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Investigation of Chitosan from Squid Pen as Scar Remover

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Abstract: Marine resources, such as crabs, shrimps and squids are tapped for food production, which result to coastal waste of canning industries. Management of coastal waste is one of the major problems of tropical countries like the Philippines. One way to solve such problem is to look for application and uses of these waste materials. The utilization of waste materials from nature could be the answer to a variety of needs in skin treatment or improving its appearance. The main thrust of this study was the investigation of chitosan from squid pen in the formulation of scar remover. The characterization of prepared chitosan was done by determining the viscosity-average molecular weight (MV), degree of deacetylation (DD) and scanning of prepared chitosan films nanostructure. Preparation of chitosan films demonstrated significantly different viscosity and average molecular weight which is one of the important parameters which could influence the performance of chitosan as scar remover. The effectiveness of the formulated product was evaluated by comparing it with a commercial product. Evaluation of the product was based on the personal perception of the respondents and not based on the assessment of panelists. The results obtained lead to the conclusion that using 10% lactic acid for decalcification and 1M NaOH for deproteination is effective as active ingredient in the formulation of scar remover cream. The quick removal of scar confirmed by the respondents/ test subjects shows that chitosans can replace commercial product formulations.

Key words: Squid pen · Chitosan · Decalcification · Deproteination · Deacetylation

INTRODUCTION

The Philippines, because of its strategic location in the tropics, is abundant in natural resources, both in land and in water. Marine resources such as crabs, shrimps and squids are tapped for food production, which result to coastal waste of canning industries. Management of coastal waste is one of the major problems of tropical countries. One way to solve such problem is to recycle these waste materials for productive application.

A wide variety of medical applications for chitin and chitin derivatives have been reported over the last three decades [1-3]. It has been suggested that chitosan may be used to inhibit fibroplasia in wound healing and to promote tissue growth and differentiation in tissue culture [4].

Much attention has been paid to chitosan as a potential polysaccharide resource. Recently, a squid-derived wound-healing gel was invented by University of Otago scientists and is attracting attention from international medical companies, due to its unique blend of properties (http://www.scoop.co.nz/stories/sco711/500054.htm).The "Chitodex" medical gel, which uses a polymer derived from squid, has been patented by researchers from the University's Department of Chemistry (http://news.softpedia.com/news/Better-Surgery-Gel-Made-from-Squid-72459.html).Researchteam leader Professor Brian Robinson says Australian medical trials show the gel possesses both anti-bleeding and anti-scarring properties.

In this study, the β-chitin in squid pen was extracted and deacetylated to form β-chitosan, characterized and examined for scar removal reactions. Traditional extraction methods using strong acid and alkali at elevated temperatures was considered as the normal procedure in the characterization of chitosan. Different methods utilizing less harsh processes to obtain a high molecular weight product was one of the objectives in this study (http://neon-otago.ac.nz/chemistry/research/paa/defauylt.html). The best method producing high viscosity

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and high molecular weight of chitosan was used as an active component in the formulation of scar remover cream.

Hyperthrophic scars should be aggressively treated with topical steroids along with the use of a moisturizer containing lanolin and 7% lactic acid for cell renewal and restoration (www.jamesbeckmanmd.com/scar.php. Scar Prevention and Treatment, Skin Health Reference Library, April 26, 2005) and the use of lactic acid for decalcification of squid pen was investigated in this study.

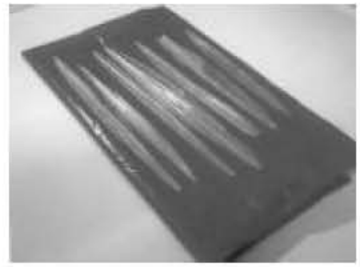

Fig. 1: Picture of Collected Squid Pens

MATERIALS AND METHODS

Collection and Preparation of the Sample: The squid pens collected from the wet market were washed with water and air-dried. The dried squid pens were cut into small pieces (1cm×1cm) before they were ground in Glen Mills MHM4 grinder using 2 mm sieve. Samples of collected squid pens were shown in Fig. 1.

Decalcification of Squid Pen: Decalcification was carried out by soaking 10 grams of ground squid pen in 150 mL of 10% lactic acid or 1N HCl for 2 h at room temperature with constant stirring using magnetic stirring motor with stirrer. The residue was filtered and washed with pH 7 buffer to about pH 6 to remove any excess acid and to prevent an acid-alkali reaction to occur during deproteination. The sample was dried in a vacuum oven at 60-70°C.

