AN ABSTRACT OF THE THESIS OF

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Both liquid and solid wastes from Pacific whiting surimi manufacturing were characterized and value-added products were recovered. A proteinase in surimi wash water (SWW) was determined to be cathepsin L with M_r 54,200 on SDS-substrate gel. Heat treatment and acidification shifted the activity zone to M_r 39,500. No evidence of calpain, cathepsin B or H activity was found. Cathepsin L from SWW was recovered by ohmic heating (55°C for 3 min), ultrafiltration, and freeze-drying with overall yield of 0.83 g protein/L SWW and 78% recovery of activity. A 5.9 purification fold was achieved by these processes. The recovered enzyme had an optimum activity at pH 4.0 and showed preferable hydrolytic activity towards casein, acid-denatured hemoglobin and myofibrils. β-Mercaptoethanol, dithiothreitol and urea enhanced the enzyme activity. The recovered proteinase showed 18.5% residual activity after 7 wk storage at 4°C.

Proteolytic activity in solid waste and digestive organs from Pacific whiting was investigated. Pepsin-like proteinase predominated in solid waste, while trypsin-like

proteinase was predominant in viscera. Carboxypeptidase b was found in both viscera and solid waste.

Protein hydrolysate was produced from Pacific whitng solid waste (PWSW) using commercial proteinase, Alcalase, under optimum hydrolysis conditions. Enzyme concentration, reaction time and waste/buffer ratio affected the hydrolysis and nitrogen recovery (NR). Correlation between the degree of hydrolysis (DH) and NR was high (R^2=0.978). Freeze-dried hydrolysate contained 79.97% protein and showed similar amino acid composition to PWSW and Pacific whiting muscle but tryptophan was reduced. With different DH (20, 30, 40, 50, 60%), surface hydrophobicity, total and surface sulfhydryl content decreased as the DH increased. The hydrolysate showed a high solubility over a wide pH range. Fat adsorption and fat binding capacity were reduced, while foam expansion was enhanced with an increased DH. Hydrolysate with DH of 30% showed highest emulsifying activity. Low emulsion stability and high foam stability were obtained in all hydrolysates tested. Hydrolysate showed antioxidant activity, but no obvious differences in activity were found with varying DH and hydrolysate concentrations.

Utilization of Wastes from Pacific Whiting Surimi Manufacturing: Proteinases and Protein Hydrolysate

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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.

Soottawat Benjakul, Author

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CONTRIBUTION OF AUTHORS

Dr. M.T. Morrissey, Dr. T.A. Seymour and Dr. H. An were involved in the experimental design, analysis and preparing the manuscript.

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UTILIZATION OF WASTES FROM PACIFIC WHITING SURIMI MANUFACTURING: PROTEINASES AND PROTEIN HYDROLYSATE

Chapter 1

Introduction

General considerations

Pacific whiting (*Merluccius productus*) represents the largest groundfish resource found off the West Coast of North America excluding Alaska. The potential average long-term yield is 245,000 MT (Radtke, 1995). In 1994, approximately 80% of the 144 million pounds of Pacific whiting landed in Oregon were used in surimi production at a recovery rate of 20-25%, producing some 90 million pounds of byproducts (Brown, 1995). The initial step of surimi processing is removal of head, viscera and bones, and subsequent mincing the remaining flesh. The meat particles are thoroughly leached to remove the water soluble substances including sarcoplasmic proteins, digestive enzymes, inorganic salts and low molecular organic substances as well as lipids and blood components (Toyoda et al., 1992). The myofibrillar proteins, which are primarily responsible for gel formation are concentrated and off-color and odor compounds are removed. During washing and dehydration, 30-40% of protein of the mince are lost into wash water, wasting usable proteins and causing organic loading of the effluent (Pedersen, 1990; Brown, 1995). The high biochemical oxygen demand (BOD) of surimi wash water creates waste disposal and environmental concerns. Wastewater disposal can be done by discharging into adjacent waterways, using municipal treatment systems, or

used for watering and fertilizing agricultural fields (Brown, 1995). To attain the full utilization of fishery harvests, there has been an increased interest to recover useful proteins before disposing wastewater. Pedersen (1990) reported the protein recovery of 80% solids in pollock processing using membrane filtration methods. Recently, Lin et al. (1995) recovered the protein from Pacific whiting wash water to lessen the impact of wastewater and to increase final surimi yield.

The alternatives for managing seafood processing wastes can include disposal and by-product recovery. By-product recovery is a viable alternative to disposal, which not only lessens disposal problems, but can be economically justifiable. The wastes can be used as a potential raw material for fish meal, fish silage, fish hydrolysate, fish feed, fish concentrate and fish oil production. Another alternative for fish carcass disposal is freezing fish carcass for crab bait, which can be highly profitable (Brown, 1995). The carcass waste has been converted into an economical and nutritionally satisfactory ration for salmonids (Crawford and McNeil, 1990). By-product recovery can include partial or complete purification of specific compounds. The recovery of proteinases from fish waste has also been demonstrated (Reece, 1988; Gildberg, 1992).

Fish Proteinases

Proteinases in fish can contribute to either improvement or loss in fish quality. The products of protein hydrolysis by tissue proteases develop the aroma, taste, color and texture of traditional products such as fish sauce (Haard, 1994). Protein hydrolysis in production of silage can be attributed to the endogenous proteinases (Raa and Gildberg, 1982; Storm and Eggum, 1981; Gildberg and Almas, 1986). Several research

efforts have focused on recovering digestive enzymes from fish processing waste for use as processing aids (Simpson and Haard, 1984; Reece, 1988; Gildberg, 1992). Haard and Simpson (1994) reviewed the application of proteinases in the seafood industry including skin and scale removal, roe production, recovery of pigment and flavor extracts. The soluble phase of acidified and autolyzed fish viscera has been used as the nitrogen source in a growth medium for bacteria (Clausen et al., 1985). Fish peptone was prepared by the autolysis of viscera followed by ultrafiltration and used for biomass production (Gildberg and Almas, 1986; Vecht-Lifshitz et al., 1990).

Proteinases can be directly responsible for the textural defects in seafood, e.g. belly burst, gaping, and mushiness (Martinez and Gildberg, 1988; Haard, 1994) and cause spoilage in fish (Mukundan et al., 1986). Proteolytic degradation of myofibrillar proteins has a detrimental effect on surimi quality (Morrissey et al., 1993). Modori-phenomenon, thermal gel degradation, has been reported as the result of heat stable alkaline proteinase (Boye and Lanier, 1988; Toyahara et al., 1990; Kinoshita et al., 1990; 1992). In Pacific whiting, catheptic enzymes play a role in modori (An et al., 1994; Seymour et al., 1994).

Muscle proteinases

Upon death of the animal, fish muscle becomes susceptible to autolysis by proteinases resulting in texture changes. The most active proteinases in fish muscle can be generally categorized into two groups: cathepsin and heat-stable alkaline proteinase (An et al., 1996). Lysosomal cysteine proteinases, i.e., cathepsin L, B and H have been shown to cause softening in chum salmon (Yamashita and Konagaya, 1990; 1991),

tilapia (Sherekar et al., 1988) and mackerel (Jiang et al., 1994). Cathepsin L was found as a major proteinase to degrade the myofibrillar proteins in Pacific whiting, resulting in the reduced gel strength of surimi (Morrissey et al., 1993; An et al., 1994). Unlike cathepsin B and H, cathepsin L has a high affinity for myosin and is not completely removed by the washing process during surimi process (An et al., 1994). The purified cathepsin L from Pacific whiting consists of a single peptide with a molecular weight of 28,800 and has a temperature optimum of 55°C (Seymour et al., 1994).

Cathepsin D, a lysosomal aspartic proteinase, has also been shown to be an important proteinase for post-mortem degradation of muscle. It can initiate the protein hydrolysis and produces peptide fragments that can then be further broken down by other cathepsins (Huang and Tappel, 1971). Cathepsin D has been purified and characterized in tilapia (Jiang et al., 1991), mackerel and milkfish (Jiang et al., 1993) and shrimp (Jiang et al., 1992).

Heat stable alkaline proteinases with high molecular weight have been identified in fish muscles (Makinodan et al., 1987; Busconi et al., 1984; Iwata et al., 1973). The molecular weight of proteinase in croaker was estimated to be 430,000 by gel filtration and composed of four different subunits with molecular weight ranging from 45,000-57,000 determined by SDS-PAGE (Makinodan et al., 1987). Hase et al. (1980) reported that molecular weight of carp proteinase was 600,000 with a complex subunit composition. These enzymes are activated by protein denaturing agents such as urea, fatty acids or detergent (Haard, 1994; Toyohara et al., 1987). Heat-stable alkaline proteinase was implicated in the textural degradation of fish gel at around 60°C (Boye and Lanier, 1988; Makinodan et al., 1985; Lanier et al., 1981).

The Ca^{2+}-activated neutral proteinases called μ-calpain (μM Ca^{2+}-dependent) and m-calpain (mM Ca^{2+}-dependent) appear to be sarcoplasmic enzymes which release thick and thin filaments from the myofilament by degrading the z-disk (Haard, 1994). m-Calpain (calpain II) isolated from carp muscle showed maximum caseinolytic activity at pH 7.0 and 25°C (Taneda et al., 1983) and the analogous enzyme from tilapia muscle exhibited optimal caseinolytic activity at pH 7.5 (Jiang et al., 1991).

Digestive proteinases

The digestive proteinases play a role in the softening of abdominal tissues during post-mortem storage of fish. These proteinases, particularly trypsin can degrade myofibrillar proteins (Martinez and Serra, 1989). The leakage of digestive proteinase into the belly cavity of fish can activate collagenase present in the connective tissue as well as directly initiate collagen degradation by digestive collagenase (Haard, 1994). Pepsin is a gastric aspartic proteinase secreted as a zymogen with molecular weight of 40,000, activated by an autolytic or HCL induced mechanism (Gildberg, 1988). The molecular weight of the active enzyme is approximately 25,000-35,000 (Gildberg and Raa, 1983; Sanchez-Chiang et al., 1987; Gildberg, 1988). Two significantly different pepsins, pepsin I and pepsin II were found in several fish species (Gildberg, 1988; Sanchez-Chiang et al., 1987; Arunchalam and Haard, 1985). Gastricin, acidic proteinase was purified from fish (*Merluccius gayi*) and showed stability at alkaline pHs (Sanchez-Chiang and Ponce, 1981). Unlike pepsins, gastricin appear to be activated by low concentration of NaCl (Haard, 1994; Sanchez-Chiang and Ponce, 1981).

Proteinases in the intestine of fish include trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase. These are normally secreted from the pyloric caeca and pancreas (Haard, 1994). The main pancreatic endopeptidases, which have been detected in pyloric caeca and intestine of fish are trypsin, chymotrypsin and elastase and the main exopeptidase, are carboxypeptidases A and B and aminopeptidase (Clark et al., 1985; Martinez and Serra, 1989). Serine proteinases, mainly trypsin and chymotrypsin, have been reported to play a decisive role in protein digestion (Martinez and Serra, 1989). Fish trypsin has molecular weight of 23,500-28,000 depending upon the species (Simpson and Haard, 1984; Martinez et al., 1988; Hjelmeland and Raa, 1982). Cohen et al. (1981) reported that the approximate molecular weights of trypsin, chymotrypsin and elastase were 25,000 and carboxypeptidase B was 34,000. Haard (1994) reviewed the properties of fish trypsins which include relatively low thermal stability, instability at low pH, and a lower content of basic amino acid residues in polypeptide chain than mammalian trypsins. Like trypsin, chymotrypsins from fish are less stable in acidic media than mammalian chymotrypsin (Haard, 1994).

Two distinct proteinases which are able to hydrolyze elastin include a typical pancreatic elastase and pancreatic metalloproteinase (Yoshinaka et al., 1983; 1984). Pancreatic elastase belong to the group of serine proteinases and were present in ten fish species tested, while metalloproteinases which require zinc for activity were found only in bluefin tuna and yellowtail (Yoshinaka et al., 1985). Digestive collagenase have been isolated from fish (Yoshinaka et al., 1978; Pan et al., 1986). These enzymes contributed to the 'honey comb' disorder in canned mackerel (Pan et al., 1986). Digestive

collagenase, a serine proteinase, differ from tissue metalloproteinase collagenase (Haard, 1994).

Protein hydrolysates

Food proteins can be modified through hydrolysis to improve the nutritional characteristics and functional properties as well as to remove off-flavors, odors as well as toxic or inhibitory ingredients (Lahl and Braun, 1994). Protein hydrolysis can be accomplished by enzymatic or chemical methods (Loffler, 1986). Generally, enzymatic hydrolysis is more advantageous since enzymes have a specificity of action and function under the milder conditions (Vieira et al., 1995). Well-defined peptide profiles can be obtained, leading to superior properties of hydrolysates (Surowka and Fik, 1992; Lahl and Braun, 1994). Acid and alkaline hydrolysis can racemize L-amino acids, forming D-amino acids and also can form toxic substances, e.g., lysino-alanine. Furthermore, these products were found to lack good functional properties (Lahl and Braun, 1994).

The enzyme or enzyme mixtures are selected based upon their ability to hydrolyze the substrate to a desired extent, termed the degree of hydrolysis. Proteinases from plants, animals or microorganisms have widely been used to prepare protein hydrolysates (Cheltel et al., 1971; Gildberg, 1993). The plant enzymes including papain, ficin and bromelain are comparatively nonselective, while microbial enzymes tend to be more selective (Lahl and Braun, 1994). The hydrolysis can be monitored to render specific product characteristics including amino acid profile and molecular weight distribution by controlling the critical parameters such as time, temperature and pH. The hydrolysis can be accelerated by increasing the enzyme concentration. After the

hydrolysis process, the enzymic reaction is terminated by adjusting the pH or by thermal inactivation. However, extreme changes in pH of the hydrolysis mixture upon neutralization can lead to excessive amounts of salt, while heat inactivation can cause nutritional loss and possibly activate undesirable reactions, such as Maillard reaction (Lahl and Braun, 1994). The hydrolysates are clarified by filtration or centrifugation to remove insoluble substrate, followed by removal of moisture. Drying including freeze drying, drum/roller drying, or spray drying is commonly used to provide a dried powdered product.

There has been an increased interest in the production of protein hydrolysate from the fish protein, agricultural wastes and under-utilized species including shark (Ondenalore and Shahidi, 1996), harp seal (Shahidi et al., 1994), capelin (Shahidi et al., 1995), herring (Hoyle and Merritt, 1994), mullet (Rebeca et al., 1991), sardine (Quaglia and Orban, 1987), crayfish (Beak and Cadwallader, 1995), hake (Cheftel et al., 1971), chicken heads (Surowka and Fik, 1992;1994), lobster (Vieira et al., 1995) and Bovine red blood cells (Synowiecki et al., 1996).

Fish protein hydrolysates have a wide spectrum of applications ranging from high value peptones and food ingredients with special functional properties to feed and fertilizer (Gildberg, 1993). The raw material, hydrolytic enzymes used and processing parameters must be considered to serve the desired application. Extensive hydrolysis renders low molecular weight products which are rich in essential amino acids, but with low functional properties. Such hydrolysates are suitable for peptones in microbial growth media (Gildberg et al., 1989). Hydrolysate technology has been reported to control food allergies (Mahmoud et al., 1992; Cordle, 1994). Protein hydrolysate can

serve as the medical foods, e.g., the basis for hypoallergenic infant formula (Schmidl et al., 1994). Protein hydrolysates from vegetable protein or yeast extract have been used as the flavor enhancers (Nagodawithana, 1992). Addition of exogenous proteases has shown to accelerate the fermentation for fish sauce production (Haard and Simpson, 1994).

Despite the potential utilization of protein hydrolysates, the development of bitter-tasting peptides has limited their use. Bitterness of hydrolysate reaches a maximum when a hydrophobic amino acid is in the nonterminal position, particularly when both ends of hydrophobic amino acid are blocked via the formation of peptide bond. The bitterness is less when the hydrophobic amino acid is in C-or N-terminal position, and is the lowest when it is a free amino acid (Pedersen, 1994). Exopeptidases including wheat carboxypeptidase (Umetsu et al., 1983) and aminopeptidase T from *Thermus aquaticus* YT-1 were used to remove the hydrophobic amino acids (Minagawa et al., 1989). However, debittering methods using exopeptidase have a limitation since the hydrophobic amino acids released may affect the taste quality of hydrolysate (Fujimaki et al., 1970). Cyclodextrin and starch have been used to mask bitterness due to their ability to wrap the hydrophobic groups of bitter peptides (Tamura et al., 1990). Plastein reaction has been shown to be a potent method to reduce bitterness (Fujimaki et al., 1970, Synowiecki et al., 1996). In addition to bitter peptide, rancidity is a major flavor problem in fish protein hydrolysates. If the product contains more than 1% fat, the removal of fat by extraction or the addition of antioxidants must be taken into consideration (Mackie, 1982).

Physicochemical and functional properties of protein hydrolysate

Food functionality is defined as any properties of a food or food ingredient except nutritional ones that affect its utilization (Pour-El, 1981). Functionality is the physio-chemical behavior which proteins exhibit when they interact with other constituents of multi-component food systems. Functional properties of proteins are governed by their characteristics, including the amino acid side groups and configurations, and can be affected by many factors including pH, ionic strength and temperature (Phillips and Beuchat, 1981; Turgeon et al., 1992a,b). A quantitative structure-activity relationship, electrical, hydrophobic and structural parameters must be taken into consideration to elucidate protein functionality (Nakai and Li-Chan, 1985). Proteinases have been used to modify protein functionality due to their role in catalyzing the cleavage of peptide bonds. Three major modifications occur: (1) an increase in the number of polar groups (NH_4^+ and COO^-) with a concomitant increase in hydrophilicity, (2) a decrease in molecular weight, and (3) a possible alternation in molecular configuration, leading to exposure of hydrophobic interior to an aqueous phase (Phillips and Beuchat, 1981).

Protein solubility

The solubility of protein hydrolysate over a wide range of pH, temperature, ionic condition is one of its most important functional properties. In general, proteins are soluble in water when electrostatic and/or hydration repulsion between molecules are greater than the driving force for hydrophobic interaction. Polar and charged groups of proteins confer solubility by their association with water molecules via ionic (hydrogen)

bonding (Phillips et al., 1994). Hydrolysis of protein substantially increases solubility of the resulting hydrolysate, particularly at the isoelectric point of the parent protein (Quaglia and Orban, 1987; Chobert et al., 1988a,b, 1989; Multilangi et al., 1996). Increase in solubility is attributed to the increase in ionizable groups during hydrolysis. The solubility of hydrolysate can be influenced by the type of enzyme used. Trypsin hydrolyzes hydrophilic sites (lysine or arginine residues) on the protein to form hydrophilic peptides, while chymotrypsin hydrolyzes hydrophobic sites produce more hydrophobic, less soluble fragments. (Multilangi et al., 1996). Monti and Jost (1978) found that heated-denatured whey protein prepared by trypsin hydrolysis showed higher solubility than that hydrolyzed by papain or neutral protease from *Bacillus subtilis*. Solubility of trypsin hydrolysate was higher than other hydrolysate treated by chymotrypsin, Alcalase and Neutrase (Multilangi et al., 1996). Solubility of capelin hydrolysate treated by Alcalase was higher than hydrolysate treated by Neutrase. Both hydrolysates showed the highest solubility at pH around 5 (Shahidi et al., 1995).

Interfacial properties

Emulsifying and foaming properties are the most important interfacial properties in food. These properties are influenced by conformation factors such as amino acid composition, primary structure, molecular flexibility, steric hindrance, amphipathic structure, hydrophobicity , molecular size, secondary structure, and net charge (Turgeon et al., 1992b; Li-Chan et al., 1984; 1985; Nakai, 1983; Townsend and Nakai, 1983).

Emulsion is the mixture of two or more immiscible liquids, such as oil and water. Proteins are used to aid emulsion formation and increase emulsion stability since

they can reduce the interfacial tension at oil-water interfaces and form an interfacial film to protect droplets against coalescence (Phillips et al., 1994). The conformational properties of a protein capable of unfolding at the interface, rather than the thickness of the adsorbed layer and surface rheological parameters indicate film stability and emulsion coalescence (Phillips et al., 1994). Emulsifying and interfacial properties of protein can be improved by partial hydrolysis (Smith and Brekke, 1985; Chobert et al., 1988a,b). A minimum molecular weight of 2,000 has been recognized as essential for good emulsifying properties (Lee et al., 1987; Turgeon et al., 1991). However, Chobert et al. (1988b) reported that the apparent molecular weight of peptides should not be lower than 5,000. Although small peptides diffuse rapidly and adsorb at the interface, these small peptides are less efficient in decreasing surface tension (Turgeon et al., 1991). Larger peptides can be produced by limiting the degree of protein hydrolysis (Alder-Nissen and Olsen, 1979) or by separation of hydrolysates using ultrafiltration (Deeslie and Cheryan, 1988; Turgeon et al., 1991; Multilangi et al., 1996).

The importance of enzyme specificity was demonstrated in having an influence on the interfacial and emulsifying properties (Chobert et al., 1988a,b; Turgeon et al.,1991). Proteinase which is specific for cleaving certain peptide bonds can either disrupt or maintain the hydrophobic clusters intact within the resulting peptides (Mahmoud, 1994). Trypsin hydrolysis provided higher molecular weight peptides, probably having a more amphipathic nature, and gave higher emulsion capacity than the chymotrypsin fraction. (Turgeon et al., 1991). Chymotrypsin cleaves peptides at the carboxyl end of aromatic amino acids (Trp, Tyr, Phe), resulting in peptides with less hydrophobic amino acids within their structure than tryptic peptides (Turgeon et al.,

1991). Gauthier et al. (1993) and Multilangi et al. (1996) found that trypsin hydrolysate had higher emulsifying capacity than chymotrypsin hydrolysate.

During adsorption at the interface, protein unfolds to form a cohesive interfacial film with hydrophobic residues interacting with oil and hydrophilic residues with water (Chobert et al., 1988b; Turgeon et al., 1992). Significant correlations between emulsifying properties with surface hydrophobicity have been reported (Nakai, 1883; Voutsinas et al., 1983; Li-Chan et al., 1984; Mahmoud et al., 1992). The decrease in hydrophobicity and emulsion activity was observed after proteolytic hydrolysis (Townsend and Nakai, 1983; Quaglia and Orban, 1990; Mahmoud et al., 1992). Consequently, the more the degree of hydrolysis, the less the hydrophobicity and emulsion activity was observed. Salt is known to enhance hydrophobic interactions. Ions interact with charged groups of proteins, decreasing electrostatic attraction between opposite charges on neighboring groups, allowing hydrophobic interaction to become predominant allowing adsorption at the interface (Wagner and Anon, 1990; Turgeon et al., 1992).

Foaming properties of protein are fundamentally related to their film-forming properties at the air-water interface (Phillips et al., 1994). The intermolecular interactions at the interface involve hydrogen bonding, and electrostatic and hydrophobic interactions. Low molecular weight and amphipathic protein molecules which are conductive to rapid foam formation may not necessarily be ideal to form the protein-protein interactions that render stable foams (Kinsella, 1981, Phillips et al., 1994). To exhibit good foaming, a protein must be capable of migrating rapidly to the air-water interface, unfolding and rearranging at the interface (Halling, 1981). Flexible protein

molecules can rapidly decrease the surface tension at the air-water interface and form foams with great volumes. For foam stability, the surface rheology properties are more important than a decrease in electrostatic repulsion and steric stabilization. (Mitchell, 1986). Generally, stronger films are obtained at pH values close to the isoelectric pH of most protein (Kinsella, 1981). Surface hydrophobicity showed good correlation with foaming properties of proteins (Kato et al., 1983). Amphiphilic proteins possess high surface hydrophobicity. They are forcefully adsorbed at the interface between air and water to cause a pronounced reduction of interfacial tension or surface tension that readily facilitates foaming (Kato et al., 1983). Dickenson (1989) suggested that the foaming capacity of protein can be improved by exposing more hydrophobic residues and by reducing the average molecular weight.

The foaming properties can be improved by increased protein concentration. The decrease in foam expansion at high concentration is believed to correspond to a reduced solubility of protein (Cherry and McWatters, 1981; Britten and Lavoie, 1992). As protein concentration increased, thicker and more rigid interfacial film was produced (Britten and lavoie, 1992).

Lipid and other low molecular weight surfactants destabilize protein foams due to their higher surface activity, leading to displacing proteins from the interface in a competitive manner (Phillips et al., 1994). This diminishes film thickness, interrupts film cohesiveness, and ultimately weakens the film resulting in decreased foam stability (Damodaran, 1989).

Antioxidant activity

Hydrolysis causes an increase in small peptide or free amino acids. The soybean protein hydrolysate produced by acid or enzymatic hydrolysis showed antioxidant activity which reached a maximum after a short period of hydrolysis and then decreased in both hydrolysis methods (Yamaguchi et al., 1975). Recently, Chen et al. (1995) reported the antioxidant activity in protease hydrolysates of a soybean protein, β-conglycinin. The antioxidant peptides were composed of 5-16 amino acid residues, including hydrophobic amino acids, valine or leucine at N-terminal positions, and proline, histidine or tyrosine in the sequences. (Chen et al., 1995). The amino acid residues existing at the N termini of dipeptides have been known to be effective antioxidants in an oil system (Kawashima et al., 1979). Histidine containing peptides showed the antioxidant activity, probably due to chelating ability and lipid radical-trapping ability of imidazole ring (Uchida and Kawakishi, 1992; Murase et al., 1993). The antioxidant activity of histidine-containing peptides was higher than histidine (Murase et al., 1993). Carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-L-1-methylhistidine) have been reported as the natural antioxidants which are present in the muscle. The antioxidant mechanism of carnosine and anserine has been postulated to be due to metal chelation or free radical scavenging (Can and Decker, 1994). The low molecular weight fraction of spermary tissue including spermine, putrescine, hypoxanthine, xanthine, and glutathione can inhibit autoxidation and iron/ascorbate-catalyzed oxidation (Sasaki et al., 1996). The antioxidant activity of polyamines was reported and their activity increased with increasing amine content. Spermine possessed the highest activity followed by spermidine and putrescine, respectively (Lovaas, 1991).

The antioxidant activity of spermine has been postulated to be due to both iron chelating and free radical scavenging (Tadolini, 1988; Lovaas, 1991).

Research objectives

The overall objective of this research was to maximize the utilization of wastes from Pacific whiting surimi production by development of alternatives in the processing of by-products. To achieve this, enzymatic compounds, particularly proteinase in both liquids and solid wastes, were recovered and characterized. Protein hydrolysate from Pacific whiting solid waste was also demonstrated as a feasible source of protein. This information can be beneficial for the surimi industry as a means to regain some of the cost for waste treatment or disposal. The specific objectives were identified as follows:

1) Identify and characterize the proteinase in Pacific whiting surimi wash water.
2) Recover the proteinase from Pacific whiting surimi wash water using a combination of methods including the ohmic heating, ultrafiltration and freeze-drying as well as characterize the recovered enzyme.
3) Investigate the proteolytic activity of Pacific whiting solid wastes and digestive organs
4) Study the production of protein hydrolysate from Pacific whiting solid waste using the commercial enzymes and determine the composition and nutritional value of the product.
5) Investigate the functional properties of protein hydrolysate from Pacific whiting solid wastes and the physicochemical-functional relationship.

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Chapter 2

Proteinase in Pacific Whiting Surimi Wash Water: Identification and Characterization

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Abstract

A proteinase in Pacific whiting surimi wash water (SWW) was characterized to be cathepsin L based on molecular mass (M_r), substrate specificity, and SDS-substrate gel electrophoresis. The proteinase was highly active on Z-Phe-Arg-NMec, and the native M_r was 27,400 based on size exclusion (SEC)-HPLC. Acidification of the SEC-HPLC fractions showed a 2-fold increase in activity on Z-Phe-Arg-NMec. SWW proteolytic activity was found at M_r 54,200 on SDS-substrate gel. However, acidification shifted the activity zone to M_r 39,500 corresponding to cathepsin L. No evidence of activity by calpain or cathepsin B or H was found in Pacific whiting SWW.

Key words: surimi, wash water, proteinase, Pacific whiting, cathepsin L

Introduction

Pacific whiting is the most abundant marine resource off the northwest coast of the contiguous U.S. Soft texture and poor keeping quality has limited its commercial utilization (Anderson, 1985; Kabata and Whitaker, 1985; Nelson et al., 1985). Muscle infected with a microscopic parasite (Kudoa sp.) showed extensive tissue proteolysis and eventual liquefaction upon cooking (Erickson et al., 1983; Kabata and Whitaker, 1985). The use of food-grade proteinase inhibitors in Pacific whiting surimi production has prevented tissue softening which had often occurred with home cooking and improved its marketing for human consumption (Morrissey et al., 1993). Increased quantities of Pacific whiting are harvested, primarily for the production of surimi (Radtke, 1995).

Washing is a critical step in the production of surimi. It removes water-soluble material, such as blood, improves color and flavor, and enhances the gel strength of surimi (Miyauchi et al., 1973; Toyoda et al., 1992). The water-soluble constituents include sarcoplasmic proteins, digestive enzymes, inorganic salts, and low-molecular-weight organic substances such as trimethylamine oxide (Toyoda et al., 1992). The majority of soluble components are rapidly removed in the first washing cycle. In general, 5-min agitation in each washing for 2 cycles at a 3:1 water-to-meat ratio has been considered adequate (Lee, 1986). Washing mince resulted in 37% loss of solids with effective removal of sarcoplasmic proteins (Adu et al., 1983). Lin et al. (1996) reported protein losses of 27% and 38% by the second and third cycles of wash, respectively, during surimi production.

SWW generated from the processing plant usually has total suspended solids (TSS) of ≈20 g/L and biochemical oxygen demand (BOD_5) of 10 - 15 g/L (Huang,

1995). The large amounts of organic matter in the SWW effluent create problems in the disposal of waste water. In Pacific whiting surimi operations, the wash water is flocculated by air flotation devices and discarded by various methods at shoreside plants. The cost of water treatment represents a major expenditure for surimi processors.

Recovery of useful byproducts from wash water can help reduce cost. Both solid and liquid waste generated during processing requires treatment. Haard et al. (1994) reviewed several seafood proteins that have biotechnological applications. These included enzymes, gelatin, and pharmaceutical proteins with antimicrobial and antitumor activities. There is potential for recovery of several active proteinases from SWW. Proteinases active in fish muscle include cathepsin D, calpains, and alkaline proteinases (Makinodan et al., 1984). During surimi manufacturing, the proteinases are removed by water washes, leaving less proteolytic activity in final surimi products (Chang-Lee et al., 1989). The proteinase activity in mechanically deboned Pacific whiting flesh was reduced to 43.7% by 2 wash exchanges in pilot-plant operations (water: mince ratio (w/w), 3:1) and refining (Chang-Lee et al., 1989). Morrissey et al. (1995) reported that the proteinase activity in washed mince was decreased by 85% during commercial washing steps. Biologically active compounds, particularly enzymes, may be applied as processing aids in the food industry and could expand the potential for utilization of seafood by-products. The objective of this study was to identify and characterize proteinases in Pacific whiting SWW to reduce organic contents and recover biologically active compounds.

Materials and Methods

Chemicals

N-carbobenzoxy-Phe-Arg 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), Z-Arg-Arg-NMec,L-Arg-NMec,1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane(E-64), pepstatin, leupeptin, phenanthroline, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), calcium chloride, cytochrome C (horse heart), carbonic anhydrase, albumin (bovine serum), β-amylase (sweet potato), blue dextran, sodium phosphate, sodium citrate, sodium azide, Tris base, 2-mercaptoethanol (βME), and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium caseinate was obtained from US Biochemical Corp. (Cleveland, OH). Iodoacetic acid and phenylmethylsulfonyl fluoride (PMSF) were purchased from Calbiochem Co. (La Jolla, CA).

SWW collection and preparation

The SWW from Pacific whiting was collected from a commercial surimi processing plant in June, 1995. The first wash water after passing through the screener was collected as a source of proteinase, kept in ice, and transferred to the Oregon State University Seafood Laboratory. The samples were used for pH and temperature profiles and inhibitor studies.

Due to the limited harvesting season of Pacific whiting, wash water was also prepared in the lab from mince. Mince without cryoprotectants was collected and stored frozen at -20°C until used. The mince (200 g) was mixed with water at 3:1 (v/w) and stirred constantly with a magnetic stirring bar for 5 min. The filtrate was collected by

straining the mixture through double layers of cheesecloth and designated as SWW. For size exclusion chromatography and activity staining, concentrated wash water was prepared by reducing the water to mince ratio to 2:1. The prepared wash water was further concentrated 2-fold using Centriprep 10 (Amicon, Inc., Beverly, MA) for 4-5 cycles at 2,200 *xg* for 10 min. The concentrate was used for proteinase characterization.

Heat-treated SWW (HTSWW) was prepared by heating the concentrated SWW at 60°C for 3 min. The precipitates were centrifuged out at 7,800 X g for 15 min, and the supernatant was collected as HTSWW.

Enzyme assay

Proteinase activity was assayed using casein as a substrate according to the method of An et al. (1994a). Activity was determined by the TCA-Lowry assay by incubating for 20 min in McIlvaine's buffer containing 1 mM β-mercaptoethanol (βME) at various pH and temperature conditions. A blank was run in the same manner, except that enzyme was added after addition of 50% trichloroacetic acid. Activity was expressed as tyrosine equivalents in TCA supernatant as measured by the Lowry assay (Lowry et al., 1951). One unit of activity (U) was defined as 1 nmol of tyrosine released/ min.

Activity of cathepsins L, B, and H were analyzed using synthetic substrates, Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and L-Arg-NMec, respectively, according to the method of Yamashita and Konagaya (1990a). Biosep Sec-S3000 fractions (25 μL) were diluted to 500 μL with 0.1% Brij 35 and preincubated in the assay buffer at ambient temperature (25°C) for 1 min. To initiate the enzymatic reaction, 250 μL of 20 μM substrate solution was added to the mixture, and incubated at 30°C for 10 min. The

reaction was stopped by adding 200 μL of 5 mM iodoacetic acid. Fluorescence due to the released methylcoumarin was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD).

Inhibitor study

SWW (100 μL) was incubated for 10 min at room temperature with 100 μL of various inhibitors, i.e., E-64, pepstatin, PMSF, phenanthroline, EDTA, leupeptin, and iodoacetic acid, at various concentrations (Table 2.1). The residual activity was analyzed by TCA-Lowry assay using casein as a substrate in the McIlvaine's buffer for 20 min under 3 different conditions: (1) pH 3.5 at 37°C, (2) pH 5.5 at temp 55°C, and (3) pH 8.0 at 65°C.

Size exclusion HPLC

Size exclusion chromatography was performed on HPLC using a Biosep Sec-S3000 column (Phenomenex, Torrance, CA) connected with a Bio-Rad HPLC pump (Model 2700, Bio-Rad Laboratories, Inc., Hercules, CA) and a UV detector (Bio-Rad Model 1706). Samples (500 μL) were injected onto the column after filtration through Whatman paper #42 (Whatman International Ltd., Maidstone, England) and centrifuged at 5,200 *x g* for 2 min (Eppendorf Micro Centrifuge, Model 5415C, Brinkmann, New York, NY) to remove large particles. The proteins were eluted isocratically with 100 mM sodium phosphate buffer, pH 6.8, at 0.8 mL/min. Eluted proteins were monitored by absorbance at 280 nm and collected in 0.4-mL aliquots for further analysis.

The proteinase was separated on SEC-HPLC, and its molecular weight was estimated by plotting relative elution volume (Ve/Vo) vs. the logarithm of M_r of the protein standards. The elution volume (Ve) was measured for each protein standard and the proteinase, and the void volume (Vo) was estimated by the elution volume of blue dextran (2,000,000). The standards included horse heart cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (66,000), and sweet potato β-amylase (200,000).

Acidification

Biosep Sec-S3000 fractions were acidified by adding two volumes of 0.05 M sodium citrate-0.05 M phosphate buffer (pH 3.3) containing 1 mM EDTA and 1 mM βME. The final pH as affected by the HPLC eluant buffer in the fraction was not measured. The mixture was incubated for 10 min at room temperature prior to the activity assay on Z-Phe-Arg-NMec, TCA-Lowry method or SDS-substrate gels.

Study of Ca^{2+} and EGTA effects on proteinase

$CaCl_2$ and EGTA were added at levels of 1, 2, and 5 mM to the Biosep Sec-S3000 fractions with the highest proteolytic activity. After incubation, the activity was analyzed by the TCA-Lowry assay at 55°C using casein as a substrate in McIlvaine's buffer, pH 5.0.

Activity staining

Biosep Sec-S3000 fractions with proteolytic activity from SWW and HTSWW were concentrated using Microcon 10 (Amicon, Inc., Beverly, MA), mixed at 3:1 (v/v) ratio with treatment buffer containing no βME, and loaded to SDS-substrate gels. For heat treatment, the concentrated Biosep Sec-S3000 fractions were held at 60°C for 1 min, and precipitates were removed by centrifugation at 5,200 *x g* for 3 min. Acidified fractions were prepared by adding an equal volume of 0.05 M sodium citrate-0.05 M phosphate buffer (pH 3.3) containing 1 mM EDTA and 1 mM βME.

The prepared fractions were separated on SDS-substrate gels (12%) at constant 100 V using a Mini-Protean II system (Bio-Rad Laboratories, Inc., Hercules, CA). Activity staining of the gel was carried out according to the modified method of García-Carreño et al. (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, pH 5.5, containing 2% casein and 1 mM βME and incubated for 1 hr at 55°C for activity zone development. Subsequently, it was stained in 0.125% Coomassie brilliant blue R-250 and destained in 25% ethanol and 10% acetic acid until zones cleared by the activity became visible. Pacific whiting cathepsin L was purified by the method of Seymour et al. (1994) and used as reference. Low-molecular-weight standards (Pharmacia Biotech, Inc., Piscataway, NJ) including phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400) were used for estimation of apparent M_r of the activity.

Results and Discussion

pH and temperature profiles of proteinase in SWW

pH activity profiles at various temperatures were studied to characterize proteolytic activity in SWW (Fig. 2.1). The optimum pH at 55°C was similar to the optimum range reported for cathepsin L, pH 5.5 (An et al., 1994a; Seymour et al., 1994). Cathepsin L in the white muscle of chum salmon had a pH optimum of 5.6 (Yamashita and Konagaya, 1990b). An activity peak was also found at pH 6.0 at 65°C with higher activity at pH 7-8 than at 55°C. Due to the activity at pH 7-8, we presumed it was a heat-stable alkaline proteinase (Fig. 2.1). A fish muscle alkaline proteinase was reported to be a factor in textural degradation in fish meat gel production, and it required unphysiologically high temperature for activity (Cheng et al., 1979; Makinodan et al., 1985).

Some activity was observed in the low pH range at 37°C, indicating possible presence of cathepsin D, which had an optimum pH around 3 (Chen and Zall, 1986; Jiang et al., 1992, 1993). Cathepsin D is important in endogenous protein degradation. It initiates protein hydrolysis and produces peptide fragments that can be further degraded by other cathepsins (Huang and Tappel, 1971). However, the activity was only 40% of the maximum activity found at 55°C.

Effects of proteinase inhibitors

E-64 showed the most efficient inhibition of proteolytic activity in SWW on the 3 conditions tested (Table 2.1). Both leupeptin (specific for cysteine and serine proteinases) and iodoacetic acid (specific for serine, cysteine, and metallo proteinases)

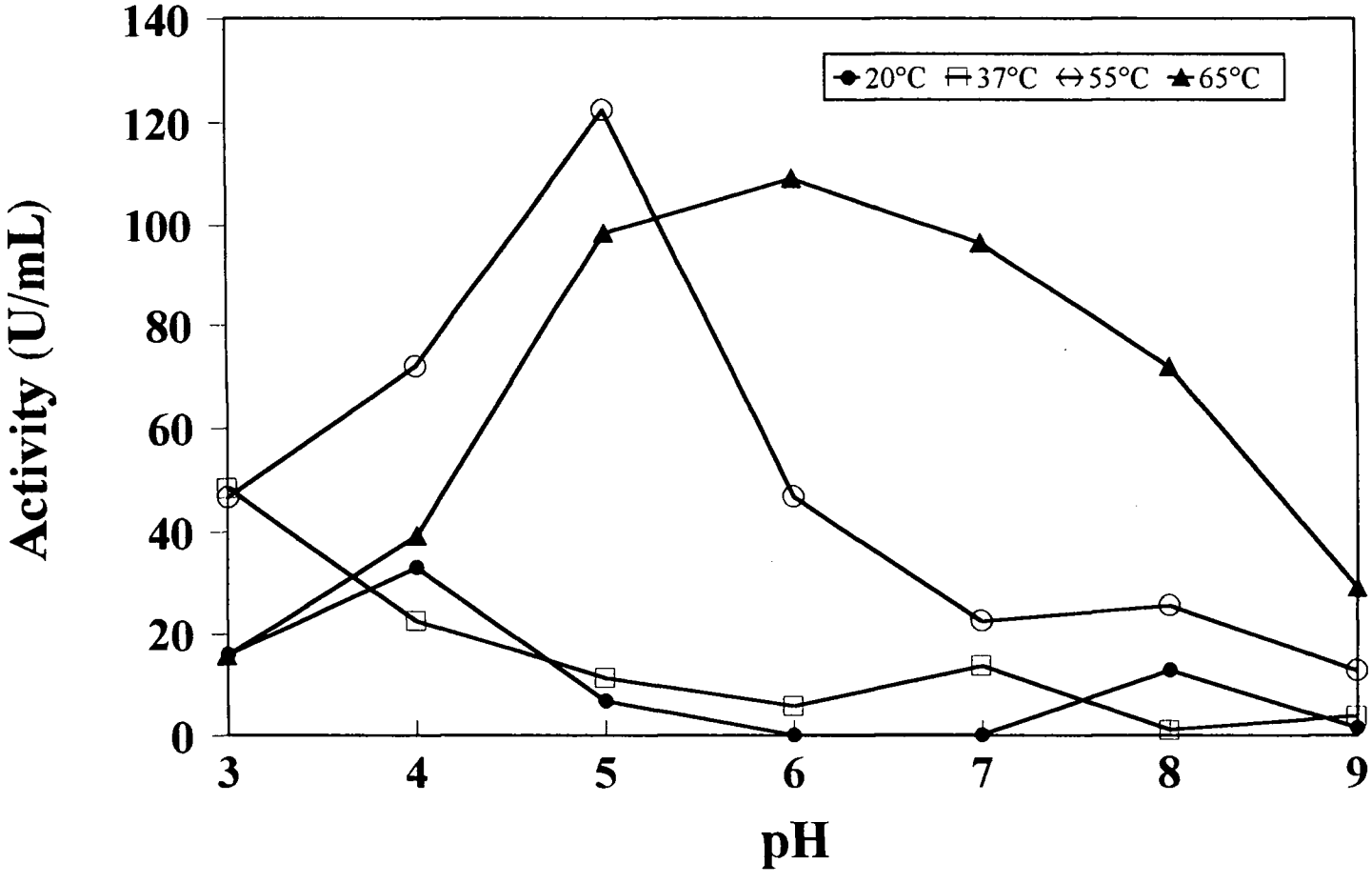

Fig. 2.1. pH activity profiles of Pacific whiting-SWW proteinase at various temperatures. SWW was tested for proteolytic activity using casein as a substrate. A unit activity was defined as 1 nmole of tyrosine released per min.

Table 2.1. Effects of inhibitors on relative activity of SWW proteinases

Protease inhibitors	Concentration	Relative activity* (%)		
		pH 3.5 37°C	pH 5.5 55°C	pH 8.0 65°C
E-64	10 μM	9.6	9.8	15.0
Pepstatin	1 mg/L	107.1	95.4	107.7
PMSF	1 mM	95.3	92.3	100.4
Phenanthroline	1 mM	105.8	98.3	110.3
EDTA	2 mM	157.0	98.5	113.1
Leupeptin	1 mM	22.9	26.7	15.7
Iodoacetic acid	1 mM	14.6	10.5	15.6

*SWW was incubated with the inhibitors for 10 min at room temperature, and the residual activity was analyzed using casein as a substrate for 20 min under the specified conditions.

also showed considerable inhibition of activity. All cysteine proteinases require that the essential sulfhydryl group in the active site be fully reduced by thiol compounds before they exhibit full activity (Kirschke and Barrett, 1987). Therefore, modification of cysteine residue at the active site can lead to complete inactivation of the proteinases. Pepstatin, phenanthroline, PMSF, and EDTA specific for aspartic acid, metallo, serine, and metallo-proteinases, respectively, had very little or no effect on activity. This result indicated that the major form of the activity in SWW was a cysteine proteinase.

