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Lab scale optimization procedures were utilized to collect a crude protease from surimi wash water. Wash water protein removal was maximized and protease losses were minimized through reduction in acidity (pH ~6), heating (60°C), and centrifugation (3000 xg for 10 min.). Attempts to increase protease purity by ultrafiltration or microfiltration with 300 kD, 1000 kD, and 0.3 μm polyethersulfone membranes were not successful. However, concentration of protease was successful utilizing 30 kD and 50 kD ultrafiltration membranes. Pre-treating wash water significantly reduced 35 – 205 kD proteins and improved overall membrane flux (2-fold). In addition, pretreatment and ultrafiltration concentration of wash water increased crude protease purity about 100-fold with an approximate 80% yield. Crude protease was characterized as having pre-dominantly cathepsin L activity with a minor amount of cathepsin B and H activity. Stability of protease decreased about 30% after 9-weeks. Decline in activity of

freeze-dried protease was not effected by storage temperature (4°C vs. −15°C vs. −80°C). Furthermore, addition of a metal chelator (0.02% and 0.1% EDTA) or cryoprotectant (0.1% and 1% phosphate) did not affect stability. Applicability of crude protease for deproteination of demineralized shrimp shell was tested. Maximum protein extraction (6.7-6.8%, dry weight basis) occurred with addition of 1% crude protease (18 mU/g shell), 55°C, 2 hrs. However, equivalent concentrations of papain (1%, 731 mU/g shell) recovered 6 times that amount of protein.

Recovery and Utilization of Catheptic Proteases From Surimi Wash Water

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Christina A. Mireles DeWitt, Author

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Chapter 1. Introduction

Commercial utilization of Pacific whiting (*Merluccius productus*) for the production of surimi occurred on the West Coast less than ten years ago. From 1990 to 1994, on shore landings of Pacific whiting increased 10-fold with the majority going into surimi production (Radtke, 1995). In 1998, 229,729 metric tons were harvested in the Pacific Northwest (U.S.), of which 40% was allocated to shoreside processors (Warren and others 1999). Prior to its use in surimi, Pacific whiting was regarded as an underutilized species for shoreside processing because of the tendency of its flesh to rapidly degrade and become soft (Pacheco Aguilar, 1986; Peters and others 1995). Poor quality was attributed to elevated levels of proteases in its flesh. These abnormal levels of proteolytic enzymes in Pacific whiting are a result of a significant proportion of the population (20-40%) being infected with a myxosporidian parasite, *Kudoa spp.* (Pacheco Aguilar, 1986). The proteases primarily responsible for degradation of functional myofibrillar proteins and gel weakening in Pacific whiting surimi are catheptic proteases (An and others 1994a; An and others 1994b; Seymour and others 1994; An and others 1995). Inhibition of these proteases can be achieved with food grade protease inhibitors such as beef plasma protein, egg white, and potato extract (Morrissey and others 1993). Currently, surimi is still the primary end product for Pacific whiting.

The production of surimi involves the concentration of myofibrillar proteins. This is achieved through removal of water-soluble (i.e. sarcoplasmic) proteins, which account for nearly 30% of total proteins (Sikorski, 1994). The first stage of surimi production involves heading, de-gutting, and filleting. Fillets are ground to form a mince. What follows is an extensive washing and dewatering regimen to remove undesirable constituents such as water and dilute salt soluble proteins, peptides, amino acids, amines, amine oxides, guanidine compounds, quaternary ammonia compounds, purines, and urea (Haard and others 1994). The number of wash cycles normally varies between 2-4 and water to meat ratios range from 12:1 to 24:1 (Lin, 1996). For a two step wash process, over 70% of protein lost is lost during the first wash operation (Jaouen and Quéméneur, 1992). In addition, protease activity in Pacific whiting mince decreases 10-fold as a result of washing and highest activity is subsequently found in wash water collected from the first wash and screw press (Morrissey and others 1995).

The washing of mince at shoreside surimi processing plants generates an average of 328.8 (± 21.9) L/min wash water with a total solids of 2.06 (± 0.12) kg/min or approximately 6 g of soluble protein per liter (Jaouen and Quéméneur, 1992; Huang, 1997). As a result, 24 hr. of processing discards close to 3 metric tons of solids into the wastewater system. The possibility of increasing production efficiencies through reclamation of wash water solids has resulted in several concerted efforts to recover wasted by-products. Lin and others (1995) used micro- and ultrafiltration to recover proteins and recycle water from the commercial

production of surimi. They found incorporation of recovered proteins into surimi did not adversely effect final quality parameters such as gel hardness, elasticity, water retention, and color. In addition, recovery of proteins increased production efficiencies by 1.7%. One of the obstacles to effective use of ultra- or microfiltration membranes is fouling and reduced flux. Bioseparation of proteins is a well-established application of ultra- and microfiltration in the dairy and food industry (Iritani, and others 1995). Jaouen and Quéméneur (1992) commented in their review of membrane filtration for wastewater protein recovery that processing of surimi wash "waters by ultrafiltration without pretreatment is not practicable." They suggested "...insoluble materials may negatively affect the operation of the unit as module channels become clogged" resulting in poor flux. In ultrafiltration, losses in initial flux are generally attributed to reversible concentration polarization (i.e. build-up of a boundary layer), pore blocking and obstruction. Long term decline results from irreversible fouling that involves solute-solute and solute-membrane interactions (Fane, 1986). Membrane fouling of surimi wash water during microfiltration was ascribed to initial pore blocking followed by cake resistance (Huang and Morrissey, 1998). Removal of protein with heat and/or reductions in pH prior to ultrafiltration is an effective means to increase flux (Jaouen and Quéméneur, 1992). Flux was increased 30% following heat pretreatment of whey (Tarnawski and Jelen, 1985).

The protease primarily responsible for tissue softening in surimi is cathepsin L (Seymour and others 1994). Cathepsin L has several unique properties,

it is stable at temperatures above 45°C and in slightly acidic solutions. Its optimum activity is at 55°C and pH 5.5 (An and others 1994a). Cathepsin L is a cysteine, lysosomal protease that is present in the extracellular fluid (sarcoplasm) of muscles. An and others (1994b) initially reported a large amount of catheptic activity in the sarcoplasmic fluid of Pacific whiting mince tissue. Benjakul and others (1996) subsequently verified the presence of cathepsin L in surimi wash water. Because of its thermostability and relative abundance in Pacific whiting, attempts were made to recover cathepsin L from surimi wash water (Benjakul, 1997; Huang, 1997). Huang and others (1997) first evaluated the feasibility of coagulating soluble proteins from wash water using ohmic heating, a novel method of direct heating. Ohmic heating occurs when an alternating current is passed through a material and its electrical resistance causes the electrical energy to be converted to heat, causing a rise in internal temperature. Benjakul and others (1997) then used ohmic heating to separate proteins from surimi wash water before attempting to recover proteases by ultrafiltration. Cathepsin L activity was observed to decrease when wash water temperature exceeded 60°C. At temperatures above 70°C, over 80% of wash water proteins are coagulated. However, at 50-55°C, only 56-64% of proteins are removed (Benjakul, 1997; Huang, 1997). Recovery attempts by Benjakul (1997) were made utilizing a spiral wound type ultrafiltration device. Recovery parameters (flux, transmembrane pressure) were not reported. Huang (1997) characterized flux decline utilizing a fabricated plate and frame device.

The objectives of this research were to: (1a) optimize parameters for reduction of protein while maintaining enzyme activity by treatment of wash water with heat and/or reduction of pH; (1b) optimize parameters for membrane filtration recovery of catheptic proteases from pretreated surimi wash water; (2) recover a crude protease fraction from surimi wash water utilizing a pilot plant, crossflow ultrafiltration, plate and frame system; (3) compare ability of recovered crude protease to hydrolyze protein from demineralized shrimp shell with a commercially obtained papain.

LITERATURE REVIEW: By-Product Utilization in the Seafood Industry

Perspective

Dwindling natural resources and competition from overseas has resulted in increased pressures to fully utilize the marine resources harvested from domestic waters (Wilcke and others 1986; De Vecchi and Coppes, 1996). For seafood, on average, less than half of the raw material harvested is utilized for human consumption (Simpson and Haard, 1987; Mandeville and others 1992; Chung and Caldwallader, 1993; Anderson and Miner, 1997; Haard, 1998; Tschersich and Choudhury, 1998). The remainder is classified as "waste" and commonly converted into low dollar value fertilizers or animal feed. Investigations into low dollar value conversions continue to create significant volumes of research concerning composting (Brodie and others 1994; Liao and others 1994), acid-preserved ensiling (Rhaghunath and McCurdy, 1990; Nicholson and Johnson, 1991; Gao and others 1992; Dapkevicius and others 1998; White and others 1998; Espe and Lied, 1999), fermented ensiling (Fagbenro and Jauncey, 1993; Zuberi, and others 1993; Fagbenro and Jauncey, 1994; Faid and others 1994; Ahmed and Mahendrakar, 1996; Faid and others 1997; Dapkevicius and others 1998; Hammoumi and others 1998; White and others 1998;), and fishmeal production (Babbitt and others 1994; Roeckel and others 1996) of fishery by-products even though these technologies are quite old (Brody, 1965). In fact, earliest recorded use of fish by-product for animal feed was in 1835 (Meehan and others 1990).

It is up to the seafood scientist to alter the fishery "waste paradigm" and find alternative, novel, value-added products, to extract or process from fishery by-products. A significant body of research has developed as scientists have searched for alternative means to recover valuable products from seafood waste. The purpose of this paper is to provide a review on the current scientific progress in the recovery of value-added products from traditional "waste" by-products produced from the seafood processing industry.

Enzymes

Extensive reviews were provided recently on the role of marine digestive (De Vecchi and Coppes, 1996), muscle (Kolodziejska and Sikorski, 1996), and cold adapted enzymes (Simpson and Haard, 1987). A key property of enzymes of a cold-adapted species is they normally have higher rates of substrate turnover compared to homologous enzymes from warm-blooded animals. In addition, enzymes from deep-water fish (due to pressure adaptation) have tighter polypeptide structure and are more resistant to proteolytic degradation. Some proteases from fish are also salt activated, while their mammalian counterparts are inactivated. Due to the unique conditions of their habitat, properties of enzymes from aquatic organisms may offer a diversity of applications for the modification of the more than 10,000 food products introduced each year (Simpson and Haard, 1987; Haard, 1990; Haard, 1998). Examples of marine enzymes applications include: the removal of skin from herring; the removal of membranes from roe sacs; their use as

fermentation aides; the recovery of protein from seafood by-products (Simpson and Haard, 1987; De Vecchi and Coppes, 1996; Haard, 1998; Tschersich and Choudhury, 1998).

Several investigators have recently published reports regarding the recovery and characterization of digestive proteases, such as trypsin, chymotrypsin, and pepsin, from shrimp (Hernández-Cortés and others 1997) and fish (Kristjansson, 1991; Kolodziejska and others 1995; Raae and others 1995; Outzen and others 1996; Xu and others 1996; El-Shemy and Levin, 1997; Pavlisko and others 1997a; Pavlisko and others 1997b; Pavlisko and others 1999; Tschersich and Choudhury, 1998). Muscle proteases were recently recovered and characterized from Pacific whiting (An, 1994a; Seymour and others 1994; An and others 1995; Porter and others 1996), arrowtooth flounder, Alaska pollock, Pacific cod (Porter and others 1996) and carp (Ogata and others 1998). An unidentified fraction of endogenous proteases from fermented prawn heads was recovered utilizing ultrafiltration by Shirai and others (1997). Recovery of non-proteolytic miscellaneous enzymes include lysozyme from squid waste (Myrnes and Johansen, 1994), alkaline phosphatase from Arctic shrimp (Ragnar and others 1991) and Atlantic cod (Asgeirsson and others 1995), chitinase from rabbitfish, seabass, and shrimp (Esaiassen and others 1996; Sabapathy and Teo, 1993) and lipase from Atlantic cod (Gjellesvik and others 1992). Both digestive proteases and miscellaneous enzymes were recovered from by-products such as the intestine, stomach, pyloric caeca or hepatopancreas. Muscle proteases were recovered from fillets, not by-products.

However, recovery of catheptic muscle proteases from by-product was reported for pork heart muscle (Artigas and others 1996). In addition, muscle proteases were also recovered (Benjakul and others 1997) and characterized (Benjakul and others 1998) from Pacific whiting surimi wash water. Pacific whiting has elevated levels of catheptic proteases as a result of a myxosporidian parasite infection. These lysosomal proteases, associated with the sarcoplasma, are extracted into the wash water during surimi processing (Morrissey and others 1995).

Enzyme recovery usually involves a combination of techniques such as centrifugation, ammonium sulfate fractionation, dialysis, ultrafiltration, diafiltration, preparative electrophoresis and/or chromatography (affinity, anion exchange, and gel). Benjakul and others (1997) simplified their recovery process by utilizing only ohmic heating and ultrafiltration. However, stability of the freeze-dried enzyme extract was reduced to less than 50% in two weeks when stored at 4°C. A one step affinity chromatography extraction of a digestive protease from Antarctic krill wastewater was also reported by Bustos and others (1999). However, researchers noted a drastic reduction in stability when enzymes were purified by affinity chromatography. Recovery of gastric proteases (trypsin, chymotrypsin, and pepsin) was also attempted from fish sauce utilizing only ultrafiltration (Gildberg and Xian-Quan, 1994). Fish sauce is a popular product in southeast Asia that involves solubilization of whole fish proteins comminuted with 30% salt. Results indicated enzymes could be extracted without affecting fish sauce development. In addition, the spray-dried protease was stable for 8 months.

Hydrolysates

Hydrolysis is a common means of utilizing "waste" fish biomass for the production of animal feed or fertilizer. Traditional means for accomplishing hydrolysis are mainly from the production of fishmeal or fish silage. Fish silage is a liquid product made by the addition of acid (normally formic) to whole fish or fish parts in order to prevent microbial growth. Endogenous enzymes (Windsor and Barlow, 1981; Raa and Gildberg, 1982) subsequently liquefy the acidified fish. A significant body of research has developed in the pursuit of enzymatic conversion of fish biomass. Haard (1994) has reviewed the use of digestive, muscle, and extracellular proteases for the production of hydrolysates. Extensive reviews on the functional modifications proteases make in foods and proteins were provided by Whitaker (1977) and Richardson (1977). A more specific review regarding marine enzymes and their modification of proteins was also provided (Haard and others 1982). Enzymatic hydrolysates are considered more acceptable for use as ingredients in foods intended for human consumption because they display improved nutritional and functional characteristics when compared to their chemically prepared counterparts (Lahl and Braun, 1994). A majority of this research involved the production of hydrolysates from underutilized species such as dogfish (Diniz and Martin, 1996), mullet (Morales de Leon and others 1990; Rebeca and others 1991), shark (Onodenalore and Shahidi, 1996), capelin (Martin and Porter, 1995), and jewfish (Yu and Tan, 1990). However, a few investigations

were recently published regarding the production of hydrolysates from "waste" products for use as ingredients in foods intended for human consumption.

Sardine

Various proteases were utilized for the solubilization and recovery of proteases from defatted sardine meal (Sugiyama and others 1991). Researchers looked at three alkaline (Acalase, Actinase, and Proleather), two neutral (Neutrase, Papain) and three acidic (Molsin, Pepsin, and Neulase) proteases. Results indicated alkaline proteases more effectively solubilized proteins. The resultant hydrolysate from alkaline proteases had higher tryptophan content and lower molecular weight. In addition, hydrolysates from Alcalase and Actinase (both alkaline) had less bitterness. Bitterness caused by excessive hydrolysis leading to formation of hydrophobic peptides was further reduced in Alcalase hydrolysates by treatment with synthetic adsorbents.

Lobster

Vieira and others (1995) prepared hydrolysates from the waste meat of lobsters. Researchers compared hydrolysates produced from papain, pepsin, and a fungal protease. Overall, panelists judged hydrolysates as either without bitterness or with very light bitterness. However, on average, hydrolysates with papain were found to be slightly more bitter than those produced from the other proteases.

Crawfish/crayfish

Baek and Cadwallader (1995) evaluated ten commercial proteases (neutral and alkaline) for hydrolysis of crayfish by-products. Three proteases were identified as having high activity relative to cost: Alcalase 2.4L, Prozyme™ 6, and Optimase™ APL-440. Of the three, Optimase™ APL-440 had the highest activity at its stated optimum conditions.

Polysaccharides

Perhaps one of the most successful examples of a value-added product from seafood waste is the production of chitosan from chitin containing shells of shrimp and crab. Chitosan is a versatile, functional biopolymer of glucosamine subunits that has found uses in the fields of medicine, pharmacology, cosmetics, and agriculture. Examples of current research include investigations into its general antifungal and antimicrobial properties (Wang, 1992; Chen and others 1996) and, more specifically, its use as an agent for the preservation of apple juice (Roller and Covill, 1999), strawberries (El Ghaouth and others 1992), cucumber and bell pepper fruits (El Ghaouth and others 1991), shrimp (Simpson and others 1997), and oysters (Chen and others 1998). Also, its film and barrier properties have continued to spur research into its application as an edible film (Butler and others 1996; Arvaitoyannis, and others 1998) and bioactive coating (El Ghaouth and others 1991; El Ghaouth and others 1992; Sapers, 1992; El Ghaouth and Wilson,

1997). A more thorough survey on food applications of chitin and chitosan was summarized by Shahidi and others (1999). Because chitosan is one of the few cationic polymers occurring in nature, its binding properties also continue to be heavily investigated. Recently, extensive reviews (Muzzarrelli, 1996; Place, 1996; Koide, 1998) and investigations (Muzzarelli and others 1996; Veneroni and others 1996; Ventura, 1996; Trautwein and others 1997; Cho and others 1998) have centered on chitosan's reputed "fat binding" and hypocholesterolemic capabilities. Moreover, binding capabilities of chitosan continue to make it a popular research subject regarding its use as a waste/wastewater treatment aide (Knorr, 1991). Specific examples of research include its use as a coagulant for processing wastewater from fishmeal (Guerrero and others 1998), cheese (Jun and others 1994; Fernandez and Fox, 1997), tofu (Jun and others 1994), food emulsion wastes (Pinotti and others 1997), and manure (Sievers and others 1994).

As a result of the myriad of applications for chitosan, investigators continue to find means that will maximize yield and polymer functionality of this valuable by-product. There are four stages involved in the production of chitosan from crustacean chitin. These include demineralization, deproteination, bleaching, and deacetylation. The first step uses a strong acid, while the second and last step use a strong base combined with elevated temperatures. Such harsh chemical treatments adversely affect polymer length and degree deacetylation of the final chitosan. Therefore researchers have sought ways to either optimize chemical treatments, that is, maximize recovery and minimize deleterious effects (Shahidi and

Synowiecki, 1991; No and Meyers, 1995; Chang and Tsai, 1997) or find alternative means for the recovery of chitin and subsequent production of chitosan. These include use of proteolytic enzymes (Gagne and Simpson, 1993; Bhuwapathapun, 1996; Wang and others 1998) and bacterial fermentations (Kungsuwan and others 1996).

Flavor compounds

Commercial seafood flavorings are produced as concentrated liquids, pastes, or spray-dried powders. Their use is increasingly being expanded in international markets such as the surimi industry (Baek and Cadwallader, 1996). Research on the recovery and utilization of flavor compounds has concentrated mainly on shellfish discards. Knorr (1991) estimated global annual production of shellfish waste at 1.44 million metric tons. A recent review, provided by Kawai (1996), outlined the importance of various volatile compounds in the flavor of freshwater, saltwater, and euryhaline fish. A review by Boyle and others (1993) specifically noted the importance of bromophenols in providing desirable sea- and iodine-like flavors to marine fish and seafoods. They reported bromophenols in seawater sediment, marine bacteria, algae, sponges, bryozoa, coelenterates, marine worms, arthropods, molluscs, and chordates. They suggested collection and utilization of bromophenols for augmentation of flavor in aquacultured species.

Oysters

Volatile compounds from fresh Atlantic (*Crasostrea virginica*) and Pacific (*Crassostrea gigas*) oysters were characterized by Josephson and others (1985). They identified (E,Z)-2,6-nonadienal and 3,6-nonadien-1-ol as the primary contributors to the "melon-like" flavor of Pacific oysters. These compounds were not found in Atlantic oysters. They attributed the planty, seaweed-like flavor of the milder Atlantic oyster to 8-carbon compounds found in both species of oysters. In general, there is little material in the literature regarding value-added utilization of waste or wastewater from oyster processing. Only one example was found after an extensive search (Shiau and Chai, 1990). In this investigation, researchers produced a concentrate from steam-jacketed heating of liquid wastes obtained from oyster shucking. The concentrate was used as an ingredient and subsequently compared with a commercial soup. Results indicated commercial and concentrate soup were not significantly different in aroma or taste.

Shrimp

Mandeville and others (1992) separated boiled commercial shrimp waste into methanolic or aqueous: acidic, neutral, and basic fractions. Shrimp notes were described in the methanolic basic, methanolic neutral, and aqueous basic concentrates. Results indicated there were 44 different functional groups (fatty acid esters, long-chain alcohols, aldehydes, and heterocyclics) in the concentrates. Lin

and Chiang (1993) looked at salt removal in order to recover free amino acids and nucleotides from a popular product produced in Asia, dried salted shrimp. Production of this product involved cooking in a 10% salt solution. Researchers were able to remove 95% of the salt with diafiltration and 85% with ultrafiltration. Free amino acid and nucleotide recovery were 50% and 70%, respectively.

Crab

Matiella and Hsieh (1990) identified the primary nonvolatile flavor compounds in boiled crab meat as glycine, alanine, arginine, glutamic acid, inosine 5'-monophospate (IMP), adenosine 5'monophosphate (AMP), and guanosine 5'monophosphate (GMP). Boiled crabmeat was found to contain higher levels of volatiles than pasteurized meat (Matiella and Hsieh, 1990). In addition, comparison of volatile components from fresh-picked blue crab meat and picking-table by-product (Chung and Cadwallader, 1993) indicated beneficial compounds, such as benzaldehyde (almond, nutty, fruity) and pyrazines (nutty, roasted), were found in equivalent or higher amounts in the by-product. However, by-product also contained higher amounts of undesirable sulfur-containing compounds such as dimethyltrisulfide. Authors noted that spoiled or rotten crabs are normally discarded into the same bin as picking discards. They suggested separation of discards to ensure high quality by-product for further processing.

Crawfish/crayfish

In the late 80s several investigations were conducted concerning the concentration (Vejaphan, and others 1988) and identification (Vejaphan, and others 1988; Tanchotikul and Hsieh, 1989) of volatile components from crawfish. In addition, No and Meyers (1989) reported on the recovery of flavor enhancing amino acids. More recently, Cha and others (1992) compared the volatile components from crawfish processing waste at two different temperatures. Aldehydes and ketones were higher in concentrates prepared from the lower temperature (85°C), while concentrates from the higher temperature (100°C) contained more nitrogenous compounds. Baek and Cadwallader (1996) evaluated the effects of an alkaline protease (Optimase APL-440) treatment and subsequent concentration methods on volatile compounds from crawfish by-products. They found more aroma active compounds were produced when hydrolysate was concentrated by atmospheric (100°C), as opposed to vacuum (60°C) evaporation. In addition, concentration of pyrazines and benzaldehyde significantly increased, while lipid decomposition products decreased after enzymatic hydrolysis. However, as with the crab by-product, levels of dimethyl disulfide and dimethyl trisulfide also increased significantly.

Lobster

Odor-active compounds for lobster were evaluated using gas chromatography/olfactometry (Cadwallader and others 1995). The most intense odorant extracted was 2-acetyl-1-pyrroline. Jayarajah and Lee (1999) optimized parameters for the concentration of lobster extract with ultrafiltration and reverse osmosis.

Connective tissue

Gelatin and Collagen

Gelatin from fish skin and bones is not as well studied as conventional gelatin from animals (Gudmunson and Hafsteinsson, 1997). This is primarily because large-scale production of collagen from mammals is more profitable (Oshima, 1998). Gelatin is produced from collagen, which exists in the skeleton, fins, and skins in fish (Sikorski and others 1984). Traditionally collagen, a fibrous protein, was extracted from demersal fish (cod, Alaska pollock, haddock, etc.) and shark skins or isinglass (swim bladders) for the production of glue or gelatin. The only distinction between gelatin and glue is the former is a very high-grade product with high jelly strength and light color that results in clear, sweet solutions. The advent of synthetic glues, however, has all but wiped-out this industry (Brody, 1965; Windsor and Barlow, 1981). Gelatin originating from fish has different

properties than those from mammalian sources. This created interesting possibilities for its application in cosmetics, photosynthesizing compounds, and as a clarifier in the brewing of beer and the fermentation of wine (Brody, 1965; Chavapil, 1979; Oshima, 1998). In addition, fish gelatin was suggested as an alternative to ethnically unacceptable sources of gelatin such as pork. As a result, there was a renewed effort concerning the extraction of gelatin from several fish skins. Gudmunson and Hafsteinsson, (1997) investigated the effect of sodium hydroxide, sulfuric acid and citric acid on yield and quality of gelatin from cod skins. Data indicated fresh cod could be kept up to 3 days at 7°C without affecting gelatin quality or odor. No difference was found between quality of gelatins from frozen or refrigerated skins. Highest yield was obtained from when 0.1-0.2% (w/v) sulfuric acid and sodium hydroxide were applied to skins followed by treatment with 0.7% (w/v) citric acid. Ciarlo and others (1997) developed a method for the production of acid-soluble type I collagen from hake (*Merluccius hubbis*) skin using 0.5 M acetic acid (1:20 w/v). Yield was estimated to be 6% on a wet basis. Nomura and others (1995) compared the renaturation properties of $\alpha 1$ chains and collagen type I from shark skin, a by-product of the tuna fisheries. Researchers reasoned that because shark collagen (I) is exclusively made of $\alpha 1$ chain, whereas the α component in collagens from land animals is composed of both $\alpha 1$ and $\alpha 2$ chains, it would display properties distinctive from mammalian collagen I. Comparison of pig skin collagen I with shark collagen I showed denaturation temperature to be 12°C lower for the latter. However, results obtained on

renaturation, intrinsic viscosity, SDS-PAGE, and molecular weight of shark skin collagen indicated it was structurally similar to collagen from mammals. They suggested shark collagen or gelatin could be used in applications requiring high solution viscosity with low gel strength. Examples given were used as an ingredient in ice cream or as a coating agent for frozen fresh fish.

