid. The solubility decreases with increasing concentration of acid. Some acids such as phosphoric, sulfuric, citric and sebacic acids are no good solvents (Kurita, 2001). Acetic and formic acids are most widely used acids for dissolving chitosan (No and Meyers, 1995).

Inorganic acids can dissolve chitosan at certain pH values after prolonged stirring and warming. Nitric acid can dissolve some chitosan, but a jelly white precipitate can be observed. With cold concentrated nitric acid, a partial collapse of the grain can be found, but dissolution is not very fast. Hydrochloric acid also requires heating and stirring for hours. Sulphuric acid does not dissolve chitosan because it forms chitosan sulphate which is a

white crystalline solid. Perchloric acid can dissolve chitosan easier (Muzzarelli, 1973).

4.2 Viscosity

Due to the high molecular weight and the linear unbranched structure of the molecule, chitosan is an excellent viscosifier in acid environments (Onsoyen and Skangrud, 1990). The mutual repulsion of the positively charged deacetylated unit ($R-NH_3^+$) supplies the uncoiling force and results in an extended conformation of polymer in solution which cause the viscous solution (Filar and Wirick, 1978; Anonymous, 1989). The viscosity as a function of chitosan concentration is shown in Figure 3.

Chitosan with a degree of deacetylation of 75 % or more dissolves readily in dilute organic acid to give solutions containing few, if any, swollen gel particles. Such solutions are clear, homogeneous and viscous. The viscosity is a function of the molecular weight of the polymer, its concentration as well as the acid and its concentration used as a solvent (Filar and Wirick, 1978). The viscosity depended on the species used for chitosan production and preparation methods (No *et al.*, 2000b). The viscosity of chitosan in acetic acid tends to increase with decreasing pH (No and Meyers, 1995).

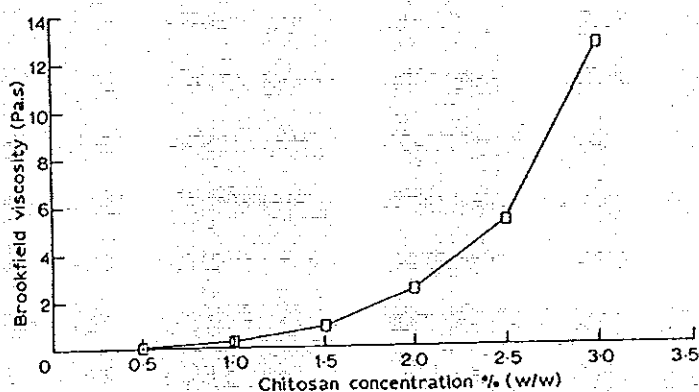


Figure 3 Viscosity as a function of chitosan concentration

Source : Onsoyen and Skangrud (1990)

The viscosity of chitosan generally ranged from 60 to 780 centipoise (cP) (Alimuniar and Zenerally, 1992; Anderson *et al.*, 1978). These ranges of viscosity also were observed by Cho *et al.* (1998) with five commercially available chitosans. It is apparent that autoclaving is an effective process to yield higher viscosity of chitosan (No *et al.*, 2000a). Rao *et al.* (1987) proposed an alternative method for deacetylation of chitin at low temperature by a novel alkali impregnation technique. However, the prepared chitosan showed a viscosity of only 290 cP.

The chitosan prepared from snow crab processing discards showed variations in their viscosity, which was closely related to the duration of the deacetylation. The decreased viscosity values may indicate depolymerization of chitosans prepared over longer treatment times (Kamil *et al.*, 2002). The repetition of deacetylation also affected the viscosity of products. More repetition of deacetylation resulted in less viscous chitosan (Benjakul and Wisitwuttikul, 1994). Chitosan from carapace showed higher viscosity and clarity than chitosan from shell, probably as a result of difference in molecular structure (Benjakul and Sophanodora, 1993). The viscosity of chitosan in acetic acid tended to increase with decreasing pH (No and Meyers, 1995).

4.3 Degree of *N* – acetylation / deacetylation

The basic repeating unit of chitin is *N* – acetyl – D – glucosamine. Although most of the C – 2 amino groups within chitin are acetylated, free amino groups are also considered to be present to some extent even in the native form (Shahidi *et al.*, 1999). Moreover, deacetylation takes place during isolation by alkaline treatment. To prepare chitin with a uniform structure or fully *N* - acetylated chitin, i.e. poly (*N*-acetyl-D-glucosamine), selective *N*-acetylation of the free amino groups is necessary (Kurita, 2001). The characteristics of chitosan, especially the degree of deacetylation, depend on the conditions employed throughout the extraction process, such as the

concentration of reagent, time, temperature and atmosphere (Bough *et al.*, 1978; Wu and Bough, 1978).

Reaction temperature plays the dominant role in deacetylation (Chang *et al.*, 1997). Other significant factors include the interaction of temperature and NaOH concentration, in the order of decreasing significance. The degree of *N*-deacetylation increased mainly with increasing temperature or NaOH concentration. However, the solution-to-chitin ratio was insignificant for deacetylation (Chang *et al.*, 1997). Benjakul and Wisitwuttikul (1994) reported that the ratio of both chitins to alkali solution (1:15 and 1:20) showed no effect on deacetylation.

Rege and Block (1999) reported that energy for the depolymerization reaction may be sufficient to hydrolyze the acetyl moiety (from the acetamido groups on the polymer) in addition to hydrolyzing the linkages between the monomers.

4.4 Molecular weight

The molecular weight of chitin and chitosan are also important factors for characterization, but poor solubility and structural ambiguities in connection with the content and distribution of acetyl groups are major obstacles to quantitatively determining molecular weight (Kurita, 2001). Furthermore, converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution, which in turn influences the agglomeration (Ravikumar, 2000).

Effect of molecular weight on the physical properties of chitosan membrane has been explored. Blair *et al.* (1987) reported that tensile strength and elongation increased proportionally with molecular weight of chitosan used. Ogawa *et al.* (1992) reported crystallinity of membrane increased with decreasing chitosan molecular weight.

Factors affecting molecular weight of chitin/chitosan

4.4.1 Conditions for chitin isolation

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 (Brzeski, 1982; Shahidi and Synowiecki, 1991).

The size of chitosan molecules is mostly affected by demineralization of the initial chitinous raw material to obtain the crude chitin. This might result in partial or extended depolymerization (Brzeski, 1982; Johnson and Peniston, 1982). The molecular weight of chitosan products are affected by deproteinization treatments of the chitinous raw material by alkali (3% NaOH) or enzymatic (Rhozyme-62) extraction (Bough *et al.*, 1978). The effects of preparation condition were also investigated. Decalcification was the most critical step determining the molecular weight of the product. The greatest depolymerization occurred when hydrochloric acid was used, followed by acetic acid and sulfurous acid. Minimal degradation occurred when EDTA was used (Brine and Austin, 1981).

4.4.2 Time of deacetylation and alkali concentration

Bough *et al.* (1978) also investigated the effect of deacetylation time on viscosity and molecular weight of chitosan. Chitosan deacetylated for 5 min with 50% NaOH at 145 – 150°C had higher viscosities (1.7 – 16.4 fold) and molecular weights (1.1 – 1.8 fold) than did chitosan deacetylated for 15 min. Kamil *et al.* (2002) found that the highest molecular weight was observed when deacetylation was carried out for 4 h, followed by those prepared over 10 and 20 h. Wu and Bough (1978) reported that molecular weight of chitosan was 1,142 kDa at the first 1 h of alkali treatment with 50% NaOH at 100°C and then decreased to 667 kDa after 5 h of treatment under the same

conditions. Hwang *et al.* (2002) found that molecular weight of chitosan significantly decreased with increasing reaction time and NaOH concentration. Tolaimate *et al.* (2000) reported that increasing the reaction time did not significantly affect the deacetylation but lowered the molecular weight.

In the presence of alkali, the polysaccharide chains were found to undergo some degradation because of high concentration of reagents used and prolonged reaction time required to obtain complete deacetylation (Whistler and Bemiller, 1958). Mima *et al.* (1983) observed that decreasing alkali concentration increased the time required to obtain soluble chitosans with a less viscous product, but increasing alkali concentration to saturation had little effect on deacetylation and viscosity. Prolonged time also increased the percentage of deacetylation and reduced molecular size (Mima *et al.*, 1983).

4.4.3 Temperature of deacetylation

Temperature plays indeed an important role in the degradation of the chitosan. Moreover, molecular weight of chitosan decreased when the temperature gradually rises (Jia and Shen, 2002). Reaction temperature has a significant influence on the degrading of chitosan by alkali treatment (Mima *et al.*, 1983). By decreasing the temperature from 120 down to 95°C and keeping the same composition of the reaction medium, an average viscometric molecular weight of 290,000 g/mol (compared to 150,000) were obtained after 2 h, confirming the role of the temperature (Tolaimate *et al.*, 2000). Rege and Block (1999) investigated the influence of temperature on depolymerization of chitosan and found that only the reaction temperature had a significant impact on the molecular weight of the resultant polymer.

4.4.4 Atmosphere

Rigby (1936) showed that free access of oxygen to chitin during deacetylation had a substantial degrading effect on the chitosan product. If oxygen is present, degradation and oxidation proceed by a free radical similar to that reported for cellulose (Hayes and Davies, 1978). Deacetylation in an atmosphere of nitrogen yielded chitosans of higher molecular weight distributions than did deacetylation in air (Bough *et al.*, 1978). Prashanth *et al.* (2002) reported that the molecular weight of chitosan prepared by carrying out *N*-deacetylation in the presence of nitrogen atmosphere was higher when compared to those of the control. This could be attributed to depolymerization of chitosan chains due to reactions as well as the possible oxidative cleavage of the glycosidic bonds (Hasegawa *et al.*, 1994). When the reaction is performed in a nitrogen atmosphere, the molecular weight is not modified. Breaking of bonds is not an oxidative process and thus the presence or absence of dioxygen has no influence on molecular weight distribution (Tolaimate *et al.*, 2000). Deacetylation was effectively achieved under the autoclaving conditions without significant degradation of the chitin structure, compared with the conventional method (No *et al.*, 2000a).

4.4.5 Use of diluents or additive

Chitin may be deacetylated to acid-soluble chitosan by treatment with concentrated aqueous NaOH in the presence of water-miscible organic solvent such as 2-propanol, 2-methyl-2-propanol or acetone (Batista and Roberts, 1990). Using such diluents enables a reduction of at least 85% of concentrated NaOH solution required. However, the extent of deacetylation and the solution viscosity of the product are less than those obtained using aqueous NaOH alone under the same conditions of time and temperature (No and Meyers, 1995).

Domard and Rinaudo (1983) proposed a new method for preparation of full deacetylated chitosan without excessive decrease in molecular weight. Such effective deacetylation was attained by the use of thiophenol during two or three successive alkali treatments for 1 h at 100°C. Prashanth *et al.* (2002) reported that the molecular weight of chitosan prepared in the presence of 1% (v/v) thiophenol was greater when compared to those of the control. Thiophenol acts as a good oxygen scavenger and possibly arrests such depolymerization reactions (Prashanth *et al.*, 2002).

Hsu *et al.* (2002) reported that after adding potassium persulfate into the chitosan at 70°C, the solution viscosity decreased rapidly in a short time. The intrinsic viscosity ($[\eta]$) of degraded solution and the corresponding average molecular weight of chitosan decreased quickly after 5 min and already reached a plateau value at only 10 min. The treatments of chitosan with H₂O₂ led to a decrease in the degree of polymerization, even at ambient temperature (Qin *et al.*, 2002). Chang *et al.* (2001) reported that low concentration of hydrogen peroxide induced random degradation of partially deacetylated chitin and chitosan. Maclaughlin *et al.* (1998) found that although the molecular weight of each chitosan decreased as the amount of sodium nitrite increased, the degree of deacetylation did not change.

5. Some applications of chitosan

5.1 Fat adsorption of chitosan

Chitosan is known to interact with hydrophobic compounds such as cholesterol, triglycerides, fatty acid and bile acid (Rockway, 2000). Chitosan binds fatty acid to form the corresponding salt (Ahmad *et al.*, 1995). Additionally, the strong positive charge carried by the chitosan molecule causes it to bind negatively charged substrates such as lipid (Ormrod *et al.*, 1998). The binding of chitosan is mainly with edible fatty acid such as oleic, linoleic, palmitic, stearic and linolenic acids. The resulting salts, after ingestion, bind additional lipids, probably due to hydrophobic interactions (triglycerides, fatty acid and bile acids; cholesterol and other sterols) and great portion of these bound lipids are excreted rather than absorbed (Muzzarelli, 1996). The hypocholesterolemic potential of a dietary fiber resides in high viscosity, polymeric nature and high water binding properties and nondigestibility in the upper gastrointestinal tract, together with low water binding in the lower gastrointestinal tract, and has a highly characteristic property in relation to other dietary plant fibers. It can bind anions such as bile acids or free fatty acids at low pH by ionic bonds, resulting from its amino group (Muzzarelli, 1996). Hwang and Damodaran (1995) reported that addition of 0.01 – 0.02% chitosan to Cheddar cheese whey at pH 4.5 resulted in formation of a chitosan-fat globule membrane complex.

Deuchi *et al.* (1994) reported that chitosan markedly increased the fecal lipid excretion and reduced the apparent fat digestibility to about a half relative to the control. Conversely, Gades and Stern (2002) reported that chitosan supplement did not increase fecal fat content and therefore did not block fat adsorption. Chitosan has viscous properties like those of viscous dietary fibers such as pectin and guar gum. The viscous fibers also sequester micellar components *in vitro*, albeit with considerably less avidity than does

cholestyramine and other commercial anion exchangers (Muzzarell *et al.*, 1996).

The mechanisms by which cholestyramine and chitosan affect lipids absorption may be different. Sugano *et al.* (1988) reported that after a 20 days feeding of 5% cholestyramine or chitosan in a cholesterol containing diet to rats, plasma and liver cholesterol levels in both groups were significantly lower than in controls. However, fecal neutral sterols were elevated only in the chitosan fed rats, suggesting a difference in the action of chitosan and cholestyramine. In a subsequent study, the feeding of chitosan caused increased fecal output of neutral sterols but not acidic steroids, while cholestyramine feeding increased mainly fecal acidic steroids. The data of Sugano *et al.* (1988) also suggest that the mechanism of chitosan action may not completely parallel to those of the viscous dietary fibers. Pectins, when fed at sufficient levels, cause some increase in the output of fecal neutral and acidic steroids, but this appears insufficient to account for their hypocholesterolemic effects. However, chitosan, which has greater hypocholesterolemic potency than pectins in cholesterol fed rats does not effectively increase bile acid excretion.

Fukada *et al.* (1991) indicated that chitosans affect the metabolism of intestinal bile acids in rats. Thus, based on evidence derived from analyses of fecal acidic and neutral steroid output, one cannot easily assign a common mechanism of action for cholestyramine, chitosan, and viscous dietary fibers. Nauss *et al.* (1983) studied the binding capacity of chitosan relative to other mixtures of bile salts, dodecyl sulfate, and a mixed microemulsion. Under experimental conditions which affect binding (e.g. altering pH and ionic strength), chitosan demonstrated a strong binding capacity for bile salts.

Han *et al.* (1999) found that the anti-obesity effects of chitin-chitosan in high-fat diet treated mice might be partly due to the inhibition of intestinal absorption of dietary fat. Consequently, chitin-chitosan might cause

improvement of the fatty liver and hyperlipidaemia in mice fed a high fat diet through inhibiting intestinal absorption of dietary fat. Kanauchi *et al.* (1995) proposed the mechanism for the inhibition of fat digestion by chitosan as shown in Figure 4. Those involved both a stomach stage and an intestinal stage.

