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Title: The Effect of Potassium Bromate on the Gel-Forming Ability of Pacific Whiting (Merluccius productus) Surimi

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The abundance and low fat content of Pacific whiting support its use for the production of surimi. The degradation of muscle proteins by myxosporidian secreted proteinase(s) has been associated with its soft texture. High residual activity is retained through the washing process used in the production of surimi and precludes the formation of a strong heat-set gel by surimi sols.

Physical, chemical and SDS-PAGE analysis defined the reinforced oxidation of free sulfhydryl groups on myofibrillar proteins to disulfide bonds by potassium bromate. SDS-PAGE demonstrated myosin degradation during heat-setting and the protection of myosin from proteinase attack by bromate. A level of 0.075% bromate inactivated 89.87% of the total proteinase activity in sols. It was assumed that cysteine proteinases were inactivated and

residual activity was associated with proteinases with a serine active site.

Major improvement in gel cohesiveness and elasticity was observed at bromate levels $\leq$0.075% with only a slight improvement at higher levels. Maximum hardness was observed at 0.150%, with no ($P>0.050$) increase at higher levels. Brittleness was improved ($P>0.050$) by bromate levels $\geq$0.100%; no maximum degree of brittleness was observed within the range ($\leq$0.250%) of concentrations investigated. An optimum folding test grade of AA was achieved by a minimum of 0.150%

Potassium bromate improved gelling characteristics of sols of Pacific whiting surimi through proteinase inactivation and reinforced disulfide formation during heat-setting. Improvement in cohesiveness and elasticity was primarily a function of proteinase inactivation. Maximum hardness and brittleness required additional oxidative capacity which was not fully required for an optimum folding test grade.

The Effect of Potassium Bromate on the Gel-Forming Ability of Pacific Whiting (*Merluccius productus*) Surimi

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INTRODUCTION

Despite the abundance of Pacific whiting off the West coast of the United States it remains an underutilized species to U.S. processors. The soft texture of whiting flesh, believed to be induced by myxosporidian proteinases, inhibits market acceptance. Its abundance suggests that it may have a potential as a protein basis for analog seafood products engineered from a surimi intermediate.

High proteolytic activity occurs in whiting muscle and minced flesh. A considerable portion of this activity is retained in the flesh after being processed into surimi. Partial hydrolysis of surimi proteins during heat processing precludes the formation of a gel matrix. The pH and thermal processing conditions at which surimi-based products are manufactured suggest that the hydrolytic enzyme(s) may be either neutral or slightly alkaline proteinase containing a cysteine residue at its active site. Such an active site would be susceptible to oxidation and inhibition by an oxidizing agent such as potassium bromate.

Enhancing the potential for the use of whiting for producing surimi-based products through a chemical and/or biochemical modification of the surimi system was the goal of this research. The objective was to elaborate on the

mechanism by which potassium bromate improves the gel-forming ability of whiting surimi.

It was assumed that the use of potassium bromate would accomplish an improvement in the gel-forming ability of the whiting surimi by any one or a combination of the following mechanisms: a) direct inhibition of the enzyme by the oxidation of the cysteine residue at the active site; b) oxidation of the cysteine residues on myosin and/or myosin hydrolysis products to form aggregates through disulfide formation (aggregates of salt solubilized proteins would be able to participate in the formation of a gel matrix during heat setting); and c) indirect inhibition of the enzyme by steric effects (formed protein aggregates would not form enzyme-substrate complexes).

LITERATURE REVIEW

The Pacific Whiting Fishery

Pacific whiting, also referred as North Pacific whiting (Federal Register, 1979) and Pacific hake (Robins et al., 1980), is listed as a member of the cod family "Gadidae" by the American Fisheries Society (Bailey et al., 1970). Whiting is a pelagic species reported from the Bering Sea to Magdalena Bay, Baja California Sur, Mexico (Frey, 1971) at depths ranging from 0 to 914 m, but generally occurs at depths >229 m (Eschmeyer and Herald, 1983). It ranges in size from 30 t0 46 cm long, with a record length of about 90 cm, and has a color ranging from gray to dusty brown with brassy overtone (Hart, 1973).

Pacific whiting first appears off the northern Oregon and Washington coast in April. It is most abundant in June through September when large feeding aggregations occur in depths of 55-550 m (Dark, 1985; Dark et al., 1980). By December, most adults have migrated south from the coast of Oregon and Washington (Fiscus, 1979). The fishery 25 years ago was characterized by domestic trawlers delivering relatively small quantities of whiting and other low-valued species to shore plants producing meal and animal food. It has evolved to become dominated by joint-venture and foreign mothership fleets processing large catches at sea for meal and human consumption. Summer surveys by U.S. and U.S.S.R.

researchers have estimated a biomass ranging from 445,000 to 3,440,000 t. Commercial catches have ranged from about 68,000 to 237,000 t annually since 1967 (Dark, 1985).

Pacific whiting constitutes the most abundant groundfish resource off the coast of California, Oregon, Washington, and British Columbia (Dark, 1985; Nelson and Larkins, 1970). A reduction in annual total catches since 1977 (Francis and Hollowed, 1985), has predicated a demand for resource information, population dynamics studies, processing and preservation research. A management modeling expanded rapidly between 1977 and 1982 as the United States and Canada extended their fisheries jurisdiction. The Pacific Fishery Management Council and Canada Department of Fisheries and Oceans have developed comprehensive management plans for the Pacific whiting and other groundfishes (Dark, 1985).

Utilization of Pacific Whiting

The Fishery Conservation and Management Act of 1976 has intensified the interest of the fishing industry in Pacific whiting as an additional food resource (Miller and Spinelli, 1982). It is still classified as an underutilized species since its current exploitation by U.S. fishermen is very limited considering its large harvest potential (Alverson and Larkins, 1969). Soft texture and poor keeping quality are the factors limiting commercial utilization of Pacific

whiting. To maintain a good quality product, it must be iced immediately after capture and processed within four days. Failure to follow these guidelines result in a mushy product which, if marketed, is not acceptable to consumers (Dassow et al., 1970). Even if whiting have been properly handled and chilled, thawing frozen whiting at room temperature can also lead to an unacceptable texture (Erickson et al., 1983).

Considerable quantities of some species of the genera Merluccius are being marketed in the form of fresh and frozen fillets, salted and dried, breaded, portions, smoked (Anonymous, 1989), and fillet blocks. They represent a valuable human food resource in countries such as the U.S.S.R., Chile, Spain, Argentina and South Africa (Crawford and Law, 1972; Crawford et al., 1979).

Despite its soft texture Pacific whiting is amenable to mechanical handling and a considerable interest has risen in the possible utilization of whiting as a raw material for the production of surimi (Groninger et al., 1985). Pacheco-Aguilar et al. (1988) and Chang-Lee (1988) reported Pacific whiting as a species having some of the characteristics required for the production of surimi and analog seafood products, now largely produced from Alaska pollock.

Texture Characteristics of Pacific Whiting Muscle

During the past fifteen years, the abnormal texture of whiting flesh, processing techniques and/or product forms that would make it possible to market whiting products having a relatively normal texture have been investigated. The solution of this problem could open the way for the increased exploitation of Pacific whiting and provide the industry with a larger resource (Kabata and Whitaker, 1986). The association of this texture problem with proteolytic activity or with the presence of parasites have not been clearly understood despite many years of investigations (Tsuyuki et al., 1982; Patashnik et al., 1982).

The mushy condition of Pacific whiting often has been observed to be in association with a microscopic myxosporidian parasite Kudoa sp. (Dassow et al., 1970). Patashnik et al. (1982) reported that the abnormal texture in Pacific whiting is caused by a myxosporidian-induced proteolysis. The latent potential for proteolytic textural softening in whiting, due to the presence of myxosporidian cysts of variable intensity appears to be an intrinsic characteristic of whiting (Patashnik et al., 1982). The muscle parasites that affect Pacific whiting are of little public health concern, but since they degrade flesh texture significantly which limits the utilization of the resource, it is a matter of technological concern (Patashnik et al.,

1982; Oppenheimer, 1962; Sinderman, 1970, 1985; Kurochkin, 1985; Krasin, 1985).

According to Kabata and Whitaker (1981, 1986) and Tsuyuki et al. (1982), the two species of Kudoa infecting Pacific whiting off North America shores are Kudoa thyrsitis and Kudoa paniformis. K. paniformis has not been previously known and is considered to be a new species. The signs of infection produced by both parasites are very similar, so that the identity of the pathogen can be determined only by examination of the spore under the compound microscope. Tsuyuki et al. (1982) reported that the final condition of the flesh might be determined by the type of Kudoa present, since K. paniformis is capable of producing more extensive infections than K. thyrsitis. These researchers observed that of 322 fish examined only 34 (10.5%) were free of Kudoa, 104 (32.3%) were infected with K. thyrsitis and 125 (38.8%) with K. paniformis. Mixed infections were found in 59 fish (18.4%).

The parasite penetrates the core of individual muscle fibers and spreads along them without damaging sarcolemma, until each fiber is filled with spores. These fibers, known as pseudocysts, isolate the parasite so that the connective tissue of the host is not provoked into reaction. The fibers are slightly distended and are externally recognizable by their pale yellowish color. It appears that the only marked defensive reaction of the fish is the deposition of melanin granules around the area invaded by

the parasite with a corresponding change in color of the fiber. Turning initially light brown, it becomes dark brown and eventually black. This melanin deposition progressively compresses and crumples spores until they are destroyed (Tsuyuki et al., 1982; Kabata and Whitaker, 1986, 1981). The pseudocysts may be closely packed or widely separated depending on the severity of the infection. They may be either uniformly or unevenly distributed through the interior flesh, on the flesh surface below the skin, or clustered in localized areas only (Patashnik et al., 1982; Patashnik and Groninger, 1964).

Willis (1949) and Patashnik et al. (1982) reported that the parasite releases a powerful extracellular enzyme that is removed in the blood stream while the host fish is alive. After the fish dies and the circulation ceases, the enzyme progressively diffuses outward from the infected focus to the noninfected flesh. Muscle portions seriously infected with the parasites have extensive tissue proteolysis accompanied by liquefaction and mushiness (Erickson et. al., 1983). The proteolytic enzyme produced by the parasites is capable of breaking the chemical bonds of the muscle fibers which are responsible for the characteristic texture of fresh fish (Miller and Spinelli, 1982; Spinelli and Steinberg, 1978; Kabata and Whitaker, 1981, 1986). The proteolysis is capable of occurring at nearly any temperature above freezing. In the raw product, this mushines could be seen after several hours of incubation,

but with improperly cooked whiting, the liquefied product could be detected immediately. Frozen products thawed at room temperature would also exhibit tissue breakdown due to this proteolysis (Erickson et al., 1983).

Pacific whiting should be promptly chilled upon catching, maintained chilled during processing, and rapidly cooked (Patashnik et al., 1982). During such conventional cooking processes as baking, broiling, and pan-frying, temperatures are reached and maintained for a time prior to achieving a temperature required for proteinase inactivation. Several methods have been recommended to prevent textural changes such as fast cooking (Patashnik et al., 1982; Spinelli and Steinberg, 1978; Dassow et al., 1970), deep-fat-fried (Crawford and Law, 1972; Crawford et al. 1972), portioned-type product, limited to about 3/8 inch in thickness, microwaved (Patashnik et al., 1982), sonication and the use of chemicals (Spinelli and Steinberg, 1978; Nelson et al., 1985), all of which have the potential for inactivating the enzyme before it damages the muscle.

Tsuyuki et al. (1982) reported that the two optimum temperatures ranges for the proteolytic activity produced by Kudoa are at 35 to 49°C (pH 3.8), and 55 to 60°C (pH 6.7). Patashnik et al. (1982) showed that rapid heating of the flesh to 70°C (158°F) for 10 min completely inactivated proteinases and preserved its textural properties. Tsuyuki et al. (1982) observed strong proteolytic activities in the acidic and neutral or slightly alkaline pH ranges.

Proteolytic activity in the acid range was found to increase greatly in muscle tissue of whiting infected with K. thyrsitis. This activity is heat labile and does not appear to degrade those structural proteins responsible for maintaining firm texture after cooking. Uninfected Pacific whiting and those infected with K. thyrsitis did not result in unacceptable soft cooked flesh texture. Neutral to slightly alkaline proteinase activity is more favorably viewed as being involved in the autolytic proteolysis of fish musculature. Muscle tissues exhibiting this strong proteolytic activity developed unacceptable soft or mushy texture during slow a cooking process. This strong proteinase activity was associated with muscle tissues infected only with white pseudocyst or the young forms of K. paniformis, either alone or as a mixed infection with K. thyrsitis. This activity was found to be relatively heat stable with an optimum at 55-60°C, a transitional temperature in the cooking process.

The development of black hair-like pseudocysts is a characteristic of older infections by either of the Kudoa species. It is considered a defense mechanism of the muscle tissues, since it eventually lead to the destruction of the parasite and the associated proteinase activity. The white pseudocyst is the younger and more proteolytically active form. This explains why some fillets with dark pseudocysts may have low proteolytic activity and a normal texture (Tsuyuki et al., 1982; Patashnik et al., 1982). Kudo et al.

(1987), reported that white pseudocysts were more closely correlated with poor cooked texture quality than were black pseudocysts. K. paniformis and mixed infections correlated well with poor sensory texture, while K. thyrsitis infections correlated poorly. Pacific whiting (Tsuyuki et al., 1982) infected with K. paniformis showed a strong positive correlation with both neutral to slightly alkaline and acid proteinase activities and a strong negative correlation with firmness. Black pseudocyst counts cannot be used as a reliable prediction of sensory texture (Kudo et al., 1987), only white pseudocysts contain the parasites that produce the proteolytic enzyme that adversely affects texture (Tsuyuki et al., 1982).

Proteolysis in Muscle Tissue

The relationship of proteinase activity and autolysis of the musculature is complex. The literature reports that the muscle tissue of many species of fish have proteinase activity in the acid to slightly alkaline range. However, detailed examination for the presence of parasites has not been an integral part of those investigations. Whether the proteinase activities associated with the parasites at these pH ranges are the same as those reported in uninfected fish is not known (Tsuyuki et al, 1982).

A vast number of proteinases have been discovered in mammalian (Asghar and Bhatti, 1987) and fish tissues (Reddi

et al., 1972; Erickson et al., 1983; Makinodan et al., 1985). Barrett and McDonald (1980) suggested the presence of at least 144 proteolytic enzymes in mammalian tissues. Although certain organs such as liver, spleen and kidney of different species are the richest sources of most of the proteolytic enzymes, skeletal muscle also contains a wide spectrum of proteinases, even though their activity is much lower than that in other organs. Some proteinases are distributed in the cytosolic fraction, while others are confined in the lysosome granules of muscle fibers. The precise origin and distribution of some proteolytic enzymes in the cellular structure of muscle is not known. Uncertainty exists as to whether they originate from the muscle per se, or they reside in other cells associated with skeletal muscle, such as fibroblast, adipocytes, mast cells, macrophages, etc. (Asghar and Bhatti, 1987). The fact that most of these cells, especially mast cells, are very rich sources of alkaline proteolytic enzymes (Ende et al., 1964; Budd et al., 1967) poses a problem in determining the subcellular localization of proteinases in skeletal muscles. In vitro investigation of the proteolytic capacity of different lysosomal and nonlysosomal proteinases revealed that almost all myofibrillar, cytoskeletal, and collagenic proteins are vulnerable to attack by these proteinase systems (Asghar and Bhatti, 1987).

Lysosomal Catheptic Proteinases

The so-called cathepsins found in the lysosomal fraction of the cytoplasm are also called acid proteinases since they generally require low pH for their activity. However, some of the cathepsins show substantial activity even at neutral pH (Iodice et al., 1972). After death, it is generally assumed that catheptic proteinases are released from lysosomes, which then diffuse in the intermyofilament space to begin protein degradation. Cathepsins B (i.e., B1), D, E, H, and L are lysosomal cathepsins whose presence has been detected in skeletal muscle (Asghar and Bhatti, 1987). Fish muscle cathepsins have not been investigated in great detail (Reddi et al., 1972) but Siebert (1958) demostrated that the cathepsin activity of fish was ten times greater than that of mammalian tissue. Siebert and Schmitt (1965) showed that fundamental differences exist between mammalian and fish cathepsins. Since fish tissues are very active in proteolysis it has to be assumed that cathepsins from fish tissues possess a different specificity from those in mammalian tissues. Moreover, their specificity should be more pronounced since fish cathepsins have shown inability to split the classical synthetic substrates (Siebert and Schmitt, 1965).

The role of cathepsins in spoilage of fish muscle has been stressed (Siebert, 1962). Reddi et al. (1972), studied the catheptic activity in winter flounder

(Pseudopleuronectes americanus) and reported the results in terms of cathepsin D activity. Although the optimum pH for hydrolysis was shown to be pH 4.0, the range of activity was between pH 3.0 to pH 7.0. This suggests that autolysis of the muscle proteins can proceed at and below pH 6.5 which is the average pH of fish muscle after capture (Amlacher, 1961). After death the pH drops further. Makinodan et al. (1985) reported considerable cathepsin D activity in white croaker muscle at pH 3.2 upon incubation at about 60°C, but at pH of the meat paste (6.8) activity was hardly observed. Tsuyuki et al. (1982) also reported that cathepsin D from carp muscle was inactive at neutral pH. Erickson et al. (1983) reported that since the major portion of proteolytic activity for whiting is in the acidic range, a secretion of cathepsins from lysosomes in phagocytic cells may indeed have occurred in response to the antigen or parasite. It was concluded from both pH and inhibitor investigations that cathepsins may be responsible for the accelerated breakdown in whiting, specifically cathepsins B and C.

Nonlysosome or Cytosolic Proteinases

Evidence suggests that some nonlysosomal proteinases are involved in cellular degradation of proteins (Asghar and Bhatti, 1987). Nonlysosome proteinases (i.e. serine proteinases and some thiol proteinases) have a wide range for activity in the neutral to alkaline pH (Barrett and

McDonald, 1980). Erickson et al. (1983), Tsuyuki et al. (1982), Makinodan and Ikeda (1969ab, 1979), Makinodan et al. (1979), Makinodan et al. (1985) and Iwata et al. (1974a,b), reported fish muscle proteinases active in the neutral to slightly alkaline pH assayed with casein as substrate. These proteinases in both crude and partially purified form possessed optimal activities in the slightly alkaline pH range and at high temperatures of around 60°C. The alkaline proteinases from the muscle of a number of fish shows little or no activity against casein at lower incubation temperature of around 35-37°C. Makinodan et al. (1985) reported several proteinases in croaker muscle. A neutral proteinase, active at pH 6.5-7.0 with loss of almost all its activity above 55°C. Calpain, called also Ca^{++}-activated neutral proteinase by Asghar and Bhatti (1987), with optimum activity around pH 7.0 and no activity above 45°C. An alkaline proteinase with an optimum activity at pH 8.0, but with considerable activity also at pH 7.0. This alkaline proteinase possessed activity at 60°C, but very weak activity at 50°C. Erickson et al. (1983) reported a major peak of proteinase activity in Pacific whiting occurs in the alkaline pH range where the pH optimum was estimated to be 7.1-7.2. These researchers suggested that a chymotrypsin-like and leucine aminopeptidase-like enzymes present in muscle extract could be the responsible for the activity at the slightly alkaline pH.

