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Separation of Membranes from Acid-Solubilized Fish Muscle Proteins with the Aid of Calcium Ions and Organic Acids

YONG LIANG* AND HERBERT O. HULTIN

Department of Food Science, Massachusetts Agricultural Experiment Station, University of Massachusetts/Amherst, Marine Station, Gloucester, Massachusetts 01930

Calcium chloride, and to a lesser extent $MgCl_2$, aided in the separation of membranes by centrifugation from cod (*Gadus morhua*) muscle homogenates solubilized at pH 3 in the presence of citric acid or malic acid but not lactic acid. Adding citric acid and Ca^{2+} before solubilizing the cod muscle homogenates was needed for the effect. At 1 mM citric acid, 70−80% of the phospholipid and 25−30% of the protein were removed at 10 mM Ca^{2+}. At 8 mM Ca^{2+}, citric acid showed an optimal effect on phospholipid removal at 5 mM with 90% of the phospholipid and 35% of the protein removed. The treatment with citric acid and Ca^{2+} was also effective in separating the membrane from solubilized herring (*Clupea harengus*) muscle homogenate. Ca^{2+} and citric acid might exert their influence by disconnecting linkages between membranes and cytoskeletal proteins.

KEYWORDS: Acid-solubilized muscle proteins; membranes; phospholipid; Ca^{2+}; citric acid

INTRODUCTION

Lipid oxidation can adversely affect the flavor, color, texture, and nutritional value of muscle-based foods, which makes it a major cause of quality deterioration (*1*). Fish has a higher susceptibility to lipid oxidation as compared to land animals, in part due to the higher content of highly unsaturated lipids. It is well-known that the most unsaturated fatty acids in fish are those in the polar lipids (phospholipids) present in membranes, which are considered to be primary substrates for lipid oxidation as compared with triacylglycerols (*2*). In certain lean fishes, the lipids of membranes represent almost all of the lipids of the cell. Membranes also have a large surface area to contact with pro-oxidants in the aqueous phase around the membranes (*3*). There is also close contact of the membrane lipids with the electron transport components of the membranes, which could produce free radicals leading to lipid oxidation. All of these factors suggest a critical role of membrane lipids in the oxidation process. Thus, if fish muscle cellular membranes could be removed from muscle proteins, the stability of the isolated proteins could be dramatically increased.

Although it had been shown that up to 60% of the muscle cellular membranes in cod or herring muscle homogenates solubilized at pH 3 could not be sedimented by centrifugation at 10000*g* for 15 min, we found that an isolated membrane fraction prepared from cod could be easily sedimented at pH 3 at a *g* force as low as 1000*g* for 15 min (*4*). In addition, most of an isolated membrane fraction could be sedimented at a *g* force of 4000*g* for 15 min after being added back to solubilized muscle protein at pH 3 (*4*). These facts might imply that the inability of membrane sedimentation in solubilized muscle homogenates involved some interaction between the membranes and the muscle proteins.

Much research has shown that calcium ions can induce aggregation or fusion of membranes/phospholipid vesicles (*5*−*10*). Calcium ions were also reported to be used for aggregation and precipitation of phospholipoproteins originating from fat globule membranes in cheese whey and thus could be used to purify whey proteins (*11*). In this paper, the effect of organic acids and Ca^{2+} (Mg^{2+}) on membrane sedimentation (at 4000*g* for 15 min) was studied under different conditions in cod and herring muscle homogenates solubilized at pH 3.

MATERIALS AND METHODS

Materials. Fresh Atlantic cod (*Gadus morhua*) was purchased from John B. Wright Fish Co., Inc. (Gloucester, MA) on the day that it was brought to port and used on the same day. Fresh Atlantic herring (*Clupea harengus*) was obtained from Cape Seafoods, Inc. (Gloucester, MA) on the day that 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 Kitchen Aid mincer (Kitchen Aid 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 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 3. The muscle homogenate solubilized at pH 3 was prepared by adjusting the pH of the muscle homogenate to 3.00 ± 0.05 using 2 N HCl.

* To whom correspondence should be addressed. Current address: 359 FSHN BLDG., Newell Drive, P.O. Box 110370, Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL 32611. Tel: 352-392-1991 ext. 501. Fax: 352-392-9467. E-mail: yliang@ufl.edu.

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Table 1. Effect of Ca^{2+} on Phospholipid and Protein Removal from Cod Muscle Homogenates Solubilized at pH 3[a,b]

	phospholipid removed (%)			protein removed (%)		
Ca^{2+} concn (mM)	0 min	30 min	60 min	0 min	30 min	60 min
0 (control)	10.4 ± 3.5	8.2 ± 1.5	9.1 ± 2.3	4.6 ± 6.8	4.8 ± 1.8	2.4 ± 1.1
0.1	14.0 ± 2.2	10.5 ± 2.3	14.2 ± 1.6	5.2 ± 2.6	5.6 ± 1.4	2.6 ± 1.6
1	14.4 ± 0.3	14.8 ± 2.6	14.8 ± 1.7	6.8 ± 2.8	6.6 ± 3.0	7.6 ± 1.1
5	19.4 ± 0.7	21.5 ± 4.2	22.0 ± 3.7	5.6 ± 3.2	8.0 ± 2.7	9.5 ± 2.0
10	20.5 ± 1.3	24.1 ± 0.9	26.5 ± 4.1	5.6 ± 1.3	7.8 ± 3.3	7.1 ± 3.7
50	33.1 ± 2.1	29.9 ± 0.9	30.7 ± 3.6	13.1 ± 2.1	12.3 ± 1.6	11.7 ± 3.0

[a] Treatment: muscle homogenate → add different amounts of $CaCl_2$ solution → incubate for 0, 30, or 60 min → adjust to pH 3 → centrifugation at 4000*g* for 15 min. [b] The phospholipid and protein analyzed were those that remained in the supernatant after centrifugation. The phospholipid and protein removed were then calculated by subtracting the percentage of phospholipid and protein remaining in the supernatant from 100%.

Determination of Protein. The protein content of the samples was measured according to the method of Markwell et al. (*12*), a modification of the Lowry procedure (*13*) for measuring samples containing membranes and lipoproteins.