Proteoglycans

Connective tissue is composed of either collagen or elastin. The cellular and fibrous components of connective tissue are surrounded by materials called ground substances. Most of these interstitial ground substances are composed of proteoglycans and glycoproteins. Proteoglycans are comprised primarily of polysaccharide sub-units with protein covalently attached as a minor constituent. Included in the proteoglycans are hyaluronic acid, chondroitin sulfate, dermatan sulfate, keraten sulfate and heparan sulfate (Goldberg and Rabinovitch, 1983). Shark cartilage chondroitin sulfate, a sulfated hexosamine, is the base ingredient for many cosmetics such as hand cream (Oshima, 1996). It is also thought to be the bioactive component of cartilage responsible for therapeutic functions such as anti-arthritis, anti-joint inflammation, and anti-cancer (Molyneaux and Lee, 1998). Although research is still open to interpretation, it is believed that chondroitin sulfate inhibits the enzymes responsible for cartilage degradation or osteoarthritis and increases synthesis of proteoglycans and collagen (Anonymous, 1999). In addition, antimutagenic properties in the water-soluble fraction from shark cartilage

were explored (Felzenszwalb and others 1998). Their data indicated cartilage-containing preparations played a scavenger role for reactive oxygen species.

Pigments

Carotenoproteins are responsible for the orange-red pigmentation of fish (salmonids) and shellfish (crustaceans). The properties, occurrence, and utilization of carotenoids in foods were reviewed (Borenstein and Bunnell, 1966; Shahidi, 1995). Among marine life, crustaceans contain astaxanthin; lobsters contain a protein complex of astaxanthin; red fish contain astaxanthin, lutein, and taraxanthin; salmon contain astaxanthin and beta-carotene; and canthaxanthin, astaxanthin, and beta-carotene are found in sea trout (Borenstein and Bunnell, 1966). Aquacultured salmonids (Arctic char, rainbow trout, and salmon) are unable to synthesize carotenoids *de novo*. As a result, pigments must be added to their diet. Carotenoids can be added to salmonid diets in the form of shrimp meal. Pigment enrichment of diets with shrimp meal resulted in variable pigment levels, rapid deterioration of raw material, bulkiness, high transportation costs, high ash, and high chitin contents. In addition, pigmentation increases in the skin/flesh of fish with diets supplemented with shrimp meal is nominal as a result of poor utilization due to the presence of chitin and calcium. An alternative to the use of shrimp meal, i.e. the enrichment of diets by carotenoids recovered with vegetable or marine oil (1:2 v/w, 60°C, 30 min.), solved many of the problems observed with the use of shrimp meal (Chen and Meyers, 1982; Shahidi and Synoweicki, 1991;

Shahidi, 1995). However, this left the diets devoid of supplementation with protein and amino acids, which serve as feed stimulants, and other valuable nutrients (Simpson and others 1992). As a result, Simpson and others (1992) developed a process for the recovery of carotenoid pigments in the form of more stable carotenoproteins from lobster (*Homarus americanus*). The process involved utilization of trypsin (0.1%) to recover protein and ethylenediamine tetraacetic acid (0.5 mM) to reduce ash. Carotenoprotein from the hydrolysate was recovered with filtration, followed by precipitation with ammonium sulfate and centrifugation (5000 xg, 30 min.). Authors concluded the product was enriched with protein and fat and depleted with chitin and ash. Furthermore, essential amino acid content adequately met dietary requirements for cultured salmonids.

Antioxidants

Degradation of carotenoids is hypothesized to be similar to lipid oxidation. Research has shown antioxidants help to maintain the red color. Li and others (1998) prepared a crude and partially purified antioxidant from shrimp shell waste by ethanol extraction and silica gel column chromatography. Whole rockfish (*Sebastolobus alascanus*) were subsequently treated with a crude antioxidant dip. Fillets (*Sebates alutus*) were treated with partially purified dips. Red color in rockfish is integrally linked to its value. Higher red color values (a*) were found in dipped fish. TBA values of dipped fish were significantly lower than controls. However, although filets treated with antioxidant from shrimp shell retained color

better than untreated samples, color retention was higher in filets treated with a commercial antioxidant, sodium erythrobate.

Fermented products

Fish fermentation is widely used in Asia for production of fish sauces and paste. The process utilizes up to 30% salt and fermentation occurs at about 30°C. The elevated temperatures helps increase rates of proteolysis. These proteases come from endogenous (viscera) and microbial sources (natural flora, contaminants). Production of fish sauce is a very slow process taking from several months to several years. Final product is liquid/semiliquid with pungent cheese-like smell and strong salty flavor. In Europe, fermented fish products are normally of Scandinavian origin made primarily from herring (*Clupea harengus*), salmon (*Salmo salar*), or trout (*Salmo trutta*). The products are Gaffelbitar (or Tidbits), Gravlax, and Rakørett. For Tidbits, whole herring is mixed with salt (15-17%), sugar (6-7%) and spices and left for spontaneous fermentation for 12-18 months. Fish are then filleted and packed in glass jars. In Gravlax and Rakørett salt and sugar are added to degutted and filleted fish. Fermentation lasts 2 weeks in a cool place. Acceleration of fermentation can be accomplished by addition of a bacterial starter culture (Morzel and others 1997) or protease (Chaveesuk and others1993). As far as the utilization of waste for the production of fish sauce, Gildberg (1992) fermented salted cod intestines to produce a fish sauce that was palatable and

comparable in taste to traditional fish sauce. He also recovered trypsin-like enzymes from the sauce utilizing ultrafiltration.

Omega-3 fatty acids

Interest in recovery of polyunsaturated fatty acids (PUFAs) has increased as a result of the myriad of putative health benefits from ω-3 fatty acids (Calder, 1996; Shahidi and Wanasundara, 1998). Garcia (1998) has even suggested fish oils can be regarded as neutraceuticals as they provide health benefits "above and beyond....simple nutrition or basic fortification." Unfortunately the recovery of these valuable, bioactive compounds from marine by-products has yet to be reported. There were a few recent reports on the recovery of oils from non-traditional species such as hoki and orange roughy (Irianto and others 1993) and shark (Bordier and others 1996). In addition, one report suggested the use of supercritical carbon dioxide as a means to recover oil high in ω-3 fatty acids so that protein residue functionality could be retained (Temelli and others 1995). The popularity of omega-3 fatty acids suggests research concerning their recovery from by-products would be valuable.

Exploring pharmaceuticals and neutraceuticals

There is growing interest and excitement concerning the recovery of nutraceuticals, pharmaceuticals, and drugs from marine resources (Halvey, 1989;

Cowan, 1997; Fenical, 1997; Oshima, 1998). However, a great many of these drugs and nutraceuticals are found in marine organisms that are not part of the traditional harvest such as: a medicine for use against a water insect from sea cucumber; various anti-tumor, anti-inflammation, and anti-viruses agents from sponges; anti-tumor agents from seaweed, antifreeze proteins from Arctic and Antarctic fish; lectin, a glycoprotein with blood coagulant, anti-viral, and nerve growth factor characteristics from giant clams, sea squirts, eels, and sea urchins; and protamine, a thermostable antibacterial agent from fish testicles (Feeney and Yeh, 1993; Feeney and Yeh, 1998; Oshima, 1998). Opportunities for commercial recovery of bioactive compounds from seafood waste are as of yet, unrealized. Our rapidly expanding knowledge about the sources and properties of bioactive compounds from marine life will benefit and help seafood scientists in their attempts to identify and recover valuable by-products.

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Chapter 2. Optimization of Parameters for the Recovery of Proteases from Surimi Wash Water

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ABSTRACT

Optimum protease activity was expressed when Pacific whiting (*Merluccius productus*) wash water acidity was reduced to pH 4 using either HCl or L-ascorbic acid. Acidification and heat (60°C) treatment of wash water resulted in maximum activities at pH 5-6 (HCl) and pH 6 (L-ascorbic acid). Permeation of protease using ultrafiltration membranes of 300 kD and 1,000 kD molecular weight cut-off and microfiltration membranes of 0.3 μm did not increase purification. However, permeation of protease was greater with heat and acid treated samples than with acid only treated samples. In addition, ultrafiltration time required for a 10-fold concentration of protease was reduced by half when wash water was heat and acid treated as opposed to treated only with acid. Ultrafiltration concentration of protease following pretreatment with acid and heat resulted in yields of 80% with 50 kD and 50% with 100 kD molecular weight cut-off membranes.

INTRODUCTION

Effective and responsible utilization of natural resources has become increasingly important as a result of society's increased awareness of the environmental consequences of waste. Pacific whiting *(Merluccius productus)* is an abundant, natural resource off the Pacific Northwest Coast. Over the past five years, annual harvests have ranged from 227,497-368,099 metric tons (U.S. and Canada), the majority of which is made into surimi, a washed fish mince (Warren, 1999). About 20-30% of the material harvested is utilized for human consumption. The remainder is considered solid or soluble waste. In the seafood industry, solid waste is usually converted to animal feed (via hydrolysates) or fishmeal. In some cases, it is ground and returned to a processing plant's waste stream.

Soluble wastes are largely generated through the surimi washing process. The production of surimi involves the concentration of myofibrillar proteins and is achieved by washing fish mince with large volumes of water to remove undesirable sarcoplasmic proteins (proteases, hemepigments, etc.) which are water soluble. Valorization of this wash water via recovery of bioactive compounds such as proteases would serve to simultaneously increase economic value while reducing waste of this important resource. Cathepsin L has been identified as the protease primarily responsible for myosin degradation in surimi made from Pacific whiting (An and others 1994b). Cathepsin L is a heat stable (optimal activity at 55°C), lysosomal, cysteine protease. In general, cathepsins are the most active proteases in muscle (Barrett and Kirshke, 1981). The food industry application of proteolytic

enzymes from marine organisms has been thoroughly reviewed by Haard (1992; 1994; 1998). Applications of proteases are found for commodities such as cereal, eggs, meats, fish, pulses, dairy, brewing, wine, and cocoa. Some examples of specific applications are protein removal from bones, hydrolysate production, tenderization, clarification, fermentation and filtration aid, etc.

Morrissey and others (1995) reported on the removal of catheptic proteases during Pacific whiting surimi processing. They showed the surimi process removes 85% of the protease activity from mince to the wash water. Lin and others (1995) reported that ultrafiltration (30 kD) significantly reduced protease in the wash water. Benjakul, and others (1996) identified and characterized cathepsin L (molecular weight 39.7 kD) as the predominant protease in Pacific whiting surimi wash water and suggested recovery of this protease might have economic benefits to the industry. Jaouen and Quéméneur (1992) in their review on the use of membrane filtration for wastewater recovery indicated the impracticability of processing by ultrafiltration without pretreatment. They suggested prefiltering, centrifugation, pH-shifting, and heat coagulation as possible treatment alternatives prior to wastewater ultrafiltration.

Attempts to combine heat-treatments with ultrafiltration on Pacific whiting surimi wash water were first conducted by Huang and others (1997). They studied the effects of ohmic heating on protein coagulation in surimi wash water and found that 92.1% of the protein could be removed utilizing ohmic heating (70°C). Benjakul (1997) recovered, purified, and characterized a crude protease from

surimi wash water. His recovery methods involved pretreatment of the wash water with ohmic heating followed by ultrafiltration and freeze-drying. Ohmic heating was utilized in order to minimize deleterious effects of prolonged temperature exposure on protease activity. A 2-fold increase in protease activity, possibly due to dis-sociation of the enzyme/inhibitor complex, was observed in surimi wash water following ohmic heating treatment (Benjakul and others 1996). However, subsequent ultrafiltration resulted in a 40% loss of total activity. It was hypothesized that losses were probably a result of high pressure (30 psi) and loss of enzyme through the membrane. Acidification of wash water is a treatment alternative to heat, as myofibrillar proteins can be removed from wash water by isoelectric precipitation. The isoelectric point of myofibrillar proteins from Pacific whiting is estimated to be in the range of 5-5.4 (Choi, 1999), while cathepsin L from Pacific whiting ranges from 4.91-4.94 (Seymour and others 1994). In addition, activity of catheptic proteases from Pacific whiting increases when acidity is increased (Benjakul and others 1996).

Previous work by Huang and others (1997) demonstrated that maximum reduction in wash water protein occurred when treatment temperatures exceeded 70°C. However, wash water protease activity is severely reduced at these temperatures (Benjakul and others 1997). Treatment at slightly lower temperatures 50-55°C removes about 40% of wash water proteins. Pederson (1990) suggested combining heat and pH treatments to recover proteins from surimi process water. When Benjakul (1997) recovered catheptic proteases, using heat to pre-treat wash

water, he noted that subsequent acidification of the recovered enzyme increased its hydrolytic activity. As a result, it was postulated in this study that combined heat and acid treatment may be more effective in removing protein from the wash water, than either alone. The objective of this study was to determine the parameters necessary for efficient recovery of proteolytic enzymes from surimi wash water utilizing heat, acid, and ultra- or microfiltration.

MATERIALS AND METHODS

Collection of mince

Mince was collected from a local processing plant and was transported to the lab in plastic bags covered with ice. It was placed in plastic block molds (~5 Kg), vacuum packaged, and stored at –20°C.

Preparation of surimi wash water (Benjakul and others 1996)

The necessary amount of frozen mince was chipped-off, vacuum packaged, and then rapidly thawed by being placed in a room temperature water bath. One part mince was weighed and combined with 3 parts water. In order to simulate the wash step in the surimi process, the resultant mince-water slurry was vigorously stirred for 10 min. at 5°C. The mince-water mixture was subsequently passed through 2-layers of cheesecloth to separate mince from wash water.

Treatment of wash water with acid

Wash water was treated with two acids, hydrochloric (HCl) and L-ascorbic. L-ascorbic was utilized to see if its antioxidant properties would provide a protective effect against oxidation of the enzyme. Pre-measured amounts of L-ascorbic acid were added to 25.0 mL wash water. Final concentration of L-ascorbic acid in the wash water corresponded to 0, 0.05, 0.1, 0.2, 0.5, and 1.0%. Alternatively, wash water (20.0g) was also treated with HCl by dropwise adjustment of pH with 1N and 0.1N solutions of the acid. Final volume was brought to 25.0 mL with distilled water (dH$_2$0). Target pH's were 7, 6.5, 6.0, 5.5, 5.0, 4.5, and 4.0. All preparations were done in duplicate. Acid treated samples were centrifuged at 3000xg for 6 min., 4°C (Sorvall RC-5B refrigerated superspeed centrifuge) and final pH was measured. Supernatant was collected by passing it through 4-layers of cheesecloth. Supernatant not utilized for subsequent heat-treatment was collected and stored at −80°C until further analysis could be performed. Further chemical analysis involved estimation of total proteins (Bradford, 1976; Sedmak and Grossberg, 1977) using the Bradford reagent from BioRad and protease activity (as expressed by cathepsin L and B activity) following the method as outlined by Barrett and Kirshke (1981).

Treatment with acid and heat

Heat-treatment was optimized in preliminary studies for a standard volume of wash water. Optimum activity was expressed when a 10 mL aliquot was placed in a 50 mL erlenmeyer capped with aluminum foil and placed in a 60°C water bath for 10 min. Acid treated supernatants were therefore heated as described, rapidly cooled in an ice bath, and re-centrifuged at 3000xg for 6 min. Supernatant was collected and stored at –80°C until further analysis (as outlined above) could be performed.

Comparison of the effect of 4 different acids and heat on final protein content

Wash water (25 mL) was adjusted to pH 6.2-6.4 with L-ascorbic, acetic, and citric acid. · In addition, 20.0 g of wash water was adjusted with 1N and 0.1N HCl to final pH 6.2-6.4. Samples were centrifuged at 3000xg, 4°C. Supernatants were collected and 10 mL aliquots were heat-treated (60°C water bath, 10 min.). Samples were re-centrifuged (3000xg) and analyzed for total protein in the supernatant as described above.

Protease analysis

Protease activity was followed by monitoring cathepsin L and B activity using the method of Barrett and Kirschke (1981) with a modification of the stopping reagent by addition of iodoacetate (An and others 1994a). Samples were

analyzed in polystyrene tubes and kept on ice before and after incubation. An 100 µL aliquot of wash water was combined with 400 µL of 0.1% Brij (35% w/v). Appropriate dilution of samples was made prior to analysis if necessary (appropriate range = 0.3 - 3.0 ng of cathepsin L). A 250 µL aliquot of assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA, 8 mM DTT, final pH 5.5) was added and tubes (covered with petrifilm) were placed in a 30°C water bath. After 1 min., 250 µL of pre-warmed substrate (20 µM Z-Phe-Arg-NMec) was added. Samples were agitated and 1 mL of stopping reagent (100 mM sodium monochloracetate, 30 mM sodium acetate, 70 mM acetic acid, 5mM iodoacetate, pH 4.3) was added at 10 min. Fluorescence of the free aminomethylcoumarin was measured (excitation = 370 nm; emission = 460 nm) in the sample and compared to the standard (0.5 µM 7-amino-4-methylcoumarin corresponded to 0.1 mU of activity in the tubes). One mU was defined as being equivalent to the release of one nmole aminomethylcoumarin/min.

Permeation/retention of protease in treated and untreated samples

Untreated wash water was designated as the control. For acid treatment, 20 mL aliquots of wash water were brought to pH 4, using 1N and 0.1N HCl, and a final volume of 25 mL. For acid plus heat, 25 mL aliquots of wash water were treated with 0.1% L-ascorbic acid, pH 6.2-6.3. All samples were centrifuged at 3000 xg for 6 min. at 4°C. For acid (L-ascorbic) plus heat-treated samples, 10 mL of supernatant (duplicate) were collected, heat-treated in a 60°C water bath for 10

min., and cooled in an ice water bath. Duplicate heat-treatments were combined and the heat-treated wash water was re-centrifuged at 3000 xg for 6 min. at 4°C. For all samples, 15 g of supernatant were weighed, in duplicate, into pre-weighed centrifugal units (Pall Filtron, Northborough, MA) with molecular weight cut-off at 300 kD and 1,000 kD, and 0.3 μm. Centrifugal units were assembled and centrifuged (Sorvall RC-5B refrigerated superspeed centrifuge) at 5000 xg for 30 min., 1 hr., or 1.5 hr. Both permeate (filtrate) and retentate (concentrate) were collected and stored at −80°C until further analysis could be performed. All samples were analyzed for total protein and protease (Z-Phe-Arg-NMec) activity.

Concentration by ultrafiltration

Aliquots of wash water (25 mL) were acidified to 0.1% L-ascorbic acid. Treated wash water was centrifuged at 3000 xg. The supernatant was collected and a 10 mL aliquot was heated in a water bath at 60°C for 10 min. Samples were rapidly cooled using an ice water bath and re-centrifuged (3000 xg, 4° C, 6 min.). A 15 g aliquot of supernatant was then transferred (in duplicate) into pre-weighed centrifugal units (Pall Filtron, Northborough, MA) with molecular weight cut-off at 30 kD, 50 kD, and 100 kD. Centrifugal units were assembled and centrifuged at 5000 xg for 30 min., 1 hr., or 1.5 hr. Both permeate (filtrate) and retentate (concentrate) were collected and stored at −80°C until further analysis for total protein and protease (Z-Phe-Arg-NMec) activity.

Statistical analysis

Statistical analysis was completed using multifactor ANOVA and variance component analysis with StatGraphics®*Plus*.

RESULTS AND DISCUSSION

Effect of acid and acid plus heat on total protein

Total protein significantly decreased when wash water acidity was reduced below pH 6 using either HCl or L-ascorbic acid (Fig. 2.1; $p < 0.05$). In addition, heat-treated samples had significantly ($p < 0.05$) lower protein content than their unheat-treated counterparts. Data also suggested that type of acid applied (HCl or L-ascorbic acid) did not significantly effect the final protein content of unheated wash water. However, in heat-treated samples more variance was attributed to L-ascorbic acid as opposed to the HCl treatments (52.22% and 29.44%, respectively). This implies that L-ascorbic acid may work synergistically with heat in reducing total protein. This is supported in the literature as L-ascorbic acid, in addition to being an antioxidant, also acts to promote non-enzymatic browning (Gregory, 1996). In this role, L-ascorbic acid reacts with amino acids to produce a wide array of polymerization products that could possibly interact and thus cause increased precipitation of the protein in the wash water. A minimum in total protein was recovered when wash water was acidified to pH ~4.5 with either
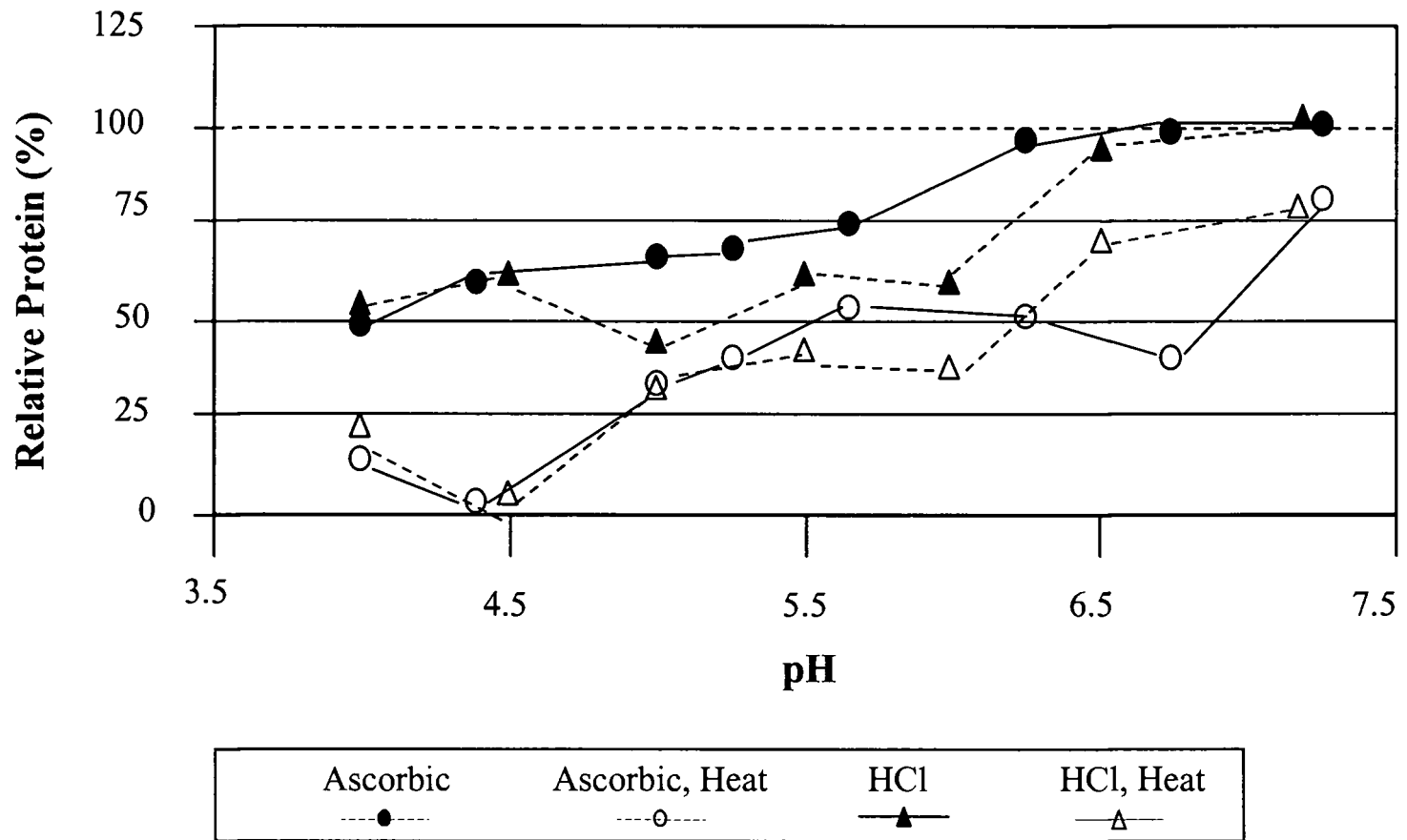
Figure 2.1. Relative effect of acid and heat (60°C for 10 min.) on protein in Pacific whiting surimi wash water.

HCl plus heat (HCl+H) or L-ascorbic plus heat (ASC+H). These conditions produced an approximate 80-fold (1889-1893 µg/L $\Rightarrow$ 24.5-27 µg/L) reduction in wash water protein. However, for the purpose of enzyme purification, the applicability of this treatment condition must be correlated with its effect on enzyme activity before any real significance can be established. Based on results from the treatment of wash water with L-ascorbic acid and HCl, a comparison was made on the effect of L-ascorbic acid, HCl, acetic acid, and citric acid on final protein of the wash water. Analysis determined that although mean total protein was lower for samples treated with L-ascorbic acid, differences between type of acid utilized were not significant (Fig. 2.2).