Several other muscle cytosolic proteinases with activity in the neutral to alkaline pH range involved in the degradation of myofibrillar proteins have been reported in several vertebrate species. These include myofibrillar serine proteinase (pH 9.0) (Nogushi and Kandatsu, 1971), group specific proteinase (pH 8.0) (Katunuma et al., 1975), ATP-activated alkaline proteinase (pH 7.8) (Etlinger and Goldberg, 1977), and Ca^{++}-activated neutral proteinase (pH 7.5) (Suzuki and Goll, 1974; Guroff, 1964; Busch et al., 1972; Huston and Krebs, 1968; Reddy et al., 1975). Even though the presence in fish muscle tissue of all these specific enzymes has not been well documented, they may indeed be part of the alkaline proteinase activity in fish muscle reported by several researchers (Montejano et al., 1983; Su et al., 1981a,b; Lanier et al., 1981; Cheng et al., 1979).

Alkaline Proteinase from Organ Tissue Contamination

The integrity of the myofibrillar proteins may also be reduced by proteolytic enzymes during heat processing by a heat-stable proteolytic factor(s), termed alkaline proteinase by several researchers (Montejano et al., 1983; Su et al., 1981ab; Lanier et al., 1981; Cheng et al., 1979). Distribution of alkaline proteinases is clearly evident in the edible flesh of various fish (Suzuki, 1981; Su et al., 1981a). Su et al. (1981a), showed that alkaline proteinases

exist in the sarcoplasmic fraction of skeletal muscle but also in the skin and internal organs of croacker. Alkaline proteinase activity in liver, alimentary canal and kidney tissues were found to be several hundred times that in muscle. Elevated alkaline proteinase activity is imparted to minced flesh by residual organ tissues. Su et al. (1981b) postulated that the lower texture ratings for products prepared from mechanically separated flesh were due, at least in part, to a significantly higher alkaline proteinase activity as a result of residual organ tissue contamination. Investigations by Cheng et al. (1979), concluded that the degradation of myosin observed in cooked fish gels was highly related to gel texture properties and that changes in muscle protein during heating were caused by alkaline proteinases. Alkaline proteinases hydrolyze muscle proteins optimally around 60°C at neutral to slightly alkaline pH and influence the functionality of fish meat gels (Cheng et al., 1979; Lanier et al., 1981; Makinodan et al., 1985). Lanier et al. (1981) reported that degradation of myosin was related to the textural strength of cooked fish gels. Under normal circumstances, rapid heating to 85°C internal temperature produces a firmer gel and more springy texture. These researchers noted that, at temperatures approaching optimum proteolytic activity, texture firmness decreased, while at temperatures above and below this region, proteolytic activity was decreased and the texture firmness of the gel was much greater.

Characterization of the Possible Endogenous Proteinases Involved in the Degradation of Pacific Whiting Muscle

Endogenous proteinases that participate in the degradation of fish are not well defined. Most of the available data result from studies concerning autolytic processes. Autolysis is a very complex event and rather complex measuring systems seem to be required. In such investigations no attention was paid to the degree to which exogenous or endogenous proteinases, or both, contribute to autolytic processes. Naturally such studies provide little evidence as to the detailed mechanism of single autolytic processes, or to the sequence of events which finally result in deterioration of fish (Siebert and Schmidt, 1965).

The specificities of fish enzymes have been reported to be different from those occurring in mammalian tissue. Siebert and Schmidt (1965) showed that fundamental differences exist between mammalian and fish cathepsins, therefore, it seems that no close analogies can be drawn from studies of one type of muscle flesh to that of any other kind. The prevention of undesirable deterioration of fish requires a thorough investigation of fish enzymes.

Tsuyuki et al. (1982), Erickson et al. (1983) and Makinodan et al. (1985) have reported proteinase activity in fish muscle in the acidic and alkaline pH ranges. Based on the results from those investigations the following is a brief characterization of the possible endogenous

proteinases involved in the degradation of muscle protein of Pacific whiting.

Leucine aminopeptidase

Erickson et al. (1983) reported a leucine aminopeptidase-like activity in muscle extract from Pacific whiting based upon activity on synthetic substrates and suggested that the enzyme may be responsible for the activity found at the slightly alkaline pH. The enzyme, an exopeptidase, cleaves the N-terminal peptide bond at which a leucine residue is present at the N terminus. Investigations with synthetic substrates show the enzyme to have a Vmax at pH 7.8-8.0 and to be activated by Mg^{++} and Mn^{++} cations (DeLange and Smith, 1971), and NaCl (Sylven and Snellman, 1962). Its activity was rapidly lost at a pH below 7.0 (Joseph and Sanders, 1966).

Leucine aminopeptidase is a true metal protein since its activity is inhibited by metal poisons such as cyanide and sulfide and inactivated by the amino acid cysteine (Smith, 1951) which is capable of forming metal complexes. The enzyme is not entirely an exoaminopeptidase, since it can also split peptide bonds which are not adjacent to the essential free amino group. How far the free amino group can be from the carbonyl of the sensitive peptide bond has not been determined (Smith, 1951). The presence of leucine aminopeptidase has been reported in the skeletal muscle of

rabbit, rat (Smith, 1948) and humans (Bury and Pennington, 1973).

Cathepsin C

This enzyme is also known as dipeptidyl aminopeptidase I, dipeptidyl peptidase, dipeptidyl arylamidase I, dipeptidyl transferase and glucagon-degrading enzyme (McDonald and Schwabe, 1977). The enzyme is responsible for removing dipeptides sequentially from the N-terminal end. Synthetic substrates have been used to identify its activity at pH 5.6 (Planta et al., 1964). A thiol group constitutes its active site (Fruton and Mycek, 1956) and chloride ions are required for its activation at pH 5-6 (McDonald et al. 1966, 1974). Cathepsin C is reported to be activated by thiol reagents (i.e cysteine and mercapthoethylamine) (Barrett, 1972). The thiol proteinases are inhibited by thiol blocking reagents such as oxidizing and alkylating agents and by heavy metals which bind to the sulfhydryl group (Barrett, 1972; Mihaly, 1972). Erickson et al. (1983) concluded from results of both pH and inhibition studies, that cathepsin C may be one of the cathepsins responsible for the accelerated muscle breakdown in Pacific whiting.

Cathepsin B

Cathepsin B is a thiol endopeptidase and was indicated by Erickson et al. (1983) as having major responsibility for the deterioration of whiting muscle. The optimum pH for cathepsin B activity varies widely (3.5-6.0), however, the Vmax has been found at pH 6.0 (Barrett 1972, 1973). The activity of this enzyme depends on the sulfhydryl group and it is unstable above pH 7.0 (Asghar and Bhatti, 1987). Erickson et al. (1983) pointed out that the presence of this enzyme may be due to its release from phagocytic cells in response to the parasite, but they also mentioned that before this proposal can be accepted, the absence of cathepsin B from unparasitized whiting must be established.

Cathepsin H

Cathepsin H is a thiol proteinase which exhibits activity at pH above 7 (Locnikar et al. 1981), but its Vmax is generally observed at pH 6 (Schwartz and Barrett, 1980). This enzyme also possesses aminopeptidase activity and Barrett (1980) considered that cathepsin H and leucine aminopeptidase may be the same enzyme. Since thiol proteinases contain a thiol group at the active center, which should be in the reduced form for the enzyme to be in the active state, the low redox potential (depletion of

oxygen) in postmortem muscles favors their activity (Asghar and Bhatti, 1987).

Ca^{++}-Activated Neutral Proteinase (CANP)

Ca^{++}-activated neutral proteinase (CANP) is distributed in the cytoplasm of both vertebrate and invertebrate species. It is reported under different names, such as kinase activating factor from skeletal muscle (Huston and Krebs, 1968), calcium-activated factor (CAF) from pig muscle (Busch et al., 1972) and calcium-activated sarcoplasmic factor (CASF) from rabbit muscle (Suzuki and Goll, 1974). Some Japanese scientists (Murachi et al. 1981a,b; Makinodan et al., 1985) preferred to call it calpain on the basis of generic nomenclature (cal stands for calcium and pain for thiol proteinases, such as papain) (Asghar and Bhatti, 1987).

CANP shows maximal activity around neutral pH (7.5) for degrading protein and requires 1-5 mM Ca^{++} for activity under reducing conditions. A thiol group is believed to constitute the active site of the enzyme (Waxman, 1981). CANP seems unique among proteinases in that it has no activity on small peptides substrates and requires fairly long peptides to express its activity. It is believed that CANP does not require any specific sequence of amino acid residues for cleaving the peptide bond (Asghar and Bhatti, 1987).

Serine Proteinases (Alkaline Proteinases)

Those endopeptidases which have a unique reactive serine residue in the active center have been classified as serine proteinases. Since these enzymes show maximum activity at a high pH (pH 8), they are also called alkaline proteinases (Bird et al., 1980). Serine proteinases are inhibited by acylation of the seryl OH group with diisopropyl fluorophosphate (DFP) (Jansen et al., 1949) or sulfonyl fluoride (Fahrney and Gold, 1963) and by alkylation of the imidazole group of histidine (Shaw et al., 1965).

Many researchers have succeeded in identifying serine proteinases with chymotrypsin-like activity in skeletal muscle of different species (Makinodan et al.,1985, 1982; Koszalka and Miller, 1960; Su et al., 1981a,b; Lanier et al., 1981; Cheng et al., 1979; Nogushi and Kandatsu 1971; Katunuma et al. 1975; Reinauer and Dahlmann, 1970) with an activity optima within a pH range from 7 to 9.

Chemical Inhibition of Proteolysis in Pacific Whiting Minced Flesh

When a change in the texture of Pacific whiting occurs, it is due to the presence of a proteinase that is capable of breaking the chemical bonds of the muscle fibers that contribute to the characteristic texture of any muscle food. Several methods of preventing this textural change have been

evaluated (Spinelli and Steinberg, 1978); among them the inactivation of proteinases with chemicals.

Several inhibitors (Miller and Spinelli, 1982; Groninger et al., 1985; Chang-Lee et al., 1988) have been evaluated for their potential for inactivating proteinases. Miller and Spinelli (1982) reported that proteinase inhibitors such as those found in egg white, potato, and soy and lima beans were ineffective as inhibitors, since none of them caused significant inhibition in concentrations that would be suitable for use in food systems. On the other hand, Groninger et al. (1983) and Chang-Lee et al. (1988) found that egg white, at a 3% addition level, has the capacity to inhibit the proteinase and improve the functionality of whiting surimi gels.

Investigations by Miller and Spinelli (1982) have demonstrated that two common oxidizing agents, hydrogen peroxide (H_2O_2) and potassium bromate ($KBrO_3$), were effective inhibitors of proteinases. Concentrations of less than 0.5% of either of these additives achieved nearly complete inhibition. These reagents mixed into ground parasitized Pacific whiting muscle were found to have inhibited proteolysis sufficiently during frozen storage and later cooking to maintain texture comparable to nonparasitized fish. The inhibition of the proteinase activity in whiting surimi by the addition of $KBrO_3$ (200-600 ppm) alone or with egg white (3%) is also well documented by Groninger et al. (1985). Proteinase inhibition studies

(Miller and Spinelli, 1982) using sulfhydryl binding compounds (i.e. iodoacetate and N-ethylmaleimide) supported a thiol proteinase as being responsible for the texture deterioration in whiting flesh.

The Myosin Molecule

Myosin is one of the principal protein components of numerous contractile systems and comprises almost 50% of the total protein in skeletal muscles (Harrington and Rodgers, 1984; Samejima et al., 1981). Although each myosin has unique properties depending on its origin, certain features are common to all. Myosin (mol wt 460,000) is a hexamer consisting of one pair of heavy chains (mol wt 200,000) and two pairs of light chains that vary in mass depending on the source (mol wt 15,000-27,000). The molecule consists of two globular head regions and a rodlike tail portion that is a "coiled coil" of alpha-helices (Figure 1). The rod portion is responsible for the assembly of myosin to form the functional thick-filament structure. The globular heads contain the enzyme active site (ATPase activity), the active actin-binding site and several divalent cation-binding sites (Millar et al., 1987). Each head also has two noncovalently bound light chains that appear to be involved in the regulation of contraction. Chemical modification of two highly reactive thiol groups (SH_1 and SH_2) in each myosin head alter the ATPase activity of the molecule (Griffiths et

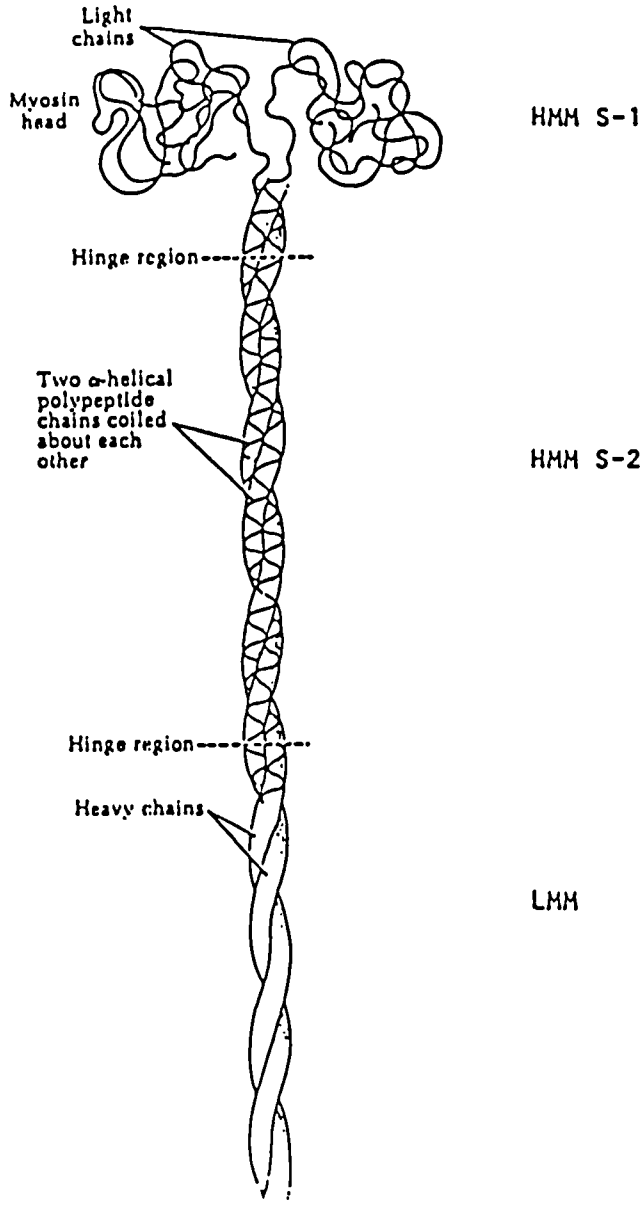


Figure 1. The myosin molecule (Lehninger, 1982)

al., 1987; Stanners and Bagshaw 1987; Harrington and Rodgers, 1984; Adelstein, 1980; Regenstein and Regenstein, 1984).

Sulfhydryl Content

All myofibrillar proteins contain sulfhydryl groups. The myosin molecule has been reported to contain 42 SH groups (Hofmann and Hamm, 1978; Buttkus, 1970, 1971). The average sulfhydryl content, expressed as moles/1 x 10^5 g protein, has been reported to be 9.1 for myofibrils from different sources, 7.3-9.0 for myosin, and 7.5-8.8 for actomyosin (Hofmann and Hamm, 1978). Some of the values determined in native myofibrils with N-ethylmaleimide (NEM), p-chloromercuribenzoate (PCMB), and disulfide 5,5´-dithiobis (2-nitrobenzoic acid) (DTNB) do not represent the total sulfhydryl content but rather the groups available under the conditions used for their determination.

Many authors have found that the number of sulfhydryl groups in myosin which are titrable with PCMB is approximately equal to the half cystine content found by amino acid analysis. Myosin probably contains no disulfide bridge. Disulfides have been found only in tropomyosin and troponin (Hofmann and Hamm, 1978).

Reactivity of Sulfhydryl Groups

Sulfhydryl and disulfide groups have been widely implicated as important functional groups in many foods proteins (Beveridge et al., 1974; Jones and Carnegie, 1969; Redman and Ewart, 1967). The amino acids cysteine and cystine are the carriers of the sulfhydryl and disulfide groups on proteins. Knowledge on the reactions of these amino acids is therefore indispensable for an understanding of the reactivity of these groups in biological systems. Cyteine and cystine content of food proteins is of great importance from the point of view of their nutritional value, sensory quality and processing characteristics. More than 95% of the sulfhydryls of myofibrils are located in the actin-myosin system. Like myosin, actin contains no disulfide bridges.

Several hundred publications deal with the role of sulfhydryl groups on myofibrillar proteins with respect to enzymatic activities, interaction with ions, substrates, another proteins, and the process of muscular contraction and relaxation. Sulfhydryl groups are important for the adenosine triphosphatase (ATPase) activity of myosin (Young, 1969; Regenstein and Regenstein, 1984). Fifty percent of the ATPase activity was lost when 5 of 42 sulfhydryl groups per molecule were blocked and inactivation was complete if 7 per molecule were blocked. These groups are located in the

head region of the molecule, probably close to the active site.

Sulfhydryl functions are usually considered the most reactive functional groups on proteins and are easily oxidized to disulfide functions under neutral or alkaline conditions according to the following reactions:

$$2\ R\text{-}SH + 1/2\ O_2 \longrightarrow R\text{-}SS\text{-}R \qquad \text{or}$$

$$2\ R\text{-}S^- - 2e^- \longrightarrow R\text{-}SS\text{-}R$$

The denaturation of a protein renders sulfhydryl groups more labile to oxidation by molecular oxygen (Ziegler, 1985; Hofmann and Hamm, 1978; Wall, 1971). On the other hand, the disulfide bond is the most labile covalent linkage common in proteins. It is readily reduced or oxidized to yield scission products. It may also undergo sulfhydryl-disulfide interchange, alkaline hydrolysis, destruction by radiation and possibly mechanical scission (Wall, 1971; Koshland and Mozersky, 1964).

Interaction-aggregation between free fatty acids and myofibrillar protein during frozen storage of fish muscle has been widely documented by several authors (Buttkus, 1970; Robinson, 1966; Anderson and Ravesi, 1970). Robinson (1966) reported that sulfhydryl groups are able to react with the double bond of unsaturated fatty acids. Furthermore, they can be oxidized by fatty acid peroxides which may be formed during frozen storage of fish, minced flesh or surimi. Malonaldehyde, a product of oxidation of

polyunsaturated fatty acids such as those present in fish lipids, reacts with sulfhydryl groups in proteins and is able to act as a cross-linking reagent between different myosin monomers (Buttkus, 1970). The presence of Cu^{2+} and some other ions, traces of heavy metal included, are known to catalyze the autoxidation of sulhydryl functions (Hofmann and Hamm, 1978). Buttkus (1971), found that trout myosin in the presence of traces of heavy metals was more susceptible to oxidation than rabbit myosin.

