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Title: QUALITATIVE EVALUATION OF THE PROTEOLYTIC ACTIVITY IN THE MUSCLE OF PACIFIC WHITING (Merluccius productus)

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The proteolytic enzymes in the muscle fluid of Pacific whiting (Merluccius productus) were studied and compared to those found in muscle fluid of true cod (Gadus macrocephalus). Preliminary studies indicated three pH optima of activity for whiting, pH 3.5-3.9, 4.3-4.6 and 7.1-7.2. Only two pH optima were found for the proteolytic activity of true cod, pH 3.2-3.6 and 7.7-8.0.

The sarcoplasmic fluid of whiting and cod muscle was studied in more detail. For both whiting and cod, no hydrolysis of the substrates hippuryl-L-phenylalanine, hippuryl-L-arginine, α-N-benzoyl-D,L-arginine p-nitroanilide (BAPA), or toluene sulfonyl arginine methyl ester (TAMA) at neutral pH's could be detected, indicating the absence of trypsin and carboxypeptidases A and B. Neither whiting nor cod contained elastase and only whiting was shown to have activity similar to that of cathepsin B. True cod was found to contain higher chymotrypsin activity than whiting at pH 7.15 using the substrate glutaryl-L-phenylalanine p-nitroanilide. Hydrolysis of the substrate glycyl-L-phenylalanine β-naphthylamide (Gly-Phe-2-naphthylamide) from pH 5 to 8 occurred to a greater extent in Pacific whiting than in true cod.

Various inhibitors and activators were used to characterize the enzymes in whiting and cod muscle hydrolyzing the substrates GPNA and Gly-Phe-2-naphthylamide. The responses to the chemicals were compared with the effects reported in the literature on the hydrolysis of the substrates by enzymes found in other animal sources.

Qualitative Evaluation of the Proteolytic Activity in the Muscle of Pacific Whiting (*Merluccius productus*)

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INTRODUCTION

Due to the large harvest potential of Pacific whiting, the development of this resource as a food commodity would be highly advantageous to both the fishing industry and the consumer. Presently, Pacific whiting is utilized only to a small degree because of consumer rejection of its mushy flesh. The latter develops rapidly if the fish are not iced immediately after capture. In addition, limitations on the preparation of the fish for the dinner table (rapid cooking, i.e., deep-fat frying) further restrict the utilization of whiting to a level far below its potential. The reason(s) for the mushy texture of whiting is not known. Thus, accumulation of knowledge about the softening of whiting flesh is necessary if appropriate measures are to be developed to minimize or at least control this defect to a level acceptable to most consumers.

A similar textural problem occurring in arrowtooth flounder was investigated by Kramer *et al.* (1977). In the raw state the flounder exhibited normal textural characteristics but upon cooking, it developed a very soft texture. The latter was attributed to a high level of proteolytic activity in the muscle. Since the arrowtooth flounder was rather heavily infected with the myxosporidian parasite, *Kudoa sp.*, Kramer and co-workers also indicated that this parasitism may have contributed to the soft texture of the cooked flesh. This seems to be related somewhat to the textural problems of Pacific whiting which also is subject to heavy myxosporidian parasitism. In contrast to arrowtooth flounder however,

Pacific whiting will develop mushy texture in the raw state if not rigidly held at low temperatures ($2^{o}C$) prior to cooking. This particular difference suggests that the proteases of Pacific whiting are released or activated much easier than those of arrowtooth flounder.

The objective of this research was to evaluate the extent to which proteases might be involved in the severe textural problem of Pacific whiting. Specific proteases in Pacific whiting were studied and the results were compared qualitatively with the proteolytic activity of true cod, a fish closely related to whiting but which has a firm texture. Proteases were identified by using specific substrates, various inhibitors and activators. Classifying a particular enzyme activity was accomplished by comparing results with those reported in the literature. Particular attention was directed to the lysosomal enzymes in an attempt to determine whether the myxosporidian parasite exerted any influence upon the catheptic activity of Pacific whiting.

REVIEW OF LITERATURE

Location and Abundance of Pacific Whiting

Pacific whiting, Merluccius productus, is found in commercial quantities in the coastal waters of California, Oregon, and Washington. A smaller but commercially important population also exists in the Puget Sound, Washington.

The whiting fish grows relatively fast, especially during its first four years. It matures at an age of 3 to 4 years and on the average will weigh about 2.68 lbs. (1.22 kg) and be 20.5 inches (52 cm) long. The length can reach 3 feet (91 cm) (Dark, 1975).

Whiting has been reported from the Gulf of Alaska to the Gulf of California but large accumulations appear to be limited to coastal areas between Baja California, Mexico and central Vancouver Island. The adult population is normally confined to waters overlying the continental slope and shelf except during the spawning season when whiting may be found several km seaward in the southern part of the range (Dark, 1975).

The size of standing stock of Pacific whiting that inhabits the waters off Oregon and Washington during summer (based on sonic survey and test fishing) has been estimated at 609.5 thousand short tons (455 million kg) by U.S. biologists and near 1,206 thousand short tons (900 million kg) by Soviet fishery scientists. The estimated sustainable yields at these population levels range from about 174 thousand to 349 thousand short tons (130 million to 260 million kg) annually. The Pacific whiting resource off California, estimated to be in the order of 3.6 million tons (2.7 billion kg), is reported to be second in size only to the northern anchovy in the California

current system. In the Puget Sound, enough Pacific whiting are present during the winter to support a yield of 6 thousand tons (4.5 million kg) per year (Alverson and Larkin, 1969).

Properties of Pacific Whiting

Proximate Chemical Composition

One of the first studies to evaluate the value of Pacific whiting as a food was done by Patashnik et al. (1970) who determined the proximate chemical composition of the fish. While factors such as the size, sex, and stage of maturity of the fish, and the season and place of capture affect the composition, these factors were not evaluated in detail owing to the limited scope of the investigation. Samples of whiting that were examined were frozen at $0^{o}C$ and thawed at $35^{o}C$. The composition of ocean whiting varied seasonally with fat varying the most. Whole fish had about 1.5 to 3.5% fat during March to July and about 4 to 6% fat during September to November. They had 13.4 to 15.0% protein during March to July and 14.4 to 15.6% protein from September to November. The average ash was about 3% during all periods. In contrast to the edible fillets, the waste portion had lower moisture, lower protein, and substantially higher fat contents.

The composition of Puget Sound whiting also varied seasonally with fat again varying the most. The fat content of whole fish was highest (6.4 to 7.4%) from about October through January. On the average for whole fish, the fat content of Puget Sound whiting was 73% higher than that of ocean whiting. This higher fat content also held true for fillets and the waste portion, being 56% and 68% higher, respectively, in the Puget Sound whiting than in the ocean whiting (Patashnik et al., 1970).

The protein content of whole fish ranged from 12.3 to 13.4% in early April to 16.1% in July. The average protein content of ocean whiting fillets was 16.5% and that in Puget Sound fillets was 16.1% (Patashnik et al., 1970).

A more detailed study of the nitrogenous constituents was undertaken by Dassow et al. (1970). The amount of total nitrogen and the distribution of the major nitrogenous constituents found in whiting caught at different locations and at various times of the year varied considerably among lots of fish. The constituents most affected were the myofibrillar (muscle fiber portion) and sarcoplasmic (fluid portion) protein fractions. The nonprotein nitrogen and stroma-protein nitrogen values did not vary significantly.

In general, smaller whiting had a lower ratio of myofibrillar protein to sarcoplasmic protein than large whiting. Those whiting caught in the Puget Sound had a higher myofibrillar content than those caught elsewhere. In addition, whiting caught elsewhere were found to have a low variable ratio of myofibrillar to sarcoplasmic protein while this ratio was higher and more uniform for the Puget Sound whiting. One factor suggested for this variation in protein content and distribution was the slow growth of the fish found in the Puget Sound (Dassow et al., 1970).

The concentration of nonprotein nitrogen in whiting is about 4.0 mg nitrogen/g or about 15% of the total nitrogen. A preliminary investigation of the components present in the nonprotein nitrogen fraction indicated that about 80% of the nonprotein fraction could be accounted for as follows: creatine, 34.6%; trimethylamine oxide, 23.4%; urea, 10%; purine base, 9.1%; free amino acids, 6.1%; volatile base, 5.5%; creatinine, 1.4% and trimethylamine, 0.01% (Dassow et al., 1970).

In comparing distributions of the major nitrogenous constituents of whiting with two other commercially important species, Pacific Ocean perch and English sole, whiting contained significantly less myofibrillar protein. Whiting contained 16 mg myofibrillar nitrogen/g protein. Pacific Ocean perch had 22.5 mg/g and English sole had 19 mg/g. This difference may partly explain why both Pacific Ocean perch and English sole show superior freeze-thaw characteristics with regard to drip-loss and ultimate product texture (Dassow et al., 1970).

Organoleptic Properties

In another study to determine the suitability of Pacific whiting as a food source, Dassow et al. (1970) studied several factors including color, odor, flavor, texture and keeping quality.

The color of the fish varied from an off grey-white to pink but was still considered to be within the normal range of color found in the various Pacific coast species filleted and marketed. The raw flesh had a neutral odor but if uniced for 1 day, whole whiting developed a strong, persistent odor on the skin. These strong odors were more evident on the ocean whiting than on the Puget Sound whiting. The flavor was bland for fresh whiting that had been properly cooked.

From a quality standpoint, tests showed that whole Pacific whiting should be iced or processed without delay, mainly because the storage life of whole whiting is very limited. Even for whiting iced immediately after catching, the fish remained in rigor up to 3 days but lost quality rapidly soon after. In the case of fillets prepared from adequately iced, fresh whiting, the fish could remain acceptable as long as 2 weeks (Dassow et al., 1970).

The flesh of uncooked whiting that had been iced immediately after capture was considered moist and tender. However, upon cooking it yielded a high incidence of an abnormally mushy texture. This texture is responsible for the unacceptability of the whiting as a marketable food product and therefore it is important to remedy the problem (Dassow et al., 1970).

Presently several treatments have been proposed to reduce this problem of softening. Dassow et al. (1970) proposed dipping fillets in a solution of 7.5% sodium tripolyphosphate plus 2% NaCl. They reported a shear resistance 1.7 times greater in the treated fillets and an improved water-holding capacity as measured by the amount of drip.

In a technical information report (Steinberg, 1977), the National Marine Fisheries Service recommended several procedures to be used in catching whiting that would serve to maintain quality. Their suggestions were to make shorter drags by bringing the whiting to the boat quickly through the warmer surface water and chill the fish as quickly as possible in refrigerated sea water or slush ice. At the most there must be only about 4 hours from the moment the fish are caught in the net on or near the bottom until they are transferred to the chilled water aboard the vessel. If there must be a short delay the fish should be wetted-down with sea water and covered with a wet tarp.

A third procedure proposed to maintain a good quality texture deals with cooking. When cooking whiting there should be a complete and rapid deep fat frying of sticks, portions and fillets. The practice of leaving the actual cooking to the consumer will result in mushy products although fully cooked deep fat fried fillets that are reheated by the consumer are acceptable (Steinberg, 1977).

The effects from these 3 treatments just described do not provide enough evidence to determine the source of the softening problem. They only serve to speculate on the root of the problem. By looking for the cause, better processing techniques may be devised to utilize the whiting food products to a greater degree than the present.

Description of Myxosporidian Parasitization

The mushy condition of whiting has often been observed in association with a microscopic myxosporidian parasite, Kudoa sp. (Dassow et al., 1970). Muscle portions seriously infected with the myxosporidian parasite appeared to have extensive proteolysis of the tissue with accompanying liquefaction and mushiness. The texture of the heavily parasitized whiting almost always was abnormal and mushy, but that of the slightly parasitized whiting was frequently normal. In the raw product, this mushiness could be seen after several hours of incubation at room temperature, and in the improperly cooked whiting, the liquefied product could be detected immediately.

In areas of the muscle that were seriously infected with the myxosporidian parasite, Dassow et al. (1970) found levels of proteolytic activity up to 14 times greater than in the normal textured, uninfected controls. In extreme cases of parasitization, they reported the proximate composition to be altered significantly. For example, the muscle from a single excessively parasitized whiting taken from Puget Sound was 11.1% protein in contrast to an average of 16.1% and a low value of 14% protein for all samples of Puget Sound whiting.

Dassow et al. (1970) observed on the average an incidence of the parasite in whiting of 10-40% of the sample. Occasionally 100% of the whiting were infected in some samples, depending on the area of the catch and the size of fish.

The myxosporidian parasite has been reported to be present in other species of fish, in particular arrowtooth flounder, Pacific halibut, dover sole, petrale sole or brill and starry flounder (Kramer et al., 1977). A more careful examination into the presence of the myxosporidian parasite in fish shows aggregate masses of the spore lying within the somatic muscle fibers and not around them (Willis, 1949). In halibut, the mature spore with the intact membrane is usually star-shaped or may appear like a cross without the membrane. It contains four capsulogenous cells, which are usually of different size, are ovoid at one end, and may or may not taper to a point on the other end. The overall size of the spore varied from 10 to 15 μ. One capsule is often considerably larger than the rest and the remaining cells are usually unequal or very similar in size. The spore tetrad is observed both with or without the spore membrane. Presumably the very thin spore membrane is either shed or not visible under certain conditions. The substance of the individual spore capsules appears to be granular but homogenous and shows no evidence of spiral thread or coiled flagella (Patashnik and Groninger, 1964).

The presence of the parasite can be commonly detected in hairlike cysts. These cysts are dark and readily visible in the heavily parasitized whiting but may be colorless and barely visible in a lightly parasitized fish. The cysts contain millions of spores, each spore being microscopic in size and ranging from 6 to 8 microns (Dassow et al., 1970).

The Kudoa sp. spore tetrad infecting halibut was found to be extremely resistant to physical destruction by daily temperature cycling between -18° to 21°C for weeks. However, cycling the temperature between 4.5 to 21°C for several days, followed by freezing at -18°C and a very slow return to

room temperature over a period of about 8 hr led to all the spore tetrads disappearing. Simultaneously with this disappearance of spores came the appearance of globular multiplicative forms displaying growth, binary fission, budding and multiple fission. Apparently under certain time-temperature conditions following frozen storage, growth and multiplication of this organism in a non-living fish substrate is possible. The viability of the resultant multiplicative forms was not tested (Patashnik and Groninger, 1964).

Description of Proteolytic Activity

It is possible that a myxosporidian infestation may bring about similar changes in muscle texture and proteolytic activity of all infected fish. To help in determining whether such a correlation exists, information recorded to date regarding the muscle proteolytic activity for these fish will be presented.

Pacific Whiting

Studies were made on the proteolytic enzymes of whiting flesh by de Koning and Papendorf (1965). The flesh of the whiting, Merluccius capensis Castelnau was ground in a Waring blender and an extract made after precipitation with ammonium sulphate. Denatured hemoglobin was used as a substrate in the proteolytic tests. Activity was measured using the Folin-Ciocalteu reagent by detecting the amino acids and dipeptides in the TCA soluble filtrate that had been cleaved from the substrate. Checking the proteolytic activity of whiting enzymes as a function of pH, these researchers found a maximum at pH 5.0. They considered a second maximum at pH 7.0 to be due to changing the buffer.