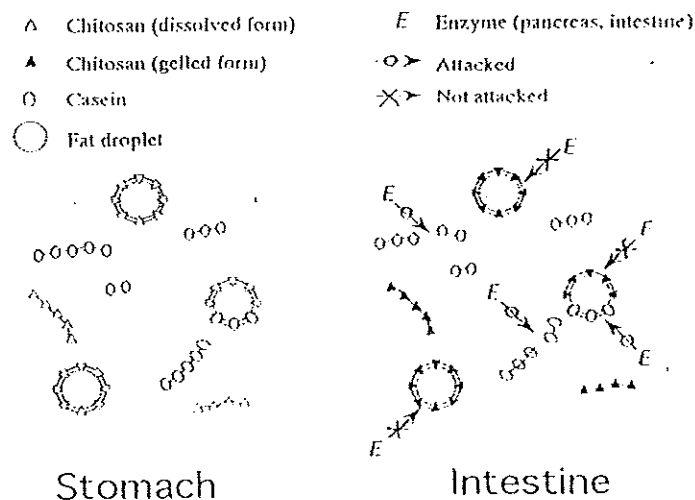


Figure 4 Mechanism for the inhibition of fat digestion by chitosan.

Source : Kanauchi *et al.* (1995)

In the stomachic stage, chitosan is dissolved in an acidic medium, and mixed well with fat by the strong ability of chitosan for emulsification (Knorr, 1982). Chitosan exists in the aqueous phase at the surface of the fat droplets as a surfactant. This mixture is transferred to the intestines. In the intestinal stage, rough emulsion of fat with chitosan immediately changes to the insoluble gel form of the entrapped fat droplets, which can not be attacked by pancreatic or intestinal enzymes. Koide (1998) reported that chitosan acts by forming gels in intestinal tract which entrap lipids and other nutrients, including fat soluble vitamins and minerals, thus interfering with their absorption. Okamoto *et al.* (2001) reported that the *in vitro* study was also performed to investigate the physical change of chitosan after treatment with artificial

gastric and intestine juices. The *in vitro* study showed that chitosan became a gel in artificial gastric juice and its weight decreased by 15%, but there were no such physical changes when it was placed in artificial intestinal juice. This study showed that chitin did not undergo any changes in weight and shape in the gastrointestinal tract, whereas chitosan did.

5.1.1 Effect of molecular properties of chitosan on fat adsorption

The relationship between hypocholesterolemic efficacy and average molecular weight of chitosan was studied in rats fed a cholesterol-enriched (0.5%) diet by Sugano *et al.* (1988). Several chitosan preparations with a comparable degree of deacetylation but differing widely in average molecular weight and viscosity, almost completely prevented the rise of serum cholesterol at the 5% dietary level. At the 2% level, chitosans with viscosities at both extremes exerted a comparable cholesterol-lowering action. The hypocholesterolemic action of chitosan is independent on their molecular weight within the tested range. Judging from the ineffectiveness of the glucosamine, it was suggested that some degree of polymerization is required to provoke a cholesterol-lowering activity (Sugano *et al.*, 1992).

The concentration of serum cholesterol at day 7 was significantly lower in rats fed chitosan with MW 5000 – 50,000 Da than in those fed cellulose. The 2000 Da chitosan did not show a hypocholesterolemic effect at day 7. All chitosan hydrolysates except for 2000 Da significantly increased fecal excretion of neutral steroids as cholesterol and coprostanol. The hydrolysates with average molecular weights above 10,000 Da were more effective in enhancing fecal excretion of neutral steroids (Ikeda *et al.*, 1993). Enomoto *et al.* (1992) noted that chitosan having molecular weight of 8000 was more effective in lowering cholesterol than chitosan having 220,000. The effect on the composition of neutral steroids varied, depending on the preparations.

Excretion of total acidic steroids was slightly increased in rats fed chitosan hydrolysates except for the 2000 Da group.

5.1.2 Effect of the viscosity or deacetylation degree of chitosan on fat adsorption

It is possible that the cholesterol-lowering effect of chitosan is due to an increase in the viscosity. Increased intestinal content supernatant viscosity is highly correlated with reduced plasma and liver cholesterol (Gallaher *et al.*, 1993) and reductions in cholesterol absorption in hamsters (Carr *et al.*, 1996). However, Sugano *et al.* (1988) found that chitosan preparation of different *in vitro* viscosities demonstrated equivalent hypocholesterolemic effect, arguing against a role for viscosity. Deuchi *et al.* (1995) reported that the viscosity of chitosan affected the apparent fat digestibility. As the viscosity of chitosan increased, the apparent fat digestibility tended to decrease.

Gallaher *et al.* (2000) determined the effect of glucomannan (G), chitosan (CH), or an equal mixture of the two (G+CH) on cholesterol absorption and fat and bile acid excretion. Total liver cholesterol was significantly reduced in G, CH and G+CH groups compared with the C group. The intestinal content supernatant viscosity of the C and the CH group was negligible, whereas both G and G+CH produced high viscosities. These results suggest that G lowered liver cholesterol by a viscosity-mediated interference of cholesterol absorption. In contrast, CH appears to lower cholesterol through a different mechanism.

Deuchi *et al.* (1995) reported that when degree of deacetylation of chitosan increased, the apparent fat digestibility tended to decrease. This result concerning the degree of deacetylation may indicate that electrostatic forces between fat and chitosan play a role as the primary driving force for rough emulsification in the stomach. Hypocholesterolemic action of chitosan

was greater with highly deacetylated than with moderately deacetylated chitosan (Sugano *et al.*, 1988).

5.1.3 Synergistic effect of chitosan on fat adsorption

Rats were fed on high-fat diets containing cellulose (control), chitosan, chitosan ascorbate, chitosan lactate and chitosan citrate salts. The presence of ascorbate caused a larger increase in the fecal fat excretion than otherwise, without considerably affecting the apparent protein digestibility. Kanauchi *et al.* (1994) carried out similar studies with 7% chitosan diet and reduced fat absorption to one third of the controls within one month.

The mechanism for the synergistic effect is considered by Kanauchi *et al.* (1995) to be (a) viscosity reduction in the stomach, (b) increase of the oil-holding capacity of the chitosan gel, and (c) reduced leaking of entrapped fat in the intestinal tract, resulting in more flexible chitosan-fat gel. Chitosan also affected the growth rate by reducing the energy intake by inhibition of lipid digestibility. About 80% of dietary fat could not be digested by supplementing chitosan with ascorbic acid. So, the rats could not intake sufficient energy when compared to the control animals. Despite feeding with a high-fat diet, the plasma triglycerides concentration and epididymal fat pad weight in the chitosan-receiving groups remained at low levels, probably because of a depressed fat intake (Kanauchi *et al.*, 1994).

5.2 Antioxidative activity of chitosan

Antioxidants are a group of chemicals used for extending the shelf-life of a wide variety of food products. The use of antioxidants was extended to a wide variety of food products including high-fat foods, cereal and even products containing very low levels of lipids. In general, antioxidants function by reducing the rate of initiation reaction in the free-radical chain reactions and are effective at very low concentration, 0.01% or less (Rajalakshmi and Narasimhan, 1996).

Lipid oxidation is a free - radical chain reaction that causes a total change in the sensory properties and nutritive value of food products. Changes in color, texture, odor and flavor, loss of vitamins and damage of protein are caused by lipid oxidation. The onset of lipid oxidation can be delayed by the addition of antioxidants (Rajalakshmi and Narasimhan, 1996).

Transition metal ions are remarkably good promoters of free-radical reaction because of singlet electron transfer during their change in oxidation stages. Transition metal ions having variable oxidation number (iron Fe^{2+} or Fe^{3+} ; copper as Cu^+ or Cu^{2+} ; Mn, Ni, Co, etc.) are implicated in enhancing the rate of oxidation (Jadhav *et al.*, 1996).

Heme (Fe^{2+}) and hemin (Fe^{3+}) proteins are widely distributed in food. Lipid peroxidation in animal tissue is accelerated by hemoglobin, myoglobin and cytochrome C. These reactions are often responsible for rancidity or aroma defects occurring during storage of fish, poultry and cooked meat (Belitz and Grosch, 1999). Decker and Hultin (1992) identified several sources of protein bound iron that exist in biological tissues, namely myoglobin, haemoglobin, ferritin, transferrin and haemosiderin. St. Angelo (1996) reported that iron bound to these proteins may be released during postharvest storage and cooking, leading to initiating lipid oxidation. There is a range of concentration of haematin compounds in muscles from different species of fish and these are present in relatively large concentrations in the muscle of

most fatty fish, especially their lateral band dark muscle (Castell and Bishop, 1969).

Autoxidation of oxymyoglobin and oxyhaemoglobin (both in the Fe^{2+} oxidant state) may also result in the formation of superoxide anion, metmyoglobin and methaemoglobin (both in the Fe^{3+} oxidant state) respectively (Sato and Shikama, 1981). Flick *et al.* (1992) reported that increased oxidation of seafoods at lower humidities may be attributed to the concentration of prooxidants such as metal ion and haemoglobin. The main source of free iron or non-haem iron in cell is ferritin, which is a soluble iron storage protein found in liver, spleen and skeletal muscle (Decker and Welch, 1990).

Both chitin and chitosan are able to form complexes with many transition metals, as well as some of those from groups 3 – 7 of the periodic table (Muzzarelli, 1973). The heavy metal complexes can be formed with chitosan. This involves the donation of nonbonding pairs of electrons from the nitrogen, and/or the oxygen of the hydroxyl groups, to the heavy metal ion (Winterowd and Sandford, 1995). *N,O*-carboxymethyl chitosan has been found to bond chemically with ion of numerous heavy metals, such as iron, copper, mercury and zinc, thus binding or sequestering them when heavy metals present at the range of 10 – 1000 ppm (Hayer, 1986).

Effectiveness of chitosan addition on oxidative stability of beef was studied by Darmadji and Izumimoto (1994). The rate of lipid oxidation in meat was reduced by addition of chitosan. St. Angelo and Vercellotti (1989) reported that *N*-carboxymethyl chitosan was effective in controlling warm over flavor (WOF) over a wide range of temperature. Use of 5000 ppm *N*-carboxymethylchitosan in ground beef resulted in a 93% inhibition of TBA and 99% reduction in the hexanal content in the products. However, Shahidi (1995) reported that *N,O*-carboxymethyl chitosan (NOCC) and its lactate, acetate and pyrrolidine carboxylate salts were effective in controlling the

oxidation and flavour deterioration of cooked meat over a nine days of storage at refrigerated temperature. NOCC and its aforementioned derivatives at 500 – 3000 ppm inhibited TBA values by 46.7, 69.9, 43.4 and 66.3%, respectively.

The mechanism by which this inhibition takes place is thought to be related to chelation of free iron which is released from hemoproteins of meat during heat processing. Therefore, catalytic activity of iron ions was inhibited. Li *et al.* (1996) added *N*-carboxymethyl chitosan to cooked pork at a level of 3000 ppm and found that this amount was sufficient to prevent the oxidative rancidity of the product. The feasibility of using chitosan powder in a fluorescence sensor for monitoring lipid oxidation in muscle foods was studied by Weist and Karel (1992). The primary amino groups of chitosan form a stable fluorosphere with volatile aldehydes such as malondialdehyde which is derived from the breakdown of fats.

Lin and Chao (2001) found that Chinese-style sausages containing 0.1% chitosan (in 1% lactic acid solution) with three molecular weights (150, 600 and 1250 kDa) were higher in TBA values (mg malonaldehyde/ kg meat) than control which could be due to lower pH. Livingstone and Brown (1981) and Renerre (1990) indicated that the myoglobin oxidation rate was accelerated at lower pH condition.

Jeon *et al.* (2003) found that the CCl_4 significantly increased the TBARS concentration but chitosan inhibited by 34.6%, compared with the CCl_4 group.

Objectives

1. To study chitosan from black tiger prawn with different molecular weights on fat adsorption of different oils.
2. To investigate the effect of some extrinsic factors influencing fat adsorption of chitosan from black tiger prawn.
3. To study chitosan from black tiger prawn with different molecular weights on antioxidative activity.
4. To study the application of chitosan powder in precooked comminuted pork.

Chapter 2

MATERIALS AND METHODS

Materials

1. Prawn shell preparation

- Black tiger prawn (*Penaeus monodon*) shell was obtained from a commercial freezing plant in Hat Yai, Thailand. The prawn shell was washed with tap water, followed by drying in a forced air oven at 65° C for 6 h. The dried shell was then ground to 1.4 – 4.0 mm particle size, placed in opaque plastic bag and stored at room temperature until used.

2. Oils / lard

Soy bean oil "Grape" brand and palm oil "Olean" brand were purchased from supermarket in Hat Yai, Songkla. For lard, the tallow was bought from the market, cut into small pieces and subjected to rendering at 180-200 °C. The oil will be filtered and used for further study.

3. Chemicals

3.1 Chemicals for determination of oil adsorption properties

- Sodium borohydride
- Ascorbic acid
- Chloroform
- Methanol

3.2 Chemicals for determination of mode of action

- Ferric chloride
- Ferrozine
- Sodium phosphate
- 2-deoxyribose

3.3 Chemical for oxidation analysis

- Thiobarbituric acid
- Hydrochloric acid
- Sodium thiocyanate
- Starch
- Acetic acid : Chloroform 3:2 (v/v)
- Iso-octane

4. Instruments

Instruments	Model	Company
Homogenizer	T25	Ultra turrax, Malaysia
Thin Layer Chromatography/ Flame Ionization Detection Analyzer (TLC-FID)	Iatroscan MK-5	Iatron Laboratories, Japan
Gas Chromatography/ Flame Ionization Detection Analyzer	HP 6850	SHIMADZU, Japan
Atomic Absorption Spectrophotometer	Analyst 100 INKUBATOR 1000	Perkin Elmer, USA Heidolph instruments, Germany
Shaker	GEL 1038	Gesellschaft fur, Germany
Shaker water bath	WB/OB 7-45	Memmert, Germany

5. Study on chemical compositions of black tiger prawn shells

Chemical compositions including, moisture, fat, ash and nitrogen content were determined by standard method (AOAC, 1999). Calcium, zinc, magnesium, iron, manganese and copper were analyzed according to the method of AOAC (1999).

6. Production of chitosan from black tiger prawn shells

6.1 Chitosan preparation

Dried shell was subjected to deproteinization with 1.0 N NaOH at 100 °C for 1 h with a solid to solvent ratio of 1:6 (w/v). Then demineralization was carried out with 0.75 N HCl at room temperature for 0.5 h with a solid to solvent ratio of 1:12 (w/v). Chitin obtained was decolorized with 3% H₂O₂ at room temperature for 12 h with a solid to solvent ratio of 1:10 (w/v).

To produce chitosan, deacetylation was then performed. Removal of acetyl group from chitin was accomplished by reacting with 50 % NaOH (w/w) in absence or presence of sodium borohydride at different concentrations (0.5, 1.0, 2.0 M). The reaction was conducted with a solid to solvent ratio of 1:15 (w/v) at 100 °C under vacuum for 45 min. The chitosan obtained was washed in water twice. The chitosan was then dried at 60 °C for 4 h.

6.2 Chemical analysis

6.2.1 Moisture, fat, ash and nitrogen contents of chitosans were determined by standard method (AOAC, 1999). Calcium, zinc, magnesium, iron, manganese and copper were analyzed according to the method of AOAC (1999).

6.2.2 Degree of deacetylation of chitosans was determined using colloidal titration method as described by Chen and Hwa (1996) (appendix 1).

6.2.3 Viscosity of chitosans was measured with a Brookfield viscometer. Chitosan solution prepared in 2% acetic acid at a concentration of 1% was subjected to analysis as described by Cho *et al.* (1998).

6.2.4 Molecular weight was determined using capillary viscometer according to the method of Huang *et al.* (2000) (appendix 1).

6.2.5 Degree of deacetylation was measured on chitosan film using infrared spectroscopy as described by Domard and Rinaudo (1983).

7. Fat adsorption properties of chitosan

7.1 Powder system

Oils (palm oil, lard, soybean oil) were percolated (15 g, with a flow rate of 1.5 g/ min) through a bed of chitosan powder (2 g, with a height of 3 cm). Both the effluent and the imbibed fraction were collected for analysis.

Extraction of oil percolated and adsorbed with chitosan

- Percolated fractions were added with 25 ml of chloroform. The mixture was filtered with Whatman No.1 filter paper and evaporated to remove the solvent at 50^oC using a rotary evaporator.

