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Title: PROTEOLYTIC ACTIVITY IN PACIFIC SHRIMP (PANDALUS JORDANI) PROCESSING WASTE: DISTRIBUTION, EFFECTS ON MUSCLE PROTEINS, AND PARTIAL CHARACTERIZATION

Abstract approved: Dr. David L. Crawford

Proteases present in shrimp processing waste are factors in the technology of shrimp. The distribution of proteolytic activity in shrimp parts was determined using hemoglobin and casein as substrates. The effects of various parameters upon activity of proteases in the inedible portion were determined using muscle protein as a substrate. Low pH active proteases from the hepatopancreas were characterized using column chromatography and inhibitors.

A crude homogenate of shrimp processing waste showed maximum proteolytic activity on casein at pH 6.25, although activity was relatively uniform between pH 5.75 and 7.5. Hemoglobin digestion was greatest between pH 3 and 3.65.

Specific activity was greatest in the foregut followed by the hepatopancreas and remainder of the inedible portion. Shrimp which contained food in their foreguts had greatest total activity in the foregut followed by the hepatopancreas. The order was reveresed for shrimp which had not been feeding. Total activity was lowest in the inedible portion with digestive organs removed. Only negligible activity was detected in the muscle.

Autolytic changes in a model system were studied where shrimp processing waste was the major protease source and muscle protein served as the major substrate. Activity data showed a major maximum at pH 3 and a minor broad maximum between pH 7 and 9. Maximum autolytic activity occurred at 50°C for pH 3 and at 55°C for pH 7.4. Incubation of the inedible portion at 65°C for 30 min was sufficient to inactivate the proteases. Proteases were unstable at low pH and 10 min on ice at pH 1.8 were required for inactivation. Autolysis at pH 3 was completely prevented by 10% NaCl while the inhibitory effects were less at pH 7.4. Protein solubility was decreased by NaCl at pH 3 and increased at pH 7.4. Heat-denatured muscle proteins were less susceptible to hydrolysis, possibly through a reduction in solubility. Changes occurred in the electrophoretic profile of soluble proteins (pH 7.4) from a muscle mixture which was incubated at 50°C.

Changes also occurred when incubation mixtures contained inedible portion and these changes were more rapid than when inedible portion was absent.

Low pH active proteases from the hepatopancreas were studied using hemoglobin as a substrate at pH 3.5. Gel filtration of a preparation from the hepatopancreas on Sephadex G-150 separated hemoglobin digesting activity in two distinct fractions. Chromatography on DEAE-cellulose also separated activity into two fractions and provided further evidence for the existence of at least two enzymes. Fractions from chromatography were unaffected by phenylmethyl sulphonylfluoride, unaffected or slightly activated by KCN, and greatly inactivated by p-chloromercuribenzoate. EDTA greatly activated all fractions. The result indicated that -SH groups are important for enzyme activity. A crude homogenate of the hepatopancreas and the major fraction from ion-exchange chromatography were most active on hemoglobin between pH 3 and 4.

Proteolytic Activity in Pacific Shrimp (*Pandalus jordani*) Processing Waste: Distribution, Effects on Muscle Proteins, and Partial Characterization

by

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INTRODUCTION

Shrimp are economically important to the seafoods industry. Frozen seafoods were worth 1.5 billion dollars in 1971 and frozen shrimp accounted for half of these sales (Roedel, 1973). Approximately 224.5 and 233.5 million pounds of shrimp, heads-off weight, were landed in the United States in 1970 and 1971, respectively (Roedel, 1973). In Oregon, a record 13.735 million pounds were delivered by 59 vessels in 1970 (23rd Annual Report of the Pacific Marine Fisheries Commission for the Year 1970).

Shrimp, like most seafoods, are highly perishable and factors responsible for their spoilage are of special interest to the seafoods technologist. Bacteria, chemical reactions such as those responsible for oxidation of lipids, and endogenous enzymes, play major roles in the spoilage mechanism. Control of these factors is important for maintaining quality and maximizing shelf life. Proteases, an important group of endogenous enzymes, are the spoilage factors considered in this study.

The digestive system contained in the heads is a rich source of proteolytic enzymes in shrimp. Major organs of the digestive system

include the hepatopancreas which secretes enzymes, and the foregut which serves as the major site of digestion. Unlike larger Gulf shrimp which are often headed at sea, smaller Pacific shrimp are kept intact after harvesting so that the digestive system and edible muscle remain in intimate contact during transportation and storage. Although autolysis does not lead directly to off odors associated with spoiled seafoods, products of protein hydrolysis promote microbial spoilage. Autolysis can also contribute to textural changes.

Once Pacific shrimp are cleaned, proteases of the digestive system are no longer available to interact with the edible muscle. However, they may still influence the technology of the inedible portion. Disposal of shrimp processing waste is a major problem in the seafoods industry. Most shrimp scrap goes into animal feeds although the supply far exceeds the demand.

Work concerning proteolytic enzymes of crustaceans has been primarily directed toward the serine proteases active in the alkaline to slightly acid pH range. Since crustaceans are primitive animals, studies of this nature have helped to elucidate the evolutionary history of proteolytic enzymes. However, few studies have been concerned with the effects of these enzymes on the technology of shrimp as a foodstuff. Moreover, enzyme characterization has been based on activity toward artificial substrates and not native proteins.

Preliminary investigations with Pacific shrimp revealed enzymes which hydrolyzed casein in the neutral pH range and those that digested hemoglobin in the acid pH range. The existence of casein digesting serine proteases in Crustacea is well established. However, reports on proteases active at low pH are rare. The above facts and findings gave rise to the following research objectives:

1. Categorize and locate major sites of proteolytic enzymes in shrimp processing waste.
2. Using shrimp proteins as a substrate, evaluate the effects of various parameters upon the activity of proteases in the inedible portion.
3. Characterize the proteolytic enzyme system active at low pH.

LITERATURE SURVEY

Digestive System of Crustacea

Vonk (1960), Lockwood (1967), and van Weel (1970) reviewed the structure and function of the crustacean digestive system. Three main sections of the gut are the foregut (stomodaeum), midgut (mesenteron), and hindgut (proctodaeum). The foregut and hindgut are lined with a chitinous layer which is not present in the midgut.

The foregut is divided into an esophagus, cardiac stomach and pyloric stomach. The cardiac stomach contains the gastric mill which is a masticatory apparatus. Food which has been ground into fine particles is passed to the pyloric stomach and then into the midgut. The midgut opens into the hindgut which finally opens at the anus. All movement of food in the gut is by peristalsis.

Closely associated with the midgut is a lateral expansion called the hepatopancreas. It consists of a mass of blindly ending tubules that join a hepatopancreatic duct opening which joins the anterior end of the midgut. The hepatopancreas is functional in absorption, digestion, and the storage of glycogen, fat, and calcium.

Digestive enzymes are produced only by the hepatopancreas (Lockwood, 1967; van Weel, 1970). Secretion is passed forward through the anterior portion of the midgut and into the cardiac stomach to initiate digestion. Early investigators found digestive enzymes

which include proteases (Krüger and Graetz, 1927; Shinoda, 1928; Mansour-Bek, 1932; Rosen, 1937), lipases (Krüger and Graetz, 1928) and carbohydrases (Wiersma and van der Veen, 1928). The main site of digestion is the foregut, although final breakdown occurs in the hepatopancreas (van Weel, 1970).

The hepatopancreas combines an absorptive function with the digestive function. Davis and Burnett (1964) showed that cells in the hepatopancreas arise from embryonic cells and that each cell is at first absorptive before becoming secretory. Absorption can also occur in the midgut. The foregut and hindgut absorb nothing other than water (Lockwood, 1967; van Weel, 1970).

In Pacific shrimp, the hepatopancreas is a large, fatty, yellow organ, while the midgut is extremely short, almost non-existent. The foregut is well defined although it is smaller than the hepatopancreas.

Proteolytic Enzymes of Crustacea

Recent investigations on the digestive enzymes of Crustacea have dealt with crayfish, crab, lobster, and shrimp.

Crayfish have been the most widely studied crustaceans. DeVillez (1965) isolated three proteolytic enzymes from the gastric juice of the crayfish Orconectes virilis and used specific synthetic substrates to characterize a trypsin, carboxypeptidase, and an

unknown protease. In another study, DeVillez and Johnson (1968) determined the molecular weight of the tryptic-component to be 24, 700. Kleine (1967a) found tryptic activity in Astacus astacus and Cambarus affinis. Kleine and Ponyi (1967) studied the exopeptidase and dipeptidase activities of the hepatopancreas and gastric juice of A. astacus and C. affinis. Carboxypeptidase and acrylamidase activities were mainly localized in the hepatopancreas. In a further study (Kleine, 1967b) the molecular weights and properties of the exopeptidases were examined in more detail. Pfleiderer et al. (1967) purified and characterized two proteases from the crayfish A. fluviatilis. One of the proteases was tryptic-like in its properties. Zwilling et al. (1969) purified the tryptic-like enzyme from A. fluviatilis and A. leptodactylus and found them to be very similar to bovine trypsin. Later, Zwilling and Tomasek (1970) used information on A. fluviatilis and A. leptodactylus in a discussion on the evolutionary history of trypsin. Tomasek et al. (1970) determined the amino acid sequence around the active serine residue of the trypsin-like protease of A. leptodactylus and found it to be identical to active serines in trypsins, chymotrypsins, elastase, and thrombin of mammalian origin.

Van Weel (1960) studied the secretion of digestive enzymes by the crab Thalamita crenata and proteolytic activity was greatest at 1 and 5-8 hours after feeding. Eisen (1967) reported on the existence of a collagenase in the hepatopancreas of the "spider crab," Libinia

emarginata, the "fiddler crab," Uca pugilator, and the "green crab," Carcinus maenas. Eisen and Jeffrey (1969) conducted a more detailed investigation on the collagenase from U. pugilator and in a recent study Eisen et al. (1973) purified the enzyme. In addition to being a collagenase, the enzyme demonstrated specificities against synthetic substrates for mammalian trypsin and chymotrypsin. Linke et al. (1969) found a tryptic-like enzyme in C. maenas which was immunologically unrelated to the crayfish trypsin of Astacus, although Herbold et al. (1971) found no difference between the two regarding cleavage specificity, effect of inhibitors on enzymatic activity, molecular weight, and hydrolysis of native proteins. Herbold et al. (1971) found no chymotryptic activity in the gastric juice of C. maenas.

Brockerhoff et al. (1970) investigated the digestive enzymes of the American lobster, Homarus americanus and found activities similar to trypsin, chymotrypsin, and carboxypeptidase A. No activities corresponding to carboxypeptidase B or leucine aminopeptidase were found. Proteolytic activity showed maximums at pH 7-8 and pH 4.

Gates and Travis (1969) purified a tryptic-like protease from the hepatopancreas of the "white shrimp," Penaeus setiferus and also isolated a carboxypeptidase A and carboxypeptidase B (Gates and Travis, 1973). DeVillez and Buschlen (1967) conducted a survey of tryptic digestive enzymes in various species of Crustacea and

Pandalus platyceros, a close relative of P. jordani, was shown to have a tryptic-like enzyme in the gastric juice. Flores and Crawford (1973) found that a crude homogenate of whole P. jordani would digest casein. Asahara (1973) studied a protease from the hepatopancreas of the shrimp, Trachypenaeus curvirostris, and found maximum activity at pH 8.4.

Proteases of Crustacea generally have their pH optimums in the slightly acid to slightly basic range. Activity in the acid range is rare and the existence of an enzyme with peptic-like specificity has not been found (Vonk, 1964; Huggins and Munday, 1968; DeVillez and Lau, 1969; van Weel, 1970). The existence of zymogens in Crustacea has not been established.