The effect of addition of the metal ions, Zn^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+}, and Mg^{2+}, sodium polymetaphosphate and sodium tripolyphosphate to the hemoglobin before mixing with whiting enzymes was investigated. It was clearly shown that none of the chemicals investigated had a significant effect. All metal ions were tested at concentrations of 10^{-3} and 10^{-4}M except for Mg^{2+} which was also tested at 10^{-2}M. Sodium polymetaphosphate was present in samples at concentrations of 0.001% and 0.01%. Sodium tripolyphosphate was only tested at 0.01% concentration (de Koning and Papendorf, 1965).

Fish Species Infected with the Myxosporidian Parasite

Kramer et al. (1977) made studies on the muscle texture and proteolytic activity of arrowtooth flounder, Atheresthes stomias. Comparing the extractable proteolytic activity of arrowtooth flounder muscle with 10 other species of flatfish, C-O sole, dover sole, English sole, flathead sole, Pacific halibut, petrale sole, rock sole, slender sole, starry flounder and sockeye salmon, showed arrowtooth flounder with a much higher muscle proteolytic activity than the other species. Generally, the extractable proteolytic activity of arrowtooth flounder was 10 times higher than the other species except for the case of the C-O sole when it was only 5 times higher.

One of the more interesting findings of the proteolytic studies conducted by Kramer et al. (1977) was that dark muscle of arrowtooth flounder had a much lower activity than light muscle. In contrast, most other species of fish had a higher activity in the dark muscle. Because this generalization is reversed for the arrowtooth flounder, this muscle could be representative of an abnormal condition.

On examining the muscle tissue of arrowtooth flounder, Kramer and associates (1977) did not find the discrete localized pockets of milky-white tissue reported by others. However, a microscopic examination of the muscle

of some fish which had high levels of proteolytic activity revealed the presence of myxosporidian spores. The spores occur as tetrads and are a species of the genus Kudoa. In a closely related species to the arrowtooth flounder, Atheresthes evermanni, the myxosporidian spores could not be found and the extractable proteolytic activity was low. With such indirect evidence, they concluded that the high muscle proteolytic activity in arrowtooth flounder was the result of this parasite. Either the myxosporidian was producing a proteinase or it caused a reaction in the fish which results in high muscle proteolytic activity.

The optimum pH for the proteolytic activity in arrowtooth flounder muscle with hemoglobin as substrate was found to be about 4 and appears to be slightly less for proteolytic activity of the dark muscle. At pH 6.0, there was very little muscle proteolytic activity and at pH 7.0, there was essentially no activity (Kramer et al., 1977).

The proteinase extracted from arrowtooth flounder by Kramer et al. (1977) remained active as low as $0^{o}C$ and as high as $70^{o}C$ and had a temperature optimum at $55^{o}C$. This enzyme has a much wider temperature range of activity and higher temperature optimum than enzymes isolated from other fish. For example, sockeye salmon has no proteolytic activity at $0^{o}C$ or $70^{o}C$ while its optimum temperature is $50^{o}C$.

By measuring muscle texture using the Ottawa Texture Measuring System (Kramer et al., 1977), it was determined that high proteolytic activity does not result in soft muscle in the raw state for arrowtooth flounder. Only one collection of samples from the same location was found to have the highest mean shear press force in addition to the highest mean proteolytic activity. In the cooked state though, high proteolytic activity did result in softer flesh for arrowtooth flounder. The elevated temperature experienced

in cooking activated the proteases present to break down the muscle. However, this softening in texture on cooking was the opposite of what occurred in petrale sole, the species of flounder used for comparison. In the case of petrale sole, cooking increased shear press force.

No correlation was found between the size of the fish and the muscle proteolytic activity for arrowtooth flounder. However, the raw muscle of small fish was significantly softer than the medium or large fish. For cooked muscle, the large fish were also found to have a firmer texture (Kramer et al., 1977).

Canadian lemon sole is another fish that is reported by Forrester (1956) to be infected with large quantities of the Kudoa sp. myxosporidian spore. However, on exploitation of the stock of fish, the incidence of milkiness decreased from 21% in 1951 to less than 1% by 1954. Since this spore requires no intermediate host, it is presumed that the incidence of infection in a stock of fish would vary with stock density as it did in the case just cited.

Fish Species Not Infected with the Myxosporidian Parasite

In addition to comparing the proteolytic activity to other infected fish, proteolytic activity of fish not infected with the parasite should be considered. A qualitative and quantitative comparison with uninfected fish will show if whiting contain higher quantities of an enzyme known to be present in all fish or whether whiting contain a unique enzyme not found in other fish. It is with the future intention of conducting such a comparison that the proteolytic activity found in uninfected fish is reported.

Makinodan and Ikeda (1969a) conducted studies on a partially purified enzyme from cultured carp that was active in the slightly alkaline pH range. The procedure used to purify the protease consisted of muscle extract preparation, heat treatment, ammonium sulfate fractionation, and chromatography on Sephadex G-200. The purified enzyme showed optimum activity at about pH 8.0 and at pH 7.0, it showed about 40% of the activity observed at pH 8.0. At pH 8.0, the optimum temperature for 2 hours incubation was about $65^{o}C$. However, there was very little or no activity below $40^{o}C$.

In determining the substrate specificity of this alkaline protease of Makinodan and Ikeda (1969a), casein and hemoglobin digestions were tested at a concentration of 50 mg/ml, and egg albumin digestion was tested at a concentration of 30 mg/ml. In each case, the sample was incubated for 2 hours at $60^{o}C$ at pH 8.0. The hydrolysis products in the TCA soluble filtrate were measured using the Folin-Ciocalteu reagent. Under these experimental conditions, casein gave the greatest activity. There was considerable activity against hemoglobin but less against egg albumin. Testing several chemicals showed that Na^{+} and K^{+} (10^{-2} and $10^{-3}M$) had no effect. Zn^{2+}, Co^{2+}, Ni^{2+} ($10^{-3}M$) and Zn^{2+} ($10^{-4}M$) inhibited the proteolytic activity considerably, whereas EDTA and KCN (10^{-3} and $10^{-4}M$) caused a marked increase of activity. Sulfhydryl compounds at concentrations of 10^{-2} and $10^{-3}M$ enhanced the activity, but $10^{-3}M$ p-chloromercuribenzoate (p-CMB) inhibited it completely. Iodoacetate ($10^{-3}M$) had no effect. Therefore, based on the effects of pH and metal ions on the proteolytic activity, the alkaline protease described by Makinodan and Ikeda is not responsible for the proteolytic activity in whiting muscle described by de Koning and Papendorf (1965). Instead, the enzymatic properties of the alkaline protease

seem to be similar to those of α-chymotrypsin when considered on the basis of inhibitor studies. The enzymatic activity was not inhibited by chicken egg white, soybean trypsin inhibitor, carp plasma, or N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), but was inhibited markedly by leupeptin, ovomucoid, N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), N-ethylmaleimide (NEM), and N-bromosuccinimide (NBS) (Iwata et al., 1974).

In another study conducted by Makinodan and Ikeda (1969b), a proteinase active in the acid range was partially purified about 200 fold from carp muscle. The purification steps included preparation of muscle extract, ammonium sulfate fractionation, acetone fractionation, and chromatography on Sephadex G-200. The enzyme showed a pH optimum for hemoglobin at 2.8-3.0. There was very little activity below pH 2.0, but at pH 4.4 and 5.0, the activity was fairly strong showing about 50% of that observed at pH 2.8. The optimum temperature for 1 hour incubation was about $50^{o}C$ while the activity at $65^{o}C$ was very low.

Since the enzyme is active in the acid pH range, it was of interest to determine if cathepsins A, B or C were present and responsible for some of the activity measured in this partially purified extract. However, cathepsins A, B and C were not shown to be present. The extracted fish enzyme was inactive against carbobenzoxy-L-glutamyl-L-tyrosine (CBZ-L-Glu-L-Tyr), benzoyl-L-arginine amide and glycyl-L-tyrosine amide, substrates for cathepsins A, B and C, respectively (Makinodan and Ikeda, 1969b).

The effect of several chemicals was tested on the activity displayed by this acid proteinase. Na^{+}, K^{+} (10^{-2} and 10^{-3}M), Zn^{2+}, Co^{2+}, and EDTA (10^{-3}M) had no effect while Ni^{2+} (10^{-2} and 10^{-3}M) increased the activity to some degree. KCN, cysteine, p-CMB (10^{-3}M) and iodoacetate (10^{-2} and 10^{-3}M)

did not stimulate the activity, but glutathione and 2-mercaptoethanol (10^{-3}M) activated the activity (Makinodan and Ikeda, 1969b).

The acid protease isolated was classified as cathepsin D since it showed a pH optimum of 2.8-3.0 against a hemoglobin substrate, not being affected by 10^{-3}M cysteine, p-CMB and EDTA, and not hydrolyzing CBZ-L-Glu-L-Tyr, benzoyl-L-arginine amide and glycyl-L-tyrosine amide.

Makinodan and Ikeda (1971) were able to detect cathepsins A, B, and C in a crude preparation of carp muscle. No purification steps had been conducted on this preparation as were undertaken in the studies on the alkaline and acid proteases (Makinodan and Ikeda, 1969a, 1969b). The only steps involved in the enzyme preparation were homogenization of muscle, centrifugation to remove supernatant and dialysis of supernatant.

The activity on the substrate CBZ-L-Glu-L-Tyr was strongest at about pH 5.0 but very weak at pH 3.0-4.0 for the crude enzyme preparation from carp. At pH 5.0, the enzyme preparation showed activity without cysteine or other SH-compounds. The activity was not affected by iodoacetamide (IAA). Such properties rule out catheptic carboxypeptidase as the principal enzyme hydrolyzing the substrate and point to the presence of cathepsin A in fish muscle.

Optimum pH for hydrolysis of benzoyl-L-arginine amide was near 6.4 for the crude enzyme extract from carp. This value is higher than that reported (4.2-5.8) for beef spleen cathepsin B by Greenbaum and Fruton (1956) but nearly equal to the value (pH 6.2) reported on beef spleen cathepsin B by Otto (1967). Activity of the carp enzyme preparation required cysteine. However, the enzyme was activated only slightly by 2-mercaptoethanol or KCN. Iodoacetamide at 10^{-3}M inhibited the activity of the carp enzyme but 10^{-4}M p-CMB had no effect. The effect of p-CMB

on beef spleen cathepsin B differs from that of the carp enzyme preparation. However, the activity of enzyme solution prepared from carp muscle agrees with the cathepsin B activity of beef spleen in respect to optimum pH, influence of cysteine, etc. From these data, the enzyme is considered to be cathepsin B (Makinodan and Ikeda, 1971).

The optimum pH for the hydrolysis of glycyl-L-tyrosine amide was near 6.4 which is similar to that of cathepsin B. However, by using this substrate, the carp enzyme was shown to be active in the absence of cysteine or another SH-compound, although the addition of cysteine (4×10^{-2}M) greatly enhanced the activity. Moreover, the enzyme was stable against heating at 60^{o}C for 20 minutes which inactivated cathepsin B completely. Accordingly, the present enzyme from the crude supernatant is clearly different from cathepsin B. Since the properties mentioned above are equal to those of beef spleen cathepsin C, the enzyme is considered to be identical to cathepsin C. (Makinodan and Ikeda, 1971).

Some of the most extensive research on fish muscle hydrolytic enzyme activity has been done by Siebert and co-workers (1958, 1963). Siebert (1958) reported that the muscles of a variety of fish including cod, herring, sole, flounder, trout and carp contained as much as 10 times the amount of cathepsin activity as found in mammalian muscle tissues. Since Siebert et al. (1963) found no cathepsins A, B, or C activity in cod muscle, another cathepsin is present to account for the high activity in the acid pH range. The specificity of a purified cod spleen cathepsin against the B chain of insulin was different from that observed for any mammalian protease (Siebert et al., 1963).

On the other hand, Groninger (1964) partially purified and characterized

an acid protease from albacore muscle which had a pH optimum of 2.4-2.5. This enzyme was active against bovine serum and the synthetic substrates used to test for cathepsins A, B and C. Thus, it remains to be determined whether the existence of cathepsins A, B, and C is a species difference or a matter of purification.

Wojtowicz and Odense (1972) ran a comparative study of the muscle catheptic activity of 13 Atlantic fish species. On examination, the hemoglobin-splitting activity was invariably greater than the autolytic activity of the same species, but the data showed a direct relationship between the hemoglobin-splitting activity and the autolytic activity for each individual.

Despite the selection of samples of a species in the same season and at the same time and the use of identical sampling and testing procedures, the extent of individual variation in animals is significant although there is considerable overlap in the ranges of different species with no evident phylogenetic distinction. For example, Atlantic cod (Gadus morhua) shows nearly the highest specific activity while white whiting (Urophycis tenuis), another gadoid, is near the bottom of the list (Wojtowicz and Odense, 1972).

The effect of the level of catheptic activity could vary among species with different muscle structures. On the other hand, within a species and at one specific time after capture, the level of catheptic activity could be directly related to the texture that one finds in the fish.

Role of Lysosomes in Muscle Breakdown

The various hydrolytic cathepsins found in fish muscle are known to be released from a group of membrane-bound cytoplasmic organelles known as lysosomes. According to Allison (1977), lysosomes perform three main

functions.

1) They digest material taken into the cell.
2) They can degrade parts of the living cell and all of it after cell death.
3) They release the enzymes they contain outside the cell, causing extracellular breakdown of macromolecules.

The lysosomes of most animal cells contain a common group of about 50 enzymes. The hydrolases acting on peptide bonds include carboxypeptidase A, B and C, dipeptidase, dipeptidyl peptidase, kininogen, elastase, neutral proteinase, plasminogen activator, cathepsin B, cathepsin D, cathepsin A, rennin, cathepsin E, cathepsin G, cathepsin C, and collagenase. Generally, the pH optima of these enzymes are in the acid range, although there are enzymes such as elastase and cathepsin G active at neutral pH.

Some of these lysosomal enzymes are reported to hydrolyze myofibrillar proteins. Cathepsin D isolated by Makinodan and Ikeda (1969b) from carp muscle hydrolyzed the myosin fraction. In addition, cathepsins A, B and C are known to degrade oligopeptides, produced by prior action of cathepsin D, to smaller oligopeptides, dipeptides and free amino acids (Iodice et al., 1966; Huang and Tappel, 1971; and Goettlich-Riemann et al., 1971).

Activities of enzymes in the lysosomal fraction are for the most part latent because enzyme substrates penetrate their membranes inefficiently. However, increased synthesis of lysosomal enzymes and increased access to substrates, either through disruption of the lysosomal membrane or selective enzyme secretion, would result in increased breakdown of the substrate. Therefore in the case of Pacific whiting, these higher levels of cathepsins released from lysosomes may cause an elevated breakdown of the muscle and a mushier texture.

One possible reason for elevated enzyme secretion in whiting may come from an allergic response to the myxosporidian parasite. Formation of complexes of antigen and antibody would cause the accumulation of phagocytic cells. These phagocytic cells (polymorphonuclear leucocytes and macrophages) have on their surfaces receptors for the activated third component of complement and for complexes of antigen and antibody. Attachment of either of the components to the receptors on leucocytes would trigger the secretion of the hydrolytic enzymes into the extracellular medium (Allison, 1977).

MATERIALS AND METHODS

Fish Samples

Pacific whiting and true cod were obtained fresh from Pacific Whiting Fish Co. in Astoria, Oregon where they had been on ice at the most one day After removal of the head and evisceration, fish were placed in Cryovac bags and frozen at -40°C. They were then stored at -10°C.