Buttkus (1970) investigated the accelerated denaturation of myosin in frozen solution and proposed a mechanism by which fish muscle toughness developed during frozen storage by myosin aggregation involving a sulfhydryl-disulfide interchange. He reported that about 10 of the sulfhydryl functional groups on myosin are very reactive and oxidize readily at 0°C, upon exposure to air, to form intramolecular disulfide bonds. The oxidation of sulfhydryl groups and the aggregation of myosin from trout and cod were found to be more extensive and rapid in comparison to myosin from rabbit.

The participation of the sulfhydryl groups in sulfhydryl-disulfide exchange reactions has been implicated in the denaturation and aggregation mechanisms of myosin (Laird et al., 1961; Tsuchiya et al., 1961; Matthews et al., 1961). Many changes in protein properties that accompany denaturation by heat treatment or other processes involve relocation of disulfide bonds. Heating a protein solution

enhances interchange since the resulting chain unfolding permits greater accessibility of sulfhydryls to disulfides (Wall, 1971). Itoh et al. (1979a) reported that the behavior of the reactive sulfhydryl functions on carp actomyosin, with respect to heating temperature, is quite compatible with the behavior of the gel-forming ability of actomyosin. These researchers concluded that the reactive sulfhydryl groups, which are exposed on the molecular surface by heating, contribute to the gel formation of actomyosin through bonding between the protein molecules.

The sulfhydryl-disulfide interchange and the reaction between two disulfides are favored by alkaline pH and by catalytic amounts of thiol (i.e. cysteine) as shown below:

$$R_1SH + OH^- \longrightarrow R_1S^- + H_2O$$

$$R_1SSR_1 + OH^- \longrightarrow R_1S^- + R_1SOH$$

$$R_2SSR_2 + R_1S^- \longrightarrow R_1SSR_2 + R_2S^-$$

$$R_1SSR_1 + R_2S^- \longrightarrow R_1SSR_2 + RS_1^-$$

Since the RS^- is the active participant in the interchange, such reactions are more rapid in alkaline media (Koshland and Mozersky, 1964; Wall, 1971; Cecil and McPhee, 1959).

Heat Induced Gelation of Proteins

Gels are differentiated from other systems in which small proportions of solid are dispersed in relatively large proportion of liquid by the property of mechanical rigidity,

or the ability to support shearing stress at rest. Rigidity is the characteristic property common to all gels. In many protein gels, including those of denatured proteins, the essential structural feature appears to be a three-dimensional network (Ferry, 1948). Gelation may be theoretically defined as a protein aggregation phenomenon in which polymer-polymer and polymer-solvent interactions and in which attractive and repulsive forces are so balanced that a well ordered tertiary network or matrix is formed (Acton and Dick, 1984).

The mechanism proposed by Ferry (1948) is still the most generally accepted for heat-induced gelation of proteins. This two-step mechanism involves an initiation step involving an unfolding or dissociation of the protein molecule followed by an aggregation step in which association and aggregation occur resulting in gel formation under appropriate conditions. Hermansson (1978) elaborated on Ferry's proposed mechanism, stating that, contrary to coagulation where aggregation of the protein molecules is random, gelation involves the formation of a continuous network exhibiting a certain degree of order. Furthermore, when aggregation is suppressed prior to denaturation, the resulting network can be expected to exhibit a higher degree of elasticity than if random aggregation and denaturation occur simultaneously, or if aggregation precedes denaturation. The slower the second step, relative to the first, the better denatureds chain are oriented and the

finer the gel network. For the formation of a highly ordered gel matrix, it is imperative that the aggregation step proceed at a slower rate than the unfolding step.

Schmidt (1981) pointed out that in addition to the effects of the size, shape and arrangement of the primary protein strands comprising the gel network, the characteristics of protein gels are affected by intra- and inter-strand crosslinking. This crosslinking combined with the fluidity of the immobilized solvent give gels their characteristic strength, elasticity and flow behavior. The degree of crosslinking must be optimal. Insufficient crosslinking or overdependency on it results in undesirable gel structure. Protein gels may be crosslinked by specific bonding at specific sites on the protein strands or by nonspecific bonding occurring along the protein strands. The nature and degree of crosslinking would vary with the type of protein and the gelation environment. Both covalent and noncovalent bonds are thought to be involved. The general types of crosslink bonds, their characteristics and proposed role in protein gels have been summarized by Schmidt (1981) (Table 1).

Investigation of covalent bonding in heat-induced protein gels have focused primarily on disulfide bridging. Several possible reactions result in disulfide bridge formation. Heat treatment can result in cleavage of existing disulfide bonds or "activation" of buried sulfhydryl groups through unfolding of the protein. These

Table 1. Proposed crosslink bonding of protein gel structures and their properties[a].

Type	Energy (Kcal/mol)	Interaction Distance	Groups Involved	Role in Gel matrix
Covalent Bonding	80-90	1-2 A°	-S-S-	Bridging; Ordering
Hydrogen Bonding	2-10	2-3 A°	-NH O=C- -OH O=C-	Bridging; Stabilizing
Hydrophobic and Related Interactions	1-3	3-5 A°	Nonspecific	Strand thickening; Strengthening; Stabilizing
Ionic Bonding and Interactions	10-20	2-3 A°	$-NH_3^+$ $-COO^-$, etc.	Solvent interactions; Salt linking

[a]From Schmidt (1981).

newly formed or activated sulfhydryl groups can form new intermolecular disulfide bonds. In some protein systems, disulfide bridging may be imperative to the formation of a highly ordered gel structure. However, a high dependence upon disulfide bonding would tend to restrict solvent immobilization and result in more aggregated gel structures (Schmidt, 1981).

The following parameters (Balmaceda et al., 1976) may be important in characterizing protein gels: gel strength or hardness (resistance to compression), adhesiveness (stickiness to other materials), cohesiveness (stickiness to itself) and elasticity (ability to regain original form after deformation under mild pressure). The single most measured parameter has been hardness because of the relative

ease in measurement. These gel parameters can be obtained using Instron methodology.

Muscle Protein Transitions During Heat Denaturation

Protein heat-denaturation is a process or sequence of processes in which the spatial arrangement of the polypeptide chain within the molecule is changed from that typical of the native protein to a more disordered arrangement. There is no alteration in the protein's primary structure during the process (Kauzmann, 1959). Denaturation can therefore be restricted to the continuous process of native protein structural changes involving secondary, tertiary, and quaternary structure in which alterations of hydrogen bonding, hydrophobic interactions, and ionic linkages occur during the transition to the denatured state (Anglemier and Montgomery, 1976).

Hamm (1966) concluded that high temperatures, characteristic of cooking, cause the helical portions of muscle proteins to unravel into random coil-type configurations. Ziegler and Acton (1984) summarized the events that may occur during the heat denaturation of actomyosin (Table 2). Transition occurring at 55°C is possibly the most crucial, since gels do not attain appreciable strength until this temperature is reached. Anglemier and Montgomery (1976) stated that proteins in a random-coil configuration more readily form aggregates.

Heat Induced Gelation of Myofibrillar Proteins

The heat-induced formation of a three-dimensional protein matrix by myosin and actomyosin, termed gelation, can be represented by two stages of reactions. Each stage involves distinct segments of the myosin molecule. More critically, the stages occur in separate temperatures regions during heating. The first stage of aggregation occurs between 30°-50°C and the second at temperatures >50°C. Thus, in the protein-protein interactions of myosin, each stage, by temperature region, can be represented independently using Ferry's two-step sequence of reactions (Acton and Dick, 1984).

Each of the myosin's molecule structural regions (Figure 1) has an important role in its heat-induced gelation. Heat-induced gelation is initiated at 30-35°C and involves aggregation of the globular head regions of the molecule. This stage of gelation is believed to be completed at 50°C. Based upon investigations involving S-1 fraction, HMM segment and myosin, aggregation is thought to be dependent on oxidation of sulfhydryl groups which are predominantly found in the globular region (Samejima et al., 1981; Ishioroshi et al., 1982). Samejima et al. (1981) suggested that the head portions associate to form "super-junctions" which provide extra crosslinking to the gel network. While the rate of sulfhydryl oxidation progressively increases from 20 to 70°C, a considerable

Table 2. Conformational changes which may occur during the thermal denaturation of natural actomyosin[a]

Temperature (°C)	Protein(s) or segment involved	Description of events
30-35	Native tropomyosin	Thermally dissociated from the F-actin backbone
38	F-actin	Super helix dissociates into single chains
40-45	Myosin	Dissociates into light and heavy chains
	"Head"	Possible some conformational change
	"Hinge"	Helix to random coil transformation
45-50	Actin, myosin	Actin-myosin complex dissociates
50-55	Light meromyosin	Helix to coil transformation and rapid aggregation
>70	Actin	Major conformational changes in the G-actin monomer

[a]From Ziegler and Acton (1984).

degree of oxidation occurs within the 20 to 50°C temperature range. It is reasonable to assume that the free sulfhydryl content decreases due to rapid head-to-head aggregation in the lower temperature region. Aggregation cross-linkages could also involve noncovalent bonding as well. Whether covalent disulfide bonding is formed or not is not critical (Acton and Dick, 1984). Since agents that block the formation of disulfide bonds did not prevent the aggregation of the myosin head, but only retarded its onset, Samejima et

al. (1981) concluded that another type of aggregation, perhaps due to intermolecular association of side chains, superimposes on the sulfhydryl-dependent reaction. Investigations by Ishioroshi et al. (1980) support the role of sulfhydryl group involvement in head-to-head aggregation as one factor of the protein-protein interactions. The role, if any, of disulfide formation in heat-induced gelation of myofibrillar proteins is still not completely clear (Ziegler and Acton, 1984).

The "setting" of fish muscle proteins at approximately 40°C, or below those temperatures at which rapid aggregation occurs, may be viewed as the process where partially denatured proteins begin to interact noncovalently to form a fine elastic network. Setting at or below 40°C prior to heating to 60-80°C allows slow "ordering" of the protein molecules and results in the formation of gels with greater firmness and cohesiveness (Lanier et al., 1982). This appears to support the suggestion of Hermansson (1978) that the denaturation of proteins prior to aggregation results in a finer gel structure, exhibiting greater elasticity than if random aggregation occurs simultaneously or prior to denaturation. This slow "ordering" effect fits step one of the Ferry's (1948) gelation mechanism (Ziegler and Acton, 1984).

The second stage of heat induced gelation is associated with changes occurring at temperatures >50°C with termination of gel formation at 65 to 70°C. Structural

changes of the helical rod segment of myosin which culminates in network formation through cross-linking of these segments occurs within this temperature region. Gels do not reach appreciable strength until the myosin tail portion has undergone helix-coil transformations and subsequent cross-linking. Bonding between myosin tail portions appears to involve noncovalent interactions, since there seems to be no participation of sulfhydryl to disulfide reaction. Samejima et al. (1981) found gelation to be effectively inhibited by urea and guanidine hydrochloride. The myosin head region is important since "super-junctions" formed at lower temperatures form a "super-thick" filament network interlink at temperatures >50°C (Siegel and Schmidt, 1979; Samejima et al., 1981).

The Role of Sulfhydryl-Disulfide Interchange and Sulfhydryl Oxidation in the Improvement of Heat-Induced Gelation of Fish Pastes

Itoh et al. (1979a) investigated the participation of sulfhydryl groups in the heat-induced gelation of carp actomyosin by determining the effect of heating temperatures up to 80°C on the content of total and reactive sulfhydryl in carp actomyosin. These researchers observed an increase in oxidized sulfhydryl content as actomyosin solutions were heated above 40°C. The maximum concentration of reactive sulfhydryl, which was believed to be exposed by unfolding of the protein, was observed at 60°C. The difference between

total and reactive sulfhydryl, which is taken as the inner molecular content, was at minimum concentration. Itoh et al. (1979a) also observed that the behavior of the reactive sulfhydryl of the actomyosin, with respect to heating temperature, was quite compatible with the behavior of the gel-forming ability of actomyosin. Itoh et al. (1979b) clarified the participation of sulfhydryl groups in gel formation by determining the effect of two sulfhydryl reagents, N-ethylmaleimide and p-CMB, on the gel formation of carp actomyosin. These researchers found that the rigidity modulus of the gels formed at 40 and 80°C was decreased by adding the sulfhydryl reagents to the actomyosin solution and that the gels containing the reagents were more soluble in 8 M urea solution than control gel. Itoh et al. (1979a,b) concluded that sulfhydryl groups, which appear on the molecular surface from heating, participate in the gel formation of carp actomyosin through disulfide bonding between protein molecules. It was believed that a similar process occurs during the heat-setting of sols of fish meat.

Itoh et al. (1979c,d) further evaluated the participation of sulfhydryls in heat-induced gelation by determining the effect of cysteine and cystine, and inorganic reducing agents (sodium nitrite, sodium thiosulfate, sodium hydrogen sulfite) on the gel formation by heating fish meat. Itoh et al. (1979c) found that cystine added to carp meat paste and to carp actomyosin

solution was reduced to cysteine upon heating. This result suggested that the effects of cysteine and cystine on gel formation were due not only to the oxidation of sulfhydryl groups to intermolecular disulfide functions by cystine, but also to the formation of mixed disulfides by interchange and to the enhancement of disulfide interchange between protein molecules by cysteine. The elastic properties of the gels containing the inorganic reducing agents (Itoh et al., 1979d) were similar to those of the gels containing cysteine, cystine, ascorbic acid, dehydroascorbic acid and potassium bromate. These researchers concluded that the inorganic reducing agents promote the formation of disulfide bonds between protein molecules via a disulfide interchange.

The use of potassium bromate to improve the gel-forming ability of fish meat pastes through the oxidation of the sulfhydryl group present in myofibrillar proteins to disulfide bond is well documented in the literature (Itoh et al. 1979d; Tabata et al., 1976; Niwa and Miyake, 1971; Groninger et al., 1985; Jiang et al., 1986; Okada and Nakayama, 1961). Jiang et al. (1986) used various reductants and oxidants to improve the quality of minced fish products. These researchers conclude that the addition of reducing agents (i.e. mercaptoethanol, cyteine, tannic acid, sodium bisulfide, and ascorbic acid) during surimi processing recovered reactive sulfhydryl groups and subsequently increased the gel strength of frozen-thawed fish meat. The addition of oxidants (potassium bromate and

hydrogen peroxide) to sols of surimi containing reducing agents yielded stronger gels that samples without oxidants. It was believed that the oxidants induced the formation of disulfide bonds which intensified the network structure of the fish gles (kamaboko). The work by Jiang et al. (1986) suggest the importance of disulfide bonds in gelation of minced fish products. The use of potassium bromate to promote the oxidation of sulfhydryl groups in fish meat proteins to a disulfide bond was also documented by Hujita and Itoh (1984), although it was suggested that sulfhydryl groups are not only oxidized to disulfide bonds, but also to sulfonic acid groups.

Surimi

Surimi is a Japanese term for minced fish flesh that has been washed with water, dewatered and mixed with cryoprotectants (sucrose and sorbitol) to extend its frozen shelf life (Figure 2). It is an intermediate product used to make kamaboko which takes the form of imitation shellfish meats (shrimp, clams, scallops, crab legs and crab meat strips), fish sausage and fish ham (Buck and Fafard, 1985; Lee, 1984; Mitchell, 1984; Dassow, 1982; Lanier et al., 1982; Suzuki, 1981). Surimi is produced by repeatedly washing mechanically separated fish flesh with chilled water (5-10°C) until it becomes essentially odorless, colorless and tasteless. Technically, the flesh is washed until most

of the water soluble protein has been removed, which imparts superior gelling ability to the washed flesh by concentration of the myofibrillar protein fraction (Lee, 1984; Mitchell, 1984; Suzuki, 1981). This fraction of muscle tissue is known to be the most active in performing the functions of texture formation or particle cohesion and binding of fat and water in many processed muscle food systems (Acton et al., 1983). Surimi is the only functional protein concentrate to be commercially produced from any animal muscle tissue (Lanier, 1986).

The most important characteristic of surimi is its ability to form highly elastic gels when mixed with salt and other ingredients (Lanier, 1986). The quality of frozen surimi, as a raw material, is graded on the basis of its chemical and visual condition and the strength of its heat-set gels or kamaboko (Lee, 1984; Suzuki, 1981; Sonkodi, 1983). To test for gelling ability, surimi is mixed with salt and sometimes potato starch, then stuffed into casings and cooked at various temperatures. The cooked gels are then submitted to a variety of tests including rheological testing such as the fold and punch tests (Lee, 1984), and the torsion test (Lanier et al., 1985). The grade of surimi produced is largely a function of the species and freshness of fish and processing procedures used (Lee, 1984).

The surimi technology could provide a use for the bulk of the now underutilized fish species and give seafood

protein a secure and substantial role in the diet of U.S. consumers (Sabella, 1985). Surimi-based shellfish products are largely made from surimi prepared from Alaska pollock, but might be prepared from other low priced species such as fresh water catfish, gulf croaker, menhaden and red hake (Hasselback, 1984). Investigations by Pacheco-Aguilar et al. (1988) concluded that Pacific whiting, an underutilized species, is a feasible species for surimi manufacturing.

Surimi technology continues to stimulate interest throughout the seafood, as well as the food industry in the United States (Lee, 1986). The U.S. production of Alaska pollock surimi has increased substantially. Although data on surimi production have only recently been developed and may be subject to inaccuracies, estimates indicates that in 1986, about 77 percent of U.S. surimi demand was satisfied by imports. In 1988, preliminary estimates indicate that U.S. production of surimi grew between 130 and 140 million pounds, and nearly 100 million pounds of surimi were exported. Apparent U.S. consumption of surimi more than doubled between 1986 and 1988, from about 22 million pounds to about 50 million pounds (Beale and Jensen, 1988).

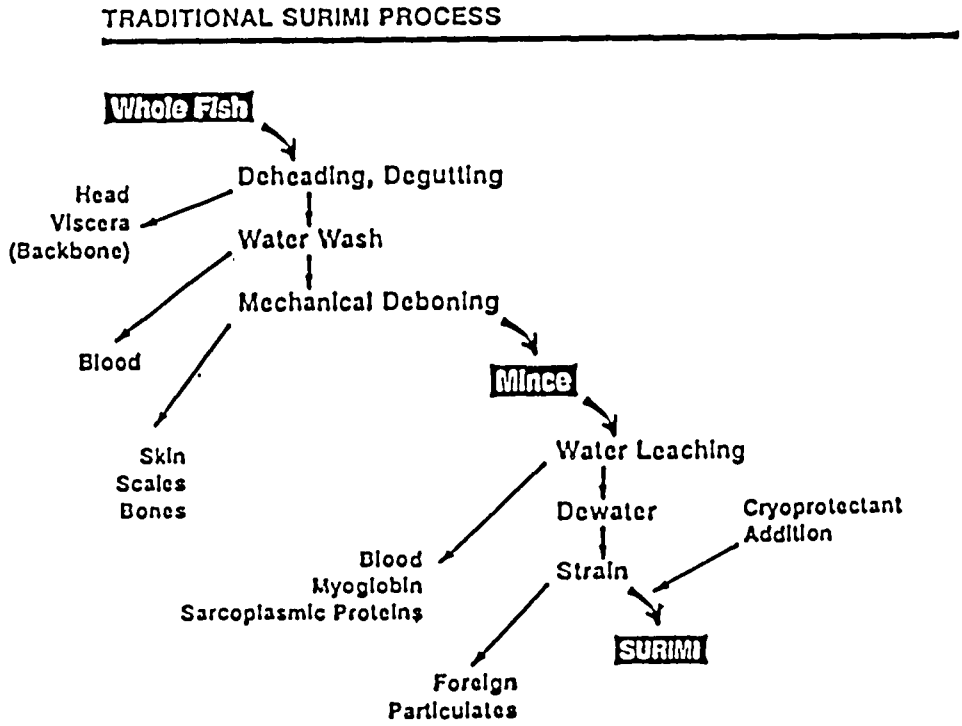


Figure 2. Traditional surimi process (Lanier, 1986)

MATERIALS AND METHODS

Processing Round Fish into Surimi

Fish Source

Fresh Pacific whiting were obtained <24 hrs post-catch from local seafood processing plants in Astoria, Oregon during the fall of 1987 and summer of 1988. Round fish were held in ice from immediately post-catch until processing.

