AN ABSTRACT OF THE THESIS OF

Diana H. Wasson for the degree of Doctor of Philosophy

in Food Science and Technology presented on March 6, 1992.

Title: Identification and Characterization of a Heat Stable Protease in Arrowtooth Flounder (*Atheresthes stomias*) and Methods of Inhibition in Surimi

Abstract approved: _____
Daniel P. Selivonchick

A heat stable protease was identified as the cause of textural degradation in cooked arrowtooth flounder (*Atheresthes stomias*) muscle. Maximum proteolytic activity in the fish muscle was observed between 55°C and 60°C and myosin heavy chain appeared to be the primary substrate for the enzyme. Degradation of this myofibrillar protein at 55°C was extremely rapid and myosin heavy chain was completely hydrolyzed to peptide fragments smaller than actin, while actin itself was unaffected.

A single strand 32kD proteolytic enzyme was extracted from the muscle and purified 125-fold. The enzyme was stable to freezing for up to 6 months. Activity of the semi-purified enzyme at 55°C was optimal against casein between pH 6.0 and 7.0. Sulfhydryl reagents *p*-chloromercuriphenylsulfonic acid, iodoacetate, iodoacetamide and cystatin were effective in inhibiting enzyme activity in casein assays. The serine

protease inhibitors phenylmethylsulfonylfluoride and trypsin-chymotrypsin inhibitor appeared to activate enzyme activity against casein. Adenosine triphosphate was also an activator.

Arrowtooth flounder was then considered as a raw material for surimi, since the surimi process provides for repeated washing of the minced muscle and a final mixing step during which inhibitory substances can be conveniently added. Arrowtooth muscle was monitored at all stages of surimi production. There was no evidence of myosin degradation on sodium dodecyl sulphate polyacrylamide electrophoretic gels at any time during surimi production or during the preparation of samples for testing. However, when the washed mince was incubated at 55°C, 12% residual proteolytic activity was observed. This level was sufficient to degrade the myosin component of surimi gels prepared from the control surimi to which no inhibitors had been added. The food grade substances tested for proteolytic inhibition were bovine blood plasma powder, egg white powder, whey protein concentrate, carrageenan and crude α_2-macroglobulin. Addition of plasma and/or egg white powders to control surimi resulted in a product that was comparable to pollock in functional properties as measured by gel strength, expressible moisture and fold tests. Electrophoretic comparison of surimi made with 1.0% or 2.0% plasma powder or egg white with surimi produced with 0.1% or 0.2% α_2-macroglobulin suggested that the plasma and egg white contributed gel enhancing effects in addition to protease inhibition. Carrageenan was not effective as either a protease inhibitor or gel enhancer.

Identification and Characterization of a Heat Stable Protease in Arrowtooth Flounder (*Atheresthes stomias*) and Methods of Inhibition in Surimi

by

Diana H. Wasson

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed March 6, 1992

Commencement June 1992

APPROVED:

Professor of Food Science and Technology in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented March 6, 1992

Typed by Tracy Mitzel for Diana H. Wasson

CONTRIBUTION OF AUTHORS

"Characterization of a Heat Stable Protease from Arrowtooth Flounder, *Atheresthes stomias*" and "Effects of Protein and Carbohydrate Additives on Proteolytic and Functional Properties of Arrowtooth Flounder Surimi"

J.K. Babbit:	Director, NOAA/NMFS Utilization Research Laboratory in Kodiak, Alaska, where thesis research was conducted. Basic funding and equipment was supplied by NMFS.
J.S. French:	Director of FITC, University of Alaska, Fairbanks in Kodiak, Alaska, which provided additional funding and equipment for the project. Dr. French was also co-advisor for thesis research.
K.D. Reppond:	Research Chemist, NOAA/NMFS Utilization Research Laboratory in Kodiak, Alaska who provided technical advice and assistance with surimi test methods.

TABLE OF CONTENTS

TABLE OF CONTENTS (cont.)

LIST OF FIGURES

LIST OF TABLES

Identification and Characterization of a Heat Stable Protease in Arrowtooth Flounder (*Atheresthes stomias*) and Methods of Inhibition in Surimi

INTRODUCTION

Arrowtooth flounder (*Atheresthes stomias*) make up the largest single species fish biomass in the Gulf of Alaska and are a rapidly increasing stock in the Bering Sea. The Gulf biomass is currently estimated to be approximately 2 million metric tons, which far outnumbers the pollock (*Theragra chalcogramma*) resource. Commercial utilization of the species, however, has been limited due to extreme textural degradation of the cooked muscle. While such textural degradation is not uncommon among fish of the Pacific Ocean, it has not been well investigated for fish from Alaska waters. A review of the literature concerning fish muscle proteases that have been implicated in heat induced myofibrillar degradation follows in Chapter 1.

Both exogenous and endogenous enzymes have been identified as responsible for the textural degradation problems in other fish species. Pacific whiting (*Merluccius productus*), for example, is well known for its soft texture when cooked, but for many years an infecting Myxosporean parasite of the *Kudoa* genus has been reported to be the source of the proteolytic enzyme responsible for muscle degradation. Several endogenous enzymes known collectively as the "alkaline proteases" have also

been identified in fish such as carp (*Cyprinus carpio*) and white croaker (*Sciena schlegeli*). These thiol protease are relatively large, composed of subunits and are optimally active at pH 8.0 or above at temperatures between 50°C and 70°C. Routine screening of homogenized arrowtooth flounder muscle for Myxosporean parasites, however, failed to yield a consistent correlation between spore density and the extent of autolytic activity at 55°C in this species. Gel filtration and electrophoretic size determination of the arrowtooth protease and pH optimum assays further indicated that the arrowtooth protease did not belong to the "alkaline protease" group. Methods of identification, purification and characterization of the arrowtooth enzyme are presented in Chapters 2 and 3.

Fortunately, the characteristics of the heat stable protease in arrowtooth flounder muscle were such that it was amenable to inhibition by blending any one of several food additives with the minced muscle before cooking. The effectiveness of these additives in a muscle homogenate and the size of the resource naturally suggested the use of this fish species for surimi. The recent decline in pollock stocks in the Gulf of Alaska and Bering Sea and restrictions on fishing seasons and quotas combined were timely developments in obtaining funding for this phase of the project. A detailed comparison of the functional properties of various treated arrowtooth flounder surimis with pollock surimi follows in the final chapter.

I. LITERATURE REVIEW

Fish Muscle Proteases and Heat-Induced Myofibrillar Degradation. A Review

Diana H. Wasson

National Marine Fisheries Service

Utilization Laboratory

2725 Montlake Blvd. E.

Seattle, WA 98112

Accepted for publication in *Journal of Aquatic Food Product Technology*

INTRODUCTION

Two very different degradative phenomena have provided most of the impetus for research in the field of fish muscle proteases--postmortem spoilage at refrigeration temperature and textural degradation at relatively high temperature, 50°C - 65°C. The latter type of softening has been observed in whole fish muscle during routine cooking and in surimi pastes during setting. Depending on species, the degree of textural deterioration (*modori*) of cooked surimi pastes (*kamaboko*) in the 50°C - 65°C range can range from mild to severe degradation of the myosin and other myofibrillar proteins, while very little proteolysis is generally observed at immediately lower or higher setting temperatures. The presence of proteases that are relatively stable at cooking temperatures and neutral pH has become a significant problem for the fishing industry since the gel strength (*ashi*) of surimi, which is largely a measure of myofibrillar cross-linking, is also a determining factor in the market price of surimi.

As the popularity of crab analogs and other products made from surimi has spread outward from Japan, interest in the *modori* phenomenon has become a focal research concern in the United States and Europe. Declining stocks of species traditionally used for surimi, such as pollock, have prompted research in the use of alternate species, such as menhaden (*Brevoorti tyrannus*), Pacific whiting (*Merluccius productus*), yellowfin sole (*Limanda aspera*) and arrowtooth flounder (*Atheresthes*

stomias). High levels of proteolytic activity have been observed in all of these fish. The latter three species are also commonly infected by Myxosporean parasites, which has raised questions regarding the source of the proteases responsible for degradation of host myofibrillar proteins.

THE ACID PROTEASES

The literature documenting the presence of acid proteases in fish muscle is extensive, although most of the enzymes active in acidic media appear to be incapable of effecting the type of textural degradation observed in certain fish products as a function of cooking. Identifications of the lysosomal proteases in fish muscle have also tended to be equivocal. Nonspecific catheptic activity has often been identified simply on the basis of hemoglobin hydrolyzing activity at low temperature and acid pH (Wojtowicz and Odense, 1972, Geist and Crawford, 1974). While all of the cathepsins in mammalian muscle have been characterized as serine, cysteine or carboxyl proteases (Goll et al., 1989), Musch et al. (1971) separated two catheptic enzymes from cod muscle that appeared to involve histidine, rather than serine or cysteine, at their active sites. However, in subsequent investigations with cod, McLay (1980) reported the presence of both cathepsins A and D while Erickson et al. (1983) tentatively identified cathepsin C.

In a series of papers representing a long and methodical research program, Makinodan and co-workers (Makinodan and Ikeda, 1969a, 1971b, 1976, Makinodan et al. 1982a, 1982d, 1983) confirmed the existence of cathepsins A,B,C and D in carp (*Cyprinus carpio*) muscle and extensively characterized cathepsin D. Cathepsin D,

which involves a carboxyl group at the active site, has considerable potential for direct involvement in postmortem degradation since it rapidly hydrolyzes myosin heavy chain up to pH 5.5 and is optimally active at higher temperatures than most other cathepsins (Zeece and Katoh, 1989). Fractions containing cathepsin D activity from flounder (*Pseudopleuronectes americanus*) (Reddi et al.,1972) and *Tilapia mossambica* (Doke et al.,1980) were active against endogenous protein fractions between pH 3.0 and 7.0, suggesting a role for cathepsin D in autolytic spoilage. Makinodan et al. (1982a), however,reported that carp muscle cathepsin D did not hydrolyze myofibrillar proteins above pH 6.0, ruling out its involvement in *modori*, since the pH of surimi is generally around 6.8. Carp muscle cathepsin A, likewise, appeared to be active only below pH 6.0 (Makinodan and Ikeda, 1976). Cathepsin A also did not hydrolyze intact myofibrillar or sarcoplasmic proteins under experimental conditions, but was shown to act synergistically with cathepsin D in using hydrolytic products as substrates for further hydrolysis.

More recently, Hara et al. (1988) observed that the activity of carp muscle cathepsin B against synthetic substrates was maximal at pH 6.0 and its ability to degrade myosin heavy chain was activated by NaCl. Cathepsin B fractions with activity up to pH 6.0 have likewise been reported for mackerel (*Scomber japonicus*) (Matsumiya et al., 1989), grey mullet (*Mujil auratus*) (Bonete et al., 1984) and Pacific whiting (Erickson et al., 1983). However, since most of the proteases extracted from the muscle of fish species that are particularly susceptible to *modori* have demonstrated pH optima higher than 6.0 (see below), it is not likely that cathepsin B

is the major enzyme involved in myofibrillar degradation. Researchers in the field of mammalian proteases (Goll et al., 1989) have documented that lysosomal cathepsins are involved in the intracellular turnover of myofibrillar proteins in living tissue up to pH 6.0, but that they have virtually no effect on myofibrils in the extra-lysosomal pH range of healthy muscle tissue, which is usually above pH 6.0. Therefore, it is unlikely that the lysosomal cathepsins initiate myofibril degradation. By analogy, the enzymes responsible for the heat activated proteolysis that occurs at pH values well above 6.0 in the postmortem muscle of various food fish probably are not lysosomal.

Konagaya and co-workers recognized that although cathepsin D could be isolated from yellowfin tuna (*Thunnus albacores*) (Konagaya, 1982, Konagaya, 1984, Konagaya and Amano, 1973) and swordfish (*Xiphias gladius*) (Konagaya, 1983), it did not break down the myofibrillar structure of the muscle at pH 5.8, where separate and non-catheptic thiol proteases were highly active. Konagaya and Aoki (1981) found that neither cathepsin B nor D was responsible for the abnormal softening of Pacific hake muscle, but rather a distinct protease that was active up to pH 7.0 and inhibited by sulfhydryl reagents. Early studies by Makinodan and Ikeda (1969a) demonstrated the presence of proteases active at 60°C - 65°C between pH 8.0 and 8.5 in eight out of 12 fish species that displayed only acid protease activity at 37°C. The discovery of proteases active in the neutral to alkaline range provided a far more plausible explanation for the myofibrillar degradation commonly observed in a large number of fish during storage and processing and these enzymes consequently have received the greater proportion of subsequent research attention.

THE ALKALINE PROTEASES

Early observations that the quality of fish products was influenced by alkaline proteases were made in Japan. Researchers and industry personnel had observed that when surimi was heat set at 60°C to 65°C the gel strength of the resulting *kamaboko* was considerably lower than when either lower or higher setting temperatures were used. It is now well known that surimi gel strength is largely a function of myosin heavy chain, which forms a cross-linked network during setting (Nishimoto et al., 1987). When low gel strength values are encountered, it is reasonable to consider denaturation or proteolytic degradation of myosin as the cause. The degradation phenomenon has been observed in the gels of numerous fish, and the earlier reports of Makinodan and Ikeda (1971) include barracuda *(Sphyraena pinguis)*, white croaker (*Argyrosomus argentatus*), lizard fish (*Saurida undosquamis*), horse mackerel (Trachurus japonicus) and cod. Iwata et al. (1974b) later reported alkaline protease activity against casein in 21 additional freshwater and marine fish species.

Carp (*Cyprinus carpio*) was used for most of the initial work of protein purification and characterization. Research by independent laboratories (Makinodan and Ikeda, 1969b; Iwata et al., 1973, 1974a) established the presence of a protease in carp that was optimally active at pH 8.0 and 65°C and which was activated by sulfhydryl reagents and inhibited by sulfhydryl modifying reagents. Purified alkaline proteases from white croaker and barracuda displayed essentially the same characteristics (Iwata et al., 1974c). Iwata and co-workers (1974d), however, observed that while the carp protease hydrolyzed carp actomyosin at elevated pH and low NaCl concentration, at 0.5M NaCl and pH 6.6 very little hydrolysis was evident. Further,

when the protease purified from carp white muscle was added back to the washed muscle, it had very little or no effect on the gel strength, expressible moisture or organoleptic score of gels subsequently prepared from the mixture. Consequently, Iwata et al. (1977) proposed a non-enzymatic mechanism for the *modori* phenomenon in carp. They proposed that *modori* was mediated by two proteins of 44kD and 50 kD molecular weight rather than the 780 kD alkaline protease (Iwata et al., 1973). When extracts containing these proteins were added to washed carp muscle, the *kamaboko* gels subsequently prepared from the mixtures and pre-incubated at 65°C scored lower on the fold test and contained a greater percentage of expressible moisture than either control gels or gels to which purified carp alkaline protease had been added. Additional experiments with horse mackerel (Iwata et al., 1979; Makinodan et al., 1987a) and croaker (Shimizu et al., 1986), however, once again clearly demonstrated the causal link between alkaline protease activity and the *modori* observed during *kamaboko* production in the 60°C - 65°C range. Most fish researchers now accept the validity of alakline proteases as causative agents in the textural degradation of fish muscle at high temperature in species not otherwise affected by additional factors such as parasites or spawning metabolism.

Hase et al.(1980) reported a carp muscle alkaline protease composed of 4 subunits that was in the 580 kD to 600 kD range. It did not hydrolyze casein below approximately 55°C, although this temperature barrier was found to be lowered by the addition of urea. Makinodan and Ikeda (1977) observed considerable activity against casein at temperatures lower than 55°C in the presence of 5 M urea. They suggested

that the alkaline protease might be involved in post-mortem degradation of fish muscle if denaturing substances analogous to urea were produced during storage, since the enzyme was shown to degrade myofibrillar and sarcoplasmic proteins at 40% and 50% the rate of casein, respectively (Makinodan et al., 1982a,1982b). The highest specific activity of the enzyme was found in the microsomal fraction of carp muscle, implicating the alkaline protease in myofibrillar turnover and ruling out any possible confusion with lysosomal (catheptic) enzymes (Makinodan et al., 1982a).

Although the alkaline protease has been shown to be capable of degrading sarcoplasmic proteins, it has been the enzyme's ability to rapidly destroy myofibrillar proteins, particularly myosin heavy chain, that has made it the center of more recent research attention. Makinodan et al. (1985) observed the unambiguous loss of myosin heavy chain in electrophoretic gels prepared from carp and white croaker surimi that had been held at 62°C. The temperature-gel strength curve for croaker gels was highly symmetrical to the temperature-autolytic activity curve for the same gels, providing strong evidence for the role of myosin degradation in the textural softening observed in surimi gels cooked in the 55°C - 65°C range. Later purification of an alkaline protease from white croaker (Makinodan et al., 1987b) demonstrated that the enzyme was similar to the carp muscle protease, in that it was composed of 4 subunits, contained a cysteine active site and was capable of rapidly degrading isolated myosin heavy chain, actin and tropomyosin at 60°C. While proof of alkaline protease activity at high temperature does not explain the enzyme's role *in vivo* at ambient temperature, it does offer a plausible mechanism for the *modori* phenomenon in *kamaboko* prepared from many different species of fish.

Protease research with different species of fish in different laboratories, however, has yielded an array of details, pointing out the difficulty of generalizing data from one species to another. Among ten different fish species including carp, rainbow trout (*Salmo irideus*), red sea bream (*Pagrus major*), frog flounder (*Pleuronichthys cornutus*), yellow tail (*Seriola quinqueradiata*), white croaker, lizardfish, horse mackerel, common mackerel (*Scomber japonicus*) and sardine (*Sardinops melanosticta*), significant differences in alkaline protease activity were observed (Makinodan et al., 1984). Large differences in activity level among individuals of the same species have also been common (Konagaya, 1980; Wasson et al., 1991). Temperature and pH optima for caseinolytic activity and hemoglobin hydrolysis have varied according to species and substrate. Deng (1981) reported optimal activity of an alkaline protease from mullet against urea denatured hemoglobin at pH 8.0 and 65°C, consistent with the carp alkaline protease. At 25°C, on the other hand, caseinolytic activity of a sulfhydryl protease extracted from yellowfin sole (Konagaya, 1980) was optimal at pH 6.5, which is more consistent with the postmortem pH of fish muscle. At 55°C, a heat stable sulfhydryl protease from arrowtooth flounder displayed maximal caseinolytic activity at pH 6.7 (Greene and Babbitt, 1990, Wasson et al., 1991).

Most of the research with carp indicated that the alkaline protease was located in the microsomal fraction of the muscle, while research with threadfin bream (*Nemipterus virgatus* and *Nemipterus bathybius*) (Lee et al., 1990, Toyohara and Shimizu, 1988, Toyohara et al., 1990) indicated that the enzyme responsible for

decreased gel strength and water holding capacity in surimi gels set at 60°C was located in the sarcoplasmic fraction extracted from threadfin mince. A separate fraction, identified as the heat-stable alkaline protease according to the criteria of Makinodan and Ikeda (1969b), did not display any proteolytic activity against myosin in gels prepared from threadfin myofibrillar proteins (Toyohara et al., 1990). The effectiveness of soybean trypsin inhibitor in inhibiting the breakdown of myosin heavy chain (Toyohara and Shimizu, 1988) argued in favor of a serine protease, in contrast to the well documented cysteine protease identified in carp and white croaker.

Ambiguity in classifying fish muscle proteases has been common in the literature. Toyohara et al. (1990b) reported a proteolytic substance in oval filefish (*Navodon modestus*) that was tightly associated with the myofibrillar component of the muscle. Soybean trypsin inhibitor was effective in inhibiting myosin heavy chain breakdown in oval-filefish at 50°C but not at 65°C, suggesting the presence of a serine protease, while leupeptin was effective at both temperatures, suggesting a cysteine protease. Taguchi et al. (1983b) had earlier reported ambiguous results using a variety of serine and cysteine enzyme blocking reagents in oval filefish gels. *N*-butyl, *n*-amyl and *n*-hexyl alcohols, on the other hand, completely inhibited *modori*. Eight to 13-fold increases in gel strength were also obtained when these alcohols were added at the 2% level. The researchers then proposed that *modori* is a function of two factors--the presence of endogenous muscle proteases and the formation of an intrinsically unstable gel structure (Taguchi et al., 1983a,1983b).

