AN ABSTRACT OF THE THESIS OF

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Title : Apparent Inhibition of Pacific Whiting Surimi-Associated Protease by Whey Protein Concentrate

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Surimi is a seafood product which is used to manufacture restructured products such as artificial crab and lobster. Surimi is produced from fish fillets by washing to remove sarcoplasmic proteins and increase the concentration of myofibrillar proteins, and mixing with cryoprotectants. A valuable attribute of surimi is its ability to form an elastic gel, the gel network being formed by the myofibrillar proteins of fish muscle. It is generally accepted that the quality of surimi gels is influenced by the activity of endogenous protease which acts on the myofibrillar proteins. The proteases in Pacific whiting surimi (*Merluccius productus*) are particularly problematic due to their high catalytic activity on muscle myosin. The addition of whey protein concentrate (WPC) to Pacific whiting surimi has been shown to enhance the gel strength of the corresponding products produced from this surimi. The mechanism through which WPC enhances the gel strength of Pacific whiting surimi has not been determined, but it has been suggested that WPC acts to inhibit surimi autoproteolysis. The objective of this study was to determine whether the incorporation of WPC into Pacific whiting surimi inhibits autoproteolysis and/or protects the myosin fraction from proteolytic degradation.

The effect of supplementing surimi with WPC, beef plasma protein (BPP) and bovine serum albumin (BSA) on its apparent autoproteolysis activity was determined.

Three WPC preparations were tested, WPC 34, 34% protein; WPC 80, 80% protein; and WPC 95, 95% protein. Each of the additives was incorporated at the 1, 2, 3 or 4% level. Proteolysis of surimi and supplemented surimi samples was allowed to occur at 55 °C. Proteolytic reaction mixtures were terminated by the addition of trichloroacetic acid (TCA). Proteolytic activity was estimated by measuring the difference in TCA-soluble peptides present in reaction mixtures of paired (identical) samples, one having been incubated at 55 °C while the paired sample was kept on ice. Peptides were quantified by the bicinchoninic acid, Lowry, dye-binding and trinitrobenzenesulfonic acid methods. Results based on the different peptide assays were compared in order to asses the reliance of results on specific assay methods.

BPP was found to have the most inhibitory activity in the autoproteolysis assays, followed by the WPC preparations and then BSA. Autoproteolysis was completely inhibited by the incorporation of 1% BPP, 3% WPC 80 and 2% WPC 95. The extent of inhibition by the WPC preparations was related to their protein content, the higher the protein content the greater the extent of inhibition per unit weight added to surimi. BSA was not an inhibitor of autoproteolysis under the conditions used in this study. The relative extents of inhibition observed for the different additives were independent of the method used to quantify the soluble peptide products.

Each of the additives was also tested for their ability to protect the myosin component of surimi from proteolytic degradation. These experiments were done as described above for the autoproteolysis assays with the exception that following the incubation period a portion of the sample, either surimi or a surimi/additive mixture, was completely solubilized in detergent solution, subjected to SDS-PAGE electrophoresis and visualized by protein staining. In these experiments the additives were incorporated at the 4% level. No apparent degradation of myosin could be detected over a 60 min reaction period for surimi samples that were supplemented with BPP, WPC 80 and WPC 95. In contrast, surimi samples incubated without additive clearly showed a loss of myosin after

15 min reaction period. Some myosin degradation was apparent following the 60 min incubation period for the WPC 34-supplemented surimi.

A further experiment was conducted to determine the mechanism through which WPC protects myosin and inhibits autoproteolysis. In this experiment WPC 95 and BPP were separately incubated at 55 °C with a crude fish protease preparation, i.e. the reaction mixture approximates that used in the autoproteolysis assays except that it contains no surimi. The results indicate that BPP and WPC 95 behave in a similar manner. However, the results were inconclusive with regard to explaining the additive's mechanism of action. Plausible mechanisms which are consistent with the results are discussed.

Apparent Inhibition of Pacific Whiting Surimi-Associated Protease by Whey Protein Concentrate

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Chapter 1 : LITERATURE REVIEW

SURIMI PRODUCTION

Surimi is a processed minced fish product which has been washed and dewatered to remove sarcoplasmic proteins. It thus has a relatively high concentration of myofibrillar proteins (Lee, 1984), and it is generally mixed with cryoprotectants to increase its shelf-life during frozen storage (Hollingworth et al., 1990).

The surimi process is shown in figure 1. Preprocessing prepares the fish for deboning. The deboning operation separates the fish flesh from the bones, fin, and skin. The washing step is an important step of the surimi production since it is a means of removing fats, oils, and fishy odor as well as making the product color lighter. It was also noticed the washing increased the product's gel strength. During the washing step several functions occurr including; (1) removal of sarcoplasmic proteins which interfere with the gel properties of surimi based products, (2) removal of fat, undesired blood, pigments, and odorous substances, (3) an increase in the concentration of myofibrillar proteins, and (4) a reduction in the susceptibility of myofibrillar proteins to freeze denaturation (Lanier and Macdonald, 1988).

Next, dewatering of the leached muscle removes excess moisture to yield a light colored, bland protein material of about the same moisture content as lean fish muscle (70-82%). Major protein losses, however, can occur in the dewatering stage. The purpose of the straining or refining step is to separate out any remaining connective tissue, skin or small bones. Some processes refine the mince prior to dewatering to reduce problems with temperature.

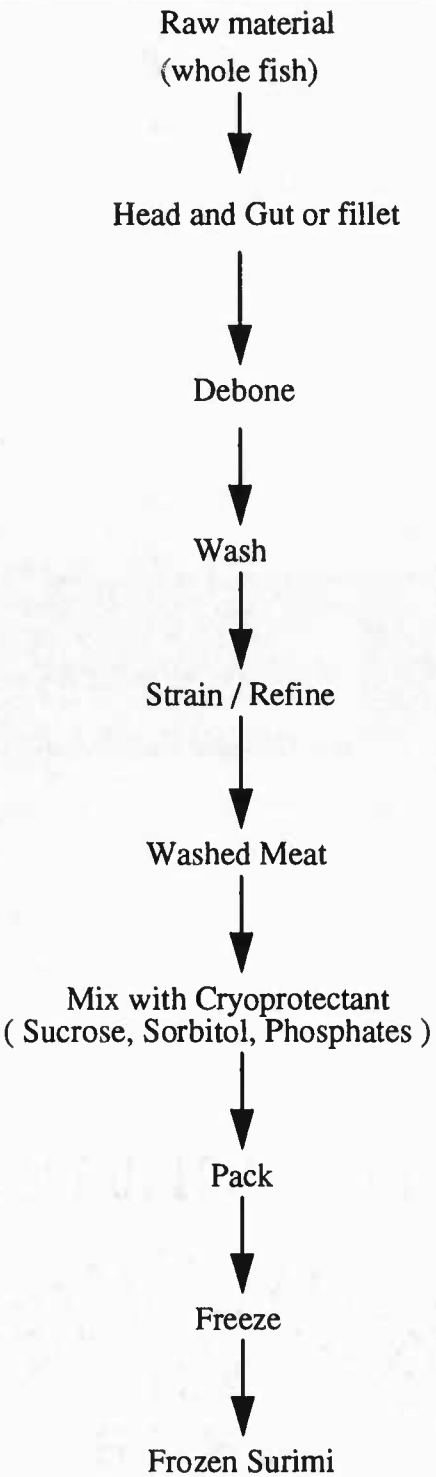

Figure 1: Diagram exhibiting production of surimi.

In the surimi making process, some compounds called cryoprotectants are added. The freezing of surimi became commercially possible after the discovery of these cryoprotectants. Fish actomyosin, a complex consisting of actin and myosin, has been found to be quite labile, and easily changed during processing and storage. During frozen storage, the actomyosin becomes progressively less soluble and the flesh becomes increasingly tough due to denaturation of proteins (Deman, 1980). Denaturation of actomyosin can occur as a result of aggregation caused by the progressive increase in the intermolecular cross-linking of myosin through hydrogen bonds, ionic bonds, and disulfide bonds. Cryoprotectants play a big role in preventing denaturation of actomyosin by increasing the surface tension of water as well as the amount of bound water. Therefore, ice crystal growth and migration of water molecules from the protein can be prevented, thus stabilizing proteins in their native form during frozen storage (Yoon and Lee, 1990). Sucrose and sorbitol have shown excellent cryoprotective effects on fish myofibrillar proteins (Akahane, 1982). Sucrose reduces the denaturation of actomyosin during frozen storage. Initially, sucrose was used, but it made the surimi too sweet, so sorbitol was substituted to lessen the sweetness. Sorbitol is a naturally occurring sugar alcohol that is 63% as sweet as sucrose. Phosphates, especially triphosphate and pyrophosphate, enhance the effect of the sugars.

After the addition of cryoprotectants, the surimi is packed and frozen to -25 °C. Some loss of functional properties will still occur in frozen storage at this temperature. A storage temperature of -30 °C is suggested as a practical goal for a long term storage of surimi.

Good quality surimi is white in color and it is capable of forming strong gel. The important quality of surimi is its gel forming ability and this can be affected by many factors. Important factors include the species of fish or animal, the action of proteolytic enzymes, the relative concentration of myofibrillar vs. water soluble and stroma proteins, and the amount of denaturation of the myofibrillar proteins. Denaturation of proteins can

be caused by many factors. Some of these include: excessive temperature, or excessive time at refrigerated temperatures; generation and introduction of denaturants, such as metal ions, formaldehyde, peroxides or salts; oxidation; low pH; and freezing and storage especially in the absence of cryoprotectants.

MECHANISM OF PROTEIN GELATION IN SURIMI

The gelling property of surimi is dependent on its protein composition. About 18-23% of fish muscle is protein. Proteins of fish muscle can be classified into three categories on the basis of their solubilities.

The first category is easily soluble proteins, i.e. sarcoplasmic proteins including most of the muscle enzymes. This fraction can be extracted from muscle with water or dilute salt solution. It accounts for 25 to 30% of the total proteins.

The second one is insoluble proteins, i.e. connective tissue proteins and membrane proteins. They are not soluble in water or in dilute or concentrated salt solutions.

The last group is contractile proteins. They are soluble in salt solutions of high ionic strength but not in water or dilute salt solutions. Most of them are structural proteins, i.e. myofibrillar proteins. The myofibril of fish muscle contains the same major proteins as mammalian muscle : myosin, actin, actomyosin, and tropomyosin. These proteins are the major contribution to muscle contraction.

In contractile proteins, myosin is the major protein of the thick filament. It contains two identical polypeptide chains called heavy chains, which are supercoiled. These chains can be separated by treating myosin with concentrated urea or guanidine solution. Each has approximately 1,800 amino acid residues. Skeletal muscle myosin has three unique amino acids: 3-Methylhistidine, ε-N-Monomethyllysine, and ε-N-Trimethyllysine (Lehniger, 1975). Additionally, the myosin molecule has two heads, which are responsible for its enzymatic (ATPase) activity and its ability to interact with actin. Associated with each globular head section are two light chains. Proteolytic

enzymes such as trypsin can cleave myosin near the head region and produce two fractions of the protein.

Many attempts to explain the gel formation have been made, and reported that the three-dimensional network theory is most closely appropriate for protein gelation (Meyer, 1960). In three-dimensional network, the compound capable of gelation is either fibrous in structure or can react with itself to form a fiber. On cooling, the fibers form a three-dimensional network by reacting either at widely separated intervals on the chain or at relatively small distances. The bonds established, which tie the fibers into the three-dimensional network, can be covalent bonds, hydrogen bonds and electrostatic bonds. Furthermore, the network formation depends on the forces of a non specific attraction between portions of the molecules or among the entire molecule. The solute molecules would attract one another at some spots but would be separated by solvent molecules that are attracted at others. The result would be gelation. If the solvent molecules were too strongly attracted to the solute, then the solute molecules would have no chance to contact one another and a network would not form. In contrast, if the attraction of the solute molecules was too strong, dispersion in the solvent would not occur. A precipitate will be formed rather than gel. Gel formation in this system must consider the temperature, concentration, pH, and salt concentration (Meyer, 1960).

The first step in gel formation involves comminuting of the muscle tissue with salt. This partially solubilizes the myofibrillar proteins and yields a thick sol, or paste. Maximum extraction of myosin from fish muscle will produce the highest ratings for firmness and springiness in finished comminuted products (Cheng et al., 1979). Then, the following step is a heating process that establishes a gelatinous structural network of proteins. This protein network is most likely stabilized by hydrogen bonding and hydrophobic interactions. The network induce extensive structural order in the entrapped water. The specific properties of gels depend on the species from which the muscle tissue is derived and on the particular muscles used. Also, processing variables such as rate of

heating, salt concentration, and pH influence the final properties of the protein gel. Morita and Ogata (1991) reported that the gel strength depends on several factors such as pH, ionic strength, protein concentration, and myosin/actin weight ratio.

Myosin is the most important component of the muscle tissue for gel-forming. Actin has an enhancing effect on myosin gelation, depending on the myosin/actin weight ratio (Choe et al., 1991). Cheng et al. (1979) stated that a whole intact myosin molecule is needed for the best performance in gel formation. The tail and head portions of the myosin molecule play different roles in the heat induced gelation of the muscle minces. In the heat-induced gelation of myosin, the myosin molecule undergoes at least two conformational changes. One is the unfolding of the helical tail portion which undergoes a partially irreversible helix-to-coil transition during heating and then participates in the formation of a three-dimensional network (Hultin, 1985). The other one is the aggregation which was due to fusion of the head portion by heat. The head portion of the myosin molecule undergoes irreversible aggregation involving the oxidation of -SH groups. This aggregation contributes to formation of the three-dimensional protein network of the gel (Ishioroshi et al., 1979; Sano et al., 1990). Because hydrophobic amino acid residues are rich in the head portion and emerge at the surface of the molecule on heating, hydrophobic interaction may be responsible for the aggregation of the head portions (Hultin, 1985). Beas et al. (1991) reported that the slower the second step (aggregation) relative to the first step (denaturation), the better the denatured chains orient themselves and the finer the gel network.

