

AN ABSTRACT OF THE THESIS OF

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(THUNNUS ALALUNGA)

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The proteolytic enzymes in the visceral organs of albacore tuna fish (Thunnus alalunga) were studied, initially as an extract of the whole viscera, then as extracts of the individual organs. Preliminary studies indicated three pH optima of activity in the whole viscera extracts pH 1.7, 3.1-3.5 and 9.5. Analysis of extracts of the individual organs revealed the alkaline proteolytic activity was present in the intestine and the pyloric caeca, with the pyloric caeca showing the highest activity. The stomach extract demonstrated high protease activity at pH 1.7 and 3.5, while the remaining organs had relative activities at least ten times lower at these pH values.

The extract obtained from pyloric caeca was studied in more detail. The approximate levels of trypsin-like and chymotrypsin-like activities were measured, and the effects of three inhibitors, phenylmethylsulfonyl-fluoride (PMSF), 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK) and L-1-tosylamide-2-phenylethylchloromethylketone (TPCK), determined. Cationic discontinuous polyacrylamide gel electrophoresis coupled with a substrate inclusion technique for localization of the separated proteases of the pyloric caeca extract revealed the presence of ten proteolytic

enzymes. One was active in acid solution (ca. pH 4) and was inhibited by PMSF and TLCK. The remaining nine proteases were active at pH 8.0. Five of the alkaline proteases were serine proteases and one of these showed trypsin-like specificity including PMSF and TLCK inhibition and activity against BANA. None of the electrophoretically separated proteases showed specificities similar to chymotrypsin.

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Tuna Fish (Thunnus alalunga)

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SOME VISCERAL PROTEASES OF ALBACORE TUNA FISH (THUNNUS ALALUNGA)

INTRODUCTION

Seafood processing results in production of substantial quantities of wastes or by-products, including the viscera which contain the endogenous proteolytic enzymes. Processing techniques involving autolysis of the by-product proteins by endogenous proteolytic enzymes have been studied and practiced commercially for many years. In addition, processes for the production of more functional fish protein concentrate by digestion with visceral proteases have been reported. In each case, however, research has involved analysis of the effects of a limited number of process variables (primarily mechanical treatment and incubation time, temperature and pH) and reported results have varied. Very limited research on the enzymes responsible for the proteolytic activity in these processes has been reported.

Recently, increased interest has been shown in the more complete utilization of protein from oilseed meals to help overcome the world protein shortage. The digestibility of these proteins is lower than animal sources due to complexing of the protein with the fibrous carbohydrate material present in plants. In addition, certain amino acids, particularly lysine and the sulfur containing amino acids, may be limiting in these proteins.

Work at the Oregon State University Seafoods Laboratory in Astoria, Oregon, has centered on use of a visceral homogenate to hydrolyze

oilseed meals for use in fish feeds. It is believed the action of the visceral proteases on the oilseed protein will increase solubility and improve its digestibility by fish. Addition of the visceral proteins which are generally high in lysine and methionine, will also provide supplementation of limiting amino acids. This combination will hopefully result in an overall increase in protein bioavailability and value of the resulting feeds.

Optimum utilization of the endogenous visceral proteases in solubilization of either fish proteins or oilseed proteins requires knowledge of the types and activities of the proteases in the visceral homogenates under various conditions, including their reaction specificities and properties. A survey of the proteolytic activity at pH 7.6 in the viscera of the commercially important fish species processed in Astoria, Oregon, indicated tuna fish, and albacore in particular, contained the highest level of activity. For these reasons a study of the visceral proteases of albacore tuna fish was initiated to: 1) provide an indication of the complex nature of proteolysis by visceral extracts of the tuna fish, and 2) provide a method which will aid in a systematic study of the individual proteases.

REVIEW OF LITERATURE

Classification of Digestive Proteases

Analysis of the digestive proteases in a wide variety of animals, both vertebrates and invertebrates, has led to various classifications of proteolytic enzymes. The simplest classification has been in respect to pH of optimum activity; the acidic and alkaline proteases.

Pepsin is the most studied and most important acidic protease in the digestive system of many animals. The enzyme has a rather broad specificity (Tang, 1963) and is generally described as the proteolytic activity at low pH (ca. 2) with hemoglobin as substrate (Anson, 1938).

Digestive proteases active in alkaline solution are a more diverse group of enzymes and classification has been extended to the basis of catalytic functionality. Serine proteases including trypsin (EC 3.4.4.4), the chymotrypsins (α , δ and π - chymotrypsins; EC 3.4.4.5) and elastase (pancreatopeptidase E, EC 3.4.4.7) in animals are easily identified by their sensitivity to diisopropylphosphofluoridate (DFP) and phenylmethylsulfonylfluoride (PMSF) (Fahrney and Gold, 1963). The enzymes are also classified as endopeptidases since they hydrolyze peptide bonds in the interior of the protein chain. Cleavage of terminal peptide bonds is inhibited by the charge on the amino or carboxyl groups of terminal residues (Walsh and Wilcox, 1970).

Exopeptidases, proteases which cleave terminal amino acids from protein or peptide chains include the carboxypeptidases and aminopeptidases. Carboxypeptidase A (peptidyl-L-aminoacid hydrolase, EC 3.4.2.1) and carboxypeptidase B (peptidyl-L-lysine hydrolase, EC 3.4.2.2)

are the primary enzymes responsible for hydrolyzing amino acids from the carboxy terminal end of the chain. These enzymes are also classified as metal containing enzymes. Each contains 1 gram-atom of Zn^{++} per mole of enzyme and is generally inhibited by chelating agents (Vallee and Neurath, 1954 and Folk et al., 1960).

Distribution of Proteases

Serine proteases present in the digestive system of animals of all types have been studied extensively. In a survey of 23 species of vertebrates, Zendzian and Barnard (1967a) reported trypsin-like and chymotrypsin-like activity in every case. The presence of elastase appears to be equally widespread in vertebrates (Shotton, 1970).

In vertebrates which have a distinct pancreas, serine proteases are present in this gland or the exocrine juice as the inactive zymogens. Activation of the enzymes, once they enter the intestine, is initiated by the enzyme enterokinase and continued by the action of active trypsin. In these organisms trypsin also activates the precursors of the carboxypeptidases, A and B (Neurath, 1964).

Teleost fishes, including the tuna fish, do not have a distinct pancreas, however. In these fish the serine proteases and carboxypeptidases have been located in the pyloric caeca (Chesley, 1934; Kitamikado and Tachino, 1960; Zendzian and Barnard, 1967a; Ooshiro, 1968 and 1971; Uchida, 1970; Overnell, 1973 and Alliot et al., 1974).

Serine proteases from the pyloric caeca were obtained only as the active enzymes by Zendzian and Barnard (1967a). More recently Overnell (1973) separated the pyloric caeca from the associated

mesentery and assayed for trypsin-like and chymotrypsin-like activities in extracts from each portion, as well as an extract from both components (total caeca). In extracts of total caeca, trypsin-like and chymotrypsin-like enzymes were present in fully active form while extracts of the associated mesentery revealed the presence of 70% to 90% of the trypsin-like and 30% to 40% of the chymotrypsin-like activities in the inactive zymogen form. Trypsin-like enzymes present in the lumen contents of the caeca were thought to cause the total activation previously noted in total caeca extracts. Extracts of mackerel pyloric caeca (apparently the total caeca) displayed activation of caseinolytic activity, both autocatalytically and by the addition of bovine trypsin (Kaľáč, 1975).

Characterization of Protease Activity

Total proteolytic activity has been analyzed by measuring the hydrolysis of a protein substrate. Anson (1938) published revised methods for the estimation of pepsin, trypsin and cathepsin using hemoglobin as substrate. The enzyme preparations were assumed to be pure and differentiation of activities was based on pH. Alkaline proteolytic activity is now more commonly analyzed by the casein digestion method of Kunitz (1947).

Extent of proteolysis was quantitated by analysis of the supernatants following precipitation of the reaction solution with trichloroacetic acid (TCA). Amino acids, dipeptides and some tripeptides are soluble in TCA and may be estimated by measurement of the absorbance at 280 nm or by use of the folin phenol reagent (Lowry et al., 1951).

Both methods of quantitation are specific for the aromatic amino acids, in particular tyrosine. These methods are therefore most sensitive to the combined action of chymotrypsin and carboxypeptidase A.

Each of the digestive proteases has a somewhat more or less specific activity associated with it. In the case of the serine proteases, the mechanism for splitting the peptide bond is the same in each case and involves three amino acid residues (serine, histidine and aspartic acid) at the active site. The conformation of the enzyme imparts specificity in terms of which bond in the chain is hydrolyzed. Trypsin-like enzymes split the bonds involving the carboxyl group of the basic amino acids lysine and arginine. Chymotrypsin-like enzymes catalyze the identical reaction on the carboxyl side of bulky hydrophobic residues. Elastase is somewhat specific for bonds involving glycine, alanine and serine residues (Stroud, 1974).

The reaction mechanism for the carboxypeptidases differs from the serine proteases and involves substrate binding to the Zn^{++} ion in the enzyme molecule (Vallee, 1964). Carboxypeptidase A can hydrolyze any amino acid except lysine, arginine or proline from the carboxyl terminus of polypeptides. Carboxypeptidase B catalyzes a similar reaction with C-terminal basic amino acids (Whitaker, 1972).

Analysis of individual digestive proteases is dependent on their specificity which allows characterization and quantitation of one in the presence of the others. Substrates specific for estimation of trypsin and trypsin-like activity include benzoyl arginine ethyl ester (BAEE) (Schwert and Takenaka, 1955), toluene sulfonyl arginine methyl ester (TAME) (Hummel, 1959), benzoyl-DL-arginine- β -naphthylamide (BANA)

(Blackwood and Mandl, 1961) and p-nitrophenyl, p'-guanidinobenzoate (NPGb) (Chase and Shaw, 1967). The first three substrates involve spectrophotometric analyses based on the differential spectra of hydrolyzed and unhydrolyzed substrate. The last, NPGb, is an active site titrant.

Specific active site inhibitors of trypsin or trypsin-like enzymes are used to characterize the specificity and reaction mechanism, confirm enzyme identity or quantitate specific activity. Classically serine proteases have been defined as those which are irreversibly inhibited by DFP. This reagent phosphorylates the serine residue at the active site destroying the catalytic activity. More recently the less toxic reagent, PMSF, has been used to irreversibly bind to the active site serine residue. Similar inhibition rate constants for DFP and PMSF were reported for inhibition of trypsin and chymotrypsin (Fahrney and Gold, 1963).

Inhibition of trypsin-like activity in the presence of other serine proteases is accomplished with 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK). This inhibitor is a chloromethylketone derivative of tosyllysine and binds covalently with the histidine at the active site of trypsin-like enzymes. Inhibition of proteolytic activity by TLCK indicates: 1) the enzyme has a binding site complementary to the lysine residue, and 2) a histidine residue in the enzyme is essential to the hydrolysis mechanism. Competitive inhibitors or denaturing agents inhibit or prevent alkylation of the active site histidine by TLCK. Alkylation of histidine residues not at the active site does not occur (Shaw et al., 1965).

Substrates for the analysis of chymotrypsin-like activity include N-acetyl-L-tyrosine ethyl ester (ATEE) (Schwert and Takenaka, 1955), benzoyl tyrosine ethyl ester (BTEE) (Hummel, 1959) and glutaryl-L-phenylalanine- β -naphthylamide (GPNA) (Blackwood et al., 1965). Specific inhibition of chymotrypsin-like activity is accomplished with L-1-tosylamide-2-phenylethylchloromethylketone (TPCK). Decrease in proteolytic activity and loss of activity against the above substrates following treatment with TPCK again indicates an active site histidine residue and a substrate binding site complementary to the hydrophobic phenylalanine residue (Schoellman and Shaw, 1963).

