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Separation of Muscle Membrane from Alkali-Solubilized Fish Muscle Proteins

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Treatment with Ca^{2+} and citric acid improved membrane removal from muscle homogenates solubilized at pH 10.5 by centrifugation at 4000*g* for 15 min. The percentage of phospholipid removed from muscle homogenates increased with increasing Ca^{2+} concentrations at 1 mM citric acid. More than 85% phospholipid and 45% protein in the muscle homogenates were removed at Ca^{2+} concentrations of >20 mM in the presence of 1 mM citric acid. At 8 mM Ca^{2+}, addition of citric acid at 5 mM improved phospholipid removal to ~78% from 58% in its absence. Because treatment with 8 mM Ca^{2+} alone can remove significant amounts of phospholipid, it is likely that Ca^{2+} played the major role in membrane removal in muscle homogenates solubilized at pH 10.5.

KEYWORDS: Alkali-solubilized muscle protein; citric acid; calcium ion; membrane; phospholipid

INTRODUCTION

Biological cellular membranes are composed of plasma membrane and membranes of cellular organelles, such as endoplasmic reticulum, lysosomes, nucleus, mitochondria, and chloroplasts. Phospholipid (PL) is a major component of the membranes, ranging from 25 to 80% in different membranes (*1*). In fish, especially in lean fish, the phospholipid in membranes is believed to be the key substrate for lipid oxidation due to its highly unsaturated fatty acid composition. Membranes have a large surface area to contact pro-oxidants in the aqueous phase of the cell, and their lipids are located near the site of electrons leaked from electron transport processes (*2*). This all suggests that membranes play a critical role in the lipid oxidation of fish. In addition, enzymes present in membranes could also cause adverse effects on fish quality, for example, trimethylamine-*N*-oxide (TMAO) demethylase (*3*, *4*).

A new process of protein isolation from muscle tissue has made it possible to separate membranes from muscle proteins (*5*, *6*). In this process, the muscle homogenate is solubilized at either alkali or acid pH, and centrifugation of the solubilized muscle homogenate sediments the membranes from the bulk of the solubilized muscle proteins. Using this process, approximately 37 and 51% of the phospholipids were removed from chicken breast muscle and from thigh and leg muscle at 15000*g* when they were solubilized at pH 2.8 (*7*). In studies with cod and herring solubilized at pH 3 or 10.5, up to 40% of the phospholipids could be removed by centrifugation at 10000*g* for 15 min (*8*). The typical centrifuges used in the food industry are those with maximal centrifugal forces of no more than 4000*g*. Centrifugation at 10000*g* or higher is not practical for membrane removal in industrial application. Improved techniques are needed for more complete removal of membranes from solubilized muscle proteins at forces as low as 4000*g*. If fish cellular membranes could be separated from the muscle proteins, the adverse effects on the quality of fish protein products could be significantly reduced.

In our studies with cod and herring solubilized at pH 3, treatment with Ca^{2+} and citric acid before solubilization of the muscle homogenate improved membrane separation (*9*). At 1 mM citric acid and 10 mM Ca^{2+}, 70−80% of the phospholipid was separated from cod muscle homogenates solubilized at pH 3, whereas 25−30% of the protein was also removed. At 8 mM Ca^{2+}, citric acid showed an optimal effect on phospholipid removal at 5 mM with 90% of the phospholipid separated, whereas 35% of the protein was removed. In this paper, treatments with Ca^{2+} and citric acid on muscle homogenates solubilized at pH 10.5 were examined for their ability to separate membranes from muscle proteins.

MATERIALS AND METHODS

Materials. Fresh Atlantic cod (*Gadus morhua*) was purchased from John B. Wright Fish Co., Inc., Gloucester, MA, on the day it was brought to port and used on the same day. Fresh Atlantic herring (*Clupea harengus*) was donated by Cape Seafoods, Inc., Gloucester, MA, on the day it was brought to port and used on the same day. The fish was kept under ice from purchase to the time used. The pH of the cod used varied from about pH 6.8 to 7.2. The pH of the herring used was in the range of 7.0−7.2.

Methods. *Preparation of Muscle Homogenate.* The fish were filleted, and the fillets were minced by a model KSM90 KitchenAid mincer (KitchenAid Inc., St. Joseph, MI). Minced muscle tissue was then homogenized by a model PT 10-35 Polytron homogenizer (Kinematica AG, Littau, Switzerland) with 9 volumes of cold distilled water at speed 5. The resultant preparation was used as the muscle homogenate. All

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of the treatments of fish samples in this paper were performed at low temperature by keeping the samples on ice throughout the process.