Protein gelation is based on interaction between protein molecules through hydrogen bonding, ionic bonding, disulfide bonding, and hydrophobic association. Thus, the type and extent of interaction between or within protein molecules would affect the textural properties of protein gel products. The formation of strong gels may result from extensive cross-linking of polypeptides. Recently, Chan et al. (1992) suggested that there was a positive correlation between the cross-linking of the myosin heavy chain and the

gel-forming ability. It was indicated that the most important sources for the temperature-induced cross-linking reaction were cysteine and cystine (Chung and Lee, 1990). Hydrogen bonds, ionic bonds, hydrophobic bonds, and disulfide bonds are believed involved with a formation of intermolecular cross-linkages (Yoon and Lee, 1990).

Gel Formation in Surimi:

Gel-forming and water-binding ability are the main functional properties in protein gel-based products (Chung and Lee, 1991). The gelation of muscle proteins occurs during heating and is due to the association of denatured proteins. The thermally induced gelation of proteins is a two-phase process consisting of unfolding and association events (Foegeding and Lanier, 1987). Gel forming ability of fish muscle depends on many factors including the freshness, fishing method, season, size, and species. Chan et al. (1992) also stated that rate and extent of aggregation is species specific.

In surimi gel formation, salt is added to extract the myofibrillar protein from fish muscle, and it forms a sol or paste. Upon heating to 50 °C, a slightly transparent gel forms with a relatively open structure of a loose net. This step is called suwari. Raising the temperature to approximately 60 °C partially degrades this gel and causes a break in the gel network. This breakdown in the gel network is related to a proteolytic activity associated with the surimi. This is called the modori phenomenon. At temperatures greater than 60 °C, a more rigid gel of fish which has an ordered net is formed. Thus, the sol-gel transition of fish meat sol has been related to the setting (suwari) and disintegration (modori) reaction. It is the heat-induced gelation which is irreversible (Yasui et al., 1979). Chan et al. (1992) suggested that formation of a good elastic gel by setting at low temperature involved initial aggregation, and then the formation of a network made up of large cross-linked myosin heavy chain aggregation. Formation of large aggregates was presumably a prerequisite to formation of a good elastic gel.

Water-binding is the other important functional property in protein gel-based products. It is based on the capability of myosin to form heat-induced gels (Morita and Ogata, 1991). In a thermally induced comminuted meat gel system, a sol-to-gel transformation involves protein hydration and heat setting. Protein hydration, through a salting-in effect of NaCl during comminution, increases water-binding capacity of myofibrillar proteins by increasing the negative charges to attract water molecules and thus enhance water binding. During thermal gelation, a three dimensional network stabilizes water physically and chemically within the gel structure. Water may interact with the protein matrix through attractive forces such as hydrogen bonding and electrostatic reaction. The hydration of protein prior to heat-induced gelation is an important step to form gels capable of immobilizing or trapping large amounts of water. The state in which water is immobilized influences the structural and textural properties of meat gel products (Chung and Lee, 1991).

PROTEOLYTIC ENZYMES

During the gelation process of surimi-based products, at a temperature between 50 °C to 60 °C, some parts of the gel network can be destroyed and cause the break in the gel network. This is the called modori phenomenon, which affects the quality of surimi gel by causing an undesirable texture of the gel. Many studies have been made to understand the mechanism of this phenomenon and prevent it. A proposed mechanism is that it results from a heat-activated proteolytic enzymes which also have an optimum temperature of about 50-60 °C. These enzymes can degrade the myofibrillar proteins (Hamann et al., 1990). There are many kinds of proteases found in the muscle of fish, including Cathepsin A, Cathepsin D, a neutral proteinase (or a sub-endopeptidase), calpain, and alkaline proteinase. The purified alkaline protease found in white muscle of horse mackerel, *Trachurus japonicus*, was reported by Iwata et al. (1978) and exhibited the ability to induce the modori phenomenon.

In carp, a protease has been isolated and purified, and it is different from cathepsins when molecular weight and other physicochemical and enzymatic properties are compared. This enzyme is classified as an alkaline protease which contributes to the modori phenomenon (Iwata et al., 1973; Makinodan and Ikeda, 1977). Carp muscle also contains a neutral protease (Makinodan et al., 1979), calpain (Toyohara et al., 1983) and cathepsin D (Makinodan et al., 1982).

In white croaker (*Micropogon opercularis*), Busconi et al. (1984) were able to isolate two kinds of alkaline proteases from fish muscle, i.e. protease I and protease II, which have the maximal activity at pH 8.5 and 9.1, respectively. Protease I showed the characteristics of a trypsin-like enzyme, and protease II showed those of SH-enzyme, like carp and Atlantic croaker protease. Folco et al. (1984) exhibited that purified myosin was readily degraded by both proteases. Additionally, Folco et al. (1988) demonstrated that protease purified from white croaker skeletal muscle was an alkaline protease, protease II, and might be a multicatalytic enzyme having at least two different active sites. Furthermore, Folco et al. (1989) showed that an alkaline protease, protease I, from white croaker (*Micropogon opercularis*) which was shown to be able to degrade contractile myofibrillar proteins was a trypsin-like serine protease since the enzyme was strongly inhibited by several serine proteinase inhibitors. Makinodan et al. (1985) reported that there were at least four proteases found in white croaker : cathepsin D, a neutral protease, calpain, and an alkaline protease. However, among them only alkaline proteases were capable of activity at the pH of meat paste and around 60 °C which resulted in a gel of meat paste to have poor elasticity (Toyohara et al., 1990).

According to Su et al. (1981), the greater texture breakdown observed in gel-type products from Atlantic croaker (*Micropogon undulatus*), can be attributed to the significantly higher protease activity measured in mechanically separated tissue of that fish. The protease was an alkaline protease which had the optimum temperature at 60 °C. Lanier et al. (1981) studied the interrelations between proteolytic activity, myosin

degradation and textural firmness in gels prepared from Atlantic croaker. Their results provided the role of a heat-stable alkaline protease in the weakening of gel integrity during processing at temperatures near 60 °C. In addition, Su et al. (1981) indicated that the integrity of myofibrillar proteins may be reduced by proteolytic enzymes, both during cold storage as the result of catheptic or bacterial action and during heating processing by heat-stable alkaline proteases.

In Pacific whiting (*Merluccius productus*), the mushy texture has been observed to be related with some myxosporidian parasite, *Kudoa* sp. (Erickson et al., 1983). The proteolytic enzymes in the sarcoplasmic fraction of Pacific whiting were studied. They exhibited two pH optima for enzyme activity : pH 3.5-3.9 and pH 7.1-7.2. Based on the results from both pH and inhibitor studies, it indicated that cathepsin may be responsible for the acceleration of muscle breakdown in whiting which caused the mushy texture (Erickson et al., 1983). Recently, the enzyme in Pacific whiting has been purified and identified as cathepsin L (Akazawa et al., 1993).

Boye and Lanier (1988) reported that one of the initial factors which might limit the conversion of fish into good quality surimi for consumption was the apparent presence of a protease in the fish muscle. Since the proteolytic activity which causes the decrease in quality of surimi-based products is a critical problem, researchers have tried to minimize this problem by using food additives as enzyme inhibitors. Hydrolyzed beef plasma (Hamann et al., 1990) and egg white (Hamann et al., 1990; Chang-Lee et al. 1989; Chang-Lee et al., 1990) showed the inhibition effect by increasing the gel hardness and elasticity when applied to surimi.

Assay of Proteolytic Enzyme Activity:

To determine the activity of proteolytic enzymes, both proteins and synthetic compounds can be used as a substrate. When proteins are used, the most widely used method is the change in the trichloroacetic acid (TCA) soluble peptides when it is

subjected to the action of a proteolytic enzyme. The most commonly used substrate proteins are casein and acid or urea denatured hemoglobin (Whitaker, 1972). Additionally, the modified casein can be used for determining proteolytic activity, for instance azocasein (Jesen et al., 1980; Martone et al., 1991) or fluorescein isothiocyanate-labeled casein (Twining, 1984). As a proteolytic enzyme acts upon a protein the amount of TCA-soluble peptides produced is proportional to the amount of enzyme and time of the reaction. The amount of TCA-soluble peptides produced can be determined by measuring the absorbance of the supernatant liquid at 280 nm or by using a color reaction such as Lowry or Dye-binding method.

WHEY PROTEINS

Whey is a waste product obtain from cheese processing. Whey has been extensively studied to develop its potential utilization. These developments are based on the protein fraction in whey. There are two major protein components in whey, i.e. β-lactoglobulin and α-lactalbumin. Whey also contains bovine serum albumin, immunoglobulins and proteose-peptones. In addition, there are many trivial proteins in whey such as lactoferrin, lactollin, glycoprotein, and blood transferrin. The composition of whey proteins is shown in Table 1. Whey proteins are sensitive to temperatures above 60 °C, except the proteose-peptone fraction which is stable. The degree of denaturation of whey proteins when heated depends on the protein component, total protein and solids concentrations, pH, ionic strength, temperature, and time of exposure.

Table 1. The Composition of Whey Proteins

	Concentration (g/liter)	% of Total Whey Protein	Molecular Weight (dalton)
β-Lactoglobulin	3.0	50	18,300
α-Lactalbumin	0.7	12	14,000
Immunoglobulins	0.6	10	15,000-1,000,000
Bovine Serum Albumin	0.3	5	69,000
Proteose-Peptone	1.4	23	4,100-40,800
Total Whey Protein	6.0	100	

From: Fox, P.F. 1989. Developments in Dairy Chemistry-4.

Two commercial whey protein products, i.e. concentrates and isolates have been produced by using ultrafiltration-reverse osmosis, gel filtration, adsorption or precipitation as polyphosphate or carboxy-methylcellulose complex. Currently, ultrafiltration is a widely used method for manufacturing whey protein concentrates with > 50% protein and ion-exchange adsorption is the preferred technology for manufacturing whey protein isolates with > 90% protein on a dry basis (Morr and Foegeding, 1990). The composition of whey protein concentrates is quite variable and dependent upon the particular whey source, i.e. acid or sweet whey, and upon the fractionation technology employed in their production, e.g. ultrafiltration, gel filtration, protein complex precipitation. When applied to food products, whey protein concentrates should not adversely affect the product's flavor, color and physical properties such as clarity, viscosity, texture, appearance, and heat stability.

Whey protein concentrates exhibit not only emulsion stabilizing properties, but also gelation properties. Morr (1982) described the gelation properties of whey protein

concentrates are affected by many factors, including types of whey, chemical properties of the proteins, protein concentration, processing condition used to produce the gel, and pH. Heat treatment changes protein solubility and the ratio of α-lactalbumin and β-lactoglobulin, and will also affect the gelation and emulsification properties of whey protein.

PROTEIN QUANTIFICATION

It is very useful to determine the amount of protein in surimi since there is a relationship between the quality of surimi and the quantity of protein in that surimi. The gel forming ability of minced-based products depends on protein concentration (Morita and Ogata, 1991). Myosin has been reported to be the most responsible protein for gel formation (Konno, 1992; Choe et al., 1991; Morita and Yasui, 1991). If the myosin is broken down to peptides that no longer have gel forming ability, it will affect the quality of the gel. Thus, it will be very useful to qualify and quantify the protein in that fish gel.

In quantitative analysis, the determination of protein content must be achieved rapidly, accurately, reproducibly, and sensitively with low cost. Many methods for determining protein concentration have been developed so far, including chemical measurement such as Biuret reaction, and direct measurement, i.e. A280. In qualitative analysis, the gel electrophoresis is widely used to identify the proteins based on the sizes of proteins.

1. Direct Spectrophotometric Methods:

Most proteins exhibit a distinct ultraviolet absorption which is caused by three amino acids: tryptophan, tyrosine and, to a small extent, phenylalanine. The extinction coefficient varies significantly from protein to protein depending on the amino acid composition (Harris and Angal, 1989). The absorbance of these amino acids is pH dependent. The peptide bond also has a strong absorbance at about 180 nm, but it is

difficult to measure at such a low wavelength. Thus, the protein quantification through use of the higher energy peptide bond is not widely used.

The advantage of measuring the absorbance at 280 nm is rapid and easily accomplished. In addition, this method is a nondestructive method, so proteins are not susceptible to denaturation. However, it is not easy to interpret the results, since proteins are considerably different in their amino acid composition, and this causes a difference in their absorption at 280 nm. Moreover, it requires the samples to be prepared so that only proteins can absorb light in the wavelength range under measurement. It is very sensitive to interference from other compounds which can absorb at 280 nm such as purine and pyrimidine ring in nucleic acids and nucleotides. Also, at low wavelengths, many of the commonly used buffer can absorb the light, thus only restricted buffer can be used. This method is consequently rather inaccurate unless the protein is relatively pure and its extinction coefficient is known, and relatively insensitive requiring 0.05 to 2 mg of protein.

2. Biuret Method:

In the biuret procedure, copper sulfate dissolved in alkaline solution is added to protein. Under the alkaline conditions, the cupric ion (Cu^{2+}) is bound to peptide nitrogen of proteins and peptides to produce a purple color with an absorption maximum at 540-560 nm, and becomes a cuprous ion (Cu^{1+}). This reaction requires at least a pair of peptide-bonded nitrogens. In the biuret reaction, approximately 1 atom of copper is bound for each 4 amino acid residues (Mehl et al., 1949). Thus, there is no interference from free amino acids and there is little dependence on amino acid composition, as the reaction occurs at peptide chains rather than at side groups. The color development with various proteins is different, however, the variation are less than other colorimetric method.

The biuret method is simple, rapid and inexpensive. Moreover, since the biuret method is based on a reaction with the peptide linkage, it provides an accurate

determination of protein concentration. The principal disadvantage of this method, which limits its applicability, is its low sensitivity. It requires milligram quantities of proteins (1-6 mg protein/mL). In addition, the color must be standardized against known protein concentrations.

3. Lowry Method:

The Lowry method has been used for many years and it is by far the most widely used procedure. The Lowry method is based on the biuret reaction of proteins with copper under alkaline conditions and follows by the reaction of copper-protein complex with the Folin phenol reagent. The Folin reagent is a mixture of phosphotungstate and phosphomolybdate. Initially, the reagent is yellow. Upon occurrence of the copper-protein complex, the bound copper ions reduce phosphomolybdate and phosphotungstate to heteropolymolybdenum and the yellow color disappears. Concurrently, an intense deep blue color appears. This blue color, which is approximately proportional to protein concentrations, can measure the absorbance at 750 nm.

