JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stabilization of Oil-in-Water Emulsions by Cod Protein Extracts

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The ability of two protein fractions extracted from cod to form and stabilize oil-in-water emulsions was examined: a high salt extracted fraction (HSE protein) and a pH 3 acid extracted fraction (AE protein). Both fractions consisted of a complex mixture of different proteins, with the predominant one being myosin (200 kDa). The two protein fractions were used to prepare 5 wt % corn oil-in-water emulsions at ambient temperature (pH 3.0, 10 mM citrate−imidazole buffer). Emulsions with relatively small mean droplet diameters ($d_{3,2} < 1\ \mu$m) and good creaming stability (> 9 days) could be produced at protein concentrations ≥0.2 wt % for both fractions. The isoelectric point of droplets stabilized by both protein fractions was pH ∼5. The emulsions were stable to droplet flocculation and creaming at relatively low pH (≤4) and NaCl concentrations (≤150 mM) when stored at room temperature. In the absence of salt, the emulsions were also stable to thermal treatment (30−90 °C for 30 min), but in the presence of 100 mM NaCl droplet flocculation and creaming were observed in some of the emulsions, particularly those stabilized by the AE fraction. The results suggest that protein fractions extracted from cod can be used as emulsifiers to form and stabilize food emulsions.

KEYWORDS: Emulsions; fish protein; cod protein; myosin; tropomyosin

INTRODUCTION

Fish is an important source of animal protein for about 1 billion people or about 17% of the world's population; more than 50% derive 20% or more of their animal protein from fish. The fishing industry currently produces a great quantity of low-value byproducts, that is, fish species or fish fractions (e.g., skin, bones, heads, tails) that are normally considered to be unsuitable for human consumption. According to the United Nations' Food and Agricultural Organization estimates of world fisheries, 97 million tonnes of a total of 131 million tonnes of fish produced in the year 2000 went directly to human consumption. This leaves almost 34 million tonnes that was used for nonfood, low-value, production (*1*). Researchers in the fishing industry are therefore seeking methods of increasing the value of these byproducts by finding new applications and markets for them. Shahidi has discussed the properties of bioactive peptides produced from various low-value fish species such as capelin and Alaska pollack. Peptides produced from the capelin were separated into different fractions, some of which had a strong antioxidant activity. Stepwise hydrolysis of Alaska pollack skin with alcalase, Pronase E, and collagenase gave after the Pronase step a peptide fraction of 1.5−4.5 kDa with a high antioxidant activity (*2*). Gildberg has pointed out the importance of enzymes and bioactive peptides produced from fish byproducts using both traditional Asian methods and modern biotechnology (*3*). In his review on value-added products from low-cost fish, Venugopal discussed the availability of such fish in India and the methods necessary for the isolation of the flesh for further processing. The end-products discussed all involve well-established methods, but the discussion shows the easy availablity of low-value raw material for further processing (*4*). Fish byproducts contain a wide variety of different proteins, including sarcoplasma protein, myosin, and actin. The early work in this area was often concerned with the bulk isolation of fish protein isolates and hydrolysates with emphasis on the undisputed nutritional qualities of the protein. As early as the mid 1970s researchers were, however, realizing that fish protein concentrates and hydolysates were not turning into the commercial success that many expected, and it was pointed out that the functional properties of fish protein could be another valuable resource (5, *6*). Fish proteins and their derivatives may therefore have functional applications in food products, as discussed by Spinelli (*7*). Specific protein fractions can be extracted from fish byproducts and utilized as functional ingredients in foods. The utilization of value-added fish-based ingredients in foods depends on the identification of the optimum functional characteristics of different protein fractions, and the establishment of the range of environmental conditions (e.g., pH, ionic strength, and temperature) over which these ingredients exhibit the desired characteristics.

Previous studies have shown that fish proteins can be used to stabilize oil-in-water emulsions under certain conditions. Cofrades and co-workers have, for example, shown that acto-

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10.1021/jf035251g CCC: $27.50
Published on Web 05/13/2004

myosin from hake had higher emulsifying activity and stability than the actomyosin from chicken and pork. Dickinson and Lopez have, on the other hand, found that sodium caseinate had better emulsifying properties than fish gelatin (*8*−*14*).