Preparation of Muscle Homogenate Solubilized at pH 10.5. The muscle homogenate solubilized at pH 10.5 was prepared by adjusting the pH of the muscle homogenate to 10.50 ± 0.05 using 2 N NaOH.

Determination of Protein. The protein content of the samples was measured according to the method of Markwell et al. (*10*), a modification of the Lowry procedure (*11*) for measuring samples containing membrane and lipoprotein. The protein analyzed was that in original homogenate and that remained in the supernatant after centrifugation. The percentage of protein removed by the centrifugation was then calculated by the difference of protein content between the original homogenate and the corresponding supernatant divided by the protein content in the original homogenate.

Determination of Phospholipid. Lipid extraction from samples was carried out according to the method of Lee et al. (*12*) with the modifications previously described (*9*). The lipid phosphorus content in the extracts was determined according to the method of Anderson and Davis (*13*). The phospholipid content in fish samples was then calculated by assuming that each lipid phosphorus (31 Da) corresponded to an average molecular mass of phospholipid of 750 Da. The phospholipid content analyzed was that in original homogenate and that remained in the supernatant after centrifugation. The percentage of phospholipid removed by the centrifugation was then calculated by the difference of phospholipid content between the original homogenate and the corresponding supernatant divided by the phospholipid content in the original homogenate.

Protein Analysis by Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). The SDS-PAGE protein analysis was run on precast linear 4−20% gradient minigels (ICN Biomedicals, Costa Mesa, CA) using a vertical PAGE Mini Device (Daiichi Scientific, Tokyo, Japan) as previously described (*9*). The samples were diluted at the same ratio with distilled water to ∼1 mg protein/mL before denaturation. The diluted samples were mixed with SDS-PAGE sample buffer (Owl Separation Systems, Woburn, MA) in a ratio of 3:1 and then denatured in boiling water for 3 min. Samples were then applied to the gel for running. After running, the gel was transferred into distilled water and agitated for 5 min before the water was changed. The agitation with distilled water was done three times. After washing with distilled water, EzBlue Gel staining reagent (Sigma Chemical Co., St. Louis, MO) was added after the distilled water had been poured out. The gel was agitated for another 1 h. After staining, the gel was agitated with distilled water overnight to increase the contrast. A model 365W densitometer analysis software program (Hoefer Scientific Instruments, San Francisco, CA) was used to calculate the percentage of each polypeptide band in the same lane. The molecular weight standard protein mixture was purchased from Sigma Chemical Co. (St. Louis, MO) and composed of peptides with molecular weights of 29, 45, 66, 96.4, 116, and 205 kDa.

Lipid Analysis by Thin-Layer Chromatography (TLC). Lipid analysis was performed with TLC as previously described (*9*). The lipids applied to the plates were from fish muscle homogenates with and without treatments and corresponded to the same amount of original fish muscle so that removal of phospholipids with the different treatments could be compared by the intensity of their phospholipid spots with the intensity of the phospholipid spots of sample without any treatment. The quantity of phospholipids removed by treatment was estimated by comparing the intensity of the spots on the plates from the treated samples with those that were not treated as described by Liang and Hultin (*9*). The TLC reference standard was purchased from Nu-Chek Prep, Inc. (Elysian, MN). The standard is a mixture of equal amounts of cholesterol, cholesteryl oleate, triolein, oleic acid, and lecithin and was prepared as 1 μg/μL in chloroform before application.

Determination of Consistency. Consistency of samples was measured using a Brookfield Syncro-lectric viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA) with a no. 2 spindle at 60 rpm (*9*).

Statistical Analysis. The data are given as mean ± standard deviation, $n = 3$, unless specifically noted. Analysis of variance (ANOVA) and Tukey's studentized range test were determined using a SAS program.

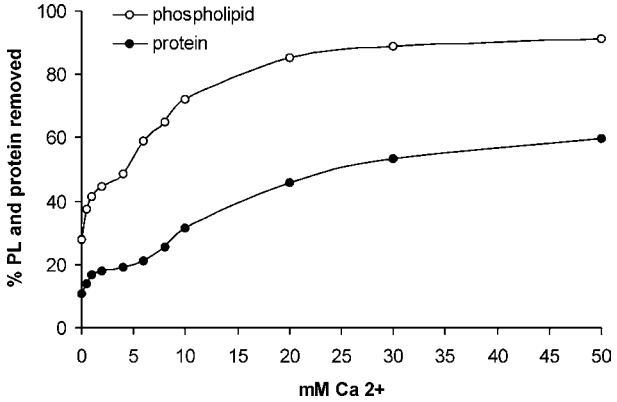

Figure 1. Effect of $CaCl_2$ concentration on membrane sedimentation in muscle homogenates solubilized at pH 10.5 in the presence of 1 mM citric acid. The pH was brought down from the original pH of 7.0 (original cod muscle pH) to pH 6.4–6.5 after the addition of 1 mM citric acid; the pH was lowered further after the adddition of different amounts of Ca^{2+} and incubation to as low as pH 5.9 at 50 mM Ca^{2+}. The protein/phospholipid analyzed was that in the original homogenate and that remained in the supernatant after centrifugation. The percentage of protein/phospholipid removed by centrifugation was calculated as (protein/phospholipid in original homogenate − protein/phospholipid in supernatant) ÷ protein/phospholipid in original homogenate × 100%.