The Lowry methods is 10-20 times more sensitive than the ultraviolet method, and up to 100 times more sensitive than the biuret method. It can detect the protein concentration from 0.1-1.0 mg protein/mL. In spite of its high sensitivity, there are still some disadvantages:

- Severely pH-dependent reaction which should be maintained to 10.5.

- The instability of the Folin phenol reagent. This reagent is stable only at acidic pH; however, the Lowry reaction occurs at pH 10. Therefore, when Folin reagent is added to the alkaline copper-protein solution, mixing must occur immediately so that the reduction can occur before the phosphomolybdic-phosphotungstate breaks down.
It is necessary to have precise timings of reagent addition and mixing with the sample to achieve accurate and reproducible results.

- A moderate variation of response to protein amino acid composition. At the same protein concentration, different proteins will yield different absorbance.

- Some substances found to interfere in this method such as amino derivatives, amino acids, buffers, detergents, drugs, lipids, sugars, nucleic acids, salts, and sulfhydryl reagents.

- The lack of linearity of the standard curves at high protein concentration (> 40 μg/mL for BSA).

- Cumbersome, lengthy and poorly suited to automation since it is a two step procedure.

4. Bicinchoninic Acid Method:

Bicinchoninic acid (BCA) method is based on the biuret reaction. It can be used for monitoring cuprous ion produced in the reaction of protein with cupric ion (Cu^{2+}) under alkali condition and results in the intense purple color with an absorbance maximum at 562 nm. The purple color produced from this reaction is stable and increases proportionally with increasing protein concentrations.

This BCA method can get rid of many problems occurred in the Lowry method which are due to the instability of Folin phenol reagent. Moreover, the BCA method is generally more tolerant to the presence of compounds that can interfere with the Lowry procedure. It is also more tolerant to simple buffer salts and a variety of detergent (anionic, non-ionic and zwitterionic) which are usually used to solubilize proteins. However, the BCA assay is more sensitive to interference from reducing sugars. Copper-chelating reagents such as EDTA also affect this assay, as do buffer solutions which change the pH optimum.

Because of the reagent stability, the BCA method is a simple, one-step assay. It has a high sensitivity with 0.1-0.2 mg protein/mL for the standard method and 0.5-10 μg

protein/mL for the micro assay procedure. In addition, it has less protein-to-protein variation than the Lowry and Dye-binding method.

5. Dye-binding Method:

Proteins can bind qualitatively under specific conditions with certain organic dyes, which can be used to determine total acidic and basic groups of proteins. The most commonly used dye is Coomassie Brilliant Blue G-250 which is commercially available. Coomassie blue reagent is prepared by dissolving the dye in an acidic solution either phosphoric or perchloric acid, the latter reagent being more stable. When added to a solution of proteins, the dye binds to the proteins and turns from a reddish brown to blue; this causes a shift in the maximum absorption of the dye from 465 to 595 nm.

Coomassie blue binds maximally to arginine residues and weakly to histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. The proteins that can bind with Coomassie blue, must have basic and aromatic functions, and macromolecular structures (Davis, 1988). Thus, the assay is useful for measuring proteins and polypeptides with M_r greater than 3,000-5,000, depending on the charge groups.

There are many factors that can affect the color development of dye including: protein amino acid composition (the number of basic amino acids and hydrophobic sites on the protein), the accessibility of these sites to the dye, the conditions favoring protein-bound dye, and interaction with soluble dye.

This procedure is very sensitive, 0.2-1.4 mg protein/mL for the standard assay and 5-10 μg protein/mL for the micro assay procedure. This dye binding method is a one step method, so it is simple and rapid. It requires no critical timing and produces a stable color. However, some detergents and basic protein buffers can interfere with the assay by chemical-protein and/or chemical-dye interactions. In addition, there is a great variability in color development among different proteins at the same protein concentrations. It can be attributed to the specificity of the dye for different amino acids and macromolecular

structure of proteins, and the disparity of color development between proteins which depends on the concentration of dye and proteins (Davis, 1988).

6. Trinitrobenzenesulfonic acid method:

The use of 2,4,6-Trinitrobenzenesulfonic acid (TNBS) to determine the primary amino groups of amino acids and peptides was developed by Satake and Okuyama in 1960. It can react specifically, under mild conditions, with free amino groups to give trinitrophenyl (TNP) derivatives. During the reaction, sulfite is displaced from TNBS by an attacking nucleophile and yields trinitrophenylated amino complexes that can measure the absorbance at 340 nm and 420 nm, depending on the presence of excess sulfite ions (Whitaker et al., 1980). Liberated sulfite can react reversibly with TNP-amino complexes and TNP-thiol groups, and causes difficulty in measuring quantity of proteins (Field, 1972; Crowell et al., 1985). Thus, the reaction mixture is acidified to dissociate the sulfite complexes before reading (Field, 1972). There is no reaction with tyrosine or with histidine side chains. In addition, Satake et al. (1960) demonstrated that TNBS reacted only with primary amino acids and peptides without any undesirable side reaction.

For TNBS method, it is rapid and simple. There is low variation in color development of various proteins compared to phenol reaction. Using TNBS for measuring rate of proteolysis has been extensively studied (Binkley et al., 1968; Lin et al., 1969; Whitaker et al., 1980; Chobert et al., 1988).

Nevertheless, the interpretation of absorption results should be cautious. This is because specific amino groups in a protein can react independently with TNBS, and must have no hypochromic or hyperchromic interactions. If the active groups are buried within the protein, any changes in conformation that occur during trinitrophenylation, may readily increase or decrease the rate of reaction of TNBS with other partly buried residues.

7. Electrophoresis:

Electrophoresis is the most powerful method available for separation and analysis of complex mixtures of charged biopolymers. It is a method whereby charged molecules in solution, chiefly proteins and nucleic acids, migrate in response to an electrical field. Their rates of migration or mobility through the electrical field depend on the strength of the field; on the net charge, size and shape of the molecules; and on the ionic strength, viscosity, and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid, and highly sensitive. It is used to study the properties of a single charged species and as a separating technique.

Gel sieving electrophoresis can also be applied to whole native proteins. However, the most effective application of gel sieving to proteins involves the use of denaturing ionic detergents, sodium dodecyl sulfate (SDS). The usefulness of SDS polyacrylamide gel electrophoresis (SDS-PAGE) is for molecular weight determinations. It depends on the ability of the detergent, SDS, to interact with and denature a wide variety of proteins. Native proteins having widely different charge, size, and shape characteristics are converted, upon reduction and SDS-binding, to SDS-protein complexes of their constituent polypeptide chains. These SDS-protein complexes have a constant charge per unit mass (since the charge of the bound SDS masks the protein charge) and identical conformations which vary in size directly in relation to the protein's subunit molecular weight. Under a given set of eletrophoretic conditions (i.e. pH, voltage, time, gel concentration, etc.), the mobility of protein depends on its charge density, size, and shape. Since most SDS-protein complexes have the same charge density and assume identical shapes, differences in their mobilities are due to differences in size. Therefore, in SDS-PAGE protein molecular weights can be determined by comparison of the electrophoretic mobilities of unknown proteins with those of standard proteins of known molecular weight.

Chapter 2 : APPARENT INHIBITION OF PACIFIC WHITING SURIMI-ASSOCIATED PROTEASE BY WPC

INTRODUCTION

Surimi is a minced fish product that has been washed to remove sarcoplasmic proteins and increase the concentration of myofibrillar proteins, and mixed with cryoprotectants for longer shelf-life. Surimi-based products are becoming more popular. Their production in the U.S. has increased rapidly, and it grew up to 110 million lbs. by 1990 (Zalke, 1992).

Pacific whiting (*Merluccius productus*) has been used for surimi production due to the abundant quantity of this species coupled with its limited usefulness in other products. However, use of Pacific whiting has been limited because of soft texture. Pacific whiting surimi typically undergoes excessive softening due to the presence of endogenous proteolytic enzymes. In surimi-based products, the ability to form highly elastic gels when mixed with salt and other ingredients is the most useful characteristic. The major component that contributes to gel forming ability is myofibrillar protein, i.e. myosin, which causes the three-dimensional network when heated. Proteolytic enzymes found in fish can degrade the myosin, and cause the soft texture, which is an adverse effect on the gel forming properties of surimi. This is called modori phenomenon. There are many proteases associated with fish muscle, many of which may be involved in the degradation of myofibrillar proteins (Iwata et al., 1973; Folco et al., 1984; Toyohara et al., 1990)

Since the proteolytic activity which causes the decrease in quality of surimi-based products is a critical problem, many attempts have been made to minimize this problem by using food additives such as protease inhibitors in order to improve the gel quality of surimi. Hamann et al. (1990) showed that addition of beef plasma hydrolysate and egg white solids in Atlantic menhaden and Alaska polluck surimi was associated with increased gel strength, and apparent protection of myosin heavy chains from proteolytic

degradation. Chang-Lee et al. (1990) demonstrated that the addition of egg white markedly improved the gel strength of surimi made from Pacific whiting. Incorporation of a mixture of potato starch and egg white has also been shown to improve the gel strength of surimi from Pacific whiting (Chang-Lee et al., 1989). Beef plasma protein, BPP (Morrissey et al., 1992) and a compound extracted from potato (Porter, 1992) have been shown to inhibit the apparent rate of proteolysis in Pacific whiting surimi.

Whey protein concentrate (WPC) is a commercial product produced from whey, a waste product obtained from cheese processing. Incorporation of WPC into Pacific whiting surimi has been shown to increase the hardness and elasticity of heat-set gels (Chang-Lee et al., 1990).

The objective of this study was to determine whether the incorporation of whey protein concentrates into Pacific whiting surimi inhibits autoproteolysis and/or protects the myosin fraction from proteolytic degradation.

MATERIALS AND METHODS

Materials:

Surimi, made from Pacific whiting (*Merluccius productus*), was obtained from American Seafoods Co., Seattle, WA. The surimi contained 4% sucrose, 4% sorbitol, 0.3% tripolyphosphates, and 0.12% monodiglycerides. Whey protein concentrates (WPC) and beef plasma protein (BPP) were obtained commercially; WPC 34 (Davisco International, Inc., Minnesota, marketed as 34% protein content), WPC 80 (Alaco Surimi Plus, New Zealand Milk Products, Inc., CA, marketed as 80% protein content), WPC 95 (BIPRO, Le Sueur Isolates, MN, marketed as 95% protein content), and BPP (AMPC Inc., Iowa, AMP 600N, Hydrolyzed Meat Protein from Beef). Bovine Serum Albumin, BSA, was purchased from SIGMA (fraction V, 98-99% Albumin).

Extent of Apparent Inhibition of Autoproteolysis by Protein Additives; Effect of Protein Additive Concentration:

The autoproteolysis assay is based on the measurement of released peptides due to the degradation of fish by its endogenous protease . To initiate the reaction, three g of fish was mixed either with 0, 30, 60, 90 or 120 mg of protein additive (0,1, 2, 3 or 4% additive, respectively), and then placed in a 55 °C water bath. After a 1 hr reaction period, the reaction was terminated by the addition of twenty two mL precipitating solution, which contained 0.9% (w/v) Trichloroacetic acid (TCA), 0.575% (w/v) anhydrous sodium acetate and 1.05% (v/v) glacial acetic acid. The mixture was incubated at 37 °C for 30 min, and then transferred to an ice bath for 15 min. The termination and precipitation procedure are based on that reported by Miles technical information (Miles laboratories, Indiana). The resulting solution was then filtered through Whatman No. 5 filter paper, and the peptide content of the filtrate was measured by the Bicinchoninic acid

(BCA) method (Smith et al., 1985). The control was as described above with the exception that it did not contain protein additives.

"Blank" samples were done for all conditions by being handled exactly like the experimental and control samples with the exception that blanks were kept on ice for the 1 hr reaction period. The difference in the absorbance (ΔA) of the experimental sample and its corresponding blank was considered directly proportional to the amount of peptides released due to proteolysis. The mass of peptides released in any given reaction period was based on BSA equivalents. The apparent inhibition associated with the addition of a protein additive in units of % Inhibition, was calculated relative to the control sample as shown in equations (1), (2) and (3);

$$\Delta A_{control} = (A_{control}) - (A_{control})_{blank} \quad \text{-------- (1)}$$

$$\Delta A_{experimental} = (A_{experimental}) - (A_{experimental})_{blank} \quad \text{-------- (2)}$$

$$\% \text{ Inhibition} = \frac{\Delta A_{control} - \Delta A_{experimental}}{\Delta A_{control}} * 100 \quad \text{-------- (3)}$$

Extent of Apparent Inhibition of Autoproteolysis by Protein Additives; Comparison of Peptide Assay Methods:

The extent of apparent inhibition of autoproteolysis by protein additive was as described above with the addition of 30 mg of protein additive (1% additive). Different peptide assay methods were compared. After filtration, the peptide content of the filtrate was measured by the Bicinchoninic acid (BCA) method (Smith et al., 1985), Lowry method (Lowry et al., 1952), Dye-binding method (Bradford, 1976) and 2,4,6-Trinitrobenzenesulfonic acid (TNBS) method (Fields, 1972). When the peptides content

of the filtrate from surimi mixed with BSA was measured by BCA method, the reaction was stopped by adding cold 5% TCA.

The apparent inhibition associated with the addition of a protein additive in units of % Inhibition, was calculated relative to the control sample as described above.