Harvesting, Processing and Utilization of Shrimp

Pacific shrimp are caught by vessels that drag the ocean with large nets. The shrimp are stored in ice aboard ship and may remain there up to four days. The shrimp are transported to the plant where they are unloaded and either stored or processed. Freshly caught shrimp are stored two days to condition them for processing. Shrimp are de-iced and separated from debris and scrap fish prior to automatic peeling. Mechanical peelers utilize counter-rotating rollers to pull and separate the inedible portion from the meat. Two types of machines are available, one which takes shrimp directly and another

which conditions the carcasses with a short steam precook. Shrimp peeled with the aid of steam are usually frozen while shrimp peeled directly are usually canned or frozen (Anonymous, 1973). Processing of Gulf shrimp is similar to Pacific shrimp except that the larger southern species are usually headed at sea to improve storage life and the heads are thrown overboard (Idyll, 1963).

A major problem facing the shrimp industry concerns wastes which are left after the extraction of meat for canning and freezing (Mendenhall, 1971). Approximately 65% of hand-cleaned shrimp is waste (Wigutoff, 1953) while 82% of machine-cleaned shrimp is waste (Jensen, 1965). Since it has been declared illegal to dump processing wastes in natural bodies of water, technologists have been trying to find saleable by-products which might be derived from the wastes (Mendenhall, 1971).

Crude waste meal, prepared by grinding and drying waste material in a heated rotary drum, is a major outlet for processing wastes. The meal is used in feeds for cattle, swine, and poultry (Morrison, 1956; Borgstrom, 1962). Shrimp meal also goes into fish feed. The red pigment of shrimp, astaxanthin, can supply the pink color characteristic of trout (Rousseau, 1960; Steel, 1971) and the Oregon moist diet can contain 4% shrimp or crab meal (Crawford and Law, 1972). Astaxanthin is readily oxidized so care must be taken when meal is prepared for fish feed (Rousseau, 1960).

Unprocessed shrimp waste in the form of a paste has been used to feed hatchery fish (Evans, 1968). Law and Crawford (1970) evaluated unprocessed shrimp scrap in fish feeds and found it could be used for feeding salmon and trout. The technology of unprocessed shrimp waste is complicated by powerful digestive enzymes which are contained in the heads. Grajcer (1972) found that digestive enzymes of Gulf shrimp were very unstable at pH 1, 8 and proposed pH adjustment as a means of stabilizing raw shrimp waste. Ground scraps were adjusted to pH 1. 8 with concentrated HCl and held 6-24 hr before readjustment to pH 7 with NaOH. This method of stabilizing scrap may have nutritive advantages over methods which use heat and could lead to the expanded use of shrimp scrap.

Chitin (polymer of B-linked N-acetyl-D-glucosamine units) shrimp waste has potential industrial value in the form of a derivative called chitosan. Chitosan can be used in moisture proof films and coatings, sizes for paper and textiles, coatings for glass fibers to permit dying, water and waste coagulants, oil well drilling additives, thickeners for foods, paints and inks, gels, ion-exchange resins, and films and encapsulation agents for pharmaceuticals (Peniston et al., 1969; Mendenhall, 1971).

A high quality protein concentrate can be made from crustacean waste (Peniston et al., 1969). The process involves extraction of protein with dilute alkali and a reduction in alkalinity before the extract

is spray dried. The product contains 90% protein.

Although numerous potential uses and means of processing shrimp waste have been investigated, the demand for waste is not nearly large enough to absorb all the waste from processing. Utilization and disposal of the waste remains a major problem for the seafoods industry.

Post-Mortem Changes in Shrimp

Post-mortem changes in shrimp are assumed similar to those that occur in fish. Factors responsible for spoilage include bacteria, digestive enzymes, and chemical reactions such as those which cause the oxidation of lipids (Bramsnaes, 1965). Bacteria are primarily responsible for the spoiled odor of seafood (Liston et al., 1963). The primary result of autolysis is softening of texture and although autolysis does not contribute directly to off odors, it may provide products that speed up microbial spoilage. Autolytic products are also capable of serving as substrate for an enzymatic browning reaction found in shrimp (Bailey and Fieger, 1954; Faulkner and Watts, 1954; Bailey et al., 1960). Phenol-amine derivatives are converted to melanins which give the shrimp an undesirable black color.

Shrimp collagen contains lower quantities of imino acids, proline and hydroxyproline which appear to be replaced by tryptophane and the ratio of unformed collagen to collagen laid down as a tissue is

much greater than for other animals (Thompson and Thompson, 1968; Thompson and Thompson, 1970a; Thompson and Thompson, 1970b). Shrimp tissues are more delicate than tissues of other animals because of the reduced hydroxyproline content and this may be the reason for the ease with which shrimp tissues are degraded by enzymes (Thompson and Farragut, 1971).

Proteolytic enzymes of fish may be found as cathepsins in tissue or located in the gut as important constituents of digestive fluid. The latter are usually the most active and their importance in spoilage is reflected by the widely recognized procedure of removing the viscera of certain fish immediately after catching. Rather dramatic effects of digestive enzymes can be experienced in species such as herring or mackerel which are left whole due to the large expense of gutting. Here, a condition known as "belly burn" can occur as the result of proteases in the digestive fluid.

The relative importance of the various spoilage factors towards the quality of shrimp depends upon how the shrimp are handled. Large Gulf shrimp are usually headed after catching so that digestive juices are removed with the guts. Here, bacteria probably play the major role in spoilage. Digestive enzymes may have a role in the spoilage of small Pacific shrimp which are not cleaned prior to storage on ice.

A complicated relationship exists between processing, quality, and autolysis of Pacific shrimp. Fresh shrimp cannot always be

efficiently machine-peeled and an aging time of two days is required to facilitate mechanized cleaning (Collins, 1960; Collins and Kelley, 1969). There is a flavor reduction in shrimp upon storage but certain palatability attributes such as texture and juiciness may improve (Flores and Crawford, 1973). It is possible that autolytic changes may be promoting conditions for bacterial spoilage at the same time certain desirable rheological attributes are being improved.

Numerous studies have been made on the biochemical changes that occur in shrimp after death. Researchers have been trying to develop numerical indices of quality by correlating biochemical changes with quality. Although trends in biochemical changes are recognized, it has been difficult to assign absolute numerical values to quality. There is an increase in pH upon storage (Bailey et al., 1956; Iyengar et al., 1960; Bethea and Ambrose, 1962; Flores and Crawford, 1973), and the numbers of bacteria increase (Green, 1949; Cambell and Williams, 1952; Fieger and Friloux, 1954; Flores and Crawford, 1973). Trimethylamine oxide decreases upon aging (Flores and Crawford, 1973). Storage on ice results in increases in trimethylamine (Collins et al., 1960; Iyengar et al., 1960; Bethea and Ambrose, 1962; Flores and Crawford, 1973). Post-mortem increases in formaldehyde and dimethylamine also occur (Flores and Crawford, 1973). Amino nitrogen decreases upon storage (Fieger and Friloux, 1954; Gagnon and Fellers, 1958; Velankar and Gouindan, 1958; Gagnon

and Fellers, 1958; Collins et al., 1960; Flores and Crawford, 1973). The carotenoid content of shrimp decreases upon storage and has been proposed as an index of quality (Faulkner and Watts, 1955; Collins and Kelley, 1969; Kelley and Harmon, 1972). Although various trends have been observed, the direct effects of post-mortem changes upon acceptance have not been clearly established.

MATERIALS AND METHODS

Source of Shrimp

Samples of Pacific shrimp were obtained through the help of commercial fishermen. Live shrimp were frozen on Dry Ice immediately after the trawl net was removed from the water. Upon landing, the shrimp were placed in polyethylene bags, which were then placed in polyethylene laminated pouches. The pouches were sealed under 29 in vacuum and stored at -30°F until used.

Dissection of Shrimp and Preparation of Crude Homogenates

Frozen whole shrimp were permitted to partially thaw before separation into edible and inedible fractions. Heads, shell, and feet in naturally occurring proportions constituted the inedible fraction while the remaining muscle represented the edible portion. For certain experiments, the heads were further dissected to separate the hepatopancreas and foregut. Homogenates were prepared by blending cold distilled water and shrimp parts for one minute with a Tissumizer blender (Tekmar Co.). Depending upon the experiment, 6 to 75 parts of water were blended with 1 part of shrimp.

Determination of Proteolytic Activity

Proteolytic activity was determined in the basic to slightly acid pH range with casein as substrate, and under acid conditions with hemoglobin as substrate.

Assay with Casein as a Substrate

The assay was a modification of a method outlined by Kakade et al. (1970). Substrate was prepared in 250 ml batches. Ten gm of sodium caseinate (J. T. Baker Co.) were dispersed in 225 ml distilled water. The mixture was placed in a boiling water bath until all the casein had been solubilized. After cooling, the pH was reduced to 6.25 by rapidly mixing in 1 N HCl, and 6.25 mg Thimerosal, N. F. (sodium ethylmercurithiosalicylate) were added before the mixture was diluted to 250 ml. The 4% sodium caseinate solution was stored at 4°C.

The assay was carried out in 25 ml Erlenmeyer flasks which were placed in a shaker bath at 37°C. Two ml of 0.4 M potassium phosphate buffer (pH 6.25), 1 ml 4% sodium caseinate (pH 6.25), and 0.5 ml distilled water were permitted to reach temperature equilibrium before the addition of 0.5 ml enzyme preparation. The reaction was terminated after 15 min by the addition of 10 ml of an acetate buffered trichloroacetic acid (TCA) solution (18 gm TCA, 18 gm

anhydrous sodium acetate, and 20 ml glacial acetic acid per liter). Approximately 30 minutes after TCA addition, the samples were filtered through Whatman No. 1 filter paper. Non-incubated blanks were prepared by the addition of TCA solution to substrate, buffer, and water followed by the addition of enzyme. Absorbance at 275 nm was measured with a Beckman D. B. spectrophotometer against a reagent blank.

Total activity was expressed as the change in absorbance for 15 min incubation while specific activity was represented as activity per 15 min per mg of shrimp protein. Protein for all work was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Assay with Hemoglobin as a Substrate

The assay was a modification of the method developed by Anson (1938). Substrate was prepared in 250 ml batches. Fifteen gm of denatured hemoglobin powder (hemoglobin standardized for protease assay -- Nutritional Biochemicals Corp.) were dispersed into 150 ml of distilled water. The mixture was dialyzed against 40 volumes of distilled water for two days at 4°C with four changes of water. After dialysis, the hemoglobin solution was diluted to approximate volume and the pH was reduced to 3.5 with 1N HCl. Thimerosal, N. F. (6.25 mg) was added before the mixture was diluted to 250 ml. The 6%

hemoglobin solution was stored at 4°C.

The final assay mixture contained 2 ml of 0.4 M acetate buffer (pH 3.5), 1 ml of 6% hemoglobin (pH 3.5), 0.5 ml distilled water, and 0.5 ml of enzyme preparation. The method of conducting the assay was exactly as that described for casein except a 10 min incubation time was used. Total activity was expressed as change in absorbance for 10 min while specific activity was expressed as change in absorbance per 10 min per mg shrimp protein.

Determination of Autolytic Activity

Shrimp were separated into edible and inedible portions and each portion was homogenized with eight parts of cold distilled water. The inedible portion was passed through a loose mat of glass wool to remove pieces of shell that might impair pipetting. The muscle and inedible portion homogenates were subdivided into 75 and 20 ml aliquots, respectively, and frozen at -30°F in beakers covered with cellophane. Sufficient homogenates were prepared to permit running of all experiments on subsamples of the same lot.

Both portions were thawed just prior to use. Determinations were carried out in 125 ml Erlenmeyer flasks which were placed in a shaker bath at 37°C. Five ml of distilled water and 12.5 ml of muscle homogenate (12.7 mg protein per ml) were permitted to reach equilibrium temperature in the flasks (5 min). Depending upon the

experiment, substances such as acid, base, or salt, were added to the reaction mixture at the expense of water while keeping the reaction mixture volume constant. To start the reaction, 2.5 ml of the inedible portion homogenate (10.2 mg protein per ml) were added and after 1 min of mixing, duplicate 2 ml aliquots were taken and each was added to 5 ml of acetate buffered TCA. These duplicates represented 0 time. Aliquots were then taken at appropriate time intervals and allowed to stand 30 min before being filtered through Whatman No. 1 filter paper. Attempts to measure protein hydrolysis as a function of change in absorbance at 275 nm failed because of erratic changes in absorbance. Breakdown products present in the filtrates were determined using Folin's reagent according to the procedure of Ting et al. (1968). Three ml of filtrate were mixed with 6 ml of 0.5 N NaOH. Two ml of diluted Folin's reagent (Phenol Reagent, Fisher Scientific Co.) were then added and the solution mixed. Exactly 8 min later, absorbance was measured at 650 nm against a distilled water reference.