Separation and characterization of proteolytic activity

The protein components of SWW and HTSWW, were separated on SEC-HPLC (Figs. 2.2 and 2.3) and tested for proteolytic activity (Fig. 2.4). The protein peak with retention time of 14.15 min was sharply reduced in HTSWW due to the denaturation and/or precipitation of proteins during heat treatment with a concomitant appearance of a peak with retention time 15.98-16.04 min. Heat treatment above 40-50°C can provide sufficient energy to disrupt the bonds and destabilize secondary and tertiary structures of proteins (Cheftel et al., 1985).

The fractions collected from SEC-HPLC were tested for proteolytic activity against casein on two conditions of pH 5.0 at 55°C and pH 7.0 at 65°C (Fig. 2.4). The highest activity was found with the SEC-HPLC fraction collected between 15.0-15.5 min for both conditions. Generally, the activity at pH 5.0 and 55°C was always higher than pH 7.0 and 65°C, suggesting the activity was due to one form of the enzyme. Molecular mass of the proteinase was estimated to be M_r 27,400 based on the relative elution volume on Biosep Sec-S3000 (Fig. 2.5).

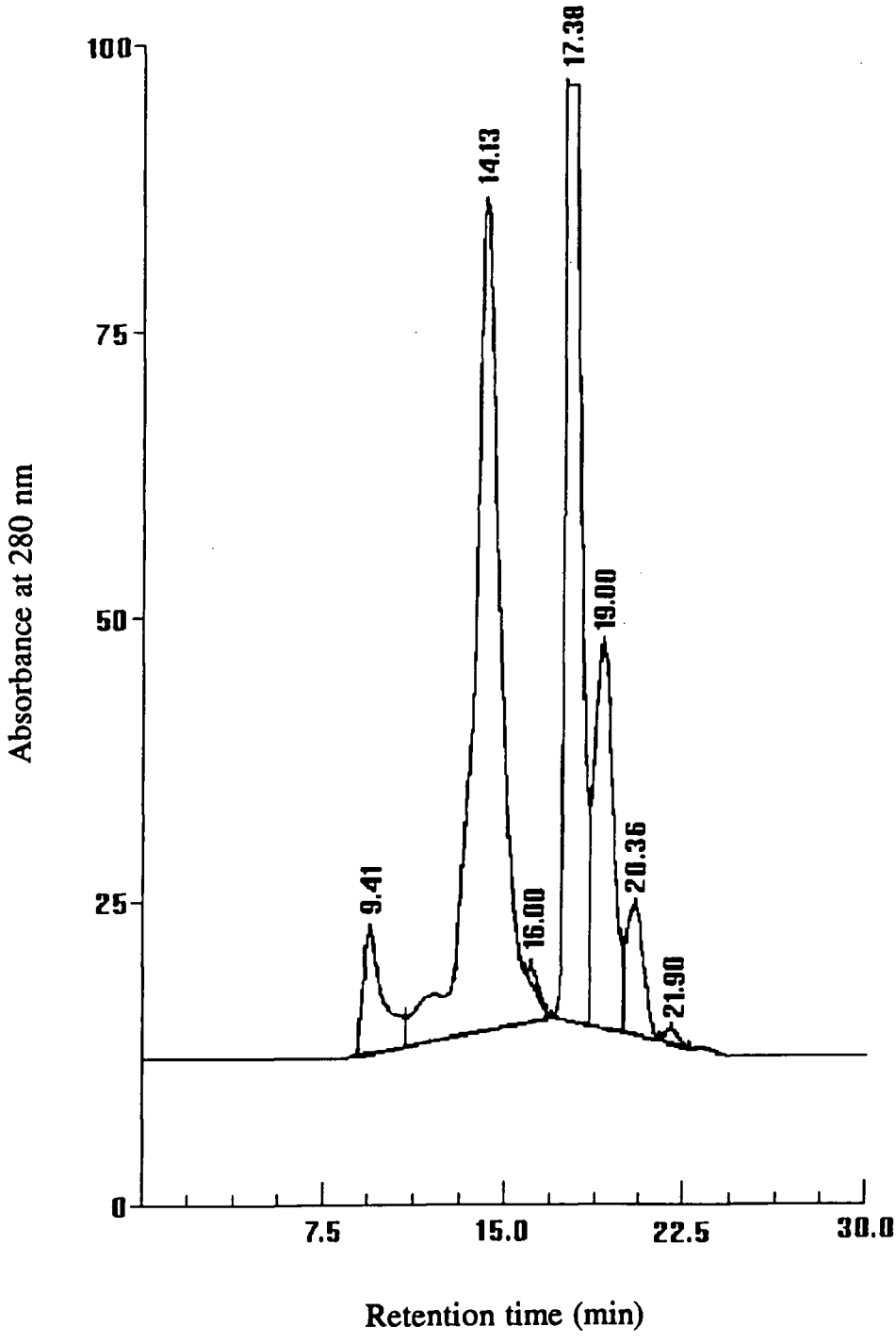

Fig. 2.2. Elution profile of SWW on Sec-HPLC. SWW proteins were separated on Biosep Sec-S3000 with 100 mM sodium phosphate buffer, pH 6.8 at 0.8 mL/min. Eluted proteins were monitored by absorbance at 280 nm.

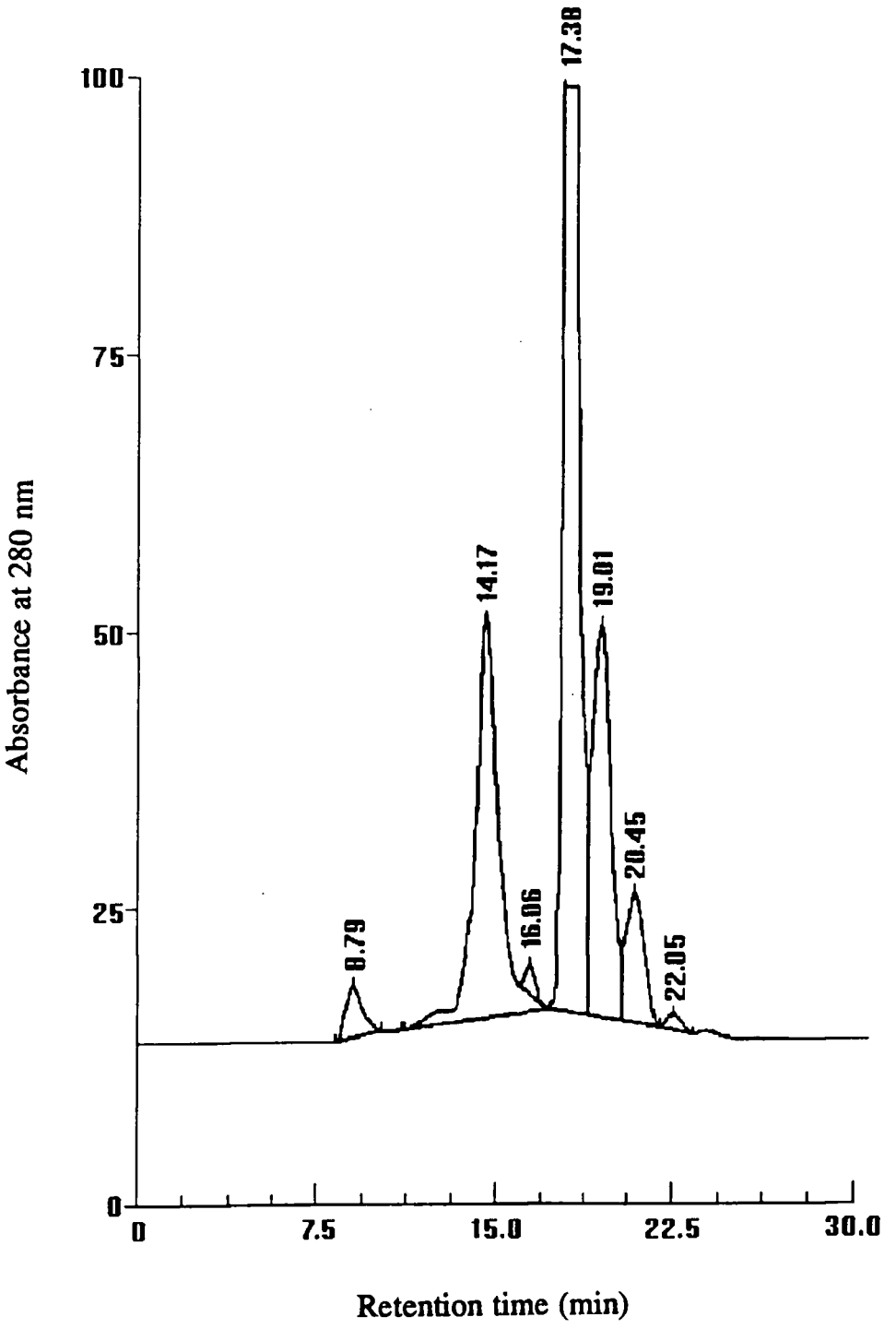

Fig. 2.3. Elution profile of HTSWW on SEC-HPLC. HTSWW were prepared by heat treatment of SWW at 60°C for 3 min. HTSWW proteins were separated on Biosep Sec-S3000 with 100 mM sodium phosphate buffer, pH 6.8 at 0.8 mL/min. Eluted proteins were monitored by absorbance at 280 nm.

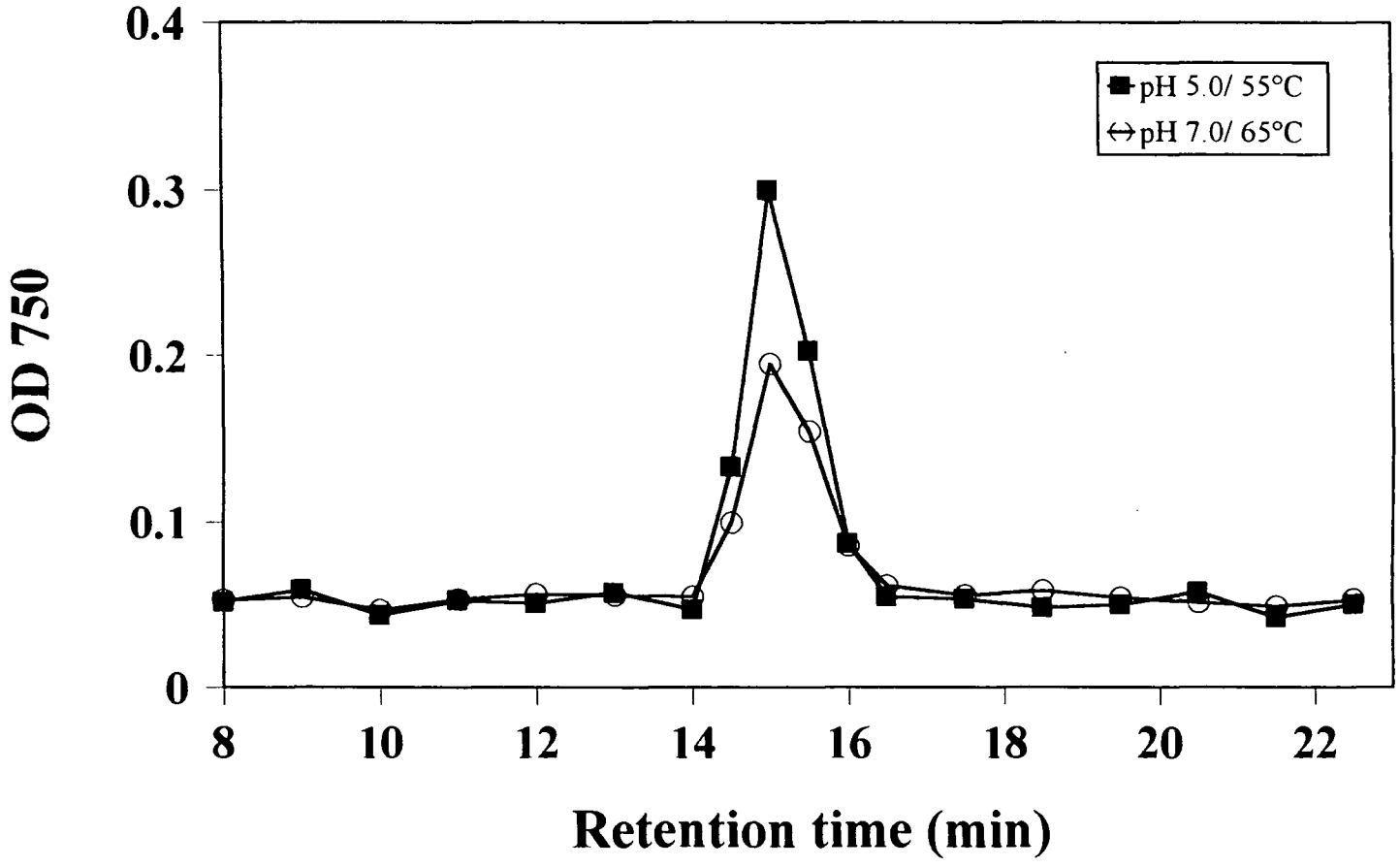

Fig. 2.4. Proteolytic activity of Biosep Sec-S3000 fractions of SWW analyzed against casein at two different conditions. Activity was estimated by absorbance at 750 nm (OD750).

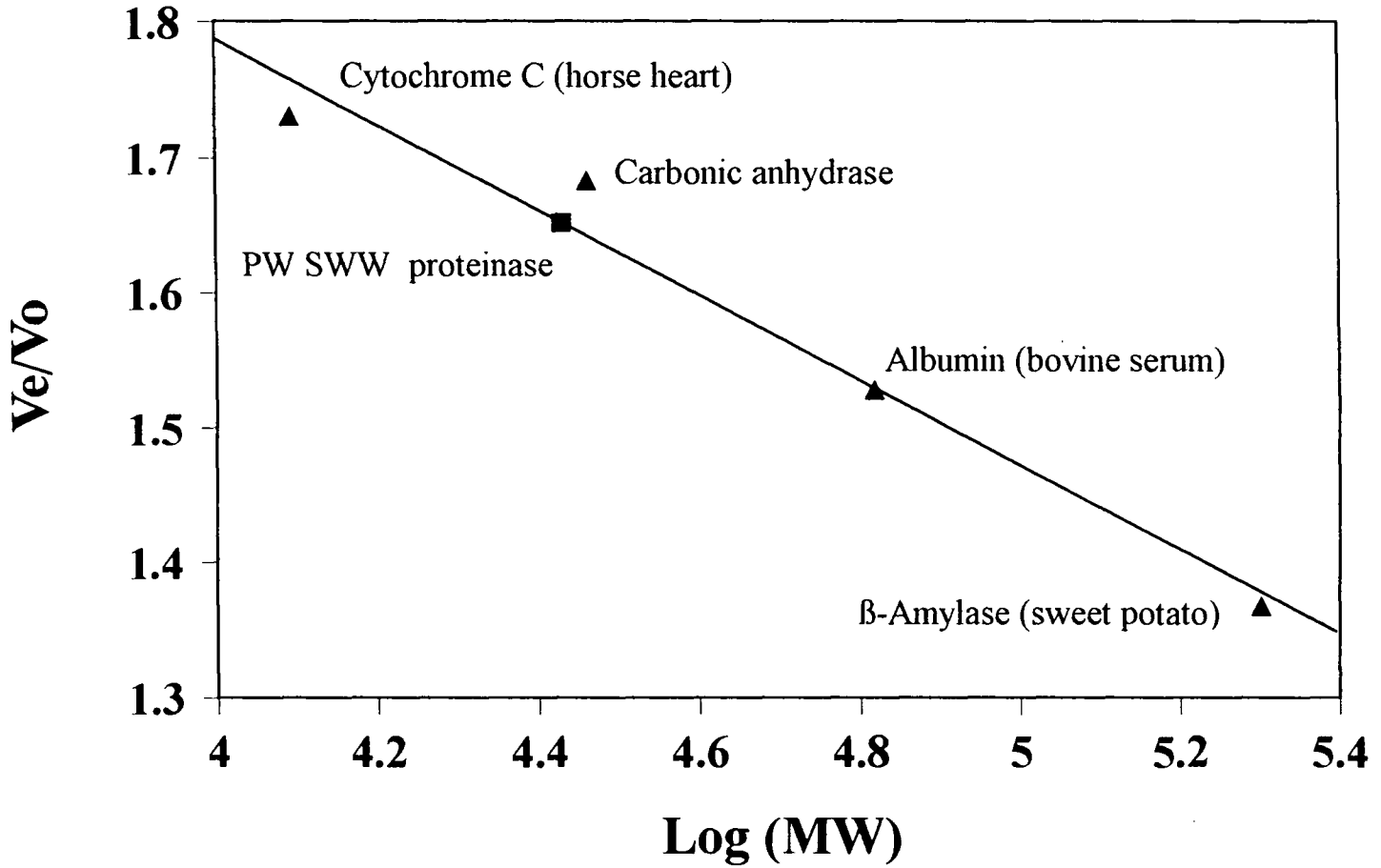

Fig. 2.5. Estimation of the native molecular weight of SWW proteinase based on elution volume on Biosep Sec-S3000. The molecular weight was calculated to be M_r 27,400.

Pacific whiting muscle contains many cysteine cathepsins (An et al., 1994a; Seymour et al., 1994); therefore, fractions were assayed with synthetic substrates for cathepsins B, H, and L to identify the enzymatic activity. The SEC-HPLC fraction at 15.0-15.5 min was most active on Z-Phe-Arg-NMec (Fig. 2.6), the specific synthetic substrate for cathepsin L (Barrett and Kirschke, 1981). Seymour et al. (1994) reported that the specific activity of two forms of cathepsin L from Pacific whiting, P-I and P-II, were 453 and 526 U/nmol of enzyme, respectively, on Z-Phe-Arg-NMec. The activity on Z-Arg-Arg-NMec, specific for cathepsin B, was low. It was reported that cathepsin B was unstable and became rapidly inactivated at neutral pH (Kirchke and Barrett, 1987). In our study, iodoacetic acid was used with the synthetic substrates in the assays to stop the reaction. However, the L-Arg-NMec-hydrolyzing activity was not terminated by addition of iodoacetic acid (specific for cysteine, serine, and metallo-proteinase) or pepstatin (specific for aspartic proteinases) (Benjakul, 1995). These inhibitors are known to be effective against endolytic proteinases. Therefore, we concluded that the L-Arg-NMec-hydrolyzing activity was due to an exopeptidase without endolytic activity. The peak showing the highest activity on L-Arg-NMec had little activity on casein. The activity was eluted from Biosep Sec-S3000 column at 14.5-15.0 min, which was ≈0.5 min earlier than the elution of casein-hydrolyzing activity.

The SEC-HPLC fraction (retention time 15.0-15.5 min) with the highest activity on casein also showed the highest activity on Z-Phe-Arg-NMec among the 3 synthetic substrates tested (Fig. 2.6), indicating that cathepsin L was the major form of proteolytic activity in SWW. Cathepsin B was the most active cysteine cathepsin in Pacific whiting mince; however, it was completely removed during surimi manufacturing with no

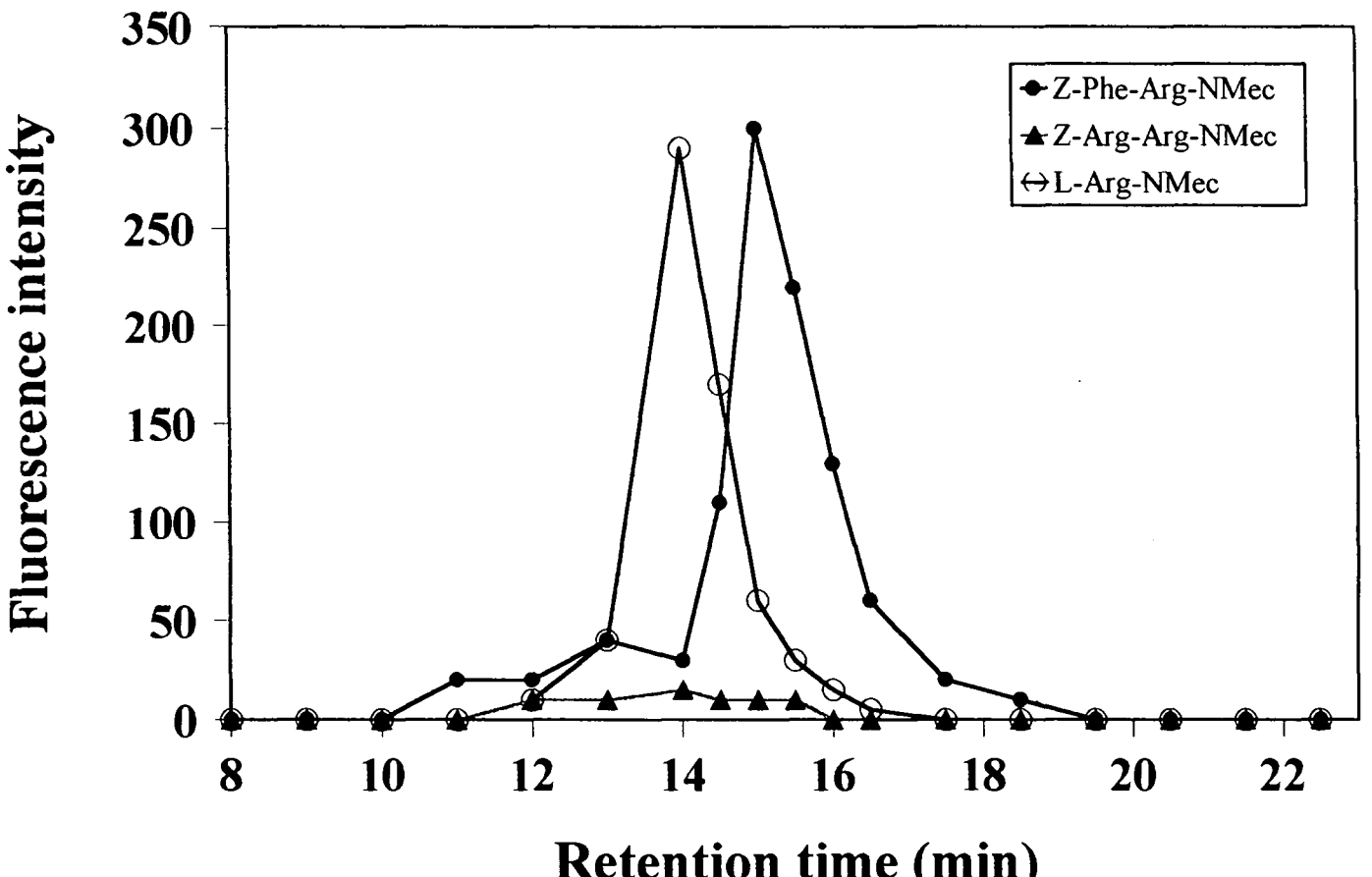

Fig. 2.6. Activity of Biosep Sec-S3000 fractions on synthetic substrates assayed for 10 min at 37°C.

activity found in final surimi (An et al., 1994a). In contrast, cathepsin L was only 60% as active as cathepsin B in mince, but it was retained in surimi at 15% of the original level (Morrissey et al., 1995) and became the most important proteinase in surimi to result in the hydrolysis of myosin at elevated temperatures (An et al., 1994a).

Effect of Ca^{2+} and EGTA on proteinase activity

The effect of Ca^{2+} and EGTA was tested on the proteinase activity to estimate the contribution of calpains in the SWW activity (Table 2.2). Calpain is an intracellular cysteine proteinase and its activity is controlled by calcium ions and calpastatin *in vivo* (Wang et al., 1992). Activities of cysteine cathepsins B, H and L are not affected by the presence of EDTA, EGTA or Ca^{2+} (Koohmaraie et al., 1988). Therefore, EGTA was also included in our study to chelate any endogenous Ca^{2+} present in the muscle.

Incubation of the SWW SEC-HPLC fractions (retention time 15.0-15.5 min) with Ca^{2+} or EGTA, a chelator of Ca^{2+}, at 1-5 mM each did not show an increase or decrease in proteolytic activity measured on casein. This result implied that the contribution of calpains to the proteolytic activity in SWW was negligible and the proteinase isolated from SWW was not Ca^{2+}-dependent. We presumed this was due to the heat lability of calpains. The optimum temperature for calpains from both tilapia and grass shrimp muscles was reported to be 30°C, and the activity was heat-denatured at temperatures above 60°C (Wang et al., 1993). Calpain from white croaker (*Sciena schlegeli*) showed no activity at 45°C and above (Makinodan et al., 1985).

Table 2.2. Effect of Ca^{2+} and EGTA on SWW proteinase activity

Treatment	Relative activity* (%)
Control	100
1 mM Ca^{2+}	105
2 mM Ca^{2+}	106
5 mM Ca^{2+}	107
1 mM EGTA	109
2 mM EGTA	105
5 mM EGTA	95

* SWW was incubated with Ca^{2+} or EGTA, and the residual activity was analyzed at 55°C, pH 5.0 using casein as a substrate.

Effect of acidification

Acidification of the SEC-HPLC fraction (retention time 15.0-15.5 min) resulted in a 2-fold increase in activity on Z-Phe-Arg-NMec and a slight increase on casein (Figs. 2.7 and 2.8), indicating that acid treatment at pH 3.3 dissociated the proteinase-inhibitor complex. This was in agreement with results reported by An et al. (1994b) that acidification of the fraction at pH 3.3 resulted in an 11-fold increase in activity of P-I on Z-Phe-Arg-NMec. Yamashita and Konagaya (1992) also reported the activity increased 22 fold by an acid treatment at pH 3.3 for 10 min at 25°C. Lysosomal

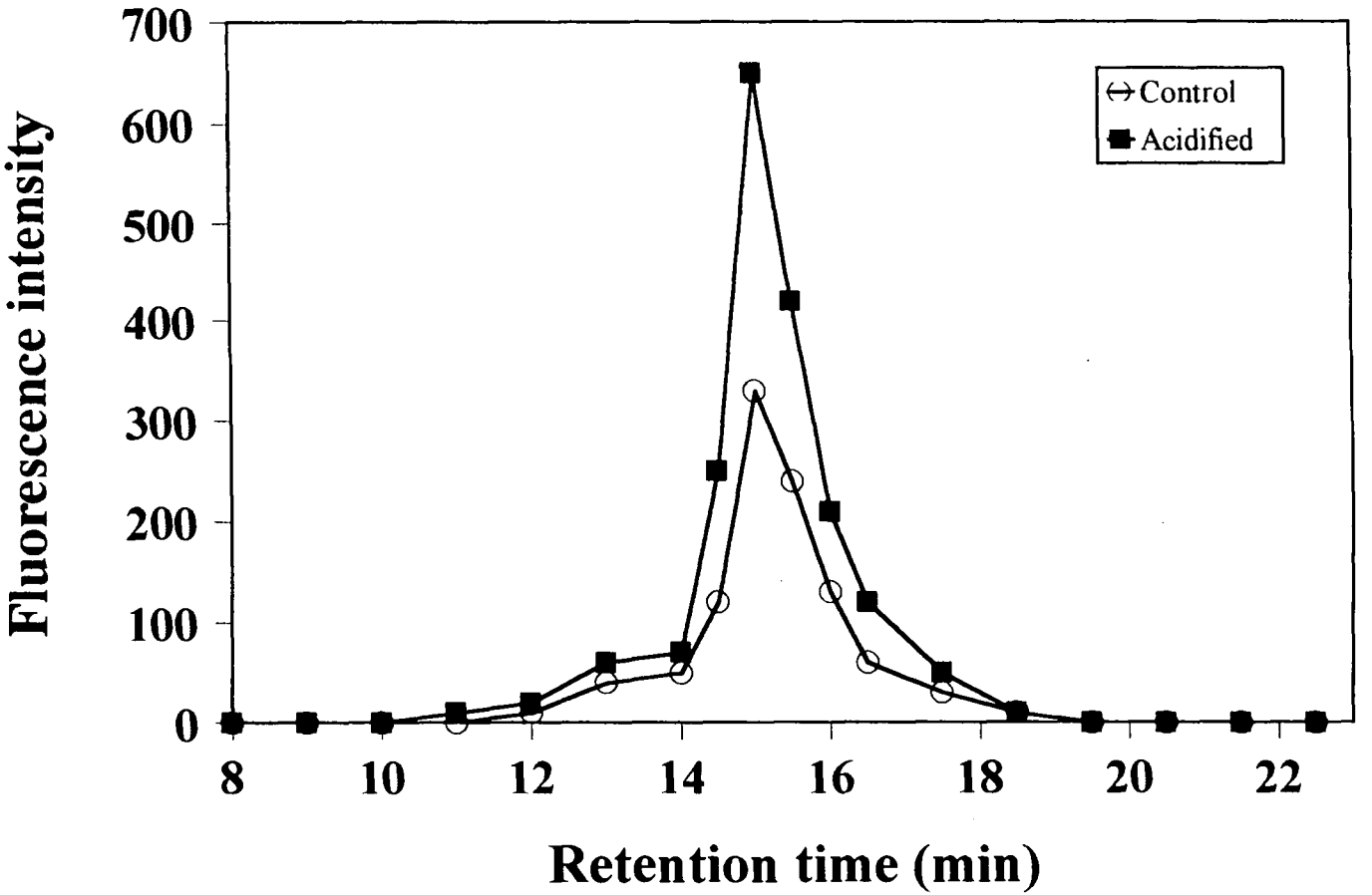

Fig. 2.7. Effect of acidification on the Z-Phe-Arg-NMec-hydrolyzing activity of Biosep Sec-S3000 fractions. The SWW fractions were incubated at pH 3.3 at ≈25°C for 10 min prior to activity assay.

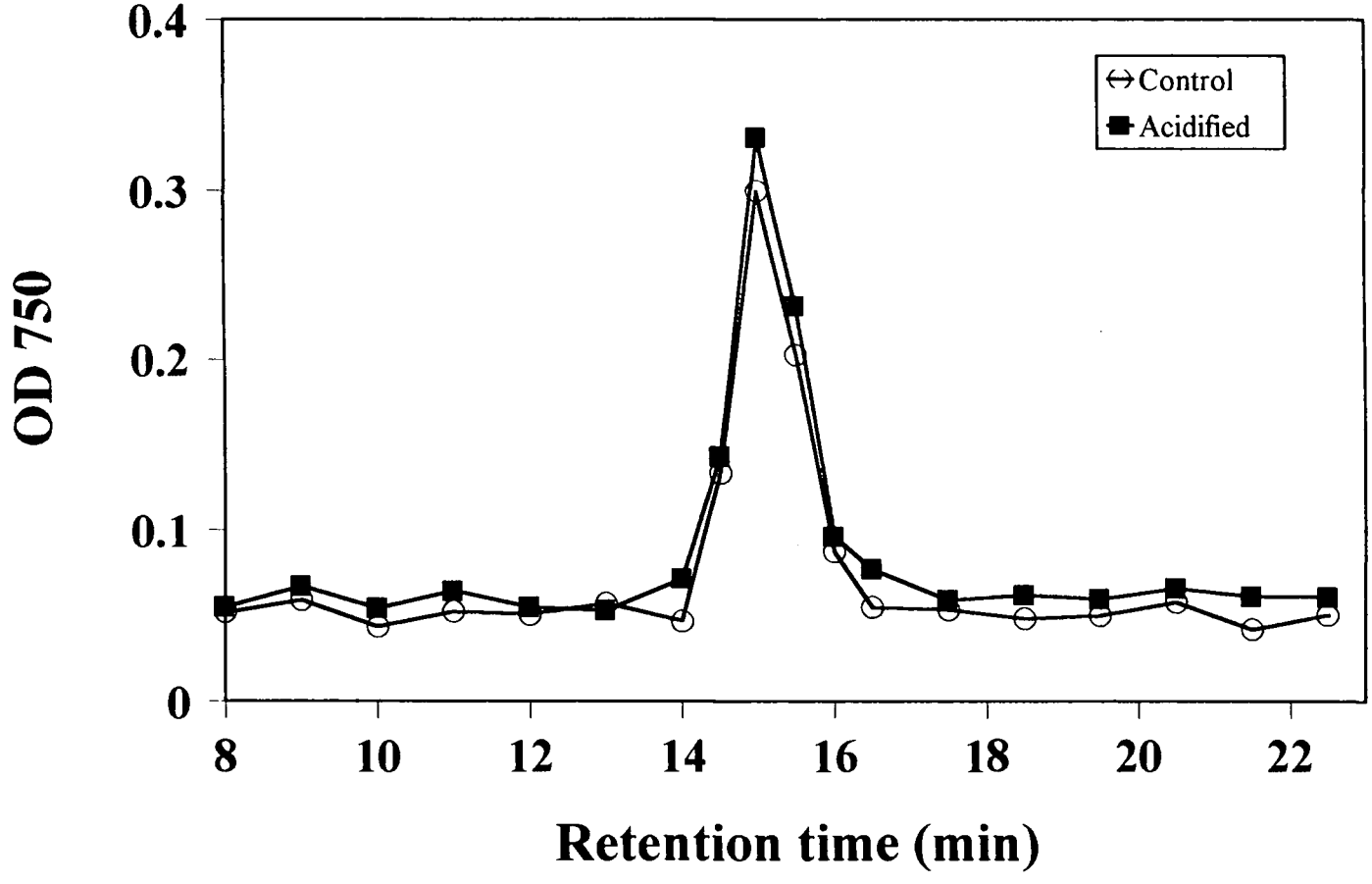

Fig. 2.8.Effect of acidification on proteolytic activity of Biosep Sec-S3000 fractions. The SWW fractions were incubated at pH 3.3 at 25°C for 10 min prior to activity assay against casein. Activity was estimated by absorbance at 750 nm (OD 750).

cysteine proteinases are among the most active in muscle (Barrett, 1986). Their activity is usually controlled *in vivo* by the presence of endogenous inhibitors, such as cystatin. This is an important inhibitor of cysteine proteinase which can bind them extremely tightly but reversibly (An et al., 1994a; Salvesen and Nagase, 1989). This result implied that the proteinases in SWW were in a latent form complexed with proteinase inhibitor.

Identification of proteinase by SDS-substrate gel electrophoresis

The proteinase in SWW and HTSWW was identified by separating the proteins on electrophoresis and staining for proteolytic activity on the SDS-substrate gel (Figs.2.9 and 2.10). The concentrated SWW showed a clearing at M_r 54,200; however, HTSWW showed two clear zones at M_r 54,200 and 39,500 (Fig. 2.9). The clearing at M_r 39,500 corresponded to purified cathepsin L used as reference. Purified cathepsin L on nonreducing condition resolved at M_r 39,500 rather than at M_r 28,800 as previously reported for Pacific whiting cathepsin L (Seymour, 1994). An et al. (1995) reported that heat treatment was the main factor affecting migration rate of purified cathepsin L by complete denaturation of the internal structure of the protein.

To test whether the band at M_r 54,200 was due to a complex with a proteinase inhibitor, the SEC-HPLC fraction (retention time 15.0-15.5 min) of SWW was acidified and heat-treated at 60°C prior to application on SDS-substrate gel followed by staining for proteolytic activity (Fig. 2.10). Acidification has been shown to effectively dissociate proteinase and inhibitor complexes with an increase in activity on synthetic substrate (An et al., 1994b). Acidified SWW showed only one clear band at M_r 39,500 with the disappearance of activity at M_r 54,200, implying that the molecular weight had

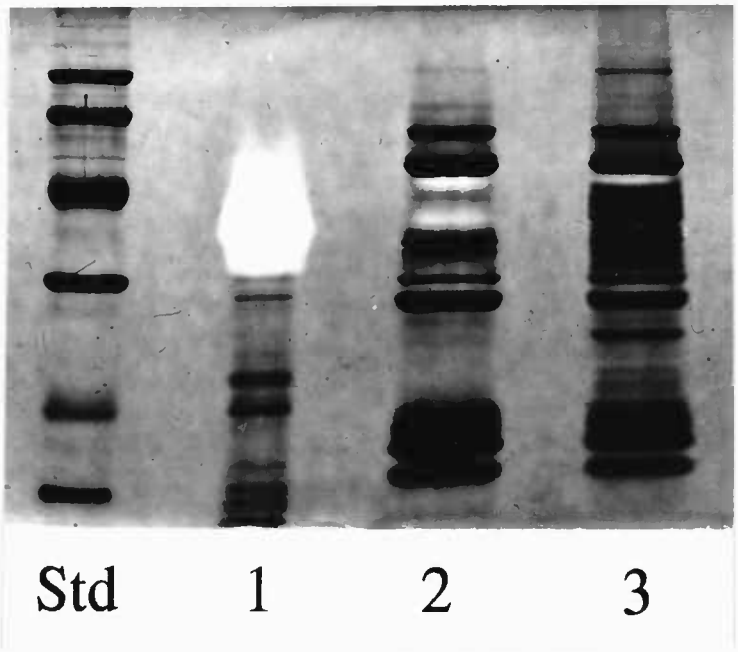

Fig. 2.9. Separation of SWW and HTSWW proteins on SDS-substrate gel electrophoresis followed by staining for proteolytic activity. Std denotes molecular weight protein standards; (1) purified cathepsin L used as a reference; (2) HTSWW proteins; and (3) SWW proteins.

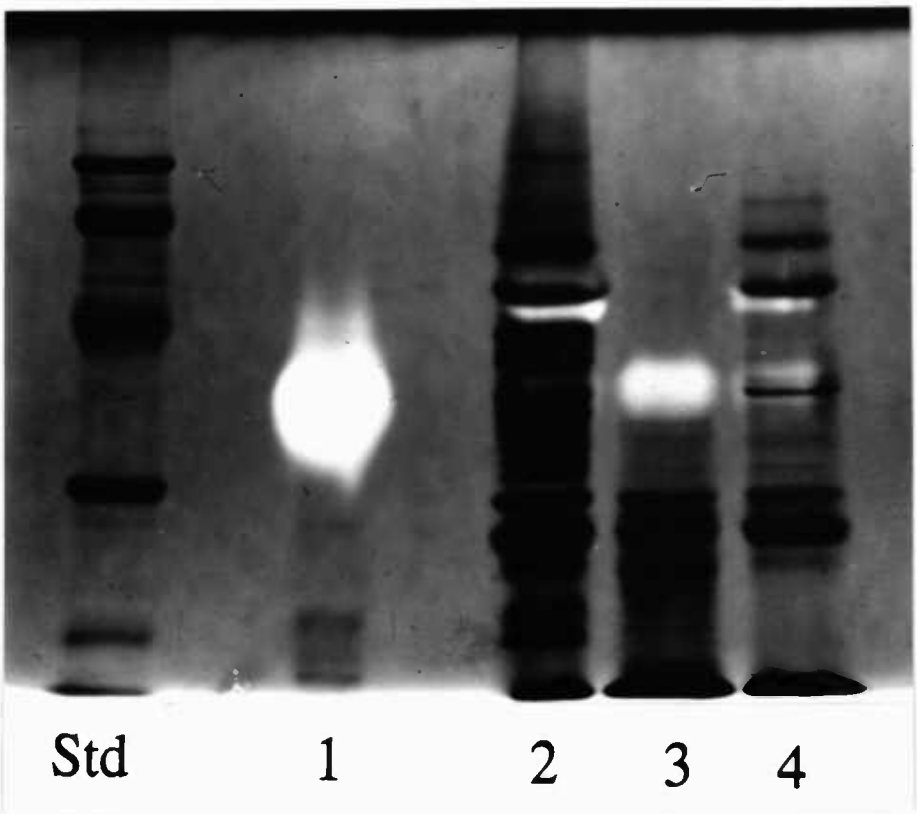

Fig. 2.10. SWW concentrated SEC-HPLC fractions analyzed on SDS-substrate gel and stained for proteolytic activity. The fractions were acidified at pH 3.3 (Lane 3) or heat-treated at 60°C (Lane 4). Purified cathepsin L was used as a reference (Lane 1), and SWW without pretreatment was used as a control (Lane 2). Std denotes molecular weight protein standards.

been reduced by dissociation of the complex. The clearing at M_r 39,500 corresponded to the activity of purified cathepsin L. The dissociation of the complex was also shown by heat treatment of the fraction to a lesser degree. The heat treatment helped partially dissociate the complex, resulting in clearings at both M_r 54,200 and 39,500. The activity zones on SDS-substrate gels of the SWW SEC-HPLC fraction, heat-treated prior to application on the gel, was similar to that observed with HTSWW (Fig. 2.9). Therefore, the clearing at M_r 54,200 is proposed to be a complexed form of cathepsin L. Results indicate that the main proteinase activity was due to cathepsin L in both SWW and HTSWW. The activity may exist in different physical forms in SWW and HTSWW due to presence of dissociation of the enzyme complex.

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Chapter 3

Recovery of Proteinase from Pacific Whiting Surimi Wash Water

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Abstract

A proteinase from Pacific whiting surimi wash water (SWW) was recovered by ohmic heating, ultrafiltration, and freeze drying with the overall yield of 0.83 g proteins/L SWW and 78% recovery of activity. Ohmic heating conditions were optimized for the maximum recovery or the enzyme. Different voltages applied (50, 70, 90 V) showed no differences in efficiency for removing protein and retaining cathepsin L activity. At 90 V, protein content decreased rapidly at temperatures above 40°C. Cathepsin L activity reached the maximum after ohmic heating to 55°C whereas cathepsin B activity decreased constantly with the increased temperature. A constant reduction in protein content was observed with the increase in heating temperatures between 45°C and 60°C and holding time up to 5 min. The highest retention of both total and specific activity of cathepsin L was obtained with the treatment at 55°C for 3 min. On this condition, 193% activity was recovered from SWW, although a large amount of the activity was lost by the subsequent steps of ultrafiltration and freeze drying.

Introduction

An important step in producing high-quality surimi is the washing of fish mince. Washing concentrates myofibrillar proteins, which play an essential role in gel formation, and improves color and flavor of products (Toyoda et al. 1992). Sarcoplasmic proteins, inorganic salts, low-molecular-weight-organic substances, lipids and blood components are removed during washing. Proteinases are also removed resulting in less activity in washed surimi product (An et al. 1994). When mechanically deboned Pacific whiting flesh was washed with two exchanges, 43.7% of proteinase activity remained in surimi (Chang-Lee et al. 1989). In commercial Pacific whiting surimi after washing and passing through screw press, the proteinase activity was reduced to 15% of the original (Morrissey et al. 1995).

There has been a large effort to recover proteins or biologically active components from seafood processing wastes. Maximum utilization of wastes from fisheries has been strongly encouraged due to limited biological resources and increased environmental concerns. Gildberg (1992) recovered pepsin from cod stomach silage preserved with formic acid and trypsin-like enzymes from fish sauce produced by salt fermentation of cod intestines for food, feed and biotechnological purposes. A review of applications of fish proteinases in the seafood industry includes skin and scale removal, roe production, membrane removal, seafood flavoring and pigment recovery (Haard and Simpson, 1994). Lin et al. (1995) recovered proteins from Pacific whiting surimi processing waste water by ultrafiltration, which was supplemented back into surimi to increase yields. This process reduced chemical and microbial loads in wash

water, resulting in a cleaner discharge from the processing plant. Watanabe et al. (1986) recovered soluble proteins from fish jelly processing using membrane technology.

Membrane separation is widely applied in food, biotechnology and petrochemical industries. Ultrafiltration has gained wide acceptance as a method of increased yields for cheese (Phillips, 1989); removing glucosinolates and concentrating proteins from rapeseed meal extract (Lewis and Finnigan, 1989); and use in the brewing industry (Finnigan and Skudder, 1989). However, fouling of membranes is a problem, contributing to a rapid decrease in the flux rate during ultrafiltration processing, though pretreatment of liquids can lead to an increase in ultrafiltration efficiency (Lewis and Finnigan, 1989). Removal of proteins in cottage cheese whey by heating and centrifugation increased the permeate flux by 40% (Tarnawski and Jelen, 1986).

Ohmic heating is known as a method of generating heat rapidly volumetrically throughout a conducting material (Stirling, 1987; Biss et al. 1989). This technique has been used to aseptically process food particulates (Parrott, 1992), or to maximize gel formation of Pacific whiting surimi (Yongsawatdigul et al. 1995) or to remove high-molecular-weight proteins in SWW (Huang et al. 1996). The objective of this study was to apply ohmic heating and ultrafiltration technology and evaluate the processes for proteinase recovery from Pacific whiting SWW.