- Adsorbed fractions was eluted with 50 ml of chloroform. The mixture was filtered with Whatman No.1 filter paper and evaporated to remove the solvent as mentioned above.

Analysis

1. Weight of oil either percolated or retained was measured.

2. Fatty acid and lipid compositions of both percolated and retained oils were determined as fatty acid methyl esters (FAME) using a gas chromatography, HP 6850 series, equipped with fused silica capillary column carbowax – PEG (30 m, 0.25 mm ID) and flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 150°C, increased to 230°C at a rate of 3°C/min, and then held at 230°C for an additional 10 min. The detector temperature was set at 240°C, while the temperature at the injection port was maintained at 240°C. Heptadecanoic acid (C17, Sigma) was added to all samples as an internal standard before preparation of FAME. Retention time of FAME standards were used to identify chromatographic peaks.

3. The lipid compositions of both fractions were determined by thin layer chromatography/Flame Ionization Detection analyzer (TLC-FID). Scanned quartz rods (silica gel powder coated Chromarod S III) were dipped in 3% boric acid solution for 5 min, dried and rescanned with the TLC-FID analyzer. The sample solution (1 µL) was spotted on the rod and the separation was performed in the mixtures of benzene:chloroform:acetic (52:20:0.7) for approximately 40 min. Then the rods were dried in an oven (105°C) for 2-3 min before applying to Flame Ionization detector. The analytical condition was H₂: flow rate of 160 mL/min, air flow rate of 2000 mL/min and scanning speed of 30 sec/scan.

7.2 emulsion system

One gram of chitosan was dissolved in 100 ml of 0.1M HCl and stirred for 2 min. Oils including palm oil, lard and soy bean oil (4 g) was then added and mixed for 1 min and then shaken at 37 °C for 1 h . The solution were sequentially dropped into 0.1M NaOH solution and small chitosan - oil gel granules were formed. These granules in the alkaline solution were shaken at 37 °C for 2 h. Chitosan-oil granules (7 g) were washed with distilled water. The mixture of chloroform and methanol (a ratio of 2:1) (100 mL) was added and homogenized for 30 sec, followed by addition of 50 ml of distilled water. The mixture was homogenized for 30 sec and centrifuged at 6500 x g for 15 min. Then chloroform layer was collected and evaporated. Oil extracted from chitosan-oil gel granule were subjected to analysis as shown in section 7.1.1.

Chitosan samples which exhibited the highest oil adsorption was chosen for further study.

7.2.1 The effect of chitosan amount on fat adsorption in emulsion system

Chitosan at different levels including 0.5, 0.75, 1.0, 1.25 and 1.5 g were dissolved in 100 ml of 0.1 M HCl and mixed with oil as described in section 7.2.

7.2.2 Synergistic effect of ascorbic acid on fat adsorption of chitosan

Ascorbic acid at different concentrations including 0.025, 0.05 and 0.1M were mixed with 0.1M HCl at a ratio of 1:1(v/v). Chitosan at a level of 1% was dissolved in those solutions. Chitosan-oil gel granules were prepared as mentioned in section 7.2. Fat adsorption capacity of chitosan dissolved in different solutions was analyzed.

Viscosity of chitosan-acid solution was determined. Ascorbic acid at a concentration of 0.025, 0.05. and 0.1 M will be mixed with 0.1 M HCl. Then 1% chitosan will be added and stirred for 3 h at 37°C. The viscosity was measured using a Brookfield viscometer.

8. Antioxidative activity off chitosan

8.1 Chelating activity of chitosan powder on Fe²⁺

The chelating activity of chitosan powder on Fe²⁺ was measured according to the method of Dinis *et al.* (1994). Chitosan powder (0, 5, 10, 15 mg) was reacted with FeCl₂ (2 mM, 0.2 ml) and ferrozine (5 mM, 0.2 mL) for 10 min, and the spectrophotometric absorbance was determined at 562 nm. A lower level of absorbance indicates a stronger chelating activity.

8.2 Scavenging effect on hydroxyl radicals

The deoxyribose degradation caused by hydroxyl radicals was determined by the formation of TBARS (Aruoma, 1994). Different amounts of chitosan powder (particle size < 1 mm) (0, 5, 10, 15, 20, 30 mg) were used (appendix 1).

9. Determination of antioxidant activity in lecithin liposome system (Frankel *et al.*, 1997 ; Yi *et al.*, 1997).

Lecithin (2.4 g) was suspended in deionized water at a concentration of 8 mg/mL by stirring with a glass rod and sonicating for 15 min. To test antioxidant activity, chitosan powder (0, 50, 200 ppm) were added to the lecithin liposome system. After addition of chitosan powder, the liposome suspension was sonicated again for 2 min. To initiate the assay, 10 μ L of cupric acetate (3.0 M) was added. The mixtures were shaken (120 rpm) at 37^o C in dark. Liposome oxidation was monitored by determining thiobarbituric acid – reactive substances (TBARS) and conjugated diene as follows:

9.1 Measurement of TBARS. Liposome sample (1 mL) was mixed with 5 mL of butylated hydroxytoluene (0.2%) and 2 mL (15% TCA / 0.375% TBA / 0.025 N HCl). The mixtures were then heated for 10 min in a boiling water bath (95 – 100^o C) to develop pink color, cooled with tap water, and centrifuged for 20 min at 5,500 x g. The supernatant containing the pink chromogen was quantified at 532 nm (Lee and Hendricks, 1997 : Duh, 1998) (appendix 1).

9.2 Measurement of conjugated diene. Liposome sample (0.1 mL) were dissolved in methanol (5.0 mL), and conjugated diene was measured at 234 nm (Frankel *et al.*, 1997) (appendix 1).

10. Application of chitosan powder in cooked ground pork.

Antioxidant efficacy of chitosan powder at different levels in cooked ground pork was studied. Raw ground pork was purchased from the supermarket in Hat Yai, Songkhla. The chitosan powder with a particle size of 250 μ m was added to raw ground pork to obtain the final concentrations of 50, 100 or 200 ppm. Samples were cooked in a water bath at 85 \pm 2^o C (internal temperature of 72 \pm 3^o C) for 40 min. The samples were stirred every

5 min with a glass rod. After cooling to room temperature, the samples were transferred into polyethylene bags and then stored under refrigerated condition at 4 ± 1 °C.

Cooked ground pork were analyzed for lipid oxidation immediately after cooking (day 0) and after storage at 4 °C for 3, 6, 9, 12 and 15 days. Lipid oxidation was assessed by TBARS (Burge and Aust, 1978), peroxide value (IUPAC, 1979) conjugated diene (Frankel and Huang, 1996). Non heme iron content was also determined (Schricker *et al.*, 1982).

11. Statistical analysis

Completely randomized design was used throughout this study. Data was subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's Multiple Range Test (DMRT). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc.).

Chapter 3

RESULTS AND DISCUSSION

1. Chemical compositions of shell and chitosan from black tiger prawn

1.1 Chemical compositions of shell from black tiger prawn

Chemical compositions of shell from black tiger prawn are shown in Table 3. Dried shells from black tiger prawn contained 8.65% moisture, 45.34% crude protein, 0.85% fat, and 31.57% ash. Among minerals analyzed, calcium was a predominant mineral. Other minerals such as iron (Fe) and Zinc (Zn) were negligibly low. A large proportion of calcium salts are the constituents of crustacean shell. Crustacean shell waste consists mainly of 30 – 40% protein, 30 – 50% calcium carbonate and 20 – 30% chitin (Johnson and Peniston, 1982). However, contents of main component in the crustacean shell vary with species, the age, sex, time and site of catching (Gamzazade *et al.*, 1989; Green and Mattick, 1979). Benjakul and Sophanodora (1993) demonstrated that the content of P, K, Ca, Mg, Fe, Mn, Zn and Cu in black tiger shrimp (*Penaeus monodon*) were 0.8%, 8.2%, 1.7%, 0.8%, 96.5, 15.3, 10.0 and 5.0 ppm, respectively for carapace and 2.1%, 0.2%, 1.0%, 0.4%, 110.6, 48.6, 32.8 and 14.8 ppm, respectively for shell.

Table 3 Chemical compositions of black tiger prawn shell

Constituents	Content
Moisture(%)	8.65 \pm 0.09
Fat (% dry weight)	0.85 \pm 0.01
Crude protein (% dry weight)	45.34 \pm 0.31
Ash (% dry weight)	31.57 \pm 0.13
Minerals	
Ca (%)	9.57 \pm 1.15
Mg (%)	0.73 \pm 0.03
Fe (ppm)	80.49 \pm 0.79
Mn(ppm)	46.34 \pm 4.32
Zn (ppm)	49.60 \pm 2.13
Cu (ppm)	20.41 \pm 3.46

Mean \pm SD from triplicate determinations

1.2 Chemical compositions of chitosan from black tiger prawn shell

The chemical compositions of chitosan produced in absence and presence of sodium borohydride at different concentrations during deacetylation process are shown in Table 4.

The residual nitrogen content in the chitosan samples was considered an effective index for protein removal (No *et al.*, 1989). The nitrogen content ranged from 7.73 to 7.94 % on a dry basis. These nitrogen values were similar to those reported by Benjakul and Sophanodora (1993) (7.7-8.4%) chitosans contained satisfactorily low ash contents. Ash content was below 1.0%, indicating the effectiveness of the demineralization process used for removal of calcium carbonate. Similar mineral compositions were obtained among chitosan samples. Fat content ranged from 1.24 to 1.43 % on a dry basis. No significant differences in ash, fat and nitrogen content were observed among chitosan prepared without and with sodium borohydride at different concentrations ($p > 0.05$). The chemical compositions of chitosan products in this study were comparable to those reported by Ornum (1992), Li *et al.* (1992) and Cho *et al.* (1998). Generally, the nitrogen content of chitosan normally ranges from 7.06 to 7.97% and ash content is less than 1% (No and Meyers, 1995). For mineral compositions, no marked differences in mineral compositions of chitosan prepared with different sodium borohydride concentrations.

From these result, it could be inferred that sodium borohydride levels had no effect on compositions of chitosan.

Table 4 Chemical compositions of chitosan prepared by deacetylation in presence of sodium borohydride at different concentrations

	Sodium borohydride concentration (M)			
	0	0.5	1.0	2.0
Moisture(%)	9.17 ± 0.66a	10.23 ± 0.19c	8.16 ± 0.21b	9.22 ± 0.22a
Fat (% dry weight)	1.24 ± 0.05c	1.35 ± 0.03c	1.39 ± 0.09c	1.43 ± 0.06c
Nitrogen (% dry weight)	7.78 ± 0.23d	7.94 ± 0.21e	7.74 ± 0.10d	7.73 ± 0.18d
Ash (% dry weight)	0.55 ± 0.01b	0.50 ± 0.04b	0.50 ± 0.04b	0.49 ± 0.01b
Minerals				
Ca (%)	1.52 ± 0.01a	1.23 ± 0.05b	1.62 ± 0.08c	1.39 ± 0.05d
Mg (%)	0.014 ± 0.004ab	0.010 ± 0.002a	0.014 ± 0.002ab	0.017 ± 0.001b
Fe (ppm)	13.71 ± 1.82a	12.49 ± 0.89a	13.22 ± 0.19a	13.84 ± 1.31a
Mn (ppm)	3.26 ± 0.22a	4.25 ± 0.56a	3.58 ± 0.58a	6.17 ± 1.66b
Zn (ppm)	8.39 ± 0.10a	9.35 ± 0.68a	15.11 ± 2.85b	10.12 ± 0.87a
Cu (ppm)	0.92 ± 0.06a	1.11 ± 0.04b	1.19 ± 0.04ab	0.96 ± 0.19a

Mean ± SD from triplicate determinations

Different letters in the same row indicate the significant difference (p<0.05)

2. Molecular properties of chitosan

2.1 The average MWs and intrinsic viscosity of chitosan

Molecular weight of chitosans were determined by measuring the relative viscosity of chitosan at various concentrations in 1% acetic acid (η_{rel}). The specific viscosity (η_{sp}) could be calculated from the following equation: $\eta_{sp} = \eta_{rel} - 1$. The intrinsic viscosity $[\eta]$ was then determined from the intercept at $C = 0$ when η_{sp}/C were plotted against C (concentrations). To calculate the approx. molecular weight (M) of the polymers, the Mark-Houwink Equation: $[\eta] = KM^a$ in which $K = 8.93 \times 10^{-4}$, $a = 0.71$ was applied (Rutherford and Austin, 1978). Molecular weight of chitosans considerably increased when sodium borohydride concentration increased ($p < 0.05$). Chitosan prepared in presence of sodium borohydride at a concentration of 2.0 M had the highest molecular weight, which was associated with highest viscosity. The increase in molecular weight was coincidental with the increased intrinsic viscosity (Table 5). Thus, concentration of sodium borohydride showed preventive effect on the reduction of molecular weight and intrinsic viscosity of chitosan ($p < 0.05$).

Sodium borohydride is a chemical reducing agent that removes or prevents formation of oxidized impurities such as carbonyls, peroxides and metal cations (Cha and Lee, 2000). Although these impurities or contaminants are normally present only at the parts-per-million (ppm) level, they are costly and difficult to remove by traditional purification methods such as distillation, hydrogenation, adsorption, or oxidation. Sodium borohydride (NaBH_4) products are effective and very selective reducing agents used in the manufacture of pharmaceuticals, intermediates and fine chemicals. The light or heat resistance of many polymers can be improved using sodium borohydride treatment during polymer production. This application is based on the

reduction of the carbonyl groups to the corresponding hydroxyl groups which are more resistant to light and heat (Simek *et al.*, 1997).

Table 5 Molecular weight of chitosan prepared by deacetylation in presence of sodium borohydride at different concentrations.

Sodium borohydride Concentration (M)	Molecular weight ($\times 10^6$ dalton)	Intrinsic viscosity [η]
0	5.19 \pm 0.13a	50.87 \pm 0.69a
0.5	5.21 \pm 0.08b	52.56 \pm 0.54b
1.0	6.52 \pm 0.13c	61.08 \pm 0.97c
2.0	6.72 \pm 0.07d	62.70 \pm 0.50d

Mean \pm SD from triplicate determinations.

Different letters in the same column indicate the significant difference ($p < 0.05$).

From the result, it can be inferred that the oxidation during deacetylation resulted in the decreased molecular weight. Reducing agent such as sodium borohydride played an essential role in prevention of degradation of chitosan polymer during such a harsh condition. Roberts and Domszy (1982) reported that chitosan was treated with sodium borohydride under acidic conditions to reduce any oxidized end groups to aldehyde groups. Molecular weight-induced conformational change was attributed to different spatial distribution forms of the chain molecule unit among larger and smaller molecular weight chitosans and/or may be due to differences in intramolecular hydrogen bonds and/or differences in charge distribution (Chen and Tsaih, 1998).

Prashanth *et al.* (2002) reported that the molecular weight of chitosan prepared in the presence of thiophenol, (123,000 Da) was greater when

compared to those of chitosans prepared by carrying out N-deacetylation in the presence of nitrogen atmosphere, (117,000 Da) and the control (96,000 Da).

2.2 Viscosity

Figure 5 shows viscosity of chitosan prepared with different sodium borohydride concentrations. Viscosities of chitosan prepared in presence of various sodium borohydride concentrations were different ($p < 0.05$). The viscosity of chitosan increased as the concentration of sodium borohydride increased. Chitosan prepared with 2.0 M sodium borohydride exhibited the highest viscosity. Without sodium borohydride, the lowest viscosity was observed.