Autolysis is used here in reference to the degradation of all types of shrimp protein in the 20 ml mixture which contained 159 mg of muscle protein and 25.5 mg of protein from the inedible portion. Autolysis for a time interval was expressed as the change in absorbance at 650 nm, obtained by subtracting the mean of 0-time duplicates from the mean of duplicates for the desired incubation period. To

assess the contribution of proteases in the muscle toward autolysis, controls were run using inedible portion which had been heat-treated (15 min in a boiling water bath). All experiments were carried out at least twice. A second experiment was run after a preliminary experiment had established the range of points needed to describe a parameter.

Polyacrylamide-Gel Electrophoresis

Autolytic mixtures (pH 7.4 and 3) of muscle and inedible portion were prepared as previously described and incubated at 50°C. Mixtures were also prepared in which water replaced the inedible portion. Aliquots were taken after 0, 10, 20, and 120 min of incubation, cooled on ice, and centrifuged (4°C) at 30, 900 x G for 15 min. The supernatants were frozen and held at -30°F for electrophoresis.

Electrophoresis was conducted with a vertical gel electrophoresis apparatus (Model EC-470, E-C Apparatus Corporation, Philadelphia, Pa.) and companion power supply (Model EC-454). The discontinuous technique as outlined in detail by Petropakis et al. (1969) was followed for proteins soluble at pH 7.4. Sample solutions were prepared by adding 1 ml of supernatant to 0.25 gm of sucrose and a minimum amount of Bromphenol Blue. Approximately 0.1 ml of sample were added to each gel slot. A 7% gel concentration was used for the plug and running gels while a 4% concentration was used for the

spacer gel.

The above technique was not satisfactory for the separation of proteins soluble in autolytic mixtures at pH 3. Protein precipitated when the pH increased and remained at the site of sample application during electrophoresis. The situation was improved by incorporating urea (5 M) and dithiothreitol (1 mM) in all gels, the sample, and electrode buffer according to the method of Petropakis (1971). In all other respects the method was identical to that used for soluble proteins from mixtures at pH 7.4 except 6% plug and running gels were used and the electrode buffer was made 0.0165 M in Tris. The pH of the electrode buffer increased to 8.7.

After electrophoresis was complete, gel slabs were stained for 30 min with dye (0.25% Amido Black 10 B, made up in 5:5:1 v/v/v, methanol, water, and acetic acid). The slabs were repeatedly bathed in a solution of methanol, water, and acetic acid (5:5:1, v/v/v) until clear patterns were obtained.

Characterization of Proteases Active at Low pH

Assay

Hemoglobin digestion as previously described was used to quantify the acid proteases. Tests were done in triplicate, unless otherwise stated, and a blank was run for each test.

Preparation of Enzymes for Chromatography

The hepatopancreas was dissected from 30-100 animals. Tissue was blended with ten parts cold 0.6 M NaCl and the homogenate was centrifuged in the cold (4°C) at 30,900 x G for 30 min. The supernatant was passed through a loose mat of glass wool to remove lipid which had accumulated on the surface. The supernatant was brought to 40% saturation with solid ammonium sulfate and held on ice for 30 min. The precipitate which formed was removed by centrifuging at 30,900 x G for 10 min. The supernatant was brought to 60% saturation with ammonium sulfate and held for 30 min on ice. The precipitate was collected by centrifuging as above and was dissolved in a minimum amount of buffer. Precipitate which did not dissolve was removed by centrifuging. When Sephadex chromatography was used, a 0.005 M potassium phosphate buffer (pH 6.5) was employed and the same buffer containing 0.15 M NaCl was used for ion-exchange chromatography. In addition, the enzyme preparation for ion-exchange chromatography was dialyzed against the dissolving buffer for 30 min on ice in a Bio-Fiber 50 Beaker (Bio-Rad Laboratories). Enzyme preparations were subjected to column chromatography immediately or were frozen since a rapid loss in activity was experienced in the concentrated state, presumably by autolysis.

Sephadex Chromatography

A column (Pharmacia, 2.5 x 50 cm) filled with Sephadex (Pharmacia, G-150) particle size 40-120 μ was equilibrated at 4°C with 0.005 M potassium phosphate buffer (pH 6.5). For the separation, 12 ml of enzyme solution were applied to the column and chromatography was performed with the equilibration buffer. A mariotte bottle was used to maintain a constant pressure head. The absorbance of effluent from the column was continuously recorded at 280 nm by a Gilson absorption meter. Ten ml fractions were collected. Single activity determinations were made on tubes collected from chromatography.

Ion-Exchange Chromatography

Pre-swollen microgranular diethylaminoethyl (DEAE) cellulose (Whatman, DE-52) was packed into a column (Pharmacia, 2.5 x 50 cm) and equilibrated with starting buffer (0.15 M NaCl in 0.005 M potassium phosphate buffer, pH 6.5). For chromatography of the enzyme, 25 ml of preparation were applied to the column and starting buffer was pumped through the column until the first protein peak had been detected with a Gilson absorption meter. A linear phosphate buffered salt gradient (800 ml) from 0.15 to 0.6 M NaCl was used to elute the column. Ten ml fractions were collected.

RESULTS AND DISCUSSION

Distribution of Proteolytic Enzymes

Weights and Proportions of Various Shrimp Parts

Twenty shrimp from each of two catches were dissected and the weights of different shrimp parts were determined (Table 1). There were no significant differences between the catches regarding hepatopancreas, total inedible portion, muscle, and body weight (t-test, $P > 0.1$). The foreguts from catch 1 (July 1973) were slightly larger (t-test, $P < 0.1$) than the foreguts from catch 2 (September 1973). Shrimp from the former appeared to have more food in their foreguts than the latter and this could account for the difference in weight. Shrimp weighed about 4.5-5 gm and the yield of waste (63-64%) agreed closely with the value of 65% reported by Wigutoff (1953). Shrimp parts and organs are pictured in Figures 1 and 2, respectively.

Evaluation of Assays

The response with casein as a substrate was not exactly linearly proportional to enzyme concentration since a slight downward curvature was observed (Figure 3). This type of behavior is common in proteolytic assays where proteins serve as substrate (Dixon and Webb, 1964). A linear approximation was used to estimate the

Table 1. Weights and proportions of various shrimp parts.[a]

Shrimp part	Catch 1 July 1973 Mean ± standard deviation	Range	% of body weight	Catch 2 September 1973 Mean ± standard deviation	Range	% of body weight
Hepatopancreas	0.39 ± 0.10	0.15-0.54	8.4	0.34 ± 0.13	0.15-0.63	6.8
Foregut[b]	0.23 ± 0.08	0.10-0.40	5.0	0.18 ± 0.09	0.07-0.34	3.6
Total inedible portion	2.93 ± 0.50	1.50-3.53	63.1	3.20 ± 0.84	1.68-4.88	63.8
Muscle	1.71 ± 0.29	0.92-2.30	36.9	1.82 ± 0.42	0.98-2.40	36.2
Body weight	4.64 ± 0.74	2.42-5.83	100.0	5.01 ± 1.21	2.76-7.28	100.0

[a]Weights are in gm per shrimp based on 20 randomly selected specimens from each catch.

[b]Catch 1 significantly different than catch 2, $P < 0.10$.

Figure 1. Shrimp parts: (A) whole shrimp; (B) inedible portion (heads and shell); (C) edible portion (muscle).

Figure 2. Shrimp digestive organs: (A) foregut; (B) hepatopancreas.

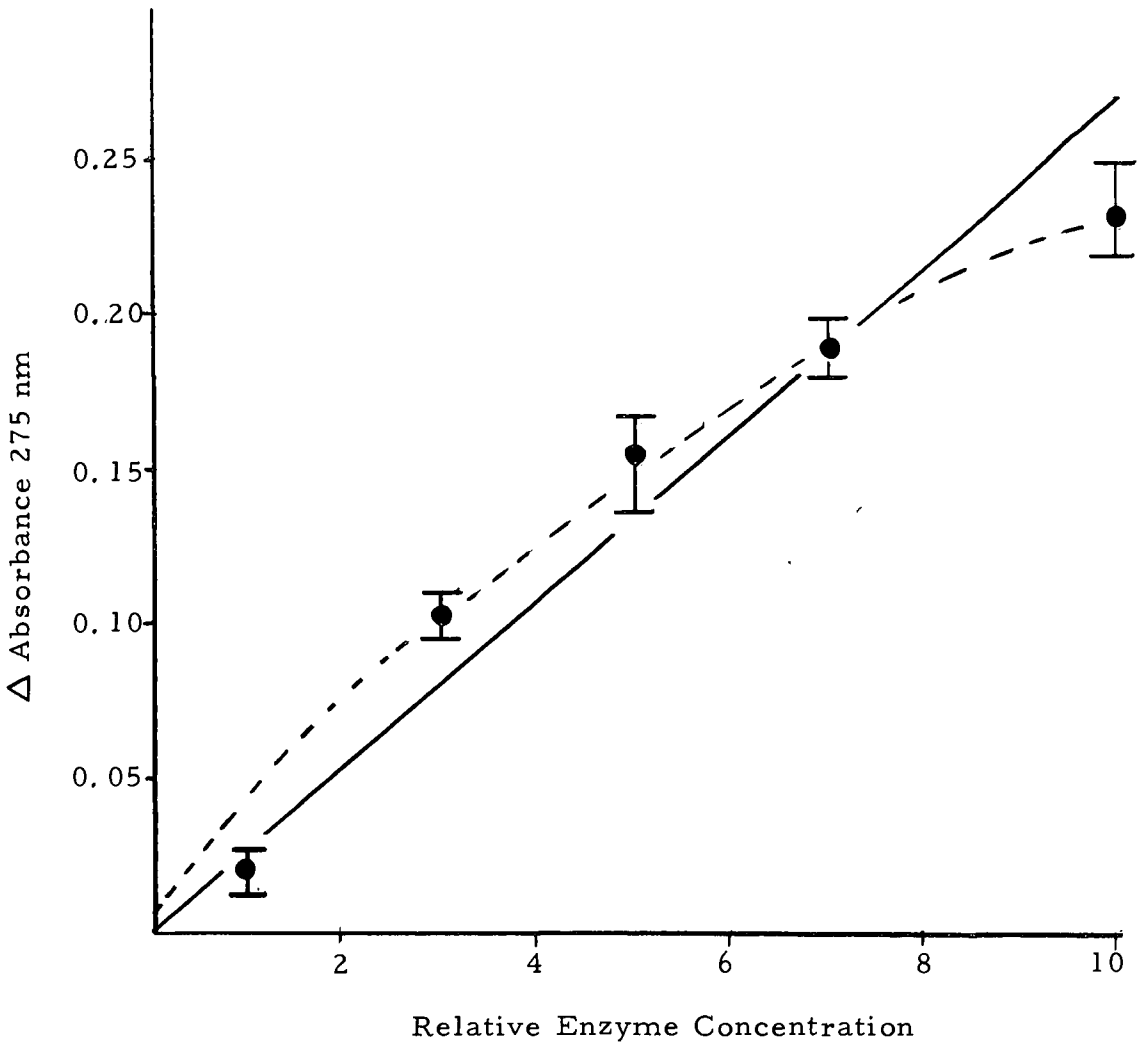


Figure 3. Activity of proteases from the inedible portion on casein, pH 6.25, as a function of enzyme concentration (10 corresponds to crude homogenate of 1 part shrimp to 6 parts water). Triplicates, I--Range, ●--Mean.

activity of crude homogenates.