Niki et al. (1984a, 1984b) dismissed the possibility that structural differences in the muscle were responsible for the *modori* phenomenon in kamaboko prepared from Peruvian hake (*Merluccius gayi peruanus*). They found no physico-chemical differences between actomyosins from hake, which generally displays intense proteolytic activity between 50°C and 60°C and Alaska pollock (*Theragra chalcogramma*). The levels of proteolytic activity observed in pollock surimi during *kamaboko* production have been extremely low by comparison. When the researchers added a water extract from hake to pollock surimi, however, the gel strength of the pollock *kamaboko* was weakened considerably. A similar experiment had been conducted by Nagahisa et al. (1981) using Pacific hake and Alaska pollock. Cysteine protease inhibitors, oxidizing agents, egg white and potato extract were all effective in inhibiting proteolytic activity, although egg white and potato extract were the least effective. Since the majority of the protease inhibitors present in egg white and potato are specific to serine proteases, these substances are generally regarded as trypsin-chymotrypsin inhibitors. However, both egg white and potato have repeatedly been reported as effective *modori* inhibitors in surimi containing known cysteine proteases. It has not yet been determined whether the comparatively small quantities of cysteine protease specific inhibitors (*e.g.* cystatin, ficin inhibitor) present in these substances are the active agents of inhibition, or whether sulfhydryl rich substances such as ovalbumin are able to effectively bind active sulfhydryl groups on the fish proteases. Chang-Lee et al. (1989, 1990) suggested that the greater percentage of sulfhydryl groups in egg white compared to whey protein concentrate might account for the

greater effectiveness of egg white when the two substances were added to Pacific hake surimi.

The alkaline protease in Atlantic croaker (*Micropogon undulatus*) is another well-studied enzyme. Cheng et al. (1979a, 1979b) observed an inverse relationship between the gel texture and the soluble protein content of cooked gels prepared from croaker, which they linked to the complete disappearance of myosin heavy chain in SDS-PAGE gels of samples that had been cooked at 70°C. The optimum temperature and pH for caseinolytic activity of a crude extract from croaker mince were 60°C and 8.0 - 8.5, respectively, consistent with Makinodan's alkaline protease. Lanier et al. (1981) found the same inverse correlation between proteolytic activity and myosin retention in the cooked gels as a function of temperature, with a peak in proteolytic activity at 60°C. Chymotrypsin inhibitor prepared from Russet potatoes was effective in increasing the hardness of cooked croaker gels, suggesting the action of a serine protease. When Lin and Lanier (1980) purified the alkaline protease from croaker skeletal muscle, however, they observed almost complete inhibition of caseinolytic activity by *p*-choloromercuribenzoic acid (*p*-CMB) and no inhibition by phenylmethyl sulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) or N-α-*p*-tosyl-L-lysine chloromethyl ketone·HCl, indicating the involvement of sulfhydryl groups instead. Lin and Lanier further characterized the enzyme as an 80kD protein that degraded myosin heavy chain at 60°C. Conflicting data was obtained for alkaline proteases isolated from Atlantic croaker liver (Su et al., 1981a, 1981b), which were unaffected by *p*-CMB, but inhibited by ovomucoid and potato protease inhibitor. Su et al. (1981a, 1981b) had suggested that contamination from

organ tissues during mechanical mincing of fish muscle could be a contributing factor to the level of enzyme activity later observed in the surimi.

Boye and Lanier (1988) considered the impact of gut contamination in the production of menhaden surimi. Since they observed the same temperature dependent degradation of myosin in the excised muscle of whole fish as in the surimi, however, they identified the muscle as the primary source of proteolytic enzyme(s). The isolated alkaline protease was optimally active against casein at 60°C at pH 7.5 - 8.0, but the optimum temperature dropped to 55°C when the pH was lowered to 7.0. Hamann et al. (1990) observed that myosin heavy chain was degraded to the greater extent in menhaden surimi gels that were pre-cooked for 30 minutes at 60°C before a 90°C final cook compared to gels that were pre-incubated at 40°C or cooked directly at 90°C. Proteolytic degradation of myosin heavy chain also occurred in low grade Alaska pollock surimi gels that had been pre-cooked at 60°C. Proteolysis in gels from both fish species was prevented by powdered plasma and egg white. Hamann et al. (1990) suggested that the agent responsible for proteolytic inhibition in both plasma and egg white might be α_2-macroglobulin, which is a known inhibitor of sulfhydryl and metallo-proteases. More recent investigation of the role of α_2-macroglobulin, however, has indicated that yet another fraction of plasma may be the primary inhibitor of proteolytic activity (Lanier, pers. comm.).

Researchers in Argentina (Busconi et al., 1984, Folco et al., 1984) have studied the proteolytic activity in muscle from a different species of white croaker, *Micropogon opercularis*. Since this work was conducted with a view to elucidating mechanisms of protein turnover and not concerned with the use of croaker as a raw

material for surimi, the researchers did not apply a heat step in purifying the muscle enzymes, as has been routine in the purification of enzymes associated with *kamaboko* production. They isolated a trypsin-like enzyme of 132 kD molecular weight and a thiol protease of 363 kD molecular weight that demonstrated pH optima of 8.5 and 9.1, respectively. Both were optimally active at 60°C. However, when heat was applied during a subsequent isolation procedure, the thiol protease was lost. In addition to the two proteases, they also found a 65 kD protease inhibitor, which showed activity against the trypsin-like protease (Folco et al., 1984). Toyohara et al. (1983) identified a trypsin inhibitor in carp of approximately the same molecular weight, 70 kD, but were unable to identify a target serine protease in the carp muscle.

Additional research data with white croaker (Folco et al., 1989) indicated that the serine protease was a 269kD protein, which was much larger than originally estimated. Further substrate and inhibitor tests with the thiol protease (Folco et al., 1988a, 1988b) indicated that the protease was multicatalytic, possessing trypsin, chymotrypsin and cysteine protease activities under different assay conditions. Folco et al. (1988b) also demonstrated that the temperature optimum of the latter enzyme against casein shifted from 60°C to 37°C - 45°C in the presence of millimolar concentrations of unsaturated fatty acids. The trypsin-like activity of the enzyme, however, was inhibited by fatty acids (Folco et al., 1988a).

NEUTRAL AND Ca^{2+}-ACTIVATED PROTEASES

Only a limited amount of effort has been directed toward understanding the proteases that are optimally active in the neutral range. Makinodan and Ikeda (1976b)

detected maximum hemoglobin hydrolyzing activity at pH 7.0 in carp and red sea bream (*Pagrus major*) muscle extracts. In cathepsin-specific substrate assays the authors also clearly ruled out any confusion of the carp neutral protease with cathepsins A, C and D. However, the neutral protease did not hydrolyze myofibrils or actomyosin isolated from carp muscle, but was primarily active against peptides liberated from hemoglobin by cathepsin D. In this regard the neutral protease may play a secondary role in the post-mortem degradation of fish muscle.

Another likely mediator of protein turnover in fish muscle is calpain II, which has been purified from carp (Toyohara et al., 1983, Taneda et al., 1983). The 100 kD enzyme displayed maximum caseinolytic activity at pH 7.0 and 25°C, and was inhibited by reagents such as iodoacetic acid and *N*-ethylmaleimide. Calpastatin isolated from carp muscle (Toyohara et al., 1983) was also effective in inhibiting caseinolytic activity. Carp calpain II degraded carp myofibrils in the presence of 5mM Ca^{2+} at 25°C, suggesting an analogous role for this enzyme in fish protein metabolism as has been documented for mammalian tissues.

PROTEASES IN PARASITIZED FISH

Myofibrillar degradation in cooked whole muscle has been a significant problem for the fishing industry, concurrent with that of *modori* in surimi products. A great deal of research in this area has revolved around the presence of parasites, specifically Myxosporeans, which have been identified in many of the fish species which display textural softening when cooked. The list of affected species is long,

and includes barracuda, tuna, numerous flatfish, salmon, herring, and most notably, Pacific whiting (Lom and Arthur, 1989).

Pacific whiting has been extensively studied due to the large proportion of fish that are infected and the widespread occurrence of visible cysts. Patashnik et al. (1982) found that the acid protease activity of parasitized whiting was 7 times higher than that of uninfected fish, but it did not correlate with the visually estimated degree of parasite cyst intensity. Nor did all the fish with visible cysts develop abnormal texture when cooked. Further, Myxosporean spores collected by bacterial filter did not generate significant proteolytic activity when incubated at 40°C for 6 hours. Kabata and Whitaker (1981) and Tsuyuki et al. (1982) explained that Pacific whiting was infected by two different species of Myxosporeans, *Kudoa thyrsitis* and *Kudoa paniformis*, depending on location. Further, they reported that while elevated levels of acid protease activity could be measured in infected muscle, the acid protease was heat labile and did not degrade structural proteins. Hake harvested from the Georgia Strait did not exhibit proteolytic activity at pH 6.7 at any temperature, despite infection by *K. thyrsitis*. All hake from the west coast of Vancouver Island, however, exhibited proteolytic activity at pH 6.7 and a temperature optimum between 55°C and 60°C. Proteolysis was greater in fish infected with *K. paniformis* than in non-infected fish.

On the microscopic level, Konagaya and Aoki (1981) demonstrated a close relationship between the degree of infection and caseinolytic activity at pH 5.5 by counting spores extracted from the muscle. Caseinolytic activity was completely inhibited by (*p*-CMB) and iodoacetic acid, suggesting a thiol protease. Acid protease

and autolytic activity were also elevated in severely infected muscle. Konagaya and Aoki used blackened photographic plates to demonstrate localized proteolysis in muscle fibers containing cysts. Nagahisa et al. (1983) correlated visible cysts in whiting with TCA-soluble nitrogen and also found that the enzyme activity was inhibited by iodoacetic acid and *p*-CMB.

Despite the reported correlations between spore or cyst numbers and proteolytic activity in the muscle tissue, ultrastructural studies of the two *Kudoa* species argue against the supposition that the spores or cysts are the source of proteolytic enzymes. Morado and Sparks (1986) and Stehr and Whitaker (1986) reported that white and black plasmodia were common to both species of *Kudoa*, but that the more mature black plasmodia were proteolytically inactive. In addition, the white plasmodia affected only those myofibrils that were in direct contact with the spore mass. None of the usual enzyme producing organelles, such as Golgi bodies or endoplasmic reticula, were observed in any of the plasmodia studies. Even in studies distinguishing between the different spore types, Kudo et al. (1987) found that white pseudocyst counts could only account for 55% of the observed variability in sensory texture ratings.

The parasite theory of protease production is clearly controversial, and contradictory evidence has been reported for other species as well. Kramer et al. (1977) reported a correlation between infection level and muscle softening in arrowtooth flounder. Cysts are less frequently visible in arrowtooth flounder, yet all fish of this species suffer severe textural degradation when subjected to temperatures in the 50°C to 65°C range. However, since hemoglobin was used as a substrate in the

study, no proteolytic activity was detected at pH 6.0. Greene and Babbitt (1990) and Wasson et al. (1991) reported high levels of autolytic and caseinolytic activity at pH 6.7, but found no clear correlation between spore density and cooked texture as measured by Instron punch tests. On the other hand, in experiments with yellowfin sole, Konagaya (1980) reported a close relationship between the number of spores and caseinolytic activity at pH 6.5. A similar type of thermal degradation in yellowtail kingfish, *Seriola lalandi*, was only observed in infected fish, while all uninfected fish maintained normal texture during cooking (Lester, 1982).

The weakness of all the parasite/protease studies lies in the absence of published information regarding the complete life cycles of all but one of the 1100 Myxosporean parasites reported in fish muscle. Wolf and Markiw (1984) reported data on *Myxosoma cerebralis* that could substantially alter current thinking about the protease problem in many fish if other Myxosporeans are found to behave similarly. *M. cerebralis* spores are abundantly present in the tissue of infected fish, yet they have never been shown to cause whirling disease when introduced in uninfected fish. However, when spores are ingested by tubificids, they are transformed into actinosporeans of the genus *Triactinomyxon*. It is in this form that the Myxosporeans then induce disease when they are ingested by the host fish. If other Myxosporeans engage in similarly complex life cycles, the presence of spores in the affected tissues may only serve as an indicator and not a measure of the disease.

CONCLUSION

Despite substantial research effort to understand the role of proteases in the thermal degradation of fish muscle, in many ways the topic has become more complex. It appears that there are at least two different categories of enzymes capable of degrading myofibrillar proteins at elevated temperature (50°C-65°C), those with pH optima in the alkaline range (8 to 9) and those which are optimally active at the post-mortem pH of fish muscle. A variety of serine and cysteine proteases have been reported within each category. As the next step, several laboratories are planning to prepare fluorescent antibodies to the proteases in different fish species. These and other studies to determine the source and potential homology among the muscle proteases have the potential to provide useful and timely information for the fishing industry.

REFERENCES

Bonete, M.J., Manjon, A., Llorca, F. and Iborra, J.L. 1984. Acid proteinase activity in fish II. Purification and characterization of cathepsins B and D from *Mujil auratus* muscle. Comp. Biochem. Physiol. 78B: 207.

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

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

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

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

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

Cheng, C.S., Hamann, D.D., Webb, N.B. and Sidwell, V. 1979b. Effects of species and storage time on minced fish gel texture. J. Food Sci. 44: 1087.

Deng, J.C. 1981. Effect of temperatures on fish alkaline protease, protein interaction and texture quality. J. Food Sci. 46: 62.

Doke, S.N., Ninjoor, V. and Nadkarni, G.B. 1980. Characterization of cathepsin D from the skeletal muscle of fresh water fish, *Tilapia mossambica*. Agric. Biol. Chem. 44: 1521.

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

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

Folco, E.J., Busconi, L, Martone, C.B. and Sanchez, J.J. 1988a. Multicatalytic proteinase in fish muscle. Arch. Biochem. Biophys. 267: 599.

Folco, E.J., Busconi, L., Martone, C.B., Trucco, R.E. and Sanchez, J.J. 1988b. Activation of an alkaline proteinase from fish skeletal muscle by fatty acids and sodium dodecyl sulfate. Comp. Biochem. Physiol. 91B: 473.

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

Geist, G.M. and Crawford, D.L. 1974. Muscle cathepsins in three species of Pacific sole. J. Food Sci. 39: 548.

Goll, D.E., Kleese, W.C. and Szpacenko, A. 1989. Skeletal muscle proteases and protein turnover. Ch. 8. In *Animal Growth Regulation*, D.R. Campion, G.J. Hausman and R.J. Martin (Eds.), p.141. Plenum Press, New York.

Greene, D.H. and Babbitt, J.K. 1990. Control of muscle softening and protease-parasite interactions in arrowtooth flounder *Atheresthes stomias*. J. Food Sci. 55: 579.

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

Hara, K., Suzumatsu, A. and Ishihara, T. 1988. Purification and characterization of cathepsin B from carp ordinary muscle. Nippon Suisan Gakkaishi 54: 1243.

Hase, J., Kobashi, K., Nakai, N., Mitsui, K., Iwata, K. and Takadera, T. 1980. The quaternary structure of carp muscle alkaline protease. Biochimica et Biophysica Acta 611: 205.

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

Iwata, K., Kobashi, K. and Hase, J. 1974a. Studies on muscle alkaline protease-II. Some enzymatic properties of carp muscular alkaline protease. Bull. Jpn. Soc. Sci. Fish. 40: 189.

Iwata, K., Kobashi, K. and Hase, J. 1974b. Studies on muscle alkaline protease-III. Distribution of alkaline protease in muscle of freshwater fish, marine fish and in internal organs of carp. Bull. Jpn. Soc. Sci. Fish. 41: 201.

Iwata, K., Kobashi, I. and Hase, KJ. 1974c. Studies on muscle alkaline protease-IV. Comparison of physicochemical and enzymatic properties of alkaline protease from white croaker and barracuda muscle with those of the enzyme from carp muscle. Bull. Jpn. Soc. Sci. Fish. 40: 1043.

Iwata, K., Kobashi, K. and Hase, J. 1974d. Studies on muscle alkaline protease-V. Effect of carp muscular alkaline protease upon *modori* phenomenon in *kamaboko* production. Bull. Jpn. Soc. Sci. Fish. 40: 1051.

Iwata, K., Kobashi, K. and Hase, J. 1977. Studies on muscle alkaline protease-VI. Purification of proteins which induce the *modori* phenomenon during *kamaboko* production and of cathepsin A from carp muscle. Bull. Jpn. Soc. Sci. Fish. 43: 181.

Iwata, K., Kobashi. K. and Hase, J. 1979. Studies on muscle alkaline protease-VII. Effect of the muscular alkaline protease and protein fractions purified from white croaker and horse mackerel on the *himodori* phenomenon during *kamaboko* production. Bull. Jpn. Soc. Sci. Fish. 45: 157.

Kabata, Z. and Whitaker, D.J. 1981. Two species of *Kudoa* (Myxosporea: Multivalvulida) parasitic in the flash of *Merluccius productus* (Ayres, 1855) (Pisces: Teleostei) in the Canadian Pacific. Can. J. Zool. 59: 2085.

Konagaya, S. 1980. Jellification and protease activity of yellowfin sole muscle in association with a Myxosporean parasite. Bull. Jpn. Soc. Sci. Fish. 46: 1019.

Konagaya, S. 1982. Separation from jellied yellowfin tuna meat of the protease responsible for jellification. Bull. Jpn. Soc. Sci. Fish. 48: 543.

Konagaya, S. 1983. Protease responsible for jellification of myxosporidia-infected swordfish meat. Bull. Jpn. Soc. Sci. Fish. 49: 919.

Konagaya, S. 1984. Studies on the jellied meat of fish, with special reference to that of yellowfin tuna. Bull. Tokai Reg. Fish. Res. Lab. 114:1.

Konagaya, S. and Amano, K. 1973. Studies on jellied meat of tuna-III. Proteolytic activity of the jellied meat of yellowfin tuna. Bull. Jpn. Soc. Sci. Fish. 39: 1169.

Konagaya, S. and Aoki, T. 1981. Jellied condition in Pacific hake in relation to Myxosporidian infection and protease activity. Bull. Tokai Reg. Fish. Res. Lab. 105: 1.

Kramer, D.E., Nordin, D.M.A. and Boyd, J.W. 1977. Studies on the muscle texture and proteolytic activity of arrowtooth flounder, *Atheresthes stomias*. Paper presented at the 28th Annual Meeting of Pacific Fisheries Technologists, Harrison Hot Springs, British Columbia, March 2-5.

Kudo, G., Barnett, H.J. and Nelson, R.W. 1987. Factors affecting cooked texture quality of Pacific whiting, *Merluccius productus*, fillets with particular emphasis on the effects of infection by the Myxosporeans *Kudoa paniformis* and *K. thyrsitis*. Fishery Bulletin 85: 745.

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

Lee, N., Seki, N., Kato, N., Nakagawa, N., Terui, S. and Arai, K. 1990. Gel forming ability and cross-linking ability of myosin heavy chain in salted meat paste from threadfin bream. Nippon Suisan Gakkaishi 56: 329.

Lester, R.J.G. 1982. *Unicapsula seriolae* n. sp. (Myxosporea, Multivalvulida) from Australian yellowtail kingfish *Seriola lalandi*. J. Protozool. 29: 584.

Lin, T.S and Lanier, T.C. 1980. Properties of an alkaline protease from the skeletal muscle of Atlantic croaker. J. Food Biochem. 4: 17.

Lom, J. and Arthur, J.R. 1989. A guideline for the preparation of species descriptions in Myxosporea. J. Fish Dis. 12: 151.

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

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

Makinodan, Y. and Ikeda, S. 1969a. Studies on fish muscle protease-I. On the existence of two kinds of proteinases active in acid and in slightly alkaline pH range. Bull. Jpn. Soc. Sci. Fish. 35: 672.

Makinodan, Y. and Ikeda, S. 1969b. Studies on fish muscle protease-II. Purification and properties of a proteinase active in slightly alkaline pH range. Bull. Jpn. Soc. Sci. Fish. 35: 749.

Makinodan, Y. and Ikeda, S. 1969c. Studies on fish muscle protease-III. Purificationand properties of a proteinase active in acid pH range. 1969. Bull. Jpn. Soc. Sci. Fish. 35: 758.

Makinodan, Y. and Ikeda, S. 1971a. Studies on fish muscle protease-IV. Relation between *himodori* of *kamaboko* and muscle proteinase. Bull. Jpn. Soc. Sci. Fish. 37: 518.

Makinodan, Y. and Ikeda, S. 1971b. Studies on fish muscle protease-V. On the existence of cathepsins A, B and C. Bull. Jpn. Soc. Sci. Fish. 37: 1002.