Materials and Methods

Preparation of surimi wash water

Pacific whiting mince was collected from a commercial surimi processing plant, kept in ice and transported to OSU Seafood Laboratory. The mince was mixed with

distilled water at 3:1 (v/w) ratio and stirred for 5 min. The mixture was filtered with two layers of cheese cloth, and the filtrate was referred to as "surimi wash water" (SWW) (Benjakul et al. 1996).

Ohmic heating of SWW

SWW was heated using an ohmic heating apparatus as described by Huang et al. (1996). SWW (250 mL, 10°C) was heated at different voltages (50, 70 and 90 V). The heated SWW was cooled down rapidly with ice water and centrifuged at 6,500 xg for 30 min at 4°C (Sorvall, DuPont Co., Newtown, CT). Supernatant was referred to as "OHSWW" and used for protein and activity analyses. To optimize the ohmic heating process, heating temperatures of 45, 50, 55, and 60°C were tested with holding time of 0, 1, 3, and 5 min at the designated temperatures.

Ultrafiltration and freeze drying of OHSWW

Lin et al. (1995) used a membrane with 30,000 molecular weight cut off for ultrafiltration to recover proteins in Pacific whiting SWW and found proteinase activity concentrated in the recovered protein fraction. The same molecular weight cut-off membrane was used in this study. To concentrate cathepsin L in OHSWW by ultrafiltration, a Cuno separation system (Commercial Intertech Corp., Norwood, MA) consisting of a processing circulation pump, ultrafiltration cartridge (cut-off M_r 30,000), and cartridge prefilter was used. OHSWW (2 L) was applied in the separation system under the pressure of 30 psi. The retentate was circulated continuously in this separation system until sample volume was reduced to 20-25% of initial volume. Both retentates

and permeates were collected for protein and activity analyses. The retentate was freeze-dried using a freeze dryer (Labconco Corp., Kansas City, MO), and dry crude enzyme powder was collected and kept at −80°C until analyzed.

Cathepsin activity assays

Synthetic substrates, N-carbobenzoxy-Phe-Arg 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec) and Z-Arg-Arg-NMec (Sigma Chemical Co., St. Louis, MO), were used to analyze activity of cathepsins L and B, respectively, according to the method of Barrett and Kirschke (1981). Sample, 100 μL, was diluted to 500 μL with 0.1% (w/v) Brij 35 and preincubated in 250 μL assay buffer at room temperature for 1 min. To the mixture, 250 μL of 20 μM substrate solution was added to initiate the reaction. The reaction was terminated precisely in 10 min by adding 200 μL of 5 mM iodoacetic acid. Methylcoumarin released was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using an Amico-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, MD). One unit (U) of activity was expressed as the amount of enzyme that can release 1 μmol of aminomethylcoumarin in 1 min.

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results and Discussion

Optimization of ohmic heating

Effect of voltages on ohmic heating was evaluated for removing proteins in SWW (Fig. 3.1). No differences in protein content was observed with the different voltages applied, i.e., 50, 70, and 90 V. Protein content decreased gradually as the temperature increased from 45°C to 65°. OHSWW after ohmic heating treatment to 65°C contained 24.7-28.0% proteins compared with SWW. OHSWW obtained from the different voltages applied also showed similar effects on cathepsin L activity over the temperatures studied between 45°C to 65°C (Fig. 3.2). The activity increased to a maximum as SWW was heated to 55°C followed by a decrease at the temperature. The most rapid loss of activity was observed between 60°C and 65°C. Specific activity of OHSWW also showed the similar trends between the different voltages applied. It increased continuously between 45°C and 60°C, showing the maximum at 60°C (Fig. 3.3). At the highest temperature tested, 65°C, the more loss of specific activity occurred with the higher voltages applied.

In general, OHSWW treated with different voltages showed a similar protein content at all the temperatures tested. Also the relative and specific activity of cathepsin L showed the similar temperature profile. Therefore, it is concluded that temperature was the most critical parameter for compositional changes of SWW. The main difference observed between the different voltages tested was with the heating rate. Rate of heating at 50 V was lower than that at 70 or 90 V, and thus it took the longest time to obtain the desired temperature at this voltage. Heating rate is shown to be dependant on the applied voltages, specific heat and conductivity of product, and temperature rise

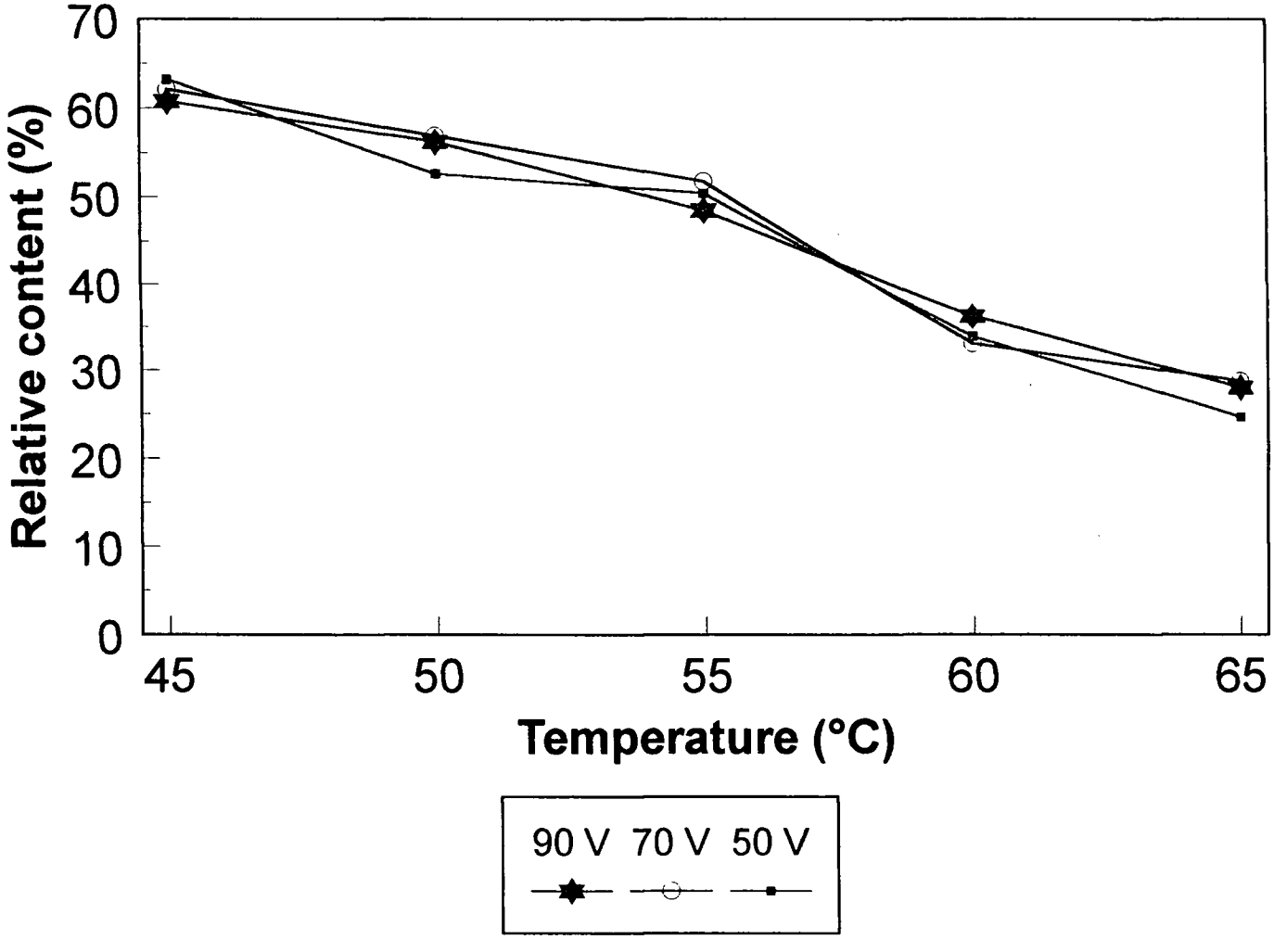

Fig. 3.1.Relative protein content of OHSWW treated at different voltages. Protein content was estimated by Lowry's method and reported as a relative content (%) compared to the original content in SWW.

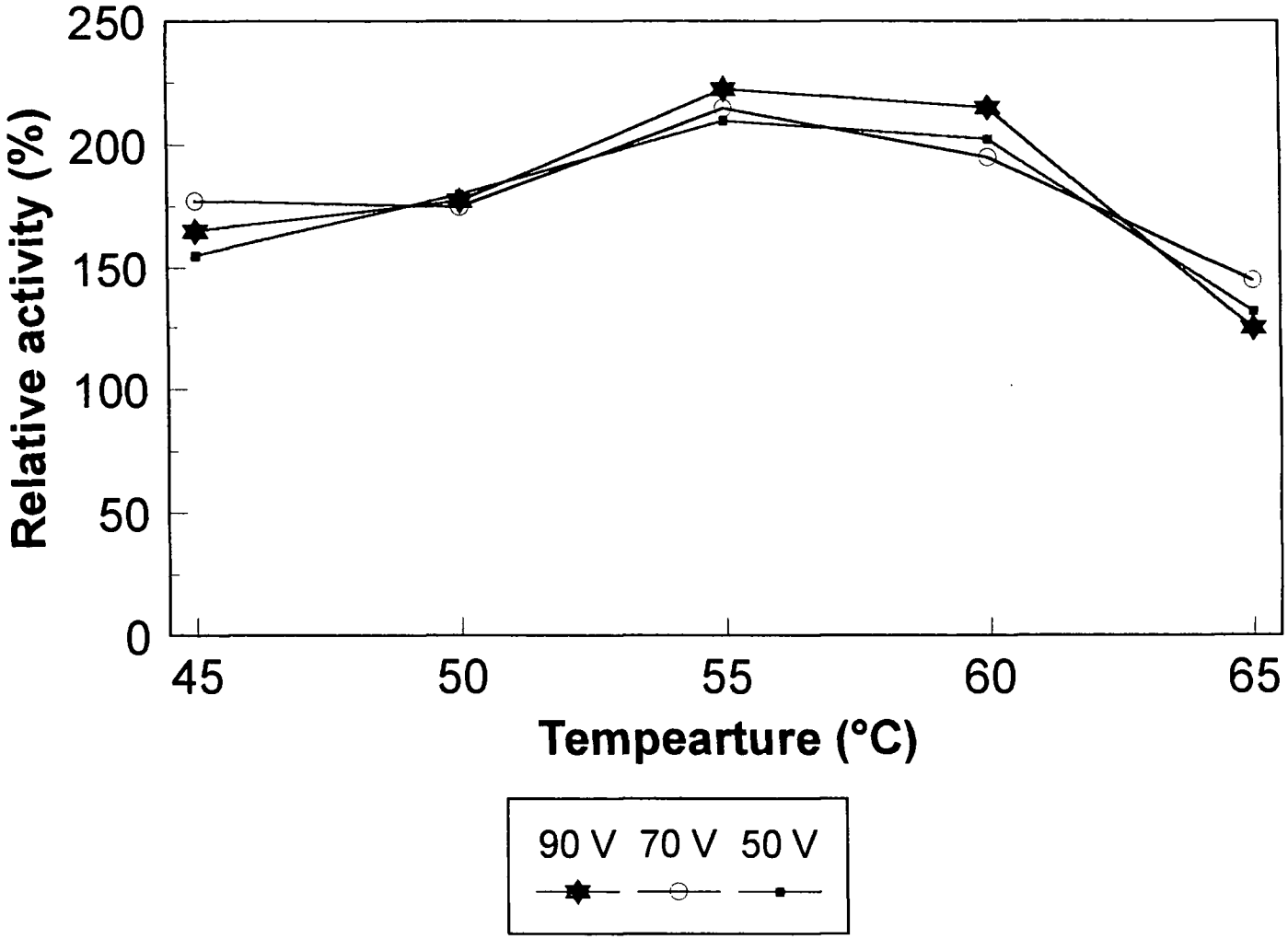

Fig. 3.2. Relative cathepsin L activity in OHSWW treated at different voltages. Activity was measured against Z-Phe-Arg-NMec and expressed as relative activity (%) compared to the original activity in SWW.

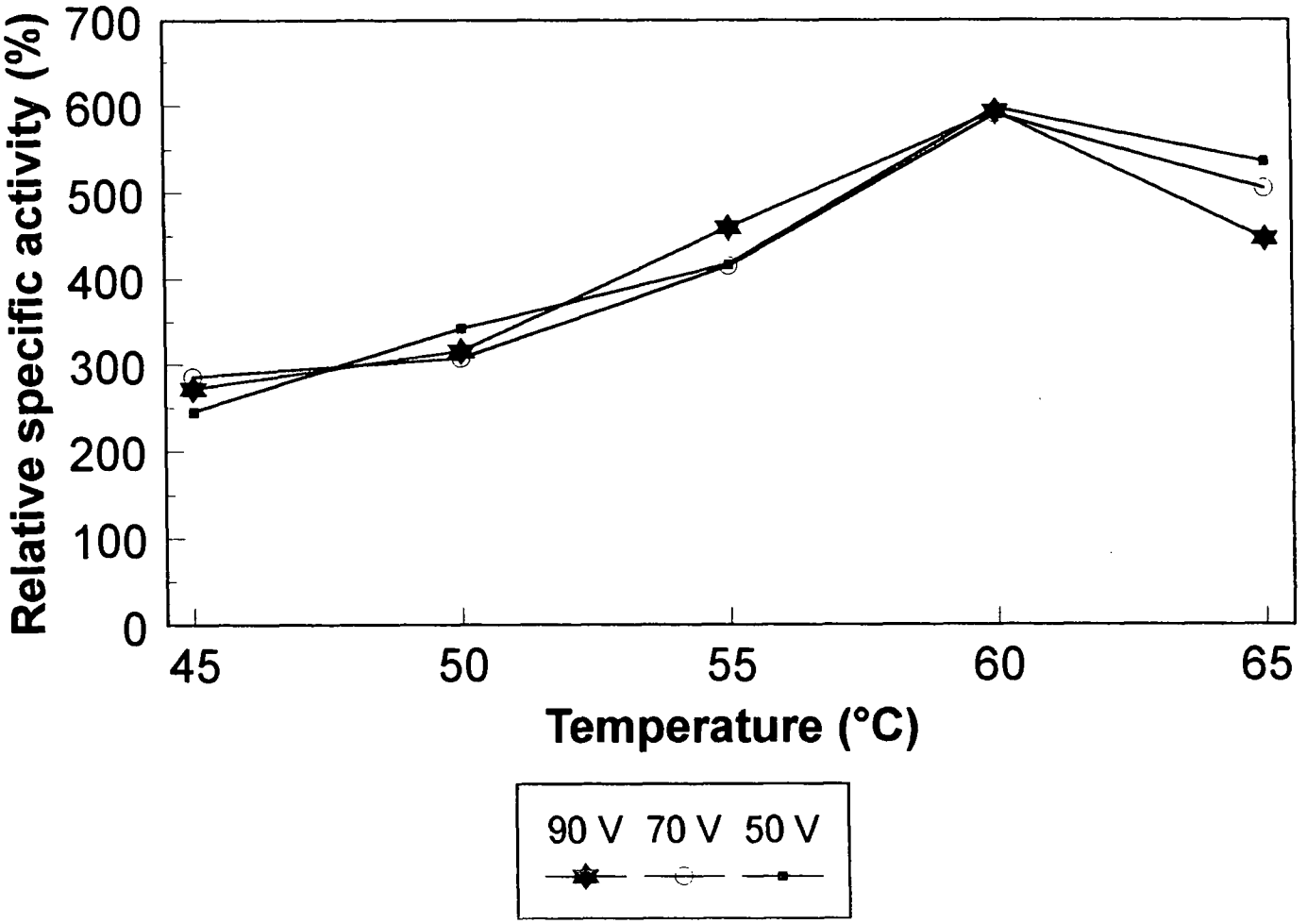

Fig. 3.3. Relative specific cathepsin activity in OHSWW treated at different voltages. Activity was measured against Z-Phe-Arg-NMec, and the protein content was determined by Lowry's method. Relative specific activity (%) was calculated as compared to that of SWW.

in product is directly proportional to (voltage)^2 (Skudder and Biss, 1987). The differences in heating time were more pronounced at the temperatures below 45°C. Sastry and Palaniappan (1992) noted that the heating rate at a constant voltage increased with time as a result of increased conductivities. In this study, 90 V was chosen based on the rapid heating rate.

Effect of ohmic heating on SWW proteins

Ohmic heating treatment at 90 V showed a dramatic reduction in protein content in SWW at the temperatures above 40°C (Fig. 3.4). Changes in protein content between 20 and 40°C were negligible. Protein content of OHSWW obtained at 70°C was reduced to 26% of initial content. Kawai et al. (1992) reported decreased solubility of sarcoplasmic proteins from carp by heating above 40°C. Heating above this temperature disruptions of intramolecular disulfide bridges, ion pair interactions and Van der Waals forces were noted which led to unfolding of proteins (Fryer et al. 1989). Intermolecular bridge can then be formed readily between the unfolded proteins, resulting in aggregation of proteins into insoluble particulates.

OHSWW obtained at 50-55°C contained 56-64% proteins compared with SWW. Apparent coagulation of high molecular-weight proteins in SWW was observed at these temperature ranges. However, the changes in protein content of OHSWW was negligible at temperatures above 65°C. These results were in agreement with Huang et al. (1996) who reported the maximum reduction in protein content, COD and total solids at temperatures above 70°C by ohmic heating of SWW. Li-Chan et al. (1984) reported that heating salt-extractable muscle proteins at 50°C or higher resulted in increased

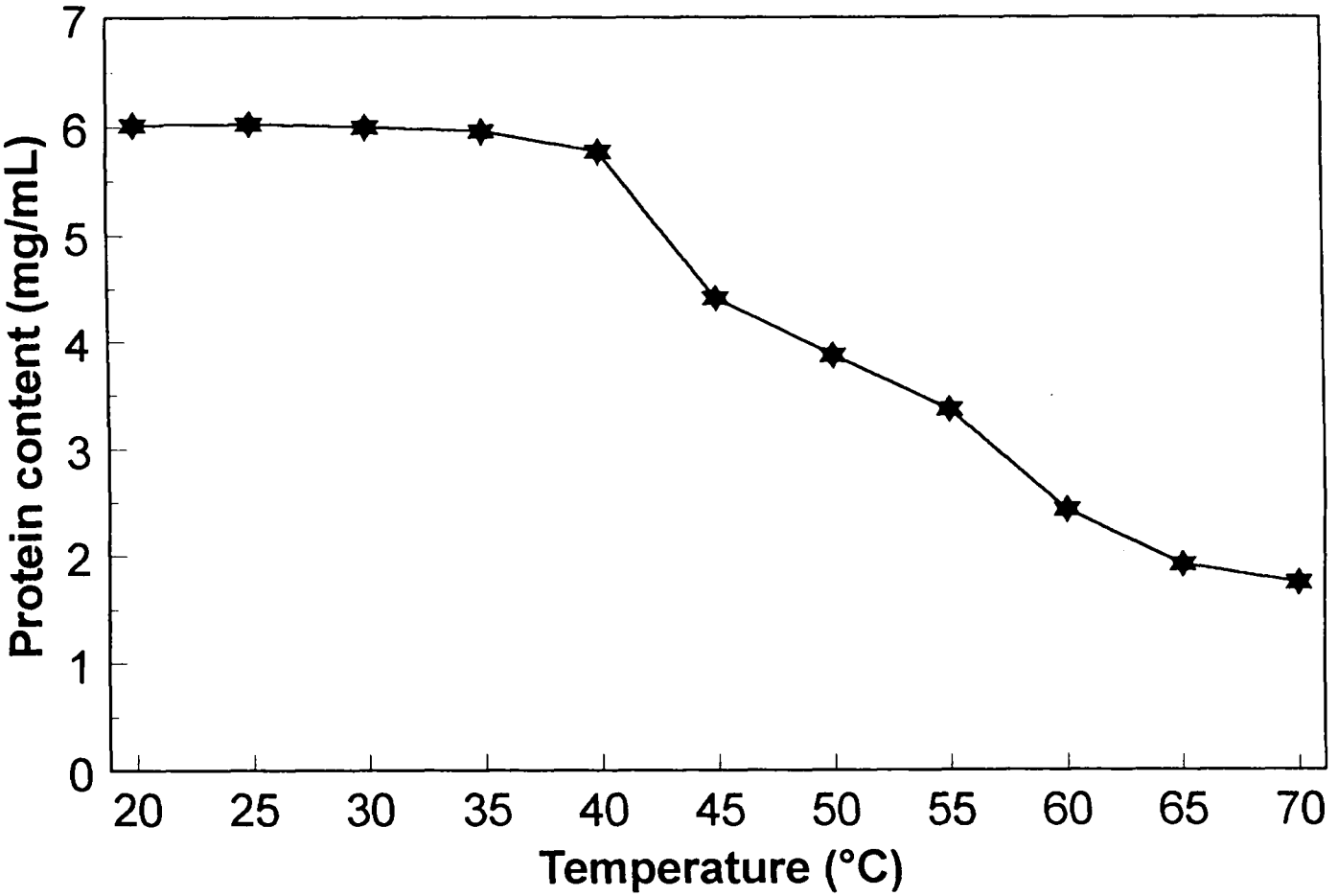

Fig. 3.4. Protein contents of OHSWW obtained at various temperatures. SWW was treated with ohmic heating at 90 V to the desired temperatures, and protein contents were determined by Lowry's method.

hydrophobicity (So) and decreased solubility. The change in high MW proteins during heating is faster than that of water-soluble proteins due to the differences in their hydrophobic amino contents (Nakai and Li-Chan, 1989).

Effect of ohmic heating on cathepsin activities

Cathepsin L activity in OHSWW was higher than that of cathepsin B throughout all temperature ranges studied (Fig. 3.5). This result was in agreement with Benjakul et al. (1996) that cathepsin L was a major proteolytic activity in Pacific whiting surimi wash water. No changes in cathepsin L activity were observed in the range of 20-40°C. Upon heating above 40°C, the activity increased rapidly, reaching the maximum relative activity of 260% at 55-60°C. The activity decreased at temperature above 60°C. At 70°C, the residual activity was 76% of that at 20°C. For cathepsin B activity, there was a gradual decrease as the temperatures increased from 20 to 55°C. No activity was found at temperatures above 55°C.

Effect of ohmic heating time and temperature

Time and temperature were optimized for ohmic heating to coagulate proteins and to maximize the enzyme activity retained. The protein content in OHSWW decreased as the heating time or temperature increased (Fig. 3.6). OHSWW preheated to and held at 45, 50, 55 and 60°C showed the larger changes in protein contents during preheating than the subsequent holding up to 5 min at each temperature. The protein contents in SWW preheated to 45, 50, 55 and 60°C were 73.2%, 61.8%, 49.7% and 35.3%, respectively. Holding at the temperatures further reduced the protein contents

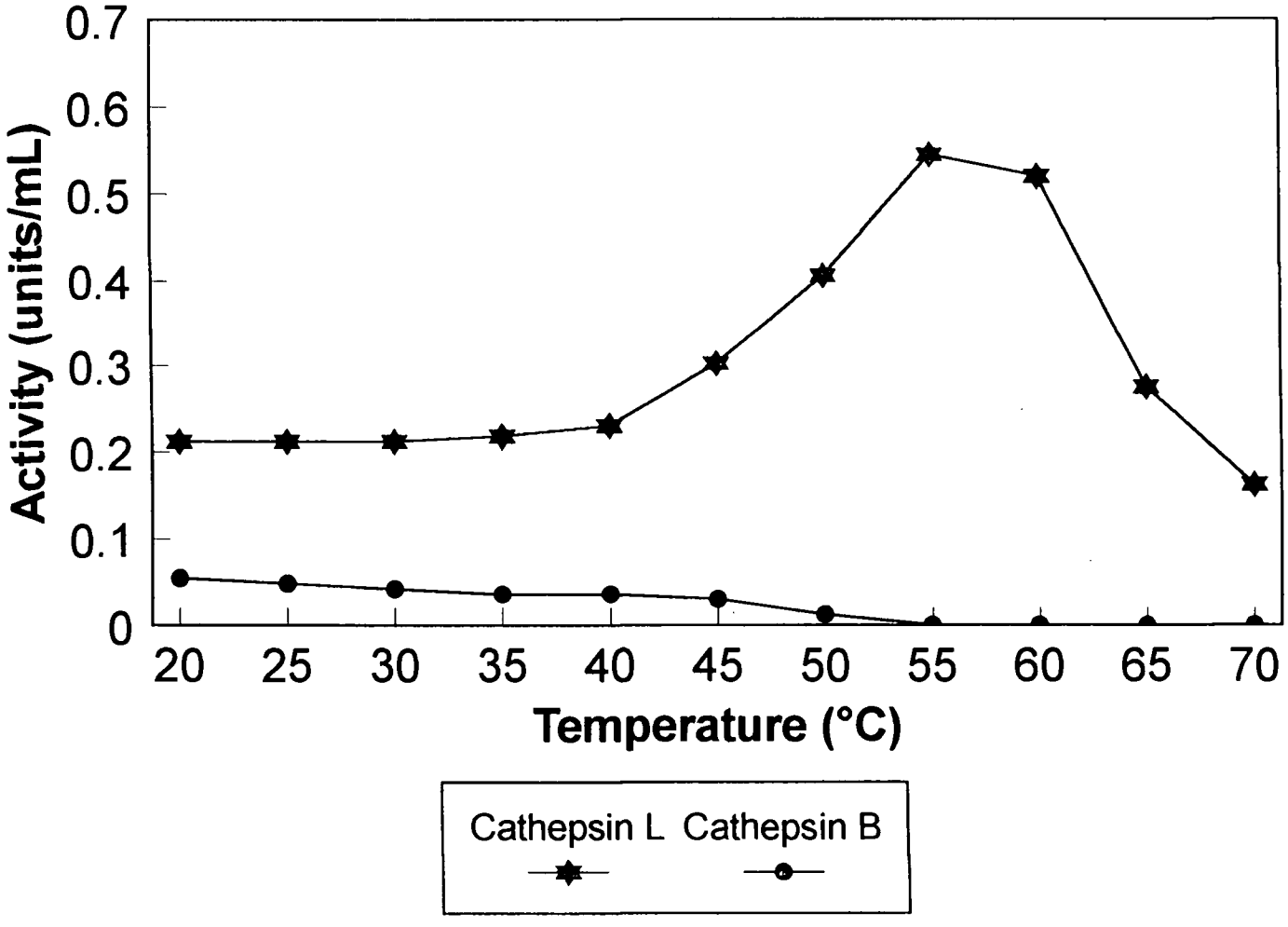

Fig. 3. 5. Cathepsin B and L activities in OHSWW at various temperatures. SWW was treated with ohmic heating at 90 V to the desired temperatures, and cathepsin B and L activities were analyzed against Z-Arg-Arg-NMec and Z-Phe-Arg-NMec, respectively.

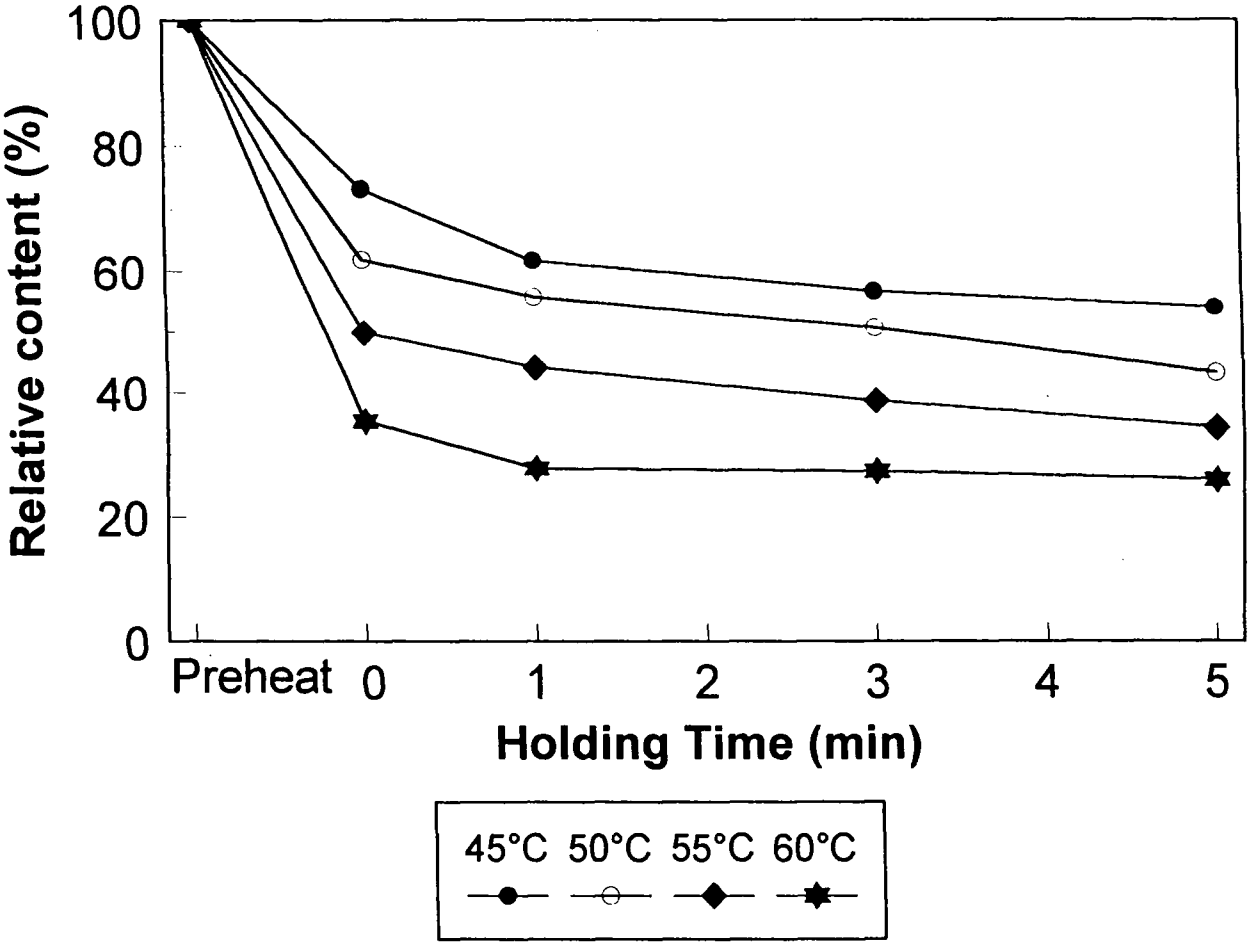

Fig. 3.6. Relative protein contents of OHSWW prepared at different temperatures and holding time. SWW was treated with ohmic heating at 90 V to the desired temperatures and held up to 5 min. Protein content was estimated by Lowry's method and reported as a relative protein content (%) compared to that of original SWW.

to 53.8%, 43.1%, 34.3%, and 26.0% respectively. Therefore, it was indicated that initial preheating was more important to affect protein contents in OHSWW than holding time at the temperatures.

Ohmic heating reduced cathepsin L activity slightly with the increase in holding time at 50 and 55°C (Fig. 3.7). The activity in OHSWW at 60°C was 69, 44, and 29% after holding for 1, 3 and 5 min, respectively. However, at 45°C the activity of OHSWW increased with the holding time, showing the maximum activity at 3 min. When specific activity was monitored on these conditions, a slightly different pattern was observed. The specific activity of cathepsin L in OHSWW at these heating conditions showed that ohmic heating increased the specific activity up to 3 min at 45, 50 and 55°C (Fig. 3.8). The specific activity was 132% of that of control (0 min) at 55°C when held for 3 min at this temperature. In contrast, specific activity decreased rapidly at 60°C, as holding time increased. At 60°C, activity decreased to 87, 57, and 39% of the control (0 min) by holding for 1, 3 and 5 min, respectively. Seymour et al. (1994) reported four-fold increase in enzyme activity by heat treatment at 60°C for 3 min presumably by a slower heating rate by the conventional method. Ohmic heating has been reported to be rapid and effective for heating rate and temperature control (Stirling, 1987; Biss et al. 1989). Since both holding time and heating temperature can affect specific activity of cathepsin L in SWW, preheating to and holding for 3 min at 55°C was chosen as the optimum condition for ohmic heating treatment to recover proteinase from SWW.

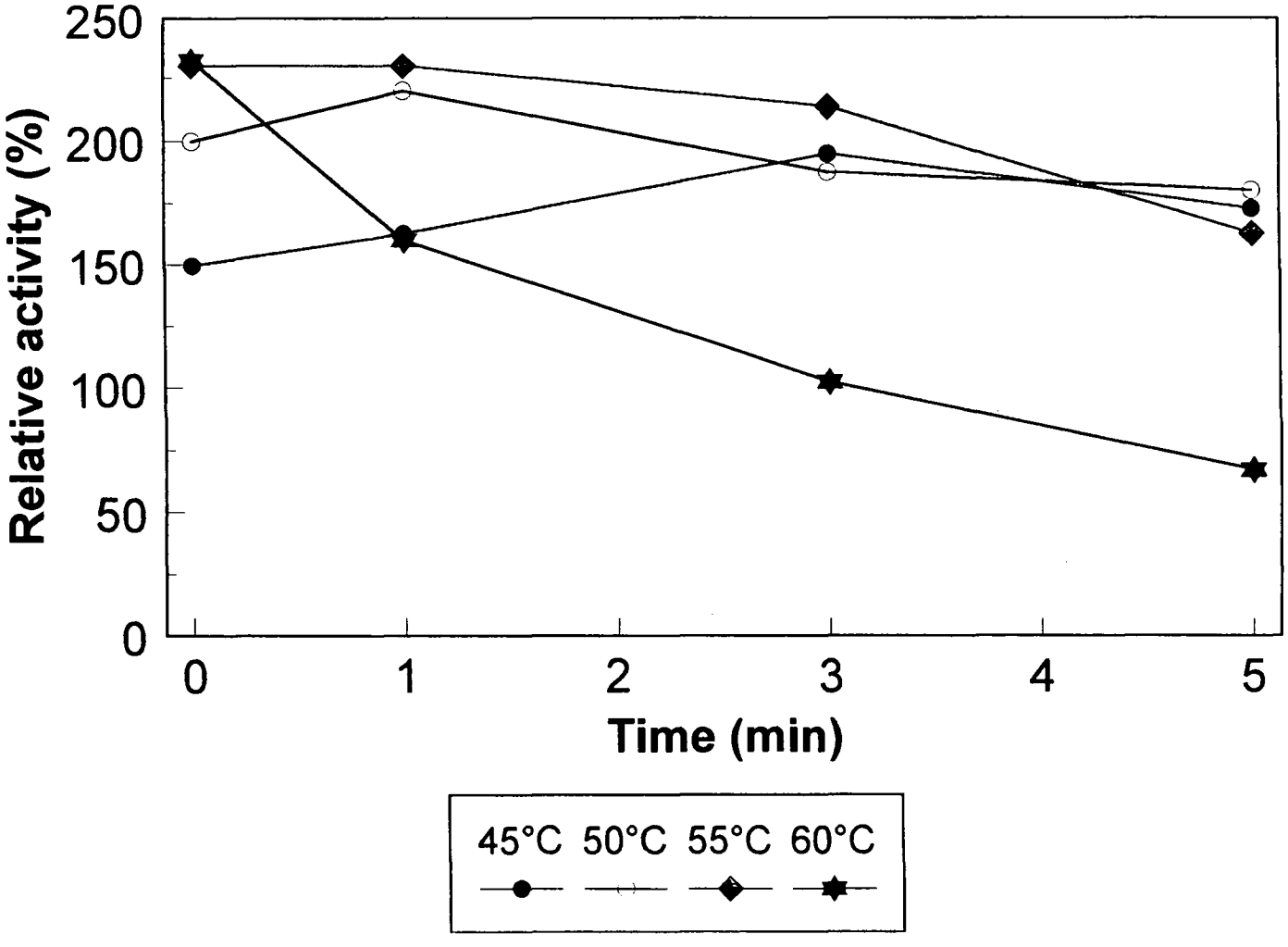

Fig. 3.7. Relative cathepsin L activity in OHSWW prepared at different temperatures and holding time. SWW was treated with ohmic heating at 90 V to the desired temperatures and held up to 5 min. Activity was measured against Z-Phe-Arg-NMec and expressed as relative activity (%) compared with that of original SWW.

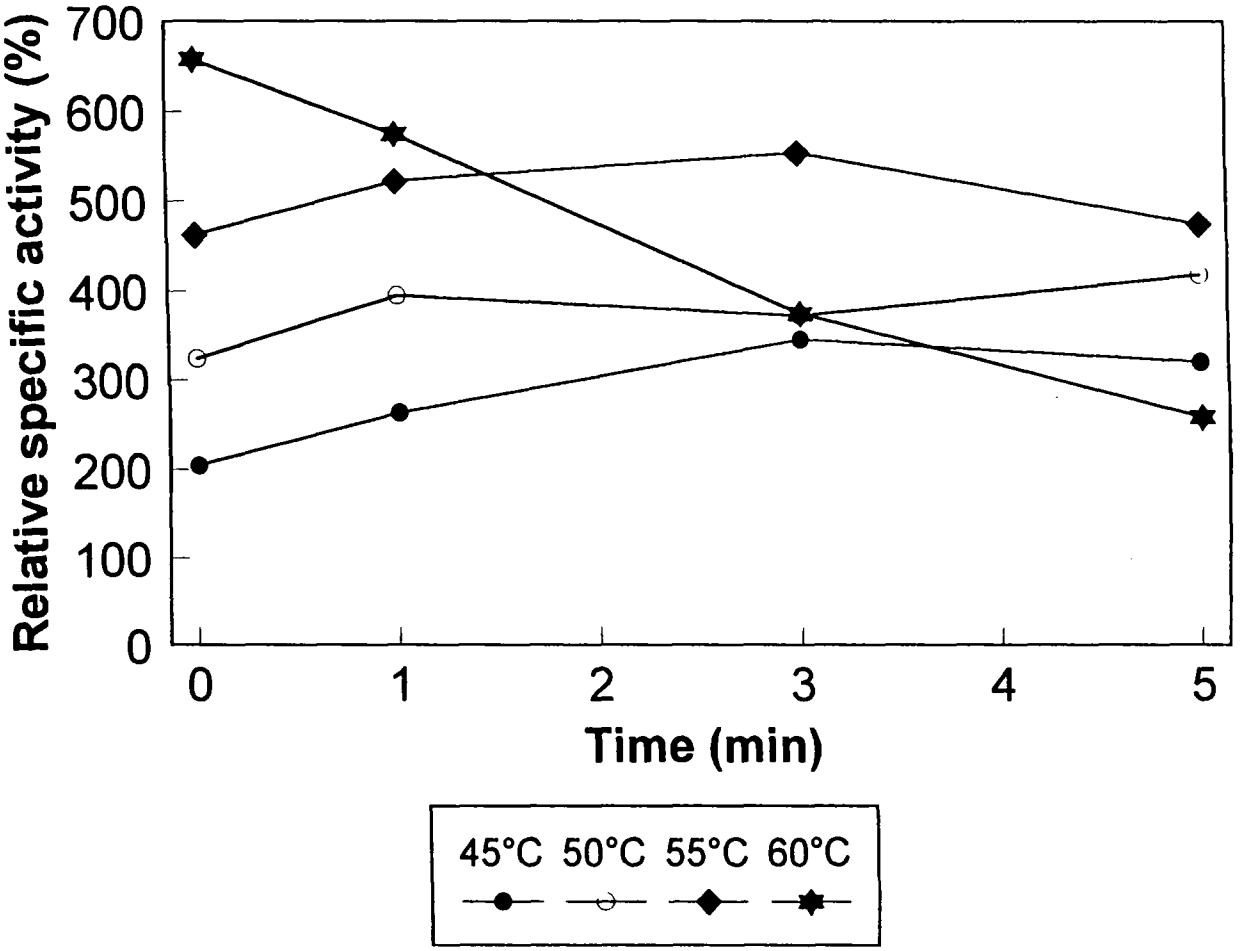

Fig. 3.8. Relative specific cathepsin activity in OHSWW prepared at different temperatures and holding time. SWW was treated with ohmic heating at 90 V to the desired temperatures and held up to 5 min. Activity was measured against Z-Phe-Arg-NMec, and the protein content was determined by Lowry's method. Relative specific activity (%) was calculated by comparing to the specific activity of SWW.

Evaluation of proteinase recovery from SWW

The yield of recovered proteinase was 0.83 g protein/L SWW. The relative recovery for each step was 193, 95, and 78% for ohmic heating, ultrafiltration and freeze-drying step, respectively (Table 3.1). Two fold increase in relative recovery was observed in OHSWW. This was postulated that heat treatment dissociated the enzyme complex and liberated the active free form (Benjakul et al. 1996). A large portion, 40.2%, of total activity in OHSWW was lost during the ultrafiltration step. The loss may be due to the high pressure applied during the precess as well as the penetration of enzyme through the membrane. Eleven percent of OHSWW activity was found in permeate. Richardson and Hyslop (1985) reported that high pressure can inactivate enzymes. By freeze drying, only 17.9% of activity was lost from the fraction concentrated by ultrafiltration. Overall, the processes used resulted in a 78% recovery of proteinases from SWW. The recovered proteinase might be applied as a processing aid in the food processing.

Table 3.1. Proteinase recovery from SWW by the processes

Recovery process	% Recovery
SWW	100
Ohmic heating	193
Ultrafiltration	95
Freeze drying	78

Acknowledgement

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Chapter 4

Characterization of Cathepsin L Recovered from Pacific Whiting Surimi Wash Water

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Abstract

Purification characteristics were studied for Pacific whiting surimi wash water (SWW) proteinase recovered by ohmic heating, ultrafiltration, and freeze-drying. By these processes, 5.9 purification fold was achieved, and the most efficient step was ohmic heating, which concentrated the proteinase by 4.8 fold. Specific activity of the recovered SWW proteinase on casein and Z-Phe-Arg-NMec was 28.2 and 0.17 U/mg protein, respectively. The SWW proteinase showed a preferable hydrolytic activity toward casein, acid-denatured hemoglobin and myofibrils. Acidification increased specific activity on all substrates tested but reduced thermal stability. β-Mercaptoethanol (βME), dithiothreitol and urea enhanced activity against Z-Phe-Arg-NMec. Proteinase activity on Z-Phe-Arg-NMec showed an optimum pH of 4.0. The recovered proteinase showed 18.5% residual activity after 7 wk storage at 4°C.

Introduction

There has been increased interest in utilizing seafood processing waste due to limited biological resources and increased environmental concerns. Attention has been given to proteinases due to their essential role in food processing, biotechnology and other industries. Proteinases are normally present in muscle cells, extracellular matrix and connective tissues surrounding muscle cells, and digestive or other organs (Haard 1994). Pepsin, trypsin and chymotrypsin have been detected in digestive organs of many fishes (Brewer et al. 1984; Hjelmeland and Raa 1982; Clark 1985; Martinez and Serra 1989). Pepsin was concentrated from an acid aqueous autolysate of cod viscera silage by ultrafiltration (Gildberg and Almas 1986; Gildberg 1992). Trypsin-like enzyme was also recovered from fish sauce produced by salt fermentation of cod intestines (Gildberg 1992). Fish muscles contain about 10 times more catheptic activities than mammalian muscles (Haard and Simpson 1994). The proteolytic activity plays a major role in muscle softening (Wasson 1990). Cathepsins have been isolated and characterized from a number of aquatic species including Pacific whiting (Seymour *et al.* 1994), chum salmon (Yamashita and Konagaya 1990a,b, 1991), mackerel (Jiang *et al.* 1994), tilapia (Sherekar *et al.* 1988), and carp (Hara *et al.* 1988).