Determination of Phospholipid. Lipid extraction from samples was carried out according to the method of Lee et al. (*14*). To extract the lipids, a muscle homogenate sample of 15 mL at pH 3.0 was first adjusted to pH 5.5 to allow the precipitation of proteins in a 250 mL glass beaker. The lipid extraction of the resultant mixture was then performed according to the method of Lee et al. with 1:2 chloroform: methanol. The lipid phosphorus content in the lipid extracts was determined by the method of Anderson and Davis (*15*). 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.

Protein Analysis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). The SDS−PAGE protein analyses were run on precast linear 4−20% gradient minigels (ICN Biomedicals, Costa Mesa, CA) using a vertical PAGE Mini Device (Daiichi Scientific, Tokyo, Japan) with a constant current of 30 mA per gel. A Hoefer GS300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) was used to scan the polypeptide bands on the gels. A model 365W Densitometer Analysis software program (Hoefer Scientific Instruments) was used to calculate the percentage of each polypeptide band in the same lane.

Lipid Analysis by Thin-Layer Chromatography (TLC). Lipid analysis was performed with TLC. The lipids extracted by Lee's method were separated on silica gel plates (Whatman Ltd., Maidstone, Kent, England) using a solvent system of hexane:diethyl ether:formic acid at 80:20:2, v/v/v (*16*). After development and drying, the plates were sprayed with 70% sulfuric acid saturated with $K_2Cr_2O_7$ and then charred by heating the plates at 120 °C for 20 min. The extracted lipids applied to the plates were from fish muscle 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. In addition, a simple method was used to quantify the spots of phospholipids approximately on the plates as follows: the extracted lipids of cod fish without any treatment were applied on one plate in amounts of 100, 80, 40, 30, 20, and 10% of the amount when they were applied to plates with the treated samples for comparison. In this way, the amounts of phospholipids remaining in the treated samples could be estimated by comparing their intensity with the intensity of the different percentages of lipids extracted from fish muscle without any treatment. The TLC reference standard was purchased form 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. The consistency of samples was measured using a Brookfield Syncro-lectric viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA) with a #2 spindle at 60 rpm. Samples were put into 250 mL Kimax beakers on ice for measurement. The volume of samples in the beakers was kept constant at about 200 mL to favor comparisons. The readings of measurement were converted to mPA·s using the manufacturer's conversion chart.

Table 2. Effect of Mg^{2+} (10 mM) on Phospholipid and Protein Removal from Cod Muscle Homogenates Solubilized at pH 3[a]

incubation time	phospholipid removed (%)	protein removed (%)
control (no Mg^{2+} added)	23.1 ± 1.3	8.4 ± 1.4
0 min	24.8 ± 0.9	10.5 ± 4.4
30 min	26.2 ± 1.5	9.2 ± 3.6
60 min	27.9 ± 2.8	11.3 ± 5.8

[a] Treatment: muscle homogenate → add $MgCl_2$ solution → incubate for 0, 30, or 60 min → adjust to pH 3 → centrifugation at 4000*g* for 15 min.

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

RESULTS

Effect of Ca^{2+} and Mg^{2+} on Membrane Sedimentation. The effect of calcium ions on membrane sedimentation was studied by adding different amounts of $CaCl_2$ solution to cod muscle homogenates. The muscle homogenates containing calcium were adjusted to pH 3 after 0, 30, or 60 min of incubation with Ca^{2+} (the time of incubation counted from the point of mixing the $CaCl_2$ solution with muscle homogenate) and then centrifuged at 4000*g* for 15 min. The phospholipid and protein remaining in the supernatants were measured. The percentages of phospholipid and protein removed from the solubilized muscle homogenates are shown in **Table 1**. The removal of membrane phospholipid increased with increasing amounts of calcium. Treatments with 50 mM Ca^{2+} removed about three times of the phospholipid and protein that removed from the control. Statistical analysis showed that the Ca^{2+} concentration affected the efficiency of phospholipid removal significantly ($\rho \leq 0.05$), while incubation time did not. Treatments with 5 and 10 mM Ca^{2+} removed significantly more membrane phospholipids than the treatments with 0.1 and 1 mM Ca^{2+}, while it removed significantly less membrane phospholipids than the treatments with 50 mM Ca^{2+}. The effect of Mg^{2+} on membrane removal was studied by adding $MgCl_2$ solution into muscle homogenates to make a final concentration of 10 mM Mg^{2+}. The muscle homogenates containing magnesium were adjusted to pH 3 after 0, 30, or 60 min incubation and then centrifuged at 4000*g* for 15 min. Not much additional membrane phospholipid was removed as compared to the control under the studied conditions (**Table 2**). No significant difference was found on phospholipid removal among the treatments with different incubation times.

Effect of Ca^{2+} and Citric Acid on Membrane Sedimentation. The effect of Ca^{2+} on membrane sedimentation was studied

Table 3. Effect of Ca^{2+} and Citric Acid (1 mM) on Phospholipid and Protein Removal from Cod Muscle Homogenates Solubilized at pH 3[a]

		phospholipid removed (%)			protein removed (%)		
Ca^{2+} concn (mM)	pH[b]	0 min	30 min	60 min	0 min	30 min	60 min
0 (control[c])	6.4	31.7 ± 4.2	31.7 ± 4.2	31.7 ± 4.2	8.4 ± 3.0	8.4 ± 3.0	8.4 ± 3.0
10	6.2	58.8 ± 7.6	72.5 ± 1.8	77.7 ± 3.2	16.0 ± 2.2	15.8 ± 3.1	19.4 ± 3.2
50	6.0	80.2 ± 4.9	80.5 ± 7.2	80.7 ± 5.1	30.9 ± 3.2	28.6 ± 2.3	28.2 ± 1.4

[a] Treatment: muscle + citric acid → homogenization → add different amounts of $CaCl_2$ solution → incubate for 0, 30, or 60 min → adjust to pH 3 → centrifugation at 4000*g* for 15 min. [b] pH: pH after adding citric acid and calcium solution to muscle homogenates. [c] Control: 1 mM citric acid added and no Ca^{2+} added.

in the presence of 1 mM citric acid. Citric acid was added to the mixture of ground cod muscle and chilled distilled water before homogenization. After homogenization, a $CaCl_2$ solution was added to the muscle homogenate to a final concentration of 10 or 50 mM. The muscle homogenates were incubated for different times and then adjusted to pH 3. After centrifugation at 4000*g* for 15 min, the phospholipid and protein remaining in the supernatants were determined. In the presence of citric acid, significant amounts of membrane phospholipid were removed from muscle homogenates at Ca^{2+} concentrations of 10 and 50 mM as compared to a control without Ca^{2+} (**Table 3**). The time of incubation had no effect at 50 mM Ca^{2+} but affected membrane removal at 10 mM Ca^{2+}. About 20% more of the original protein (an increase of about 3.6 times) was sedimented with the phospholipid at a Ca^{2+} concentration of 50 mM as compared to the control without Ca^{2+}. Adding citric acid (1 mM) lowered the pH of muscle homogenates from 7.1 to 6.4−6.5. Adding calcium lowered the pH further to 6.0−6.3.