The casein assay called for the use of an acetate buffered TCA solution (pH 4.3), while most protease assays use unbuffered TCA solutions. Preliminary investigations showed that use of the buffered TCA solution increased sensitivity by giving greater absorbance readings, probably through the solubilization of more products absorbing at 275 nm.

The response using hemoglobin as substrate, although linearly proportional to enzyme concentration, passed slightly above the origin as the enzyme concentration decreased (Figure 4). This phenomenon was noted by Gianetto and deDuve (1955) and by Whiting (1973) when hemoglobin was used as substrate in a protease assay. A linear response starting at the origin was assumed when estimating the activity of crude homogenates. Enzyme concentrations were manipulated to give readings in the 0.15-0.25 absorbance range to minimize error.

Effect of pH Upon Activity

The pH of reaction mixtures was varied by the addition of HCl or NaOH in place of water. Although the pH was sometimes outside the effective range of the buffers, the pH of the mixtures did not change during the assay.

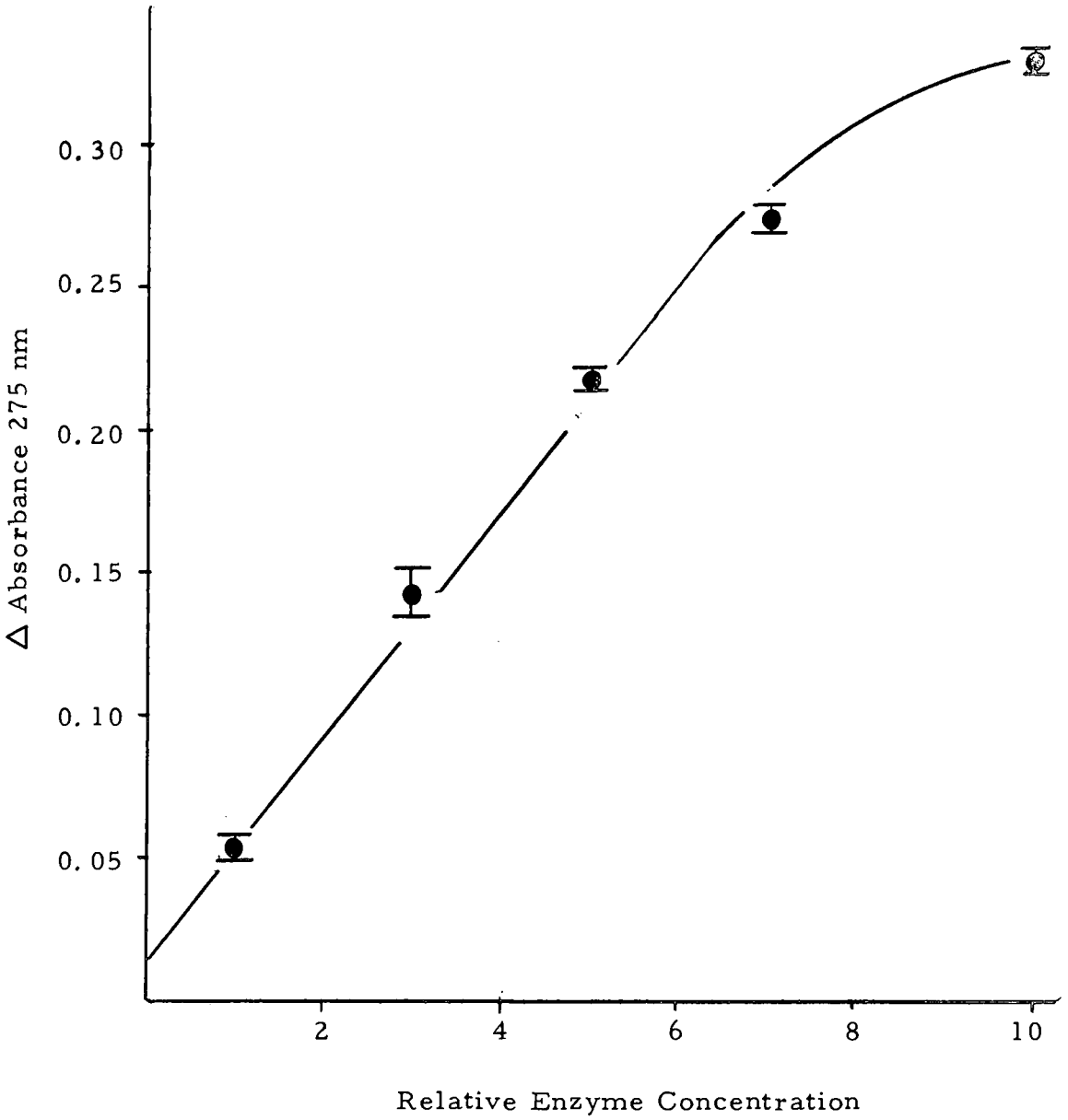


Figure 4. Activity of proteases from the inedible portion on hemoglobin, pH 3.5, as a function of enzyme concentration (10 corresponds to crude homogenate of 1 part shrimp to 8 parts water). Triplicates, I--Range, ●--Mean.

A crude homogenate of the inedible portion showed an optimum toward casein near pH 6.25, although activity was relatively uniform between pH 5.75 and 7.5 (Figure 5). The digestive enzymes of nearly all crustaceans show activity toward casein under basic to slightly acid conditions (DeVillez and Buschlen, 1967; van Weel, 1970).

Activity toward hemoglobin was maximum between pH 3 and 3.65 (Figure 6). Proteases active at low pH are unusual for Crustacea (Brokerhoff et al., 1970).

Location of Activity

The shrimp parts from two catches (Table 1) were analyzed for activity. It was necessary to determine activity immediately after dissection since a rapid loss in activity occurred upon standing. Results of activity on casein are presented in Table 2. Specific activity was greatest in the foregut followed by the hepatopancreas while low specific activity was found in the remainder of the inedible portion. Activity was negligible in the muscle. Total activity of the foregut of catch 1 was greater than that of the hepatopancreas while the reverse was true for catch 2. Shrimp of catch 1 contained more food in their foreguts, and greater activity is expected in foreguts actively engaged in digestion. For catch 1, total activity of the inedible portion minus digestive organs was slightly less than that of the hepatopancreas. For catch 2, the inedible portion minus digestive

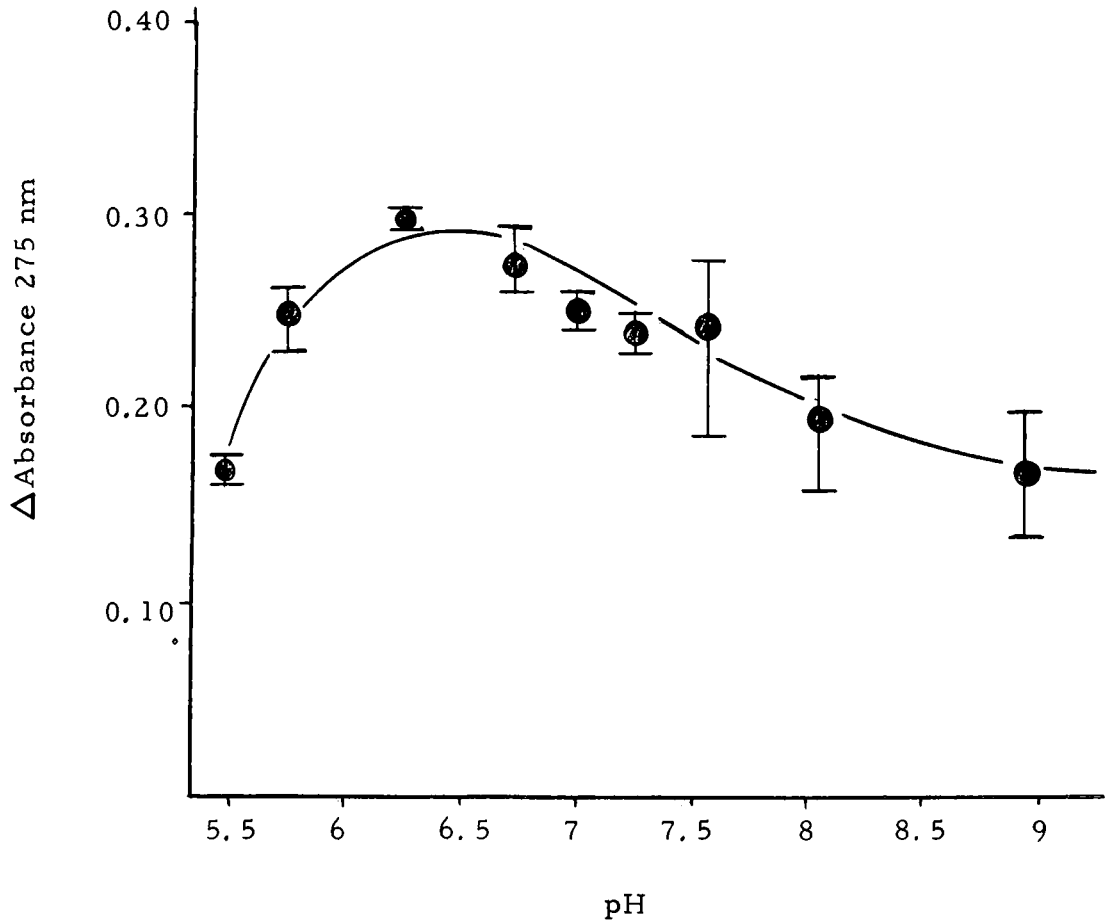


Figure 5. Activity of proteases from the inedible portion on casein as a function of pH. Crude homogenate of 1 part shrimp to 8 parts water. Triplicates, I--Range, ●--Mean.

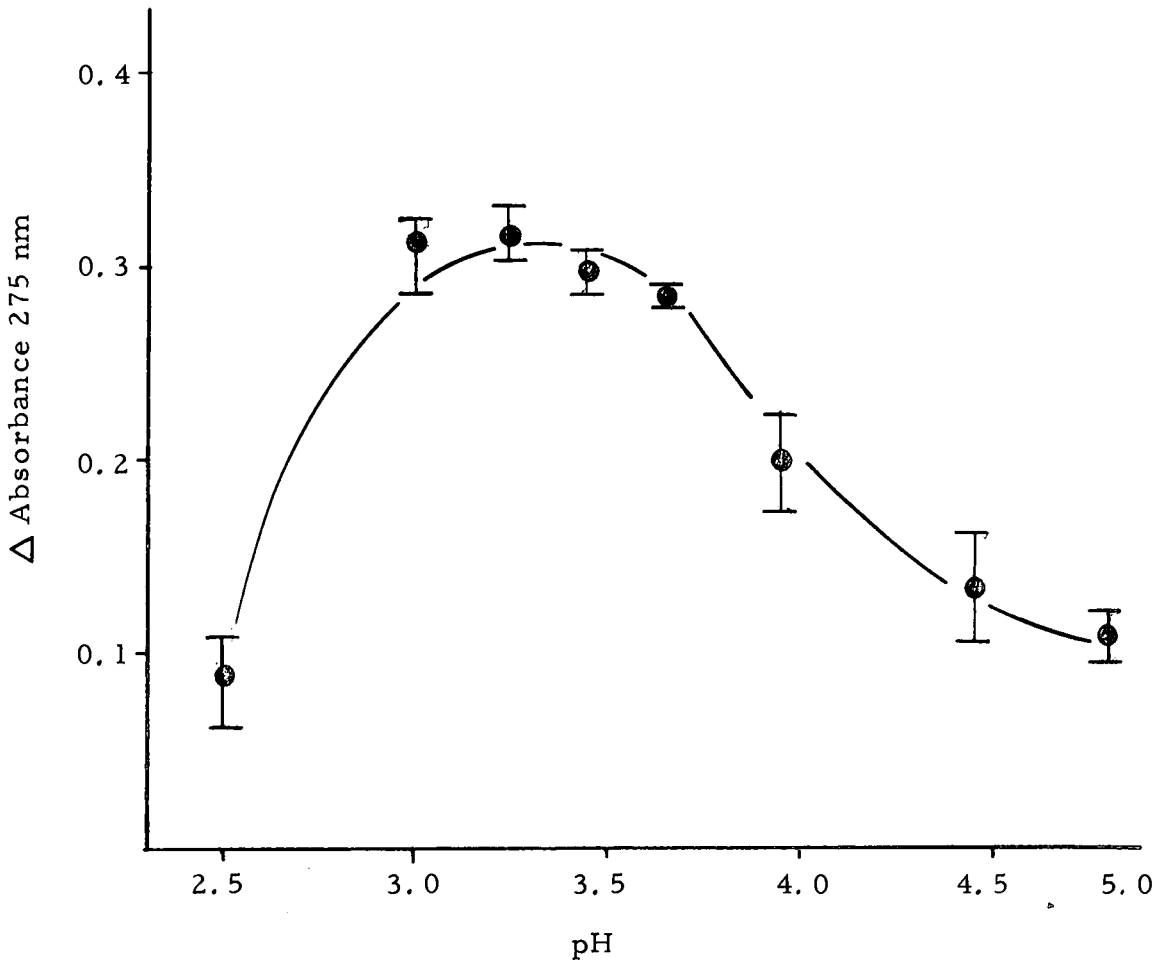


Figure 6. Activity of proteases from the inedible portion on hemoglobin as a function of pH. Crude homogenate of 1 part shrimp to 10 parts water. Triplicates, I--Range, ●--Mean.

organs had slightly less total activity than the foreguts.

