Characterizations of Chitosan Sponges Prepared from Shrimp Shell (*Penaeus monodon*) and Squid Pen (*Loligo formosana*)

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Abstract

Chitosan is a deacethylated product of chitin which is a major component of prawn shell and squid pen. Structurally, the linear polymer chains of chitin from prawn shell are arranged by anti-parallel fashion, α-form while the chains from squid pen are arranged by parallel fashion, β-form. Since the compatible biopolymers exhibit anti-bacterial, anti-fungal, enhance wound healing and hemostatic activities, various forms of product have been modified to use in medical applications. Unfortunately, most of available reports were confined with the α- form, but rarely information was seen for another one. Therefore, these studies aim to compare characteristics of the sponge prepared from chitosan derived from the two sources. To each source, preparation was carried out by freez-drying known properties of chitosan in acid solution at different concentration, and at different volumes. Surface study with scanning electron microscope (SEM) revealed that the sponge from prawn shell chitosan showed the higher porosity with the more compact structure than that from the squid pen. Moisture content and concentration of residual acid was influenced by amounts of chitosan used in preparation rather than source and acid volume. Solubility examination showed that all solution at pH around 7. However, they became insoluble after drying and sterilizing. Interestingly, the concentration of residual acid was increased in the sponge from prawn chitosan after heating whereas the opposite was found in another one. Therefore, degree of solubility as well as residual acid concentration in the sponges might be modified by optimizing drying period.

Keywords: Chitosan Chitin Chitosan sponge Sponge characteristics

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คุณลักษณะของแผ่นพรุนไคโตซาน ที่เตรียมจากเปลือกกุ้งและกระดองปลาหมึก

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บทคัดย่อ

ไคโตซานเป็นผลิตผลที่ได้จากการกำจัดหมู่อะซิติลออกจากโครงสร้างของไคตินซึ่งเป็นส่วนประกอบสำคัญของเปลือกกุ้งและกระดองปลาหมึก สารชีวภาพนี้มีคุณสมบัติในการต้านจุลชีพทั้งแบคทีเรียและเชื้อรา ตลอดจนช่วยเร่งการสมานแผล จึงเหมาะที่จะนำมาประยุกต์ใช้ทางการแพทย์ อย่างไรก็ตามโครงสร้างของสารโพลิเมอร์ชีวภาพที่สกัดจากสองแหล่งนี้ มีลักษณะการจัดเรียงตัวแตกต่างกันคือ ไคตินจากเปลือกกุ้งจัดเรียงตัวเป็นอัลฟ่าฟอร์ม แต่ไคตินจากกระดองปลาหมึกเป็นเบต้าฟอร์ม ดังนั้นคุณสมบัติหลายประการจึงแตกต่างกันด้วย เนื่องจากเปลือกกุ้งเป็นของเหลือจากกระบวนการผลิตกุ้งแช่เยือกแข็งที่สามารถจัดหามาสกัดในระดับอุตสาหกรรมได้ง่าย ดังนั้นรายงานที่เกี่ยวกับการประยุกต์ใช้ไคติน และไคโตซานส่วนใหญ่จึงจำกัดขอบเขตเฉพาะอัลฟ่าฟอร์ม ในขณะที่ข้อมูลเกี่ยวกับเบต้าฟอร์มมีน้อยมาก งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาคุณลักษณะของแผ่นพรุนที่เตรียมโดยการระเหิดสารละลายไคโตซานจากเปลือกกุ้งเปรียบเทียบกับกระดองปลาหมึก ผลการตรวจด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบสแกนพบว่า แผ่นพรุนของไคโตซานจากเปลือกกุ้งมีรูพรุนเป็นจำนวนมาก และมีเส้นใยแน่นมากกว่าที่พบในกระดองปลาหมึก ความชื้นและความเข้มข้นของกรดที่คงเหลือในแผ่นพรุน ขึ้นอยู่กับปริมาณของไคโตซานที่ใช้เตรียมตัวอย่าง แผ่นพรุนที่เตรียมใหม่ทั้ง 2 ชนิดสามารถละลายได้ดีในน้ำและน้ำเกลือ แต่ละลายได้น้อยมากในสารละลายบัฟเฟอร์ที่ pH ประมาณ 7 แต่จะไม่ละลายในสารละลายเหล่านี้เมื่อนำไปอบแห้งด้วยความร้อน หรือเมื่อนำไปผ่านการนึ่งฆ่าเชื้อ เมื่อเพิ่มปริมาณไคโตซานในการเตรียมแผ่นพรุนให้สูงขึ้นความเข้มข้นกรดที่เหลือจะเพิ่มขึ้นด้วย อย่างไรก็ตามเมื่อเปรียบเทียบความเข้มข้นของกรดระหว่างแผ่นพรุนไคโตซานอบแห้งที่เตรียมจากสองแหล่งพบว่า แผ่นพรุนที่เตรียมจากเปลือกกุ้งมีความเข้มข้นเพิ่มขึ้น ในขณะที่แผ่นพรุนไคโตซานที่เตรียมจากกระดองปลาหมึกมีความเข้มข้นต่ำลง ดังนั้นคุณสมบัติการละลายของแผ่นพรุนและความเข้มข้นของกรดที่เหลือ สามารถปรับให้สอดคล้องกับการประยุกต์ใช้ โดยกำหนดระยะเวลาการอบแห้งให้มีความเหมาะสม