Effect of acid and acid plus heat on protease activity

Protease activity levels did not significantly increase until wash water pH was reduced below pH 5.3 for either HCl or L-ascorbic acid treated samples. Maximum activity for both treatments occurred at pH <4.5 (Fig 2.3). When acidified wash water samples were heat-treated, the maximum (or optimum) for protease activity shifted (with both HCl and L-ascorbic acid) to the right on the pH scale. This shift in pH optimum is most likely attributed to the combined effects of acidification and heat. Acidification, by itself, causes the unfolding (or denaturing) of the protein as a result of electrostatic repulsion. Additional unfolding occurs upon heating as a result of further weakening of ionic bonds (Damodaran, 1996). Previous research on Pacific whiting (Seymour, and others 1994; An, and others
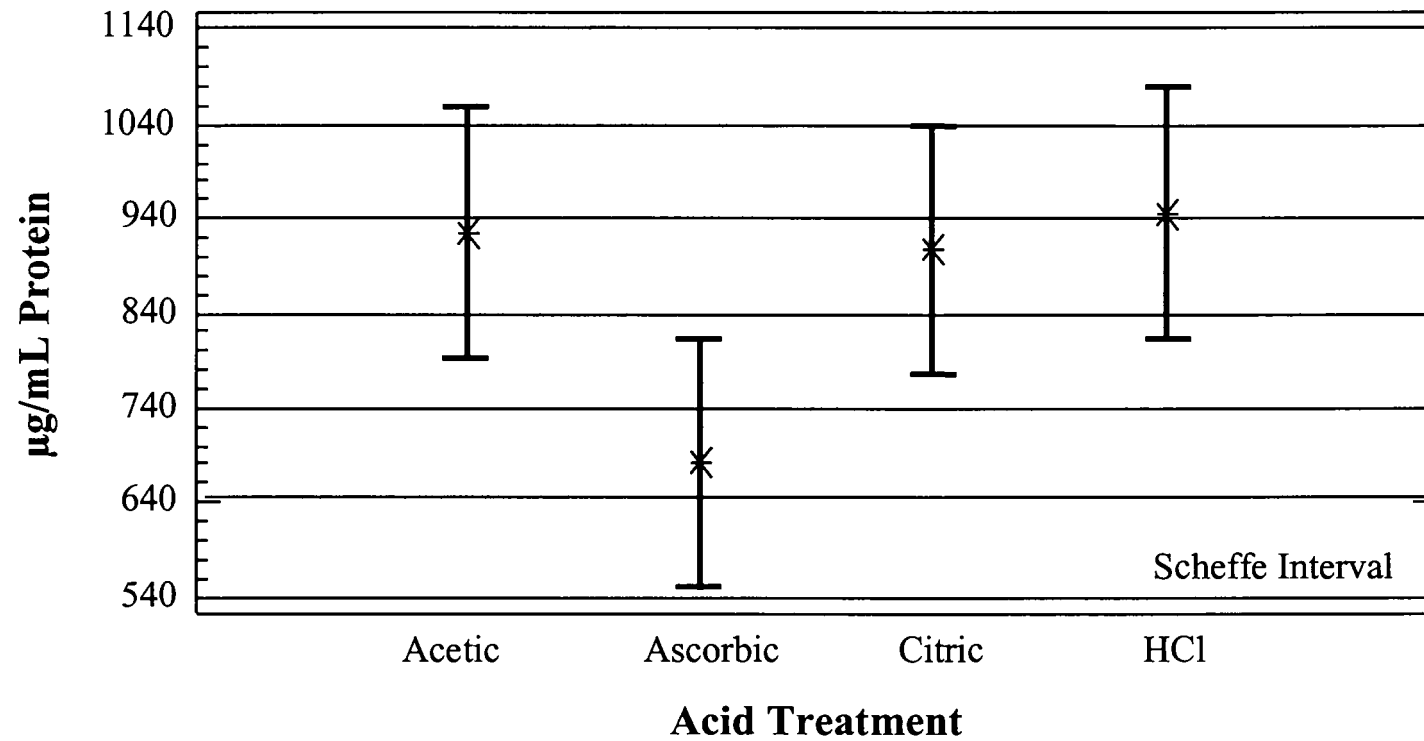
Figure 2.2. Effect of different acid treatments on total protein in surimi wash water at pH ~ 6.2.
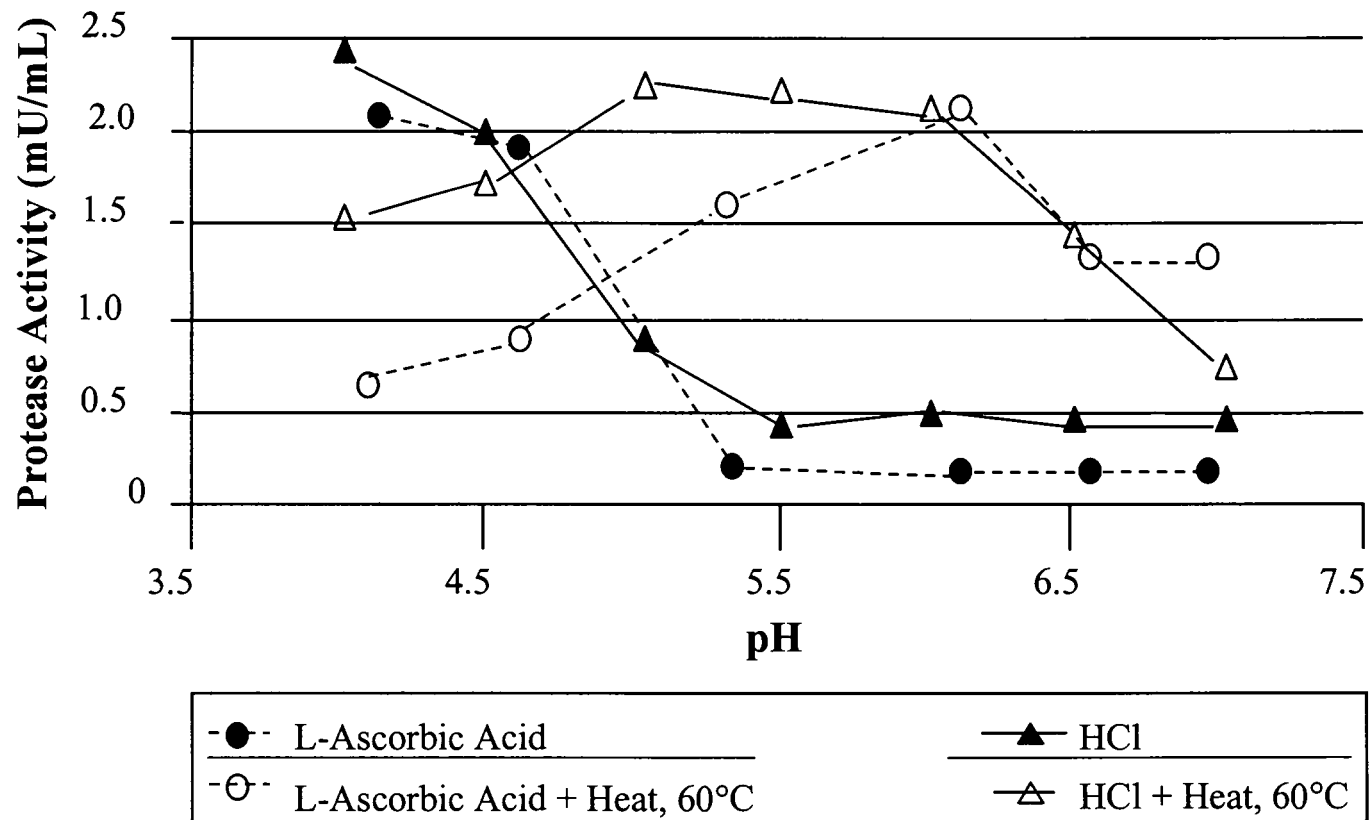
Figure 2.3. Treatment effect on protease activity in surimi wash water.

1995; Benjakul and others 1996) has indicated that heat or acid treatment will cause an increase in expressed protease activity as a result of the denaturation of a less stable, indogenous inhibitor. Results indicated that the combined effects of heat and acid caused the inhibitor to denature at a higher pH. The subsequent decrease observed in protease activity at lower pH values was probably attributed to either excessive denaturation and thus inactivation of the enzymes active site or precipitation of the enzymes as a result of association of heat stable hydrophobic sites (Whitaker, 1996). The range for maximum protease activity for ASC+H was much narrower than for the HCl+H treated samples. This may be explained by interaction of L-ascorbic acid with amino acids to produce polymerization products that cause either an inactivation of the active sight or precipitation of the enzymes.

Effect of membrane size on protease permeation/retention

Using the data obtained from the previous analysis, optimum treatment conditions were chosen and then tested to determine the best filter membrane size for permeation/retention of the protease in the wash water. Ideally, conditions for treatment of the wash water prior to ultrafiltration would reduce protein without reducing enzymatic activity. Optimum expression of protease activity was achieved utilizing either acid (L-ascorbic or HCl) at pH 4. Optimum expression of protease activity in acid plus heat-treated samples occurred with HCl at pH ~5-6 and L-ascorbic acid at pH ~6. In addition, preliminary analysis to test the permeation of protease from heat-treated samples through a 100 kD membrane had

indicated that 75% of the enzymes remained in the retentate (unpublished data). Therefore, the permeability of protease from wash water treated with HCl, pH 4 (HCl-4), and L-ascorbic acid plus heat, pH ~6 (ASC+H-6), was tested on Pall Filtron centrifugal units with membrane sizes of 300 kD, 1000 kD, and 0.3μm. Untreated wash water was used as a control.

The determination of centrifugation time was based on empirical evidence. Preliminary experiments had indicated that units with acid plus heat (ASC+H-6) would require 30 min., acid only (HCl-4) 1 hr., and untreated (control) 1.5 hr. Fig. 2.4-2.6 show percentage of protease activity remaining in each of the fractions collected, permeate and retentate. Unaccounted for protease activity, calculated from mass balance of original, permeate, and retentate protease activity levels, were reported as the "membrane" fraction. It was assumed losses to the membrane fraction were attributed to adsorption/precipitation of protease onto the membrane (Belter and others 1998). However, it is also possible the observed reductions in activity were a result of losses in protease stability over time (Benjakul and others 1997). In general, almost all protease activity for ASC6+H passed to the permeate. For HCl-4, distribution of protease between the permeate and retentate was approximately equivalent. For the control, the amount of protease in the permeate increased as membrane size increased. Results indicated that better permeation was achieved from untreated than acid treated, HCl-4, wash water using either the 1000 kD or 0.3μm membranes. Proteins remaining in acid treated wash water are closer to their isoelectric point than those in untreated wash water. Ehsani and others
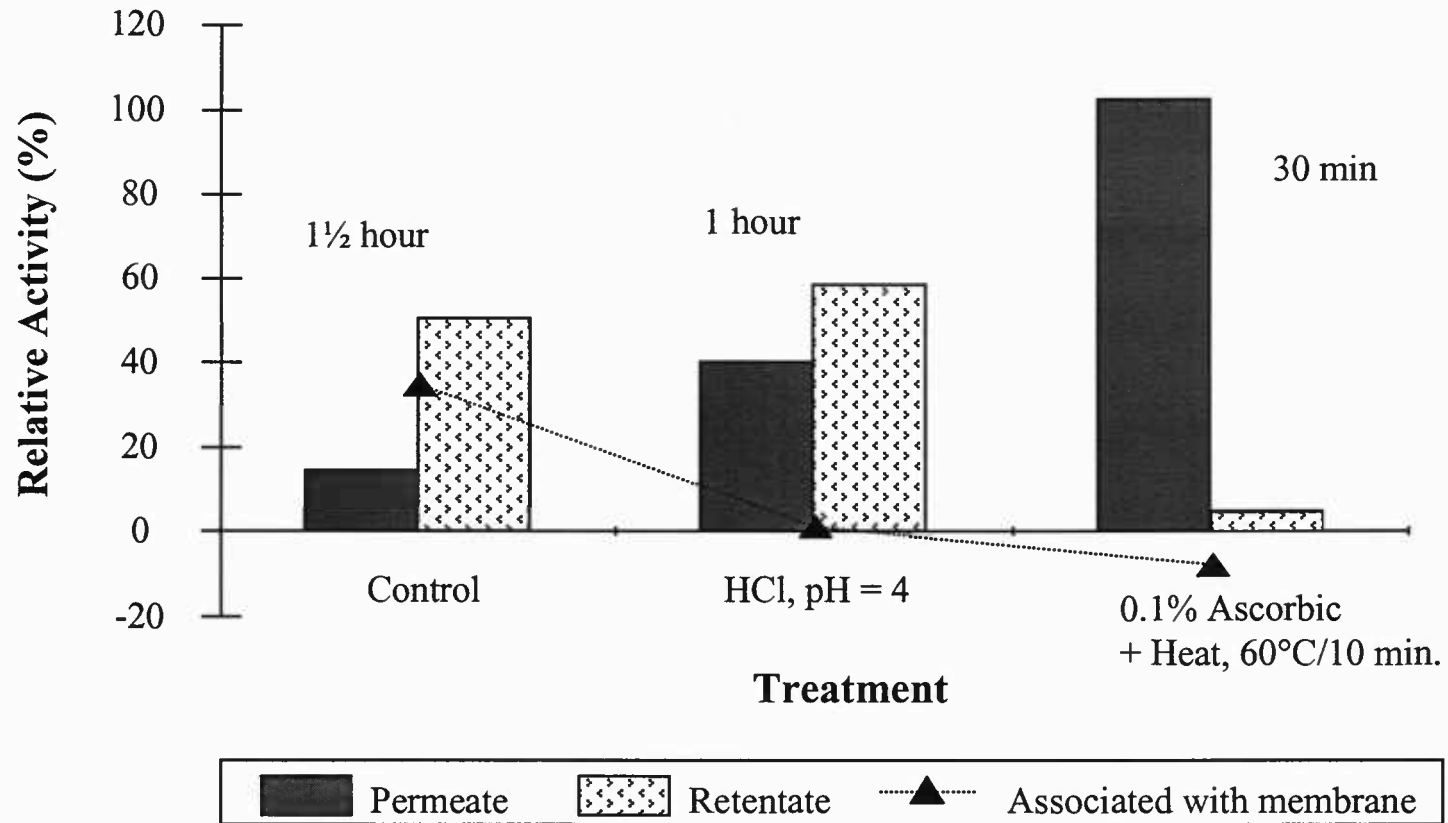
Figure 2.4. Permeation and retention of wash water protease acitivity through a 300 kD centrifugal unit.
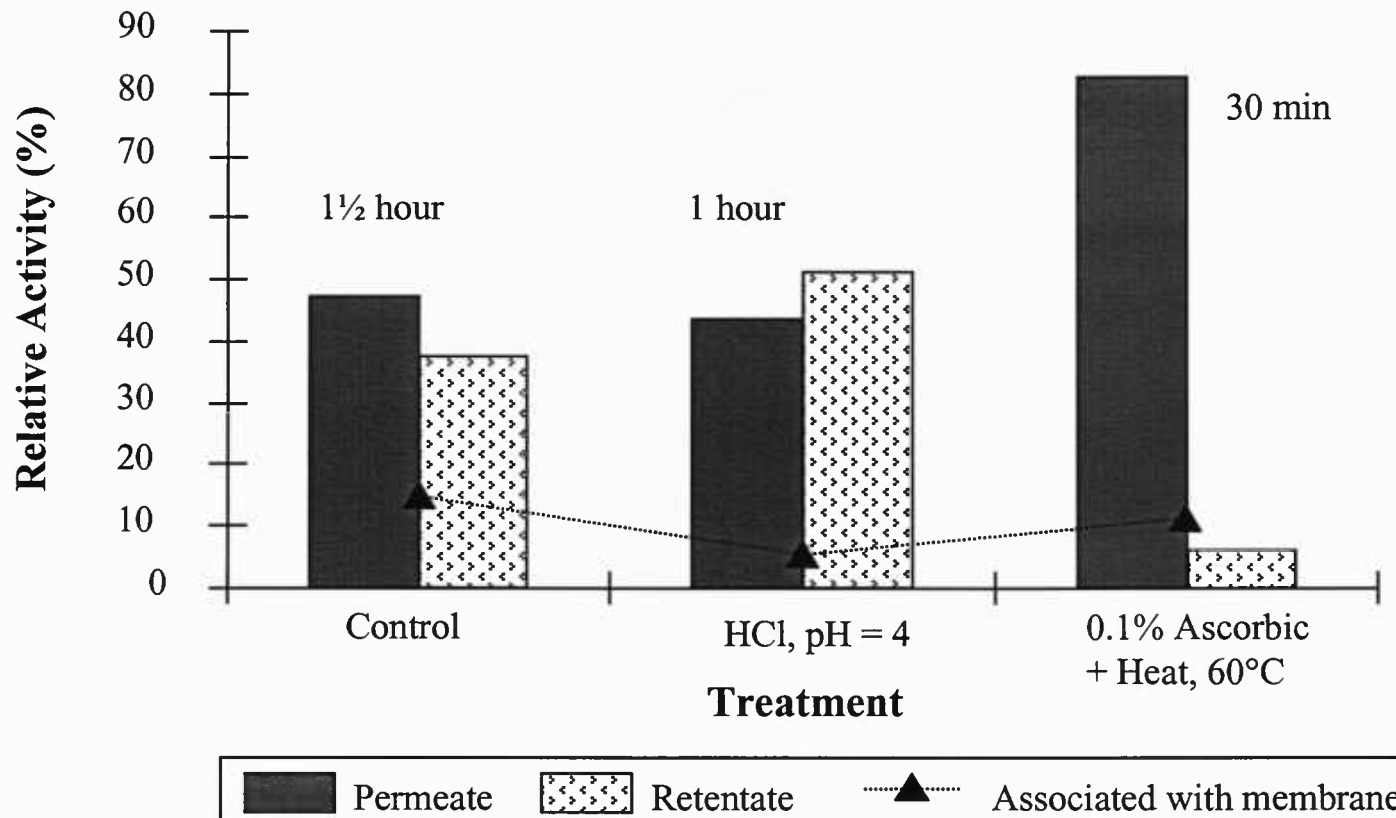
Figure 2.5. Permeation and retention of wash water protease activity
through a 1000 kD centrifugal unit.
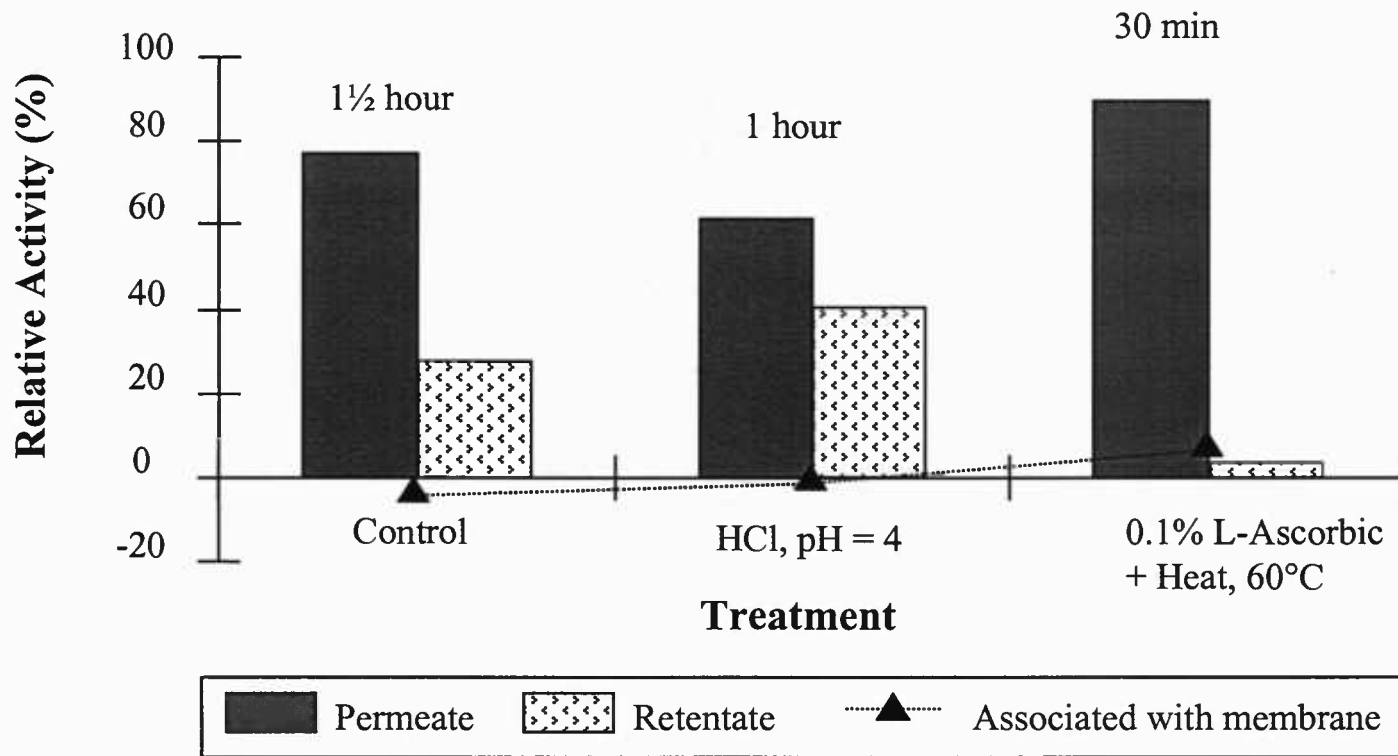
Figure 2.6. Permeation and retention of wash water protease activity
through a 0.3μm centrifugal unit.

(1996) indicated that permeate flow is at its minimum when proteins are at their isoelectric point and in their most compact form, resulting in the formation of a more densely packed dynamic membrane.

The data from Fig. 2.4-2.6 also indicate ease of flux of the wash water and permeation/retention of the protease. However, no indication of the actual work being performed by the membranes is given. In Fig. 2.7, the concentration of the enzymes, as expressed by mU/mg BSA, prior to and after ultrafiltration indicates that membrane filtration did not enhance purification. Improvement in protease purity only occurred for the ASC+H-6 treated sample with the 300 kD and 1000 kD membranes and that improvement was minimal. As a result, it was determined that further separation of enzymes from proteinaceous material via permeation through ultrafiltration/microfiltration membranes would not be feasible. Therefore, a new approach was taken to determine if smaller (30 kD, 50 kD, and 100 kD) ultrafiltration membranes could be effectively utilized to remove water and increase protease concentration.