Cathepsins in Pacific whiting have been shown to cause post-harvest muscle softening (An et al., 1994b). However, a substantial amount of enzymes are removed during the washing process in surimi production (An *et al.* 1994b; Morrissey *et al.* 1995; Chang-Lee *et al.* 1989). The remaining proteolytic activity has been controlled by use of food-grade inhibitors such as BPP, egg white, potato powder, whey protein concentrate (Morrissey *et al.* 1993; Weerasinghe *et al.* 1996). Recently, a major

proteinase from Pacific whiting SWW was characterized as cathepsin L with an apparent M_r of 39,500 (Benjakul *et al.* 1996). Recovery processes of the proteinase which included ohmic heating, ultrafiltration and freeze-drying with the yield of 0.83 g/ L and activity recovery of 78% were developed (Benjakul *et al.* 1997). The objective of this study was to characterize the biochemical properties and the stability of the recovered proteinase from Pacific whiting SWW.

Materials and methods

Chemicals

N-carbobenzoxy-Phe-Arg 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), azocasein, hide powder azure, hemoglobin, βME, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium caseinate was purchased from US Biochemical Corp. (Cleveland, OH). Iodoacetic acid was obtained from Calbiochem Co. (La Jolla, CA)

Recovery of SWW proteinase

SWW was prepared according to the method of Benjakul *et al.* (1996). The proteinase was recovered according to the method of Benjakul *et al.* (1997) by ohmic heating at 55°C for 3 min at 90 V, concentrated by ultrafiltration at 30 psi using a Cuno separation system (Commercial Intertech Corp., Norwood, MA) and freeze-dried to produce crude enzyme powder (Labconco Corp., Kansas City, MO).

Cathepsin activity assays

Cathepsins L activity was analyzed by the method of Barrett and Kirschke (1981) using Z-Phe-Arg-NMec as a substrate. Sample, 100 μL, was diluted to 500 μL with 0.1% (w/v) Brij 35 and preincubated in 250 μL assay buffer at room temperature for 1 min. To the mixture, 250 μL of 20 μM Z-Phe-Arg-NMec was added to initiate the reaction. The reaction was terminated precisely in 10 min by adding 200 μL of 5 mM iodoacetic acid. Methylcoumarin released was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using an Amico-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, MD). One unit of activity was expressed as the amount of enzyme that can release 1 μmol of aminomethylcoumarin in 1 min.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Substrate specificity

Various protein substrates, i.e., azocasein, hide powder azure, casein, hemoglobin, acid-denatured hemoglobin, BSA, and Pacific whiting myofibrils, were tested for specificity of enzyme hydrolysis. For each reaction mixture, 2 mg of the substrates were added to MacIlvaine's buffer, pH 5.5 (625 μL) and the final volume was adjusted to 1.15 mL with deionized water. The mixture was preincubated at 55°C prior to adding 100 μL enzyme or acidified enzyme solution as described in the section "Acidification

of enzyme". The enzyme solutions were prepared at 1% (w/v) in MacIlvaine's buffer, pH 5.5. The activity was analyzed by TCA-Lowry assay (An *et al.* 1994a). With azocasein, the TCA-soluble supernatant (800 μL) was added with 60 μL of 10 N NaOH, and the activity was reported as Δ_{450}. Hide powder azure assay was carried out according to the modified method of Rinderknecht *et al.* (1968). Ten mg of substrate was added to the reaction buffer prepared as described above. The reaction was terminated by adding TCA, and activity was expressed as Δ_{595}. Blank was prepared by adding 50% (w/v) TCA prior to initiation of reaction.

Acidification of enzyme

Acidified enzyme solution (1%, w/v) was prepared according to the method of An *et al.* (1995). The recovered SWW enzyme powder was dissolved in a 0.05 M sodium citrate-0.05 M phosphate buffer, pH 3.3 and incubated at room temperature for 10 min prior to activity assay.

Thermal stability of enzyme

Acidified and non-acidified enzyme solutions (1%, w/v) were added to cathepsin L assay buffer preincubated at 0, 25, 35, 45, 55, 65, and 75°C and held at the temperatures for 10 min. The mixture was rapidly cooled in ice water. The residual activity was assayed against Z-Phe-Arg-NMec.

pH stability of enzyme

Enzyme powder was dissolved in deionized water at 2% (w/v) and was mixed with a buffer at the ratio of 1:1 (v/v). For pH 3 through 8, MacIlvaine's buffer was used; and for pH 9, 0.2 M Tris-HCl was used. The reaction mixtures were added with or without 20 mM βME and incubated at 25°C for 30 min. The residual activity was measured against Z-Phe-Arg-NMec at pH 5.5 as described above.

Effect of chemicals on enzyme activity

Solutions of βME (10, 20, and 40 mM) and dithiothreitol (10, 20, and 40 mM), urea (1.0, 2.0, and 5.0 M), and NaCl (0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 M) were prepared and mixed at 1:1 ratio (v/v) with the crude enzyme solution in water at 2% (w/v). The mixtures were kept at ambient temperature for 15 min, and residual activity was analyzed against Z-Phe-Arg-NMec.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) using 4% stacking gel and 12.5% separating gel. SDS was added to the samples of SWW, OHSWW, retentate, and permeate to give the final concentration of 5% (w/v), and were heated at 85°C for 1 hr. To remove the undissolved debris, the solutions were centrifuged at 5,000 *xg* for 5 min (Eppendorf Micro Centrifuge, Model 5415C, Brinkmann, New York, NY) at ambient temperature. Supernatant was mixed at 1:1 (v/v) ratio with the treatment buffer containing 1.5 M βME and boiled for 3 min. The samples, 60 μg, were applied on the

gel except for permeate. Only 10 μg was loaded for permeate due to the low protein content. The gel was stained with 0.125% Coomassie brilliant blue R-250 in 25% ethanol and 10% acetic acid and destained with 25% ethanol and 10% acetic acid. Molecular weight was estimated using high and low molecular weight standards. High molecular weight standards (Sigma Chemical Co., St. Louis, MO) included rabbit muscle myosin (205,000), *E.coli* β-galactosidase (116,000), rabbit muscle phosphorylase b (97,000), BSA (66,000), ovalbumin (45,000) and bovine erythrocytes carbonic anhydrase (29,000). Low molecular weight standards (Phamacia Biotech, Inc., Piscataway, NJ) included phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Results and discussion

Efficiency of enzyme recovery

Crude SWW enzyme powder was obtained with the yield of 0.83 g of protein/L and the recovery of 78% total activity by procedures listed in Table 4.1. Ohmic heating increased the total activity to 193%, resulting in a 4.8-fold purification. The increase in total activity was due to dissociation of cathepsin L complex by heat treatment (Benjakul *et al.* 1996). During the subsequent ultrafiltration step, a large portion, 40.2%, of the total activity in OHSWW was lost, although a 1.4-fold purification was achieved when the specific activities of OHSWW and retentate were compared. Eleven percent of the total activity found in OHSWW was found in the permeate, indicating some loss of the enzyme through the membrane. The final enzyme preparation showed an overall 5.9-fold purification. The estimated concentration factors based on protein substrates, i.e.,

Table 4.1. Purification and recovery analyses

Recovery process	Amount	Total proteins (g)	Total activity* (U)	Specific activity (U/mg)	% Recovery	PF'
SWW	2,000 mL	12.3	360.0	0.029	100	1.0
OHSWW	1,880 mL	1.9	695.6	0.139	193	4.8
Retentate	450 mL	1.8	342.0	0.187	95	6.5
Permeate	1,230 mL	0.59	73.8	0.125	20	4.3
Enzyme powder	3.91 g	1.65 g**	281.5**	0.171	78	5.9

* Activity was assayed using Z-Phe-Arg-NMec as a substrate.
**Enzyme solution was prepared at 1% (w/v) in MacIlvaine buffer, pH 5.5 to determine protein content and activity.
'Purification fold

casein and acid-treated hemoglobin, were 2.50 and 1.75-fold, respectively (Table 4.2). As was observed with Z-Phe-Arg-NMec, the highest purification in these procedures was achieved by ohmic heating, resulting in a 2.00 and 2.35-fold purification as estimated with casein and acid-treated hemoglobin, respectively.

Protein patterns in SWW during enzyme recovery process

Types of proteins present in SWW, OHSWW, the retentate, and the permeate were analyzed by SDS-PAGE (Fig. 4.1). SWW contained a range of proteins with M_r 10,300-205,000 (Lane 1). The high-molecular-weight proteins larger than M_r 94,000 detected in SWW were presumed to be myosin heavy chain (M_r 205,000) and its degradation products, which were removed during the washing process. Lin *et al.* (1995) reported that a large amount of myofibrillar proteins were lost during washing and dewatering.

In OHSWW, Proteins with high molecular weight were removed. Proteins with M_r 52,220 and 49,380 were absent, and proteins with M_r 64,120 and 27,180 were reduced (Lane 2). Huang *et al.* (1996) reported that the SWW protein with approximately M_r 40,000 began to disappear after ohmic heating to 60°C. In general, high molecular weight proteins were more affected by heating. Cheng and Parrish, Jr. (1979) reported that heavy and light chains of myosin were heat labile and were easily precipitated at 55°C, while actin, troponin, and tropomyosin were suspended in the solution until they precipitated at 70-80°C. Normally, fish myosin is unstable compared with that of mammals. The stability also can vary among species of fishes (Ogawa et al. 1993).

Table 4.2. Specific activity of SWW enzyme during recovery process.

Recovery process	Specific activity (U/mg protein)		
	Casein	Acid-treated hemoglobin	Z-Phe-Arg-NMec
SWW	11.3	12.4	0.029
OHSWW	22.7	29.1	0.139
Retentate	26.4	22.1	0.187
Crude enzyme (freeze-dried)*	28.2	21.7	0.171

* The enzyme was prepared at 1% (w/v) in MacIlvaine buffer, pH 5.5

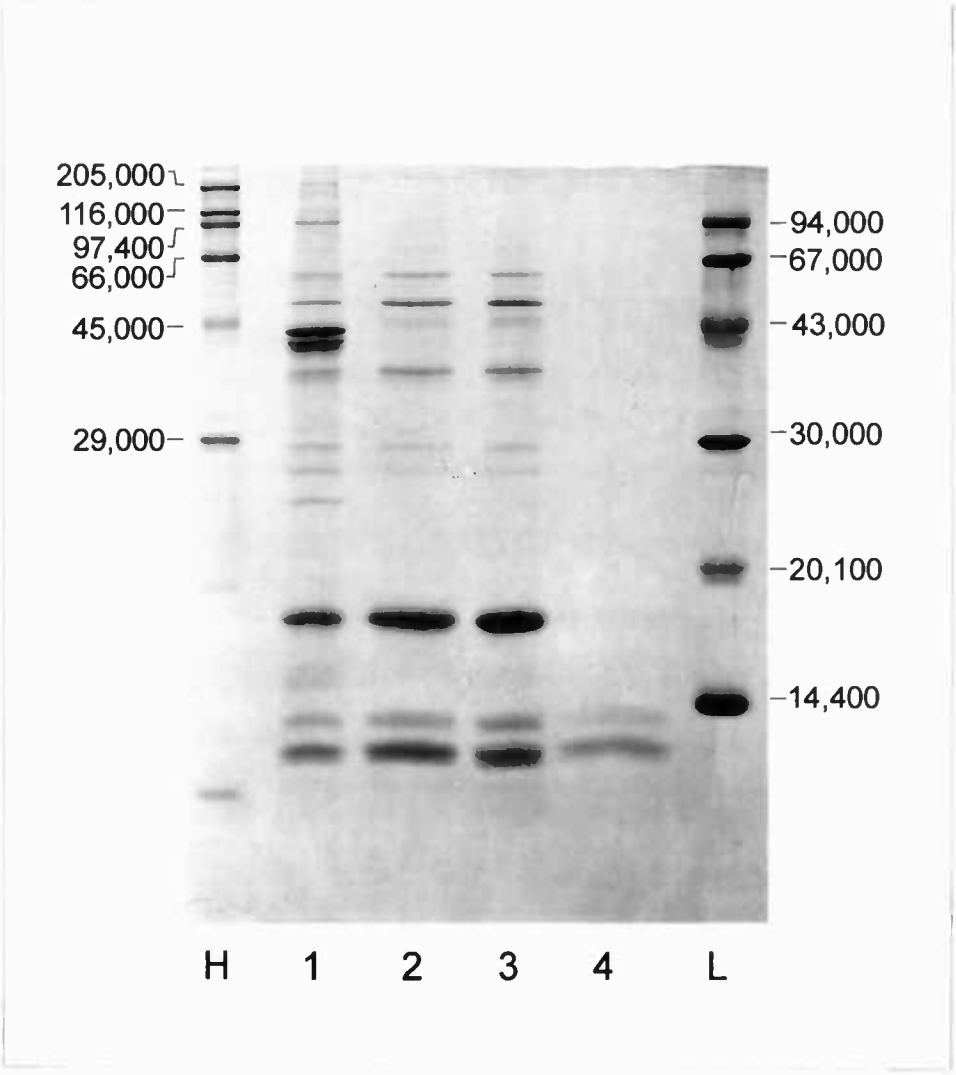

Fig. 4.1. SDS-PAGE analysis of SWW proteins on the various steps of the recovery process. Sixty μg protein was applied on 15% polyacrylamide gel for SWW, OHSWW and retentate. Ten μg protein was applied for permeate. (H) the high molecular weight standards; (1) SWW; (2) OHSWW; (3) retentate; (4) permeate; and (L) low molecular weight standards.

The ohmic heating treatment did not remove the low molecular weight proteins (M_r 17,370; M_r 11,740; and M_r 10,300). This result was in agreement with Kawai *et*al. (1992), who found that a M_r 12,000 protein in carp sarcoplasmic proteins was still soluble at 70°C or above and was postulated to be parvalbumin. Sarcoplasmic proteins from Pacific mackerel remained above 60°C (Morioka and Shimizu 1992).

The retentate of ultrafiltration showed the identical protein pattern as that of OHSWW (Lane 3), while the permeate showed only small molecular weight proteins with M_r 11,740 and 10,300 (Lane 4). The proteins with M_r 17,370 or above were not filtered through the membrane even though membrane with molecular weight cut-off of 30,000 was used. This may be due to the fouling of proteins, which can reduce the pore size. This result indicates that the large activity loss encountered by the ultrafiltration step was not due to the loss of enzyme by permeation through the membrane.

Substrate specificity of the recovered enzyme

The recovered SWW enzymes showed some differences in substrate hydrolysis (Table 4.3). Casein, acid-denatured hemoglobin and myofibrils were hydrolyzed more readily than native hemoglobin or BSA. Yamashita and Konagaya (1991) reported that myofibrillar proteins, which has undergone denaturation, were more susceptible to hydrolysis by enzymes. The recovered enzyme also showed a hydrolytic activity on hide powder azure. Cathepsin L displays a higher activity than other lysosomal enzymes in degrading various protein substrates (Barrett and Kirschke 1981; Okitani *et al.* 1980). Cathepsin L was reported to have high proteolytic activity towards azocasein, hemoglobin, serum albumin (Yamashita and Konagaya 1990a) and major components

Table 4.3. Substrate specificity of the recovered SWW enzyme

Substrates	Non-acidified	Acidified
Casein[1]	9.75	16.10
Hemoglobin[1]	3.31	5.53
Acid-denatured hemoglobin[1]	8.98	12.32
BSA[1]	5.17	8.28
Myofibril[1]	8.49	10.89
Azocasein[2]	0.123	0.247
Hide powder azure[3]	0.193	0.386

[1] Activity is expressed as μmol tyrosine/g enzyme/min.
[2] Activity is reported as Δ_{450}.
[3] Activity is expressed as Δ_{595}.

of myofibrils, such as connectin, nebulin, myosin, α-actinin and troponin (Yamashita and Konagaya 1991). Purified Pacific whiting cathepsin L hydrolyzed myofibrils, myosin,and native or heat-denatured collagens (An *et al.* 1994b). Cathepsin L was responsible for muscle autolysis causing the extensive muscle softening in Pacific whiting (Morrissey *et al.* 1993;An *et al.* 1994b) and chum salmon (Yamashita and Konagaya 1990c, 1992). Acidification of recovered enzyme increased hydrolytic activity on all the substrates tested.

Thermal stability of recovered enzyme

Acidified enzyme showed lower thermal stability than non-acidified (Fig. 4.2). It was postulated that the free enzyme dissociated from the complex was more susceptible to denaturation. The activity of acidified enzyme was 2.7-fold higher than the non acidified at 55°C. Both acidified and non-acidified enzyme activity was reduced substantially at temperatures above 55°C and completely disappeared at 75°C. For the non-acidified enzyme, a two-fold increase in activity was observed at 55°C compared with 0°C. However, 60% residual activity was observed for acidified enzyme at the same temperature. Benjakul *et al.* (1996) reported that Pacific whiting SWW proteinase existed in a complex form with an inhibitor, and the apparent molecular mass of the complex was M_r 54,200. The complex was easily dissociated by heat treatment to the free form with an apparent M_r 39,500.

Effect of chemicals on recovered enzyme

βME, dithiothreitol and urea increased activity of the recovered enzyme (Table 4.4). Dithiothreitol showed 2.6 to 3.7-fold higher enhancement of activity than βME at 5-20 mM. In this range, activity increased with the increased concentration of reducingagents. Increase in relative activity of 204 and 537% was observed with 20 mM βME and dithiothreitol, respectively. Cathepsin L is a cysteine proteinase and requires the sulfhydryl groups to be reduced for catalytic activity (Asghar and Bhatti 1987).

The SWW proteinase activity was highly enhanced by urea (Table 4.4). The activity was increased to 138% by addition of 2.5 M urea, although the effect of

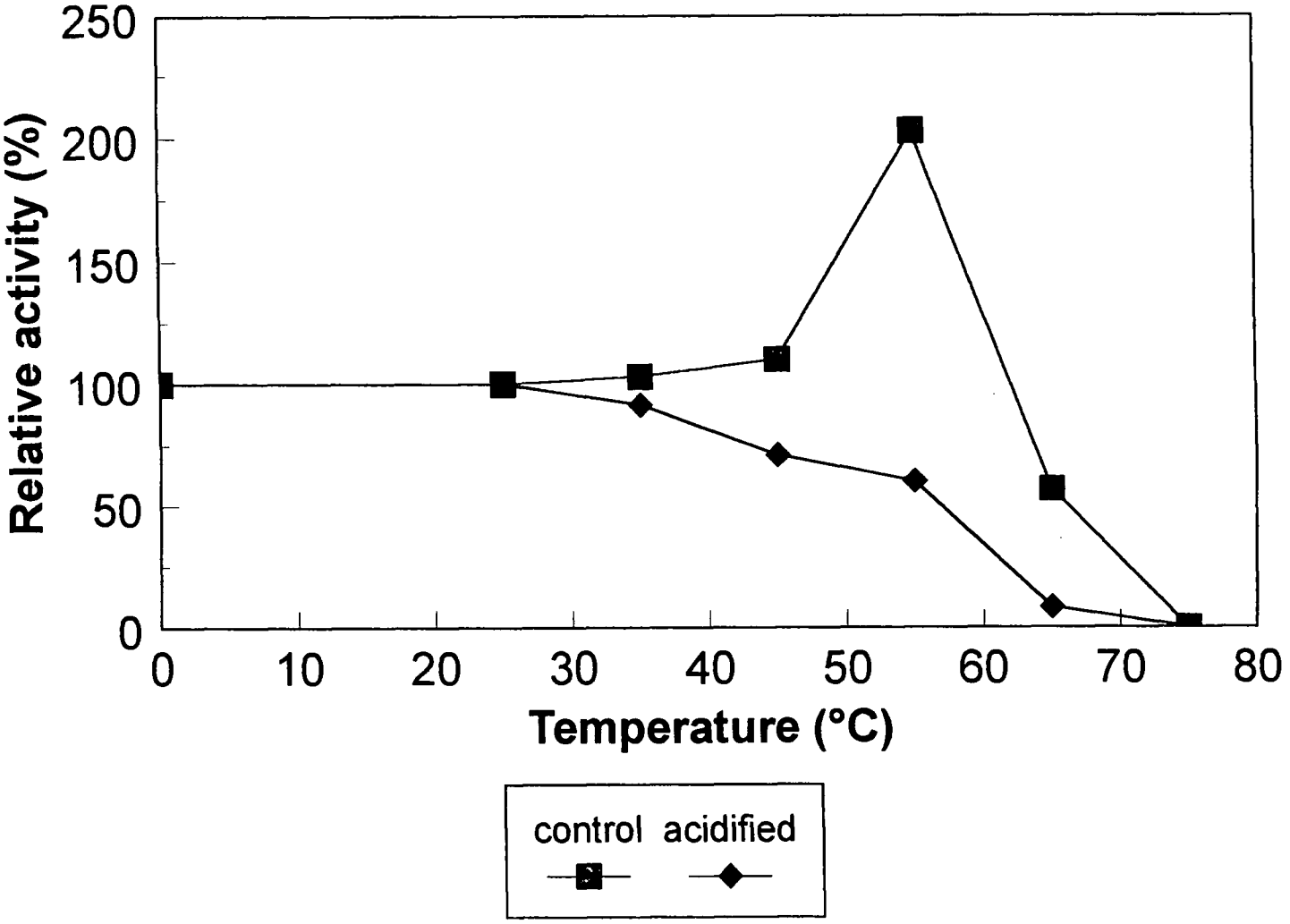

Fig. 4.2. Thermal stability of the recovered SWW enzyme at various temperatures. Acidified and non-acidified enzyme solutions were incubated at various temperatures for 10 min, and activity was assayed against Z-Phe-Arg-NMec.

Table 4.4. Effect of chemicals on the recovered SWW enzyme activity

Chemicals	Concentration (mM)	Relative activity (%)
βME	5	116
	10	144
	20	204
Dithiothreitol	5	438
	10	468
	20	537
Urea	500	119
	1,000	122
	2,500	138
NaCl	10	100
	25	100
	50	104
	100	104
	250	102
	500	104
	1,000	95

concentration was not as much as that of dithiothreitol or βME. This result was in agreement with Bromme *et al.* (1989) that the rate of azocasein degradation by cathepsin L was increased in the presence of 3 M urea, which was a unique characteristic to differentiate cathepsin L and B. NaCl in the range of 0.01-1.0 M did not affect the activity of the enzyme.

pH stability of the recovered enzyme

The maximum activity was found at pH 4.0 in the presence and absence of 20 mM βME (Fig. 4.3). The addition of βME increased activity at all pH ranges studied. The activity at pH 4.0 increased by 67% with the addition of 20 mM βME. Benjakul *et al.* (1996) demonstrated that acidification of partially purified cathepsin L from Pacific whiting SWW resulted in a two-fold increase in activity. This result agrees with An *et al.* (1995) that non-acidified cathepsin L had maximum activity at pH 4.5 due to combination effects of optimum pH and maximal dissociation of enzyme-inhibitor complex. These data indicate that cathepsin L in muscle homogenate is mainly present in a complex form with an inhibitor.

The activity of enzyme decreased considerably with increase in storage time (Fig. 4.4). More than 50% of activity was lost during the first two wk of storage. After 7 wk storage, the enzyme had only 18.5% residual activity. Further study is needed to stabilize the recovered enzyme.

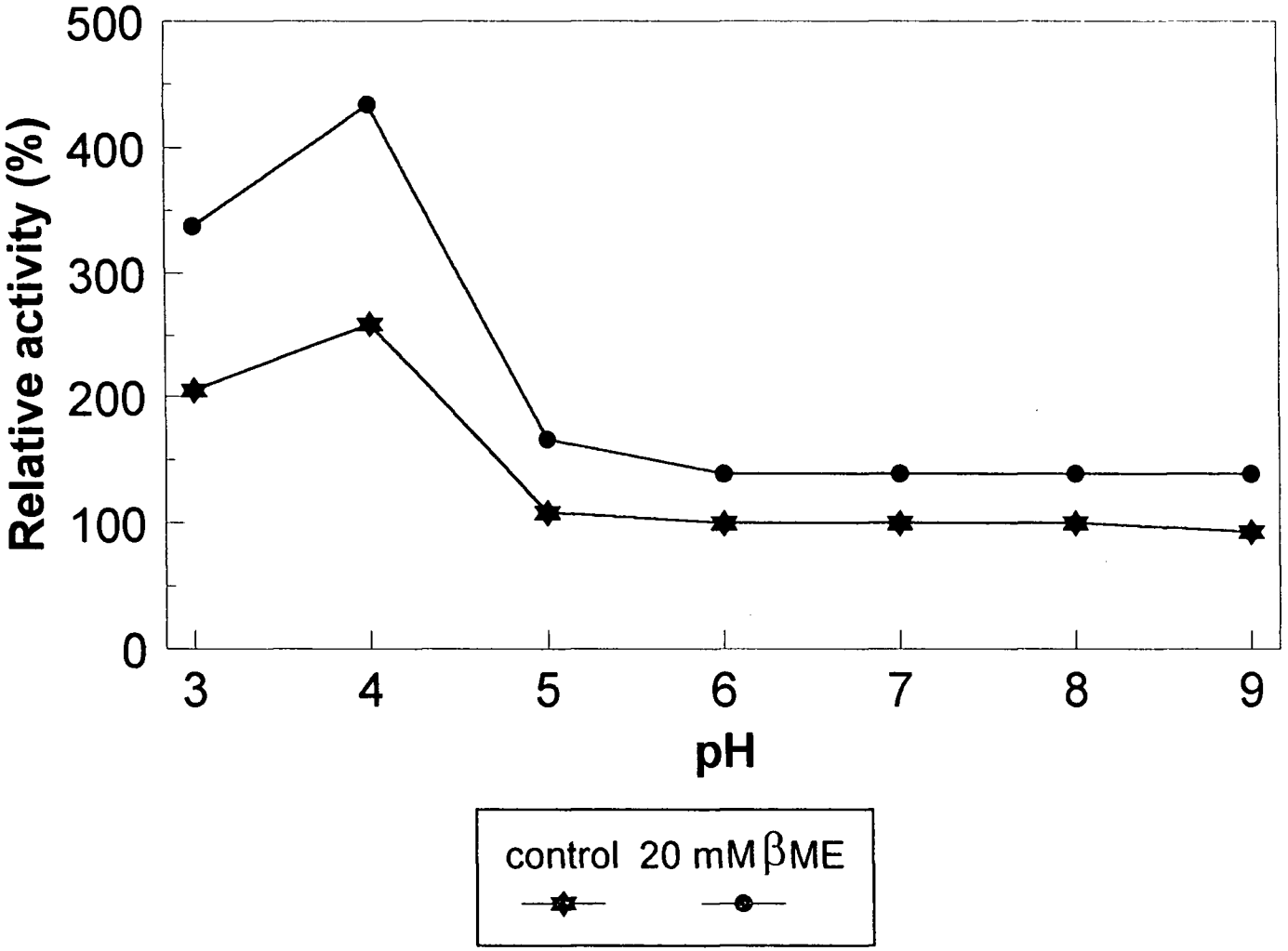

Fig. 4.3. pH stability of the recovered SWW enzyme. Enzyme solution was incubated at various pH at ambient temperature for 30 min. The residual activity was assayed against Z-Phe-Arg-NMec.

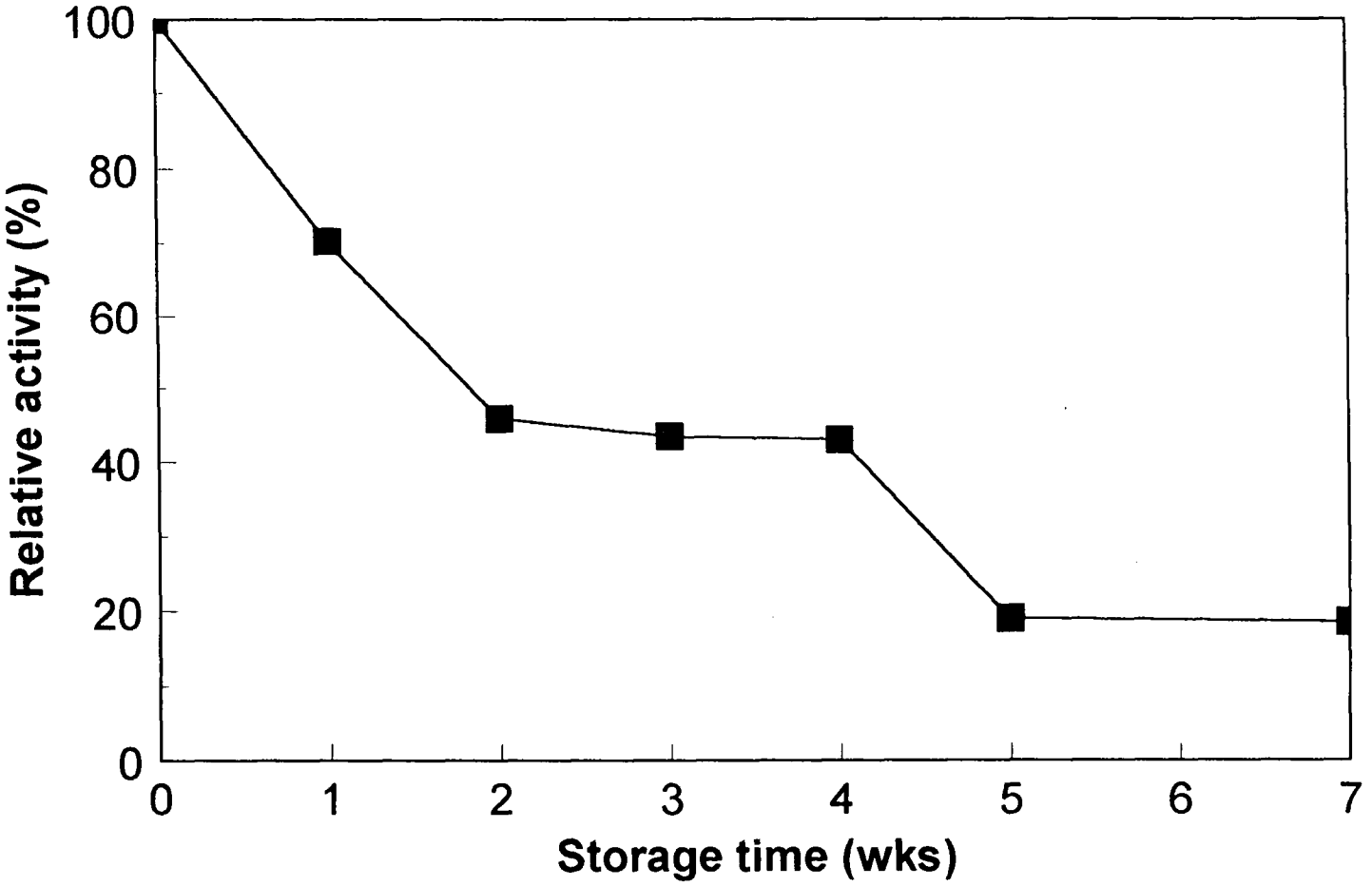

Fig. 4.4. Stability of the recovered SWW enzyme. Crude enzyme powder was kept at 4°C, and residual activity was measured against casein.

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Chapter 5

Proteolytic Activities in Solid Wastes and Digestive Organs of Pacific Whiting

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Abstract

Proteases in digestive organs and solid wastes from Pacific whiting were identified based on the inhibitory study, substrate specificity and SDS-substrate gel electrophoresis. Trypsin-like proteinase with apparent M_r of 25,000 on SDS-substrate gel was the predominant enzyme in the viscera and showed the highest activity at 55°C, pH 8.5 against hemoglobin and pH 7.5 against casein. Trypsin inhibitors and TLCK efficiently inhibited activity of proteinase band at M_r 25,000. A pepsin-like proteinase was found as the predominant enzyme in solid wastes but showed a minor activity in digestive tract. An optimum activity was observed at pH 2.5 and 45°C with hemoglobin. ATP and molybdate did not affect the pepsin-like activity. The presence of carboxypeptidase b was also detected in both viscera and solid wastes.

Key words: digestive enzyme, proteinase, viscera, waste, Pacific whiting

Introduction

Pacific whiting is the most abundant marine resource off the northwest coast of the contiguous United States. The potential average long term yield is 245,500 MT per year (Radtke, 1995). Approximately 80% of 144 million pounds of Pacific whiting landed in Oregon in 1994 were used in surimi production at a recovery rate of 20-25%, producing 90 million pounds of waste (Brown, 1995). During the surimi process, a large amount of water is used for washing, leading to the removal of soluble compounds and some myofibrillar proteins (Toyoda et al., 1992; Lin et al., 1995). Solid wastes including viscera, head , skin and bone are another crucial source of wastes and can be as high as 80% of the original raw material. Waste disposal can include grinding to a specific particulate size and dumping in the ocean or removal to landfills. This type of disposal has the disadvantages, not utilizing usable by-products and stressing the environment. By-product recovery is a viable alternative that can provide economic gain and avoid disposal problems.

Viscera is an important source for digestive proteases which include both gastric proteases and intestinal proteases (Haard, 1994). These enzymes can be a potential source of processing aids in the seafood and food industry. Haard and Simpson (1991) reviewed the applications of proteases in the seafood industry which include the selective removal of skin, hydrolysis of membranes and supportive tissues that envelope roe and recovery of pigment and flavor. Enzymes from digestive tracts and organs have been investigated for anchovy (Martinez et al., 1988; Martinez and Gildberg, 1988; Martinez and Sera, 1989), carp (Cohen et al., 1981), cod and herring (Stoknes et al., 1993), yellow-tail (Yoshinaka, et al., 1977) and catfish (Yoshinaka et al., 1981). The recovery

of digestive enzymes from cod, mackerel and salmon viscera was demonstrated (Reece, 1988; Gildberg, 1992). However, the proteolytic enzymes from Pacific whiting digestive tract and solid wastes have not yet been characterized. The objective of this study was to identify and characterize the proteolytic enzymes in digestive organs and solid wastes from Pacific whiting.

Materials and Methods

Reagents

N_α-benzoyl-DL-arginine p-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), N-acetyl-Alanine-Alanine-Alanine p-nitroanilide ($AcAla_3NA$), hyppuryl-arginine, hyppuryl-phenylalanine, N_α-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), ethylenediaminetetraacetic acid (EDTA), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), pepstatin, leupeptin, phenanthroline, aprotinin, chicken egg white trypsin inhibitor type IV-O, type III-O, sweet potato β-amylase, yeast alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, horse heart cytochrome C, blue dextran, hemoglobin, adenosine 5'-triphosphate (disodium salt) and sodium molybdate were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium caseinate was obtained from US Biochemical Corp. (Cleveland, OH). Iodoacetic acid and phenylmethylsulfonyl fluoride (PMSF) were purchased from Calbiochem Co. (La Jolla, CA)

Sample collection and preparation

Pacific whiting were obtained from a local surimi processing plant less than 24 hr after harvest. Fish were kept in ice and transported to Oregon State University Seafood Laboratory. Fifteen fish were collected and whole digestive organs excluding liver were removed, pooled and kept at -80°C until used. Ground Pacific whiting solid wastes including head, skin, bone and viscera, were collected from a local fish waste processing plant. The samples were vacuum-packed and kept at -80°C.

Preparation of crude extracts

Frozen samples were thawed in running water before extraction. Pooled digestive organs were cut into small pieces, then, homogenized in 2 vol of ice-cold, deionized water for 2 min with a blender. The homogenates were centrifuged at 15,000 xg for 20 min. The supernatant was kept at -80 °C and was referred to as 'digestive organs extract' (DOE). The 'solid wastes extract' (SWE) was prepared in the same manner.

Enzyme assay

Proteinase activity was determined using hemoglobin as a substrate according to the method of An et al. (1994). The reaction mixture consisted of 100 μL crude extract (5-10 folds diluted), 625 μL assay buffer and 525 μL substrate solution containing 2 mg protein. Activity was assayed for 20 min at various pH (McIlvaine's buffer (pH 1.0-8.0) or Tris-HCl buffer (pH 8.5-11.0)) and temperatures as specified in the text. The reaction was terminated by adding 200 μL of 50% (w/v) trichloroacetic acid. The mixture was centrifuged at 8,100 xg for 3 min to remove precipitates. The blank was carried out as

above except that crude extract was added after the addition of 50% trichloroacetic acid. One unit of activity was defined as the amount of enzyme releasing 1 nmol of tyrosine per min. The concentration of oligopeptides in supernatant was measured by Lowry's method (Lowry et al., 1951) using tyrosine as a standard.

Trypsin activity was determined using BAPNA as a substrate according to the method of Erlanger et al (1961). Elastase activity was determined by using $AcAla_3NA$ as a substrate (Feinstein et al, 1973). Specific activity of trypsin and elastase were calculated as ΔA_{410}/min x 1,000 x vol / (8,800 x mg protein) when 8,800 is the extinction coefficient of ρ-nitroaniline and vol is the volume of reaction mixture (mL) (Eranger et al.,1961; Simpson et al., 1990) . Chymotrypsin activity was analyzed using BTEE as a substrate (Roa and Lombardi, 1975) and specific activity was calculated as ΔA_{256}/min x 1,000 x vol / (964 x mg protein) where 964 equals molar extinction coefficient for N-benzoyl-DL-tyrosine (Worthington Biochemical Corporation, 1971). Carboxypeptidase A and B were measured using Hipp-L-Phe (Bermeyer et al., 1974) and Hipp-L-Arg (Appel, 1974) as a substrate, respectively. Specific activity was calculated as ΔA_{254}/min / (AI x mg protein) where AI stands for the molar absorbancy index of hippuric acid, 0.360 and 0.349 for carboxypeptidase A and B, respectively (Warthington Biochemical Corporation, 1971).

Chemical inhibitor study

Various inhibitors, including E-64, pepstatin, PMSF, phenanthroline, leupeptin, iodoacetic acid, trypsin inhibitor type III-O and IV-O, TLCK, TPCK, EDTA and aprotinin were used to classify the proteinases in DOE and SWE. Crude extract (100

μL) was incubated with each inhibitor at the concentration listed in Table 1. The residual activity of DOE was determined using hemoglobin as a substrate at 55°C, pH 8.5 and 25°C, pH 2.0. For SWE, the inhibition study was performed at 45°C and pH 2.5.

Separation of enzymes by gel filtration

Separation of proteins in DOE and SWE was carried out using Superose 12 HR (Pharmacia LKB Biotechnology, Uppsala, Sweden) connected with a Bio-Rad HPLC pump (Model 2700, Bio-Rad Laboratories, Inc., Hercules, CA) and a UV detector (Bio-Rad Model 1706). The crude extract was filtered using syringe filters (0.2 μm pore size) (Gelman Sciences, Ann Arbor, MI). Samples (200 μL) were injected to column and eluted isocratically with 100 mM sodium phosphate buffer, pH 7.2 at the flow rate of 0.5 mL/min. Absorbance at 280 nm was used to monitor the eluted proteins. The fraction of 0.5 mL was collected for enzymic activity determination.

Molecular weight of proteins was estimated by plotting relative elution volume (Ve/Vo) vs. the logarithm of molecular weight of protein standards. The void volume (Vo) was estimated by the elution volume of blue dextran. The protein standards including sweet potato β-amylase (M_r 200,000), yeast alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 29,000) and horse heart cytochrome C (M_r 12,400).

Effect of ATP and sodium molybdate on enzyme activity

To differentiate activity of pepsin from that of cathepsin D, the modified method of Pillai and Zull (1985) was applied. ATP and sodium molybdate were added to final

concentration of 0, 100, 200, 400, 600, 800 and 1,000 μM. The activity was analyzed by TCA-Lowry assay (An et al., 1994) using hemoglobin as a substrate at 25°C, pH 2.0 for DOE and 45°C, pH 2.5 for SWE.

SDS-Substrate gel

DOE fractions with proteolytic activity were pooled and mixed with treatment buffer containing no βME at the ratio of 3:1 (v/v) and loaded onto a 15% SDS-substrate gel. To study the effect of some inhibitors on activity band of proteases, inhibitors were added to DOE and incubated at ambient temperature for 15 min before addtion of treatment buffer. Chemical inhibitors included 1 mM leupepsin, 1 mg/mL TLCK and 2mg/mL trypsin inhibitors type III-O and IV-O. Activity staining was performed according to modified method of Garcia-Carreno et al. (1993) as reported by An et al. (1995). The separation of enzymes on gels was carried out using a Mini-Protean II system (Bio-Rad Laboratories, Inc, Hercules, CA) at the constant 150 V. The gel was immersed in 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7.5 at 0°C for 1 hr with constant agitation to accelerate the penetration of casein into gel. The gel with casein solution was subsequently incubated in McIlvaine's buffer, pH 7.5 at 55°C for 1 hr. The gel was rinsed with deionized water and stained in 0.125% Coomassie brilliant blue R-250 and destained in 25% ethanol and 10% acetic acid until activity zone appeared. Low molecular weight standards (Pharmacia Biotech, Inc., Piscataway, NJ) including phosphorylase b (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrous (M_r 30,000), trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400) were applied to estimate the apparent molecular weight.

Results

pH and temperature profile of proteases in solid wastes and digestive organs

Maximum activity of DOE with hemoglobin as a substrate was found at 50°C at pH 8.0 and 55°C at pH 7.0 (Fig. 5.1). At pH 5.5, the optimum activity was also observed at 50°C, but was considerably lower than activity measured at pH 7.0 or 8.0. Enzymatic activity decreased readily at temperatures above 60°C and there was a complete loss of activity at all pHs studied at the temperature 75°C or above. For samples tested at pH 3.0, activity remained constant between 25-50°C but became heat-inactivated at temperatures above 50°C.

The proteolytic activity in SWE with hemoglobin was very prominent at pH 3.0 (Fig. 5.2) and showed a similar pattern to the DOE enzymes under the same pH condition with maximum activity at 45°C. The loss of activity was observed at temperatures above 50°C. At other pH ranges, the activity was considerably lower although the highest activity was found at 60-65°C. The activity loss of proteinases active under neutral/alkaline pHs was postulated to be due to the low stability of the enzymes. Simpson et al. (1990) reported that cod trypsin are generally unstable at acidic or neutral pH but stable at alkaline pH. These results indicated that an acidic proteinase was the major enzyme in Pacific whiting solid wastes.

DOE showed the optimum pH at 7.5 and 8.5 on casein and hemoglobin, respectively as analyzed at 55°C (Fig. 5.3). At the optimum pH for each substrate, enzymes showed 3-fold higher activity on casein than hemoglobin. The activities of acidic proteinase assayed against hemoglobin at pH 1.0-4.0 and 25°C were lower than

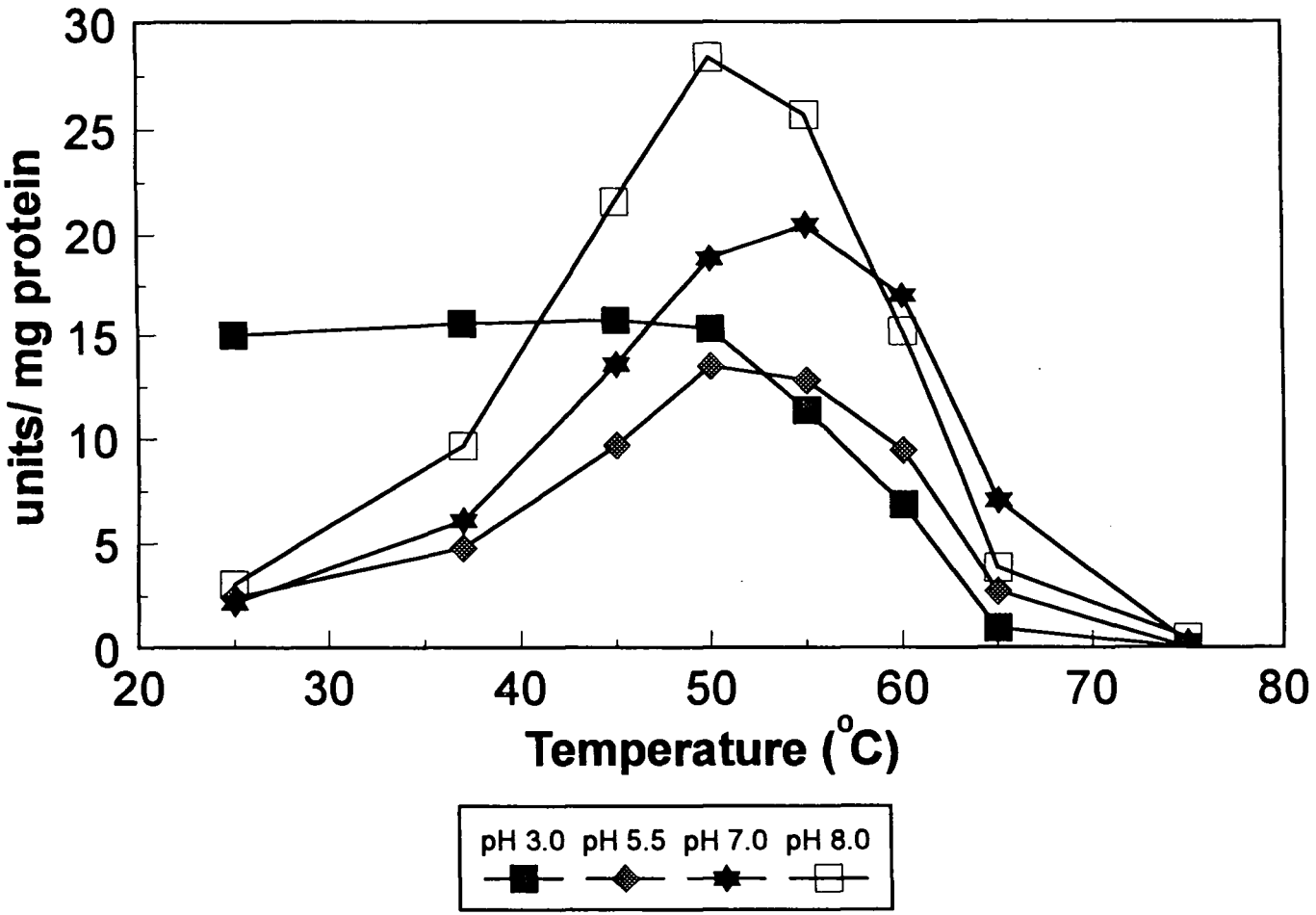

Fig. 5.1. pH activity profiles of DOE proteases at various temperatures. Proteolytic activity was tested using hemoglobin as substrate. A unit activity was defined as the amount of enzyme releasing 1 nmol of tyrosine per min.