RESULTS

Effect of Ca^{2+} Concentration on Membrane Sedimentation at pH 10.5 in the Presence of 1 mM Citric Acid. Membrane sedimentation was studied by adding different amounts of $CaCl_2$ (0−50 mM) to muscle homogenates in the presence of 1 mM citric acid. After incubation with Ca^{2+} and citric acid for 60 min, the samples were adjusted to pH 10.5 and then centrifuged at 4000*g* for 15 min. The pH was brought down from the original pH of 7.0 (original cod muscle pH) to 6.4−6.5 after the addition of 1 mM citric acid. The pH was lowered further after the addtion of different amounts of Ca^{2+} and incubation to as low as 5.9 at 50 mM Ca^{2+}. The phospholipid and protein removed by the centrifugation increased with the increase of Ca^{2+} concentration (**Figure 1**). The phospholipid remaining in the supernatant after the treatment with 50 mM Ca^{2+} was lowered to 9% of the original phospholipid, but more than half of the muscle protein was also removed at the same time.

Effect of Citric Acid Concentration on Membrane Sedimentation at pH 10.5 in the Presence of 8 mM Ca^{2+}. To study the effect of citric acid concentration on membrane sedimentation, the concentration of Ca^{2+} was set at 8 mM. This is because at this Ca^{2+} concentration, the amount of phospholipids removed was reasonably high, whereas the protein removed was not unreasonably high when 1 mM citric acid was used (**Figure 1**). This Ca^{2+} concentration should allow phospholipid removal to be sensitive to different citric acid concentrations. This study was conducted by adding different amounts of citric acid (0−10 mM) to muscle homogenates in the presence of 8 mM Ca^{2+}. After incubation for 60 min and pH adjustment to 10.5, the samples were centrifuged at 4000*g* for 15 min. The phospholipid and protein removed are shown in **Figure 2**. In the absence of citric acid and calcium (the lower points at 0 mM citric acid in **Figure 2**), the phospholipid removed was ∼26%. The phospholipid removed was raised to 58% at 8 mM Ca^{2+} in the absence of citric acid. The amount of phospholipid removed increased to 65% at 1 mM citric acid and further to 78% at 5 mM and then declined somewhat at 8−10 mM. The presence of citric acid showed a slight synergistic effect on

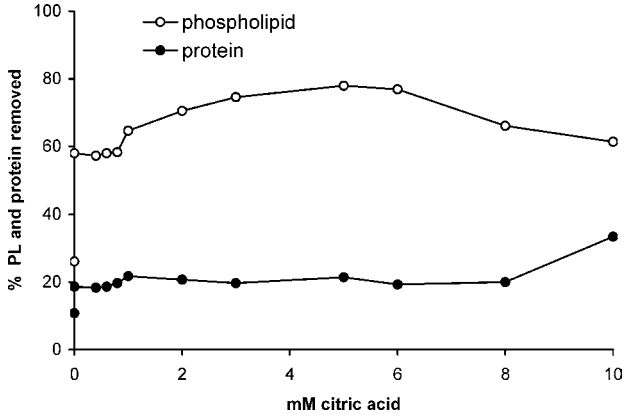

Figure 2. Effect of citric acid concentration on membrane sedimentation in muscle homogenates solubilized at pH 10.5 in the presence of 8 mM Ca^{2+}. The lower points of phospholipid and protein removed at 0 mM citric acid correspond to treatment in the absence of Ca^{2+}. The upper points of phospholipid and protein removed at 0 mM citric acid correspond to treatment in the presence of 8 mM Ca^{2+}. The protein/phospholipid analyzed was that in the original homogenate and that remained in the supernatant after centrifugation. The percentage of protein/phospholipid removed by centrifugation was calculated as (protein/phospholipid in original homogenate − protein/phospholipid in supernatant) ÷ protein/phospholipid in original homogenate × 100%.