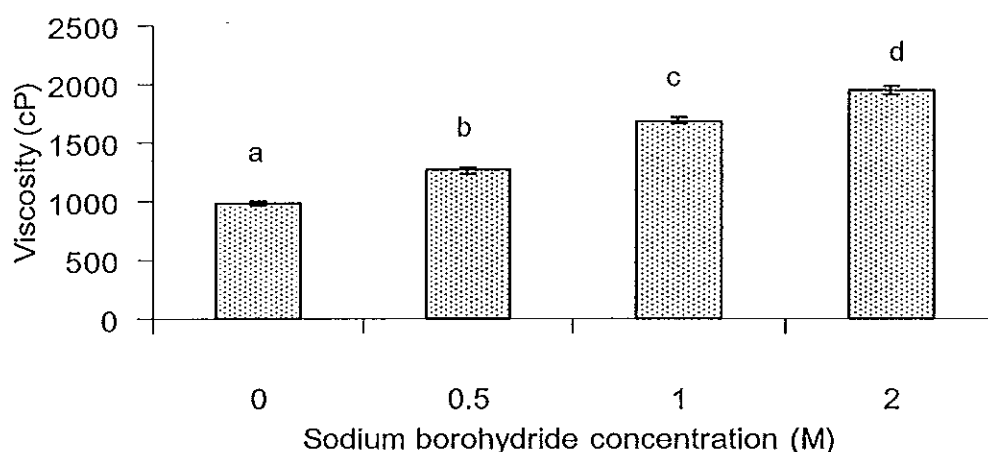


Figure 5 Viscosity of chitosan prepared by deacetylation in presence of sodium borohydride at different concentrations. 1% chitosan was dissolved in 1% acetic acid and viscosity was measured by a Brookfield viscometer at 25°C.

* Different letters indicate the significant difference ($p < 0.05$).

The result suggested that the addition of reducing agent mainly prevented the depolymerization of chitosan molecule. Prashanth *et al.* (2002) found that chitosan prepared with thiophenol had the higher viscosity, when compared to the control. Furda (1983) reported that the degree of polymerization of glucosamine unit is the major factor in viscosity, when the extent of deacetylation is comparable. Jeon *et al.* (2002) found that the viscosity of chitosan was also dependent on molecular weights. Chitosan with the viscosity of 360, 57 and 14 cP had the molecular weight of 1.8×10^6 , 9.6×10^5 and 6.6×10^5 Da, respectively.

2.3 Degree of deacetylation

Colloidal titration method

The effect of sodium borohydride concentrations on degree of deacetylation of chitosan was determined using colloidal titration method is shown in Figure 6. Degree of deacetylation of chitosans prepared with various sodium borohydride concentrations was not significantly different ($p > 0.05$). Degree of deacetylation of chitosan ranged from 82.12 to 83.21%. Therefore, it can be concluded that sodium borohydride had no influence on degree of deacetylation, but mainly on molecular weight (Table 5). In other words, sodium borohydride did not affect the removal of acetyl group from C-2 position of chitin molecule. The degree of deacetylation is defined as the ratio of the number of amino groups in chitosan to the sum of the amino and acetyl groups (Huang *et al.*, 2000).

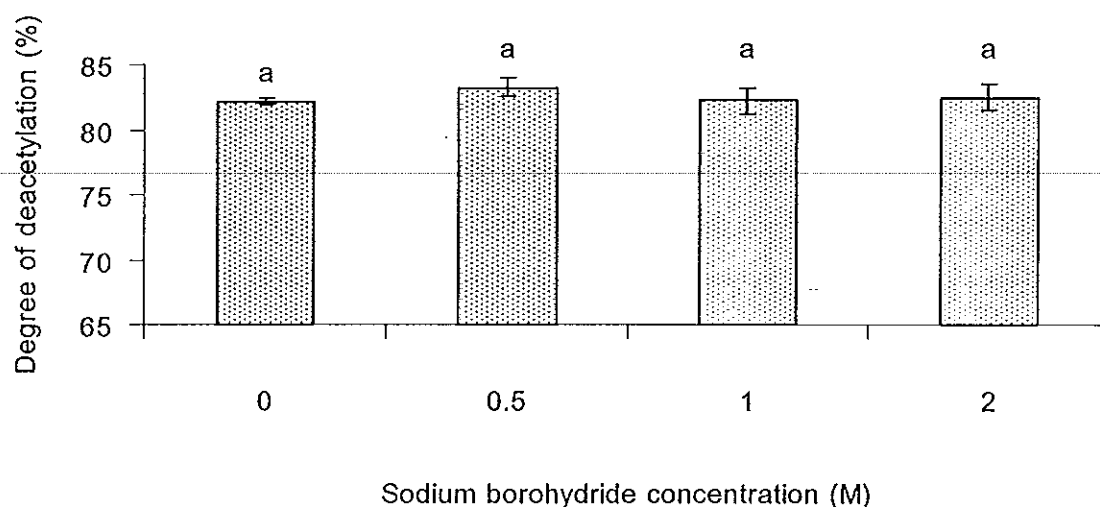


Figure 6 Degree of deacetylation of chitosans prepared by deacetylation in presence of sodium borohydride at different concentrations determined using colloidal titration method.

* Different letters indicate the significant difference ($p < 0.05$).

Brine and Austin (1981) and Rutherford and Austin (1978) reported variations in the acetyl values of chitins with different species and preparation methods. The acetyl value (19.6%) of the crawfish chitin indicates that it has been partially deacetylated to 7.5%, naturally or during preparation, based on the theoretical acetyl value for chitin of 21.2% (No *et al.*, 1989). Benjakul and Wisitwuttikul (1993) reported that the optimal deacetylation attained by intermittently washing the intermediate product in water two times after alkali treatment. However, chitosan products from both carapace and shell of black tiger shrimp (*Penaeus monodon*) showed 31.89 and 43.21 % N – acetylation, respectively.

Infrared spectroscopy

The degree of deacetylation is a distinguishable feature, which can be followed through infrared spectroscopy (Monteiro and Airoidi, 1999). Table 6 shows degree of deacetylation of chitosans prepared with and without sodium borohydride at different concentrations determined using infrared spectroscopy. From the result, no differences in degree of deacetylation between chitosan prepared with and without sodium borohydride during deacetylation process (Table 6). This result was in agreement with that determined by colloidal titration (Figure 6).

The main change in the spectra is related to two distinct regions: from 3300 to 2900 cm^{-1} and from 1700 to 1300 cm^{-1} (appendix 2). The peak found at 3450 cm^{-1} indicates the OH group of the polymer. The peak located at 1655 cm^{-1} (amide I band) can be attributed to the presence of the CO - NH groups (Monteiro and Airoidi, 1999). Two different i.r. bands have been proposed as internal standards depending on the range of acetyl content. The first is best used for degree of acetylation > 10% and the OH band at 3450 cm^{-1} is used as a reference; the acetyl content (%) is defined from the ratio of absorbances: $(A_{1655 \text{ cm}^{-1}} / A_{3450 \text{ cm}^{-1}}) \times 100 / 1.33$ (Domard and Rinaudo, 1983).

Spectrum of chitosan sample with 99% degree of deacetylation had a clear amide I band but the band decreased in intensity when degree of deacetylation decreased (Mima *et al.*, 1983). Duarte *et al.* (2002) reported that chitin and chitosan from different sources with different structures, different isolation and deacetylation processes normally have different intensity of i.r. spectrum. From the result, no differences in infrared spectra between all samples were observed (appendix 2).

Table 6 Degree of deacetylation of chitosans prepared by deacetylation in presence of sodium borohydride at different concentrations determined using infrared spectroscopy.

Sodium borohydride concentrations (M)	Degree of deacetylation (%)
0	82.039 \pm 0.958a
0.5	82.818 \pm 0.257a
1.0	82.470 \pm 1.074a
2.0	80.701 \pm 1.578a

Mean \pm SD from triplicate determinations.

Different letters in the same column indicate the significant difference ($p < 0.05$).

3. Fat adsorption properties of chitosan in powder system

Amount of oil adsorbed in the dry chitosan powder prepared in absence or presence of sodium borohydride at different concentrations is depicted in Figure 7. When oil was percolated through a bed of chitosan powder, the amounts of oil adsorbed in the dry powder varied, depending on the type of oil ($p < 0.05$). Soybean oil and lard were more adsorbed in chitosan than palm oil. No significant differences in oil adsorbed were observed among chitosan prepared with different sodium borohydride concentrations ($p > 0.05$).

From the result, sodium borohydride added during deacetylation had no effect on oil adsorption of chitosan powder. Thus, it was presumed that oil was physically entrapped between chitosan powder

and chemical compositions of chitosan, particularly molecular weight of chitosan molecule, had no influence on oil adsorption.

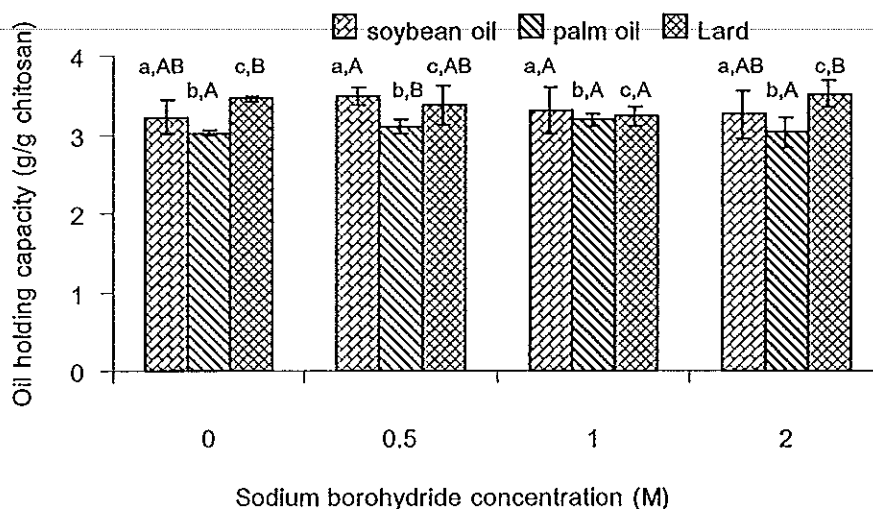


Figure 7 Oil adsorption of chitosan powder prepared by deacetylation in presence of sodium borohydride at different concentrations.

* Different letters in the same oil indicate the significant difference ($p < 0.05$). Different letters (capital) in the same sodium borohydride concentration indicate the significant difference ($p < 0.05$).

When olive oil was percolated through a bed of chitosan, both *N*-lauryl chitosan and *N*-dimethylaminopropyl chitosan, the amounts of oil retained in the dry powder varied, depending on the chemical functions of the polysaccharide (Muzzarelli *et al.*, 2000). When 15 g of oil were percolated at 25°C 5.5 g were retained in the *N*-dimethylaminopropyl chitosan, while 4.0 g were retained in the chitosan or *N*-lauryl chitosan (Muzzarelli *et al.*, 2000). No *et al.* (2000b) reported that fat binding capacity of chitosans also differed considerably with products, ranging from 217 to 403%. Overall, higher fat binding capacity values (314 – 535%) observed in this study was comparable to that reported by Cho *et al.* (1998).

Muzzarelli *et al.* (2000) reported that chitin had a high olive oil holding capacity. The chitin which did not undergo any treatments intended to depress its degree of crystallinity. Therefore, it might be expected that fully amorphous chitins could have higher holding capacity. The fat uptake of chitin, microcrystalline chitin (redispersible chitin), and chitosan ranged from 170 to 215% (Knorr, 1982).

Fatty acid and lipid compositions

The fatty acid compositions of oils including soybean oil, palm oil and lard percolated and adsorbed with chitosan powder are shown in Table 7.

The fatty acid compositions of the oil was not appreciably altered, as a consequence of the contact or absorption with chitosans used. The same fatty acid profile in the percolated and the adsorbed fractions of all types of oil was observed. The results suggested no specific chemical interaction between fatty acids in oil and chitosan powder. Therefore, no differences in fatty acids in both percolated and adsorbed fraction were found, compared to the original oils.

Muzzarelli *et al.* (2000) found that the concentrations of 12 fatty acids retained substantially the same in the percolated and in the retained fractions for all systems tested, in spite of the different amine nitrogen content, moisture content and hydrophobicity of the powders, and regardless of the degree of unsaturation of the fatty acids.

Lipid compositions of oil percolated and adsorbed through chitosan column are shown in Table 8. Lipid compositions of oil percolated and adsorbed were mostly triglyceride. Diglyceride and phospholipid were found as the minor constituents. Triglyceride in percolated and adsorbed

Table 7 Fatty acid compositions of oil percolated and adsorbed in chitosan powder.

	Fatty acid (mg/g oil)											
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0
Soybean oil			62.84		17.60	122.45	359.91	76.47	0.91			1.31
Percolated fraction			63.37		17.53	122.18	355.98	75.92	0.96			1.31
Adsorbed fraction			63.96		17.44	120.92	355.86	74.75	0.91			1.19
Palm oil	7.17	10.44	199.28		17.20	281.51	78.06		0.90			
Percolated fraction	7.28	10.32	203.59		17.75	271.27	79.31		0.96			
Adsorbed fraction	7.11	10.67	205.78		18.24	287.76	76.43		0.99			
Lard		10.27	105.94	12.20	41.56	160.77	105.52	12.24		8.92	9.31	
Percolated fraction		11.23	103.75	12.55	37.50	157.36	99.82	11.05		8.04	8.50	
Adsorbed fraction		10.37	103.85	12.19	38.98	167.94	103.38	11.18		7.90	8.96	

At neutral pHs, amino groups did not have the positive charge, which can form ionic interaction with negative charge of phosphate group in phospholipid or carboxyl group of fatty acid esterified with triglyceride or diglyceride. As a result, no differences in lipid compositions were observed between percolated and adsorbed fractions.

Table 8 Lipid compositions of oil (soybean oil, palm oil, and lard) percolated and adsorbed in chitosan powder.

	Lipid content (%)			
	Triglyceride	Diglyceride	Phospholipid	Free fatty acid
Soy bean oil	98.8	0.40	0.80	ND
Percolated fraction	98.84	0.45	0.71	ND
Adsorbed fraction	98.90	0.39	0.75	ND
Palm oil	97.05	2.15	0.80	ND
Percolated fraction	97.30	2.07	0.64	ND
Adsorbed fraction	97.18	2.12	0.70	ND
Lard	99.03	0.40	0.32	0.25
Percolated fraction	99.07	0.37	0.28	0.28
Adsorbed fraction	99.01	0.40	0.30	0.29

ND : Not detectable

4. Fat adsorption properties of chitosan in emulsion system

Oil adsorption of chitosans in emulsion system is shown in Figure 8. Chitosans prepared in presence of various sodium borohydride concentrations showed significantly different oil adsorption for soybean oil and lard ($p < 0.05$). However, no significant differences in oil adsorption of chitosan prepared in presence of various sodium borohydride concentrations in emulsion system for palm oil were observed ($p > 0.05$).

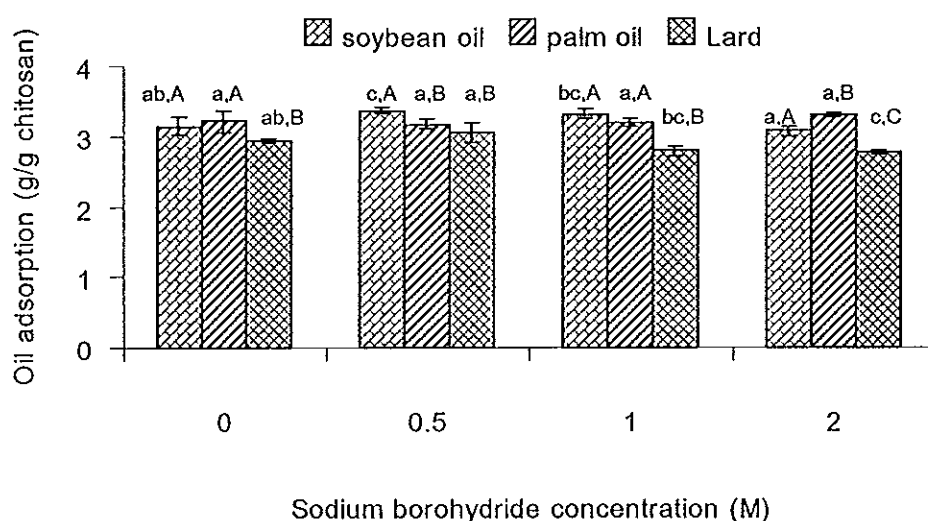


Figure 8 Oil adsorption of chitosan prepared by deacetylation in presence of sodium borohydride at different concentrations in emulsion system.