Proteases in Tuna Fish

Analysis of a partially purified pyloric caeca extract of tuna (Thunnus secundodorsalis) by Zendzian and Barnard (1967a, b) revealed relatively low levels of trypsin-like, chymotrypsin-like and total protease activity at pH 7.6. Trypsin-like activity was measured as the esterolytic activity on BAEE and was inhibited completely by both DFP and TLCK within 2 hrs incubation at pH 7.2 and 25⁰C. Chymotrypsin-like activity was measured with BTEE and was effectively inhibited by DFP and TPCK suggesting substantial similarities in the tuna and bovine enzymes. TPCK-treated pyloric caeca extract showed a 25% reduction in casein digestion while treatment with TLCK resulted in a 14% reduction. Treatment of the extract with either DFP or a combination of TPCK and TLCK decreased the activity against casein 35%. At least some of the activity remaining against casein was attributed to the presence of carboxypeptidase A in the extract.

A similar extract of tuna (Thunnus secundodorsalis) pyloric caeca was treated with [carbonyl- ^{14}C] TPCK (Barnard and Hope, 1969). One molecule of histidine was alkylated at N-3 of the imidazole ring per molecule of chymotrypsin-like enzyme. The same basic active site structure in tuna and bovine chymotrypsin was therefore assumed.

Reaction rates on a number of substrates and inhibitors for the tuna and bovine chymotrypsin activity were determined to establish molecular and kinetic properties of these enzymes (Möckel and Barnard, 1969). Alkylation of the tuna enzyme with TPCK was rapid and similar to the same reaction with bovine chymotrypsin. Inhibition with N-tosyl-L-leucine chloromethylketone (TLeuCK) was much slower for the tuna BTEE hydrolysis as compared to that measured for the bovine, and only 80% complete in the case of the tuna.

A protease from the pyloric caeca of tunny (Thunnus orientalis) was isolated and crystallized by Katsumata et al. (1974). The activity against casein substrate was optimum at pH 7.6 to 7.8. Complete stability for 24 hrs at 30°C was noted between pH 5 and 7, but became less stable outside this range. The enzyme was stable for 10 min at 40°C , but only 10% of the original activity remained after incubation at 60°C for 10 min. Complete inactivation occurred at 70°C . The enzyme was activated by Co^{++} and Ca^{++} and significantly inhibited by Fe^{++} , Zn^{++} , Hg^{++} , EDTA, PCMB and iodoacetic acid.

Polyacrylamide Gel Electrophoresis and Protease Detection

Polyacrylamide gel electrophoresis is an excellent and well documented method for the separation of macromolecules, including

proteins. A number of variations in the method exist, but in each case separation is based on a combination of the size and charge of the macromolecule. For a thorough explanation of the theoretical basis and considerations of polyacrylamide gel electrophoresis refer to Ornstein (1964), Chrambach and Rodbard (1971) and Maurer (1971). Procedural considerations are described in detail by Davis (1964).

Enzyme Detection

Three basic techniques have been developed for analysis of electrophoretically separated enzymes (Gabriel, 1971 and Maurer, 1971). The elution method involves slicing the gel and crushing each section in a small volume of buffer. Extended incubation allows migration of the enzyme into the buffer. Time of incubation depends on the extent of gel crosslinking and the molecular weight of the enzyme. Any applicable assay may be run on the eluate.

The substrate inclusion technique is used for enzymes which have large molecular weight substrates (including proteases). The substrate is incorporated into the gel and the sample separated under conditions in which the enzyme is stable but inactive. Following electrophoresis these conditions (usually pH and temperature) are altered so that the enzyme becomes active. Detection of activity depends on the reaction, substrates and products involved. A variation of this technique is the contact print method. The substrate and any necessary cofactors are polymerized into a gel. The sample is separated on a standard gel and following electrophoresis this gel is placed in contact with the substrate-containing gel and incubated to allow the reaction to

proceed.

In cases where small molecular weight substrates are used the gel may be incubated, following electrophoresis, in the appropriate substrate solution. The reaction products are either colored or may be coupled to a color producing reaction to allow localization of the enzyme in situ. This technique is termed the gel staining method and is often a modification of a histochemical method.

Localization of Proteases

Since proteases are active against very large molecular weight substrates, non-specific assays for electrophoretically separated proteases involve use of the substrate inclusion or contact print techniques. Merkel (1966) used a contact print method in conjunction with cellulose acetate electrophoresis to determine the number of proteases in bacterial culture filtrates. Agar plates were poured which contained algal chromoproteins. The cellulose acetate strip, following electrophoresis, was placed "face down" on the plate. Protease activity was associated with decolorized zones. Kaminski and Bushuk (1968) separated a number of protease preparations in starch gels containing 0.4% hemoglobin. Following electrophoresis the pH of the starch block was adjusted so that the particular enzyme was active and incubation was continued until the hemoglobin was digested. Since electrophoresis was conducted at pH 3.4 the method worked well with all proteases studied except pepsin. This enzyme digested the substrate as it migrated through the starch block.

Andary and Dabich (1974) developed a method with three variations

which was used to detect submicrogram quantities of protease in polyacrylamide gels. In the case of trypsin and chymotrypsin, as little as 50 pg and 500 pg, respectively, could be detected. The primary method involved incorporation of 0.1% (w/v) casein substrate in the acidic gels used for electrophoretic separation of the extract. This method proved to be most sensitive and provided a uniform dispersion of substrate which neither migrated during electrophoresis nor affected migration of the proteases in the gel.

In the second variation, hemoglobin (0.1% w/v) was similarly incorporated into gels. This substrate resulted in lower sensitivity which could be increased somewhat by binding nigrosin to the undigested hemoglobin. Sensitivity was still greatest in the casein substrate inclusion gels.

Casein was allowed to migrate into gels following electrophoresis in the third variation of the method. This reportedly resulted in less even distribution of the substrate, but would be the method of choice to use in conjunction with anionic electrophoresis since incorporated substrate would be digested during electrophoretic separations.

Using the substrate inclusion method, Andary and Dabich (1974) detected four protease bands from partly purified seminal plasma. Only three fractions could be separated by column chromatography. They also reported that using the hemoglobin inclusion method they were able to detect two distinct protease fractions in a crude mouse blastocyst homogenate.

Protease Identification

Identification of proteases, which have been located by the formation of clear zones in substrate containing gels, requires determination of specificity. Use is made of a number of specific substrates and inhibitors, including those mentioned earlier, to provide an indication of the type of enzyme.

Zwilling et al. (1969) reported on a method to determine trypsin-like activity directly in polyacrylamide gels using the specific substrate BANA. BANA hydrolysis was associated with BAEE hydrolysis of the protease eluted from a duplicate gel. Using this method they were able to demonstrate two electrophoretically distinct trypsin-like enzymes in a preparation shown to be pure by means of ion-exchange chromatography and gel filtration.

Hagenmaier (1975) discussed the methodology and sensitivity of in situ amino acid naphthylamide assays in polyacrylamide gels. Bovine trypsin was detectable at 0.3 μg per gel by the BANA assay, while ca. 3 μg of bovine chymotrypsin was required for a positive reaction with the specific substrate GPNA. Using the substrate N-acetyl-phenylalanine- β -naphthyl ester (APNE), bovine chymotrypsin was detected at a level of 0.1 μg per gel. However, APNE was reported to give a positive reaction with trypsin and esterases (Ravin et al., 1954).

Confirmation of a specific protease activity determined in polyacrylamide gels was accomplished using specific inhibitors; analogous to similar reactions in solution (Andary and Dabich, 1974). Hydrolysis of BANA was inhibited by incubating the gel in a solution of TLCK prior

to incubation in substrate, but not by incubation in TPCK. Similarly, TPCK treatment of the gels prevented hydrolysis of GPNA. GPNA hydrolysis was resistant to TLCK treatment. Hydrolysis of casein in substrate inclusion gels by trypsin and chymotrypsin was also inhibited by incubation of the gels in TLCK and TPCK, respectively.

A partially purified pyloric caeca extract of mackerel (Scomber scombrus) was analyzed by polyacrylamide gel electrophoresis (Kaľáč, 1975). Incubation of gels in BANA solution resulted in formation of three bands with apparent trypsin activity and two additional bands were produced on reaction with GPNA. A duplicate gel was sectioned and the protein eluted. Confirmation of trypsin-like and chymotrypsin-like activities determined in situ was provided by TAME and BTEE hydrolysis, respectively, in eluates of corresponding gel sections. Inhibition of activity in the gel or corresponding eluates was not determined with specific inhibitors, however.

To date limited work has been reported on the complement of proteases present in the viscera of fish and particularly tuna fish. This study was initiated to: 1) provide an indication of the complex nature of proteolysis by visceral extracts of the tuna fish, and 2) provide a method which will aid in a systematic study of the individual proteases.

MATERIALS AND METHODS

Preparation of Extracts

Samples of albacore tuna fish (Thunnus alalunga) viscera were obtained soon after evisceration from Bumble Bee Seafoods Company, Astoria, Oregon. The stomach, intestine, pyloric caeca and associated mesentery, spleen and liver were placed in separate cryovac bags, sealed and frozen and held at -30°C until extraction.

Extraction of all organs was performed at 5°C according to the method of Zendzian and Barnard (1967a). After partially thawing at 5°C for approximately two hrs, the tissue was weighed, sliced and passed through a hand meat chopper. The ground tissue was placed in a Waring blender jar with two parts (w/v) precooled 0.02 M sodium acetate buffer, pH 5.0. The mixture was blended for fifteen seconds in three five second intervals and transferred to a beaker with another volume of cold 0.02 M sodium acetate buffer, pH 5.0. pH of the resulting suspension was adjusted to 5.0 with HCl and was allowed to stand for one hr at 5°C .

The suspension was transferred to 250 ml capacity centrifuge bottles and centrifuged for 20 min at $10,000 \times G$ (5°C). The supernatant, subsequently referred to as the protease extract, was decanted carefully through glass wool. The volume was recorded and the extract frozen and stored at -30°C . Prior to the following analyses an aliquot of the extract was allowed to completely thaw in a tap water bath (ca. 30 min) before centrifugation for 20 min at $20,000 \times G$ (5°C) to remove any insoluble material.

Total Proteolytic Activity

Protease activity at pH 7.6 was determined by the casein digestion method of Kunitz (1947). Substrate solution was prepared by suspending 1.00 g of casein (Hammarsten quality, National Biochemicals Corp.) in approximately 75 ml of 0.10 M TRIS-HCl buffer, pH 7.6 at 37°C (pH 7.88 at 25°C). The suspension was held in a boiling water bath for 15 min, with occasional stirring, to dissolve the casein. After cooling to 25°C the pH was readjusted to 7.88. Thimerosal (2.5 mg) was added as a preservative and the volume made to 100 ml with the same buffer. This substrate was stored at 5°C and the unused portion discarded after one week.

Caseinolytic assays were carried out at 37°C in a thermostatically controlled water bath. Two ml of 1% casein, 1.0 ml of 0.10 M TRIS-HCl buffer (pH 7.6 at 37°C) and 0.80 or 0.90 ml of water was pipetted into 15 x 150 mm test tubes and incubated at 37°C for five min. At time zero, 0.20 or 0.10 ml of appropriately diluted protease extract was pipetted into the substrate solution and thoroughly mixed. Following a reaction time of exactly ten min, 6.0 ml of 5% trichloroacetic acid (TCA) were pipetted rapidly into the solution and mixed to stop the reaction and to precipitate the undigested protein. Thirty min after the addition of TCA, the solution was filtered through Whatman #1 paper and the absorbance at 280 nm was measured in a Beckman Model DB spectrophotometer against a water blank.

Reaction blanks consisted of the substrate solution incubated ten min at 37°C. The appropriate volume of protease extract was pipetted

into the solution followed immediately by 6.0 ml of 5% TCA. The absorbance of the supernatant was measured at 280 nm against a water blank.