Preparation of Muscle Supernatants

Samples of Pacific whiting or true cod were removed from the freezer and thawed at room temperature. The time required for thawing was 8 hrs. Upon thawing, the fish were filleted and the skins removed. The muscle was cut into small pieces to fit into 45 ml conical tubes which were centrifuged at 3015 x G for 30 min. Neither was the fish ground nor an extracting solution used. The liquid portion (supernatant) was drained off and collected. The combined supernatants were stored in an Erlenmeyer flask at 4°C and samples to be used in enzyme assays were taken from this stock. Preliminary experiments indicated that the proteolytic activity of the stock to be stable for 4 days. These supernatants will be referred to in the future as the enzyme supernatant or enzyme solution. The protein concentration of the supernatant was determined using the Lowry procedure (Lowry et al., 1951; Layne, 1957).

Proteolytic Activity as Affected by Temperature

Casein (Hammarsten quality, National Biochemicals Corp.) was the substrate used to determine the effect of temperature on the proteolytic

activity of the supernatant sample collected from the whiting muscle. Ten ml of 1% casein solution, 5 ml of 0.1M Tris-HCl buffer, pH 7.6 and 5 ml of the supernatant were added to an Erlenmeyer flask and incubated at one of the following temperatures: 25^{o}, 35^{o}, 45^{o}, 50^{o}, 55^{o} and 65^{o}C. The pH of the mixture was measured using a Corning pH meter, Model 125. During a 2 hr assay period, 2 ml samples were removed from the incubating mixture at 30 min intervals and 3.5 ml of 5% trichloroacetic acid (TCA) was added immediately. The blank contained 10 ml of the 1% casein solution and 5 ml of 0.1M Tris-HCl buffer, pH 7.6. At the sampling intervals, a 3-ml sample was removed from the blank, 1 ml of the supernatant solution was added, followed immediately by 7 ml of 5% TCA. After 30 min of the addition of TCA, the precipitate was removed from the solutions using Whatman No. 1 filter paper. The absorbancy of the filtrate at 280 nm was then measured.

Proteolytic Activity as Affected by pH

A hemoglobin (2x Crystallized, Sigma Chemical Co.) substrate was used to determine the effect of pH on the proteolytic activity of supernatant samples collected from the whiting muscle. Two ml of a 2.5% hemoglobin solution, 1.0 ml of buffer and 0.5 ml of the supernatant enzyme solution were added to a test tube and incubated for 30 min at 50^{o}C. At the end of the incubation period, 6 ml of 5% TCA was added to each tube. For the blank, the supernatant solution was added immediately preceding the precipitation with TCA. Thirty min following the addition of TCA, the solution was filtered through Whatman No. 1 filter paper and the absorbance at 280 nm was recorded on the filtrate.

The pH of the assay solution was checked with a Corning pH meter, Model 125. Adjustment to the desired pH was accomplished by adding either 0.1M NaOH or 0.1M HCl. Various buffers were used only within the range at which they had buffering strength. The buffers and ranges in which they were used were as follows: 0.2M glycine-HCl buffer, pH 2.2-3.6; 0.1M citric acid-sodium citrate, pH 3.0-6.2; 0.2M Na_2HPO_4-NaH_2PO_4 buffer, pH 5.8-8.0; 0.1M Tris-HCl buffer, pH 7.1-8.9; and 0.2M glycine-NaOH buffer, pH 8.6-10.6.

Determination of Specific Proteolytic Activities

The next stage of the study involved attempts to identify the various enzymes present in the whiting and true cod supernatants. Table 1 lists the enzyme assays that were run on whiting and true cod samples. Column 3 lists whether activators and inhibitors were included in the assay to characterize the enzyme activity detected. Activators and inhibitors used in this study were obtained from Sigma Chemical Co. and Mallinckrodt, Inc.

Carboxypeptidase A Activity

The determination of carboxypeptidase A activity in the muscle supernatant was based upon the method of Folk and Schirmer (1963). By measuring the increase in absorbancy at 254 nm, the hydrolysis of hippuryl-L-phenylalanine by carboxypeptidase A can be determined.

The procedure consisted of pipetting 2.9 ml of substrate (0.001M hippuryl-L-phenylalanine in 0.025M Tris-HCl, pH 7.5 with 0.5M NaCl) into each cuvette (1 cm quartz cells). These cuvettes were incubated in the spectrophotometer for 3-4 min to reach temperature equilibration (no thermostatted compartment was used). At zero time, 0.1 ml of a diluted supernatant sample was added to the sample cuvette and the increase in

Table 1. List of enzymes assayed in Pacific whiting and true cod muscle extracts.

Enzyme Assay	Species of Fish Sampled	Inhibitors and Activators Examined
Carboxypeptidase A	Whiting, True Cod	None
Carboxypeptidase B	Whiting, True Cod	None
Elastase	Whiting, True Cod	None
Neutral Collagenase	Whiting	None
Catheptic Collagenase	Whiting	None
Trypsin	Whiting, True Cod	None
Chymotrypsin	Whiting, True Cod	Cysteine, PCMB, iodoacetamide, $CuSO_4$, EDTA, PMSF, TLCK, TPCK, $CaCl_2$
Cathepsin B	Whiting, True Cod	None
Cathepsin C	Whiting, True Cod	NaCl, EDTA, cysteine, iodoacetamide, PMSF, B-mercaptoethylamine, TLCK, p-CMB
Cathepsin A	Whiting, True Cod	Cysteine, iodoacetamide, EDTA, $ZnCl_2$

absorbance at 254 nm was recorded for 3-5 min. Each sample was assayed in duplicate.

Carboxypeptidase B Activity

Carboxypeptidase B activity was measured by the spectrophotometric method of Folk et al. (1960) using hippuryl-L-arginine as the substrate. Hydrolysis of the substrate was measured by the increase in absorbancy at 254 nm.

The procedure consisted of pipetting 2.9 ml of substrate (0.001M hippuryl-L-arginine in 0.025M Tris-HCl, pH 7.65 containing 0.1M NaCl) into cuvettes (1 cm quartz cells). These cuvettes were incubated in the spectrophotometer for 3-4 min to reach temperature equilibration. At zero time, 0.1 ml of diluted supernatant was added to the sample cuvette and the increase in absorbancy at 254 nm was recorded for 3-4 min. Each sample was assayed in duplicate.

Elastase Activity

The method of Sachar (1955) was used to detect the presence of elastase. In this method, elastase solubilizes elastin impregnated with orcein and the amount of dye released is measured colorimetrically. The procedure consisted of placing 20 mg of orcein-impregnated elastin in a test tube. To each tube of dyed elastin, 1.0 ml of 0.2M Tris buffer, pH 8.8 was added. The control flask contained only 2.0 ml of the buffer and no elastin. At zero time, 1.0 ml of the supernatant sample was added to each tube. The tubes were incubated at 40^{o}C and shaken occasionally. After 2 hr, the reaction was stopped by adding 2.0 ml of 0.5M phosphate buffer, pH 6.0. The mixture

was filtered, the sides of the tube were washed with 1.0 ml of water and the absorbancy of the filtrate was measured at 590 nm.

Neutral Collagenase

Collagen was hydrolyzed at neutral pH using a modification of the procedure of Mandl et al. (1953). Twenty-five mg of collagen was weighed into each test tube and 5 ml of 0.05M Tris buffer, pH 7.5 with 0.36mM $CaCl_2$ was added to the tubes. The reaction was started by adding 0.1 ml of the enzyme supernatant to the appropriate tubes. One of the controls did not contain collagen, while the other control had 0.1 ml of distilled water in place of the enzyme solution. After incubating at 40°C for periods from 5 to 15 hr, the contents of the tube were filtered on Whatman No. 1 filter paper to remove the residual collagen.

The method used for the determination of hydroxyproline was a procedure reported by Bergman and Loxley (1963). The following reagents were required during the assay. An oxidant solution contained 1 part of a 7% w/v aqueous solution of Chloramine T (N-chloro-p-toluene-sulfonamide sodium) and 4 parts of 0.1M acetate/citrate buffer, pH 6.0. The Ehrlich's reagent solution contained 3 volumes of solution (a) and 13 volumes of isopropanol. Solution (a) was prepared by dissolving 2 gms of p-dimethylaminobenzaldehyde to 3 ml of 60% perchloric acid.

Once the filtrate from the collagenase assay was obtained, a sample (0.2 ml) was transferred to a test tube and 0.2 ml of conc. HCl was added. Test tubes were sealed with a Teflon-lined cap, and the hydrolysis was performed at 110°C for 16 hr. After cooling, 9.6 ml of 0.04 M citric acid-0.32M NaOH solution was added to each tube. The hydrolysate mixture was

monitored with a pH meter to maintain the pH between 5 and 6. Then a 1 ml portion of this faintly acid solution was pipetted into a test tube. To this tube was added 3 ml of isopropanol and 1 ml of the oxidant solution. The new solution was mixed well and allowed to stand for 4 (±1) min at room temperature. Then 13 ml of Ehrlich's reagent solution was added with mixing and the tube heated for 25 min (±15 sec) at 60°C in a water bath. The tubes were cooled for 2 to 3 min in running tap water and the absorbance against water was measured within 30 min at 558 nm.

Catheptic Collagenase

The procedure of Anderson (1969) was used to hydrolyze collagen in acidic pH's while the procedure of Bergman and Loxley (1963) was used to measure the hydroxyproline produced during the assay.

Bovine tendon collagen was suspended at 10 mg/ml in a solution of 0.35% acetic acid in 25% (v/v) glycerol. The collagen was dispersed with a homogenizer.

The collagenolytic cathepsin was assayed at pH 3.5 in test tubes. Each tube contained 0.4 ml of the collagen suspension, 0.6 ml of 0.2M citric-citrate buffer containing 10mM cysteine and 0.2 ml of the enzyme supernatant sample. Tubes were incubated at 40°C for periods between 1 and 15 hr. At the end of the incubation period, the contents of the tube were filtered with Whatman No. 1 filter paper to remove residual collagen. Blank readings were obtained from two different assay mixtures. One mixture contained the enzyme solution but had 0.4 ml of a solution of 0.35% acetic acid in 25% (v/v) glycerol in place of the collagen suspension. The other mixture

contained collagen but had 0.2 ml of distilled water in place of the enzyme solution. The method used for the determination of hydroxyproline was described previously in the section for the neutral collagenase assay.

A standard curve for hydroxyproline was prepared by diluting a 400 ppm hydroxyproline (Eastman Kodak Co.) standard solution to 3 different concentrations between 10 and 40 ppm. Samples from these standards (0.2 ml) were then assayed as described for the determination of hydroxyproline.

Trypsin-like Activity

The method of Hummel (1959) was used for the first assay to detect trypsin-like activity. This activity is distinguished by its narrow specificity, which is almost exclusively directed toward L-lysyl and L-argininyl bonds of polypeptides. Since trypsin is classified as a pancreatic enzyme, it would not exist in muscle extracts except as a contaminant. Consequently substantial hydrolysis of the trypsin substrate at neutral pH's would indicate the existence of a trypsin-like enzyme.

The substrate solution was prepared by adding 19.7 mg of p-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAME·HCl) to 50 ml 0.04 M Tris-HCl buffer (pH 8.1) containing 0.01 M $CaCl_2$.

Then 3 ml of the substrate solution was placed in the reference and sample cuvettes. Approximately 3 min were allowed for the cuvettes to reach temperature equilibration in the compartment. Before starting the reaction the absorbance of the two cuvettes (1 cm quartz) was balanced at 247 nm. Then 0.1 ml of water was added with mixing to the sample cuvette. Immediately following the addition of the enzyme, the absorbance at 247 nm was recorded continuously for the next 5 min.

The second procedure used to detect trypsin-like activity was a modification of the method of Erlanger et al. (1961). In this method, the hydrolysis of the substrate α-N-benzoyl-D,L-arginine-p-nitroanilide (BAPA) was measured by estimating spectrophotometrically the quantity of p-nitroaniline present.

The substrate stock solution was prepared by dissolving 43.5 mg DL-BAPA in 1 ml of dimethylsulphoxide. This solution was brought to 100 ml with 0.05M Tris buffer, pH 8.2, containing 0.02M $CaCl_2$.

To start the reaction, 5 ml of the substrate stock solution and 1 ml of enzyme solution were placed in a test tube. The mixture was incubated for 2 hr at 40°C at which time the reaction was stopped with 1 ml of 10% TCA. After 30 min, the solution was filtered through Whatman No. 1 paper and the absorbance of the filtrate was read at 410 nm. The control had 1 ml of distilled water in place of enzyme solution.

Chymotrypsin-like Activity

The method of Hummel (1959) was used for the first assay to detect chymotrypsin-like activity. This activity is distinguished by its preferential catalysis of peptide bonds involving L-isomers of tyrosine, phenylalanine and tryptophan. Since chymotrypsin is classified as a pancreatic enzyme, it would not exist in muscle extracts except as a contaminant. Consequently substantial hydrolysis of the chymotrypsin substrate at neutral pH's would indicate the existence of a chymotrypsin-like enzyme.

A solution of 0.001M N-benzoyl-L-tyrosine ethyl ester (BTEE) in 50% (w/w) aqueous methanol was prepared as the substrate solution. The procedure consisted of placing 1.5 ml of the substrate solution and 1.5 ml

of the buffer (0.10M Tris-HCl, pH 7.8 containing 0.10M $CaCl_2$) in the reference cuvette (1 cm quartz). The sample cuvette contained 1.5 ml of substrate solution and 1.4 ml of buffer. Solutions in the cuvettes were allowed 5 min to reach thermal equilibrium in the cell compartments. Then the instrument was zeroed at 256 nm. At zero time, 0.1 ml of the diluted enzyme solution was added to the assay cuvette. The absorbancy difference was recorded continuously for a period of about 5 min.

The second procedure used to detect chymotrypsin-like activity was a modification of the method of Erlanger et al. (1966). In this method, hydrolysis of the substrate glutaryl-L-phenlyalanine-p-nitroanilide (GPNA) was measured by estimating spectrophotometrically the quantity of p-nitroaniline released.

The substrate stock solution was prepared by dissolving with warming 20 mg of GPNA in 1.0 ml of methanol. The solution was then brought to 50 ml with 0.05M Tris-HCl buffer, pH 7.6 containing 0.02M $CaCl_2$. To start the reaction 5 ml of the substrate stock solution and 1 ml of the enzyme solution were placed in a test tube. The mixture was incubated at $40^{o}C$ for 1 hr after which the reaction was stopped with 1 ml of 10% TCA. After 30 min, the solution was filtered through Whatman No. 1 paper and the absorbance of the filtrate was read at 410 nm. The control had 1 ml of distilled water in place of the enzyme solution.

Cathepsin B Activity

The first assay to be described that was used to measure cathepsin B activity was taken from the procedure of Barrett (1972a). The reagents required and their preparation were as follows. The buffered activator was made daily by dissolving 32.3 mg anhydrous cysteine base in 100 ml buffer, pH 6.0, containing 12.0 gm KH_2PO_4, 3.22 gm $Na_2HPO_4 \cdot 7H_2O$ and 0.495 gm

$Na_2EDTA \cdot 2H_2O$ per liter. The substrate stock solution contained 40 mg benzoyl-D,L-arginine 2-naphthylamide HCl (BNA) per ml of dimethylsulphoxide. (This stock was stored at 4^oC). The coupling reagent was prepared within 30 min of being used by mixing the following solutions (a) and (b) in equal parts. Solution (a) was 10mM 4-chloromercuribenzoic acid in 50mM of EDTA. Solution (b) contained Fast Garnet GBC freshly dissolved in 4% (w/v) aqueous Brij-35, at a concentration of 0.5 mg/ml.