Extent of Apparent Inhibition of Autoproteolytic Myosin Degradation; Electrophoretic Analysis:

Myosin was visualized by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis as described by Wasson et al. (1992). Three g of surimi was mixed with 120 mg of protein additive and incubated at 55 °C for 15, 30 and 60 min. To terminate the reaction, 27 mL of 95 °C SDS solution (15 mL of 10% SDS and 12 mL of distilled water) was added with mixing. For the blank, the SDS solution was added at 0 time, prior to incubation. Terminated reaction mixtures and blanks were then incubated in an 80 °C water bath until completely solubilized. Solubilized samples were then heated with treatment buffer (0.0625 M Tris-Cl pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol), at ratio 1:4 for 90 sec, and 50 µl of 0.02% Bromophenol Blue (BPB) was added. The sample was run on SDS-PAGE (10% Acrylamide gel), according to method of Hoefer Scientific laboratory manual (Hoefer Scientific Instruments, 1986), and visualized by protein staining with Coomassie Brilliant Blue G-250. Premixed SDS-PAGE molecular weight standards including Myosin, ß - Galactosidase, Phosphorylase B, Serum albumin, Ovalbumin, Carbonic anhydrase, Trypsin inhibitor, Lysozyme and Aprotinin (SDS-PAGE Molecular Weight Standards, Broad Range, BIORAD) were applied to all gels.

RESULTS

Extent of Apparent Inhibition of Autoproteolysis by Protein Additives; Effect of Protein Additive Concentration:

The effect of additives, such as WPC and BPP, on the proteolytic activity of surimi is important relative to the functional properties of the end-products produced from that surimi. The experimental data presented in Table 2 describes the relationship between supplementation of surimi with WPCs, BPP and BSA and the apparent proteolytic activity of the surimi as measured by autoproteolysis using BCA to quantify solubilized peptides. It is clear that incorporation of each of the WPC preparations into surimi decreased the apparent proteolytic activity associated with that surimi. Of the WPCs tested, the lowest percent inhibition was consistently associated with WPC 34. The higher protein content WPCs, WPC 80 and WPC 95, had essentially the same affect on apparent proteolysis. The BPP preparation, which is widely reported to act as an inhibitor of endogenous proteolysis in surimi (Hamann et al., 1990; Morrissey et al., 1992), inhibited essentially 100% of the proteolytic activity at each level tested. In contrast, BSA supplementation had little to no inhibitory effect under these assay conditions. "Inhibition" in the context of this assay refers to a decrease in the BCA reactive/TCA-soluble peptides produced during the incubation period. Consequently, the apparent inhibition associated with a given additive may not necessarily correlate with an actual decrease in proteolytic events in terms of the number of peptide bonds hydrolyzed.

Extent of Apparent Inhibition of Autoproteolysis by Protein Additives; Comparison of Peptide Assay Methods:

The extent of inhibition observed in the autoproteolysis assay is dependent on several factors, including the assay used to measure the TCA-soluble peptides resulting from proteolysis. Peptide and protein solubility in any given TCA-based precipitating

reagent is dependent on the final composition of the precipitating solution, and on the amino acid composition and size of the peptides and proteins. Proteolytic events resulting in peptide products that are soluble in the precipitating reagent are assumed to be detected. However, the actual quantity of solubilized peptides/amino acids measured will be somewhat dependent on the assay used for quantification. The data presented in Table 3 compares the percent inhibition associated with each of the additives when using different assay methods to quantify the TCA-soluble peptides/amino acids. The percent inhibition observed due to the incorporation of 1% BPP was consistently high for each of the assay methods (Figure 2, 3, 4, and 5). With respect to the WPC preparations, the percent inhibition values obtained by the BCA, Lowry, Dye-binding and TNBS assays (Figure 2, 3, 4, and 5, respectively) were similar. The apparent inhibition values observed for BSA supplemented surimi is very low, when measured by both BCA and TNBS assays (Figure 2 and 5, respectively).

Extent of Apparent Inhibition of Autoproteolytic Myosin Degradation; Electrophoretic Analysis:

The apparent decrease of autoproteolysis that was associated with the inclusion of the WPCs and BPP in surimi suggests that these additives may act to protect the myofibrillar proteins in surimi from proteolytic degradation. The relevance of this protective effect was tested qualitatively by visually following the change in the intensity of SDS-PAGE gel bands associated with myosin, tropomyosin and actin from surimi incubated with and without the incorporation of these additives. Each of the additives was incorporated at the 4% level, which insured approximately 100% inhibition of autoproteolysis for each additive based on the data of Table 2. Figure 6, lane b, shows that actin, myosin and tropomyosin are clearly visible when non-supplemented surimi is assayed prior to incubation at 55 °C, and that essentially all of the myosin and tropomyosin fractions were degraded over the subsequent sixty minute incubation period.

Incorporation of either WPC 34 or WPC 80 resulted in a significant protection of these fractions. The WPC 80 sample showed no significant loss of either myosin or tropomyosin over the time frame of this study, while WPC 34 sample showed some partial degradation of these fractions at sixty minutes of incubation time (Figure 6). Figure 7 illustrates that the addition of either WPC 95 or BPP similarly protected each of these myofibrillar protein fractions. In contrast, the addition of BSA into surimi did not show the protection of these myofibrillar protein fractions (Figure 8). The actin fraction appeared to be far less susceptible to proteolytic degradation than either myosin or tropomyosin, such that changes in this fraction were not detectable in any of the treatment groups.

DISCUSSIONS

The results indicate that the addition of WPC to Pacific whiting surimi protects the myofibrillar proteins from proteolytic degradation through a mechanism which is manifested as a decrease in the apparent rate of surimi autoproteolysis. The electrophoretic data clearly indicates a prolonged lifetime for the myofibrillar proteins of surimi supplemented with WPC. The protection of myosin and tropomyosin may occur by one or a combination of several mechanisms. The percent inhibition of autoproteolysis, as reported herein, is based on results generated using common, empirical, protease assays. These assays are based on measuring the apparent rate of production of TCA soluble peptides, the peptides resulting from the proteolysis of substrate proteins which are themselves not soluble in TCA. By definition, the assays will only measure proteolytic activity which results in soluble peptides. It is clear, however, that some proteolytic events may occur which do not produce a TCA soluble product. The peptides solubilized in this assay protocol are typically quantified by an empirical colorimetric peptide/protein assay. It is well established that in these assays structurally unique peptides/proteins may have different color yields per unit mass. Therefore, two reaction mixtures with equivalent proteolytic activities, in mg peptide solubilized per unit time, may not show equivalent activities in a particular assay if the solubilized peptides unique to each reaction mixture have different color yields per unit mass. These ambiguities, dependence on TCA solubility and non-absolute peptide quantification, complicate the interpretation of these traditional protease assays. For this reason, the inhibition observed in autoproteolysis assays must be considered as an "apparent" inhibition. The major advantage in using the autoproteolysis assay to monitor changes in proteolytic activity is that the reaction is allowed to progress insitu, such that artifacts associated with the fractionation of this complex product are avoided.

The importance of the colorimetric method used to assay solubilized peptides may be tested by comparing the results from several chemically distinct quantification methods.

The different quantitative methods used in this study all gave similar percent inhibition value (Table 3). The Lowry and BCA methods are both based on the biuret reaction of peptides in alkaline solution, hence one may predict that these methods would yield similar results. However, the dye-binding and TNBS assays are each unique, both based on chemistry distinctly different from the biuret reaction. The similarity of results obtained with the different peptides quantification assays strongly suggests that each assay in itself is a reasonable measure of the relative mass of protein solubilized. This is particularly true considering that for any given protein the color yield relative to a standard reference protein is expected to be different for each of the assays used to quantify peptides. The assays evaluating BSA-supplemented surimi also included two distinct TCA-based termination methods. This was necessary because BSA is soluble in the TCA/acetic acid-based terminating solution used throughout this study for control, WPC-supplemented and BPP-supplemented samples. Autoproteolysis reaction mixtures containing BSA-supplemented surimi were terminated using higher TCA concentrations. This BSA-specific termination scheme applied to assays in which product peptides were measured by the BCA, Lowry and dye-binding methods. The higher TCA concentration was not necessary when peptides solubilized from BSA-supplemented surimi were determined by the TNBS method since the TNBS assay is based on the liberation of a new amino group for each proteolytic event. The general agreement between the two assays used for BSA-supplemented surimi (Table 3), which differed with respect to the TCA precipitation methods and the peptide quantification chemistry, suggests that the results of this study are not assay specific.

The apparent inhibition of autoproteolysis by WPC is consistent with the observed protection of myosin by WPC. WPC may protect the myofibrillar proteins of surimi by acting as a true inhibitor or by serving as an alternative substrate which effectively decreases the proteolytic activity on myosin per se. The alternative substrate mechanism would be consistent with the data presented here if the products resulting from the

proteolytic degradation of WPC were not soluble in the TCA solution used to terminate the reaction. The extent to which WPC preparations inhibited autoproteolysis appeared to correlate with their total protein content. This, of course, suggests that the extent of inhibition of autoproteolysis is dependent on the amount of WPC protein added to the surimi. It follows that supplementation with relatively low levels of WPC protein may not significantly modify the extent of autoproteolysis. This result was obtained in the study of Akazawa et al. (1993), in which low levels of WPC (.25% and .50%) were shown to have only a minimal effect on Pacific whiting surimi autoproteolysis.

The observed protection of myofibrillar proteins by WPC indicates that WPC-supplemented surimi may form a stronger heat-set gel than a corresponding non-supplemented product. The rational for this statement comes from data which suggests that myosin is the principle gel-forming component of surimi (Choe et al., 1991; Konno, 1992). Two recent studies have demonstrated this relationship, one reporting a relative increase in shear stress and shear strain (Chung and Morrissey, 1993) and the other a general improvement in gel texture (Akazawa et al., 1993) due to supplementation of Pacific whiting surimi with WPC.

Table 2: Apparent inhibition of surimi autoproteolysis by incorporation of protein additives.

Protein additives	Apparent inhibition (%)[a]				
(%)	WPC 34	WPC 80	WPC 95	BPP	BSA
1%	31.70±4.70	76.60±0.14	78.80±0.15	100.00±0.00	16.50±2.72
2%	57.70±5.94	99.50±0.55	100.00±0.00	100.00±0.00	0.00±0.00
3%	82.80±0.35	100.00±0.00	100.00±0.00	100.00±0.00	6.90±6.89
4%	93.30±5.46	100.00±0.00	100.00±0.00	100.00±0.00	3.50±3.49

[a] apparent inhibition is reported as Mean±S.E.M. (n=2, assayed in duplicate).

Table 3: Apparent inhibition of surimi autoproteolysis by incorporation of 1% (wt/wt) protein additives. Comparison of methods used to quantify solubilized peptides.

Protein additives	Apparent inhibition (%)[a]				
	BCA	Lowry	Dye-binding	TNBS	n
WPC 34	44.05±4.23	46.64±2.18	34.23±4.71	48.16±5.50	5
WPC 80	72.93±5.49	69.94±6.13	70.65±4.06	63.09±4.96	6
WPC 95	89.00±4.76	84.59±4.33	83.91±4.94	75.55±6.02	6
BPP	98.49±1.07	99.09±0.58	72.60±12.87	98.89±0.69	6
BSA	4.39±1.96[b]	-	-	3.69±1.56	6

[a] apparent inhibition is reported as Mean±S.E.M.

[b] autoproteolysis was terminated by addition of cold 5% TCA.

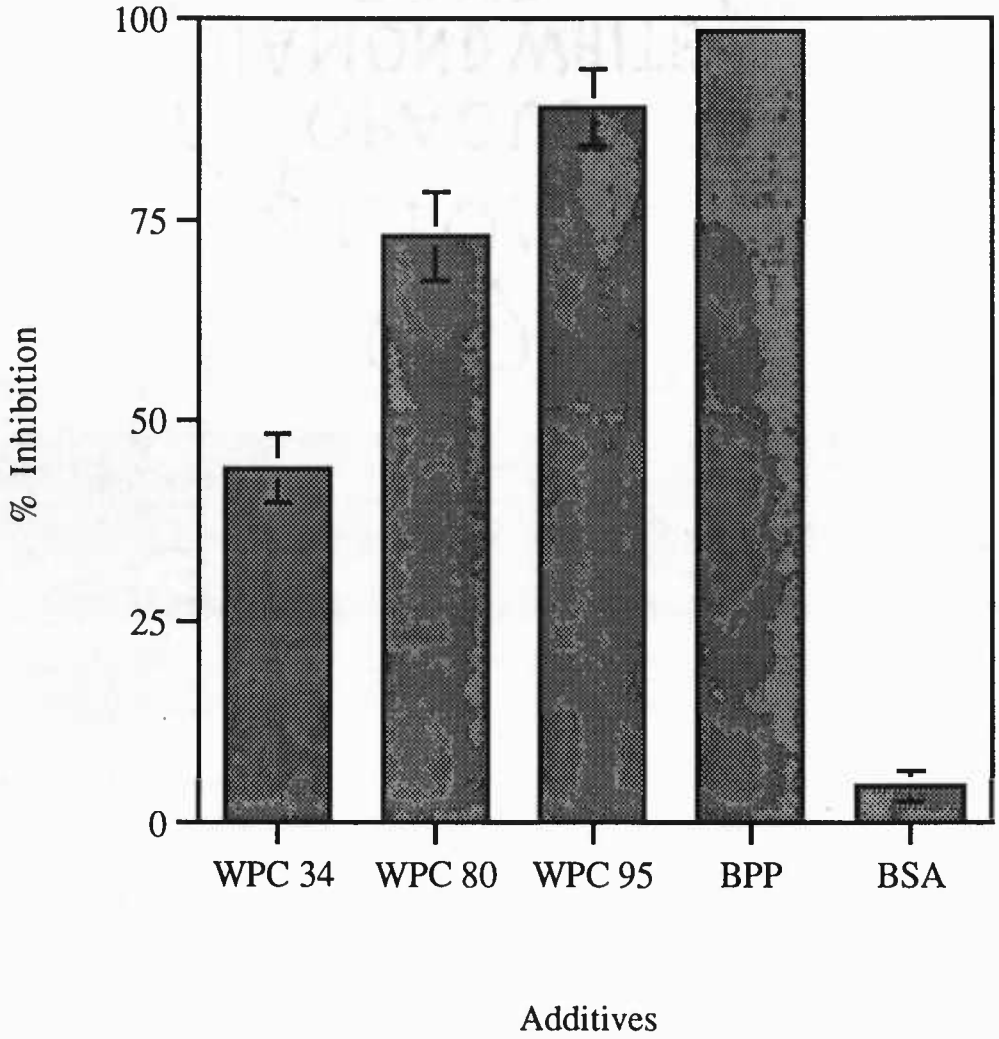

Figure 2 : Apparent inhibition (%) of 1% different protein additives measured by BCA assay.

