Response Surface Optimization and Kinetics of Isolating Chitin from Pink Shrimp (*Solenocera melantho*) Shell Waste

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Response surface methodology has been applied to the isolation of chitin from pink shrimp shell powder. The optimal deproteinization condition occurs at 75 °C, 2.5 N NaOH. A minimal solution to solid ratio of 5 mL/g is required to maintain fluidity during deproteinization. Deproteinization exhibits two-stage first-order reaction kinetics. The maximum deproteinization rate constant approaches 0.1 min^{-1} when the protein content is decreased from 16% to slightly above 7%. After the first 30 min, the deproteinization rate constant could decrease up to 2 orders of magnitude. The optimal demineralization condition is around 1.7 N HCl, with an acid solution to solid ratio of 9 mL/g at ambient temperature. Demineralization could be described as a pseudo-first-order reaction. The demineralization rate constant ranges from 0.00020 to 0.017 min^{-1}.

Keywords: *Chitin; deproteinization; demineralization; optimization; kinetics*

INTRODUCTION

Chitin can be found in crustacea, insects, molluscan organs, and fungi (Knorr, 1984). It is more abundant than any other natural biopolymers except cellulose. The production, properties, and application of chitin and its derivatives have attracted worldwide attention (Austin et al., 1981; Brine et al., 1992; Li et al., 1992). The potentials of using chitin and chitosan in water treatment, agricultural and food processing, cosmetics, pharmaceuticals, and biotechnology have been extensively investigated for years and summarized in recent reviews (Brine et al., 1992; Li et al., 1992). To date, the major source of industrial chitin comes from crustacean shells (Knorr, 1984). Seafood processing and consumption generate thousand tons of shellfish wastes in Taiwan each year. A practice in resource recovery is to grind and dry the shrimp shells and use the powder for animal or fish feed. The shrimp shell powder is of low economic value but readily available in Taiwan. A better alternative is to recover chitin from shrimp shells or their powder. This provides a renewable resource for various value-added materials such as chitosan, modified chitin/chitosan, and chito-oligomers (Chen and Jin, 1995; Li et al., 1992; Roberts, 1992).

The isolation of chitin from crustacean shell wastes consists mainly of the deproteinization and demineralization steps (No et al., 1989; Shahidi and Synowiecki, 1991). Although the production of chitin and chitosan was commercialized for decades, little has been reported about the optimization or kinetics of the preparation process. Sannan et al. (1977) reported that the deacetylation of chitin behaved as a pseudo-first-order reaction. Their result was derived from a semilogarithmic analysis of the volume of liquid used in the titration of deacetylated product. Scattered information on the preparation conditions and time course studies abounds in the literature (Bough et al., 1978; No et al., 1989; Roberts, 1992; Shahidi and Synowiecki, 1991; Shimahara and Takiguchi, 1988; Wu and Bough, 1978). Nevertheless, a large variation exists for the conditions of preparing either chitin or chitosan (Roberts, 1992). Obviously, there has been no consensus on the ideal combinations of variables for making these useful biomaterials. This is partly due to the one-variable at a time approach in the literature reporting optimal conditions.

This research investigates the response surface optimization of preparing chitin from pink shrimp shell powder. Kinetics during preparation is also analyzed with respect to the deproteinization and demineralization processes.

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MATERIALS AND METHODS

Raw Material and Preparation. Shells of pink shrimps (*Solenocera melantho*) were collected from a local fish market in Keelung, Taiwan. The shrimp shells were soaked in 0.5 N sodium hydroxide solution for 6 h at ambient temperature. They were then washed and flushed with distilled water at room temperature (20−25 °C). This was conducted to remove the organic compounds or protein loosely associated on the surface of the shells. Approximately $^2/_3$ of the protein originally present in raw shrimp shell was removed by alkaline soaking and water washing. The remaining shells were oven-dried at 30 °C for 48 h. The shells were then ground with a laboratory scale hammer mill and screened to 60−80 mesh (0.177−0.250 mm) powder. The powder was prepared to serve as a single-species, uniform-size simulant of the commercial shrimp shell powder.