Each sample was assayed in duplicate or triplicate and the corresponding blank was in duplicate. Total proteolytic activity was expressed as the average absorbance difference between samples and blanks per minute per gram of wet tissue ($\Delta A/\text{min/g}$ wet tissue). Corrections were made for the dilution of the extract.

A modification of the casein digestion assay was utilized to determine the temperature-activity characteristics of the pyloric caeca proteases. For each incubation temperature, 35.0 ml of 1% casein in 0.10 M TRIS-HCl buffer, 17.5 ml of 0.10 M TRIS-HCl buffer and 17.5 ml of water were placed in a beaker and the pH adjusted to 7.6 at the temperature of incubation. Fifteen ml of the substrate were pipetted into each of four test tubes and placed in a constant temperature water bath. At time zero, 0.50 ml or 1.00 ml of appropriately diluted protease extract was added to tubes 1 and 2 and each mixed thoroughly. At ten min intervals, for 60 min, 2.0 ml were pipetted from each of the four tubes into separate test tubes. Three ml of 5% TCA were immediately added to the 2.0 ml aliquots from tubes 1 and 2. To the 2.0 ml aliquots from tubes 3 and 4, the appropriate quantity of protease extract was added followed immediately by 3.0 ml of 5% TCA (blanks). Precipitation of the protein by TCA was allowed to continue for 30 min, and after filtration absorbance at 280 nm was measured against a water blank.

Due to the insolubility of casein at low pH, the pH profile of the

visceral protease extract was determined using hemoglobin as substrate. A 2.5% solution of denatured hemoglobin (hemoglobin standardized for protease assay, National Biochemicals Corp.) was prepared by dissolving 6.25 g of hemoglobin in ca. 200 ml of water. This solution was dialyzed two days against four changes of distilled water and diluted to a total volume of 250 ml (Whiting, 1974). Thimerosal (6.2 mg per 250 ml) was added as a preservative.

Prior to analysis, 10.0 ml of 2.5% hemoglobin solution and 4.0 ml of water were pipetted into a beaker. NaOH or HCl (1.0 N) plus water were added to attain a total volume of 15.0 ml and the approximate pH desired. The substrate was incubated at 37°C for at least five min.

One ml of the appropriately diluted protease extract was pipetted into each of five test tubes. After allowing five min for temperature equilibration, 3.0 ml of the substrate solution were pipetted into tubes 1-3 and thoroughly mixed. During the 20 min incubation the pH of each sample was measured and recorded. Six ml of 5% TCA were added to halt the reaction and to precipitate the undigested protein. Following incubation of the extract in tubes 4 and 5 (reaction blanks) for 20 min, 3.0 ml of the hemoglobin substrate were added, followed immediately by 6.0 ml of 5% TCA. The protease activity was calculated and reported as $\Delta A/\text{min/g wet tissue}$.

For assays conducted at pH 1.7, 2.0 ml of 2.5% hemoglobin and 0.5 ml 0.30 N HCl were mixed and incubated at 37°C. Protease extract (0.20 ml) was added to each of five test tubes and at time zero 1.0 ml of the above substrate mixture was added to tubes 1-3. The reaction was stopped with 5.0 ml of 5% TCA after exactly ten min. Reaction blanks,

tubes 4 and 5, were prepared as before and proteolytic activity reported as $\Delta A/\text{min/g}$ wet tissue.

At pH 3.5 the reaction solution contained 4.0 ml of 2.5% hemoglobin, 1.0 ml of 1.35 M acetic acid - 0.02 M $(\text{NH}_4)_2\text{SO}_4$ adjusted to pH 3.5 with 1 N NaOH, 1.0 ml of water and 1.0 ml of appropriately diluted protease extract. Incubation time was exactly ten min and 9.0 ml of 5% TCA was used to stop the reaction and precipitate the undigested protein.

Determination of Specific Protease Activities

Determination of trypsin-like activity was performed according to the method of Hummel (1959) using TAME (Sigma Chemical Co.) as substrate. TAME (9.85 mg per 25 ml) was prepared fresh each day in 0.04 M TRIS-HCl buffer (pH 8.1) containing 0.01 M CaCl_2 . Rate of hydrolysis of this substrate was measured spectrophotometrically in a thermostatted cell compartment. Three ml of TAME solution were pipetted into the reference and sample cells and both were equilibrated at 30.0°C in the compartment of the spectrophotometer.

To the blank, 0.10 ml of water was added and thoroughly mixed. One tenth (0.10) ml of diluted protease extract was added to the sample cell, mixed, and the increase in absorbance at 247 nm was recorded for at least five min. The activity was reported as μmoles TAME hydrolyzed per minute per gram wet tissue using the following formula:

$$\text{TAME units/g} = \frac{(\Delta A/\text{min}) (3.1 \text{ ml}) (\text{dilution}) (\text{vol. extract prepared})}{(0.1174 \text{ A}/\mu\text{mole TAME}) (0.10 \text{ ml}) (\text{wt. wet tissue})}$$

Chymotrypsin-like activity was determined by the method of Hummel (1959) using BTEE (Sigma Chemical Co.) as substrate. Substrate solution

was prepared fresh each day by dissolving 7.8 mg BTEE in 15 ml of methanol (spectral grade) and diluting to a total volume of 25 ml with water. This solution was mixed with an equal volume of 0.10 M TRIS-HCl buffer (pH 7.8) containing 0.10 M CaCl_2 . The assay procedure for TAME hydrolysis was followed except that the hydrolysis of BTEE was measured at 256 nm. The chymotrypsin-like activity was reported as moles BTEE hydrolyzed per minute per gram of wet tissue using the following formula:

$$\text{BTEE units/g} = \frac{(\Delta A/\text{min}) (3.1 \text{ ml}) (\text{dilution}) (\text{vol. extract prepared})}{(0.02714 \text{ A}/\mu\text{mole BTEE}) (0.10 \text{ ml}) (\text{wt. wet tissue})}$$

Carboxypeptidase A-like activity was determined as described by Whitaker et al. (1966). The substrate, 1×10^{-3} M N-carbobenzoxy-glycyl-L-phenylalanine (CGP) (Sigma Chemical Co.) was dissolved in equal volumes of 1 N NaOH and 0.05 M TRIS-HCl buffer (pH 7.50) containing 0.45 M KCl. The pH was readjusted to 7.50 with 1 N HCl and 3.0 ml were pipetted into the sample cell. Three ml of the buffer were pipetted into the reference cell and both were equilibrated at 25.0°C in the spectrophotometer compartment. A wavelength was selected so that the absorbance difference between substrate and buffer was 1.8 (222-223 nm). Diluted extract, 0.10 ml, was added to both cells, mixed thoroughly, and the absorbance decrease recorded as a function of time.

Carboxypeptidase B-like activity was determined by the method of Folk et al. (1960). Hippuryl-L-arginine (HA) (Sigma Chemical Co.), 1×10^{-3} M, dissolved in 0.025 M TRIS-HCl (pH 7.65) containing 0.10 M NaCl, was used as substrate. Three ml of substrate were pipetted into both the reference and sample cells and the temperature was equilibrated at 25.0°C . Increase in absorbance at 254 nm following addition of 0.05 ml of the protease extract was recorded to obtain a plot of absorbance as

a function of time. The carboxypeptidase B-like activity was reported as μ moles HA hydrolyzed per minute per gram wet tissue.

$$\text{HA units/g} = \frac{(\Delta A/\text{min}) (3.05 \text{ ml}) (\text{dilution}) (\text{vol. extract prepared})}{(0.12 \text{ A}/\mu\text{mole HA}) (0.05 \text{ ml}) (\text{wt. wet tissue})}$$

Protein concentration in the extracts was estimated by the method of Lowry et al. (1951). Bovine serum albumin was used as standard.

Discontinuous Polyacrylamide Gel Electrophoresis and Protease Assays

Discontinuous Polyacrylamide Gel Electrophoresis

The apparatus used for electrophoresis was constructed as described by Davis (1964). The constant voltage power source used was the LKB Power Supply, Type 3371C.

Round plastic containers, 12.5 cm in diameter, were used as electrode chambers. Nine holes, 9.5 mm in diameter, were drilled in the bottom of the upper chamber, one in the center and eight equally spaced about the center. A single hole, 2.0 cm in diameter, was drilled in the center of the lower chamber.

The graphite cores from size "D" batteries were used as electrodes. A one cm section of rubber tubing was fitted around the end of one electrode and inserted into the center hole of the upper chamber. A six mm hole was bored 3/4 of the way through a No. four rubber stopper and the other electrode was inserted. The rubber stopper was then fitted tightly into the hole in the lower chamber. A frame was constructed to hold the electrode chambers level and in place, one above the other. The bottom of the upper chamber was 2.0 cm above the top of the lower chamber, thereby allowing the gel tubes to extend into both chambers.

The cationic system of Reisfeld et al. (1962) as modified by Williams and Reisfeld (1964) was used in this study. The following stock solutions were prepared and used in the preparation of the gels. With the exception of the electrode buffer, each was stored at 4°C in tightly sealed brown glass bottles.

1. Electrode buffer, pH 4.5; 31.2 g of β -alanine (U.S. Biochemicals Corp.) was dissolved in distilled water (ca. 1 liter) and the pH was adjusted to 4.5 with acetic acid. This required 8.0 ml of glacial acetic acid and the total was diluted to a volume of 10.0 liters.
2. Small-pore buffer, pH 4.3; 48.0 ml of 1 N KOH, 4.0 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) (E. C. Apparatus Corp.) and ca. 25 ml of water were combined and the pH was readjusted to 4.3 with glacial acetic acid (ca. 17 ml). The total was diluted to 100 ml.
3. Large-pore buffer, pH 6.3; 48.0 ml of 1 N KOH, 0.46 ml of TEMED and 2 ml of glacial acetic acid were combined and diluted to ca. 80 ml. Glacial acetic acid was added dropwise to pH 6.3 and this solution was diluted to a total volume of 100 ml.
4. 48.0 g of Cyanogum 41 (95% acrylamide and 5% bisacrylamide) (E.C. Apparatus Corp.) were dissolved in water and diluted to 100 ml. The solution was filtered through Whatman #1 paper.
5. 100 ml of 16% Cyanogum 41 (w/v) was prepared as in (4) above.
6. 4.0 mg of riboflavin (Eastman Organic Chemicals) were dissolved in water and diluted to 100 ml.

Immediately prior to pouring the gels, these solutions were mixed

in the following proportions to obtain the separating and spacer gel solutions for eight tubes.

Separating gel - pH 4.3

2.0 ml solution 2

4.0 ml solution 4

2.0 ml water or 0.8% casein (precipitated
and sonicated)

8.0 ml ammonium persulfate (0.028 g/10 ml,
prepared fresh each day)

Spacer gel - pH 6.3

1.0 ml solution 3

2.0 ml solution 5

1.0 ml solution 6

4.0 ml water

The polyacrylamide columns were prepared in glass tubes 5 mm I.D. and 75 mm in length. One end of each tube was sealed with parafilm and placed vertically in a rack. The separating gel was prepared as indicated and pipetted into each tube to a height of 60 mm. Water was carefully layered on each column. A faint opalescence, indicating that gelling had occurred, appeared after ca. 20 min. The gels were left undisturbed for 30 min, however, to ensure complete polymerization.

The water layer was removed, the spacer gel solution prepared and 0.20 ml was pipetted on top of each separating gel. The spacer gel was carefully overlaid with water and a fluorescent lamp was placed ca. 15 cm from the tubes to promote photopolymerization. Gelling time was again 20 min as evidenced by the loss of the yellow color of riboflavin.

Once the gels were prepared the parafilm was removed from the bottom of each tube. One cm sections of rubber tubing were placed in each of the sample holes of the upper chamber as gaskets and the gel tubes were inserted. The lower chamber was filled with electrode buffer and the upper chamber was put in place and filled with buffer.