These studies show that the effectiveness of a fish protein emulsifier depends on the species it was extracted from, the method used to extract it, and any subsequent modifications made to the protein structure (e.g., by heating, hydrolysis, or pH treatment). If a protein ingredient is going to find widespread application as an emulsifier in the food industry, it is important to establish the range of environmental conditions under which it can successfully exhibit its functional properties, for example, pH, ionic strength, and temperature (*15*−*20*). In this study, we examined the effectiveness of protein fractions extracted from cod flesh to act as emulsifiers in oil-in-water emulsions and examined the influence of environmental conditions on emulsion stability.

MATERIALS AND METHODS

Materials. All general chemicals used were of analytical grade purchased from Sigma Chemical Co. (St. Louis, MO). The oil used was Mazola corn oil (ACH Food Co., Inc.) stored at −80 °C prior to use. The cod protein was isolated from fresh cod fillets (*Gadus morhua*) bought the same day from the local fish market (Bread & Circus, Hadley, MA) using the method described below. Deionized and distilled water was used for the preparation of all solutions.

Isolation of Fish Protein Extracts. The method for the isolation of protein from the cod fillet was based on a method described previously (*21*, *22*). Two protein fractions were isolated: a high salt extracted fraction (HSE protein) and a pH 3 acid extracted fraction (AE protein) in the following manner. Fish and solvents were stored at 4 °C prior to the extractions being carried out, and all vessels were kept on ice during the extractions. The tail end of four cod fillets, 890 g (158 g of protein based on 17.8% protein content), was ground through a 5 mm plate using a food grinder. The ground flesh was immediately added to 2 L of cold 0.200 M KCl, 0.0200 M phosphate buffer (29.82 g of KCl, 28.60 g of K_2HPO_4, 4.872 g of KH_2PO_4, pH 7.4) and stirred mechanically on ice for 20 min.

The mixture was centrifuged at 16300*g* and <5 °C for 25 min using a Sorvall Ultra 80 centrifuge (Kendro Laboratory Products, Newtown, CT). The HSE protein was contained within the supernatant (1.6 L) that was poured off and diluted 7-fold by pouring it into 10 L of cold distilled water and the solution acidified with hydrochloric acid to the myosin isoelectric point of pH 5.5. This was left in a 2−5 °C cold room overnight. The precipitated protein was isolated by centrifugation at 16300*g* for 20 min, and the jelly-like product was freeze-dried to give 28.0 g or 18% of total protein. This is the HSE protein.

The precipitate remaining after the high-salt solubilization step mentioned above was poured into 2 L of cold distilled water and acidified to pH 3 with dilute hydrochloric acid. This mixture was stirred mechanically on ice for 30 min and then centrifuged at 16300*g* for 30 min. The supernatant was poured off, and the pH was raised to 5.5 using 6 and 1 M sodium hydroxide solutions. This was stored overnight at 4 °C. The precipitated acid extracted protein (AE protein) was isolated by centrifugation at 16300*g* for 20 min, and the grit-like wet product was freeze-dried to give 22.0 g or 14% of total protein.

Composition of Fish Protein Extracts. The composition of the fish protein extracts was investigated by SDS-PAGE (Bio-Rad Laboratories). Small aliquots (10 μL) of the 1.5 mg/mL protein fraction in pH 3 buffer (see below) were placed on an electrophoresis cell and run at 25 mA for 70 min. The resulting gels were stained, and the molecular weight of each protein constituent was determined by comparing its position to those of protein standards of known molecular weight run using the same electrophoresis conditions.

Solubility of Fish Protein Extracts. The solubility of the AE extract was determined by placing 100 mg of freeze-dried protein in 20 mL of an aqueous solution of the appropriate pH and sodium chloride concentration and leaving it at 4 °C for 3 days. The protein concentration remaining in the supernatant of the solution was then determined using the biuret method and bovine serum albumin as a standard (**Table 1**).