Deproteinization. Sodium hydroxide solution was used to remove protein from shrimp shell powder. A central composite rotatable design for three variables (Mullen and Ennis, 1979) was applied to select the deproteinization conditions. The ratio of NaOH solution to shrimp shell powder (solution to solid ratio) had levels of 5.0, 8.0, 12.5, 17.0, and 20.0. The NaOH concentrations were 0.50, 0.91, 1.50, 2.09, and 2.50 N. Deproteinization temperatures were 25, 40, 62, 84, and 99 °C. The design consists of 20 experiments (Table 3) and includes six replicates of the central points. A portion of the samples was removed after reacting for 5, 10, 15, 20, 25, 30, 60, 120, 180, 240, 300, and 360 min, respectively. These samples were analyzed in triplicate for their protein contents.

Demineralization. Hydrochloric acid solution was used to remove minerals. Demineralization conditions for the shrimp shell powder was also selected according to the central composite design (Table 5). The solution to solid ratios varied

S0021-8561(96)00687-5 CCC: $14.00

Table 1. Chemical Composition of Pink Shrimp Shell Powder[a]

composition	wet weight basis (%)	dry weight basis (%)
water	5.7 ± 0.2	
crude protein[b]	16 ± 1.2	16.4
crude fat	7.9 ± 0.4	8.4
ash	40 ± 0.6	42.4
calcium	7.6 ± 0.2	8.1
chitin	22 ± 0.5	23.3

[a] Average of three measurements. [b] Crude protein = (total nitrogen − chitin nitrogen) × 6.25.

from 3.0, 5.4, 9.0, 12.6, to 15.0 mL/g. Hydrochloric acid solution had concentrations of 0.20, 0.56, 1.10, 1.64, and 2.00 N. The treatments proceeded at 30, 44.2, 65.0, 85.8, and 100 °C. Sampling periods were 5, 15, 30, 60, 120, 180, 240, 300, and 360 min after the start of each test. Triplicate measurements were conducted to determine the contents of calcium.

Chemical Analysis. Water, fat, and ash contents were determined by standard methods (AOAC, 1984). Kjeldahl method was used to determine the nitrogen content. Crude protein content was calculated by multiplying the corrected nitrogen content by 6.25. The corrected nitrogen content was the nitrogen content of shrimp shell powder minus the nitrogen content of chitin (1.18%) determined by the same procedure. For the determination of calcium content, 0.5 g of sample was mixed into 5 mL of sulfuric acid and 5 mL of nitric acid. The solution was digested in a microwave digester until it became clear. Then the solution was diluted to a concentration below 50 ppm. The calcium content was measured with a Hitachi atomic absorption spectrometer. The standard curve was established by using ultrapure (99.95%) calcium carbonate solution at 0, 5, 10, 25, and 50 ppm. The chitin content was determined as described by Hackman and Goldberg (1971).

Statistical Analysis. The general linear model (GLM) procedure was used to analyze the effect of variables on deproteinization and demineralization (SAS, 1985). Time course data were subjected to the least-squares fit with a worksheet software program on a personal computer.

RESULTS AND DISCUSSION

Chemical Composition of Pink Shrimp Shell Powder. Raw shrimp shells had 48% ± 3.8% (five replicates) crude protein on a dry weight basis (Tsai, 1996). Table 1 shows the chemical compositions of partially extracted, dried, and ground pink shrimp shell powder on both wet weight and dry weight basis. The pink shrimp shell powder contains 22% chitin by wet weight. The crude protein is 16%, ash is 40%, and calcium content is 7.6%. To remove surface meat from kuruma prawn shells, Chen and Yang (1994) soaked the shells in 0.5 N NaOH at 20 °C for 8 h. Their results indicated that kuruma prawn shells have 14−54% ash and 1.8−13.8% calcium at different molt stages. No et al. (1989) reported that there were 16.9% crude protein, 23.6% chitin, 63.6% ash, and 24.8% calcium in crawfish shells washed with hot water. Shahidi and Synowiecki (1992) found that Newfoundland pink shrimp had a calcium content of 15.3%. Our results in ash and calcium content are within the range reported by Chen and Yang for prawn exoskeleton samples prepared by similar procedure. The chitin and protein content are comparable to that of dried crawfish shell (16.9%) after hot water washing reported by No et al. (1989). The crude protein content is also about that of kuruma prawn cuticles (15.4% and 16.9%) after autoclave sterilization (Shimahara et al., 1984). Considerable differences in composition apparently exist for crustacea. It is caused by their variations in species, harvest location, molt stage, feed, and other biological or environmental factors. However, it appeared that about 16% of the shell weights of several crustacean species are contributed by more strongly bound protein.

