Recovery and Functionality of Wash Water Protein from Krill Processing

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Microfiltration followed by ultrafiltration was used to concentrate soluble proteins in the wash water of cephalothorax and krill muscle and to remove organic substances and microorganisms. Wash water, concentrate, and ultrafiltrate were characterized. Most of the proteins from muscle thus extracted were less than 67 kDa, while from cephalothorax there was a large amount of 100−150 kDa proteins. Filtrate exhibited low levels of chemical oxygen demand (COD) and total viable count (TVC). Emulsion properties were assayed for different protein concentrations in freeze-dried cephalothorax and muscle concentrates. The first one presented poor emulsion stability in contrast to the second one. Measurements of emulsion activity index (EAI) and stability in the muscle concentrate emulsion were virtually unaffected by ionic strength (0−3% NaCl) and slightly affected by pH.

Keywords: *Ultrafiltrate; microfiltrate; wastewater; protein emulsion; krill*

INTRODUCTION

Fish meal industries use a large amount of water to produce surimi and/or wash minced fish muscle to remove fat and water-soluble substances such as sarcoplasmic proteins, pigments, amines, vitamins, and enzymes in order to concentrate myofibrillar proteins. Santos et al. (1995) estimate that 167 000−526 000 L of freshwater is used per day to produce approximately 25 tons of surimi. High-quantity wastewater is produced in other fish industry processes such as mechanical peeling of shellfish (Bustos et al., 1996). Krill are peeled by these processes that involve a large amount of water, loosing substances from cephalothorax. On the other hand, because of its small size, krill muscle is not destined for direct consumption and for this purpose should be restructured. This process sometimes involve washing steps so that sarcoplasmic and some myofibrillar soluble proteins are lost in wash water.

Recent studies indicate that a significant portion of myofibrillar proteins are solubilized in low ionic strength (near zero) solutions (Stefanson and Hultin, 1994; Lin and Park, 1995). These proteins lost in the wastewater account for 15−30% of the total protein of minced fish, and some of them could be highly functional. Moreover, loss of minced fish solids, in soluble or insoluble form, during washing and dewatering (Watanabe et al., 1982, Adu et al., 1983, Pacheco-Aguilar et al., 1989, Lin et al., 1995) generates a bulky liquid effluent with a high organic content, constituting a huge contaminating load. Some authors have studied the recovery of this wastewater in order to avoid ecological hazards (Martí et al., 1994; Rodriguez-Estrada et al., 1994).

Methods for fish protein recovery from wastewater of fish meal and surimi factories have included evaporation and drying, precipitation, centrifugation, filtration, or microfiltration or a combination of centrifugation, microfiltration, and ultrafiltration, depending on the raw material. It has been found that with microfiltration and/or ultrafiltration, the functional properties can be retained much better than with other processes (Nishioka and Shimizu, 1983; Green et al., 1984; Swafford, 1987; Pedersen et al., 1987, 1989; French and Pedersen, 1990; Rodriguez-Estrada et al., 1994).

The aim of the present work was to recover the proteins from cephalothorax and minced krill wash water by means of a microfiltration/ultrafiltration process and to examine their emulsifying properties for application to products for human consumption, as well as reducing chemical and microbiological loads in wash water, to avoid associated pollution problems.

MATERIALS AND METHODS

Sample Preparation. The fish used in these experiment was antarctic krill (*Euphasia superba dana*). Frozen krill was tempered overnight and then skinned. Cephalothorax and tail muscle were separated. Minced muscle was held for 10 min at 0−3 °C in a pH 7 aqueous solution of 0.5% bicarbonate, proportion 3:1 (solution:minced muscle), with constant stirring. The cephalothorax was processed in the same way to obtain a similar dilution of solutes, although in the industry far larger amounts of water are used in the process. The solution was then microfiltered through a serum capsule of 1.2 μm supplied by Gelman Sciences to eliminate a major portion of microorganism content. Later the solution was concentrated by ultrafiltration. For this purpose, a tangential flow membrane filter (30 kDa) was placed in a Minessette hardware filter holder and a pump provided recirculating flow. Minessette and filter were supplied by Filtron Technology Corp. and the pump was a Millipore variable speed tubing pump (Bedford, MA).