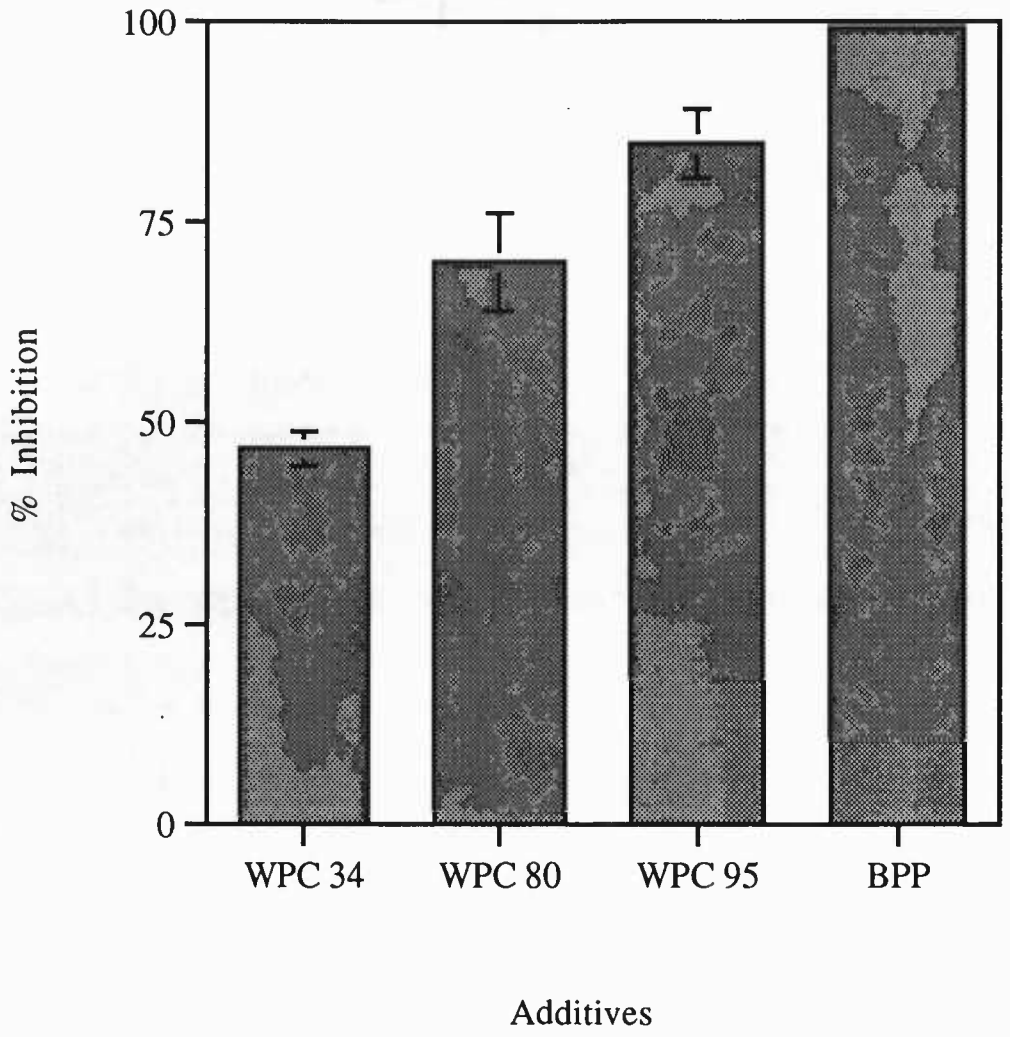

Figure 3 : Apparent inhibition (%) of 1% different protein additives measured by Lowry assay.

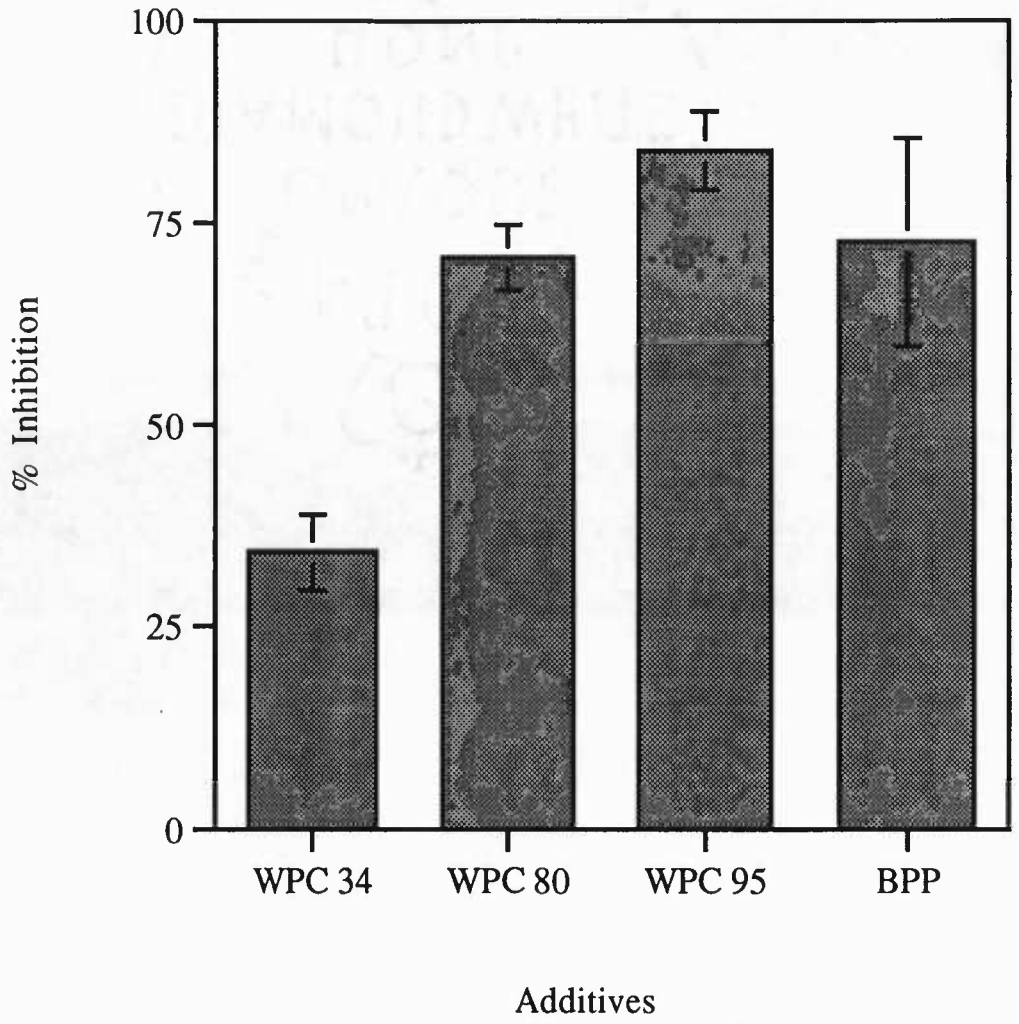

Figure 4 : Apparent inhibition (%) of 1% different protein additives measured by Dye-binding assay.

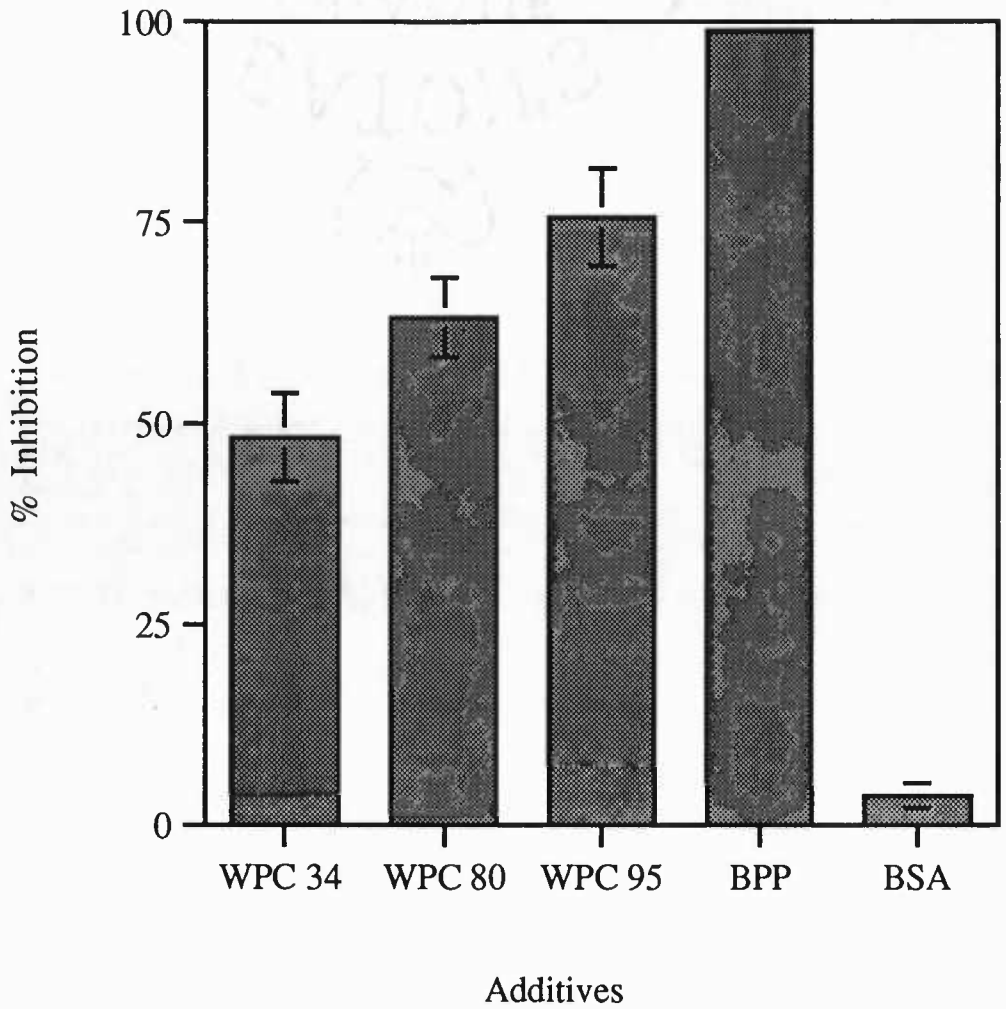

Figure 5 : Apparent inhibition (%) of 1% different protein additives measured by TNBS assay.

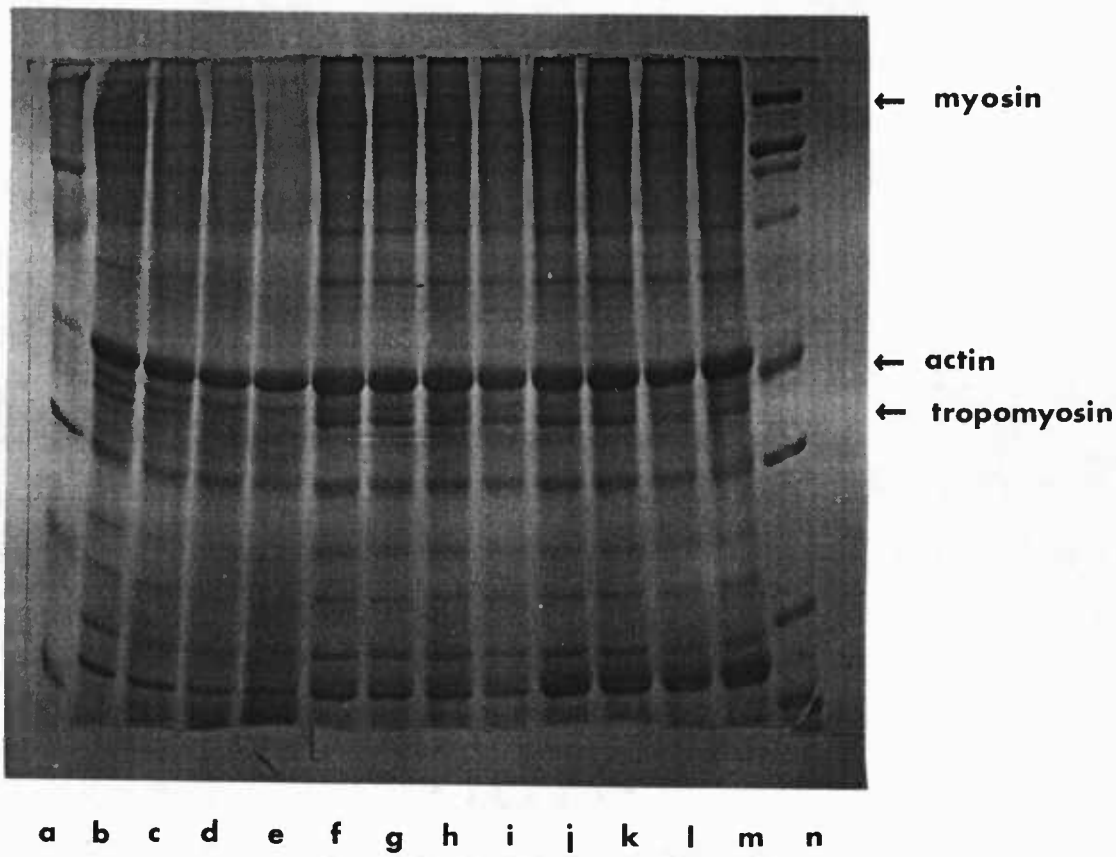

Figure 6 : SDS-PAGE of myosin degradation of surimi (Pacific whiting) in presence and absence of WPC 34 or WPC 80 at 4% additives, as a function of incubation time (0, 15, 30, and 60 min) at 55 °C. Lane a, n: protein standard. Lane b, c, d, e: surimi at 0, 15, 30, and 60 min respectively. Lane f, g, h, i: surimi+WPC 34 at 0, 15, 30, and 60 min respectively. Lane j, k, l, m: surimi+WPC 80 at 0, 15, 30, and 60 min respectively.