Adding Ca^{2+} or Mg^{2+} alone to muscle homogenates followed by pH adjustment to 3 did not lead to efficient membrane removal at the studied concentrations (**Tables 1** and **2**), while adding Ca^{2+} in the presence of 1 mM citric acid caused sedimentation of most of the membranes in muscle homogenates solubilized at pH 3 under the same conditions (**Table 3**). Because the treatment of muscle homogenate with citric acid alone (control in **Table 3**) did not produce efficient membrane removal, it was concluded that the observed membrane removal effect needed the involvement of both citric acid and Ca^{2+}.

Comparison of Ca^{2+}, Mg^{2+}, and Na^{+} on Membrane Sedimentation in the Presence of Citric Acid. The effect of Ca^{2+}, Mg^{2+}, and Na^{+} on membrane sedimentation in cod muscle homogenates was studied at final concentrations of 10, 10, and 30 mM, respectively, in the presence of 1 mM citric acid. After incubation, the pH of the muscle homogenates was adjusted to 3, and the homogenates were centrifuged at 4000*g* for 15 min. The phospholipid removed as a function of incubation time is shown in **Figure 1**. Little additional removal of phospholipid with time of incubation was observed with the Na^{+} treatment as compared with the control, while the percentage of phospholipid removed increased with an increase of incubation time for both the Mg^{2+} treatment and the Ca^{2+} treatment. The ion type, incubation time, and interaction of ion type and incubation time affected phospholipid removal significantly ($\rho \leq 0.05$). The percentages of membrane phospholipid removed were significantly different from each other among the treatments with the different ions and among the treatments with the different incubation times.

NaCl at 30 mM gives the same ionic strength as $CaCl_2$ and $MgCl_2$ at 10 mM. If the role of Ca^{2+} and Mg^{2+} in membrane removal from solubilized muscle homogenates is due to charge neutralization of the membranes, treatment with NaCl at the same ionic strength should have shown a similar membrane removal effect. The inability of Na^{+} to affect membrane removal indicates that the sedimentation of membranes was not related solely to ionic strength.

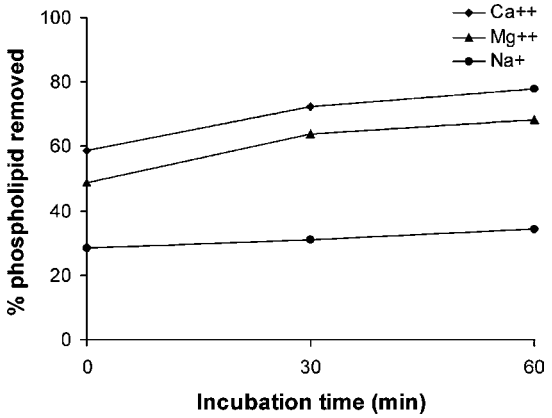

Figure 1. Effect of Ca^{2+}, Mg^{2+}, and Na^{+} on phospholipid removal in the presence of 1 mM citric acid. $CaCl_2$, $MgCl_2$, and NaCl were added at 10, 10, and 30 mM, respectively. The pH of the mixtures was adjusted to 3 after 0, 30, or 60 min incubation. Centrifugation was at 4000*g* for 15 min.

Effect of Ca^{2+} without Citric Acid on Membrane Removal at pH 6.4. The above studies showed that adding Ca^{2+} in the presence of citric acid could remove phospholipid efficiently from a solubilized muscle homogenate while adding Ca^{2+} in the absence of citric acid could not. Because adding citric acid at 1 mM lowered the pH of cod muscle homogenate to around 6.4, phospholipid removal might be due to the low pH caused by adding citric acid. To determine if the membrane removal effect was due to the low pH caused by citric acid, the pH of muscle homogenates was lowered to pH 6.4 by HCl, followed by adding $CaCl_2$ solution to a final concentration of 10 mM. After incubation, muscle homogenates were adjusted to pH 3 and centrifuged at 4000*g* for 15 min. Although there are significant differences in membrane phospholipid removal among the treatments and the control ($\rho \leq 0.05$), only a relatively small amount of additional phospholipid was removed by the treatments as compared to the untreated control (no $CaCl_2$ added) also initially adjusted to pH 6.4 with HCl (**Figure 2**). This shows that the role of citric acid in membrane removal was not solely due to the low pH that it caused.

Effect of Initial pH on Membrane Sedimentation by Ca^{2+} and Citric Acid. Cod muscle homogenates were lowered to pH 6.4 by HCl. Citric acid (1 mM) and $CaCl_2$ (10 mM) were then added. After incubation for 1 h, the pH of the muscle homogenates was adjusted to pH 3. The phospholipid and protein removed after centrifugation are shown in **Figure 3**. Adding citric acid lowered the pH from 6.4 to 5.9 and adding Ca^{2+} lowered the pH further to 5.5. Although selective membrane removal was observed by this treatment, the percentage of phospholipid removed was lower than when citric acid was added to the muscle homogenate at native pH. This could mean that the presence of Ca^{2+} at an appropriately high pH

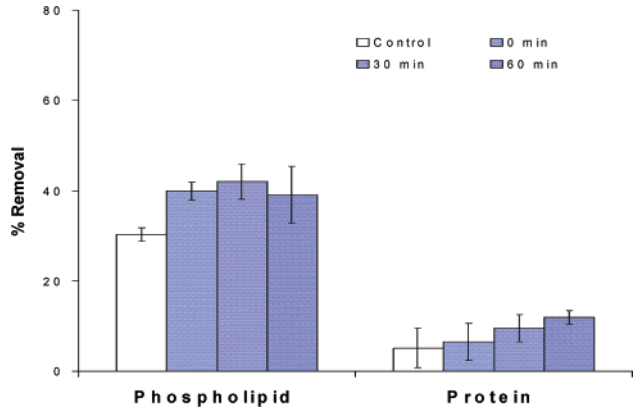

Figure 2. Effect of Ca^{2+} without citric acid on membrane removal with or without incubation after adjustment to pH 6.4 with HCl. Control: No citric acid added and no Ca^{2+} added. Treatment: Homogenized muscle → lower the pH to 6.4 by HCl → add Ca^{2+} solution (10 mM) → incubation → adjust to pH 3 → centrifugation at 4000*g* for 15 min.

