Isolation and characterization of chitosan from fish scale waste

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Abstract. This research aims to study the isolated conditions that affect the characteristics of chitosan from fish scale waste which was received from fermented fish factory. The chitosan was isolated by three steps which are deproteinization, demineralization, and deacetylation. In deacetylation steps, the chitosan from fish scale was produced under conditions of 35, 45, and 55°C with 2 M NaOH for 5 hr and a ratio between chitin and NaOH equal to 1:4, 1:6, and 1:8 (w/v). The experimental results showed that the ratio between fish scale and NaOH and deacetylation temperature influenced on yield, L*, b*, and deacetylation degree of the chitosan. The yield values of the chitosan were in range of 24.94-49.84%. The chitosan had deacetylation degree was in the range of 19.17-21.64%. The viscosity of the chitosan solution was approximately 15.66 cP. The outer surface of the chitosan is rough. Moreover, the Staphylococcus aureus inhibit activity of the chitosan was not presented.

1. Introduction

Fermented fish is one of traditional food products in Thailand. In fermented fish process, a lot of fish scale are discarded as waste. These fish scale wastes causes environmental pollution such as flies and unwanted odour problems. However, fishery wastes such as fish scales are normally source of chitin [1, 2] which is natural polymer that consist of N-acetyl-D-glucosamine unit. Chitin can be changed into chitosan by alkali deacetylation. During alkali deacetylation, part of N-acetyl-D-glucosamine are broken with the formation of D-glucosamine units which contain a amine group [3, 4].

Chitosan is non-toxic biodegradable polymer [5]. It is one of important materials due to its good properties such as antimicrobial, adsorption, and film-forming properties [6]. Chitosan is usually used in the wastewater treatment, food and beverages, cosmetics and toiletries, bio-pharmaceutics, medical, and agricultural applications. Especially in food and agricultural fields, chitosan can be used for many applications such as clarifying agent, enzymatic browning inhibitor, antioxidant, edible film, seed coating, fruit and vegetable coating, time releasing agent of fertilizers into the soils, antimicrobial agent, etc. [2, 7].

Normally, chitosan isolation process consists of three basic steps which are deproteinization, demineralization, and deacetylation [5]. However, the isolated conditions in each step is different due to the difference of raw material type. Chitosan characteristic distribution is influenced by isolated conditions. Therefore, this research aims to isolate chitosan from fish scale waste from fermented fish industry. The isolation conditions were studies. Finally, the chitosan characteristics were investigated.
2. Material and Methods

2.1. Material
Java fish scale were collected from Baan Nern Hi meat processing community enterprise factory, Muang, Prachinburi province, Thailand. Before using, the fish scales were washed thoroughly with tap water. Then, the fish scales were dried at 60°C for 12 hr.

2.2. Experimental procedures

2.2.1. Chitosan isolation. The production of chitosan involved deproteinisation, demineralisation, and deacetylation steps. The fish scale was soaked in 2 M NaOH for 5 hr at 40°C with a ratio between fish scale and NaOH equal to 1:8 (w/v). Then, the fish scales were washed with water until their pH is neutral. The next step was accomplished by treating the fish scale with 2 M HCl for 5 hr at 40°C with a ratio between fish scale and HCl equal to 1:8 (w/v). The residue was then washed, filtered and dried at 60°C for 14 hr in a hot air oven to receive chitin.

The deacetylation step was achieved by treating chitin under conditions of 35, 45, and 55°C with 2 M NaOH for 5 hr and a ratio between chitin and NaOH equal to 1:4, 1:6, and 1:8 (w/v). The resulting chitosan was then collected, washed, and dried at 50°C for 12 hr.

2.2.2. Yield of chitosan. The yield of chitosan was calculated by equation (1) as follows:

\[ \text{Yield (\%)} = \frac{\text{Weight of chitosan (g)}}{\text{Weight of initial dry scale (g)}} \times 100 \]  

2.2.3. Determination of chitosan color. Color measurements of the chitosan were performed with a MiniScan EZ 4500L Spectrophotometer (Hunter lab, USA). The measurement was taken at an illumination condition of medium daylight (D65) with a 10° standard observer. The results were expressed as L*, a*, b*. Where, L* is the measure of brightness from black (0) to white (100), a* indicates the degree of redness (+a*) to greenness (-a*) and b* is the measure of yellowness (+b*) to blueness (-b*).

2.2.4. Determination of deacetylation degree of chitosan. The deacetylation degree of chitosan was determined by the titration method modified from Kumari and Rath [5]. 0.2 g of chitosan was dissolved in 20 ml of 0.1 M HCl and 25 ml of distill water. This solution was then titrated with a 0.1 M NaOH. The deacetylation degree of chitosan was calculated by equation (2) as follows:

\[ \text{Deacetylation degree (\%)} = \frac{2.03(V2-V1)}{m+0.0042(V2-V1)} \]  

Where \( m \) was mass of the chitosan and \((V2-V1)\) was the difference between two volume values of NaOH between the two points.