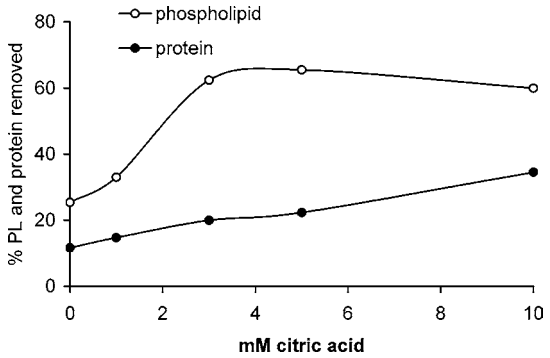

Figure 3. Effect of various concentrations of citric acid on membrane sedimentation in muscle homogenates solubilized at pH 10.5 in the presence of 8 mM $MgCl_2$. The protein/phospholipid analyzed was that in the original homogenate and that remained in the supernatant after centrifugation. The percentage of protein/phospholipid removed by centrifugation was calculated as (protein/phospholipid in original homogenate − protein/phospholipid in supernatant) ÷ protein/phospholipid in original homogenate × 100%.

membrane sedimentation. Optimal removal of phospholipid was observed at 5−6 mM citric acid.

Effect of Mg^{2+} and Citric Acid on Membrane Sedimentation at pH 10.5. The effect of the citric acid (0−10 mM) and $MgCl_2$ (0−30 mM) at a constant concentration of the other component on membrane sedimentation after incubation of the muscle homogenates for 60 min followed by pH adjustment to 10.5 was studied. The samples were then centrifuged at 4000*g* for 15 min for study of phospholipid and protein removal. The phospholipid and protein removed are shown in **Figures 3** and **4**. When citric acid was not included, treatment with Mg^{2+} at 8 mM did not give the phospholipid removal effect that treatment with Ca^{2+} at 8 mM did (**Figure 3**). Only 25% of the phospholipid was removed with 12% of the protein by the treatment of 8 mM Mg^{2+} and no citric acid. The phospholipid removed could be increased to 60−70% either at 3−5 mM citric acid in the presence of 8 mM Mg^{2+} or at 20−30 mM Mg^{2+} in

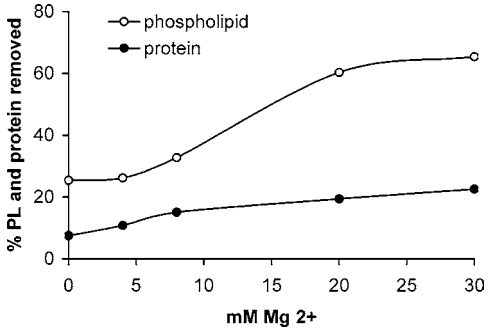

Figure 4. Effect of various concentrations of $MgCl_2$ on membrane sedimentation in muscle homogenates solubilized at pH 10.5 in the presence of 1 mM citric acid. The protein/phospholipid analyzed was that in the original homogenate and that remained in the supernatant after centrifugation. The percentage of protein/phospholipid removed by centrifugation was calculated as (protein/phospholipid in original homogenate − protein/phospholipid in supernatant) ÷ protein/phospholipid in original homogenate × 100%.

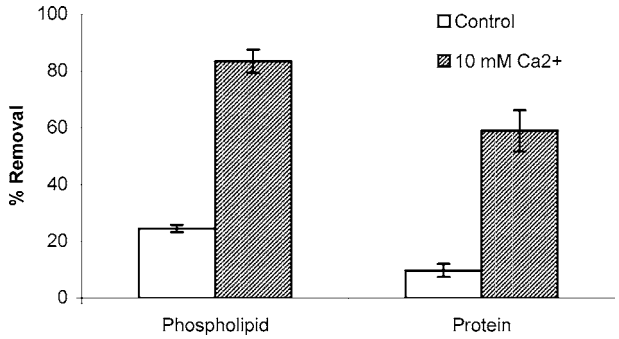

Figure 5. Effect of Ca^{2+} on membrane sedimentation by adding Ca^{2+} to muscle homogenates solubilized at pH 10.5. Control: no citric acid added, no $CaCl_2$ added. Treatment: muscle homogenate → add citric acid (1 mM) → adjust to pH 10.5 → add Ca^{2+} (10 mM) → 1 h of incubation → centrifugation at 4000*g* for 15 min. The protein/phospholipid analyzed was that in the original homogenate and that remained in the supernatant after centrifugation. The percentage of protein/phospholipid removed by centrifugation was calculated as (protein/phospholipid in original homogenate − protein/phospholipid in supernatant) ÷ protein/phospholipid in the original homogenate × 100%.

the presence of 1 mM citric acid with 20−35% protein loss (**Figures 3** and **4**).