Flesh Separation

Round fish were processed into skinless fillets by hand and throughly washed. Visceral and blood contamination was carefully avoided. Individuals with musculature visually infested with black hair-like pseudocysts and those with extremely soft or mushy texture were discarded. Fillets were ground through a 3/8 inch plate using a Autio Grinder Model 601HP (Autio Company, Astoria, OR).

Washing, Dewatering and Refining

Minced flesh was washed twice with cold water (mixture of ice and tap water at a 1:5 ratio) using a water:minced flesh ratio of 3:1 by continuously stirring the mixture for five min followed by another five min with no stirring.

Dewatering was carried out in a Model SD-8 Screwpress Dehydrator (Ikeuchi/Sato, Japan) using the maximum speed to dewater the flesh after the first wash.
The lowest possible speed after the second wash was used to achieve the lowest possible moisture content. After washing and dewatering the flesh was chilled in a blast freezer for a short period of time prior to refinig.

Refining was carried out in a Model S1 Ikeuchi Flesh Strainer (Ikeuchi Tekkosho/Akashi, Japan). Refined flesh was chilled immediately in a blast freezer for a short period of time prior to surimi preparation.

Surimi Preparation

The refined flesh was mixed with cryoprotectants to yield the following final composition: refined flesh, 91.7%; sorbitol, 4%; sucrose, 4% and Brifisol S-1 (a mixture of food-grade phosphates by BK Ladenburg Corp., N. Hollywood, CA) 0.3%. Mixing was carried out in a Model VCM Hobart Silent Cutter (The Hobart Manufacturing Co., Troy. OH). Temperature during the whole procedure was kept below 10°C to prevent or reduce protein denaturation. Aliquots of surimi were packed into individual plastic trays (10.5 X 8.5 X 5.5 cm) of approximately 600 g each, and vacuum sealed in moisture-vapor proof film (20 X 25 cm) using a Model 6250-B Cryovac Vacuum Chamber Machine (W.R. Grace & Co., Cryovac

Div., Woburn, MA). Samples were frozen and held at -30°C prior to evaluation.

Processing Documentation

A materials balance for each unit operation used in processing round fish into surimi was determined. Material weights did not include product held-up in processing equipment (refiner and screw press).

Samples of minced, washed and pressed and refined flesh and formulated surimi were randomly obtained through the processing operation. Samples were frozen and held at -30°C prior to compositional analysis.

Preparation of Heat Set Gels

Sol Formulation and Preparation

Surimi was partially thawed overnight at 2-4°C, cut into small pieces and weighed into working lots of 404 g. Sol formulation contained 2.8% NaCl and eight different concentrations of $KBrO_3$ (0.000, 0.025, 0.050, 0.075, 0.100, 0.150, 0.200 and 0.250%). Potassium bromate replaced an equal amount of NaCl in formulation to maintain an equal NaCl plus $KBrO_3$ content of 2.8%. Potassium bromate (J.T. Baker Chemical Co., Phillisburg, N.J.) was dissolved in the cold water complement of formulations and added at the same

time as solid NaCl at the beginning of the mixing operation. Water added (approximately 66% as ice) to formulation adjusted sol moisture contents to 78%. The sum of formulation components equaled 100% and a 450 g working preparation was used.

Sols were prepared by blending the working formulation in a Model KFP Kitchen-Aid Food Processor (Hobart Corp., Troy, OH) for 2-3 min or a time which allowed the sol to achieve a temperature of no more than 4°C as recommended by Babbitt and Reppond (1988). Each sol was packed into two trays (12 X 7.5 X 2.5 cm) and vacuum sealed in moisture-vapor proof film with a Model 6250-B Cryovac Vacuum Chamber Machine (W.R. Grace & Co., Cryovac Div., Woburn, MA).

Heat-Setting

A sample of each sol was heat-set under the following heating conditions: a) 40 min at 90°C, and b) 60 min at 40°C followed immediately by 20 min at 90°C. The time from initiation of sol preparation to the time sols were placed in the water bath was maintained below 15 min. Heat-set gels were immediately chilled in ice-water and held overnight at 2-4°C prior to texture evaluation.

Gel Replication

Three separated sols were prepared containing each of the 8 levels of $KBrO_3$. Each sol was heat-set under two different heating regimes. A total of 48 gels were prepared (3 replications x 8 levels of $KBrO_3$ x 2 heat-setting treatments).

Texture Evaluation

Texture Profile Analysis

Fish gels were tempered to ambient temperature (21-23°C) prior to analysis. Six cylindrical shaped sample specimens of uniform dimensions (1.5 cm diam. X 1 cm long) were cut from each gel. Textural characteristics of the gels were measured by the Texture Profile Analysis (TPA) method using a Model TM-M Instron Universal Testing Machine (Instron Corp., Canton, MA) with a 22.7 Kg (50 lb) load cell according to the recommendations of Breene (1975). The crosshead and chart speeds were set at 50 mm/min and 500 mm/min, respectively. Compression forces at 90% and 50% of the original gel sample lengths were determined. Results were reported in the texture terms of hardness, brittleness, cohesiveness, and elasticity as described by Abbot (1972). Measurements under 90% compression with 50 lb full scale (single bite analysis) were used to compute hardness and

brittleness and under 50% compression with 2 lb full scale (double bite analysis) to compute cohesivenees and elasticity.

The results were calculated according to Bourne (1968). The force (peak height) to compress each sample to 90% of its original length was reported in Newtons/gram (N/g) as hardness; the force (N/g) to cause the first gel failure under 90% compression was reported as brittleness. Cohesiveness was calculated as the ratio of the area of the second compression peak to that of the first compression peak. Area of the peaks was measured using a Model 62-0015 Polar Planimeter (Keuffel and Esser Co., USA). Elasticity was calculated as the ratio of the base length of the second peak to that of the first compression peak. Cohesiveness and elasticity were expressed in arbitrary units and reported as percent. For each textural parameter, three sample specimens per gel per replicate were evaluated. Mean and standard deviation were calculated for statistical purposes.

Folding Test

Gel characteristics were also evaluated using the folding test described by Kudo et al. (1973). This subjective test measured the flexibility and elasticity of heat-set gels. The test was conducted by folding a 3 mm thick by 2.5 cm diameter slice of gel between the thumb and index finger.

If no cracking occurred along the fold, the slice was folded again perpendicular to the first fold and the fold examined for cracking. The numerical rating scale for the folding test, as shown below, was based upon the degree of cracking occurring along the folds as described in the Japanese Grading System (Nishiya, 1963; Nippon Suisan Kaisha, Ltd. 1980). Ten sample specimens per gel per replicate were evaluated. Mean and standard deviation were calculated for statistical purposes.

Score	Condition
5 = SA or AA	No crack after quarter fold
4 = A	No crack after folding in half
3 = B	Cracks gradually when folded in half
2 = C	Cracks immediately when folded in half
1 = D	Breaks by finger pressure

Composition and pH Determination

Proximate compositions were determined in duplicate according to A.O.A.C. methodology (1984). Total protein, lipid, ash and moisture contents for minced, pressed and refined flesh and surimi were determined. Ten g samples (sols) were homogenized with 90 ml of water and the pH was measured using a Model M240 Digital pH Meter (Corning Science Products, Corning, NY).

Protein Extractability

Protein Soluble in 0.6M KCl

Protein was extracted according to Jiang et al. (1985a,b) with some modifications. Twenty g of sample (sol) were homogenized with 180 mL of chilled 0.6M KCl solution (pH 7.2) with a Kinematica CH-6010 Kriens-LU Homogenizer (Brinkman Instruments, Co., Westbury, NY) with the speed set at 4, for two consecutive periods of 2 min each. An ice bath was used to prevent heating of the protein solution during homogenization. The solution container was filled near to the rim and fitted with parafilm M (American Can Co., Greenwich, CT) to prevent foaming during extraction. The homogenate was centrifuged at 10,000 rpm at 0°C for 30 min. The supernatant was brought to 300 mL with chilled 0.6M KCl. Protein concentration was determined by the Biuret method (Gornall et al, 1979) using bovine serum albumin as a standard. Extractability was expressed as mg protein/g of sample. Three replicate extractions of each treatment were carried out.

Actomyosin

Actomyosin was extracted using the method described by Jiang et al. (1985ab) with some modifications. Twenty g of samples (sols) were homogenized with 180 mL of chilled 0.6M

KCl solution (pH 7.2) with a Kinematica CH-6010 Kriens-LU Homogenizer (Brinkman Instruments, Co., Westbury, NY) with the speed set at 4, for two consecutive periods of 2 min each. An ice bath was used to prevent heating of the protein solution during homogenization. The solution container was filled near to the rim and fitted with parafilm M (American Can Co., Greenwich, CT) to prevent foaming during extraction. The homogenate was centrifuged at 10,000 rpm at 0°C for 20 min. Actomyosin was precipitated from the supernatant by diluting with three volumes chilled distilled water and collected using the centrifuge operating conditions described above. The residue was then redissolved in a equal volume of 1.2M KCl solution and stirred at 0°C for 30 min using a magnetic stirrer. Insoluble residue was separated by centrifugation (10,000 rpm for 20 min at 0°C) and the supernatant diluted to 300 mL with 0.6M KCl solution. Protein concentration was determined by the Biuret method (Gornall et al., 1979). Extractability was expressed as mg of actomyosin/g of sample. Three replicate extractions for each treatment were carried out.

Determination of Enzyme Activity

Ca++-ATPase Activity of Actomyosin

ATPase activity was determined using the method described by Jiang et al. (1985b) using the 6 min incubation period recommended by Scott et al. (1988), to achieve measurable levels of released inorganic phosphate. To 1 mL of actomyosin solution (1-5 mg/mL), 0.5 mL 0.5M Tris-maleate buffer (pH 7.0), 2.2 mL 2.0M KCl, 0.5 mL 0.1M $CaCl_2$, and 5.3 mL distilled water were added in order. Finally, 0.5 mL of 20mM Na-adenosine 5'-triphosphate (Na-ATP) (Sigma Chemical Co., St Louis, MO) at pH 7.0 was blown into solution. The reaction mixture was incubated for 6 min at 25°C. The reaction was terminated with the addition of 5 mL of 15% trichloroacetic acid. Inorganic phophorous was determined using the method of Bartlett (1959). Total Ca++-ATPase activity was expressed as uM Pi/min/mg actomyosin.

Proteolytic Activity

Proteolytic activity in surimi sols was determined by the autolysis procedure outlined by Groninger et al. (1985). Four 3.0 g samples of each sol were weighed into 25 mL Corex tubes. To each 3.0 mL of cold (2-4°C) 0.1M NaCl were added and mixed thoroughly with a stirring rod. At exactly 10 min after the initiation of sol preparation, 3 of the samples

were incubated in a water bath with agitation at 60°C for 30 min. The remaining sample (control) was held in ice. At the end of the incubation period 6.0 mL of cold (2-4°C) 10% TCA solution was added to each tube to terminate the reaction. The trichloroacetic acid treated mixtures were held for 30 min at 2-4°C, then filtered through Whatman No.1 filter paper. The quantity of tyrosine in each filtrate was determined using the Folin and Ciocalteu phenol reagent procedure described by Ceriotti and Spandrio (1957). Proteolytic activity was expressed as ug tyrosine released/min/g protein.

Determination of Sulfhydryl Groups

Total and reactive sulfhydryl groups were determined for actomyosin extracted from the surimi sols for each level of bromate employed. The method used was based upon the work of Ellman (1958) and Buttkus (1971). Modifications to the method with regard to the incubation of the reaction mixture were made based upon preliminary work during this investigation. Results were reported as mols/1 x 10^5 g actomyosin.

Total Sulfhydryl

To 0.5 mL of actomyosin solution (1-5 mg/mL), 2.4 mL of extraction solution (0.1M phosphate buffer pH 7.0, 6mM EDTA, 0.6M KCl, 8M urea) and 0.1 mL of the Ellman's reagent (Sigma Chemical Co., St Louis, MO)(0.004M 5,5'-dithiobis-2-nitrobenzoic acid in extraction solution) were added. After the reaction was allowed to proceed for 30 min at room temperature, the initial absorbance at 412 nm was determined and recorded. To calculate the corrected absorbance, two blanks were prepared as recommended by Hofmann and Hamm (1978); a dye blank (all the reagents but the sample) and a sample blank (all the reagents but the Ellman's reagent). The corrected absorbance was computed by subtracting the absorbance of the two blanks from the initial absorbance. The corrected absorbance and appropriate dilution corrections were divided by 13,600 (molar extinction coefficient) to obtain the molar concentration (Ellman, 1958).

Reactive Sulfhydryls

To 0.5 mL of actomyosin solution (1-5 mg/mL), 2.4 mL of extraction solution (0.1M phosphate buffer pH 7.0, 6mM EDTA, 0.6M KCl) and 0.1 mL of the Ellman's reagent (0.004M 5,5'-dithiobis-2-nitrobenzoic acid in extraction solution) were added. The reaction was allowed to proceed at room

temperature for 30 min. Reactive content was determined using procedures identical to those described for total sulfhydryls.

Estimation of Disulfide Bond Formation in Heat-Set Gels

The effect of the addition $KBrO_3$ (0.025% and 0.05%) on the promotion of disulfide bond formation during heat-setting of sols was evaluated by measuring the quantity of disulfide bonds reduced in heat-set gels by sodium borohydride. Ten g of gel (with and without bromate) was extracted with 90 mL of the extraction solution used in the determination of total sulfhydryl content. Gels were homogenized for 2 min with a Kinematica CH-6010 Kriens-LU Homogenizer (Brinkman Instruments, Co., Westbury, NY) with the speed set at 4. The homogenate was centrifuged at 10,000 rpm for 10 min at 0°C. The total sulfhydryl content of supernatants from gels heat-set with and without $KBrO_3$ were determinated and computed as previously described. The percentage of reduction in total sulfhydryl content was used as an estimate of the effect of $KBrO_3$ on the oxidation of sulfhydryl groups to disulfide functions during heat-setting. The relative degree of disulfide bond formation in gels heat-set with and without $KBrO_3$ was estimated by treating extracts with $NaBH_4$. To 5 mL of extract (7 mg protein/ml), 4 mL of 0.6M $NaBH_4$ in 8M urea and 2 drops of octylalcohol (to prevent foaming) were added.

After the mixture was allowed to stand for 2 hr, excess reductant was destroyed by the addition of 1.1 mL of 2N HCl. The pH of the reaction mixture was brought to neutrality with 0.9 mL of 2N NaOH. Total sulfhydryl was determined and computed as previously described. After consideration of dilution factors the recovery in free sulfhydryl was computed.

Viscosity

Viscosity was determined using a homogenate of 40 g of surimi sol in 160 ml of 3% NaCl solution (0-4°C). The homogenate was prepared with a Kinematica CH-6010 Kriens-LU Homogenizer (Brinkman Instruments, Co., Westbury, NY) in a 200 mL container chilled in an ice bath and fitted with parafilm M (American Can Co., Greenwich, CT) to prevent foaming. The mixture was homogenized at speed 4 for 8 min. The homogenate was transferred to a 250 mL beaker and the viscocity measured using a Model RTV 100 Brookfield Viscometer (Brookfield Engineering Laboratories, Stoughton, MA) operated at speed of 10 rpm and equipped with a No. 3 spindle. The viscometer readings were converted to centipoise units using a factor appropriate for the spindle.

SDS-Polyacrylamide Gel Electrophoresis

Sample Preparation

To 4 g of gel, 43 mL of extraction solution (same as that used for total sulhydryl determination) and 1 mL of 2-mercaptoethanol (ME) (Bio-Rad Laboratories, Richmond, CA) were added. The mixture was homogenized for 2 min with a Kinematica CH-6010 Kriens-LU Homogenizer (Brinkman Instruments, Co., Westbury, NY). This treatment completely solubilized gels from all treatments. To 1 mL of this solution (10 mg prot/ml), 3 ml of sample buffer solution (2.5 ml distilled water, 2.5 mL 1M phosphate buffer pH 7.2, 0.8 ml glycerol, 1.6 mL 10% sodium dodecyl sulfate, 0.4 mL 2-mercaptoethanol and 0.05% bromophenol blue) were added and the whole mixture was boiled for 3 min. The final total protein concentration was adjusted to 2.25 ug/uL. A protein standard containing myosin, beta-galactosidase, phosphorylase b, bovine serum albumin and ovalbunin, with their corresponding molecular weights of 200,000, 116,000, 97,400, 66,200 and 42,699 daltons respectively, (Bio-Rad Laboratories, Richmond, CA), was diluted 1:100 with sample buffer solution and also boiled for 3 min.

Gel Preparation

Polyacrylamide gel electrophoresis (PAGE) using a dissociating buffer system (sodium dodecyl sulfate) (SDS) was performed according to Weber and Osborne (1969) using a Bio-Rad Model 150A tube gel apparatus (Bio-Rad Laboratories, Richmond, CA). All chemicals were from Bio-Rad Laboratories (Richmond, CA). A 5 percent gel was prepared by mixing the reagents in the following proportions: 10 ml of SDS-phosphate buffer pH 7.2 (7.8 g $NaH_2PO_4.H_2O$ + 18.6 g Na_2HPO_4 + 2 g SDS, in 1000 mL final volume), 4.5 mL of acrylamide solution (22.2 g acrylamide + 0.6 g NN´methylene-bis acrylamide in distilled water to a final volume of 100 mL), 1 mL of ammonium persulfate solution (35 mg in 10 mL of distilled water prepared just before use), 0.032 mL of N,N,N´,N´-tetramethylethylenediamine (TEMED), and 4.5 mL of distilled water. All the composite gel solutions except TEMED were mixed and degassed for 5-10 min. After degassing, TEMED was added and immediately the gel solution was carefully transferred into the gel tubes (12 cm length X 5 mm ID) with a Pasteur pipet. A few drops of water were placed on the top to produce a flat surface and prevent dehydration and gel cracking.

Sample Load and Running Conditions

After complete polymerization of the gels, 30 microliters of high range SDS-PAGE molecular weight standard solution (Bio-Rad Laboratories, Richmond, CA), and 50 microliters of sample (122 ug) were applied to the top of the gels. Electrophoresis was performed at room temperature at 3 mA per tube for 20 min after which the current was increased to 8 mA per tube for approximately 6 hrs using a Model No. 3-1014A Regulated Power Supply Unit (Buchler Instruments, Fort Lee, NJ). The gels were removed from the tubes and placed into a fixing solution (25% propanol, 10% glacial acetic acid) for 3 hrs. Gels were then stained at room temperature for 12 hrs with a 0.01% Coomassie Blue R-250 solution. The gels were rinsed and destained in a solution of 10% acetic acid and 5% methanol. After 72-96 hours, sufficient destaining was achieved and the gels were stored in a 7.5% glacial acetic acid solution.