In both cases the wash water was concentrated down to a third of the initial volume. Wash water, filtrate, and concentrate were frozen and stored for subsequent analysis. The concentrate was freeze-dried.

Analyses Performed: Water and Protein Recovery Characterization. To characterize the samples, crude protein and ash were determined by AOAC methods (1984). Crude fat was analyzed according to Bligh and Dyer (1959). Chemical oxygen demand (COD) was measured according to

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Table 1. Proximate Composition of Cephalothorax and Muscle Wash Waters

	cephalothorax	muscle
ash (%)	0.67 ± 0.03	0.65 ± 0.03
crude protein (%)	2.02 ± 0.03	1.98 ± 0.02
crude fat (%)	1.67 ± 0.16	0.71 ± 0.03

AOAC (1984). Total viable counts (TVC) were performed on plate count agar (Oxoid, Hampshire, England). Plates were incubated at 30 °C for 72 h. Water proteolytic activity was measured at 37 °C /1 h and pH 7. Wash water, concentrate, and filtrate were used as enzyme substrate. Six milliliters of the samples was used and enzymatic activity was stopped by addition of 12 mL of 0.1 M trichloroacetic acid. Peptide concentration was determined according to Lowry et al. (1951). Tyrosine was used to prepare the standard curve. Proteolytic activity at 37 °C and pH 7 was expressed as milligrams of Tyr released per gram per hour. All determinations were made in quadruplicate. Soluble protein concentration was determined according to Lowry et al. (1951). Results of all the assays were averages of three determinations. TVC was made in duplicate.

SDS−Polyacrylamide Gel Electrophoresis. Soluble protein was analyzed by SDS−PAGE in a Phastsystem horizontal apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) using 12.5% polyacrylamide gels. The protein load was 2 mg/mL. Samples were treated according to Hames (1985) (2% SDS, 5% mercaptoethanol, and 0.002% bromophenol blue) and then heated for 5 min in a boiling water bath. Electrophoresis conditions were 4 mA/gel, 250 V, and 3 W. The protein bands were stained with Coomassie Brilliant Blue. As reference for molecular weights, a standard high molecular weight reference kit (Pharmacia LKB Biotechnology) was used: ferritin half-unit 220 000, albumin 67 000, catalase subunit 60 000, lactate dehydrogenase subunit 36 000, and ferritin subunit 18 500.

Emulsifying Properties. Samples were prepared by homogenizing the freeze-dried wash water concentrate from cephalothorax and muscle with distilled water to the different target concentrations (5, 10, 15, 20, and 25 mg/mL). Effect of NaCl (0%, 1%, 2%, and 3% NaCl) and pH (3, 4, 5, 6, 7, 8, and 9) at a protein concentration of 25 mg/mL of muscle concentrate wash water were analyzed.

Emulsion activity index (EAI, square meters per gram) was determined by the method of Pearce and Kinsella (1978) as modified by Li-Chan et al. (1984).

Emulsion stability was tested by centrifuging 25 g of the emulsion prepared for EAI determination for 10 min at 4000*g*. The aqueous plus fatty fraction (AF), which was not retained in the emulsion, was collected in capsules and dried to constant weight; these were expressed as oil released [(dry weight of AF × 100)/initial weight of sample] and percent water released [[(weight of AF − dry weight of AF) × 100]/initial weight of sample]. The precipitate (the emulsion remaining) was expressed as percent emulsion stability [(weight of precipitate x 100)/initial weight of emulsion]. Three replications were performed for all determinations.

Statistical Analysis of Data. Analysis of variance was carried out for the different samples using the Statgraphics computer program (STSC Inc., Rockville, MD). The difference of means between pairs was resolved by means of confidence intervals using least significant difference (LSD) range test. Level of significance was set for $P \leq 0.05$.

RESULTS AND DISCUSSION

The amount of proteins present in the concentrate water was 25 mg/mL. In each case protein concentration was adjusted to the desired level with water. The cephalothorax lot was dark in color and smelled strongly, as reported by Lin et al. (1995). However, the tail muscle lot was clear and smelled only mildly.