Since combined acid plus heat-treatment, ASC+H-6, was most effective in reducing the concentration time, subsequent studies on smaller pore size membranes employed this condition. Fig. 2.8 and 2.9 compare permeation/ retention and protease purity, respectively, using centrifugal units ranging in membrane size from 30 kD to 0.3μm. In Fig. 2.8, protease was distributed almost equally between permeate and retentate when using a filter with a 100 kD molecular weight cut-off. When filters were greater than 100 kD, protease passed
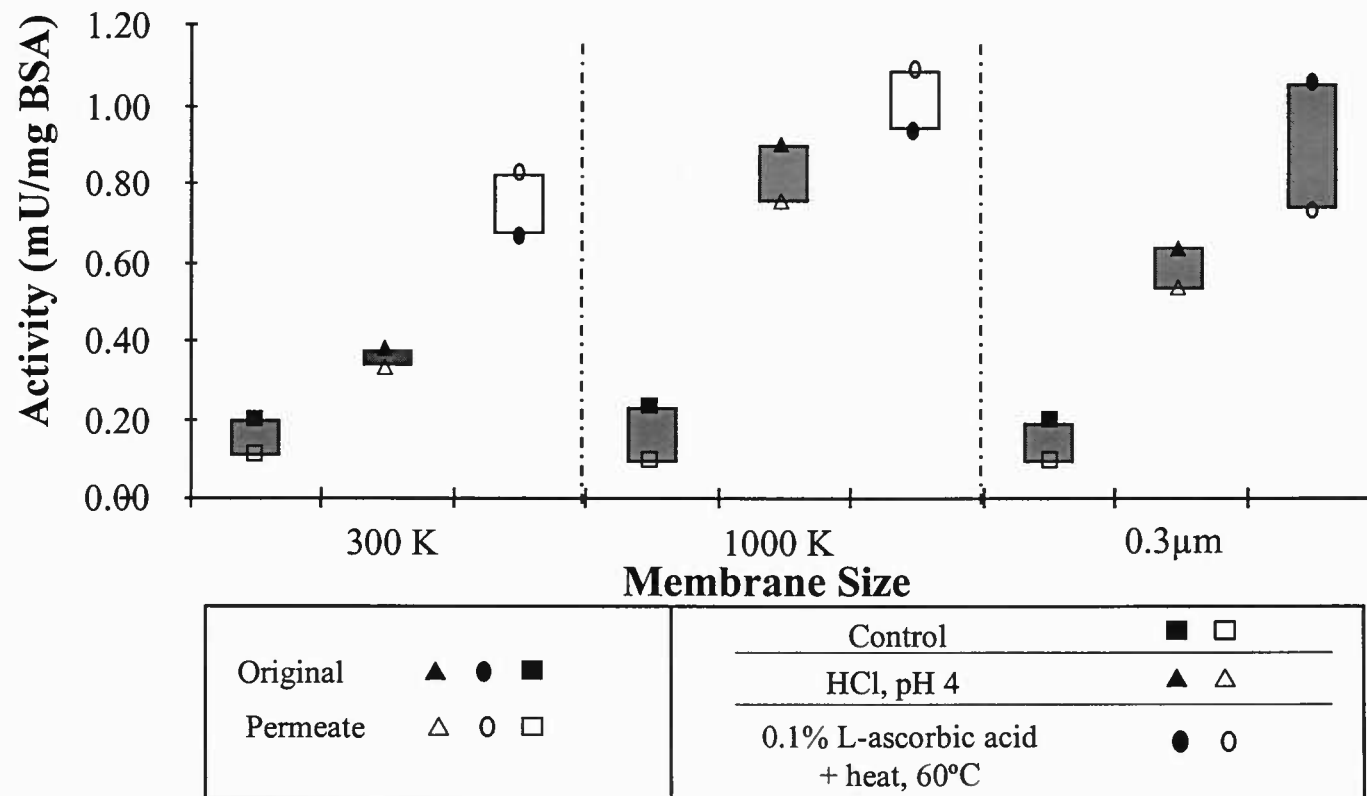
Figure 2.7. Effect of membrane size and treatment on protease purity.
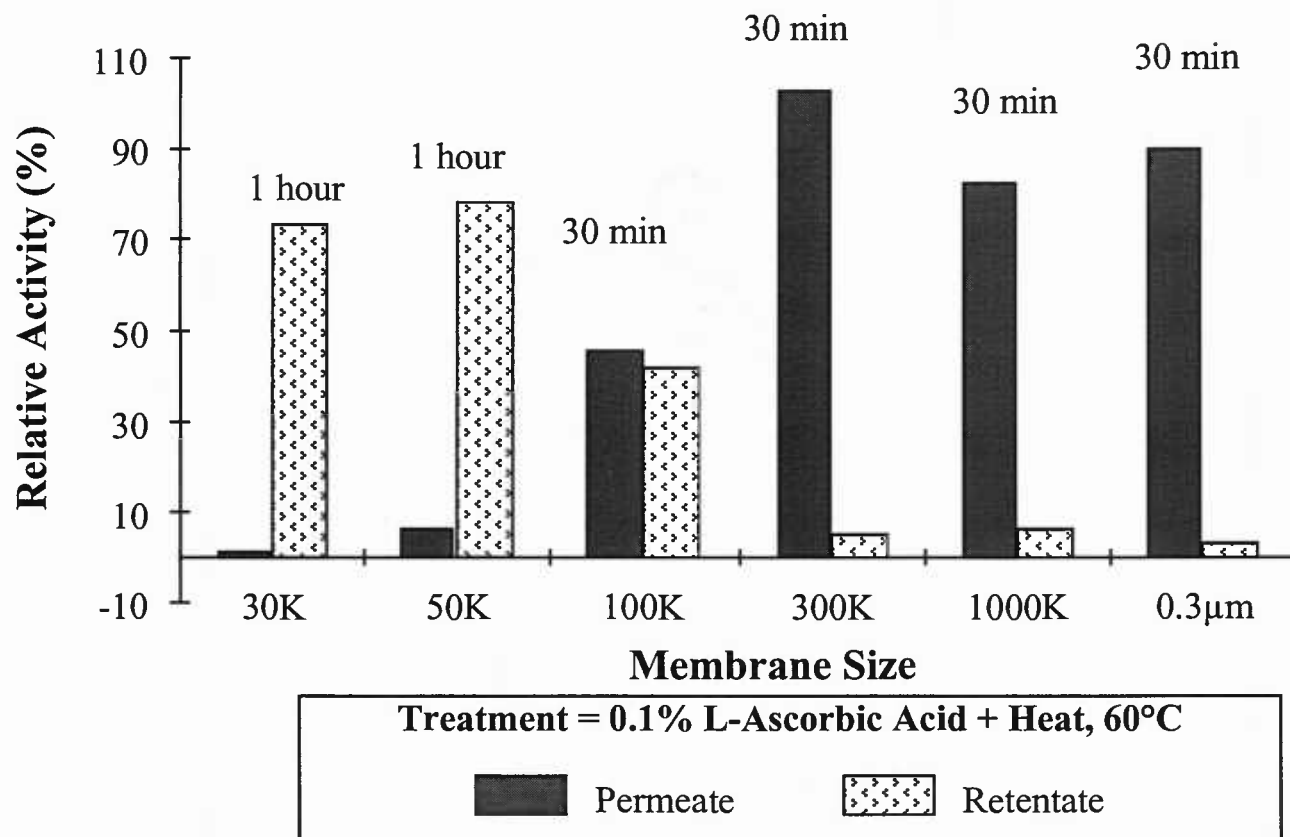
Figure 2.8. Effect of membrane size on permeation or retention of protease activity.
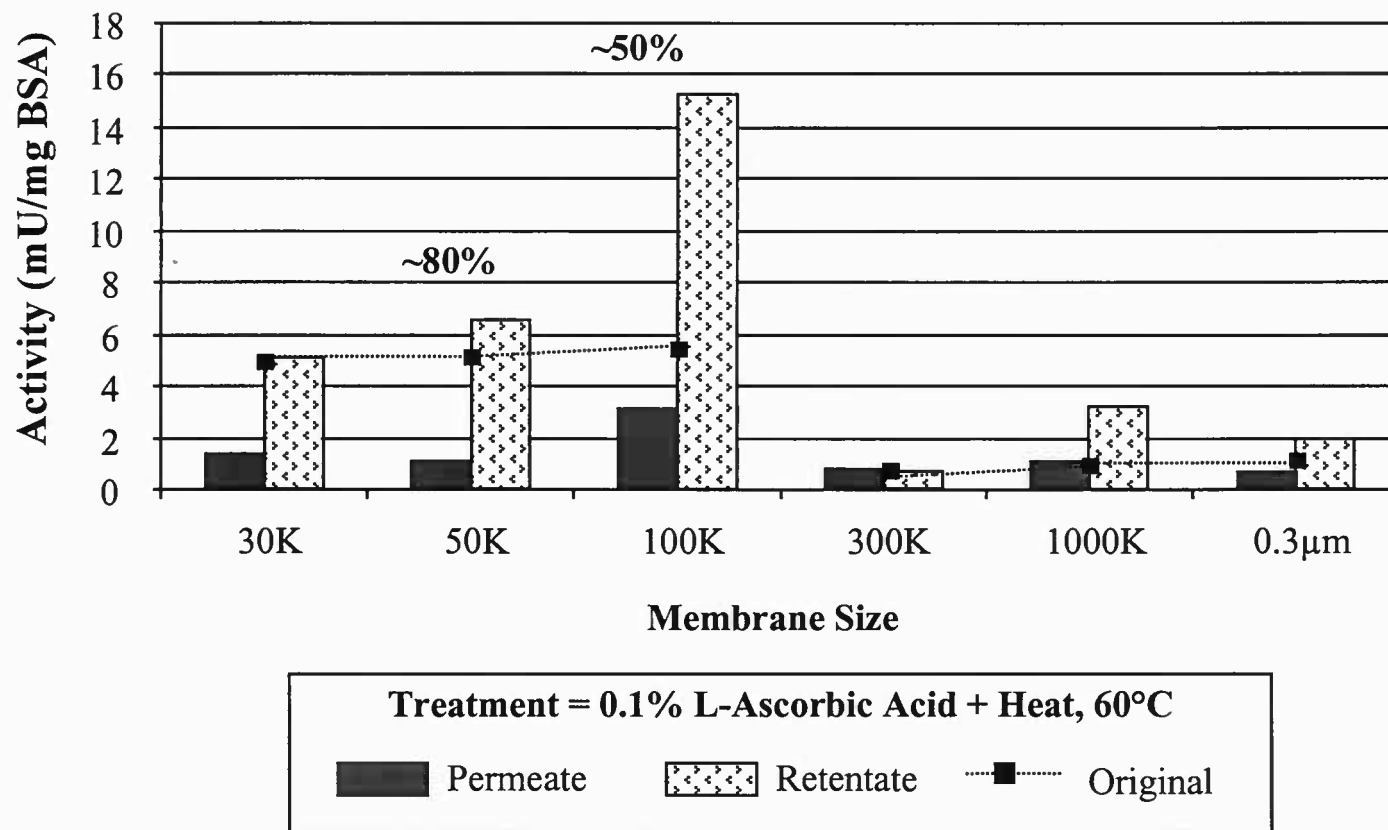
Figure 2.9. Effect of membrane size on protease purity.

into the permeate. When they were smaller, protease remained in the retentate. Fig. 2.9 gives an indication of enzyme purity. Highest purification occurred with a filter size of 100 kD, followed by 50 kD, and 30 kD, respectively. Less protease was recovered in the retentate with the 100 kD than the 50 kD membrane. However, higher purification was obtained using 100 kD (~9x) as opposed to 50 kD (~7x) membranes. In addition, concentration occurred more rapidly with the larger 100 kD membrane (30 min.) than the smaller 50 kD membrane (1 hr.).

CONCLUSION

Pretreatment of wash water effectively reduced interfering proteins and endogenous inhibitors of the protease targeted for recovery. The data suggested that acid plus heat was the most effective treatment in achieving both of those goals. In addition, utilization of microfiltration or ultrafiltration membranes to separate the protease from the bulk of the proteinaceous material was not successful. However, significant concentration of the enzymes following treatment was possible.

ACKNOWLEDGMENT

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Chapter 3. Pilot Plant Recovery of Catheptic Proteases from Surimi Wash Water

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ABSTRACT

Pretreatment (heat at 60°C and pH ~6.2) of Pacific whiting (*Merluccius productus*) surimi wash water doubled ultrafiltration membrane flux. Pretreatment significantly reduced 35–205 kD proteins in surimi wash water. Two bands of significance remained at 36 kD and 50 kD. Cathepsin L has been associated with the former. Concentrated crude protease obtained from wash water contained predominantly cathepsin L activity. Enzyme purity was increased ~100-fold and yield was about 80%. Stability (frozen and freeze-dried protease) was maintained for 9 weeks at 4°C, –15°C, -80°C. Freeze-dried preparations were stable for 9 weeks at refrigeration temperatures. A simple, effective method was developed for the simultaneous reduction of total proteinaceous material (i.e. purification) and recovery of a stable crude protease fraction from surimi wash water using pilot plant equipment.

INTRODUCTION

An issue of particular relevance to the seafood industry is waste reduction and maximized utilization of resources. The production of surimi from Pacific whiting (*Merluccius productus*) results in the utilization of approximately 20% (Wendel, 1999) of the original product. This means that about 80% of the product is considered waste or potential by-product material. In Oregon, surimi processing facilities either ship their solid waste for conversion to fishmeal, hydrolysates, or grind and return the material back to the environment. Soluble wastes from surimi wash water are returned to the environment either by direct discharge into the Columbia River or shipped and sprayed over farmers' fields. Increasing concerns, however, about the negative impact that direct discharge of waste may have on the environment may eventually pressure processors to find alternative methods of disposal.

As opposed to the traditional conversion of seafood waste into fertilizer or animal feeds/diets, more novel approaches to waste management and by-product utilization involve the identification and recovery of bioactive compounds. Perhaps the best example in the food industry concerning recovery and utilization of a bioactive product involves whey, a functional protein and by-product of cheese or casein production. Similarities in protein composition makes liquid whey (0.8– 0.9% protein: Charles and Radjai, 1977; Modler and others 1980; Shay and Wegner, 1986) a satisfactory model when considering the effect of various treatments on protein reduction and/or concentration in Pacific whiting surimi wash

water (0.6% protein: Huang, 1997). Whey protein is typically modified or concentrated by one of five processes (Grindstaff, 1974): gel filtration, electrodialysis, polyphosphate precipitation, heat coagulation, and ultrafiltration. Electrodialysis membranes function to remove ions and are normally used to produce a demineralized whey protein concentrate or modified whey (Stribley, 1963). Gel filtration is used to separate small molecules (lactose) from large molecules (protein). Polyphosphate precipitation is accomplished by forming an insoluble metaphosphate complex with whey proteins at a suitable acid pH (Hidalgo and others 1973). Heat coagulation (15 min at 85 – 100°C) denatures and produces a precipitate of lactalbumin. The precipitate from these processes is normally recovered by settling and decantation or centrifugation (Zall, 1992). Ultrafiltration is the system of choice when it comes to producing a concentrate with good functional properties. Ultrafiltration works by passing low molecular weight products (lactose, salts, water) while retaining high molecular weight products such as protein (Horton and others 1972). An early problem with ultrafiltration involved severe fouling of membranes. However, development of chemical and heat stable polysulphone membranes has increased the feasibility of the technology (Morr, 1976). A system described by Crocco (1975) was capable of processing 40,000 lbs of whey per hr. into whey protein concentrate.

Investigations into the use of pretreatments of whey prior to ultrafiltration are numerous. In general, investigators have demonstrated that heat pretreatment can effect concurrent improvements in flux and reductions in membrane fouling

(Modler and Emmons, 1975). Additional improvements in flux are obtained when protein is removed by centrifugation prior to ultrafiltration (Tarnawski and Jelen, 1985). A comprehensive review concerning the effect of pretreatments (clarification, heating, pH adjustment, demineralization, or preconcentration) on membrane processing of whey can be found by Muller and Harper (1979). Although the vast majority of protein recovery literature originates from whey reclamation efforts, several investigations were found concerning the recovery of soluble and insoluble proteins from surimi wash water.

Production of quality surimi from Pacific whiting involves extensive washing and dewatering steps. Washing removes compounds such as sarcoplasmic proteins, inorganic salts, low-molecular weight substances, lipids, and blood components. Green and others (1984) made the first investigations into the use of ultrafiltration to remove proteins from surimi processing wash water. Their main goal, however, was to reduce water usage by recycling the wash water. Lin and others (1995) recovered myofibrillar protein from surimi wash water utilizing ultrafiltration. Recovered protein was returned to the process to increase yield. In their review on membrane applications in the seafood industry, Jaouen and Quéméneur (1992) noted processing of surimi wash water by ultrafiltration without pretreatment was not practical. Huang and Morrissey (1998) characterized the microfiltration membrane fouling of surimi wash water. They determined that fouling occurred initially as a result of pore blocking resistance followed by cake resistance.

Attempts to combine ohmic heat-treatments with ultrafiltration on surimi wash water were first made by Huang and others (1997). They studied the effects of ohmic heating on protein coagulation in surimi wash water. Ohmic heating was utilized in order to minimize deleterious effects of prolonged temperature exposure on protease activity. Huang found that 92.1% of the wash water protein could be removed utilizing this treatment (70°C). Benjakul (1997) recovered, purified, and characterized a protease (cathepsin L) from surimi wash water. His recovery methods involved pretreatment of the wash water with ohmic heating followed by ultrafiltration and freeze-drying. Ohmic heating caused a 2-fold increase in wash water protease activity. Benjakul and others (1996) previously postulated this was a result of dissociation of the enzyme/inhibitor complex to its free dissociated form, however, subsequent ultrafiltration resulted in a 40% loss of total activity. Ultrafiltration was completed utilizing a 30 kD membrane (Cuno separation system) with pre-filter. It was hypothesized that losses were probably a result of high pressure (30 psi) and loss of enzyme through the membrane. It was the objective of this study to test previously determined (Ch. 2) lab scale optimizations for the recovery of proteases from surimi wash water utilizing pilot plant equipment.

MATERIALS AND METHODS

Collection of wash water

Surimi wash water was collected from the first dehydrator (Fig. 3.1) of Point Adams Packing Co. (Hammond, OR). It was immediately transported to the OSU Seafood Laboratory on ice and kept in a 4°C walk-in cooler until treatment. A portion of the untreated wash water was collected and stored at -80° C. Treatment and processing of surimi wash water began within 2 hr. of its collection.

Treatment of wash water

Wash water (20.0 kg) was weighed and transferred to a modified 60 L steam jacketed kettle (Fig. 3.2). Acidity of the wash water was reduced to pH 6 with 1:1 concentrated HCl. Upon adjustment of the pH, steam was introduced into the jacketed container. Two thermocouples (copper-constantan) were wired to a datalogger (CR10, Campbell Scientific Inc., PC208w software) and used to measure/monitor the internal temperature of the wash water. When the wash water reached 60°C, the steam valve was switched to deliver tap water. At the same time, -30°C antifreeze was pumped into copper coils submerged in the wash water to further enhance the cooling rate. Sample was cooled to at least 15° C before being centrifuged at 3000xg for 10 min at 4°C using a Sorvall RC-5B refrigerated superspeed centrifuge with GS-3 rotor. The supernatant (SUP) was collected by passing it through four layers of cheesecloth. Fractions of pH treated; pH and heat

Surimi processing flow chart

	Whole Fish	**100%**

Viscera 15-30% ← **Gutting**

Gutted Fish 70-85%

Heads 14-20% ← **De-Heading**

Headed & Gutted 56-71%

Frames 17% ← **Filleting**

Fillet Skin-on 39-47%

Skin/Bones 8-10% ← **Mincing**

Mince 32-36%

Soluble Protein 14-16% ← **Washing** → **Dehydrators 2-3 cycles**

Washed Mince 19-30%

Connective Tissue 4-8% ← **Refining** → **Screw press**

Washed Mince 19-30%

Mixing ← ← ← **Cryoprotectants**

Surimi 15-22%
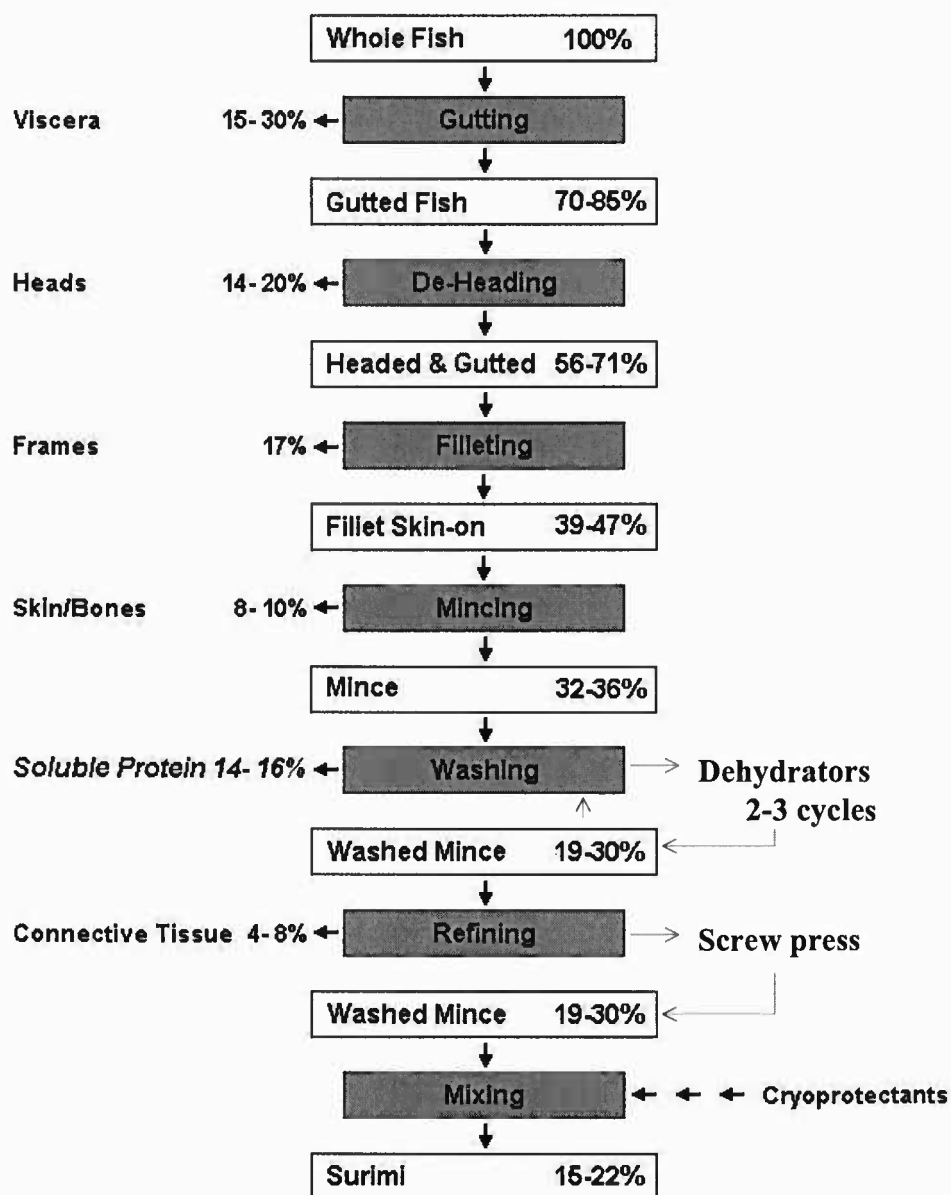
Figure 3.1 Surimi flow-diagram (adapted from Wendel, 1999)
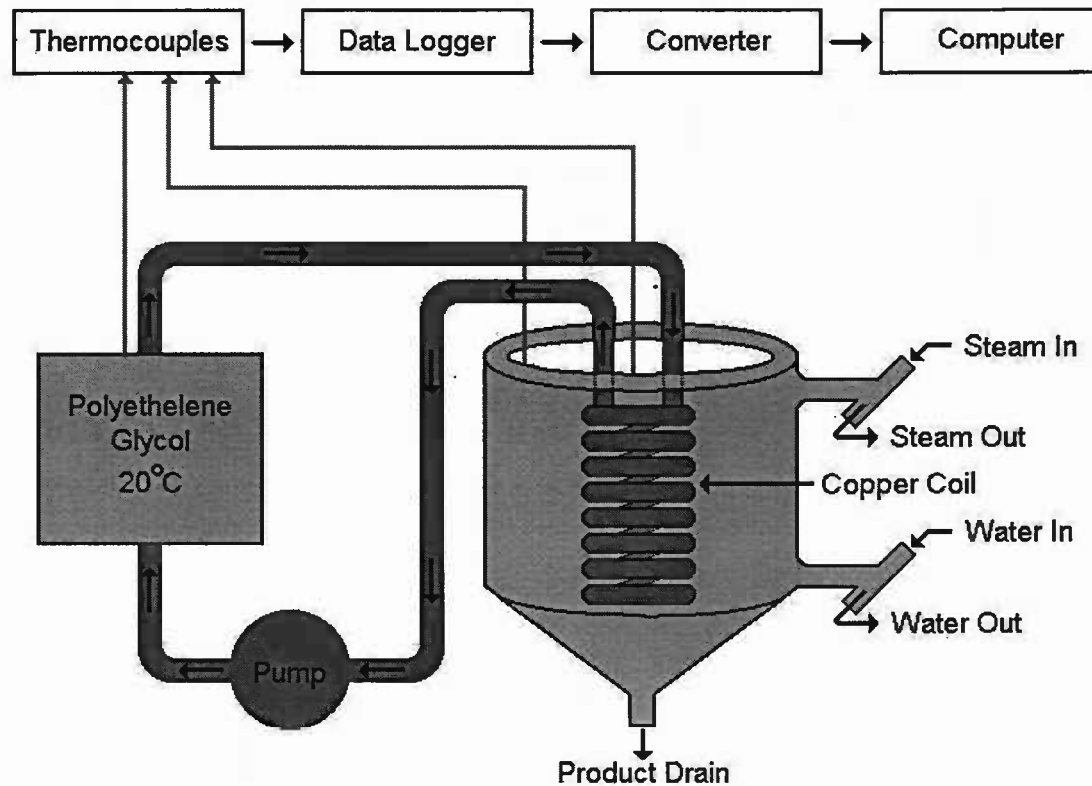
Figure 3.2. Schematic of steam-jacketed kettle set-up

treated; and pH, heat, and centrifuged (i.e., SUP) samples were collected and stored at -80° C for further analysis.