Makinodan, Y. and Ikeda, S. 1976a. Studies on fish muscle protease-VI. Separation of carp muscle cathepsins A and D, and some properties of carp muscle cathepsin A. Bull. Jpn. Soc. Sci. Fish. 42: 239.

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

Makinodan, Y. and Ikeda, S. 1976b. Studies on fish muscle protease-VIII. On the existence of protease active in neutral pH range. Bull. Jpn. Soc. Sci. Fish. 42: 665.

Makinodan, Y., Kyaw, N. N. and Ikeda, S. 1982b. Intracellular distribution of fish muscle alkaline proteinase. Comp. Biochem. Physiol. 73B: 785.

Makinodan, Y., Kyaw, N.N. and Ikeda, S. 1982c. Classification of carp muscle alkaline proteinase and its action against intracellular proteins. Bull. Jpn. Soc. Sci. Fish. 48: 479.

Makinodan, Y., Kitagawa, T., Toyohara, H. and Shimizu, Y. 1987a. Participation of muscle alkaline proteinase in *himodori* of *kamaboko*. Nippon Suisan Gakkaishi 53: 99.

Makinodan, Y., Toyohara, H and Ikeda, S. 1982d. Intracellular distribution of carp muscle cathepsin D and an electron micrograph of muscle lysosomal fraction. Bull. Jpn. Soc. Sci. Fish. 48: 1501.

Makinodan, Y., Toyohara, H. and Ikeda, S. 1983. Combined action of carp muscle cathepsins A and D on proteins. Bull. Jpn. Soc. Sci. Fish. 49: 1153.

Makinodan, Y., Toyohara, H. and Ikeda, S. 1984. Comparison of muscle proteinase activity among fish species. Comp. Biochem. Physiol. 79B: 129.

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

Makinodan, Y., Yokoyama, Y., Kinoshita, M. and Toyohara, H. 1987b. Characterization of an alkaline proteinase of fish muscle. Comp. Biochem. Physiol. 87B: 1041.

Matsumiya, M., Mochizuki, A and Otake, S. 1989. Purification and characterization of cathepsin B from ordinary muscle of common mackerel *Scomber japonicus*. Nippon Suisan Gakkaishi 55: 2185.

McLay, R. 1980. Activities of cathepsins A and D in cod muscle. J. Sci. Food Agric. 31: 1050.

Morado, J.F. and Sparks, A. 1986. Observations on the host-parasite relations of the Pacific whiting, *Merluccius productus* (Ayres), and two myxosporean parasites, *Kudoa thyrsitis* (Gilchrist, 1924) and *K. paniformis* Kabata & Whitaker, 1981. J. Fish Dis. 9: 445.

Musch, K., Siebert, G., Davies, J.V. and Ebert M. 1971. Differenzierung von zwei kathepsinen aus dorschmuskel. Hoppe-Seyler's Z. Physiol. Chem. 352: 878.

Nagahisa, E., Nishimuro, S. and Fujita, T. 1981. Kamaboko-forming ability of the jellied meat of Pacific hake. Bull. Jpn. Soc. Sci. Fish. 49: 901.

Nagahisa, E., Yasuda, S., Nishimuro, S. and Aoki, S. 1983. Jellification of Myxosporidia-infected Pacific hake muscle and its protease activity. Nippon Suisan Central Research Laboratory Report 12:15.

Niki, H., Deya, E. and Igarashi, S. 1984a. Peruvian hake meat having a lack of kamaboko-forming property. Bull. Jpn. Soc. Sci. Fish. 50: 1917.

Niki, H., Kato, T. and Igarashi, S. 1984b. Properties of Peruvian hake meat and preparation of purified surimi. Bull. Jpn. Soc. Sci. Fish. 50: 2043.

Nishimoto, S., Hashimoto, A., Seki, N., Kimura, I., Toyoda, K., Fujita, T. and Arai, K. 1987. Influencing factors on changes in myosin heavy chain and jelly strength of salted meat paste from Alaska pollock during setting. Nippon Suisan Gakkaishi 53: 2011.

Patashnik, M., Groninger, H.S., Barnett, H., Kudo, G. and Koury, B. 1982. Pacific whiting, *Merluccius productus*: I. Abnormal muscle texture caused by myxosporidian-induced proteolysis. Marine Fisheries Review 44: 1.

Reddi, P.K., Constantinides, S.M. and Dymsza, H.A. 1972. Catheptic activity of fish muscle. J. Food Sci. 37: 643.

Shimizu, Y., Nomura, A. and Nishioka, F. 1986. *Modori* (Fish gel degradation occurring at around 60°C)-inducing property of croaker myosin preparation. Bull. Jpn. Soc. Sci. Fish. 52: 2027.

Stehr, C. and Whitaker, D.J. 1986. Host-parasite interaction of the myxosporeans *Kudoa paniformis* Kabata & Whitaker, 1981 and *Kudoa thyrsitis* (Gilchrist, 1924) in the muscle of Pacific whiting, *Merluccius productus* (Ayres): an ultrastructural study. J. Fish Dis. 9: 505.

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

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

Taguchi, T., Tanaka, M. and Suzuki, K. 1983a. Effect of alcohols on *himodori* (thermally induced gel disintegration) in oval filefish meat paste. Bull. Jpn. Soc. Sci. Fish. 49: 1149.

Taguchi, T., Tanaka, M. and Suzuki. 1983b. *Himodori* (thermally induced disintegration) of oval filefish myosin gel. Bull. Jpn. Soc. Sci. Fish. 49: 1281.

Taneda, T., Watanabe, T. and Seki, N. 1983. Purification and some properties of a calpain from carp muscle. Bull. Jpn. Soc. Sci. Fish. 49: 219.

Toyohara, H., Kinoshita, M. and Shimizu, Y. 1990a. Proteolytic degradation of threadfin-bream meat gel. J. Food Sci. 55: 259.

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

Toyohara, H., Sakata, T., Yamashita, K., Kinoshita, M and Shimizu, Y. 1990b. Degradation of oval-filefish meat gel caused by myofibrillar proteinase(s). J. Food Sci. 55: 364.

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

Tsuyuki, H., Williscroft, S.N, Kabata, Z. and Whitaker, D.J. 1982. The relationship between acid and neutral protease activities and the incidence of soft cooked texture in the muscle tissue of Pacific hake (*Merluccius productus*) infected with *Kudoa paniformis* and/or *K. thyrsitis* and held for varying times under different pre-freeze chilled storage conditions. Canadian Technical Report of Fisheries and Aquatic Sciences No. 1130.

Wasson, D.H., Babbitt, J.K. and French, J.S. 1991. Characterization of a heat stable protease from arrowtooth flounder, *Atheresthes stomias*. Bull. Jpn. Soc. Sci. Fish., in review.

Wojtowicz, M.B. and Odense, P.H. 1972. Comparative study of the muscle catheptic activity of some marine species. J. Fish. Res. Bd. Canada 29: 85.

Wolf, K. and Markiw, M.E. 1984. Biology contravenes taxonomy in the Myxozoa: New discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449.

Zeece, M.G. and Katoh, K. 1989. Cathepsin D and its effects on myofibrillar proteins: a review. J. Food Biochem. 13: 157.

II. CONTROL OF MUSCLE SOFTENING AND PROTEASE-PARASITE INTERACTIONS IN ARROWTOOTH FLOUNDER *Atheresthes Stomias*

D.H. Wasson and J.K. Babbitt[1]

National Marine Fisheries Service

Utilization Laboratory

2725 Montlake Blvd. E.

Seattle, WA 98112

Published in *Journal of Food Science*

55(2):579-580 (1990)

[1] Co-author Babbitt's address is National Marine Fisheries Service Utilization Research Laboratory, P.O. Box 1638, Kodiak, AK 99615

ABSTRACT

Distinct proteolytic activity was observed in the incubated muscle tissue of arrowtooth flounder. Absorbance measurements of the TCA filtrates indicated maximum proteolytic activity between 55°C and 60°C, and minimum activity below 40°C. Instron puncture tests of raw, baked, steamed, deep-fat fried and microwaved samples of arrowtooth demonstrated that rapid inactivation of the protease(s) by microwave cooking significantly improved textural properties. Myxosporean parasites tentatively classified as *Kudoa thyrsitis* were identified in all but one of the arrowtooth samples. However, no clear relationship between parasite density and textural deterioration of the cooked fillets was observed.

INTRODUCTION

Estimates of the arrowtooth flounder (*Atheresthes stomias*) resource in the Gulf of Alaska range as high as 65% of the flatfish biomass, excluding halibut (Rose, 1987). The potential harvest of over one million metric tons of this species, however, has been hampered by excessive softening of the muscle tissue when cooked. Similar softening problems have been observed in Pacific whiting (*Merluccius productus*) (Kabata and Whitaker, 1981; Patashnik *et al.*, 1982; Tsuyuki *et al.*, 1982; Erickson *et al.*, 1983; and Kudo *et al.*, 1987) and Peruvian hake (*Merluccius gayi peruanus*) (Niki *et al.*, 1984ab). It has generally been assumed that muscle degradation of this type is the result of a protease, presumably secreted by infecting Myxosporean parasites (Willis, 1949; Patashnik and Groninger, 1964; Lom, 1970; Konagaya, 1980).

The objectives of the study were to document the presence of Myxosporean parasites in arrowtooth, to examine any possible correlation between the extent of infection and autolytic activity at cooking temperatures and to investigate methods to arrest proteolytic activity within that temperature range.

MATERIALS AND METHODS

Seventeen trawl caught arrowtooth flounder (*Atheresthes stomias*) from the Gulf of Alaska were obtained from a commercial trawler approximately 48 hours post-capture. The fish were immediately filleted and held at 4°C during subsequent testing.

Quantification of Myxosporean parasites was done by spreading a loop of homogenate (10:1/ground fish: 0.1 M NaCl) with one drop distilled water within 1 cm^2 on duplicate microscope slides. After heat fixing and staining with methylene blue, the number of spores was counted at 500x magnification.

Autolysis was measured by the method of Miller and Spinelli (1982) for ground fish with the following modifications. Three gram samples were incubated at 5°C and 20°C increments over a temperature range of 0° to 100°C for 30 minutes and the reactions stopped by the addition of 27 mL 5% trichloroacetic acid (TCA). Aliquots of the resulting TCA supernatants were monitored both at 280nm and 562nm with a Hitachi Model 100-60 dual beam spectrophotometer. The latter measurements were conducted after incubating the TCA supernatants with bicinchoninic acid (BCA) protein assay reagents (Pierce Chemical Company, Rockford, Illinois).

An Instron Model 1000 was used to measure the yield point of raw and cooked fish samples. The fillets were cut transversely into 10 cm x 1 cm x 4 cm sections and each section was punched twice on each side of the lateral line with a 1 cm diameter flat surface probe at 55 mm/min crosshead speed and 5 kg force load cell. Since no consistent differences in yield point between sections from the top and bottom fillets were observed, these values were combined in determining the means.

Cooked samples were treated by: 1) baking uncovered for 20 min at 190°C, 2) steaming in foil covered aluminum trays for 20 min, 3) microwaving on HI setting (Kenmore Model No. 566.887 86821, 1400 Watts) for 1.5 min and 4) deep-fat frying of breaded portions in vegetable oil at 190°C for 3.5 min. All samples were allowed to reach 21°C after cooking and breading was removed from the deep-fat fried sections before puncture testing.

RESULTS AND DISCUSSION

Examination of arrowtooth fillets under a dissecting microscope revealed no cysts or any other indications of muscle abnormalities, in contrast to the visible cysts reported for Pacific whiting (Kabata and Whitaker, 1981; Tsuyuki *et al.*, 1982; Morado and Sparks, 1986; Stehr and Whitaker, 1986; and Kudo *et al.* 1987). Myxosporean spores were observed in all but one of the arrowtooth samples and were identified as belonging to the genus *Kudoa* based on the morphology of Kabata and Whitaker (1981).

The following spore densities were observed: 3 fillets were in the 25-50 spores/cm^2 range, 1 was in the 12-25/cm^2 range, 12 had less than 6 spores/cm^2 and in 1 sample no spores were observed. These numbers are extremely low compared to parasite densities recorded for other species of flatfish which share the same habitat in the Gulf (Greene, 1988), and exhibit no textural abnormalities despite the presence of parasites. No significant correlation between parasite density and texture emerged when cooked samples from the same fillets were subject to Instron testing.

Samples of ground incubated arrowtooth flounder revealed a sharp A_{280} peak at 55°C, with almost no activity below 40°C (Fig. II.1). Use of the BCA reagent to test for protein fragments in the TCA supernatants shifted the peak toward 60°C, and showed minimal proteolytic activity occurring between 20°C and 40°C. The rather narrow spike of activity in the 55°C to 60°C range corresponds to that reported for West Coast hake by Tsuyuki and co-workers (1982) and for alkaline protease activity in a variety of fish species (Makinodan *et al.*, 1984).

The sharp drop in autolytic activity observed above 60°C suggested that rapid cooking would minimize tissue damage by denaturing the enzyme(s) before widespread proteolysis could occur. This hypothesis was confirmed by the Instron punch tests, which demonstrated that slow oven cooking was the most detrimental to arrowtooth texture, causing a severe drop in resistance to force compared to the raw fillet (Fig. II.2). Microwaving, on the other hand, effected a 2.8 fold increase in force required at the initial point of failure compared to baking, and a 1.6 fold increase over steaming. The latter treatment, with faster heat transfer than the dry air convection of the oven, also effected a 1.8 fold increase in force observed at the yield point compared to baking. While deep-fat frying has been reported to be effective for minimizing softening in whiting (Patashnik *et al.*, 1982), Instron values for the deep-fried arrowtooth samples showed little improvement over baking. In a comparative test, the mean Instron value for five fish was 0.30 ± 0.20 kg force, compared to 0.26 0.05 kg force for baking.

The use of microwaves to arrest proteolytic activity is a unique application since microwaving does not improve the texture of normal fish that do not display proteolysis when incubated. Studies in this laboratory with both Dover and rock soles (data not shown) have repeatedly demonstrated that the force at yield point decreases after microwaving compared to steaming or baking. The method also has an obvious advantage over the use of inhibitors, such as hydrogen peroxide and potassium bromate (Miller and Spinelli, 1982; Spinelli and Steinberg, 1978) in that microwaving maintains muscle integrity in the whole fillet, while inhibitors are only effective with ground muscle.

CONCLUSION

Autolytic data in the present study indicated the presence of a protease active at physiological pH in all arrowtooth flounder examined. While Myxosporean spores of the genus *Kudoa* were observed in all but one arrowtooth sample, Instron punch testing provided a more reliable means of assessing textural problems than enumerating parasites. Of the four heat treatments used, microwaving preserved the integrity of arrowtooth muscle to the greatest extent. Since proteolysis was shown to be a problem only at temperatures above 40°C, the use of microwaving in the secondary processing of this species for the prepared foods market is recommended.

Figure II.1 Effect of temperature on the autolytic activity of arrowtooth flounder muscle.

Figure II.1

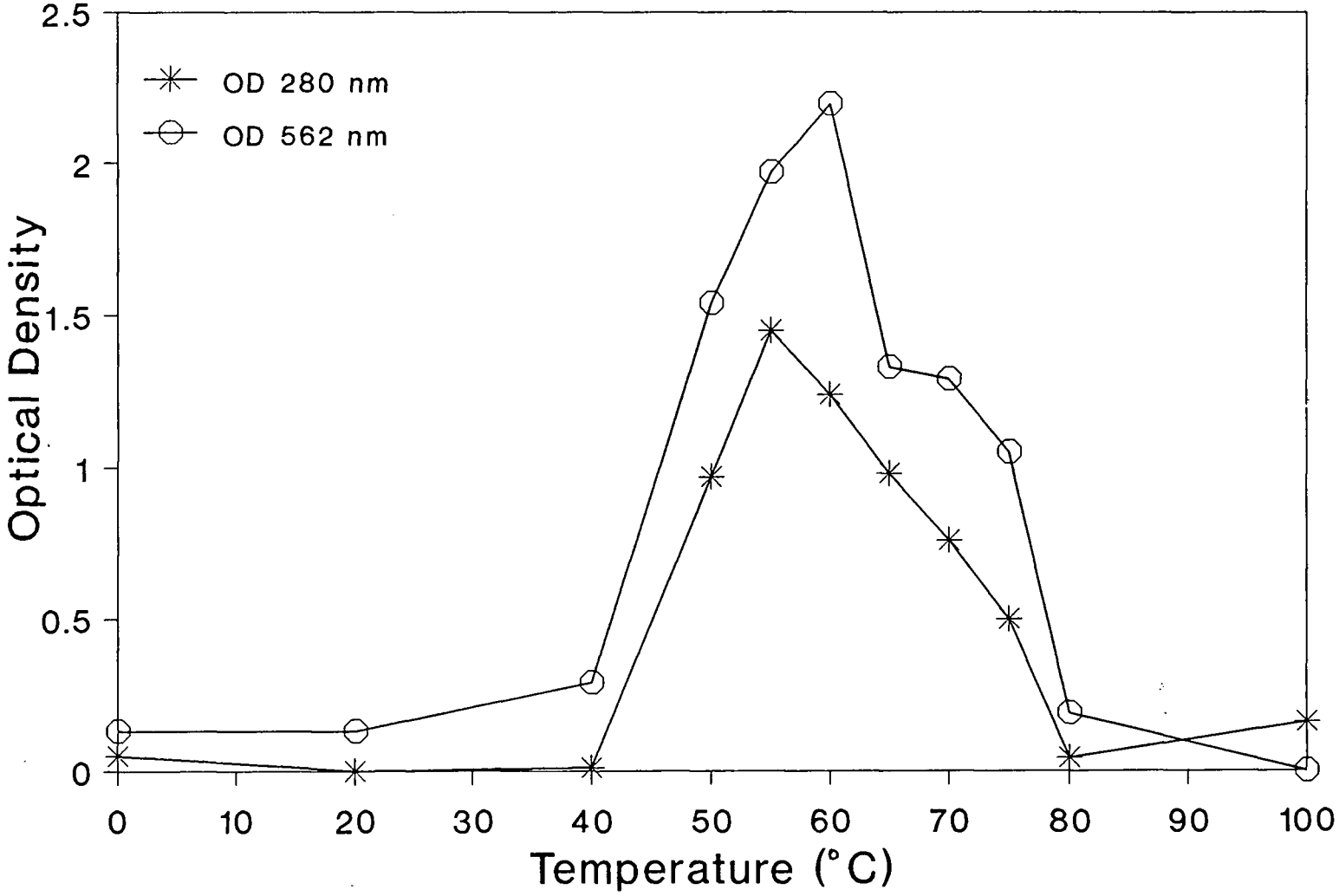
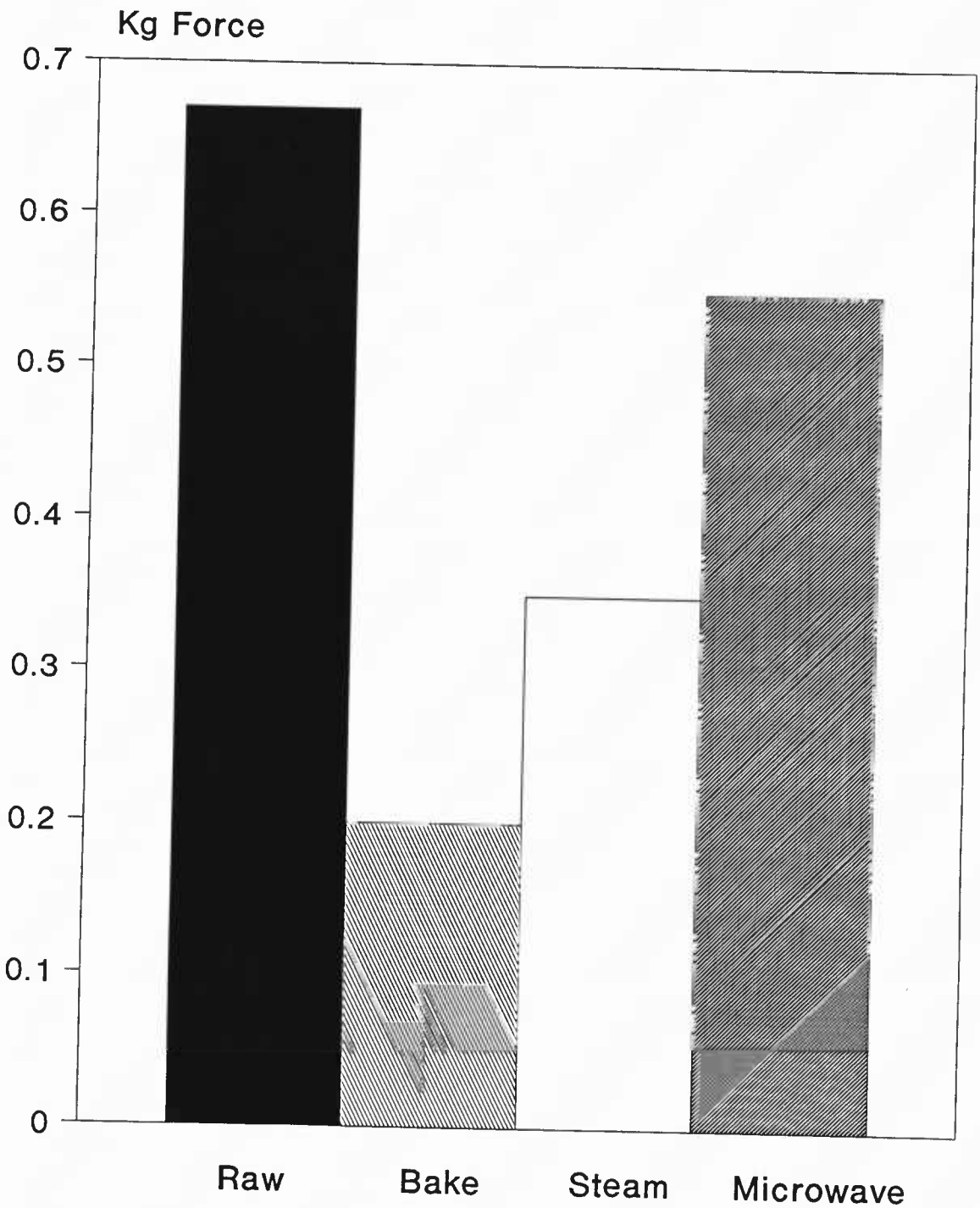

OD 280 nm
OD 562 nm
Optical Density
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

Fig. II.2. Effect of Cooking Procedures on the Yield Point of Arrowtooth Flounder Muscle Subjected to Instron Testing. The vertical bars represent the following mean values (±SD): (raw) 0.67 ± 0.17; (baked) 0.20 ± 0.07; (steamed) 0.35 ± 0.12; (microwaved) 0.55 ± 0.18.