Proximate Composition of Wash Water. Ash and crude protein were similar in cephalothorax and tail muscle wash water (Table 1). However, crude fat was higher in cephalothorax wash water because the fat content is higher in the cephalothorax than in the tail muscle (Suzuki, 1981).

Table 2 shows a similar protein concentration in wash waters from cephalothorax and muscle and a similar pattern of increase in both concentrates. However, there was a considerably larger amount of protein in the cephalothorax filtrate than the muscle filtrate, probably because of a higher level of endogenous proteolysis. There was a pronounced decrease of muscle protease activity (2.12 μg of Tyr/g·h) after washing (1.23 μg of Tyr/g·h); some muscle proteases remained in the washed muscle because they were not completely extracted. This finding agrees with that of Lin et al. (1995). Proteolytic activity mainly increased in the concentrate from tail muscle wash water, rather than from cephalothorax, with respect to the values found in wash water. Nevertheless, there was a higher level of proteolytic activity in the ultrafiltrate from the cephalothorax lot. This could mean that low molecular weight proteins from the cephalothorax have more proteolytic activity than those of muscle. Suzuki (1981) observed that there was more proteolytic activity in cephalothorax than in muscle of krill, which would account for the virtual absence of proteolytic activity and the lower protein concentration in the muscle filtrate. The washing of tail muscle and cephalothorax caused an increase of organic substances and microorganisms in the wash water, giving high values of COD and TVC (Table 2). After micro- and ultrafiltration, these parameters decreased sharply in the ultrafiltrates, and the TVC increased in the concentrates. Many authors have reported this effect in various different minced fish washing processes (Lin et al., 1995; Martí et al., 1994; Ninomiya et al., 1985), although the values were lower. Microfiltration was applied to reduce the amount of microorganisms in tail muscle and cephalothorax wash water before ultrafiltration.

Electrophoretic Study of Wash Water Proteins. The electrophoretic profiles from cephalothorax and muscle wash waters were different (Figure 1). The same profiles were observed in wash water and concentrated wash water for each sample. In the samples from cephalothorax there was a large amount of proteins of molecular weight between 67 and 150 kDa and some traces of proteins with molecular weight lower than 38 kDa. The samples from tail muscle also exhibited some

Table 2. Protein Concentration, Proteolytic Activity, Chemical Oxygen Demand, and Total Viable Count

	wash water		concentrate		filtrate	
	cephalothorax	muscle	cephalothorax	muscle	cephalothorax	muscle
PC[a] (mg/mL)	12.9 ± 1.1	12.5 ± 1.0	30.2 ± 3.0	29.8 ± 2.8	0.22 ± 0.00	0.14 ± 0.00
PA[italb] (mg of Tyr/g·h)	1.03 ± 0.04	0.64 ± 0.07	1.14 ± 0.05	1.00 ± 0.37	0.38 ± 0.03	0.08 ± 0.00
COD[c] (1000 mg/L)	7544 ± 99	1055 ± 50	-	-	193 ± 33	150 ± 21
TVC[d] (1000 cfu/mL)	104500 ± 2500	136000 ± 4000	155000 ± 500 23100 ± 35[e]	181000 ± 1000 25300 ± 24[e]	16.0 ± 0.2	0.8 ± 0.0

[a] Protein concentration. [b] Proteolytic activity. [c] Chemical oxygen demand. [d] Total viable count. [e] After microfiltration of wash water.

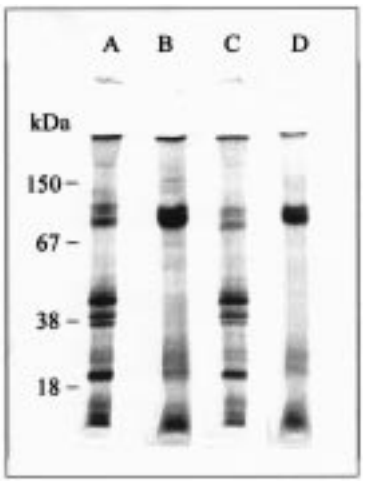

Figure 1. SDS−PAGE of (A) tail muscle concentrated wash water, (B) cephalothorax concentrated wash water, (C) tail muscle wash water, and (D) cephalothorax wash water.