Table 2. Total and specific activities of various shrimp parts on casein.[a, b, c]

Shrimp part	Catch 1 July 1973		Catch 2 September 1973	
	Total[d] activity	Specific[e] activity	Total[d] activity	Specific[e] activity
Hepato-pancreas	132 ± 10	0.187 ± 0.014	167 ± 4	0.160 ± 0.004
Foregut	238 ± 3	0.558 ± 0.008	111 ± 10	0.261 ± 0.022
Inedible minus foregut and he-patopancreas	88 ± 15	0.022 ± 0.004	107 ± 30	0.023 ± 0.007

[a]Values represent the mean ± standard deviation of four analytical replicates made on the pooled organs of 20 shrimp.

[b]Activity in the muscle was too low to be meaningful.

[c]When analyzing homogenates of hepatopancreas for protein by the method of Lowry et al. (1951), it was necessary to centrifuge assay mixtures just prior to absorbance measurement at 700 nm. A slight haze was present which gave abnormally high readings due to light scattering.

[d]$\Delta A^{275\ nm}_{1\ cm}$/15 min/20 shrimp

[e]Activity/mg protein

The results of activity toward hemoglobin were similar to those found for casein (Table 3). Activity was found primarily in the hepato-pancreas and foregut while the ratio of activity between the digestive organs depended upon the feeding status of the animal.

Table 3. Total and specific activities of various shrimp parts on hemoglobin.[a, b]

Shrimp part	Catch 1 July 1973		Catch 2 September 1973	
	Total[c] activity	Specific[d] activity	Total[c] activity	Specific[d] activity
Hepato-pancreas	153 ± 10	0.217 ± 0.014	186 ± 12	0.178 ± 0.011
Foregut	317 ± 7	0.744 ± 0.015	138 ± 3	0.325 ± 0.008
Inedible minus foregut and hepato-pancreas	121 ± 33	0.029 ± 0.008	130 ± 9	0.029 ± 0.002

[a]Values represent the mean ± standard deviation of four analytical replicates made on the pooled organs of 20 shrimp.

[b]Activity in the muscle was too low to be meaningful.

[c]$\Delta A \frac{275\ nm}{1\ cm}$/10 min/20 shrimp

[d]Activity/mg protein

Negligible activity in the edible portion does not imply the absence of muscle cathepsins. Muscle cathepsins have been found for the white shrimp, Penaeus setiferus (Bauer and Eitenmiller, 1974; Eitenmiller, 1974). The short incubation times employed here were designed for the highly active digestive enzymes. An incubation period of several hours with a concentrated enzyme extract is required for measurement of weak muscle catheptic activity.

Proteolytic enzymes in the hepatopancreas originate from that organ. Activity in the foregut is due to enzymes that originate in the

hepatopancreas although it is possible for the ingested food to contain proteases. Activity in the remainder of the inedible portion may be the result of cathepsins present in other tissues (ovaries, gills, etc.).

The hepatopancreas should be relatively free of bacteria while the foregut and exterior portion of the shrimp may contain significant numbers of microbes. Results of studies conducted by Flores and Crawford (1973) suggested that bacteria do not make a major contribution to proteolytic activity. The casein digesting activity of iced Pacific shrimp decreased upon storage while the total numbers of bacteria increased.

Caution must be exercised when drawing conclusions regarding the biological functions of proteases which are studied from crude homogenates of a digestive organ. Activity is the result of enzymes present in the digestive fluid as well as those present in the cells of the tissue. The latter may be cathepsins which are never secreted and are not involved in the digestion of food. Studies concerned with enzymes involved strictly in digestion of food should be conducted on digestive fluid rather than on a homogenized organ. Existence, rather than biological function, is the most important consideration for studies on post-mortem quality changes.

Autolytic Activity

Effect of pH on Autolysis

Autolysis was measured from pH 2 to 9 in increments of 1 $\pm$ 0.15 pH units. The pH of the water homogenate was 7.4. Under these conditions, only negligible activity was observed where reaction mixtures contained heat-treated inedible portion (Figure 7). Autolysis was due almost entirely to proteases in the inedible portion. A major maximum of activity was found near pH 3 and a minor broad maximum was found at pH 7-9. The proteases of shrimp were capable of digesting native proteins over a wide pH range.

Effect of Time on Autolysis

The effect of time on autolysis was determined at pH 3 and 7.4. The degradation products of mixtures containing heat-treated and nonheat-treated inedible portion were determined at various intervals in a 2 hr incubation period. Approximately 50% of the autolysis for the 2 hr incubation period occurred within the first 20 min (Figure 8). Although negligible digestion occurred within the first 20 min for mixtures with heat-treated inedible portion, the presence of muscle proteases was evident after longer incubation periods. Activity was greater at pH 3 than 7.4. The methods used here could be modified to study muscle cathepsins. An extended incubation period and a

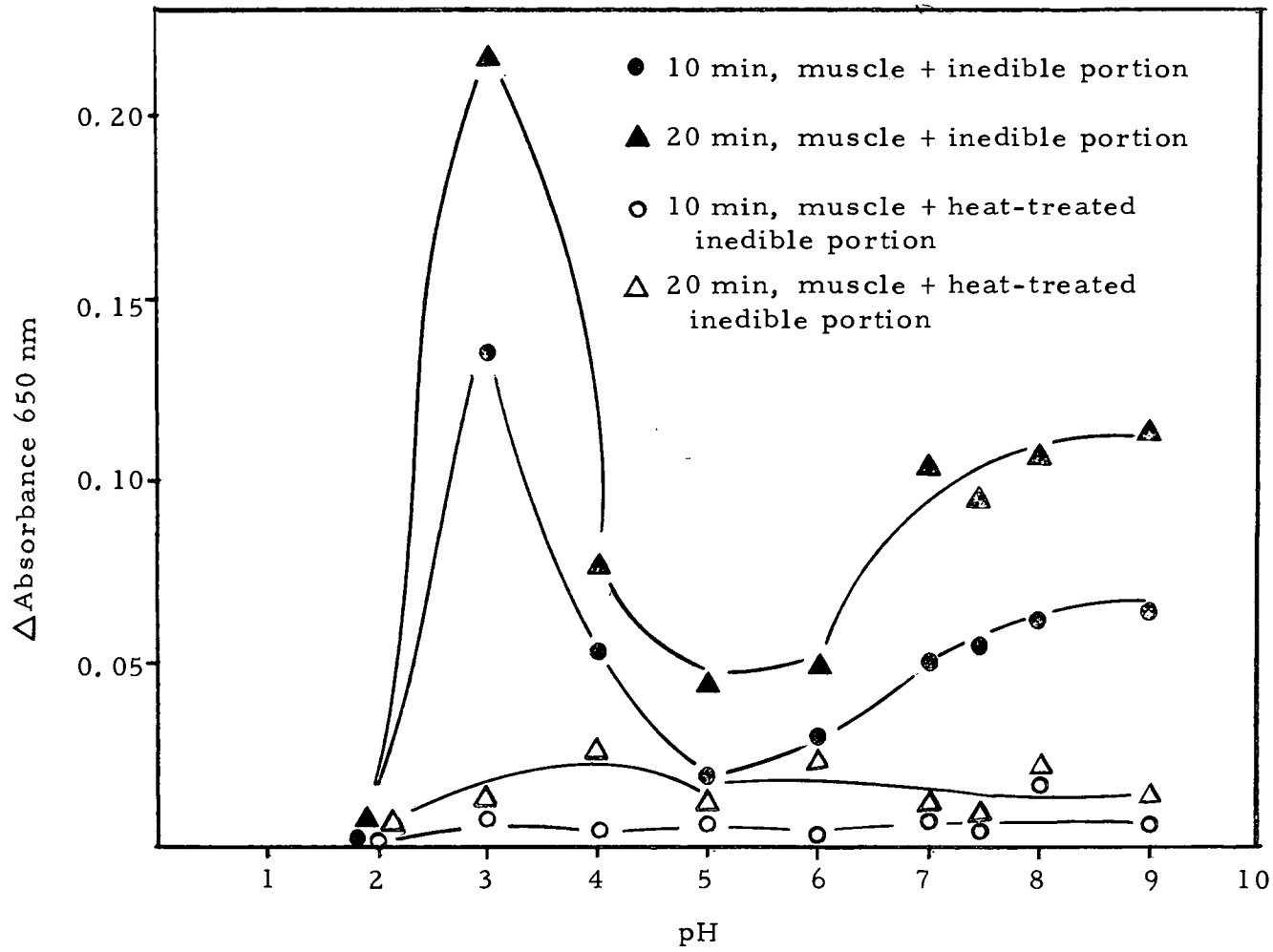


Figure 7. Effect of pH on autolysis.