Effect of Ca^{2+} on Membrane Sedimentation by Adding Ca^{2+} to Muscle Homogenates Solubilized at pH 10.5. Muscle homogenates (citric acid added to a final concentration of 1 mM) were adjusted to pH 10.5. $CaCl_2$ solution was then added to a final concentration of 10 mM. After 1 h of incubation, the samples were centrifuged at 4000*g* for 15 min. The phospholipid and protein removed are shown in **Figure 5**. Most of the phospholipid and a large amount of the protein were removed from the supernatants.

Effect of Ca^{2+} and Sodium Citrate on Membrane Sedimentation at pH 10.5. In the study with muscle homogenates solubilized at pH 3, we found that membrane removal efficiency may be related not only to the concentration of Ca^{2+} and the citrate group used but also to the pH value at which they exert their effect (*9*). Adding 5 mM citric acid can lower the pH of fish from 7.1 to 5.1−5.2, whereas adding sodium citrate will not significantly change the pH of cod muscle homogenates. In this sector, we studied the effect of Ca^{2+} and the citrate group on membrane removal at pH 7.1 (the pH of fresh cod tissue) before adjusting the pH of muscle homogenates to 10.5. Ten

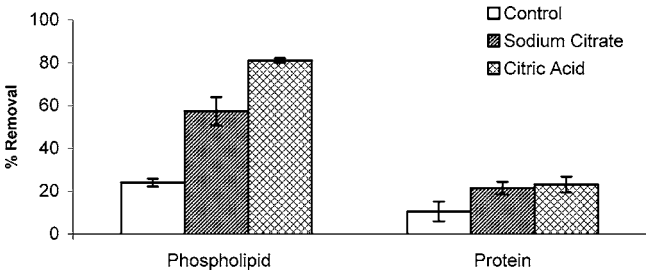

Figure 6. Effect of sodium citrate versus citric acid on membrane sedimentation in pH 10.5 solubilized muscle homogenates in the presence of 8 mM $CaCl_2$. Control: muscle homogenates solubilized at pH 10.5; no citric acid or sodium citrate added, no $CaCl_2$ added. Sodium citrate treatment: muscle homogenate (pH 7.1–7.2) → 5 mM sodium citrate (pH 7.1–7.2) → 8 mM Ca^{2+} (pH 6.9–7.1)→ 1 h of incubation → adjust to pH 10.5 → centrifugation at 4000*g* for 15 min. Citric acid treatment: muscle homogenate (pH 7.1–7.2) → 5 mM citric acid (pH 5.1–5.2) → 8 mM Ca^{2+} (pH 4.9–5.0) → 1 h of incubation → adjust to pH 10.5 → centrifugation at 4000*g* for 15 min. The protein/phospholipid analyzed was that in the original homogenate and that remained in the supernatant after centrifugation. The percentage of protein/phospholipid removed by centrifugation was calculated as (protein/phospholipid in original homogenate − protein/phospholipid in supernatant) ÷ protein/phospholipid in original homogenate × 100%.

grams of cod muscle tissue was mixed with 90 mL of 5.6 mM sodium citrate solution at pH 7.1. After homogenization, $CaCl_2$ solution was added to the muscle homogenates to a final concentration of 8 mM. After 1 h of incubation, the samples were adjusted to pH 10.5 and then centrifuged at 4000*g* for 15 min. The phospholipid and protein removed are compared with a treatment of $CaCl_2$ and citric acid done at the same time (**Figure 6**). Although considerable phospholipid was removed on preincubation with sodium citrate and Ca^{2+} prior to adjustment to pH 10.5 and centrifugation, the phospholipid removed (57%) was significantly lower ($p < 0.05$) compared to that removed (81%) by the treatment of $CaCl_2$ and citric acid. The difference might reflect the effect of the incubation pH on membrane removal. About 10% more protein was also removed by the treatment with either citric acid or sodium citrate compared with the control. The pH of muscle homogenates with sodium citrate was 7.1−7.2, and adding 8 mM Ca^{2+} lowered the pH to 6.9−7.1.

SDS-PAGE Analysis. Samples of muscle homogenates with or without citric acid and Ca^{2+} treatments at pH 10.5 were analyzed by SDS-PAGE to see the effect of the treatments on the change of muscle proteins in the supernatants after centrifugation of the solubilized proteins (details in **Figure 7**). The samples were applied at a volume that corresponded to the same amount of starting muscle homogenate, so a direct comparison can be made as to the relative concentrations of the polypeptides among the bands in the different lanes. The 105 kDa band (possibly the Ca^{2+}-ATPase of the membranes) of the sample with citric acid and Ca^{2+} treatment (sample C) had a lighter density compared to the other samples. Estimated by scanning, the quantity of the 105 kDa polypeptide in sample C represented ∼36.4% of that in sample A. This figure is higher than the amount (22.2% for the treatment with 5 mM citric acid and 8 mM Ca^{2+} in **Figure 2**) of phospholipid remaining in the supernatants with the same treatments measured by a phospholipid determination.