Table 1. Assignment of SDS-PAGE Bands to Protein Components

molecular weight		
HSE protein	AE protein	protein
200 (major fraction)	200 (major fraction)	myosin
130	130 (minor)	C-protein
	115–120	
40	40	actin
35	35	tropomyosin single strand
	30–35	

Emulsion Preparation. The buffer solution used to prepare the emulsions was a 10 mM citrate−imidazole buffer (1.92 g of citric acid and 0.680 g of imidazole in 1.00 L of water) adjusted to pH 3 with hydrochloric acid solutions (concentrated and 1 M). An emulsifier solution was prepared by dissolving the desired amount (0.1−1 wt %) of protein into buffer solution and stirring overnight at 4 °C.

Oil-in-water emulsions were prepared by blending 5 wt % corn oil and 95 wt % emulsifier solution together using a high-speed hand-held blender for 2 min (Bio Spec Products, Inc., Bartlesville, OK). This coarse emulsion was then passed through either a high-pressure double-stage valve homogenizer twice at 5000/500 psi [APV two-stage Mini-Lab homogenizer, model APV-1000 (APV Americas, Wilmington, MA)] or four times at 5000 psi and once at 500 psi using a single-stage homogenizer (Rannie high pressure, APV-Gaulin, model Mini-Lab 8.30H, Wilmington, MA).

Creaming Stability Measurements. Ten grams of emulsion was transferred into a glass test tube, tightly sealed with a plastic cap, and then stored at room temperature. After storage, a number of emulsions separated into an opaque layer at the top and a turbid or transparent layer at the bottom. We defined the serum layer to be the sum of the turbid and transparent layers. The total height of the emulsion (H_E) and the height of the serum layer (H_S) were measured. The extent of creaming was characterized by a creaming index $= 100(H_S/H_E)$. The creaming index provided indirect information about the extent of droplet aggregation in an emulsion.

Particle Size Determination. The particle size distribution of the emulsions was measured using a laser diffraction instrument (Mastersizer S, version 2.18. Malvern Instruments, Ltd., Southborough, MA). The prepared emulsions were diluted ~500-fold using buffer solution to avoid multiple scattering effects prior to analysis. Particle size measurements are reported as the volume-average mean diameter, $d_{3,2}$ ($=\Sigma n_i d_i^3/\Sigma n_i d_i^2$, where n_i is the number of particles with diameter d_i). The standard deviation was typically within 5% of the mean for nonflocculated emulsions and within 20% of the mean for flocculated emulsions.

ζ-Potential Measurements. The electrical charge (ζ-potential) on the particles was measured using a particle electrophoresis instrument (ZEM5003, Zetamaster, Malvern Instruments, Worcestershire, U.K.). The 5 wt % oil-in-water emulsions were diluted ~500-fold with buffer solution at the appropriate pH prior to measurements. The diluted emulsion was mixed thoroughly and then injected into the measurement chamber of the instrument. The ζ-potential of each individual sample was calculated from the average of five measurements on the diluted emulsion. Standard deviations were always better than $\pm$5 mV.

RESULTS AND DISCUSSION

Composition of Protein Fractions. SDS-PAGE measurements indicated that the HSE fraction consisted of a complex mixture of proteins with at least four major constituents: 200 kDa (major fraction), 130 kDa, 40 kDa, and 35 kDa. Partial assignment of these values was done by comparison with literature data and is shown in **Table 1** (*23*, *24*).

Influence of Initial Protein Concentration on Emulsion Formation and Stability. A preliminary experiment was carried

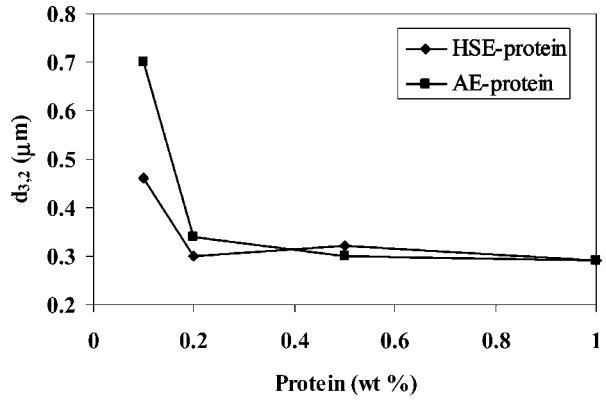

Figure 1. Influence of protein concentration on mean particle size.