Table 2. GLM Regression Results between the Protein Content after 6 h and the Deproteinization Variables

	df	parameter estimate C_i, C_{ii}, or C_{ij}	probability[a]
intercept		6.0660	0.0001***
X_1[b,c]	1	−0.0232	0.8021
X_2	1	−0.7668	0.0001***
X_3	1	−0.7057	0.0001***
X_1X_1	1	−0.0716	0.4329
X_2X_2	1	0.1866	0.0592
X_3X_3	1	0.3497	0.0026**
X_2X_1	1	0.0138	0.9093
X_3X_1	1	0.2573	0.0537
X_3X_2	1	0.1580	0.2091
R^2		0.9416	

[a] *P < 0.05; **P < 0.01; ***P < 0.001. [b] X_1, dimensionless solution to solid ratio = (solution to solid ratio − 12.5 mL/g)/(16.959 mL/g − 12.5 mL/g); X_2, dimensionless NaOH concentration = (NaOH concentration − 1.5 N)/(2.09 N − 1.5 N); X_3, dimensionless reaction temperature = (reaction temperature − 62 °C)/(84 °C − 62 °C). [c] Protein content (%) = C_0 + (C_1X_1) + (C_2X_2) + (C_3X_3) + ($C_{11}X_1X_1$) + ($C_{22}X_2X_2$) + ($C_{33}X_3X_3$) + ($C_{21}X_2X_1$) + ($C_{31}X_3X_1$) + ($C_{22}X_3X_2$).

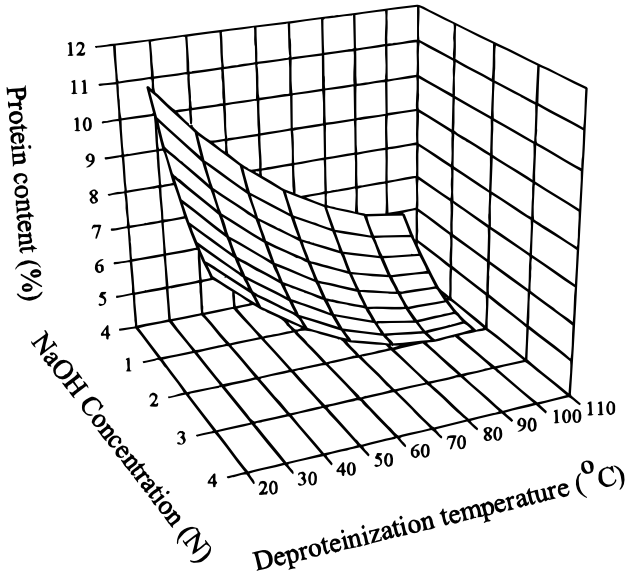

Figure 1. Protein content of shrimp shell powder after deproteinization for 6 h with a solution to solid ratio of 5 mL/g.