Lipid Analysis by TLC. Samples of muscle homogenates with or without citric acid and Ca^{2+} treatment at pH 10.5 were prepared the same as before (details in **Figure 8**). The samples

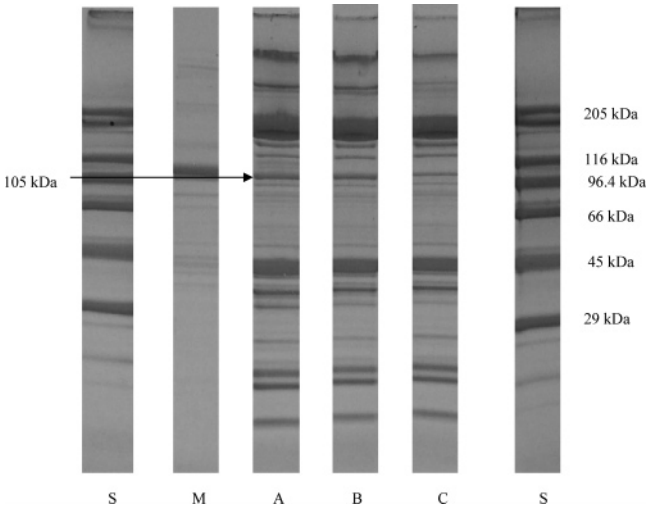

Figure 7. SDS-PAGE analysis of proteins remaining in the sample supernatants with or without citric acid and Ca^{2+} treatments. S, molecular weight standard; M, isolated membrane preparation; A, sample of muscle homogenate (no pH adjustment, no centrifugation); B, supernatant after treatment of muscle homogenate → pH 10.5 → centrifuge; C, supernatant after treatment of muscle homogenate → 5 mM citric acid + 8 mM Ca^{2+} → pH 10.5 → centrifuge. All of the centrifugations were done at 4000*g* for 15 min.

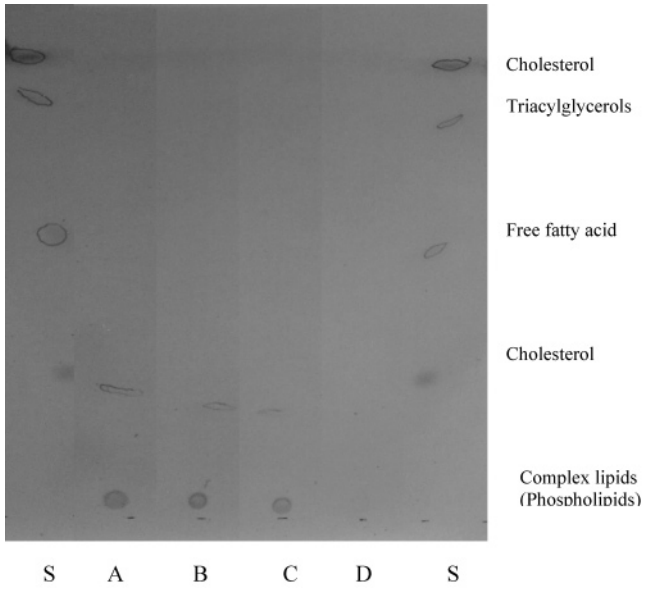

Figure 8. TLC analysis of lipids in the sample supernatants with or without citric acid and Ca^{2+} treatments. S, lipids standard; sample A, sample of muscle homogenate (no pH adjustment, no centrifugation); sample B, sample of muscle homogenate at pH 10.5 (no centrifugation); sample C, supernatant after the treatment of muscle homogenate → pH 10.5 → centrifuge; sample D, supernatant after the treatment of muscle homogenate → 5 mM citric acid + 8 mM Ca^{2+} → pH 10.5 → centrifuge. The cholesterol spots of samples A–C were too light to be scanned, so the spots were marked by pencil to highlight them. All of the centrifugations were done at 4000*g* for 15 min.

were then extracted with 1:2 chloroform/methanol overnight. Chloroform aliquots of each sample were applied for TLC analysis. The samples were applied at equal volumes so that they corresponded to the same amount of starting muscle homogenate. Thus, direct comparisons among the lanes of samples on the plate reflect the effect of treatments on the lipids. The lipids in samples A−C were mostly phospholipids with

Table 1. Consistency of Solubilized Muscle Homogenates in the Presence and Absence of Citric Acid and Ca^{2+} ($n = 6$)[a]

	consistency (mPa·s)
no citric acid, no Ca^{2+}	36.0 ± 3.4 a
1 mM citric acid + 10 mM Ca^{2+}	102.9 ± 12.0 b
5 mM citric acid + 8 mM Ca^{2+}	133.3 ± 9.7 c

[a] Muscle homogenates in the presence or absence of citric acid and Ca^{2+} → incubate for 1 h → adjust pH to 10.5 → measure the consistency. Values with different letters are significantly different from each other.