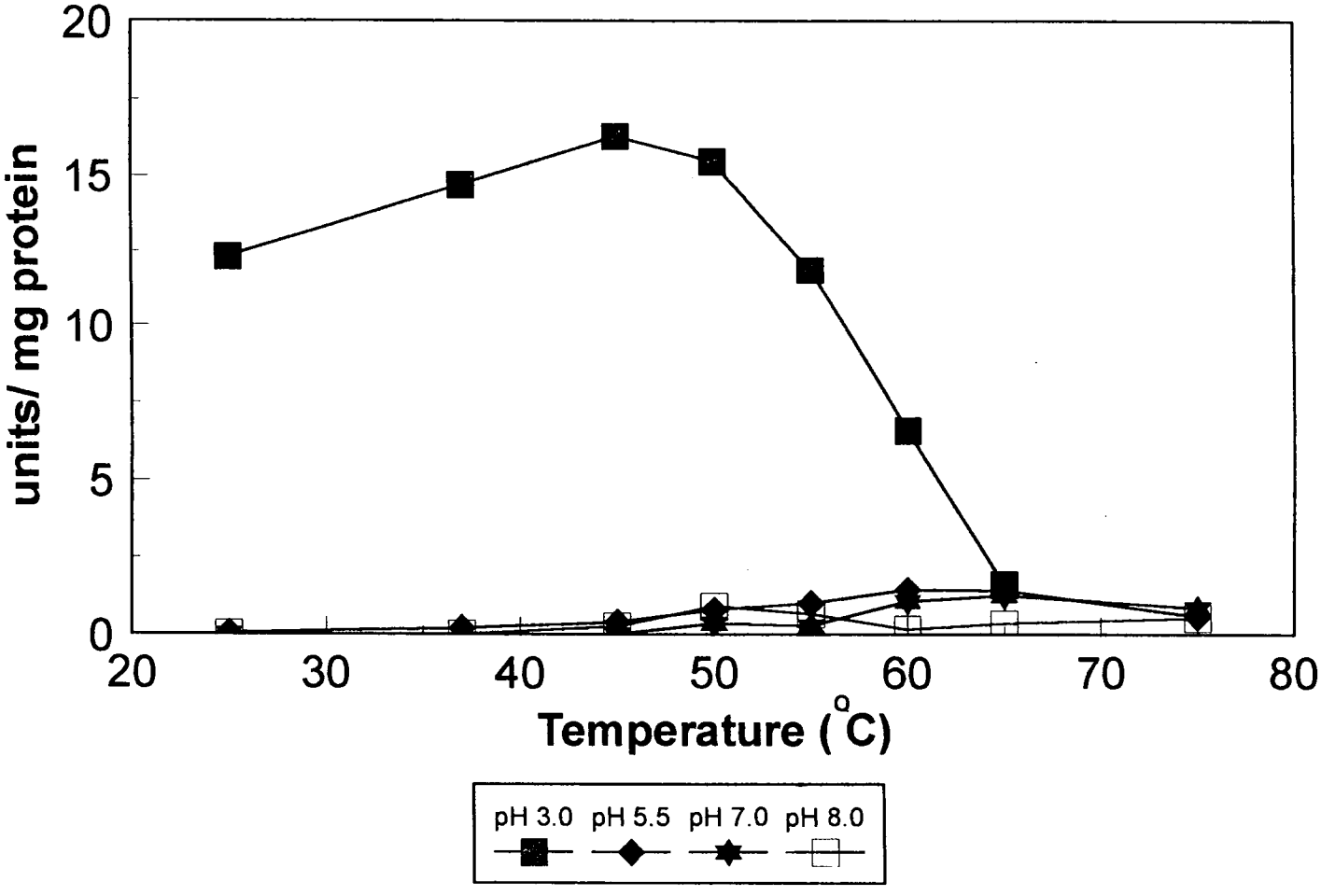

Fig. 5.2. pH activity of SWCE proteases at various temperatures. Proteolytic activity was tested using hemoglobin as substrate. A unit activity was defined as 1 nmol of tyrosine released per min.

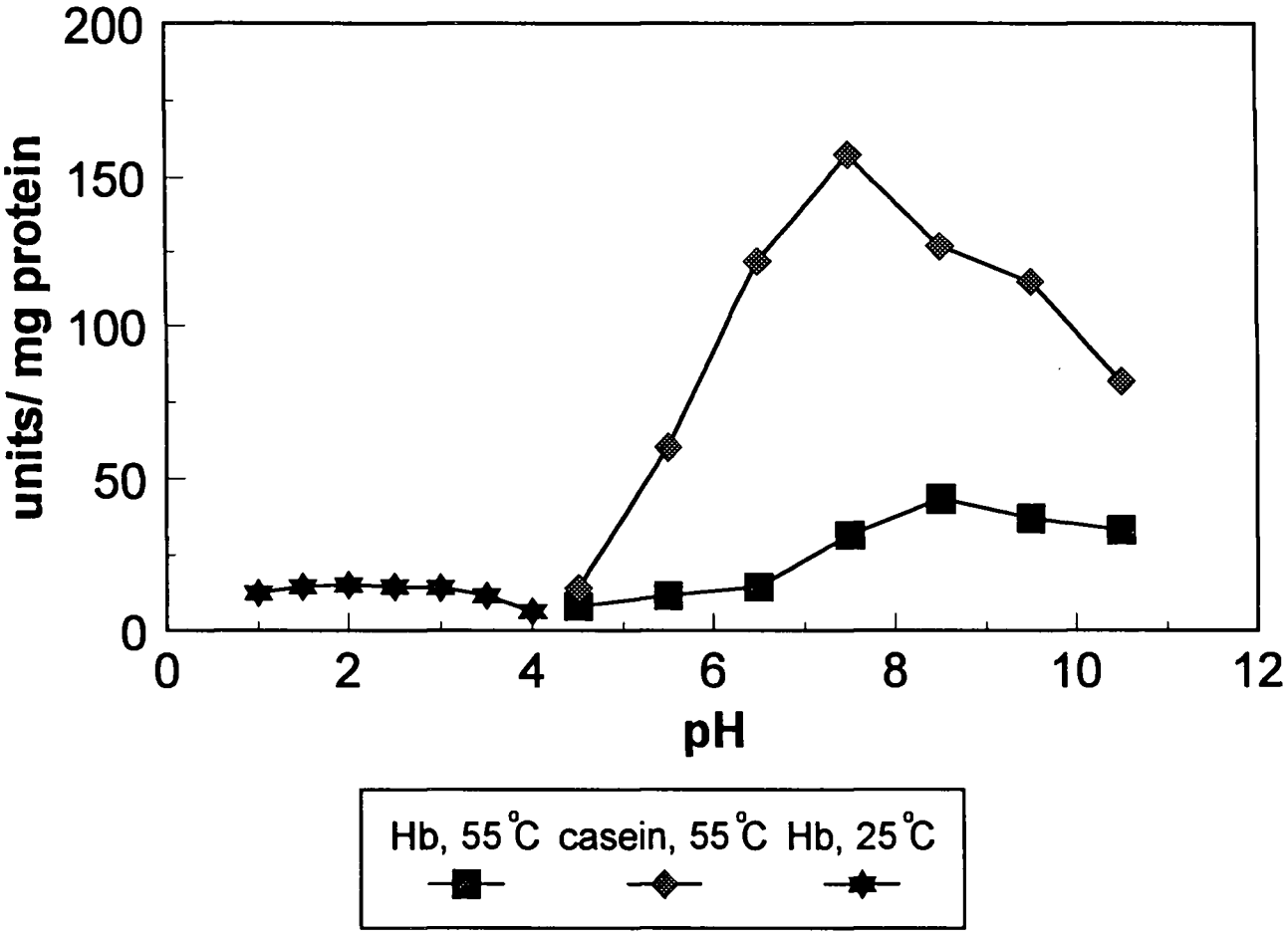

Fig. 5.3. pH activity profiles of DOE proteases. Proteolytic activity was tested using hemoglobin and casein as substrates at 55°C for pH ranges of 4.5-10.5. Activity was also determined using hemoglobin as substrate at 25°C for pH ranges of 1.0-4.0. A unit activity was defined as the amount of enzyme releasing 1 nmol of tyrosine per min.

those of proteinase analyzed at pH 7.5-10.5 and 55°C. This indicated that proteinases which active under alkaline conditions predominated in DOE. The activity of SWE was apparently low under neutral/alkaline pHs but was dominant between pH 1.0-5.0 (Fig. 5.4). The maximum activity against hemoglobin was found at pH 2.5 and showed 15-fold higher than that observed at pH 6.5-10.5. When comparing the hydrolytic activity of SWE with both substrates, casein was more hydrolyzed than hemoglobin. Therefore, casein was a preferable substrate for both DOE and SWE.

Effect of chemical inhibitors

The effect of various proteinase inhibitors on proteolytic activity was studied using hemoglobin as a substrate (Table 5.1). Under alkaline assay conditions, TLCK (specific for trypsin) showed the highest inhibitory effect on proteinases in DOE. Leupeptin (specific for cysteine and serine proteinases), aprotinin (specific for serine proteinase) and trypsin inhibitor showed almost the same degree of inhibition. However, PMSF (specific for serine proteinase) showed low inhibition. Pepstatin (specific for aspartic acid proteinase), phenanthroline and EDTA (specific for metallo proteinases), E-64 (specific for cysteine proteinases), iodoacetic acid (specific for serine, cysteine and metallo proteinases), TPCK (specific for chymotrypsin) showed very little or no inhibitory effect on proteinases from digestive organs. Therefore, serine proteinases, particularly a trypsin-like enzyme, was the predominant proteinase in Pacific whiting digestive organs. Martinez and Serra (1989) found that PMSF and SBTI were the most effective inhibitors for proteolytic activity of anchovy viscera crude extract. Additionally,

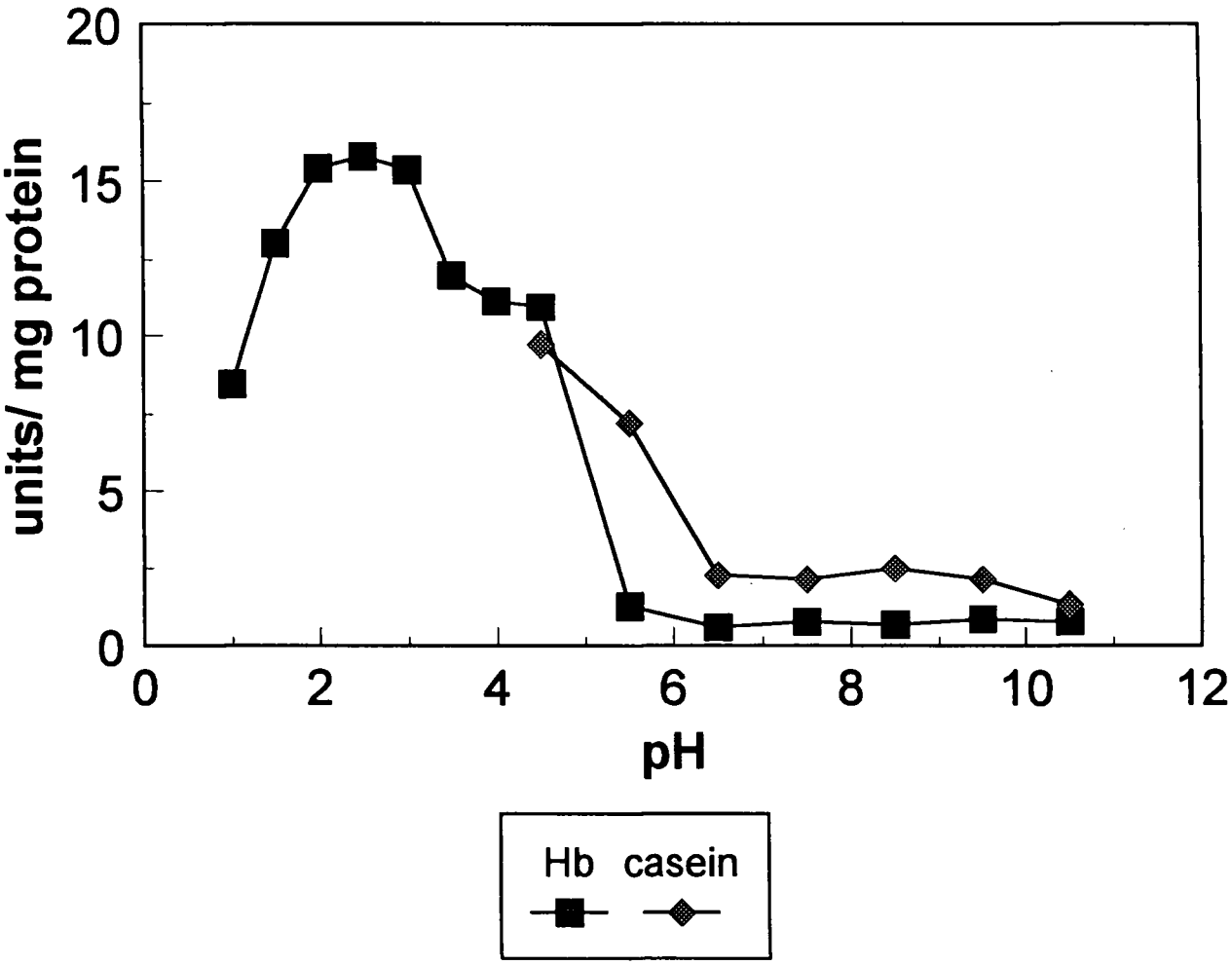

Fig. 5.4. pH activity profile of SWE proteases. Proteolytic activity was tested using hemoglobin and casein as substrates at 45°C. A unit activity was defined as the amount of enzyme releasing 1 nmol of tyrosine per min.

Table 5.1 Effect of proteinase inhibitor on activity of Pacific whiting DOE and SWE.

PI	concentration	Relative activity (%)		
		DOE 55°C, pH 8.5	DOE 25°C, pH 2.0	SWE 45°C, pH 2.5
E-64	10 μM	105.12	107.63	93.13
pepstatin	1 mg/mL	101.10	1.73	26.02
PMSF	1 mg/mL	85.35	78.37	91.08
phenanthroline	1 mg/mL	100.69	95.00	93.45
leupeptin	1 mM	55.79	101.29	93.61
iodoacetic acid	1 mM	98.90	104.26	93.45
trypsin inhibitor	2 mg/mL	60.01	107.93	98.35
TLCK	1 mg/mL	47.74	72.70	75.44
TPCK	1 mg/mL	83.05	70.99	74.59
EDTA	10 mM	83.45	88.69	97.34
Aprotinin	2 μg/mL	60.158	108.59	97.04

Martinez et al. (1988) reported that TLCK completely inhibited the activity of purified trypsin, trypsin A and B from digestive tract of anchovy.

Under acidic assay conditions for DOE, pepstatin showed almost complete inhibition. Other proteinase inhibitors had no or minor effect on the enzyme activity. It implies that aspartic acid proteinases such as pepsin or cathepsin D are present in digestive organs in addition to serine such as trypsin-like or chymotrypsin-like proteinase. For inhibitory study of SWE under acidic pH, pepstatin showed the highest inhibition with a relative remaining activity of 26.02%, indicating the presence of aspartic acid proteinase in the extract.

Substrate specificity of proteinases

DOE showed a higher activity on BAPNA which is specific for trypsin than SWE (Table 5.2). A low activity of chymotrypsin, elastase and carboxypeptidase A was observed, whereas a higher activity of carboxypeptidase B was found in both DOE and SWE. Serine proteinases (trypsin, chymotrypsin and elastase) have been reported as the important enzymes in digestive tract of fishes (Jany, 1976; Cohen et al., 1981; Martinez and Serra, 1989). Carboxypeptidase A and B and leucine aminopeptidase were found in the guts of anchovy (Martinez and Serra, 1989) and viscera of Dover sole (Clark et al., 1986).

Effect of ATP and molybdate on protease activity

A pepsin-like enzyme and cathepsin D in the extract were differentiated by using ATP and molybdate. ATP and molybdate did not affect the activity of proteases in DOE

Table 5.2 Specific activity of Pacific whiting proteases in DOE and SWE.

Enzymes	Specific activity (units/g protein)	
	DOE	SWE
Trypsin	4.770	1.273
Chymotrypsin	0.057	0.149
Elastase	0.282	0.373
Carboxypeptidase A	ND	ND
Carboxypeptidase B	2.113	3.840

*ND: not detectable

and SWE (data not shown). The aspartic proteinases are a class of endopeptidase active at acidic conditions. Pepsin and cathepsin D are the only aspartic proteinases in fish and invertebrates (Gildberg, 1988). Pepsin has an extracellular function as the major gastric proteinase, whereas cathepsin D is a lysosomal enzyme active in intracellular protein turnover. Pepsin is secreted as a zymogen (pepsinogen), which is activated by the acid in stomach to the active form of enzyme (Clarks et al., 1985). Both enzymes hydrolyze peptide bonds on the amino side of aromatic amino acids or those with bulky side chains (Barrett, 1977). Cathepsin D-catalyzed proteolysis was activated by ATP in the 0.1-1.0 mM range (Pillai et al., 1983; Pillai and Zull, 1985). Watabe et al.(1979) also found that bovine spleen cathepsin D activity was enhanced by ATP at 10 mM. It was proposed

that ATP binds to cathepsin D and probably alters the geometry of some interactions in a fashion which increases its catalytic efficiency (Pillai and Zull, 1985). However, pepsin-catalyzed hydrolysis is not activated by ATP (Pillai and Zull, 1985).

Molybdate is a phosphate analogue and can inhibit enzyme by binding with phosphate containing compounds, particularly nucleotide triphosphate. Therefore, it can reduce proteolysis via interaction with substrates instead of inhibition of cathepsin D (Pillai and Zull, 1985). Since both ATP and molybdate did not affect the proteolytic activity, it is postulated that pepsin-like proteinase, but not cathepsin D is mainly involved in proteolysis at acidic pH.

Separation of proteolytic activity

Proteins in DOE and SWE were separated using a size-exclusion HPLC (Fig. 5.5 and 5.6). The proteolytic activity in each DOE fraction was tested against casein at pH 7.5, 55°C and hemoglobin at pH 8.5, 55°C and pH 2.0, 25°C (Fig. 5.7). The fractions collected from DOE showed a broad peak on casein at pH 7.5 and 55°C with retention time between 24-30 min. However, a narrow peak (25 min fraction) was found when hemoglobin was used as a substrate on the same assay condition. Molecular weight of proteinases active on casein were estimated to be 39,680 and 20,650 with retention time of 26 and 28 min, respectively. For the proteinase that was capable of hydrolyzing hemoglobin, the molecular weight was estimated to be 55,030 with retention time of 25 min. Proteinases which were active under the alkaline condition played an important role in proteolytic activity in DOE. The fraction eluted at 36 min showed the proteolytic

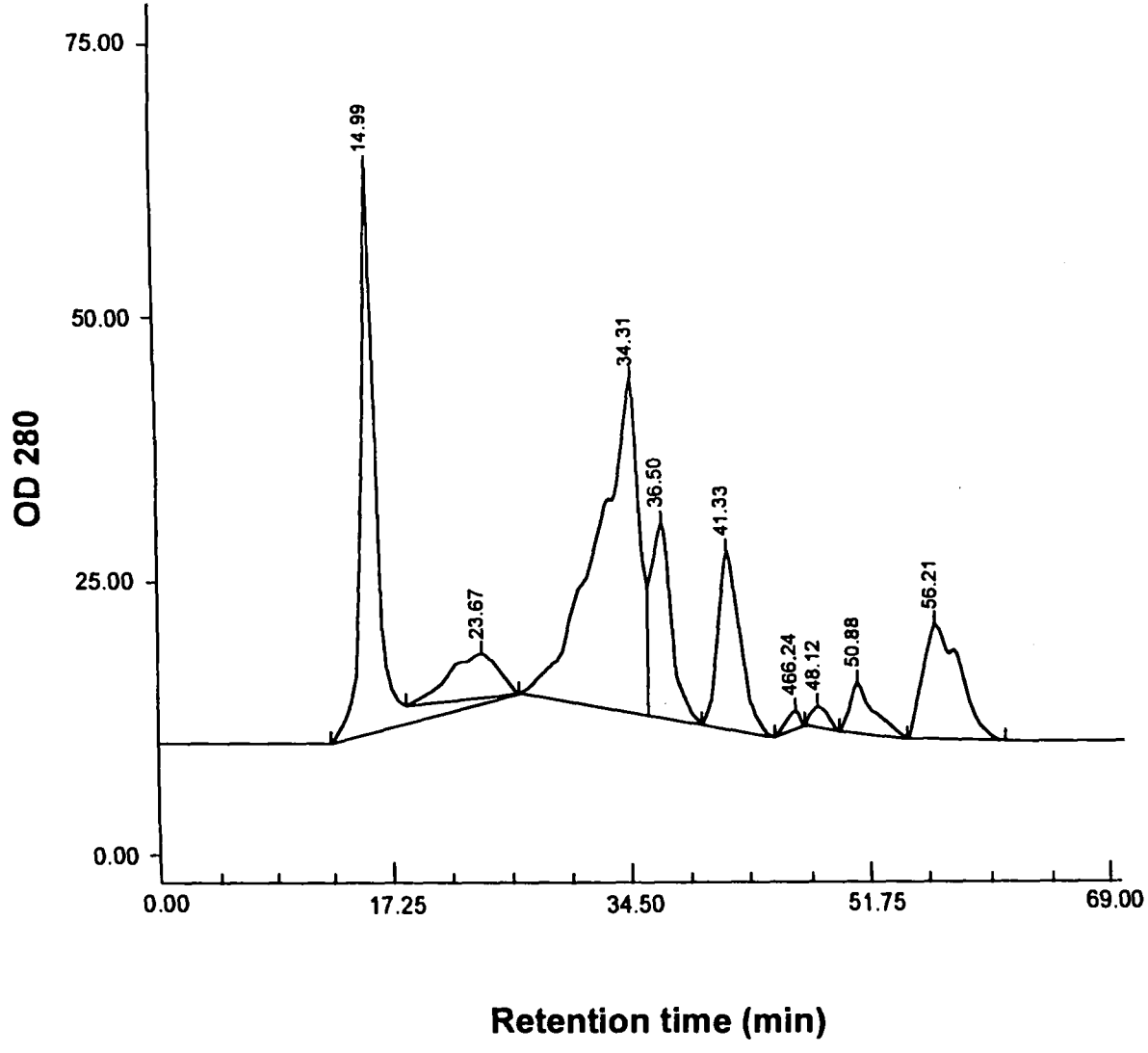

Fig. 5.5. Elution profile of DOE on superose 12 HR-HPLC. DOE proteins were separated on Superose 12 HR with 100 mM sodium phosphate buffer, pH 7.2 at 0.5 mL/min. Eluted proteins were monitored by absorbance at 280 nm.

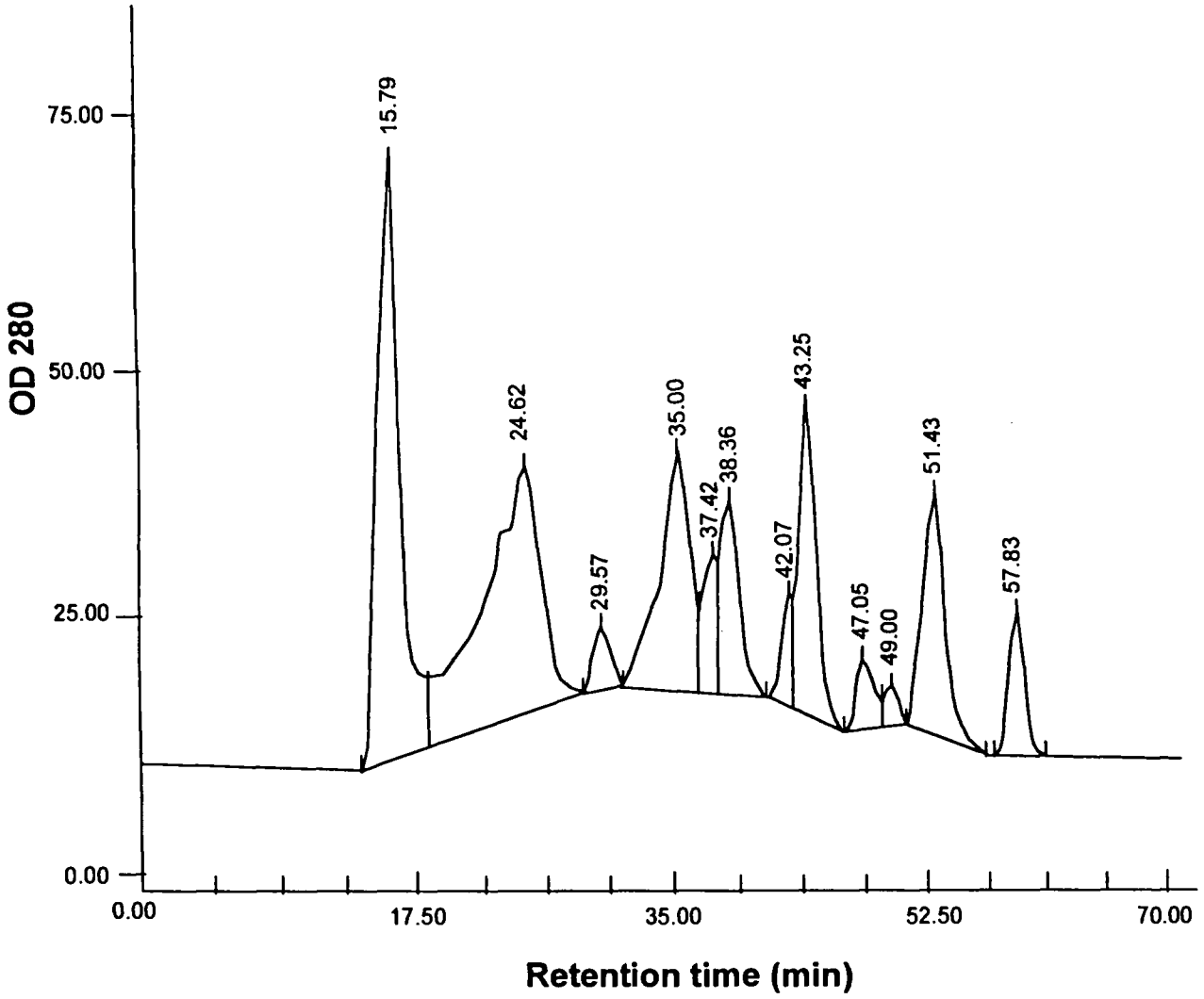

Fig. 5.6. Elution profile of SWE on superose 12 HR-HPLC. SWE proteins were separated on Superose 12 HR with 100 mM sodium phosphate buffer, pH 7.2 at 0.5 mL/min. Eluted proteins were monitored by absorbance at 280 nm.

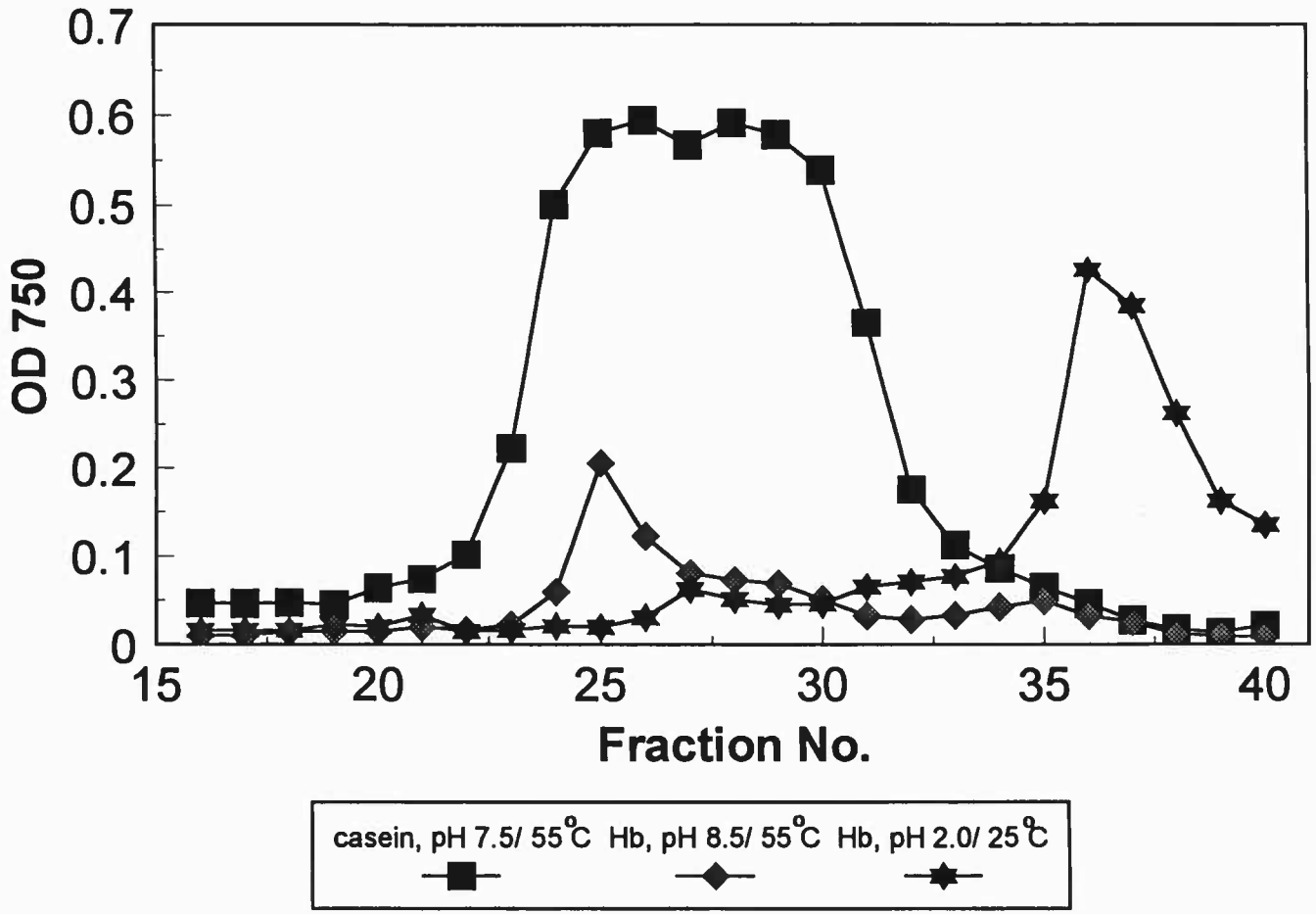

Fig. 5.7. Proteolytic activity of Superose 12 HR fractions of DOE determined against casein and hemoglobin at different conditions. Activity was expressed as ΔOD750.

activity when fractions were assayed against hemoglobin at pH 2.0 and 25°C, indicating the presence of an acidic proteinase.

Due to low activity of SWE on both casein and hemoglobin at pH 6.5 or above (Fig. 5.4), the fractions were tested under only acidic condition (pH 2.5 and 45°C). Two activity maxima were found at retention time of 31 min and 36 min with the apparent molecular weight estimated to be 7,750 and 1,510, respectively (Fig. 5.8). The protein eluted at 31 min showed the higher activity against hemoglobin. This results was in agreement with Sanchez-Chiang and Ponce (1981); Gildberg and Raa (1983) who found that fish pepsins M_r, estimated from gel filtration, was too low. The retarded elution was presumed to be caused by interaction between carbohydrate residues of fish pepsins and the polysaccharide of the gel (Gildberd et al., 1990). Cod pepsins have been found to be glycoproteins containing 1-5% carbohydrate (Gildberg et al., 1990). This supported our conclusion that two groups of acidic proteinases were the major proteinases in SWE.

SDS-substrate gel electrophoresis

Proteolytic activity of proteinase from pooled DOE fractions (fractions 24-30) were analyzed on SDS-substrate gel (Fig. 5.9). The major clear band with M_r of 25,000 was observed on the gel loaded with pooled fractions. This band had M_r in the range of fish trypsins (Cohen et al., 1981; Hjelmeland and Raa, 1982; Simpson and Haard, 1984; Martinez et al., 1988). A clear band with a M_r of 17,500 was also found on the SDS-substrate gel. Additionally, small clear bands were prevalent in the M_r range of 93,000-25,000, coinciding with the result of gel filtration.

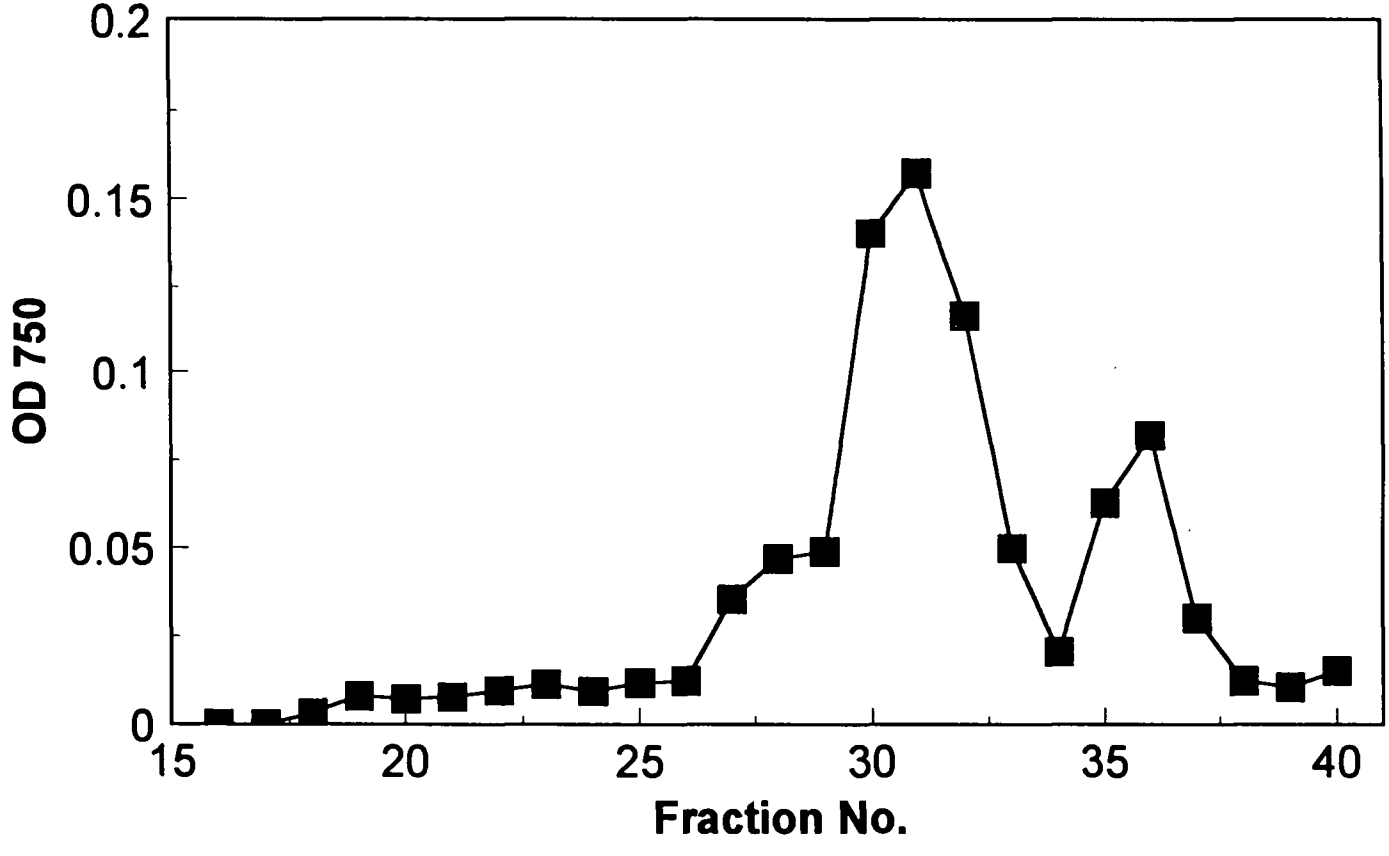

Fig. 5.8. Poteolytic activity of Superose 12 HR fraction of SWE determined against hemoglobin at pH 2.5 and 45°C. Activity was expressed as ΔOD750.

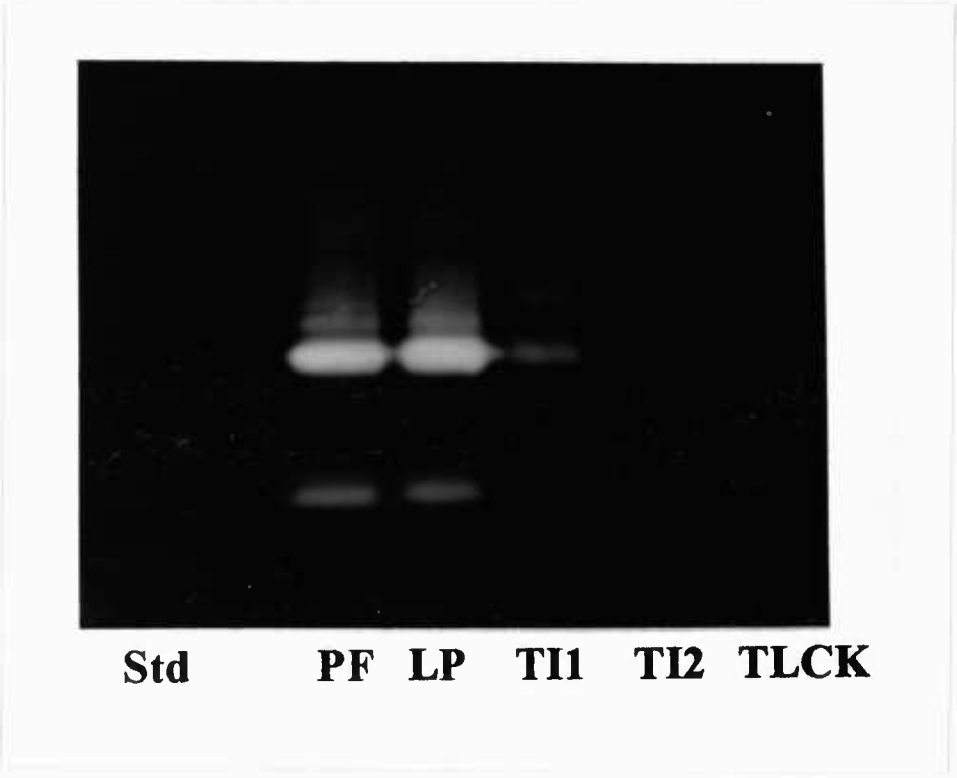

Fig. 5.9. Separation of DOE proteins on SDS-substrate gel electrophoresis and effect of chemical inhibitors on proteolytic activity. Pooled fractions of DOE were treated with no inhibitors (PF), 1 mM leupeptin (LP), 2 mg/mL chicken egg white trypsin inhibitor, type III-O (TI1), 2 mg/mL chicken egg white trypsin inhibitor, type IV-O (TI2) and 1 mg/mL TLCK. Std denotes low molecular weight standards.

Chemical inhibitors were applied to confirm the type of proteases which showed the clearing of SDS-substrate gel (Fig. 5.9). Leupeptin had no effects on activity, probably due to the revisible inhibitory mechanism during the separation of enzyme on SDS-substrate gel electrophoresis. Trypsin inhibitor, typeIV-O showed a higher inhibition effect on proteolytic activity than trypsin inhibitor, type III-O. Trypsin inhibitor, type IV-O completely eliminated the clearing on the gel whereas trypsin inhibitor, type III-O partially reduced activity of proteinase at M_r of 25,000 but totally abolished the clearing at M_r of 17,500. TLCK almost completely inhibited activity of proteinase band at M_r 25,000 and completely removed the clearing at M_r 17,500. These results verified that trypsin-like proteinase was the predominant enzyme in the fractions which were active on casein under alkaline conditions.

Discussion

Proteases were widely distributed in tissue and viscera of marine species. These enzymes show different optimum conditions for proteolysis. Based on pH and temperature profile, the activity of DOE was found at pH 3.0 with the temperature ranging between 25-50°C but the loss of activity was obtained when the temperature was above 50°C. On the other hand, the low activity was observed at pH 5.5, 7.0 and 8.0 as assayed at 25-37°C (Fig. 5.1 and 5.2). Therefore, two groups of enzymes, acidic and neutral/alkaline proteases were postulated as endogenous enzymes in digestive organs of Pacific whiting. This result was in agreement with Martinez and Serra (1989) who reported that crude extracts from anchovy digestive tracts contained protease with optimum activity at pH 3.0 and 9.5-10.

Fish trypsin are generally unstable at acid pH but stable at alkaline pH (Vithayathill et al., 1961). Two trypsin-like enzymes, Enzyme I and II isolated from the gut of capelin had an optimum pH at 8-9 on BAPNA with the apparent optimum temperature at 42°C (Hjelmeland and Raa, 1982). Simpson et al.(1990) reported that Atlantic cod trypsin was most active at pH 7.5 and 40°C with BAPNA. In Dover sole, the optimum activity at pH 7.0-8.0 resulted from trypsin and chymotrypsin-like enzymes while elastase caused the optimum activity at pH 9.5-10.5 (Clarks et al., 1985). The optimum pH for casein hydrolysis by Greenland cod trypsin was 9-9.5 in contrast to a pH optimum of 8.0 by bovine pancreas trypsin (Simpson and Haard, 1984). Our result showed that the actvity of DOE was high at pH 7.5-8.5 at 55°C (Fig. 5.3 and 5.4), coinciding with the optimum condition for trypsin-like enzyme.

Pepsin-like protease with an optimum pH value of 1.7 predominated in the stomach region of Dover sole (Clarks et al., 1985). Haard (1986) found that maximum initial rate of hemoglobin digestion of Atlantic cod pepsin was maximum at 35°C, pH 1.9. Fish pepsins could digest hemoglobin much faster than casein, myofibrillar protein and sarcoplasmic protein (Gildberg and Raa, 1983; Squires et al., 1986). Guerard and Le Gal (1987) reported that a hexapeptide is the smallest substrate to be hydrolyzed by fish pepsins. Most fish species contain two or three major pepsins with an optimum for hemoglobin digestion at pH between 2 and 4 (Noda and Murakami., 1981; Gildgerg and Raa, 1983; Twinning et al., 1983; Gildberg et al., 1990). Gilberg et al. (1990) found that the affinity of the cod pepsin, specially pepsin I towards hemoglobin was much lower at pH 2 than at pH 3.5. Furthermore, pH optimum was extremely dependent on the substrate concentration. Pepsin I and II showed similar pH optimum at pH 3.0 with very

high hemoglobin concentration, whereas pepsin I had a maximum activity in pH range 3.5-4 at lower substrate concentration (Gildberg et al., 1990). The apparent activites observed in both DOE and SWE in the acidic ranges (pH 1.0-4.0) indicated the presence of acidic protease, particularly pepsin-like enzyme (Fig. 5.3 and 5.4).