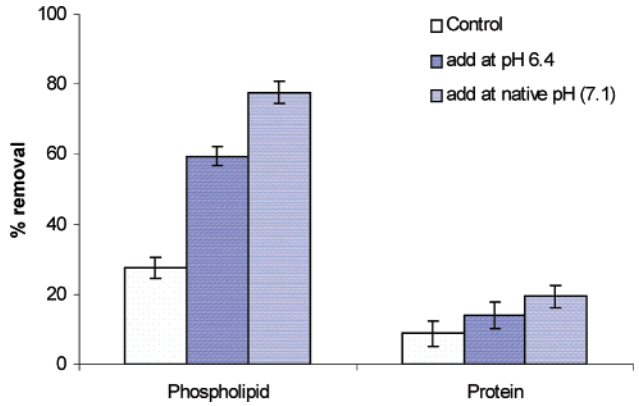

Figure 3. Effect of initial pH on membrane sedimentation by Ca^{2+} and citric acid. Control: No $CaCl_2$ added and no citric acid added. The pH of muscle homogenate (pH 7.1) was adjusted to pH 3 directly, and the samples were then centrifuged at 4000*g* for 15 min. Treatment: Muscle homogenate → lower pH to 6.4 by HCl or without pH adjustment → add 1 mM citric acid → add 10 mM $CaCl_2$ → 1 h incubation → adjust to pH 3 → centrifugation at 4000*g* for 15 min.

was required. It could also mean that a specific ionized form of citric acid was required; the third carboxyl group of citric acid has a pK_a of 5.41 (*17*) giving it a preponderance of three negative groups at pH 6.4 but about 2.5 negative groups around pH 5.5.

Effect of Sodium Citrate on Membrane Sedimentation. Ten grams of cod muscle tissue was mixed with 90 mL of 5.6 mM trisodium citrate solution at pH 7.1 (pH of fresh cod tissue). 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 3 and then centrifuged. The phospholipid and protein removed are compared with the usual treatment of adding citric acid and $CaCl_2$ to homogenized muscle in **Figure 4**. Almost no additional membrane removal was observed with the sodium citrate treatment as compared with the control while good separation was attained by the treatment with citric acid and $CaCl_2$. More than two times of protein was separated with membrane phospholipid by the treatment with citric acid and $CaCl_2$ as compared with the treatment with sodium citrate and $CaCl_2$. 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.

Effect of Ca^{2+} and Mg^{2+} Added after Adjustment of Muscle Homogenates to pH 3. $CaCl_2$ or $MgCl_2$ were added to

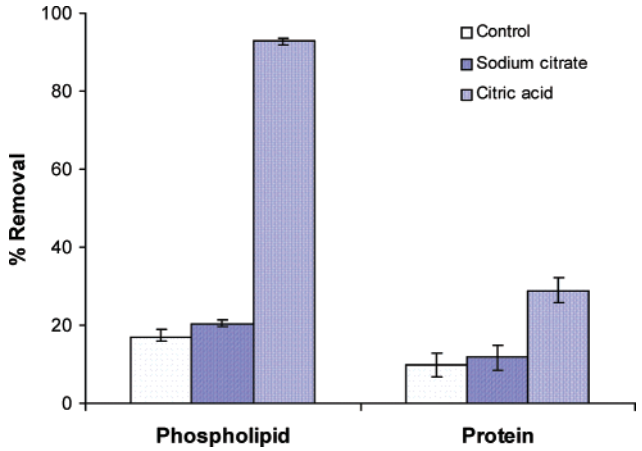

Figure 4. Comparison of sodium citrate vs citric acid on membrane separation in solubilized cod muscle homogenates in the presence of 8 mM $CaCl_2$. Control: Muscle homogenates solubilized at pH 3, no citric acid or sodium citrate added, and no $CaCl_2$ added. Sodium citrate treatment: Muscle homogenate (5 mM sodium citrate) → 8 mM Ca^{2+} → 1 h incubation → adjust to pH 3 → centrifugation at 4000*g* for 15 min. Citric acid treatment: Muscle homogenate (5 mM citric acid) → 8 mM Ca^{2+} → 1 h incubation → adjust to pH 3 → centrifugation at 4000*g* for 15 min.

a final concentration of 10 mM to muscle homogenates at pH 3 (adjusted by HCl after adding citric acid at 1 mM). The pH was readjusted to 3 if necessary after the addition of the salts. After incubation, the samples were centrifuged at 4000*g* for 15 min. $CaCl_2$ and $MgCl_2$ were not effective in enhancing membrane separation when added at pH 3 instead of at a pH of about 6.4.

Effect of Ca^{2+} and Mg^{2+} on Membrane Sedimentation when Adding Citric Acid after Incubation and Adjustment of Muscle Homogenate to pH 3. After 1 h of incubation with Ca^{2+} or Mg^{2+} (10 mM), muscle homogenates were adjusted to pH 3. Citric acid was then added to muscle homogenates to a final concentration of 1 mM. After centrifugation, the phospholipid and protein remaining in the supernatants were measured. Poor separation of membrane phospholipid was attained by these treatments. The finding together with the result that $CaCl_2$ and $MgCl_2$ were not effective in enhancing membrane separation when added at pH 3, suggests that a pH environment higher than 3 is required for whatever process(es) is (are) responsible for efficient membrane separation from solubilized muscle homogenates by citric acid and Ca^{2+} (Mg^{2+}).