* Different letters in the same oil indicate the significant difference ($p < 0.05$). Different letters (capital) in the same sodium borohydride concentration indicate the significant difference ($p < 0.05$).

From the result, chitosan prepared with 0.5 M sodium borohydride had significantly higher oil entrapment in the gel ($p < 0.05$), especially for soybean oil. Conversely, less lard was trapped in chitosan gel as the sodium borohydride increased ($p < 0.05$). High viscosity of lard, when

compared with palm oil and soy bean oil, seemed to be an obstacle to the formation of an emulsion in acid solution. This might account for the difference in oil adsorption between the lard, palm oil and soy bean oil.

It was found that chitosan with high molecular weight tended to exhibit the lower oil adsorption capacity. This might be associated with the high viscosity, leading to the difficulty to make the homogenous emulsion and form the uniform gel which could trap oil inside. However, high viscosity may be primarily responsible for the inhibition of cholesterol and fatty acid absorption by chitosan as in the case of guar gum and pectin (Ikeda *et al.* 1993). Furda (1983) reported that the degree of polymerization of the glucosamine unit is the major factor in viscosity, when the extent of deacetylation is comparable. Ikeda *et al.* (1993) reported that a longer-chain chitosan could trap dietary fat more easily as a chitosan-fat composite in the small intestine and then gel the composite. Sugano *et al.* (1988) also found that the apparent fat digestibility tended to decrease in the presence of chitosan (Deuchi *et al.*, 1995).

Fatty acid and lipid compositions of oils in chitosan gel

Fatty acid compositions of soybean oil, palm oil and lard in chitosan gels are shown in Table 9. Fatty acid compositions in oil extracted from chitosan gels were not different, compared with those of the original oils. Therefore, no specific binding of chitosan toward any particular fatty acids in the oil was found. In general, oils were more likely to be trapped in the chitosan gels without any chemical bondings. Deuchi *et al.* (1994) reported that the fatty acid composition of the fecal lipid fairly well reflected that of the dietary fat. Chitosan was found to reduce plasma triacylglycerol, cholesterol and free fatty acid, which were elevated in the high-fat diet-fed control mice (Han *et al.*, 1999). Ritva *et al.* (2002) reported that negatively charged molecules in stomach attach strongly to the positive charged tertiary amino group ($-\text{NH}_3^+$) of chitosan. Therefore, chitosan

Table 9 Fatty acid compositions of oil in chitosan gel

	Fatty acid (mg/g oil)											
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0
Soybean oil			62.84		17.60	122.45	359.91	76.47	0.91			1.31
Oil in chitosan gel			62.23		16.96	119.25	357.01	74.24	0.91			1.26
Palm oil	7.17	10.44	199.28		17.20	271.51	73.06		0.90			
Oil in chitosan gel	7.04	10.49	202.91		18.08	269.58	73.72		0.96			
Lard		10.27	105.94	12.20	41.56	160.77	105.52	12.24		8.92	9.31	
Oil in chitosan gel		10.83	105.19	12.42	39.38	158.09	100.02	11.47		8.45	9.76	

reduces fat absorption from gastrointestinal tract by binding with anionic carboxyl groups of fatty acid and bile acids, and it interferes with emulsification of neutral lipids (i.e. cholesterol, other sterols) by binding them with hydrophobic bonds.

Differences in lipid compositions of oil imbibed in chitosan gel were observed (Table 10). Phospholipid content of all types of oil in chitosan gel increased when compared with that found in original oil. In emulsion system, chitosan possessing the positive charge was postulated to preferably adsorb phospholipid, resulting in the higher phospholipid content in the gel. At pH ~ 5, amino group at C-2 of chitosan chain exhibits the positive charge and effectively bind with phosphate group in phospholipid. In addition to enhancing cholesterol excretion, this ionic bonding resulted in excretion of mixed micelles, along with associated phospholipids, monoglycerides and fatty acids (Furda, 1983; Nauss, 1983).

Table 10 Lipid compositions of oil (soybean oil, palm oil, and lard) in chitosan gel.

	Lipid content (%)			
	Triglyceride	Diglyceride	Phospholipid	Free fatty acid
Soybean oil	98.80	0.40	0.80	ND
Chitosan gel	98.84	0.21	0.95	ND
Palm oil	97.04	2.15	0.80	ND
Chitosan gel	96.34	2.32	1.34	ND
Lard	99.55	0.40	0.32	0.25
Chitosan gel	98.65	0.13	0.49	0.27

ND : Not detectable

5. Effect of chitosan amount on fat adsorption in emulsion system

The effect of different amounts of chitosan prepared in presence of 0.5 M sodium borohydride is shown in Figure 9. When various chitosan amounts were added, it was found that chitosan at a level of 0.5% chitosan showed the highest oil adsorption. When chitosan levels added increased, the oil adsorption of chitosan significantly decreased ($p < 0.05$). The similar results were observed among all oils tested. With a higher level of chitosan, more viscous solution was obtained, leading to the less emulsion formed. This may retard the consistent dispersion of oil droplet in the system. As a result, efficacy of oil entrapment in chitosan gels decreased. The use of a high viscosity chitosan at 5% w/w of diet also reduced the apparent fat digestibility of rats to about 50% (Deuchi *et al.*, 1994).

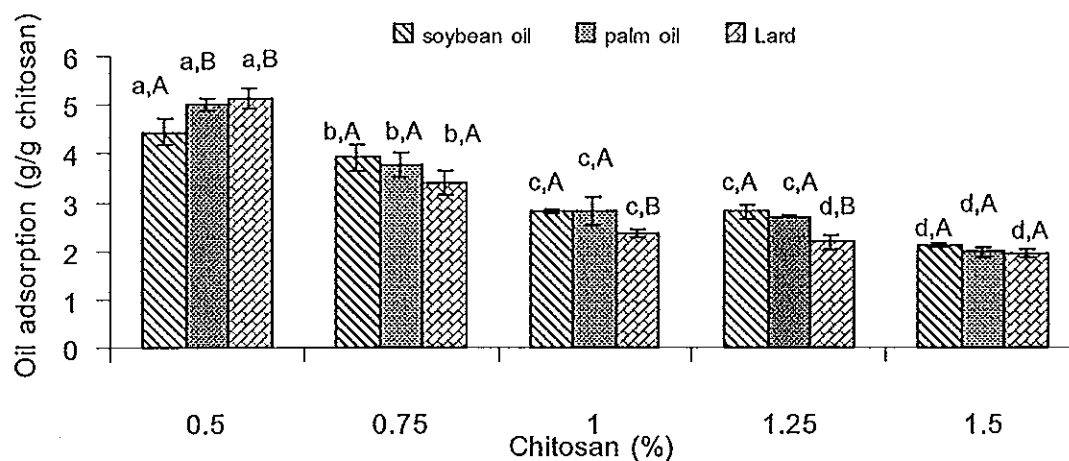


Figure 9 Oil adsorption of chitosan at various concentrations in emulsion system.

* Different letters in the same oil indicate the significant difference ($p < 0.05$).

Different letters (capital) in the same chitosan concentration indicate the significant difference ($p < 0.05$).

Therefore, the appropriate viscosity of chitosan solution to form fat-chitosan emulsion should be an essential factor determining the efficacy of fat entrapment in chitosan gel.

6. Effect of organic acids on fat adsorption of chitosan in-emulsion system

Differences in the oil adsorption of chitosan gel made with organic acids are observed (Table 11). The oil content of chitosan-AsA-HCl gel was higher than that of the chitosan-HCl gel ($p < 0.05$), but that of chitosan-citric-HCl and chitosan-acetic-HCl gels did not differ from that of chitosan-HCl gel for all oils samples tested ($p > 0.05$). Although, acetic acids could dissolve chitosan well in our *in vitro* experiment, it could not enhance the oil adsorption of chitosan. Synergistic effect of ascorbic acid on the oil adsorption was not acid-dependent but a characteristic of ascorbic acid itself (Kanauchi *et al.*, 1994). The increase in oil adsorption was possibly due to the reduced viscosity of chitosan-oil system, resulting in the uniformity of oil dispersed to form the gel. This result was in agreement with Kanauchi *et al.* (1995) found that ascorbic acid caused the viscosity reduction in the stomach. Among all oils tested, lower lard content was adsorbed in the chitosan gels prepared with all organic acids, compared with soybean oil and palm oil.

Kanauchi *et al.* (1994) reported that the apparent fat digestibility in the chitosan-AsA group was significantly lower than that in the chitosan group, although two organic acids (lactic acid and citric acid) did not decrease the apparent fat digestibility, compared to chitosan alone.

Table 11 Oil adsorption of chitosan prepared in presence of 0.5 M sodium borohydride in different organic acids solution in emulsion system.

Acid solution	Oil adsorption (g/g chitosan)		
	Soybean oil	Palm oil	Lard
0.1 M HCl	3.080 ± 0.179aA	3.058 ± 0.138aA	2.520 ± 0.109bA
0.1 M Ascorbic acid	2.530 ± 0.093aB	2.552 ± 0.230aB	1.936 ± 0.072bB
0.1 M Acetic acid	2.415 ± 0.096aB	2.528 ± 0.125aB	1.972 ± 0.143bB
0.1 M Citric acid	2.999 ± 0.185aA	2.873 ± 0.085aA	2.479 ± 0.153bA
0.1 M HCl + 0.1 M Ascorbic acid [*]	3.584 ± 0.053aC	3.462 ± 0.135aC	2.887 ± 0.083bC
0.1 M HCl + 0.1 M Acetic acid [*]	2.919 ± 0.100aA	2.941 ± 0.171aA	2.548 ± 0.249bA
0.1 M HCl + 0.1 M Citric acid [*]	3.032 ± 0.155aA	2.997 ± 0.264abA	2.646 ± 0.058bAC

Mean ± SD from triplicate determinations.

Different letters in the same row indicate the significant difference ($p < 0.05$). Different letters (capital) in the same column indicate the significant difference ($p < 0.05$).

^{*} Acid solutions were mixed at a ratio of 1:1 (v/v).

Deuchi *et al.* (1995) reported that the synergistic effect of AsA on fecal fat excretion seemed to depend on its ability to reduce the viscosity of a chitosan solution in the stomach, which could then easily mix dietary fat with chitosan. Chitosan-AsA-HCl gel entrapped 6.3 g of fat/g of chitosan. This was about 1.1 times higher than that found in HCl-chitosan gel *in vitro* (Kanauchi *et al.*, 1995). Thus, the use of chitosan in combination with ascorbic acid could enhance the fat or oil entrapment, leading to the less availability to be absorbed by the body.

The viscosity of chitosan in different organic acids, and different acid mixtures is shown in Table 12. Highest viscosity was found with sample dissolved in acetic acid. Chitosan in HCl showed the lowest viscosity. When ascorbic acid was added to HCl, the viscosity of chitosan was markedly lowered.

Therefore, the use of ascorbic acid was shown to reduce the viscosity of chitosan, resulting in the improvement of oil dispersion in chitosan gel.

Table 12 Viscosity of 0.5% chitosan in an acidic solution.

Solutions ¹⁾	Viscosity (cP) at 37°C
0.1 M HCl	66.33 ± 1.53 (1.06) ²⁾
0.1 M Ascorbic acid	86.33 ± 0.58 (2.30)
0.1 M Acetic acid	93.00 ± 1.00 (2.46)
0.1 M Citric acid	70.12 ± 1.00 (2.01)
0.1 M HCl + 0.1 M Ascorbic acid [*]	46.67 ± 0.58 (1.30)
0.1 M HCl + 0.1 M Acetic acid [*]	71.33 ± 0.58 (1.30)
0.1 M HCl + 0.1 M Citric acid [*]	60.00 ± 1.53 (1.28)

¹⁾ Chitosan (1.5 g) was dissolved in 300 mL of 0.1 M acidic solution and incubated at 37°C for 3 h before the viscosity was measured.

²⁾ pH of acidic solution

^{*} Acid solutions were mixed at a ratio of 1:1 (v/v).

Kanauchi *et al.* (1994) reported that chitosan was partially dissolved in 200 volumes of 0.1 M citric acid, but 0.1 M ascorbic acid and lactic acid dissolved it completely, resulting in high viscosity. When a 1% chitosan solution in 0.1 M ascorbic acid was diluted with an equal volume of 0.1 M HCl, the viscosity was reduced to less than 50% of its initial value. Sodium ascorbate could not dissolve chitosan, so the viscosity of chitosan in an sodium ascorbate solution could not be determined. When sodium ascorbate was added to HCl, the viscosity of chitosan was markedly lower than that of the HCl solution (Kanauchi *et al.*, 1995). Thus, the addition of ascorbic acid at a proper level could reduce viscosity of chitosan, resulting in the increased emulsification. As a result, more oil was more trapped in the chitosan gel.

7. Antioxidative activity of chitosan

7.1 Chelating activity of chitosan powder on Fe^{2+} ion.

Fe^{2+} binding activity of chitosan powder, prepared in presence of various sodium borohydride concentrations, at various amounts is shown in Figure 10. The absorbance of reaction mixture decreased with increasing amount of chitosan powders. This was caused by the complex between Fe^{2+} and chitosan powder. Thus, Fe^{2+} binding activity of the chitosan powder was increased with increasing amount of the chitosan powder. Similar result was observed with all samples tested. At a level of 5 mg, chitosan prepared with 2.0 M sodium borohydride showed the highest chelating activity. However, at a level of 15 mg, chitosan prepared with 0.5 M sodium borohydride rendered the highest chelating activity. From the result, chitosan prepared without sodium borohydride showed the lower chelating activity, compared to other samples. From the result, chitosan exhibited Fe-chelating activity in a concentration dependent manner. Nieto *et al.* (1992) reported that chitosan-Fe (III) complex was prepared by stirring chitosan powder overnight in 1.5 M ferric chloride at 30°C. Fe (III) is adsorbed from its dilute solutions by chitosan (Muzzarelli *et al.*, 1973) and formation of a liquid gel has been reported when interacting chitosan with concentrated solutions of ferric chloride (Muzzarelli, 1977). The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxide to reactive free radicals via Fenton type reactions (Miller *et al.*, 1990).

Fe^{3+} ions also produce radicals from peroxide, although the rate is ~ 10 - fold less than that of Fe^{2+} ions (Miller, 1996). Fe^{2+} ion is the most powerful prooxidant among various species of metal ions (Jadhav *et al.*, 1996). Yen *et al.* (1999) found that rosemary extracts exhibited stronger inhibitory effects in the Fe^{3+} -induced peroxidation system than in the Fe^{2+} -induced peroxidation system.

The result revealed that chitosan powder acted as Fe^{2+} -chelator. The chelating activity of chitosan powder on Fe^{2+} ion might play an important role in preventing lipid oxidation induced by Fe^{2+} .