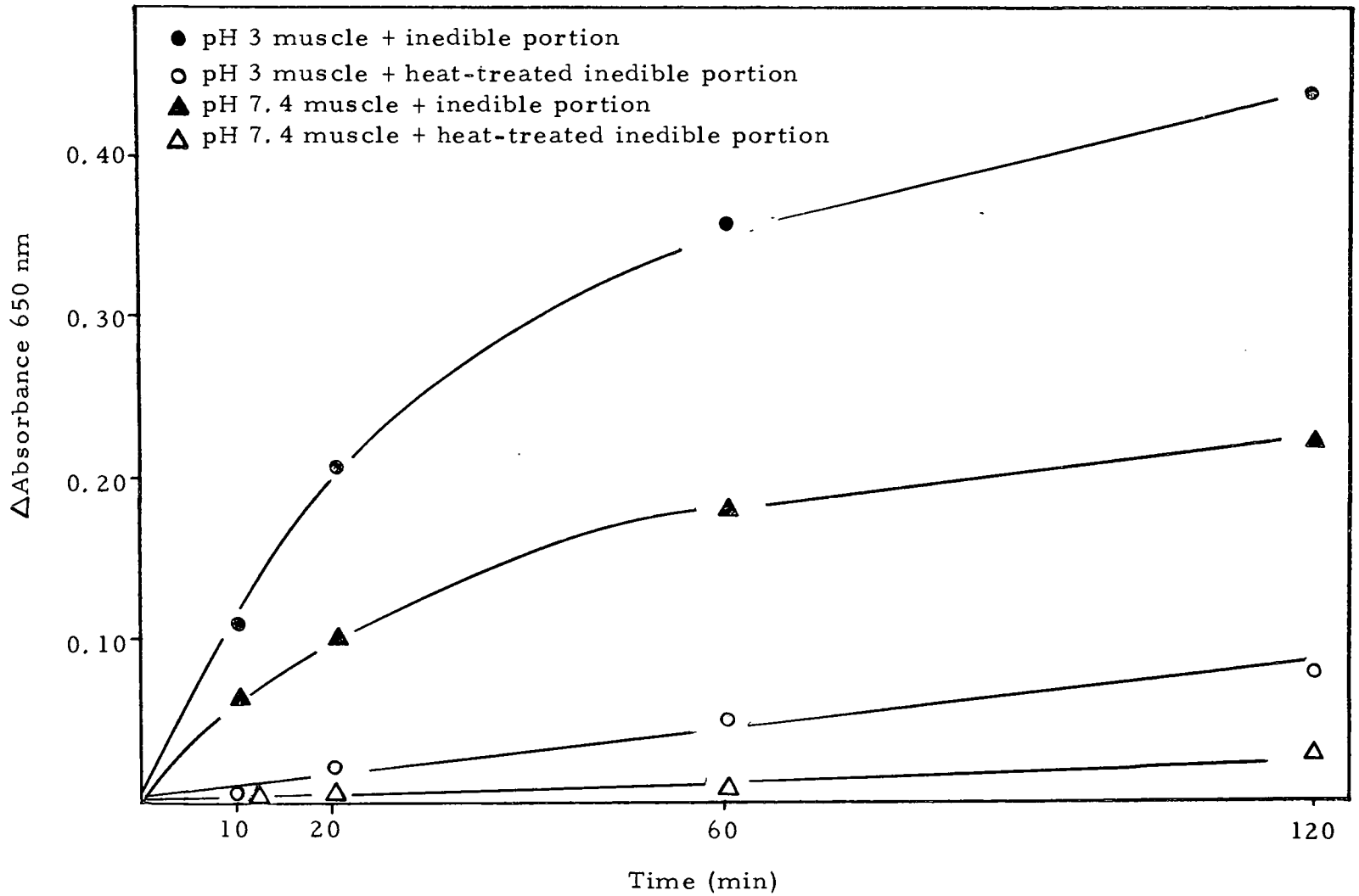


Figure 8. Effect of time on autolysis.

more concentrated muscle preparation would be required.

Effect of Substrate Concentration on Autolysis

The amount of protein in reaction mixtures was changed by varying the amount of muscle while maintaining constant pH. The effect of substrate concentration on activity at pH 3 is illustrated by Figure 9 and at pH 7.4 by Figure 10. Mixtures containing 7.5 ml of muscle homogenate were saturated with protein. Substrate was limiting near the naturally occurring ratio of muscle to inedible portion (2.5 ml muscle:2.5 ml inedible portion). Enough protein was present in the inedible portion to give detectable autolysis when no muscle was present and considerably higher activity was found at pH 3 than 7.4 under these conditions.

Effect of Enzyme Concentration on Autolysis

Autolysis was determined at different concentrations of inedible portion for 10 and 20 min incubation periods at pH 3 and 7.4. Activity was proportional to enzyme concentration at each pH (Figures 11 and 12). The data are replotted for fixed incubation periods (Figure 13). For 10 min at pH 3, autolysis was linearly proportional to concentration of the inedible portion. For 10 min at pH 7.4 and 20 min at pH 3 and 7.4, the relationship between autolysis and enzyme concentration deviated from linearity. Reasons for nonlinearity of a proteolytic

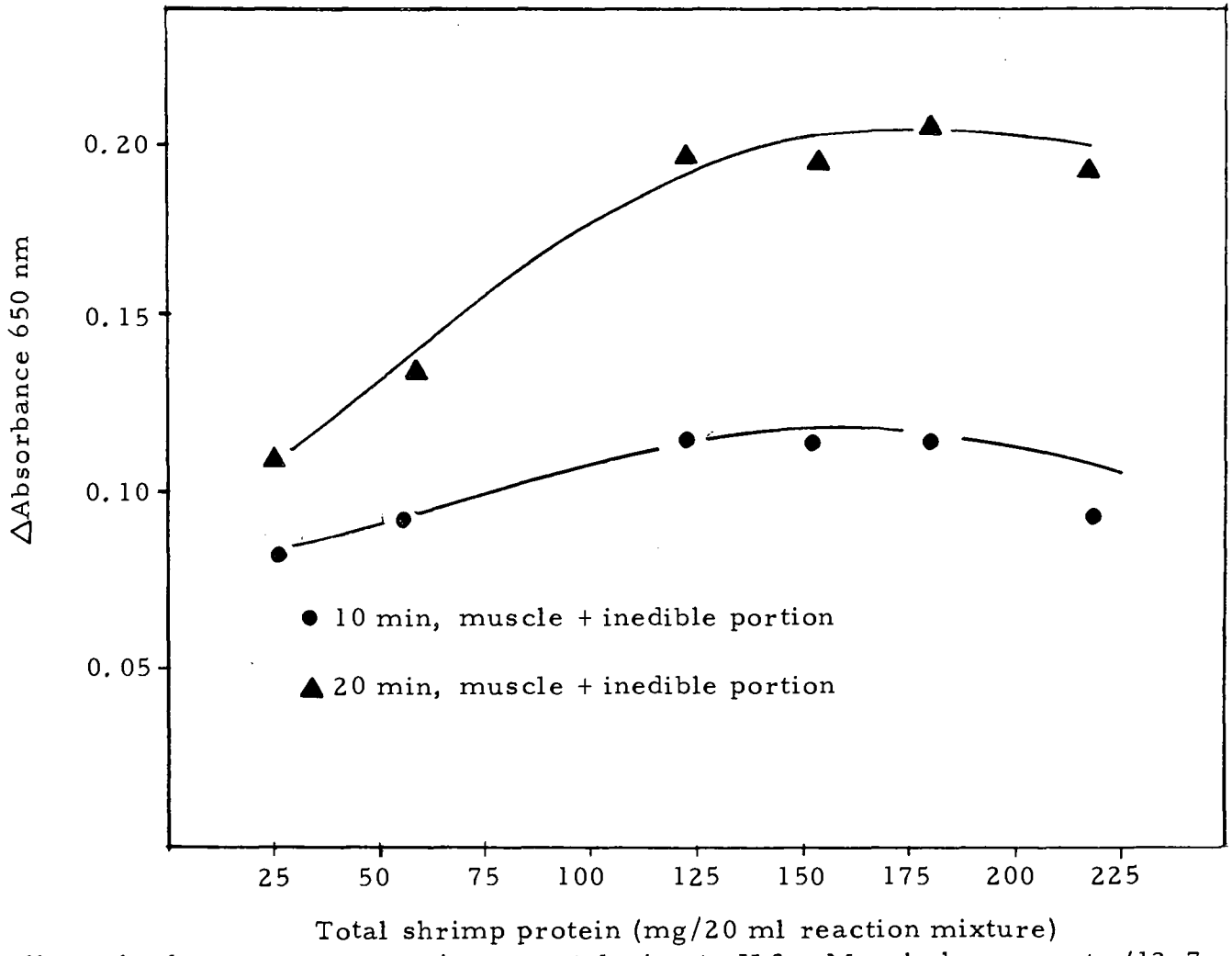


Figure 9. Effect of substrate concentration on autolysis at pH 3. Muscle homogenate (12.7 mg protein/ml); inedible portion (10.2 mg protein/ml).

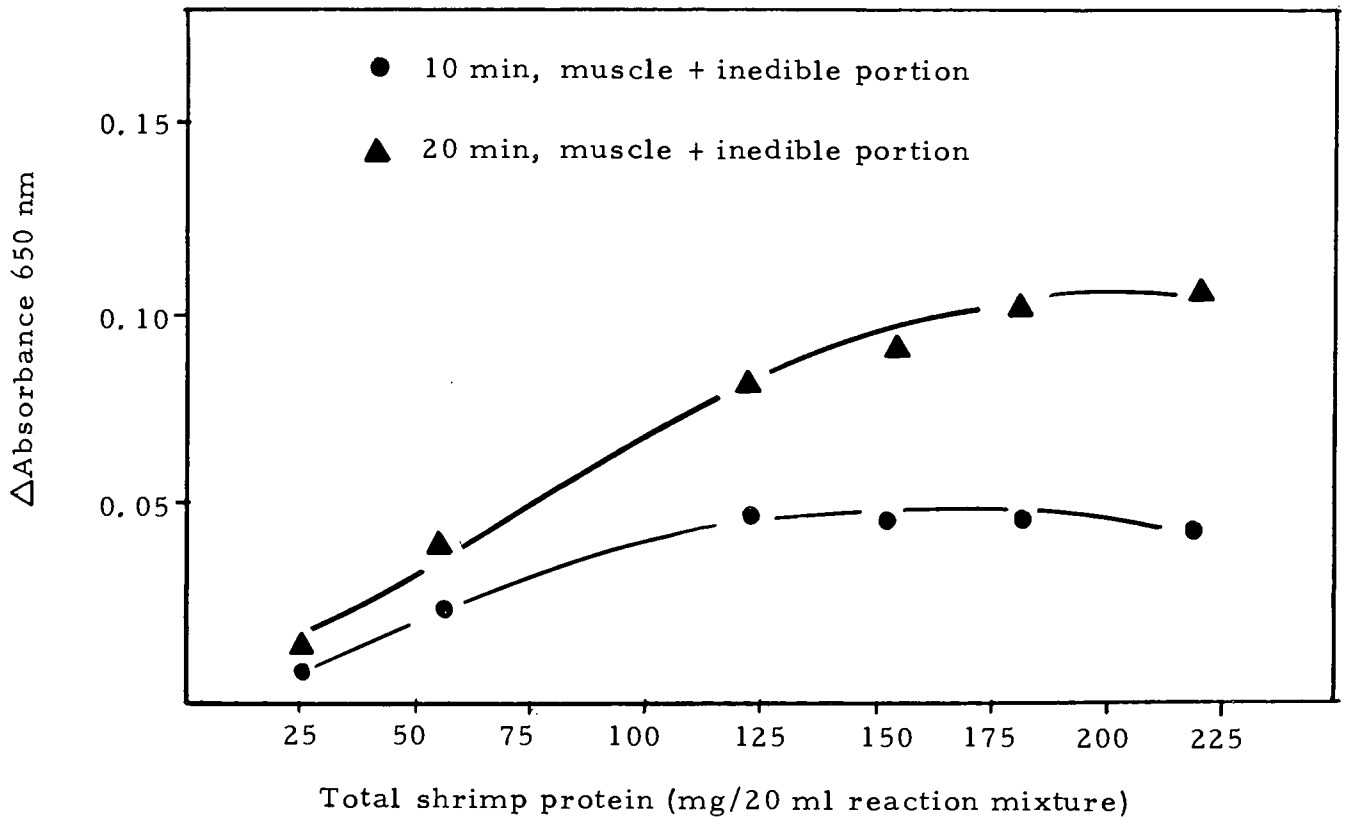


Figure 10. Effect of substrate concentration on autolysis at pH 7.4. Muscle homogenate (12.7 mg protein/ml); inedible portion (10.2 mg protein/ml).