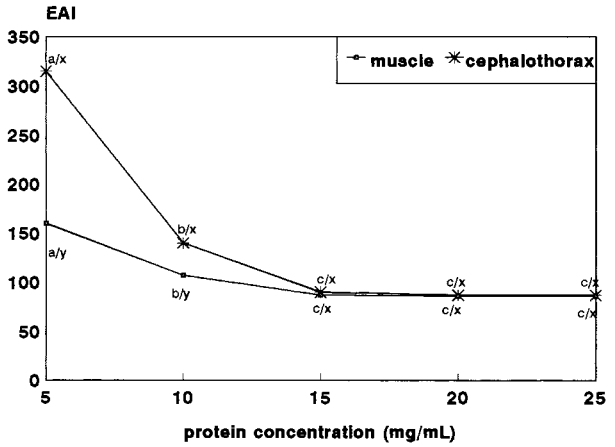

Figure 2. Emulsion activity index (EAI) of freeze-dried concentrated wash water of tail muscle and cephalothorax at different protein concentrations. Different letters a, b, ... indicate significant differences as a function of protein concentration; different letters x, y, ... indicate significant differences between muscle and cephalothorax concentrated wash water.

bands corresponding to proteins between 67 and 150 kDa, but in this case there was a wide variety of proteins between 45 and 18 kDa, which correspond to actin, tropomyosin, and troponins, indicating that the muscle wash water contained sarcoplasmic and also myofibrillar proteins. This would account for the lower protein concentration in the filtrate. This finding agrees with that of Lin et al. (1995), who reported that considerable quantities of myofibrillar proteins (myosin and actin) were dissolved to some extent when the muscle was washed with water several times and dewatered by mechanical force; myosin and actin were found after the second wash, while sarcoplasmic proteins appeared in the first wash water.

Emulsifying Properties. Figure 2 shows emulsifying properties of protein recovered from concentrated freeze-dried wash water of cephalothorax and tail muscle diluted to different concentrations. The EAI of freeze-dried wash water concentrate decreased in each lot with increased protein concentration, especially in the cephalothorax sample at low protein concentrations. This is in agreement with Cofrades et al. (1996) for actomyosin from hake, chicken, and pork. Huidobro et al. (1998) describe the same effect in emulsifying capacity of freeze-dried concentrate of sardine wash water. This could be related to protein aggregation, which could interact with the two phases, thus creating a barrier to coalescence, as frequently occurs in the drying process (Cheftel et al., 1985). Kinsella (1976) reported that an increase in protein concentration causes a rise in polypeptide chains and as a result, fewer molecules act as an interface in the emulsion. This effect has been demonstrated in fish myofibrillar proteins by Borderias et al. (1985) and in soluble collagen by Montero and Borderias (1991).