Three variations in the procedure were undertaken. In the first variation, 1.5 ml of buffered activator was added to 0.5 ml of the enzyme sample. The mixture was preincubated for 5 min at 40^oC, then 50 µl of the BNA stock was added to start the reaction. Ten min later, 2.0 ml of coupling reagent was added with mixing, and after another 10 minutes, the sample was extracted with 4 ml n-butanol to remove the particulate material. The development of the crimson color was measured at 520 nm.

In the second variation, 1.5 ml of buffered activator was added to 1.0 ml of the enzyme sample. After preincubation for 5 min at 40^oC, 100 µl of the BNA stock was added. The reaction continued for 30 min at which time it was stopped with 2.0 ml of coupling reagent. After allowing 10 min for color development, 3.0 ml of n-butanol was used to extract the azo dye from the turbid solution. The absorbance was measured at 520 nm.

In both the variations just described, controls were prepared by adding the enzyme sample after the coupling reagent.

For the third variation, 3.0 ml of buffered activator and 1 ml of the enzyme sample were mixed. A 5 min pre-incubation period was followed by the addition of 100 µl of substrate. After 30 min of incubation, the reaction was stopped with 1 ml of 17.5% TCA and allowed to sit another 30

min before filtering on Whatman No. 1 filter paper. Then 0.5 ml of the filtrate was transferred to a clean test tube and 1.0 ml of 0.1% sodium nitrite solution was added. Exactly 3 min later 1.0 ml of 0.5% ammonium sulfamate was added and 2 min later, 2.0 ml of NED reagent (0.5 mg N-(1-naphthyl) ethylenediamine dihydrochloride/ml in 95% ethanol) was added. Absorbance was measured 10 min later at 580 nm. The control contained p-hydroxymercuriphenyl sulfonic acid monosodium salt in 0.1M KH_2PO_4-Na_2HPO_4 buffer, pH 6.0. The substrate reagent was first prepared as a stock solution containing 32.75 mg/ml of α-N-benzoyl-D,L-arginine p-nitroanilide (BAPA) in dimethylsulfoxide and stored at 4°C. When the working solution of substrate was required, the stock was warmed and 0.2 ml of it was diluted to 10 ml with 0.1M KH_2PO_4-Na_2HPO_4 buffer, pH 6.0.

To start the assay, 0.8 ml of enzyme, 0.2 ml of activating solution, and 2 ml of BAPA reagent were added to a test tube. The control had 0.8 ml of distilled water in place of the enzyme solution. The mixture was incubated for 2 hr at 40°C and stopped with 2 ml of 17.5% TCA. Thirty min later, the turbid mixture was filtered with Whatman No. 1 paper and the absorbance at 410 nm was read on the filtrate.

Cathepsin C Activity

The assay used in the detection of cathepsin C activity was based upon the method of Goldbarg and Rutenberg (1958). The basic principle of this assay is to detect free 2-naphthylamine released from Gly-Phe-2-naphthylamide colorimetrically.

The following reagents were used in the assay for cathepsin C activity. The activating reagent contained 50mM mercaptoethylamine hydrochloride in

0.1M KH_2PO_4-Na_2HPO_4, pH 6.0. The substrate solution contained 0.5mM of Gly-Phe-2-naphthylamide freshly dissolved in 0.1M potassium sodium phosphate buffer, pH 6.0. The NED reagent contained 0.5 mg N-(1-naphthyl)ethylene-diamine dihydrochloride per ml of 95% ethanol. The assay procedure consisted of mixing 1.6 ml of the supernatant sample with 0.4 ml of activating reagent. Following 5 min of preincubation at 40°C, 2.0 ml of substrate solution was added. The reaction was stopped after 90 min with 2.0 ml of either 10% or 17.5% TCA. At least 30 min were allowed to pass before measuring the extent of the reaction. The control contained distilled water in place of the supernatant solution. To detect the free 2-naphthylamide, the samples are filtered on Whatman No. 1 filter paper. Then 0.5 ml samples of the filtrate were transferred to clean tubes. With precise timing, 1.0 ml of 0.1% sodium nitrite solution was added to each tube, followed 3 min later by 1.0 ml of 0.5% ammonium sulfamate. After a further 2 min, 2.0 ml of NED reagent was added. The blue color was measured at a wavelength of 580 nm after 10 min.

Cathepsin A Activity

The method used to detect the presence of cathepsin A was based on that of Iodice et al. (1966) whereby amino acids liberated from the peptide substrate are measured by the ninhydrin procedure of Rosen (1957).

Incubation mixtures contained 0.2 ml of 0.20M sodium acetate-acetic acid buffer, pH 3.6, 0.5 ml of 30.4mM carbobenzoxy-glutamyl-tyrosine (CBZ-Glu-Tyr) in 0.2M sodium acetate-acetic acid buffer, pH 5.0 and 0.3 ml enzyme sample. Incubation mixtures for the controls consisted of 0.35 ml of 0.20 sodium acetate-acetic acid buffer, pH 3.6, 0.35 ml of 0.20M sodium acetate-acetic acid buffer, pH 5.0, and 0.3 ml of enzyme sample.

The reaction proceeded for 2 hr before being stopped with 1 ml of 10% TCA. The mixture was returned to the waterbath for 10 min and then filtered with Whatman No. 1 filter paper. Fifty µl aliquots of the filtrate were run in the ninhydrin reaction as follows. Each sample was diluted to 1.0 ml with deionised water and 0.5 ml of cyanide-acetate reagent which consisted of 2.0 ml of 0.01M sodium cyanide and 98 ml of acetate-acetic acid buffer, pH 5.3-5.4. The ninhydrin reagent consisted of 3% ninhydrin in peroxide-free methyl cellosolve. After addition of these reagents to the sample, the tubes were heated for 15 min in a boiling water bath. They were then removed from the bath and 5.0 ml of 50% aqueous isopropanol diluent was immediately squirted into each from an automatic pipette. The reaction mixture was cooled in a bath of cold water, and its E_{570} measured. In those cases where the E_{570} exceeded 0.8, an additional 5.0 or 10.0 ml of diluent was added to the sample.

Due to the high absorbance of the control, dialysis on the supernatant sample was undertaken using a Bio-Fiber 50 device (Bio-Rad Laboratories). The sample was placed in the beaker jacket and the dialysate (deionized water) was passed through the fibers. Dialysis proceeded for 1 hr.

Inhibition Studies

Various activators and inhibitors were included in the assay solution to determine their effect on the hydrolysis of the substrate by the enzymes present. Some of the chemicals tested were iodoacetamide, EDTA, $CuSO_4$, cysteine and pCMB. They were dissolved separately in the substrate solution to give a final concentration of 0.5 or 1.0mM. No preincubation of these chemicals with the supernatant solution was used. The various assays were run as described previously.

The chemicals TLCK, TPCK and phenylmethylsulfonylfluoride (PMSF) required variations in the assay procedure. The various buffers used to prepare the inhibitor solutions were either the buffer used in the cathepsin C assay or the buffer used in the chymotrypsin-like assay.

To test the effect of TLCK on the enzyme activity, 5 or 10 mg of TLCK was added to 5 ml buffer. After dissolution, the TLCK solution was mixed with the enzyme supernatant in equal proportions followed by incubation at room temperature for 2 hr.

The procedure used to test the effect of TPCK on the enzyme activity was similar to that described for TLCK. The major variation was in preparing the TPCK solution. One ml of methanol was added to 1.5 mg of TPCK to dissolve the chemical. Then 1.5 ml of buffer was added. Equal quantities of TPCK solution and enzyme supernatant were mixed and then incubated for 2 hr at room temperature.

In testing for inhibition by PMSF, the inhibitor solution was prepared by dissolving 1 mg of PMSF in 0.5 ml of 2-propanol and diluting with 4.5 ml of buffer. Enzyme supernatant and inhibitor solution were mixed in equal proportions, proceeded by incubation for 20 min at room temperature.

After incubation, either 5.0 ml of chymotrypsin-like substrate solution was added to 1.0 ml of the TLCK, TPCK or PMSF inhibitor-enzyme solution, or 2.0 ml of cathepsin C substrate solution was added to 1.6 ml of the TLCK, TPCK or PMSF inhibitor-enzyme solution. Blanks for the inhibitor studies were prepared by replacing enzyme supernatant with distilled water. Due to the dilution of enzyme supernatant with inhibitor solution, controls were prepared whereby the enzyme supernatant was diluted to one-half its concentration with buffer (or buffer and methanol) and then incubated for

either the required 2 hr or 20 min. These controls were used to determine the percentage of inhibition by the chemicals TLCK, TPCK or PMSF.

RESULTS AND DISCUSSION

Preparation of Extracts

The general practice used to study catheptic enzymes involves homogenization of cells or tissue. The degree of mincing and homogenization will determine the degree of purity of the catheptic enzymes.

Gentle homogenization of cells or tissue minimizes physical damage to lysosomes. Otherwise, lysosomes will rupture and release their contents, including the catheptic enzymes. If lysosomes are not ruptured, they may be separated from other subcellular particles by centrifugation. The result of lysosome isolation is a concentrated source of lysosomal enzymes (Beaufay, 1972).

In other cases, tissues have been homogenized in Waring blenders with the intention of rupturing the lysosomes (Wojtowicz and Odense, 1972). The sensitivity of detection of enzymes is decreased in this preparation over that of a concentrated lysosomal preparation and the final result is a crude determination of the amount of catheptic enzymes present.

It was the intention of this study to examine the fluid contents of the fish muscle without any homogenization. The purpose was to identify, qualitatively and quantitatively, the enzymatic activity present in the sarcoplasmic solution that would be capable of degrading muscle tissue in the intact fish. In cases where one fish had higher amounts of enzymatic activity than another fish, one or both of the following could be implicated: an increase in the number of lysosomes present in the tissue or an increase in the fragility of the lysosomal membrane, both of which could cause more lysosomal enzymes to be released in the muscle fluid. By avoiding homogeni-

zation, damage to intact lysosomes would be minimized and the enzymes enclosed in those lysosomes would remain latent due to the lack of substrate contact. However, if these lysosomal enzymes were to be released through homogenization, a quantitative analysis of the enzymatic activity could not be related to the muscle damage imposed by the proteinases present in the intact fish.

Extracting solutions of 1% NaCl or 2% KCl were not used in sample preparation to avoid dilution of the enzymes already present. Dilution particularly of non-lysosomal enzymes would cause a decrease in the sensitivity for detecting these enzymes.

Proteolytic Activity as Affected by Temperature

Figure 1 shows the effect of temperature on the proteolytic activity in whiting muscle. The number of protease units is equivalent to the ΔA_{280}/min/gm protein. Depending on sampling time, the optimum temperature varied. At 30 min, the optimum temperature was 65°C. At 60, 90, and 120 min, the optimum temperature was at 55°C. While higher temperatures increase enzyme activity, they also cause an increase in the irreversible denaturation of enzymes (Berk, 1976). Consequently at higher temperatures, the decrease in the quantity of active enzyme eventually offsets the advantage of a faster hydrolysis.

One of the disadvantages in running this temperature study was assaying for activity at only one pH. (Limitations in time prevented extending this study to include several pH's.) If a number of enzymes were present in the enzyme supernatant, each would tend to operate in its own pH range. Different enzymes may denature at different temperatures as well as show different

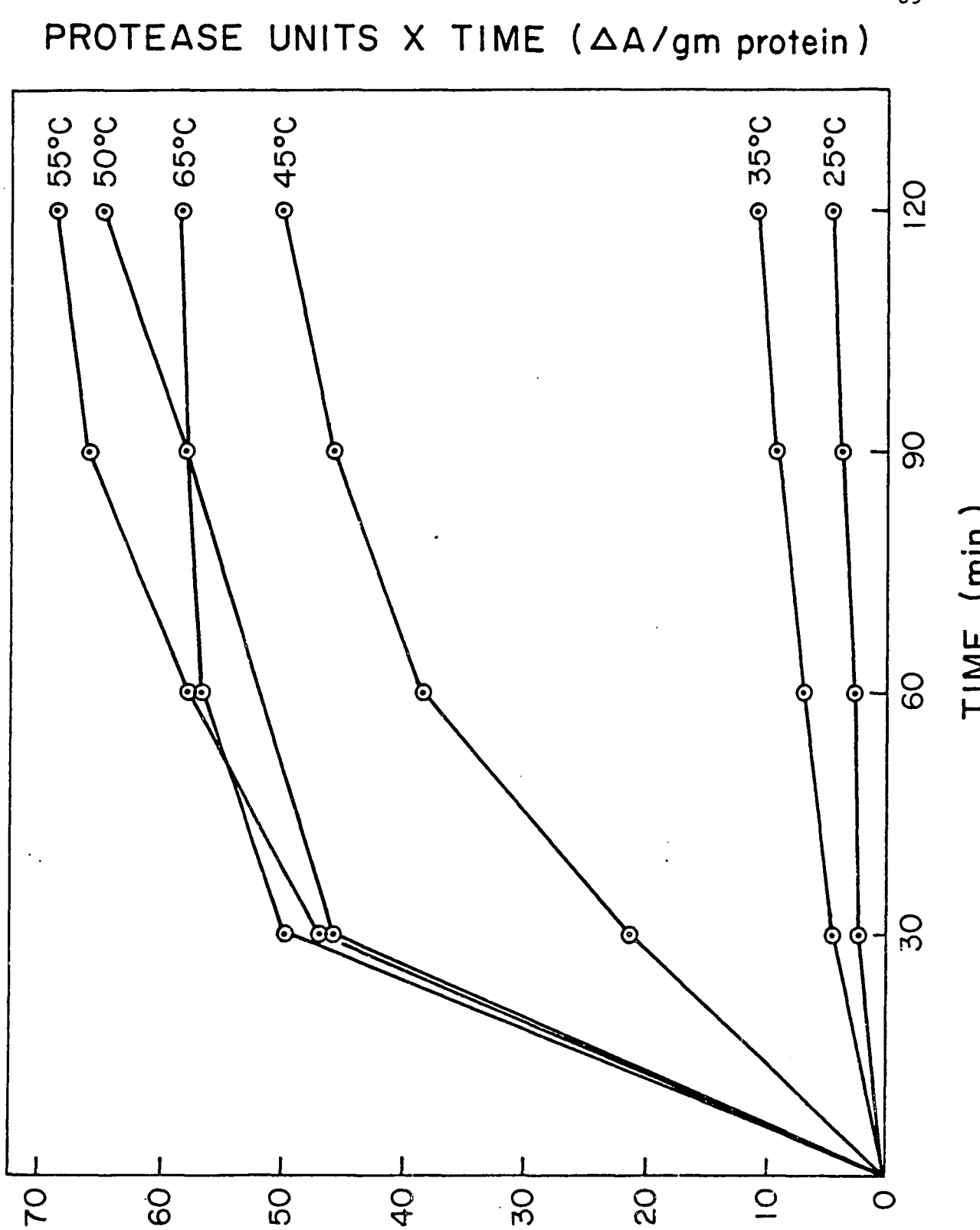

Figure 1. Effect of temperature on the activity of Pacific whiting proteases. Data obtained from the hydrolysis of casein substrate at pH 7.3.

rates of enzyme activity with increases in temperature. Therefore, it is possible that enzymes acting at low pH's would not give the same temperature optimum as those acting at neutral pH.