Qualitative Analysis of Gels

The resulting gels were analyzed by comparing the densitometric tracings of the protein bands using a Model 1020A Quick Scan Densitometer (Helena Laboratories, Beamount, TX). The approximate molecular weights of protein bands were calculated as described by Weber and Osborn (1969).

Statistical Analyses

Statistical analyses were carried out using ANOVA and simple regression analysis with the STAT Plus Program, a general statistics package for the Apple II/IIe computers (Madigen and Lawrance, 1984). Duncan's Multiple Range Test (Bruning and Kintz, 1977) was used for multiple comparison of means. A 2 X 8 factorial design (Petersen, 1985) was used to evaluate interaction between heat-setting treatment and $KBrO_3$ levels. A level of significance of 5% was used to test the significance of all statistics.

RESULTS AND DISCUSSION

Processing Pacific Whiting into Surimi

Pacific whiting was processed into surimi for investigational purposes taking into consideration three different, but highly correlated aspects. The process was designed to (1) prepare and maintain a highly functional surimi; (2) achieve maximum yield; and (3) use a minimum amount of water for washing that was consistent with producing a high quality washed and pressed flesh.

Machine deboning of manually separated skin-free fillets from round whiting yielded 31.6% minced flesh (Table 3). Minced flesh yield was slightly lower than those reported by Crawford et al. (1979) (35.5%), Pacheco-Aguilar et al. (1988) (32.42%) and Chang-Lee (1988) (34.7%) using mechanical separation of flesh from manually separated planks (fish musculature with bone less head, tail, backbone and viscera). Manual separation of skin-free fillets in this investigation was more labor-intensive, but allowed a better means of culling fish with myxosporidian infestation and soft texture. More efficient culling of fish and a less efficient separation of the musculature over planking contributed to the lowered observed yield of minced flesh.

Conventional surimi processing (Lee, 1984) requires an extensively washed flesh. This procedure results in a high quality surimi but produces low yields and considerable

water soluble waste materials. Water requirements for the washing unit operation in this investigation amounted to only 6 times the weight of minced flesh which was a reduction of 60% from the conventional process described by Lee (1984).

Yield of washed and pressed flesh (25.84%) (Table 3) was superior to those obtained by Chang-Lee (1988) (21.2%) under the same washing conditions and Pacheco-Aguilar (1988) (24.5%) using half the amount of water and only one washing exchange. Refining recovered 93.53% of the washed and pressed flesh.

Table 3. Yield and composition of Pacific whiting flesh through processing into surimi.

		Flesh			
	Round Fish	Minced	W/P[1]	Refined	Surimi
Weight (Kg)	435.27	137.55	112.49	95.39	104.03
Yield (% round)	100.00	31.60	25.84	21.92	23.89
Adjusted Yield[2]	100.00	31.60	25.84	24.17	26.35
Moisture (%)		82.85	85.99	86.05	78.62
Protein (%)		16.24	14.06	13.34	12.52
Lipid (%)		0.64	0.66	0.71	0.67
Ash (%)		1.08	0.28	0.23	0.50

[1]Washed and pressed flesh.
[2]Adjustment of refined and surimi yield accounts for 23.1 lb pressed not refined.

Recovered refined flesh yielded 26.35% surimi (containing 8% cryoprotectant and 0.3% condensed phosphate) based upon round fish weight (Table 3). This was superior to yields (22%) reported by Thrash (1983) for conventional surimi processing. It is believed that elimination of soft

and infected fish during processing played a role in the high surimi yield observed by reducing solids losses during washing and dewatering operations. Surimi composition (78.62% moisture; 12.52% protein) was consistent with that observed by Chang-Lee (1988) using a similar processing protocol.

Interaction of Potassium Bromate with Surimi Sol Proteins

Introduction

Pacheco-Aguilar et al. (1988) reported that Pacific whiting lacked strong gel forming ability, regardless of the degree to which its flesh was washed. A myxosporidian-induced proteolysis in whiting muscle has been blamed for this defect (Kabata and Whitaker, 1986, 1981; Erickson et al. 1983; Patashnik et al., 1982; Tsuyuki et al., 1982) even though the exact source of the proteinase activity in muscle has not been completely established (Tsuyuki et al., 1982).

Several additives in formulation of whiting surimi sols have been used to achieve stronger heat-induced gelation. Addition of dry egg white (2.5-5.0%) alone or in combination with potato starch (5.0%) has resulted in SA or AA grade gels using Japanese standards based on the folding test (Chang-Lee, 1988; Pacheco-Aguilar et al., 1988). Both egg white (Holt, et al., 1984; Matsuda et al., 1981; Shimada and

Matsushita, 1980; Siegel et al., 1979) and potato starch (Wu, et al., 1985) were able to induce gelation under the formulation and heating conditions used in these investigations and improve the textural characteristics (Iso, et al., 1985; Tanikawa et al., 1969; Lee and Kim, 1985; Yamashita and Yoneda, 1985). The improved gel forming ability of such surimi sols containing additives was not achieved through an improvement of the functional properties of the fish protein system, but the result of a filling and re-enforcing effect provided to the whole system by the gelation properties of both additives.

Schmidt (1981) observed that the intact myosin molecule is required to produce strong and elastic gels and that such characteristics are affected by the degree of intra- and inter-strand cross-linking. Therefore, maintaining the original molecule size of myosin (heavy chain) is the foremost concern. The proteinase activity present in whiting muscle breaks down the myosin molecule rendering smaller polypeptides that are unable to form enough intra- and inter-strand cross-linking to support gel formation.

Once the infected fish is dead (Erickson et al., 1983; Patashnik, et al., 1982; Willis, 1949) muscle degradation is initiated. Further proteolysis is promoted during the heat treatment involved in the manufacturing of surimi-based gel type products. Muscle proteolysis appears to be an intrinsic characteristic of Pacific whiting muscle and surimi produced from its washed flesh. Focus on means for

maintaining the myosin molecule through heat-induced gelation would appear to be a logical means of achieving required gel strength characteristics. Two approaches can be proposed to achieve this goal: (1) employ an oxidizing agent to promote the formation of disulfide bonds (covalent) among intact myosin molecules and/or polypeptides resulting from proteolysis; and (2) a biochemical modification of the surimi system through the use of inhibitor of the enzyme(s) responsible for the degradation of the myosin molecule.

Employing an oxidizing agent could promote the improvement of gelation capacity of the surimi system in four different, but highly correlated, mechanisms of action. (1) The addition of an oxidizing agent to surimi during sol formation could promote disulfide formation among polypeptides (products of proteolysis) and increase chain length. These longer polypeptides, could form more complex linear polypeptide aggregates during sol formation and participate in gelation upon heat-setting. (2) An oxidizing agent would promote a highly cross-linked three-dimensional network through a strengthening of disulfide bond formation during heat induced gelation. (3) Enzyme(s) activity could be inhibited by precluding the formation of an enzyme-substrate complex through steric effects produced by the formation of higher molecular weight aggregates during sol formation. (4) Direct inactivation could be accomplished through the oxidation of the sulfhydryl group present at the active site on the enzyme.

Investigations were carried out to determine the effect of $KBrO_3$ on the solubility and chemistry of the myosin molecule. Potassium broamte was added to prepared (2-4°C) sols of surimi at the 0.00, 0.025, 0.050, 0.100, 0.150 and 0.200% levels. Analysis was completed within 2 hrs after $KBrO_3$ was introduced into the sol. Sols were maintained at 2-4°C during this period.

Protein Extractability

It was assumed that added $KBrO_3$ would promote the oxidation of sulfhydryl groups to disulfide bonds to form larger protein aggregates in the sol and reduce the extractability of sol proteins. Extractable salt soluble protein ($r = -0.9862$) and actomyosin ($r = -0.9685$) were reduced in a linear manner directly related to the $KBrO_3$ concentration in the sol at (2-4°C) (Table 4). Results did not mean that $KBrO_3$ reduced protein extractability, since protein was already in the sol form. It was clear that no heat was required for the onset of the oxidation of sulfhydryl groups by bromate to disulfide functions, since a weak elastic gel was observed after 2-3 hrs at refrigerated temperature before heat-setting. The extractability of actomyosin was more affected than salt soluble protein because of its higher sulfhydryl content. The greater reduction in extractability with respect to $KBrO_3$ concentration for actomyosin ($m = -516$) in comparison to

salt soluble protein (m = -386.7) agrees with the observations of Hofmann and Hamm (1978) that more than 95% of the sulfhydryl content of myofibrils is located in the actin-myosin system. Actomyosin is considered to be the protein group responsible for the gel strength, or ashi, of minced fish products (Jiang et al., 1987; Jiang and Lee, 1985).

Viscosity

Viscosity is defined (Johnson, 1988) as a measure of the internal friction or the resistance of a fluid to movement. A reduction in the viscosity of fish protein homogenates has been reported to be a good measure of the aggregation of fish muscle proteins during frozen storage (Kim et al., 1986; Borderias et al., 1985; Groninger et al., 1983; Matsumoto, 1980). Hydrophobic, hydrophilic, electrostatic and covalent interactions among protein chains with a subsequent reduction in the number of bonds between the proteins and the medium have been proposed as being involved in aggregation. The formation of disulfide bonds was suggested by Kotodziejska and Sikorski (1980) to be a likely aggregation mechanism.

The viscosity of whiting surimi sol homogenates was assumed to be an indicator of the starting linearity and length of the protein molecules in solution. It was hypothesized that protein aggregate formation through the

oxidative action of $KBrO_3$ would disrupt linearity by forming sphere-like aggregates. This transformation would be reflected by a reduced viscosity of surimi sol homogenates. Observed viscosities for sols containing varying levels of homogenate agree with the hypothesis (Table 4.). The initial increase in viscosity of sols containing 0.025 and 0.050% $KBrO_3$ over the control (0.000% $KBrO_3$) indicated that low levels induced a form of protein aggregation that enhanced linearity. This may have been accomplished through the aggregation of short polypeptides generated by proteinase activity. This transformation was optimized at 0.025% $KBrO_3$. Potassium bromate levels of 0.050% and 0.100% reduced ($P<0.05$) viscosity from the highest viscosity level observed at 0.025% $KBrO_3$. Viscosity did not vary ($P>0.05$) for sols containing $KBrO_3$ levels of $\geq$ 0.100%. The degree of aggregation possible at (2-4°C) that was reflected in viscosity measurements appeared to be completed at a $KBrO_3$ level of 0.100%.

Ca^{++}-ATPase Activity

Sulfhydryl groups in the globular head of the myosin molecule have been reported (Taylor, 1972; Young, 1969) to be involved in the ATPase activity of the actomyosin complex. According to Seki and Hasegawa (1978), the loss of Ca^{++}-ATPase activity is due to a modification of the actin-myosin complex through the oxidation of sulfhydryl

Table 4. Effect of varying levels of $KBrO_3$ on the protein extractability, sulfhydryl status, viscosity and Ca^{++}-ATPase activity of surimi sols at 2-4°C.

$KBrO_3$ Level (%)	SSP[1,7]	AM[2,7]	T-SH[3,7]	R-SH[4,7]	Viscosity[5,7]	ATPase[6,7]
0.000	117.45[g] (5.3)	105.22[g] (10.4)	3.72[g] (.08)	2.62[g] (.05)	3770[g] (42.4)	11.25 (1.34)
0.025	108.54[g] (6.9)	77.02[h] (4.4)	3.23[h] (.09)	2.08[h] (.06)	5415[h] (91.9)	2.50 (3.53)
0.050	109.90[g] (7.4)	77.90[h] (6.2)	3.07[hi] (.06)	1.99[h] (.02)	4220[i] (14.1)	0.00
0.100	85.95[h] (8.5)	38.45[i] (3.9)	2.92[i] (.08)	2.04[h] (.06)	3430[j] (113.1)	0.00
0.150	66.53[i] (3.5)	7.08[j] (.35)	2.43[j] (.06)	1.55[i] (.02)	3395[j] (120.2)	0.00
0.200	39.73[j] (3.0)	6.23[j] (.46)	1.90[k] (.04)	1.30[j] (.02)	3330[j] (42.4)	0.00

[1]Salt soluble protein (mg/g sample), n = 3.
[2]Actomyosin (mg/g sample), n = 3.
[3]Total sulfhydryl (mol SH/1x10[5]g actomyosin), n = 2.
[4]Reactive sulfhydryl (mol SH/1x10[5]g actomyosin), n = 2.
[5]Viscocity (Cp), n = 2.
[6]Ca^{++}-ATPase activity (uM Pi/min/mg actomyosin), n = 3.
[7]Significance ($P<0.001$).
Figures in columns with different exponent letter varied ($P<0.05$).
() = S.D.

groups. Complete ATPase inactivation was reported to occur if seven sulfhydryl groups per molecule were oxidized. The inactivation of Ca^{++}-ATPase by $KBrO_3$ in this investigation demonstrated its involvement in the oxidation of sulfhydryl groups to disulfide bonds in surimi sols (Table 4). ATPase activity in sol containing 0.025% $KBrO_3$ was 22.2% of the control and completely inactivated in sols containing 0.050%. It was concluded from these results that ATPase was

inactivated through the oxidation of sulfhydryl groups of myosin by $KBrO_3$.

Status of Actomyosin Total and Reactive Sulfhydryl Groups

The total sulfhydryl content of 3.72 mols/l x 10^5 g actomyosin determined in whiting surimi sols, although determined by a different procedure, was well below the level reported by Hofmann and Hamm (1978) for fish (6.7 mols/l x 10^5 g actomyosin). This apparent low level of total sulfhydryl was a function of either an intrinsic property of whiting muscle actomyosin or the 6 months storage (-34°C) period that surimi was subjected to prior to the investigation. The loss of sulfhydryl function in fish muscle proteins through oxidation has been documented by Jiang et al. (1986), Kotodziejska and Sikorski(1980), and Buttkus (1970).

Under mild conditions, sulfhydryl functions can undergo chemical oxidation to disulfides through an ionic mechanism (Cecil and McPhee, 1959). In this investigation, both the total ($r = -0.9795$) and reactive ($r = -0.9335$) sulfhydryl content of actomyosin in surimi sols was shown to decrease in a linear manner with respect to $KBrO_3$ content (Table 4). The probability of significant further oxidation of the newly formed disulfide bonds by $KBrO_3$ to yield sulfinic (RSO_2H) and/or sulfonic (RSO_3H) acids (Cecil and McPhee,

1959) was considered to be low based upon the extractability and viscosity observed for surimi sols.

The interaction of $KBrO_3$ with whiting surimi sols reduced salt-soluble protein, soluble actomyosin content, total and reactive sulfhydryl content, viscosity and Ca^{++}-ATPase activity in a concentration dependent manner at 2-4°C. These results were consistent with the promotion of the oxidation of sulfhydryl groups to disulfide functions by $KBrO_3$. It is believed that this formation of disulfide bonds during sol preparation at low temperatures coupled with the strengthening of a similar reaction during heat-setting will result in longer polypeptide chains resulting in a stronger and more continuous and ordered gel network.

Oxidation of Free Sulfhydryl Groups on Surimi Proteins to Disulfide Bonds

Oxidation of Salt Soluble Proteins by Heat

To clarify the participation of sulfhydryl groups in heat-induced gelation, the effect of varying heating temperatures up to 80°C on the sulfhydryl status of salt-soluble protein (6 mg/mL) from Pacific whiting surimi was examined. The oxidative status of the sulfhydryl content was estimated from change in determined total and reactive contents observed after heating.

Incubation of salt-soluble proteins at 40°C for 30 min resulted (Table 5) in a reduction of the total sulfhydryl content to 97.15% of that observed at 20°C. Protein unfolding liberated inner sulfhydryl functions increasing reactive sulfhydryl content. The total sulfhydryl content distribution to inner, reactive and oxidized functions was altered from a ratio of 1.000:1.089:0.000 (inner:reactive:oxidized) at 20°C to a ratio of 1.000:2.491:0.102 at 40°C. Heating at 60°C produced a total sulfhydryl content that was 83.33% of the original, but maximized protein unfolding (Ziegler and Action, 1984) and freed a sufficient number of inner sulfhydryl functions to alter the inner:reactive:oxidized ratio to 1.000:33.012:6.804. Unfolding of proteins yielding surface reactive sulfhydryl functions was favored over oxidation to disulfide bonds at 60°C. Incubation at 80°C reduced the total sulfhydryl content to 59.10% of that observed at 20°C (100%). Oxidation of sulfhydryls to disulfide bonds was favored at 80°C and an inner:reactive:oxidized distribution ratio of the total sulfhydryl content of 1.000:4.412:3.745 was observed. Proteins underwent a degree of aggregation sufficient to enhance the inner sulfhydryl content of the proteins. Temperatures >60°C appeared to be required for a major degree of oxidative transformation of sulfhydryl functions to disulfide bonds.

The observed heat induced changes in the determined content and composition of sulfhydryls of salt soluble

proteins support their contribution to gel formation through oxidation to inter-polypeptide disulfide bonds. The transformations of sulfhydryl functions of salt soluble proteins in Pacific whiting surimi observed in this investigation agreed with those reported by Itoh et al. (1979a) for salt soluble proteins from pollock surimi. In addition, Opstvedt et al. (1984) reported a linear decrease in the content of sulfhydryls in ground fillets of frozen Alaska pollock upon heating at temperatures from 50 to 115°C with a concomitant increase in the disulfide bond content.

Table 5. Relative sulfhydryl status (%) of salt soluble proteins from Pacific whiting surimi sols incubated[1] at various temperatures.

Sulfhydryl content distribution	Initial composition	Incubation Temperature (°C) 40	60	80
Total[2,3]	100.00	97.15	83.33	59.10
Oxidized	0.00	2.85	16.67	40.90
Reactive	52.13	69.32	80.88	48.18
Inner	47.87	27.83	2.45	10.92

[1]30 min.
[2]Initial total sulfhydryl = total + oxidized sulfhydryl.
[3]Initial total sulfhydryl = oxidized + reactive + inner sulfhydryl.

Sulfhydryl Recovery with Thiol Reducing Agents

Sulfhydryl groups and disulfide bonds play important roles in functional properties of proteinaceous foods. Disulfide cross-linking in proteins may occur through the oxidation of sulfhydryl groups and by sulfhydryl-disulfide interchange (Opstvedt, et al., 1984; Sawyer, 1969).

Investigators (Jiang et al., 1986; Itoh, 1979a,b,c,d; Tabata et al, 1976) have reported that the reductants cysteine and 2-mercaptoethanol can improve the gel-forming ability of sols prepared from minced fish flesh and/or surimi. The mechanism proposed (Cecil and McPhee, 1959; Kuninori and Sullivan, 1968; Beveridge, et al. 1974; Wall, 1971) for this effect of cysteine and other sulfhydryl reagents (thiols) is through a sulfhydryl-disulfide interchange between cysteine and disulfide bonds present in a protein or a protein aggregate. The reaction between two disulfides, also favored by catalytic amounts of thiols, is essentially the same as a sulfhydryl-disulfide interchange (Koshland and Mozersky, 1964).