Any bubbles in the top and bottom of the gel were removed by flushing with electrode buffer and 0.05 ml of the sample, containing 10% sucrose and a minimum quantity of methylene green (ICN Pharmaceuticals Corp.), was layered on top of the spacer gel with a syringe. The anode (+) lead from the power supply was attached to the electrode in the anode chamber (upper) and the cathode (-) lead was attached to the electrode in the lower chamber. Electrophoresis was initiated at 50V (ca. 2 ma/tube) for 1/2 hr and was then increased to 100V (ca. 3 ma/tube) for an additional 2-2 1/2 hrs.

Following electrophoretic separation, the gels were removed from the tubes by carefully rimming between the gel and tube with a flattened syringe needle. The length of each gel and the migration distance of the tracking dye (methylene green) were measured and recorded. The gels were placed in 13 x 100 mm test tubes containing protein stain (Amido Black 10B), specific substrates or buffer as required.

Protease Detection

The location of proteases following electrophoretic migration under the specified conditions was determined by a modification of the method of Andary and Dabich (1974).

Casein substrate (0.8% in TRIS-HCl buffer) was prepared by the

method of Kunitz (1947). Glacial acetic acid was added dropwise to the isoelectric point of casein and a very fine dispersion of the precipitated casein was obtained by probe sonication, as follows. Approximately five ml of the casein suspension, following isoelectric precipitation, were placed in a 15 x 150 mm test tube and held at 0°C in an ice bath. The sample was sonicated for three one min intervals using the Bronwill Biosonik III sonicator equipped with the intermediate probe (BP-111-12T) and operated at 105 watts/cm².

In formulations of the running gel this solution replaced the added water to produce a polymerized gel containing 0.1% casein substrate. The very fine particles of casein produced by sonication resulted in an even distribution of the casein substrate throughout the gel and prevented settling during the time required for polymerization.

Following electrophoretic separation of the proteins in the pyloric caeca extract, the gels were incubated at 37°C in 0.1 M TRIS-HCl buffer (pH 8.0), containing 0.01 M CaCl₂. Raising the pH of the gel allowed the proteases present to digest the casein in the gel producing clear zones to mark their location. Once these clear zones had become visible (ca. 45 min) the buffer was removed and the gels were submerged in 12% TCA to stop the reaction and stabilize the pattern of digested and undigested portions of the gel. Each gel was scanned at 280 nm in a Gilford scanning densitometer to provide a record of the location of the proteases.

Relative migration (R_m) of each band was determined to compare data collected on different electrophoretic runs. The data required was the length of the gel and tracking dye migration immediately following

electrophoresis and the length of the gel and protease migration measured on the densitometer scan. The R_m of a specific protease was calculated as follows:

$$R_m = \frac{(\text{gel length before incubation}) (\text{protease migration on scan})}{(\text{dye migration before incubation}) (\text{gel length on scan})}$$

Amino Acid Naphthylamide Assays

To determine the position of trypsin-like and chymotrypsin-like activities in the gel, the specific substrates, benzoyl-DL-arginine- β -naphthylamide (BANA) (U. S. Biochemicals Corp.) and glutaryl-L-phenylalanine- β -naphthylamide (GPNA) (National Biochemicals Co.), respectively, were used.

Methods used were developed by Zwilling et al. (1969) and further described and modified by Hagenmaier (1975). The specific substrate (0.5 mg/ml) was dissolved in 1.0 ml of N,N-dimethylformamide followed by addition of 9.0 ml of 0.10 M TRIS-HCl, 0.01 M CaCl_2 buffer (pH 8.0). The gel was submerged in the substrate solution and incubated at 37°C. After incubation for 30 min the substrate was removed and the gel rinsed briefly in distilled water. Detection of zones where the amino acid naphthylamide was split was accomplished by addition of Fast Blue B salt (o-dianisidine, tetrazotized, Sigma Chemical Co.) (1 mg/ml), prepared in 0.10 M TRIS-HCl, 0.01 M CaCl_2 buffer (pH 8.0) immediately before use. The gels were submerged in this solution until the orange red diazo dye was formed by reaction with the free naphthylamine. The position of the dye band was recorded by scanning the gel at 490 nm in the Gilford scanning densitometer. The R_m of the diazo dye band, relative to methylene green, was calculated as before.

Inhibitor Studies

Further evidence for the identity of the electrophoretically separable proteases was provided by use of active site inhibitors. Incubation of the gels in solutions of inhibitors following electrophoresis (Andary and Dabich, 1974) was found to be impractical due to solubility problems and the requirement for large quantities of the inhibitors. Preincubation (i.e. prior to electrophoretic separation) of solutions of bovine chymotrypsin and porcine trypsin with the three inhibitors PMSF, TLCK and TPCK demonstrated the expected sensitivity or resistance. Preincubation of the pyloric caeca extract with inhibitors was, therefore, determined to be most satisfactory.

The serine proteases were inhibited by incubating the following solution for one hr at room temperature prior to electrophoresis; 0.5 mg PMSF (Sigma Chemical Co.) dissolved in 0.20 ml isopropanol, followed by addition of 0.50 ml of 0.04 M TRIS-HCl buffer (pH 7.0) containing 0.02 M CaCl_2 , 0.10 ml of water and 0.20 ml of the pyloric caeca extract which was previously adjusted to pH 7 with 1 N NaOH.

The reaction of TPCK (Sigma Chemical Co.) with chymotrypsin-like enzymes followed the method of Schoellman and Shaw (1963). TPCK (0.3 mg) was dissolved in 0.05 ml of methanol, followed by addition of 0.50 ml of 0.04 M TRIS-HCl buffer (pH 7.0) containing 0.02 M CaCl_2 . Water (0.25 ml) and 0.20 ml of pyloric caeca extract were added and the solution incubated at room temperature for ca. two hrs.

TLCK (Sigma Chemical Co.) was used to inhibit proteases with trypsin-like specificity (Shaw et al., 1965). The inhibitor (1.0 mg)

was dissolved in a solution prepared with 0.50 ml of 0.04 M TRIS-HCl buffer (pH 7.0) containing 0.02 M CaCl_2 , 0.30 ml of water and 0.20 ml of pyloric caeca extract. The addition of TLCK to the active site histidine residue of trypsin-like enzymes was allowed to continue for two hrs.

RESULTS AND DISCUSSION

pH-Activity Profile of Whole Viscera Extract

The effect of pH on protease activity was determined and the data plotted as protease units ($\Delta A/\text{min/g}$ wet tissue) vs pH (Figure 1). Three pH optima were noted at pH 1.5, 3.1 to 3.5 and ca. 9.5. Data used to generate the pH profile indicated the location of pH optima. The absolute activities in different fish at each pH varied.

Comparison of the activity against hemoglobin and casein in alkaline solution showed the effect of substrate on total protease activity. Referring again to Figure 1, the ratio of proteolytic activity using casein or hemoglobin as substrate, from pH 7 to 9, was at least 7 to 1. This may have been the result of increased substrate solubility, substrate denaturation or number and availability of susceptible peptide bonds in casein relative to hemoglobin.

Survey of Protease Activity

The location of the pH optima in the whole viscera extract indicated that measurement of proteolytic activity at pH 1.7, 3.5 and 7.6, as described by Anson (1938) and Kunitz (1947), would provide useful information on the distribution and relative levels of proteases in the visceral organs of albacore tuna fish. The results are tabulated in Table 1.

The single extraction technique employed provided an indication of relative activity and variability in protease levels in different fish. This variability was probably due to natural variation between

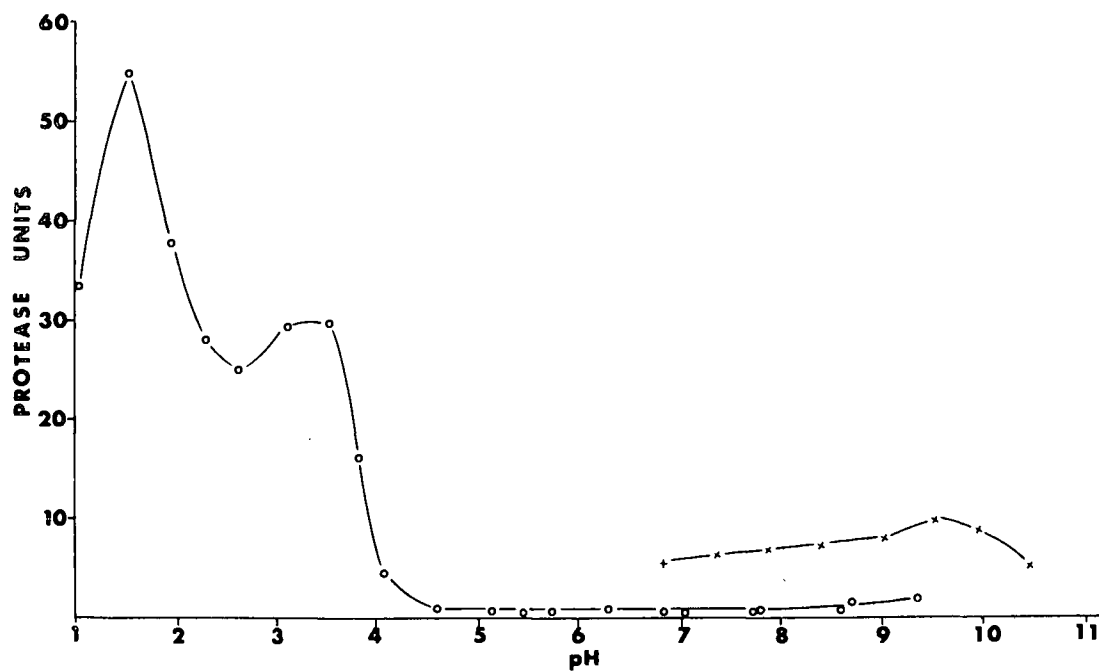


Figure 1. pH - activity curve of total viscera extract. Data represents the hydrolysis of hemoglobin (o) and casein (x) for 20 min at 37°C.

Table 1. Protease activity in extracts of the visceral organs of tuna fish.^a

Organs	Wt. of organ	ml extract per gram tissue	mg protein per gram tissue	pH 7.6 casein _b units	pH 3.5 hemoglobin _c units	pH 1.7 hemoglobin units
Pyloric caeca	136.6 ± 13.1	4.16 ± 0.07	40.3 ± 1.4	29.9 ± 5.9	1.3 ± 0.4	0.1 ± 0.04
Liver	106.6 ± 20.2	3.60 ± 0.19	42.8 ± 5.1	0.5 ± 0.1	2.0 ± 0.2	0.6 ± 0.1
Stomach	155.2 ± 19.8	3.39 ± 0.14	33.1 ± 1.2	1.2 ± 0.3	61.0 ± 11.9	67.4 ± 7.9
Spleen	12.5 ± 2.1	3.36 ± 0.16	26.9 ± 1.3	0.4 ± 0.2	6.1 ± 1.0	0.8 ± 0.2
Intestine	31.7 ± 7.8	3.60 ± 0.17	25.8 ± 1.7	11.5 ± 4.4	1.0 ± 0.1	<0.1

^aAll values are means of five organs ± standard error.

^bCasein units are ΔA_{280} /min/gram of wet tissue.

^cHemoglobin units are ΔA_{280} /min/gram of wet tissue.

individual fish and possibly the stage of feeding for each fish.

Kashiwada (1952) reported on the seasonal variation of proteolytic activity in skipjack tuna (Katsuwonus vagans). The activity in the pyloric caeca rose in the spring, declined slowly in the summer and rose again in the fall. The increase in activity corresponded to increased feeding activity. Other researchers have noted the variation in proteolytic activity as a function of feeding (Chesley, 1934 and Overnell, 1973). Variations in proteolytic activity in pyloric caeca were correlated with the stage of growth of trout (Kitamikado and Tachino, 1960).

From the mean values listed in Table 1 the location of protease activity was determined. The digestive proteases active at pH 7.6 were located in the pyloric caeca, their point of origin, and the intestine where these proteases actively digest ingested food. The liver, stomach and spleen showed relatively low levels of these enzymes.

