JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Chitosan and Chitin on the Separation of Membranes from Proteins Solubilized by pH Shifts Using Cod (*Gadus morhua*)

YONG LIANG,*,§ HERBERT O. HULTIN,§ AND SE-KWON KIM#

Massachusetts Agricultural Experiment Station, Department of Food Science, University of Massachusetts/Amherst, Marine Station, Gloucester, Massachusetts 01930, and Department of Chemistry, Pukyong National University, Pusan, Korea

Studies with isolated membranes and isolated membranes suspended in muscle proteins solubilized at pH 3 showed that mixing chitosan and membranes at this low pH followed by a pH adjustment to 10.5 could sediment membranes effectively at 4000*g*. In the solubilized muscle homogenate, the effectiveness of membrane removal by chitosan at 4000*g* for 15 min was molecular weight dependent. About 80% of the phospholipids and 28% of proteins were sedimented from solubilized muscle homogenate by mixing muscle homogenate (10 g of muscle tissue homogenized with 90 mL of distilled water) with 10 mL of MW 310−375k chitosan (10 mg/mL in 0.1 N HCl) before solubilizing it at pH 10.5, whereas 55% of the phospholipids and 12% of proteins were sedimented by mixing muscle homogenate with the MW 310−375k chitosan before solubilizing the homogenate at pH 3. Low molecular weight chitosans (at MW 1k or 33k) showed little effect on membrane sedimentation under the same conditions. Chitin was not useful for removing membranes at either pH 3 or 10.5, whether added before or after pH adjustment.

KEYWORDS: Chitosan; membranes; phospholipids; solubilized muscle proteins

INTRODUCTION

The phospholipids (PL) in muscle membranes represent most of the lipids present in certain lean fishes and are believed to play a critical role in lipid oxidation in these fishes (*1*). Recently, a new protein isolation procedure made it possible to remove membrane phospholipids from muscle protein to reduce lipid oxidation (*2*, *3*). By solubilizing muscle tissue at either low or high pH (e.g., pH 3 or 10.5), impurities including membrane PL could be removed from solubilized muscle protein by centrifugation. The "purified" protein is then recovered by precipitation at its isoelectric point (pH 5.5). Undeland and others (*4*) reported that up to 30% of the phospholipids could be removed from herring light muscle homogenate acidified at pH 2.7 by a centrifugation at 18000*g* for 20 min. In another study, Liang and Hultin (*5*) showed that about 37−42% of the PL in cod and herring muscle homogenates solubilized at pH 3 or 10.5 could be sedimented by centrifugation at 10000*g* for 15 min, whereas 25−36% of the PL could be removed by centrifugation at 4000*g* for 15 min. Using high-speed centrifugation to sediment membranes from solubilized fish muscle proteins is obviously inefficient and also not practical for industrial operation. Improved techniques have been developed to facilitate the sedimentation of phospholipds from solubilized muscle proteins by adding chemicals. In the presence of calcium ions and citric acid, as much as 90% of the PL could be removed by centrifugation at 4000*g* for 15 min (*6*, *7*).

Chitin is a polysaccharide of $\beta(1\rightarrow4)$-2-deoxy-2-acetamido-D-glucopyranose units derived from crustacean shells (*8*). Chitosan is a partially deacetylated product of chitin. The protonation of its $-NH_2$ group makes it a positively charged polymer. Due to its property of being positively charged, chitosan has been extensively studied as a coagulating agent for the recovery of suspended solids from processing wastewater (*9*, *10*). Most recently, many researchers have become interested in the coagulation of phospholipid vesicles and membranes by chitosan (*11*−*13*). Hwang and Damodaran (*11*) reported that chitosan (1% in 10% acetic acid) added to cheese whey at pH 6.2 followed by adjustment of the pH of cheese whey to 4.5 formed insoluble complexes with milk fat globule membrane fragments. Almost all of the milk fat globule membrane fragments were sedimented by adding 0.01−0.016% chitosan to the cheese whey followed by centrifugation at 1116*g* for 5 min. The authors suggested that the complex of chitosan and milk fat globule membrane fragments had a zero surface charge at pH 4.5. The zero surface charge allowed the flocculation and precipitation of the complexes.

It was of interest to see if chitosan could interact with the negatively charged phospholipids in muscle membranes allowing

* Address correspondence to this author at 359 FSHN Bldg., Newell Dr., Department of Food Science and Human Nutrition, University of Florida, P.O. Box 110370, Gainesville, FL 32611 [telephone (352) 392-1991, ext. 501; fax (352) 392-9467; e-mail yliang@ufl.edu].
§ University of Massachusetts/Amherst.
# Pukyong National University.

10.1021/jf0708084 CCC: $37.00
Published on Web 07/11/2007

sedimentation by low *g* force centrifugation. In this paper, the interaction between chitosan/chitin and fish membranes has been investigated, and membrane removal from solubilized cod muscle homogenates (at 4000*g* for 15 min) was examined.

MATERIALS AND METHODS

Materials. Fresh Atlantic cod (*Gadus morhua*) was purchased from John B. Wright Fish Co., Inc., Gloucester, MA, and kept on ice until used the same day. Chitosan (C-3646, minimum 85% deacetylated) and chitin were purchased from Sigma Chemical Co., St. Louis, MO. High molecular weight chitosan (MW 310−375 kDa, at least 80% deacetylated) and low molecular weight chitosan (MW 50−190 kDa, 75−85% deacetylated) were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Chitosan oligosaccharides (MW 1 and 33 kDa, 90% deacetylated) were prepared as described (*14*).

Methods. *Turbidity Study of Chitosans.* Chitosan solutions were first brought to pH 3 by HCl and then titrated by NaOH to pH 10.5 in the absence or presence of phosphate. The turbidity changes were recorded at 500 nm during the titration process as a function of pH. Adding phosphate (Na_2HPO_4) at a concentration of 8.5 mM to chitosan solutions, which is approximately equal to the phosphate concentration found in cod muscle homogenate, was done to determine if phosphate might interact with chitosan in the pH range studied.

Preparation of Isolated Membranes. Isolated membranes from cod muscle were prepared according to the method of Apgar and Hultin (*15*) with some modifications. Fresh cod fillets were minced with a model KSM90 KitchenAid mincer. Four volumes of histidine buffer (0.12 M KCl, 5 mM histidine, pH 7.3) was added to the minced cod muscle. The mixture was then homogenized by a model PT 10-35 Polytron homogenizer at speed 5 for two bursts of 30 s. The homogenized muscle tissue was centrifuged at 6000*g* for 20 min (Beckman L8-M Ultracentrifuge with TY 19 rotor, Beckman Instruments, Inc., Palo Alto, CA) with temperature setting between 0 and 10 °C. The resultant supernatant fraction was centrifuged again at 50000*g* for 20 min (Beckman L8-M Ultracentrifuge with TY 45 rotor). The sediment was then resuspended in 0.6 M KCl by a Potter-Elvehjem tissue grinder with a Teflon pestle and glass tube and centrifuged at 50000*g* for 20 min (Beckman L8-M Ultracentrifuge with TY 45 rotor) to reduce actomyosin contamination. The resultant sediment was resuspended in the histidine buffer (pH 7.3) with the Potter-Elvehjem tissue grinder and used as the isolated membrane preparation. All of the treatments in this paper were performed at low temperature by keeping the samples on ice throughout the process.