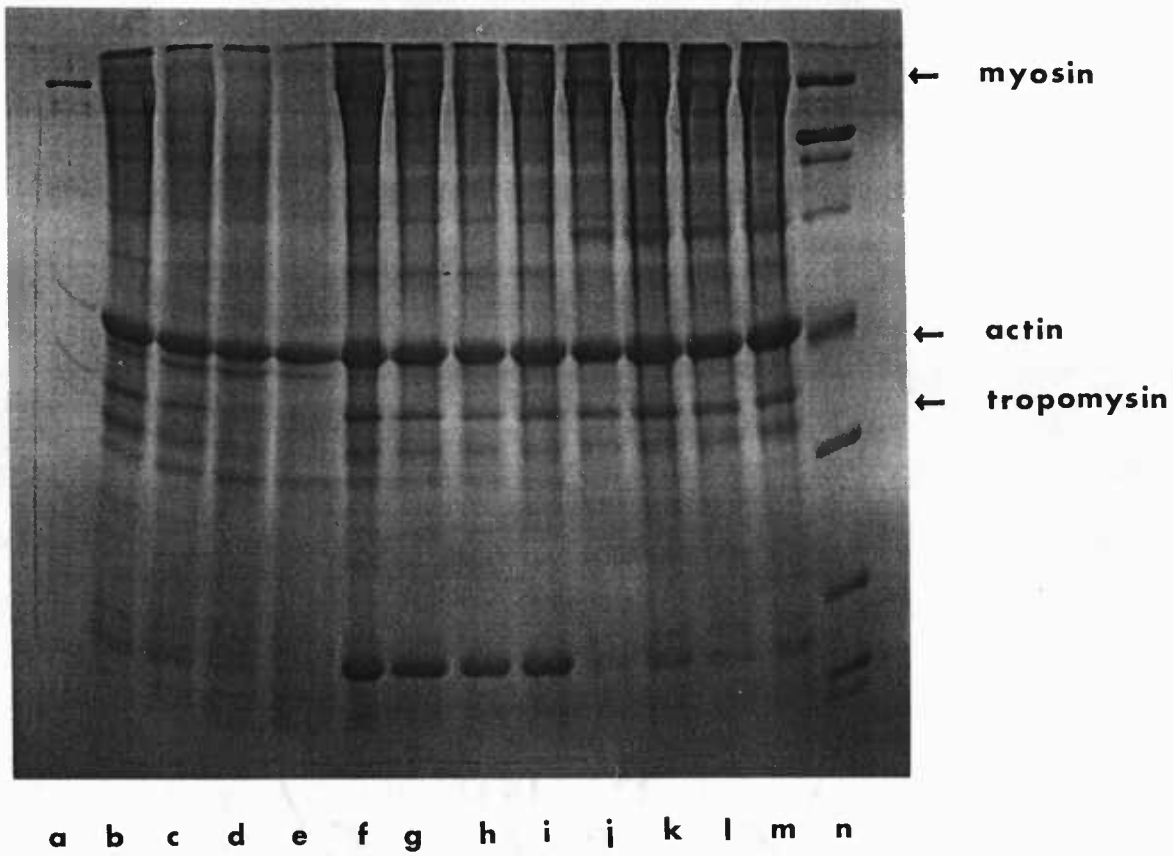

Figure 7 : SDS-PAGE of myosin degradation of surimi (Pacific whiting) in presence and absence of WPC 95 or BPP at 4% additives, as a function of incubation time (0, 15, 30, and 60 min) at 55 °C. Lane a: myosin extract. Lane b, c, d, e: surimi at 0, 15, 30, and 60 min respectively. Lane f, g, h, i: surimi+WPC 95 at 0, 15, 30, and 60 min respectively. Lane j, k, l, m: surimi+BPP at 0, 15, 30, and 60 min respectively. Lane n: protein standard.

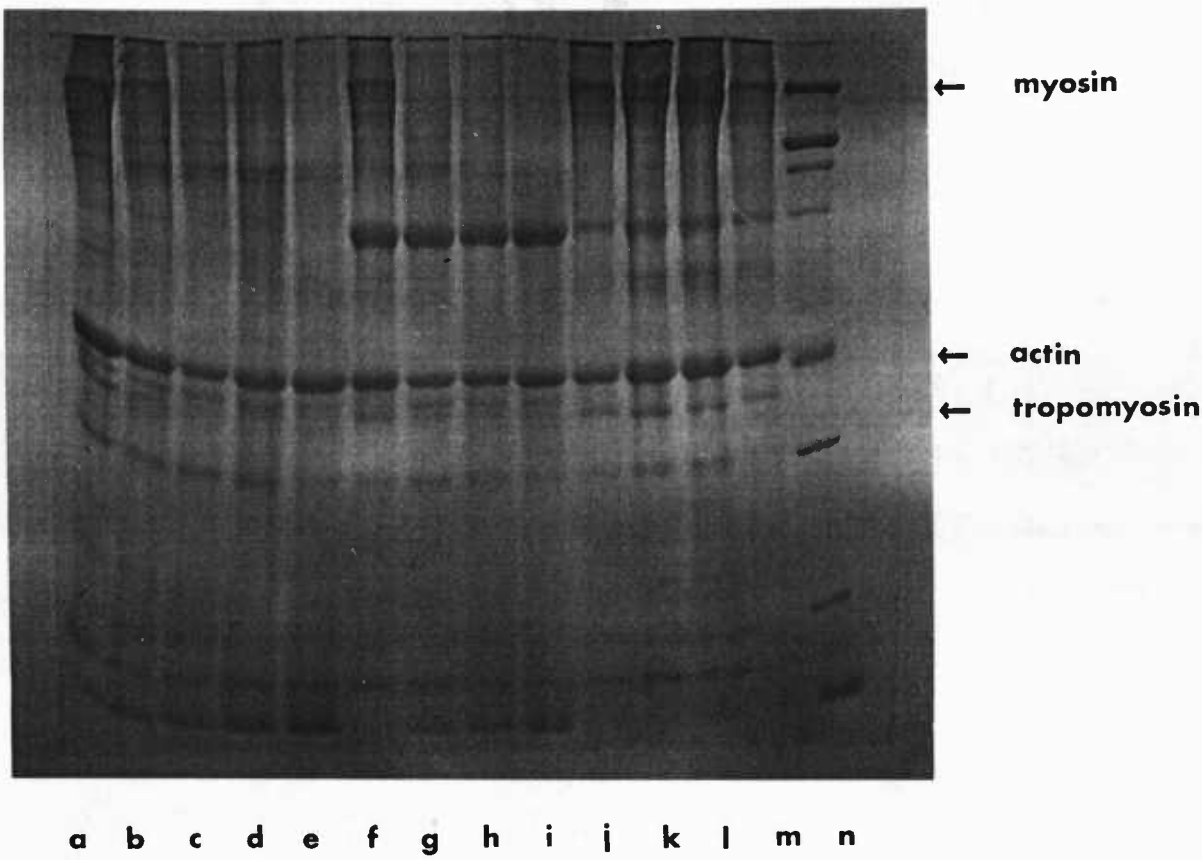

Figure 8: SDS-PAGE of myosin degradation of surimi (Pacific whiting) in presence and absence of BSA or BPP at 4% additives, as a function of incubation time (0, 15, 30, and 60 min) at 55 °C. Lane a: surimi without incubation. Lane b, c, d, e: surimi at 0, 15, 30, and 60 min respectively. Lane f, g, h, i: surimi+BSA at 0, 15, 30, and 60 min respectively. Lane j, k, l, m: surimi+BPP at 0, 15, 30, and 60 min respectively. Lane n: protein standard.

Chapter 3 : MECHANISM OF WPC INHIBITION

INTRODUCTION

The data presented in the previous chapter indicates the protection of myosin by WPC. WPC may act through two different mechanisms to protect myosin from proteolytic degradation. One mechanism is that a component of WPC acts as a true inhibitor, i.e. proteolysis does not occur. In the other mechanism, the protein of WPC serves as an alternative substrate, i.e. proteolysis still occurs but the substrate is no longer myosin. WPC may protect myosin by either one or a combination of these mechanisms. The data of chapter 2 is consistent with the inhibitor mechanism. However, the assay used in this study may not detect an alternative substrate. Therefore, the challenging question is to determine whether WPC acts as an inhibitor or an alternative substrate.

The distinguishability between these mechanisms is not trivial since it is a complex system. The determination of protease activity is based on TCA precipitation and measurement of TCA-soluble peptides. Therefore, it may not correlate with the actual proteolytic activity in terms of peptide bonds hydrolyzed. Some proteolytic events may occur which do not produce a TCA soluble product. In addition, solubilized products resulting from proteolytic activity on different substrates can not be differentiated by the peptide assays. Hence the assays measure solubilized peptides but tell nothing about the origin of the peptides.

This chapter presents the results from an experiment designed to provide information on the mechanism of myosin protection by WPC. The experimental approach was to extract the protease and determine whether WPC and other additives could serve as a substrate in the absence of myofibrillar proteins.

MATERIALS AND METHODS

Materials:

Pacific whiting (*Merluccius productus*) fillets were obtained from Astoria Seafood Sales, Inc., Astoria, OR. Whey protein concentrates (WPC) and beef plasma protein (BPP) were obtained commercially; WPC 95 (BIPRO, Le Sueur Isolates, MN, marketed as 95% protein content) and BPP (AMPC Inc., Iowa, AMP 600N, Hydrolyzed Meat Protein from Beef). Bovine Serum Albumin, BSA, was purchased from SIGMA (fraction V, 98-99% Albumin).

Protease Enzyme Preparation:

Pacific whiting fillets were chopped into mince, centrifuged at 5000 rpm for 30 min, and the supernatant decanted and kept on ice. The supernatant, referred to as "fish juice", served as a crude enzyme preparation for this study.

Extent of Apparent Rate of proteolysis by Fish Juice:

To initiate the reaction, 1 mL of fish juice was mixed with a protein additive (final concentration = 1 and 3 mg/mL) in phosphate buffer at pH 7, and then a 3 mL reaction mixture was placed in a 55 °C water bath. After a 1 hr reaction period, the reaction was terminated by 3 mL cold 5% trichloroacetic acid (TCA), and then transferred to a refrigerator for 15 min. The resulting solution was then filtered through Whatman No. 5 filter paper, and the peptide content of the filtrate was measured by the Bicinchoninic acid (BCA) method (Smith et al., 1985).

"Blank" samples were done for all conditions by being handled exactly like the experimental samples with the exception that blanks were kept on ice for the 1 hr reaction period. The difference in the absorbance of the experimental sample and its corresponding blank was considered directly proportional to the amount of peptides released due to

proteolysis. The mass of peptides released in any given reaction period was based on BSA equivalents.

RESULTS AND DISCUSSIONS

This experiment was designed to test whether the active protease in Pacific whiting was able to use WPC, BPP or BSA protein as a substrate. When designing this experiment, the following potential results were anticipated. It was assumed that the reaction mixture containing only protease (fish juice) would show negligible protease activity. Reaction mixtures containing protease plus an additive would show either negligible activity, if the additive was an inhibitor or it would show an increase in activity if the additive served as an alternative substrate. The actual results of this experiment are presented in Table 4 and 5. An unexpected result included in the table is that the fish juice sample showed relatively high activity when compared to reaction mixtures containing only additives. The simplest interpretation of this aspect of the data is that the additives themselves contained negligible protease activity, as may be expected. The relatively high proteolytic activity in the fish juice sample may be due to either autoproteolysis of the protease itself or due to the proteolysis of soluble fish proteins that had been coextracted with the protease. The latter of these two scenarios seems most plausible in that the protein content of fish juice was approximately 30 mg/mL based on the BCA protein assay using BSA as a standard. The relatively high activity observed for the fish juice sample alone made the interpretation of additive effects more complicated since, as discussed above, the experiment was designed assuming that the proteolytic activity in the fish juice sample alone would be negligible.

The apparent proteolytic activities in reaction mixtures containing fish juice, fish juice plus myosin or fish juice plus BSA were essentially the same. This applied to reaction mixtures supplemented with either 1 or 3 mg/mL. The most obvious interpretation is that the additives had no effect on proteolytic activity. One reason for this no effect may be that these additives simply act as an inert reaction mixture component. However, a second explanation would be that if the protease in the fish juice sample were already acting under saturating conditions (V_{max}), then supplementing with

additive may have little to no effect on apparent proteolytic activity even if the additive was serving as an alternative substrate. The assay used in this study does not provide information on the source of the soluble peptides produced due to proteolysis. Hence, the results from this experiment do not allow us to differentiate between the "inert substance" and "alternative substrate" scenarios.

The effects of supplementing the fish juice sample with either WPC or BPP were similar, in that both additives showed the same trends. In both cases, addition of the lower level of additive (1 mg/mL) significantly reduced the apparent proteolytic activity. The fact that the activities did not drop to the levels shown for the additives incubated alone without fish juice suggests that some proteolytic activity remained in each of the reaction mixtures, the activity being higher in the WPC supplemented sample compared to the BPP supplemented sample. The addition of higher levels of WPC or BPP (3 mg/mL) resulted in an increase in the apparent rates of proteolysis relative to the samples supplemented at 1 mg/mL. In each case, the WPC and BPP supplemented samples showed lower proteolytic activities than that for the fish juice acting alone. In this experiment, the ratio of the activities of the BPP and WPC samples remained constant when comparing the two levels of supplementation; this emphasizes that the same trend in proteolytic activity was observed due to supplementation with either of these two additives. Based on this similarity, it appears reasonable that a working hypothesis upon which to base further studies would suggest that the two additives modulate proteolytic activity by a similar mechanism. It has been widely assumed that BPP decreases proteolysis through the action of inhibitors endogenous to beef plasma, the most commonly mentioned inhibitors being α_2-macroglobulin (Hamann et al., 1990) and cystatin (Akazawa et al., 1993). The results of this experiment suggest that studies attempting to identify/isolate the active component of WPC, which may be a protease inhibitor, are warranted. It is clear, however, that the existence of a protease inhibitor in WPC is not certain and that further studies are necessary in order to determine the mechanism through which WPC lowers the apparent

rates of proteolysis in Pacific whiting surimi. Certainly, future studies would benefit from the use of a more purified protease preparation and the testing of protein fractions isolated from the complex mixture of proteins which make up WPC.