Figure II.2



REFERENCES

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

Greene, D.H. 1988. Flatfish utilization II. Alaska Fisheries Development Foundation. Anchorage, Alaska.

Kabata, Z. and Whitaker, D.J. 1981. Two species of *Kudoa* (Myxosporea: Multivalvulida) parasitic in the flesh of *Merluccius productus* (Ayres, 1855) (Pisces: Teleostei) in the Canadian Pacific. Can. J. Zool. 59: 2085.

Konagaya, S. 1980. Jellification and protease activity of yellowfin sole muscle in association with a Myxosporidian parasite. Bull. Jpn. Soc. Sci. Fish. 46: 1019.

Kudo, G., Barnett, H.J. and Nelson, R.W. 1987. Factors affecting cooked texture quality of Pacific whiting, *Merluccius productus*, fillets with particular emphasis on the effects of infection by the Myxosporeans *Kudoa paniformis* and *K. thyrsitis*. Fish. Bull. 85:745.

Lom, J. 1970. Protozoa causing diseases in marine fishes. In "A Symposium on Diseases of Fishes and Shellfishes," S.F. Snieszko (Ed.), p. 101. Am. Fish. Soc. Spec. Publ. No. 5. Washington, D.C.

Makinodan, Y., Toyohara, H. and Ikeda, S. 1984. Comparison of muscle proteinase activity among fish species. Comp. Biochem. Physiol. 79B: 129.

Miller, R. and Spinelli, J. 1982. The effect of protease inhibitors on proteolysis in parasitized Pacific whiting, *Merluccius productus*, muscle. Fish. Bull. 80: 281.

Morado, J.F. and Sparks, A. 1986. Observations on the host-parasite relations of the Pacific whiting, *Merluccius productus* (Ayres), and two Myxosporean parasites, *Kudoa thyrsitis* (Gilchrist, 1924) and *K. paniformis* Kabata & Whitaker, 1981. J. Fish Dis. 9: 445.

Niki, H., Deya, E. and Igarashi, S. 1984a. Peruvian hake meat having a lack of *kamaboko* forming property. Bull. Jpn. Soc. Sci. Fish. 50: 1917.

Niki, H., Kato, T. and Igarashi, S. 1984b. Properties of Peruvian hake meat and preparation of purified surimi. Bull. Jpn. Soc. Sci. Fish. 50: 2043.

Patashnik, M. and Groninger, H.S. 1964. Observations on the milky condition in some Pacific coast fishes. J. Fish. Res. Bd. Canada 21: 335.

Patashnik, M., Groninger, H.S., Barnett, H., Kudo, G. and Koury, B. 1982. Pacific whiting, *Merluccius productus*: I. Abnormal muscle texture caused by myxosporidian-induced proteolysis. Mar. Fish. Rev. 44: 1.

Rose, C.S. 1987. Flatfish. In "Condition of Groundfish Resources of the Gulf of Alaska Region As Assessed in 1987," p. 185, (Ed.) Major, R.L. and Wilderbuer, T.K., NWAFC/NMFS/NOAA, Seattle, WA.

Spinelli, J. and Steinberg, M. 1978. Inhibition of proteolysis in Pacific hake fillets. Mar. Fish. Rev. 40: 52.

Stehr, C. and Whitaker, D.J. 1986. Host-parasite interaction of the myxosporeans *Kudoa paniformis* Kabata & Whitaker, 1981 and *Kudoa thyrsitis* (Gilchrist, 1924) in the muscle of Pacific whiting, *Merluccius productus* (Ayres): an ultrastructural study. J. Fish. Dis. 9: 505.

Tsuyuki, H. Williscroft, S.N., Kabata, Z. and Whitaker, D.J. 1982. The relationship between acid and neutral protease activities and the incidence of soft cooked texture in the muscle tissue of Pacific hake (*Merluccius productus*) infected with *Kudoa paniformis* and/or *K. thyrsitis* and held for varying times under different prefreeze chilled storage conditions. Can. Tech. Rep. Fish. Aquat. Sci. No. 1130.

Willis, A.G. 1949. On the vegetative forms and life history of *Chloromyxum thyrsitis* Gilchrist and its doubtful systematic position. Aust. J. Sci. Res. (Series B) 2: 379.

ACKNOWLEDGEMENT

Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

III. CHARACTERIZATION OF A HEAT STABLE PROTEASE FROM ARROWTOOTH FLOUNDER *Atheresthes Stomias*

D.H. Wasson, J.K. Babbitt[1] and J.S. French[2]

National Marine Fisheries Service

Utilization Laboratory

2725 Montlake Blvd. E.

Seattle, WA 98112

Submitted to *Bulletin of the Japanese Society of Scientific Fisheries*

[1] National Marine Fisheries Service Utilization Research Laboratory, P.O. Box 1638, Kodiak, AK 99615

[2] University of Alaska--Fairbanks, Fishery Industrial Technology Center, 900 Trident Way, Kodiak, AK 99615

ABSTRACT

A single strand proteolytic enzyme of approximately 32,000 molecular weight was extracted from arrowtooth flounder muscle and purified 125 fold. Activity of the semi-purified enzyme at 55°C was optimal against hemoglobin at pH 3.0 - 3.5 and against casein at pH 6.0 - 7.0. Incubation with chemical reagents indicated the involvement of sulfhydryl groups in enzyme activity. Sodium dodecyl sulfate gel electrophoresis revealed rapid proteolytic degradation of myosin heavy chain, while actin was unaffected.

INTRODUCTION

A number of endogenous proteases have been investigated as possible agents of post-mortem degradation of fish muscle (Makinodan and Ikeda 1969a,1969b, Makinodan et al., 1982a). Among these, the so-called alkaline proteases have been implicated in the textural degradation of fish meat gels at relatively high temperature (Makinodan et al., 1987a). The enzymes as a group are optimally active around pH 8.0 and between 50°C and 70°C, but appear to differ in size and active site according to species. The alkaline proteases in carp (*Cyprinus carpio*) (Iwata et al., 1974, Makinodan et al., 1982c, 1985) and white croaker (*Sciaena schlegeli*) (Makindoan et al., 1987b) were identified as relatively large thiol proteases, 600,000 molecular weight (MW) (Hase et al., 1980) and 780,000 MW (Iwata et al., 1973) for carp and 430,000 MW for white croaker. In contrast, Lin and Lanier (1980) reported a considerably smaller cysteine protease in Atlantic croaker (*Micropogon undulatus*), 80,000 MW. However, the effectiveness of trypsin inhibitors in preventing proteolytic degradation in Atlantic croaker (Lanier et al., 1981) and oval-file fish (Toyohara and Shimizu, 1988, Toyohara et al., 1990) has suggested the involvement of serine proteases in these species. Studies with white croaker (*Micropogon opercularis*) (Busconi et al., 1984, Folco et al., 1984, 1989) have identified both serine and thiol alkaline proteases in fish skeletal muscle.

Research efforts to use currently underutilized fish species for the production of surimi have highlighted the limiting role of the heat stable proteases in fish utilization. The activity of a heat stable protease responsible for actomyosin degradation in cooked

gels has been reported for Peruvian hake (*Merluccius gayi peruanus*) (Niki et al., 1984a, 1984b), Pacific hake (*Merluccius productus*) (Konagaya and Aoki, 1981, Nagahisa et al., 1981, Chang-Lee et al., 1989, 1990) and Atlantic menhaden (*Brevoorti tyrannus*) (Boye and Lanier, 1988). Greene and Babbitt (1990) recently reported a heat stable protease in the skeletal muscle of arrowtooth flounder (*Atheresthes stomias*) from the Gulf of Alaska.

The purpose of this study was to isolate, purify and further characterize the heat-stable protease from arrowtooth flounder in order to gain a clearer understanding of the problems inherent in future utilization of this species for surimi.

MATERIALS AND METHODS

Electrophoretic analysis of arrowtooth muscle

Trawl caught arrowtooth flounder were obtained fresh from a local processor in Kodiak, AK. Skinned fillets were minced and assayed for proteolytic activity (Greene and Babbitt, 1990). Mince from fish displaying the highest activity was chosen for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Three g samples of mince were placed in each of 13 test tubes. Twenty-seven mL pre-measured and heated (95°C) SDS solution (15 mL 10% SDS and 12mL distilled H_2O) were immediately added to one of the tubes to serve as control. After homogenizing for 1 minute, the resulting solution was then held in an 80°C water bath until completely solubilized. The remaining 12 tubes were incubated in a 55°C water bath, and every 5 min up to 1 hr another tube was removed and treated in the same manner as above. Solubilized samples were then heated with sample buffer (1:8) and run on 12% acrylamide gels. The buffer system of Laemmli (1970) was employed.

Preparation of semi-purified protease extracts

Several extracts were prepared from fresh arrowtooth mince to supply sufficient enzyme for subsequent analyses. In each case mince from different fish was analyzed for proteolytic activity (Greene and Babbitt, 1990) prior to extraction, and mince displaying the highest activity was chosen for purification. The procedure of Deng (1981) was followed with several modifications. Since the 65°C heat step recommended by Deng resulted in very low yields from arrowtooth flounder, the

protocol was changed to bring the diluted supernatant up to a final temperature of 52°C over 5 minutes. Proteins precipitated at both 40% and 65% ammonium sulfate saturation were collected separately, dissolved in 0.05 M $Na_2B_4O_7$ - 0.1 M HCl, pH 7.3 (Buffer A), dialyzed against Buffer A and filtered through a 0.45 m membrane before freezing at -34°C. Both fractions were assayed for proteolytic activity against casein (see below) before and after freezing.

Determination of molecular weight

The molecular weight of the heat-stable protease was estimated by gel filtration using the following series of gel types for greater resolution: Sephadex G-200, 40 - 120 bead size (1 x 50 cm)(Sigma Chemical Co., St. Louis, MO), Bio-Gel P-100, 100-200 mesh (1.5 x 100 cm) and Bio-Gel P-60, 100-200 mesh (1.5 x 100 cm) (Bio-Rad Laboratories, Richmond, CA). In each case, flow rate through the column was 11 mL/hr, void volume was determined with blue dextran, and a standard curve was generated by plotting elution fraction vs $\log_{10}$ (MW) for 5 known proteins, bovine thyroglobulin (670 kd), bovine gamma globulin (158 kd), chicken ovalbumin (44 kd), horse myoglobin (17 kd) and cyanocobalamin (1.35 kd). Standards and the semi-purified protease extract were eluted with Buffer A and 1.8 mL fractions were collected. Each fraction was monitored for protein by measuring the absorbance at 280 nm, then assayed for protein content using bicinchoninic acid (BCA) protein assay reagents (Pierce Chemical Company, Rockford, IL). Bovine serum albumin (BSA) was used as a standard. The proteolytic activity of every other fraction containing protein was assayed against casein as described below.

Fractions containing proteolytic activity were pooled and concentrated using 10kd MW cut-off ultrafilters (Bio-Rad, Richmond, CA) in preparation for SDS-PAGE as above. Protein standards were run simultaneously on the gels and a curve generated by plotting $\log_{10}$ MW vs R_f (R_f = distance migrated by protein distance migrated by dye). Myosin (22 kd), ß-galactosidase (116 kd), phosphorylase b (97 kd), BSA (66 kd), chicken ovalbumin (43 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21 kd) and lysozyme (14 kd) were employed as standards (Bio-Rad Laboratories, Richmond, CA). Gels were stained with Coomassie Brilliant Blue R-250, then destained in a 10% methanol/7.4% acetic acid solution.

Proteolytic activity assay

Extracts from successive steps during enzyme purification and fractions collected during gel filtration were tested for proteolytic activity against casein, using a modification of the method of Makinodan and Ikeda (1969b). The reaction mixture contained 1.5 mL Buffer A, 0.5 mL 5% (w/v) sodium caseinate dissolved in Buffer A and 0.5 mL semi-purified enzyme solution. The mixture was incubated 1 hr at 55°C then stopped by addition of 2.5 mL 5% (w/v) trichloroacetic acid (TCA). The blank in each case was prepared by incubating the casein separately from the buffer and enzyme then combining the two component tubes at the end of 1 hr after adding TCA. Solutions were filtered through Whatman GF/C and absorbance measured at 280 nm and 562 nm with a Hitachi Model 100-60 dual beam spectrophotometer. The latter measurements were made on TCA filtrates that had been incubated with BCA protein assay reagents.

Determination of pH optimum

Proteolytic activity of the semi-purified protease extract was assayed over a pH range from 2.5 to 8.1 using McIlvaines's citric acid-phosphate buffer. The basic method of Iwata et al. (1973) for cathepsin D was followed with these modifications. Duplicate reaction tubes were prepared in order to test proteolytic activity against both casein and acid-denatured hemoglobin over the full pH range. Tubes were incubated at 55°C, the reaction was stopped by adding 5% TCA, and the absorbance of the filtrate was read at 280 nm. BCA protein reagents were added to an aliquot of each reaction filtrate, incubated and measured for absorbance at 562 nm.

Effects of Serine and Cysteine Protease Inhibitors

Solutions containing serine and cysteine protease inhibiting reagents were added to incubation tubes containing 125 L semi-purified enzyme extract and 0.5 mL 5% casein solution. Borate buffer (Buffer A), pH 7.3, was added to bring the final volume to 2.5 mL. Duplicate controls were prepared by adding only Buffer A to the enzyme and casein solution. The tubes were incubated 1 hr at 55°C, stopped with 2.5 mL 5% TCA, then filtered through Whatman GF/C paper. Absorbance of the resulting supernatant was read at 280 nm. Absorbance at 562 nm was read after subsequent incubation with BCA protein assay reagents. The following reagents were evaluated for inhibitory properties: 1 mM p-chloromercuriphenylsulfonic acid (p-CMP), 1 mM iodoacetic acid (IAA), 1 mM iodoacetamide (IAM), 25 g/mL cystatin (CYS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 250 g/mL trypsin-chymotrypsin inhibitor (TCI) and 1, 5 and 10 mM adenosine triphosphate (ATP)

(Sigma Chemical Company, St. Louis, MO). Inhibition was evaluated as percent activity compared to the control, based on absorbance at 562 nm.

RESULTS AND DISCUSSION

Electrophoretic analysis of incubated arrowtooth mince (Figure III.1) revealed that myosin heavy chain (MHC) was the main target of proteolytic attack. Myosin degradation was evident after only 5 min heat treatment, and the MHC band disappeared completely by 20 min. Actin, on the other hand, was unaffected during the incubation period. Since the gel strength of surimi is largely a function of MHC aggregation (Lee and Toledo, 1976, Cheng et al., 1979, Nishimoto et al., 1987, Numakura et al., 1987), the degradation of this myofibrillar protein by endogenous protease(s) must be prevented in order to utilize arrowtooth flounder for surimi production.

Successive extractions with 0.5% KCl during the purification process also demonstrated that washing only partially removed the enzyme from the minced flesh and even low levels of residual protease effected rapid degradation of MHC when the mince was incubated (data not shown). This resistance to removal by washing and the extremely rapid rate of myosin degradation observed in the SDS gels suggested that the protease is closely associated with myosin, as was found to be the case with Pacific whiting (Niki et al., 1984b). During repeated extractions, it was found that if 65°C was used as the final temperature during the heat step, all of the proteolytic activity was found in the 65% ammonium sulfate precipitate, but the yield was extremely low. As the temperature was lowered to 52°C the overall yield increased, but more of the activity started appearing in the 40% ammonium sulfate precipitate. Since the specific activity (Table III.1) of the 40% pellet (1.50) was higher than that

of the 65% pellet (0.42), this fraction was always chosen for gel filtration. However, since more than 8.0% of the total activity was recovered in the 65% pellet, a considerably higher final yield would be obtained if this fraction were also purified by column chromatography. Freezing of the dissolved pellets for up to 6 months at -34°C did not affect activity as measured by casein assay.

The gel filtration results (Table III.2) indicate that the heat-stable protease is a protein of approximately 31,000 - 32,000 MW. On both the Bio-Gel P-100 and P-60 columns we observed that as the activity against casein decreased in successive fractions, the protein content of the same fractions began to increase. This protein, however, was inactive against casein, suggesting that a slightly smaller MW protein was co-purifying with the protease. When the fractions were later concentrated and run on duplicate gels, as expected, two bands were visible (Figure III.2). The band at approximately 31,000 (band 1) in each case increased in intensity corresponding to the increase in proteolytic activity of the fractions. The 12,500 MW band (band 2), on the other hand, increased inversely to caseinolytic activity but proportionally to a second protein peak. Since band 2 always appeared as a protein of approximately 12,500 MW, regardless of the column used or the SDS-PAGE run conditions, and the MW ratio between bands 1 and 2 was always approximately 2.5, it is probable that the co-purifying protein is a dimer of about 25,000 MW, and the heat stable protease is a single strand protein.

Full range pH assays for both substrates, casein and hemoglobin, were conducted in duplicate, using semi-purified enzyme solution from both the 40% and 65% ammonium sulfate fractionations (Fig. III.3). The curves obtained in both cases

were identical. Two pH optima were observed, depending on the substrate used. With hemoglobin, the enzyme appeared optimally active at pH 3.0, and almost no activity was detected in the normal range of arrowtooth muscle, i.e. pH 6 - 7. With casein as the substrate, however, a much higher peak of activity was observed in the pH range 6 to 7, with activity dropping to lower levels with increasing alkalinity. Konagaya (1980) reported similar pH curves for protease extracts from yellowfin sole, *Limanda aspera* and identified two distinct proteases corresponding to each of the different activity curves. From the arrowtooth gel filtration and SDS-PAGE data, it would be reasonable to assume that the 25,000 MW protein was responsible for activity against hemoglobin at acid pH, although this was not tested. Most of the heat-stable proteases previously reported in other fish species have had a clear pH optimum in the alkaline range, about pH 8.0 (Makinodan and Ikeda 1969a, 1969b; Iwata et al., 1974; Cheng et al., 1979; Deng, 1981; Busconi et al., 1984; Folco et al., 1984, 1989; Makinodan et al., 1985, 1987b; Boye and Lanier, 1988). That the arrowtooth protease was optimally active in the lower range of physiological pH may explain the severe textural degradation observed in this fish compared to other species.