Determination of Protein. The protein content of the samples was measured according to the method of Markwell et al. (*16*).

Determination of Phospholipid. Lipid extraction and measurement of lipid phosphorus were carried out as described by Liang and Hultin (*5*). The lipid in isolated membranes, muscle homogenates, or supernatant of solubilized muscle homogenates was extracted with 1:2 chloroform/methanol (*17*). The lipid phosphorus content in the lipid extracts was then determined according to the method of Anderson and Davis (*18*). The PL content in fish samples was calculated by assuming that 31 g of lipid phosphorus was equivalent to 750 g of phospholipid.

Preparation of Muscle Homogenates and Solubilized Muscle Proteins. Fresh cod fillets were ground by a model KSM90 KitchenAid mincer (Kitchen Aid Inc., St. Joseph, MI). Ground cod muscle tissue was then homogenized at speed 5 by a model PT 10-35 Polytron homogenizer (Kinematica AG Littau, Switzerland) with 9 volumes of cold distilled water. This material was used as the muscle homogenate. To prepare the solubilized muscle proteins, the muscle homogenates were adjusted to pH 3 with hydrochloric acid. The pH-adjusted muscle homogenates were centrifuged at 10000*g* for 30 min. The resultant supernatants were used as the solubilized muscle proteins.

Membrane Sedimentation in the System of Isolated Membranes Suspended in Solubilized Muscle Proteins. Membrane sedimentation in the absence and presence of chitosan was studied in the mixture of isolated membranes and the pH 3 solubilized muscle proteins as described in **Figure 2**.

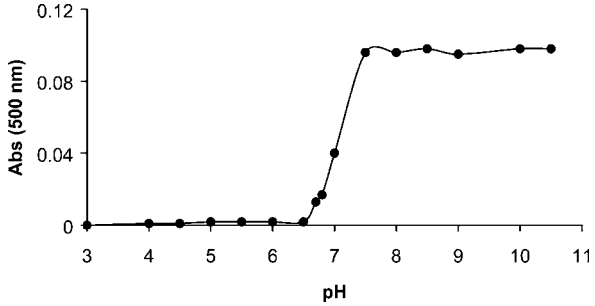

Figure 1. Titration curve of Sigma C-3646 chitosan. The absorbance at 500 nm was used to measure the turbidity of the chitosan as an indicator of its insolubility.

Statistical Analysis. The data are given as mean ± standard deviation ($n = 3$). Analysis of variance (ANOVA) was done using Microsoft Excel software (Microsoft Corp., Redmond, WA).

RESULTS

Effect of Chitosan on Sedimentation of Isolated Membranes. The two pH values of interest in the membrane sedimentation study are pH 3 and 10.5, because muscle proteins usually are solubilized at pH values near these and solubilization of muscle proteins provides the opportunity to separate membranes from muscle proteins. In a previous study, it was shown that isolated membranes can be easily sedimented at pH 3 but not at pH 10.5 (*5*). Being a polycationic polymer, chitosan has been extensively studied as a coagulating agent. Because the isolated membranes should be negatively charged, it was of interest to see if chitosan could coagulate the negatively charged isolated membranes at pH 10.5 and aid in their sedimentation.

A Sigma chitosan (C-3646) was used for the studies in this section. The chitosan was prepared from crab shells with a minimum of 85% deacetylation and an unknown molecular weight. Chitosan is insoluble in water and alkali but soluble in most organic acid solutions and some dilute inorganic acids. The most widely used acids for dissolving chitosan are acetic and formic acids (*19*). We studied the turbidity of the chitosan solution in acetic acid (10 mg/mL in 1 M acetic acid) as a function of pH (**Figure 1**). Using the turbidity of the chitosan as an index of its insolubility, the chitosan started to become insoluble above pH 6.5.

The effect of chitosan on the sedimentation of isolated membranes originally at pH 10.5 was first studied by adding a soluble chitosan preparation to the isolated membranes. The pH of the soluble chitosan preparation (10 mg/mL in 1 M acetic acid) was about 2.9. Six samples (10 mL) of isolated membranes each prepared from 40 g of muscle tissue were adjusted to pH 10.5 before treatment. One of them was centrifuged directly at 4000*g* for 15 min as a control. The other five samples were each mixed with 1 mL of chitosan preparation. One of them was centrifuged directly without pH adjustment (pH was 3.8 after mixing), and the other four were adjusted to pH 4.5, 6.5, 8.5, and 10.5, respectively, before centrifugation. After centrifugation at 4000*g* for 15 min, the protein remaining in the supernatants was determined. The percentage of protein removed by the treatments was calculated as an indicator of membrane removal (**Table 1**). The protein removed by the chitosan treatments was >90% of the original protein in the isolated membrane preparation at all of the studied pH values between 3.8 and 10.5.

The effect of an insoluble chitosan preparation on the sedimentation of isolated membranes at pH 10.5 was next studied. To make the insoluble chitosan preparation, the chitosan

Table 1. Effect of Soluble Chitosan Preparation on Sedimentation of Isolated Membranes Originally at pH 10.5

final pH after mixing[a,b]	% protein removed from the supernatant
control (no chitosan added, directly to pH 10.5)	8.8 ± 6.1
3.8	95.9 ± 0.4
4.5	96.0 ± 0.3
6.5	95.1 ± 0.2
8.5	95.5 ± 0.3
10.5	91.7 ± 0.5

[a] Control: 10 mL of isolated membranes prepared from 40 g of muscle tissue adjusted to pH 10.5 → centrifuged at 4000*g* for 15 min. [b] Treatments: 10 mL of isolated membranes prepared from 40 g of muscle tissue adjusted to pH 10.5 → mixed with 1 mL of chitosan preparation (10 mg/mL in 1 M acetic acid), pH at 3.8 after mixing → either no more pH adjustment (pH 3.8) or pH adjusted to pH 4.5, 6.5, 8.5, and 10.5, respectively → centrifuged at 4000*g* for 15 min.

Table 2. Effect of Insoluble Chitosan Preparation on Sedimentation of Isolated Membranes Originally at pH 10.5

chitosan added[a,b] (mg)	% protein removed from the supernatant
control (no chitosan added, directly to pH 10.5)	8.8 ± 6.1
0.5	12.8 ± 1.6
1	9.5 ± 4.3
5	14.4 ± 4.6
10	11.6 ± 9.2

[a] Control: 10 mL of isolated membranes prepared from 40 g of muscle tissue adjusted to pH 10.5 → centrifuged at 4000*g* for 15 min. [b] Treatments: 10 mL of isolated membranes prepared from 40 g of muscle tissue adjusted to pH 10.5 → mixed with insoluble chitosan preparation (10 mg/mL in 1 M acetic acid, adjusted to pH 10.5) at 0.5, 1, 5, and 10 mg, respectively → centrifuged at 4000*g* for 15 min.

preparation (10 mg/mL in 1 M acetic acid) was adjusted to pH 10.5. The isolated membrane samples (10 mL each, prepared from 40 g of cod fish, adjusted to pH 10.5) were mixed with different volumes of the insoluble chitosan preparation that contained 0.5, 1, 5, and 10 mg of chitosan, respectively. Because the pH adjustment of the chitosan preparation to 10.5 changed the concentration of the chitosan preparation, the amount of added chitosan preparation is expressed as mass instead of volume here. The protein removed after the centrifugation is shown in **Table 2**. Over the concentration range studied, the insoluble chitosan preparation was not effective in the sedimentation of isolated membranes.