The effect of the reductants cysteine and 2-mercaptoethanol on heat oxidized (70°C for 30 min) salt soluble proteins (6 mg/ml) from Pacific whiting surimi was evaluated using SDS-PAGE. The formation of high molecular weight aggregates was observed by SDS-PAGE through the loss of myosin (Figure 3a and 3b) and the formation of higher molecular weight aggregates (Figure 3b). The relative amount (integration of peak area) of myosin in the oxidized protein solution (Figure 3b) was only 12% of that in the unheated protein solution (Figure 3a).

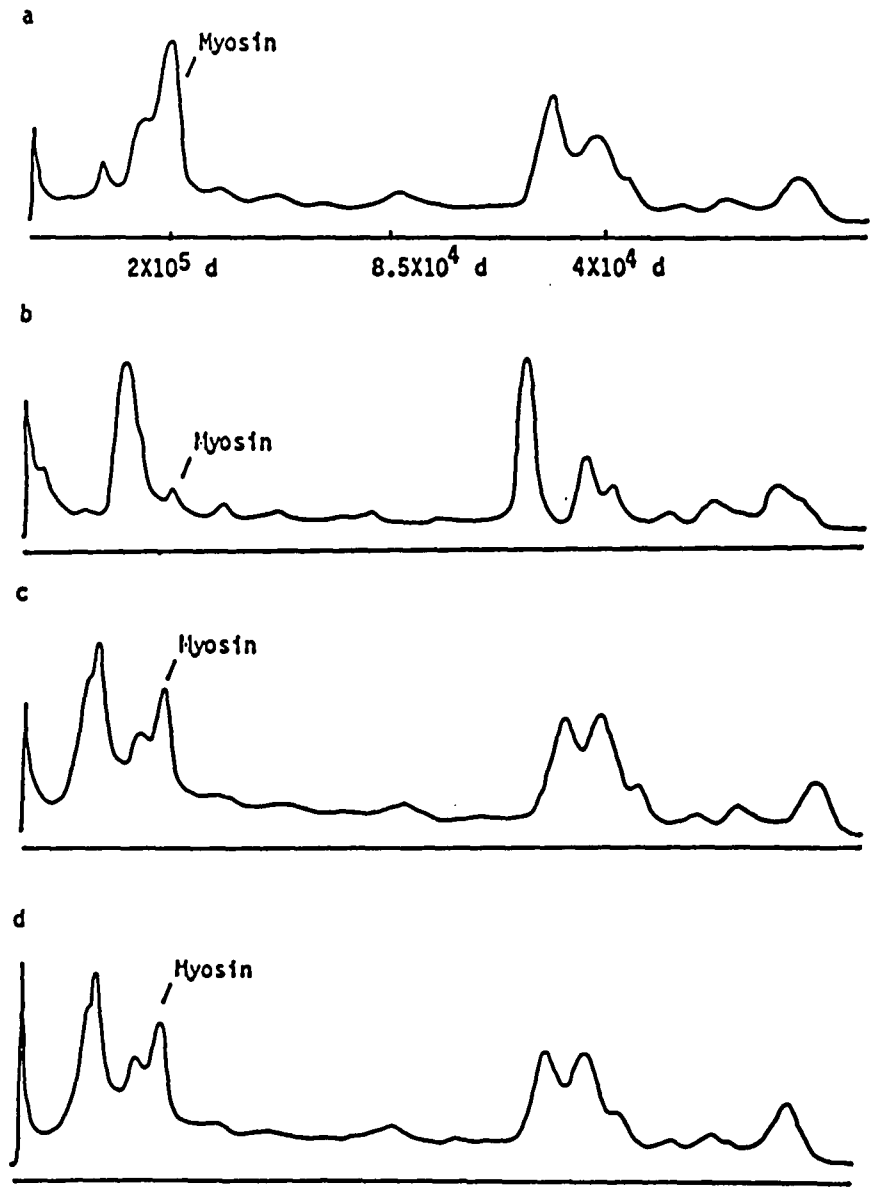
Some of the protein aggregation was assumed to be a result of disulfide cross-linking among protein chains. Koshland and Mozersky (1964) reported that the addition of cysteine and 2-mercaptoethanol (0.1% wt./vol.) to an

oxidized solution of protein promoted a sulfhydryl-disulfide interchange. They assumed that the interchange produced mixed disulfides (cysteine-SS-protein or 2-mercaptoethanol-SS-protein) and single chain polypeptides with free sulfhydryl groups breaking down the high molecular weight aggregates formed by heating. SDS-PAGE turned out to be a very useful tool to prove this hypothesis for a similar mode of action on heat oxidized salt-solubilized proteins from whiting surimi. Treatment of oxidized whiting surimi proteins with cysteine (Figure 3c) and 2-mercaptoethaonl (Figure 3d) (0.1% wt./vol.) yielded an estimated 62% recovery of myosin base upon SDS-PAGE for both reductants. Incomplete recovery of myosin from higher molecular weight aggregates was either a function of too low of a thiol addition or the presence of heat induced covalent bonds other than disulfide.

Jiang et al. (1986) added cysteine to a surimi formulation and reported an increased recovery of sulfhydryl groups and higher levels of extractable actomyosin after sol formation. Sols of this surimi produced stronger heat-set gels. According to these investigators cysteine acted as a reducing agent and inhibited disulfide formation during frozen storage. The usefulness of cysteine as a "reducing agent" in surimi is doubtful, since the reactants (mainly the proteins) are not in solution. During the frozen storage of surimi, cysteine is more likely to act as a cryoprotectant (Suzuki, 1981) functioning as a chelator

Figure 3. SDS-PAGE of heated (30 min at 70°C) protein solutions treated with cysteine and 2-mercaptoethanol: (a) protein solution (control); (b) heated protein solution (oxidized); (c) oxidized solution + cysteine; (d) oxidized solution + 2-mercaptoethanol.

Figure 3



against certain metals such as Cu^{2+} and/or Fe^{3+} (Regenstein and Regenstein, 1984) or even as a direct antioxidant agent through a free radical mechanisms (Cecil and McPhee, 1959). All these functional capabilities of cysteine during frozen storage would limit protein aggregation. The results observed by Jiang et al. (1986) could be explained through the combined effects of cysteine for inhibiting protein aggregation during frozen storage and reducing sulfhydryl bonds and/or promoting sulfhydryl-disulfide interchange during salt solubilization and heat-set gelation of the surimi sols. The latter effect was supported by the observation of Itoh et al. (1979c) that cysteine addition to sols results in the formation of mixed disulfides by sulfhydryl-disulfide interchange. This enhanced interchange between protein molecules improved the gel forming ability of fish meat during heat-induced gelation.

Sulfhydryl Recovery with Ascorbic Acid

Ascorbic acid and its sodium salt are presently used in a variety of food systems to improve functional, nutritional, and/or organoleptic characteristics (Borenstein, 1987; Liao and Seib, 1987; Tompkin et al., 1978; Bauernfeind and Pinkert, 1970; Bauernfeind, 1953). It can function as a reducing agent, antioxidant and/or a metal sequestering agent in specific food substrates (Borenstein, 1987). Antioxidant capabilities are associated with its

ability to scavenge oxygen and protect double bonds. It decreases the oxidation state of many metals, reducing catalytic activity. Ascorbic acid is a moderately strong reducing agent (Bauernfeind and Pinkert, 1970). If added to a particular medium, it lowers the oxidation potential. This reaction frequently protects other compounds sensitive to oxidation through the preferential oxidation of ascorbic acid.

Borenstein (1965) and Bauernfiend and Pinkert (1970) observed that one of the roles that ascorbic acid fulfilled as antioxidant was to maintain sulfhydryl groups in their reduced form. Jiang et al. (1986) and Itoh et al. (1979d) reported that ascorbic acid improved the gel-forming ability of fish meat and surimi. The oxidation-reduction action of ascorbic acid on sulfhydryl function and disulfide bonds of salt soluble protein from Pacific whiting surimi sols was investigated. Ascorbic acid concentrations ranging from 0.25 to 3.00% at pH levels ranging from 5.5 to 8.0 were examined. Both unheated (control) and oxidized (heated 30 min at 70°C) protein solution (6 mg/ml) were treated with ascorbic acid.

The reducing power of ascorbic acid was highly dependent on concentration, solution pH and the condition of the protein substrate (oxidized or no heat-induced oxidation). Addition of ascorbic acid to control solutions (no heating) in concentrations from 0.25% to 1.00% reduced the total sulfhydryl content of soluble proteins at all

levels pH (Table 6). A degree of reduction by ascorbic acid was shown only for control solutions containing 2.00% at pH 6.0 and 3.00% at pH 6.0 and 6.5. No reduction was observed under more basic conditions at these two levels of ascorbic acid.

Table 6. Relative (%)[1] total sulfhydryl content of non-heated and heated salt soluble protein solutions treated with varying levels of ascorbic acid under a range of pH conditions.

	pH					
Treatment	5.5	6.0	6.5	7.0	7.5	8.0
Control (C)	82.4	65.6	85.1	96.9	100.0	94.3
C + 0.25% AA[2]	62.6	64.9	50.8	53.8	56.9	49.2
Oxidized (O)	36.6	30.5	31.7	33.2	35.5	36.6
O + 0.25% AA	40.8	27.5	29.0	29.8	35.5	33.6
Control	86.0	88.7	82.4	96.4	100.0	91.7
C + 0.50% AA	69.9	54.8	73.5	76.2	79.5	73.3
Oxidized	19.6	13.7	24.4	25.6	25.9	24.7
O + 0.50% AA	41.7	36.9	40.8	37.2	40.8	42.3
Control		85.8	93.3	82.2	100.0	90.9
C + 1.00% AA		71.5	71.1	77.1	79.1	75.9
Oxidized		34.4	41.1	37.2	38.3	37.2
O + 1.00% AA		44.7	51.8	53.0	54.9	54.2
Control		94.2	90.6	97.5	100.0	94.0
C + 2.00% AA		95.6	83.8	69.6	92.3	87.9
Control		90.3	88.3	93.4	100.0	79.6
C + 3.00% AA		97.7	99.8	92.3	97.1	92.4

[1]Relative to the total sulfhydryl content of control protein solutions at pH 7.5.
[2]Ascorbic acid.

A reducing effect for ascorbic acid on oxidized solutions was only observed at pH 5.5 for a concentration of 0.25%. A reducing effect for ascorbic acid at levels >0.25%

was observed at all levels of pH. Ascorbic acid concentrations in the oxidized solution >1.00% were not investigated, since it was assumed that a similar reducing action would be present.

The results strongly suggest that the reducing role of ascorbic acid in maintaining sulfhydryl groups in the reduced form is not by a direct reduction of disulfides, but through an indirect action involving any of the mechanisms previously described. Ascorbic acid displays strong reducing properties only in an acidic medium where the enediol group is present (Bauernfeind, 1953). The observation that ascorbic acid only exerted a reducing effect on control whiting surimi protein (not heated) solutions containing 2.00% ascorbic acid at pH 6.0 and solutions containing 3.00% ascorbic acid at pH 6.0 and 6.5 was compatible with the chemical properties of ascorbic acid. When ascorbic acid was added to control protein solutions (unheated), it was oxidized by the dissolved oxygen in the system to dehydroascorbic acid. Reduction was observed only at sufficiently high concentrations of ascorbic acid to allow a strong enough redox potential after dissolved oxygen was depleted and at pH levels low enough to maintain residual ascorbic acid in the enediol form. Under conditions of pH and concentration not favoring reduction, ascorbic acid was oxidized to dehydroascorbic acid and oxidized sulfhydryls to disulfide function reducing the total sulfhydryl content of protein solutions.

When ascorbic acid was added to oxidized protein solutions (heated), where dissolved oxygen was largely depleted, reduction occurred at lower concentrations of ascorbic acid and under more basic conditions than control solutions (not heated). The redox potential of solutions containing 0.25% ascorbic acid were only sufficiently strong enough at pH 5.5. However, at concentrations $\geq$0.50% the redox potential was sufficient up to pH 8.0 to reduce disulfide functions to free sulfhydryl groups. The degree to which sulfhydryl groups were recovered from oxidized protein by ascorbic acid was controlled by the concentrations of sulfhydryl groups and dehydroascorbic acid that were generated. Produced dehydroascorbic acid would oxidize sulfhydryls and recovered sulfhydryl groups could reduce formed dehydroascorbic acid to ascorbic acid (Bauernfiend, 1953) and form disulfide functions when the redox potential was sufficiently reduced.

Jiang et al. (1986) reported an increased recovery of reactive sulfhydryl groups, extractability of actomyosin and gelation capability with the addition of ascorbic acid (0.05-0.1%) to surimi formulations. The enhanced recovery of sulfhydryl groups and the increase in actomyosin extractability were explained by a direct reducing action of ascorbic acid on disulfide bonds. Based upon the evidence developed in this investigation, ascorbic acid more likely functioned through a combination of the following two mechanisms to promote stronger gel formation. Protein

aggregation during the frozen storage of surimi was retarded through a combination of the antioxidant and metal binding effects of ascorbic acid. During heat treatment of sols, residual ascorbic acid would have oxidized to dehydroascorbic acid and promoted the oxidation of sulfhydryl groups to disulfide bonds to form a stronger gel. This observation is supported by the stronger gel forming ability reported by Itoh et al. (1979d) for fish pastes containing added dehydroascorbic acid.

Heat and Potassium Bromate Induced Disulfide Formation in Gels

The formation of disulfide functions through heat-setting (90°C for 40 min) was examined through the determination of total sulfhydryl content in sols and gels. Gels containing 0.000, 0.025 and 0.050% $KBrO_3$ were investigated. The recovery of sulfhydryl groups from heat-set gels containing $KBrO_3$ was estimated after reduction with $NaBH_4$ (Jiang et al., 1988; Opstvedt et al., 1984; Hofmann and Hamm, 1978). Only gels with low levels of $KBrO_3$ (0.025 and 0.05%) were investigated since higher concentrations yielded gels that would not dissolve without the use of 2-mercaptoethanol.

The reduction in total sulfhydryl content in gels over their respective sols illustrated the promotion of disulfide formation by heat and the strengthening of this reaction by $KBrO_3$ (Table 7). Within the range of $KBrO_3$ levels

investigated, the degree of disulfide formation was concentration dependent. The marked reduction in total sulfhydryl content of gels containing $KBrO_3$ over gels not treated (control) was likely a result of two possible actions; (1) inactivation of proteinases during sol formation and (2) strengthening of the oxidation of sulfhydryl groups to disulfide function during heat-setting. Sols without added $KBrO_3$ (controls) produced gels of extremely low

Table 7. Relative (%)[1] total sulfhydryl content of heat-set gels containing varying levels of $KBrO_3$ before and after treatment with $NaBH_4$.

Treatment	Level of $KBrO_3$		
	0.000%	0.025%	0.050%
A[2]	87.7	-	-
B[3]	-	26.0	13.8
C[4]	-	46.9	25.1

[1]Relative to sol containing no $KBrO_3$ prior to heat-setting.
[2]Sol containing no $KBrO_3$ heat-set at 90 °C for 40 min.
[3]Sol containing indicated level of $KBrO_3$ heat-set at 90 °C for 40 min.
[4]Sol containing indicated level of $KBrO_3$ heat-set at 90 °C for 40 min then reduced with $NaBH_4$.

strength characteristic of the high residual proteinase activity intrinsic to Pacific whiting surimi. Conversely, sols containing $KBrO_3$ yielded relatively high gel strengths. Proteinase activity during heat-setting would have degraded sol proteins and precluded a more continuous interaction between proteins and diminish disulfide formation. The recovery of sulfhydryls from gels through reduction with

$NaBH_4$ further demonstrated that the loss of total sulfhydryl content with heating in the presence of $KBrO_3$ was a function of disulfide formation. The incomplete recovery of sulfhydryl content as compared to gels not treated with $KBrO_3$ was likely a function of the nonspecific reducing characteristics of $NaBH_4$ in this highly oxidized system.

Improvement of the Gel-Forming Ability of Pacific Whiting Surimi Proteins through Biochemical and Chemical Modification with $KBrO_3$

It is now widely accepted that a complete myosin molecule is necessary for attaining appreciable continuity in a food protein gel matrix (Taguchi et al., 1987; Acton and Dick, 1984; Samejima et al., 1969). Shimizu et al. (1983) and Taguchi et al. (1983, 1986) concluded that the difference in the gel forming ability of fish pastes prepared from various fish species was dependent upon the properties of their myosin content. Pacific whiting minced flesh and surimi lack heat gelling properties. It is probable that this is not an inherent property of the whiting myosin, but related to the very active proteinases found in its muscle after death and in washed flesh used to prepare surimi. It is believed that hydrolysis of myosin occurs mainly during heat-setting precluding the formation of a strong gel network.

Prior investigations provided experimental proof that sulfhydryl groups are directly involved in the

polymerization among monomeric myosin molecules or their products of hydrolysis through the formation of disulfide bonds. Results from these investigations provided useful information for focusing effort on the improvement of the gel-forming ability of Pacific whiting surimi through direct chemical and biochemical modification of the protein system.

The main objective of this investigation was to overcome the lack of gel forming characteristic of whiting surimi by protecting the myosin molecule against proteolysis and by restoring gel forming properties to proteinase modified myosin. The oxidative action of $KBrO_3$ at low concentrations was employed to achieve both proteinase inhibition and an intensification of disulfide bond formation during heat-setting to accomplish this objective. Potassium bromate is currently being used in the U.S. food industry to impart desired functional and organoleptic properties to foods (Miller and Spinelli, 1982). It is currently used in breadmaking to improve the physical properties of dough (Maningat et. al., 1988; Wu et. al., 1988;). Commercial applications to meat or fish products have not been reported.

Inhibition of the Proteinase Activity

The design of this investigation involved the assumption that Pacific whiting used to prepare surimi was infected to a degree common to the resource. While highly

parasitized whiting fillets exhibiting hair-like pseudocysts and/or mushy texture were discarded from the single lot of fish subjected to processing, it was assumed that flesh with a degree of parasitization was incorporated into surimi. It was considered likely that relatively few fish may have possessed very high levels of proteinase activity. The homogeneous distribution of these relatively small amounts, but highly active tissues, throughout the entire lot was assumed to be difficult. Equipment limitations precluded mixing of the entire lot of washed flesh prior to surimi preparation. In addition, the time frame and mixing action that would have been required may have damaged surimi proteins through mechanical action and excessively elevated temperatures. Regardless of these limitations, flesh was mixed very throughly during processing. Minced flesh from individual fillets was mixed during coarse grinding. Randomly selected 20 kg sublots of minced flesh and washed flesh subjected to one wash exchange were throughly mixed during washing. In total, 20 kg sublots were mixed in water twice. In addition, random sublots were mixed in a silent cutter during surimi preparation.

Proteinase activity in different 600 g sublots of the surimi confirmed activity variability (Table 8). The distribution of flesh with high proteinase activity within the surimi lot, to a degree demonstrated the incidence of infected fish. Considering the degree of mixing that occurred during washing and surimi formulations, the

observed variation demonstrates that proteinase activity is a highly variable characteristic of whiting surimi. The high level of proteinase activity observed was not surprising and corresponded to levels of activity for whiting surimi reported by Chang-Lee (1988). While the proteinase activity in unwashed minced flesh was not measured in this investigation, Chang-Lee (1988) reported that whiting flesh subjected to identical washing unit operations and refining contained 46.4 to 60.5%, respectively, of its original proteinase activity. The efficiency of removing proteinases from the minced flesh of Pacific whiting by washing appears to be roughly similar to that for other species of fish. Mikinodan and Ikeda (1971) reported that flesh of white croaker and lizard fish subjected to ordinary water bleaching retained about 70% of their respective proteinase activities.