คำสำคัญ: ไคโตซาน ไคติน แผ่นพรุนไคโตซาน ลักษณะของแผ่นพรุน

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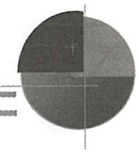
an engineered tissue was also examined using light and scanning electron microscope. Their blood coagulation and wound healing activities will be further revealed.

Methods

Shrimp shell (*Penaeus monodon*) and squid pen (*Loligo formosana*) were freshly collected from Kiang Huat Seagull Trading Frozen Food Public Company and Thepitak Seafood Co., Ltd., Thailand, respectively. Chitosan from both sources was prepared essentially similar procedures as described by Chandumpai *et al.* (23). In brief, chitin from shrimp shell powder was obtained by demineralization with 1.0 M HCl (15:190; w/v) for 1.5 h, and deproteination with 1.0 M NaOH (1:13; w/v) at 50 °C for 5 h. Similar was performed for the squid pen powder, but demineralization process was omitted. In order to make comparable results, chitin from both sources was deacethylated with 50% NaOH using solid to alkaline solution of 1:15 (w/v), and was carried out at 60 °C under nitrogen atmosphere for 6 h.

Essential properties of the raw materials, chitosans from both sources were analyzed for the contents of nitrogen and ash as described in A.O.A.C. (26). Degree of deacetylation was determined using spectroscopic method (27), while the average molecular weights (Mv) were analyzed by viscoscopic method as recommended by Bronswijk (28). Maximum solubility in 1.0% acetic acid was evaluated by slowly adding a known weight of the sample in 10 ml of the solvent with gently stirring until no further dissolving was observed.

Sponges in these studies were prepared by lyophilized a solution of the chitosan that was dissolved in 1% acetic acid. Preliminary study showed that there were some technical preparation problems which reflected product characteristics on using the different sources and concentrations. For instance, chitosans from prawn and squid pen were solubilized in the acid solvent with different saturation levels. Furthermore, at high concentration, air-bubble always appears and difficult to remove from the viscous solution eventhough gently stirring was performed, while the opposite gave a very soft product. Hence, sponges from each source were prepared at 3 concentration levels, i.e. 0.75, 1.25 and 1.75% for prawn shell and 0.30, 0.50 and 0.70% for squid pen. Since it was presumped that lyophilized period and volume of the acid solution might effect on properties of sponges, 25 and 50 ml from each concentration was also compared. Prior to pour into a glass block (5.5x8.6x2 cm) air bubble in the chitosan solution was removed under vacuum pump. They were frozen at -25 °C, then left in freeze-drier chamber (Dura-Dry™ μP, U.S.A.) until a constant weight of the products was obtained. The sponges



were immediately kept in plastic bag and left in desiccator during waiting for further analysis.