The effect of chitosan at different pH values on the sedimentation of isolated membrane at 10.5 was studied by adding chitosan at pH 2, 6, and 10.5 to isolated membranes. Because acetic acid has a pK_a value of 4.76 (*20*), it has a buffering effect during pH adjustment around this value. To avoid the interference of this buffering effect, the chitosan preparation was prepared as 10 mg/mL in 0.1 N HCl (pH around 2) in this study. The chitosan preparation was divided into three parts. One part was adjusted to pH 6 and one part to pH 10.5, whereas the pH of the other part was not pH adjusted (pH 2.0). The isolated membranes (10 mL each, prepared from 40 g of cod fish, adjusted to pH 10.5) were mixed with chitosan preparations (10 mg of chitosan in each) of pH 2, 6, and 10.5, respectively. The pH of each mixture was recorded, and the mixtures were adjusted to pH 10.5 and centrifuged. The protein removed is shown in **Table 3**. Over 80% of the protein was still suspended in the supernatants after the centrifugation.

Table 3. Effect of Chitosan at Different pH Values on the Sedimentation of Isolated Membranes Initially at pH 10.5

pH of chitosan added[a,b]	pH after mixing	% protein removed from the supernatant
control (no chitosan added, directly to pH 10.5)		8.8 ± 6.1
10.5	10.5	11.6 ± 9.2
6	9.9	13.6 ± 6.0
2	8.8	20.1 ± 9.8

[a] Control: 10 mL of isolated membranes prepared from 40 g of muscle tissue adjusted to pH 10.5 → centrifuged at 4000*g* for 15 min. [b] Treatments: 10 mL of isolated membranes prepared from 40 g of muscle tissue adjusted to pH 10.5 → mixed with chitosan preparation (10 mg in the form of 10 mg/mL in 0.1 N HCl, adjusted to pH 2.0, 6.0, and 10.5, respectively) → readjust the mixture to pH 10.5 → centrifuged at 4000*g* for 15 min.

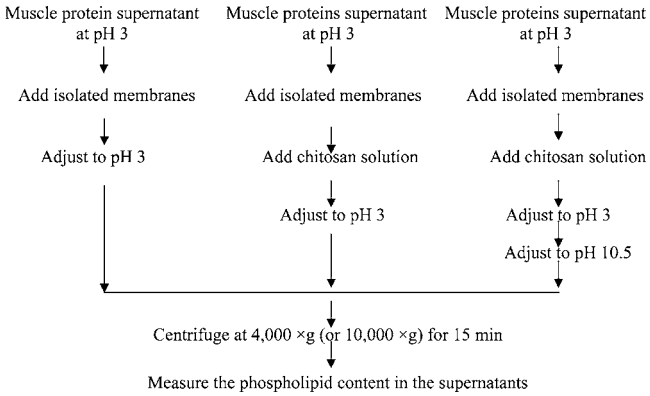

Figure 2. Schematic outline of membrane sedimentation study in the system of isolated membranes suspended in acid-solubilized muscle proteins in the absence and presence of chitosan.

Effect of Chitosan on Sedimentation of Isolated Membranes Suspended in Solubilized Muscle Proteins. To investigate the membrane sedimentation behavior in a more real environment while being able to monitor both the membranes and muscle proteins in the process, we studied membrane sedimentation in the presence of chitosan in a model system of isolated membranes suspended in solubilized muscle proteins. The solubilized muscle proteins were the supernatant fraction of solubilized cod muscle homogenate (pH 3) after a centrifugation at 10000*g* for 30 min to remove the readily sedimented membranes. The cod muscle used to prepare muscle proteins solubilized at pH 3 and that used to prepare the isolated membranes to be added were at a ratio of 1:3. This ratio was chosen because we typically recover 25−35% of the total phospholipid of cod muscle tissue in the isolated membrane fraction. The final volume of the mixture was around 150 mL. The membrane sedimentation was first studied in the absence of chitosan (**Figure 2**; **Table 4**). The pH of the isolated membrane and solubilized muscle protein mixture was brought to 3.0 after mixing. The mixture was then centrifuged at 4000*g* or 10000*g* for 15 min. The amounts of PL in the solubilized muscle protein fraction, the added isolated membranes, and the supernatants after centrifugation are shown in **Table 4**. The amounts of PL remaining in the supernatants after centrifugation at 4000*g* were higher than the amounts of PL originally in the solubilized muscle protein fraction, which means that not all of the added isolated membranes sedimented during the centrifugation. The amounts of PL remaining in the supernatants after centrifugation at 10000*g* were similar to those originally in the solubilized muscle proteins.

Table 4. Membrane Sedimentation in the System of Isolated Membranes Suspended in Solubilized Muscle Proteins at pH 3, of Isolated Membranes Suspended in Solubilized Muscle Proteins in the Presence of Chitosan at pH 3, and of Isolated Membranes Suspended in Solubilized Muscle Proteins in the Presence of Chitosan at pH 10.5

	no chitosan, pH 3[a]		chitosan, pH 3[b]		chitosan, pH 10.5[c]	
	4000*g*	10000*g*	4000*g*	10000*g*	4000*g*	10000*g*
PL[d] in 140 mL of solubilized muscle protein at pH 3[e] (mg)	60.6 ± 3.9	60.6 ± 3.9	77.5 ± 5.6	75.5 ± 5.1	77.5 ± 5.6	75.5 ± 5.1
PL in 10 mL of isolated membranes (mg)	72.7 ± 3.2	72.7 ± 3.2	102.8 ± 2.5	78.8 ± 2.7	102.8 ± 2.5	78.8 ± 2.7
PL remaining in the supernatant after centrifugation of the mixture[f] (mg)	69.7 ± 4.7	61.3 ± 4.8	86.2 ± 2.1	74.4 ± 4.6	56.0 ± 6.4	39.8 ± 2.8
% of total PL removed[g]	47.7 (87.5)[h]	54.0 (99.1)	52.2 (91.5)	51.8 (100)	68.9	74.2
% of initial PL in solubilized muscle proteins removed after centrifugation[i]					27.8	47.3

[a] Muscle protein fraction (pH 3) + isolated membranes → readjusted to pH 3 → centrifuged at 4000*g* or 10000*g* for 15 min. [b] Muscle protein fraction (pH 3) + isolated membranes → chitosan added → readjusted to pH 3 → centrifuged at 4000*g* or 10000*g* for 15 min. [c] Muscle protein fraction (pH 3) + isolated membranes → chitosan added → readjusted to pH 3 → adjusted to pH 10.5 → centrifuged at 4000*g* or 10000*g* for 15 min. [d] PL, phospholipids. [e] The solubilized muscle protein at pH 3 was the supernatant fraction prepared by centrifugation of solubilized cod muscle homogenates (pH 3) at 10000*g* for 30 min. The PL concentration was between 40.5 and 59.4 mg/mL. [f] PL remaining in the supernatant after centrifugation of the mixture was the supernatant after centrifugation (at 4000*g* or 10000*g* for 15 min) of the mixture of solubilized muscle protein at pH 3 and isolated membranes, with or without treatment of chitosan. [g] Percent of PL removed is calculated as follows: [1 − PL remaining in the supernatant/(PL in solubilized muscle proteins + PL in isolated membranes)] × 100%. [h] The numbers in parentheses are the percent of isolated membranes removed, which is calculated as follows: [1 − (PL remaining in the supernatant − PL in solubilized muscle proteins)/PL in isolated membranes] × 100%. The calculation is based on the assumption that none of the original phospholipids in the solubilized muscle proteins was removed during the centrifugation. [i] Percent of initial PL in solubilized muscle proteins removed after centrifugations, calculated as follows: (1 − PL remaining in the supernatant/PL in solubilized muscle proteins) × 100%. The calculation is based on the assumption that all of the phospholipid in the isolated membranes was removed during the centrifugation.