Effect of Ca^{2+} on Membrane Sedimentation in the Presence of Malic Acid or Lactic Acid. Cod muscle samples were homogenized in the presence of 1 mM lactic acid, 2 mM lactic acid (to match the concentrations of carboxylic group between lactic and malic acids), 1 mM malic acid, or lactic acid at 1 mM with HCl to bring the pH of the muscle homogenate down to 6.4 after adding lactic acid (to lower pH to the same pH as adding 1 mM citric acid), respectively. $CaCl_2$ solution was then added to each sample at 10 mM. After 1 h of incubation and pH adjustment to 3, the treated muscle homogenates were centrifuged at 4000*g* for 15 min. The phospholipid and protein removed and the pH changes during the treatments are shown in **Table 4**. The treatment with malic acid removed significantly more phospholipids as compared with the other treatments listed in **Table 4**, while there were no significant differences in the percentages of phospholipid removed among the lactic acid treatments. Malic acid and citric acid have more than one carboxylic group while lactic acid only has one carboxylic

Table 4. Effect of Ca^{2+} in the Presence of Malic Acid or Lactic Acid on Phospholipid and Protein Removal from Cod Muscle Homogenates Solubilized at pH 3[a]

	phospholipid removed (%)	protein removed (%)	pH before adding Ca^{2+}	pH after adding Ca^{2+} and incubation
control[b]	25.0 ± 2.9	9.3 ± 7.2		
1 mM malic acid	77.3 ± 0.5	19.2 ± 2.0	6.5	6.2
1 mM lactic acid	29.3 ± 3.0	14.0 ± 1.4	7.1	6.8
2 mM lactic acid	31.4 ± 1.9	17.0 ± 9.0	6.9	6.6
1 mM lactic acid + HCl[c]	29.6 ± 1.7	14.9 ± 4.6	6.4	6.2

[a] Treatment: muscle homogenate (malic acid or lactic acid added) → add Ca^{2+} (10 mM) → 1 h incubation → adjust to pH 3 → centrifugation at 4000*g* for 15 min. [b] Control: no malic acid or lactic acid added and no Ca^{2+} added. [c] 1 mM lactic acid + HCl: HCl was used to lower the pH of muscle homogenate (after 1 mM lactic acid was added) to 6.4 before adding Ca^{2+}.

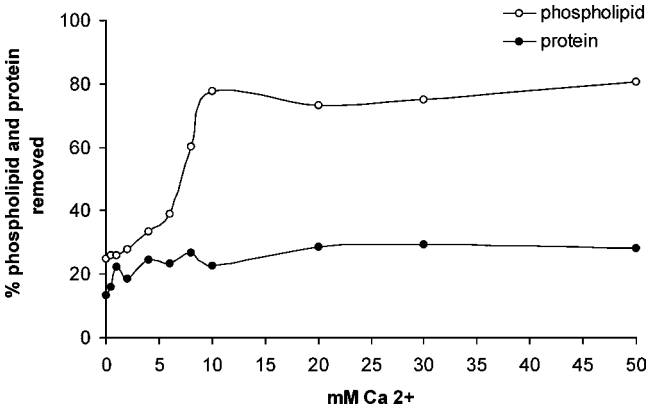

Figure 5. Effect of Ca^{2+} concentration on membrane sedimentation in cod muscle homogenates solubilized at pH 3 in the presence of 1 mM citric acid.

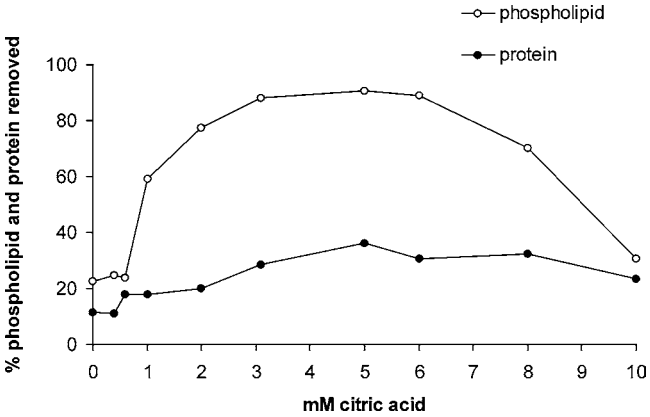

Figure 6. Effect of citric acid concentration on membrane sedimentation in homogenized muscle solubilized at pH 3 in the presence of 8 mM Ca^{2+}.

group. It is possible that the organic acids exerted their effect on membrane removal via their polycarboxylic groups. In addition, the percentage of protein removed is rather similar for all of the acids. This may suggest that it is the calcium ion that is the substance, which is mainly responsible for protein removal.

Effect of Ca^{2+} Concentration on Membrane Sedimentation in the Presence of 1 mM Citric Acid. The effect of Ca^{2+} concentration (0−50 mM) on membrane sedimentation from muscle homogenates solubilized at pH 3 after incubation for 60 min is shown in **Figure 5**. About 60% of the original phospholipid was removed at 8 mM Ca^{2+} and about 70−80% at 10 mM Ca^{2+} and above. The relatively quick change of phospholipid removal occurred at Ca^{2+} concentrations between 6 and 10 mM. About 23−30% of the original protein was removed at Ca^{2+} concentrations of 8 mM and above as compared with about 13% of the original protein removed in the control.

Effect of Citric Acid Concentration on Membrane Sedimentation in the Presence of 8 mM Ca^{2+}. In the above study, it was shown that membrane removal efficiency increased quickly at 1 mM citric acid as the Ca^{2+} concentration increased between 6 and 10 mM (**Figure 5**). The percentage of phospholipid removed reached a relatively steady figure at 10 mM $CaCl_2$ and above. In the study of the effect of citric acid concentration on membrane sedimentation, membrane removal efficiency might not fully reflect the effect of citric acid concentration if Ca^{2+} concentration is higher than 10 mM. Because the treatment with 8 mM Ca^{2+} showed considerable phospholipid removal but had not reached the maximal value in the presence of 1 mM citric acid, a Ca^{2+} concentration of 8 mM was chosen for the study of the effect of citric acid concentration on membrane sedimentation. The citric acid concentration was varied from 0 to 10 mM. After incubation for 1 h followed by pH adjustment to 3, the samples were centrifuged at 4000*g* for 15 min. The phospholipid and protein removed are shown in **Figure 6**. The pH changes are shown in **Table 5**. Citric acid showed an optimal concentration for phospholipid removal (90% removal) around 3−6 mM at the conditions of this study. However, about 25% more of the original protein was also removed with 5 mM citric acid treatment as compared with the control (8 mM Ca^{2+}, no citric acid added). The sample with 10 mM citric acid added was pastelike after centrifugation and actually did not separate into distinct supernatant and sediment fractions. The phospholipid and protein concentrations measured were those in the upper layer of the paste. Although distinct supernatant and sediment phases appeared in the sample with 8 mM citric acid added, the volume of the supernatant phase was low. Addition of 5 mM citric acid lowered the pH of muscle homogenate from 7.1 to 5.2. Addition of 8 mM calcium ions lowered the pH another 0.2−0.3 units.