Table 4: Apparent rate of proteolysis in fish juice/additive reaction mixture[a]. The final additive concentrations were 1 mg/mL.

Reaction mixture	Peptides produced (μgmL^{-1})[b]
Fish juice	1231.5±48.5
Myosin	9.0±1.0
BSA	0.0±0.0
BPP	18.0±18.0
WPC 95	0.5±2.5
Myosin+Fish juice	1406.0±99.0
BSA+Fish juice	1250.0±10.0
BPP+Fish juice	114.5±8.50
WPC 95+Fish juice	486.0±55.0

[a] final additive concentrations were 1 mg/mL for BSA, BPP, WPC 95, and 0.3 mg/mL for myosin.

[b] peptides produced are reported as Mean±S.E.M. (n=2, assayed in duplicate).

Table 5: Apparent rate of proteolysis in fish juice/additive reaction mixture[a]. The final additive concentrations were 3 mg/mL.

Reaction mixture	Peptides produced (μgmL^{-1})[b]
Fish juice	1231.5±48.5
Myosin	9.0±1.0
BSA	0.0±0.0
BPP	0.0±0.0
WPC 95	3.0±8.0
Myosin+Fish juice	1406.0±99.0
BSA+Fish juice	1174.0±25.0
BPP+Fish juice	262.0±22.0
WPC 95+Fish juice	1035.5±35.5

[a] final additive concentrations were 3 mg/mL for BSA, BPP, WPC 95, and 0.3 mg/mL for myosin.

[b] peptides produced are reported as Mean±S.E.M. (n=2, assayed in duplicate).

FUTURE STUDIES

1. Measurement of the reaction rate in the presence of WPC over time. The reaction rate can be determined by the loss of myosin on SDS-PAGE or the released amount of the unique amino acids of myosin. If WPC is an inhibitor, the reaction rate should be consistent over reaction time. If WPC is an alternative substrate, the reaction rate should be changed over reaction time. After proteolytic enzyme degrades all WPC, it will start to degrade the other substrate and cause the change in the reaction rate.

2. Measurement of some kinetic parameters. Those kinetic parameters can be determined by using the purified protease in the presence and absence of WPC in order to identify whether WPC is an inhibitor or an alternative substrate.

3. Identification of the active component in WPC that can inhibit the protease. If WPC is an inhibitor, each fraction of WPC will be tested to determine the ability of protease inhibition.

Chapter 4 : BIBLIOGRAPHY

Akahane, T. 1982. Freeze denaturation of fish muscle protein. Sc. D. thesis, Sophia Univ., Tokyo, Japan.

Akazawa, H., Miyauchi, Y., Sakurada, K., Wasson, D. H. and Reppond, K. D. 1993. Potential inhibitors for Pacific whiting surimi. In press.

Beas, V. E., Wagner, J. R., Anon, M. C. and Crupkin, M. 1991. Thermal denaturation in fish muscle proteins during gelling: Effect of spawning condition. *J. Food Sci.* 56: 281.

Binkley, F., Leibach, F. and King, N. 1968. A new method of peptidase assay and the separation of three leucylglycinases of renal tissue. *Arch. Biochem. Biopsy.* 128: 397.

Boye, S. W. and Lanier, T. C. 1988. Effects of heat-stable alkaline protease activity of Atlantic menhaden (*Brevoorti tyrannus*) on surimi gels. *J. Food Sci.* 53(5): 1340.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* 72: 248.

Busconi, L., Folco, E. J., Martone, C., Trucco, R. E. and Sanchez, J. J. 1984. Identification of two alkaline proteases and trypsin inhibitor from muscle of White croaker (*Micropogon opercularis*). *FEBS Let.* 176(1): 211.

Chan, J. K., Gill, T. A., and Paulson, A. T. 1992. Cross-linking of myosin heavy chains from cod herring and silver hake during thermal setting. *J. Food Sci.* 57: 906.

Chang-Lee, M. V., Lampia, L. E. and Crawford, D. L. 1990. Yield and composition of surimi from Pacific whiting (*Merluccius productus*) and the effect of various protein additives on gel strength. *J. Food Sci.* 55(1): 83.

Chang-Lee, Pacheco-Aguilar, R., Crawford, D. L. and Lampia, L. E. 1989. Proteolytic activity of surimi from Pacific whiting (*Merluccius productus*) and heat set gel texture. *J. Food Sci.* 54(5): 1116.

Cheng, C. S., Hamann, D. D. and Webb, N. B. 1979. Effect of thermal processing on minced fish gel texture. *J. Food Sci.* 44: 1080.

Chobert, J. H., Sitohy, M. Z. and Whitaker, J. R. 1988. Solubility and emulsifying properties of caseins modified enzymatically by *Staphylococcus aureus* vs. protease. *J. Agric. Food Chem.* 36: 220.

Choe, I., Morita, J., Yamamoto, K., Samejima, K. and Yasui, T. 1991. Heat-induced gelation of myosin/subfragments from chicken leg and breast muscles at high ionic strength and low pH. *J. Food Sci.* 56: 884.

Chung, K. H. and Lee, C. M. 1991. Water-binding and ingredient dispersion pattern effects on surimi gel texture. *J. Food Sci.* 56: 1263.

Chung, K. H. and Lee, C. M. 1990. Relationships between physicochemical properties of nonfish protein and textural properties of protein-incorporated surimi gel. *J. Food Sci.* 55: 972.

Chung, Y. C. and Morrissey, M. T. 1993. Effect of whey protein concentrates on gel strength of Pacific whiting surimi. IFT Annual Meeting Technical Program Abstract. July 10-14, 1993.

Crowell, E. A., Ough, C. S. and Bakalinsky, A. 1985. Determination of alpha amino nitrogen in musts and wines by TNBS method. *Am. J. Enol. Vitic.* 36(2): 175.

Davis, E. M. 1988. Protein assays: A review of common techniques. *American Biotechnology Laboratory.* July: 28.

Deman, J. M. 1980. In "Principle of Food Chemistry." The AVI Publishing Company INC. Westport, Connecticut.

Dixon, M. and Webb, E. C. 1964. In "Enzymes." Academic Press Inc., Publishers. New York.

Erickson, M. C., Gordon, D. T. and Anglemier, A. F. 1983. Proteolytic activity in the sarcoplasmic fluids of parasitized Pacific whiting (*Merluccius productus*) and unparasitized True Cod (*Gadus macrocephalus*). *J. Food Sci.* 48: 1315.

Fields, R. 1972. The rapid determination of amino groups with TNBS. *Method Enzymol.* 25: 464.

Foegeding, E. A. and Lanier, T. C. 1987. The contribution of non muscle proteins to texture of gelled muscle protein foods. *Cereal Foods World.* 32(2): 202.

Folco, E. J., Busconi, L., Martone, C., Trucco, R. E. and Sanchez, J. J. 1984. Action of two alkaline proteases and a trypsin inhibitor from White croaker skeletal muscle (*Micropogon percularis*) in the degradation of myofibrillar proteins. *FEBS Lett.* 176(1): 215.

Folco, E. J., Busconi, L., Martone, C. B, and Sanchez, J. J. 1988. Multicatalytic proteinase in fish muscle. *Arch. Biochem. Biophy.* 267(2): 559.

Folco, E. J., Busconi, L., Martone, C. B, and Sanchez, J. J. 1989. Fish skeletal muscle contains a novel serine proteinase with an unusual subunit composition. *Biochem. J.* 263: 471.

Fox, P. F. 1989. In "Developments in Dairy Chemistry-4. Functional Milk Proteins." Elsevier Applied Science. London and New York.

Hamann, D. D., Amato, P. M., Wu, M. C. and Foegeding, E. A. 1990. Inhibition of modori (gel weakening) in surimi by plasma hydrolysate and egg white. *J. Food Sci.* 55(3): 665.

Harris, E. V. and Angal, S. 1989. In "Protein Purification Methods." A Practical Approach. IRL Press Limited. Oxford.

Hoefer, P. 1986. Separation of proteins on the basis of molecular weight SDS-gel electrophoresis. Hoefer Scientific Instruments. p.102-107.

Hollingworth, T. A., Wekell, M. M., Sullivan, J. J., Torkelson, J. D. and Throm, H.R. 1990. Chemical indicators of decomposition for raw surimi and flaked artificial crab. *J. Food Sci.* 55: 349.

Hultin, H. O. 1985. In "Characteristics of Muscle Tissue, Food Chemistry." Marcel Dekker, INC. New York and Basel.

Ishioroshi, M., Samejima, K. and Yasui, T.1979. Heat-induced gelation of myosin: Factors of pH and salt concentrations. *J. Food Sci.* 44: 1280.

Iwata, K., Kobashi, K. and Hase, J. 1973. Studies on muscle alkaline protease-I: Isolation, purification and some physicochemical properties of an alkaline protease from carp muscle. *Bull. Jap. Soc. Sci. Fish.* 39(12): 1325.

Iwata, K., Kobashi, K. and Hase, J. 1978. Studied of muscle alkaline protease-VII: Effect of the muscular studies of muscle alkaline protease and protein fractions purified from White croaker and Horse mackerel on the "Himodori Phenomenon" during kamaboko production. *Bull. Jap. Soc. Sci. Fish.* 45(2): 157.

Jensen, S. E., Fecycz, I. T., Stemke, G. W. and Campbell, I. N. 1980. Demonstration of a cell-associated, inactive precursor for an exocellular protease produced by *Pseudomonas aeruginosa. Can. J. Microbiol.* 26: 87.

Konno, K. 1992. Suppression of thermal denaturation of myosin subfragment-1 of Alaska polluck (*Theragra chalcogramma*) by sorbitol and accelerated inactivation by pyrophosphate. *J. Food Sci.* 57: 261.

Lanier, T. C., Lin, T. S., Hamann, D. D. and Thomas, F. B. 1981. Effects of alkaline protease in minced fish on texture of heat-processed gels. *J. Food Sci.* 46: 1643.

Lanier, T. C. and Macdonald, G. A. 1988. Surimi technology workshop notes. The DSIR Fish Technology Section. Nelson, New Zealand.

Lee, C. M. 1984. Surimi process technology. *Food Technol.* 38 (11): 69.

Lehniger, A. L. 1975. In "Biochemistry." Worth Publishers, New York.

Lin, Y., Means, G. E. and Feency, R. E. 1969. The action of proteolytic enzymes on N,N-dimethyl proteins. *J. Biol. Chem.* 244: 789.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193: 265.

Makinodan, Y., Akasaka, T., Toyohara, H. and Ikeda, S. 1982. Purification and properties of carp muscle cathepsin D. *J. Food Sci.* 47: 647.

Makinodan, Y., Hirotsuke, M. and Ikeda, S. 1979. Neutral proteinase of carp muscle. *J. Food Sci.* 44: 1110.

Makinodan, Y. and Ikeda, S. 1977. Alkaline proteinase of carp muscle: Effects of some protein denaturing agents on the activity. *J. Food Sci.* 42(4): 1026.

Makinodan, Y., Toyohara, H. and Niwa, E. 1985. Implication of muscle alkaline proteinase in the textural degradation of fish meat gel. *J. Food Sci.* 50: 1351.

Martone, C. B., Busconi, L., Folco, E. E. and Sanchy, J. J. 1991. Detection of a trypsin-like serine protease and its endogenous inhibitor in Hake skeletal muscle. *Archives of Biochem and Biophysics*. 289(1): 1.

Meyer, L. H. 1960. In "Food Chemistry." Reinhold Publishing Corporation. New York.

Miles laboratories. Technical information for spectrophotometric acid protease.

Morita, J. and Ogata, T. 1991. Role of light chains in heat-induced gelation of skeletal muscle myosin. *J. Food Sci.* 56: 855.

Morita, J. and Yasui, T. 1991. Involvement of hydrophobic residues in heat-induced gelation of myosin tail subfragments from rabbit skeletal muscle. *Agric. Biol. Chem.* 55(2): 597.

Morr, C. V. 1982. In "Functional Properties of Milk Proteins and Their Use as Food Ingredients in Developments in Dairy Chemistry - 1." Applied Science Publishers. London and New York.

Morr, C. V. and Foegeding, E. A. 1990. Composition and functionality of commercial whey and milk protein concentrates and isolates: A status report. *Food Tech.* 44(4): 100.

Morrissey, M. T., Wu, J. W., Lin, D. D. and An, H. 1992. Effect of food grade protease inhibitors on autolysis and gel strength of surimi. Submitted to *J. Food Sci.*

National Fisheries Institute. 1991. In "A Manual of Standard Methods for Measuring and Specifying the Properties of Surimi." University of North Carolina Sea Grant College Program, Raleigh, N. C.

Porter, R. W. 1992. Use of potato inhibitor in Pacific whiting surimi. In Pacific Whiting : Harvesting, Processing, Marketing and Quality Assurance, G. Sylvia and M. T. Morrissey (Ed.), 33-35. Oregon Sea Grant Publication, Corvallis, Oregon.

Sano, T., Noguchi, S. F., Matsumoto, J. J. and Tsuchiya, T. 1990. Thermal gelation characteristics of myosin subfragments. *J. Food Sci.* 55: 55.

Satake, K., Okuyama, T., Ohashi, M. and Shinoda, T. 1960. The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-trinitrobenzene 1- sulfonic acid. *J. Biochem.* 47(5): 654.

Sedmark, J. J. and Grossberg, S. E. 1977. A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* 79: 544.

Smith, P. K., Krohn, R. T., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150: 76.

Su, H., Lin, T. S. and Lanier, T. C. 1981. Contribution of retained organ tissues to the alkaline protease content of mechanically separated Atlantic croaker (*Micropogon undulatus*). *J. Food Sci.* 46: 1650.

Su, H., Lin, T. S. and Lanier, T. C. 1981. Investigation into potential sources of heat-stable alkaline protease in mechanically separated Atlantic croaker (*Micropogon undulatus*). *J. Food Sci.* 46: 1654.