Optimal Condition for Deproteinization. The results of GLM regression analysis are listed in Table 2 for the residual protein content after 6 h of deproteinization. The concentration of NaOH solution and reaction temperature play dominant roles in removing protein. Increasing NaOH concentration or temperature lowers the protein content after deproteinization. Alkaline concentration is more potent in affecting the residual protein content at low temperatures. The quadratic term of temperature contributes positively to the protein content. The combined effect of the first- and second-order term of temperature leads to an optimal temperature around 75 °C. The regression equation is plotted into a response surface (Figure 1). The regression results and the response surface suggest that the optimal deproteinization condition occurs at 2.5 N NaOH and 75 °C. The influence of solution to solid ratio is insignificant within the experimental range. Nevertheless, a minimal solution to solid ratio of 5 mL/g is required to maintain fluidity during deproteinization. For a confirmation test at this condition (with a solution

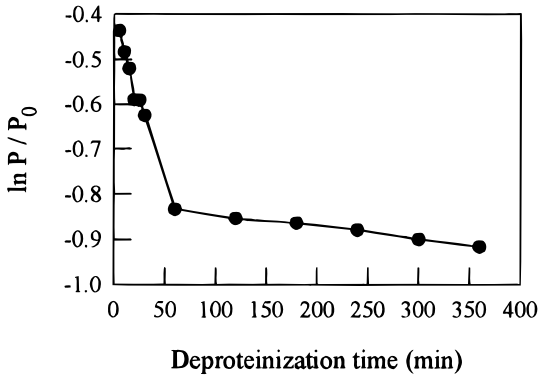

Figure 2. Semilogarithmic plot of protein content during deproteinization with a solution to solid ratio of 8.0 mL/g, NaOH concentration of 2.09 N at 40 °C.

to solid ratio of 12.5 mL/g), the protein content decreased from 15.5% to 5.2% within 6 h.

The minimal protein content achieved in this study is approximately 5%. This is higher than the 2.1% and 2.3% reported for Newfoundland pink shrimp (Shahidi and Synowiecki, 1991). Nevertheless, it is comparable to the 0.4−5.6% reported by Austin et al. (1982) for different crustacean species. The difference between the results from different laboratories could be caused by the difference in species, the nature of chitin−protein complexes, or the isolation procedure. However, we suspect that the analytical procedure used in determining protein content might also contribute to the difference. Austin et al. (1981) reported that some covalently bound proteins could only be removed after fractionating the chitin−protein complexes with 1 N NaOH at 100 °C for 48 h. Brine (1982) reported that a considerable fraction of protein remained bound with chitin even after extensive treatment. On the contrary, the Kjeldahl method is based on the reaction between reactive amino group and sulfuric acid. Its analytical results of the residual protein content therefore might be higher than those results obtained by the analysis of extracted amino acids.

Kinetics of Deproteinization. Deproteinization from shrimp shell powder appears to have two-stage first-order reaction kinetics (Figure 2). The change in reaction rates occurs after reacting for 30−60 min. The inflection point is located between 6.4% and 10.41% protein content, depending on the treatment severity. The deproteinization process can be described by a first-order reaction equation $dP/dt = -kP$, where P represents the protein content, t the treatment time, and k the reaction rate constant. When the protein contents are above 7%, rate constants are relatively large. Values as high as 0.094 and 0.088 min^{-1} are achieved for conditions near the optimum (Table 3). After the first 30 min, deproteinization rate constants decrease to 0.000 268−0.001 35 min^{-1}. The dramatic decline in deproteinization rate suggests that there is a change in mechanism during deproteinization.

One possible reason for the change in deproteinization rates or mechanism is the nature of chitin−protein complex. Deproteinization was frequently referred to as the extraction of protein from crustacean shells. A large body of evidence, however, indicated that chitin−protein complexes exist in the shells (Roberts, 1992). Several possible covalent bonds might be involved in the chitin−protein link. Brine (1982) differentiated the types of bonding into four major groups. The Schiff base type linkages, acetal (*O*-glycosidic), and amide (*N*-glycosidic and *N*-acylglucosaminyl) type bonds contribute about 68%−91% of the protein fractions of four different crab species. Residual strong covalent bonds constitute the 32%−9% fraction that could not be removed after extensive extraction. The residual protein content in this study is 32% of the initial amount. This is close to the data for Horseshoe crab in Brine's work. Furthermore, our kinetic data seem to confirm Brine's findings by a different approach. It is probable that when $P > 10.4\%$, only the loosely bound proteins are removed from the pink shrimp shells. When $5\% < P < 10.4\%$, deproteinization could involve the amide type bonds. Stronger chitin−protein covalent bonds make it difficult to reduce the protein content to below 5% even after extensive treatment for 6 h.