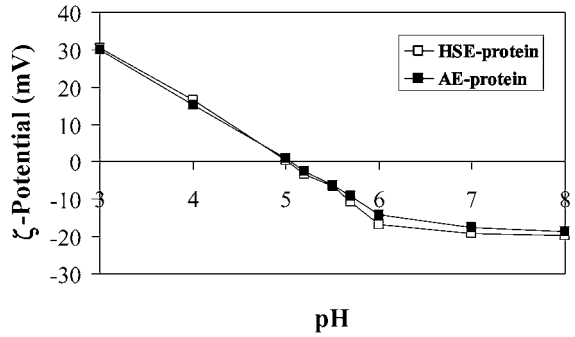

Figure 2. Influence of pH on the ζ-potential of oil-in-water emulsions stabilized by fish proteins.

out to determine the minimum amount of fish protein extract required to form stable emulsions. Corn oil-in-water emulsions (5 wt % oil) were prepared with different initial protein concentrations (0.1−1 wt %) and protein types (AE and HSE), and then the mean particle diameter was measured after homogenization (**Figure 1**).

For both protein types, there was a steep decrease in mean particle diameter when the protein concentration was increased from 0.1 to 0.2 wt %, after which a relatively constant particle size was reached ($d_{3,2} \sim 0.3$ μm). No creaming and no significant change in mean particle diameter were observed in emulsions containing 0.2−1 wt % protein after storage at 4, 20, and 37 °C for 9 days (data not shown), suggesting that these emulsions were fairly stable to droplet aggregation. On the other hand, an appreciable amount of creaming was observed in emulsions containing 0.1 wt % protein (creaming index = 74−85%) after they were stored under the same conditions, which can be attributed to the relatively large mean droplet diameter in these emulsions (**Figure 1**). In all subsequent experiments 0.5 wt % protein was used to prepare the emulsions (i.e., a protein-to-oil ratio of 1:10 g/g), because this protein concentration enabled us to produce emulsions containing relatively small droplets that were stable to creaming.

Influence of pH on Emulsion Stability. The influence of pH on droplet charge (ζ-potential), particle size, and creaming stability of 5 wt % corn oil-in-water emulsions stabilized by AE protein and HSE protein was measured (**Figures 2−4**).

The pH dependence of the ζ-potential of the emulsion droplets was similar for AE and HSE protein fractions (**Figure 2**). The ζ-potential was highly positive (∼+30 mV) at pH 3, became less positive with increasing pH until it reached a value of zero around pH 5, and then became increasingly negative as the pH was increased further, until it reached a value of ∼−19 mV at pH 8. These measurements suggested that the isoelectric points

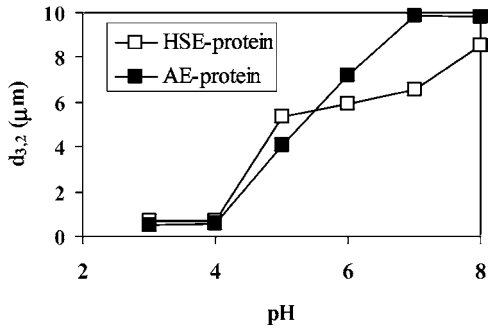

Figure 3. Influence of pH on mean particle size of emulsions.