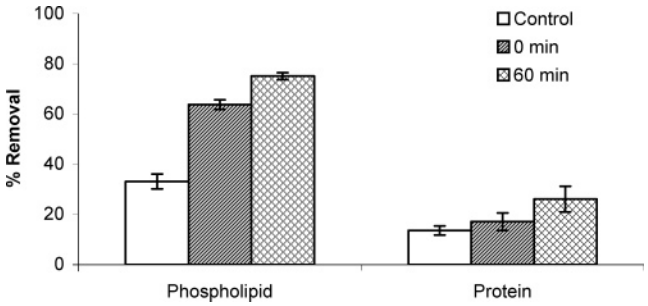

Figure 9. Effect of $CaCl_2$ and citric acid on membrane sedimentation in herring muscle homogenates solubilized at pH 10.5. Control: herring muscle homogenates solubilized at 10.5, no citric acid added, no $CaCl_2$ added. Treatment, herring muscle homogenate (5 mM citric acid) → add 8 mM Ca^{2+} → incubate for 0–60 min → adjust to pH 10.5 → centrifugation at 4000*g* for 15 min. Total lipids were extracted with 1:2 chloroform/methanol. The protein/phospholipid analyzed was that in the original homogenate and that remained in the supernatant after centrifugation. The percentage of protein/phospholipid removed by the centrifugation was calculated as (protein/phospholipid in original homogenate − protein/phospholipid in supernatant) ÷ protein/phospholipid in the original homogenate × 100%.

cholesterol as a minor component. The phospholipid spot of sample D was very light, and no cholesterol spot was detected. Using the simple approximation method, the phospholipids in sample D were ~10% or less of those in sample A.

Consistency Study. The consistency of solubilized (at pH 10.5) muscle homogenates in the presence or absence of citric acid and Ca^{2+} is shown in **Table 1**. The consistency of the muscle homogenates increased severalfold on treatment with 1 mM citric acid and 10 mM Ca^{2+} or treatment with 5 mM citric acid and 8 mM Ca^{2+} followed by pH adjustment to 10.5. Because both of the treatments increased consistency while improving phospholipid removal, it can be concluded that Ca^{2+} and citric acid aided in membrane sedimentation by mechanisms other than lowering the consistency of the system, allowing for easier sedimentation.

Effect of Ca^{2+} and Citric Acid on Membrane Sedimentation in Solubilized Herring Muscle Homogenates at pH 10.5. Membrane sedimentation in solubilized (at pH 10.5) herring muscle homogenates was studied in the presence of 5 mM citric acid and 8 mM Ca^{2+}. After incubation with citric acid and Ca^{2+} for 0 and 60 min, the pH of muscle homogenates was adjusted to 10.5. The phospholipid and protein removed after centrifugation at 4000*g* for 15 min are shown in **Figure 9**. Incubation for 60 min significantly increased ($p < 0.05$) phospholipid removal compared to that without incubation (0 min) treament. The phospholipid removed in solubilized herring muscle homogenate treated with Ca^{2+} and citric acid at pH 10.5 was about the same compared to that removed in solubilized cod muscle homogenate. Upon 1 h of incubation with 8 mM Ca^{2+} and 5 mM citric acid, the phospholipid removed was about 75% of the original for herring and 78% for cod (**Figures 2** and **9**).

DISCUSSION

Certain polyvalent ions, especially Ca^{2+}, have the ability to induce aggregation/fusion in model systems made of phospholipid vesicles. Ca^{2+} is believed to induce close contact between the vesicles through the formation of an anhydrous calcium−phospholipid complex (*14*, *15*). Calcium chloride was also studied for separating fat globule membranes from cheese whey by aggregation of lipoproteins through calcium binding and heat treatment (*16*). Our studies showed that Ca^{2+}, in the presence of citric acid, could selectively remove muscle membrane phospholipids from muscle homogenates solubilized at pH 3 by adding them prior to the solubilization (*9*).