Ultrafiltration of wash water

Collected SUP was weighed and transferred to a 20 kg plastic "retentate" reservoir (Fig. 3.3). The temperature of the SUP was monitored with a thermocouple and kept below 15°C during filtration with ice packs previously stored in a –50°C freezer. SUP was pumped from the feed tank to the filtration unit (Maximate™-EXT, Pall Filtron, Northborough, MA) at 3.75 – 4 GPM (14– 5 L/min) under an average transmembrane pressure (TMP) of 20-21.5 psi by a ballasted, double diaphram pump (All Flo Pump Co., Mentor, OH).

$$TMP = \frac{P_{feed} + P_{retentate}}{2} - P_{filtrate}$$

Where P_{feed} = pressure at feed inlet, $P_{retentate}$ = pressure at retentate outlet and $P_{filtrate}$ = pressure at filtrate outlet. The filtration system utilized consisted of stainless steel hardware and a 30 or 50 kD (Omega Industrial Maximate™-EXT, Pall Filtron, Northborough, MA) open channel, polyethersulfone cassette. The Omega membrane from Pall Filtron is specifically modified to minimize protein binding to the surface and interstitial binding. Total membrane area was 4 ft^2 (60 mL). Sample flux data was generated by measuring the weight of filtrate collected over time utilizing a loadcell (Model CLC-454G, OMEGA Engineering, Inc., Stamford, CT) wired to a datalogger (Model CR10, Campbell Scientific, Inc.,
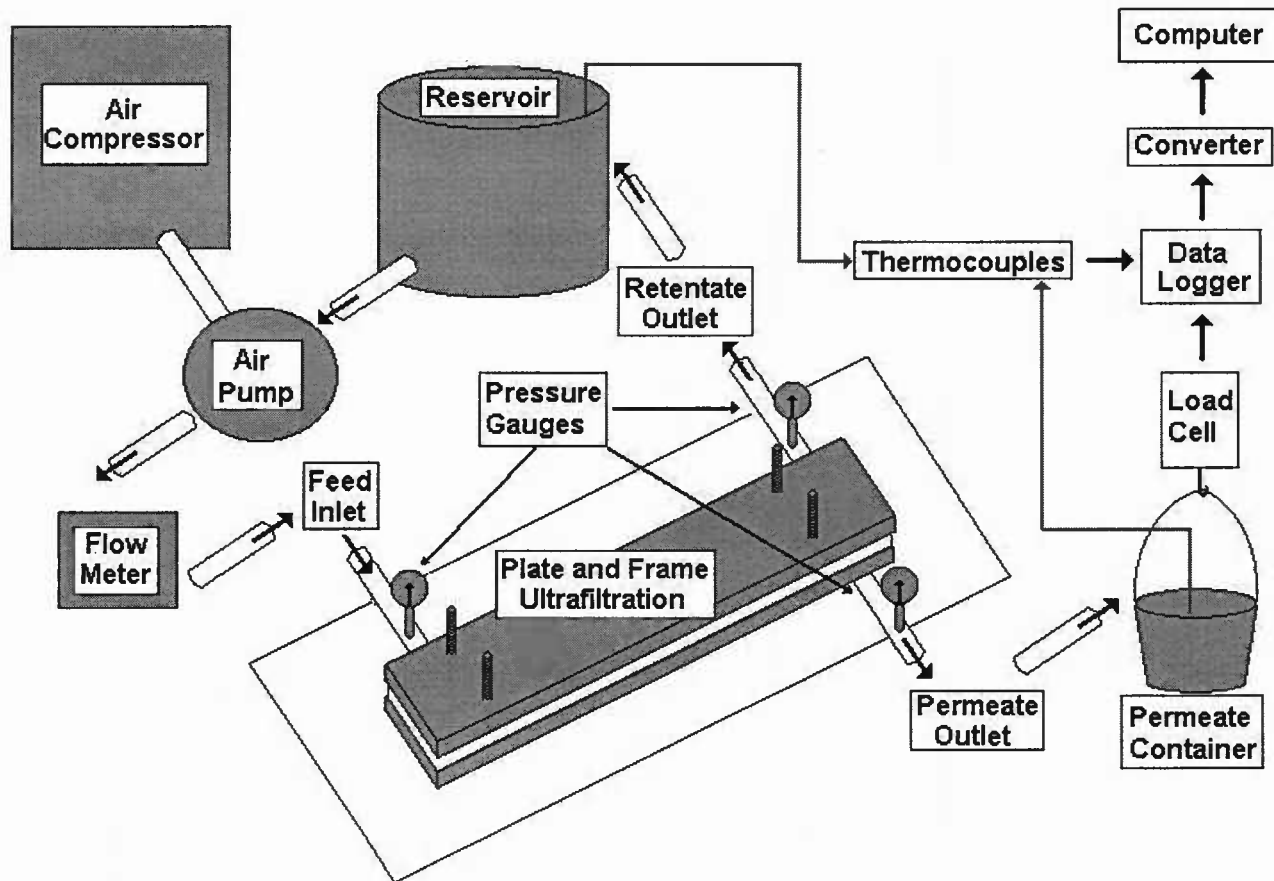
Figure 3.3. Flow diagram of ultrafiltration set-up

Logan, UT) connected through an I/O port to a portable PC. The datalogger was programmed (PC 208w software, Campbell Scientific, Inc., Logan, UT) to collect data at 5 sec. intervals for 10 min., 30 sec. intervals for the next 30 min., and 2 min. intervals for the remainder of the ultrafiltration time. Concentration of the SUP was taken as far as possible and stopped immediately once foaming began to initiate as a result of air (as opposed to sample) being introduced into the pump intake. Retentate and permeate fractions were collected and stored at $-80°C$. Treatment and ultrafiltration was conducted the same day as sample collection. The process was conducted, in triplicate, for each membrane used.

Proximate analysis

Moisture, protein, and ash were determined following established AOAC methods (AOAC, 1995).

Protease activity

Analysis for protease was determined by monitoring cathepsin L and B activity as determined according to the method of Barrett and Kirschke (1981) with a modification of the stopping reagent by addition of iodoacetate (An and others 1994). Samples were analyzed in polystyrene tubes and kept on ice before and after incubation. An 100 µL of sample was combined with 400 µL of 0.1% Brij (35% w/v). Appropriate dilution of samples was made before hand if necessary

(appropriate range = 0.3 - 3.0 ng of cathepsin L as determined by active site titration). A 250 μL aliquot of assay buffer (340 mM sodium acetate; 60 mM acetic acid; 4 mM disodium ethylene diamine tetraacetic acid, EDTA; 8 mM dithiothreitol, DTT; final pH 5.5) was added and tubes (covered with petrifilm) were placed in a 30°C water bath. After 1 min., 250 μL of pre-warmed substrate (20 μM Z-Phe-Arg-Nmec) was added. Samples were agitated and 1 mL of stopping reagent (100 mM sodium monochloracetate, 30 mM sodium acetate, 70 mM acetic acid, 5mM iodacetate, pH 4.3) was added at 10 min. Flourescence of the free aminomethylcoumarin was measured (excitation = 370 nm; emmision = 460 nm) in the sample and compared to the standard (0.5 μM 7-amino-4-methylcoumarin corresponded to 0.1 mU of activity in the tubes). One mU was defined as being equivalent to the release of 1 nmole aminomethylcoumarin/min.

Measurement of specific protease activity (Cat L, Cat B, Cat H)

The retentate fractions were measured for cathepsins L, B, and H activity following the methods of Barrett and Kirschke (1981) using the fluorogenic substrates Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and Arg-NMec, respectively, as specific substrates. For measurement of activity with the substrate Z-Phe-Arg-NMec samples were prepared with the appropriate dilution (100-fold) before analysis. Samples were not diluted before measurement of activity with the substrates Z-Arg-Arg-NMec and Arg-NMec. Substrate solutions were prepared to 20 μM concentrations. Assay conditions were similar to that prescribed for

cathepsin L, except assay buffer for cathepsin B contained 88 mM KH_2PO_4, 12 mM Na_2HPO_4, and 1.33 mM disodium EDTA (pH 6.0). The buffer was made to 2.7 mM L-cysteine (free base) daily. Assay buffer for cathepsin H contained 200 mM KH_2PO_4, 200 mM NaH_2PO_4, and 4 mM disodium EDTA (pH 6.8). The buffer was made to 40 mM L-cysteine (free base) daily.

Measurement of enzyme concentration by active site titration with E-64

A modified method by Barrett and Kirshke (1981) was used to estimate absolute molarity of the retentate fraction (as well as papain) by stoichiometric titration with E-64, L-*trans*-epoxysuccinlleucylamido(4-guanidino) butane, a powerful inhibitor of cysteine proteases. The sample was diluted 10-fold and 25 µL was combined with 25 µL of 100-1000 nM E-64 and 50 µL of the cathepsin L assay buffer (pH 5.5) without DTT. The mixture was incubated in a 30°C water bath for 5 min. (Wu, 1994). Remaining proteolytic activity was determined using the procedure for cathepsin L analysis. A linear plot of activity (Δ fluorescence units) against E-64 molarity reaches zero at the molarity of the enzyme solution.

SDS-PAGE electrophoresis

Comparison of protein bands were made between the original wash water collected (ORIG), pH and heat-treated supernatant (SUP), retentate (RET), and filtrate (FIL). Samples were combined 1:4 or 1:2 with sample buffer and heated at

95°C for 4 min. (Laemmli, 1970). Sample, 40 µg protein, were loaded and fractions separated on SDS-substrate gels (12%) at constant 200 V using a Mini-Protean II system (Bio-Rad Laboratories, Inc., Hercules, CA). The gel was stained in 0.125% Coomassie blue R-250 and destained in 25% ethanol and 10% acetic acid. Low and high molecular weight standards (Sigma-Aldrich, Co) were used for the estimation of apparent M_r of the protein bands.

The high molecular weight standard included myosin, rabbit muscle (205,000), β-galactosidase, *E. coli* (116,000), phosphorylase b, rabbit muscle (97,000), fructose-6-phosphate kinase, rabbit muscle (84,000), albumin, bovine serum (66,000), glutamic dehydrogenase, bovine liver (55,000), ovalbumin, chicken egg (45,000), and glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36,000). The low molecular weight standard included albumin, bovine serum (66,000), glutamic dehydrogenase, bovine liver (55,000), ovalbumin, chicken egg (45,000), glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36,000), carbonic anhydrase, bovine erythrocytes (29,000), trypsinogen, bovine pancreas (24,000), trypsin inhibitor, soybean (20,000), α-lactalbumin, bovine milk (14,200), and aprotinin, bovine lung (6,500).

Activity staining

Retentate fractions collected from the 50 kD and 30 kD membranes were combined at a 3:1 ratio with sample buffer or with sample buffer containing no β-mercaptoethanol (βME). Samples with βME were heat-treated at 95°C for 4 min.

before loading (20μL) on a SDS-substrate gel (12%). Samples without βME were not heat-treated prior to loading (20μL) on the gel. Fractions were separated at constant 100 V using a Mini-Protean II system. Activity staining of the gel was carried out according to the modified method of García-Carreño and others (1993). The gel was immersed in 2% casein in 50 mM Tris-HCl buffer, pH 7.5, and kept on ice for 1 hr. with constant agitation. The gel was transferred to McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M sodium citrate), pH 5.5, containing 2% casein and 1 mM βME. The gel was incubated for 1 hr. at 55°C for activity zone development. Following incubation the gel was stained in 0.125% Coomassie blue R-250 and destained in 25% ethanol and 10% acetic acid. Low and high molecular weight standards were used for the estimation of apparent M_r of the activity.

Stability study of freeze-dried retentate

Samples were treated with high and low levels of a cryoprotectant (sodium phosphate) or a cryprotectant and metal chelator (EDTA) prior to freeze-drying. Untreated retentate (liquid and freeze-dried) was used as a negative control. Both treated samples and negative controls were stored at refrigeration (4°C) and freezer (-15°C) storage temperatures. Samples stored at –80°C were used as positive controls. Treated samples had either 0.2% or 1% cryoprotectant. Samples with both cryoprotectant and metal chelator were treated at either 0.2% cryoprotectant and 0.02% EDTA or 1% cryoprotectant and 0.1% EDTA. Protease activity was

measured using Z-PHE-ARG-NMec (Barrett and Kirschke, 1981) at least weekly for at least 60 days. All samples were stored under nitrogen.

RESULTS AND DISCUSSION

Ultrafiltration

Cross-flow ultrafiltration, a technique involving the perpendicular sweep of solution across a membrane, was used to minimize the development of a filter cake or concentration polarization at the membrane surface (Belter and others 1988). Previous benchtop analysis had demonstrated that effective retention of protease was achieved utilizing 30 kD and 50 kD membranes. Data had also indicated that little difference existed between 50 kD and 30 kD membranes as virtually all protease was retained by both. Analysis, however, was performed with centrifugal as opposed to tangential flow units. Therefore, it was expected that actual performance of pilot plant scale cross flow membranes would show differences. Initial pilot plant tests were conducted with the 50 kD membrane. Lab scale optimization studies had previously predicted losses of ~20% protease. An accounting of protease flow in the fractions collected before and after pilot plant ultrafiltration indicated that similar amounts (~20%) of protease also passed through the membrane into the permeate. However, tests conducted with the 30 kD membrane showed that more protease (~90%) was recovered than predicted by lab scale test (~75%). The centrifugal units used in lab scale tests behaved more like

dead-end than crossflow filtration devices. Precipitation of protein on membranes was more evident with the lab scale units. This probably accounts for differences in results between lab and pilot plant tests with the smaller 30 kD membrane.

Effect of acid and heat-treatment on flux

A load cell connected to a datalogger was utilized to measure wash water flux. Flux is a measure of the solvent velocity through the membrane and can be used to calculate concentration polarization. Fig. 3.4 and 3.5 are examples of fluxes obtained using the 30 kD and 50 kD membranes. These figures indicate there was a substantial decrease in initial flux in the first 10 min. after which apparent steady state equilibrium (albeit slightly decreasing over time) was achieved. Huang and others (1997) hypothesized that rapid decline in initial permeate flux of surimi wash water was a result of pore blocking and subsequent decline was related to the boundary layer near and the cake layer deposited on the surface.

Preliminary trials were conducted to determine the flow of untreated surimi wash water though membranes. Table 3.1 compares flux (kg/m^2hr) of untreated wash water with wash water treated by acidification to ~pH 6.2, heating to 60°C, cooling, and centrifugation. Results indicated the flux of untreated wash water was just under half that of treated wash water. This was reasonable as solids (mainly protein) were reduced by 50% as a result of treatment (Tables 3.2 and 3.3). Reduction in protein reduces cake formation and thus cake resistance, thereby
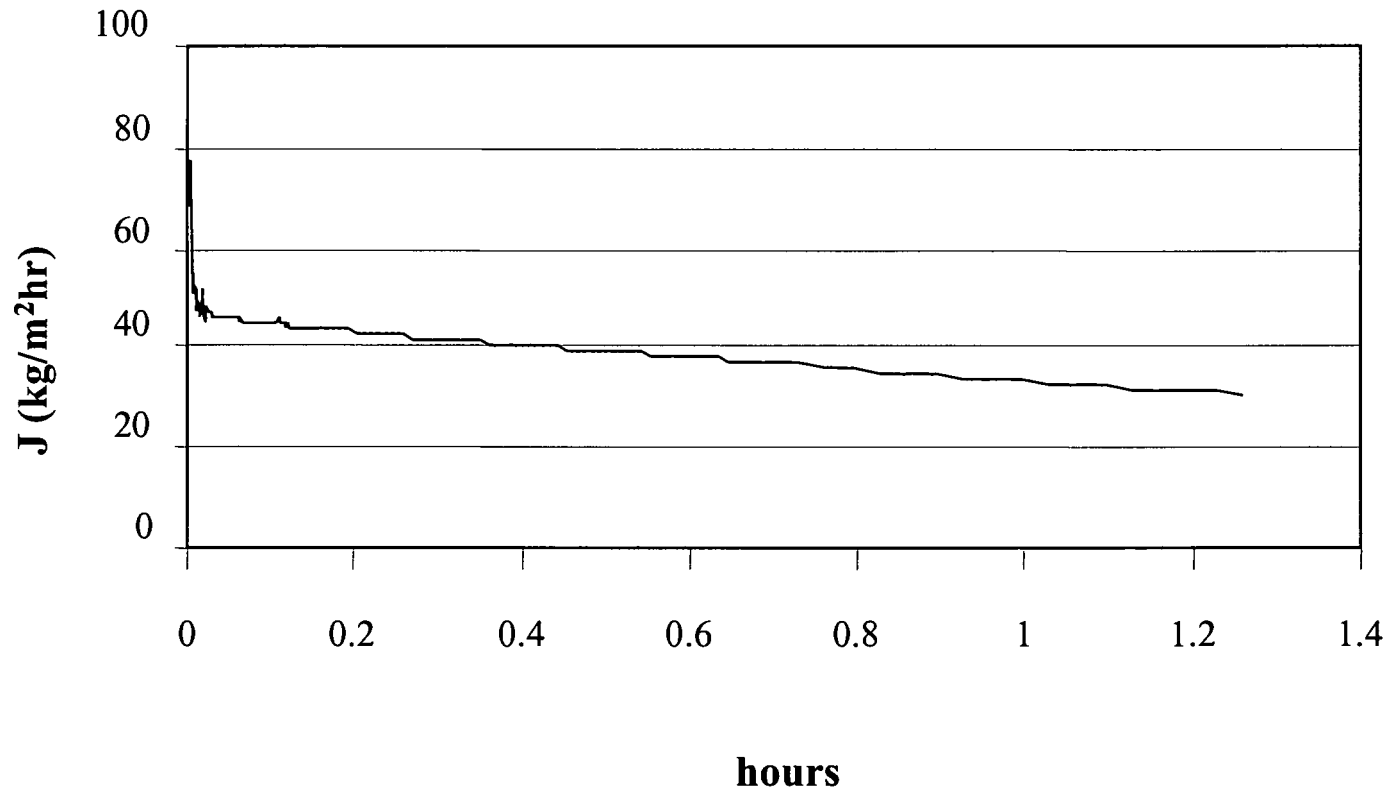
Figure 3.4. Flux of acid and heat treated surimi wash water through a 30 kD Omega ultrafiltration membrane.
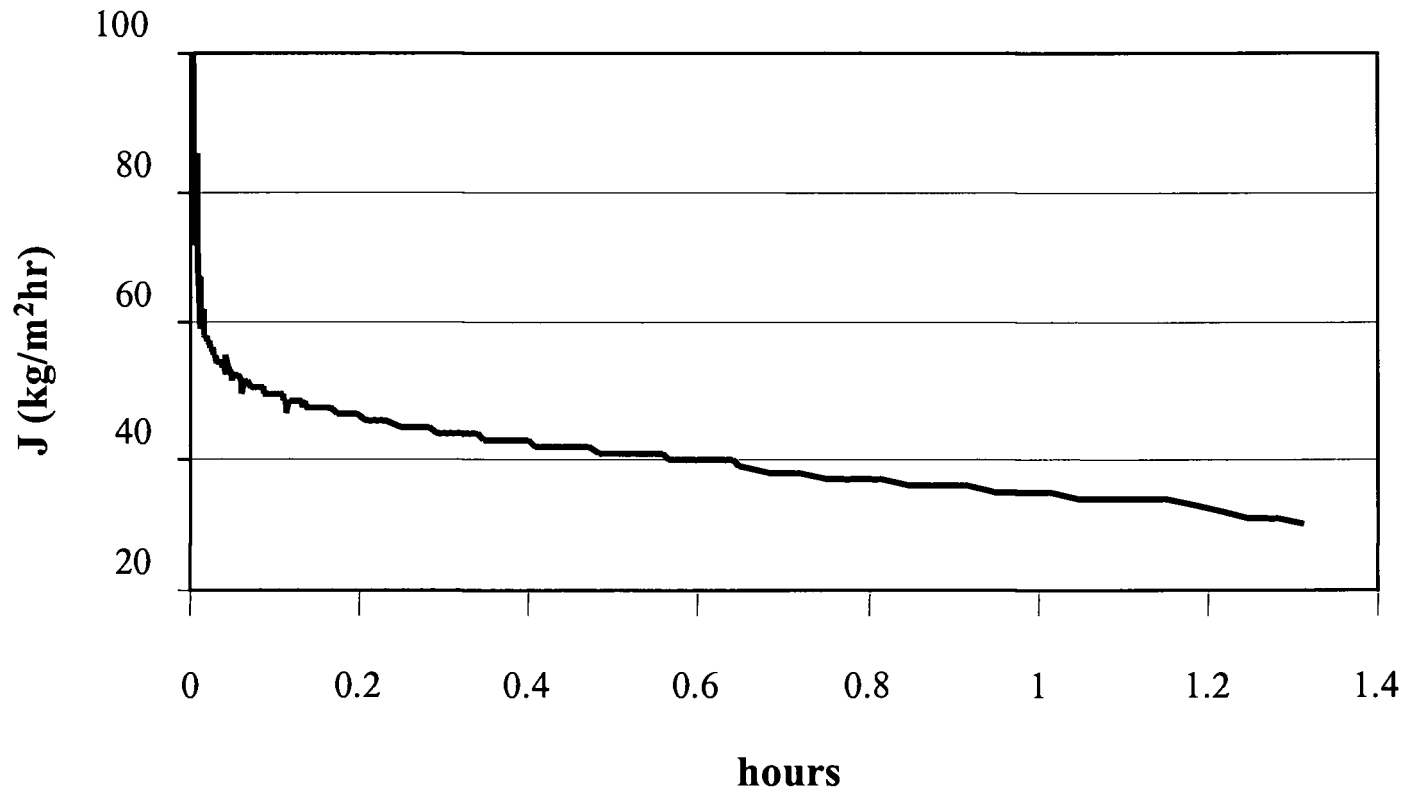
Figure 3.5. Flux of acid and heat treated surimi wash water through a 50 kD Omega ultrafiltration membrane.

Table 3.1. Flux at 1 min. and 1 hr. for untreated and treated surimi wash water.

Membrane size	1 min. (kg/m^{2}hr)	1 hr. (kg/m^{2}hr)
30 kD treated	52.74 (10.46)	39.57 (6.57)
50 kD treated	51.75 (9.48)	32.40 (2.68)
50 kD untreated	24.86 (.572)	14.91 (.744)

Treated surimi wash water was acidified to ~pH 6.2, heated to 60°C, and centrifuged to remove precipitated proteins prior to ultrafiltration. Untreated surimi wash water was passed through 4-layers cheesecloth to remove suspended solids prior to ultrafiltration. Values in parenthesis represent standard deviations.

Table 3.2. Proximate analysis of original, supernatant, retentate, and permeate fractions collected during 30 kD ultrafiltration.

Sample	Solids (mg/mL)	Ash (mg/mL)	Protein (mg/mL)	Calculated Fat+CHO (mg/mL)
Original	23.31 (8.10)	3.67 (1.18)	19.19 (5.96)	0.45 (1.11)
Supernatant	11.84 (4.16)	3.67 (1.25)	9.13 (3.68)	-0.97 (0.91)
Permeate	8.93 (3.07)	3.51 (1.13)	7.10 (2.71)	-1.68 (0.91)
Retentate	24.38 (9.11)	3.74 (1.05)	19.69 (7.76)	0.95 (0.46)

Values in parenthesis represent standard deviations.

Table 3.3. Proximate analysis of original, supernatant, retentate, and permeate fractions collected during 50 kD ultrafiltration.

Sample	Solids (mg/mL)	Ash (mg/mL)	Protein (mg/mL)	Calculated Fat+CHO (mg/mL)
Original	26.37	3.56	22.01	0.81
	(7.62)	(0.51)	(8.07)	(1.45)
Supernatant	13.62	3.66	9.91	0.13
	(3.84)	(0.37)	(2.83)	(1.66)
Permeate	10.72	3.46	8.22	-0.95
	(2.81)	(0.44)	(2.50)	(0.29)
Retentate	23.25	3.67	18.70	0.88
	(3.69)	(0.51)	(3.06)	(0.40)

Values in parenthesis represent standard deviations.

increasing flux. Further illustration of improved flux was evident by differences in time required for a near 10-fold concentration of wash water between untreated (3 hr.) and treated (1.5 hr.) wash water. In addition, no significant advantage in rate of flux was obtained through use of the larger 50 kD as opposed to the 30 kD membrane.

Profiles taken of the retentate and permeate over time indicated that temperature was maintained below 15°C (data not shown). Jaouen and Quéméneur (1992) recommended in their review on membrane ultrafiltration in the seafood industry that processing be conducted at 15°C or less to avoid protein denaturation.

Proximate analysis

Composition of the various fractions collected before and during membrane processing is given in Tables 3.2 and 3.3. Average concentration of the retentate fraction for the 30 kD membrane was 9.23-fold (±1.58) and 8.09-fold (±0.19) for the 50 kD membrane. Mass balance of the solids and protein, taking into account concentration, showed good correlation. For example, average initial solids (mg/mL) in the SUP for the 30 kD membrane was 11.84, while total calculated solids from PER and RET was 11.94 (Table 3.4). In general, results indicated treatment of surimi wash water with heat and acid reduced total solids/protein at least 50%. Large variation in wash water initial total solids (1.3–2.9% for 30 kD and 1.8-3.5% for 50 kD) made it difficult to find significant differences in membrane performance. However, a comparison of means suggested that more

Table 3.4. Mass balance of supernatant, permeate, and retentate fractions.

Membrane size	Fraction	Solids (mg/mL)	Ash (mg/mL)	Protein (mg/mL)
30 kD	Supernatant	11.84	3.67	9.13
	Permeate + Retentate*	11.94	3.97	9.53
50 kD	Supernatant	13.62	3.66	9.91
	Permeate + Retentate**	13.24	3.86	10.24

* Retentate concentrated on average 8.1-fold by ultrafiltration.
** Retentate concentrated on average 9.2x-fold by ultrafiltration.

solids/protein passed into the permeate when a 50 kD membrane was used for filtration.