Casein assays conducted with chemical reagents clearly indicated the involvement of sulfhydryl group(s) in the mechanism of proteolytic attack. All four sulfhydryl modifying reagents, p-CMP, I-Ac, I-Am and CYS, inhibited the activity of the enzyme (Table III.3), and I-Ac effected almost complete inhibition (98.4%). On the other hand, PMSF and TCI, known to be inhibitory for serine proteases, appeared to activate the enzyme. The effects of different concentrations of ATP were investigated since Makinodan et al. (1987b) reported that the cysteine protease isolated

from white croaker was inhibited by increasing concentrations of ATP. Cathepsin D, which was identified in winter flounder as a 32,000 MW protein likewise was found to be inhibited by increasing concentrations of ATP, which Reddi et al. (1972) suggested might be a means of *in vivo* regulation. When added to the arrowtooth flounder protease, however, ATP was clearly an activator, although this did not appear to be concentration dependent. Above 5 mM, activity dropped off to approximately the same level as observed at 1 mM (125%). Although cathepsin D activity has been reported up to 50°C (Makinodan et al., 1982a) and a protein of similar molecular weight was found in another species of flounder, the results of the p-CMP and PMSF assays clearly argue against the identification of the heat-stable protease with cathepsin D. P-CMB normally is without effect against cathepsin D, and PMSF is usually inhibitory. With arrowtooth flounder, p-CMB was inhibitory, while PMSF was slightly activating.

CONCLUSIONS

The presence of a heat stable cysteine protease in arrowtooth flounder muscle that is capable of rapid myosin heavy chain degradation severely limits the use of this species for surimi production without the application of sulfhydryl modifying agents. While chemical reagents demonstrated that the enzyme is not catheptic, extensive pH testing suggested that at least two substrates should be assayed when using a semi-purified enzyme extract. Temperature is also a critical factor in assaying proteolytic activity. Previous data (Greene and Babbitt, 1990) demonstrated that at 37°C, the temperature at which many pH assays are run, the arrowtooth protease exhibits minimal activity. The main characteristics of the enzyme of importance to potential processors of this species are its heat resistance, its rapid and complete destruction of myosin heavy chain at 55°C and the involvement of sulfhydryl group(s) at the active site.

Fig. III.1. SDS-PAGE analysis of myosin degradation in arrowtooth flounder muscle as a function of heat treatment and time. Lanes L and H = low and high molecular weight standards, respectively; lanes a through m represent 5 min intervals from t_0 through t_{60}, all loaded at 10 L. Lane m = t_{60} loaded at 12.5 L; lane o = t_0.

Figure III.1

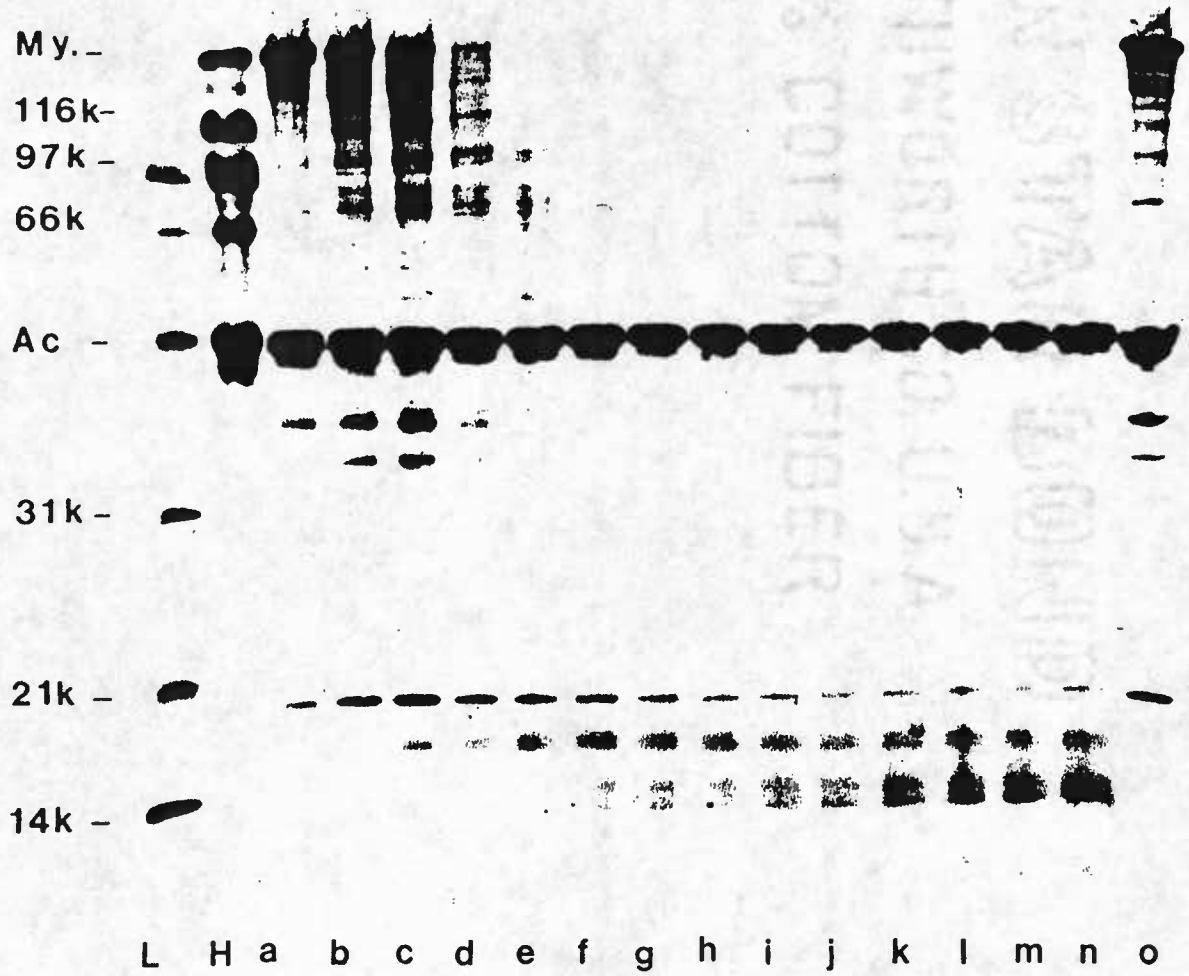


Fig. III.2. SDS-PAGE analysis of Bio-Gel P-100 filtrate containing proteolytic activity. "L" = low molecular weight standards. Lanes a,b,c,d,e represent the following pooled fractions: 36 & 37, 38 & 39, 40 & 41, 42 & 43, and 44 & 45, respectively.

Figure III.2

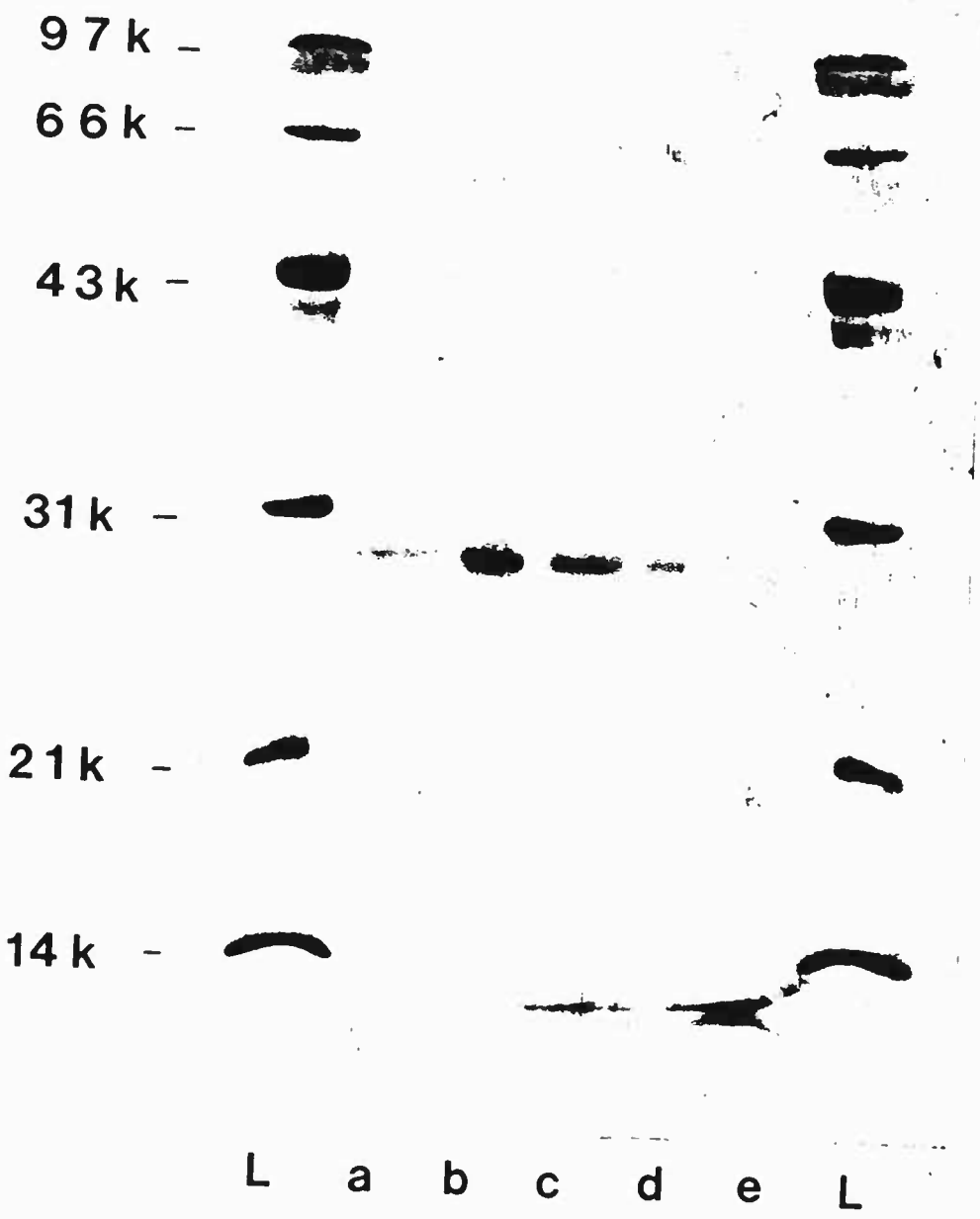


Fig. III.3. Proteolytic activity of the semi-purified arrowtooth enzyme against hemoglobin and casein as a function of pH.

Figure III.3

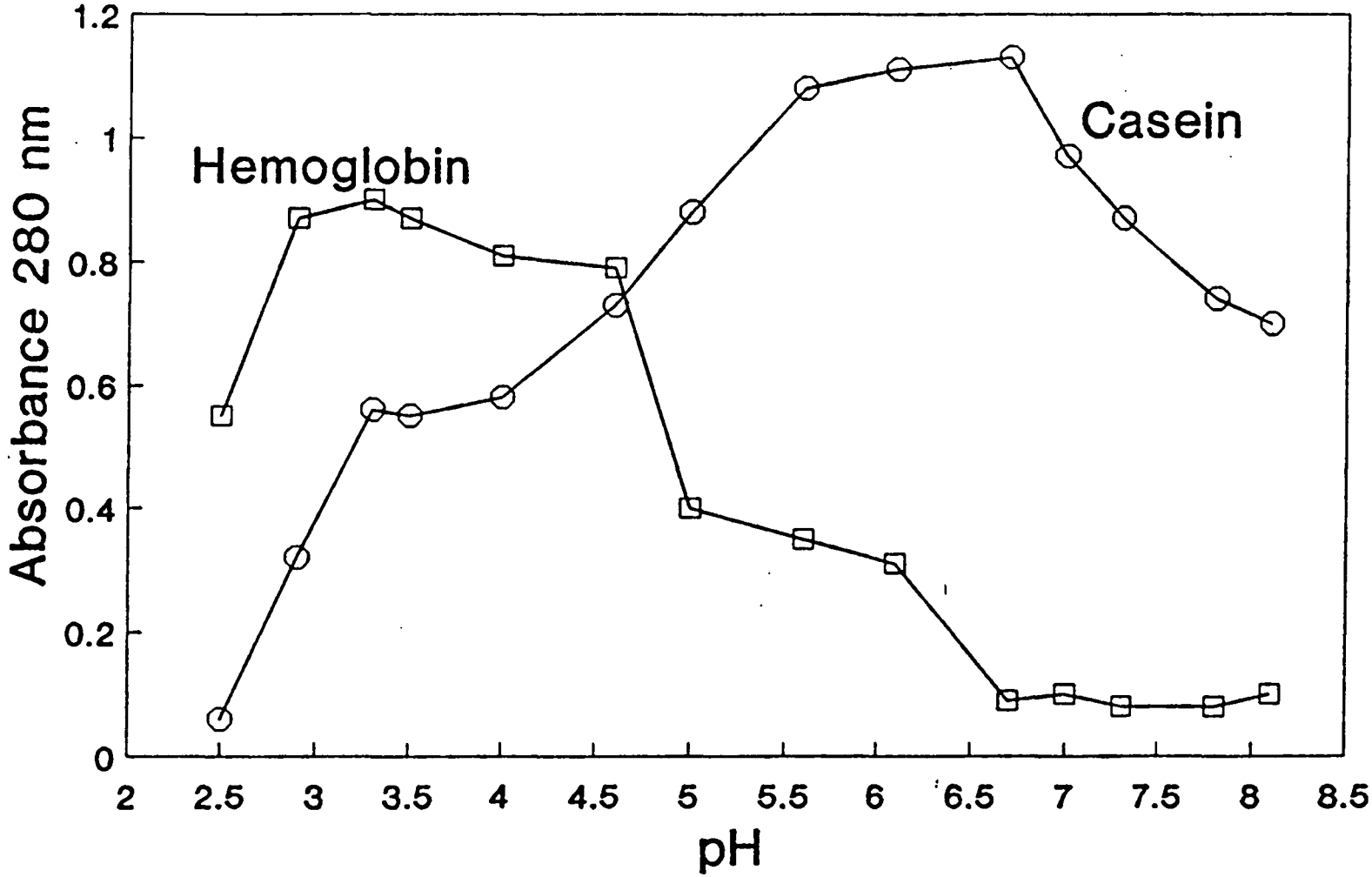

Absorbance 280 nm
1.2
1
0.8
0.6
0.4
0.2
0
Hemoglobin
Casein
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
pH

Table III.1. Purification of heat-stable protease from arrowtooth flounder.

Table III.1

Fraction	Volume (mL)	Units[a] mL	Protein (mg/mL)	Total Units Activity	Specific Activity	Yield (%)	Purification (fold)
Crude Extract	376	1.48	5.47	556.48	0.27	100.0	1.0
40%$(NH_4)_2SO_4$ Pellet	10	2.53	1.69	25.30	1.50	4.5	5.5
65%$(NH_4)_2SO_4$ Pellet	18	2.60	6.21	46.87	0.42	8.0	1.6
Bio-Gel P-60 (40% Pellet)	19.8	0.34	0.01	6.73	34.00	1.2	126.0

[a] Absorbance of casein assay supernatant at 562nm after incubating with BCA protein assay reagents.

Table III.2. Molecular weight determination of heat stable protease from arrowtooth flounder.

Table III.2

Gel Filtration		SDS-PAGE[a] (band ratio)	
Sephadex G-200	29,400		
Bio-Gel P-100	32,300	30,200 12,200	(2.5)
		32,800 12,600	(2.6)
Bio-Gel P-60	33,100	31,000 12,900	(2.4)
		29,000 12,100	(2.4)

[a] SDS-PAGE analysis of the concentrated gel filtration eluate always resulted in the appearance of 2 bands.

Table III.3. Effect of chemical reagents on activity of heat-stable protease from arrowtooth flounder.

Table III.3

Chemicals	Final Concentration	Relative Activity (%)
Control		100.0
P-CMP	1mM	3.6
I-Ac	1mM	1.6
I-Am	1mM	8.7
CYS	25 g	11.0
PMSF	1mM	118.5
ATP	1mM	124.6
ATP	5mM	163.4
ATP	10mM	130.7
TCI	250 g	122.0

REFERENCES

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

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

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

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

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

Deng, J.C. 1981. Effect of temperatures on fish alkaline protease, protein interaction and texture quality. J. Food Sci. 46: 62.

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

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

Greene, D.H and Babbitt, J.K. 1990. Control of muscle softening and protease-parasite interactions in arrowtooth flounder (*Atheresthes stomias*). J. Food Sci. 55: 579.

Hase, J., Kobashi, K. Nakai, N., Mitsui, K., Iwata, K. amd Takadera, T. 1980. The quaternary structure of carp muscle alkaline protease. Biochim. Biophys. Acta 611: 205.

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

Iwata, K., Kobashi, K. and Hase, J. 1974. Studies on muscle alkaline protease--II. Some enzymatic properties of carp muscular alkaline protease.Bull. Jpn. Soc. Sci. Fish. 40: 189.

Konagaya, S. 1980. Jellification and protease activity of yellowfin sole muscle in association with a Myxosporidian parasite. Bull. Jpn. Soc. Sci. Fish. 46: 1019

Konagaya, S. and Aoki, T. 1981. Jellied condition in Pacific hake in relation to Myxosporidian infection and protease activity. Bull. Tokai Reg. Fish. Res. Lab. 105: 1.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680.

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

Lee, C.M. and Toledo, R.T. 1976. Factors affecting textural characteristics of cooked comminuted fish muscle. J. Food Sci. 41: 391.

Lin, T. and Lanier, T. 1980. Properties of an alkaline protease from the skeletal muscle of Atlantic croaker. J. Food Biochem. 4: 17.

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

Makinodan, Y. and Ikeda, S. 1969a. Studies on fish muscle protease--I. On the existence of two kinds of proteinases active in acid and in slightly alkaline pH range. Bull. Jpn. Soc. Sci. Fish. 35: 672.

Makinodan, Y. and Ikeda, S. 1969b. Studies on fish muscle protease--II. Purification and properties of a proteinase active in slightly alkaline pH range. Bull. Jpn. Soc. Sci. Fish. 35: 749.

Makinodan, Y., Kitagawa, T., Toyohara, H. and Shimizu, Y. 1987a. Participation of muscle alkaline proteinase in himodori of kamaboko. Nippon Suisan Gakkaishi. 53: 99.

Makinodan, Y., Kyaw, N.N. and Ikeda, S. 1982b. Classification of carp muscle alkaline proteinase and its action against intracellular proteins. Bull. Jpn. Soc. Sci. Fish. 48: 479.

Makinodan, Y., Kyaw, N.N. and Ikeda, S. 1982c. Intracellular distribution of fish muscle alkaline proteinase. Comp. Biochem. Physiol. 73B: 785.

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

Makinodan, Y., Yokoyama, Y., Kinoshita, M. and Toyohara, H. 1987b. Characterization of an alkaline proteinase of fish muscle. Comp. Biochem. Physiol. 87B: 1041.

Nagahisa, E., Nishimuro, S. and Fujita, T. 1981. Kamaboko-forming ability of the jellied meat of Pacific hake. Bull. Jpn. Soc. Sci. Fish. 49:901.

Niki, H., Deya, E. and Igarashi, S. 1984a. Peruvian hake meat having a lack of kamaboko-forming property. Bull. Jpn. Soc. Sci. Fish. 50: 1917.

Niki, H., Kato, T. and Igarashi, R. 1984b. Properties of Peruvian hake meat and preparation of purified surimi. Bull. Jpn. Soc. Sci. Fish. 50: 2043.

Nishimoto, S., Hashimoto., A., Seki, N., Kimura, I., Toyoda, K., Fujita, T., Arai, K. 1987. Influencing factors on changes in myosin heavy chain and jelly strength of salted meat paste from Alaska pollock during setting. Bull. Jpn. Soc. Sci. Fish. 53: 2011.

Numakura, T., Seki, N., Kimura, I., Toyoda, K., Fujita, T., Takama, K., and Arai, K. 1987. Changes in the SDS-gel filtration patterns of muscle proteins in saltedfish meatpaste during setting (suwari). Bull. Jpn. Soc. Sci. Fish. 53: 2045.

Reddi, P.C., Constantinides, S.M. and Dymsza, H.A. 1972. Catheptic activity of fish muscle. J. Food Sci. 37: 643.