From inhibition study, proteinase in DOE was classified as serine proteinase when analyzed under alkaline condition and as aspartic acid proteinase when assayed under acidic condition. An aspartic acid proteinase was identified as a major enzyme in SWE. EDTA, which acts as a chelator of the active site zinc ion in metallo-proteases, such as carboxypeptidase A and B, showed slightly inhibited the activity (Table 5.1) and carboxypeptidase B was also found in the DOE and SWE extracts (Table 5.2). Carboxypeptidase B is exopeptidase and catalyzes hydrolysis of basic amino acid, lysine and arginine, from the carboxyl terminal position in polypeptides (Folk and Gladner, 1958). Therefore, it was presumed that both endopeptidases and exopeptidases played an essential role in both DOE and SWE.

To identify the proteinase, the substrate specificity was tested (Table 5.2). Our result indicated that a high activity of trypsin was found in DOE. Moreover, M_r of proteinase calculated from size exclusion (Fig. 5.5 and 5.7) and SDS-substrate gel electrophoresis (Fig. 5.9) was in the ranges of trypsins which have been reported. Molecular weight of trypsin ranges from 20,000 to 24,000 (Keil, 1971). Hjelmeland and Raa (1982) found two trypsins from Arctic fish capelin with M_r of about 28,000. For Greenland cod trypsin, M_r of 23,500 was reported by Simpson and Haard (1984). Trypsin A and B from anchovy had M_r of 27,000 and 28,000, respectively (Martinez et al., 1988). Cohen et al.(1984) reported that carp trypsin M_r was 25,000. In addition, the

inhibitors used, particularly trypsin inhibitors and TLCK, showed a high inhibition on the activity. Therefore, it can be concluded that trypsin-like enzyme was the major enzyme in DOE.

Due to precipitation of casein at low pH, the SDS-substrate gel was not able to be used to determine activity of acidic proteinases. Hemoglobin was used as substrate on a SDS substrate gel at low pH but the fixation of protein in the gel was excessive and difficult to remove. Thus, the gel could not be destained and the clearings was not observed.

Conclusion

Trypsin-like proteinase was the major enzyme in digestive organs and showed the highest activity at 55°C, pH 8.5 against hemoglobin and pH 7.5 towards casein. Pepsin-like proteinase predominated in solid wastes but showed minor activity in digestive organs of Pacific whiting. Its activity maximum was at pH 2.5 and 45°C.. These proteinases may have potential for use as processing agents.

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Chapter 6

Protein Hydrolysates from Pacific Whiting Solid Wastes

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Abstract

Alcalase and Neutrase showed optimum activity against Pacific whiting solid wastes (PWSW) at pH 9.5, 60°C and pH 7.0, 55°C, respectively. Alcalase had a higher proteolytic activity than Neutrase. Enzyme concentration, reaction time and waste/buffer ratio significantly affected the hydrolysis and nitrogen recovery (NR) ($p<0.05$). Optimum conditions for PWSW hydrolysis were 20 AU Alcalase/kg, 1 hr reaction time, waste/buffer ratio of 1:1 (w/v). Correlation between the degree of hydrolysis (DH) and NR (R^2=0.969-0.978) was high. Freeze-dried hydrolysate was brownish yellow in color (L*54.59, a*6.70, b*27.89) and contained 2.77% moisture, 79.97% protein, 13.44% ash and 3.83% lipid. Amino acid composition of freeze-dried hydrolysate was similar to that of PWSW and Pacific whiting muscle but tryptophan was reduced to 21.5% and 14.74%, respectively.

Introduction

Due to the abundance of Pacific whiting (*Merluccius productus*) off the west coast of the United States, it has been exploited as a raw material for surimi production (Morrissey et al., 1996). During processing, solid wastes including viscera, head, skin, bone and some muscle tissue are generated and can be as high as 70% of the original raw material. Normally, these wastes have been used as fish meal or fertilizer. Novel means of processing are required to convert the underutilized wastes into more marketable and acceptable forms. To upgrade protein by-products, proteases from plant, animal and microbial origin have been applied to convert seafood processing wastes and under-utilized species into protein concentrate (Onodenalore and Shahidi, 1996; Shahidi et al., 1994; 1995; Beak and Cadwallader, 1995; Hoyle and Merritt, 1994; Rebeca et al., 1991; Quaglia and Orban, 1987; Cheftel et al., 1971). Commercial enzymes were also used for protein hydrolysate production from chicken heads (Surowka and Fik, 1992; 1994) and veal bone (Linder et al., 1995; 1996). Autolysis caused by endogenous enzyme can contribute to the protein hydrolysis, however, the rate of hydrolysis is difficult to control due to several factors including the fish species, seasonality as well as the type and amount of enzymes (Sikorski and Naczk, 1981). Consequently, application of exogenous enzyme is more common, particularly for protein hydrolysate production since the hydrolysis and properties of resultant product can be manipulated. The objective of this study was to study the production and composition of hydrolysate from Pacific whiting solid waste using the commercial enzymes.

Materials and Methods

Reagents

2,4,6-Trinitrobenzenesulfonic acid (TNBS), sodium sulfite, and L-Leucine were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Laboratories, Hercules, CA. Boric acid was obtained from Mallinckrodt, Inc., St. Louis, MO. Alcalase 2.4 L (a declared activity of 2.4 AU/g and a density of 1.18 g/mL) and Neutrase 0.5 L (a declared activity of 0.5 AU/g and a density of 1.25 g/mL) were provided by Novo Nordisk Biochem North America, Inc. (Franklinton, NC).

Materials

Ground Pacific whiting solid wastes (PWSW) including head, skin, bone, viscera and muscle tissue were obtained from Point Adams Packing Co. (Hammond, OR). The samples were transported to OSU Seafood Laboratory, vacuum-packed in polyethylene bags and kept at -20°C until used.

pH and temperature profile for Alcalase and Neutrase on PWSW

Optimum pH of Alcalase and Neutrase against PWSW was studied at 60°C and 50°C, respectively. PWSW (5g) was added with 0.2 M McIlvaine buffer (pH 4.5-8.0) or 0.2 M borate buffer (pH 8.5-11.5) at the ratio of 1:2 (w/v) and pH of mixture was rechecked and adjusted with 6 N NaOH or 6 N HCl. The mixtures were incubated and well-shaken at reaction temperature for 10 min before the

reaction was initiated by adding 20 μL of enzymes. After 10 min, a 500 μL aliquot was mixed with 2.0 mL of 1% hot SDS solution (85°C) and placed in water bath at 85°C for 15 min.

The studies of optimum temperature for Alcalase and Neutrase on PWSW were carried out under optimum pH for each enzyme. α-amino acid released was measured and expressed as L-Leucine. Increased amount of α-amino acid was determined by subtracting the α-amino acid at 0 min from that of hydrolyzed PWSW at 10 min.

Enzymic hydrolysis of PWSW

Two levels of enzyme concentration (5 and 10 AU/Kg) were used to compared the hydrolytic activity between Alcalase and Neutrase. The reaction were held at optimum conditions, pH 9.5, 60°C for Alcalase and pH 7.0, 55°C for Neutrase. Increased α-amino acid was analyzed at different reaction times. Hydrolytic curves for each enzyme were obtained by plotting increased α-animo acid vs reaction time.

To study the effect of enzyme concentration on DH, different amounts of enzyme were added to the suspension of PWSW in buffer (1:2 ratio, w/v) and the reaction was carried out under optimum conditions. After 30 min, reaction was stopped by heating at 90°C for 5 min. The supernatant was obtained by centrifuging at 3,000 xg for 10 min.

Effect of PWSW and buffer ratio on α-amino acid and NR was investigated. PWSW was mixed with buffer at a ratio of 1:0.5, 1:1, 1:2, 1:3, 1: 5 and 1:8 (w/v). Enzyme (20 AU/Kg) was added and reaction was maintained for 30 min under optimum condition. α-amino acid and NR were determined as mentioned above.

Electrophoresis

Alcalase or Neutrase (10 AU/Kg) were added to the mixture of PWSW and buffer (ratio 1:2 (w/v)). The reaction was carried out under optimum condition at different times (5, 10, 30 and 60 min). After the exact reaction time, SDS was added to obtain the final concentration of 5% (w/v) and the mixture were heated at 85°C for 1.5 hr. The undissolved debris was removed by centrifuging at 3,500 xg for 5 min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run according to the method of Laemmli (1970) using 4% stacking gel and 15% separating gel. Sixty μg of protein determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard was applied to the gel. Proteins were stained in 0.125% Coomassie brilliant blue R-250 and destained in 25% ethanol and 10% acetic acid. M_r of protein bands was estimated using protein standards. High molecular weight standards (Sigma Chemical Co., St. Louis, MO) included rabbit muscle myosin (205,000), E. Coli β-galactosidase (116,000), rabbit muscle phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), and bovine erythrocytes carbonic anhydrase (29,000). Low molecular weight standards (Phamacia Biotech, Inc., Piscataway, NJ) contained phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrous (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Determination of optimum condition for PWSW hydrolysate production using Alcalase

Three parameters were studied to optimize the hydrolysate process as follows: enzyme concentration (10, 20, 40 AU/Kg), PWSW/ 0.2 M borate buffer ratio (1: 0.25.

1: 0.5, 1:1, 1:3 (w/v)) and reaction time (30, 60, 120 min). The hydrolysate obtained from different conditions was determined for NR. The liquid hydrolysate produced under optimum conditions was freeze-dried using a freeze dryer (Labconco Corp., Kansas City, MO).

Determination of Degree of hydrolysis (DH)

Modified methods of Adler-Nissen (1979) and Crowell et al. (1985) were used to determine α-amino acid content. Properly diluted samples (125 μL) were mixed thoroughly with 2.0 mL of 0.2125 M phosphate buffer, pH 8.2 followed by the addition of 1.0 mL of 0.01% TNBS solution. The mixtures were then placed in a water bath at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled down at ambient temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid was expressed in term of L-Leucine. The DH was determined using the modified method of Beak and Cadwallader (1995) and defined as follows:

$$DH = [(L_t-L_0)/(L_{max}-L_0)]x100$$

Where L_t corresponded to the amount of α-amino acid released at time t. L_0 was the amount of α-amino acid in original PWSW. L_{max} was the maximum amount of α-amino acid in PWSW obtained after acid hydrolysis. PWSW suspension (500 μL) was mixed with 4.5 mL of 6 N HCl. The tube with sample mixtures was flashed with nitrogen gas and sealed tightly with screw-cap. The hydrolysis was run at 100°C for 24 hr (Beak and Cadwallader, 1995). The acid-hydrolyzed sample was filtered through Whatman paper

#1 to remove the unhydrolyzed debris. The supernatant was neutralized with 6 N NaOH before α-amino acid determination.

Determination of Nitrogen recovery (NR)

After hydrolysis reaction, the dense lipid layer was skimmed using two-layer cheese cloth. The volume of soluble fraction was recorded and total nitrogen in supernatant was determined using AOAC method (1984). NR was calculated using the following equation:

NR (%) = [Total nitrogen in supernatant (mg)/Total nitrogen in substrate (mg)]x100

Composition analyses

Chemical compositions of samples including freeze-dried hydrolysate, PWSW and Pacific whiting muscle were determined as described below. Moisture content was measured by oven drying at 105°C until a constant weight (AOAC, method 24.002, 1984). Protein and ash were determined according to method 24.027 and 18.025, respectively (AOAC, 1984). Lipid was assayed using a modification of method 18.043 (AOAC, 1984).

Amino acid composition was determined after hydrolysis at 115°C for 20 hr in 6 N HCl with 0.05% mercaptoethanol and 0.02% phenol. The amino analysis was performed by post-column derivatization with ninhydrin using a Beckman System 6300 amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA). Cysteine was determined as cysteic acid. Performic acid oxidation was run at 50°C for 15 min prior

to acid hydrolysis (Hirs, 1967). Tryptophan was measured by alkaline hydrolysis at 135°C for 48 hr (Hugli and Moore, 1972).

Color measurement

L* (lightness), a* (redness,+ or greenness,-) and b* (yellowness,+ or blueness,-) of samples including freeze-dried hydrolysate, PWSW and Pacific whiting muscle were measured using a Minolta Chroma meter CR-310 (Minolta Corp., Ramsey, NJ). A Minolta calibration plate (Y_{CIE} = 94.5, x_{CIE} = 0.3160, y_{CIE} = 0.330) and a Hunter Lab standard plate (L* = 82.13, a* = -5.24, b* = -0.55) were used to standardize the instrument with D65 illuminant and 2° observer.

Statistical analyses

Data was analyzed using the analysis of variance procedure. Mean difference was determined using the least significant difference (LSD) multiple range test (Statgraphics Version 6.0, Manugistics Inc., Rockville, MD). All experiments were done in duplicate.

Results and Discussion

pH and temperature profile of Alcalase and Neutrase on PWSW

The pH activity curves of Alcalase and Neutrase are shown in Fig. 6.1. The optimum pH for Alcalase and Neutrase on PWSW were 9.5 and 7.0, respectively. Alcalase showed a broad activity in alkaline pH range. However, a sharp decrease in activity was observed at pH 11.5. Alcalase was more active at alkaline pH and remains active to pH 6.0 (Adler-Nissen, 1986). For Neutrase , the activity reached the maximum

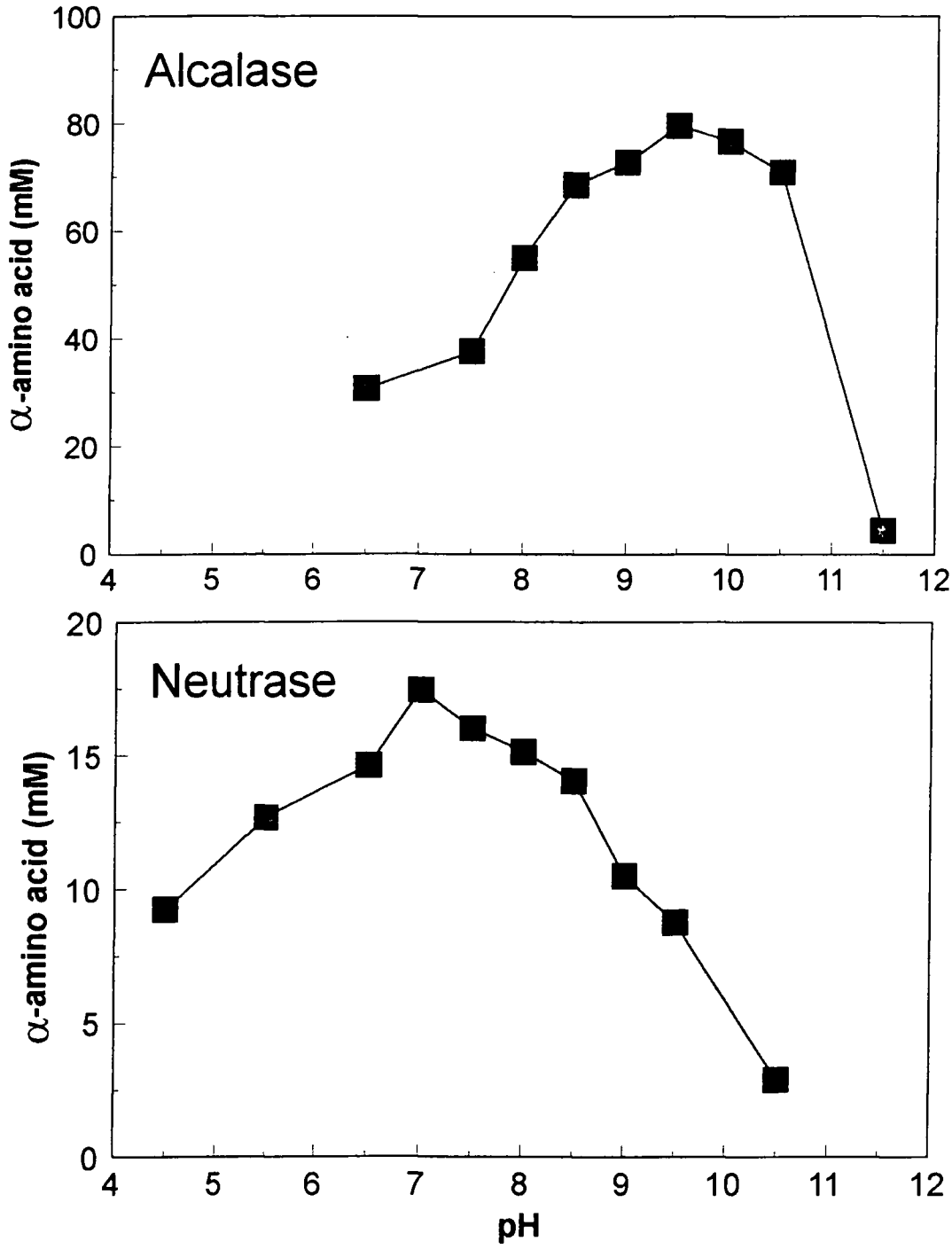

Fig. 6.1. pH profile of Alcalase and Neutrase on PWSW. The hydrolytic reaction was run for 10 min at 60° and 50°C for Alcalase and Neutrase, respectively. The hydrolysis product was expressed as α-amino acid (mM).

at pH 7.0. The activity was high in pH range of 6.5-8.5 but showed considerable loss of activity at pH 10.5. A change in pH affects both the substrate and enzyme by changing the charge distribution and conformation of the molecules (Adler-Nissen, 1986). Most enzymes undergo irreversible denaturation in very acid and alkaline solution, causing the loss of stability (Whitaker, 1994). The pH also effects the ionization of prototrophic groups in the active site of enzyme which are involved in maintaining proper conformation of active site of enzyme, binding of substrate to enzyme and transformation of substrate to product (Whitaker, 1994).

The effect of temperature on enzyme activity is shown in Fig. 6.2. Alcalase showed a high activity in the high temperature range (55-70°C) with an optima at 60°C. However, a appreciable decrease in enzyme activity was observed above 70°C. In general, Alcalase was less active at low temperature. At 20°C, the activity was approximately 4-fold lower than that obtained at 60°C. Adler-Nissen (1986) reported that the activity of Alcalase doubled for every temperature rise of 12°C. Beak and Cadwallader (1995) found that optimum temperature of Alcalase on crayfish processing by-products was 70°C. This difference was probably due to the different substrate and reaction condition. For Neutrase, the activity increased to 55°C followed by a sharp decrease in activity. No activity was found at 80°C. At high temperatures, most enzymes will be irreversibly denatured. The temperature activity curves indicates that Alcalase was more heat stable and active at a higher temperature for PWSW hydrolysis.

Enzymatic hydrolysis of PWSW

Hydrolytic curves of PWSW by Alcalase and Neutrase are compared (Fig. 6.3).

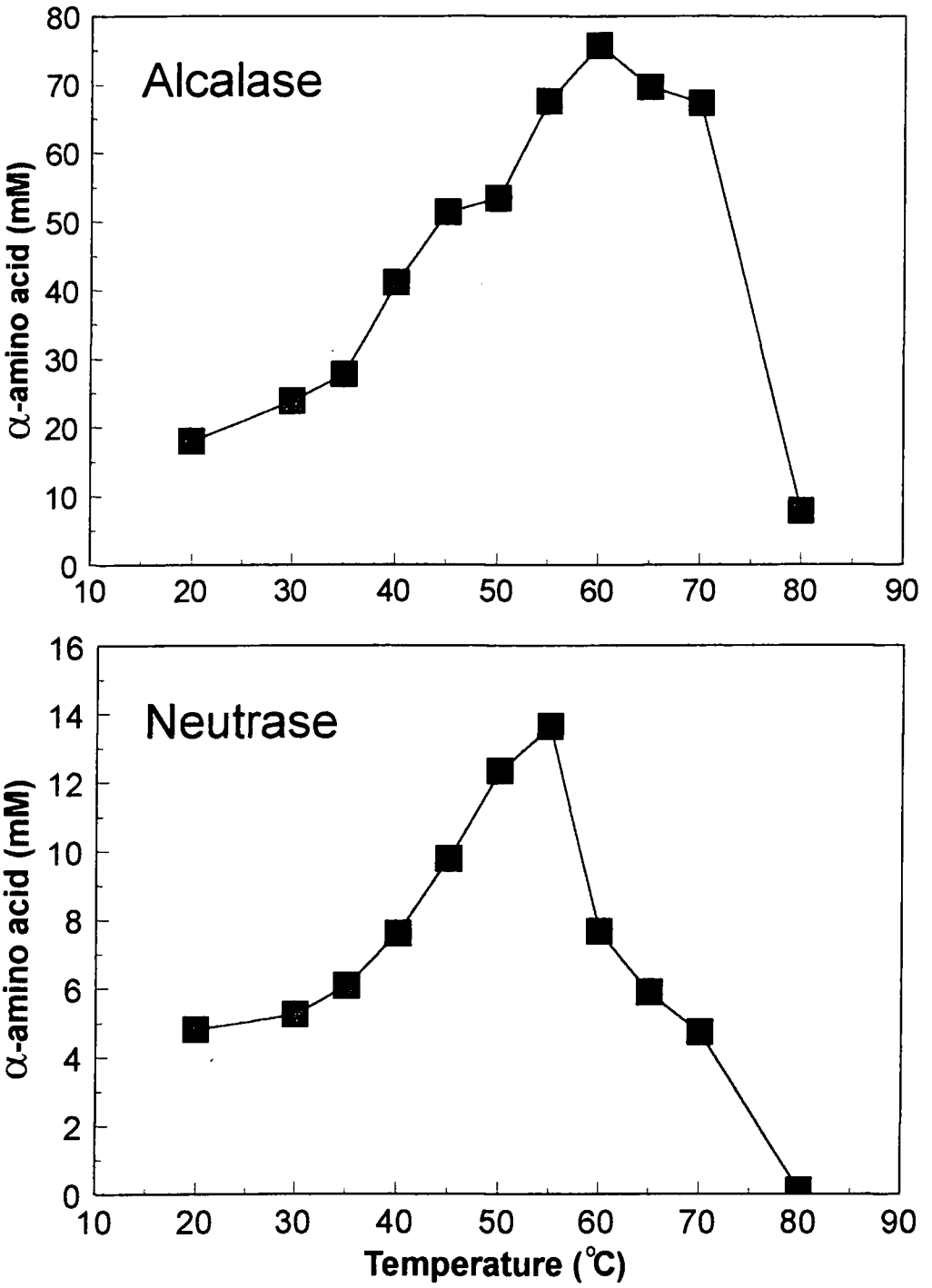

Fig. 6.2. Temperature profile of Alcalase and Neutrase on PWSW. The hydrolytic reaction was run for 10 min at pH 9.5 and 7.0 for Alcalase and Neutrase, respectively. The hydrolysis product was expressed as α-amino acid (mM).

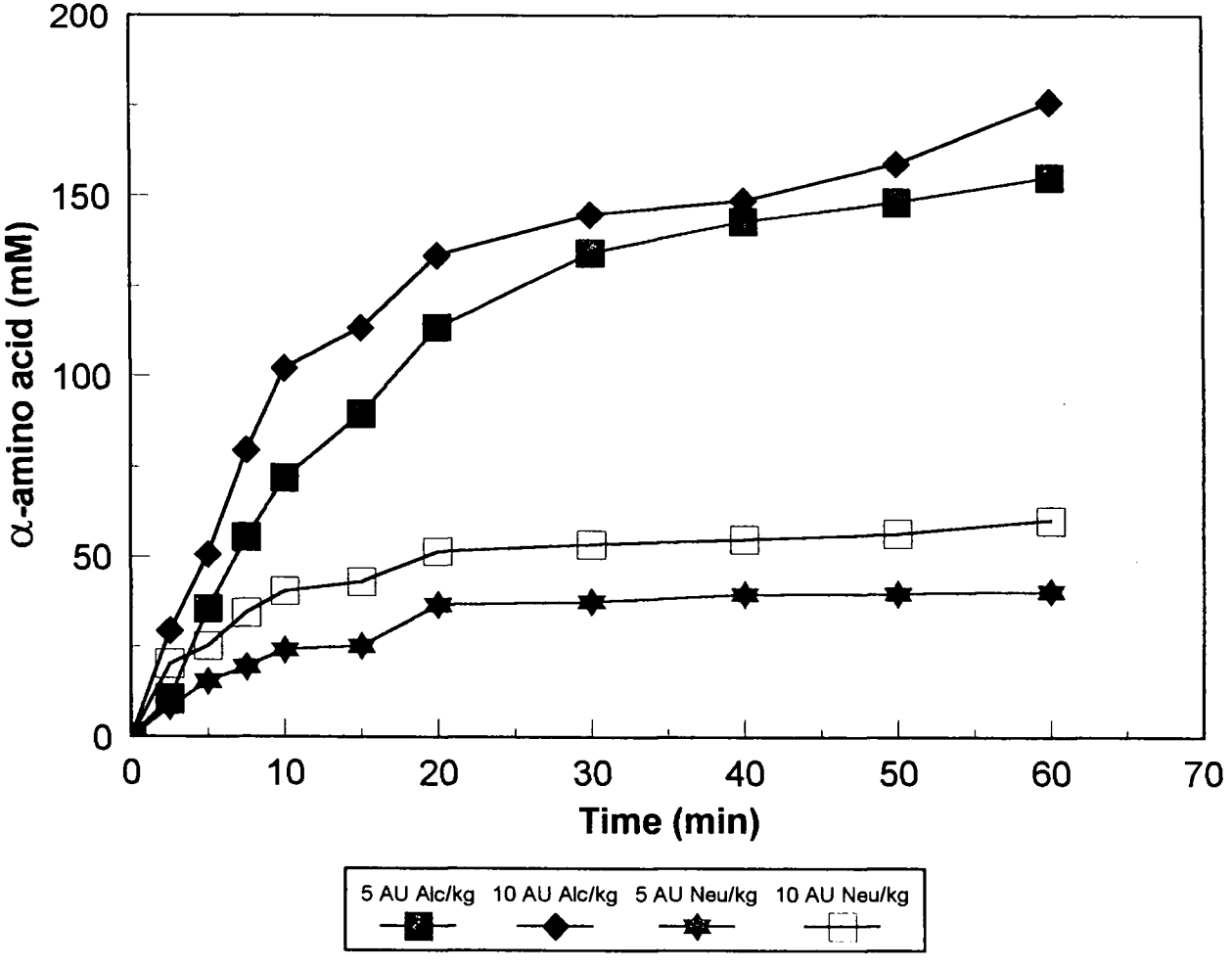

Fig. 6.3. Proteolytic activity of Alcalase and Neutrase on PWSW. The hydrolytic reaction was run at pH 9.5, 60°C and pH 7.0, 55°C for Alcalase and Neutrase, respectively. The hydrolysis product was expressed as α-amino acid (mM).

For each enzyme, α-amino acid in PWSW treated with 10 AU/kg was slightly higher than that treated with 5 AU/kg. At the same level of enzyme, Alcalase showed a higher hydrolytic activity than Neutrase. A rapid reaction rate was obtained in the first 10 min, then the rate of hydrolysis subsequently decreased . A constant rate was observed with PWSW treated with Neutrase after 20 min of reaction time, suggesting that the enzymatic reaction reached the steady-state phase. A similar curve was reported for the enzymatic hydrolysis of crayfish by-product (Beak and Cadwallader, 1995), capelin (Shahidi et al., 1995), sardine (Quaglia and Orban, 1987) and veal bone (Linder et al., 1996). Generally, the enzyme absorbs rapidly onto the insoluble protein particles followed by cleaving the polypeptide chains that are loosely bound to the surface. The more compacted core proteins are hydrolyzed more slowly. The rate of enzymic cleavage of peptide bond controls the overall rate of hydrolysis (Archer et al., 1973).

Hydrolysis pattern on SDS-PAGE

SDS-PAGE of control and enzyme treated PWSW is shown (Fig. 6.4). In the control sample (C), protein bands with M_r ranging from 82,000 to 111,700 were observed. These bands were postulated to be the native proteins or the degradation products from myosin due to an autolysis during handling or waste generation process. Actin with M_r 45,000 was prominent in PWSW. Benjakul et al. (1997) reported that forty-five percent of Pacific whiting myosin heavy chain (MHC) was degraded within 8 days iced storage, but no noticeable difference was observed in actin. A broad range of low M_r bands was also noted. After treatment of PWSW with Alcalase, high M_r bands were totally removed and the low M_r bands were observed. No obvious difference was

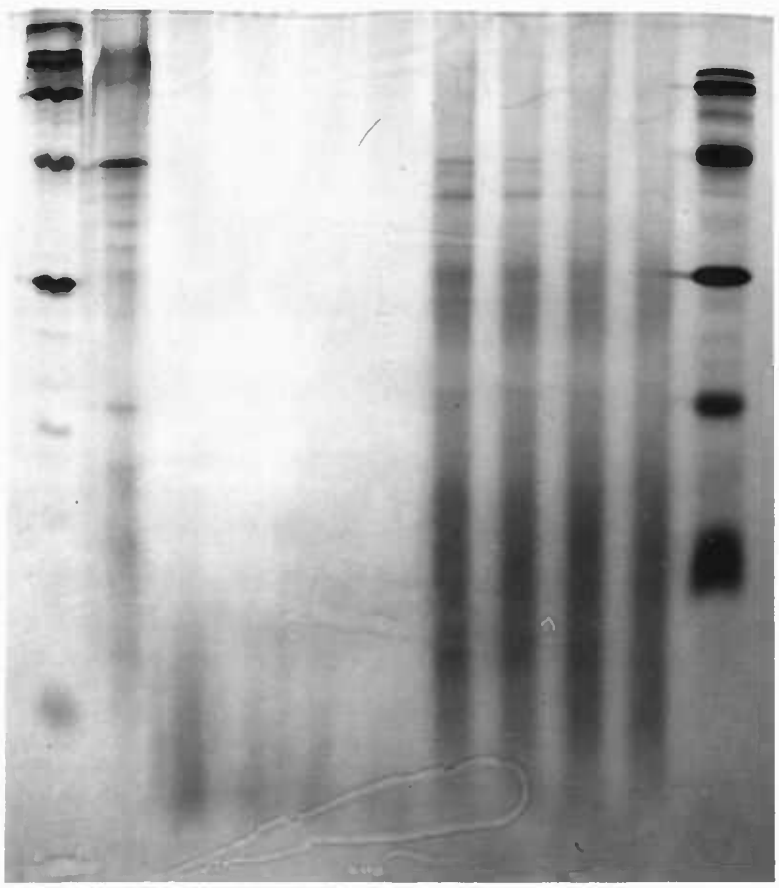

Fig. 6.4. SDS-PAGE patterns of PWSW proteins during hydrolysis by Alcalase (A) and Neutrase (N). Numbers designate the reaction time (min). HMW, LMW and C stand for high molecular weight standards, low molecular weight standards and control (PWSW in the absence of enzyme).

found with different reaction time. Different hydrolytic patterns of PWSW proteins were noted after treatment with Neutrase. After Neutrase treatment, the bands ranging from M_r 82,000 to 117,000 entirely disappeared while an increase of bands at M_r 30,000 and M_r lower than 17,000 were observed. At 5 min (N_5), a decreasing intensity of band with Mr 45,000 was found and band intensity was decreased as time increased. This band totally disappeared after 60 min (N_{60}). From SDS-PAGE results, it can be inferred that Alcalase showed a considerably higher hydrolytic activity on PWSW when compared with Neutrase. The result coincided with a higher amount of α-amino acid in hydrolysate obtained after treatment of PWSW with Alcalase (Fig. 6.3).

Effect of enzyme concentration on DH and NR

When the enzyme concentration was increased, DH and NR of PWSW treated with both Alcalase and Neutrase increased (Fig. 6.5, 6.6). Significant changes in DH and NR occurred with the enzyme treatment at concentrations ranging from 0-34 AU/kg ($p<0.05$). Increase in DH was less in the low concentration range when Neutrase was applied. However, no significant increases for both DH and NR were found with treatment of enzyme at concentration above 57 AU/kg ($p>0.05$). PWSW hydrolysate treated with Alcalase showed appreciably higher DH and NR than that treated with Neutrase. The value of DH in this study was similar to that obtained in crayfish processing by-product hydrolysate treated with Alcalase (Beak and Cadwallader, 1995). Cheftel et al. (1971) found that an increased amount of protein solubilized was due to the increase in enzyme concentration.

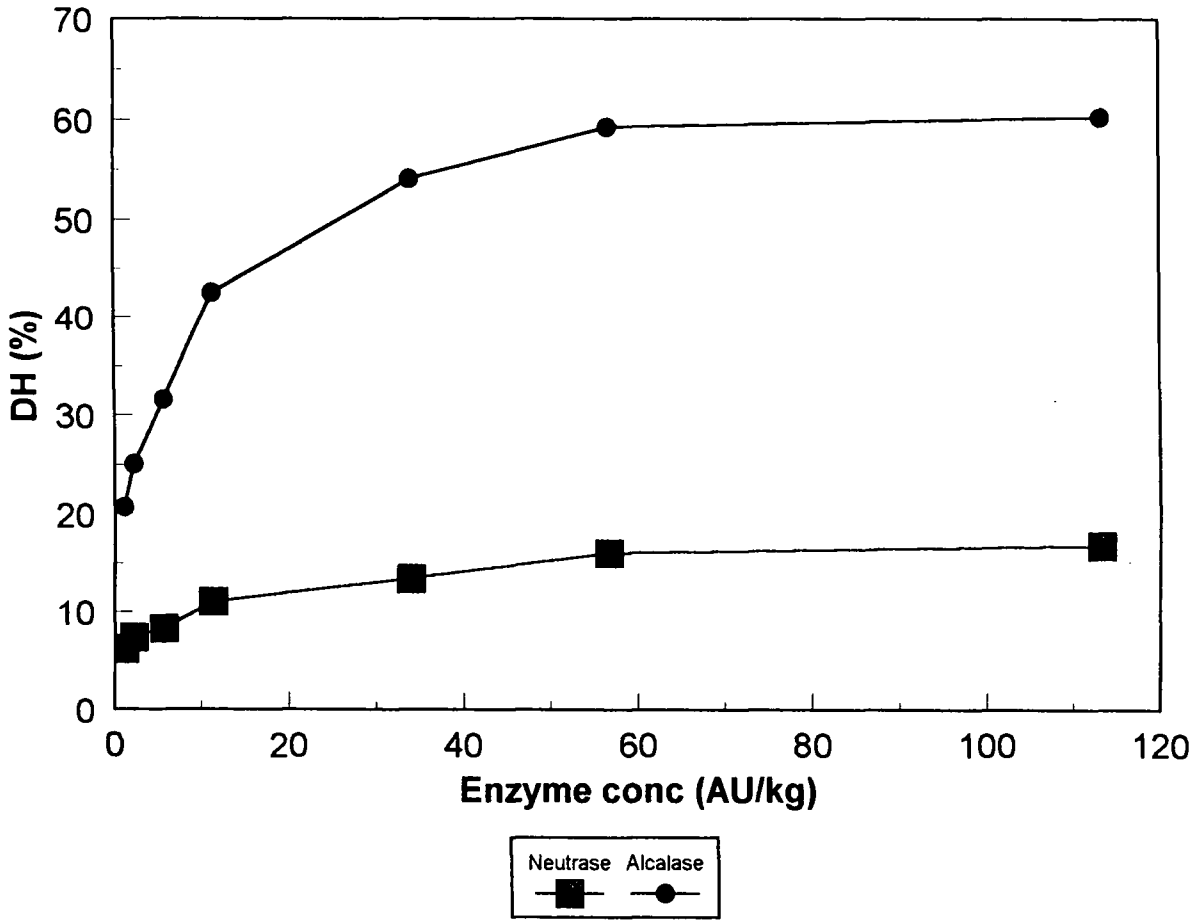

Fig. 6.5. Effect of Alcalase and Neutrase concentration on DH of PWSW.

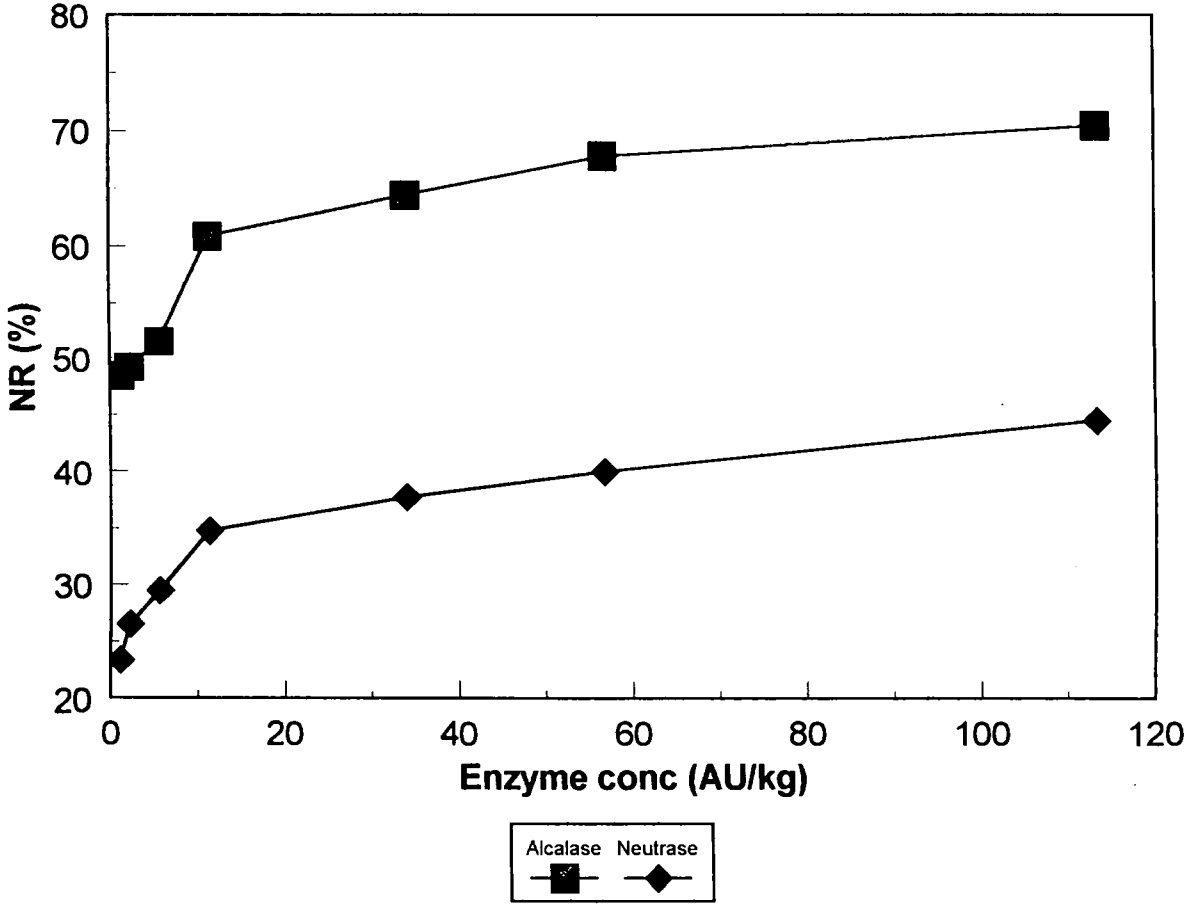

Fig. 6.6. Effect of Alcalase and Neutrase concentration on NR of PWSW.

When plotted Log_{10}(enzyme concentration) vs DH , A linear relationship was observed (Fig. 6.7). The correlation coefficient of R^2=0.978 and 0.972 were obtained for Alcalase and Neutrase, respectively. This result was in agreement with Beak and Cadwallander (1995) who reported that the relationship between log_{10}(protease amount) and DH for enzymatic hydrolysis of crayfish processing by-products was linear. Hale (1969) found that a logarithmic plot of digestion ratio vs enzyme concentration for enzymatic hydrolysis of fish protein substrate powder was linear. From this relationship, the exact concentration of enzyme required to hydrolyze PWSW to a required DH in 30 min under optimum condition can be calculated.

Our results showed that NR was direct proportional to DH (Fig. 6.8, 6.9). The correlation coefficient of R^2=0.978 and 0.969 were obtained for Alcalase and Neutrase, respectively, indicating the close relationship existed between DH and NR. At higher DH, there was a higher concentration of soluble peptides, leading to an increase in NR. Shahidi et al. (1995) reported that considerable soluble protein was released during initial phase and no increase in soluble hydrolysate was observed when additional enzyme was added during the stationary phase of hydrolysis. The rate of hydrolysis and NR was reduced with a high concentration of soluble peptides in reaction mixtures. In our study, the increase in enzyme concentration caused an increase in DH and soluble peptides, leading to a higher NR.

Effect of substrate/buffer ratio on hydrolysis and NR

The effect of substrate/buffer ratio on hydrolysis and NR of PWSW by Alcalase and Neutrase is shown (Fig. 6.10, 6.11). In general, an increase in waste/buffer ratio

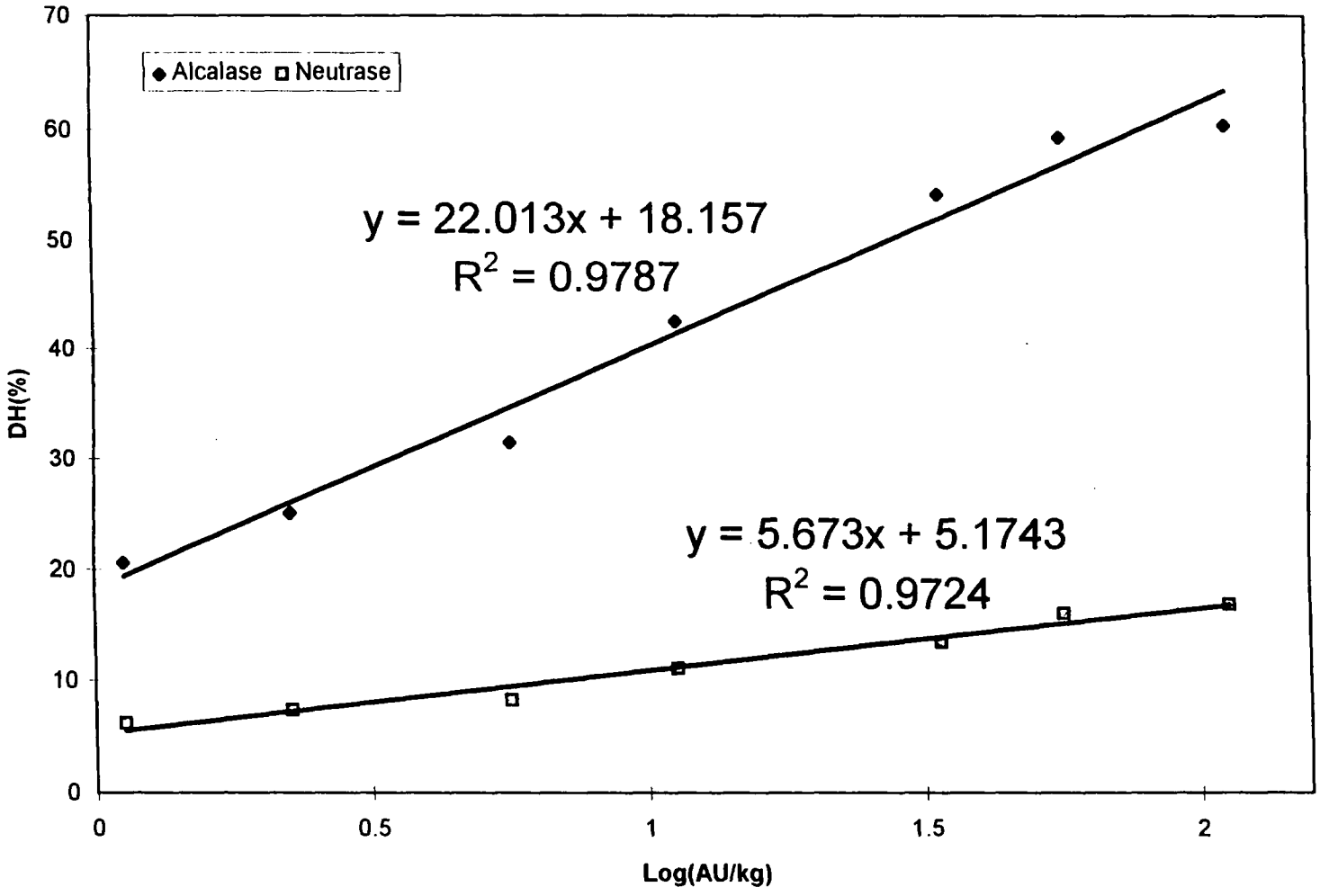

Fig. 6.7. Relation between Log_{10}(enzyme concentration) and DH for PWSW treated with Alcalase or Neutrase.