Another precaution to be taken in interpreting these data is that one is not only measuring the hydrolysis of casein but also that of endogenous protein. In preparing the blank, the enzyme supernatant was added immediately preceding the addition of TCA. No incubation of the enzyme supernatant was possible and therefore hydrolysis of endogenous proteins could not be subtracted.

Proteolytic Activity as Affected by pH

Figure 2 shows the effect of pH on the hydrolysis of hemoglobin by both whiting and true cod enzyme supernatants. No whiting examined off the Northern Oregon Coast could be found that were not parasitized. In view of this fact, true cod was used as the unparasitized control. Since both fish are gadoids, it was hoped that species differences in enzymatic activity for the unparasitized fish would be negligible. Thus, any differences in enzymatic activity could then, with caution, be attributed to the presence of the parasite.

Two independent samples of each species were measured for effect of pH on hydrolysis of hemoglobin. No attempt was made to average these effects but rather to show the variation that could result from samples taken at different times of the year.

As observed in Figure 2, the largest pH optimum of the whiting samples was in the range of 3.5-3.9. The first whiting sample could also have had an optimum closer to pH 3.5, since no assays were run at pH's between

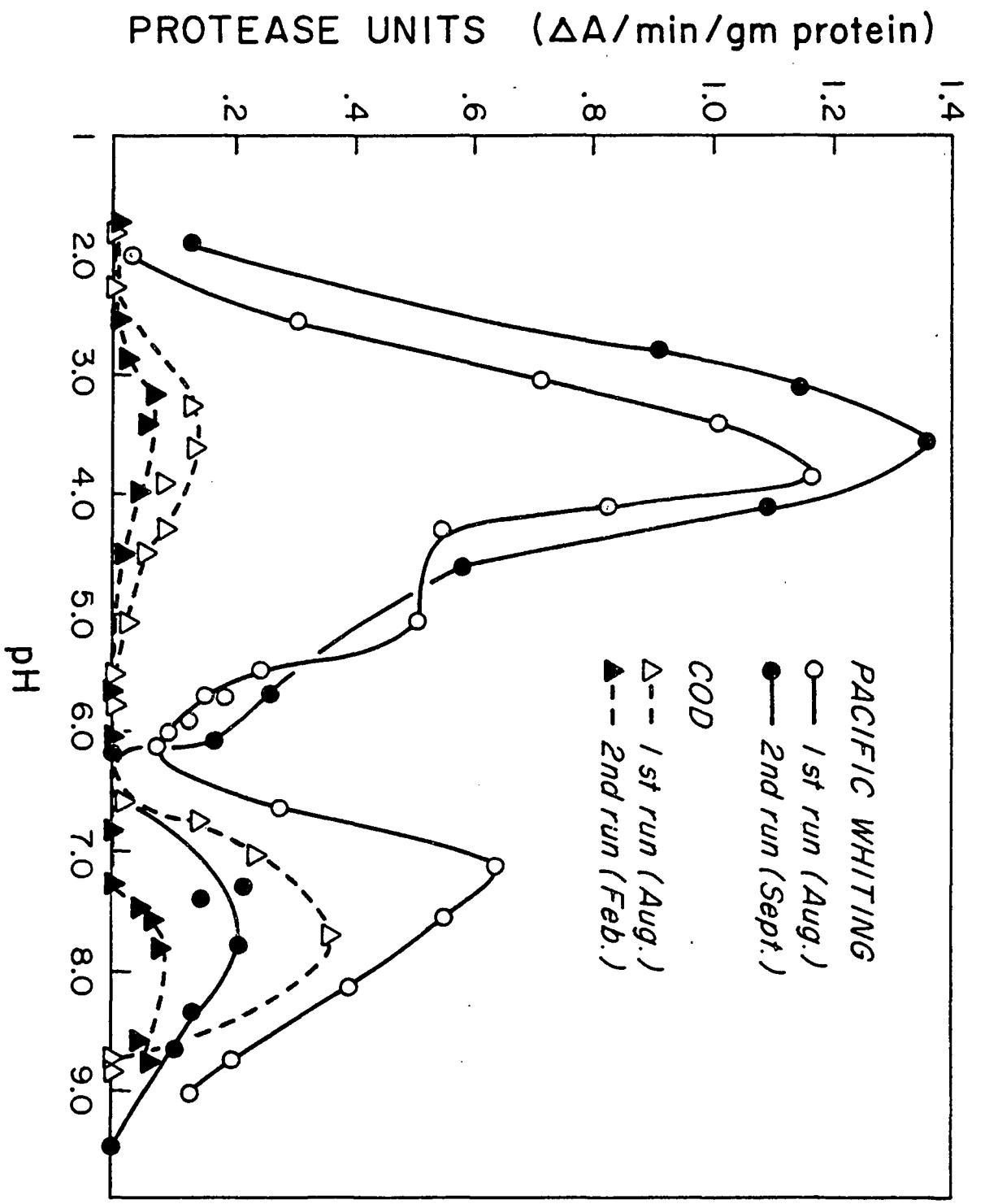

Figure 2. Effect of pH on the activity of Pacific whiting true cod proteases. Data represents the hydrolysis of hemoglobin for 30 min. at 50°C.

3.4 and 3.85. A pH optimum at 3.5 would agree with that of several other investigators who characterized acidic proteases in various other fish specimens. Atlantic mackerel is reported to have a pH optimum range of 3.2-3.8, white hake, 3.0-3.6, and Atlantic herring, 3.2-3.6 (Wojtowicz and Odense, 1972). The pH optimum for cod in Figure 2 is in the range of 3.2-3.6.

A second pH optimum for whiting occurs in the alkaline range at approximately 7.1-7.2, for cod it was approximately 7.7-8.0. One other possible optimum for whiting was approximately pH 4.3-4.6 as indicated in sample No. 1. A noticeable change in the rate of decrease in activity occurs with increasing the pH from 4.0 to 5.5 which may be due to the presence of a enzyme different from those creating the other two optima. In sample No. 2, however, it is difficult to distinguish the same event occurring in this range mainly due to the selection of pH's sampled.

One distinguishing feature in Figure 2 is the large difference in activities of the whiting samples vs. the true cod samples, particularly in the acid range. Quantitative comparisons of the average proteolytic activities in whiting and true cod at several pH's are reported in Table 2. The pH's selected have significance only in regard to distinguishing which enzymes may be important in a particular sample. For example, the activity in the pH range of 3-4.5 could be due largely to cathepsin D (Lapresle and Webb, 1960). The activity of whiting is approximately 13-18 times greater than true cod in this range.

The optimum pH of cathepsin C is approximately 5.5 (Tallon et al., 1952). At this pH, the relative activity of whiting to true cod is 102:1. It may be possible that the increased release of cathepsins C and D from the

Table 2. Ratios of proteolytic activity of Pacific whiting to true cod at varying pH's.

pH	Pacific Whiting:True Cod
3.6	13.6:1
3.9	17.6:1
4.6	18.4:1
5.0	31.7:1
5.5	102:1
7.7	1.6:1
8.8	4.2:1

lysosomes of whiting would be responsible for the increased proteolytic activity observed. Molecular weight determinations of the products of digestion show that the protease cathepsin D initiates protein hydrolysis and produces peptide fragments that can then be further digested by the peptidase cathepsin C, which is not active against intact protein (Huang and Tappel, 1971). The fact that very little activity was observed in the cod samples at pH 5.5 would indicate the absence of cathepsin C. This assumption agrees with the findings by Siebert (1963) who found no cathepsin C activity in cod, as well as no cathepsin A or B activity.

Referring again to Figure 2, an increased level of activity close to neutrality would be very important as enzymes would be able to hydrolyze proteins at the physiological pH of the fish muscle in contrast to cathepsins which act optimally at lower pH's. However, it would appear based on these 4 samples that whiting does not always give an increased level of activity, and thus enzymes which act optimally in this range would not be the major cause for the softening in Pacific whiting.

Going to a more alkaline pH, approximately pH 8.8, higher proteolytic activities were again consistently found for Pacific whiting. Elastase operating optimally at this pH could be degrading stroma proteins and thus have significant effects on the texture (Partridge and Davis, 1955).

One of the major limitations in making any definite conclusions from these results is the fact that native hemoglobin was used instead of acid denatured hemoglobin. Native hemoglobin dissociates reversibly into 2 subunits at about pH 4.5 and is irreversibly degraded to 4 subunits near pH 3 (Rossi Fanelli et al., 1964). These alterations could cause alterations in the ease of digestion of the substrate. Therefore, it is difficult to say

with any certainty that catheptic enzymes were present in greater quantities than enzymes acting in the neutral range.

Another limitation in the assay procedure used to determine the effect of pH on proteolytic activity was in the detection of hydrolysis products. Not only could amino acids and small peptides be absorbing light at 280nm but also nucleic acids (Segal, 1976). Therefore, the variation of the nuclease activity with pH would interfere with the interpretation of the effect of pH on proteolytic activity. Limitations in time prevented repeating the pH study using the Lowry procedure to measure hydrolysis products in the TCA soluble filtrate. The Lowry procedure involves the complexing of copper in alkaline solution with peptide linkage of proteins and hence, nucleic acids present are not measured.

One comment should be made on the temperature selected for the incubation of the samples. To minimize the risk of heat denaturation of the enzyme, samples were not incubated at 65^{o}C (the temperature optimum found after incubating 30 min with casein). Instead a lower temperature, 50^{o}C, was selected which still gave sufficient hydrolysis of the protein substrate to be measured.

Specific Proteolytic Activities in Whiting and True Cod

Carboxypeptidases A and B

To help clarify the effects of pH observed, specific proteolytic activities were studied by using selected substrates. For carboxypeptidases A and B, no activity was detected using substrates described for their detection in materials and methods. These results are not to suggest that these enzymes or other enzymes capable of hydrolyzing the substrates were

not present. Rather, the assay requires dilution of the enzyme to a degree where the initial absorbance of the assay solution can be read spectrophotometrically. The enzyme supernatant was diluted to 1/9.3 of its original concentration for the carboxypeptidase A assay and to 1/8.14 of its original concentration for the carboxypeptidase B assay. Such dilutions may render it impossible to detect any enzymes capable of hydrolyzing the substrates, hippuryl-L-phenylalanine or hippuryl-L-arginine.

Elastase

Another enzyme that could not be detected in whiting or cod muscle supernatants was elastase. Elastase is distinct in that it acts upon elastin (Partridge and Davis, 1955).

Although whiting samples gave small absorbance readings in the elastase assay, the values were outside the range of reliability of the spectrophotometer. Table 3 shows the extent of hydrolysis for various concentrations of a purified commercial elastase enzyme. With the 3 concentrations sampled, a correlation coefficient of 0.999 was obtained indicating that solubilization by elastase was linear in this range. Therefore, it may be reported that if present in whiting or cod, there is less than 0.023 units of elastase.

Collagenases

Two different assays were used to detect collagenases in the enzyme supernatant from whiting. The first assay was used to detect an enzyme active at a pH optimum of approximately 7.2. Under the conditions described for the assay, no enzyme hydrolyzing collagen at a neutral pH was detected in the whiting extracts. The assay was not conducted on the cod supernatant.

Table 3. Activity of porcine pancreatic elastase for estimating the lower limit of detection of the elastase assay.[a]

Elastase units[b]	pH	A_{590}	Average A_{590}
0.23	8.51	(1) 0.197 (2) 0.198	0.198
0.077	8.54	(1) 0.056 (2) 0.061	0.058
0.023	8.52	(1) 0.012 (2) 0.018	0.015

[a]Elastase was purchased from Sigma Chemical Co. (2 x crystallized aqueous suspension).

[b]One elastase unit will solubilize 1 mg of elastin in 20 min at 37°C, pH 8.8.

A major limitation in the assay for neutral collagenase was the insolubility of the substrate. Interaction of the substrate with the enzyme if it was present remained limited. Consequently, a very high level of collagenase would be needed before its presence would be detected.

To arrive at the net absorbance given in both the neutral collagenase and the catheptic collagenase results, absorbance readings obtained from two separate blanks, where one blank contained only the substrate and the other had only enzyme, were each subtracted from the average absorbance of the sample containing both enzyme and substrate. These subtractions eliminated any absorbances contributed by either the enzyme or substrate. Thus, the net absorbance represents only the action of collagenase on collagen.

The second assay employed to measure collagenase activity was undertaken only on the whiting enzyme supernatant (Table 4). At each of the incubation times listed, collagen was shown to be hydrolyzed at pH 3.5. The amount of hydroxyproline produced in these samples was determined directly from the standard curve (Figure 3). Further studies need to be conducted that will aid in characterizing the activity of this enzyme. Some of the activators and inhibitors to use include cysteine, p-CMB, TLCK, iodoacetamide, Cu^{2+}, EDTA and diisopropylphosphofluoridate. The effects of these chemicals on catheptic collagenase have all been reported in the literature. Cysteine and EDTA activate the enzyme while p-CMB, TLCK, iodoacetamide and Cu^{2+} inhibit the hydrolysis of collagen. The addition of diisopropylphosphofluoridate has no effect on the activity (Etherington, 1972).

Verification of the presence of catheptic collagenase in the muscle fluid of whiting will point to the release of the enzyme from lysosomes, where it is generally found (Frankland and Wynn, 1962). If in addition the

Table 4. Catheptic collagenase activity of Pacific whiting muscle extract.[a]

Incubation time (hr.)	Net A_{558}	Hydroxyproline Produced (μmoles)	Collagenase Units[b]	Specific Activity
1	0.112	0.0785	1.3×10^{-3}	3.8×10^{-5}
2	0.130	0.0846	0.70×10^{-3}	2.0×10^{-5}
3	0.238	0.122	0.68×10^{-3}	2.0×10^{-5}

[a]Average of triplicate assays.

[b]One collagenase unit is the amount of enzyme that will release 1 μmole of hydroxyproline in 1 min at 40°C, pH 3.5.