Unless otherwise stated, the examined properties were from lyophilized sponges. The remaining moisture was determined by heating the sponge samples in forced air oven at 100±3 °C until a constant weight was obtained. Subsequently, moisture readsorption was periodically monitored after leaving the dried samples at ambient temperature for 12 h intervals over 240 h.

The sponge samples prepared from 50 ml of similar concentrations (7.5 and 7.0 mg/ml of chitosan from squid pen and prawn shell, respectively) were cross sectioned for morphological examination with stereomicroscope (Olympus CH-2, U.S.A.). The same sample was also processed to investigate microstructural characteristics using scanning electron microscope at 15 kV (SEM; Jeol 35CF, Japan).

As preliminary studied showed that solubility of spong in aqueous solution was effected by heating, which in turn may be contributed by residual acid in sponges. These were evaluated after leaving the samples in forced air oven at 100±3 °C for 92 h. The properties after sterilizing at 121 °C under a pressure of 15 lbs/inch^2 for 15 min were also examined. These samples were left in desiccator during waiting for further studying.

Solubility of the sponges in aqueous solutions which are commonly involved in biological system was examined. Four mediums were selected, namely, 0.9% NaCl, 16 mM sodium phosphate buffer pH 7.4, 16 mM sodium phosphate buffer pH 7.4 with 0.9% NaCl. This was performed at ambient temperature by dissolving 4.0 mg of a sponge portion in 1 ml of the solutions for 1 h, the degree of solubility was ranked in 5 levels. Comparison was also made with those of the heated and sterilized samples as above described conditions.

As acidity remaining in sponges might effect on their physicochemical properties as well as their biochemical activities, it was evaluated by dissolving an accurate known weight of approximately 2x2 cm sponge in 10 ml deionized distilled water (Milli Q). Following sonication about 10 seconds, pH of the solution were determined. The concentration of residual acid liberated from sponge was calculated on the basis of sample weight which was assumed to be solely chitosan content. Comparison was also made with those of the heated and sterilized samples which were insoluble in water. They were examined by emmerging a known weight of samples in deionized distilled water.

Results

Table 1 shows some essential pro perties of the chitosan derived from prawn shell and squid pen which was used for preparation of sponge throughout this study.

Table 1 Some properties of chitosan derived from prawn shell and squid pen used in this study. The presented data are mean ± S.D. of 5 replications.

Properties	Squid pen	Prawn shell
Nitrogen (%)	8.04±0.02	8.09±0.05
Ash (%)	0.17±0.18	0.46±0.06
Degree of deactylation (%)	92.24±0.19	73.10±0.80
Averaged molecular weight (daltonsx10^6)	6.35±0.32	2.69±0.48
Maximum solubility in 1.0% acetic acid (mg/ml)	10	25

Chitosan from both sources comprising of similar nitrogen contents (app. 8%). It appeared that chitosan from prawn shell comprising of the higher ash contents (0.46%) than that of the squid pen (0.17%), whereas an averaged molecular weight of former source was almost 3 times lower than the latter, i.e. 2.69 x106 and 6.35x106 daltons, respectively.

Figure 1a and 1b show surface morpho logy of the sponges from both sources which were observed under stereomicroscope. It appeared that no markedly different between the samples prepared from similar concentration and the same volume of chitosan was observed.Gross investigation under scanning electron microscope revealed that the sponges from both sources were opened pore microstructure with a high degree of interconnectivity (Fig. 2a and 2b). In comparison, however, an arrangement of network in the sponge from prawn shell exhibited the more compact fibril with irregular in sizes and shape than another one of which a pore size of approximately 100 m was seen. Furthermore, it was found that the higher amounts of the same chitosan were used, the more compact structure with the smaller pore size was obtained.

Regardless of the source, moisture contents in sponge seem depended on the amounts of chitosan used in preparation. The higher amount held the more moisture contents, and moisture readsorption reached saturation levels within 12h after leaving the dried samples at ambient temperature. Again, the moisture readsorption behavior seems depended on the amounts of chitosan used in sponge preparation.