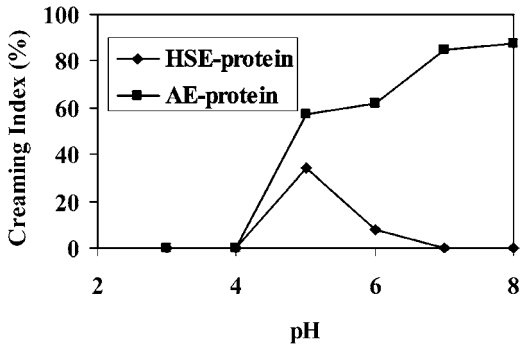

Figure 4. Influence of pH on creaming stability of emulsions.

of the adsorbed proteins were around pH 5, which is close to the value expected for non-adsorbed fish proteins (see ref *5*). The pH dependence of the mean particle diameter was also similar for emulsions stabilized by AE and HSE protein fractions (**Figure 3**).

The mean particle diameter was relatively small ($d_{3,2} \sim 0.5$ μm) at pH 3 and 4 but was appreciably greater ($d_{3,2} > 4$ μm) at higher pH values.

The emulsions stabilized by HSE protein were stable to creaming at low pH values (pH 3 and 4), unstable at intermediate pH values (pH 5−6), and more stable at high pH values (pH 7 and 8) (**Figure 4**). On the other hand, the emulsions stabilized by AE protein were stable at low pH values (pH 3 and 4) but unstable at all higher pH values. The increase in droplet size and creaming instability at higher pH values suggests that extensive droplet aggregation occurred.

The most likely reason for droplet flocculation is the decrease in the magnitude of the electrical charge on the droplets (at intermediate pH values), as well as the decrease in the solubility of the proteins (at high pH values). We expect that emulsion droplets stabilized by fish proteins will become unstable to flocculation under solution conditions (e.g., pH, ionic strength, and temperature) where fish proteins dissolved in aqueous solutions tend to precipitate. The high creaming index observed for both proteins at pH values close to their isoelectric points (pH 5) can therefore be attributed to the fact that the emulsion droplets have a small electrical charge, so that the electrostatic repulsion is no longer sufficient to prevent droplet flocculation. On the other hand, the extensive creaming observed in the AE-stabilized emulsions at high pH can be attributed to the low solubility of this protein under these solution conditions.

Influence of Salt on Emulsion Stability. The influence of NaCl concentration on particle size and creaming stability of 5 wt % corn oil-in-water emulsions stabilized by 0.5 wt % AE protein or HSE protein at pH 3 was measured (**Figures 5** and **6**). Emulsions stabilized by both protein fractions exhibited

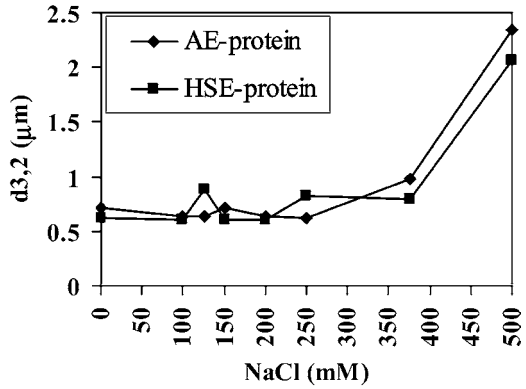

Figure 5. Influence of NaCl on mean particle size of emulsions.

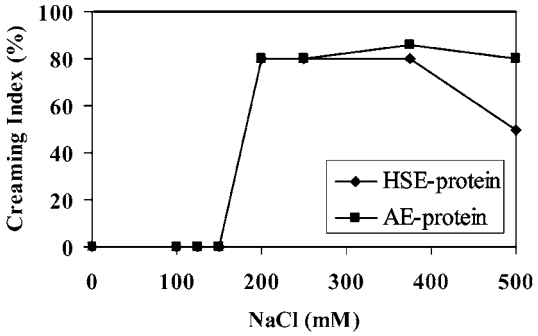

Figure 6. Influence of NaCl on creaming stability of emulsions.

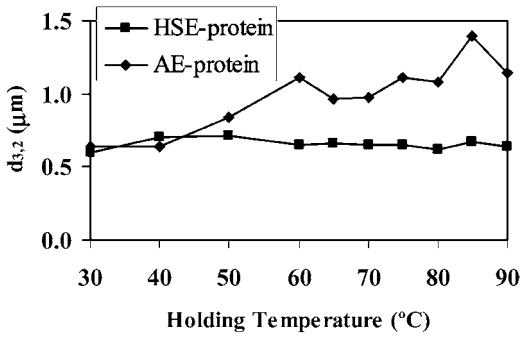

Figure 7. Influence of temperature on mean particle size of emulsions. The emulsions were stored at the holding temperatures for 30 min and then cooled to ambient temperature before their particle sizes were measured.