Toyohara, H., Sakata, T., Yamashita, K., Kinoshita, M. and Shimizu, Y. 1990. Degradation of oval-filefish meat gel caused by myofibrillar proteinase(s). J. Food Sci. 55: 364.

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

ACKNOWLEDGEMENTS

Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

IV. EFFECTS OF PROTEIN AND CARBOHYDRATE ADDITIVES ON PROTEOLYTIC AND FUNCTIONAL PROPERTIES OF ARROWTOOTH FLOUNDER SURIMI

D.H. Wasson, K.D. Reppond[1], J.K. Babbitt[1] and J.S. French[2]

National Marine Fisheries Service

Utilization Laboratory

2725 Montlake Blvd. E.

Seattle, WA 98112

Submitted to *Bulletin of the Japanese Society of Scientific Fisheries*

[1] National Marine Fisheries Service Utilization Research Laboratory, P.O. Box 1638, Kodiak, AK 99615

[2] University of Alaska--Fairbanks, Fishery Industrial Technology Center, 900 Trident Way, Kodiak, AK 99615

ABSTRACT

Arrowtooth flounder was found to be a suitable raw material for the production of surimi. Proteolytic activity was not observed during the production process itself, and the 12% residual activity in the mince after washing was effectively inhibited by bovine plasma powder, egg white and α_2-macroglobulin. Plasma and egg white exhibited additional gel enhancing effects when added at levels higher than necessary to prevent proteolytic activity. Carrageenan was not effective as either a protease inhibitor or gel enhancer.

INTRODUCTION

Arrowtooth flounder, *Atheresthes stomias*, is the most abundant commercial fish species in the Gulf of Alaska yet the least utilized due to extreme degradation of the musculature when the fish is cooked. Greene and Babbitt (1990) and Wasson et al. (1991) reported on the presence and characteristics of a heat stable enzyme in arrowtooth muscle, which they found to be a cysteine protease capable of extremely rapid degradation of myosin heavy chain at elevated temperature, particularly in the 50°C-60°C range. The size of the biomass, which is estimated to be between 1 and 2 million metric tons, and the relative inactivity of the enzyme below 20°C consequently suggested the use of this species for surimi. Prior pilot scale production of arrowtooth surimi in the laboratory demonstrated that washing did not completely remove the enzyme from the mince (unpublished data), and residual activity was sufficient to destroy the gel-forming properties of the surimi. In order to maintain the ability to form a strong enough gel for analog production, it was apparent that inhibitory substances would have to be incorporated in the surimi.

Haga et al. (1980) documented the use of 3% dried egg white in improving the gel strength of surimi made from Pacific whiting, *Merluccius productus*, and *Atheresthes evermanni*, a related species of arrowtooth flounder found primarily in the Bering Sea. Chang-Lee et al. (1990) likewise reported the superiority of 3% egg white for improving the functional characteristics of Pacific whiting surimi compared to equal levels of either whey protein concentrate or soy protein isolate. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns clearly

showed the inhibitory effect of egg white in preventing myosin degradation by a similar protease in the whiting muscle (Chang-Lee et al., 1989).

Ueno et al. (1984) demonstrated textural improvement of surimi prepared from low quality Alaska pollock, *Theragra chalcogramma*, and sardine by adding blood plasma and/or lactalbumin. Roussel and Cheftel (1988) obtained similar results by replacing potato starch in gels made from sardine, *Sardina pilchardus*, surimi with bovine serum albumin, egg white or soy protein isolate. Hamann et al. (1990) used dried plasma hydrolysate and egg white solids in surimi made from top quality Atlantic menhaden, *Brevoorti tyrannus*, which, like whiting and arrowtooth flounder, retains proteolytic activity even after the washing steps of the surimi process. Their data indirectly argued for protease inhibition by plasma and egg white.

This paper reports on the characteristics of *Atheresthes stomias* gels prepared from unwashed mince and surimi. Five different protein and starch additives were evaluated for their effects on proteolytic activity, functionality and other physical parameters. Various levels of plasma powder and egg white were also evaluated as a function of when they were added to the surimi, either at the time of surimi manufacture but before freezing (A) or after freezing, when the surimi was tempered for testing (C). These two times of addition were chosen for evaluation since it is often more practical and economical to incorporate additives during analog production rather than during surimi production, which is usually either at sea or at a remote shore-based facility.

MATERIALS AND METHODS

Sample Preparation

Arrowtooth flounder for unwashed mince gels was obtained from a local processor in Kodiak, AK within 24 hr of harvest. Fish for surimi preparation were landed within 12 hr of harvest, filleted and skinned at All Alaskan Seafoods (Kodiak, AK) then processed into surimi at Alaska Pacific Seafoods (Kodiak, AK) within 48 hr of landing. A procedural flow chart listing the equipment used is shown in Fig. IV.1. The surimi was vacuum sealed in 3 kg blocks, plate frozen then stored at -18°C.

Bovine plasma powder (AMP)(AMP-600, American Meat Protein Corp., Ames, Iowa), dried egg white (EW) (P-21, P-110, P-1100, Henningsen Foods, Omaha, NE), whey protein concentrate (WPC) (Cal-Pro 7502, Golden Cheese Company of California, Corona, CA) and carrageenan (XP-8009) (Gelcarin XP-8009, FMC Corp., Philadelphia, PA) were screened for proteolytic inhibition in unwashed mince gels. AMP, EW, XP-8009 and crude α_2 macroglobulin from beef plasma (F-13) (Factor 13, Life Technologies, Inc., Gaithersburg, MD) were subsequently selected for incorporation in surimi gels.

When the surimi was produced, the following test batches were prepared from control surimi before freezing: (Group A) 2% AMP, 3% AMP, 2% AMP + 2% EW, 3% EW. The required amount of each additive was calculated as a percentage of press cake weight (product obtained from the screw press, Fig. 1) before addition of cryoprotectants. In a replicate set of samples (Group B) water was added along with each of the Group A additives to adjust the final moisture content of the surimi to equal that of the control, 74%. Control surimi contained only the following

cryoprotectants: 4% sorbitol, 4% sucrose and 0.3% blended phosphates (Brifisol 450 Super, BK Ladenburg, Cresskill, NJ).

Gel Preparation

Gels from unwashed arrowtooth mince were prepared by homogenizing each of the following additives with 25 g minced flounder: 0.5%, 1.0% and 1.5% AMP, 1.0% and 1.5% each P-21, P-110 and P-1100, 1.0% WPC and 0.5% and 1.0% XP-8009. Distilled water up to a final volume of 50 mL was added to each sample to facilitate blending. The required amount of each additive was calculated as a percentage of the final weight of the homogenate, 50 g. Three g of each mixture were incubated in duplicate test tubes in a 55°C water bath for 1 hr. One set of tubes was used for autolysis assay and the other for SDS-PAGE.

Surimi gels were prepared from surimi within 2 wk of manufacture using a Stephan Vacuum Chopper/Mixer (Model UM 12) according to the method of Babbitt and Reppond (1988). This was arbitrarily designated as "0 months" frozen storage for comparison with product that was held in frozen storage for 3 mo. All gels were blended with 3% NaCl at the time of chopping and cooked in a 90°C water bath for 40 min within 15 - 20 min of stuffing into casings. The cooked gels were then cooled in an ice water bath.

At the time of the first surimi testing, which commenced 1 week after production, gels were also prepared by adding the following ingredients to tempered control surimi: a) all of the Group A ingredients, in order to test the effect, if any, of adding these ingredients at a time after surimi manufacture instead of during

production, b) 2% AMP + 1% XP-8009, and c) 0.1% and 0.2% F-13. At 3 mo, Group A and Group B samples were re-tested as well as plasma and egg white at the following reduced levels: 1% AMP, 1% AMP + 1% EW, and 2% EW.

Protein and Moisture Determination

The protein content was determined in triplicate by the Kjeldahl method using Buchi digestion (model 420) and distillation (model 322) equipment and a Metrohm Dosimat automatic titrator (model 665). The moisture content (5 replicates) was determined with a CEM AVC-80 microwave moisture analyzer.

SDS-PAGE and Autolysis Assays

Samples from mince and surimi gels were solubilized as previously reported (Wasson et al., 1991) then diluted 1:4 and 1:8, respectively, with sample buffer. Aliquots containing approximately 20 g protein were run on 12% acrylamide gels. The buffer system of Laemmli (1970) was employed. Autolytic activity was assayed according to the method of Greene and Babbitt (1990). Trichloroacetic acid (TCA) filtrates were incubated with bicinchoninic acid protein assay reagents (Pierce Chemical Company, Rockford, IL) and monitored at 562 nm with a Hitachi Model 100-60 dual beam spectrophotometer.

Punch, Expressible Moisture and Fold Tests

Cooked surimi gels were equilibrated to 20°C by holding at ambient temperature for approximately 2 hr after cooling in ice water. All gels were tested on

the day of preparation. Punch force and punch deflection at failure were recorded using a Model 302-B Food Checker rheometer (Sunkagaku Co., Ltd.), as reported by Reppond et al. (1987).

Expressible moisture (EM) was determined using an Instron Model 1000. A 30 mm long sample of the cooked gels was placed between two sheets of Whatman No. 1 filter paper and subjected to 90% deformation at a crosshead speed of 50 mm/min. The Instron head was immediately elevated after the compression, and the weight gain in the paper was divided by the original weight of the gel to obtain the percent expressible moisture. Four replicates were measured for each sample.

Cooked gels were cut into 3 mm thick slices for fold evaluation using the following scale for scoring: 1) no fold possible, 2) breaks at 1st fold, 3) folds in half without cracking, 4) folds twice with cracking, 5) folds into quarters without breaking.

Color Evaluation

Uncooked surimi batters and cooked surimi gels prepared from Group A samples were evaluated for CIE L*a*b* values using a Minolta Chroma Meter II Reflectance. In the CIE system, L* is a measure of light intensity, a* values represent the chromatic scale from green (negative a* values) to red (positive a*), and b* values represent the chromatic scale from blue (negative b*) to yellow (positive b*).

Statistical Evaluation

One way analysis of variance (ANOVA) was used to determine whether the various treatments had a significant effect on experimental results. If significant

differences existed, the least significant difference test was used to determine which values were different. The Student t-test was used to determine if any significant changes in expressible moisture occurred between 0 and 3 months. All calculations were computed using programs by Norusis (1988).

RESULTS AND DISCUSSION

Gel Electrophoretic Analysis and Autolysis Tests

SDS-PAGE analysis of gels made from unwashed arrowtooth mince clearly demonstrated the protective effect of AMP in inhibiting the proteolysis of myosin heavy chain, which is represented by the 200,000 molecular weight band in Figure IV.2. On the other hand, myosin degradation was evident after 60 minutes in the sample to which 1% WPC had been added, and the extent of myosin degradation in the XP-8009 samples appeared to be even greater than the control. In the case of egg white powder, myosin degradation varied according to the egg white preparation used (Table IV.1). Results of the autolysis assays (Table IV.1) closely paralleled the patterns of myosin degradation in the SDS gels, confirming earlier data that identified myosin degradation as the major proteolytic event in the textural softening of arrowtooth flounder (Wasson et al., 1991). There appeared to be no advantage to increasing the level of AMP from 1.0% to 1.5%, since there was very little difference in relative activity between the two samples, 12.5% and 13.3%, respectively. Likewise, there was very little difference in relative activity between 1% and 1.5% egg white for any of the powdered egg white samples. P-21 was used in subsequent surimi tests, despite the greater effectiveness of P-1100, since the latter contains 25% sucrose. WPC was dropped from the experiment since it was much less effective at inhibiting proteolysis than either AMP or EW. XP-8009, on the other hand, was later incorporated in surimi along with AMP in order to determine if the carrageenan could function as a gel enhancer in a situation where the protease was controlled by another additive, in this case plasma powder. During surimi production, samples of unwashed

mince and press cake were also taken for SDS-PAGE and autolysis tests. Absorbance readings of the TCA filtrates obtained after incubating the mince and press cake revealed an 88% reduction in proteolytic activity as a result of the washing procedure. The remaining 12% relative activity, however, was sufficient to cause extensive myosin degradation when the surimi was cooked, as evidenced in SDS polyacrylamide gels (Figure IV.3, compare lanes d and e). As predicted by temperature-activity data for the protease reported earlier (Greene and Babbitt, 1990), there was no evidence of proteolytic activity during the production of surimi, since temperatures were kept below 10°C throughout most of the process, with brief periods up to 15°C during refining and later mixing with cryoprotectants. Lanes b, c & d (Figure IV.3) show no change in the myosin band among the three mixing stages, press cake (b), press cake plus cryoprotectants (c) and press cake plus cryoprotectants plus NaCl (d). Only during the heat-set stage was myosin degradation evident. AMP at 2% (lanes f & g) and 3% (lanes h & i) showed no change in the myosin band as a function of heating, nor did 3% EW (j & k) or 2% AMP + 2% EW (l & m). Comparison of lane o, prepared from control surimi to which 2% AMP was added at the time of testing, with lane g, in which 2% AMP had been added at the time of manufacture, indicates that AMP was equally effective in preventing myosin degradation whether added before or after freezing.

Surimi Punch Test Results

Punch test results from the first test period are presented in Figure IV.4. The 3% AMP sample from Group A was lost for comparative purposes since 4.3% NaCl

was inadvertently added to the surimi instead of the standard 3.0%. There were no significant differences in gel strength between the remaining Group A additives that had been mixed before freezing and those which were blended with tempered surimi after freezing. However, there were significant differences among additive treatments. When added along with the cryoprotectants before freezing, 2% AMP and 3% EW effected equally significant improvements in gel strength compared to the control, while the increase in gel strength resulting from the addition of 2% AMP + 2% EW was significantly greater than either of these two.

A similar pattern was observed when the additives were blended with tempered surimi at the time of testing. The following gel strength order was observed ($p<0.05$): (2% AMP + 2% EW) > (2% AMP) = (3% AMP) > (3% EW) = (2% AMP + 1% XP-8009) = (0.2% F-13) > (0.1% F-13). The addition of carrageenan (XP-8009) to surimi containing 2% AMP appeared to interfere with the gel forming ability of the plasma, resulting in lower gel strength than that observed for the surimi containing only 2% AMP. The gel strength of the 3% EW sample was significantly less than that of the 2% AMP sample in this group. Electrophoretic gels (not shown) did not show any myosin degradation in either of the 3% EW samples, indicating that the time of addition did not affect inhibitory ability, but rather that the additional gel forming properties of the egg white differed between the two treatment times. Similarly, there was no evidence of myosin degradation in electrophoretic gels of the 0.2% F-13 sample. Since F-13 does not form a gel, in contrast to AMP and EW, it appears that the maximum gel strength for this lot of arrowtooth surimi with only a protease blocker such as F-13 was in the 500 g.cm - 600 g.cm range. All higher values

observed for the AMP, EW and AMP + EW blends were probably a function of further gel formation by the additives themselves.

All treatments resulted in significantly higher gel strengths than the control. Addition of water (Group B), however, resulted in lower gel strength values (Figure IV.4). Water appeared to impact the egg white treatment groups more than the plasma, such that there was no significant difference in mean gel strength between 2% AMP + H_2O and 2% AMP + 2% EW + H_2O, despite the higher protein content of the latter surimi (Table IV.2). Iso et al. (1985) reported similar observations in pollock surimi, where the gel enhancing effects of added egg white were largely cancelled when water was added. Since gel electrophoretic analysis of all treatment groups represented in Table IV.2 indicated all additives were fully effective in preventing proteolysis of myosin, the protein and moisture data in Table IV.2 strongly suggest that the differences between plasma and egg white powder were not a function of protein and moisture content, but rather basic differences in the way these proteins interacted with the fish myofibrillar proteins. For example, although the 3% EW + H_2O surimi had the same moisture content (72.9%) and a higher protein content than the 2% AMP surimi (18.51 and 18.06, respectively), the 3% EW + H_20 sample had a much lower mean gel strength (612 $\pm$ 57 compared to 712 $\pm$ 46, respectively). Conversely, there was no significant difference in gel strengths between 2% AMP + H_2O and 2% AMP + 2% EW + H_2O, 655 $\pm$ 35 and 696 $\pm$ 99, respectively, despite the higher moisture and lower protein content of the 2% AMP + H_2O surimi.

At 3 mo, no significant changes in gel strength (punch force x punch deflection) were observed for the control, 2% AMP or 3% EW surimis (Table IV.3).

The gel strength of the 2% AMP + 2% EW surimi, however, decreased significantly from 923 $\pm$ 93 to 768 $\pm$ 43. Loss in elasticity, as reflected in a lower punch deflection value, was responsible for the decrease. Lesser decreases in punch deflection values were observed for the other Group A samples (Table IV.3). When the 3 month samples were analyzed by SDS-PAGE and compared to 0 month samples (not shown), no changes in the myosin band were evident for any of the treatments, suggesting that the observed decreases in punch deflection were probably due to normal protein denaturation during frozen storage. Since the lower levels of group A additives, 1% AMP, 2% EW, and 1% AMP + 1% EW, all prevented proteolysis of myosin as determined by SDS-PAGE, the punch force and punch deflection values for these 3 treatments provide additional evidence that higher levels of AMP and EW function as gel enhancers. Such ability to restore elasticity to surimi made from low quality pollock was the basis for the Ueno et al.(1984) patent, prescribing the use of plasma in frozen surimi. However, the arrowtooth surimi results contrast the conclusions of Hamann et al. (1990), who suggested that plasma and egg white proteins function strictly as inhibitors of proteolytic activity.

Expressible Moisture and Fold Test Data

Expressible moisture or, conversely, water holding capacity, have long been considered useful indicators of textural strength. Lee and Toledo (1976) reported an inverse correlation between the amount of expressible moisture from cooked mackerel gels and shear strength, compressive strength, modulus of elasticity and resilience. At the same time, the quantity of expressible fluid increased rapidly as the percentage of

water in the formula was increased. Cheng et al. (1979) further correlated loss of water holding capacity with proteolytic degradation of myofibrillar proteins in Atlantic croaker. A similar positive correlation between proteolytic activity and expressible moisture was observed in Pacific whiting (Chang-Lee et al., 1989). The same general effects were observed in the arrowtooth surimis in Table IV.4, although textural differences between treatments were not always reflected by the expressible moisture due to the presence of additives. At 0 mo, control EM was significantly higher than any of the other treatments tested at that time, reflecting both lower texture scores and proteolytic activity (Figure IV.4). However, there were no significant differences in EM between Group A and Group B (H_20 added) samples, despite significant differences in gel strength. All treatments except the control at both 0 mo and 3 mo received a fold test score of 5, despite significantly higher EM values observed for the 1% AMP, 1% AMP + 1% EW and 2% EW samples than for the other treatments tested at 3 mo.

Color Evaluation

Uncooked arrowtooth flounder surimi was exceptionally white, as were the cooked gels from the control (Table IV.5). L* values for the cooked gels were not significantly affected by the addition of 2% AMP or 3% EW, although CIE L* values for the 3% AMP and 2% AMP + 2% EW surimis were significantly lower than the other treatment groups. There were statistically significant differences in a* values as well, but the range was small. The greatest differences in chromaticity were observed in the b* scale, which served as the best single indicator of color changes that resulted

from the incorporation of additives. A noticeable increase in off-white tones resulted from the addition of plasma powder. Egg white also increased the b* value of the cooked gels, although to a lesser extent than plasma.

CONCLUSION

Results of this study indicated that a commercial grade surimi can be produced from arrowtooth flounder provided that the residual proteolytic activity in the press cake is controlled by the addition of inhibitory substances. Bovine plasma powder and egg white powder both proved to be effective additives. When incorporated in the arrowtooth surimi at levels in excess of that necessary to prevent proteolytic activity, these ingredients were also found to have gel enhancing effects. The addition of plasma powder adversely affected the color of the arrowtooth surimi as measured on the CIE b* scale, although the magnitude of difference would probably be reduced by dilution in most analog product applications.