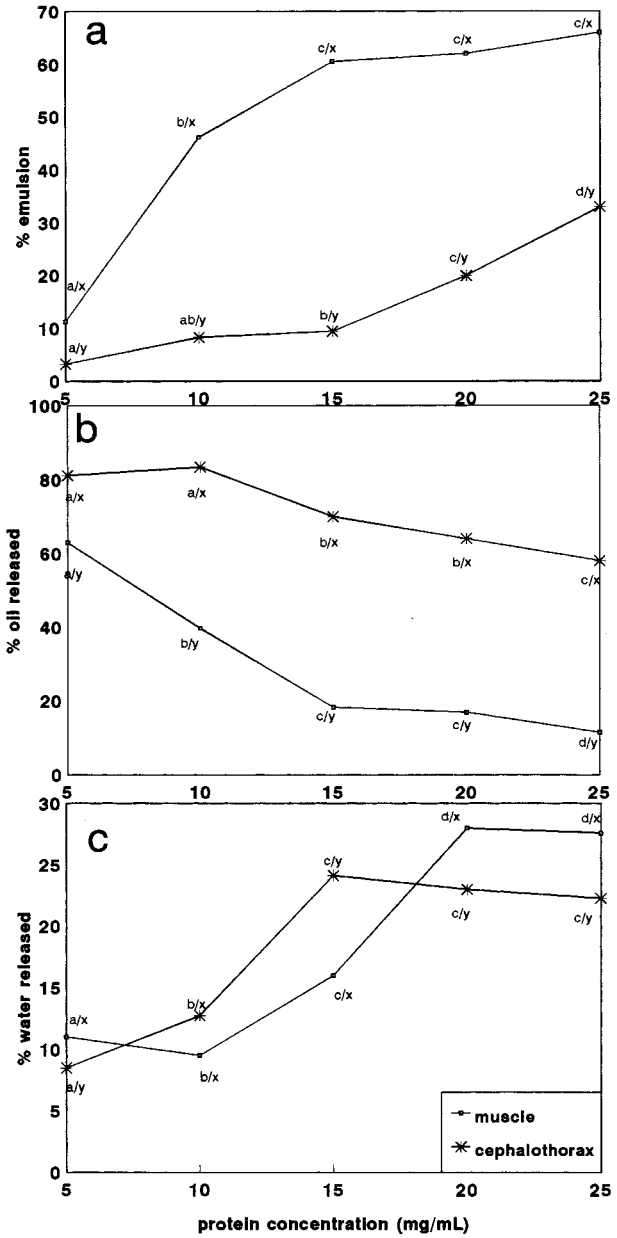

Figure 3. Emulsion stability [(a) % emulsion, (b) % oil released, and (c) % water released] of freeze-dried concentrated wash water of tail muscle and cephalothorax at different protein concentrations. Different letters a, b, ... indicate significant differences as a function of protein concentration; different letters x, y, ... indicate significant differences between muscle and cephalothorax concentrated wash water.

Emulsion stability increased ($P \leq 0.05$) in samples from cephalothorax and muscle with increased protein concentration (Figure 3), but evolution was very different in the two samples. The cephalothorax wash water did not in fact make a stable emulsion. This could be

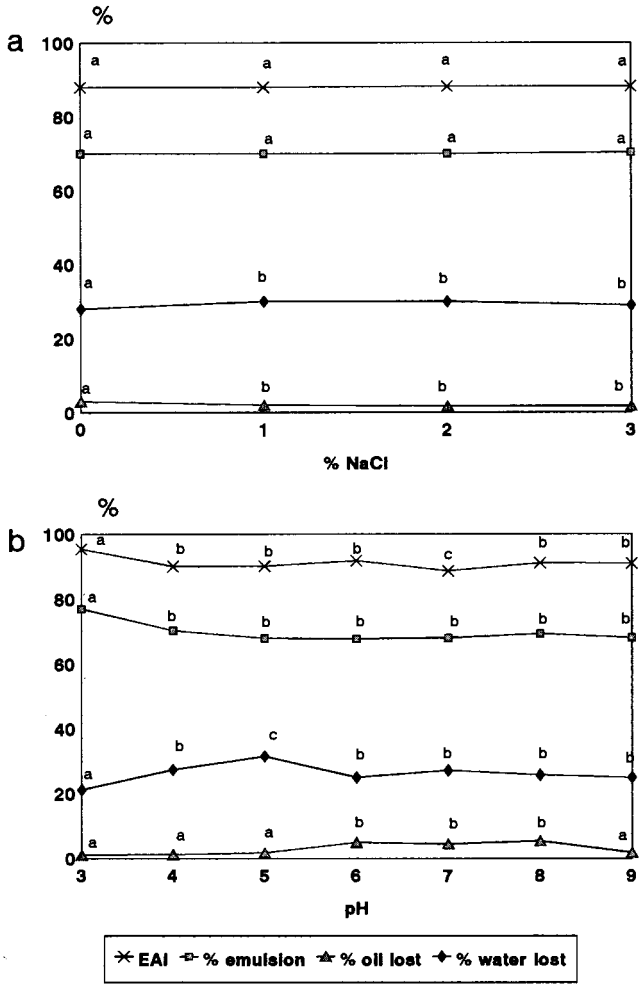

Figure 4. Emulsion stability (% emulsion, % oil released, and % water released) of freeze-dried concentrated wash water of tail muscle (25 mg/mL protein concentration) at different NaCl concentrations (panel a) and pHs (panel B). Different letters a, b, ... indicate significant differences.