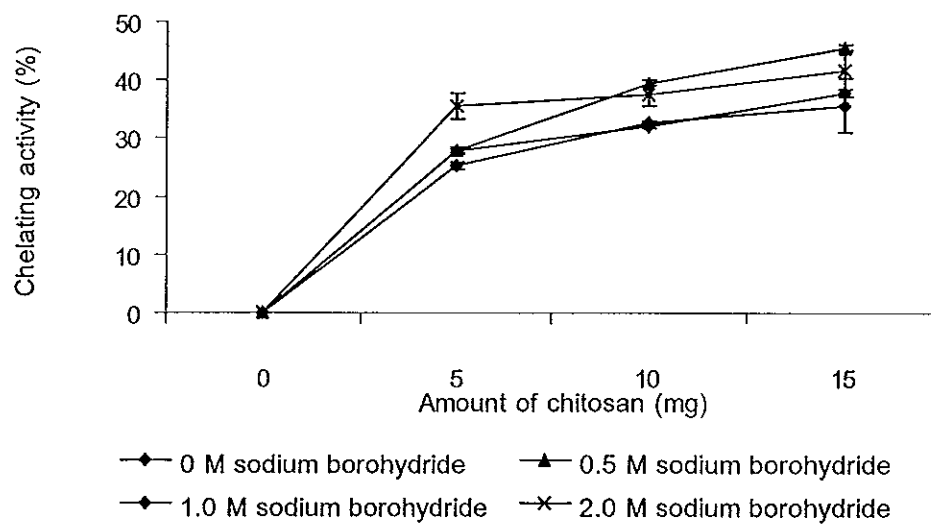


Figure 10 Chelating activity of different amounts of chitosan powder on Fe^{2+} ion

7.2 Hydroxyl radical (OH^\bullet) scavenging activity of chitosan powder

Hydroxyl radical scavenging activity of chitosan with different molecular weights at different concentrations is presented in Table 13. Chitosan powder exhibited hydroxyl radical scavenging activity in a concentration-dependent manner up to a concentration of 30 mg. Xie *et al.* (2001) and Xue *et al.* (1998) showed that the scavenging effect of chitosan on hydroxyl radicals inhibits lipid peroxidation of phosphatidylcholine and linoleate liposomes *in vitro*. Xie *et al.* (2001) reported that the scavenging activities of chitosan derivatives against OH^\bullet may be derived from some or all of the following : (a) The hydroxyl groups in the polysaccharide unit can react with OH^\bullet by the typical H-abstraction reaction. (b) OH^\bullet can react with the residual free amino groups NH_2 to form stable macromolecule radicals. (c) The NH_2 groups can form ammonium groups (NH_3^+) by absorbing H^+ from the solution, then reacting with OH^\bullet through addition reaction. Shon *et al.* (2002) found that chitosan oligosaccharide exhibited *in vitro* superoxide and hydroxyl radical scavenging activities. The results demonstrated that chitosan powder inhibited deoxyribose degradation. Hydroxyl radicals are known to be responsible for the breakdown of deoxyribose (Ueda *et al.*, 1996). Therefore, it indicated that chitosan powder had hydroxyl radical scavenging activity.

Table 13 Scavenging effects of chitosan with different molecular weights at different concentrations on the hydroxyl radical in the deoxyribose assay.

Na-borohydride (M)	Amount of chitosan powder (mg)	Absorbance at 523 nm	Hydroxyl radical-scavenging activity(%)
0	0	0.566 ± 0.006a	0.000a
	5	0.488 ± 0.006b	13.722 ± 1.136b
	10	0.446 ± 0.001c	21.260 ± 0.102c
	15	0.439 ± 0.007c	22.438 ± 1.237c
	20	0.426 ± 0.005d	24.097 ± 0.907d
	30	0.405 ± 0.002e	28.504 ± 0.408e
0.5	0	0.566 ± 0.006a	0.000a
	5	0.451 ± 0.009b	20.377 ± 1.632b
	10	0.425 ± 0.004c	24.853 ± 0.714c
	15	0.406 ± 0.003d	28.327 ± 0.510d
	20	0.393 ± 0.002e	30.506 ± 0.368e
	30	0.385 ± 0.002e	31.920 ± 0.270e
1.0	0	0.566 ± 0.006a	0.000a
	5	0.458 ± 0.010b	19.140 ± 1.770b
	10	0.426 ± 0.004c	24.735 ± 0.707c
	15	0.417 ± 0.003c	26.325 ± 0.467c
	20	0.406 ± 0.005d	28.269 ± 0.935d
	30	0.394 ± 0.003e	30.448 ± 0.445e
2.0	0	0.566 ± 0.006a	0.000a
	5	0.466 ± 0.003b	17.727 ± 0.445b
	10	0.436 ± 0.012c	22.968 ± 2.149c
	15	0.414 ± 0.003d	26.796 ± 0.540d
	20	0.403 ± 0.006d	28.740 ± 1.065d
	30	0.391 ± 0.005e	30.919 ± 0.916e

Mean ± standard deviation from triplicate determination.

Different letters in the same column under the same sodium borohydride concentration indicate significant difference ($p < 0.05$).

8. Antioxidant activity of chitosan in lecithin liposome system.

The antioxidant activity of chitosan powder were estimated by determining 2- thiobarbituric acid and conjugated diene in lecithin liposomes oxidized at 37°C in the presence of cupric acetate. The chitosans with different molecular weights were active antioxidant in lecithin liposomes (Figure 11). Lipid oxidation in the control (without chitosan) was much higher than system containing chitosan powder. After 1 day of incubation, significant differences in TBARS between samples added with the chitosan powder and the control were observed ($p < 0.05$). At day 2 and day 3, differences in TBARS between samples added with chitosan powder at level of 50 and 200 ppm were observed. Samples added with 200 ppm showed lower TBARS than those with 50 ppm. This suggested that antioxidation activity of chitosan was dependent on concentrations used.

The formation of conjugated diene in the liposome system increased as the incubation time increased (Figure 12). At the same level of chitosan, no differences in conjugated diene values of samples added with the chitosan powder of different molecular weight were observed. However, it was noted that the control had the highest conjugated diene, compared to the samples added with chitosan throughout the storage.

Transition metal ions, e.g. Fe, Cu, Co, which possess two or more valence states with a suitable oxidation-reduction potential affect both the speed of autooxidation and the direction of hydroperoxide breakdown to volatile compounds (Gordon, 2001). The capacity of chitosan for binding metal ions such as those of copper, chromium, zinc, lead and iron has been demonstrated (Winterowd and Sandford, 1995). Schmuhl *et al.* (2001) reported that chitosan forms chelates with copper ions by releasing hydrogen ions. From the result, chitosan might function as the copper chelator as well as radical scavenger in the lecithin liposome system as evidenced by the lower lipid oxidation.

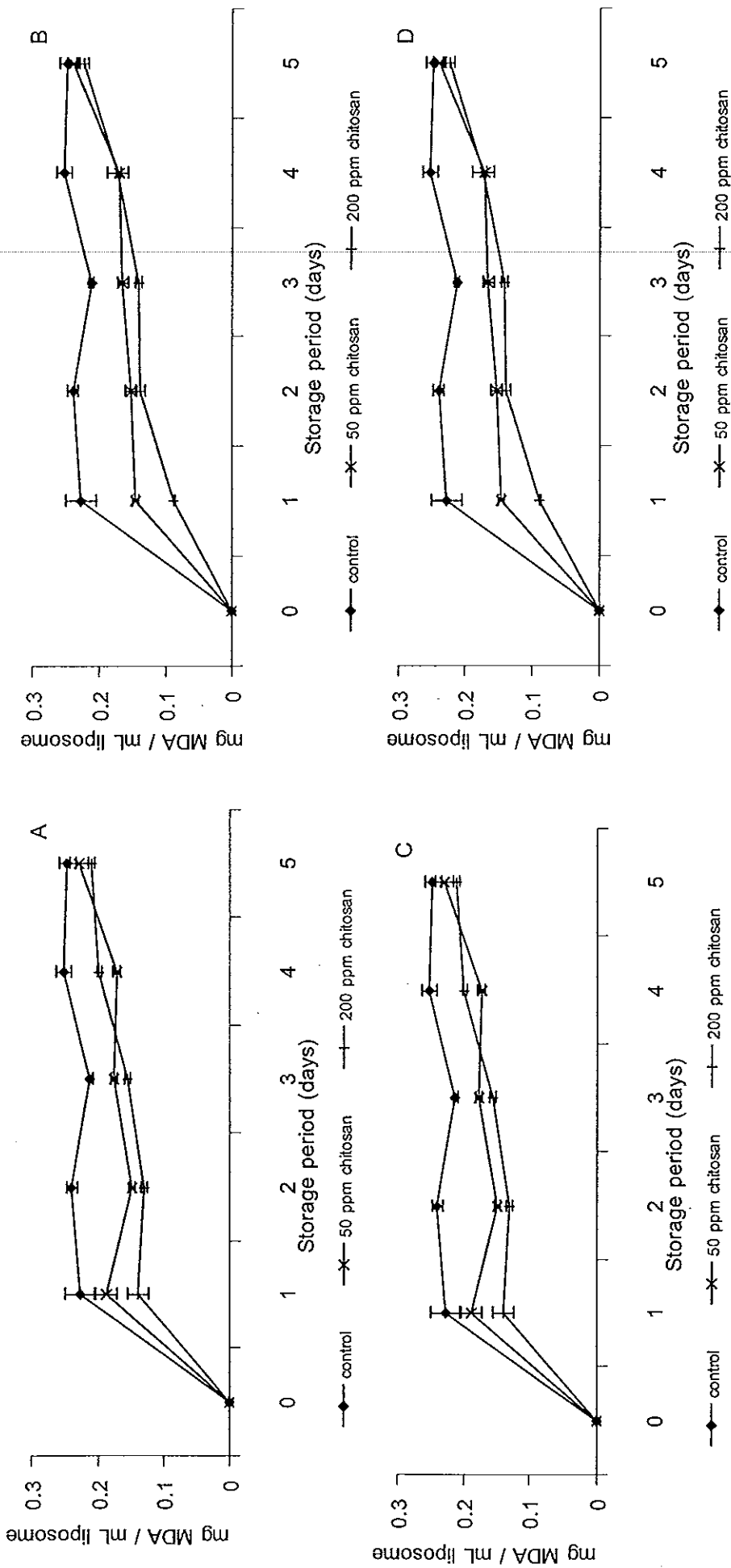


Figure 11 Effect of chitosan with different molecular weights at different concentrations on TBARS formation in lecithin liposome system. (A : 0 sodium borohydride ; B : 0.5 M sodium borohydride ; C : 1.0 M sodium borohydride, and D : 2.0 M sodium borohydride)

* Bars indicate standard deviation from triplicate determinations.

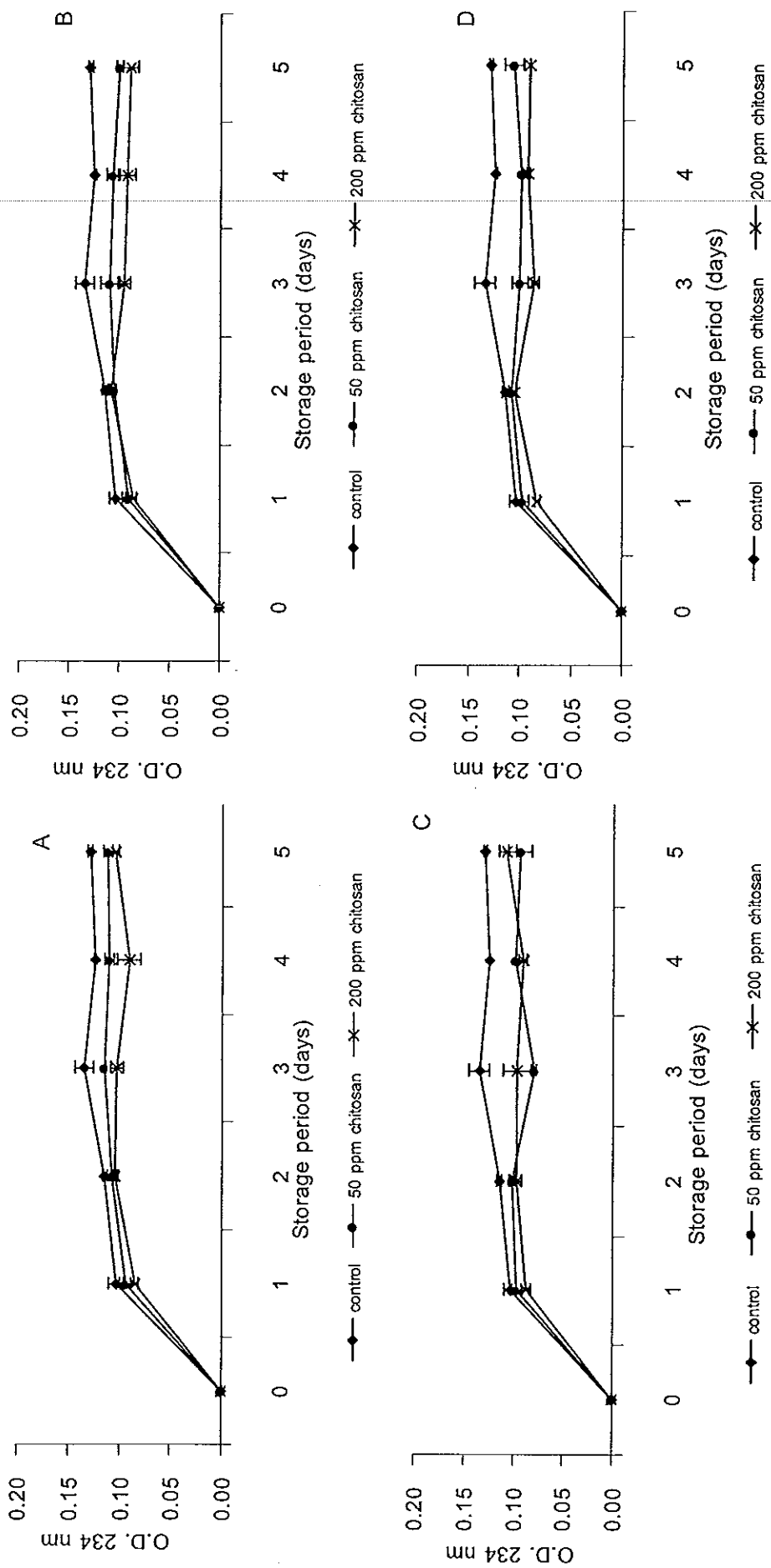


Figure 12 Effect of chitosan with different molecular weights at different concentrations on conjugated diene in lecithin liposome system. (A : 0 M sodium borohydride ; B : 0.5 M sodium borohydride ; C: 1.0 M sodium borohydride, and D : 2.0 M sodium borohydride)

* Bars indicate standard deviation from triplicate determinations.

9. Application of chitosan powder in cooked ground pork.

9.1 Peroxide values

The peroxide values of cooked ground pork added with chitosans with different molecular weights at levels of 50, 100 and 200 ppm are presented in Figure 13. The control (without chitosan) had an increased peroxide value up to 9 days of storage. Subsequently, no marked changes in peroxide value were observed.

As shown in Figure 13, lower peroxide values were observed in all samples added with chitosan, compared to the control throughout the storage. Lower peroxide values were evident for cooked ground pork samples containing 200 ppm chitosan, compared to samples added with lower amount of chitosan. At the same level, no differences in peroxide value of cooked pork samples containing different molecular weights chitosan were observed ($p>0.05$).

After 12 days of storage, peroxide values of pork samples added with the chitosan at the level of 200 ppm were 22.38-31.66% lower than that of the control. Kamil *et al.* (2002) reported that herring samples treated with the 14 cP chitosan, regardless of the concentration used, had lower peroxide values than those of 57 and 360 cP chitosans. Peroxide values of herring and cod samples added with chitosan were ~ 48 – 63% lower than that of the control (Jeon *et al.*, 2002).