Table 8. Proteinase activity variation in sublots surimi prepared from a single lot of Pacific whiting.

Sample number	Proteinase activity (ug tyrosine/g protein/min)
1	187.8 (6.7)[1]
2	259.5 (5.1)
3	248.1 (7.1)

() = S.D.
[1]n = 3 determinations/600 g sublot.

Incorporation of varying levels of $KBrO_3$ up to 0.075% into surimi sols inhibited proteinase activity in a concentration dependent manner (Table 9). Higher levels of

$KBrO_3$ did not produce significant ($P>0.05$) further inhibition of activity. Potassium bromate levels of $\geq 0.075\%$ inhibited 90-94% of the total proteinase activity observed under the conditions used for estimating activity. Miller and Spinelli (1982) reported 47% and 63% proteinase inhibition for 0.025 and 0.050% levels of $KBrO_3$, respectively, in ground whiting muscle. Groninger et. al. (1985) added 200 ppm (0.020%), 400 ppm (0.040%) and 500 ppm (0.050%) $KBrO_3$ to whiting surimi batter and, after incubation, found proteinase inhibition of 40%, 77% and 86%, respectively. Results from this investigation were very similar to those reported by Groninger et. al. (1985) considering the variability in activity possible. The difference from inhibition reported by Miller and Spinelli (1982) was most likely a function of the larger amount of substrate in unwashed ground whiting muscle subject to oxidation.

The observed proteinase inhibition by $KBrO_3$ can be explained by the following possible mechanisms, either functioning alone, or in combination: (1) direct inhibition through the oxidation of the cysteine residue at the active site of the proteinase; (2) indirect inhibition through covalent bond formation between sulfhydryl groups on the proteinase and a surimi protein; and (3) indirect inhibition of the proteinase through steric effects (formed protein aggregates would not form enzyme-substrate complexes). Potassium bromate was shown in this investigation to be

capable of oxidizing myosin (or polypeptides) sulfhydryl groups to disulfide functions and form higher molecular weight aggregates. It seems likely that the predominant mechanism by which $KBrO_3$ inhibits proteinases in whiting surimi was through oxidation of thiol active sites.

Based upon the level of inhibition achieved with $KBrO_3$ (90-94%), thiol proteinases would appear to be the predominant proteinase system in whiting surimi. This finding was supported by the observation of Miller and Spinelli (1982) that the enzyme producing the characteristic softening of whiting muscle was a thiol proteinase. These researchers observed inhibition with iodoacetate and N-ethylmaleimide, both sulfhydryl binding compounds (Hames and Rickwood, 1981), as well as with oxidizing agents such as potassium bromate, hydrogen peroxide and disodium and dipotassium phosphate peroxides.

Under the conditions used in this investigation to determine proteinase activity, complete inhibition of activity (6-10%) was never achieved even with the highest level (0.250%) of $KBrO_3$ employed. The lack of significant additional inhibition at $KBrO_3$ levels >0.075% supports the presence of other proteinases in whiting surimi with other than thiol active sites that were not inhibited by the oxidative action of $KBrO_3$.

Groninger et al. (1985) suggest that a second enzyme involved in the proteolysis of whiting muscle could indeed be a serine or alkaline proteinase. These researchers

Table 9. Effect of the addition of $KBrO_3$ on the inhibition of total proteinase activity present in surimi sols prepared from Pacific whiting surimi.

$KBrO_3$ (%)	Proteinase activity (ug tyrosine/min/g protein)[1,2]	Inhibition (%)
0.000	231.80 (38.5)[a]	0.00
0.025	63.77 (12.6)[b]	72.49
0.050	38.87 (10.6)[c]	83.23
0.075	23.47 (3.7)[cd]	89.87
0.100	22.10 (8.5)[cd]	90.47
0.150	13.13 (3.1)[d]	94.34
0.200	14.90 (4.5)[d]	93.57
0.250	14.43 (7.0)[d]	93.77

Regression of proteinase activity (y) in sol on $KBrO_3$ level (x): R^2 = .9517 (best fit = logarithmic (y=a+blnx)

[1]n = 3; [2]P<0.001.
Figures within column with same exponent letter did not vary (P>0.05).

postulated that its activity, which involves a nucleophilic attack of the peptide bond, could be promoted by the oxidizing conditions introduced by the addition of $KBrO_3$. Such an enzyme might be very active during heat processing at temperatures below 60°C even at $KBrO_3$ levels of 0.075%.

Groninger et. al. (1985) compared the proteinase inhibition effects of varying levels of $KBrO_3$ (200, 400, and 600 ppm) and egg white (1% and 3%) in surimi batters, both alone and in combination (3% egg white plus 500 or 600 ppm $KBrO_3$). These researchers determined that mixtures produced more effective inhibition of the enzyme and improvement of the functional properties of surimi. A 95% inhibition of the enzyme was observed when both mixtures were used against an 82% and 86% inhibition, respectively, when 3% egg white and 500 ppm (0.050%) $KBrO_3$ were employed alone. The

proteinase inhibition observed by Groninger et al. (1985) for egg white could have been accomplished by the following mechanisms, either alone, or in combination: (1) direct action of the egg white trypsin inhibitor on alkaline (serine) proteinase; (2) direct inhibition of thiol proteinases through a sulfhydryl-disulfide interchange between the thiol proteinase and ovalbumin; and (3) indirect inhibition of both proteinases (thiol and alkaline) by steric effects of high molecular weight aggregates of egg white and surimi proteins. This latter mechanism is supported by the observation of Matsudomi et al. (1986) that ovalbumin can participate in heat-induced aggregation with other proteins, such as fish myofibrillar proteins, by a sulfhydryl-disulfide interchange.

The enhanced proteinase inactivation observed by Groninger et. al. (1985) with the employment of egg white in combination with $KBrO_3$ seems likely to be a function of two mechanisms. The more rapid inactivation of thiol proteinases through a direct $KBrO_3$ oxidation of thiol active sites and development of steric effects through $KBrO_3$ induced aggregation between surimi proteins and/or egg white and surimi proteins.

The inhibitory effect of $KBrO_3$ on proteinases and the preservation of the myosin molecule was clearly illustrated by SDS-PAGE of whiting surimi sols and heat-set gels treated with SDS, 2-mercaptoethanol and urea (Figure 4a-4i). The electrophoresis of surimi sol proteins (Figure 4a) showed a

broad peak in the molecular weight range from 221,107 d to 161,000 d in which the myosin heavy chain (mol. wt. 200,000 d) was contained. The peak observed with a molecular weight higher than 200,000 d could be a result of covalent protein aggregation, other than by disulfide bond formation (i.e., formaldehyde and malonaldehyde mediated), occurring during the frozen storage of the surimi as proposed by Matthews et al. (1961), Laird et al., (1961), and Buttkus (1970). Peaks to the right of myosin may be polypeptides resulting from early hydrolysis taking place at low temperatures. Heat treatment (40°C/60 min followed by 90°C/20 min) of the sol containing no $KBrO_3$ resulted in the broad peak containing myosin to largely disappear (Figure 4b). In addition, a number of lower molecular weight peaks in the range 162,000 d to 92,000 d appeared as the result the intense proteolysis occurring during heat-setting.

The effect of addition of $KBrO_3$ to sol formulation was clearly demonstrated by the recovery of proteins in the molecular weight range from 200,000 d to 100,000 d from heat-set gels treated with SDS, 2-mercaptoethanol and urea. A distinct myosin peak appeared with addition of 0.025% $KBrO_3$ to sols with six peaks directly adjacent to its right (Figure 4c). A comparison of the height of myosin peak with the six peaks adjacent to the right revealed myosin to be present in relatively minor quantities. As the $KBrO_3$ level was increased from 0.025% to 0.250% in sols, the

Figure 4. SDS-PAGE of 2-mercaptoethanol- and urea-treated sols and heat-set gels (40°C/60 min followed by 90°C/20 min) containing varying level of $KBrO_3$. (a) Surimi sol; (b) control gel (0.000% $KBrO_3$); (c) Gel with 0.025% $KBrO_3$; (d) Gel with 0.050% $KBrO_3$; (e) Gel with 0.075% $KBrO_3$; (f) Gel with 0.100% $KBrO_3$; (g) Gel with 0.150% $KBrO_3$; (h) Gel with 0.200% $KBrO_3$; (i) Gel with 0.250% $KBrO_3$

Figure 4

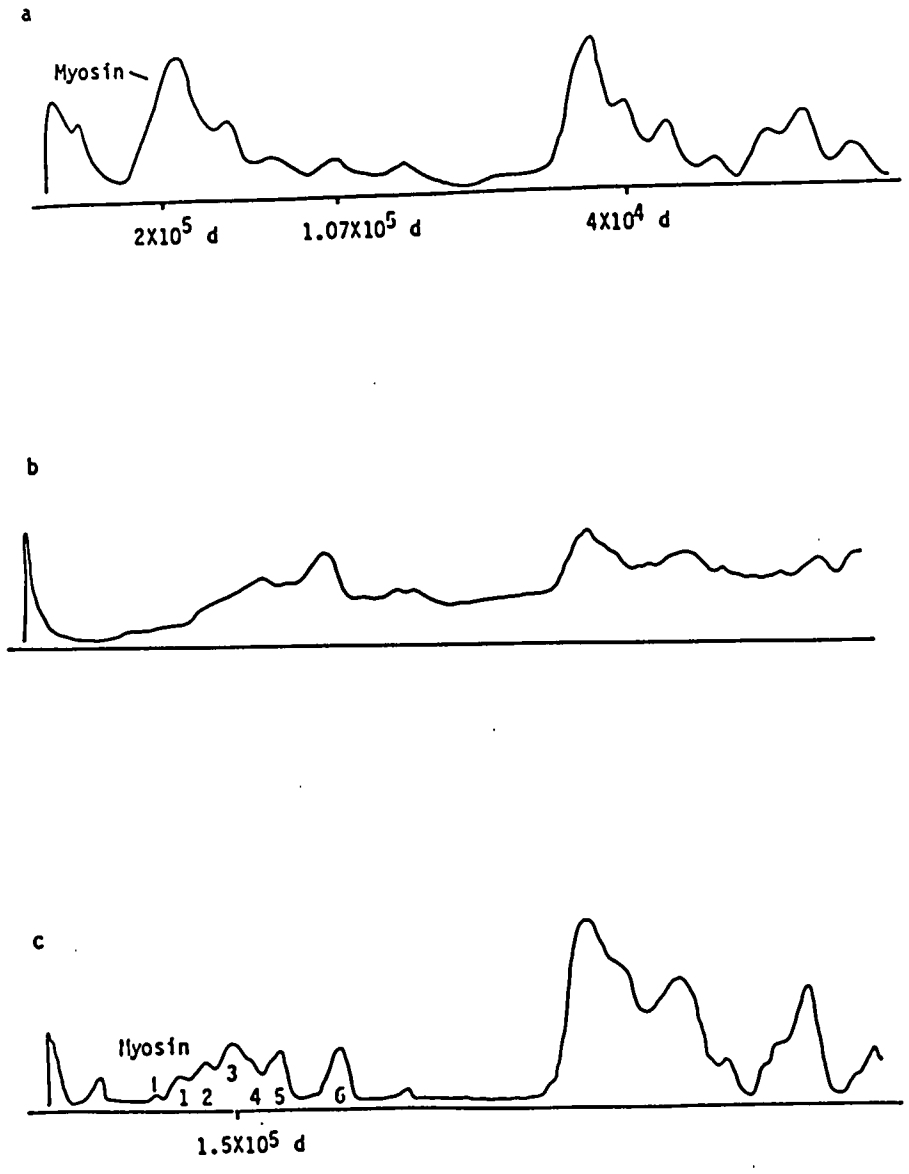
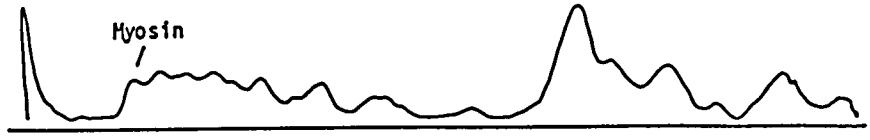


Figure 4 (continued)

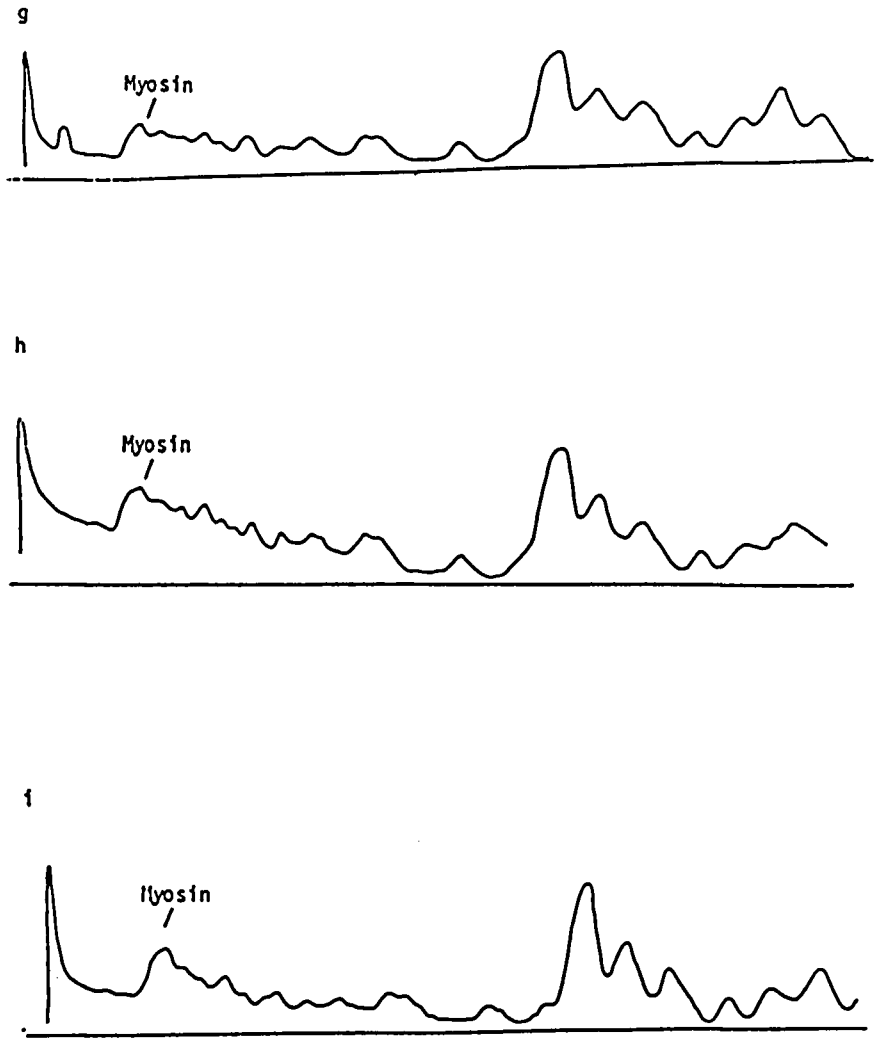
d

e



f

Figure 4 (continued)



quantity of myosin recovered from heat-set gels was elevated while the six adjacent peaks declined (Figures 4c-i). At $KBrO_3$ concentrations $\geq$0.15% recovered myosin clearly exceeded the concentrations of the six adjacent peaks to the right of myosin. This result strongly suggests that proteins composing peaks 1 to 6 (Figure 4c) were large protein fragments that originated through proteolysis of myosin. It was assumed that the height of the myosin peak in Figures 4c-i was proportional to the myosin recovery after the two stages heat treatment. Based upon this assumption, a 6.3 higher fold recovery of myosin from heat-set gels was observed as $KBrO_3$ levels were elevated from 0.000% to 0.250% in sol formulation (Table 10).

Table 10. SDS-PAGE recovery of myosin from gels[1] containing varying levels of $KBrO_3$ dissolved with urea and 2-mercaptoethanol.

$KBrO_3$ (%)	Recovery (fold)[2]
0.000	1.0
0.025	1.3
0.050	2.1
0.075	3.4
0.100	3.7
0.150	3.8
0.200	6.6
0.250	6.3

[1]40 °C for 60 min + 90 °C for 20 min.
[2]Peak height of myosin recovered from $KBrO_3$ treated gels/peak height of myosin recovered from gels containing no $KBrO_3$ (control).

Strengthening Gel Forming Capacity

Previous investigation demonstrated that the oxidative action of low levels of $KBrO_3$ on whiting surimi proteins produced chemical and biochemical modifications that improved their potential for forming stronger heat-set gels. The promotion of disulfide bond formation by surimi proteins through the action of heat and $KBrO_3$ was demonstrated by the loss of total and reactive sulfhydryls (Table 4 and 5). Disulfide formation was confirmed through the recovery of sulfhydryls from heated salt soluble proteins (Figure 3a-d) (Table 6) and heat-set gels (Table 7) using thiol reducing agents. The inhibition of proteinase activity afford by the oxidative action of $KBrO_3$ (Table 9) was shown to preserve myosin during heat-setting (Figure 4a-i).

The effect of these beneficial modification to whiting surimi proteins on gel forming capacity were determined using Texture Profile Analysis methods and a subjective folding test employed by the surimi industry as a quality control test. Eight levels of $KBrO_3$ (0.000, 0.025, 0.050, 0.075, 0.100, 0.150, 0.200 and 0.250%) in sols heat-set under a one stage (40 min at 90°C) and two stages (60 min at 40°C followed by 20 min at 90°C) temperature regimes were used to evaluate gel strength.

In general, the level of $KBrO_3$ in sols defined improvements in the gel-forming ability of whiting surimi. Factorial analysis of variance (2 x 8) revealed that varying

levels of $KBrO_3$ affected ($P<0.001$) the Texture Profile Analysis parameters of hardness (Table 11), brittleness

Table 11. Hardness (N/g) of gels heat-set under two different temperature regimes and treated with varying levels of $KBrO_3$.

$KBrO_3$ (%)	Heat-Setting Regime	
	One stage[1]	Two stage[2]
0.000	65.8^{b}	46.3^{a}
0.025	69.8^{b}	59.1^{ab}
0.050	67.4^{b}	72.0^{b}
0.075	73.3^{b}	89.4^{c}
0.100	92.1^{c}	108.2^{d}
0.150	117.7^{do}	129.9^{of}
0.200	128.6^{of}	135.5^{fg}
0.250	130.8^{of}	148.8^{g}

Analysis of Variance (2x8 Factorial Design)

	Significance
Heat Treatment (H)	$P>0.050$
$KBrO_3$ Level (%) (B)	$P<0.001$
Interaction (HxB)	$P=0.014$

[1]90 °C for 40 min.
[2]40 °C for 60 min + 90 °C for 20 min.
Mean values in both columns with same exponent letter did not vary ($P>.05$).