The level of proteolytic activity at pH 3.5 was similar in the pyloric caeca, liver and intestine. The mean level of activity in the spleen was three times as great as that in the above three organs and proteolytic activity at pH 3.5 was exceptionally high in the stomach.

The relatively high proteolytic activity at pH 3.5 measured in the stomach extract was originally thought to be the result of a wide pH optimum for pepsin. The pH profile of the extract from the whole viscera, however, indicated a sharp activity optimum at pH 1.5 and a relatively high activity at pH 3.5 (Figure 1). Taken together, these data indicated the presence of at least one additional acidic protease in the stomach which was maximally active at pH 3.1 to 3.5.

The proteolytic activity at pH 1.7 was associated almost exclusively with the stomach. Although direct comparison of proteolytic activity against casein and hemoglobin was not possible, the activity of the acidic proteases in the stomach appeared to be significantly higher than the activity at pH 7.6 in the other visceral organs. Norris and Mathies (1953) reported that the specific activity of crystalline tuna pepsins, including the yellowfin, albacore and bluefin tuna was higher than other pepsins studied. The high level of acidic protease activity was also indicated by the pH profile of enzymatic activity in the whole viscera extract (Figure 1).

Effect of Temperature on Pyloric Caeca Proteases

Despite the apparently lower activity measured at pH 7.6 relative to the acidic protease activity, the proteases active in neutral and moderately alkaline solution were chosen for further study. Their use in digestion of proteins involves relatively mild reaction conditions, they display a wide pH optimum and the necessity of extensive acidification with its associated problems (Tarky et al., 1973) is avoided. The pyloric caeca was chosen as the organ containing the richest source of alkaline proteases.

The effect of temperature on the activity of the proteases extracted from the pyloric caeca was determined by following the digestion of casein for one hr at 10°C intervals. The results are summarized in Figure 2. At 50°C no significant inhibition of proteolytic activity occurred during the one hr incubation. At 60°C, however, there was a definite decrease in reaction rate after 30 min and at 70°C nearly

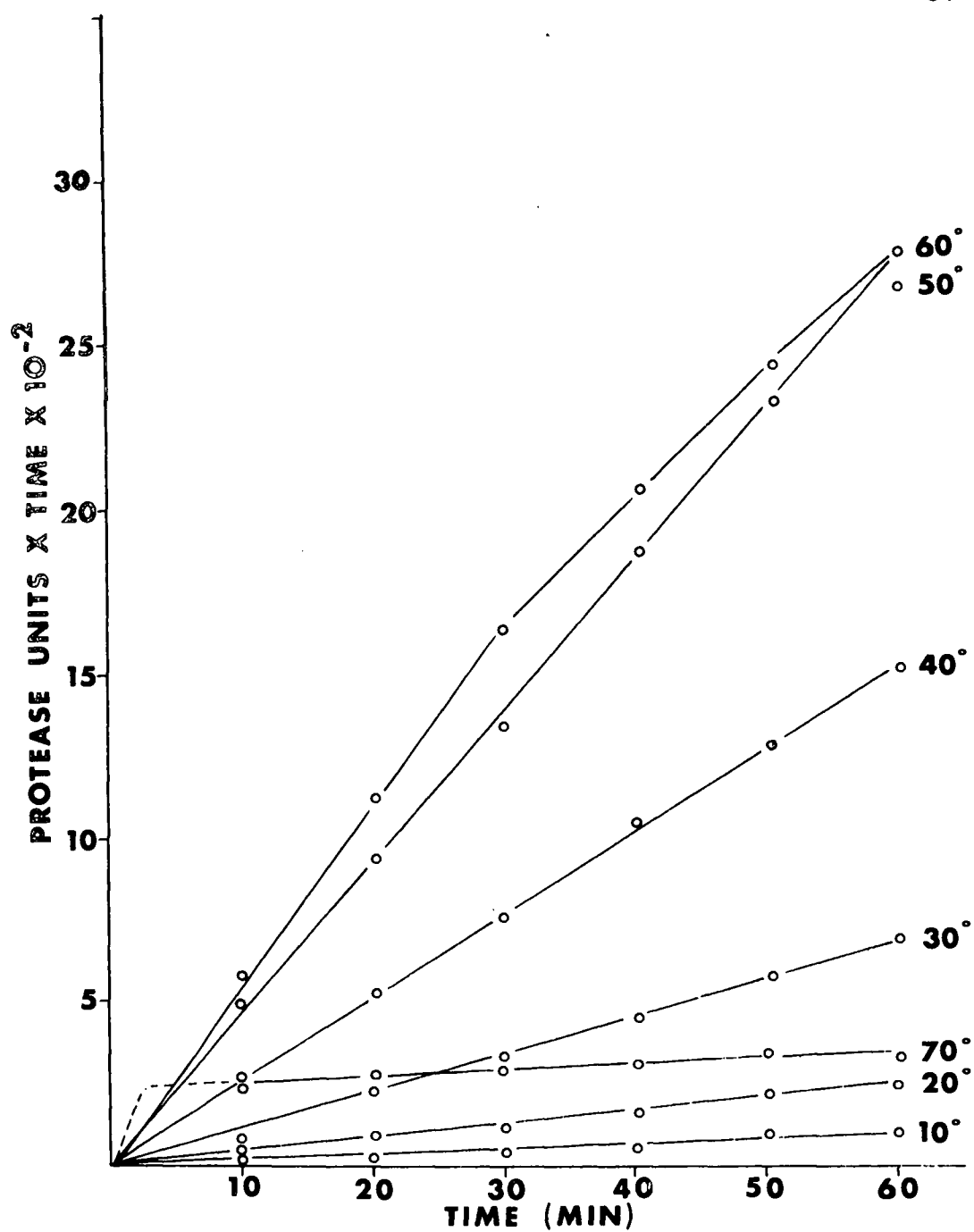


Figure 2. Effect of temperature on the activity of pyloric caeca proteases. Data obtained from the hydrolysis of casein substrate at pH 7.6.

all proteolytic activity was destroyed within 10 min. The temperature coefficient, Q_{10} , calculated between 10°C and 40°C varied between 2.3 and 2.8. The corresponding activation energy calculated for the protease mixture was 14,560 cal/mole.

Pyloric Caeca Protease Activity and Inhibition

The approximate levels of protease activity present in the pyloric caeca extract used for the temperature stability and electrophoresis studies is presented in Table 2. No activity was measured against CGP, although activity against hippuryl- β -phenyllactic acid, another substrate specific for carboxypeptidase A, was reported in similar extracts from tuna (Zendzian and Barnard, 1967a). Lack of CGP hydrolysis in our extract may have been due to a difference in specificity or differences in reaction conditions required by the tuna enzymes. CGP hydrolysis was measured in chum salmon (Uchida, 1970) and cod (Overnell, 1973) pyloric caeca.

The action of three inhibitors (PMSF, TLCK and TPCK) was determined by measuring the activity against casein, TAME and BTEE in solution. Data is presented in Table 3 and indicates nearly half of the apparent digestion of casein was resistant to PMSF. This serine protease inhibitor completely destroyed activity against the trypsin-specific substrate, TAME, and the chymotrypsin-specific substrate BTEE.

The action of the chymotrypsin inhibitor, TPCK, on the pyloric caeca extract revealed a 32% decrease in activity against casein substrate, however, the activity against BTEE was not completely destroyed. The nearly 20% activity remaining suggested the presence

Table 2. Approximate protease activity in the pyloric caeca extract.

Protein concentration ^b	Activity Measured ^a				
	Casein units	TAME units	BTEE units	CGP units	HA units
38.3	22	70	340	0	40

^aActivity reported as units per gram wet tissue.

^bmg protein extracted per gram wet tissue.

Table 3. Effect of inhibition of pyloric caeca proteases.

Inhibitor ^a	% Activity Remaining		
	Casein	TAME	BTEE
PMSF (2.87×10^{-3} M)	48	0	0
TPCK (8.52×10^{-4} M)	68	81	19
TLCK (2.71×10^{-3} M)	88	0	110
TPCK (8.52×10^{-4} M)			
+ TLCK (2.71×10^{-3} M)	69	0	31

^aIncubation conditions as reported in materials and methods.

of a PMSF-sensitive protease or esterase capable of hydrolyzing BTEE in addition to a true chymotrypsin-like enzyme. TPCK treatment also resulted in a 19% reduction in TAME hydrolysis indicating the possibility of cross reactivity of TPCK with a component of the trypsin-like enzymes.

The trypsin inhibitor, TLCK, resulted in a 12% reduction in casein digestion as measured by absorption of the TCA filtrate at 280 nm. The hydrolysis of TAME was completely destroyed by this reagent. Activity against BTEE was increased slightly relative to the control. The apparent activation of chymotrypsin-like activity was probably due to decreased autolysis in the extract during the two hr incubation at pH 7 by the trypsin-like enzymes present.

When the extract was treated with a combination of TPCK and TLCK the activity against casein was decreased 31%, TAME hydrolysis was destroyed and 69% of the BTEE hydrolysis was inhibited. The remaining BTEE hydrolysis, we believe, was due to a combination of a TPCK-resistant fraction and decreased destruction of the TPCK-resistant fraction by the TLCK sensitive proteases. Decreased autolysis combined with the cross-reactivity of the chymotrypsin inhibitor (TPCK) with a portion of the trypsin-like activity (TAME hydrolysis) may have accounted for the higher hydrolysis of casein measured for the combined action of TPCK and TLCK, relative to the hydrolysis that would be estimated from data on the action of TPCK and TLCK used separately.

Zendzian and Barnard (1967a) reported similar inhibition of caseinolytic activity in the partially purified pyloric caeca extract by TPCK and TLCK. However, TPCK and TLCK used together resulted in the

same level of inhibition as did DFP treatment or the sum of the inhibitions by TPCK and TLCK when used separately. TPCK and TLCK treatment completely inhibited BTEE and BAEE (a substrate specific for trypsin-like enzymes) hydrolysis, respectively. BAEE hydrolysis was unaffected by TPCK, and BTEE hydrolysis was unchanged by incubation of the partially purified pyloric caeca extract with TLCK. The discrepancy in the results reported by Zendzian and Barnard (1967a) and in our study may be due to species variation or the partial purification of the pyloric caeca extract performed by these investigators.

Electrophoretic Separation and Characterization of Proteases

Preliminary electrophoretic experiments were made with the pyloric caeca extract using the cationic system of Reisfeld et al. (1962) to determine satisfactory conditions for electrophoresis. Use of Cyanogum 41, which contains 95% acrylamide and 5% bisacrylamide, required lowering the total gel concentration (% T) from 15% T, as suggested, to 12% T. This provided increased migration of the proteins and prevented precipitation of the stock Cyanogum 41 solution during refrigerated storage.

The quantity of protein applied to the gels was varied between ca. 50 and 700 μg . Detection of proteases in gels containing 0.1% casein substrate was optimal when ca. 150 μg of protein was applied to each gel and incubation at 37°C and pH 8.0 was continued for 45 min. This corresponds to 0.05 ml of extract diluted 1:5. The clearest pattern of proteins stained with Amido Black 10B was obtained when

ca. 350 μ g of protein (0.05 ml of extract diluted 1:2) was applied to each gel.

Protein Resolution

The densitometer scan at 600 nm of a gel stained with Amido Black 10B is reproduced in Figure 3. Counting peaks and prominent shoulders there are at least 15 distinct proteins present. The relative migration (R_m) of each was determined relative to the methylene green tracking dye for comparison with similar data for protease bands.

Protease Detection

The location of proteases in the gel following electrophoretic separation is shown in Figure 4D. Absorbance minimums (i.e. valleys) on the scans at 280 nm were associated with proteases, however, the scans were inverted so that proteases could be associated with peaks.