Analysis for associated proton in the lyophelyzed sponges revealed that the concentrations were notably influenced by an amount of chitosan used in preparation rather than sources and volume of acid solvent (Table 2).

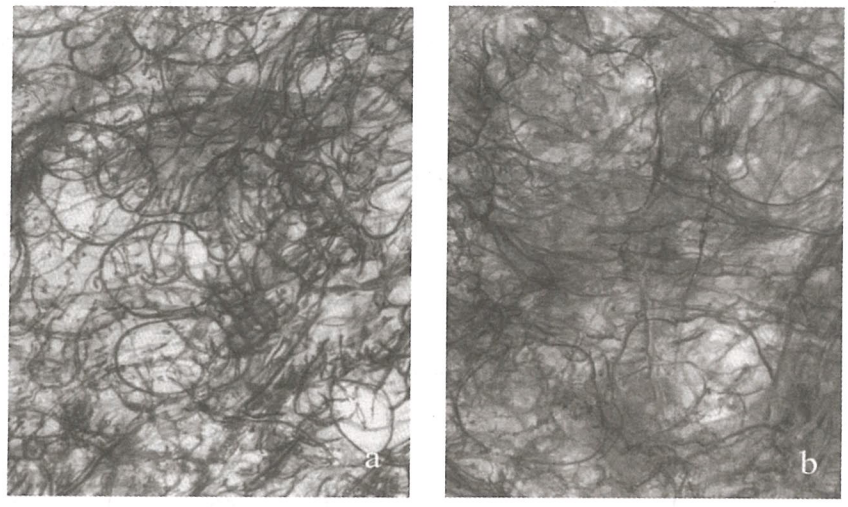


Figure 1 Surface morphology of sponge investigated under stereomicroscope, a) prawn shell chitosan and b) squid pen chitosan.

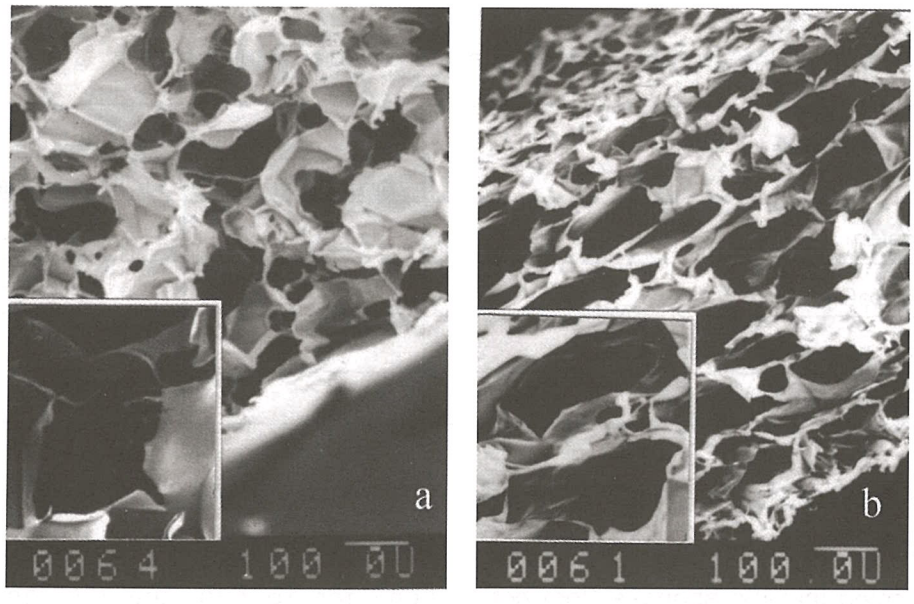


Figure 2 Surface morphology of sponge investigated under scanning electron microscope, a) prawn shell chitosan and b) squid pen chitosan.

Table 2 The residual acid (proton concentrations; μ mole/gm of sponge) in lyophilized sponges prepared from different concentrations and volumes of chitosan derived from squid pen and prawn shell. The presented data is mean of 3 replications.