Three milliliters of chitosan (Sigma C-3646, 10 mg/mL in 1 M acetic acid) was added to the mixtures at pH 3 (about 150 mL), which were then either readjusted to pH 3 or adjusted to pH 10.5 before centrifugation at 4000*g* and 10000*g* for 15 min, respectively (**Figure 2**). The membrane sedimentation effect of chitosan at pH 3 and 10.5 is shown in **Table 4**. For the treatment with chitosan at pH 3, the results are similar to what happened with the system at pH 3 in the absence of chitosan; that is, the amounts of phospholipid remaining in the supernatants after centrifugation at 4000*g* were higher than the amounts of phospholipid originally in the solubilized muscle proteins, which means that some of the added isolated membranes did not sediment during the centrifugation. The amounts of PL remaining in the supernatants after centrifugation at 10000*g* were similar to that originally in the muscle supernatant fraction.

Chitosan treatment of the solubilized muscle proteins and isolated membranes mixture at pH 10.5 lowered the PL content in the resultant supernatants to values that were lower than either that originally in the solubilized muscle proteins or that in the added isolated membranes. This means that both the membranes originally in the muscle supernatant and those in the added isolated membranes were removed during the treatment.

Membrane Sedimentation in Solubilized Muscle Homogenates with Chitosan Treatments. *Turbidity of Different Molecular Weight Chitosans as a Function of pH.* Different molecular weight chitosan solutions (MW = 1 and 33 kDa, 10 mg/mL in distilled water; MW = 50−190 and 310−375 kDa, 10 mg/mL in 0.1 N HCl) were titrated from pH 3 to 10.5. The 50−190 and 310−375 kDa chitosans were dissolved in 0.1 N HCl instead of 1 M acetic acid to avoid the pH buffering interference of acetic acid during the titration and treatment of muscle homogenates with chitosan. The titration curves of chitosans in the absence and presence of phosphate at a concentration approximately equal to that found in the cod muscle homogenate, that is, 8.5 mM, are shown in **Figures 3** and **4**. Because the concentration of phosphorus in cod muscle is about 102 mM (*21*) and the preparation of muscle homogenate (10 g of fish muscle homogenized with 90 mL of distilled water) dilutes the concentration of phosphorus about 12-fold, the phosphate present in cod muscle homogenate is about 8.5 mM. The turbidity of MW = 1 kDa chitosan did not change throughout the pH range studied whether or not phosphate was present. The turbidity of MW = 33, 50−190, and 310−375 kDa chitosans started to increase at pH 7.5, 6.8, and 6.7, respectively, in the absence of phosphate and at pH 7.5, 6.9, and 6.8 in the presence of phosphate. Using turbidity as an index of the insolubility of chitosans, the chitosan with a higher MW began to become insoluble at a lower pH. Although the pH values at which chitosan began to be insoluble were similar for each molecular weight chitosan in the absence and presence of

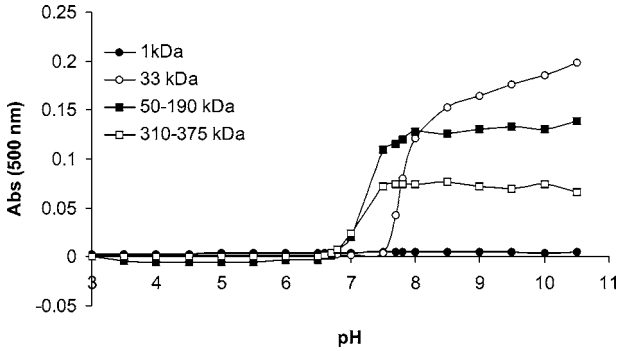

Figure 3. Titration curves of different molecular weight chitosans in the absence of phosphate. The absorbance at 500 nm was used to measure the turbidity of the chitosans as an indicator of their insolubility.

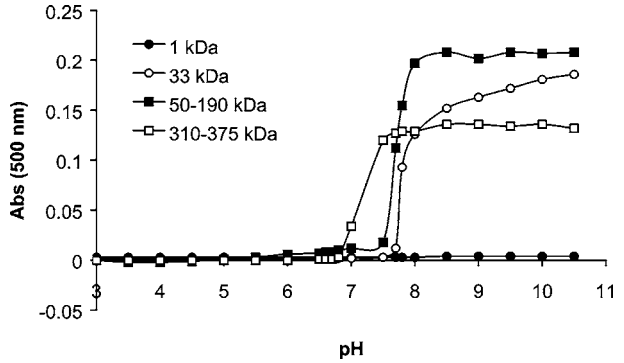

Figure 4. Titration curves of different molecular weight chitosans in the presence of phosphate (8.5 M). The absorbance at 500 nm was used to measure the turbidity of the chitosans as an indicator of their insolubility.

Table 5. Phospholipid and Protein Removed from Solubilized Muscle Homogenate by Adding Different MW Chitosans at pH 6 before pH Adjustment[a]

	chitosan added to 100 mL (mL)	add before pH 3[b]				add before pH 10.5[c]			
		MW 1k	MW 33k	MW 50k[d]	MW 310k[e]	MW 1k	MW 33k	MW 50k[d]	MW 310k[e]
PL removed (%)	0 (control)	21.5 ± 2.3	24.5 ± 2.3	27.9 ± 1.4	26.6 ± 2.1	29.8 ± 1.8	25.9 ± 1.5	36.2 ± 3.0	29.4 ± 2.4
	1	24.0 ± 1.5	24.5 ± 2.1	41.5 ± 1.6	45.9 ± 3.1	33.7 ± 1.1	26.9 ± 4.2	46.8 ± 2.7	46.3 ± 0.9
	2	24.2 ± 0.8	26.6 ± 1.5	45.4 ± 2.9	46.3 ± 2.0	39.6 ± 2.9	30.4 ± 4.4	57.4 ± 2.9	49.2 ± 1.8
	5	23.9 ± 1.5	26.3 ± 1.4	44.9 ± 1.8	48.0 ± 2.7	38.4 ± 1.7	33.6 ± 4.9	63.0 ± 3.9	67.0 ± 3.9
	10	24.8 ± 1.8	26.5 ± 0.3	48.5 ± 2.8	55.3 ± 9.0	38.9 ± 4.7	38.6 ± 2.8	78.4 ± 1.2	80.5 ± 0.8
protein removed (%)	0 (control)	10.6 ± 1.7	9.4 ± 1.8	7.7 ± 1.0	7.1 ± 1.5	7.0 ± 2.8	9.2 ± 1.5	9.8 ± 0.9	7.5 ± 3.6
	1	8.5 ± 1.6	9.7 ± 3.1	8.0 ± 3.2	11.3 ± 2.5	13.7 ± 2.5	10.4 ± 2.2	13.8 ± 1.9	19.6 ± 1.1
	2	13.4 ± 4.0	11.6 ± 1.0	9.4 ± 1.0	10.7 ± 2.1	13.9 ± 3.0	11.5 ± 3.5	13.6 ± 1.3	18.5 ± 6.3
	5	11.1 ± 6.4	10.3 ± 1.8	8.3 ± 3.9	12.7 ± 3.6	13.6 ± 2.9	12.3 ± 6.8	16.2 ± 3.0	19.7 ± 1.1
	10	16.3 ± 4.0	10.4 ± 0.6	10.2 ± 3.4	12.2 ± 3.6	14.3 ± 0.8	11.5 ± 3.6	24.8 ± 2.1	28.2 ± 3.8