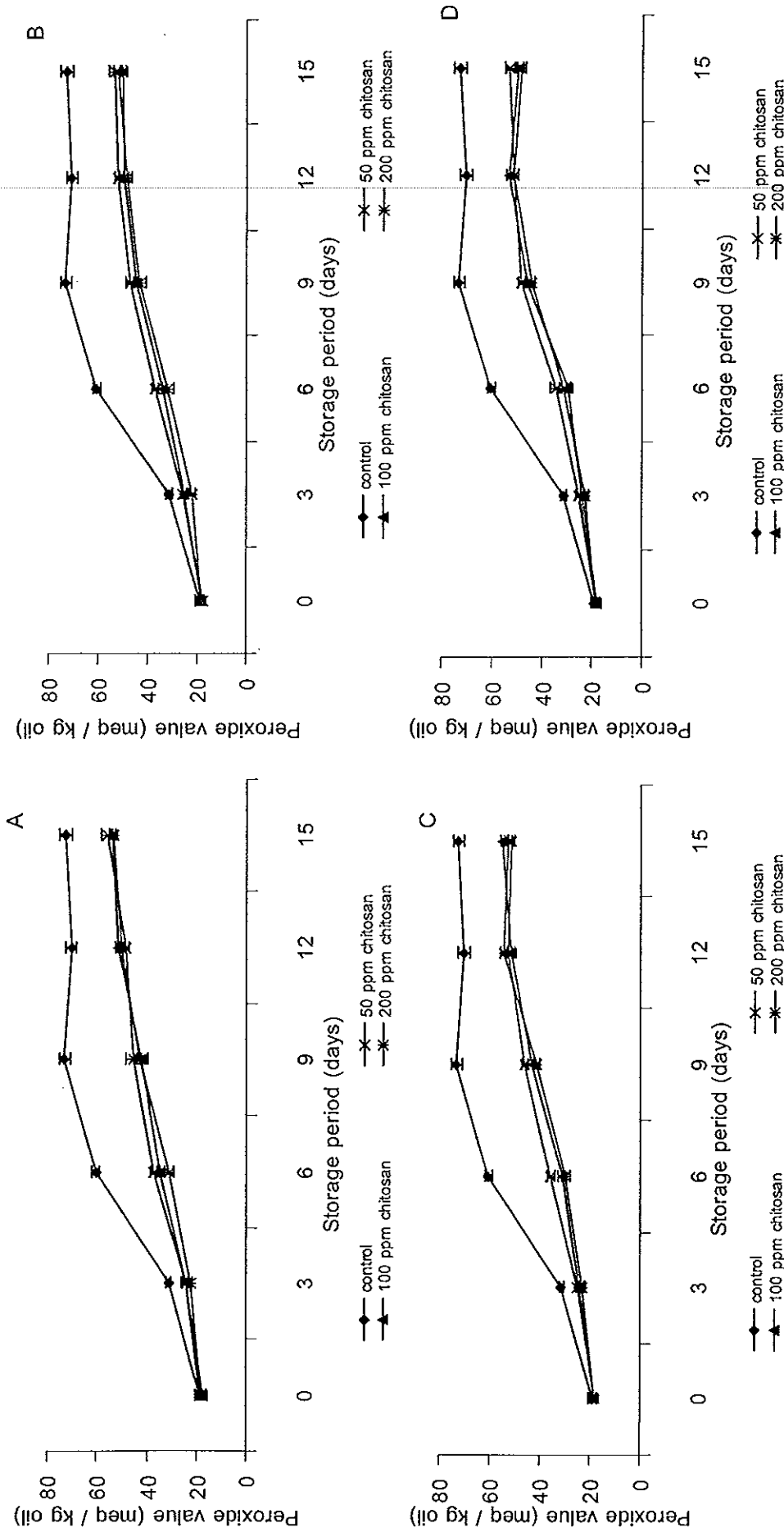


Figure 13 Effect of chitosan with different molecular weights at different concentrations on peroxide value in a cooked comminuted pork model system. (A : 0 sodium borohydride ; B : 0.5 M sodium borohydride ; C: 1.0 M sodium borohydride, and D : 2.0 M sodium borohydride)

* Bars indicate standard deviation from triplicate determinations.

9.2 Conjugated diene values

Conjugated diene values of cooked ground pork added with chitosans, prepared by deacetylation in presence of sodium borohydride at different concentrations were also monitored during extended storage as shown in Figure 14 . The changes in conjugated diene values for cooked ground pork were similar to that of TBARS values of the corresponding samples. Generally, conjugated diene value of ground pork added with the chitosan prepared without sodium borohydried at level of 200 ppm increased by ~ 1.8 fold at the end of a 15-day storage period, whereas the increase in conjugated diene for the control samples was 2.8 fold. After 15 days of storage, conjugated diene of chitosan (without sodium borohydride) at 200 ppm were 45.17% lower than that of the of the control. The corresponding values of samples added with chitosan prepared by deacetylation in presence of sodium borohydride at concentrations 0.5, 1.0 and 2.0 M, at 200 ppm were 44.35%, 43.61% and 42.30% lower than that of the control, respectively.

Sklan *et al.* (1983) determined the content of conjugated dienes, trienes, and tetraenes, referred to as total conjugated products of oxidation, in total lipid extracts of turkey meat during a 60-day storage at 4°C. Level of both dienes and trienes increased with increasing storage time. Medina *et al.* (1999) reported that conjugated diene values in cooked tuna stored at both 40 and 60°C were increased continuously during 4 days of storage. Jeon *et al.* (2002) reported that herring and cod samples treated with 14 cP chitosan showed higher conjugated diene values than those treated with 57 and 360 cP chitosans under similar experimental condition. Conjugated diene among reduced-fat Chinese-style sausage containing 0.1% chitosan with three different molecular weights were not different (Lin and Chao, 2001).

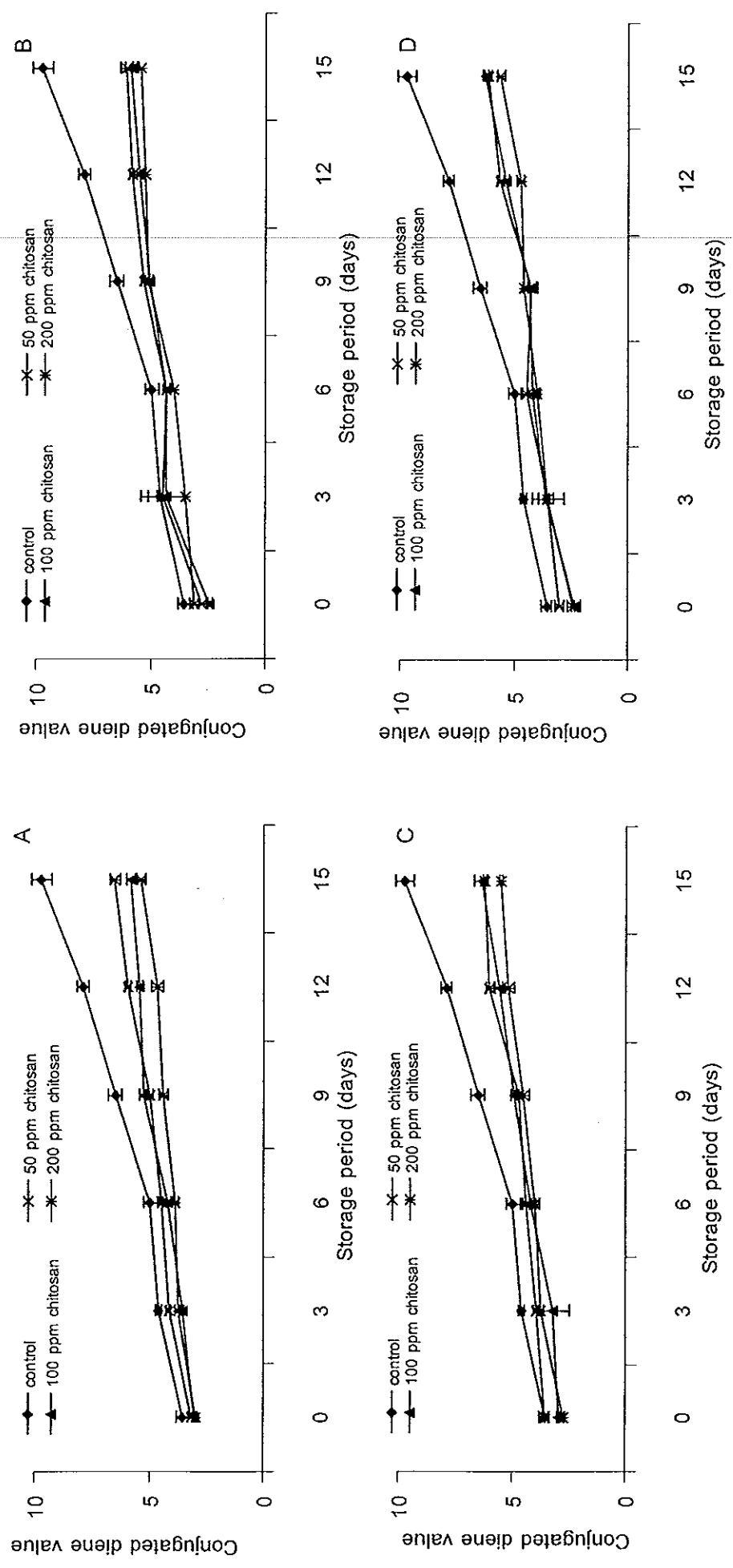


Figure 14 Effect of chitosan with different molecular weights at different concentrations on conjugated diene in a cooked comminuted pork model system. (A : 0 sodium borohydride ; B : 0.5 M sodium borohydride ; C: 1.0 M sodium borohydride, and D : 2.0 M sodium borohydride)

* Bars indicate standard deviation from triplicate determinations.

9.3 TBARS values

The rate of lipid oxidation in cooked ground pork was reduced by addition of chitosan, as indicated by lower TBARS formation in cooked pork added with chitosan during storage at 4°C (Figure 15). Generally, TBARS values of samples added with chitosans increased over the entire storage period, but at slower rates than the control sample. The differences in TBARS values of samples containing different molecular weight chitosans were not significant over the entire storage period ($p>0.05$).

Addition of chitosans with all molecular weights tested at 50, 100 and 200 ppm levels resulted in lower TBARS values, compared to the control (without chitosan). The inhibition of oxidation was dependent on concentrations used. The higher chitosan level, the more inhibitory activity on lipid oxidation was observed.

Effectiveness of chitosan treatment on oxidative stability of beef was studied by Darmadji and Izumimoto (1994). The addition of chitosan at a level of 1% resulted in a decrease of 70% in the 2-thiobarbituric acid (TBA) values of meat after 3 days of storage at 4°C. After 6 days of storage, the TBARS values of herring samples containing 14 cP chitosan were approximately 49-56% lower than that of the control (Kamil *et al.*, 2002). Jeon *et al.* (2002) reported that the content of TBARS in chitosan-coated herring and cod samples were lower than those of the uncoated samples throughout the storage period. Lee and Toledo (1977) also found that cooking significantly increased the TBA values of minced mullet (*Mugil spp.*) during refrigerated storage. N-carboxymethylchitosan (NCMC) could prevent warmed-over flavour (WOF) in uncured meat with 93 and 99% inhibition of TBARS and hexanal content in ground beef, respectively (St. Angelo and Vercellotti, 1989). Similarly, Shahidi (1995) reported that NCMC and its derivatives were effective in controlling the oxidation and flavour deterioration of pork during storage for nine days at refrigerated temperatures.

Xue *et al.* (1998) reported that the liposoluble marine polysaccharides, hexanoyl chitin and *N*- benzoylhexanoyl chitosan solutions, retarded the accumulation of hydroperoxides from methyl linoleate by effectively trapping peroxy radicals in organic solvent when the radical chain reaction had been initiated by 2,2'-azobis (2,4-dimethylvaleronitrile). Moreover, Xue *et al.* (1998) reported that water-soluble chitosans chelate metals or combine with lipids.

Chitosans with different molecular weights did not show different antioxidant effect in cooked ground pork. From the result, the use of chitosan from black tiger prawn could retard the lipid oxidation in cooked ground pork effectively. Thus, chitosan can be used as an alternative natural antioxidant to prolong the shelf life of lipid containing foods.

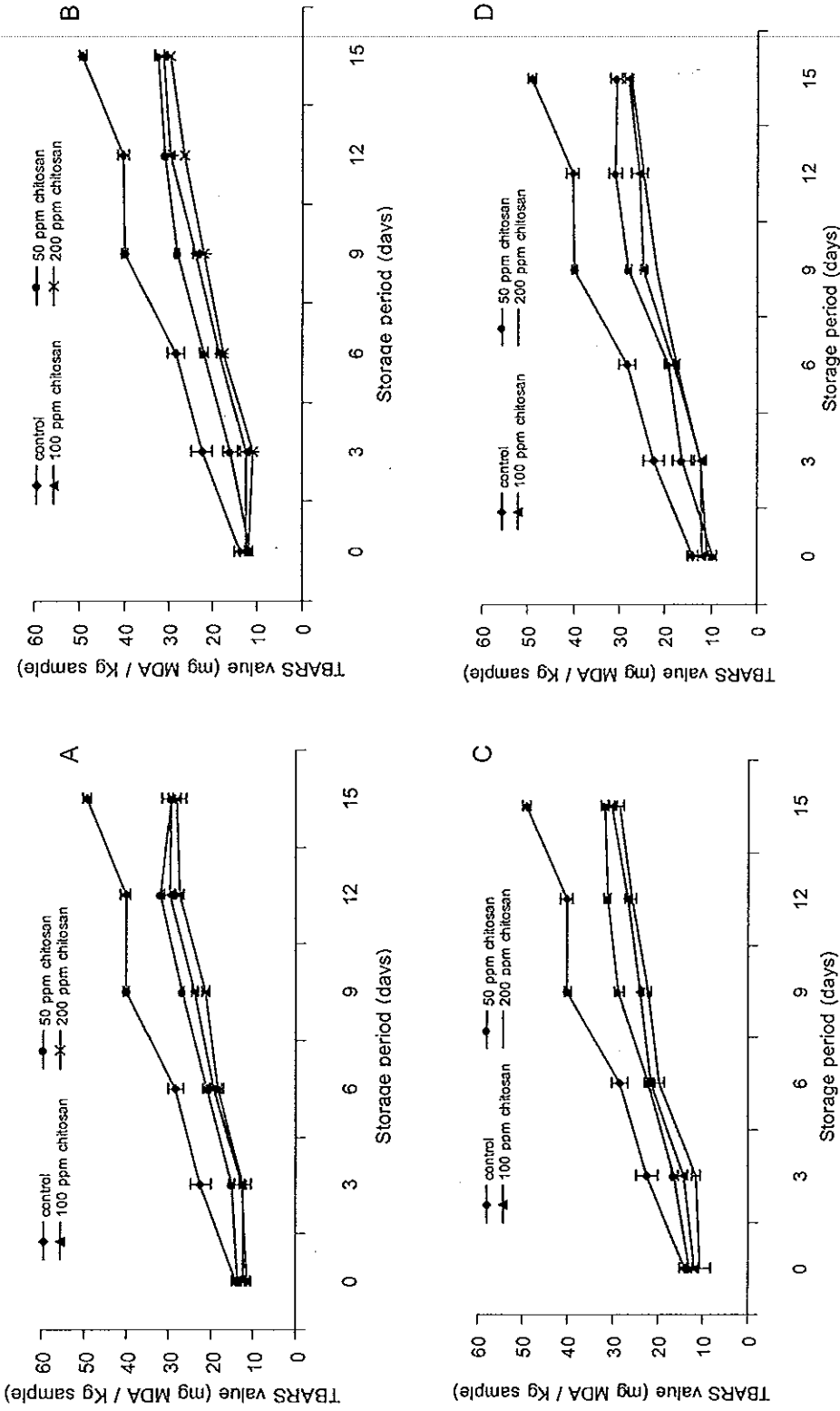


Figure 15 Effect of chitosan with different molecular weights at different concentrations on TBARS formation in a cooked comminuted pork model system. (A : 0 sodium borohydride ; B : 0.5 M sodium borohydride ; C: 1.0 M sodium borohydride, and D : 2.0 M sodium borohydride)

* Bars indicate standard deviation from triplicate determinations.

9.4 Non-heme iron content

Effects of different molecular weights and amount of chitosan powder on non-heme iron content of cooked comminuted pork are shown in Figure 16. The non-heme iron content of all samples treated with chitosan were significantly lower than that of the control ($p < 0.05$). No significant differences in non-heme iron content of cooked ground pork added with chitosan with different molecular weight at same concentration were observed. After 15 days of storage, non-heme iron in cooked pork treated with 200 ppm level was significantly lower than that found in sample added with chitosan at 50 ppm level with all molecular weights tested ($p < 0.05$).

The cooking process disrupts the muscle membrane system, thereby exposing the lipid components to oxygen and/or other reaction catalysts such as iron (Love and Pearson, 1976). In cooked meats, there has also been much debate about the relative prooxidant roles of heme and nonheme iron in lipid oxidation (Monahan *et al.*, 1993). Igene *et al.* (1979) found that iron was released from heme pigments during cooking and proposed that the resultant increase in nonheme iron was responsible for the rapid oxidation of stored cooked meats. Furthermore, the increase in non-heme iron content of muscle was probably due to the degradation of insoluble hemoprotein or other iron-containing proteins, resulting in the release of non-heme iron (Benjakul and Bauer, 2001). In addition, the oxidation intermediates or products, particular superoxide anion, can induce the release of iron from some compounds (Benjakul and Bauer, 2001).