Toyahara, H., Kinoshita, M. and Shimizu, Y. 1990. Proteolytic degradation of threadfin-bream meat gel. *J. Food Sci.* 55(1): 259.

Toyohara, H., Makinodan, Y., Tanaka, K. and Ikeda, S. 1983. Detection of calpastatin and trypsin inhibitor in carp muscle. *Agric. Biol. Chem.* 47(5): 1151.

Toyohara, H. and Shimizu, Y. 1988. Relation between modori phenomenon and myosin heavy chain breakdown in threadfin-bream gel. *Agric. Biol. Chem.* 52(1): 255.

Twining, S. S. 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal. Biochem.* 143: 30.

Wasson, D. H., Babbitt, J. K. and French, J. S. 1992a. Characterization of a heat stable protease from Arrowtooth flounder, *Atheresthes stomias*. *J. Aquatic Food Product Technol.* 1: 167.

Wasson, D. H., Reppond, K. D., Babbitt, J. K. and French, J. S. 1992b. Effects of additives on proteolytic and functional properties of Arrowtooth flounder surimi. *J. Aquatic Food Product Technol.* 1: 147.

Whitaker, J. R. 1972. In "Principle of Enzymology for the Food Science." Marcel Dekker, INC. New York.

Whitaker, J. R. and Tannenbaum, S. R. 1977. In "Food Proteins." AVI Publishing Company, INC.

Whitaker, J. R., Granum, P. E. and Asen, G. 1980. Reaction of ammonia with trinitrobenzene sulfonic acid. *Anal. Biochem.* 108: 72.

Yasui, T., Ishioroshi, M., Nakano, H. and Samejima, K. 1979. Changes in shear modulus, ultrastructure and spin-spin relaxation times of water associated with heat-induced gelation of myosin. *J. Food Sci.* 44: 1201.

Yoon, K. S. and Lee, C. M. 1990. Cryoprotectant effects in surimi and surimi/minced-based extruded products. *J. Food Sci.* 55:1 210.

Zalke, J. 1992. Global markets for surimi-based products. In Pacific Whiting : Harvesting, Processing, Marketing and Quality Assurance, G. Sylvia and M. T. Morrisey (Ed.), 33-35. Oregon Sea Grant Publication, Corvallis, Oregon.

APPENDICES

APPENDICES

MODELING THE PROTECTION OF MYOSIN BY WPC

Proteases found in fish have the ability to degrade myosin. In the simplest case, myosin degradation may be assumed to follow the Michaelis-Menten kinetics, these being described by equation (1) (Dixon and Webb, 1964).

$$v = \frac{V_{max}[Myosin]}{K_M + [Myosin]} \qquad \text{------ (1)}$$

v = velocity of the reaction

V_{max} = maximum velocity

K_M = Michaelis-Menten constant

The data of this thesis shows that the incorporation of WPC into Pacific whiting surimi serves to protect the myosin component of that surimi from proteolytic degradation. We have suggested that this protection may be due to WPC protein(s) acting either as an inhibitor or as an alternative substrate. The rate of myosin degradation in the presence of WPC, assuming either of these mechanisms, may be described by equation (2).

$$v = \frac{V_{max}[Myosin]}{K_M(1 + \frac{[WPC]}{K_{WPC}}) + [Myosin]} \qquad \text{------ (2)}$$

In the presence of WPC, the rate of myosin degradation decreases since the apparent K_M for myosin increases. From equation (2), if WPC acts as a real inhibitor, the K_{WPC} is equivalent to K_I for WPC. If WPC acts as an alternative substrate, the K_{WPC} is equivalent to K_M for WPC. The important point is that myosin protection would occur in either case. The difference is that as a real inhibitor, the concentration of WPC would remain constant, while in the case of an alternative substrate, the WPC concentration would decrease over time.

In the autoproteolysis assay as used in this study, the protease activity is determined by measuring the quantity of TCA-soluble peptides resulting from the proteolytic degradation of surimi or surimi/additive samples. The velocity of the reaction is considered in terms of total product, i.e. total peptides and amino acids solubilized over a given period of time. When protein additives are present, the possibility arises that the surimi-associated protease may act on either of the two protein sources, surimi or additives. Therefore there will be competition between the two substrates. The apparent protease activity may be considered as the sum of the activities for both substrates, as described by equations (3) and (4) (Dixon and Webb, 1964).

$$v_t = V_A + V_B \qquad \text{------ (3)}$$

$$v_t = \frac{V_A\alpha + V_B\beta}{1+\alpha+\beta} \qquad \text{------ (4)}$$

While $\alpha = [A]/K_A$; A = Myosin

$\beta = [B]/K_B$; B = Protein additives

If the K_m for the protein additive (K_B) is very large compared to the K_m for myosin (K_A), i.e. $K_B >> K_A$, and [A] = [B], then the protein additive will have little influence on the apparent protease activity. The apparent proteolytic activity will depend solely on the reaction rate of myosin, as shown in equation (5).

$$v_t \approx \frac{V_A\alpha}{1+\alpha} \qquad \text{------- (5)}$$

As described in this study, the addition of BSA to surimi does not appear to protect the myosin component, and the apparent proteolytic activity of surimi was not significantly changed by the addition of BSA. One explanation for this is that BSA acts as an alternative substrate with a relatively high K_m.

In theory, the addition of a protein additive having a relatively high affinity for the enzyme ($K_B << K_A$), and a relatively low V_{max} ($V_B \approx 0$), would result in a sharp decrease in the apparent proteolytic activity, as shown in equation (6).

$$v_t \approx \frac{V_B\beta}{1+\beta} \approx 0 \qquad \text{-------- (6)}$$

Hydrolyzed beef plasma is known to contain at least two protein inhibitor; α_2-macroglobulin (Hamann et al., 1990) and cystatin (Akazawa et al., 1993), both of which are known to inhibit a wide range of proteases. Thus, it may be that the kinetic expression for a BPP/surimi mixture will reduce to equation (6).

Considering WPC, two hypothesis are proposed. If WPC has a K_m equal to the K_m for myosin ($K_{WPC} = K_{Myosin}$) and if $V_{WPC} << V_{Myosin}$, then when [WPC] ≈ [Myosin], the observed proteolytic activity will decrease relatively to that of a non-supplemented surimi, as shown in equation (7). If V_{WPC} is very small, such that the turnover of this substrate is negligible in the time frame of this assay, then WPC is essentially a competitive inhibitor.

$$v_t = \frac{V_A[A]+V_B[B]}{K_m+[A]+[B]} \approx \frac{V_A[A]}{K_m+[A]+[B]} \qquad \text{-------- (7)}$$

In another case, if $K_{WPC} << K_{Myosin}$ and $V_{WPC} << V_{Myosin}$, then when [WPC] ≈ [Myosin], the observed activity will essentially equal the proteolytic activity associated with WPC degradation, as shown in equation (8).

$$v_t \approx \frac{V_B\beta}{1+\beta} \qquad \text{-------- (8)}$$

Using either of these two scenarios for WPC, the observed result would be that WPC acts as an inhibitor.

BCA PROTEIN ASSAY

A. Reagents:

1. Reagent A: 1000 mL contain - Sodium carbonate
   - Sodium bicarbonate
   - BCA (bicinchoninic acid)

   in 0.1 N NaOH
2. Reagent B: 25 mL 4% Copper sulfate solution

These reagents are stable at least 12 months at room temperature.

B. Working reagent:

mix - 50 parts of Reagent A
- 1 parts of Reagent B

(stable 1 week at room temperature)

C. Assay Procedure:

1. 0.1 mL sample or each standard (blank use 0.1 mL diluent)
2. 2.0 mL BCA working reagent
3. mix well
4. incubate at 37 C, 30 min
5. cool down to room temperature
6. measure Absorbance at 562 nm

D. Preparation of protein standard (BSA 2 mg/mL):

tube	vol. of BSA(mL)	vol. of H_2O(mL)	conc. (µg/mL)
1	0.0	0.1	0
2	0.01	0.09	200
3	0.02	0.08	400
4	0.03	0.07	600
5	0.04	0.06	800
6	0.05	0.05	1000

LOWRY PROTEIN ASSAY

A. Reagents:

1. Reagent A: 100 g Na_2CO_3 in 1 liter of 0.5 N NaOH
2. Reagent B: 1 g $CuSO_4 \cdot 5H_2O$ in 100 mL H_2O
3. Reagent C: 2 g potassium tartate in 100 mL H_2O
4. Reagent D: 15 mL Reagent A
   0.75 mL Reagent B
   0.75 mL Reagent C
5. Reagent E: 5.0 mL of 2 N Folin-phenol to 50 mL H_2O

B. Assay Procedure:

1. use 1.0 mL sample or each standard
2. add 1.0 mL of reagent D, vortex the tubes to mix.
3. incubate the tubes for 15 min at room temperature.

4. prepare reagent E while the tubes are incubated.

5. after the incubation period, add 3.0 mL of reagent E and vortex the tubes immediately.

6. incubate the tubes at room temperature for 45 min.

7. determine the absorbance at 750 nm

C. Preparation of protein standard (BSA 0.3 mg/mL):

tube	vol. of BSA (mL)	vol. of H_2O (mL)	conc. (µg/mL)
1	0.0	1.0	0
2	0.1	0.9	30
3	0.2	0.8	60
4	0.3	0.7	90
5	0.4	0.6	120
6	0.5	0.5	150
7	0.6	0.4	180
8	0.7	0.3	210
9	0.8	0.2	240
10	0.9	0.1	270
11	1.0	0.0	300

TNBS PROTEIN ASSAY

A. Reagents:

1. Reagent A: 100 mL of 0.1 M Na_2SO_3 (make fresh weekly)

dissolve Na_2SO_3 12.6 g in H_2O 100 mL

2. Reagent B: 1 liter of 0.1 M NaH_2PO_4

   dissolve NaH_2PO_4 13.8 g in H_2O 1 liter

3. Reagent C: 1 liter of $Na_2B_4O_7$ in 0.1 M NaOH

   dissolve NaOH 4 g in H_2O 1 liter

   dissolve $Na_2B_4O_7$ 38.14 g in H_2O 1 liter

4. Reagent D: 1.5 mL of solution A plus 98.5 mL of solution B

   (make fresh daily)

5. Reagent E: 1 mL of 1.1 M TNBS

   dissolve 0.3225 TNBS in H_2O 1 mL

   add 0.200 mL H_2O for each 100 mg TNBS.

B. Assay Procedure:

1. use 0.5 mL sample or each standard.
2. mix with 0.5 mL of borate buffer (solution C).
3. add 20 µl of 1.1 M TNBS (solution E).
4. mix rapidly using a vortex mixer.
5. after exactly 5 min, add 2.0 mL of solution D.
6. measure the absorbance at 420 nm.

(10 samples may be run together by adding the TNBS at 30 sec)

C. Preparation of protein standard (glycine 200 μM):

tube	vol. of glycine(mL)	vol. of H_2O(mL)	conc. (mg/L)
1	0.00	0.10	0.0
2	0.25	0.75	0.7
3	0.50	0.50	1.4
4	0.75	0.25	2.1
5	1.00	0.00	2.8

DYE-BINDING PROTEIN ASSAY

A. Assay Procedure:

1. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water. Filter through Whatman #1 filter to remove particulates. This reagent can be used for 2 weeks when kept at room temperature.
2. Pipet 100 μl of each standard and sample solution into a clean, dry test tube.
3. Add 5.0 mL of diluted dye reagent to each tube and vortex.
4. Incubate at room temperature for at least 5 minutes.
5. Measure absorbance at 595 nm.
6. Prepare three to five solution of a protein standard, using BSA 0.2 to 0.9 mg/mL.

SDS-PAGE METHOD

A. Reagent:

1. Monomer solution (30%T 2.7% C_{Bis})

2. 4X Running gel buffer (1.5 M tris-Cl pH 8.8)

3. 4X Stacking gel buffer (0.5 M tris-Cl pH 6.8)

4. 10% SDS

5. 10% Ammonium persulfate

6. 2X Treatment buffer containing 0.125 M tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol

7. Tank buffer (0.025 M tris pH 8.3, 0.192 M glycine, 0.1% SDS)

8. Stain solution (0.125% coomassie blue R-250, 50% methanol, 10% acetic acid)

9. Destain solution I (50% methanol, 10% acetic acid)

10. Destain solution II (7% acetic acid, 5% methanol)

B. Assay Procedure:

1. Preparation of the separating gel : Assemble the slab gel unit in the casting mode. Prepare the separating gel solution according to the table shown below. Leave out the ammonium persulfate and the TEMED. Degas the solution under vacuum. Add ammonium persulfate and TEMED. Pipet the solution into the sandwich about 11 cm height, and put the water layer on the top. Allow the gel to sit for several hours.

2. Preparation of the stacking gel : Prepare the stacking gel solution according to the table shown below. Leave out the ammonium persulfate and the TEMED. Degas the solution under vacuum. Add ammonium persulfate and TEMED. Rinse the surface of the separating gel with the stacking gel solution, then fill the sandwich with stacking gel solution. Insert the comb into each sandwich. Allow the gel to sit gel at least a half hour.

	Separating Gel 10%T 207%C	Stacking Gel 4%T 2.7%C
30%T 2.7%C (1)	20 mL	2.66 mL
Buffer (2)	15 mL	-
Buffer (3)	-	5.0 mL
10% SDS (4)	0.6 mL	0.2 mL
H_2O	24.1 mL	12.2 mL
Ammonium persulfate (5)	300 µl	100 µl
TEMED	20 µl	10 µl

3. Loading and running the gel : Remove the comb, and fill each well with tank buffer. Put the sandwich into the chamber, filled with tank buffer. Use a syringe to load the sample into each well. Turn the power supply by using 30 mA for stacking gel and 60 ma for separating gel.

4. Staining and destaining the gels : Disassemble the sandwich and put the gel into the stain solution with gently shaking for 4-8 hours. Remove the gels and put them into destain solution I, and shake for 1 hour. Transfer the gels into destain solution II, and shake until the background is clear.