Deproteination of Squid Pen: Deproteination was done using 1M NaOH solution (150 ml/10g of demineralized squid pen) or 2000 ppm papain at room temperature for 2 h with constant stirring. Then, several washes were carried out up to pH 8 to the filtered chitin and dried in vacuum oven at 60-70°C.

Deacetylation of Chitin: The N-deacetylation of chitin was done following the procedure of Hirano [5] with little modification. Seven grams of prepared chitin was treated with 210 mL 40% NaOH aqueous solution at 120°C for 1 h using soxhlet apparatus and the precipitate was washed with water. The precipitate was further washed with phosphate buffer 8. The chitosan was dried in a vacuum oven at 60-70°C. The dried chitosan was kept in dessicating cabinet with silica gel.

Characterization of Prepared Chitosan

Determination of Viscosity-Average Molecular Weight (M_v): One percent (w/v) of the chitosan was prepared by dissolving 0.5g of purified chitosan in 50 mL of 1% acetic acid with stirring using magnetic stirring bar and motor for about 1 h. The viscosity of the solution was determined using Brookfield viscometer with RV number 6 spindle. The viscosity-average molecular weight was calculated using Eq. (1):

$$[\eta] = K_m M_v^a \quad (1)$$

Eq. (1) shows the relationship of viscosity and average molecular weight where $[\eta]$ is the intrinsic viscosity, Km = 1.81×10^{-3} and a = 0.93 are the empirical Mark-Houwink viscometric constants that are specific for a given polymer [6].

Analysis of the Degree of Deacetylation (DD): The chitosan films were prepared according to the method of Khan *et al.* [7] with slight modifications. Chitosan films were prepared by casting 1.0% w/v of purified chitosan in 1% acetic acid, followed by drying in a vacuum oven at 60°C for 12 h. The chitosan films were deprotonated by washing 3-4 times with 1:1 methanol:ammonium hydroxide followed by distilled water and methanol. Chitosan films were heated in a vacuum oven at 80°C for 3-16 h and kept in a desiccating cabinet with silica gel prior to scanning. The spectra of chitosan films were obtained using Impact 400 Nicolet FTIR with a frequency range of 4000-400 cm^{-1}. The degree of deacetylation (DD) of the chitosan films was calculated using baseline (a), which was proposed by Domszy and Roberts [8] as internal standard to correct for film thickness or for differences in chitosan concentration. The computation equation for the degree of deacetylation (DD) using baseline (a) is shown in Eq. 2.

$$DD=100-[(A_{1655}/A_{3450})/1.33][100-\text{baseline}(a)] \quad (2)$$

Where, A_{1655} and A_{3450} were the absorbances at 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band as an internal standard.

Scanning of Prepared Chitosan Films Nanostructure: The nanostructure of the prepared chitosan films were scanned using Olympus CK40 phase contrast microscope coupled with camera at 20×magnification.

Formulation of Scar Remover: The prepared chitosan was used as active ingredient and thickener. One gram of chitosan was dissolved in 100 mL of 1% glycolic acid in a beaker. One gram of paragon II was added to the mixture. In another beaker, 4.0 grams of stearic acid, 4.0 grams of stearyl alcohol, 4.0 grams of manro CS, 5.0 grams of mineral oil, 1.0 gram of cremophor A25 and 0.5 gram of cremophor A6 were mixed. Both beakers were heated at 80°C. The oil phase was added to the aqueous phase and mixed thoroughly. The pH was adjusted to 6 by adding triethanolamine (TEA).

Statistical Treatment of Data: To determine how effective is the formulated scar remover compared to a known commercial product, the two-tailed type of t-test was applied [9]. The volunteers rated their evaluation in terms of a.) quick removal of scar, b.) odor, c.)non-irritating effect, d.) spreadability and e.) moisturizing effect as follows: 4-excellent, 3-very good, 2-good, 1-needs improvement. The evaluation forms were tallied, analyzed and interpreted.

RESULTS AND DISCUSSION

Effects of Decalcification and Deproteination Processes on the Characterization of Chitosan: The viscosity and average molecular weight of the prepared chitosan samples were shown in Table 1. The chitosan prepared using 10% lactic acid for decalcification and 1M NaOH for deproteination gave the highest viscosity and average molecular weight. The values were very much higher compared to chitosan prepared using the standard procedures of decalcification and deproteination [10]. The chitosan prepared using 10% lactic acid for decalcification and 2000ppm papain for deproteination gave the lowest viscosity and average molecular weight. These results showed that enzymatic method using papain was less effective than chemical method in preparing high molecular weight chitosan. Compared with viscosity average molecular weight of commercially available chitosan (usually prepared from shrimp shell) estimated to be 1.00 +/- 0.04×10^6 Da [11], all the prepared chitosan samples had higher molecular weights. The high intrinsic viscosity of squid-derived chitosan samples produced in this study led to interesting and useful property of polymers which is high film forming property.