Characterization of proteins by SDS-PAGE

Fractions collected during membrane processing were analyzed by SDS-PAGE to determine the effect of treatment and ultrafiltration on distribution of protein. Band patterns for both ultrafiltration membranes, 30 kD and 50 kD, were qualitatively similar (Fig. 3.6 and 3.7). Treatment of surimi wash water followed by centrifugation significantly reduced the presence of bands from 205 kD to 35 kD. Lin and Park (1996) identified the major proteins in this range as myosin heavy chain (205 kD) and actin (44.5 kD). Table 3.5 shows calculated molecular weight based on the retention factor of the furthest edge of migration for each significant band. Based on these values and a comparison band patterns, myosin and actin appear to be included in bands 8 and 5, respectively (Fig. 3.8). Two major bands (1 and 6) remain in the SUP following pretreatment. Band 1 contains β-tropomyosin and troponin T (Lin and Park, 1996). Benjakul and others (1996) stated activity staining of heat-treated cathepsin L, in non-reducing conditions produced clearing zones at 39.5 kD. Based on this, cathepsin L would also be associated with β-Tropomyosin/troponin in band 1 (Fig. 3.8). Measurement of band thickness indicated an increase in concentration of only 3.4 and 2.7-fold as a result of ultrafiltration with 30 kD and 50 kD membranes, respectively. Although this method of estimating concentration supports conclusions that more protease/
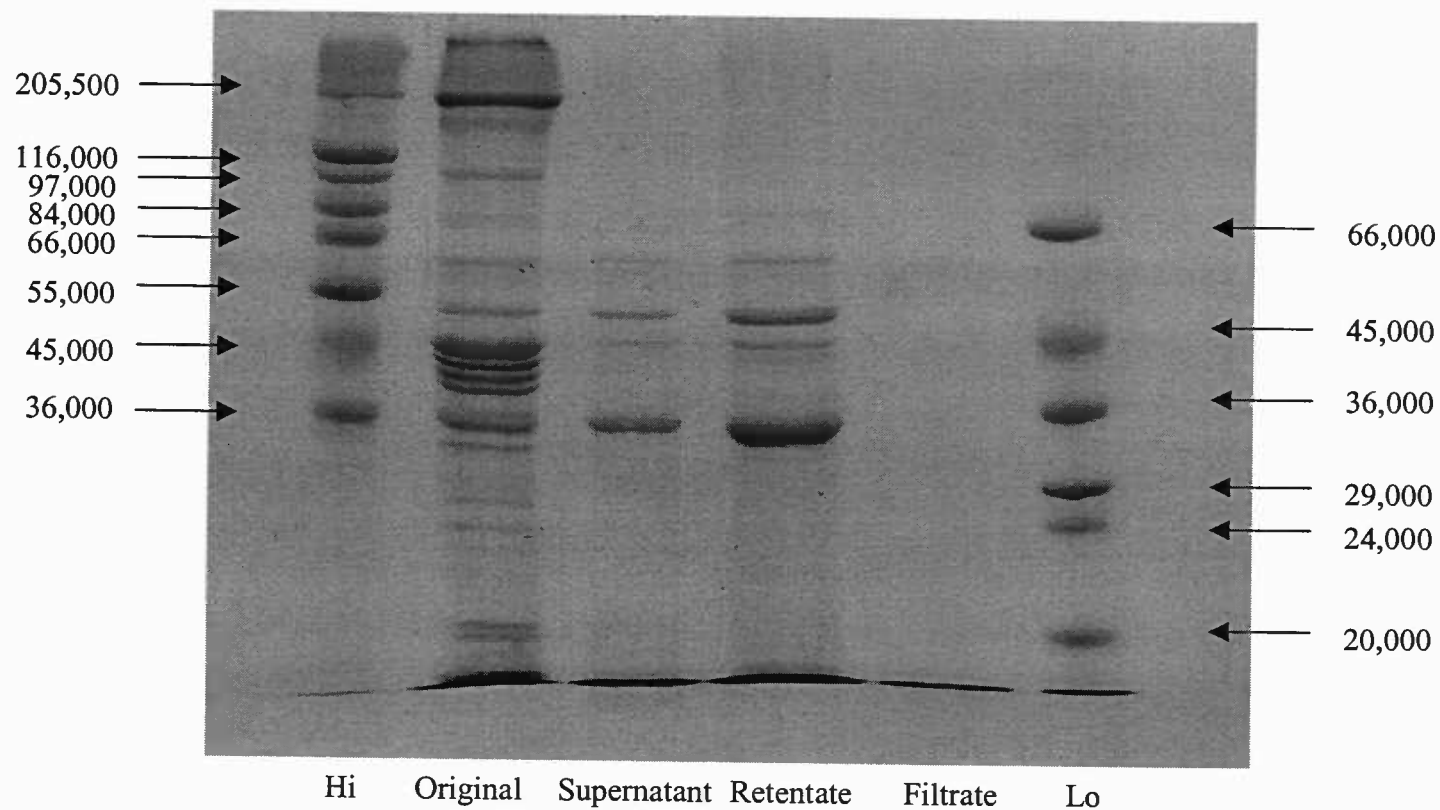
Figure 3.6. SDS-PAGE pattern of surimi wash water fractions before and after ultrafiltration with a 30 kD membrane. Hi=high molecular weight standard. Lo=low molecular weight standard.
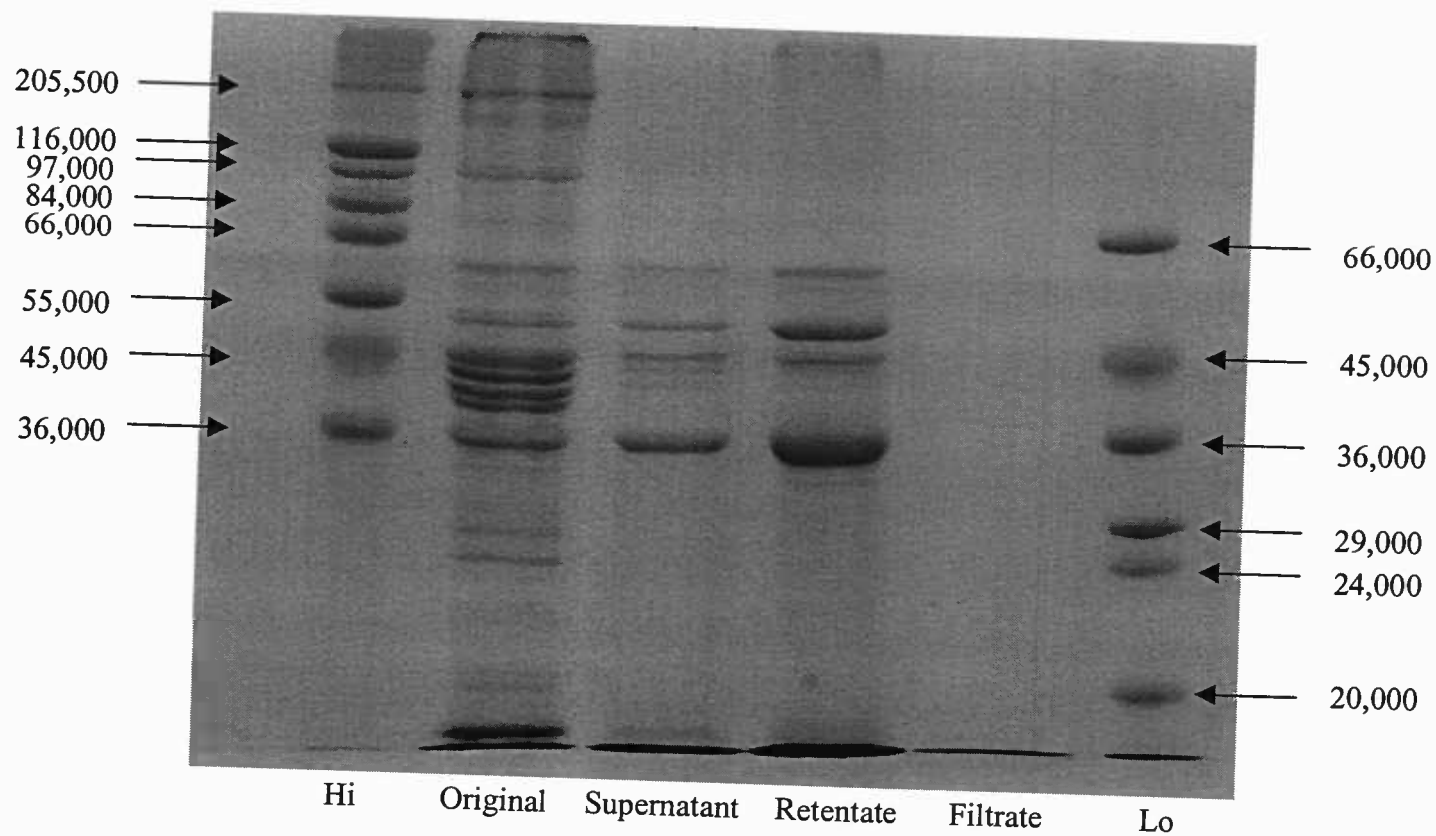
Figure 3.7. SDS-PAGE pattern of surimi wash water fractions before and after ultrafiltration with a 50 kD membrane. Hi=high molecular weight standard. Lo=low molecular weight standard.

Table 3.5. Molecular weights of significant unknown bands calculated from R_f.

Fraction	Unknown Band	50 kD (Molecular Weight)	30 kD (Molecular Weight)
Supernatant	1	33000 (200)	34000 (3000)
	5	46000 (500)	48000 (2000)
	6	50000 (1000)	53000 (2000)
	7	67000 (2000)	70000 (1000)
Retentate	1	32000 (500)	33000 (2000)
	5	45000 (700)	50000 (3000)
	6	50000 (1000)	52000 (4000)
	7	67000 (1000)	69000 (2000)
Original	1	33000 (800)	34000 (2000)
	2	37000 (700)	37000 (1000)
	3	40000*	38000 (600)
	4	42000*	40000 (200)
	5	48000 (300)	41000 (900)
	8	161000 (10000)	162000 (10000)

Values in parenthesis represent standard deviations.
*Based on one measurement as a result of smearing of bands in duplicate gels.
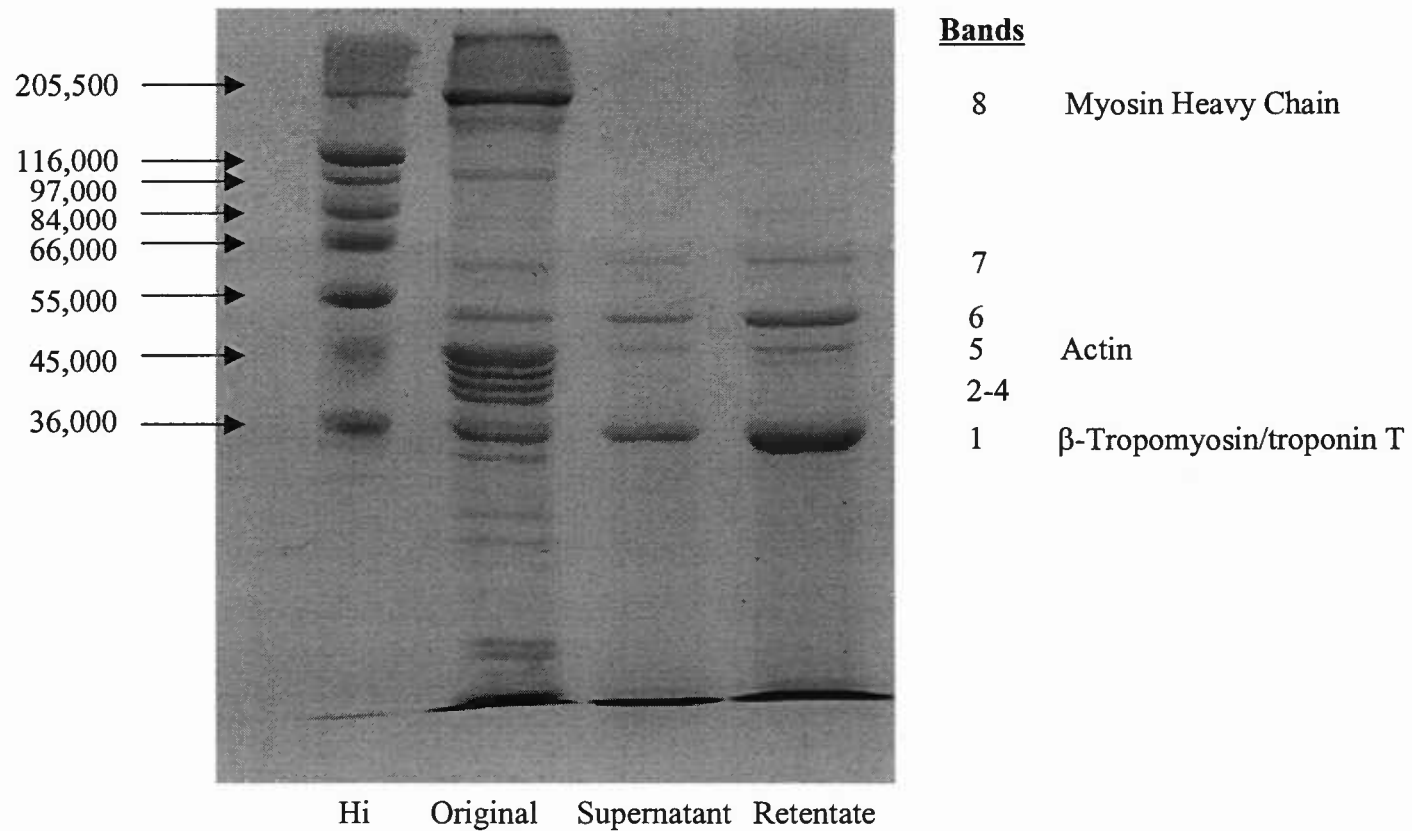
Figure 3.8. Numbers assigned to significant bands in Table 3.5. Hi=high molecular weight standard.

was retained by the 30 kD as opposed to 50 kD membrane, it underestimates actual measured concentration of the retentate (Table 3.4).

Protease analysis/characterization

An and others (1994) determined optimum pH and temperature conditions (5.5 and 55°C, respectively) for protease analysis. Seymour and others (1994) isolated the protease responsible for tissue softening and weakening of surimi gel strength. They confirmed optimal assay conditions and established two forms of cathepsin L (P-I and P-II) were predominant. The identification and characterization of protease in the surimi wash water from Pacific whiting has also been well established. Morrissey and others (1995) found a significant amount of protease was removed from the mince during the washing process. Benjakul (1997) recovered, purified, and characterized the main protease from surimi wash water as cathepsin L. Since cathepsin L was well established as the predominant protease in wash water from Pacific whiting, analysis were conducted only for confirmation of this information. The methods of Barrett and Kirschke (1981) were used for the fluorogenic assay of cathepsin L, B, and H (Table 3.6). Assays under these conditions indicated specific activity of cathepsin L was 70-fold (30 kD membrane) and 120-fold (50 kD membrane) greater than cathepsin B. Specific cathepsin L activity was also greater when compared to cathepsin H (200-fold, 30 kD membrane; 230-fold, 50 kD membrane). Tables 3.7 and 3.8 show protease purity in the retentate was increased with a 50 kD as opposed to 30 kD membrane.

Table 3.6. Cathepsin B, H, and L activity in the retentate.

Protease	30 kD (mU/mg)	50 kD (mU/mg)
Cathepsin L	0.9900 (0.3199)	1.4529 (0.3083)
Cathepsin B	0.0142 (0.0089)	0.0123 (0.0035)
Cathepsin H	0.0050 (0.0028)	0.0064 (0.0026)

Values in parenthesis represent standard deviations.

Table 3.7. Purification and recovery analysis for 30 kD ultrafiltration.

Collected fraction	Protein (mg/mL)	Activity (mU/mL)	Specific activity (mU/mg)	Purity fold	Yield
Original	19.19 (5.96)	0.17 (0.07)	0.011 (0.009)	1	
Supernatant	9.13 (3.68)	2.47 (0.64)	0.298 (0.091)	26.8 (13.1)	100
Permeate	7.10 (2.71)	0.06 (0.06)	0.022 (0.015)	2.10 (0.77)	5.61 (2.68)
Retentate	19.69 (7.76)	17.65 (3.92)	0.990 (0.320)	109 (41.7)	88.5 (6.03)

Values in parenthesis represent standard deviations. Activity/Specific activity defines cathepsin L type activity. Purity is calculated from Specific activity. Yield is calculated from activity in and concentration of the retentate following ultrafiltration, 8.1x.

Table 3.8. Purification and recovery analysis for 50 kD ultrafiltration.

Collected fraction	Protein (mg/mL)	Activity (mU/mL)	Specific activity (mU/mg)	Purity fold	Yield
Original	22.01 (8.07)	0.28 (0.05)	0.012 (0.002)	1	
Supernatant	9.91 (2.83)	4.73 (2.60)	0.452 (0.131)	33.1 (9.56)	100
Permeate	8.22 (2.50)	1.14 (0.89)	0.124 (0.085)	10.6 (7.27)	20.8 (12.2)
Retentate	18.70 (3.06)	27.65 (9.82)	1.45 (0.308)	126 (33.2)	78.6 (5.58)

Values in parenthesis represent standard deviations. Activity/Specific activity defines cathepsin L type activity. Purity is calculated from Specific activity. Yield is calculated from activity. Yield is calculated from activity in concentration of the retentate following ultrafiltration, 9.2x.

This could indicate that the 50 kD membrane was able to separate some of the proteinaceous material from the protease. However, statistical analysis indicated these apparent differences were not significant ($p>0.05$). Membrane size, however, did have a significant effect on yield with runs utilizing the 30 kD membrane recovering a larger proportion (~10%) of the protease from the supernatant.

Activity staining further confirmed conclusions that the majority of proteolytic activity was associated with band 1. In Fig. 3.9 and 3.10, comparison of heat denatured with native retentate fractions demonstrate significant protein degradation in bands 2-7 as a result of protease activity (background is highly contrasted in order to see individual bands and clearing zones). In addition, protein degradation resulted in the formation of new bands at lower molecular weights. This was most clearly illustrated in Fig. 3.9. Band 1, however, was largely unaffected by proteolytic activity. Active endpoint titration was used to estimate cathepsin L concentration in the retentate. Analysis indicated almost equivalent activity per mole enzyme in the retentate from either the 30 kD (8.54 U/μmol) or 50 kD (8.04 U/μmol) membrane.

Protease stability

Fig. 3.11 and 3.12 show stability at typical commercial refrigeration (4°C) and freezer (-15°C) temperatures. As expected, liquid retentate rapidly deteriorated and lost enzyme activity when stored at refrigeration temperatures. However, an

Figure 3.9. SDS-PAGE activity stain patterns of surimi wash water retentate fraction collected from ultrafiltration with a 30 kD membrane. Hi=high molecular weight standard. Lo=low molecular weight standard.
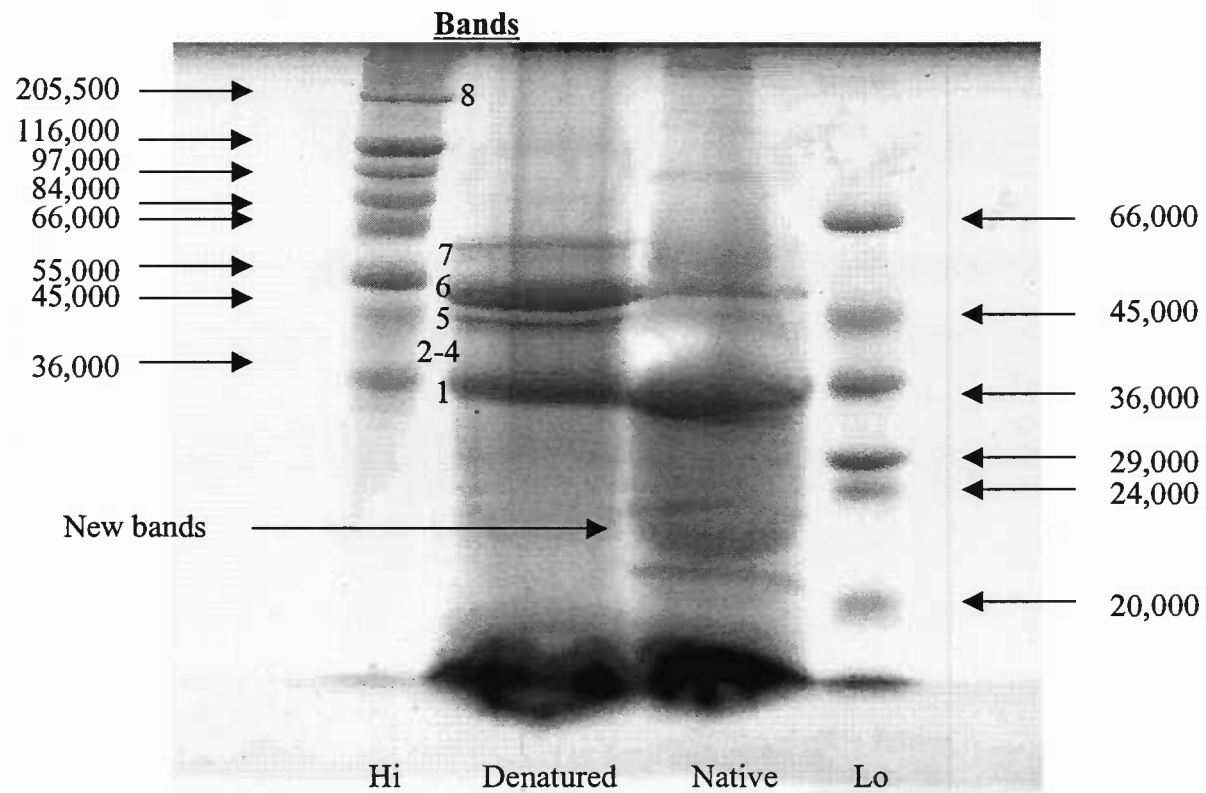
Figure 3.10. SDS-PAGE activity stain patterns of surimi wash water retentate fraction collected from ultrafiltration with a 50 kD membrane. Hi=high molecular weight standard. Lo=low molecular weight standard.

Figure 3.11. Stability of retentate at 4°C. FD=freeze dried. NT=not treated with P or E. Lo P=0.2% phosphate. Lo E=0.02% EDTA. Hi P=1% phosphate. Hi E=0.1% EDTA.
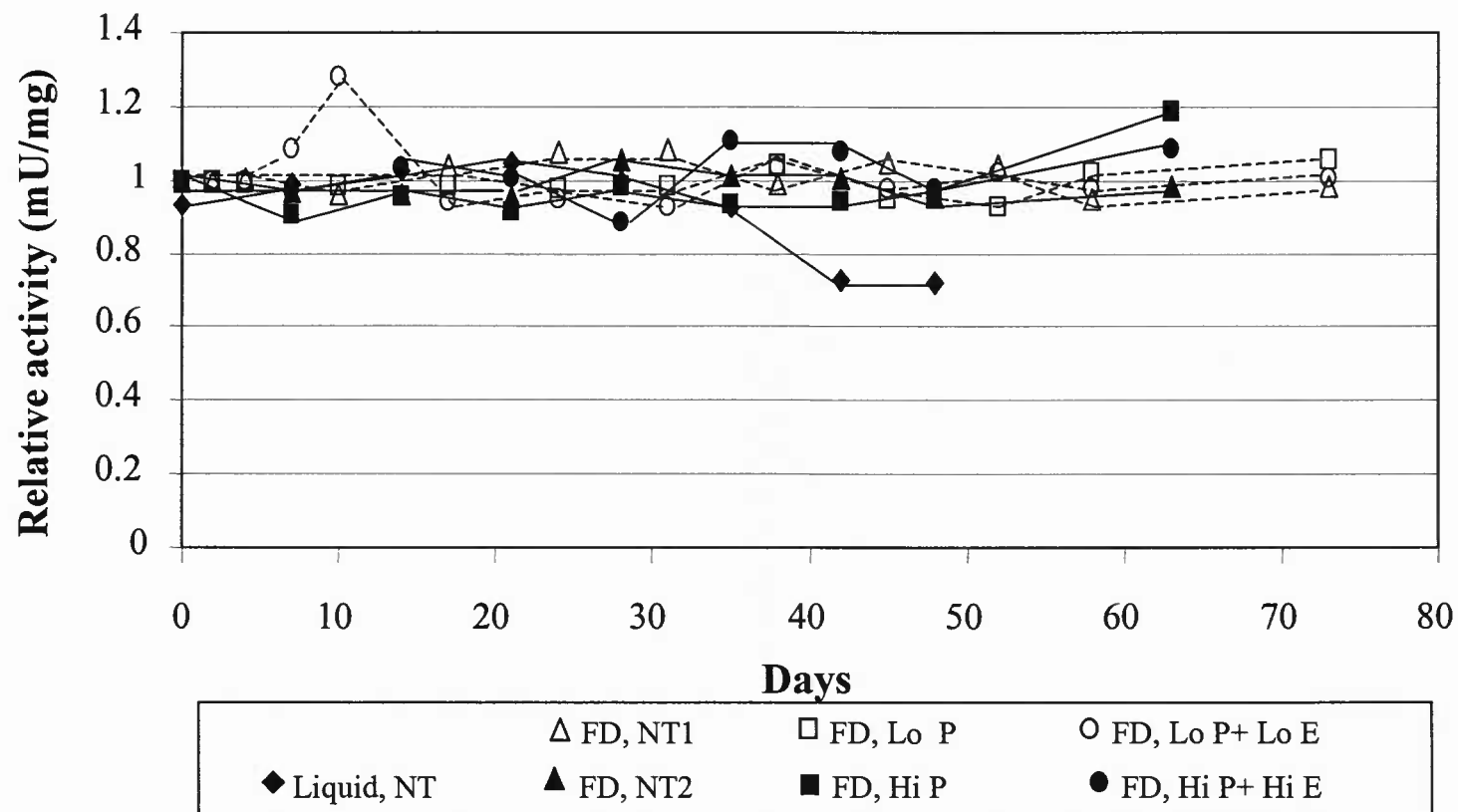
Figure 3.12. Stability of retentate at -15°C. FD=freeze dried. NT=not treated with P or E. Lo P=0.2% phosphate. Lo E=0.02% EDTA. Hi P=1% phosphate. Hi E=0.1% EDTA.