Source	Chitosan concentration (mg/ml)	Volume (ml)	Residual acid (µmole/gm)
Squid pen	3.00	25	61.00
		50	35.40
	5.00	25	28.25
		50	18.10
	7.00	25	20.40
		50	12.38
Prawn shell	7.50	25	14.70
		50	12.52
	12.5	25	8.60
		50	4.56
	17.5	25	5.59
		50	2.58

Table 3 Solubility of the chitosan sponge in some aqueous solutions.

Medium	Solubility of sponge	
	Squid pen	Prawn shell
Deionized water	+++++	+++++
Saline (0.9% NaCl)	+++++	+++++
16mM sodium phosphate buffer pH 7.0	+	+
16mM sodium phosphate buffer pH 7.0 + saline	+	+

Table 3 shows degree of solubility of the chitosan sponge in some aqueous solutions. These indicated that the sponges from both sources were highly soluble in deionized distilled water and normal saline solution (0.9% NaCl). In contrast, they were sparingly soluble in the mediums containing 16mM sodium phosphate buffer pH 7.0 in both with and without 0.9% sodium chloride salt.

After heating at 100 ± 3 °C for 96h and sterilizing at 121 °C under a pressure of 15 lbs/inch2 for 15 min, the sponge became insoluble in all the examined mediums (Table 3). Analysis for the residual acid indicated that heating increased the content in the sponge from prawn shell chitosan, whereas the reverse was true for another one (Figure 3a and 3b).

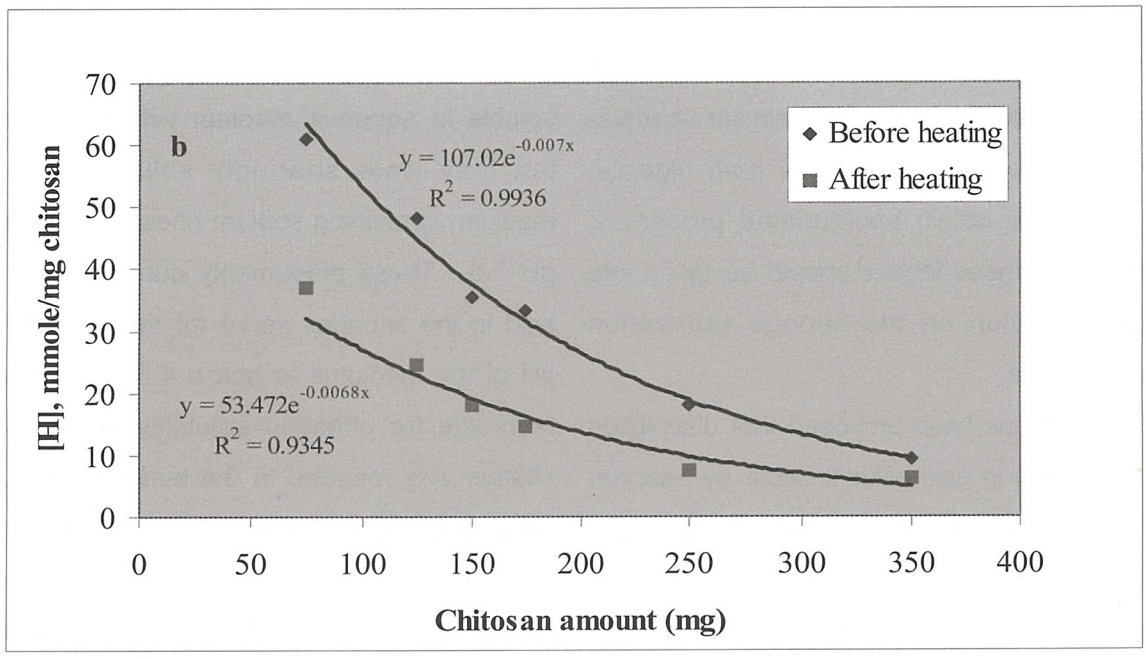


Figure 3 Relation between the amounts of chitosan used for sponge preparation and the concentration of residual acid; a. prawn chitosan, b. squid chitosan.