Fig. IV.1. Arrowtooth surimi production flow chart

Figure IV.1

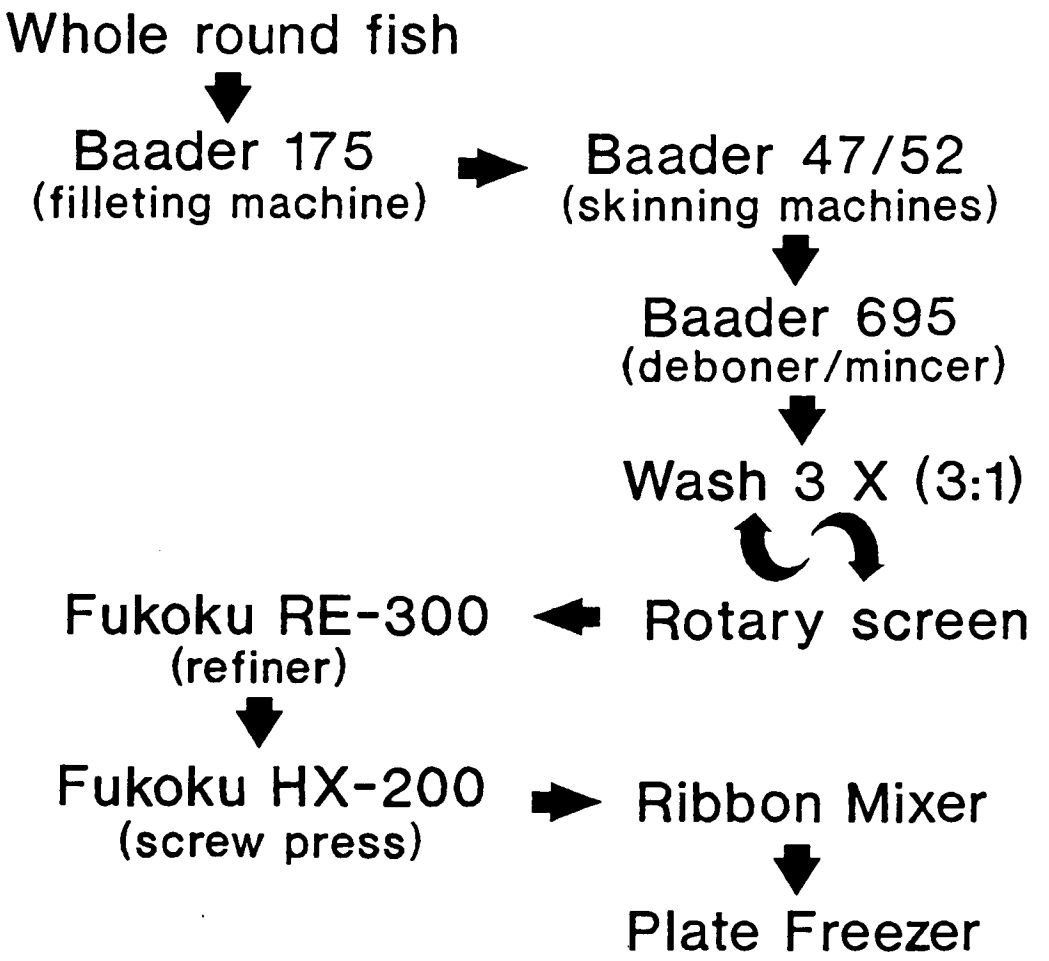

Whole round fish
Baader 175
(filleting machine)
Baader 47/52
(skinning machines)
Baader 695
(deboner/mincer)
Wash 3 X (3:1)
Rotary screen
Fukoku RE-300
(refiner)
Fukoku HX-200
(screw press)
Ribbon Mixer
Plate Freezer

Fig. IV.2. SDS-PAGE analysis of unwashed arrowtooth mince incubated at 55°C. "H" and "L" = high and low molecular weight standards, respectively. Lanes a-c = control; lanes d-f = 1.0% AMP; lanes g-i = 1.0% WPC; lanes j-l = 0.5% XP-8009; lanes m-o = 1.0% XP-8009. Each series of 3 lanes represents sequential sampling at 0, 30 and 60 minutes, respectively.

Figure IV.2

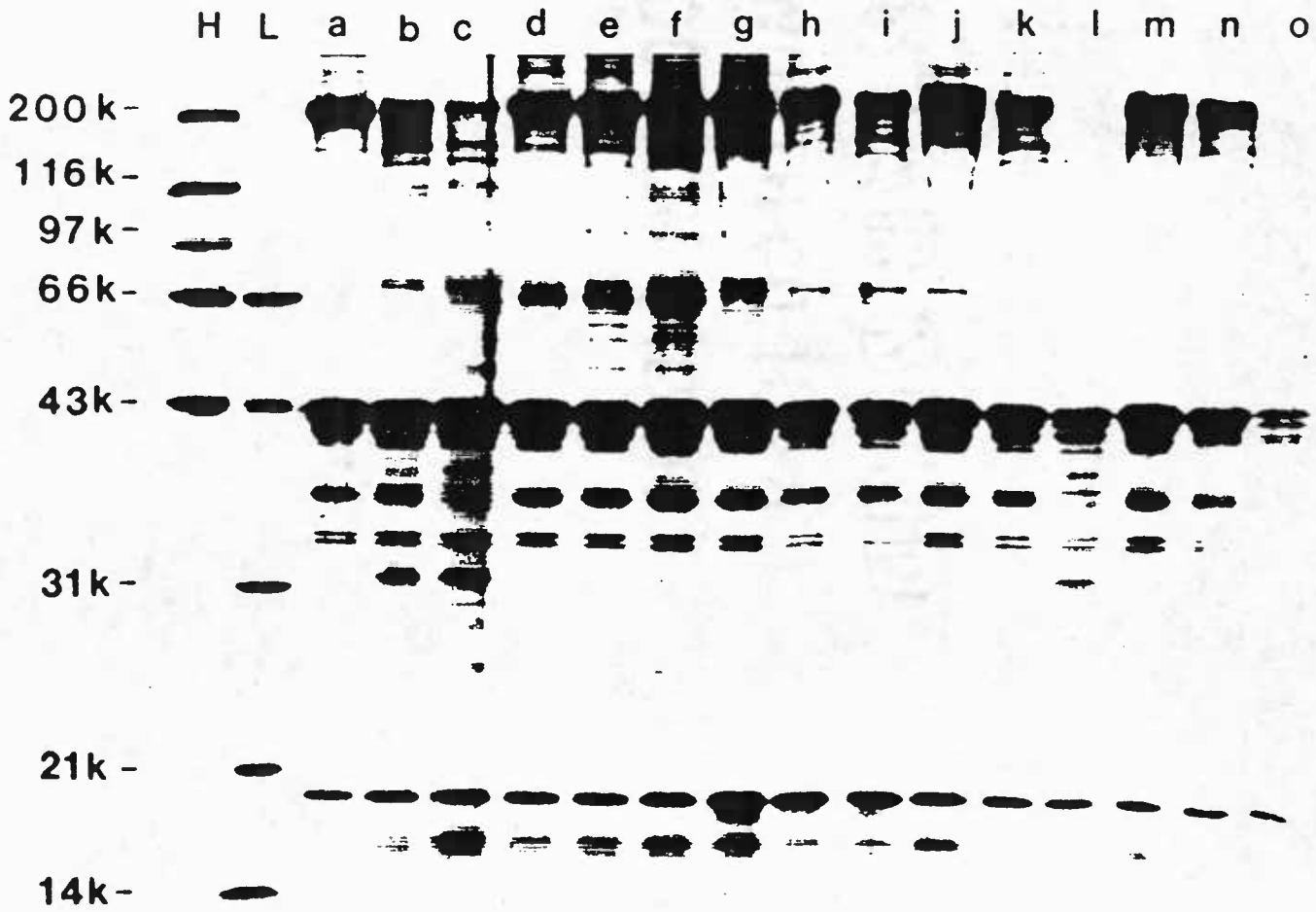


Fig. IV.3. SDS-PAGE analysis of proteolytic activity in arrowtooth flounder muscle during surimi production and gel strength testing at 0 mo. "L" and "H" = low and high molecular weight standards, respectively. "a" = unwashed mince, "b" = press cake, "c" = control surimi (with cryoprotectants only), "d" = control surimi after addition of NaCl (Control), uncooked (U), "e" = control, cooked (C), "f" = 2.0% AMP (U), "g" = 2.0% AMP (C), "h" = 3.0% AMP (U), "i" = 3.0% AMP (C), "j" = 3.0% EW (U), "k" = 3.0% EW (C), "l" = 2.0% AMP + 2.0% EW (U), "m" = 2.0% AMP + 2.0% EW (C), "n" = 2.0% AMP added to control after freezing (U), "0" = 2.0% AMP added to control after freezing (C).

Figure IV.3

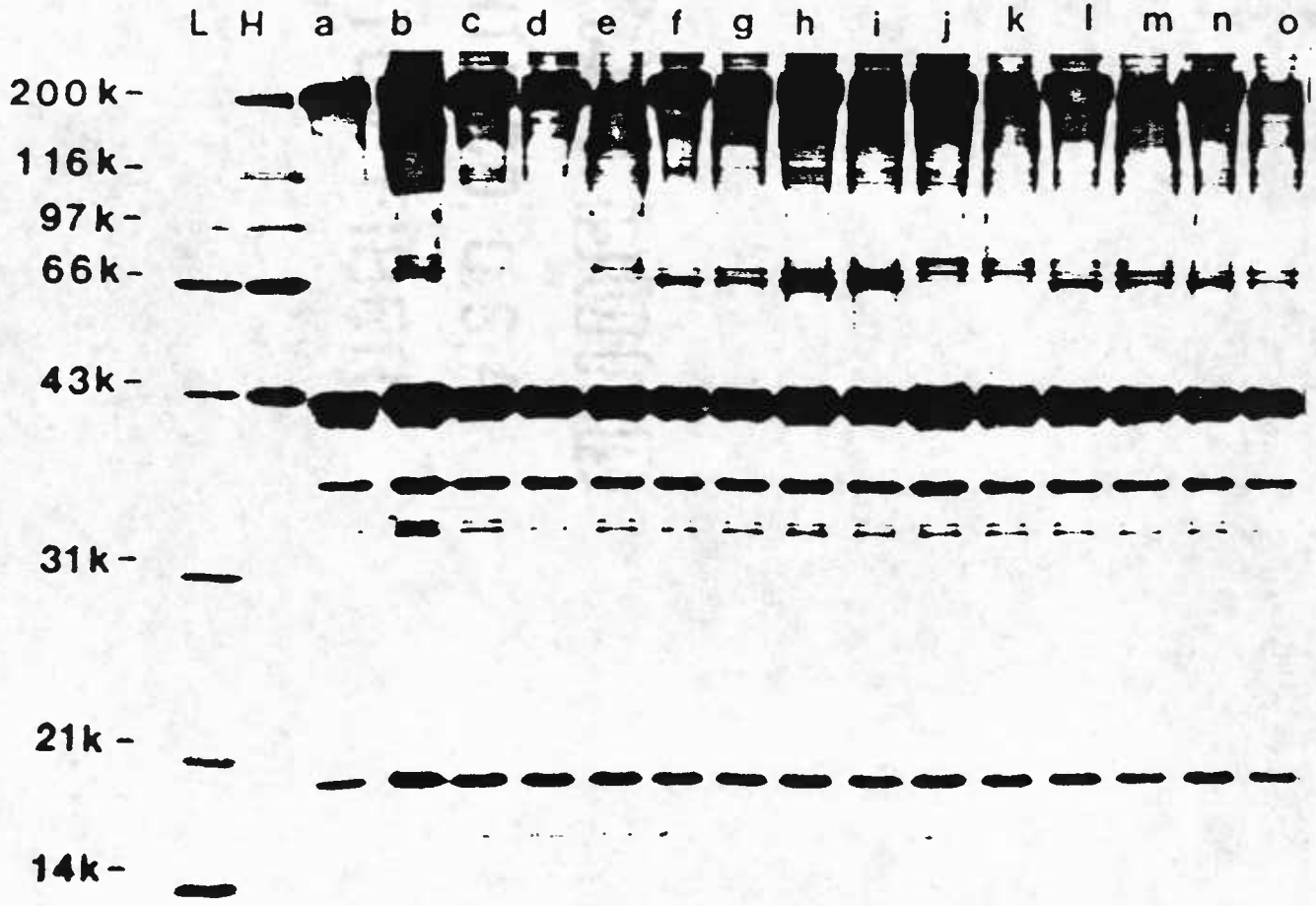


Fig. IV.4. Gel strength values (g x cm) of arrowtooth surimis tested at 0 mo.

Figure IV.4

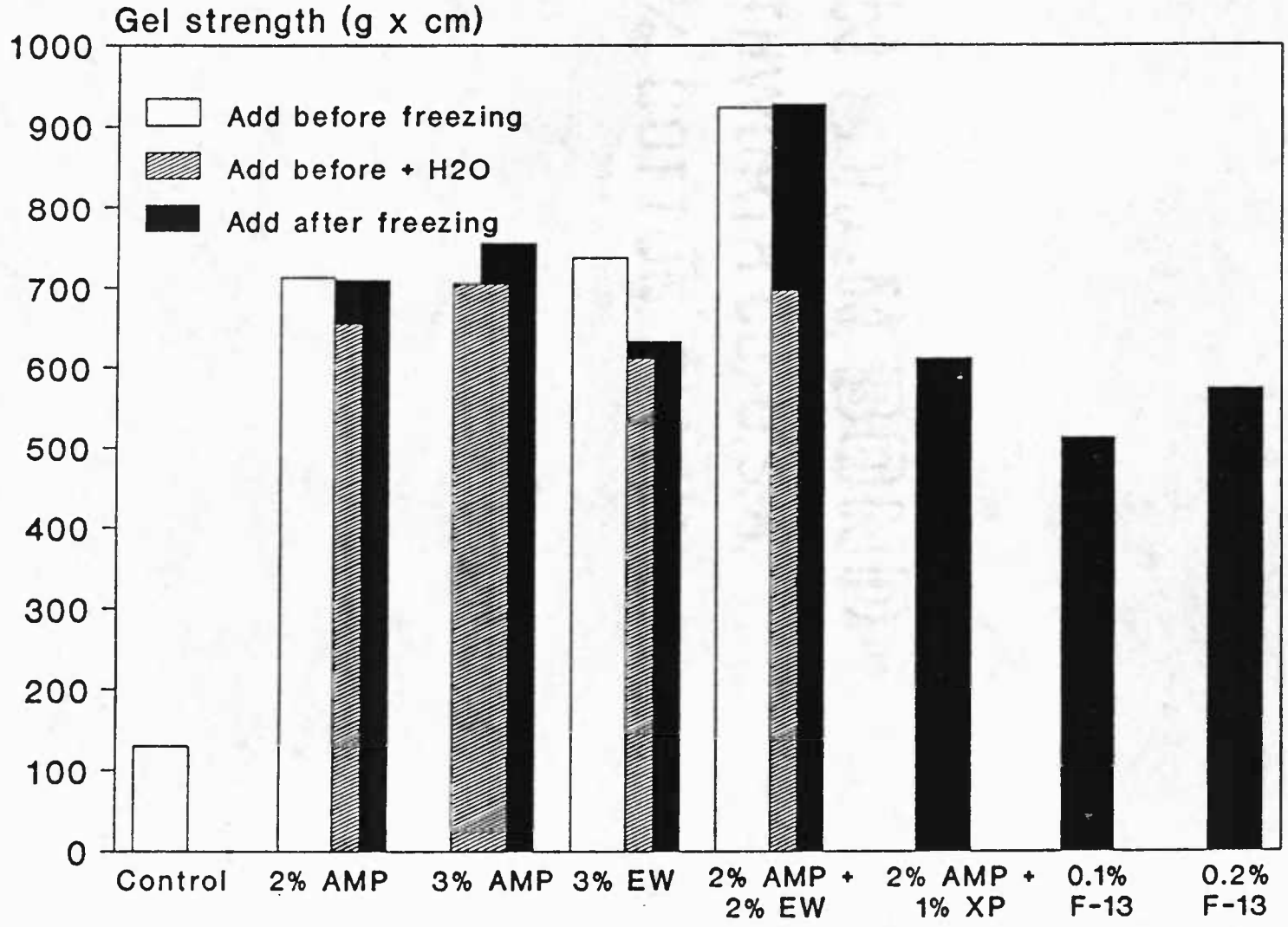

Gel strength (g x cm)
1000
900
800
700
600
500
400
300
200
100
0
Add before freezing
Add before + H2O
Add after freezing
Control
2% AMP
3% AMP
3% EW
2% AMP + 2% EW
2% AMP + 1% XP
0.1% F-13
0.2% F-13

Table IV.1. Effect of additives on autolytic activity of minced arrowtooth flounder gels.

Table IV.1

Additive	Concentration (%)	Relative Activity (%)
Control		100.0
AMP	0.5	26.6
	1.0	12.5
	1.5	13.3
EW (P-21)	1.0	22.1
	1.5	23.7
EW (P-110)	1.0	33.0
	1.5	20.7
EW (P-1100)	1.0	16.0
	1.5	17.8
WPC	1.0	31.1
XP-8009	0.5	97.6
XP-8009	1.0	85.4

Table IV.2. Moisture and protein content of arrowtooth surimi treatment groups.

Table IV.2

Additive	Moisture (%)[a]	Protein (%)[b]
Control	74.2 ± 0.07^{A}	16.05 ± 0.13^{A}
2% AMP	72.9 ± 0.08^{C}	18.06 ± 0.39^{B}
2% AMP + H_2O	73.4 ± 0.13^{B}	17.85 ± 0.20^{B}
3% AMP	72.3 ± 0.06^{E}	18.67 ± 0.25^{C}
3% AMP + H_2O	72.8 ± 0.00^{C}	18.11 ± 0.20^{B}
3% EW	72.5 ± 0.06^{D}	18.84 ± 0.03^{D}
3% EW + H_2O	72.9 ± 0.21^{C}	18.51 ± 0.29^{C}
2% AMP + 2% EW	71.9 ± 0.15^{F}	19.08 ± 0.06^{D}
2% AMP + 2% EW + H_2O	72.6 ± 0.15^{D}	18.78 ± 0.13^{C,D}
ANOVA F	139.9	37.97
P	<0.001	<0.001

[a] Values represent the mean of 5 replicates.
[b] Values represent the mean of 3 replicates.
[A-F] Means within a column not sharing a common letter were significantly different ($p < 0.05$).

Table IV.3. Punch force and punch deflection values of surimi at 0 and 3 mo.

Table IV.3

Additive	Punch Force (g)		Punch Deflection (mm)	
	0 Months	3 Months	0 Months	3 Months
Control	184 ± 16^{A}	251 ± 22^{A}	7.0 ± 0.3^{A}	6.6 ± 0.4^{A}
2% AMP	651 ± 26^{B}	749 ± 28^{E}	10.9 ± 0.3^{B}	10.4 ± 0.3^{F}
3% AMP		750 ± 39^{E}		10.1 ± 0.3^{E}
3% EW	690 ± 57^{C}	723 ± 28^{D}	10.7 ± 0.3^{B}	10.0 ± 0.4^{D,E}
2% AMP + 2% EW	780 ± 40^{D}	785 ± 25^{F}	11.9 ± 1.3^{C}	9.8 ± 0.3^{D}
1% AMP		617 ± 22^{C}		9.8 ± 0.4^{D}
2% EW		584 ± 34^{C}		8.7 ± 0.5^{B}
1% AMP + 1% EW		626 ± 23^{C}		9.0 ± 0.3^{C}
ANOVA F	495.7	353.4	84.65	105.8
P	<0.001	<0.001	<0.001	<0.001

$^{A-F}$ Means within a column not sharing a common letter were significantly different ($p<0.05$).

Table IV.4. Expressible moisture of arrowtooth surimi treatment groups.

Table IV.4

Additive	0 Months (%)	3 Months (%)
Control	1.34 ± 0.11^{A}	1.26 ± 0.13^{A}
2% AMP	1.07 ± 0.05^{B}	$1.08 \pm 0.05^{C,D}$
2% AMP + H_2O	1.01 ± 0.08^{B}	0.97 ± 0.11^{D}
3% AMP		0.96 ± 0.13^{D}
3% AMP + H_2O	1.02 ± 0.16^{B}	$1.04 \pm 0.06^{C,D}$
3% EW	0.97 ± 0.09^{B}	0.95 ± 0.10^{D}
3% EW + H_2O	1.06 ± 0.10^{B}	$1.13 \pm 0.08^{A,B,C}$
2% AMP + 2% EW	1.01 ± 0.05^{B}	$1.06 \pm 0.03^{C,D}$
2% AMP + 2% EW + H_2O	0.96 ± 0.03^{B}	0.98 ± 0.07^{D}
1% AMP		$1.21 \pm 0.24^{A,B}$
1% AMP + 1% EW		1.24 ± 0.12^{A}
2% EW		$1.16 \pm 0.08^{A,B,C}$
ANOVA F	7.922	4.976
P	<0.001	<0.001

$^{A-D}$ Means within a column not shring a common letter were significantly different ($p<0.05$).