Degree of Deacetylation: The spectrum of the prepared chitosan film using HCl-papain indicating the degree of deacetylation is shown in Fig. 2. In the infrared spectrum of the chitosan film, a broad peak at 3450 to 3000 cm^{-1} observed was assigned to the OH stretch vibrational frequency. An amide band was observed at 1650 cm^{-1}. These two peaks are important in the calculation of the degree of deacetylation using baseline a in Eq. 2.

Table 2 showed the results for the degree of deacetylation of the chitosan produced using different methods of decalcification and deproteination. The chitosan prepared using 1M HCl and 2000ppm papain gave the highest degree of deacetylation. The value is higher compared to the chitosan prepared using standard reagents of deacetylating chitin [8]. Though the % deacetylation of the chitosan prepared using 10% lactic acid and 1M NaOH was lower than the standard procedure, this is because of the presence of more acetyl

Table 1: Viscosity and average molecular weight of prepared chitosan samples

Decalcification	Deproteination	Ave. Viscosity (mPas)	M_v (Dalton)
1M HCl	1M NaOH	50,000	8.33×10^6
10% Lactic Acid	1M NaOH	112,500	1.77×10^7
1M HCl	2000 ppm Papain	62,500	1.02×10^7
10% Lactic Acid	2000 ppm Papain	12,500	4.4×10^6

Table 2: Degree of deacetylation of prepared chitosan samples

Decalcification	Deproteination	% Degree of Deacetylation (DD)
1M HCl	1M NaOH	53
10% Lactic Acid	1M NaOH	43
1M HCl	2000 ppm Papain	63
10% Lactic Acid	2000 ppm Papain	28

Degree of Deacetylation

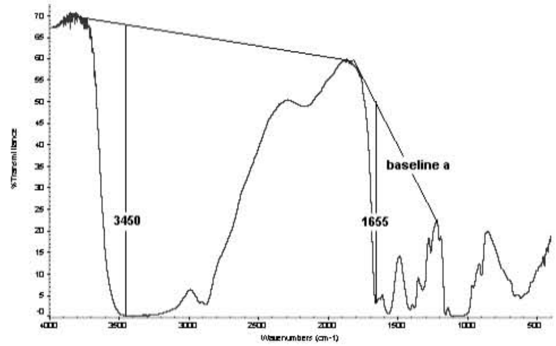

Fig. 2: I.R. Spectrum of the Prepared Chitosan with Highest Percentage

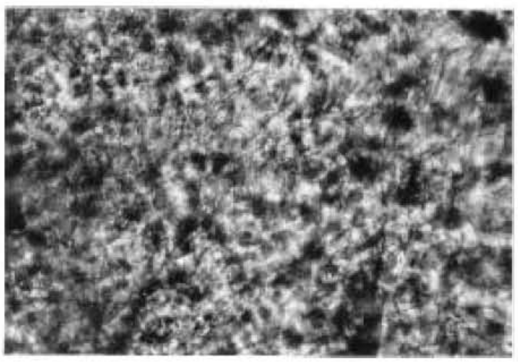

Fig. 3: Film from Chitosan Prepared from Lactic Acid-Papain Method
Photograph of chitosan film prepared from Lactic Acid-Papain method. Thickness of film: 20.67 μm; magnification of phase contrast microscope: 20

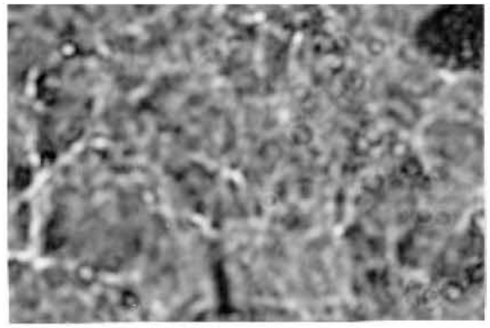

Fig. 4: Film from Chitosan Prepared from NaOH-HCl Method
Photograph of chitosan film prepared from NaOH-HCl method. Thickness of film: 3.67 μm; magnification of phase contrast microscope: 20

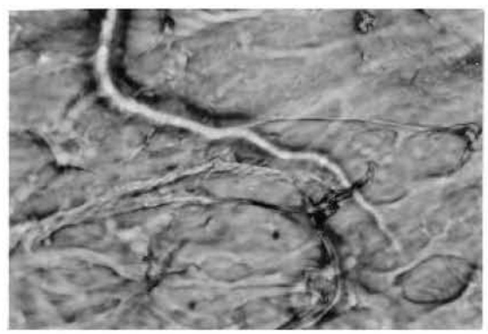

Fig. 5: Film from Chitosan Prepared from Lactic acid-NaOH Method
Photograph of chitosan film prepared from Lactic acid-NaOH method. Thickness of film: 11.33 μm; magnification of phase contrast microscope: 20

groups to be cleaved in the strand of the sample which has very high molecular weight. Thus, greater time and reagent are needed to deacetylate the sample compared to the others. The chitosan prepared using 10% lactic acid and 2000ppm papain gave the lowest degree of deacetylation. This suggests a modification of the structure of chitosan that prevents the deacetylation of the amide band.