Again, the effects were rather influenced by the amounts of chitosan used in preparation which regression lines were exponentially correlated as the following equations:

Sponge from prawn chitosan

Before heating: $Y = 22.807\ e^{-0.0032x}$ R = 0.9769

After heating: $Y = 28.266\ e^{-0.0023x}$ R = 0.9569

Sponge from squid chitosan

Before heating: $Y = 107.02\ e^{-0.007x}$ R = 0.9936

After heating: $Y = 53.472\ e^{-0.0068x}$ R = 0.9345

Where X and Y represent the amounts of chitosan used for sponge preparation and proton concentrations (μ mole/gm chitosan), respectively.

In addition, it was noted that the samples turned from white to brown color after heating. The longer heating period the higher color intensity was observed.

Discussion

Nitrogen and ash contents of the chitosan from prawn shell and squid pen which were used as raw material in this study, were comparable to the results reported by Chandumpai et al. (23). It should be noted, however, that averaged molecular weights (Mv) of the samples were markedly higher than those of commonly found in literatures concerning medical and pharmaceutical applications. This presumably due to the deacetylation process was performed at low temperature and under nitrogen atmosphere as previously discussed (23).

Surface morphology study suggested that pore size and degree of porosity of the chitosan sponges was not only influenced by an amount of chitosan used in preparation,

but it was also depended on source. Cohen and Shapiro (29) reported a similar observation for the sponge prepared from alginate. In order to obtain appropriated properties, therefore, these factors should be taken into consideration on the sponge fabrication procedures

It has been proposed that dissolution of chitosan in acid solvent occur by association of proton to amino group therein, and this disrupt intermolecular H-bonding. It could be deduced, therefore, that the free polymer chains of chitosan derived from both α and β - chitins should be associated by relatively similar proton contents. Consequently, the concentrations of residual acid in sponge were influenced by an amount of chitosan used in preparation rather than source of chitosan. Heating exerted significant effects not only on their solubility but also on the concentration of the residual acid. It was interesting to note that the proton concen trations in the sponge from prawn shell chitosan were increased after heating, but the opposite was found for another one. This is presumably due to the different in polymer chain arragement. It is likely that as moisture evaporated from the samples with similar rate, the higher acid content was held in compact structure of chitosan derived from -chitin than that from β-chitin.

The newly lyophelyzed sponges were soluble in aqueous medium without buffer, but they were sparingly soluble in the medium containing sodium phosphate buffer pH 7.0. These presumably due to residual acid in the sponges serve for decreasing in pH of the mediums to below 4.5 which was favorable for chitosan solubility, while the change was retarded in the buffer solution. Also, heating and sterilizing brought the samples became insoluble in all the examined mediums. Similar results have been reported in the chitosan sponge prepared from prawn shell (21). These probably due to the effects were not only decrease the moisture content but it also increased evaporation of the volatile acid. Consequently, the more compact structure with the stronger intermolecular hydrogen bonding was enhanced after heating and sterilizing, whereas a portion of acid was evaporated to a level which was insufficient for their solubility. This suggested, therefore, that degree of solubility of the sponges might be modified by optimizing either drying or sterilizing period. It could be concluded from this investigation that characteristics of the sponges prepared from chitosan derived from prawn shell and squid pen exhibited both similarity and different in some respects. For similarity,the newly prepared sponges from both chitosan sources were soluble in water insoluble in aqueous medium containing buffer

at pH about 7. However, they became insoluble in aqueous solvents after heating and sterilizing. Most properties such as moisture content, moisture readsorption, concentration of residual acid and pore size depended on the amounts of chitosan used in preparation rather than source and the volume of acid solvent. For different, the sponge prepared from prawn shell chitosan exhibited the more compact fibril with irregular in sizes and shape than from squid pen. In addition, heating the sponge from prawn chitosan liberated more acid to aqueous medium than the one from squid pen.

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