explained by assuming that the nature of the protein in either sample was different, as the electrophoretic profiles suggested, and that there were more proteolytic enzymes in cephalothorax. Emulsion stability in freeze-dried concentrate of tail muscle wash water exhibited a very sharp increase ($P \leq 0.05$) between 5 and 10 mg/mL. From 15 mg/mL, emulsion stability was similar. In this connection, Cofrades et al. (1996) reported that protein concentration did not influence emulsion stability in actomyosin isolated from hake muscle (*Merluccius merluccius*) at concentrations between 5 and 15 mg/mL. These differences in emulsion stability could be attributed not only to be species dependent but also to differences in molecular composition.

In both samples, the oil release fraction of the emulsion decreased with increasing protein concentration, although an increase in water loss was detected. In contrast, Huidobro et al. (1998) reported that the aqueous fraction in concentrated wash water from sardine tended to decrease gradually as protein concentration increased, whereas oil release was stable.

The influence of ionic strength and pH on emulsion formation and stability was studied in tail muscle wash water concentrate at a protein concentration of 25 mg/mL. Ionic strength had no significant influence ($P \leq 0.05$) on EAI and emulsion stability values (Figure 4a). A positive effect of addition of NaCl on emulsion stability was reported by Huidobro et al. (1998) in freeze-dried sardine wash water, suggesting again that the emulsifying properties could be species-dependent.

The influence of pH on EAI was slight but significant ($P \leq 0.05$) (Figure 4b). The EAI values were highest at pH 3, reaching a minimum at pH 7. In this connection, Cofrades et al. (1996) reported that pH had a direct linear effect on EAI, which was significant in chicken and hake but not in pork within a pH range of 5.2−7.5. Emulsion stability at pH 3 was significant higher than at pHs 4−9. The pH affected the oil loss fraction, which was significantly smaller at low pHs (3−5) and at pH 9; the aqueous loss fraction was highest at pH 5.

Conclusions. These results suggest that microfiltration followed by ultrafiltration is a suitable procedure for recovery of krill wash water proteins, as it reduces the contamination in the filtrate while the concentrate has possible applications in emulsion-type products for human consumption, since it shows a high stability in a wide range of pH and NaCl concentrations.

LITERATURE CITED

Adu, G. A.; Babbit, J. K.; Crawford, D. L. Effect of washing on the nutritional and quality characteristics of dried minced rockfish flesh. *J. Food Sci.* **1983**, *48*, 1053−1060.

AOAC. *Official Methods of Analysis of the Association of Official Analytical Chemists*; AOAC: Washington, DC, 1984.

Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **1959**, *37*, 911−917.

Borderías, A. J.; Jiménez-Colmenero, F.; Tejada, M. Viscosity and emulsion ability of fish and chicken muscle protein. *J. Food Technol.* **1985**, *1* (20), 31−42.

Bustos, R. O.; Romo, C. R; Healy, M. Stabilisation of trypsin-like enzymes from Antarctic Krill: effects of polyols, polysaccharides and proteins. *J. Chem. Technol. Biotechnol.* **1996**, *65*, 193−199.

Cheftel, J. C.; Cuq, J. L.; Lorient, D. *Proteines Alimentaires*; Technique et Documentation, Lavoisier: Paris, France, 1985.

Cofrades, S.; Carballo, J.; Careche, M.; Jiménez-Colmenero, F. Emulsifying properties of actomyosin from several species. *Lebensm. Wiss. Technol.* **1996**, *29*, 379−383.

French, J. S.; Pedersen, L. D. Properties and stability of surimi combined with process-water protein concentrates. In *IIR, Commission C2, Chilling and freezing of new fish products*; International Institute of Refrigeration: Aberdeen, U.K., 1990; pp 95−101.

Green, D. P.; Tzou, L.; Chao, A. C.; Lanier, T. C. Strategies for handling soluble waste generated during minced fish (surimi) production; *Proceedings of the 39th Industrial Waste Conference*, Purdue University, West Lafayette, IN; Butterworth Publishers: Boston, Ma, 1984; p 565.

Hames, B. D. Gel electrophoresis of proteins: A practical approach; IRL Press: Oxford, England, 1985; pp 1−91.