Table 3. First-Order Reaction Rate Constants during Deproteinization

deproteinization condition				
mL of solution/g of solid	NaOH concn (N)	reaction temp (°C)	k^a (10^{-2} min^{-1}) (protein > 7%)	k^b (10^{-4} min^{-1}) (t >30 min)
8.0	0.91	40	*c*	5.86
8.0	0.91	84	1.79	5.63
8.0	2.09	40	1.39	2.68
8.0	2.09	84	9.42	8.66
17.0	0.91	40	0.314	13.5
17.0	0.91	84	1.68	3.97
17.0	2.09	40	0.712	10.8
17.0	2.09	84	5.70	6.07
20.0	1.5	62	2.63	6.19
5.0	1.5	62	2.58	6.73
12.5	2.5	62	5.73	7.25
12.5	0.5	62	*c*	4.48
12.5	1.5	99	8.79	4.17
12.5	1.5	25	*c*	3.47
12.5	1.5	62	2.72	7.08
12.5	1.5	62	3.76	6.42
12.5	1.5	62	1.73	6.34
12.5	1.5	62	2.55	8.50
12.5	1.5	62	2.77	3.82
12.5	1.5	62	2.77	3.86

[a] Calculated using the equation for first-order reaction, $k = \ln 2/t_{1/2}$, where $t_{1/2}$ is the half-life of the reaction when the protein content reaches half of the initial value. [b] Obtained from semilogarithmic regression of time course data. Correlation coefficients range from 0.91 to 0.99. [c] After 6 h of deproteinization, the protein content in sample remains above half of the initial value.

Optimal Condition for Demineralization. Table 4 shows the GLM regression analysis results for calcium content after 6 h of demineralization. The influences of HCl concentration and solution to solid ratio were significant, but the effect of temperature was insignificant. The quadratic term of solution to solid ratio is also significant. Increasing HCl concentration or solution to solid ratio decreases the residual calcium content (Figure 3). The solution to solid ratio has more influence at low acid concentration. Similarly, the HCl concentration is more influential at low solution to solid ratio. From the regression equation and Figure 3, the optimal condition for calcium removal by HCl treatment is around 1.70 N HCl and 9.00 mL/g solution to solid ratio. At treatment conditions around the optimum, demineralization proceeds so fast that the calcium content is reduced to around 0.05% in 1 h. A confirmation test after 6 h under the optimal condition at 30 °C leads to a residual calcium content of 0.0004%. Due to the relatively high water temperature during this test, the optimal temperature represents a controllable temperature close to the ambient. In reality, that means the ambient temperature is the optimal temperature. The above results are close to those reported for

Table 4. GLM Regression Results between the Calcium Content after 6 h and the Demineralization Variables

	df	parameter estimate C_i, C_{ii}, or C_{ij}	probability
intercept		0.1161	0.7994
X_1[b,c]	1	−1.4098	0.0007***
X_2	1	−1.3794	0.0009***
X_3	1	0.0726	0.8108
X_1X_1	1	1.1271	0.0029**
X_2X_2	1	0.3771	0.2186
X_3X_3	1	0.1303	0.6598
X_2X_1	1	1.1919	0.0114*
X_3X_1	1	−0.0527	0.8939
X_3X_2	1	−0.1222	0.7578
R^2		0.8760	

[a] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. [b] X_1, dimensionless solution to solid ratio = (solution to solid ratio − 9 mL/g)/(12.567 mL/g − 9 mL/g); X_2, dimensionless HCl concentration = (HCl concentration − 1.1 N)/(1.64 N − 1.1 N); X_3, dimensionless reaction temperature = (reaction temperature − 65 °C)/(85.8 °C − 65 °C). [c] Calcium content (%) = $C_0 + (C_1X_1) + (C_2X_2) + (C_3X_3) + (C_{11}X_1X_1) + (C_{22}X_2X_2) + (C_{33}X_3X_3) + (C_{21}X_2X_1) + (C_{31}X_3X_1) + (C_{32}X_3X_2)$.