Table IV.5. CIE L*a*b* values of cooked gels from arrowtooth flounder surimi.

Table IV.5

Treatment	L*	a*	b*
Control	83.8^{C} ±0.1	-1.9^{D} ±0.2	2.3^{A} ±0.1
2% AMP	81.9^{C} ±0.6	-3.4^{A} ±0.1	5.3^{C} ±0.2
3% AMP	79.9^{A} ±0.4	-2.4^{C} ±0.2	6.2^{D} ±0.1
3% EW	83.8^{C} ±0.4	-2.0^{D} ±0.1	3.7^{B} ±0.1
2% AMP + 2% EW	79.2^{A} ±0.7	-3.0^{B} ±0.2	6.0^{D} ±0.1
ANOVA			
F	62.39	72.94	632.8
P	<0.001	<0.001	<0.001

$^{A-D}$ Means within a column not sharing a common letter were significantly different ($p<0.05$).

REFERENCES

Babbitt, J.K. and Reppond, K.D. 1988. Factors affecting the gel properties of surimi. J. Food Sci. 53: 965.

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

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

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

Greene, D.H. and Babbitt, J.K. 1990. Control of muscle softening and protease-parasite interactions in arrowtooth flounder *Atheresthes stomias*. J. Food Sci. 55: 579.

Haga, H., Shigeoka, R. and Yamauchi, T. 1980. Method for processing fish contaminated with sporozoa. U.S. patent 4,207,354, Jun. 10.

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

Iso, N., Mizuno, H., Saito, T., Lin, C.Y., Fujita, T. and Nagahisa, E. 1985. The effects of additives (egg white and soybean protein) on the rheological properties of kamaboko. Bull. Jpn. Soc. Sci. Fish 51: 485.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680.

Lee, C.M. and Toledo, R.T. 1976. Factors affecting textural characteristics of cooked comminuted fish muscle. J. Food Sci. 41: 391.

Norusis, M.J. 1988. The Statistical Package for the Social Scientist, PC+ Version 3.0, Chicago, IL.

Reppond, K.D., Edson, S.A., Babbitt, J.K. and Hardy, A. 1987. Observations on the functional properties of U.S. land-processed surimi. J. Food Sci. 52: 505.

Roussel, H. and Cheftel, J.C. 1988. Characteristics of surimi and kamaboko from sardines. International J. of Food Sci. and Tech. 23: 607.

Ueno, R., Kanayama, T., Tomiyasu, K., Fujikami, A. and Nakashima, T. 1984. Processes for production of refrigerated minced fish flesh and fish paste product having improved quality. U.S. patent 4,464,404, Aug. 7.

Wasson, D.H., Babbitt, J.K. and French, J.S. 1991. Characterization of a heat stable protease from arrowtooth flounder, *Atheresthes stomias*. In review.

ACKNOWLEDGEMENTS

Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

BIBLIOGRAPHY

Babbitt, J.K. and Reppond, K.D. 1988. Factors affecting the gel properties of surimi. J. Food Sci. 53: 965.

Bonete, M.J., Manjon, A., Llorca, F. and Iborra, J.L. 1984. Acid proteinase activity in fish II. Purification and characterization of cathepsins B and D from *Mujil auratus* muscle. Comp. Biochem. Physiol. 78B: 207.

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

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

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

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

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

Cheng, C.S., Hamann, D.D., Webb, N.B. and Sidwell, V. 1979b. Effects of species and storage time on minced fish gel texture. J. Food Sci. 44: 1087.

Deng, J.C. 1981. Effect of temperatures on fish alkaline protease, protein interaction and texture quality. J. Food Sci. 46: 62.

Doke, S.N., Ninjoor, V. and Nadkarni, G.B. 1980. Characterization of cathepsin D from the skeletal muscle of fresh water fish, *Tilapia mossambica*. Agric. Biol. Chem. 44: 1521.

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

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

Folco, E.J., Busconi, L, Martone, C.B. and Sanchez, J.J. 1988a. Multicatalytic proteinase in fish muscle. Arch. Biochem. Biophys. 267: 599.

Folco, E.J., Busconi, L., Martone, C.B., Trucco, R.E. and Sanchez, J.J. 1988b. Activation of an alkaline proteinase from fish skeletal muscle by fatty acids and sodium dodecyl sulfate. Comp. Biochem. Physiol. 91B: 473.

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

Geist, G.M. and Crawford, D.L. 1974. Muscle cathepsins in three species of Pacific sole. J. Food Sci. 39: 548.

Goll, D.E., Kleese, W.C. and Szpacenko, A. 1989. Skeletal muscle proteases and protein turnover. Ch. 8. In *Animal Growth Regulation*, D.R. Campion, G.J. Hausman and R.J. Martin (Eds.), p.141. Plenum Press, New York.

Greene, D.H. 1988. Flatfish utilization II. Alaska Fisheries Development Foundation. Anchorage, Alaska.

Greene, D.H. and Babbitt, J.K. 1990. Control of muscle softening and protease-parasite interactions in arrowtooth flounder *Atheresthes stomias*. J. Food Sci. 55: 579.

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

Hara, K., Suzumatsu, A. and Ishihara, T. 1988. Purification and characterization of cathepsin B from carp ordinary muscle. Nippon Suisan Gakkaishi 54: 1243.

Hase, J., Kobashi, K., Nakai, N., Mitsui, K., Iwata, K. and Takadera, T. 1980. The quaternary structure of carp muscle alkaline protease. Biochimica et Biophysica Acta 611: 205.

Iso, N., Mizuno, H., Saito, T., Lin, C.Y., Fujita, T. and Nagahisa, E. 1985. The effects of additives (egg white and soybean protein) on the rheological properties of kamaboko. Bull. Jpn. Soc. Sci. Fish 51: 485.

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

Iwata, K., Kobashi, K. and Hase, J. 1974a. Studies on muscle alkaline protease-II. Some enzymatic properties of carp muscular alkaline protease. Bull. Jpn. Soc. Sci. Fish. 40: 189.

Iwata, K., Kobashi, K. and Hase, J. 1974b. Studies on muscle alkaline protease-III. Distribution of alkaline protease in muscle of freshwater fish, marine fish and in internal organs of carp. Bull. Jpn. Soc. Sci. Fish. 41: 201.

Iwata, K., Kobashi, I. and Hase, KJ. 1974c. Studies on muscle alkaline protease-IV. Comparison of physicochemical and enzymatic properties of alkaline protease from white croaker and barracuda muscle with those of the enzyme from carp muscle. Bull. Jpn. Soc. Sci. Fish. 40: 1043.

Iwata, K., Kobashi, K. and Hase, J. 1974d. Studies on muscle alkaline protease-V. Effect of carp muscular alkaline protease upon *modori* phenomenon in *kamaboko* production. Bull. Jpn. Soc. Sci. Fish. 40: 1051.

Iwata, K., Kobashi, K. and Hase, J. 1977. Studies on muscle alkaline protease-VI. Purification of proteins which induce the *modori* phenomenon during *kamaboko* production and of cathepsin A from carp muscle. Bull. Jpn. Soc. Sci. Fish. 43: 181.

Iwata, K., Kobashi. K. and Hase, J. 1979. Studies on muscle alkaline protease-VII. Effect of the muscular alkaline protease and protein fractions purified from white croaker and horse mackerel on the *himodori* phenomenon during *kamaboko* production. Bull. Jpn. Soc. Sci. Fish. 45: 157.

Kabata, Z. and Whitaker, D.J. 1981. Two species of *Kudoa* (Myxosporea: Multivalvulida) parasitic in the flash of *Merluccius productus* (Ayres, 1855) (Pisces: Teleostei) in the Canadian Pacific. Can. J. Zool. 59: 2085.

Konagaya, S. 1980. Jellification and protease activity of yellowfin sole muscle in association with a Myxosporean parasite. Bull. Jpn. Soc. Sci. Fish. 46: 1019.

Konagaya, S. 1982. Separation from jellied yellowfin tuna meat of the protease responsible for jellification. Bull. Jpn. Soc. Sci. Fish. 48: 543.

Konagaya, S. 1983. Protease responsible for jellification of myxosporidia-infected swordfish meat. Bull. Jpn. Soc. Sci. Fish. 49: 919.

Konagaya, S. 1984. Studies on the jellied meat of fish, with special reference to that of yellowfin tuna. Bull. Tokai Reg. Fish. Res. Lab. 114:1.

Konagaya, S. and Amano, K. 1973. Studies on jellied meat of tuna-III. Proteolytic activity of the jellied meat of yellowfin tuna. Bull. Jpn. Soc. Sci. Fish. 39: 1169.

Konagaya, S. and Aoki, T. 1981. Jellied condition in Pacific hake in relation to Myxosporidian infection and protease activity. Bull. Tokai Reg. Fish. Res. Lab. 105: 1.

Kramer, D.E., Nordin, D.M.A. and Boyd, J.W. 1977. Studies on the muscle texture and proteolytic activity of arrowtooth flounder, *Atheresthes stomias*. Paper presented at the 28th Annual Meeting of Pacific Fisheries Technologists, Harrison Hot Springs, British Columbia, March 2-5.

Kudo, G., Barnett, H.J. and Nelson, R.W. 1987. Factors affecting cooked texture quality of Pacific whiting, *Merluccius productus*, fillets with particular emphasis on the effects of infection by the Myxosporeans *Kudoa paniformis* and *K. thyrsitis*. Fishery Bulletin 85: 745.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680.

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

Lee, C.M. and Toledo, R.T. 1976. Factors affecting textural characteristics of cooked comminuted fish muscle. J. Food Sci. 41: 391.

Lee, N., Seki, N., Kato, N., Nakagawa, N., Terui, S. and Arai, K. 1990. Gel forming ability and cross-linking ability of myosin heavy chain in salted meat paste from threadfin bream. Nippon Suisan Gakkaishi 56: 329.

Lester, R.J.G. 1982. *Unicapsula seriolae* n. sp. (Myxosporea, Multivalvulida) from Australian yellowtail kingfish *Seriola lalandi*. J. Protozool. 29: 584.

Lin, T.S and Lanier, T.C. 1980. Properties of an alkaline protease from the skeletal muscle of Atlantic croaker. J. Food Biochem. 4: 17.

Lom, J. 1970. Protozoa causing diseases in marine fishes. In "A Symposium on Diseases of Fishes and Shellfishes," S.F. Snieszko (Ed.), p. 101. Am. Fish. Soc. Spec. Publ. No. 5. Washington, D.C.

Lom, J. and Arthur, J.R. 1989. A guideline for the preparation of species descriptions in Myxosporea. J. Fish Dis. 12: 151.

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

Makinodan, Y. and Ikeda, S. 1969a. Studies on fish muscle protease-I. On the existence of two kinds of proteinases active in acid and in slightly alkaline pH range. Bull. Jpn. Soc. Sci. Fish. 35: 672.

Makinodan, Y. and Ikeda, S. 1969b. Studies on fish muscle protease-II. Purification and properties of a proteinase active in slightly alkaline pH range. Bull. Jpn. Soc. Sci. Fish. 35: 749.

Makinodan, Y. and Ikeda, S. 1969c. Studies on fish muscle protease-III. Purification and properties of a proteinase active in acid pH range. 1969. Bull. Jpn. Soc. Sci. Fish. 35: 758.

Makinodan, Y. and Ikeda, S. 1971a. Studies on fish muscle protease-IV. Relation between *himodori* of *kamaboko* and muscle proteinase. Bull. Jpn. Soc. Sci. Fish. 37: 518.

Makinodan, Y. and Ikeda, S. 1971b. Studies on fish muscle protease-V. On the existence of cathepsins A, B and C. Bull. Jpn. Soc. Sci. Fish. 37: 1002.

Makinodan, Y. and Ikeda, S. 1976a. Studies on fish muscle protease-VI. Separation of carp muscle cathepsins A and D, and some properties of carp muscle cathepsin A. Bull. Jpn. Soc. Sci. Fish. 42: 239.

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

Makinodan, Y. and Ikeda, S. 1976b. Studies on fish muscle protease-VIII. On the existence of protease active in neutral pH range. Bull. Jpn. Soc. Sci. Fish. 42: 665.

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

Makinodan, Y., Kyaw, N. N. and Ikeda, S. 1982b. Intracellular distribution of fish muscle alkaline proteinase. Comp. Biochem. Physiol. 73B: 785.

Makinodan, Y., Kyaw, N.N. and Ikeda, S. 1982c. Classification of carp muscle alkaline proteinase and its action against intracellular proteins. Bull. Jpn. Soc. Sci. Fish. 48: 479.

Makinodan, Y., Kitagawa, T., Toyohara, H. and Shimizu, Y. 1987a. Participation of muscle alkaline proteinase in *himodori* of *kamaboko*. Nippon Suisan Gakkaishi 53: 99.

Makinodan, Y., Toyohara, H and Ikeda, S. 1982d. Intracellular distribution of carp muscle cathepsin D and an electron micrograph of muscle lysosomal fraction. Bull. Jpn. Soc. Sci. Fish. 48: 1501.

Makinodan, Y., Toyohara, H. and Ikeda, S. 1983. Combined action of carp muscle cathepsins A and D on proteins. Bull. Jpn. Soc. Sci. Fish. 49: 1153.

Makinodan, Y., Toyohara, H. and Ikeda, S. 1984. Comparison of muscle proteinase activity among fish species. Comp. Biochem. Physiol. 79B: 129.

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

Makinodan, Y., Yokoyama, Y., Kinoshita, M. and Toyohara, H. 1987b. Characterization of an alkaline proteinase of fish muscle. Comp. Biochem. Physiol. 87B: 1041.

Matsumiya, M., Mochizuki, A and Otake, S. 1989. Purification and characterization of cathepsin B from ordinary muscle of common mackerel *Scomber japonicus*. Nippon Suisan Gakkaishi 55: 2185.

McLay, R. 1980. Activities of cathepsins A and D in cod muscle. J. Sci. Food Agric. 31: 1050.

Miller, R. and Spinelli, J. 1982. The effect of protease inhibitors on proteolysis in parasitized Pacific whiting, *Merluccius productus*, muscle. Fish. Bull. 80: 281.

Morado, J.F. and Sparks, A. 1986. Observations on the host-parasite relations of the Pacific whiting, *Merluccius productus* (Ayres), and two myxosporean parasites, *Kudoa thyrsitis* (Gilchrist, 1924) and *K. paniformis* Kabata & Whitaker, 1981. J. Fish Dis. 9: 445.

Musch, K., Siebert, G., Davies, J.V. and Ebert M. 1971. Differenzierung von zwei kathepsinen aus dorschmuskel. Hoppe-Seyler's Z. Physiol. Chem. 352: 878.

Nagahisa, E., Nishimuro, S. and Fujita, T. 1981. Kamaboko-forming ability of the jellied meat of Pacific hake. Bull. Jpn. Soc. Sci. Fish. 49: 901.

Nagahisa, E., Yasuda, S., Nishimuro, S. and Aoki, S. 1983. Jellification of Myxosporidia-infected Pacific hake muscle and its protease activity. Nippon Suisan Central Research Laboratory Report 12:15.

Niki, H., Deya, E. and Igarashi, S. 1984a. Peruvian hake meat having a lack of kamaboko-forming property. Bull. Jpn. Soc. Sci. Fish. 50: 1917.

Niki, H., Kato, T. and Igarashi, S. 1984b. Properties of Peruvian hake meat and preparation of purified surimi. Bull. Jpn. Soc. Sci. Fish. 50: 2043.

Nishimoto, S., Hashimoto, A., Seki, N., Kimura, I., Toyoda, K., Fujita, T. and Arai, K. 1987. Influencing factors on changes in myosin heavy chain and jelly strength of salted meat paste from Alaska pollock during setting. Nippon Suisan Gakkaishi 53: 2011.

Norusis, M.J. 1988. The Statistical Package for the Social Scientist, PC+ Version 3.0, Chicago, IL.

Numakura, T., Seki, N., Kimura, I., Toyoda, K., Fujita, T., Takama, K., and Arai, K. 1987. Changes in the SDS-gel filtration patterns of muscle proteins in saltedfish meatpaste during setting (suwari). Bull. Jpn. Soc. Sci. Fish. 53: 2045.

Patashnik, M. and Groninger, H.S. 1964. Observations on the milky condition in some Pacific coast fishes. J. Fish. Res. Bd. Canada 21: 335.

Patashnik, M., Groninger, H.S., Barnett, H., Kudo, G. and Koury, B. 1982. Pacific whiting, *Merluccius productus*: I. Abnormal muscle texture caused by myxosporidian-induced proteolysis. Marine Fisheries Review 44: 1.

Reddi, P.K., Constantinides, S.M. and Dymsza, H.A. 1972. Catheptic activity of fish muscle. J. Food Sci. 37: 643.

Reppond, K.D., Edson, S.A., Babbitt, J.K. and Hardy, A. 1987. Observations on the functional properties of U.S. land-processed surimi. J. Food Sci. 52: 505.

Rose, C.S. 1987. Flatfish. In "Condition of Groundfish Resources of the Gulf of Alaska Region As Assessed in 1987," p. 185, (Ed.) Major, R.L. and Wilderbuer, T.K., NWAFC/NMFS/NOAA, Seattle, WA.

Roussel, H. and Cheftel, J.C. 1988. Characteristics of surimi and kamaboko from sardines. International J. of Food Sci. and Tech. 23: 607.

Shimizu, Y., Nomura, A. and Nishioka, F. 1986. *Modori* (Fish gel degradation occurring at around 60°C)-inducing property of croaker myosin preparation. Bull. Jpn. Soc. Sci. Fish. 52: 2027.

Spinelli, J. and Steinberg, M. 1978. Inhibition of proteolysis in Pacific hake fillets. Mar. Fish. Rev. 40: 52.

Stehr, C. and Whitaker, D.J. 1986. Host-parasite interaction of the myxosporeans *Kudoa paniformis* Kabata & Whitaker, 1981 and *Kudoa thyrsitis* (Gilchrist, 1924) in the muscle of Pacific whiting, *Merluccius productus* (Ayres): an ultrastructural study. J. Fish Dis. 9: 505.

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

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

Taguchi, T., Tanaka, M. and Suzuki, K. 1983a. Effect of alcohols on *himodori* (thermally induced gel disintegration) in oval filefish meat paste. Bull. Jpn. Soc. Sci. Fish. 49: 1149.

Taguchi, T., Tanaka, M. and Suzuki. 1983b. *Himodori* (thermally induced disintegration) of oval filefish myosin gel. Bull. Jpn. Soc. Sci. Fish. 49: 1281.

Taneda, T., Watanabe, T. and Seki, N. 1983. Purification and some properties of a calpain from carp muscle. Bull. Jpn. Soc. Sci. Fish. 49: 219.

Toyohara, H., Kinoshita, M. and Shimizu, Y. 1990a. Proteolytic degradation of threadfin-bream meat gel. J. Food Sci. 55: 259.

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

Toyohara, H., Sakata, T., Yamashita, K., Kinoshita, M and Shimizu, Y. 1990b. Degradation of oval-filefish meat gel caused by myofibrillar proteinase(s). J. Food Sci. 55: 364.

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

Tsuyuki, H., Williscroft, S.N, Kabata, Z. and Whitaker, D.J. 1982. The relationship between acid and neutral protease activities and the incidence of soft cooked texture in the muscle tissue of Pacific hake (*Merluccius productus*) infected with *Kudoa paniformis* and/or *K. thyrsitis* and held for varying times under different pre-freeze chilled storage conditions. Canadian Technical Report of Fisheries and Aquatic Sciences No. 1130.

Ueno, R., Kanayama, T., Tomiyasu, K., Fujikami, A. and Nakashima, T. 1984. Processes for production of refrigerated minced fish flesh and fish paste product having improved quality. U.S. patent 4,464,404, Aug. 7.

Wasson, D.H., Babbitt, J.K. and French, J.S. 1991. Characterization of a heat stable protease from arrowtooth flounder, *Atheresthes stomias*. Bull. Jpn. Soc. Sci. Fish., in review.

Wojtowicz, M.B. and Odense, P.H. 1972. Comparative study of the muscle catheptic activity of some marine species. J. Fish. Res. Bd. Canada 29: 85.

Wolf, K. and Markiw, M.E. 1984. Biology contravenes taxonomy in the Myxozoa: New discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449.

Zeece, M.G. and Katoh, K. 1989. Cathepsin D and its effects on myofibrillar proteins: a review. J. Food Biochem. 13: 157.