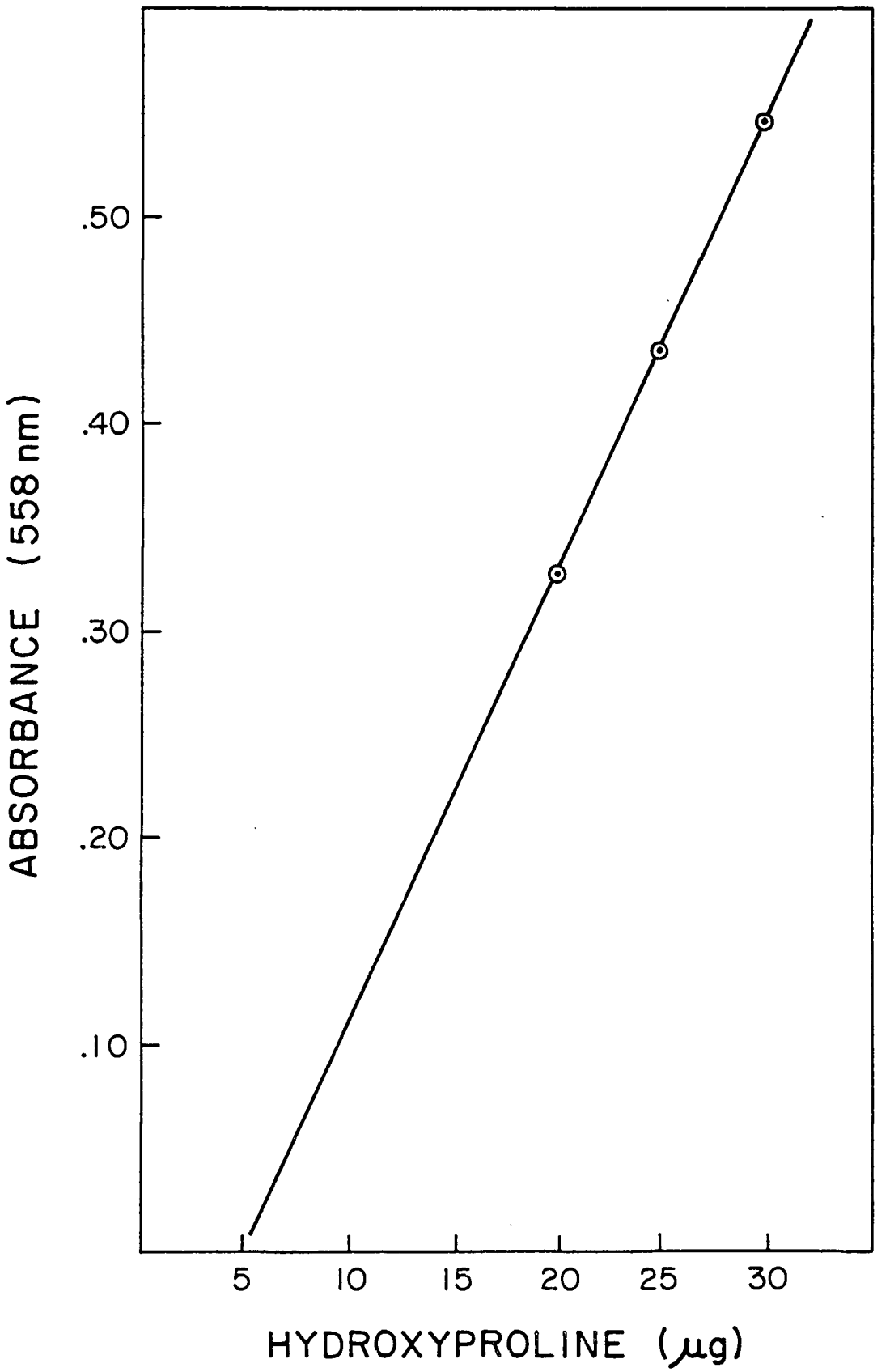

Figure 3. Hydroxyproline standard curve.

enzyme is shown to be present in cod, comparisons of the levels of activity between whiting and cod could be made. Those fish with higher levels of catheptic collagenase than other fish would be indicative of a greater release of catheptic enzymes from the lysosomes. Thus, if whiting were shown to have an accelerated release compared to cod, the large levels of cathepsins including catheptic collagenase could be responsible for the muscle breakdown in whiting.

Trypsin

Detection of trypsin-like activity was attempted using two different substrates, TAME and BAPA. In Table 5, the following equation was used to calculate the specific activity, the number of TAME units/mgm protein:

$$\text{TAME units/mgm protein} = \frac{\Delta A/\text{min (dilution factor)}}{(0.1174\ A/\mu\text{mole TAME})\ (0.1\text{ml})\ (\text{protein conc.})}$$

(Sullivan, 1976). One unit of TAME activity is defined as the amount of trypsin catalyzing the hydrolysis of 1 micromole of TAME/min (Walsh, 1970). The protein concentration for whiting and cod was 51.4 and 55.3 mg/ml, respectively, and the dilution factor for both fish samples was 9.33. Two hundred forty-seven TAME units are equivalent to 1.0 mg of trypsin (Walsh, 1970).

No trypsin activity could be detected in whiting or cod using the substrate BAPA. However, at the same time a 1/100 dilution of the trypsin standard (10mg/100ml) gave an absorbance of 0.425 at 410nm. These results demonstrate that if the enzyme supernatants from either fish contained trypsin, they contained less than 1µg trypsin/ml. Contradictory to these conclusions were the results from the assay using TAME. Whiting and cod

Table 5. Trypsin activity of Pacific whiting and true cod muscle extracts using TAME substrate.

Whiting	pH	ΔA/min[a]	% of Control	Specific activity	mg trypsin/ gm protein
No inhibitors (control)	8.03	0.245	100	3.789	15.3
Cysteine (1mM)	7.99	0.197	80.4	3.046	12.3
EDTA (1mM)	7.87	0.163	66.5	2.520	10.2
Iodoacetamide (1mM)	7.94	0.367	149.8	5.674	23.0
Cod	**pH**	**ΔA/min**	**% of Control**	**Specific activity**	**mg trypsin/ gm protein**
No inhibitors (control)	8.07	0.352	100	5.059	20.5
Cysteine (1mM)	8.03	0.260	73.9	3.736	15.1
EDTA (1mM)	7.91	0.213	60.5	3.061	12.4
Iodoacetamide (1mM)	7.98	0.525	149.1	7.545	30.5

[a]Average of triplicate assays.

were found to have 15.3 and 20.5 mg trypsin/gm protein respectively. These results imply that 765µg of trypsin placed in the assay sample was unable to hydrolyze the BAPA substrate. Yet, at the same time 1µg of standard trypsin did show activity. It must be assumed therefore that the results of the TAME assay are misleading and invalid. The positive results were probably caused by the increase in turbidity which formed in the cuvettes during measurement. An enzyme blank without substrate should have been run in conjunction with each set of samples. The fact that the addition of inhibitors or activators gave differences in the rate of absorbance change can only be used to indicate that they were influencing the formation of turbidity or influencing the enzyme working on its own endogenous protein.

Chymotrypsin

The formation of turbidity could also have been responsible for the unbelievably high activity observed for hydrolysis of the substrate BTEE (Table 6). Even crystallized chymotrypsin does not hydrolyze BTEE to that high a degree (Walsh and Wilcox, 1970). However, the possibility can not be ruled out that another enzyme exists in muscle which is capable of hydrolyzing BTEE at a higher reaction velocity than chymotrypsin. The following equation was used to determine the specific activity:

$$\text{the number of BTEE units/mg} = \frac{(\Delta A/\text{min})\ (\text{dilution factor})}{(0.02714\ A/\mu\text{mole BTEE})\ (0.10\text{ml})\ (\text{protein conc.})}$$

(Sullivan, 1976). The dilution factor was 26 while the protein concentrations for both cod and whiting were the same as used in the TAME equation.

The presence of chymotrypsin-like behavior was confirmed in the assay using GPNA as substrate. The results of this assay for whiting and cod are

Table 6. Chymotrypsin activity of Pacific whiting and true cod muscle extracts using BTEE substrate.

Whiting		ΔA/min[a]	% of Control	Specific activity
	pH			
No inhibitors (control)	7.83	5.02	100	935.6
Cysteine (1mM)	7.87	3.53	70.3	657.9
EDTA (1mM)	7.71	3.72	74.1	693.3
Iodoacetamide (1mM)	7.87	5.63	112.2	1049.3
Cod		**ΔA/min[a]**	**% of Control**	**Specific activity**
	pH			
No inhibitors (control)	7.81	3.71	100	642.7
Cysteine (1mM)	7.85	4.41	118.9	764.0
EDTA (1mM)	7.69	2.50	67.4	433.1
Iodoacetamide (1mM)	7.85	5.19	139.9	899.1

[a]Average of triplicate assays.

given in Table 7. Although whiting is shown to contain a higher activity than cod at pH 7.8, the opposite case holds true at pH 7.15. Consequently, more samples of fish will need to be examined before this enzyme can be linked to the accelerated muscle protein breakdown in whiting.

To check the linearity of the chymotrypsin-like assay, the enzyme supernatant from cod was diluted to various degrees (Figure 4). As the amount of enzyme in the sample was increased, the rate of increase in absorbance declined. One possibility that might explain this rate decrease is that the substrate became limiting. In order to estimate with accuracy the differences between samples in the amount of enzyme present, it will be necessary to dilute samples to within the range of linearity for this assay. Presently this range only extends from 0.3 to 0.6.

Figure 5 shows the effect of pH on the hydrolysis of GPNA for both whiting and true cod. The pH optimum for whiting was approximately 7.25 and for true cod approximately 7.15. These optima are slightly lower than that of 7.8 determined for chymotrypsin on GPNA by Erlanger et al. (1966).

To characterize the enzyme responsible for the activity on GPNA, several chemicals were added to the assay solution to determine their effect. The results of this study are presented in Table 8. Several of the chemical effects agree with those reported for the chymotrypsin. Chymotrypsin was found to be inhibited by Cu^{2+} (Wilcox, 1970), TLCK (Shaw, 1967), and phenylmethylsulfonylfluoride (Sullivan, 1976). These chemicals also inhibited the activity in both whiting and cod samples. In addition, $CaCl_2$ activated the enzyme in cod as it does for chymotrypsin (Chervenka, 1959).

Beyond these similarities to chymotrypsin, there is no agreement whether the enzyme present hydrolyzing GPNA is a serine protease, a

Table 7. Chymotrypsin activity of Pacific whiting and true cod muscle extracts using GPNA substrate.[a]

	Specific Activity at pH 7.15[b]	Specific Activity at pH 7.8[b]
Whiting	8.74×10^{-3}	5.88×10^{-3}
True cod	20.0×10^{-3}	3.67×10^{-3}

[a]Average of triplicate assays.

[b]Specific activity is the number enzyme units per mg protein. One unit of enzyme activity is that amount causing transformation of 1.0 micromole of GPNA per minute at 40°C.

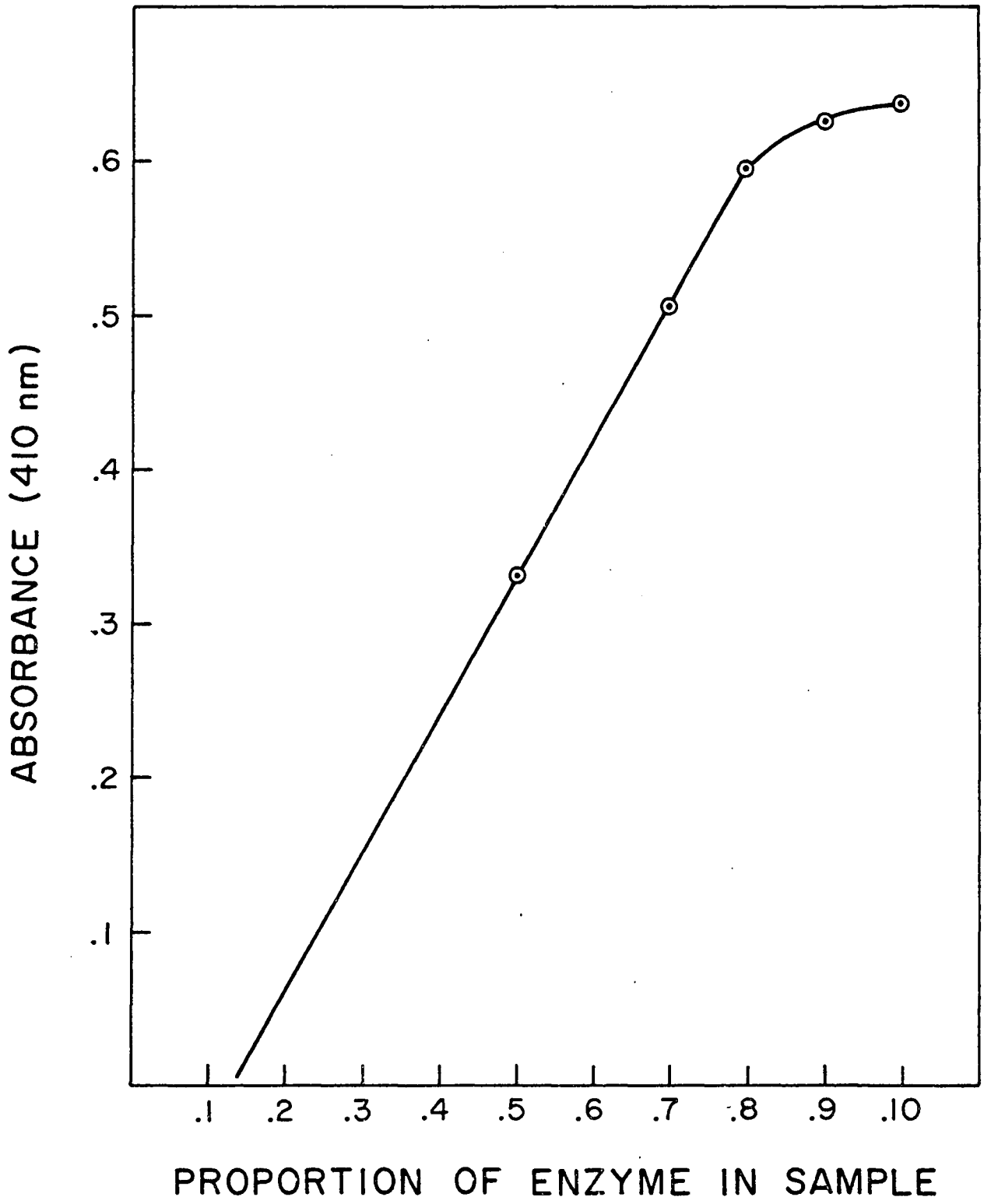

Figure 4. Linearity of chymotrypsin activity of true cod muscle extracts.

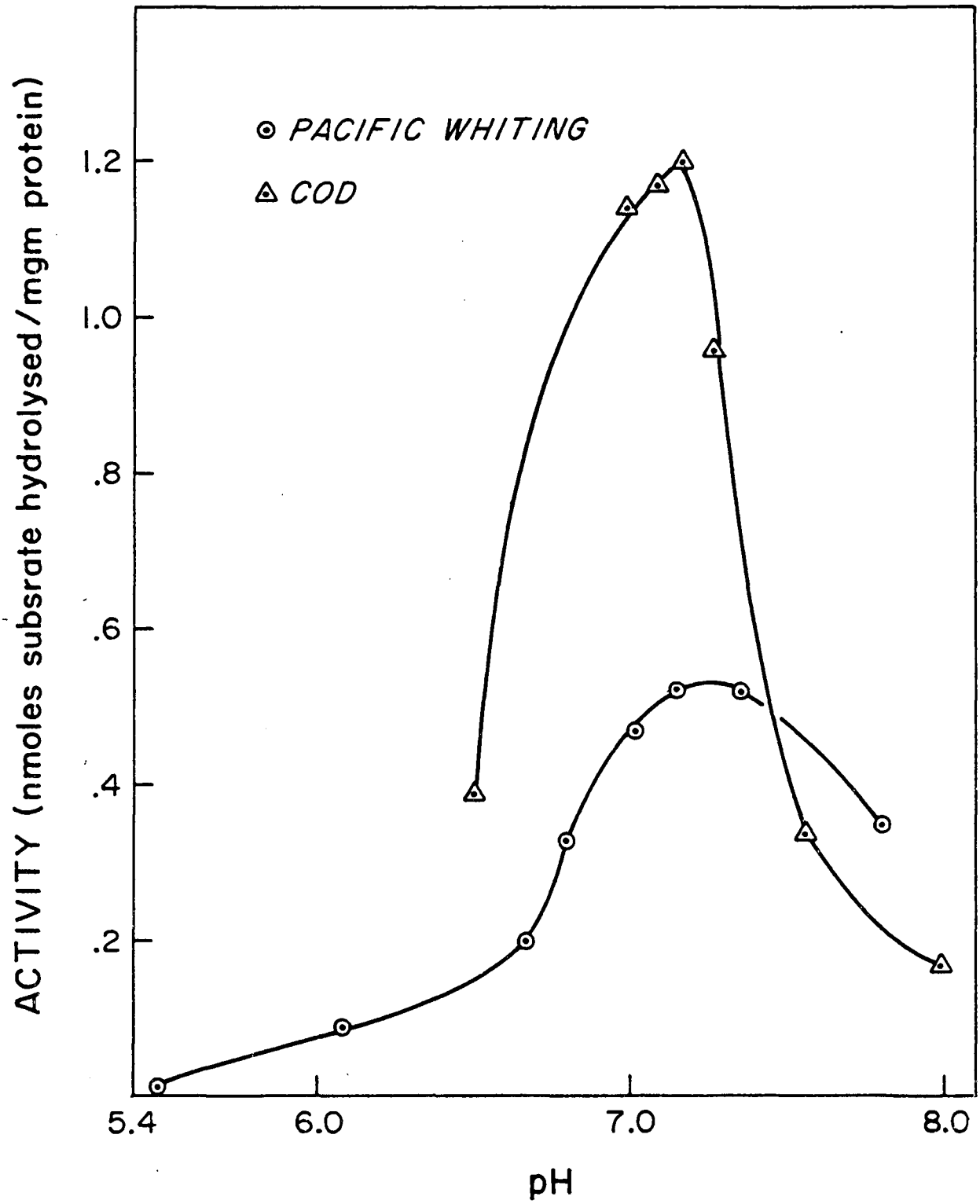

Figure 5. Effect of pH on the hydrolysis of GPNA by chymotrypsin-like enzymes of Pacific whiting and true cod muscle extracts.