Huidobro, A.; Montero, P.; Borderías, A. J. Emulsifying properties of an ultrafiltered protein from minced fish wash water. *Food Chem.* **1998**, *61* (3), 339−343.

Kinsella, J. E. Functional properties of proteins in food: a survey. *Crit. Rev. Food Sci. Nutr.* **1976**, *7*, 219−296.

Li-Chan, E.; Nakai, S.; Wood, D. F. Hydrophobicity and solubility of meat proteins and their relationship to emulsifying properties. *J. Food Sci.* **1984**, *49*, 345−350.

Lin, T. M.; Park, J. W. Solubility of myofibrillar proteins during surimi processing. Effects of ionic strength and washing. Presented at the annual meeting of Institute of Food Technologists, Anaheim, CA, June 3−7, 1995.

Lin, T. M.; Park, J. W.; Morrisey, M. T. Recovered protein and reconditioned water from surimi processing waste. *J. Food Sci.* **1995**, *60* (1), 4−9.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265−275.

Martí, C.; Roeckel, M.; Aspe, E.; Kanda, H. Recovery of proteins from fishmeal factory wastewaters. *Process Biochem*. **1994**, *29*, 39−46.

Montero, P.; Borderías, A. J. Emulsifying capacity of collagenous material from the muscle and skin of hake (*Merluccius merluccius* L.) and trout (*Salmo irideus* Gibb): effect of pH and NaCl concentration. *Food Chem*. **1991**, *41*, 251−267.

Ninomiya, K.; Ookawa, T.; Tsuchiya, T.; Matsumoto, J. J.. Recovery of water soluble proteins in wastewater of fish processings plants of ultrafiltration. *Nippon Suisan Gakkaishi* **1985**, *51* (7), 1133−1138.

Nishioka, F.; Shimizu, Y. Recovery of proteins from washings of minced fish by pH shifting method. *Bull. Jpn. Soc. Sci. Fish*. **1983**, *49*, 795−799.

Pacheco-Aguilar, R.; Crawford, D. L.; Lampila, L. E. Procedures for the efficient washing of minced whiting (*Merluccius productus*) flesh for surimi production. *J. Food Sci.* **1989**, *54* (2), 248−252.

Pedersen, L. D.; Rose, W. W.; Brewbaker, D. L.; Merlo, C. A. Hyperfiltration technology for recovery and utilization of protein materials in surimi process waters. Final Report Phase I; National Food Processors Assn.: Dublin, CA., 1987.

Pedersen, L. D.; Rose, W. W.; Deniston, M. F.; Merlo, C. A. Hyperfiltration technology for recovery and utilization of protein materials in surimi process waters. Final Report Phase II; National Food Processors Assn.: Dublin, CA, 1989.

Pierce, K. N.; Kinsella, J. E. Emulsifying properties of proteins: evaluation of a turbidimetric technique. *J. Agric. Food Chem*. **1978**, *26*, 716−723.

Rodríguez-Estrada, M. T.; Chung, S.; Chinachoti, P. Solids extraction of cod frame and effects on ultrafiltration of the aqueous extract. *J. Food Sci.* **1994**, *59* (4), 799−803.

Santos, M. R.; Huang, L.; Singh, R. P.; Park, J. W.; Morrisey, M. T. Accounting of water use and wastewater generation in the seafood industry. Presented at the annual meeting of Institute of Food Technologists, Anaheim, CA, June 3−7, 1995.

Stefansson, G.; Hultin, H. G. On the solubility of cod muscle proteins in water. *J. Agric. Food Chem*. **1994**, *42*, 2656−2664.

Suzuki, T. *Fish and krill protein: processing technology*; Applied Science: London, UK, 1981.

Swafford, T. C. Separation−recovery of soluble/insoluble proteins from surimi processing washwaters. Proceedings of the Pacific Fisheries Technology Meeting, Monterey, CA, 1987.

Watanabe, H.; Takai, R.; Sekigawa, A.; Hasegawa, H. An estimation of the amount of protein lost in the effluent from frozen surimi manufacture. *Nippon Suisan Gakkaishi* **1982**, *48* (6), 869−871.

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