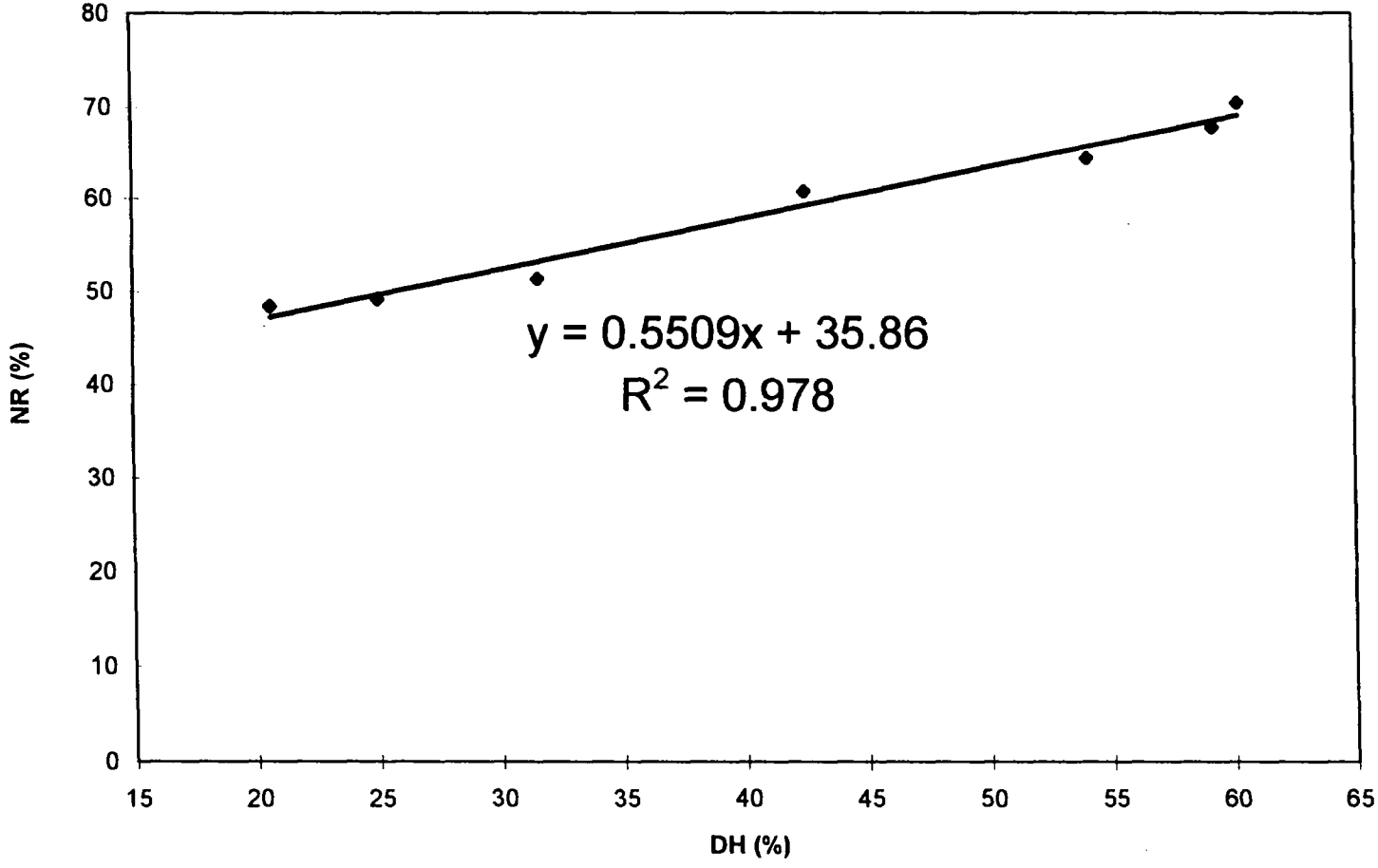

Fig. 6.8. Relation between DH and NR for PWSW treated with Alcalase.

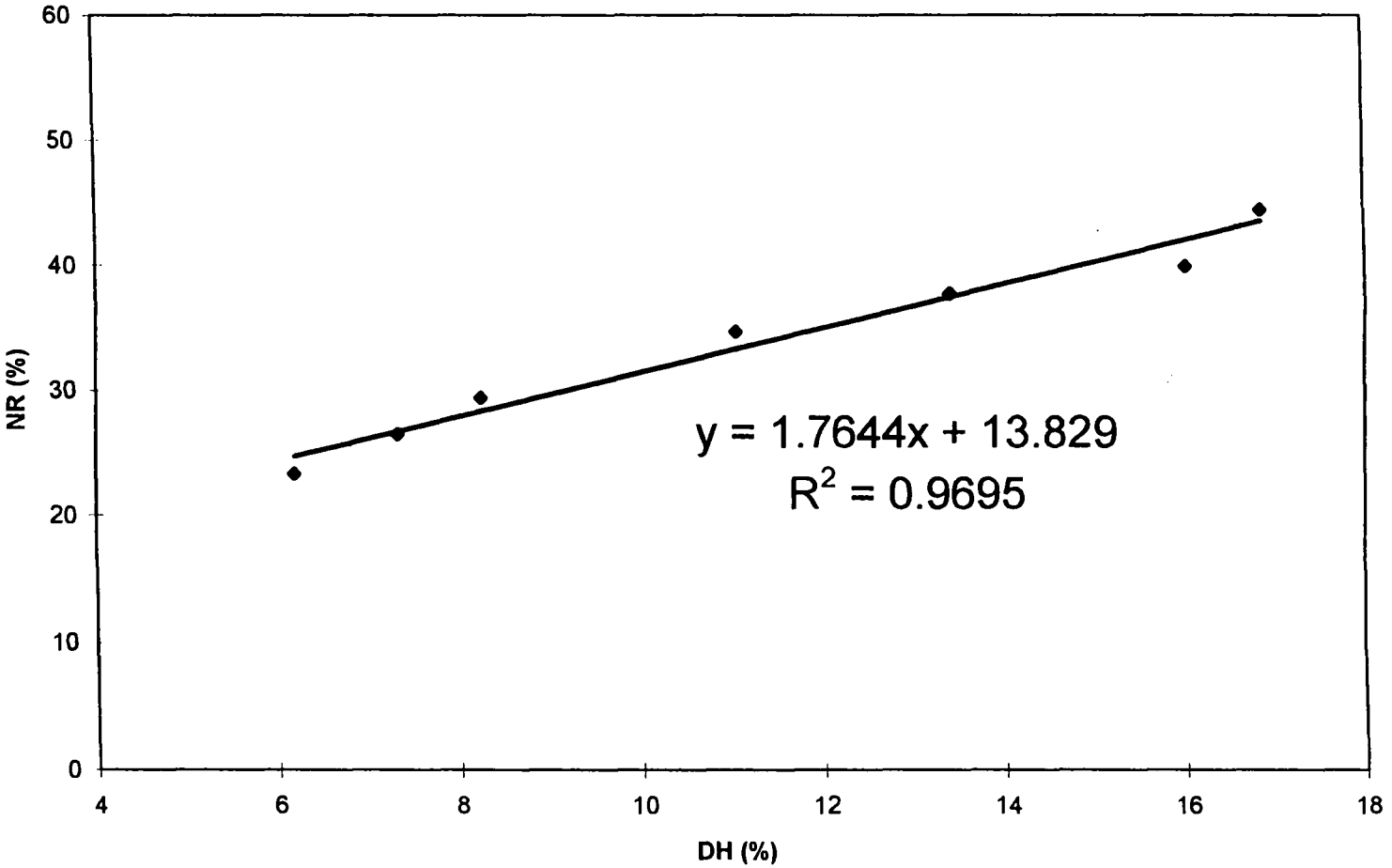

Fig. 6.9. Relation between DH and NR for PWSW treated with Neutrase.

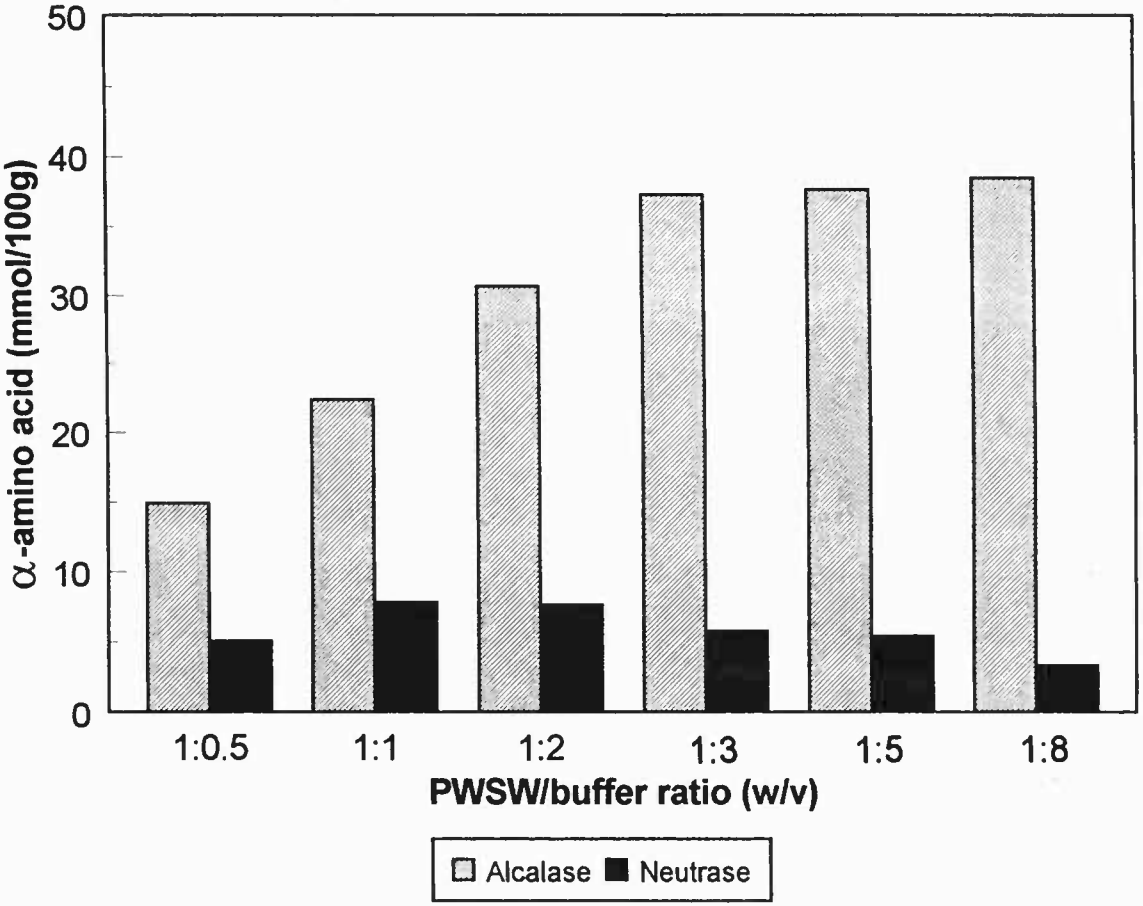

Fig. 6.10. Effect of waste/buffer ratio on hydrolysis of PWSW treated with Alcalase or Neutrase. The hydrolysis rate was expressed as α-amino acid (mmol/ 100 g sample).

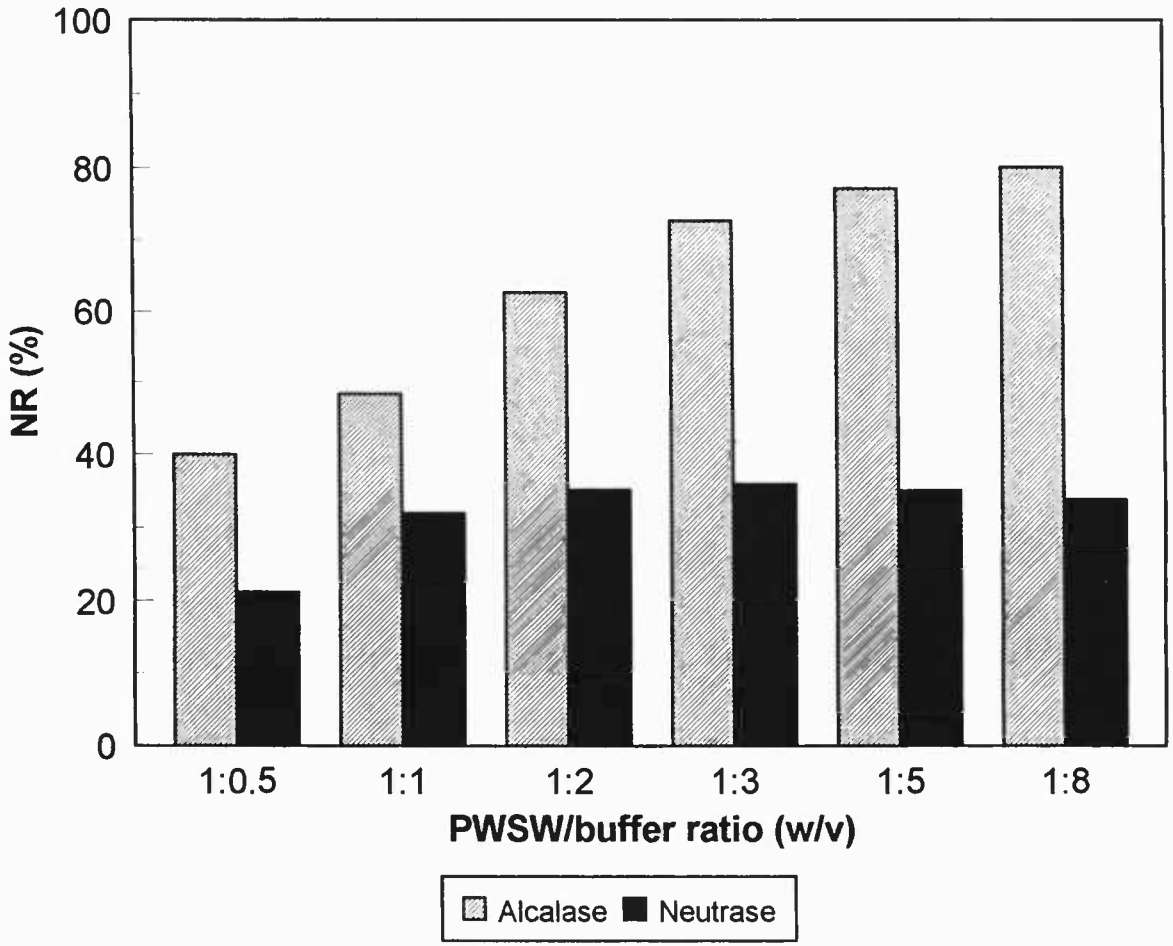

Fig. 6.11. Effect of waste/buffer ratio on NR for PWSW treated with Alcalase or Neutrase.

resulted in an increase in α-amino acid concentration as well as NR. For Alcalase, the increase in ratio up to 1:3 significantly increased both α-amino acid concentration and NR ($p<0.05$). However, no significant changes were observed at the ratio higher than 1:3 ($p>0.05$). For Neutrase, the ratio above 1:1 did not cause a significant increase in NR ($p>0.05$). Sufficient buffer provided buffering capacity for the reaction and worked as a media for enzyme dispersion and was considered as an important factor for PWSW hydrolysis. From these results, waste/buffer ratio of 1:2 and 1:3 (w/v) was sufficient for enzymatic reaction. Increased water added to substrate enhanced enzyme homogeneity, promoted tissue swelling and reduced the localized concentration of hydrolysis products (Surowka and Fik, 1994). This result was in accordance with Surowka and Fik (1992; 1994) who applied pepsin and neutrase to recover the proteinaceous substances from chicken heads and found that an increase in the ratio of added water resulted in an increase in non protein nitrogen.

Optimum condition for PWSW hydrolysis by Alcalase

Due to the high activity of Alcalase in PWSW hydrolysis, it was chosen for hydrolysate optimization tests. In these tests, yield is represented by NR instead of α-amino acid concentration. All three parameters including enzyme concentration, waste/buffer ratio and reaction time affected NR (Fig. 6.12). To minimize the cost of enzyme and energy for water removal to produce dry hydrolysate as well as to reduce the reaction time, the conditions for enzymic hydrolysis of PWSW with Alcalase 2.4L were selected as pH 9.5, 60°C, 1 hr reaction time, waste/0.2 M borate buffer ratio of 1:1 (w/v) and 20 AU/kg waste.

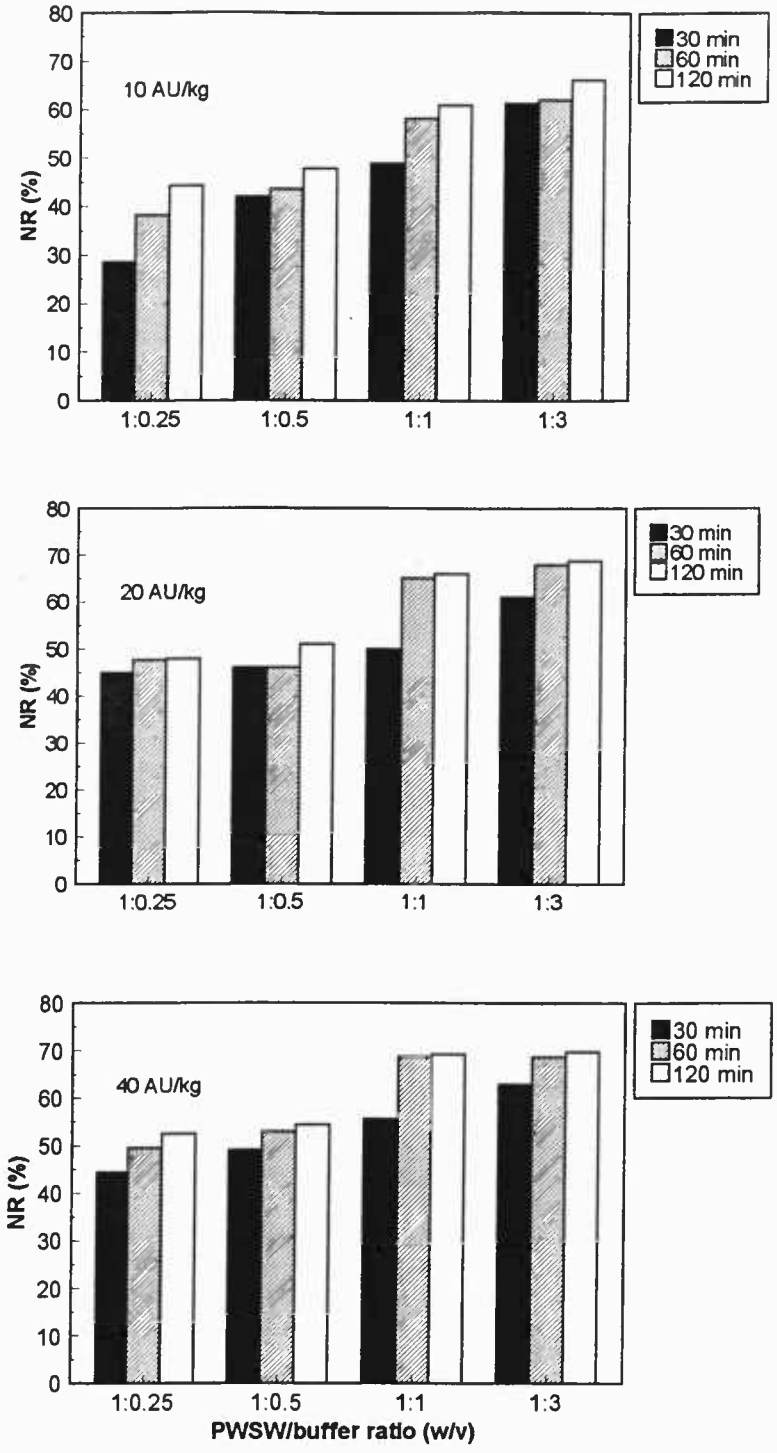

Fig. 6.12. Effect of enzyme concentration, reaction time and waste/buffer ratio on NR for PWSW treated with Alcalase

Compositions of PWSW hydrolysate

The freezed-dried hydrolysate, PWSW and whole Pacific whiting muscle contained 2.77, 81.10 and 84.62% moisture, respectively. On dry weight basis, freeze-dried hydrolysate contained higher protein content but lower ash and lipid content than PWSW (Table 6.1). The high protein content was a result of the solubilization of protein during hydrolysis, the removal of insoluble undigested non-protein substances and partial removal of lipid after hydrolysis. The inorganic substances reported in the hydrolysate were possibly due to the addition of borate buffer during the enzymatic reaction. However, inorganic compounds were lower than PWSW. The main source of minerals in PWSW was the bone and scale which were ground together with the whole waste. Removal of the fat layer after hydrolysis caused a low lipid content in hydrolysate. During the hydrolysis process, the muscle cell membranes tend to round up and form insoluble vesicles, leading to the removal of membrane structured lipids (Shahidi et al., 1995). Reduced lipid content was reported in harp seal (Shahidi et al., 1994), capelin (Shahidi et al., 1995) and shark hydrolysates (Onodenalore and Shahidi, 1996). Hoyle and Merritt (1994) reported hydrolysate preparation with a low lipid content by using the ethanol-extracted herring. For Pacific whiting muscle, a considerably high protein content with a low ash and lipid content were observed compared with PWSW and freeze-dried hydrolysate.

Freeze-dried hydrolysate was brownish yellow in color (L* 54.59, a* 6.70, b* 27.89) and lighter than PWSW whose color was dark probably due to the oxidation of myoglobin and the melanin pigment in the skin (L* 45.94).

Table 6.1 Chemical composition of Freeze-dried hydrolysate, PWSW and Pacific whiting muscle*

Compositions (% dry weight basis)	Freeze-dried hydrolysate	PWSW	Pacific whiting muscle
Protein	82.25 ± 0.05	69.36 ± 3.28	98.05 ± 3.97
Lipid	3.94 ± 0.13	20.31 ± 0.74	2.53 ± 0.75
Ash	13.82 ± 0.08	20.74 ± 4.34	6.50 ± 0.65

* Freeze-dried hydrolysate, PWSW and Pacific whiting muscle contained 2.77 ± 0.12, 8.10 ± 0.29 and 84.62 ± 0.28% moisture, respectively

The amino acid compositions of freeze-dried hydrolysate were similar to those of PWSW and Pacific whiting muscle (Table 6.2). Freeze-dried hydrolysate contained a lower amount of glutamic acid and tryptophan than PWSW and the muscle. Tryptophan in hydrolysate was reduced to 14.74-21.5% of that in PWSW and muscle, respectively. Shahidi et al. (1995) reported that the tryptophan in capelin hydrolysate was reduced by approximately 60% when compared with the original muscle. Freeze-dried hydrolysate and PWSW had a similar amount of glycine which was approximately 2-fold higher than the muscle. These differences were possibly due to the variation between the compositions of soluble proteins and unhydrolyzed residues. PWSW contained skin and other connective tissues, leading to the differences in amino acid composition from the muscle. From this result, freeze-dried hydrolysate contained a

comparable amount of amino acids when compared with Pacific whiting muscle. In future studies, the functional properties of hydrolysate will be tested.

Table 6.2 Amino acid compositions of freeze-dried hydrolysate, PWSW and Pacific whiting muscle

Amino acid compositons	% of Total protein		
	Freeze-dried hydrolysate	PWSW	Pacific whiting muscle
Alanine	6.53	6.45	5.51
Arginine	7.29	7.71	7.28
Aspartic acid	10.10	9.72	10.50
Cysteine	0.92	0.82	1.13
Glutamic acid	13.80	18.50	15.00
Glycine	7.88	8.09	3.87
Histidine	2.10	2.19	2.35
Isoleucine	4.30	4.28	4.97
Leucine	7.16	7.08	8.05
Lysine	8.33	8.19	10.20
Methionine	3.02	2.96	3.20
Phenylalanine	3.80	3.87	4.06
Proline	6.00	6.25	4.04
Serine	5.33	5.35	4.80
Threonine	5.12	4.86	4.86
Tryptophan	0.14	0.65	0.95
Tyrosine	3.50	3.38	4.18
Valine	4.72	4.61	4.94

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Chapter 7

Physicochemical and Functional Properties of Pacific Whiting Solid Waste Hydrolysate

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Abstract

Pacific whiting solid waste (PWSW) hydrolysates were prepared using Alcalase. The influence of degree of hydrolysis (DH = 20, 30, 40, 50, 60%) on the physicochemical, functional properties and antioxidant activity was investigated. Surface hydrophobicity, total and surface sulfhydryl content increased as the DH increased. The hydrolysates showed a high solubility over a wide pH range. Fat adsorption and fat binding capacity were reduced with an increased DH. The highest emulsifying activity was obtained in hydrolysate with DH of 30%. However, the low emulsion stability was found in all hydrolysates. The foam expansion was increased when the DH was increased and high foam stability was observed in all hydrolysates except that with DH of 20%. Hydrolysates showed antioxidant activity but no obvious differences in activity were noted with different DH and hydrolysate concentration.

Introduction

Waste utilization has become increasingly important due to the environmental concerns and the focus of the seafood industry on full utilization of what is harvested. Hydrolysis processes or the production of hydrolysate have been developed to convert the underutilized by-products into value-added products (Linder et al., 1995; Shahidi et al., 1994; 1995; Quaglia & Orban, 1987). Hydrolysis under controlled conditions can improve the functional properties of proteins (Multilangi et al., 1996; Quaglia & Orban, 1990; Smith & Brekke, 1985). The peptides produced by proteolysis possess smaller molecular sizes and less secondary structure than native proteins, leading to increased solubility near the isoelectric point, decreased viscosity and significant changes in the foaming, gelling and emulsifying properties (Chobert et al., 1988a,b). The role of structural and physicochemical properties on functional behavior of food proteins have been reported (Wagner & Anon, 1990; Hayakawa & Nakai, 1985; Li-Chan et al., 1984; 1985; Nakai & Li-Chan, 1985; Nakai, 1983; Townsend & Nakai, 1983; Voutsinas et al., 1983).

Antioxidative properties of enzymatic hydrolysate of some proteins have been described (Yamaguchi et al., 1975; Tsuge et al., 1991; Chen et al., 1995). Histidine-containing peptides have been shown as the primary antioxidant (Murase et al., 1993; Uchida & Kawakishi, 1992). These peptides can be used as a potential source of the natural antioxidant.

The objective of this study was to determine the influence of degree of hydrolysis of Pacific whiting solid wastes on physicochemical and functional properties as well as the antioxidant activity of the resultant hydrolysate.

Materials and Methods

Reagents

Alcalase 2.4 L (a declared activity of 2.4 AU/g and density of 1.18 g/mL) was provided by Novo Nordisk Biochem North America, Inc. (Franklinton, NC). 8-anilino-1-naphthalene sulfonic acid (ANS), 5,5'-dithio-bis(2-nitrobensoic acid), linoleic acid, trans-β-carotene were purchased from Sigma Chemical Co. (St. Louis, MO). Boric acid was obtained from Mallinckrodt, Inc. (St. Louis, MO). Phosphoric acid meta was purchased from Mallinckrodt, Inc. (Paris, KY).

Preparation of hydrolysate

Pacific whiting solid wastes (PWSW) was obtained from Point Adams Packing Co. (Hammond, OR) and kept at -20°C until used. PWSW hydrolysates with different degree of hydrolysis (DH) (20, 30, 40, 50 and 60%) were prepared according to the method of Benjakul and Morrissey (1997). PWSW was mixed with 0.2 M borate buffer, pH 9.5 at the ratio of 1:2 (w/v). The pH was rechecked and adjusted with 6 N NaOH or 6 N HCl. The mixture was incubated at 60°C before adding enzyme at different concentration to obtain the required DH. After 30 min, the enzymatic reaction was stopped by heating the mixture at 90°C for 5 min followed by rapid cooling in ice water. The undigested debris was removed by centrifuging at 3,000 xg for 15 min. The supernatant was freeze-dried using a freeze dryer (Labconco Corp., Kansus City, MO).

Hydrophobicity

PWSW hydrolysate was dissolved and serially diluted with 10 mM phosphate buffer, pH 7.0 or 10 mM phosphate buffer, pH 7.0 containing 0.2 M NaCl to obtain the equivalent concentration of 0.1-0.5 mg/mL (Mahmoud et al., 1992). Protein surface hydrophobicity was determined by the method of Li-Chan et al. (1985). The diluted protein (2 mL) was mixed with 10 μL of 8 mM 1-anilinonaphthalene-8-sulfonic acid (ANS) in 10 mM phosphate buffer, pH 7.0 or 10 mM phosphate buffer, pH 7.0 containing 0.2 M NaCl. The relative fluorescence intensity of ANS-protein conjugates was measured with an Aminco Bowman spectrofluorometer (American Instrument Co., Silver Spring, MD) at excitation and emission wavelength of 374 and 485 nm, respectively. The fluorescence intensity reading was calibrated using an ANS standard (2 mL methanol added directly to 10 μL of ANS) and the reading was adjusted to 80% of full scale. The blank was run by measuring fluorescence intensity in the sample without ANS. Protein hydrophobicity was calculated as the slope of the plot of relative intensity against protein concentration (%, w/v).

Total and surface sulfhydryl content (SH)

Total SH content was measured using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) according to the method of Sompongse et al. (1996a) with a slight modification. PWSW hydrolysate solution (5 mg/mL) was prepared using 0.2 M Tris-HCl buffer, pH 6.8, containing 8 M urea, 2% SDS, and 10 mM EDTA. To 4 mL of mixture, 0.4 mL of 0.1% DTNB solution was added, mixed and incubated at 40oC for 25 min. Absorbance was measured at 412 nm wavelength with a spectrophotometer (Beckman Instrument,

Inc., Redmond, WA). Sample and reagent blank was run for each determination. SH content was calculated from the absorbance using molar extinction of 13,600 M^{-1} cm^{-1} and expressed as mol/10^5g protein. For surface SH content, it was determined in the same manner as the total SH group but 0.2 M Tris-HCl containing 0.6 M KCl was used and the reaction with DTNB was run at 15°C for 1 h (Sompongse et al., 1996b).

Solubility

Solubility of PWSW hydrolysate was determined according to the modified method of Chobert et al. (1988b). The pH of PWSW hydrolysate solution (10 mg/mL) was adjusted from 1.0-11.0 with 6 N HCl and 6 N NaOH. After 30 min equilibration, the sample was centrifuged at 5,500 rpm for 15 min. The protein content in the supernatant was measured by the method of Lowry et al. (1951) using BSA as a standard. The solubility was expressed as percent soluble protein at a given pH.

Fat adsorption

Fat adsorption was measured with the method of Wang and Kinsella (1976) with some modifications. Mazola corn oil (Best Foods Div., CPC International Inc., Englewood Cliffs, NJ) (1.2 mL) was added to 150 mg PWSW hydrolysate in the centrifuge tube. The mixture was stirred and sonicated with a Bransonic ultrasonic cleaner Model 121OR-MT (Branson ultrasonic corp., Danbury, CT) for 5 min to disperse the sample. After holding at room temperature for 30 min, the mixture was centrifuged at 2,000 xg for 25 min. The oil retained in the protein pellet was expressed as fat adsorbed in ml of oil per g of sample.

Fat binding capacity

Fat binding capacity was determined using the method of Voutsinas and Nakai (1983). To 40 mg protein of freeze-dried PWSW hydrolysate, 1.5 mL of Mazola corn oil was added. The mixture was mixed, sonicated for 3 min and centrifuged at 3,000 xg for 20 min after being held at room temperature for 30 min. The free oil was pipetted off and 2 mL of distilled water was added. The precipitate was gently scraped from the bottom of centrifuge tube to release the oil that was entrapped beneath the precipitate. To the mixture, 1 mL of 0.1 N metaphosphoric acid was added and the tube was centrifuged at 4500 xg for 15 min. The supernatant was pipetted off and the precipitate was washed with distilled water (4 mL) without dispersing the pellet. The supernatant was removed and the tube wall was cleaned with the wipers (Kimberly-Clark corp., Atlanta, GA). To the precipitate, 0.3 mL of distilled water was added and mixed thoroughly followed with 20 mL digestive medium (7 M urea in 50% H_2SO_4). The mixture was homogenized for 30 sec with a Polytron at speed 3. The mixture was held at room temperature for 30 min and the absorbance was measured at 600 nm. The volume of oil bound was determined from the standard curve. The protein content in the combined supernatant removed during the analysis was measured using the method of Lowry et al. (1951). This protein content was substracted from 40 mg and net protein content was used to calculated the fat binding capacity. Fat binding capacity was expressed as volume of oil (mL) bound by 100 g of protein sample. The standard curve was run by adding corn oil ranging from 0-100 μL to 40 mg protein samples. To the mixture, 0.3 mL of distilled water was added followed by the addition of 20 mL

digestive medium. The mixture was homogenized and kept at room temperature. The absorbance was taken at 600 nm.

Emulsifying activity

Emulsifying activity was measured according to method of Pearce and Kinsella (1978) modified by Vaghela and Kilara (1996). A 30 mL of 10 mg/mL PWSW hydrolysate solution was mixed with 10 mL Mazola corn oil (oil volume fraction, Φ = 0.25). The mixture was homogenized using a Polytron (Brinkmann Instruments, Westbury, NY) at speed 4 for 2 min at room temperature. The aliquot of 100 μL was pipetted from the emulsion and diluted 500 fold into 0.1% (w/v) SDS in 0.1 M NaCl, pH 7.0. The volumetric flasks were inverted five times to obtain the homogeneous mixtures, followed by OD measuring at 500 nm. The emulsifying activity was expressed as the emulsifying activity index (EAI) as follows:

$$EAI = 2T/\Phi c$$

where T (turbidity) = 2.3A/l (A = absorbance at 500 nm; l = pathlength in meters), Φ (oil phase volume) = 0.25, c (protein concentration) = 1% (10 mg/mL)

Emulsion stability

Emulsion stability was determined according to the method of Chobert et al. (1988a). The stock emulsion prepared above was heated at 80°C for 30 min, followed by cooling to room temperature. After thorough stirring, the aliquot was diluted and turbidity was measured as mentioned above (EAI, 80oC). The emulsion stability index was expressed as ΔEAI and was calculated as given below:

$$ESI = (EAI_{RT}-EAI_{80°C})x\ 100/EAI_{RT}$$

where EAI_{RT} is the EAI measured before heating and $EAI_{80°C}$ is EAI obtained after heating at 80°C for 30 min.

Foaming capacity

Foaming capacity was determined using the method of Shahidi et al. (1995) with a slight modification. PWSW hydrolysate was dispersed in 10 mL distilled water to obtain the protein concentration of 3 and 6% (w/v) and the mixture was homogenized using Polytron at speed 4. The mixture was poured into the 25-mL cylinder and the total volume was noted. The foaming capacity was expressed as foam expansion (FE) (Britten and Lavoie, 1992). FE was calculated as follows:

$$FE\ (\%) = (v_t/v_o)\ x\ 100$$

where v_t is the total volume and v_o is the original volume before whipping.

Foam stability

Foam stability (FS) was measured by the modified method of Shahidi et al. (1995). The foam stability was calculated as the volume of foam remaining after 30 min at room temperature. ES was calculated as follows:

$$FS\ (\%) = (v_{30}/v_0)\ x\ 100$$

where v_{30} is the remained volume after 30 min and v_0 is the total volume after whipping at 0 min.

Antioxidant activity

The antioxidant activity was carried out according to the method of Miller (1971) with a slight modification. The substrate emulsion was prepared by mixing 50 μL linoleic acid, 200 mg Tween 40 and 0.5 mL of 0.5 mg/mL β-carotene in chloroform. After removal of chloroform using nitrogen flash, deionized water was added with vigorous stirring. The final volume was adjusted to 50 mL using deionized water. The antioxidant assay was initiated by adding 5 mL of substrate emulsion to 100 μL of sample. The OD was measured at 470 nm every 10 min. The antioxidant activity was expressed as a decrease in absorbance at 470 nm during 80 min. Tenox® was used as the positive control while the water was used as the negative control.

Statistical analysis

Data was analyzed using the analysis of variance procedure. Mean difference was determined using the least significant difference (LSD) multiple range test (Statgraphics Version 6.0, Manugistics Inc., Rockville, MD). All experiments were done in duplicate.

Results and Discussion

Hydrophobicity

The freeze-dried PWSW hydrolysate samples prepared with varying DH showed the different surface hydrophobicity ($p<0.05$) (Fig. 7.1). The same pattern was obtained between both solvents, water and 0.2 M NaCl. The surface hydrophobicity decreased as DH was increased except for DH of 20%. This result was in accordance with Quaglia & Orban (1990) who reported that surface hydrophobicity of sardine protein

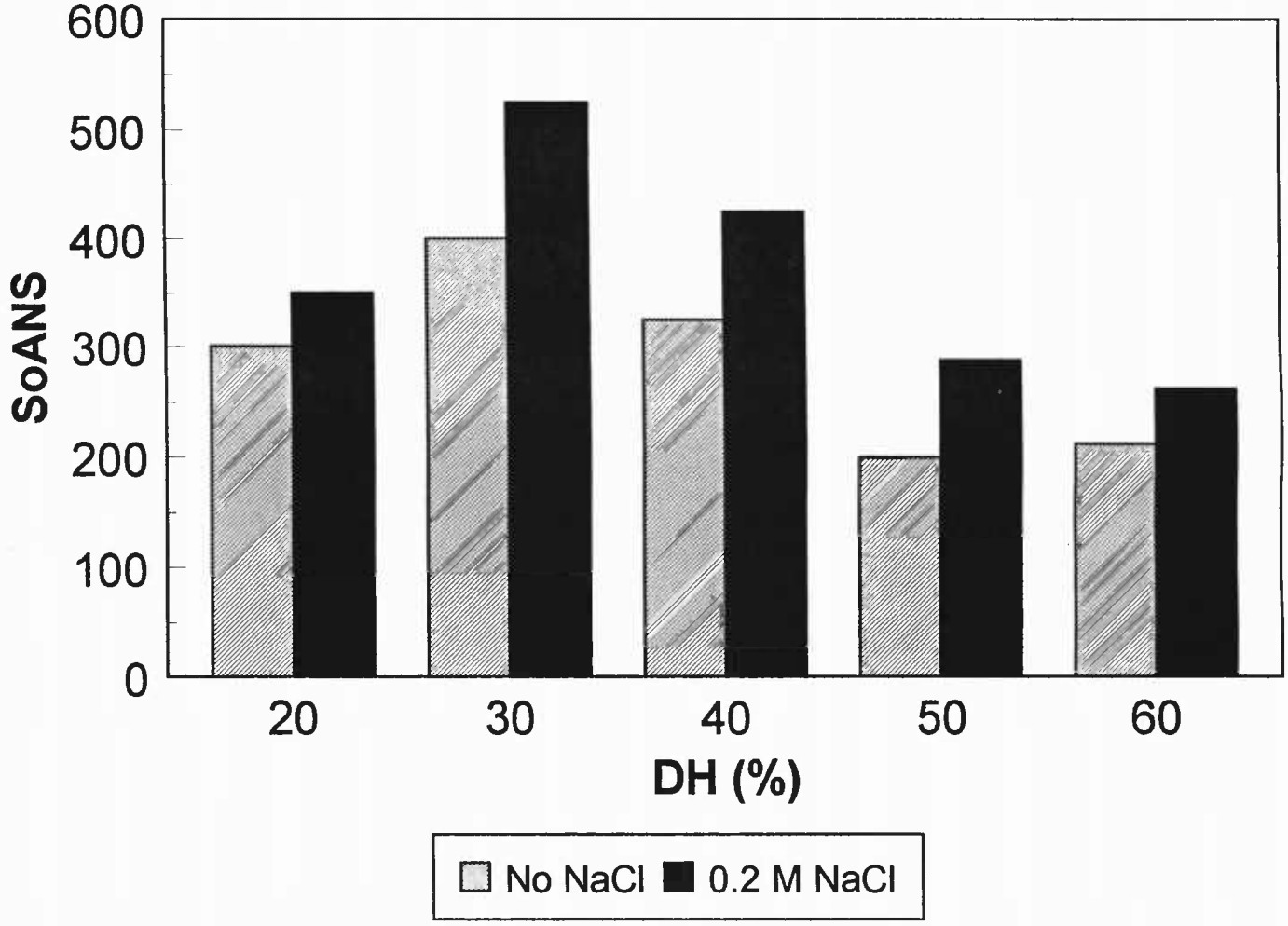

Fig. 7.1. Surface hydrophobicity (SoANS) of freeze-dried PWSW hydrolysate with different DH in the presence of 0 and 0.2 M NaCL.

hydrolysate increased with decreased hydrolysis. Mahmoud et al. (1992) found that hydrophobicity of casein decreased with increased DH. A low surface hydrophobicity at DH of 20% probably was a result of incomplete hydrolysis of protein, leading to a low protein solubilization of polypeptides. Therefore, only sarcoplasmic proteins or very small peptides were recovered after centrifugation process. On the other hand, sufficient solubilization with a soluble long chain peptides was postulated at DH of 30% and the shorter chain peptides were obtained with the higher DH.

Hayakawa & Nakai (1985) concluded that protein hydrophobicity can be classified into the aliphatic and aromatic hydrophobicity and protein functionality is contributed by these hydrophobic amino acids. Some aromatic amino acids which are more hydrophobic in nature may reside at the surface and contribute to surface hydrophobicity of the molecule. The high surface hydrophobicity at DH of 30% was postulated as a result of exposure of the interior hydrophobic side chain as well as the conformational changes of protein. Moreover, the decrease in surface hydrophobicity with increased DH was probably due to the increase in net charge density. When the polypeptide was cleaved into smaller peptides and amino acids, a greater increase in net charge density was obtained, leading to the decrease in hydrophobicity. Mahmoud et al. (1992) stated that enzymatic hydrolysis produced amino acids and polypeptides, resulting in an increase in zeta potential which can overcome the exposure of the hydrophobic residues. Paulson & Tung (1987) reported that a decreased surface hydrophobicity was observed with the increased surface charge on succinylated canola protein isolate, as a result of increased degree of succinylation. Therefore, it is assumed that the increase in net charge density played a profound role in the decreased surface hydrophobicity of

PWSW hydrolysate, especially at the higher DH. A higher frequency of charged groups results in a lower frequency of non-polar groups on the surface, with a resultant decrease in surface hydrophobicity.

Hydrophobicity of freeze-dried PWSW hydrolysates with all DH in 0.2 M NaCl was higher than that in water (Fig. 7.1). This result agrees with Wagner & Anon (1990) who showed that soy protein isolates had a higher surface hydrophobicity in 0.2 M NaCl when compared with water. In presence of NaCl, charge frequency on the protein molecules decreased with a resultant increase in the number of hydrophobic groups exposed at the interface (Paulson & Tung, 1987).

Total and surface sulfhydryl content

Both total and surface SH content in freeze-dried PWSW hydrolysates decreased as the DH was increased ($P<0.05$) (Fig. 7.2). The hydrolysate with DH of 20% showed the highest SH content whereas 60% DH hydrolysate showed the lowest SH content. Total SH content in all samples was higher than surface SH group content. The decrease in SH content was hypothesized as a result of the oxidation of SH group at high temperature during hydrolysis and enzyme inactivation. Nakai & Li-Chan (1985) found that sulfhydryl content decreased through disulfide bonding mechanism, especially when heated at high temperature for a long time. However, Nakai & Li-Chan (1985) reported that pepsin treatment of whey protein concentrate did not appreciably change the SH and SS content or hydrophobicity. This difference can be the result of the variation of protein used and the condition applied for hydrolysis. Wagner & Anon reported a tendency for a decreased solubility with an increase in the number of free SH groups.