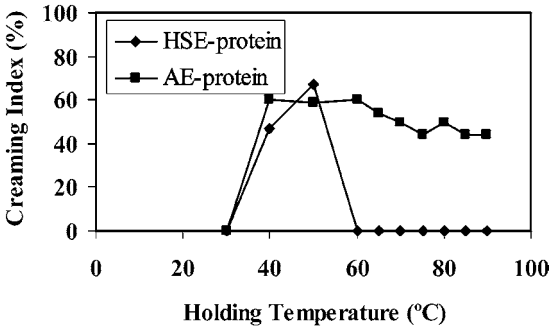

Figure 8. Influence of heat treatment on creaming stability of emulsions.

similar behavior. The mean particle diameter of the emulsions was relatively small ($d_{3,2} \sim 0.6-0.9$ μm) at low salt concentrations (0−375 mM NaCl) but increased appreciably at the highest salt concentration (500 mM NaCl). The emulsions were stable to creaming at low salt concentrations (0−150 mM NaCl) but were highly unstable at higher salt concentrations (200−500 mM NaCl), suggesting that extensive droplet flocculation occurred. The reason that extensive droplet flocculation was not observed in the particle size measurements (**Figure 5**) was probably because the droplets in the flocs were held together by relatively weak attractive interactions that were disrupted during the 500-fold dilution step required in laser diffraction analysis.

This highlights the importance of confirming laser diffraction measurements of particle aggregation with measurements made using another technique that does not involve sample dilution.

Influence of Thermal Processing on Emulsion Stability. The influence of holding temperature on the particle size and creaming stability of 5 wt % corn oil-in-water emulsions stabilized by 0.5 wt % AE protein or HSE protein was measured (**Figures 7** and **8**). Emulsions were held at the specified temperatures for 30 min, then cooled to room temperature, and stored prior to analysis. The particle size determination was carried out after the emulsions had been diluted (500-fold) in the light scattering instrument. In the absence of salt (0 mM NaCl), there was no significant change in the mean particle diameter of emulsions after any of the heat treatments ($d_{3,2} \sim 0.6-0.7$ μm). In the presence of salt (100 mM NaCl), the mean particle diameters of the emulsions stabilized by HSE protein were fairly similar ($d_{3,2} \sim 0.6-0.8$ μm) for all heat treatments, but the emulsion stabilized by AE protein showed some evidence of droplet aggregation at temperatures >40 °C ($d_{3,2} \sim 1.0-1.4$ μm) (**Figure 7**).

In the absence of salt (0 mM NaCl), there was no evidence of creaming in the emulsions stabilized by both protein fractions after any of the heat treatments (creaming index = 0%). In the presence of salt (100 mM NaCl), the emulsion stabilized by HSE protein exhibited creaming after heating to 40 and 50 °C but was stable for all other heat treatments (**Figure 8**). On the other hand, the emulsion stabilized by AE protein showed extensive creaming instability at all temperatures >40 °C. It is possible that the adsorbed AE proteins undergo some form of conformational change upon heating above a critical temperature that increases the attraction between the droplets, for example, due to increased exposure of hydrophobic groups.

The behavior of the HSE-protein-stabilized emulsions is more difficult to explain. Studies of the rheology of fish protein fractions have shown that there is a pronounced maximum in the shear modulus around 40−50 °C (*12*), which suggests that a conformational change may occur that could promote droplet flocculation.