[a] Chitosan preparation (10 mg/mL in 0.1 N HCl). Homogenized muscle: 10 g of cod muscle tissue + 90 g of distilled water → homogenization. Centrifugation was done at 4000*g* for 15 min. [b] Add before pH 3: pH 6.3 homogenized muscle + pH 6 different MW chitosans → adjust to pH 3 → centrifugation. [c] Add before pH 10.5: pH 6.3 homogenized muscle + pH 6 different MW chitosans → adjust to pH 10.5 → centrifugation. [d] MW 50k* = MW 50–190 kDa. [e] MW 310k* = MW 310–375 kDa.

Table 6. Effects of Different MW Chitosans at pH 6 on Membrane Sedimentation by Adding Them after pH Adjustment of Muscle Homogenates[a]

		control	MW 1 kDa	MW 33 kDa	MW 50–190 kDa	MW 310–375 kDa
PL removed (%)	add after pH 3[b]	15.6 ± 5.5	15.7 ± 2.2	20.7 ± 0.5	23.2 ± 0.2	29.6 ± 2.8
	add after pH 10.5[c]	17.2 ± 0.2	20.1 ± 1.6	21.9 ± 1.4	28.7 ± 1.5	24.1 ± 4.4
protein removed (%)	add after pH 3[b]	7.3 ± 1.3	8.6 ± 3.2	9.1 ± 1.3	11.3 ± 2.7	13.5 ± 0.7
	add after pH 10.5[c]	8.0 ± 1.8	8.7 ± 3.3	12.0 ± 4.8	12.1 ± 1.3	9.2 ± 1.3

[a] Homogenized muscle: 10 g of cod muscle tissue + 90 g of distilled water → homogenization. Ten milliliters of chitosan solution (10 mg/mL in 0.1 N HCl) was added for each treatment. The centrifugation was done at 4000*g* for 15 min. [b] Add after pH 3: homogenized muscle → pH 3 → add different MW chitosans (10 mL) at pH 6 → centrifugation. [c] Add after pH 10.5: homogenized muscle → pH 10.5 → add different MW chitosans (10 mL) at pH 6 → centrifugation.

phosphate, the readings of absorbance were higher in the presence of phosphate than in its absence for each molecular weight chitosan.

Effects of Different MW Chitosans at pH 6 on Membrane Sedimentation When Added before pH Adjustment of Muscle Homogenates. In the above sedimentation studies of isolated membranes with chitosan, it was hypothesized that soluble chitosan is necessary to interact with membranes. In this study of the effects of different MW chitosans on membrane sedimentation in solubilized muscle homogenates, chitosan preparations (10 mg/mL in 0.1 N HCl) were adjusted to pH 6 and the muscle homogenates (homogenate of 10 g of muscle + 90 mL of distilled water) were adjusted to pH 6.3 before mixing so that the chitosans would still be soluble after mixing. After mixing, the suspensions were adjusted to pH 3 or 10.5. The phospholipid and protein removed by the treatments were measured after centrifugation at 4000*g* for 15 min (**Table 5**). There was no membrane removal when chitosan of molecular weight 1 or 33 kDa was added before the muscle homogenate was solubilized at pH 3. However, PL removal increased from 27−28% (control) to 49% when 50−190 kDa chitosan was used and to 55% when 310−375 kDa chitosan was added.

For the treatments of homogenates at pH 10.5 with 50−190 and 310−375 kDa chitosans, the PL removed increased as the amounts of chitosan added to the muscle homogenates increased. By adding 10 mL of MW 50−190 and 310−375 kDa chitosan preparations at pH 6, only about 20% of the original PL remained in the supernatants. At the same time, 25−28% of the original protein (versus 8% removal for the control) was also removed (**Table 8**). The high MW chitosans removed considerable PL from solubilized muscle homogenates under these conditions, and increasing the chitosan increased the removal. Treatments at pH 10.5 with the high MW chitosans removed more PL and protein than the chitosan treatments at pH 3.

Effects of Adding Different MW Chitosans at pH 6 after pH Adjustment on Membrane Separation. The effects of adding different MW chitosans (10 mg/mL in 0.1 N HCl) on membrane sedimentation were studied by adding 10 mL of pH 6 chitosan solutions to a muscle homogenate (homogenate of 10 g of muscle + 90 mL of distilled water) solubilized at pH 3 or 10.5 followed by centrifugation at 4000*g* for 15 min. The phospholipid and protein removed by the treatments are shown in **Table 6**. The membrane removal observed by adding MW 50−190 and 310−375 kDa chitosans after pH adjustment of muscle homogenates was not as effective as adding the chitosans before pH adjustment as shown in **Table 5**.

Effects of Different MW Chitosans at pH 3 on Membrane Sedimentation Added before pH Adjustment of Muscle Homogenates. Because chitosan was chosen for membrane removal study basically due to its polycationic polymer property, chitosan having more positive charges might cause more interaction of chitosan with the membranes and remove more membranes. The pK_a value of the chitosan glucosamine residues is about 6.3 (*11*), so chitosan at pH 3 should be more positively charged than at pH 6. In this study, the effects of different MW chitosans (10 mg/mL in 0.1 N HCl) on membrane sedimentation were studied by adding 10 mL of chitosan solutions at pH 3 to muscle homogenates (homogenate of 10 g of muscle + 90 mL of distilled water) at pH 6.3. The mixtures of chitosan and muscle homogenate were then adjusted to pH 3 or 10.5, respectively, and centrifuged at 4000*g* for 15 min. The phospholipid and protein removed by the treatments are shown in **Table 7**. At 10 mL of chitosan, PL removal on treatment with chitosans at pH 3 (as shown in **Table 7**) was not significantly different ($p \leq 0.05$) from that with the treatment of chitosans at pH 6 (as shown in **Table 5**) no matter whether the muscle homogenate was solubilized at pH 3 or 10.5 after mixing with the chitosans.

Effect of Chitin on Membrane Sedimentation in Solubilized Muscle Homogenates. The effect of chitin on membrane

Table 7. Effects of Different MW Chitosans at pH 3 on Membrane Sedimentation by Adding Them before pH Adjustment of Muscle Homogenates[a]

		control	MW 1 kDa	MW 33 kDa	MW 50–190 kDa	MW 310–375 kDa
PL removed (%)	add before pH 3[b]	32.0 ± 1.7	37.0 ± 2.1	39.8 ± 1.6	45.5 ± 1.9	55.2 ± 2.0
	add before pH 10.5[c]	30.6 ± 1.0	35.1 ± 2.4	38.7 ± 0.5	84.4 ± 1.9	86.2 ± 2.2
protein removed (%)	add before pH 3[b]	7.9 ± 1.0	7.4 ± 4.6	8.3 ± 2.0	9.4 ± 1.5	14.0 ± 2.8
	add before pH 10.5[c]	6.1 ± 4.2	8.0 ± 5.9	7.8 ± 2.5	30.0 ± 3.8	38.8 ± 1.8

[a] Homogenized muscle: 10 g cod muscle tissue + 90 g distilled water → homogenization. Ten milliliters of chitosan solution (10 mg/mL in 0.1 N HCl) was added for each treatment. The centrifugation was done at 4000*g* for 15 min. [b] Add before pH 3: pH 6.3 homogenized muscle + pH 3 different MW chitosans → adjust to pH 3 → centrifugation. [c] Add before pH 10.5: pH 6.3 homogenized muscle + pH 3 different MW chitosans → adjust to pH 10.5 → centrifugation.