To determine the effect of time on proteolysis and to provide gels with reasonably well resolved zones of digestion, the time of incubation at 37°C and pH 8.0 was varied. At 15 min intervals the protease activity was stopped in one of four gels by submersion in 12% TCA. Figure 4, as well as other data obtained from longer incubation times, indicated 45 min at 37°C and pH 8.0 was optimum. All subsequent densitometer scans at 280 nm represent gels incubated in this manner.

The data in Figure 4 also indicated the presence of a protease of high molecular weight, or low net charge at the pH of electrophoresis, which actively digested casein at a pH of ca. 4.

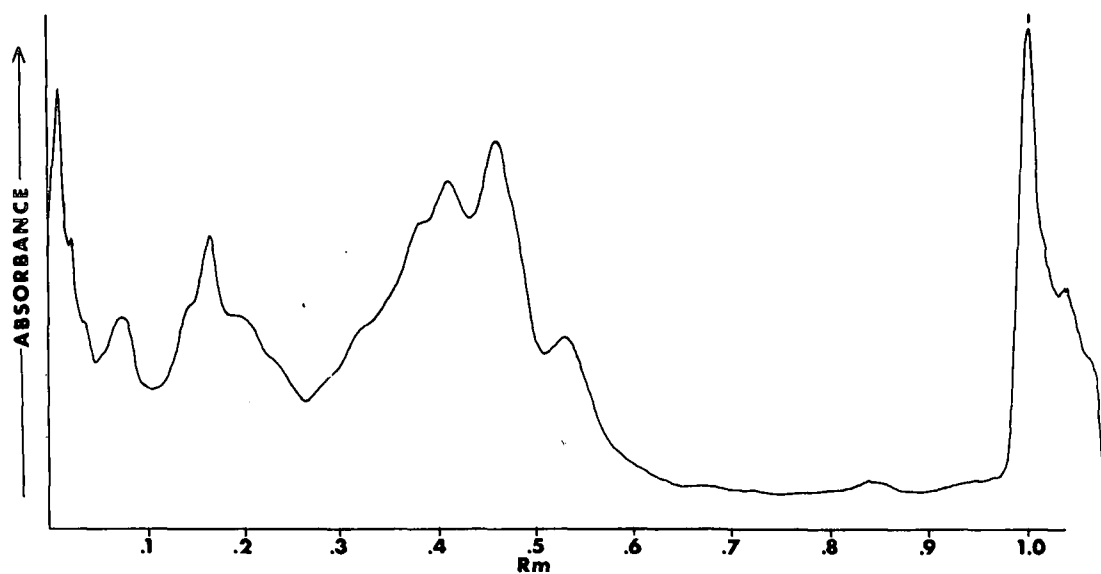


Figure 3. Electrophoretic pattern of pyloric caeca extract proteins. Electrophoresis was performed for 2.5 hr using the cationic system of Reisfeld et al. (1962). Protein was stained for 30 min with Amido Black 10B, destained and scanned at 600 nm. Abscissa values indicate R_m relative to the methylene green tracking dye (Peak I).

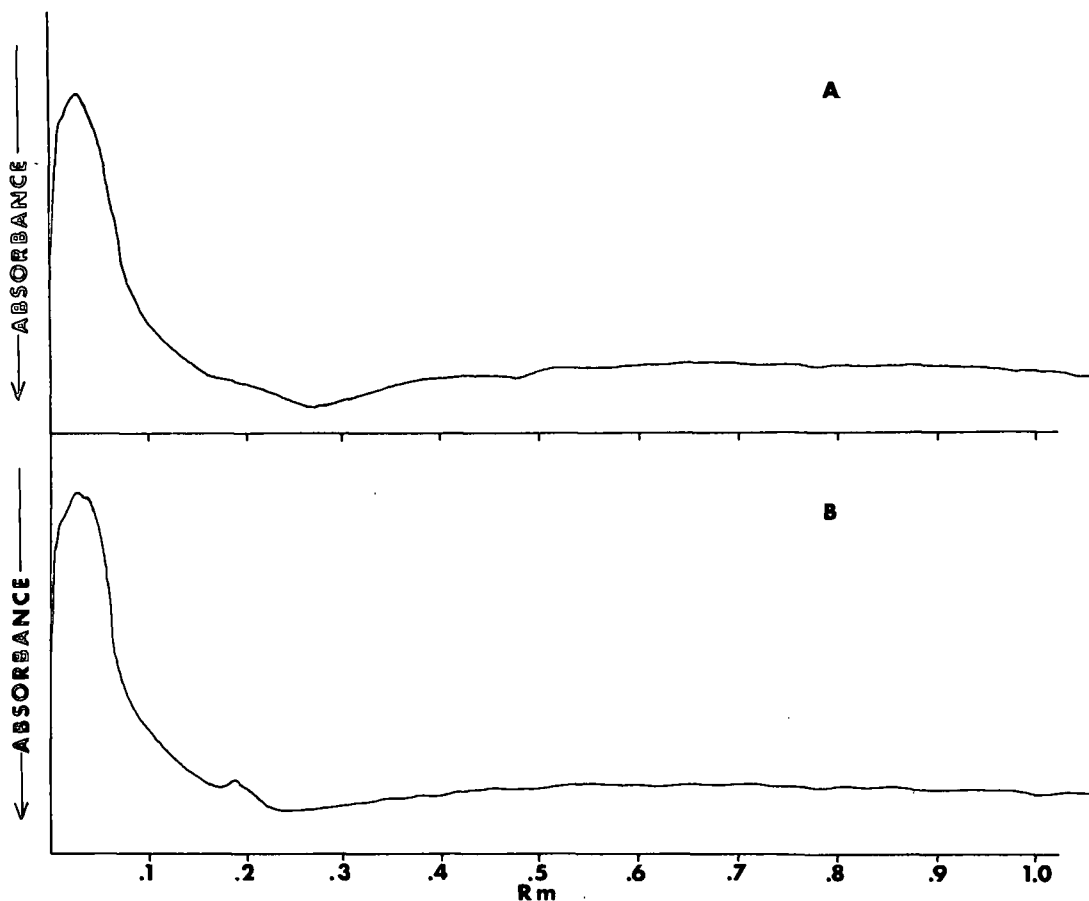


Figure 4. Effect of time on casein digestion in polyacrylamide gels. Electrophoresis was performed for 2.5 hrs using the discontinuous cationic system of Reisfeld *et al.* (1962). Gels contained 0.1% sonicated casein as substrate. Figure 4A represents a gel submerged in 12% TCA without prior incubation at pH 8.0. 4B, C and D were incubated at 37°C in 0.10 M TRIS-HCl, 0.01 M CaCl_2 , pH 8.0, for 15, 30 and 45 min, respectively, prior to addition of 12% TCA. Scans were made at 280 nm in a Gilford scanning densitometer.

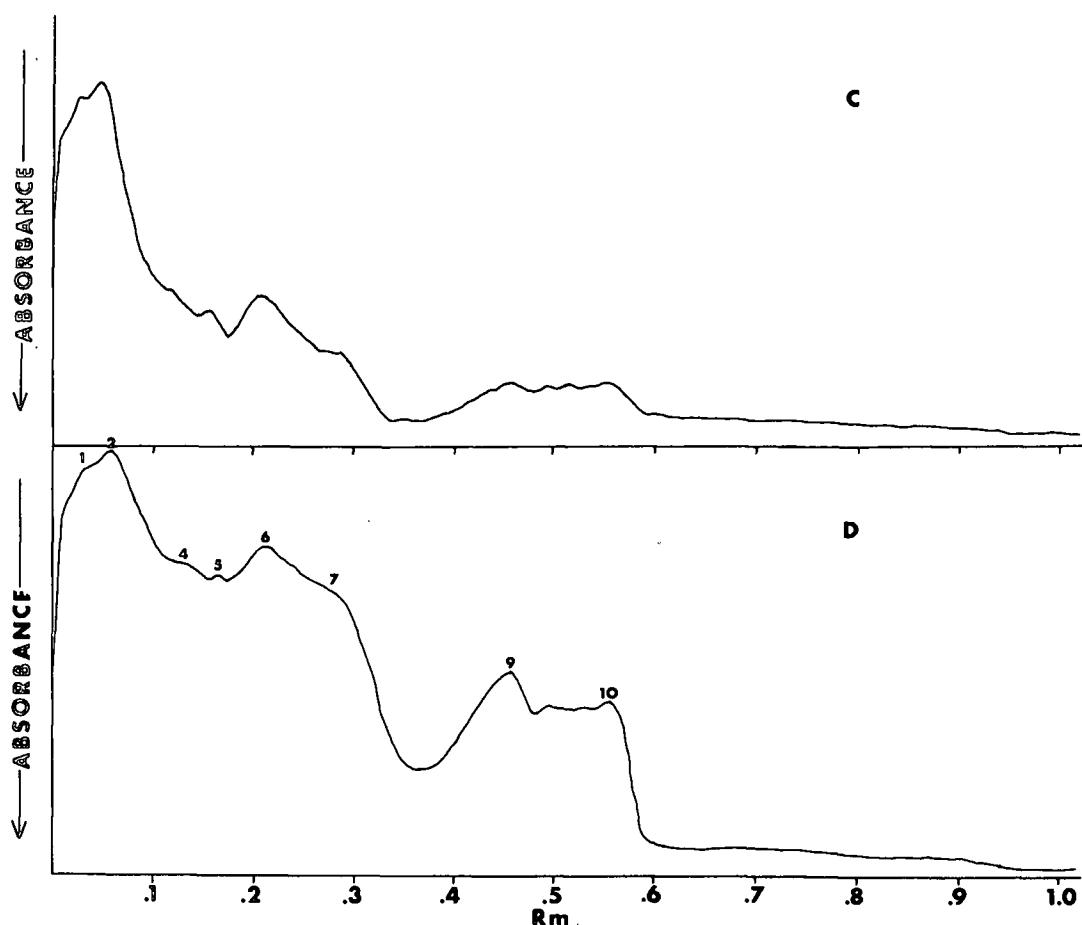


Figure 4. Effect of time on casein digestion in polyacrylamide gels. Electrophoresis was performed for 2.5 hrs using the discontinuous cationic system of Reisfeld *et al.* (1962). Gels contained 0.1% sonicated casein as substrate. Figure 4A represents a gel submerged in 12% TCA without prior incubation at pH 8.0. 4B, C and D were incubated at 37°C in 0.10 M TRIS-HCl, 0.01 M CaCl_2 , pH 8.0, for 15, 30 and 45 min, respectively, prior to addition of 12% TCA. Scans were made at 280 nm in a Gilford scanning densitometer.

Inhibition of Protease Activity

Analysis of Figure 4D indicated the presence of at least eight distinct proteolytic enzymes. Each protease was identified with its calculated R_m and a number between one and ten. In order to determine some characteristics of these enzymes, the effects of the three inhibitors PMSF, TPCK and TLCK were noted.

In Figure 5 the effect of preincubating the serine protease inhibitor, PMSF, with the pyloric caeca extract, relative to incubation in the PMSF solvent blank, is shown. Four proteolytic enzymes are resistant to PMSF inhibition, while the remainder are sensitive to this inhibitor. The protease active at the pH of electrophoresis (peak #1), previously referred to, is PMSF-sensitive.

The densitometer scans of gels containing uninhibited and PMSF-treated samples of pyloric caeca extract produced additional information. Comparison of Figures 5A and 5B indicates the presence of a protease with $R_m = 0.10$ (peak #3), as a distinct protease in the PMSF-treated gels. This protease is not distinguishable in the uninhibited system, being overshadowed by adjacent PMSF-sensitive proteases.

Preincubation of the extract with the chymotrypsin inhibitor TPCK revealed no protease was completely inhibited by this reagent (Figure 6). The action of the trypsin inhibitor TLCK, however, indicated the presence of two proteases with trypsin-like specificity (Figure 7). The protease active at low pH and sensitive to PMSF (Peak #1), was inhibited by TLCK and there was some indication of inhibition at $R_m = 0.18$ (peak #5).