Solubility Characteristics of Fish Protein Extracts. We postulated that the ability of fish protein fractions to stabilize oil-in-water emulsions would depend partly on protein solubility. We therefore examined the influence of pH and ionic strength on the solubility of selected protein fractions. Three different concentrations of sodium chloride were investigated and six different pH values. The AE protein, which contains mainly myosin but also a considerable amount of five other proteins, was used for this study. The protein solutions were prepared by placing 100 mg of freeze-dried protein in 20 mL of an aqueous solution of the appropriate pH and sodium chloride concentration and leaving it at 4 °C for 3 days. The protein concentration remaining in the supernatant of the solution was then determined using the biuret method and bovine serum albumin as a standard (**Table 2**).

The results in **Table 2** show that the solution conditions that give the highest protein solubility were pH 3 and no added salt. Increasing the pH resulted in a sharp decrease in the solubility

Table 2. Solubility of AE Protein at Different pH Values and Salt Concentrations As Determined by the Biuret Method[a]

	[NaCl] = 0 M		[NaCl] = 0.15 M		[NaCl] = 0.8 M	
pH	A_{540}	protein (mg/mL)	A_{540}	protein (mg/mL)	A_{540}	protein (mg/mL)
3	0.337	3.85	0.088	1.01	0.011	0.13
4	0.051	0.58	0.039	0.45	0.031	0.35
5	0.035	0.40	0.031	0.35	0.073	0.83
6	0.043	0.49	0.057	0.65	0.084	0.96
7	0.066	0.75	0.067	0.77	0.096	1.10
8	0.066	0.75	0.071	0.81	0.098	1.12

[a] The concentration was determined from the formula for a best line for standard solutions containing 0–5.00 mg/mL protein. The formula for the best line was $y = 11.425x$ with $R^2 = 0.9948$, where $x = A_{540}$ and $y =$ mg of protein.

with a minimum around pH 5, which is consistent with the known isoelectric point of myosin, p*I* 5.2 (see ref 5). Above the isoelectric point the total protein solubility started to increase again but had reached only ~20% of the maximum value at pH 8. Interestingly, the tropomyosin single chain, which has practically no solubility at pH 4, 5, and 6, appears again at pH 7 and 8 and is by far the most soluble protein at these more basic conditions. This protein is also quite soluble at pH 3 and 8 with 0.15 M NaCl but not at pH 4 and 5. At low pH the protein solubility decreased sharply with increasing salt concentration. The poor stability of the AE-protein-stabilized emulsions at relatively high pH (≥5) and NaCl (≥200 mM) may therefore be due at least partly to the decrease in the solubility of the proteins.

Conclusions. This work has shown that fish protein, which can be isolated by simple methods from cod muscle, is very effective in stabilizing oil-in-water emulsions. The thermal stability is particularly noteworthy for both the HSE and AE proteins. This is important because the acid extraction makes available a much larger part of the total protein. Emulsions stabilized by both proteins were stable to salt up to ~200 mM NaCl but unstable to creaming at higher salt concentrations. It is clear from the results presented here that these proteins are most suitable for emulsions in acidic conditions. The solubility at pH >3 and up to pH 8 in a salt-free solution is so low that it would restrict the effective preparation of emulsions. More basic conditions were not investigated. The stability of emulsions prepared at pH 3 when the pH was raised was also low, which can probably be explained by the low solubility of the protein at intermediate pH values. Evidence was obtained to show that the stability of the AE-protein-stabilized emulsions was increasing again at higher pH, 7 and 8. This increased stability may be associated with the increased solubility of tropomyosin at neutral pH, which the SDS-PAGE shows clearly.

The work on the solubility characteristics of fish protein extracts clearly points to effective conditions to isolate tropomyosin-rich fractions by using either pH 8 and no added salt or pH 3 with 0.15 M sodium chloride. This will be investigated further.

ACKNOWLEDGMENT

S.P. gratefully acknowledges the hospitality of the Department of Food Science, University of Massachusetts, during the spring term of 2003. We thank Prof. Herbert Hultin for advice regarding the protein extraction and Dr. Shuming Ke for help with the SDS-PAGE experiments.

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Received for review October 25, 2003. Revised manuscript received March 16, 2004. Accepted April 14, 2004. S.P. gratefully acknowledges the financial support of the Fulbright Organization and the University of Akureyri.

JF035251G