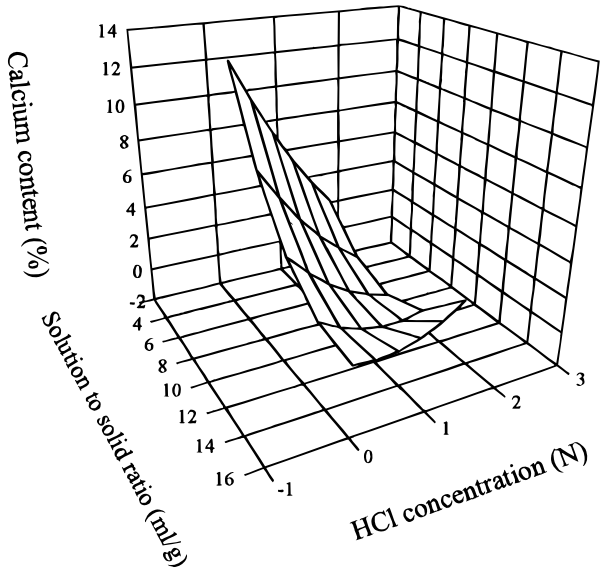

Figure 3. Calcium content of shrimp shell powder after 6 h of demineralization at 30 °C.

crawfish by No et al. (1988) and for Newfoundland pink shrimp by Shahidi and Synowiecki (1991).

Kinetics of Demineralization. Demineralization from shrimp shell powder observes a pseudo-first-order reaction kinetics. Its reaction equation can be described as $dC/dt = -kC$, where C represents the calcium content. The rate constant ranges from 0.0002 to 0.017 min^{-1} (Table 5).

The differences in crustacean species may cause variations in the results of chitin isolation (Austin et al., 1982; Brine, 1982; Shimahara et al., 1984). It is unexpected that the optimal conditions obtained in this study by the response surface methodology are close to those reported for crawfish by No et al. (1988) and for Newfoundland pink shrimp by Shahidi and Synowiecki (1991). However, results from the response surface and kinetic studies should provide more valuable information to the preparation of chitin from other crustacean shells. For instance, high deproteinization temperature could compensate for a low NaOH concentration and a low alkaline solution to solid ratio. The optimal solution to solid ratio of 9 mL/g for demineralization implies that a further increase in acid amount would be an unnecessary burden to the environment. A compromise in deproteinization or demineralization rate could also reduce the amount of alkaline or acid solution used during the isolation of chitin. These findings are useful when one hopes to improve the isolation efficiency or minimize the environmental impact of the alkaline or acid solution used in recovering chitin from crustacean wastes.

Table 5. First-Order Reaction Rate Constants during Demineralization[a]

demineralization condition			
mL of solution/g of solid	HCl concn (N)	reaction temp (°C)	rate constant k (10^{-3} min^{-1})
5.4	0.56	44.2	0.403
5.4	0.56	85.8	0.203
5.4	1.64	44.2	6.17
5.4	1.64	85.8	2.86
12.6	0.56	44.2	1.92
12.6	0.56	85.8	3.37
12.6	1.64	44.2	11.0
12.6	1.64	85.8	11.0
15.0	1.1	65	11.0
3.0	1.1	65	1.06
9.0	2.0	65	17.0
9.0	0.2	65	1.56
9.0	1.1	100	15.0
9.0	1.1	30	6.02
9.0	1.1	65	4.55
9.0	1.1	65	2.76
9.0	1.1	65	5.94
9.0	1.1	65	2.35
9.0	1.1	65	8.49
9.0	1.1	65	3.75

[a] Obtained from semilogarithmic regression of time course data. Correlation coefficients range from 0.86 to 0.98.

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Received for review September 9, 1996. Revised manuscript received February 25, 1997. Accepted February 28, 1997.[⊗] This work was supported by the National Science Council of the Republic of China, Grant NSC 84-2321-B-019-031.

JF9606870

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.