Preincubation of the extract in the inhibitor solvent blanks

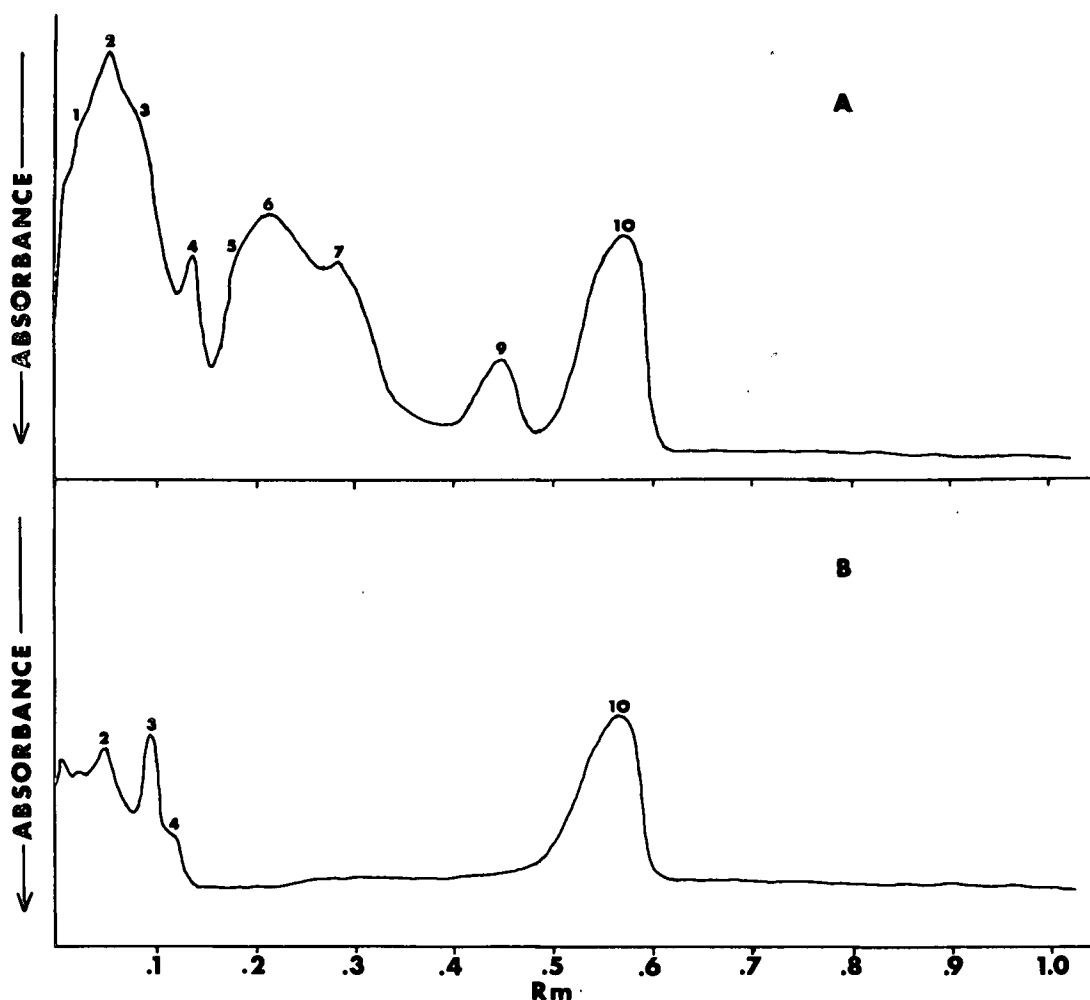


Figure 5. Effect of PMSF on electrophoretically separated pyloric caeca proteases. Figure 5A (control) represents the pattern of proteases in pyloric caeca extract preincubated in the PMSF solvent (20% isopropanol, 0.02 M TRIS-HCl, 0.01 M CaCl₂, pH 7.0) for 1 hr prior to electrophoresis. Figure 5B represents the pattern of proteases in extract preincubated in 2.87×10^{-3} M PMSF for 1 hr. Electrophoresis was performed for 2.5 hrs using the discontinuous cationic system of Reisfeld et al. (1962). Gels contained 0.1% sonicated casein as substrate. Post-electrophoretic incubation at pH 8.0 was for 45 min prior to addition of 12% TCA. Scans were made at 280 nm in a Gilford scanning densitometer.

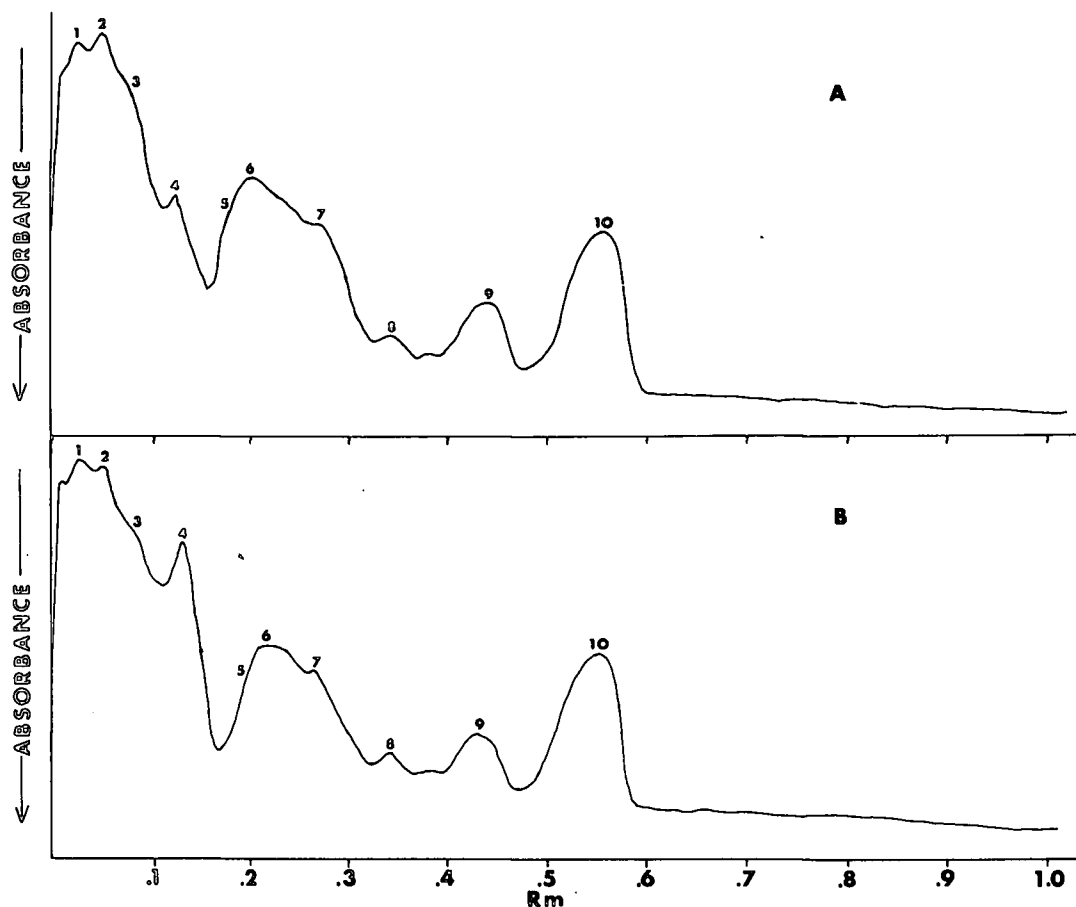


Figure 6. Effect of TPCK on electrophoretically separated pyloric caeca proteases. Figure 6A (control) represents the pattern of proteases in pyloric caeca extract preincubated in the TPCK solvent (5% methanol, 0.02 M TRIS-HCl, 0.01 M CaCl_2 , pH 7.0) for 2 hrs prior to electrophoresis. Figure 6B represents the pattern of proteases in extract preincubated in 8.52×10^{-4} M TPCK for 2 hrs. Electrophoresis was performed for 2.5 hrs using the discontinuous cationic system of Reisfeld *et al.* (1962). Gels contained 0.1% casein as substrate. Post-electrophoretic incubation at pH 8.0 was for 45 min prior to addition of 12% TCA. Scans were made at 280 nm in a Gilford scanning densitometer.

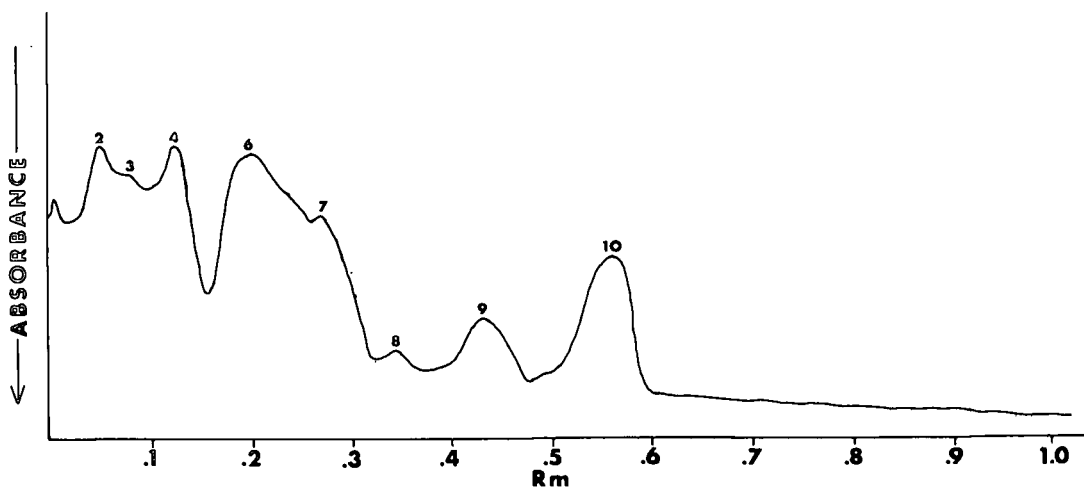


Figure 7. Effect of TLCK on electrophoretically separated pyloric caeca proteases. Figure represents the pattern of proteases in pyloric caeca extract preincubated in 2.71×10^{-3} M TLCK (in 0.02 M TRIS-HCl, 0.01 M CaCl_2 , pH 7.0) for 2 hrs prior to electrophoresis. Electrophoresis was performed for 2.5 hrs using the discontinuous cationic system of Reisfeld *et al.* (1962). Gels contained 0.1% sonicated casein as substrate. Post-electrophoretic incubation at pH 8.0 was for 45 min prior to addition of 12% TCA. Scans were made at 280 nm in Gilford scanning densitometer.

(20% isopropanol or 5% methanol at pH 7.0) resulted in the apparent loss of activity between the proteases with $R_m = 0.45$ and $R_m = 0.56$ (peaks 9 and 10) (compare Figure 4D with Figures 5A and 6A). Resolution of a protease with $R_m = 0.35$ (peak #8) was also provided by incubation of the pyloric caeca extract in the above solvent blanks, TPCK or TLCK prior to incubation (compare Figure 4D with Figures 5A, 6A, 6B and 7). The assays of untreated pyloric caeca extract combined with assays of inhibitor-treated extract, therefore, indicated the presence of at least ten electrophoretically distinct proteases in the pyloric caeca of albacore.

Amino Acid Naphthylamide Assays

Gels, following electrophoresis, were assayed using the specific substrate GPNA (chymotrypsin-like activity) and BANA (trypsin-like activity). Reaction with BANA resulted in one band with $R_m = 0.19$ (Figure 8A) corresponding closely with the PMSF- and TLCK-sensitive band at $R_m = 0.18$ (Figures 5B and 7). TLCK-treatment of the pyloric caeca extract destroyed the activity against BANA (Figure 8B), while TPCK treatment resulted in no inhibition of BANA hydrolysis in the gel (Figure 9).