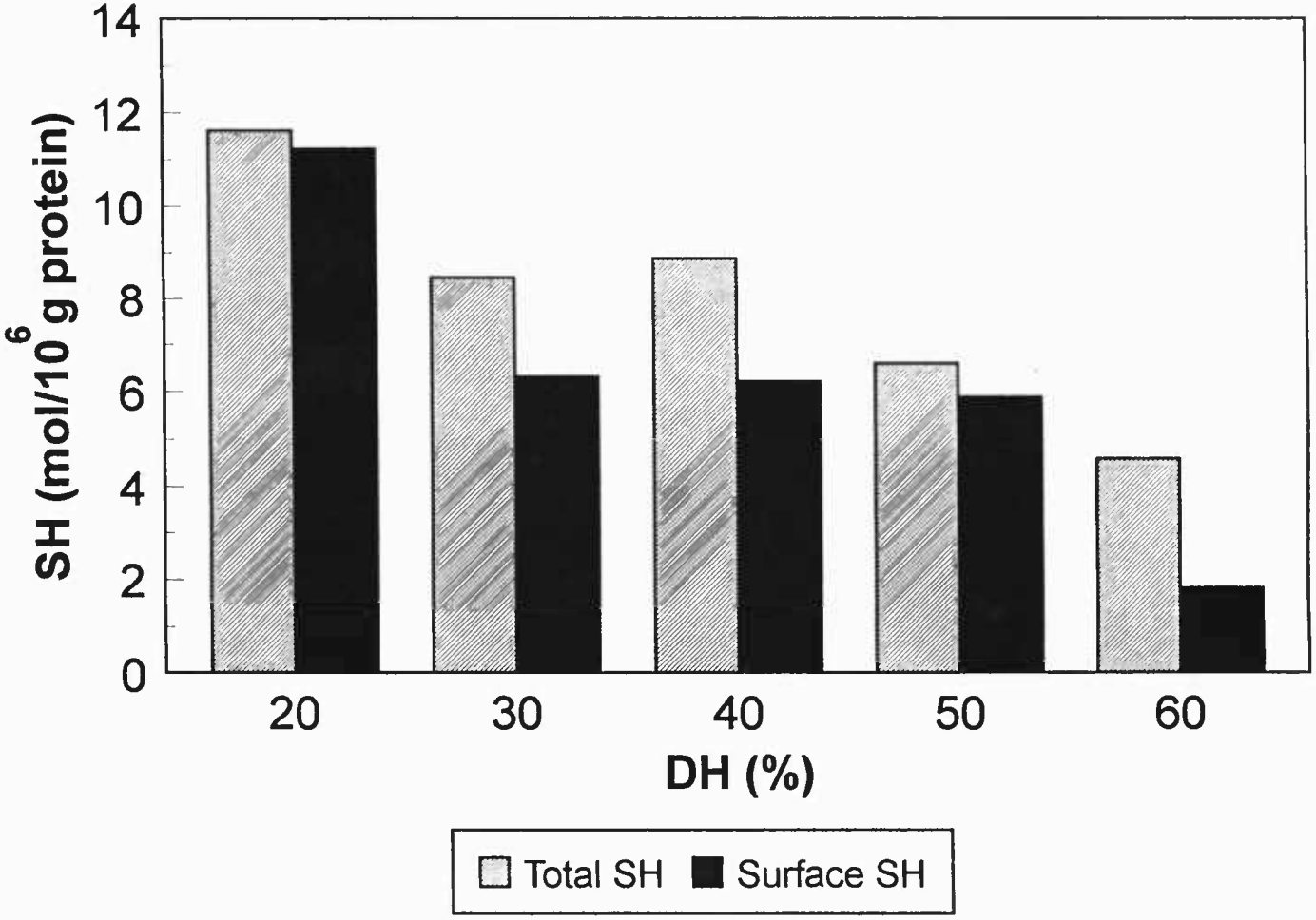

Fig. 7.2. Total and surface sulfhydryl group content in freeze-dried PWSW hydrolysate with different DH.

Solubility

Solubility of freeze-dried PWSW hydrolysate over a range of pH 1-11 is presented in Fig. 7.3. All hydrolysates with different DH showed high solubility over the entire pH range. These results were in agreement with Shahidi et al. (1995) who reported that protein hydrolysate from capelin prepared by Alcalase treatment had high solubility(> 90%). The degradation of proteins into smaller peptides generally leads to more soluble products. Smith & Brekke (1985) stated that limited proteolysis increased solubility by disrupting the aggregated, denatured structure of mechanically deboned fowl myofibrillar proteins. From these results, no obvious differences in solubility were observed among the hydrolysates with different DH. However, Linder et al. (1996) reported that veal bone hydrolysate with higher DH showed increases in solubility. After partial hydrolysis of casein with trypsin or *S. aureus* V8 protease, solubility in pI range increased (Chobert et al., 1988a,b). For partial hydrolyzed whey proteins, the increased solubility was noted at all pH ranges (Chobert et al., 1988b). Protein with high solubility over wide pH ranges are frequently used in some applications, e.g. in soup or gravy mixes, beverages, frozen desserts or other fluid products (Sekul et al., 1978).

Fat adsoption and fat binding capacity

The highest value of fat adsorption and fat binding capacity was observed in the freeze-dried PWSW hydrolysate with DH of 30% and was significantly different from the hydrolysate with other DHs ($P<0.05$) (Fig. 7.4). Except for DH 20%, fat adsorption and fat binding capacity decreased as DH increased. This results coincided with the

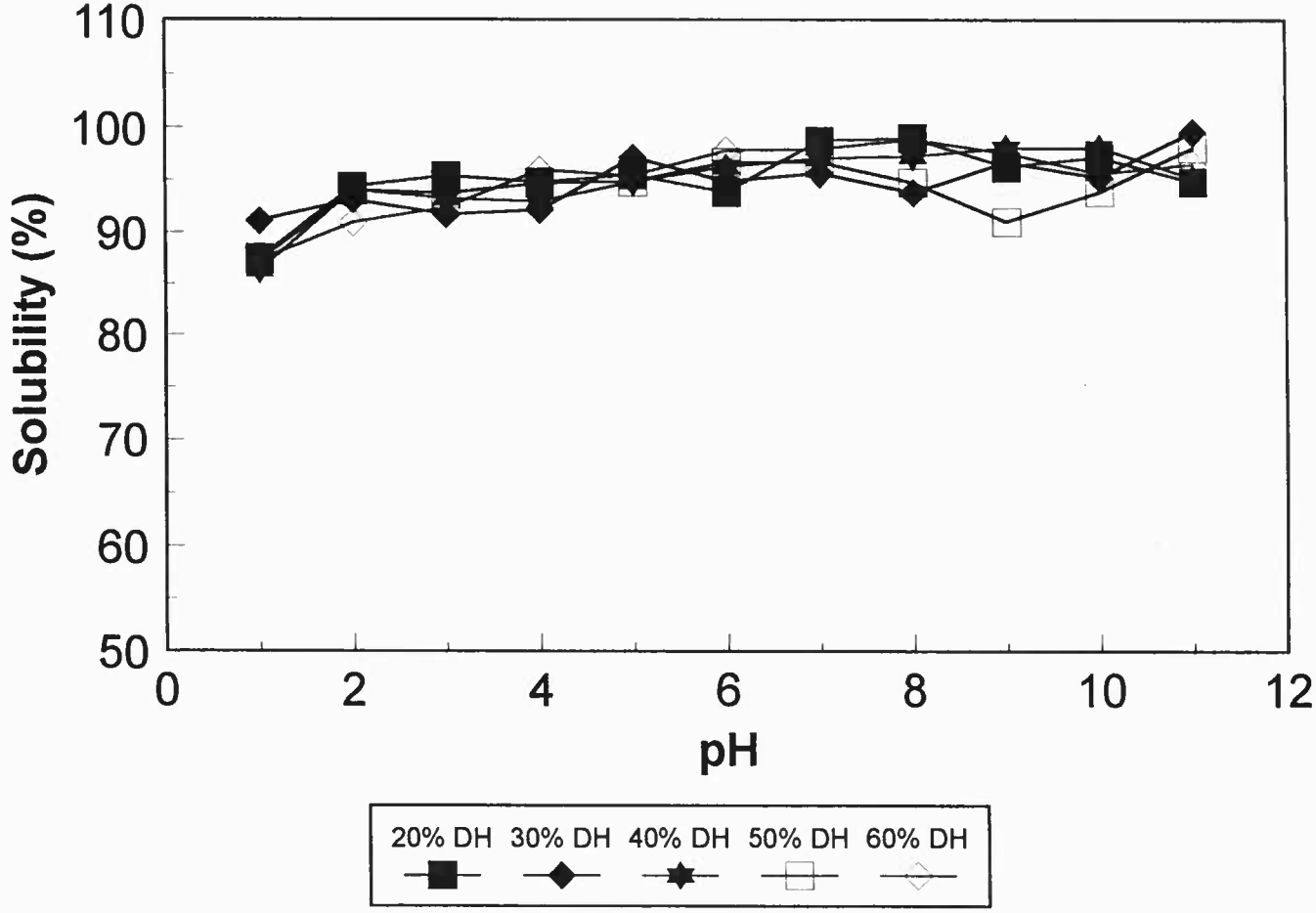

Fig. 7.3. Solubility of freeze-dried PWSW hydrolysate with different DH in the pH range of 1-11.

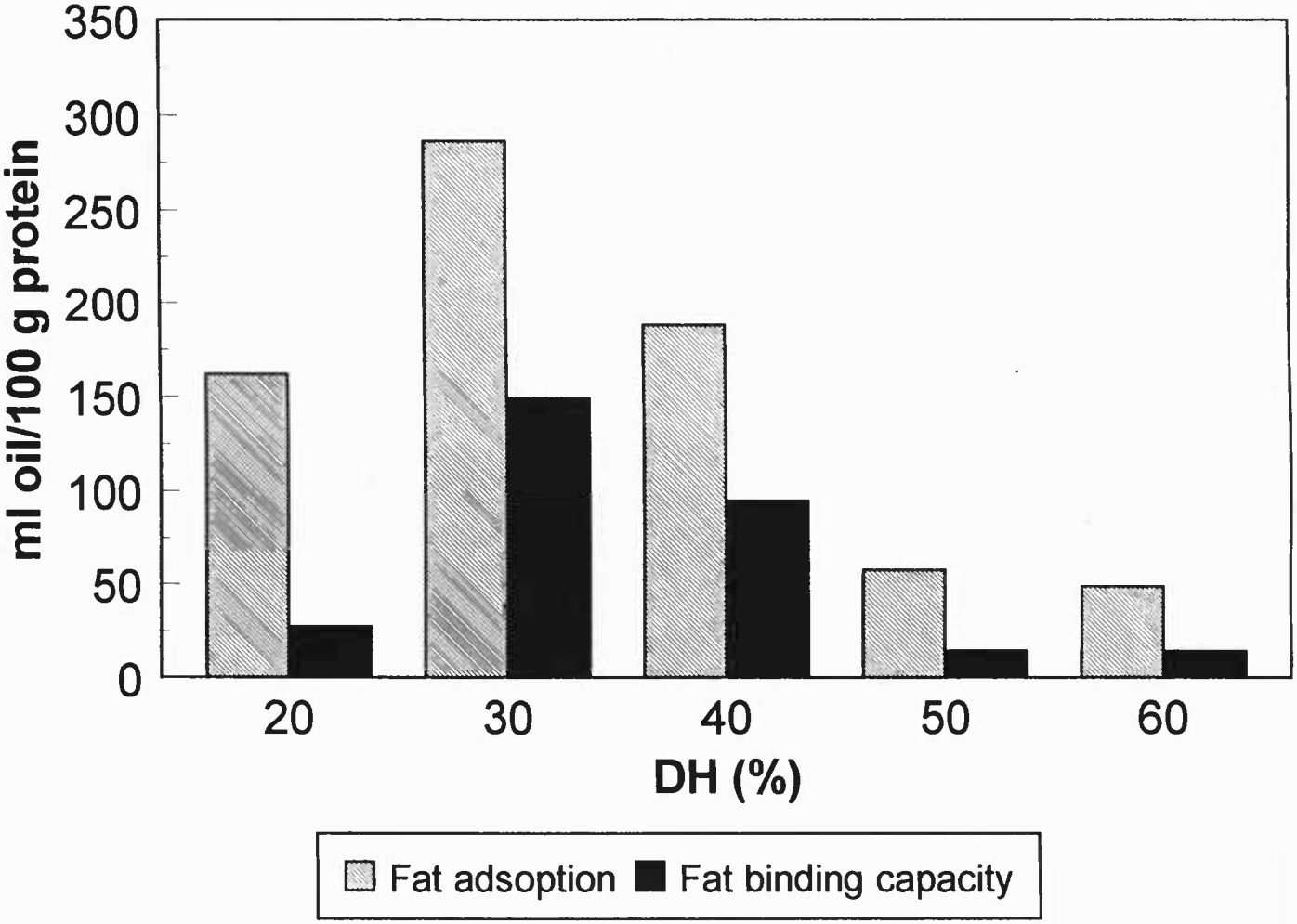

Fig. 7.4. Fat adsorption and fat binding capacity of freeze-dried PWSW hydrolysate with different DH.

decrease in surface hydrophobicity (Fig. 7.1). However, fat adsoption and fat binding capacity of hydrolysate with DH of 50 and 60% were similar. This result was in accordance with Sheen & Sheen (1988) who demonstrated that prolonged treatment with acid or protease rendered hydrolyzed protein with high solubility but reduced fat adsorption. Generally, fat adsorption of all samples was higher than fat binding capacity. Fat adsorption was mostly due to physical entrapment of oil while fat binding capacity indicates the amount of oil truely bound to the protein (Voutsinas & Nakai, 1983). Wang & Kinsella (1976) reported high correlation between bulk density and fat adsorption (R^2 = 0.95). Fat binding capacity was positively affected by surface hydrophobicity (Voutsinas & Nakai, 1983). From our result, fat adsorption ranged between 162-286% for hydrolysate with DH of 20-40% whereas the value of 49-58% was obtained for those with DH of 50-60%. Shahidi et al. (1995) reported that protein hydrolysate from capelin with DH of 12% had a fat adsorption of 171%. The difference the two studies was probably the result of variation in protein composition and different hydrolysis conditions. Factors affecting the protein-lipid interaction include protein conformation, protein-protein interactions and the spatial arrangement of lipid phase resulting from lipid-lipid interaction (Hutton & Campbell, 1981). Hydrophobic, electrostatic and hydrogen bonding are of primary importance in lipid-protein interaction, whereas hydrogen bonding is less involved (Karel, 1973).

Emulsifying activity

The EAI value of freeze-dried PWSW hydrolysate with DH of 30% showed approximately 2-fold higher than those of other hydrolysate (Fig. 7.5). The higher EAI

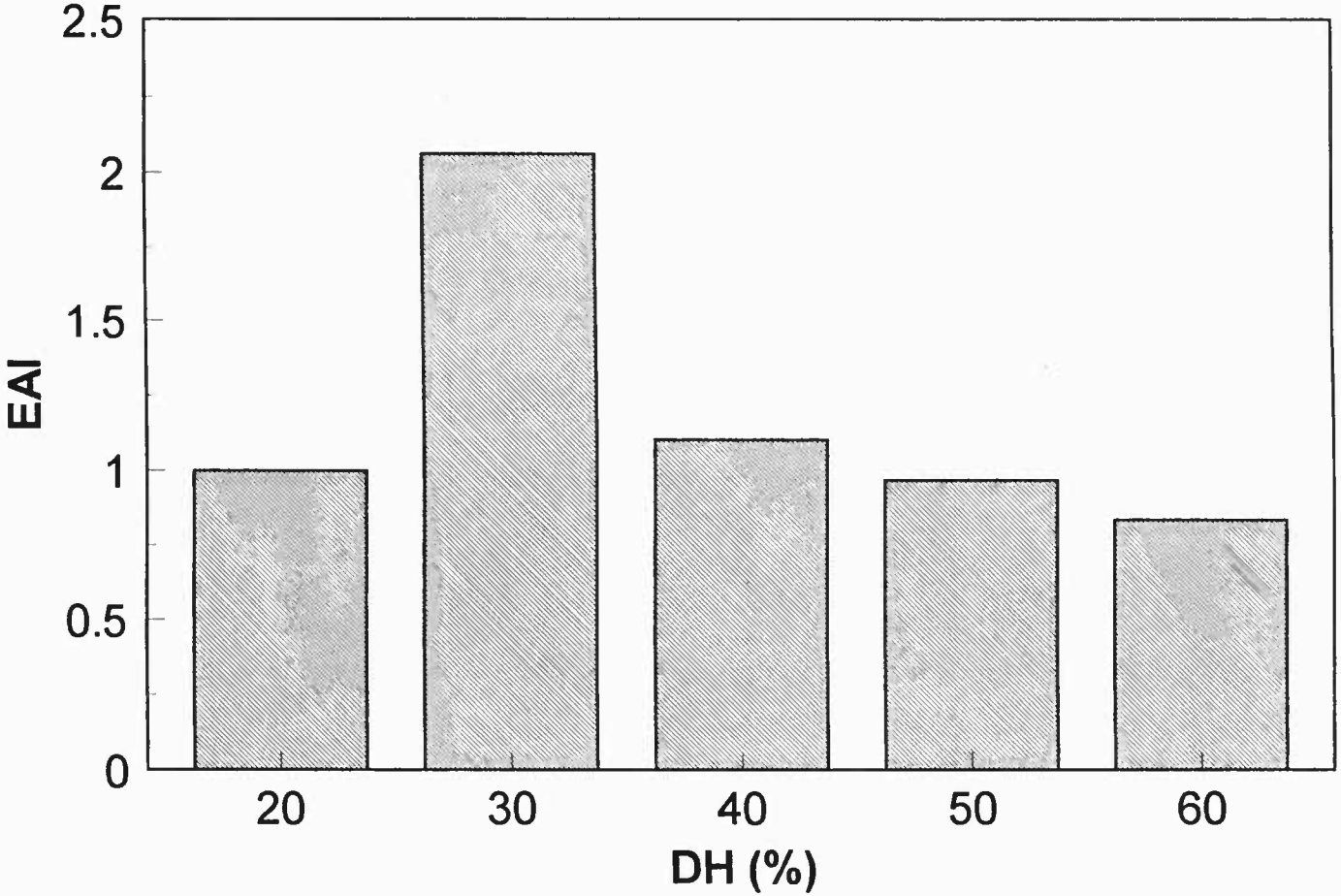

Fig. 7.5. Emulsifying activity index of freeze-dried PWSW hydrolysate with different DH.

of 30% DH hydrolysate accompanied the high surface hydrophobicity (Fig. 7.1). Surface hydrophobicity is important for predicting emulsifying properties (Li-Chan et al.,1984). More hydrophobic proteins which lower interfacial tension contribute to superior binding of lipophilic materials (Kato & Nakai, 1980). From our result, The EAI tended to correlate with surface hydrophobicity. This indicates the role of surface hydrophobic moieties on binding with the fat globules. With high DH, EAI of PWSW hydrolysate was reduced. Quaglia & Orban (1990) found that the lower the DH of sardine protein, the higher the emulsifying capacity was obtained. Mahmoud et al. (1992) illustrated the inverse linear relationship (R^2=0.96) between EAI and DH and high correlation between EAI and hydrophobicity (R^2=0.89). In general, PWSW hydrolysate showed a lower EAI when compared with other protein reported (Pearce & Kinsella, 1978). This was probably due to the differences in protein structure and properties. Furthermore, the hydrolysate used in this experiment contained some lipids (Benjakul & Morrissey, 1997). Many investigators have improved the emulsifying and foaming properties of proteins by reducing the lipid (Karleskind et al., 1996; Blecker et al., 1997). In addition, low EAI for PWSW hydrolysate, particularly at high DH indicates that smaller peptides are not able to form a stable film surrounding the fat globules. The apparent M_r of peptide should not be lower than 5,000 to maintain good emulsifying properties (Chobert et al., 1988b). Mahmoud et al. (1992) suggested that peptides produces by enzymatic hydrolysis had diminished adsorption at the oil-water interface. Chobert et al. (1988b) stated that a limited DH was necessary to improve emulsifying activity, since extensive hydrolysis may lead to nonamphiphilic peptides. Protein with amphiphilic nature are adsorbed to the interface between oil and water, causing a pronounced reduction of

interfacial tension that readily facilitates emulsification (Horwitz & Piatigorsky, 1980; Li-Chan & Nakai, 1991).

Solubility of protein is a prerequisite for film formation. Soluble protein diffuses to and concentrates at oil water inteface (Chobert et al., 1988b). From our result, hydrolysate showed high solubility. Therefore, low EAI possibly resulted from the poor protein conformation to form a strong film at the interface, instead of the low solubility of protein.

Emulsion stability

Freeze-dried PWSW hydrolysate samples showed low emulsion stability (ESI = 96.4-98.8%) (data is not shown). No differences were observed among the hydrolysates with different DH ($P>0.05$). Shahidi et al. (1995) reported a low emulsion stability of protein hydrolysate from capelin (ESI = 92.0%). The low emulsion stability was probably caused by the small size of peptides and the destabilization effect of lipid molecules that remained in the hydrolysate. The reduction of protein amount to form a strong film at the interface was postulated. Protein stabilization of emulsion or foam is based upon the formation of a stiff viscoelastic, adsorbed layer at the oil-water or air-water interfaces. In presence of fats, the lipid components adsorb with proteins, resulting in the decreased interaction between protein molecules and the reduced strength of the adsorbed layer (Blecker et al., 1997). Chobert et al. (1988a) reported that small peptides were not able to form a stable film surrounding the fat globules. Morr & Ha (1993) reviewed the factors affecting emulsion stability which include interfacial tension,

characteristics of adsorbed interfacial film, magnitude of electrical changes, size and surface-volume ratio of dispersed globules and viscosity of dispersing phase.

Foaming capacity

Besides hydrolysate with DH of 20%, increased foam expansion of freeze-dried PWSW hydrolysate was obtained as the DH increased ($P<0.05$) (Fig. 7.6). The hydrolysates with higher protein concentration (6%) showed a slightly higher foam expansion than those with a lower protein concentration (3%). Britten & Lavoie (1992) found that foam expansion increased with concentration up to a limit value over which it decreased. The greater the protein concentration, the stiffer the foam was obtained. Generally, most proteins display maximum foam ability at 2-8% concentration (Damodaran, 1996). However, Britten & Lavoie (1992) reported that the effect of protein concentration on foaming properties is dependent upon the type of protein used to stabilize the foam. From our result, hydrolysate with higher DH rendered a higher foam expansion, indicating the superior ability of hydrolysate with higher DH to decrease the surface tension. Phillips et al. (1994) stated that the extent of protein formation is related to the ability to decrease surface tension, while foam stability is dependent upon the nature of film, reflecting the protein-protein interaction within the film matrix. Generally, proteins must migrate rapidly to air-water interface, unfolding and rearranging at the interface (Halling, 1981). The differences in molecular size was postulated to contribute the different foaming capacity. Multilangi et al. (1996) suggested that larger molecular weight peptides exerted an inhibitory effect on foaming characteristics of the low molecular weight peptide. From our result, the foam

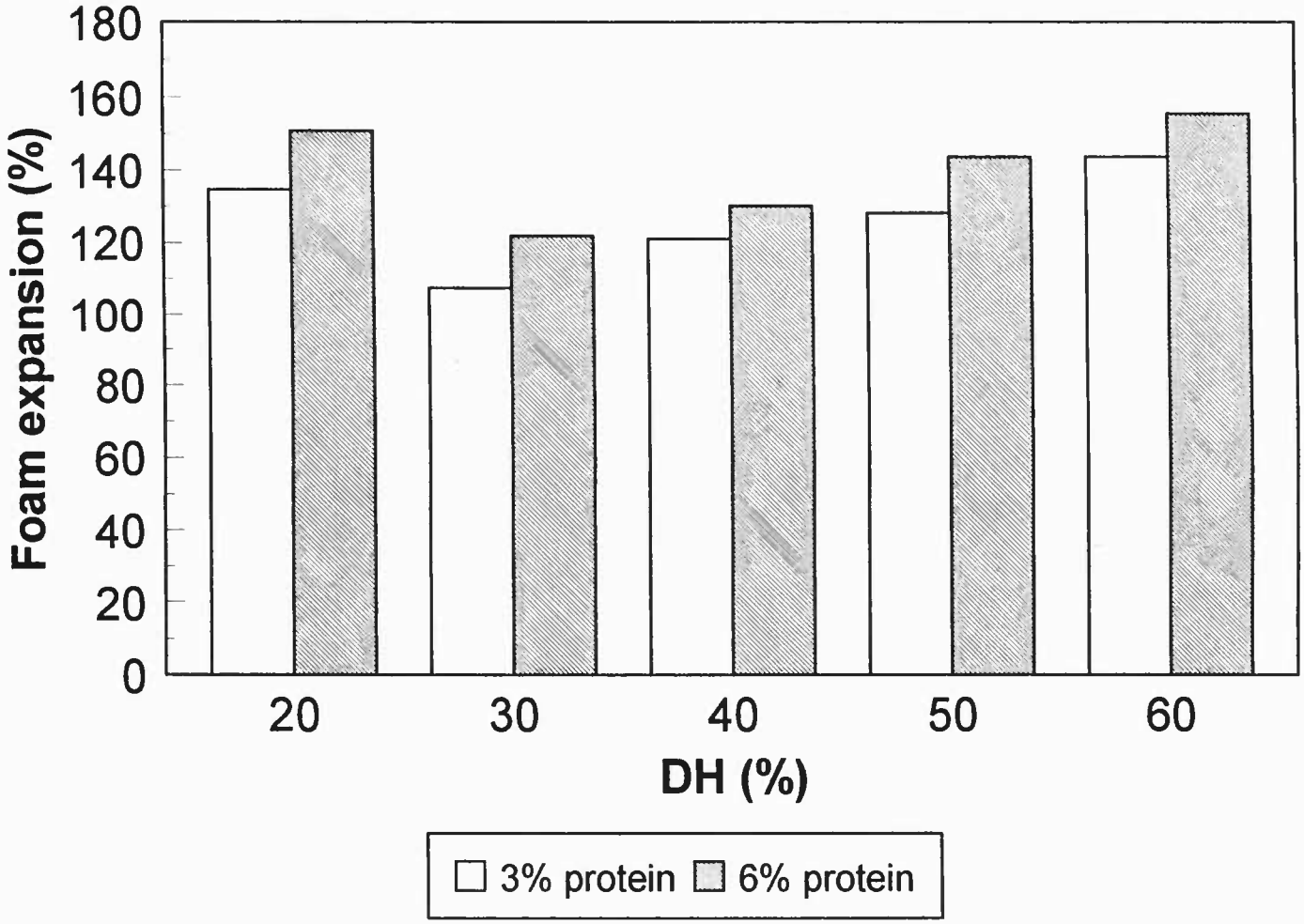

Fig. 7.6. Foam expansion of freeze-dried PWSW hydrolysate with different DH.

expansion had no positive correlation with surface hydrophobicity, which was in agreement with Multilangi et al. (1996).

Foam stability

No differences in foam stability was observed between hydrolysates with different DH except with DH of 20% which was significantly lower ($P<0.05$) (Fig. 7.7). The increase in protein concentration did not affect the stability. Although hydrolysate with DH of 20% showed high foam expansion, it provided low stability. The foam stability ranged from 96.58-99.42% for hydrolysate with DH of 30-60%, while foam stability of 72.30-75.02% was obtained for hydrolysate with DH of 20%. This indicates the difference in the protein components and its conformation which can exhibit the optimum intermolecular interactions at the air-water interface and form a continuous cohesive film. The intermolecular interactions at the interface include hydrogen bonding, electrostatic and hydrophobic interactions (Phillips et al., 1994). Proteins with high net charge exert strong electrostatic repulsion at the interface, leading to the poorer formation of cohesive film (Phillips et al., 1994).

Antioxidant activity

The antioxidant activity of freeze-dried PWSW hydrolysate with different DH is shown in Fig. 7.8. The comparison of antioxidant activity are based on minimizing β-carotene loss in an emulsion aqueous, coupled oxidation of linoleic acid and β-carotene (Marco, 1968). Antioxidant activity was found in the hydrolysates but showed

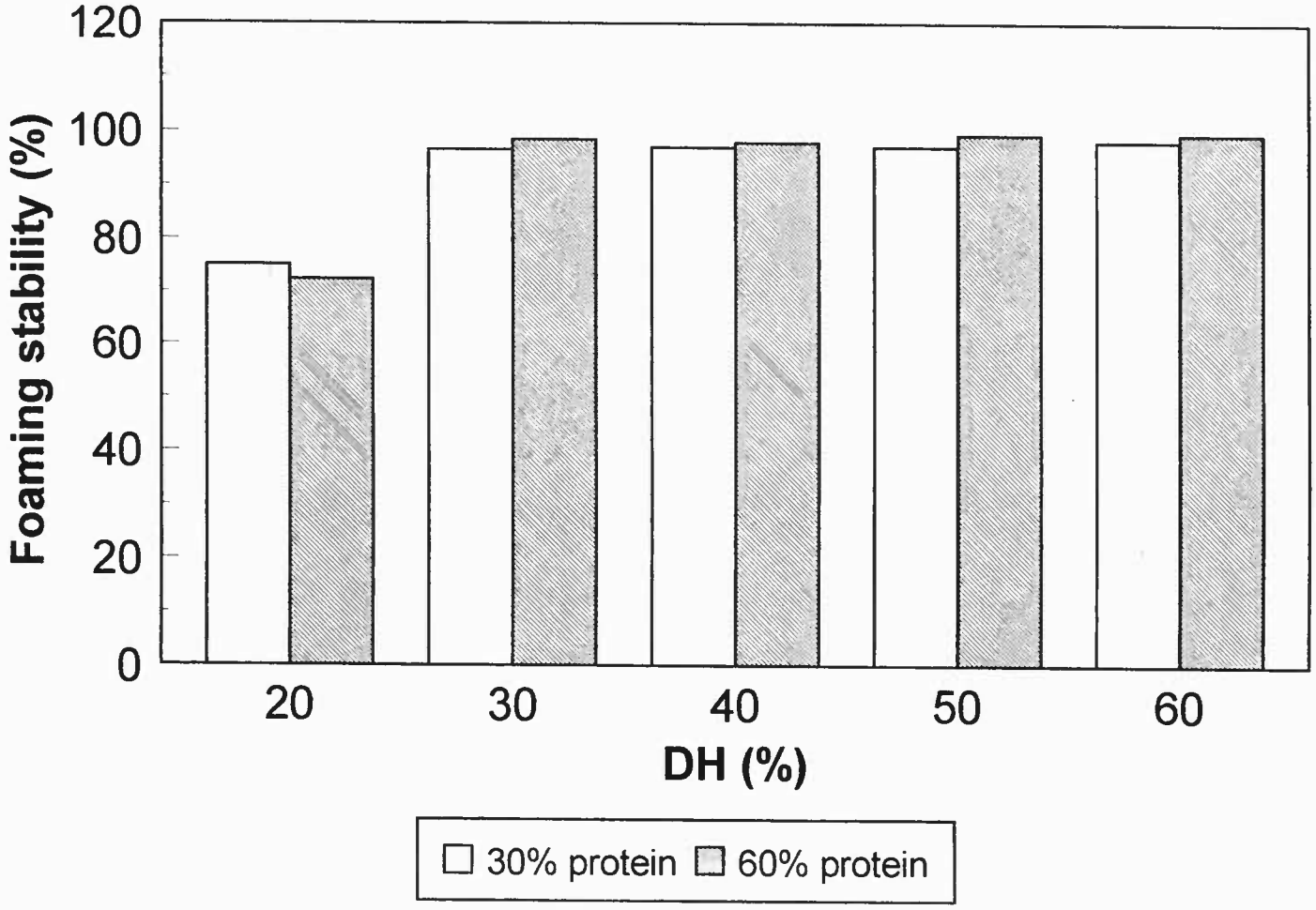

Fig. 7.7. Foam stability of freeze-dried PWSW hydrolysate with different DH.

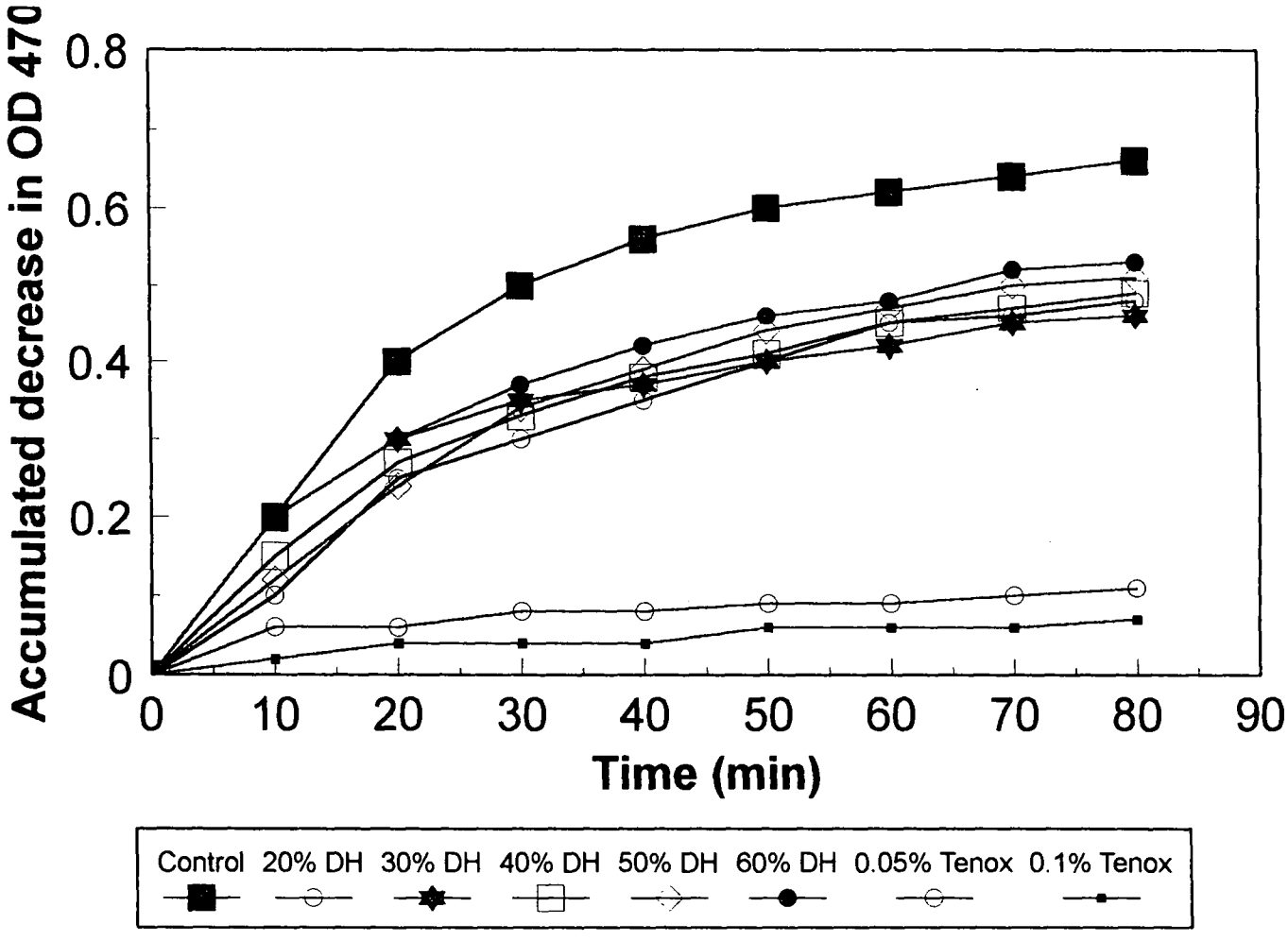

Fig. 7.8. Antioxidative activity of freeze-dried PWSW hydrolysate with different DH.

a lower activity when compared with Tenox®. Tenox® at level of 0.1% rendered a higher activity than that of 0.05%. From our results, no obvious differences in antioxidant activity was observed in the hydrolysates with different DH. However, hydrolysate with lower DH , especially DH of 30% tended to show a higher activity than those with high DH. Chen et al. (1995) reported that antioxidative peptides from soybean hydrolysate were composed of hydrophobic amino acids, valine or leucine at the N terminus. It is possible that the high activity of hydrolysate with DH 30% is the result of the high hydrophobic amino acid content (Fig. 7.1). The amino acid residues at N terminal of dipeptides showed an antioxidant activity in an oil system (Kawashima et al., 1979). This amino acids residues possibly play a role in increasing the interaction between peptides and fatty acids (Chen et al., 1995). In addition, histidine-containing peptides have been reported as antioxidant (Uchida & Kawakishi, 1992; Murase et al., 1993). The activity of these peptides seems to be attributed to the lipid peroxyl radical-trapping ability of His moiety (Kohen et al., 1988). Recently, Sasaki et al. (1996) reported that LMW fraction of spermary tissue inhibited both metallic- and non metallic-catalyzed lipid oxidation.

Due to the superior functionality and antioxidant activity, hydrolysate of 30% DH was used to study the effect of concentration on antioxidant activity (Fig. 7.9). No large difference was observed between the concentrations used. Nevertheless, the concentration of 50 mg/mL tended to show the highest activity. Due to the contamination of other components, particularly iron and heme containing proteins or some oxidized lipids, which can catalyze the decomposition of lipid peroxides, purification should be applied to obtain the high antioxidative substances.

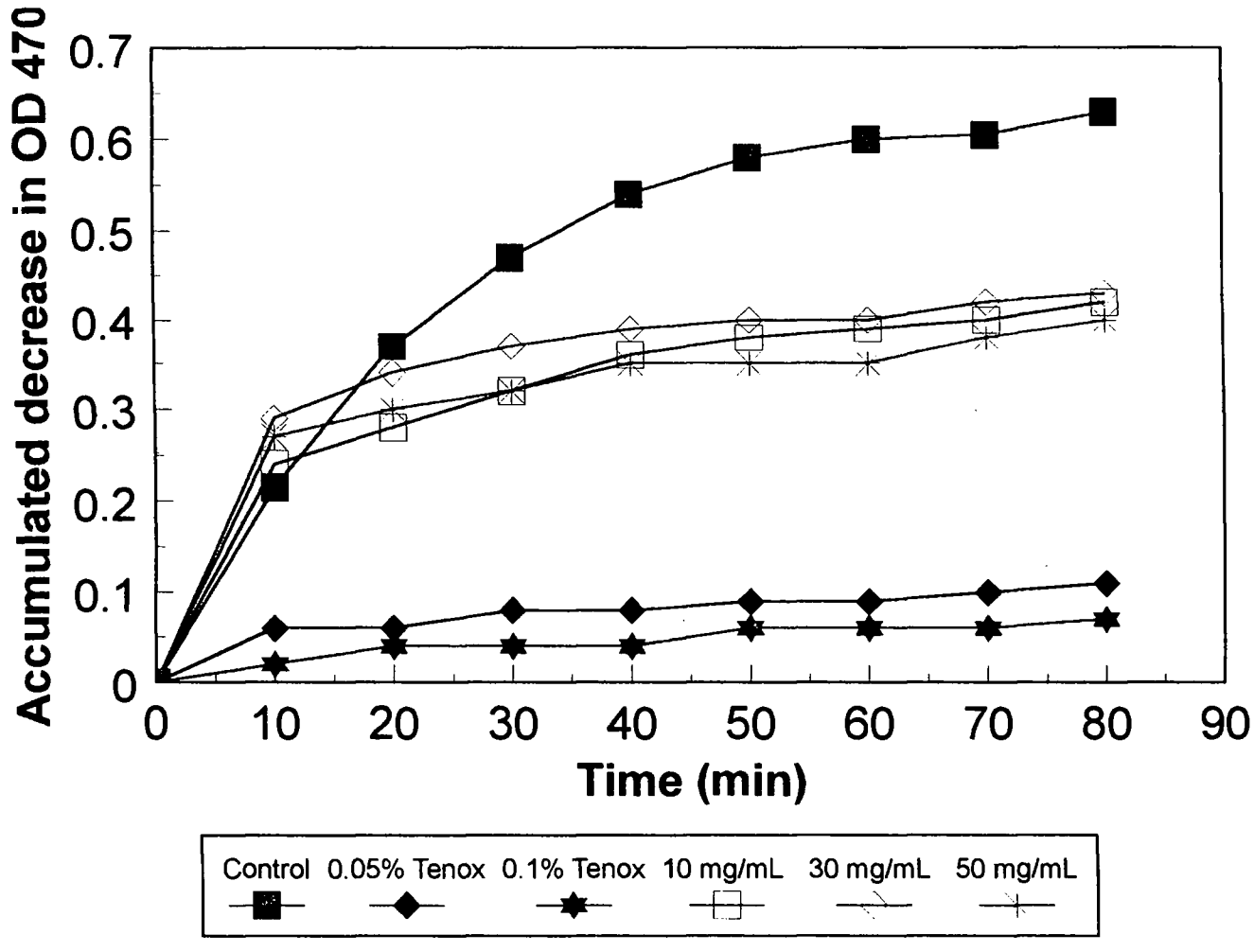

Fig. 7.9. Effect of concentraion of freeze-dried PWSW hydrolysate with DH of 30% on ıtioxidant activity.

To increase the specific functional properties of PWSW hydrolysate, the chemical or enzymatic modification of protein is suggested for future investigation. The removal of lipid after or before hydrolysis is also recommended for quality improvement of hydrolysate.

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Chapter 8

Summary

Cathepsin L was shown to be the major proteinase in Pacific whiting surimi wash water (SWW). A complexed form of cathepsin L with M_r 54,200 on SDS-substrate gel was identified. Heat treatment and acidification caused the dissociation of the cathepsin L complex into smaller components. Acidification shifted the enzyme activity zone to M_r 39,500 while heat treatment partially separated the complex, resulting in clearing at both M_r 54,200 and 39,500. Proteinase from SWW was recovered by ohmic heating, ultrafiltration and freeze-drying with overall yield of 0.83 g proteins/ L SWW and 78% recovery. Activity of the cathepsin L was increased 193% by ohmic heating (55°C for 3 min) recovery step. Subsequent steps of ultrafiltration and freeze-drying diminished the enzyme activity. The freeze-dried proteinase powder with optimum pH of 4.0 on Z-Phe-Arg-NMec showed a preferable hydrolytic activity towards casein, acid-denatured hemoglobin and myofibrils. Acidification increased specific activity on all substrates tested but reduced the thermal stability. β-Mercaptoethanol, dithiothreitol and urea enhanced the enzyme activity. The recovered proteinase showed 18.5% residual activity after 7 wk storage at 4°C.

A trypsin-like proteinase was the predominant enzyme in viscera and showed the highest activity against hemoglobin and casein at 55°C with pH 8.5 and 7.5, respectively. A pepsin-like proteinase was found as the major enzyme in Pacific whiting solid waste

(PWSW) with an activity maximum on hemoglobin at pH 2.5 and 45°C. The carboxypeptidase b was found in both viscera and solid waste.

Hydrolysate from Pacific whiting solid wastes (PWSW) was produced using Alcalase and Neutrase. Alcalase and Neutrase showed optimum activity against PWSW at pH 9.5, 60°C and pH 7.0, 55°C, respectively. Alcalase had a higher proteolytic activity than Neutrase. All factors including enzyme concentration, reaction time and waste/buffer ratio significantly affected the hydrolysis and nitrogen recovery (NR) ($p<0.05$). The optimum condition for PWSW hydrolysis were 20AU Alcalase/kg, 1 hr reaction time and waste/buffer ratio of 1:1 (w/v). Freeze dried hydrolysate contained 2.77% moisture, 79.97% protein, 13.44% ash and 3.83% lipid. Amino acid composition of freeze-dried hydrolysate was similar to that of PWSW and Pacific whiting muscle but tryptophan was reduced.

Degree of hydrolysis of PWSW (DH=20, 30, 40, 50, 60%) affected the functional properties of hydrolysate. Surface hydrophobicity, total and surface sulfhydryl content decreased as the DH increased. The hydrolysates with different DH possessed high solubility over wide pH range. Fat adsorption and fat binding capacity were decreased with the increased DH. The hydrolysate with DH of 30% showed the highest emulsion activity, however, all hydrolysates had a low emulsion stability. The foam expansion increased when DH was increased and all hydrolysates showed a high foam stability, except for samples with DH of 20%. Hydrolysates showed antioxidant activity. No obvious difference in antioxidant activity was observed with different DH and hydrolysate concentrations.

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