2.2.5. Determination of viscosity of chitosan. To determine of viscosity of chitosan, 1 g of chitosan was dissolved in 100 ml of 0.1 M acetic acid. The viscosity of the chitosan was measured in a viscometer (RVDVE230, Brookfield, USA).

2.2.6. Analysis of chitosan morphology. Analysis of the morphology of chitosan was carried out using scanning electron microscope (JSM 6400, JEOL, Japan).
2.2.7. Determination of antimicrobial activity of chitosan. To study on antimicrobial activity of chitosan by disc diffusion method, *Staphylococcus aureus* from stock culture was transferred to TSB and incubated at 37°C for 18 hr. After incubating, *Staphylococcus aureus* was diluted with buffer peptone water (10^6 CFU/ml) and spread on MHA plate. Then, 6 mm in diameter of filter paper which was soaked into 15 μl of chitosan solution (1 g of chitosan in 100 ml of 0.1 M acetic acid) was placed on the plates. After keeping at 4°C for 2 hr, the plate was incubated at 37°C for 24 hr. Finally, the diameter of clear zone of inhibition was evaluated as follow: Antimicrobial index (AI) = (diameter of clear zone of inhibition – diameter of filter paper)/(diameter of filter paper).

2.3. Statistical analysis
The variance was determined by ANOVA and the difference of mean values was determined by Duncan’s multiple range tests at a statistically significant level of 0.05.

3. Results and Discussions

3.1. Yield of chitosan
Yield values of chitosan isolating with ratio between fish scale and NaOH equal to 1:4, 1:6, and 1:8 (w/v) at deacetylation temperature of 35, 45, and 55°C are shown in table 1. The results showed that the yield values were in range of 24.94-49.84%. The ratio between fish scale and NaOH and deacetylation temperature affected on the yield values. The yield values decreased with increasing of the ratio between fish scale and NaOH. However, the yield values increased when deacetylation temperature increased. The results implied that the yield values may depend on deacetylation conditions.

| Ratio (Scale:NaOH) | Deacetylation temperature (°C) | Yield* (%) |
|-------------------|-------------------------------|------------|
| 1:4               | 35                            | 34.04±0.57 |
|                   | 45                            | 44.04±0.34 |
|                   | 55                            | 49.84±0.72 |
| 1:6               | 35                            | 32.71±1.22 |
|                   | 45                            | 33.15±0.94 |
|                   | 55                            | 37.36±0.74 |
| 1:8               | 35                            | 24.94±0.89 |
|                   | 45                            | 27.64±0.77 |
|                   | 55                            | 30.55±0.67 |

*Different superscripts in the same column indicate statistical difference (p ≤ 0.05)

3.2. Color of chitosan
Table 2 showed L*, a*, and b* values of chitosan isolating with ratio between fish scale and NaOH equal to 1:4, 1:6, and 1:8 (w/v) at deacetylation temperature of 35, 45, and 55°C. L*, a*, and b* values of the chitosan were in range of 43.94-49.64, 2.92-6.60, and 24.54-32.65, respectively. This result indicated that the chitosan had a dark reddish yellow color. The ratio between fish scale and NaOH and deacetylation temperature non significantly affected on a* value. However, the ratio of between fish scale and NaOH presented the inconstant trend of L* and b* values. For deacetylation temperature, L* and b* values decreased with increasing of deacetylation temperature. The chitosan was darker when the deacetylation temperature was rise. The results implied that the color of the chitosan may depend on deacetylation conditions. Muley *et al.* [8] who studied the extraction and characterization of chitosan.
from prawn shell waste found that the color of chitosan was affected by alkaline treatment and extraction time.

Table 2. L*, a*, and b* values of chitosan.

| Ratio (Scale:NaOH) | Deacetylation temperature (°C) | L*       | Color* |
|-------------------|-------------------------------|----------|--------|
|                   | 35                            | 49.31 ±2.03 | 4.97 ±2.04 | 31.26 ±2.72 |
| 1:4               | 45                            | 49.44 ±1.06 | 3.62 ±1.40 | 26.12 ±0.89 |
|                   | 55                            | 48.25 ±1.44 | 2.92 ±2.19 | 24.54 ±2.58 |
| 1:6               | 35                            | 49.44 ±1.43 | 6.18 ±0.64 | 35.89 ±1.04 |
|                   | 45                            | 45.65 ±0.97 | 6.04 ±1.13 | 27.59 ±0.97 |
|                   | 55                            | 43.94 ±1.32 | 5.54 ±2.34 | 30.11 ±1.84 |
| 1:8               | 35                            | 49.64 ±2.13 | 6.60 ±2.52 | 32.65 ±1.33 |
|                   | 45                            | 47.92 ±2.05 | 5.64 ±1.76 | 27.88 ±1.45 |
|                   | 55                            | 47.25 ±0.89 | 4.63 ±2.29 | 26.07 ±1.80 |