Table 8. Effect of Chitin on Membrane Sedimentation by Adding It before and after Adjustment of the pH of Muscle Homogenates to 3[a]

treatment	% PL removed	% protein removed
control (no chitin)	25.8 ± 4.8	6.5 ± 1.6
add before pH 3[b] (100 mg)	26.5 ± 2.2	9.5 ± 1.6
add before pH 3[b] (20 mg)	26.8 ± 1.9	9.2 ± 2.1
add after pH 3[c] (100 mg)	29.8 ± 4.4	8.2 ± 3.9
add after pH 3[c] (20 mg)	25.4 ± 4.0	9.8 ± 2.1

[a] Homogenized muscle: 10 g of cod muscle tissue + 90 g of distilled water → homogenization. The centrifugation was done at 4000*g* for 15 min. [b] Add before pH 3: homogenized muscle + chitin → pH 3 → centrifugation. [c] Add after pH 3: homogenized muscle → pH 3→ chitin added → pH 3 → centrifugation.

Table 9. Effect of Chitin on Membrane Sedimentation by Adding It before and after Adjustment of the pH of Muscle Homogenates to 10.5[a]

treatment	PL removed (%)	protein removed (%)
control (no chitin)	30.0 ± 6.4	6.3 ± 3.4
add before pH 10.5[b] (100 mg)	37.0 ± 2.1	10.6 ± 1.1
add before pH 10.5$_b$ (20 mg)	36.0 ± 5.6	7.4 ± 2.8
add after pH 10.5[c] (100 mg)	36.7 ± 1.2	6.9 ± 2.4
add after pH 10.5[c] (20 mg)	36.2 ± 2.2	6.6 ± 3.6

[a] Homogenized muscle: 10 g of cod muscle tissue + 90 g of distilled water → homogenization. The centrifugation was done at 4000g for 15 min. [b] Add before pH 10.5: homogenized muscle + chitin → pH 10.5 → centrifugation. [c] Add after pH 10.5: homogenized muscle → pH 10.5→ chitin added → pH 10.5 → centrifugation.

sedimentation was studied by adding 20 and 100 mg of chitin powder before or after the proteins of muscle homogenates (homogenate of 10 g of muscle + 90 mL of distilled water) were solubilized at pH 3 or 10.5. The chitin masses of 20 and 100 mg are equal to the masses of chitosan in 2 and 10 mL of the solutions (10 mg/mL) used in the above studies with chitosan. The PL and protein removed by the treatments are shown in **Tables 8** and **9**. Additional membrane removal compared to a control was negligible at pH 3 and small at pH 10.5.

DISCUSSION

The membrane sedimentation effect of chitosan was studied at three levels, that is, its effect on sedimentation of isolated membranes, of isolated membranes suspended in solubilized muscle proteins, and of the membranes naturally present in solubilized muscle homogenate.

A soluble chitosan preparation (dissolved in 1 M acetic acid) was effective in sedimenting isolated membranes originally at pH 10.5 over a pH range of 3.8−10.5 (**Table 1**), whereas adding an insoluble chitosan preparation was not (**Table 2**). The pH of the mixture after the addition of the soluble chitosan preparation (pH 2.9) to the isolated membranes at pH 10.5 was 3.8. It was shown in our previous studies that isolated membranes that experienced a low pH treatment could be easily sedimented (*5*). Thus, the sedimentation of isolated membranes by treatment with a soluble chitosan preparation might be solely due to the pH changes caused by adding the chitosan solution even though the pH was subsequently raised to as high as 10.5. Alternatively, a synergistic effect of the low pH and chitosan interaction with isolated membranes at low pH could have been responsible for the sedimentation of isolated membranes. When the chitosan solution was added at pH 2 (dissolved in 0.1 N HCl) to isolated membranes at pH 10.5 (**Table 3**), little sedimentation of isolated membranes was observed. In this case, the pH of the mixture was 8.8 instead of the pH attained when chitosan was dissolved in acetic acid (pH 3.8). Because the Sigma chitosan started to be insoluble at pH 6.5−6.7 (**Figure 1**), the chitosan should have been insoluble at pH 8.8. The insoluble form of chitosan might not interact with isolated membranes and thus had no effect on the sedimentation of the isolated membranes.

Taking all of the facts above into consideration, it is proposed that it is not the pH of the chitosan added, but the pH of the mixture after the addition of chitosan that played the critical role in the sedimentation of isolated membranes in this study. A low pH is needed to sediment the isolated membranes due to either the normal effect of low pH on membrane sedimentation and/or the effect of low pH on the solubilization of the chitosan, which is necessary for chitosan to interact and aggregate with membranes, that makes the removal of the membranes from suspension easier. A high pH renders the chitosan noncharged and insoluble, which prevents its interaction with membranes and causes it to have no effect on the sedimentation of isolated membranes.

When membrane sedimentation was studied at the second level, that is, in the system of "isolated membranes suspended in solubilized muscle proteins", in the presence of chitosan at pH 3, results were similar to that obtained in the system without chitosan (**Table 4**). The presence of chitosan had little effect at pH 3. However, adding chitosan to the system at pH 3 followed by pH adjustment of the resulting mixture to 10.5 removed 69−75% of the total membranes (isolated membranes + membranes in the solubilized muscle protein fraction) in the system (**Table 4**). The data indicate that not only the added isolated membranes but also some of the membranes in the solubilized muscle protein fraction were removed. If it is assumed that all of the added isolated membranes were removed, then 28 and 47% of the membranes originally in the solubilized muscle protein fraction were removed at 4000*g* and 10000*g* for 15 min, respectively. The solubilized muscle protein fraction had been previously centrifuged at 10000*g* for 30 min.

Because the membranes in the solubilized muscle protein fraction could not be sedimented efficiently by a centrifugation at 4000*g* at pH 10.5 (*5*), the removal of membranes from the solubilized muscle protein fraction in the presence of chitosan at pH 10.5 should be due to the interaction between the chitosan

and membranes in the solubilized muscle protein fraction. This interaction could cause the cosedimentation of the chitosan and membranes in the solubilized muscle protein fraction after adjustment of the pH of the mixture to 10.5. As the membranes of the solubilized muscle protein fraction (those present before the isolated membranes were added) could not be sedimented at 4000*g* for 15 min if the chitosan was added to the system at pH 3 without further adjustment to pH 10.5 (**Table 4**), the interaction between chitosan and membranes itself was not enough to sediment these membranes. A later pH adjustment to 10.5 was probably required to insolubilize a chitosan and membranes complex.

The importance of the information obtained here is that it aids in designing techniques for removing membranes from muscle homogenates with chitosan; that is, the chitosan and muscle homogenates should be mixed at a low pH (pH <6.5) that will not insolubilize the chitosan. This is the prerequisite for the interaction between chitosan and membranes. The pH of the mixture should then be adjusted to a pH that allows the cosedimentation of membranes and chitosan from the solubilized muscle homogenate.