Table 8. Effect of inhibitors on chymotrypsin activity of Pacific whiting and true cod muscle extracts.

Inhibitor	Concentration (mM)	% Activity of Control	
		Whiting	Cod
Iodoacetamide	1	64.4	72.6
Cysteine	5	35.0	45.8
$CuSO_4$	1	12.9	39.9
EDTA	1	98.0	101.8
PCMB	1	0.0	0.0
PMSF	0.2	31.0	30.4
TLCK	1	34.0	12.0
TPCK	0.034	64.5	50.0
$CaCl_2$	20	-[a]	112.7

[a]Not determined.

sulfhydryl protease or a heavy metal enzyme. The inhibition by cysteine might suggest that the enzyme could have a requirement for some metal ion. However, at the same time, EDTA, another chelator of metal ions, did not affect activity. Possibly the higher concentration of cysteine was responsible for destabilizing the enzyme to a greater degree than EDTA.

Another inconsistency in the results was the effect of pCMB, cysteine and iodoacetamide. Iodoacetamide and pCMB are sulfhydryl inhibitors. yet a sulfhydryl activator, cysteine, did not activate the enzyme. If the enzyme was a serine protease like chymotrypsin, sulfhydryl inhibitors would have had no effect on it (Sizer, 1945). All of these inconsistencies suggest that there may be more than one enzyme present acting on the substrate. Purification studies would help to resolve this question.

Cathepsin B

The presence of cathepsin B in the whiting supernatant might be verified by the hydrolysis of the substrate, benzoyl-D,L-arginine 2-naphthylamide (Table 9). Using a naphthylamide standard curve (Figure 6), the specific activity of the whiting sample was determined. However, the supernatant from true cod did not appear to contain cathepsin B. After incubation of the cod samples, no naphthylamide could be detected using either the Fast Garnet detection (Barrett, 1972a) or the detection procedure proposed by Goldbarg and Rutenberg (1958).

The greater sensitivity of the assay when using the naphthylamide substrate instead of the nitroanilide substrate is attributable to two factors: (1) the much larger value for V_{max} when cathepsin B acts on BANA rather than BAPA and (2), the greater molar extinction coefficient

Table 9. Cathepsin B activity of Pacific whiting muscle extract using naphthylamide substrate.[a]

A_{580}	Naphthylamide present (μmoles)	Enzyme Units[b]	Specific Activity
0.066	0.138	4.6×10^{-3}	1.02×10^{-4}

[a]Average of triplicate assays.

[b]One unit of enzyme activity is that amount causing transformation of 1.0 micromole of naphthylamide substrate per minute at 40°C, pH 6.

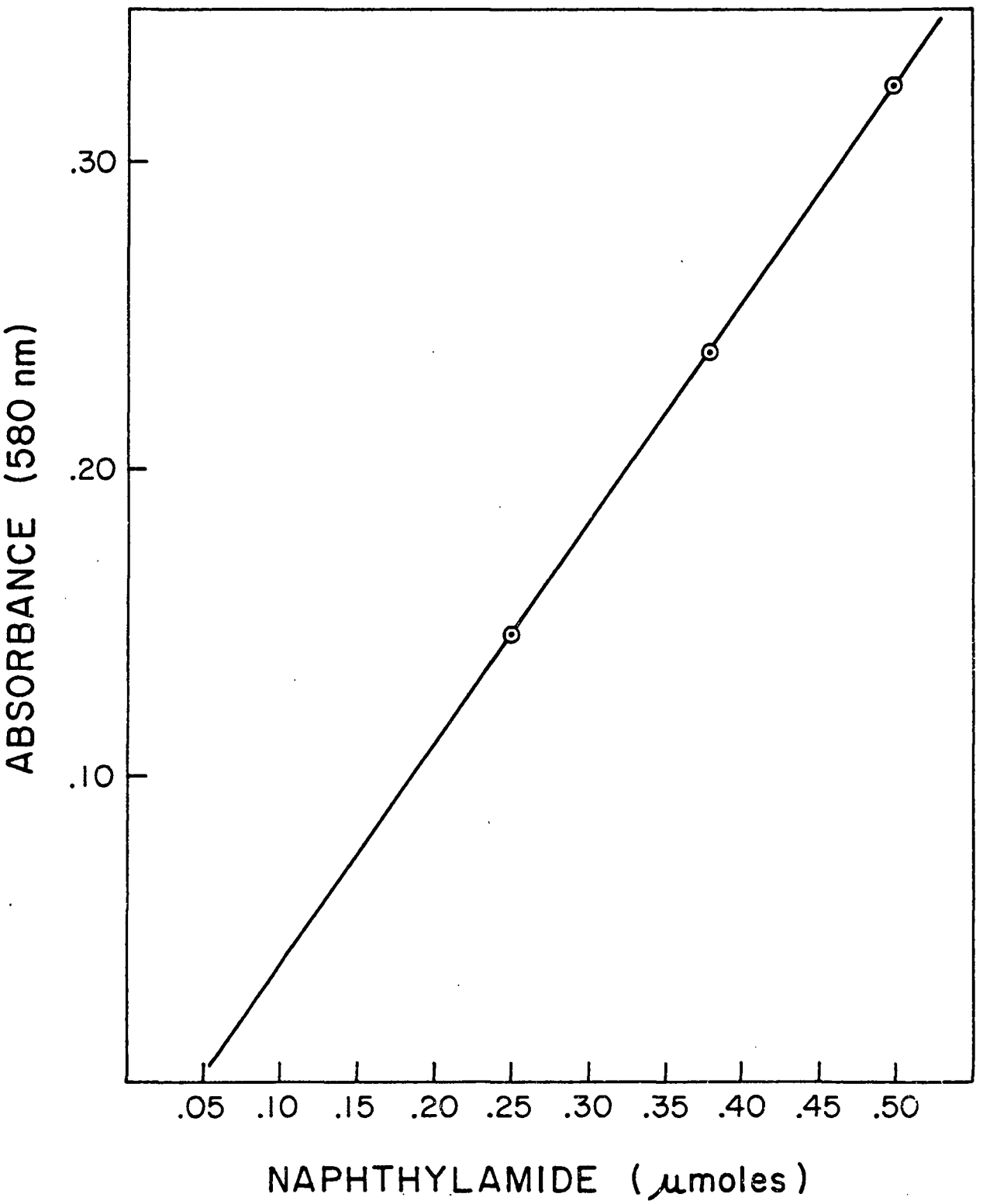

Figure 6. Naphthylamide standard curve.

of the reaction product in the BANA procedure, i.e., 24,000 (Barrett, 1972a) as compared with 8,800, the value for 4-nitroaniline (Erlanger et al., 1961). These factors were most likely responsible for the inability to detect cathepsin B in whiting or cod when the nitroanilide substrate was used.

In the procedure using the naphthylamide substrate, Barrett (1972a) had recommended the incubation period be limited to 10 min because of the instability of the reagents. Increasing the incubation period to 30 min did not cause an increase in the absorbance of the blank signifying that the substrate had not undergone spontaneous hydrolysis. In addition, the activators cysteine and EDTA were used in other assays for incubation periods greatly exceeding 10 min (Vanha-Perttula et al., 1965; Sylven and Snellman, 1962). Therefore, the limitation of 10 min incubation can be eliminated. In fact, by increasing the incubation period to one or two hours, it may be possible to detect cathepsin B activity in cod. The greater quantity of naphthylamide released by the whiting enzymes will also allow the sensitivity to increase in determining the effects of activators and inhibitors. Some of the chemicals which would be of interest to study include iodoacetamide, cysteine, EDTA, Cu^{2+}, TLCK pCMB and diisopropyl-phosphofluoridate. Iodoacetamide, Cu^{2+}, TLCK and pCMB are reported to inhibit cathepsin B activity (Misaka and Tappel, 1971; Barrett, 1972b) while cysteine and EDTA activate the enzyme (Snellman, 1969). Diisopropyl-phosphofluoridate has no effect on cathepsin B. With the more detailed study of the enzyme responsible for hydrolyzing BANA, more evidence might be found that cathepsin B is actually present.

One of the difficulties in using a chemical assay to detect naphthylamide is that of determining the effect other chemicals might have on the formation of the colored complex. Tests were therefore run using naphthylamide standards to measure whether the activators and inhibitors added to the cathepsin assay mixture would interfere with the final absorbance reading. Three different concentrations of naphthylamide were used while the chemicals tested were all present at the same concentration they were used in the cathepsin B and C assays. Results of these tests are given in Table 10.

Only B-mercaptoethylamine and dithiothreitol interfered significantly with the naphthylamide detection. Therefore, it is necessary to be aware of the problem of detecting naphthylamide when these chemicals are used in enzyme activation and inhibition studies.

Cathepsin C

Following the procedure of Goldbarg and Rutenberg (1958), hydrolysis of the substrate Gly-Phe-2-naphthylamide was detected (Table 11). Since these results were determined from only one sample for each species of fish, it is necessary to warrant caution in saying that whiting contains more activity than cod. It is not even safe to assume that it is the same enzyme in the two fish samples responsible for the hydrolysis of the substrate. In the study looking at the effect of pH on hydrolysis, different pH optima were found for whiting and for cod (Figure 7). The optimum pH for whiting was approximately 7 and for cod it was approximately 6. Based on this evidence, the enzyme in whting may be leucine aminopeptidase (Schwabe, 1969) while that in cod might be cathepsin C (Tallon et al., 1952).

Table 10. Inhibition by compounds in naphthylamide detection.

Chemical	% of Control
Iodoacetamide	108.1
EDTA	100.9
PCMB	100.1
$CuSO_4$	97.3
Cysteine	96.4
TLCK	108.1
PMSF	98.7
NaCl	98.0
β-mercaptoethylamine	86.8
Dithiothreitol	58.8

Table 11. Hydrolysis of Gly-Phe-2-naphthylamide by Pacific whiting and true cod muscle extracts.

	Specific Activity[a]	nmoles naphthylamide released/min/gm fish
Whiting	1.07×10^{-4}	1.08
True cod	1.00×10^{-4}	0.75

[a]Specific activity is the number of enzyme units per mg protein. One unit of enzyme activity is that amount causing transformation of 1.0 micromole of naphthylamide per minute at 40°C, pH 6.

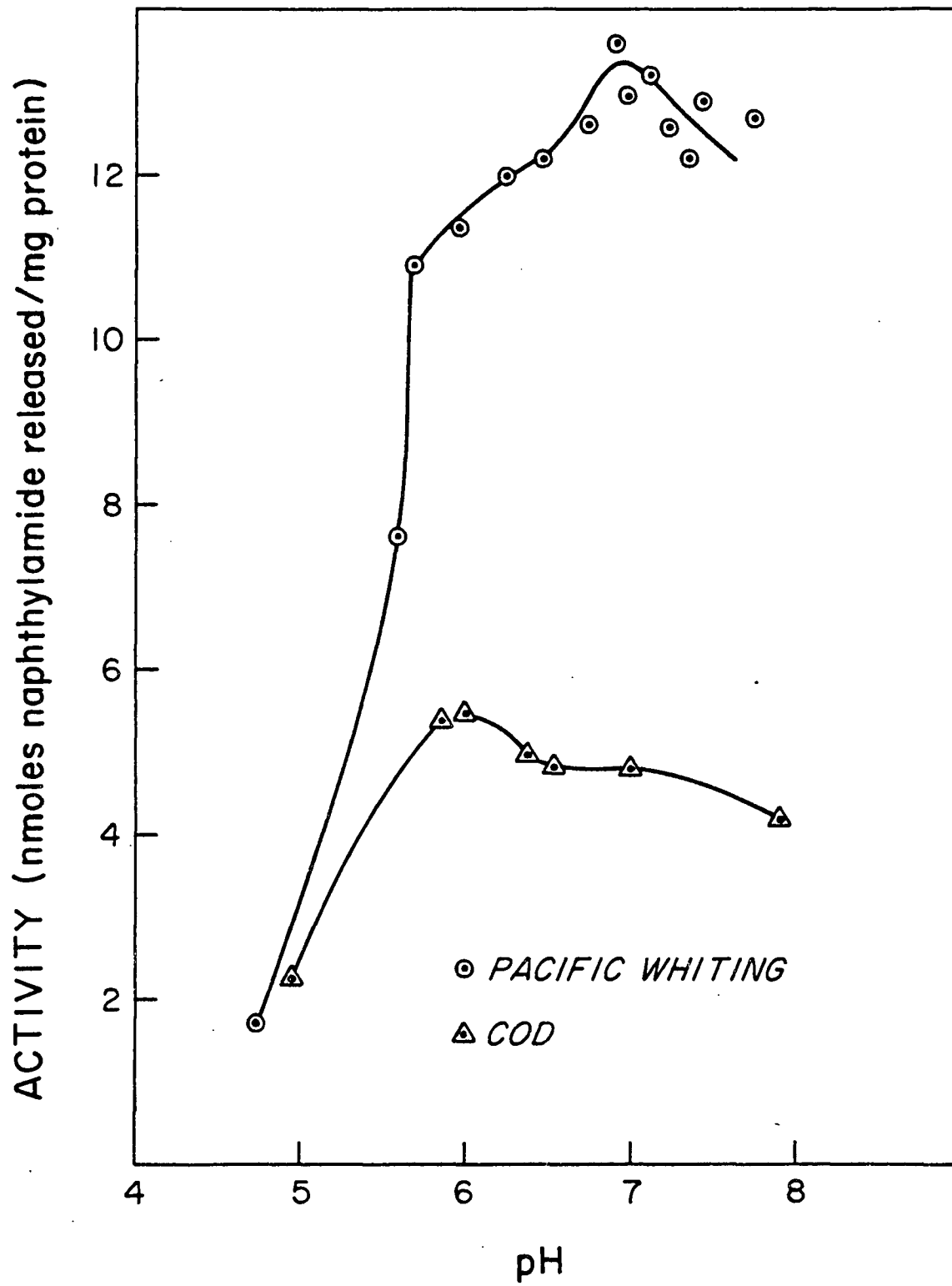

Figure 7. Effect of pH on Gly-Phe-2-naphthylamide hydrolysis by Pacific whiting and true cod muscle extracts.