Table 5. pH Changes of Cod Muscle Homogenates during the Study of the Effect of Citric Acid Concentration on Membrane Sedimentation

citric acid concn (mM)	pH after adding citric acid	pH after adding 8 mM Ca^{2+} and incubation
0	7.1	6.9
0.4	6.9	6.7
0.6	6.8	6.6
1	6.5	6.2
2	6.0	5.8
3	5.6	5.3
5	5.2	4.9
6	5.0	4.8
8	4.7	4.6
10	4.6	4.5

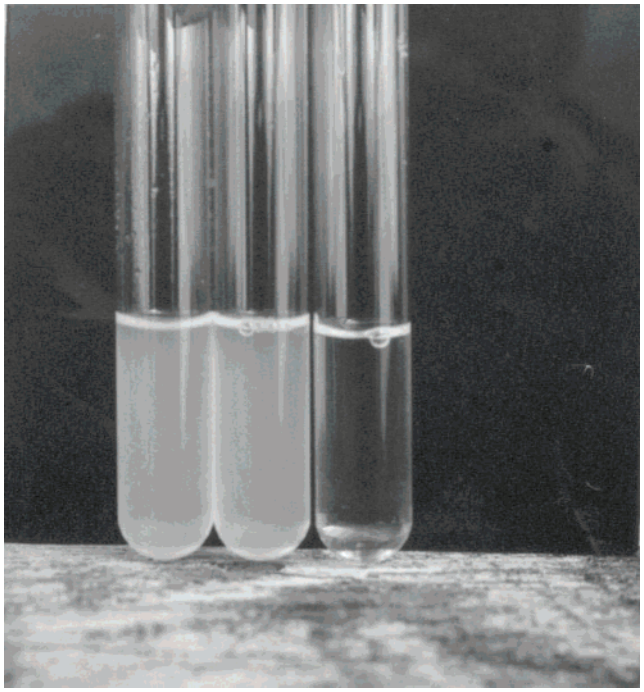

Figure 7. Picture of the supernatants with or without citric acid and Ca^{2+} treatment. The tubes containing supernatants after the following treatments are numbered from left to right. Tube 1: Muscle homogenate (no citric acid, no Ca^{2+}) → pH 3 → centrifuge at 4000*g* for 15 min. Tube 2: Muscle homogenate (no citric acid, 8 mM Ca^{2+}) → pH 3 → centrifuge at 4000*g* for 15 min. Tube 3: Muscle homogenate (5 mM citric acid, 8 mM Ca^{2+}) → pH 3 → centrifuge at 4000*g* for 15 min.

Visual appearance of some supernatant samples with or without the citric acid and Ca^{2+} treatments was recorded (**Figure 7**). The supernatant in tube 3 (5 mM citric acid, 8 mM Ca^{2+}) was clear as compared to the supernatants in tubes 1 (no citric acid, no Ca^{2+}) and 2 (no citric acid, 8 mM Ca^{2+}). The supernatant (tube 3) after treatment with 5 mM citric acid and 8 mM Ca^{2+} contained about 10% of the original phospholipid and 65% of the original protein of the muscle homogenate. The supernatants in tubes 1 and 2 contained about 74−77% of the original phospholipid and 88−90% of the original protein. In the study of removing fat globule membranes from cheese whey, turbidity was used as an index of how many fat globule membranes were present in the cheese whey (*18*). The clearer the cheese whey was, the fewer fat globule membranes remained. It is possible that the clearness of the supernatant after the treatment with 5 mM citric acid and 8 mM Ca^{2+} (tube 3 in **Figure 7**) could also have been a reflection of a low quantity of membranes present.

SDS−PAGE Analysis. Muscle homogenates solubilized at pH 3 with or without treatment with citric acid and Ca^{2+} were centrifuged at 4000*g* for 15 min. Samples of the supernatants were analyzed by SDS−PAGE, and the polypeptides were compared with those of the original muscle homogenate (**Figure 8**). The separated samples were applied on the same percentage basis as the starting muscle homogenate, so that direct comparison among the lanes of samples on the gel reflects the effect of treatments on the polypeptides of the samples. The results showed that the polypeptide profiles of samples that had been solubilized at pH 3 (treatments B and C) did not have the three high molecular weight polypeptides (molecular weights higher than 205 kDa) that were present in the original homogenate (treatment A). The samples solubilized at pH 3 (treatments B and C) had polypeptides at 136, 130, and 75 kDa that the original homogenate did not have. The band of the 105 kDa polypeptide was much lighter in the sample treated with citric acid and Ca^{2+} (treatment C) as compared to the original homogenate (treatment A) or the sample solubilized and centrifuged but without the citric acid and Ca^{2+} treatment (treatment B). Most likely, the 105 kDa polypeptide is the Ca^{2+}-adenosine triphosphatase (Ca^{2+}-ATPase), which is the characteristic protein of the sarcoplasmic reticulum (*19*). Thus, SDS−PAGE analysis probably could be used as a simple method for the study of membrane loss in muscle supernatants prepared by solubilization of muscle homogenates followed by centrifugation. This would represent an additional method of measuring the amount of membrane in any sample along with phospholipid measurement. Estimated by scanning, the quantity of the 105 kDa polypeptide in the citric acid/Ca^{2+} treated sample was about 21% of that in the original homogenate. This estimate of membrane removal (79%) is less than what was estimated by a phospholipid determination (90.8%, **Figure 6**). 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 (*20*). The higher percentage of phospholipid removal with the treatment as compared with the percentage of 105 kDa polypeptide removal might be an indication of easier removal of Z-disk phospholipids than membrane phospholipids.