The proteolytic enzyme at the top of the gel ($R_m = 0.03$) showed no reaction with BANA at either pH 8.0 or pH 4.5. Inhibition of this enzyme with PMSF and TLCK indicated the presence of an active site serine and an active site histidine residue, and TLCK sensitivity and TPCK resistance suggests binding specificity similar to trypsin. Hydrolysis of a substrate specific for trypsin-like enzymes is needed as

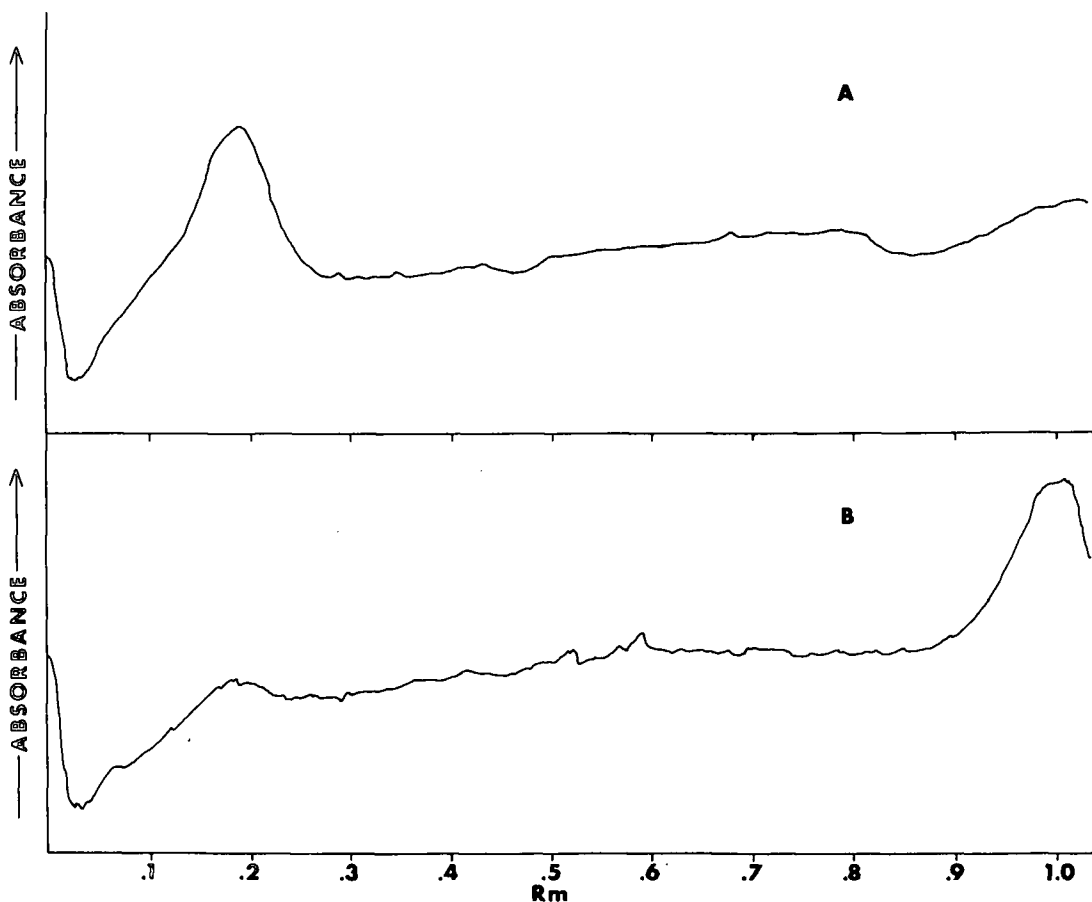


Figure 8. Effect of TLCK on in situ BANA assay of electrophoretically separated pyloric caeca proteases. Figure 8A represents the BANA-hydrolyzed zone in electrophoretically separated extract. Figure 8B demonstrates the lack of BANA hydrolysis in extract preincubated in 2.71×10^{-3} M TLCK (in 0.02 M TRIS-HCl, 0.01 M CaCl_2 , pH 7.0) for 2 hrs prior to electrophoresis. Electrophoresis was performed for 2.5 hrs using the discontinuous cationic system of Reisfeld et al. (1962). Scans were made at 490 nm in a Gilford scanning densitometer.

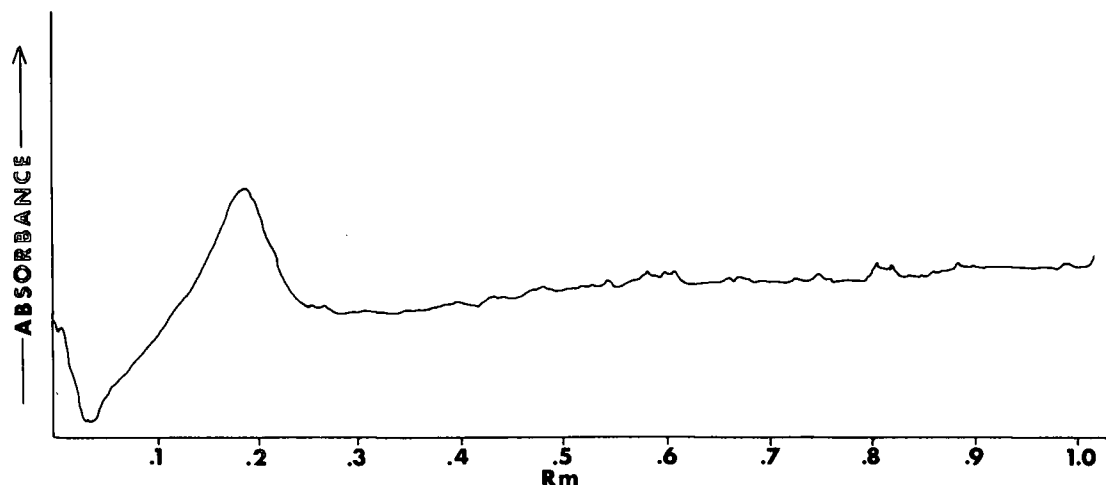


Figure 9. Effect of TPCK on in situ BANA assay of electrophoretically separated pyloric caeca proteases. Figure represents the zone of BANA hydrolysis in pyloric caeca extract preincubated in 8.52×10^{-4} M TPCK (in 0.02 M TRIS-HCl, 0.02 M CaCl_2 , pH 7.0) for 2 hrs prior to electrophoresis. Electrophoresis was performed for 2.5 hrs using the discontinuous cationic system of Reisfeld *et al.* (1962). Scans were made at 490 nm in a Gilford scanning densitometer.

confirmatory evidence for this enzyme's specificity, however.

Activity against GPNA was negative. The TPCK resistance of all protease bands, as previously noted, is in agreement with this result.

Experiments to Locate Chymotrypsin-Like Activity in Gels

The absence of TPCK sensitivity and GPNA hydrolysis was unexpected. As reported in Table 2, significant activity against BTEE was measured in the pyloric caeca extract. The hydrolysis of BTEE could be inhibited by both PMSF and TPCK (Table 3) providing substantial evidence for the presence of a chymotrypsin-like enzyme. Incubation of an aliquot of the pyloric caeca extract in a solution of GPNA (0.5 mg/ml in 0.10 M TRIS-HCl, 0.01 M CaCl_2 buffer, pH 8.0, containing 10% (v/v) N,N-dimethylformamide) for 15 min followed by addition of Fast Blue B salt (1 mg per ml of solution) resulted in formation of the orange red diazo dye confirming activity against this substrate.

A number of experiments were run to determine the cause for the lack of chymotrypsin-like activity in the gels. Bovine chymotrypsin (Sigma Chemical Co.) was subjected to electrophoresis and the gel was assayed for GPNA hydrolysis. One band was observed indicating that the procedure would detect chymotrypsin activity.

To further investigate the lack of chymotrypsin-like activity in the gels, the elution technique of enzyme detection in polyacrylamide gels was performed. Following standard electrophoresis the gel was sliced into one cm sections, placed in 0.50 ml of phosphate buffer, pH 6.5, crushed and held overnight at 4°C to allow elution of the protein. No activity against BTEE was present in any of the fractions.

Two experiments were run in an attempt to increase the quantity of the cyymotrypsin-like enzyme applied to the gel. Extract containing between 150 and 700 μg of protein was applied to a series of gels and separated electrophoretically. Activity against GPNA was negative in all gels. To further increase the quantity of BTEE-reactive enzyme applied to the gel, a partial purification of the extract was performed as described by Zendzian and Barnard (1967a). The protein was precipitated by the addition of 2 volumes of cold acetone and the precipitate was dissolved in water and subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation. The fraction precipitated between 40% and 60% saturation had the highest activity against BTEE and was subjected to electrophoresis. Reaction with GPNA in all gels was negative. Therefore, increasing the concentration of the BTEE-reactive enzyme in the extract did not reveal the chymotrypsin-like activity in the gels.

Since any protein with an isoelectric point below pH 4.5 would not migrate into the gel of the cationic system used, anionic electrophoretic separation of the extract was performed. The method described by Taylor (1970) was adapted to the tube electrophoresis apparatus used in our study. The system consisted of a 10% T running gel (0.38 M TRIS-HCl, pH 9.0), a 4% T spacer gel (0.062 M TRIS-HCl, pH 6.7) and electrode buffer at pH 8.75 (0.0165 M TRIS-0.0390 M glycine). Amino acid naphthylamide assays revealed one band with activity against BANA, but no activity against GPNA. This indicated that a very low isoelectric point for the chymotrypsin-like enzyme did not alone account for the inability to locate the enzyme on gels run in the cationic system.

The explanation for lack of chymotrypsin-like activity in the gels

is, therefore, still not known. Two factors not fully investigated were sensitivity of the chymotrypsin-like enzymes to the pH of electrophoresis or degradation by other proteases present.

Andary and Dabich (1974) reported similar findings in their analysis of mouse blastocyst extracts. Although GPNA hydrolysis was determined fluorometrically in solution, no activity against this substrate or reaction with TPCK could be determined in the gels following electrophoresis.

SUMMARY

Preliminary analysis of the total viscera extracts of the albacore tuna fish indicated three pH values of optimum activity; pH 1.5, 3.1-3.5 and 9.5. Extracts of individual organs revealed that the activity in acid solution, both at pH 1.7 and 3.5, was located primarily in the stomach. Alkaline protease activity was highest in the pyloric caeca extracts.

This study was directed primarily toward the alkaline proteases present in the pyloric caeca. Their use in protein hydrolysis provides mild reaction conditions, eliminates the need for acidification and minimizes the effects of acid reported in pepsin hydrolyzates. The literature reports the presence of enzymes with specificities similar to trypsin, chymotrypsin and the carboxypeptidases in tuna species. In no case, however, was a study of the full complement of proteolytic enzymes present in the pyloric caeca reported.

Our study of the trypsin-like and chymotrypsin-like activity, in solution, indicated that a very complex enzyme system was present in the pyloric caeca of tuna fish. Using discontinuous polyacrylamide gel electrophoresis, ten proteases were resolved and located by the casein substrate inclusion technique. A number of specific inhibitors and substrates were used in an attempt to identify some of the proteases present, or at least determine some properties of the enzymes.

Six of the proteases were serine proteases since their activity was inhibited by the action of PMSF. The serine proteases had R_m values; 0.03, 0.18, 0.22, 0.29, 0.35 and 0.45. One band ($R_m = 0.18$) was

similar in specificity to trypsin using the criteria of PMSF and TLCK inhibition, as well as activity against the specific amino acid naphthylamide substrate, BANA. The protease with $R_m = 0.03$ showed similarities to trypsin, being inhibited by PMSF and TLCK. However, this enzyme was active at a pH of ca. 4 and did not hydrolyze the specific substrate BANA indicating substantial differences between this enzyme and trypsin.

The methods used failed to detect the presence of a chymotrypsin-like enzyme in the polyacrylamide gels despite its presence in the pyloric caeca extracts applied to the gels.

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