unexpected result was the overall stability of the remaining crude enzyme extracts. Benjakul and others (1998) previously reported that when stored at 4°C, a crude freeze-dried enzyme preparation lost 50% of its activity after 2 weeks when measured against casein. In this study, when activity was measured at 30°C with a fluorogenic substrate, stability was fairly consistent over a 60 day period for freeze-dried samples regardless of treatment. For the untreated frozen liquid (-15°C), after 40 days about one fourth of the original activity, relative to the control at −80°C, was lost. Stability of the positive controls (storage at −80°C) indicated there was a gradual loss in activity over time (Fig. 3.13). One possible explanation for differences in results is differences in temperature assay conditions (30°C vs. 55°C). Whitaker (1996) reported that temperature affects not only catalytic velocity, but stability of an enzyme as well. It is possible storage causes conformational changes that make the enzyme more susceptible to heat denaturation. Another possible explanation for the differences in stability between the two studies is how samples were stored. All samples in this study were kept flushed with nitrogen to minimize oxidative denaturation. Certain amino acids are more susceptible to oxidation than others. These include cysteine and cystine (Damodaran, 1996). Cysteine can be oxidized to form mono- and disulfone derivatives incapable of being reduced back to L-cysteine. Catheptic proteases are classified as cysteine type proteases. This means their active site contains a cysteine residue. Oxidation of the cysteine residue would result in loss of enzyme activity (Palmer, 1995). Fig. 3.13 shows stability at −80°C (positive controls).
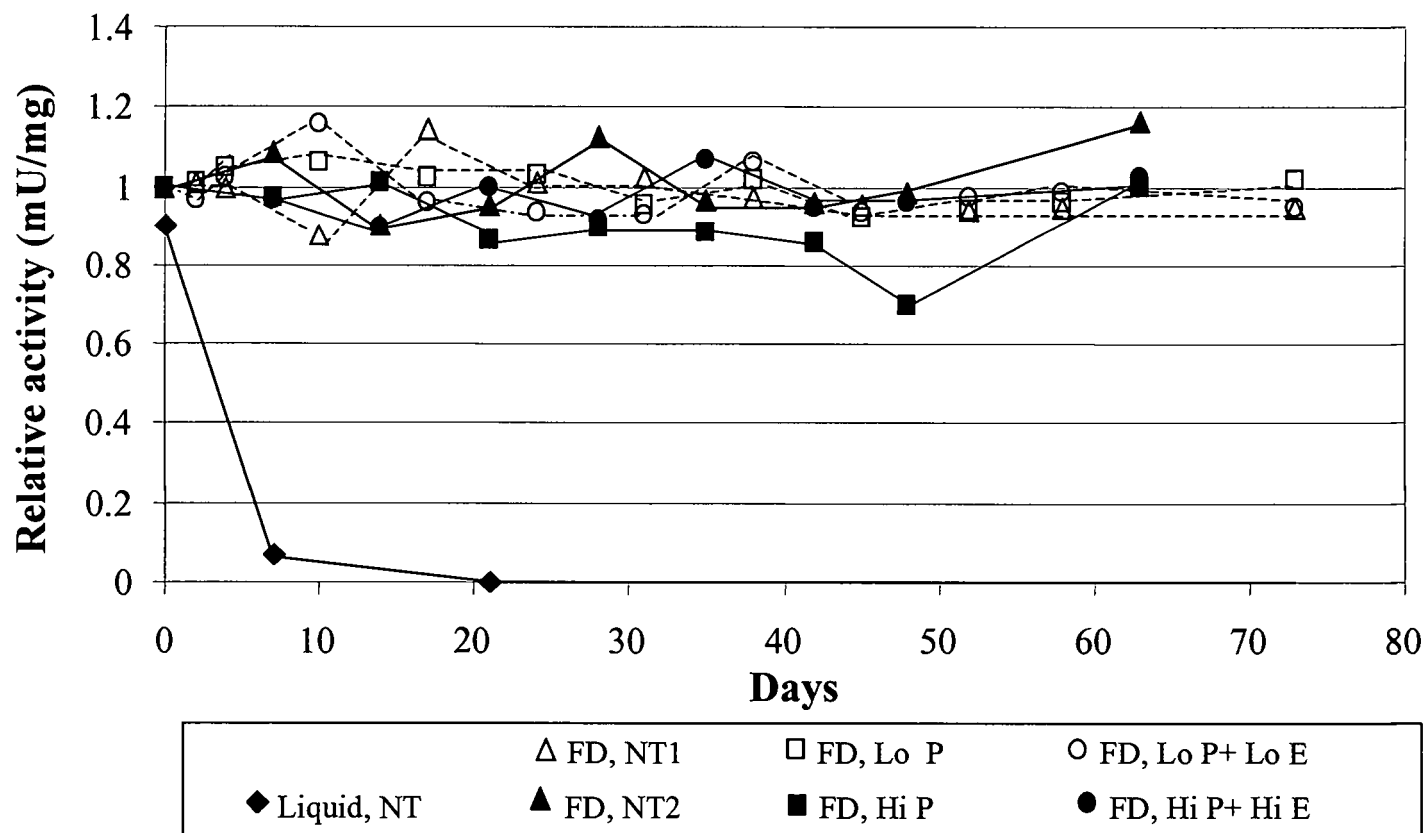
Figure 3.13. Stability of retentate at -80°C. FD=freeze dried. NT=not treated with P or E. Lo P=0.2% phosphate. Lo E=0.02% EDTA. Hi P=1% phosphate. Hi E=0.1% EDTA.
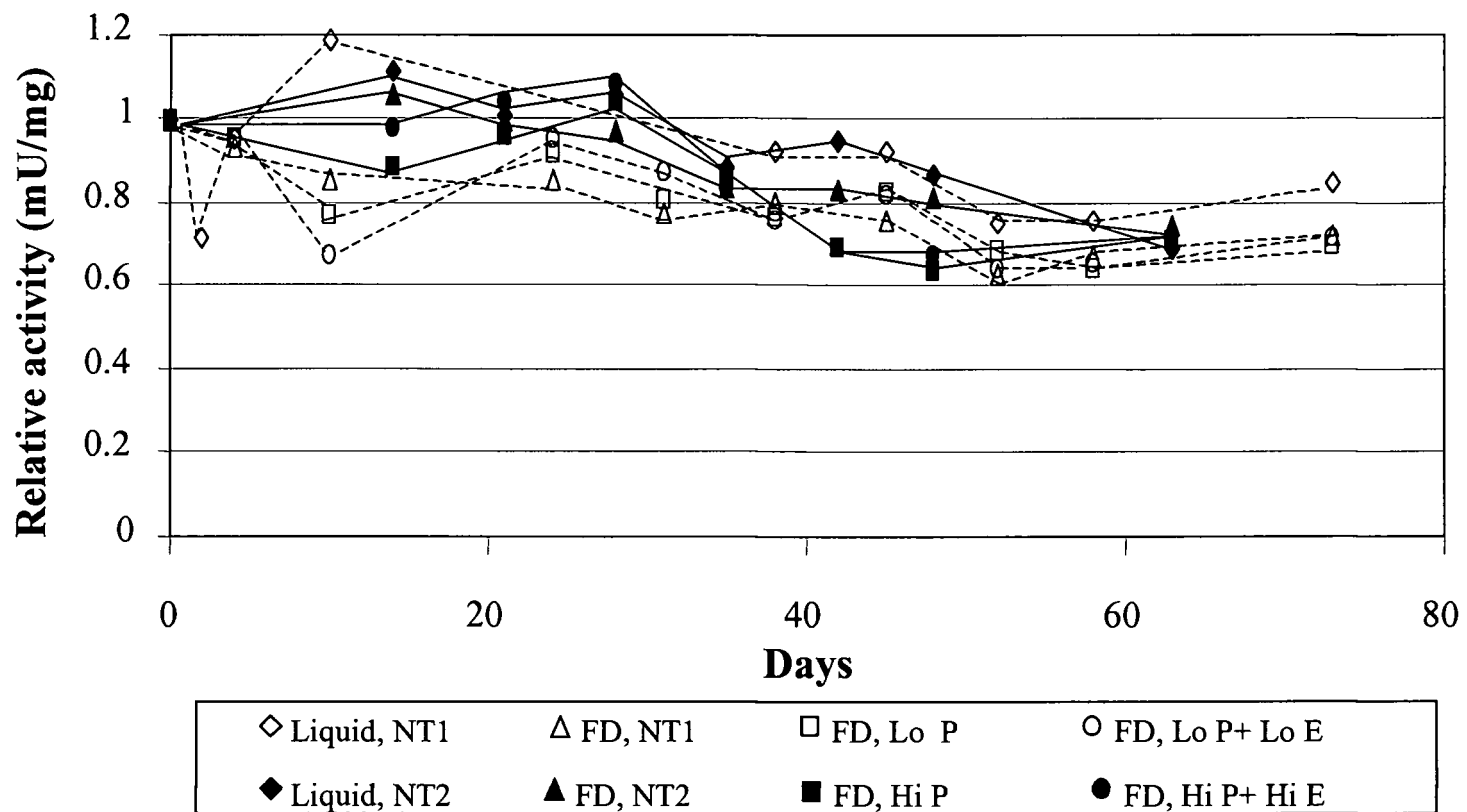
Large variations indicate week to week assay conditions have a significant effect on analysis results. Mean relative activity decreased for the first (0.952), second (0.828), and third (0.711) months of the study.

CONCLUSION

The objective of this study was to recover purified protease from surimi wash water using pilot plant scale equipment. Bench-top optimization studies had indicated either a 30 kD or 50 kD membrane would be most effective for ultrafiltration and recovery of protease from treated wash water. Data obtained in this study demonstrated that utilization of a pilot plant 50 kD membrane resulted in losses of protease into the permeate that were similar to that predicted by bench-top optimization studies. Therefore, comparisons were made between 30 kD and 50 kD membranes. In general, use of the 50 kD membrane did not appear to hold any advantage over the 30 kD membrane. Neither filtration time, solvent flux, nor protease purity was significantly increased as a result of using the larger 50 kD membrane. Protease recovery, however, was significantly improved by ultrafiltration with the smaller, 30 kD membrane. As a result, use of the 30 kD membrane was determined to be better than the 50 kD membrane for protease recovery.

Activity measurements helped identify cathepsin L as the predominant protease recovered from the retentate. Protease had better stability than initially predicted. During the 9-week storage period only 30% of the original activity was

lost. Addition of cryoprotectant or metal chelator did not enhance stability. Freeze-dried retentate was more stable than liquid concentrate at either 4°C or – 15°C.

ACKNOWLEDGMENT

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Chapter 4. Deproteination of Demineralized Shrimp Shell (*Pandalus jordani*) with Catheptic Proteases Extracted from Pacific Whiting (*Merluccius productus*) Surimi Wash Water and Commercial Papain.

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Michael T. Morrissey

ABSTRACT

Protein from demineralized shrimp shell was extracted using a crude catheptic protease recovered from Pacific whiting (*Merluccius productus*) surimi wash water, a commercial papain, and 3.5% NaOH. Hydrolysis with 3.5% NaOH (1:10 w/v, 55°C) produced a hydrolysate with 41% protein (dry weight basis). Hydrolysis with 1% papain (731 mU/ g shell) produced a hydrolysate with nearly the same amount of protein, 36.3%. However, when crude protease was added at equivalent concentrations, 1%, the resulting hydrolysate contained only 6.7-6.8% protein. Enzyme activity to waste ratio (E/W) of papain was approximately 40x greater than 1% crude protease (18 mU/ g shell) at optimum pH (5.5) and temperature (55°C) conditions for crude protease. Papain, at equivalent E/W, hydrolyzed twice as much protein from the shell than crude protease.

INTRODUCTION

Proteases are used in a wide range of food applications. Examples include use of chymosin in cheese production to gel milk and addition of papain or ficin to tenderize meat. Advantages cited for the use of proteases, in general, include their: catalysis of specific reactions; activity at low concentrations under mild conditions of temperature and pH; and ease of inactivation (Simpson and Haard, 1987). The use of marine proteases as food processing aides was recently advocated by several investigators (e.g. Simpson and Haard, 1987; Gildberg and Xian-Quan, 1994; De Vecchi and Coppes, 1996; Benjakul and others 1997; Haard, 1998; Bustos and others 1999). Specifically, Simpson and Haard (1987) suggested the potential for recovery of enzymes from fish processing waste. They indicated that proteases from marine sources, as a result of organisms living and adapating to enormously diverse environments, offer unique physicochemical properties that cannot be obtained from conventional sources of proteases. Recovery of valuable biochemicals, such as proteases, would simultaneously address concerns about full-utilization of our resources and the disposal problems generated as a consequence of their inefficient use.

Collaborative research by academia and industry has helped establish successful, onshore processing of surimi from Pacific whiting. However, as with most fish processing, increased success has resulted in an increased volume of offal. Pacific whiting contains elevated levels of catheptic protease activity (An and others 1994a) which causes post-harvest degradation of myofibrillar proteins

(An and others 1994b). Research has shown that a significant portion of catheptic proteases are removed during the surimi washing process (Morrissey and others 1995). The primary protease from Pacific whiting surimi wash water is cathepsin L (Benjakul and others 1997; Benjakul and others 1998). Cathepsins are some of the most active proteinases in the body (Barrett and Kirschke, 1981). They are capable of degrading nearly all proteins (e.g., cytosolic proteins, collagen, and elastin), activating some precursors, such as proplasminogen activator and inactivating several enzymes, e.g., glucose-6-phosphate dehydrogenase (Mort, 1998). As a result, the effectiveness of a crude catheptic protease, previously recovered from surimi wash water (Ch. 3), in hydrolyzing protein from demineralized shrimp shell was measured and compared to a commercial papain. Papain was chosen because it is currently one of the most commonly used proteases in the food industry. In addition, papain is from the same clan of proteases as cathepsins (clan CA; Barrett, 1998). Both the crude protease recovered from Pacific whiting surimi wash water and papain are cysteine proteases that are capable of hydrolyzing a large number of peptide bonds (Barrett and Kirschke, 1981). In addition, both and are heat and acid stable. Cathepsin L from Pacific whiting has a pH optimum at 5.5 and temperature optimum at 55°C (An and others 1994a; Benjakul and others 1996). Papain is active and stable at pH's from 4-10 and at temperatures up to 80°C (Ménard and Storer, 1998).

Several researchers have proposed the use of proteases in the production of chitosan (Broussignac, 1968; Bhuwapathapun, 1996; Wang and others 1998). In

the seafood industry, chitosan is produced from the demineralization, deproteination, and deacetylation of chitin obtained from the shells of crustacea. Bade and Wick (1988) demonstrated that a more "predictable" chitosan is produced when demineralization proceeds deproteination. Demineralization is normally accomplished with a dilute acid such as HCl while deproteination is achieved through addition of a dilute solution of hot alkali such as NaOH. For shrimp and prawn shells, reported deproteination conditions range from 1-5% NaOH at a ratio of 1:1 to 1:30 for 0.5-2 hr. at 65 -100°C (No and Meyers, 1995).

Use of strong alkali and acids not only produces waste disposal problems for chitosan production facilities, but it has also been suggested that prolonged treatments with such harsh chemicals (especially NaOH) can cause partial deacetylation of chitin and hydrolysis of the polymer. This produces a lower molecular weight chitosan with inconsistent properties (Muzzarelli, 1977; No and Meyers, 1995; Wang and others 1998). As a result, several investigations were conducted to study the use of enzymes as alternatives for chitin deproteination. Broussignac (1968) suggested the use of papain, pepsin, or trypsin. More recently, researchers looked into the effectiveness of proteases from bacteria and fungi (Bhuwapathapun, 1996; Wang and others 1998). While these enzymes did not modify chitin, complete removal of protein was not attained. It has been suggested that catheptic proteases may act more effectively on their own natural substrates (i.e. meat from fish/shellfish, beef, or pork) and produce fewer undesirable degradation products than proteases from other sources (Artigas and others 1996).

The objective of this study was to determine if a crude preparation of catheptic proteases from Pacific whiting surimi wash water would extract protein from demineralized shrimp shell as well as a commercial papain.

MATERIALS AND METHODS

Demineralized shrimp shell

Demineralized shrimp shell (*Pandalus jordani*) was obtained from a local chitosan processing facility (Natural Biopolymer, Inc., Raymond, WA). Shrimp shell was rinsed with water through 4-layers of cheesecloth until pH of the wash water was >7. Excess water was gently pressed out. Shrimp shell was vacuum-packed and stored at –50°C. Total solids were determined following established AOAC (1995) methods.

Enzymes

A crude protease (CP) was obtained from surimi wash water processed from Pacific whiting. Surimi wash water was collected from Point Adams Packing Co. (Hammond, OR). Wash water acidity was reduced to pH 6 with 1:1 HCl, heated to 60°C, and centrifuged (Ch.3). Supernatant was passed through 30 kD and 50 kD tangential flow ultrafiltration membranes (Omega Industrial Maximate(-EXT, Pall Filtron, Northborough, MA). The crude protease from 3 separate runs (retentate)

was collected and stored at -80°C. The other enzyme utilized in this study was a commercially available semi-refined papain (Specialty Enzymes, Chino, CA).

Analysis of proteolytic activity

Protease activity was followed by monitoring cathepsin L according to the method of Barrett and Kirschke (1981) with a modification of the stopping reagent by addition of iodoacetate (An, 1994a). Samples were analyzed in polystyrene tubes and kept on ice before and after incubation. An 100 μL sample was combined with 400 μL of 0.1% Brij (35% w/v). Appropriate dilution of samples was made prior to analysis if necessary (appropriate range = 0.3 - 3.0 ng of cathepsin L as determined by active site titration). A 250 μL aliquot of Barrett's assay buffer (340 mM sodium acetate; 60 mM acetic acid; 4 mM disodium ethylene diamine tetraacetic acid, EDTA; 8 mM dithiothreitol, DTT; final pH 5.5) was added and tubes (covered with petrifilm) were placed in a 30°C water bath. After exactly 1 min., 250 μL of pre-warmed substrate (20 μM Z-Phe-Arg-Nmec) was added. Samples were agitated and at 10 min., 1 mL of stopping reagent (100 mM sodium monochloracetate, 30 mM sodium acetate, 70 mM acetic acid, 5mM iodacetate, pH 4.3) was added. Flourescence of the free aminomethylcoumarin was measured (excitation = 370 nm; emmision = 460 nm) in the sample and compared to the standard (0.5 μM 7-amino-4-methylcoumarin corresponded to 0.1 mU of activity in the tubes). One mU was defined as being equivalent to the release of 1 nmole aminomethylcoumarin/min.

Effect of L-cysteine on protease activity

L-cysteine was added at 0, 1, 10, 100, and 1000 mM in place of DTT in the Barrett's assay buffer. Analysis for protease activity proceeded as described above.

Deproteination of shrimp shell with NaOH

Pre-warmed 3.5% NaOH (0.87 N) was added to demineralized shrimp shell (25 g) at a ratio of 1:10 (w/v). Samples were placed in a water bath at 55°C. Reaction was stopped by neutralizing 3 mL aliquots of liquid (taken through 4-layers of cheesecloth) at 0, 5, 10, 20, 30, 40, 60, 80, 100, and 120 min. intervals with 3 mL 0.87 N HCl. Protein was determined by Kjeldahl (AOAC, 1995) and Lowry (Lowry and others 1951).

Deproteination of shrimp shell with protease

Papain was added to demineralized shrimp shell (25 g) at approximately 1%, w/w (730.8 mU/g shell) or 0.04%, w/w (18 mU/g shell) concentration. CP was added at approximately 1% concentration (18 mU/g shell). Enzyme activity was measured immediately before utilization for hydrolysis. Total volume of liquid was brought to 250 mL with pre-warmed Barrett's buffer, modified by the addition of 10 mM L-cysteine in place of DTT. Samples were incubated at 30°C or 55°C for 2 hr. Reaction was terminated by transferring 3 mL aliquots of sample (taken through 4-layers of cheesecloth) at 0, 5, 10, 20, 30, 40, 60, 80, 100, and 120 min.

intervals to tubes in an 80°C water bath containing 3 mL, 1% sodium dodecyl sulfate (SDS). Tubes were capped and held at 80°C for an additional 20 min. to insure inactivation of the enzyme. Protein in the sample was measured using the Lowry method (Lowry and others 1951). Alpha-amino acid content was measured using the Fluram™ reagent from Roche Diagnostics (Nutley, N.J.). Protein in the residue recovered after filtration was measured by digesting the wet residue with 250 mL 3.5% NaOH at 55°C for 2 hr. The liquid was collected through 4-layers of cheesecloth and protein was measured by the Kjeldahl method (AOAC, 1995).

RESULTS

Effect of L-cysteine on hydrolysis

Papain and cathepsin L exhibit overlapping temperature and pH stability. The crude protease previously recovered and utilized in this study was characterized as exhibiting primarily cathepsin L activity (Ch. 3). Experimental conditions for hydrolysis were tailored for conditions that would optimize cathepsin L activity. Barrett and Kirschke (1981) used a synthetic fluorescent substrate for analysis of cathepsin L, tailoring the constituents in the buffer for optimized activity measurement. Therefore, Barrett's buffer was utilized as a dilution buffer for hydrolysis experiments with demineralized shrimp shell. However, the presence of DTT in the buffer as the sulfhydryl reducer is not very feasible for industrial use. DTT is non-GRAS (Generally Recognized As Safe),

unstable at room temperature, and expensive, $11.68/g (Aldrich® Chemical, Milwaukee, WI). A suitable replacement for the reducer in the buffer is L-cysteine, a GRAS, room temperature stable, and relatively inexpensive additive ($0.12/g; Ajinomoto U S A, Teaneck, NJ). In Fig. 4.1 activity increased until concentration of L-cysteine reached 10 mM. Higher concentrations did not significantly enhance activity. In addition, protease activity as measured by DTT was not significantly different than activity when L-cysteine was utilized for a sulfhydryl substitute.

Protein extraction

In order to determine maximum hydrolysis of protein from shrimp shell, demineralized shrimp shell was deproteinated with 3.5% NaOH under equivalent time (2 hr.) and temperature (55°C) conditions utilized for enzyme hydrolysis. Protein extraction from demineralized shrimp shell reached a maximum after 40 min. of hydrolysis (Fig. 4.2). Protein extraction was estimated to be about 41.0% (dry basis) using Lowry and 36.9% (dry basis) using Kjeldahl (Table 4.1). Reported values of protein from crustacean shell ranged from 30-48% (No and Meyers, 1995; Chang and Tsai, 1997)

Initially, it was determined that utilization of enzymes at concentrations above 1% would make the process too cost prohibitive to be of practical use to processors. In addition, previous work with papain and similar proteases had demonstrated optimal recovery typically occurred near an enzyme/waste (E/W) ratio of 0.5-1% (Gagne and Simpson, 1993; Wang and others 1998). As a result,
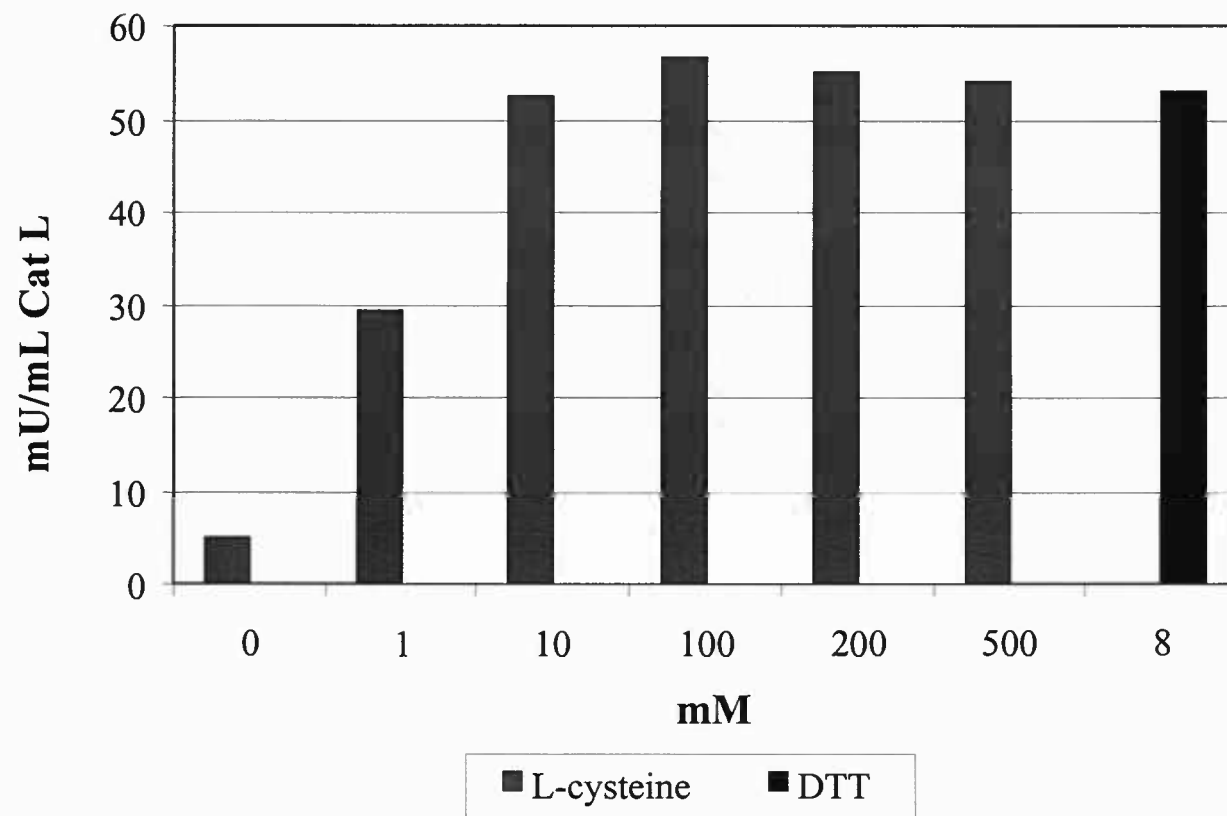
Figure 4.1. Effect of L-cysteine on cathepsin L activity.
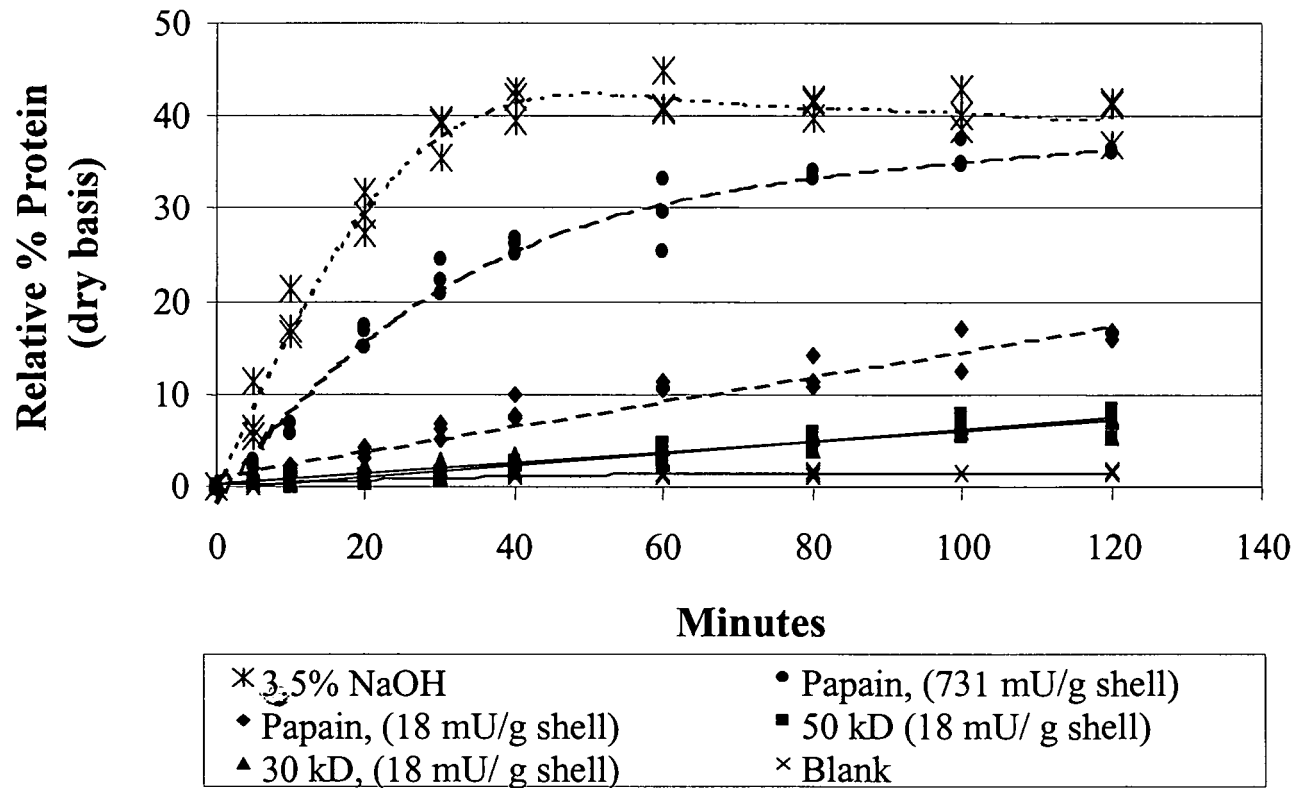
Figure 4.2. Hydrolysate % protein from extraction of demineralized shrimp shell, 55°C.