One of the chemicals included in the original assay was mercaptoethylamine. Mercaptoethylamine was included in the cathepsin C assay to satisfy the thiol and halide requirements of the enzyme (Barrett, 1972a). Data presented in Table 12, however, indicate that mercaptoethylamine inhibits the activity in both the cod and whiting samples. The interference of mercaptoethylamine in the naphthylamide detection does not justify completely the lower amount of naphthylamide measured. It is, therefore, unfortunate that the cod samples contained mercaptoethylamine when testing for the effects of other chemicals. For example, the effect of iodoacetamide is uncertain in cod because the sulfhydryl inactivator would have only removed the effect of mercaptoethylamine.

No conclusive evidence is presented in Table 12 that points to the presence of leucine aminopeptidase or cathepsin C in the whiting and cod samples. For example, cysteine had no effect on the hydrolysis in the whiting samples. This observation neither satisfies the effect for cathepsin C (Vanha-Perttula et al., 1965) nor leucine aminopeptidase (Sylven and Snellman, 1962). As shown in Table 12, no activation occurred with NaCl which is required for leucine aminopeptidase (Sylven and Snellman, 1962).

The defense for the presence of cathepsin C in whiting rests on the effects of $CuSO_4$ and iodoacetamide (Misaka and Tappel, 1971). Both chemicals showed inhibition when mercaptoethylamine was absent. The fact that they showed activation when mercaptoethylamine was present suggests that a similar occurrence may be happening in cod. The removal of mercaptoethylamine from the cod assays may also result in inhibition by $CuSO_4$ and iodoacetamide. Regardless of the agreement with cathepsin C regarding the

Table 12. Comparison of the effects of inhibitors on cathepsin C activity of Pacific whiting and true cod muscle extracts with those reported in the literature.[a]

	Whiting pH 7 Mercaptoethylamine absent in control	Whiting pH 6 Mercaptoethylamine present in control	Whiting pH 6 Mercaptoethylamine absent in control	Cod pH 6 Mercaptoethylamine present in control	Cathepsin C in literature	Leucine Aminopeptidase in literature
NaCl (50mM)	99.6	111.24[b]	-	101.1[b]	Activates (McDonald et. al. 1966)	-
EDTA (1mM)	95.4	107.9	103.5	98.6	Activates slightly (Perttula et. al. 1965)	Inhibits about 30-40% (Sylven & Snellman, 1962)
Cysteine (50mM)	100.4	-	95.9	-	Activates (Perttula et. al., 1965)	Inhibits (Sylven Snellman, 1962)
$CuSO_4$ (1mM)	28.2	117.5	88.0	128.1	Strong inhibition (Misaka & Tappel, 1971)	Inhibits (Schwabe, 1969)
Iodoacetamide (1mM)	85.1	130.2	94.8	102.8	Inhibits (Misaka & Tappel, 1971)	No inhibition
Mercaptoethylamine (50mM)	6.85	23.2[b]	-	50.6[b]	Activates (Greenbaum & Fruton, 1957)	-
PMSF (0.2mM)	82.7	-	4.55	-	No inhibition (Fruton & Mycek, 1956)	-
TLCK (1mM for whiting 0.5mM for cod)	106.7	97.1	-	60.3	-	-
pCMB (1mM)	-	82.5	91.6	92.9	Inhibits (Greenbaum & Fruton, 1957)	No inhibition (Smith & Spackman, 1955)

[a]Average triplicate assays.

[b]Control for these samples contained no mercaptoethylamine.

effects of these two chemicals, it cannot be the basis for saying cathepsin C is present. First, it must be shown that leucine aminopeptidase is not present.

Cathepsin A

Confirmation as to the presence of cathepsin A was complicated by several factors. A high content of ninhydrin-positive material in both whiting and cod samples necessitated the addition of a large volume of isopropanol diluent. While dialysis relieved this problem somewhat, it did not remove it completely. Consequently, the dilution reduced the sensitivity of the assay towards detecting the hydrolysis of the peptide. The solution to this problem seemed to be to increase the incubation period to a point where peptide hydrolysis could be measured. Unfortunately this point could not be reached. As shown in Table 13 for whiting, the controls that contained no substrate resulted in the production of a larger amount of ninhydrin-positive materials than samples containing substrate. The simplest explanation for this phenomenon is that the peptide is inhibiting the enzyme directly or indirectly. To inhibit indirectly, the enzyme would have to have endopeptidase activity as has been suggested by Lichenstein and Fruton (1960). Endogenous proteins present in the whiting sample could then serve as substrate. With longer incubation times, more amino acids could be liberated through the action of the enzyme on the protein instead of the peptide. Consequently, the presence of the peptide limits the attack of the enzyme on the protein by competitive inhibition. The result of this sequestering is the lower production of amino acids.

Table 13. Cathepsin A activity of dialyzed samples of Pacific whiting and true cod muscle extracts.[a]

	Whiting		Cod	
	A_{570}	Difference	A_{570}	Difference
No inhib. or activ.	0.188		0.200	
		-0.070		0.057
No inhib., activ., or substrate	0.258		0.143	
Cysteine (4mM)	0.248		0.310	
		-0.134		0.090
Cysteine and no substrate	0.382		0.220	
Iodoacetamide (1mM)	0.152		0.210	
		-0.056		0.080
Iodoacetamide and no substrate	0.170		0.130	
EDTA (1mM)	0.254		0.279	
		+0.024		0.145
EDTA and no substrate	0.310		0.134	
$ZnCl_2$ (1mM)	0.138		0.188	
		+0.024		0.074
$ZnCl_2$ and no substrate	0.114		0.114	

[a]Average of triplicate assays.

Even if cathepsin A were not to have endopeptidase activity, the endogenous proteins could still serve as substrates for the removal of single amino acids from their carboxyl end (Taylor and Tappel, 1974). If the enzyme has a lower V_{max} on the peptide than it does on the protein, the presence of peptide would inhibit the enzyme from operating maximally on the protein. Thus, a higher production of amino acids could be produced from the protein alone than when the protein and peptide were present together.

The fact that cod did not follow the example of whiting in having higher controls than samples containing substrate may be an indication that cathepsin A in whiting is a unique enzyme. The uniqueness would arise if the whiting enzyme had lower K_m's and higher V_{max}'s for proteins than for peptides while cathepsin A's from other fishes demonstrate the opposite case. It is important to look more closely at the kinetics of the enzyme because increased binding to and hydrolysis of proteins by cathepsin A from whiting would cause a higher muscle protein breakdown. By purifying cathepsin A from the endogenous proteins in the enzyme supernatant, the enzyme from whiting and cod could be characterized.

Review of Specific Proteolytic activities examined

The various substrates examined for hydrolysis by whiting and true cod extracts are summarized in Table 14. While the specific enzyme hydrolyzing a particular substrate could not be identified through inhibitor studies, the major enzyme(s) suspected is listed. However results of inhibitor studies on crude enzyme preparations are of limited value because differences may occur in a chemical's effect on the hydrolysis of proteins and hydrolysis of peptides.

Table 14. Review of the specific proteolytic activities examined

Substrate	Hydrolysis		Suspected Enzyme(s)
	Whiting	True Cod	
Hippuryl-l-phenylalanine	No	No	Carboxypeptidase A
Hippuryl-L-arginine	No	No	Carboxypeptidase B
Elastin	No	No	Elastase
Collagen (neutral pH)	No	-	Neutral Collagenase
Collagen (acidic pH)	Yes	-	Catheptic Collagenase
TAME·HCl	No	No	Trypsin
α-N-Benzoyl-D,L-arginine p-nitroanilide (BAPA) (neutral pH)	No	No	Trypsin
N-Benzoyl-L-tyrosine ethyl ester (BTEE)	Yes	Yes	Chymotrypsin
Glutaryl-L-phenylalanine-p-nitroanilide (GPNA)	Yes	Yes	Chymotrypsin
Benzoyl-D,L-arginine-α-naphthylamide HCl (BNA)	Yes	No	Cathepsin B
BAPA (acidic pH)	No	No	Cathepsin B
Gly-Phe-α-naphthylamide	Yes	Yes	Cathepsin C; leucine aminopeptidase
Carbobenzoxy-glutamyl-tyrosine	?	Yes	Cathepsin A

Factors Affecting the Quantitative and Qualitative Composition of Enzyme in Fish

There are numerous factors affecting enzyme activity that will have to be considered in interpreting data for any of these enzymes examined. Lysova and Chernogortsev (1968) observed seasonal variations in the total proteolytic activity of fish, while Shenderyuk and Dvoretskaya (1967) reported regional differences. However, Wojtowicz and Odense (1972) found that despite the selection of samples of a species in the same season, at the same time of day and the use of identical sampling and testing procedures, significant individual variation occurred in the animals with considerable overlap in the ranges of different species. These variations could be due to the age of the fish sampled (Asano et al., 1979), body size (Fujii et al., 1951) and physiological state of the fish. The various conditions in animals known to affect catheptic activity are starvation (Creach et al., 1969), protein deficiency (Soto et al., 1978), tissue vitamin E levels (Koszalka et al., 1961) and ATP levels (Reddi et al., 1972). Creach et al. (1969) found a dimunition in the catheptic activity of carp tissues during a prolonged period of starvation. For rats maintained on a prolonged protein-poor ration, the stability of the lysosomal membranes was disturbed resulting in a higher solubilization of lysosomal enzymes in vitro (Soto et al., 1978). Vitamin E deficiency is associated with an increase in the autolytic activity of rat skeletal muscle (Koszalka et al., 1961) while ATP inhibits the catheptic activity (Reddi et al., 1972). Therefore, after capture when the ATP level declines, the inhibitory effect upon cathepsin may be removed.

Factors to be controlled during sampling include the method of capture and the handling of the fish after capture. Subjecting fish to stress during capture will diminish the fish's supply of ATP faster than without stress. Consequently, the fish may exhibit greater enzyme activity even in in vitro studies because less ATP is present in the crude enzyme supernatant. In addition, stress could affect the degree to which lysosomes have been ruptured. Stress accelerates the process of rigor mortis and tenderization. Therefore Ca^{++} ions are released from the sarcoplasmic reticulum sooner than if the fish had not been in stress. These Ca^{++} ions could stabilize the lysosomes from further rupture. Whiting et al. (1975) found that the presence of $CaCl_2$ reduced the solubilization of cathepsin from a lysosome suspension.

The factors that need to be controlled during handling include the temperature and the medium in which the fish will be held. During handling, an increase in temperature may bring about a greater release of cathepsins from the lysosomes (Rahman, 1964) while the practice of a brine soaking may serve to control autolytic spoilage by inactivating cathepsins (Reddi et al., 1972).

After obtaining the fish samples, consistent selection of muscle type will eliminate variation. Groninger (1964) found higher activity in the red muscle of albacore than in the white muscle. The method of freezing and thawing will also have to be controlled. Freezing and thawing cause lysosomes to be ruptured (Doi et al., 1974) and in order to measure with some degree of accuracy the level of cathepsins previously released, this rupturing must be minimized. Fast and intermediate rates of freezing result in lower lysosomal enzymatic activities than are observed after slow freezing

(Luhovy and Kravencherko, 1976; Geromel and Montgomery, 1980). In addition lower storage temperatures have been found to decrease the rate of release of acid lipase from the lysosomal fraction (Geromel and Montgomery, 1980).

The final source of variation that may affect the value of the data is the sample number. Assays should be run at least in triplicate with enzyme extracts from two or more different animals. Bodwell and Pearson (1963) found that the activity of different enzyme preparations from tissues derived from the same or different animals varied considerably using denatured hemoglobin as well as natural substrates.

Besides conducting assays in triplicate, the only other factor to be controlled in the present experiments was the process of freezing and thawing. Thus, quantitative as well as qualitative variations in the enzyme activity for different samples could be due to the other factors described that affect enzyme activity. Even, so, the use of a crude extract made it difficult to confirm the identity of the enzymes responsible for hydrolyzing the substrates. Consequently, it is impossible to determine with certainty what variations in enzyme levels may exist between Pacific whiting and the control fish, true cod.

SUMMARY

Analysis of crude muscle supernatants from whiting and true cod showed whiting to have a higher proteolytic activity on hemoglobin at acidic pH's than true cod. At neutral and alkaline pH's, the two species overlapped in their range of proteolytic activities. Based on these findings, if enzymatic activity is responsible for the accelerated muscle breakdown in whiting, the cathepsins operating optimally at the acidic pH's would have to be suspected.

Results from both pH and inhibitor studies indicate that cathepsins may be responsible for the accelerated muscle breakdown in whiting. Specifically cathepsin B was found in whiting and not in cod. Also, larger amounts of cathepsin C were found in whiting than in true cod. Therefore, these results suggest that there had been a greater release of cathepsins from lysosomes in whiting than in true cod. These differences in the level of cathepsins could reflect differences in the release of enzymes from lysosomes before freezing. The release of cathepsins during thawing could be considered to occur to the same extent in both species of fish by having been frozen and thawed in the same manner. Consequently, it could be speculated that the higher catheptic levels in whiting before freezing might result from an immunological response to the myxosporidian spore.

Results from the catheptic collagenase assay do not provide much support for the role of cathepsins in the accelerated muscle breakdown in whiting. While catheptic collagenase was demonstrated to be present in whiting, its presence in true cod was not detected. Therefore it is uncertain if catheptic collagenase follows the pattern of the cathepsins B

and C in being present to a greater extent in whiting than in true cod.

With regard to cathepsin A, a question remains about its occurrence in whiting. The presence of the peptide substrate in whiting resulted in the production of less ninhydrin-positive materials than in its absence. Thus, it was impossible to determine if cathepsin A acted on the substrate using the crude muscle extract. For subsequent studies, cathepsin A should be purified to remove the endogenous proteins which should allow for better detection of the hydrolysis of the peptide.

The chymotrypsin-like enzyme detected in whiting and true cod by the hydrolysis of BTEE and GPNA does not appear to be responsible for muscle softening in whiting. At pH's near the physiological pH, true cod had considerably more activity than whiting. Consequently, if the enzyme had been active in the intact muscle, true cod would have a mushier texture than whiting. Since this was not the case, the chymotrypsin-like enzyme can be eliminated from the list of suspects causing the softening problem in whiting.

In conclusion, this preliminary study has shown that whiting contained higher levels of the catheptic enzymes than true cod. Even though these findings were obtained from a limited number of fish samples, they suggest that the lysosomes in whiting have released their contents prior to freezing to a greater extent than in the case of true cod. Consequently, the probability of whiting muscle being subjected to hydrolysis by cathepsins is increased. Confirmation of the effect of cathepsins on muscle texture requires correlating texture measurements with the level of catheptic activity in the sarcoplasmic fluid. Also, the cause of the earlier release of enzymes from lysosomes in Pacific whiting remains to be determined.

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