Chitosan may retard lipid oxidation by chelating ferrous ions present in the system, thus eliminating their prooxidant activity. Furthermore, amino groups in chitosan may participate in the chelation of metal ions (Peng *et al.*, 1998 ; Winterowd and Sandford, 1995). Chitosan was shown to have antioxidant activity in cooked ground beef to inhibit warmed-over flavour (WOF) when mixed with the meat samples (St. angelo *et al.*, 1988).

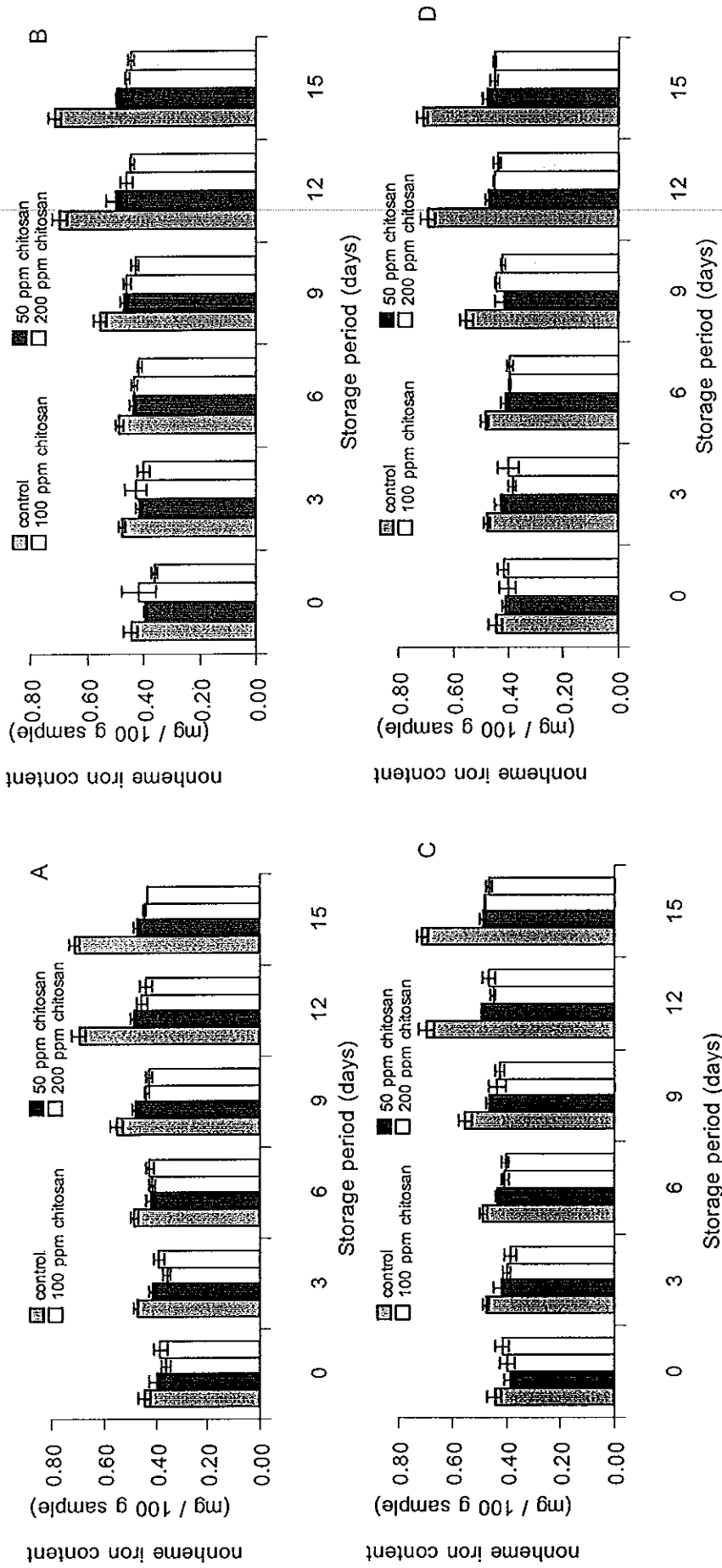


Figure 16 Effect of chitosan with different molecular weights at different concentrations on nonheme iron content in a cooked comminuted pork model system. (A : 0 sodium borohydride ; B : 0.5 M sodium borohydride ; C: 1.0 M sodium borohydride, and D : 2.0 M sodium borohydride)

* Bars indicate standard deviation from triplicate determinations.

Chapter 4

CONCLUSION

1. The addition of sodium borohydride during deacetylation process increased molecular weight of chitosan, suggesting its capacity to reduce depolymerization of chitosan.
2. In chitosan powder system, no differences in oil adsorption capacity were observed among chitosan with different molecular weight. In emulsion system, chitosan prepared by deacetylation in presence of 0.5 M sodium borohydride showed the highest oil adsorption capacity.
3. Ascorbic acid was shown to function as synergist on oil adsorption capacity of chitosan via decreasing the viscosity of chitosan. Antioxidant activity of chitosan powder increased with an increasing concentration of chitosan powder.
4. Chitosan powder possessed scavenging activity toward hydroxyl radical in a concentration-dependent manner.
5. Chitosan powder acted as Fe^{2+} chelator in a concentration-dependent manner.
6. Chitosan powder retarded the increase in peroxide, conjugated diene, nonheme and TBARS value in cooked ground pork during storage at 4°C for 15 days. No differences in antioxidant activity of chitosan powder with different molecular weights were observed.

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Appendix 1
ANALYTICAL METHODS

1. Determination of peroxide value (IUPAC, 1979).

Chemicals

- Acetic acid : chloroform (3:2, v/v)
- Saturated potassium iodide solution
- 0.01 N Sodium thiosulphate solution
- 1% Starch solution

Method

Sample (≈ 1 g) was mixed with a mixture of acetic acid and chloroform (75 mL), follow by addition of 1.0 mL of potassium iodine. The reaction mixture was left to stand for 5 min in the dark. Distilled water (75 mL) was added to the mixture. The mixture was titrated with sodium thiosulfate solution and shaken vigorously until color of mixture was light yellow, then 0.5 mL of starch solution was added. The mixture had blue color. Sodium thiosulphate was used to tritrate until clear solution was obtained. Peroxide value was expressed as meq / kg oil using:

$$\text{Peroxide value (meq/kg oil)} = \frac{(a-b) \times N \times 1000}{W}$$

Where

- a = Volume (mL) of sodium thiosulphate of blank
- b = Volume (mL) of sodium thiosulphate of sample
- N = Concentration of sodium thiosulphate (Normal)
- W = Weight of sample

2. Triobarbituric acid-reactive substance (TBARS) (Buege and Aust, 1978).

Chemicals

- TBARS solution : 0.375 g of triobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of hydrochloric were mixed thoroughly in 100 mL of distilled water.

Method

Ground sample (0.5 g) was dispersed in 2.5 mL of TBA solution. The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 x g for 20 min and the absorbance was measured at 532 nm. A standard curve was prepared with malonaldehyde bis (dimethyl acetal) (MDA) at concentration ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malondialdehyde / kg sample.

3. Measurement of conjugated diene (Frankel and Huang, 1996)

Chemicals

- Iso-octane

Method

Sample (0.1 g) was dissolved in 5.0 mL of iso – octane and the absorbance was measured at 234 nm. Conjugated diene was calculated as an increase in absorbance per 0.2 mg oil.

4. Determination of non-heme iron content (Schricker et al., 1982).

Chemicals

- 0.39% (w/v) Sodium nitrite
- A mixture of 40% trichloroacetic acid and 6 N HCl (ratio of 1:1 [v/v], prepared freshly)
- Non-heme iron color reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of (1) bathophenanthroline (0.162 g, dissolved in 100 mL of double deionized water with 2 mL thioglycolic acid [96-99%]); (2) double-deionized water; and (3) saturated sodium acetate solution.

Method

The chopped sample (1.0 g) was weighed into a screw cap test tube and 50 μL of 0.39% (w/v) sodium nitrite was added. A mixture (4 mL) of 40% trichloroacetic acid and 6 N HCl (ratio of 1:1 [v/v]), prepared freshly) was added. The tightly capped tubes were placed in an incubator shaker at 65°C for 22 h and then cooled down at ambient temperature for 2 h. The supernatant (400 μL) was mixed with 2 mL of the non-heme iron color reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 450 nm. The non-heme iron content was calculated from iron standard curve. The iron standard solution, ranging from 0 to 2 ppm (400 μL) was mixed with 2 mL of the non-heme iron color reagent. The concentration of non-heme iron was expressed as mg/100 mg sample.

5. Determination of degree of deacetylation (Chen and Hwa, 1996)

Chemical

- 5% (v/v) acetic acid
- toluidine blue (indicator)
- n/400 PVSK (potassium polyvinylsulfate)

Method

0.5 g of chitosan was dissolved in 100 mL 5% (v/v) acetic acid. 1.00 g of chitosan/ acetic acid solution was mixed with 30 mL distilled water. After adding 2 to 3 drops of 0.1% toluidine blue (indicator), the solution was titrated with n/400 PVSK (potassium polyvinylsulfate). The degree of deacetylation was calculated as follow :

$$DD(\%) = [X/161 / (X/161 + Y/203)] \times 100$$

$$X = 1/400 \times 1/1000 \times f \times 161 \times V$$

$$Y = 0.5 \times 1/100 - X$$

Where V : Titrated volume (mL) of n/400 PVSK; f : Factor of n/400 PVSK solution.

6. Determination of scavenging effect on hydroxyl radical (Aruoma, 1994)

Chemical

- 33.6 mM deoxyribose
- 17.28 mM H₂O₂
- 1.2 mM ascorbic acid
- KH₂PO₄-KOH buffer pH 7.4
- Stock solution [1% (w/v) TBA in 50 mM sodium hydroxyl plus 2.8% (w/v) TCA]

Method

The reaction mixture were 17.28 mM H₂O₂, 33.6 mM deoxyribose, 1.2 mM ascorbic acid, and KH₂PO₄-KOH buffer pH 7.4 of final phosphate concentration 120 mM. To perform the deoxyribose assay 0.1 mL deoxyribose, 0.7 mL water, 0.1 mL H₂O₂, 0.1 mL iron salt (where indicated), 0.1 mL of buffer and 0.1 mL ascorbate were mixed with chitosan powder. The mixture was incubated for 10 min at 37°C. One mL stock solution was added to the reaction mixture. It was then heated for 10 min in the boiling water bath, cooled with tap water, and the absorbance of the pink chromogen was read at 532 nm.

7. Determination of molecular weight (Huang *et al.*, 2000)

Chemical

- 1% (w/w) acetic acid

Method

The molecular weight (M) of polymers was measured by the viscometer based the well-known Mark-Houwink equation $[\eta] = KM^a$. Molecular weight of chitosans were determined by measuring the relative viscosity of chitosan at various concentrations in 1% acetic acid (η_{rel}). The specific viscosity (η_{sp}) could be calculated from the following equation: $\eta_{sp} = \eta_{rel} - 1$. The intrinsic viscosity $[\eta]$ was then determined from the intercept at $C = 0$ when η_{sp}/C were plotted against C (concentrations). To calculate the approx. molecular weight (M) of the polymers, the Mark-Houwink Equation:

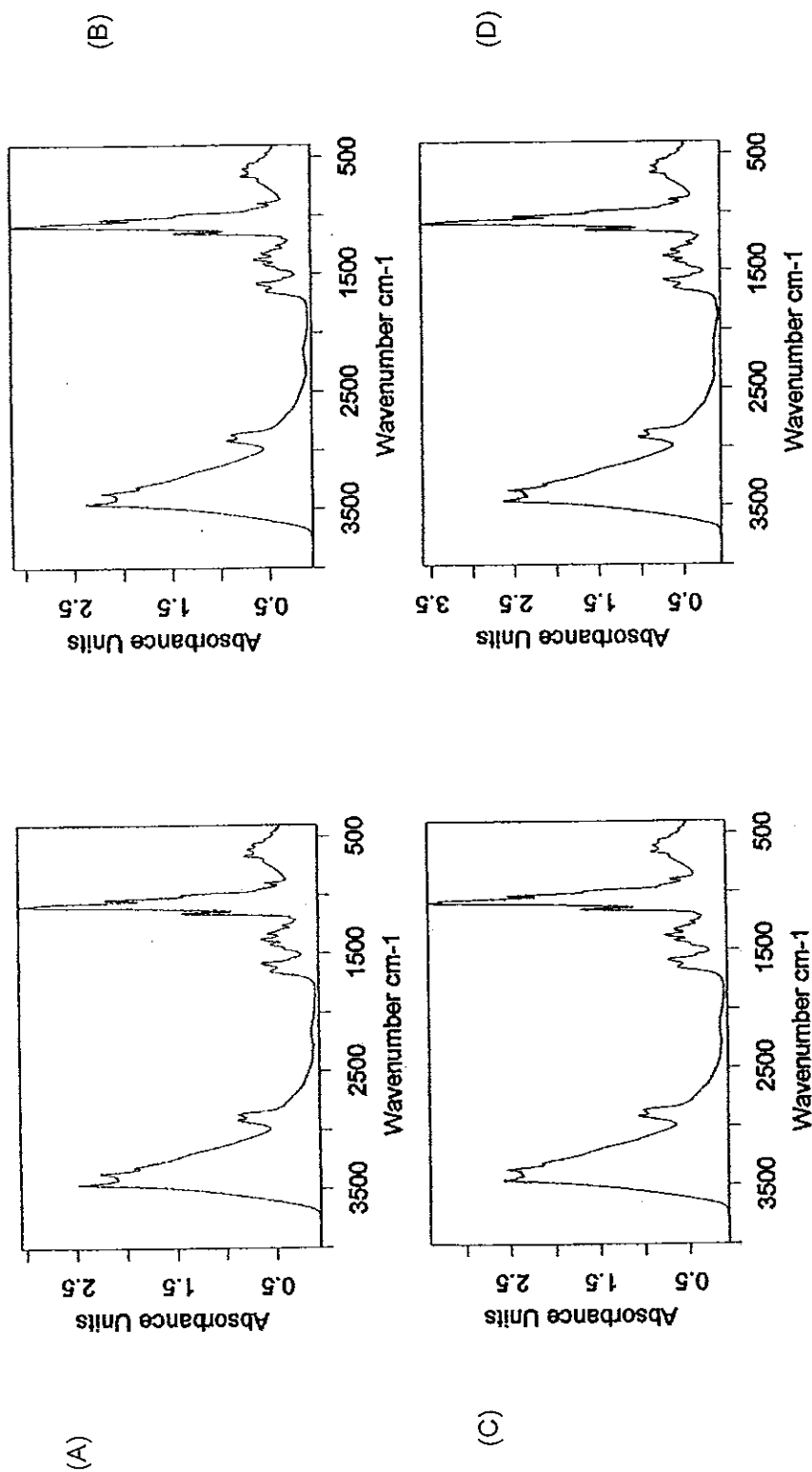
$$[\eta] = KM^a$$

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

in which $K = 8.93 \times 10^{-4}$, $a = 0.71$

Appendix 2

Infrared spectrum of chitosan



Infrared spectrum of chitosan prepared by deacetylation in presence of sodium borohydride at different concentrations. (A : 0 sodium borohydride; B : 0.5 M sodium borohydride; C : 1.0 M sodium borohydride, and D : 2.0 M sodium borohydride).

Calculation of degree of deacetylation (Domard and Rinaudo, 1983)

Degree of deacetylation (DD) = 100 - acetyl content (%)

$$\text{acetyl content (\%)} = (A_{1655 \text{ cm}^{-1}} / A_{3450 \text{ cm}^{-1}}) \times 100 / 1.33$$

Where A : absorbances

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