Table 4.1 Moisture and protein results.

Sample	% Protein (Kjeldahl)	Std. Dev.	% Protein (Lowry)	Std. Dev.
3.5% NaOH	45.6-50.8* (100)**		41.0 (100)**	1.93
1% Papain	37.9 (71-87)*	1.77	36.3 (84-93)	0.17
Residue	10.3 (19-24)*	0.84	2.6-6.8* (6-17)*	

*Calculated values.
**Assumed values. Percent yields are in parenthesis. Protein was calculated on a dry basis. Demineralized shrimp shell total solids were 18.8 ± 0.87%.

initial analysis was conducted with 1% (w/w) protease. Protein extraction for both proteases was not complete. A significant portion of the protein from the shell was recovered with 1% papain (Fig. 4.2). Protein extraction with papain (36.3%) was almost equal to that achieved with 3.5% NaOH (41.0%). However, protein extraction with 1% CP (6.7% 30 kD; 6.8%, 50 kD) was almost 6x less effective than with 1% papain. When papain and CP were added at equivalent activity levels (18 mU/g shell), papain extracted 2.5x the amount of protein at 55°C (Fig. 4.3) and a little over 7.5x the amount of protein at 30°C as CP (Fig. 4.4).

Gagne and Simpson (1993) optimized conditions for hydrolysis of protein utilizing response surface methodology. They looked at three temperatures (25°C, 32°C, and 40°C), three pH's (6.5, 7.8, and 9.1), and five E/W ratios (2:1000; 4:1000; 6:1000; 8:1000; and 10:1000 mg/mg). Results indicated that conditions for maximum recovery of protein (23%) with papain occurred at 40°C, pH of 8.7 and E/W ratio of 10:1000. Total hydrolysis time was 72 hr. Protein remaining in the residue was 2.1%. Residual protein was recovered using 2N NaOH (1:25 w/v) at 70°C for 1 hr. and measured by a modified Lowry method. In this study, using equivalent levels of papain, calculated residual protein based on Lowry analysis indicated remaining protein in the residue ranged from 3-7 % (Table 4.1). Protein remaining in the residue was also measured directly after following the enzyme hydrolysis with an alkali hydrolysis. Average protein recovered after hydrolysis with 1% papain was 37.9% (Kjeldahl). Protein recovered from subsequent alkali hydrolysis was 10.3% (Kjeldahl). Estimates measured directly with Kjeldahl or
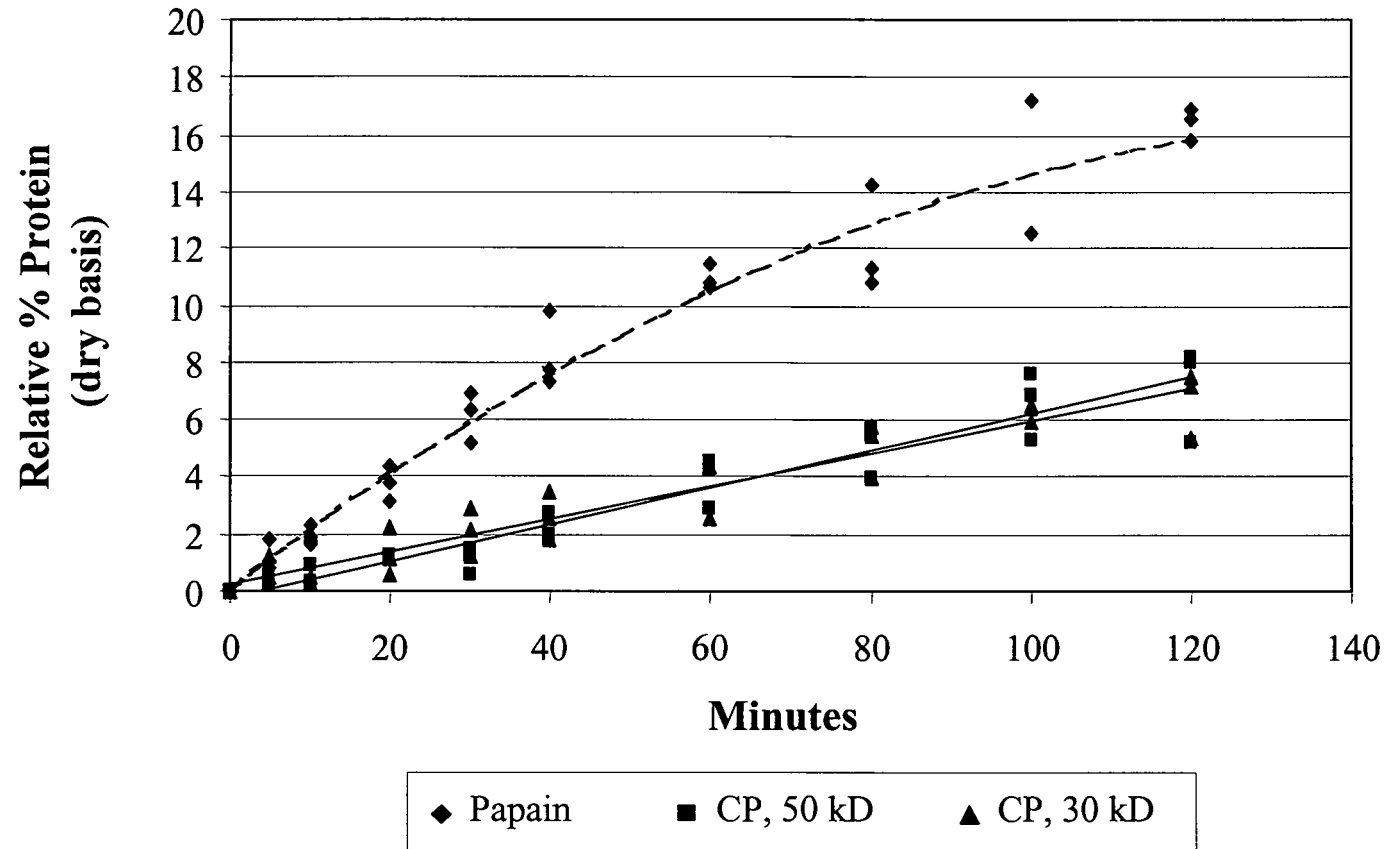
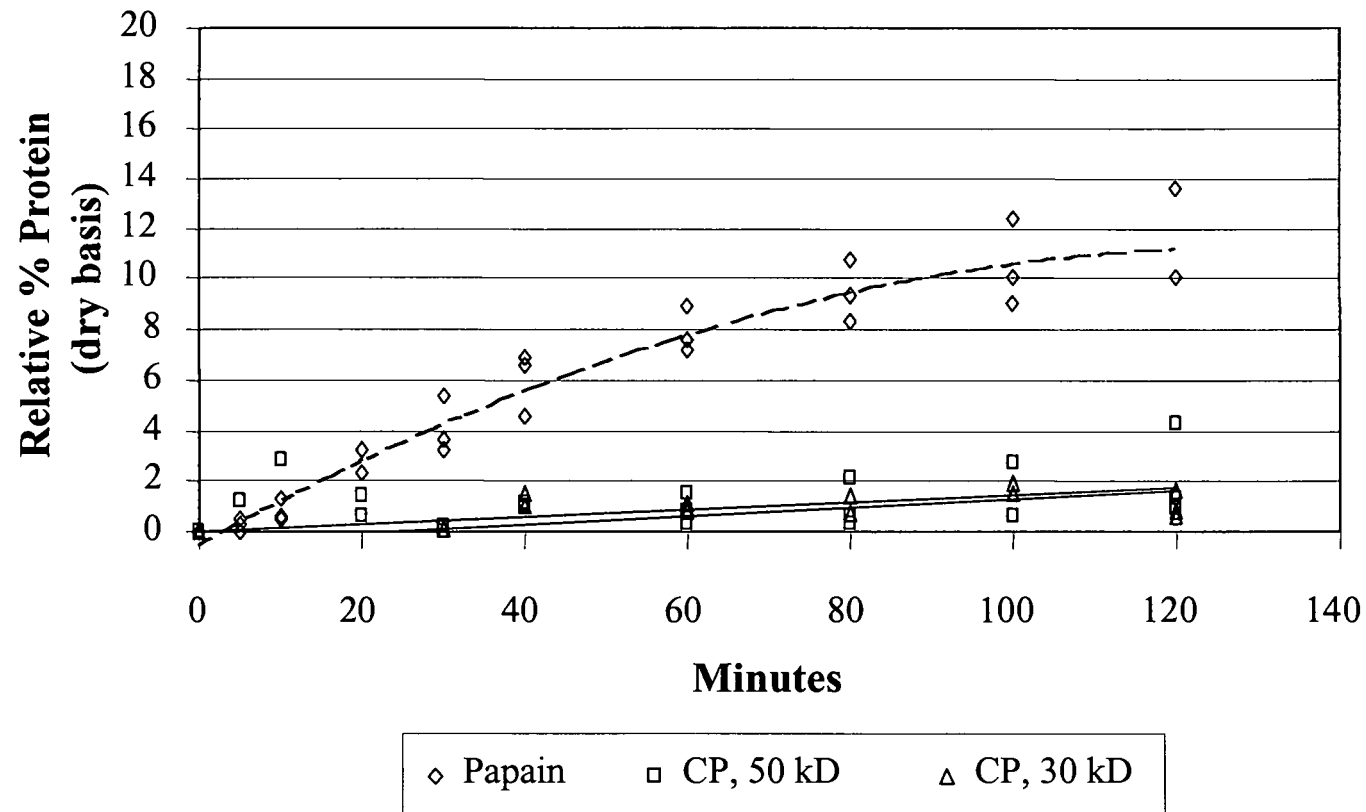Figure 4.3. Hydrolysate % protein from extraction of demineralized shrimp shell (55°C, 18 mU/g shell).

Figure 4.4. Hydrolysate % protein from extraction of demineralized shrimp shell (30°C, 18 mU/g shell).

indirectly with Lowry roughly agreed. Variations are probably due to lack of homogeneity of the waste from batch to batch. Although residual protein was higher under the conditions tested in this study than those reported by Gagne and Simpson (1993), it should be noted that hydrolysis was allowed to proceeded for only 2 hr. Since incubation times were significantly less than the 72 hr. utilized by Gagne and Simpson, it appears that an increase in incubation times could offer significant advantages in time efficiencies when using the prescribed conditions from this study (i.e. Barrett's buffer, pH 5.5, and 55°C).

α-amino acid

Fig. 4.5 illustrates the rate α-amino acid increased as hydrolysis progressed using either papain or CP at 55° or 30°C, 18 mU/g shell. One-way ANOVA (StatGraphics® Plus, version 3.0) identified papain as producing the largest change in α-amino acid over time. The 50 kD CP at 55°C and papain at 30°C were identified as statistically similar and produced the next largest change in α-amino acid content. Hydrolysis with 50 kD CP produced a greater amount of amino acids than hydrolysis with 30 kD CP, regardless of temperature. In addition, production of α-amino acids in relation to amount of protein hydrolyzed (h) indicated that, over time, 50 kD CP maintains an h that is equivalent to that at time 0. However, 30 kD CP and papain (30°C and 55°C) did not (Fig. 4.6). The difference in hydrolysis action between 50 kD CP and 30 kD CP could indicate the presence of a
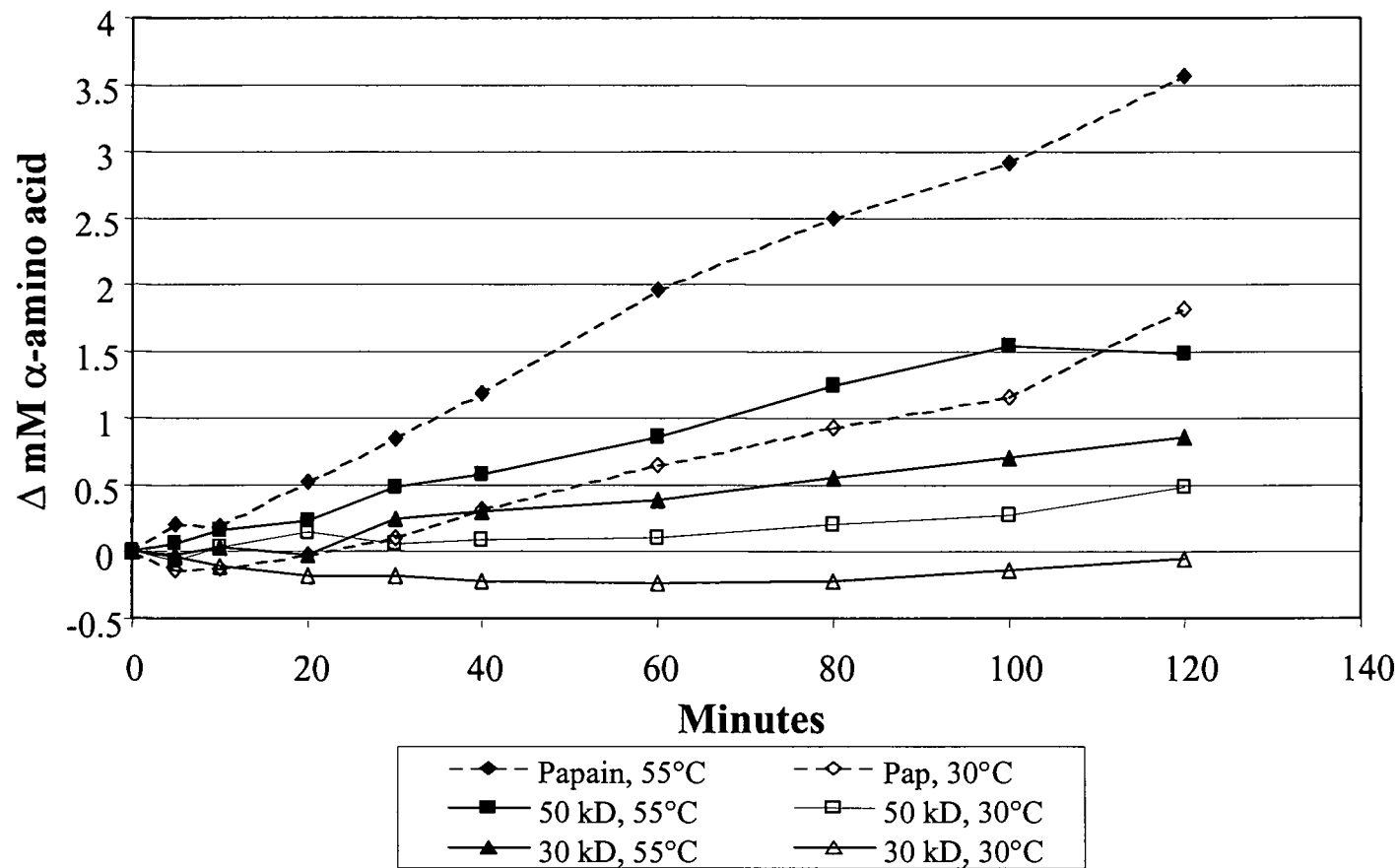
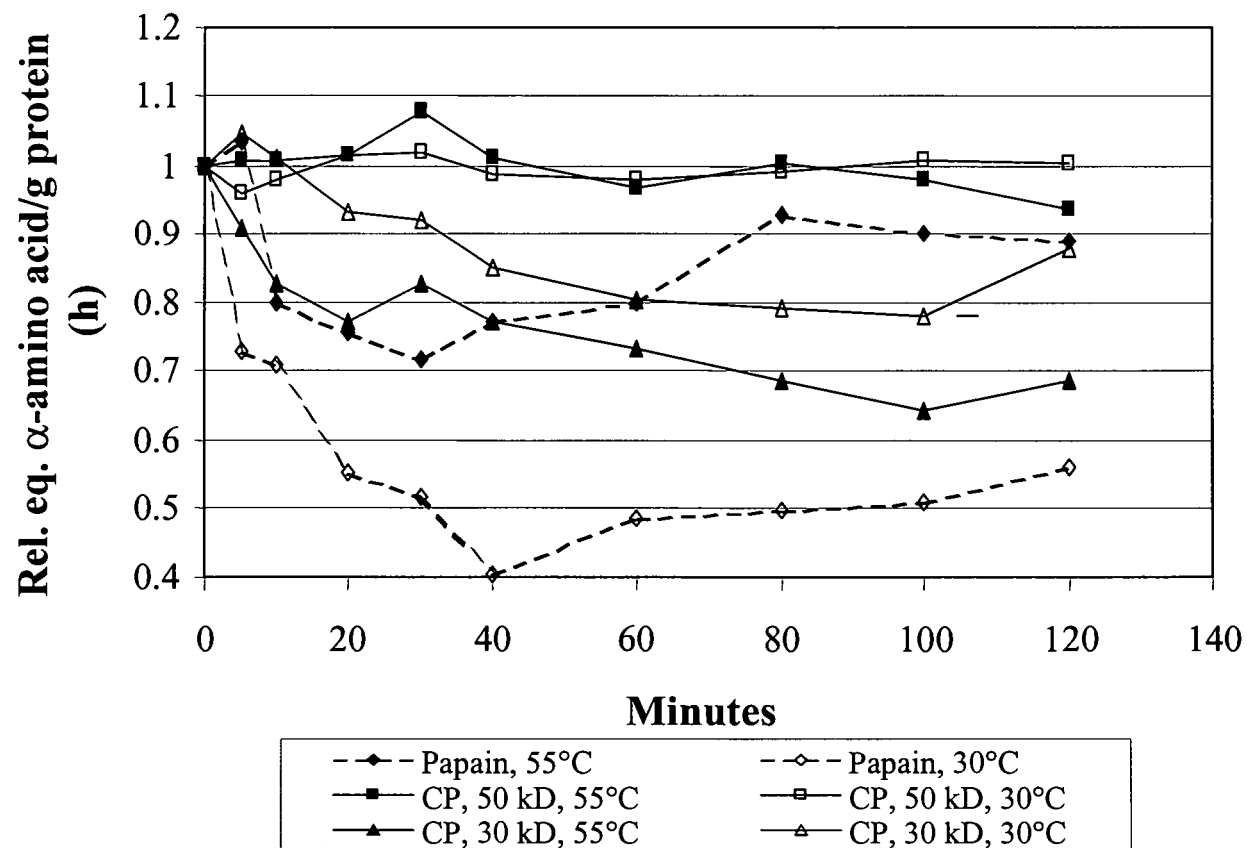Figure 4.5. Increase in α-amino acids with respect to time.

Figure 4.6. Relative ratio of hydrolysate α-amino acid to protein.

protease not previously identified. Since both CPs were recovered from processing wash water and not highly purified, presence of other proteases is likely.

As previously reported (Ch. 3), CP contains several catheptic proteases (specifically L, B, and H). Previous work by Benjakul and others (1996) demonstrated cysteine-type proteases were responsible for the majority of proteolytic activity observed in surimi wash water. However, they noted hydrolysis of L-Arg-NMec was not terminated by addition of either iodoacetic acid (specific for cysteine, serine, and metallo-proteinase) or pepstatin (specific for aspartic proteases), both of which are also effective inhibitors against endolytic proteases. They suggested that surimi wash water contained an exopeptidase. In general, 30 kD CP contained almost twice the amount of initial α-amino acid as 50 kD CP. In addition, the 30 kD CP also contained a higher initial concentration of proteins/peptides. These peptides and/or proteins already in solution can serve as competitive substrate with the protein from the shrimp shell. Exopeptidase activity on suspended proteins would compete less with catheptic proteases for shell protein, thus allowing catheptic proteases from the 50 kD CP to work more efficiently.

CONCLUSION

Commercial crude papain was 40-times more active than CP. In addition, papain was capable of hydrolyzing more protein despite being added at the same E/W as CP. It is likely that improved performance by the crude papain is a result

of it acting more broadly on protein on the shrimp shell than CP. Although CP did

not perform as well as expected, there is still potential for its application in food.

At 30°C, proteins were extracted much more effectively with papain than CP. One

of the problems with the utilization of papain as a meat tenderizer is its action is so

ubiquitous that the tenderization process typically exceeds desired results (Simpson

and Haard, 1987). It is possible that Pacific whiting CP's reduced activity at low

temperatures may be advantageous for applications such as meat tenderization.

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Chapter 5. Summary

Parameters for laboratory scale recovery of catheptic proteases from Pacific whiting (*Merluccius productus*) surimi wash water were optimized for pretreatment with acid and heat. Combined acid and heat-treatment was more effective in reducing wash water proteins than either heat or acid alone. Maximum protease activity was expressed at pH 5-6 with HCl and pH 6 with L-ascorbic acid when wash water was heat-treated. Further purification of protease in untreated, acid treated, and acid plus heat-treated wash water by ultra- and microfiltration was not successful using benchtop 300 kD, 1000 kD, and 0.3 μm centrifugal filtration units. However, results from these studies indicated improved membrane fluxes were achieved when using acid plus heat-treated wash water. As a result, concentration studies were subsequently run on acid plus heat-treated wash water employing 30 kD, 50 kD, and 100 kD centrifugal filtration units. Maximum recovery of protease in the concentrate occurred with 30 kD and 50 kD membranes. However, purity was improved most using 100 kD membranes.

Optimized laboratory scale parameters were used for pilot plant protease recovery efforts. Pretreatment of wash water by acidification (pH 6), heat (60°C), and centrifugation improved steady-state flux 2-fold over untreated wash water. Pretreatment removed proteins ranging from 35-205 kD. Two significant bands at about 34 kD and 50 kD remained in the wash water following pretreatment. Cathepsin L is associated with the former. Recovered surimi wash water protease

was characterized as having predominantly cathepsin L activity. Cathepsin B and H were present as minor constituents. Stability of freeze-dried retentate was not effected by storage temperature (4°C, -15°C, and -80°C) or addition of cryoprotectant or metal chelator. Stability decreased 30% after 9-weeks. Subsequent comparison of recovered protease with a commercial papain demonstrated the commercial papain was more effective in extracting protein from demineralized shrimp shell.

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