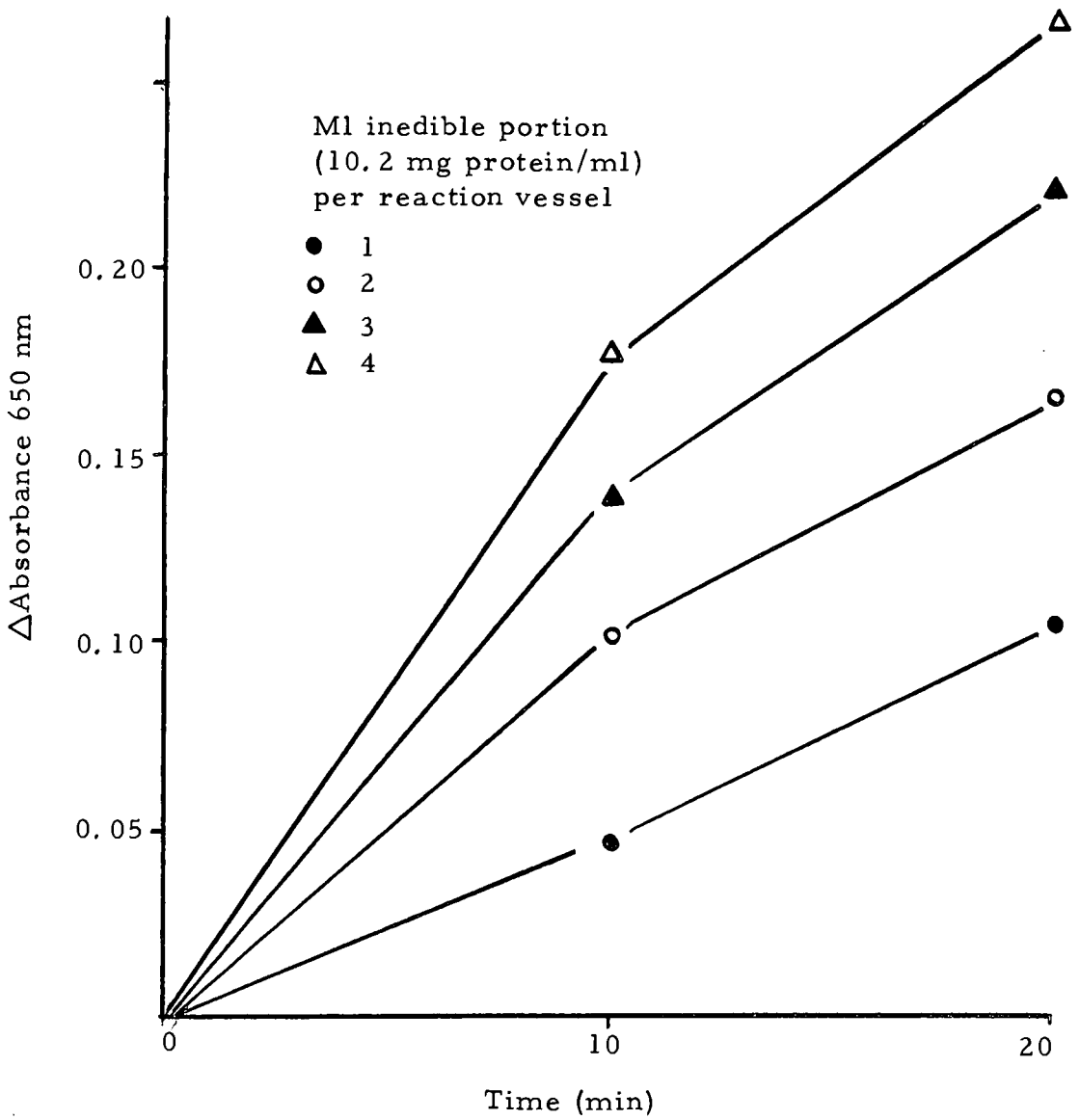


Figure 11. Effect of enzyme concentration on autolysis for 10 and 20 min incubation periods at pH 3.

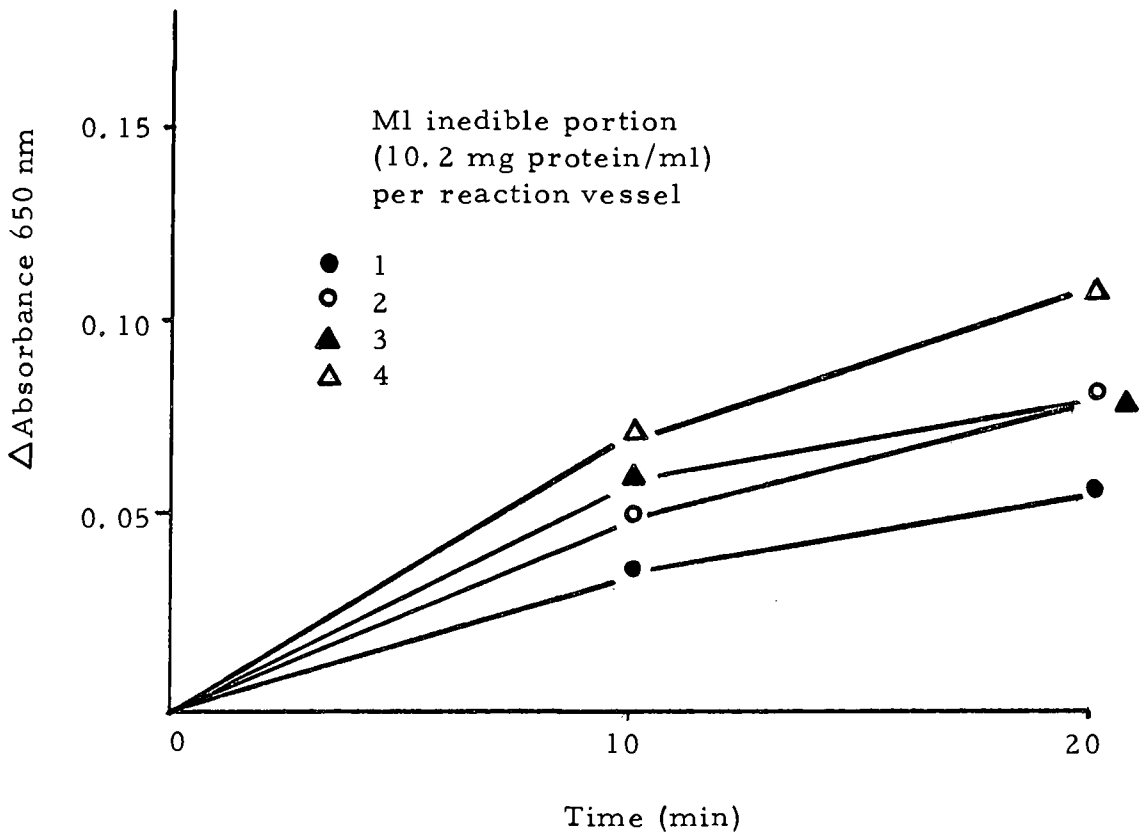


Figure 12. Effect of enzyme concentration on autolysis for 10 and 20 min incubation periods at pH 7.4.

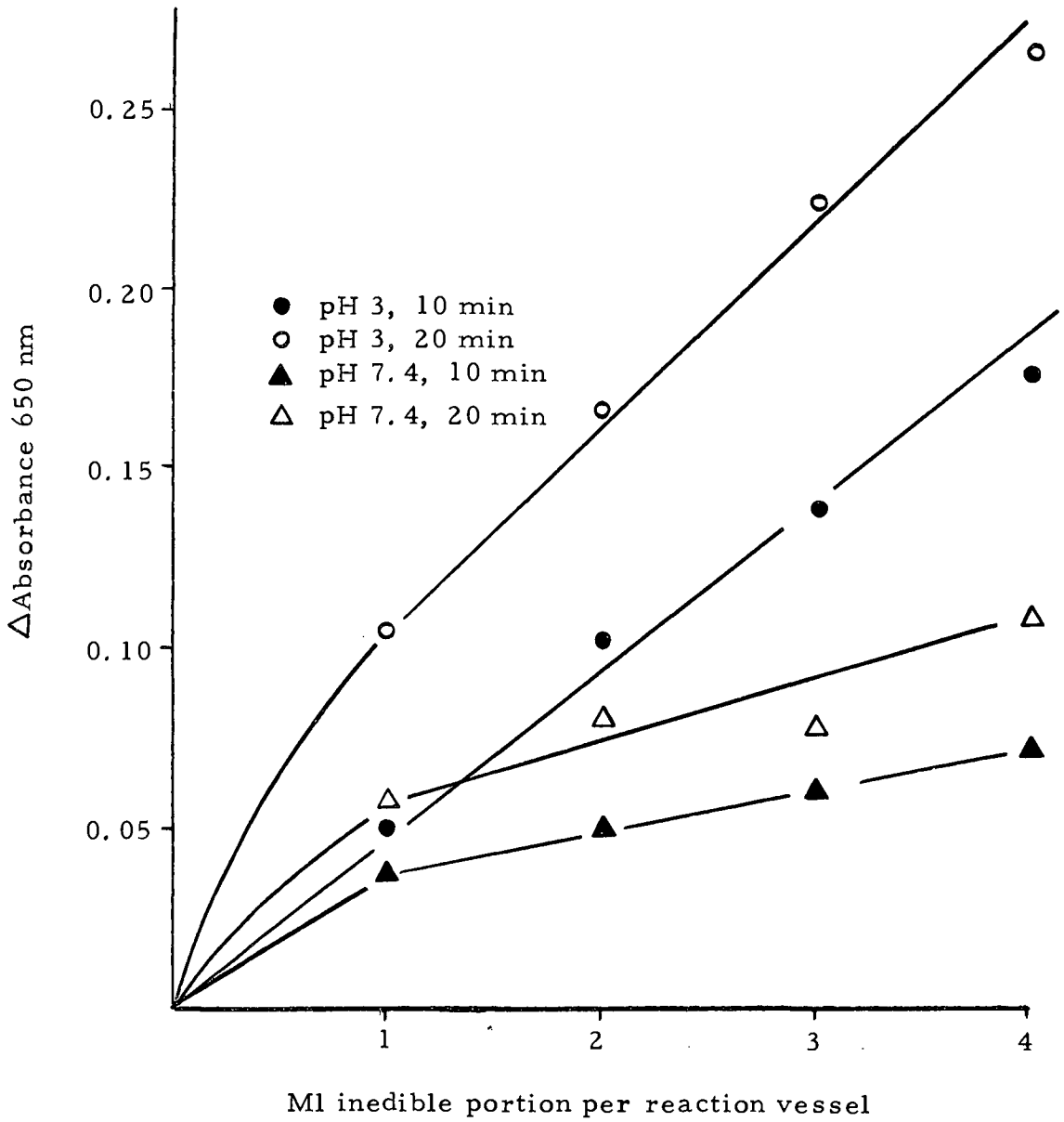


Figure 13. Effect of enzyme concentration on autolysis for a fixed incubation period.

assay include presence of inhibitors, time required for diffusion of substrate to the enzyme, peptide bonds which differ in susceptibility to attack, and errors in estimating initial velocity by determining product produced for a fixed time period (Dixon and Webb, 1964).

Effect of Temperature on Autolysis

The effect of temperature on autolysis was determined at pH 3 (Figure 14) and 7.4 (Figure 15). Maximum autolysis occurred near 50°C at pH 3 but decreased rapidly above 55°C. At pH 7.4, activity was greatest near 55°C while a gradual reduction occurred at higher temperatures. Preliminary experiments gave similar results when casein and hemoglobin were used as substrates at pH 7.5 and 3.5, respectively.

These findings may help explain the results of a study conducted by Collins and Kelley (1969). They evaluated different precook processes as substitutes for holding P. borealis on ice to precondition the shrimp for machine peeling. Peeling properties were improved by dipping the shrimp in a water bath at 110°F (43°C) or 130°F (54°C) for 2 min. These temperatures occur within the optimum range for autolysis. They found that heating at 150°F (66°C) for 2 min did not improve peelability. Autolysis did not occur at this temperature for pH 3 and was minimal at pH 7.4. They did find peeling improvement at 150°F or at higher temperatures when the precook was 15 seconds.