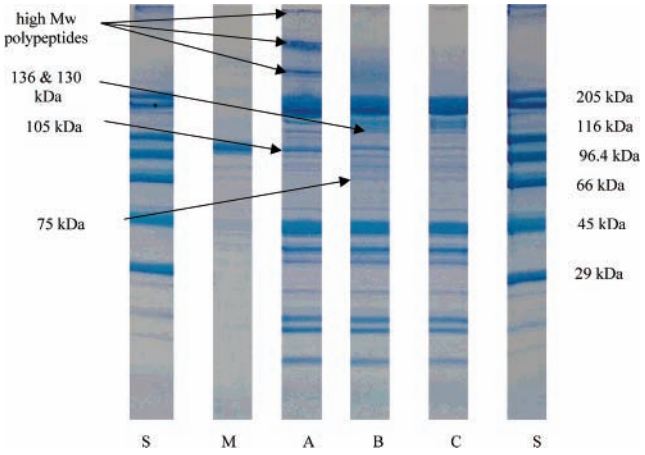

Figure 8. SDS–PAGE analysis of proteins in the samples with or without citric acid and Ca^{2+} treatments. S, molecular weight standard; M, isolated membrane preparation; A, sample of muscle homogenate (no pH adjustment and no centrifugation); B, supernatant after the treatment of "muscle homogenate → pH 3 → centrifuge"; C, supernatant after the treatment of "muscle homogenate → 5 mM citric acid + 8 mM Ca^{2+} → pH 3 → centrifuge". All of the centrifugations were done at 4000*g* for 15 min.

Lipid Analysis by TLC. Samples of cod muscle homogenates for the TLC analysis were prepared as described in **Figure 9**. The resultant samples were extracted with 1:2 chloroform: methanol overnight. Chloroform aliquots of each sample were analyzed by TLC. The samples were applied at the same volume, which corresponds to the same amount of starting muscle homogenate, so that direct comparison among the lanes of samples on the plate reflects the effect of treatments on the lipids. The lipids in samples A, B, and C were mostly phospholipids with 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 described in the Methods section, the phospholipids in sample D were about 10% or less of those in sample A. This is consistent with the phospholipid analysis in **Figure 6**.

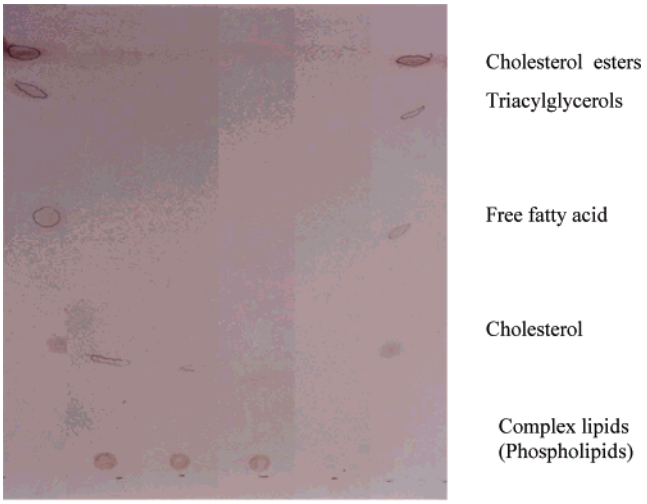

Figure 9. TLC analysis of lipids in the samples with or without citric acid and Ca^{2+} treatments. S, lipid standards; sample A, sample of muscle homogenate (no pH adjustment and no centrifugation); sample B, sample of muscle homogenate at pH 3 (no centrifugation); sample C, supernatant after the treatment of "muscle homogenate → pH 3 → centrifuge"; sample D, supernatant after the treatment of "muscle homogenate → 5 mM citric acid + 8 mM Ca^{2+} → pH 3 → centrifuge". The cholesterol spots of samples A, B, and C were too light to be scanned so that the spots were marked by pencil to highlight them. All of the centrifugations were done at 4000*g* for 15 min.

Table 6. Consistency of Cod Muscle Homogenates Solubilized at pH 3 in the Presence and Absence of Citric Acid and Ca^{2+} [a]

treatment	consistency
no citric acid, no Ca^{2+}	47.2 ± 10.8 mPa s
1 mM citric acid + 10 mM Ca^{2+}	52.1 ± 14.7 mPa s
5 mM citric acid + 8 mM Ca^{2+}	223.5 ± 27.2 mPa s

[a] Muscle homogenates in the presence or absence of citric acid and Ca^{2+} → incubate for 1 h → adjust pH to 3 → measure the consistency.

Consistency Study. The consistency of the solubilized muscle homogenate system is one of the factors that might affect the sedimentation of the membranes. A low consistency aids in the sedimentation of membranes theoretically. It would be interesting to know if the efficient removal of membranes with the treatment of citric acid and Ca^{2+} is due to their ability to lower the consistency of the system. The consistency of cod muscle homogenates solubilized at pH 3 in the presence or absence of citric acid and Ca^{2+} is shown in **Table 6**. As compared with the muscle homogenate in the absence of citric acid and Ca^{2+}, there was no change in consistency in the muscle homogenate on treatment with 1 mM citric acid and 10 mM Ca^{2+} followed by pH adjustment to 3 and a large increase in consistency on treatment with 5 mM citric acid and 8 mM $CaCl_2$ under the same conditions. This result shows that the effect of citric acid and Ca^{2+} on membrane removal is not due to their ability to lower the consistency of the system.

Effect of Ca^{2+} and Citric Acid on Membrane Sedimentation in Herring Muscle Homogenates Solubilized at pH 3. Herring was chosen as representative of fatty fish for the study of the effect of citric acid and Ca^{2+} treatment on membrane sedimentation. Membrane sedimentation in herring muscle homogenates solubilized at pH 3 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 or 60 min, the pH of muscle homogenates was adjusted to pH 3. The phospholipid and protein removed after the centrifugation at 4000*g* for 15 min are shown in **Figure 10**. Treatment with 5 mM citric acid and 8 mM Ca^{2+} removed considerable membrane phospholipids from herring muscle homogenate solubilized at pH 3. A 60 min incubation increased membrane removal by a small amount. The effect of citric acid and Ca^{2+} on membrane removal in solubilized herring muscle homogenate was not as complete as that in solubilized cod muscle homogenate at these concentrations of Ca^{2+} and citric acid. However, it should be indicated that the conditions used may not be optimal for membrane separation from solubilized herring homogenate.