Four different MW chitosans were used in the studies of level three, that is, the studies with cod muscle homogenate. A solubility study of the chitosans as a function of pH showed that the chitosan of MW 1000 was soluble throughout the studied pH range, whereas the three higher MW chitosans started to precipitate at pH values at 6.7 or higher (**Figures 3** and **4**). Because the pK_a value of the glucosamine residues is about 6.3 (*11*), chitosan loses its charges rapidly when the pH increases around the pK_a. Most likely, loss of charge causes the precipitation of high MW chitosans. The higher readings of the turbidity of chitosans in the presence of phosphate compared with the readings in its absence could be due to the coprecipitation of chitosan and phosphate (**Figures 3** and **4**). Possibly, the positively charged chitosan was cross-linked by the negatively charged phosphate at low pH and the insolubilization of chitosan at high pH caused the phosphate to precipitate with the polymer during the titration from pH 3 to 10.5. Because the phosphate groups of membrane PL also bear negative charges, it is reasonable to postulate that the phosphate groups of membrane PL can interact with chitosan at low pH and then cosediment with chitosan at high pH.

The effect of chitosans on membrane sedimentation in solubilized muscle homogenates was studied by mixing muscle homogenates at pH 6.3 with chitosan solutions at pH 6, so that the chitosans were still soluble after the mixing. Adding 1 and 33 kDa chitosans at pH 6 did not remove much membrane phospholipids at the concentrations studied whether the pH of the muscle homogenates was adjusted to 3 or 10.5 after the addition of chitosans (**Table 5**). Adding 50−190 and 310−375 kDa chitosans at pH 6 to muscle homogenates at pH 6.3 caused considerable membrane removal after pH adjustment. The pH adjustment of the mixture of the two high MW chitosans and muscle homogenates to 10.5 removed more membrane phospholipids than pH adjustment to 3 (**Table 5**). Hwang and Damodaran (*11*) reported that adding chitosan (1% in 10% acetic acid) to cheese whey at pH 6.2 followed by adjustment of the pH of cheese whey to 4.5 formed insoluble complexes between chitosan and milk fat globule membrane fragments. Almost all of the milk fat globule membrane fragments were sedimented by adding 0.01−0.016% chitosan to the cheese whey followed by centrifugation at 1116*g* for 5 min. The authors suggested that the complex of chitosan and milk fat globule membrane fragments had a zero surface charge at pH 4.5. The zero surface charge allowed the flocculation and precipitation of the complexes. About 5.8% of the total protein was lost during the chitosan treatment. These unrecovered proteins were not major proteins of the cheese whey. Although the cheese whey system is very different from muscle homogenates solubilized at pH 3 or 10.5, the study with cheese whey showed that chitosan could interact with membranes selectively in the presence of milk proteins.

Although charge neutralization is an explanation for chitosan-induced aggregation of certain negatively charged particles, the flocculation of *Escherichia coli* with chitosan was attributed to the bridging effect of chitosan (*22*). In addition, in the studies of polymer-induced flocculation, it was proposed that the flocculation induced by low molecular weight charged polymers is mainly attributed to charge neutralization, whereas flocculation induced by high molecular weight charged polymers normally is due to a bridging mechanism (*13*, *23*). The efficiency of flocculation induced by charge neutralization does not depend on the molecular weight of the polymer, whereas the efficiency of flocculation induced by bridging is related to the molecular weight of the polymer (*13*). A polymer with high molecular weight is normally a better bridging agent than a polymer with low molecular weight. Because the efficiency of membrane removal from muscle homogenates was dependent on the molecular weight of the chitosans, it may indicate that the chitosans effected this removal by a bridging mechanism. It is possible that a portion of the membranes in the muscle homogenates aggregated to a critical size through the "bridges" of high molecular weight chitosan and sedimented after the muscle homogenates had been solubilized at pH 3. The higher efficiency of high molecular weight chitosan than low molecular weight chitosan could be attributed to its higher total charge per molecule and larger radius of gyration, which increased its chance to interact with more of the negatively charged membranes.

When the pH is adjusted to 10.5 after mixing chitosans at pH 6 with muscle homogenates at pH 6.3, a much greater quantity of membrane phospholipids was removed with MW 50−190 and 310−375 kDa chitosans than when the pH was adjusted to 3 after the mixing. What was observed here might be similar to what happened in the study with the model system of isolated membranes suspended in solubilized muscle proteins in the presence of chitosan; that is, the soluble chitosan interacted with membranes at low pH and then induced membrane sedimentation when the chitosan became insoluble at pH 10.5. Because low molecular weight chitosan has a higher solubility compared with high molecular weight chitosan at pH 10.5, not much membrane removal was observed by the treatment with 1 and 33 kDa chitosans. About 78−80% of the original PL and 25−30% of the original protein were removed by adding 10 mL of MW 50−190 or 310−375 kDa chitosans to muscle homogenates (10 g of muscle tissue homogenized with 90 mL of distilled water) followed by pH adjustment to 10.5 compared with 30−35 and 8−10% removal of the original PL and protein in the control. This shows that membrane removal by treatment with the high MW chitosans was selective for PL versus protein.

Adding chitosans at pH 6 after muscle homogenates had been solubilized at pH 3 did not remove much membrane phospholipid under the studied conditions (**Table 6**). Most likely, the chitosan did not interact with membrane PL at pH 3 as extensively as it interacted with membrane PL at pH near 6.3. One possibility is that membranes might not have sufficient negative charge to interact with chitosan at pH 3. When chitosan

at pH 6 was added after muscle homogenate had been solubilized at pH 10.5, small insoluble globs of chitosan were found at the bottom of the centrifuge bottles after centrifugation. Most likely, the chitosan precipitated after being added to the muscle homogenate at pH 10.5. The aggregated chitosan may have physically trapped some of the muscle homogenate. Because the chitosan showed there is no membrane removal effect if it was added to the muscle homogenate (pH 6.3) after the homogenate had been solubilized at either pH 3 or 10.5, this may suggest that chitosan requires a more or less intact membrane with which to react initially. Perhaps the membranes were aggregated at low pH and broken up with solubilized protein and PL at high pH, which prevented them from interacting with chitosan. This might also explain why the high molecular weight chitosans were more efficient in sedimenting membranes than the low molecular weight ones. The low molecular weight chitosans are too small to cover a significant portion of the membrane, giving therefore very limited, if any, protection. The complex of membrane and high molecular weight chitosan at high pH might be a stabilizing effect.

Because chitosan is more positively charged at pH 3 than at pH 6, it might be expected that chitosan at pH 3 should be more reactive with membranes and result in more membrane sedimentation compared to pH 6. However, the study showed that there was no significant difference ($p \leq 0.05$) in membrane removals by adding chitosan at pH 3 (**Table 7**) or at pH 6 (**Table 5**). The pH after mixing 10 mL of a pH 3 chitosan preparation (10 mg/mL) and a muscle homogenate sample at pH 6.3 (10 g of muscle tissue homogenized with 90 mL of distilled water) was about 5.9 and only slightly lower (about 0.2 pH unit) than that of mixing 10 mL of a pH 6 chitosan preparation with a muscle homogenate at pH 6.3. This is most likely due to the high buffering capacity of the muscle homogenate. It might be that the pH of the mixture of chitosan and muscle homogenate instead of the pH of the added chitosan was more important in the interaction between membranes and chitosan. Another possibility is that the charge of chitosan at pH 6 is sufficient to cause an electrostatic interaction between the chitosan and muscle protein. According to the Henderson−Hasselbach equation, about 76% of the $-NH_2$ groups of chitosan are ionized at pH 6. This is based on the estimation of the pK_a of chitosan being about 6.5.