*Different superscripts in the same column indicate statistical difference (p ≤ 0.05)

3.3. Deacetylation degree of chitosan

Deacetylation degree determines the content of free amino groups in the polysaccharide. Normally, deacetylation degree of chitosan influence the properties of chitosan [9, 10]. Deacetylation degree of chitosan isolating with ratio between fish scale and NaOH equal to 1:4, 1:6, and 1:8 (w/v) at deacetylation temperature of 35, 45, and 55°C are shown in table 3. The isolated chitosan from this experiment showed that the deacetylation degree values were in the range of 19.17-21.64%. However, commercial chitosan usually has deacetylation degree in range of 70-95% [11]. Moreover, the ratio between fish scale and NaOH and deacetylation temperature slightly affected on the deacetylation degree. Prashanth et al. [12] explained that, during deacetylation, the acetyl groups of chitin cannot be removed if polysaccharide chains do not degradation by high temperature alkaline. In addition, No and Mayers [13] suggested that the change of chitin to chitosan is generated when deacetylating with concentrated NaOH solution (40-50%) usually at 100°C or higher. Therefore, the results implied that chitin that was deacetylated at ratio between fish scale and NaOH equal to 1:4, 1:6, and 1:8 (w/v) and deacetylation temperature of 35, 45, and 55°C could not be completely changed to be the chitosan. The reason may be unsuitable of NaOH concentration and deacetylation temperature.

Table 3. Deacetylation degree of chitosan.

| Ratio (Scale:NaOH) | Deacetylation temperature (°C) | Deacetylation degree* (%) |
|-------------------|-------------------------------|--------------------------|
| 1:4               | 35                            | 21.64 ±0.53              |
|                   | 45                            | 20.72 ±0.54              |
|                   | 55                            | 20.41 ±0.93              |
| 1:6               | 35                            | 20.72 ±0.54              |
|                   | 45                            | 19.79 ±0.54              |
|                   | 55                            | 19.17 ±0.54              |
| 1:8               | 35                            | 20.41 ±0.93              |
|                   | 45                            | 19.17 ±0.55              |
|                   | 55                            | 17.60 ±0.94              |

*Different superscripts in the same column indicate statistical difference (p ≤ 0.05)
3.4. Viscosity of chitosan
The viscosity of chitosan isolating with ratio between fish scale and NaOH equal to 1:4, 1:6, and 1:8 (w/v) at deacetylation temperature of 35, 45, and 55°C was determined. The result showed that the viscosity of the chitosan produced from various isolation conditions was not different. The viscosity of the chitosan was approximately 15.66 ± 0.57 cP. The chitosan showed less viscous. Normally, the polymer chain length determines its viscosity [2]. The chitosan that has short molecular chain usually has low viscosity. If chitosan is high viscous, it can be effectively used in agriculture applications such as seed coating, fruit and vegetable coating, etc.

3.5. Morphology of chitosan
The morphology of the obtained chitosan was revealed by SEM as shows in figure 1. The electron micrograph (50X) and (200X) indicated the outer of chitosan was rough and had some little parts on the chitosan skin.

![Figure 1](image1.png)

**Figure 1.** Morphology of chitosan (A) 50x (B) 200 x.

3.6. Antimicrobial activity of chitosan
To determine antimicrobial activity of chitosan on *Staphylococcus aureus*, disc diffusion method was used to find clear zone of inhibition. However, the result found that the clear zone of inhibition did not appear. The result indicated that the chitosan synthesized from fish scale with ratio between fish scale and NaOH equal to 1:4, 1:6, and 1:8 (w/v) at deacetylation temperature of 35, 45, and 55°C could not inhibit *Staphylococcus aureus*. The mechanism of antimicrobial activity of chitosan is unknown. However, some hypothesis has been suggested that the interaction between positive charges of chitosan and negative charges of microbial cell membrane changes cell membrane permeability resulting to leak of proteinaceous and other intracellular constituents of the microbial cell [14, 15, 16]. The positive charge of the chitosan is one of important factors for the chitosan’s antimicrobial activity. In this research, the chitosan had low deacetylation degree. The chitosan with low deacetylation degree normally has low positive charge. Therefore, the *Staphylococcus aureus* inhibit activity of the chitosan was not presented.

4. Conclusion
In this research, the chitosan can be isolated by three steps which are deproteinization, demineralization, and deacetylation. During deacetylation, the ratio between fish scale and NaOH and deacetylation temperature influenced on yield, L*, b*, and deacetylation degree of the chitosan. The chitosan had low deacetylation degree. The viscosity of the chitosan was approximately 15.66 ± 0.57 cP. In addition, the chitosan could not inhibit *Staphylococcus aureus*.
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