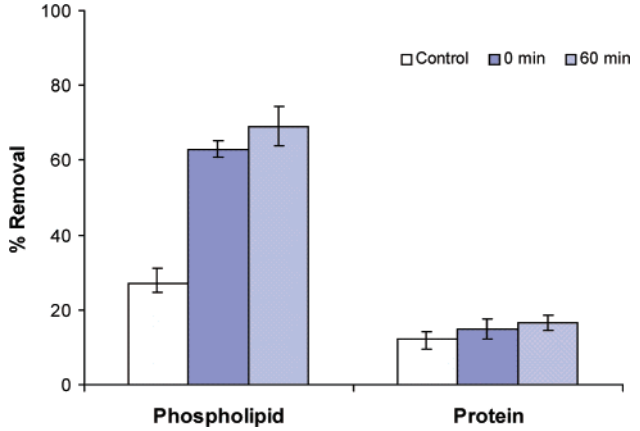

Figure 10. Effect of Ca^{2+} and citric acid on membrane sedimentation in herring muscle homogenates solubilized at pH 3. Control: Herring muscle homogenates solubilized at pH 3, no citric acid added, and no $CaCl_2$ added. Treatment: Herring muscle homogenate (5 mM citric acid) → add 8 mM Ca^{2+} → incubate for 0 or 60 min → adjust to pH 3 → centrifugation at 4000*g* for 15 min.

DISCUSSION

Polyvalent ions, especially Ca^{2+}, have been extensively studied for their ability to induce aggregation/fusion in model systems made of phospholipid vesicles. It has been suggested that Ca^{2+} induces close contact between the vesicles through the formation of an anhydrous calcium−phospholipid complex (*6*, *21*). Magnesium ion has also been shown to be effective in inducing aggregation/fusion of phospholipid vesicles but could not induce fusion of pure phosphatidylserine vesicles that could be induced by Ca^{2+} (*7*). In addition, the binding constant of Mg^{2+} to phosphatidylserine is lower than that of Ca^{2+}. Therefore, a threshold concentration of 4.5 mM at pH 7.0 is required for vesicle aggregation and fusion by Mg^{2+} while that for Ca^{2+} is 1.0 mM at pH 7.0 (*22*). To remove fat globule membranes from cheese whey, $CaCl_2$ was added to the whey up to a calcium content of 1.2 g (30 mmol)/kg at 2 °C followed by pH adjustment to 7.3 and heating at 50 °C for 8 min (*11*). This treatment caused the formation of a white, fine precipitate and reduced the phosphorus content of the cheese whey by 70% after separation of the precipitate. About 11% of the original nitrogen was also removed from cheese whey during the treatment. The authors suggested that the removal of fat globule membranes using this treatment was due to the aggregation of lipoproteins through calcium binding and heat treatment. These findings of Ca^{2+}-induced membrane/vesicle aggregation and fusion motivated the studies of removing membranes from muscle homogenates by treatments with Ca^{2+}.

In our previous study with isolated cod muscle cellular membranes, it was shown that the isolated membranes could

aggregate and sediment easily at pH 3 at a centrifugal force as low as 1000*g* for 15 min (*4*). However, more than 60% of the membranes in muscle homogenates solubilized at pH 3 could not be sedimented at a *g* force as high as 10000*g* for 15 min. Because most of the isolated membranes could still be sedimented when added to muscle proteins solubilized at pH 3, the inability of membrane sedimentation in solubilized muscle homogenates should not be solely due to the high viscosity of the solubilized muscle proteins. We determined here that conditions that improved phospholipid removal either had no effect on or increased the consistency of the protein solutions. Most likely, there are some interactions between membranes and proteins preventing the membranes from sedimentation. In muscle tissue, membranes are known to be connected with myofibrillar proteins through cytoskeletal proteins. Niggli (*23*) suggested that many cytoskeletal proteins might interact with membrane phospholipids in vitro and in situ. Electrostatic interactions between the acidic phospholipids of membranes and the basic amino acid residues of the cytoskeletal proteins are major interactions between them. It is possible that this connection between membranes and cytoskeletal proteins, which links membranes further to myofibrillar proteins, is the reason membranes do not sediment easily from solubilized muscle homogenates. It is also possible that the membranes sedimented during centrifugation were membranes that were physically disconnected from the cytoskeletal proteins during homogenization, while many of the membranes that could not be sedimented were the fraction of membranes whose links to the cytoskeletal proteins were not broken.

One possible explanation for the effect of citric acid and Ca^{2+} in allowing separation of membranes from the solubilized proteins is that these compounds might disconnect the linkage between membranes and cytoskeletal proteins. The release of membranes could make it possible for membranes to aggregate and sediment at 4000*g* for 15 min. It is difficult to know exactly how citric acid and Ca^{2+} produce the results given in this paper. However, it should be noted that much research has shown that Ca^{2+} at concentrations higher than that in the cytosol can disassemble the intermediate filaments involved in meat tenderization (*24*−*27*). Hydrolysis of cytoskeletal proteins induced by Ca^{2+} could contribute to the disassembly of intermediate filaments (*25*, *28*). It is possible that in the treatment of muscle homogenates with citric acid and Ca^{2+}, the presence of Ca^{2+} at high concentration helps to speed up the release of membrane phospholipids from attached cytoskeletal proteins through a mechanism similar to what happens in meat tenderization as mentioned above. Citric acid might play a role as a binding agent to the basic amino acid residues of cytoskeletal proteins competing with the acidic phospholipids of membranes (*29*, *30*). The carboxylic groups of citric acid might be the groups interacting with the basic amino acid residues of the cytoskeletal proteins. Because cytoskeletal proteins normally interact with membrane phospholipids through the polyanionic groups of polyphosphoinositides, the polycarboxylic groups of citric (or malic) acid might be needed for the successful competition for the points on the cytoskeletal proteins, which interact with membrane phospholipids. Malic acid and citric acid aided in membrane removal while lactic acid did not. The single negative charge of lactic acid may not be sufficient to have an effect. The membranes released from the cytoskeletal proteins could then aggregate or fuse due to either the final low pH (charge neutralization) as occurred during the studies with isolated membranes (*4*) or by cross-linking induced by Ca^{2+}. The aggregation or fusion of membranes could have been responsible for the sedimentation of the membranes from the solubilized muscle homogenates during centrifugation.

In this paper, it has been shown that treatment with citric acid and Ca^{2+} could aid in removal of membrane from cod muscle homogenates solubilized at pH 3. However, the mechanism of the treatment is not well understood. A clear understanding of what happens is not easy due to the complexity of the muscle homogenate system. Knowledge such as how muscle proteins interact with membranes at different pH values, whether citric acid and Ca^{2+} exert their effect on membrane sedimentation through their interaction with membranes, proteins, or both, and how pH affects the membranes themselves is needed for a better understanding of what happens during the treatment with citric acid and Ca^{2+}.

ABBREVIATIONS USED

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

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