In general, the membrane sedimentation effect of chitosan is molecular weight dependent. The higher the molecular weight of the chitosan used, the more membrane was sedimented. Oligosaccharides of chitosan had limited effect on membrane sedimentation. Chitosan showed the membrane removal effect only if it was added to the muscle homogenate (pH 6.3) before the homogenate was solubilized at either pH 3 or 10.5. Most likely, the chitosan interacted with the membranes by electrostatic interaction and/or bridging effect, and then they aggregated to a critical size that could be sedimented at 4000*g* after solubilization of the muscle protein at pH 3. The insolubilization of chitosan at pH 10.5 facilitated the sedimentation of the aggregated chitosan and membranes even better than at pH 3 when the muscle protein was solubilized at pH 10.5 after treatment with chitosans. To compare to the treatment with Ca^{2+} and citric acid (*6*, *7*), the treatment with MW 310−375 k chitosan at 10 mL showed good and similar results in membrane sedimentation as did treatment with Ca^{2+} and citric acid (e.g., 10 mM Ca^{2+} and 1 mM citric acid) when the homogenate was solubilized at pH 10.5, but the chitosan was not as good when the muscle homogenate was brought to pH 3. However, it should be noted that 100 mg of chitosan (10 mL of 10 mg/mL) is a lot of chitosan considering that this is a treatment for 10 g of muscle tissue (10 g of muscle tissue + 90 mL of distilled water). The markedly high cost of chitosan may severely limit the use of this treatment to remove membrane phospholipids. In addition, the time required to solubilize chitosan with acid and the difficulty of handling the highly viscous chitosan solutions are additional drawbacks to its use. However, despite these disadvantages, chitosan could still be useful in removing phospholipids from muscle proteins as a laboratory procedure.

LITERATURE CITED

(1) Hultin, H. O. Role of membranes in fish quality. In *Fish Quality—Role of Biological Membranes*; Jessen, F., Ed.; Nordic Council of Ministers: Copenhagen, Denmark, 1995; pp 1−35.

(2) Hultin, H. O.; Kelleher, S. D. Process for isolating a protein composition from a muscle source and protein composition. U.S. Patent 6,005,073, Dec 21, 1999.

(3) Hultin, H. O.; Kelleher, S. D. High efficiency alkaline protein extraction. U.S. Patent 6,136,959, Oct 24, 2000.

(4) Undeland, I.; Kelleher, S. D.; Hultin, H. O. Recovery of functional proteins from herring (*Culpea harengus*) light muscle by an acid or alkaline solubilization process. *J. Agric. Food Chem.* **2002**, *50*, 7371−7379.

(5) Liang, Y.; Hultin, H. O. Effect of pH on sedimentation of cod muscle membranes. *J. Food Sci.* **2005**, *70*, C164−C172.

(6) Liang, Y.; Hultin, H. O. Separation of membranes from acid-solubilized fish muscle proteins with the aid of calcium ions and organic acids. *J. Agric. Food Chem.* **2005**, *53*, 3008−3016.

(7) Liang, Y.; Hultin, H. O. Separation of muscle membrane from alkali-solubilized fish muscle proteins. *J. Agric. Food Chem.* **2005**, *53*, 10012−10017.

(8) Cho, Y.-W.; Jang, J.; Park, C. R.; Ko, S.-W. Preparation and solubility in acid and water of partially deacetylated chitins. *Biomacromolecules* **2000**, *1*, 609−614.

(9) Bough, W. A. Coagulation with chitosan-an aid to recovery of by-products from egg breaking wastes. *Poult. Sci.* **1975**, *54*, 1904−1912.

(10) Bough, W. A.; Shewfelt, A. L.; Salter, W. L. Use of chitosan for the reduction and recovery of solids in poultry processing waste effluents. *Poult. Sci.* **1975**, *54*, 992−1000.

(11) Hwang, D.; Damodaran, S. Selective precipitation and removal of lipids from cheese whey using chitosan. *J. Agric. Food Chem.* **1995**, *43*, 33−37.

(12) Fang, N.; Chan, V.; Mao, H.; Leong, K. W. Interactions of phospholipid bilayer with chitosan: effect of molecular weight and pH. *Biomacromolecules* **2001**, *2*, 1161−1168.

(13) Strand, S. P.; Vandvik, M. S.; Varum, K. M.; Ostgaard, K. Screening of chitosans and conditions for bacterial flocculation. *Biomacromolecules* **2001**, *2*, 126−133.

(14) Jeon, Y. J.; Kim, S. K. Continuous production of chitooligosaccharides using a dual reactor system. *Process Biochem.* **2000**, *35*, 623−632.

(15) Apgar, M. E.; Hultin, H. O. Lipid peroxidation in fish muscle microsomes in the frozen state. *Cryobiology* **1982**, *19*, 154−162.

(16) Markwell, M. A. K.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *87*, 206−210.

(17) Lee, C. M.; Trevino, B.; Chaiyawat, M. A simple and rapid solvent extraction method for determining total lipids in fish tissue. *J. AOAC Int.* **1996**, *79*, 487−492.

(18) Anderson, R. L.; Davis, S. An organic phosphorus assay which avoids the use of hazardous perchloric acid. *Clin. Chim. Acta* **1982**, *121*, 111−116.
(19) Li, Q.; Dunn, E. T.; Grandmaison, W. E.; Goosen, M. F. A. Applications and properties of chitosan. In *Application of Chitin and Chitosan*; Goosen, M. F. A., Ed.; Technomic Publishing: Lancaster, PA, 1997; pp 7−8.
(20) Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. In *Data for Biochemical Research*; Dawson, R. M. C., Elliott, D. C., Elliott, W. H., Jones, K. M., Eds.; Oxford University Press: New York, 1969; p 66.
(21) Nettleton, J. A. Nutrition composition of seafoods. In *Seafood Nutrition: Facts, Issues and Marketing of Nutrition in Fish and Shellfish*; Nettleton, J. A., Ed.; Osprey Books: Huntington, NY, 1985; p 211.
(22) Baran, A. A. Flocculation of cellular suspensions by polyelectrolytes. *Colloid Surf.* **1988**, *31*, 259−264.
(23) Baran, Sh.; Gregory, D. Flocculation of kaolin suspensions by cationic polyelectrolytes. *Colloid J.* **1996**, *58*, 9−14.

Received for review March 19, 2007. Revised manuscript received May 25, 2007. Accepted June 1, 2007. This study was supported in part by the Cooperative State Research, Extension, Education Service, U.S. Department of Agriculture, Massachusetts Agricultural Experiment Station, under Project MAS00834; by Grant 2002-01659 of the USDA National Research Initiative Competitive Grants Program; and by the U.S. Department of Commerce−NOAA, under Agreement NAJRG0074, M.I.T. Sea Grant College Program Grant 5700000741. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing official policies or endorsements, either expressed or implied, of the Department of Commerce−NOAA or the U.S. government.

JF0708084