(Table 12), elasticity (Table 13) and cohesiveness (Table 14).

Gel hardness was a linear function of $KBrO_3$ concentration for both one- ($R^2 = 0.8656$) and two-stage ($R^2 = 0.8939$) heat-setting regimes (Table 16). The hardness of gels heat-set using one stage regime were not altered ($P>0.050$) by $KBrO_3 \leq 0.075\%$. Gel hardness was increased incrementally ($P<0.050$) in a concentration dependent manner

for 0.075, 0.100 and 0.150% levels of $KBrO_3$. The hardness for gels containing $\geq$150% did not vary ($P>0.05$).

Table 12. Brittleness (N/g) of gels heat-set under two different temperature regimes and treated with varying levels of $KBrO_3$.

$KBrO_3$ (%)	Heat-Setting Regime	
	One stage[1]	Two stage[2]
0.000	0.6[a]	0.5[a]
0.025	2.5[a]	2.3[a]
0.050	3.9[ab]	5.0[ab]
0.075	5.2[ab]	9.7[ab]
0.100	10.3[ab]	17.5[bc]
0.150	26.9[c]	62.8[c]
0.200	43.5[d]	93.3[f]
0.250	69.3[e]	112.8[g]

Analysis of Variance (2x8 Factorial Design)

	Significance
Heat Treatment (H)	$P<0.001$
$KBrO_3$ Level (%) (B)	$P<0.001$
Interaction (HxB)	$P<0.010$

[1]90 °C for 40 min.
[2]40 °C for 60 min + 90 °C for 20 min.
Mean values in both columns with same exponent letter did not vary ($P>0.05$).

Conversely, the hardness of gels heat-set using the two stage regime were significantly altered by $KBrO_3$ levels >0.050% in a $KBrO_3$ concentration dependent manner to a concentration of 0.150%. Levels of $KBrO_3$ >0.200% did not produce harder gels ($P>0.05$).

The brittleness of gels heat-set by either time and temperature regime was not altered ($P>0.050$) between $KBrO_3$ levels of 0.000 to 0.100%. At levels >0.100% gels became

more brittle ($P<0.05$) in an manner directly related to $KBrO_3$ concentration. The regression of brittleness values on $KBrO_3$ concentration for one- ($R^2 = 0.9310$) and two- stage ($R^2 = 0.9111$) heat-setting regimes conformed best to a power function (Table 16).

Gel elasticity was a logarithmic function of $KBrO_3$ concentration for both one- ($R^2 = 0.9534$) and two-stage

Table 13. Elasticity (%) of gels heat-set under two different temperature regimes and treated with varying levels of $KBrO_3$.

$KBrO_3$ (%)	Heat-Setting Regime	
	One stage[1]	Two stage[2]
0.000	49.1^{b}	39.7^{a}
0.025	84.9^{cd}	83.5^{c}
0.050	85.2^{cd}	86.2^{cdo}
0.075	85.0^{cd}	88.2^{of}
0.100	86.7^{do}	89.2^{of}
0.150	92.1^{gh}	93.3^{ghi}
0.200	90.7^{fg}	94.2^{hi}
0.250	95.0^{i}	96.5^{i}

Analysis of Variance (2x8 Factorial Design)

	Significance
Heat Treatment (H)	$P>0.050$
$KBrO_3$ Level (%) (B)	$P<0.010$
Interaction (HxB)	$P<0.010$

[1]90 °C for 40 min.
[2]40 °C for 60 min + 90 °C for 20 min.
Mean values in both columns with same exponent letter did not vary ($P>0.05$).

($R^2 = 0.9771$) heat-setting regimes (Table 16). Major improvement ($P<0.050$) in the elasticity of gels heat-set under both time-temperature regimes was observed with the

addition of only 0.025% $KBrO_3$. Relatively small improvements in gel elasticity were observed for $KBrO_3$ levels >0.025% and ≤0.100%. Gels containing $KBrO_3$ levels >0.100% were more elastic ($P<0.05$), but the degree of improved elasticity was small compared the that initially achieved by a concentration of 0.025% over the control.

Table 14. Cohesiveness (%) of gels heat-set under two different temperature regimes and treated with varying levels of $KBrO_3$.

$KBrO_3$ (%)	Heat-Setting Regime	
	One stage[1]	Two stage[2]
0.000	32.7^{b}	23.3^{a}
0.025	60.5^{c}	57.8^{c}
0.050	77.8^{d}	78.5^{de}
0.075	78.3^{de}	81.0^{def}
0.100	81.8^{efg}	84.2^{g}
0.150	88.5^{hi}	91.7^{ij}
0.200	85.5^{gh}	90.3^{ij}
0.250	91.9^{ij}	93.2^{j}

Analysis of Variance (2x8 Factorial Design)

	Significance
Heat Treatment (H)	P>0.050
$KBrO_3$ Level (%) (B)	P<0.010
Interaction (HxB)	P<0.010

[1]90 °C for 40 min; [2]40 °C for 60 min + 90 °C for 20 min. Mean values in both columns with same exponent letter did not vary (P>0.05).

The cohesiveness of gels heat-set by both one and two stage regimes was improved ($P<0.05$) in a concentration dependent manner up to a $KBrO_3$ content of 0.050%. Potassium bromate levels >0.050% yielded more cohesive gels with a maximum level of cohesiveness being achieved by levels

$\geq$0.150%. The overall effect of $KBrO_3$ level on the cohesiveness of gels heat-set with one- (R^2 = 0.8199) and two-stage (R^2 = 0.8514) heating regimes conformed to a logarithmic function (Table 16).

Gels heat-set under the two-stage regime were more ($P<0.001$) brittle (Table 12) than gels set with a one-stage heating. This result was a function of the more ($P<0.050$) brittle gels formed by the two-stage regime at $KBrO_3$ levels >0.100% over the one stage and was the source of the significant ($P<0.001$) interaction of heat-setting regime with $KBrO_3$ level. Heat-setting regime interacted with $KBrO_3$ level to affect hardness ($P=0.014$), elasticity ($P<0.010$) and cohesiveness ($P<0.010$) values as a result of the lower ($P<0.050$) values for control (0.000% $KBrO_3$) gels from the two-stage regime over the one stage. The interaction effect was strengthened, in some cases, by higher values for the two stage regime at $KBrO_3$ levels >0.075%.

The two-stage heat-setting regime overall produced gels yielding a higher folding test grade ($P=0.019$) (Table 15) than the one-stage. The level of $KBrO_3$ employed in sol formulations altered ($P<0.001$) folding test grades in a positive manner which conformed to linear function (Table 16) with respect to gels heat-set with both the one- (R^2 = 0.8787) and two-stage (R^2 = 0.8384) regimes. The improvement in folding test grade observed for both heat-setting regimes was incremental ($P>0.050$) with respect to $KBrO_3$ concentration up to a level of >0.100%; levels

≥0.150% did not alter (P>0.050) folding test grades. The overall higher grade observed for gels heat-set with the two- stage regime was a result of significantly better grades at 0.075% $KBrO_3$ and higher, but non-significant, grades for 0.100, 0.150 and 0.200% $KBrO_3$. Unlike TPA parameters (hardness, brittleness, elasticity and cohesiveness) heat-setting regime and $KBrO_3$ level did not interact (P>0.05) to affect folding test grades. This subjective test was not sufficiently sensitive to detect the differences between the two heat-setting regimes observed for the brittleness of gels containing $KBrO_3$ levels

Table 15. Folding test score for gels heat-set under two different temperature regimes and treated with varying levels of $KBrO_3$.

$KBrO_3$ (%)	Heat-Setting Regime	
	One stage[1]	Two stage[2]
0.000	1.00[a]	1.00[a]
0.025	1.50[b]	1.53[b]
0.050	2.16[c]	2.20[c]
0.075	2.53[c]	3.30[d]
0.100	3.67[d c]	4.07[c]
0.150	4.60[f]	4.87[f]
0.200	4.80[f]	4.97[f]
0.250	4.97[f]	4.97[f]

Analysis of Variance (2x8 Factorial Design)

	Significance
Heat Treatment (H)	P=0.019
$KBrO_3$ Level (%) (B)	P<0.001
Interaction (HxB)	P>0.050

[1]90 °C for 40 min.
[2]40 °C for 60 min + 90 °C for 20 min.
Mean values in both columns with same exponent letter did not vary (P>0.05).

≥0.150%, which were responsible for the interaction.

Potassium bromate improved the gel characteristics of whiting surimi sols through the inactivation of proteinases and strengthening of disulfide formation. The negative power function correlation (R^2 = 0.8546) (Table 16) of proteinase activity with myosin recovery from heat-set (two stage) gels treated with urea and 2-mercaptoethanol using SDS-PAGE (Table 10) documented the protection afforded myosin by $KBrO_3$. The relationship of myosin recovery to gel strength characteristics supported the positive action of $KBrO_3$ on gel strength. Myosin recovery was shown to be a power function of hardness (R^2 = 0.9472), brittleness (R^2 = 0.9320) and folding test grade (R^2 = 0.9353) and a logarithmic function of cohesiveness (R^2 = 0.7970) and elasticity (R^2 = 0.6157). The enhancement of gel strength characteristics by $KBrO_3$ levels in excess (>0.075%) of those required for proteinase inactivation supported a strong role for the oxidative action of $KBrO_3$ in strengthen disulfide formation. The relative importance of these two mechanisms varied with respect to $KBrO_3$ concentration and, to a lesser degree, to heat-setting regime.

Optimization of the elasticity (Table 13) and cohesiveness (Table 14) functions of gel strength relied on the maintenance of only a portion of the intact sol proteins from proteinase attack. Major improvement in these functions occurred at very low levels of $KBrO_3$ (0.025%) that afforded only a 72.49% (Table 9) inactivation of proteinases

and an estimated 1.3 higher fold recovery of myosin (Table 10) from heat-set gels. The majority of improvement observed occurred at $KBrO_3$ level $\leq$0.075% where proteinase inactivation occurred. A $KBrO_3$ level of 0.075% inactivated 89.87% of the proteinases in sols and was the minimum level required that achieved the highest ($P<0.050$) degree of inactivation observed for $KBrO_3$ (Table 9). Only a 3.4 higher fold recovery of myosin was estimated from heat-set gels treated with 0.075% $KBrO_3$. Overall, elasticity (R^2 = 0.9361, one-stage; 0.9501, two-stage) and cohesiveness (R^2 = 0.9384, one-stage; 0.9524, two-stage) were exponential functions of proteinase activity (Table 16). Improvements in the elasticity and cohesiveness of gels were relatively minor at $KBrO_3$ levels >0.075%, where strengthening of disulfide formation was the remaining potential mechanism for gel strength improvement. These results suggest that proteinase inactivation was the primary mechanism for improving elasticity and cohesiveness. The strengthening of disulfide formation played a relatively smaller role, but was required for full optimization of these functions of gel strength at $KBrO_3$ levels $\geq$0.150%.

Conversely, the hardness (Table 11) and brittleness (Table 12) functions of gel strength were only optimized if sol proteins were protected from proteinase attack and a strengthening of disulfide formation was accomplished by levels of $KBrO_3$ in excess of those required for proteinase inactivation. Overall, hardness (R^2 = 0.5267, one-stage;

0.8153, two-stage) and brittleness (R^2 = 0.7995, one-stage; 0.8480, two-stage) were power functions of proteinase activity (Table 16). These functions were optimized only at $KBrO_3$ levels $\geq$0.075% where proteinase inactivation had occurred (Table 9) and where $\geq$3.4 fold recovery of myosin from heat-set gels was observed (Table 10). A maximum level of hardness possible with $KBrO_3$ was observed at $\geq$0.150% while no maximum degree of brittleness was observed within the range of $KBrO_3$ levels investigated.

Folding test grade was a function of proteinase inactivation and, to a smaller degree, reinforced disulfide formation. Overall, folding test grade (R^2 = .7858, one stage; .8575, two stage) was a power functions of proteinase activity (Table 16). Optimization was achieved with $KBrO_3$ levels $\geq$0.150% where a $\geq$94.34% proteinase inactivation (Table 9) and a $\geq$3.8 fold recovery of myosin (Table 10) from heat-set gels were observed. Optimization did not require either the hardness or the brittleness levels observed for gels containing $KBrO_3$ levels $\geq$0.200%. This suggests that the degree of disulfide formation afforded by $KBrO_3$ levels $\geq$0.200% was in excess of that required for optimum whiting surimi gel grade.

The time and temperature relationship used to heat-set gels altered the proteinase inactivation and disulfide formation reinforcing functions of $KBrO_3$. Sols containing no $KBrO_3$ yielded lower levels ($P<0.05$) of hardness (Table 11), elasticity (Table 13) and cohesiveness (Table 14) when

heat-set with the two stage regime. This was the result of a more rapid heat inactivation of proteinases by the one stage regime and the extended time at low temperature employed by the first stage (60 min at 40 °C) of the two stage regime. Even with partial inactivation of proteinase ($KBrO_3$ levels >0.025%), gels heat-set with the two stage regime yielded better gel strength characteristics. These gel strength characteristics were generally better correlated as functions of both $KBrO_3$ level and proteinase activity (Table 16).

It is believed that the time at low temperature afforded by the first stage of the two stage heat-setting regime allowed proteins to assume a more ordered conformation. This more ordered configuration allowed for a more active and effective covalent cross-linking to occur. Under the more rapid heat-setting produced by the one-stage regime, the formation of these covalent cross-links, in combination with a more rapid rate of aggregation, would have locked proteins into a less desirable network structure. The more effective covalent cross-linking in gels heat-set by the two-stage regime is supported by the more ($P<0.05$) brittle gels observed at $KBrO_3$ level $\geq$0.150% (Table 12), harder ($P<0.05$) gels at $KBrO_3$ levels of 0.075 and 0.100% (Table 11), more ($P<0.050$) elastic gels at 0.075% (Table 13) and gels with a higher ($P<0.05$) folding test grade at a $KBrO_3$ level of 0.075% (Table 15). All of these differences occurred at $KBrO_3$ levels $\geq$0.075% where

proteinases were largely inactivated and reinforced disulfide formation was the remaining mechanism available for gel strengthening. Under these conditions of optimum maintenance of surimi proteins, the most efficient degree of ordering was possible.

Table 16. Coefficients of determination (R^2) for the regression of Texture Profile Analysis indices, folding test scores, proteinase activity[1] and myosin recovery[2] on $KBrO_3$ levels and other variables.

	Factor (x)				
	$KBrO_3$ level[3]		Proteinase activity[3]		Myosin recovery[3]
Factor (y)	One stage	Two stage	One stage	Two stage	Two stage
Hardness (N/g)	.8656[a]	.8939[a]	.5267[b]	.8153[b]	.9472[b]
Brittleness (N/g)	.9310[b]	.9111[b]	.7995[b]	.8480[b]	.9320[b]
Cohesiveness (%)	.8199[5]	.8514[5]	.9384[7]	.9524[7]	.7970[5]
Elasticity (%)	.9534[5]	.9771[5]	.9361[7]	.9501[7]	.6157[5]
Folding test	.8787[a]	.8384[a]	.7858[b]	.8575[b]	.9353[b]
Myosin recovery		.9260[a]			
Proteinase activity					.8546[b]

[1]Activity in sol (ug tyrosine/min/g protein).
[2]Peak height of myosin recovered from $KBrO_3$ treated gels/peak height of myosin recovered from gels containing no $KBrO_3$ (control).
[3]n=24.
[a]Best fit: linear ($y=mx+b$).
[5]Best fit: logarithmic ($y=a+b\ln x$).
[b]Best fit: power ($y=ax^b$).
[7]Best fit: exponential ($y=ae^{bx}$).

These results observed for the interaction of $KBrO_3$ with surimi proteins suggest that a level of 0.150% was required to achieve the biochemical and chemical modifications necessary for strong heat-set gels. Levels >0.150% produced stronger gels as measured by Texture

Profile Analysis, but this additional strengthening of gels did not appear to be necessary for achieving an optimum folding test grade. Indeed, levels of $KBrO_3$ >0.150% may induce sufficient disulfide cross-linking to impart a strength to some gel attributes that may not be optimum. Levels would have to be reflect the textural requirements of the a particular food product or analog.

SUMMARY AND CONCLUSIONS

Strong, residual proteinase activity in Pacific whiting surimi precludes its use for the manufacturing of seafood analogs which require a high level of protein functionality. Proteinase activity, either endogenous or derived from myxosporidian infestation, degraded the myosin complement of surimi proteins during heat-set gelation and precluded gel formation.

The oxidative action of low levels of $KBrO_3$ on whiting surimi proteins produced chemical and biochemical modifications that improved their potential for forming stronger heat set gels. The promotion of disulfide bond formation was demonstrated by the loss of reactive and total sulfhydryl functions. Disulfide formation was confirmed through their recovery from heated salt soluble proteins and heat-set gels using thiol reducing agents. SDS-PAGE of sols and heat-set gels solubilized with urea and thiol reducing agents confirmed the degradation of myosin during heat-setting and its protection from proteinase attack by $KBrO_3$.

Potassium bromate levels of 0.075% inactivated 89.9% of the total proteinase activity in whiting surimi. Inactivation was most likely accomplished through the oxidation of the sulfhydryl active sites of the proteinase(s). Proteinases with a serine active site, which would not be subject to inactivation by $KBrO_3$, were believed

to be the source of residual activity ($\leq$10.13% of total activity) observed at $KBrO_3$ >0.075%.

Improved gel strength characteristics afforded by $KBrO_3$ were a function of proteinase inactivation and strengthening of disulfide bridge fromation during heat-setting. Major improvement in the texture profile analysis parameters of cohesiveness and elasticity occurred at $KBrO_3$ concentrations $\leq$0.075%. Optimization of these parameters appeared to rely most on the inactivation of proteinases. Conversely, hardness and brittleness were only optimized if sols were protected from proteinase attack and a strengthening of disulfide formation was accomplished by levels of $KBrO_3$ in excess of those required for proteinase inactivation. Hardness was optimized at $KBrO_3$ $\geq$0.150%, while no maximum degree of brittleness was observed within the range of $KBrO_3$ levels investigated.

An optimum folding test grade of SA or AA was achieved by $KBrO_3$ levels $\geq$0.150% and was a function of proteinase inactivation and, to a smaller degree reinforced disulfide fromation. The highest grade of surimi gel did not require the hardness or brittleness of gels containing $KBrO_3$ levels $\geq$0.200%. Levels of $KBrO_3$ $\geq$0.150% may have produced sufficient disulfide cross-linking to impart a strength to some gel attributes that may not be optimum for some applications.

Investigations directed toward reducing the amount of $KBrO_3$ needed in whiting surimi sols to achieve sufficient

gel strength should be conducted. Addition of mixtures of cysteine-rich proteins, such as egg white and wheat proteins, in combination with $KBrO_3$ might achieve this purpose. The capability of dehydroascorbic acid or a combination of ascorbic acid and $KBrO_3$ to oxidize cysteine residues should also be investigated. Reduced amounts of $KBrO_3$ would minimize the possibility of excess oxidation and deleterious effect on the bioavailability of certain amino acids.

The results of this investigation demonstrated a procedure by which sols of surimi prepared from Pacific whiting can develop a strong gel strength. This could be a catalyst for the emergence of the Pacific whiting surimi industry in the West Coast of the United States.

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