In this paper, it has been shown that phospholipids could also be removed from muscle homogenates solubilized at pH 10.5 by adding Ca^{2+} and citric acid prior to the solubilization. However, the membrane sedimentation behavior observed in muscle homogenates solubilized at pH 10.5 was different from that in homogenates solubilized at pH 3. First of all, in the presence of 8 mM Ca^{2+} alone, the percentage of phospholipid removed from muscle homogenates solubilized at pH 10.5 was improved to ~58% compared with 26% in the control (**Figure 2**). On treatment with 8 mM Ca^{2+} alone, almost no additional phospholipid was removed from muscle homogenates solubilized at pH 3 compared with its control (*9*). Most of the phospholipid that would be eventually separated into the sediment could be sedimented at pH 10.5 when the Ca^{2+} was added at that pH (**Figure 5**). In the acid process, the Ca^{2+} had to be added before the proteins were solubilized to be effective. Sodium citrate, added to the homogenized muscle tissue, was very effective in the later separation of phospholipid. In the acid separation process, sodium citrate functioned very poorly (*9*).

High phospholipid removal from muscle homogenates solubilized at pH 10.5 was always accompanied with high protein removal. In the presence of 1 mM citric acid, an increase of Ca^{2+} concentration kept increasing phospholipid removal in muscle homogenates solubilized at pH 10.5, whereas an increase of Ca^{2+} concentration over 10 mM did not improve phospholipid removal in muscle homogenates solubilized at pH 3 (*9*). Although greater phospholipid removal was obtained in muscle homogenates solubilized at pH 10.5 than at pH 3 in the presence of 8 mM Ca^{2+} and citric acid concentrations lower than 1 mM, more phospholipids were removed from muscle homogenates solubilized at pH 3 than at pH 10.5 at citric acid concentrations of 2−8 mM and 8 mM Ca^{2+} (*9*). Overall, it seems that Ca^{2+} played the major role in membrane sedimentation in muscle homogenates solubilized at pH 10.5, with citric acid having a slight synergistic effect, whereas membrane sedimentation in muscle homogenates solubilized at pH 3 depended on both citric acid and Ca^{2+}. More muscle proteins were concurrently sedimented with the phospholipid by treatment of muscle homogenates solubilized at pH 10.5 compared to those at pH 3. When Mg^{2+} was studied for replacement of Ca^{2+}, less phospholipid was removed compared to the treatments with Ca^{2+} and citric acid at the same conditions, and the protein was also removed at a lower percentage, especially when the ions were compared at 20 and 30 mM.

The SDS-PAGE analysis showed that the 105 kDa polypeptide (Ca^{2+}-ATPase) decreased on treatment with citric acid and Ca^{2+} compared to samples solubilized at pH 10.5 without this treatment. The intensity of the 105 kDa polypeptide in the

muscle homogenates solubilized at pH 10.5 after the treatment indicated that ~64% of this polypeptide was removed from the supernatant after the centrifugation. The amount of membrane removed estimated by phospholipid measurement was 78% (**Figure 2**). The difference is about the same for the measurements in muscle homogenates solubilized at pH 3 after treatment with citric acid and Ca^{2+} (*9*). Recently, it was reported that the phospholipids of muscle tissue are present not only in membranes but also in the Z-disks in the form of an amorphous matrix (*9*). The difference between 105 kDa polypeptide removal and phospholipid removal may be partly due to the presence of phospholipids in Z-disks and their easier removal compared with the phospholipid in membranes (*9*).

The membranes in muscle homogenates taken to pH 10.5 might experience a similar change initially as the membranes in muscle homogenates taken to pH 3 when they are treated with Ca^{2+} and citric acid; that is, the membranes are released from their original links to cytoskeletal proteins with the help of Ca^{2+} and citric acid through a process of their competition for the electrostatic binding sites on the acidic phospholipids of membranes and the basic amino acid residues of cytoskeletal proteins. This process might also have a role in meat tenderization (*9*). Whereas the released membranes may have aggregated or fused due to either the final low pH (*9*) or cross-linking induced by Ca^{2+} in the muscle homogenates solubilized at pH 3, the released membranes in the muscle homogenates solubilized at pH 10.5 probably aggregated or fused through the cross-linking induced by Ca^{2+}.

In general, cellular membranes could be selectively sedimented at low *g* force from muscle homogenates solubilized at pH 10.5 by treatment with citric acid and Ca^{2+}. However, the membrane removal behavior in muscle homogenates solubilized at pH 10.5 was quite different from that observed in muscle homogenates solubilized at pH 3. This might be related to the different aggregation mechanism of membranes and/or different solubilities of Ca^{2+} at different pH values.

ABBREVIATIONS USED

SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; PL, phospholipids; Ca^{2+}-ATPase, Ca^{2+}-adenosine triphosphatase.

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