Morphology of the Chitosan Films: The phase contrast microscope photographs of the chitosan films revealed the differences of the nanostructure of the films, which are dependent on the molecular weights of the prepared chitosan samples. The film of chitosan prepared using 10% lactic acid and 2000ppm papain (Fig. 3) appeared fibrous and the globular clusters are smaller compared to the film of chitosan prepared from 1M HCl and 1M NaOH (Fig. 4). The film of chitosan prepared using 10% lactic acid and 1M NaOH (Fig. 5) showed smooth surface and the globular clusters are larger as compared to the film of chitosan prepared using 1M HCl and 1M NaOH (Fig. 4). The film of chitosan prepared using HCl and 2000ppm papain (Fig. 6) appeared similar to the film prepared using lactic acid and 1M NaOH but the globular clusters are smaller. These photographs support the results of the molecular weights of the chitosan samples.

Evaluation of Prepared Scar Remover Cream: The formulated scar remover as well as one commercial product were evaluated by eleven respondents whose ages ranged from seventeen to fifty years old. The formulated scar remover was applied on identified prominent scars twice a day every after bath and before

Table 3: Comparison of the perception of the subjects on the effectiveness of the formulated scar remover and the known commercial product

	Formulated		Commercial			
Effects	($\bar{x}$)	SD	($\bar{x}$)	SD	t-value	Significance
1.Quick Removal of Scar	3.27	0.65	2.09	0.83	-5.22	P=0.00<0.01
2.Odor	3.27	0.47	2.27	1.10	-3.03	P=0.01<0.05
3.Non-irritating effect	3.45	0.69	2.81	0.87	-1.75	P=0.11>0.05
4.Spreadability	2.45	0.93	2.91	0.70	1.05	P=0.32>0.05
5.Moisturizing effect	3.00	0.63	2.36	0.81	-1.88	P=0.89>0.05
Overall	3.09	0.43	2.49	0.71	-2.43	P=0.04<0.05

*Probability Value>0.05 is not significant; if < 0.05 is significant; result is very satisfactory if the value is < 0.01 level of significance [9]

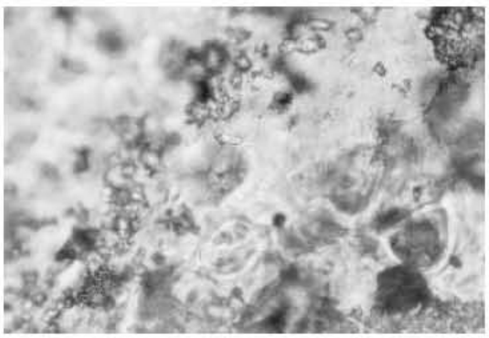

Fig. 6: Film from Chitosan Prepared from HCl -Papain Method
Photograph of chitosan film prepared from HCl-Papain method. Thickness of film: 8.33 μm; magnification of phase contrast microscope: 20

bedtime for a period of one month. The skin condition of volunteers who used the formulated product was monitored for a week to assess the safety of the formulated cream. No one among the volunteers had complained of irritation or had experienced allergic reaction in using the prepared product. A remarkable thinning of the keloid scar after 10 days of using the formulated product was observed from one of the volunteers. The same procedure was followed using the commercial product. Evaluation form was given to each respondent after the use of both products to collect data for analysis.

Table 3 showed that in terms of quick removal of scar, the formulated product had a very significant difference compared to a known commercial product. This means that the prepared scar remover is more effective than the commercial product. The non-irritating effect, spreadability and moisturizing effect showed no significant differences, but the odor showed significant difference. This means that the odor of the prepared scar remover cream is more preferred than the commercial product.

CONCLUSIONS

This study has successfully found productive applications of the chitosan produced from squid pen. These are production of polymer films with high average molecular weight and degree of deacetylation; and the application of the polymer produced as active ingredient in the formulation of scar remover. Using HCl for decalcification and papain for deproteination suggest that this combination of purifying processes produce chitosan with high molecular weight and high degree of deacetylation.

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