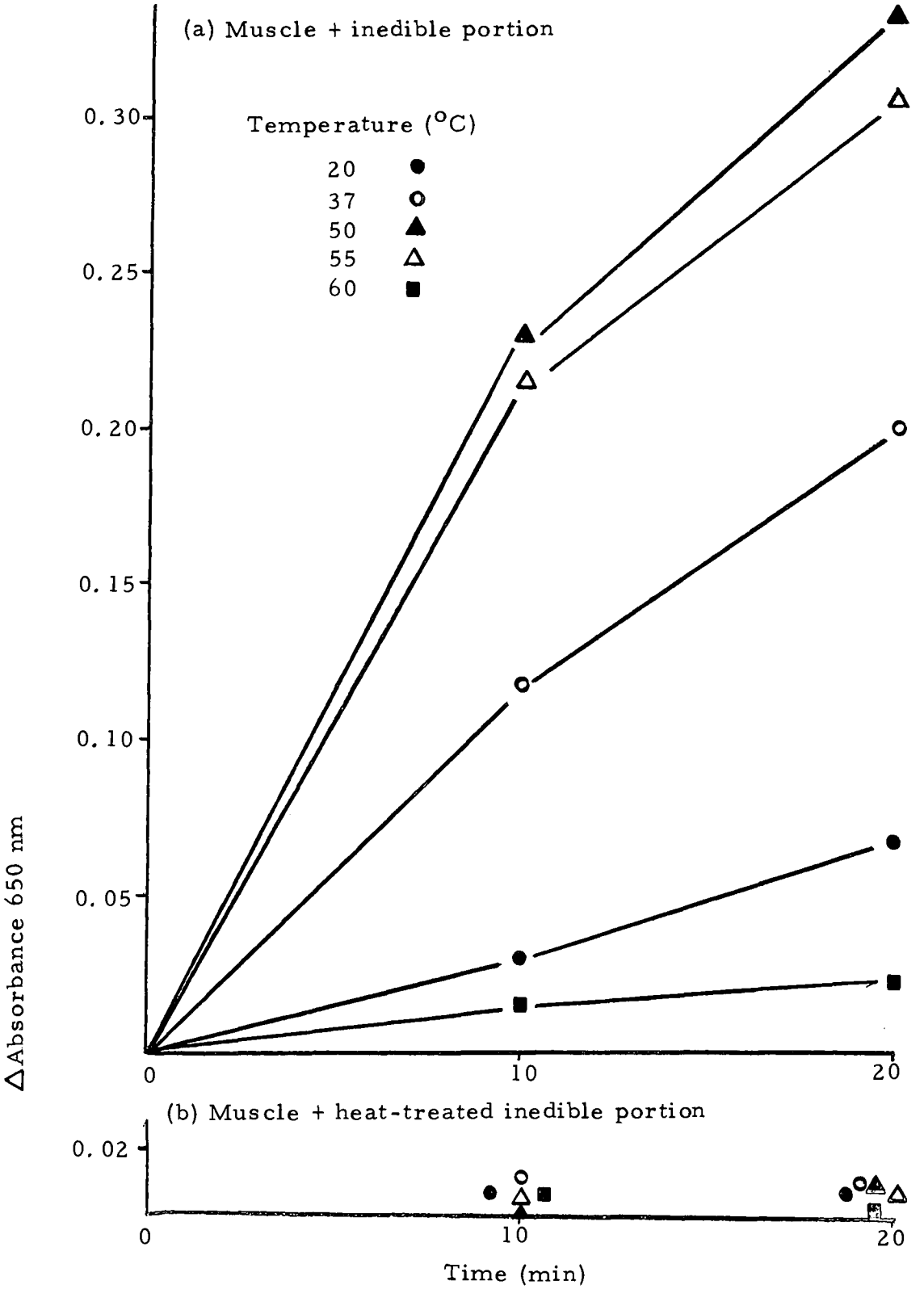


Figure 14. Effect of temperature on autolysis for 10 and 20 min incubation periods at pH 3.

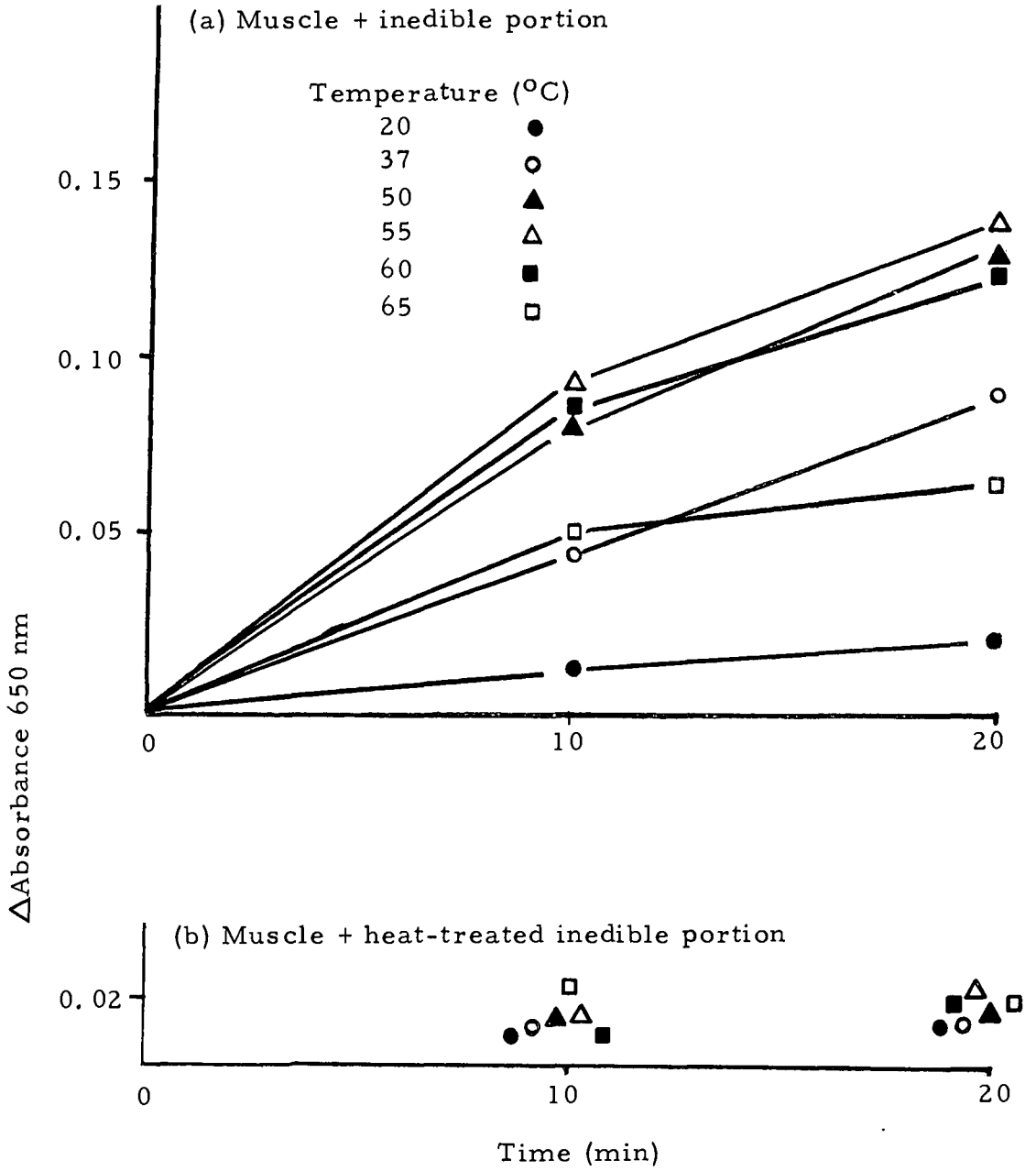


Figure 15. Effect of temperature on autolysis for 10 and 20 min incubation periods at pH 7.4.

It is unlikely that shrimp would reach the precook temperature in this short period of time and the internal temperature of the shrimp could have been in the optimum range for autolysis.

The temperature optimum of an enzyme is an operational parameter rather than a true characteristic (Whitaker, 1972). Product formation is usually constant at lower temperatures. However, at higher temperatures the enzyme undergoes denaturation resulting in a rate decrease with increasing reaction time. Different temperature optima will be obtained depending upon the time interval selected for activity measurement. Here, several time intervals were selected. The measurement of autolysis reflects total protein degradation for a given time period and is not necessarily an accurate estimate of initial reaction velocity.

Thermal Stability of Proteases from the Inedible Portion

A 250 ml Erlenmeyer flask was prewarmed in a shaker bath at 65°C before 25 ml of the inedible portion were added to the flask. Aliquots were taken after 10, 20 and 30 min of incubation and were cooled on ice before activity on muscle was determined for a 20 min incubation period at pH 3 and 7.4. The results are presented in Figure 16 and a correction was made for the slight activity in the muscle. Nearly 30 min were required to completely inactivate the proteases although a large reduction in autolysis occurred after

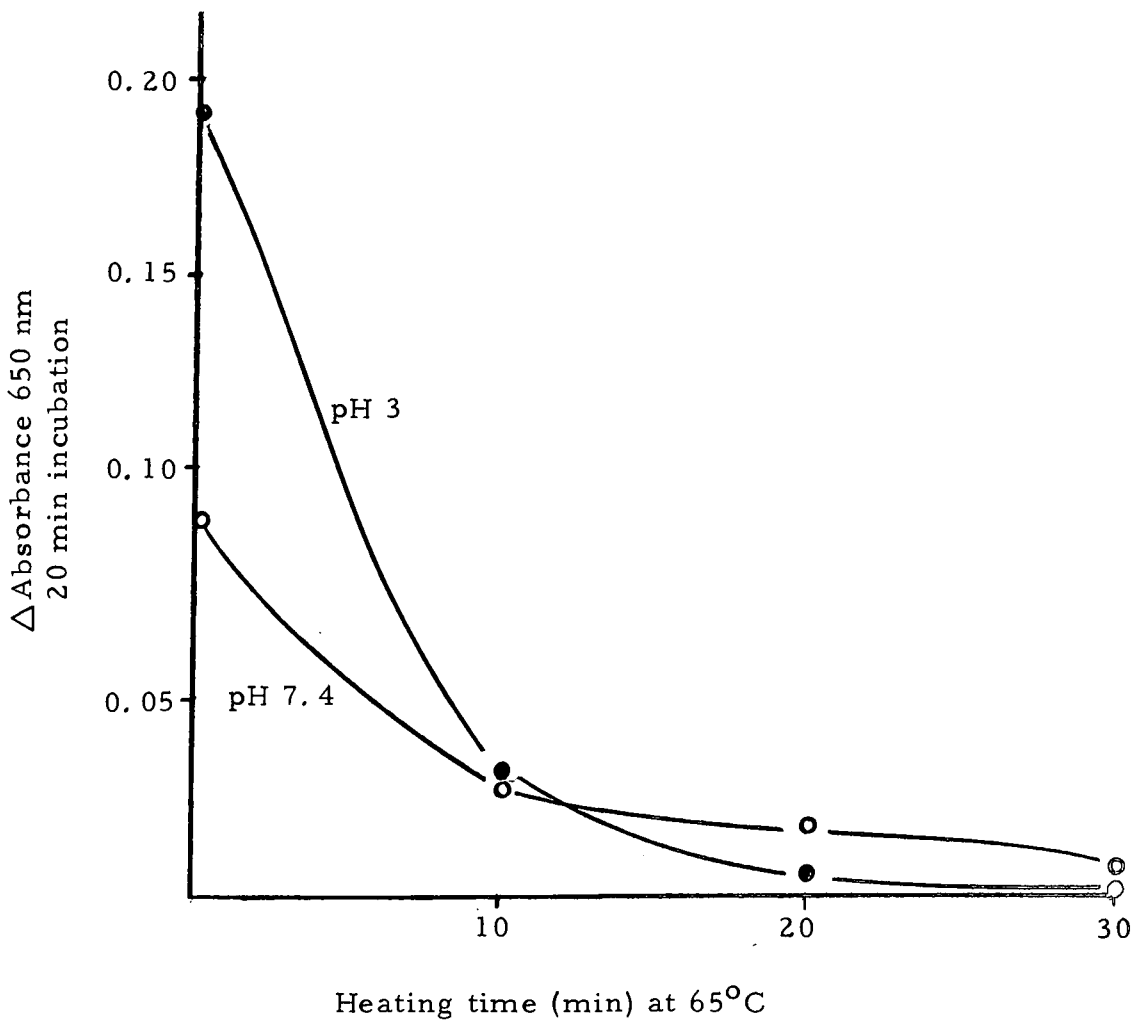


Figure 16. Heat stability of proteases in a crude homogenate of the inedible portion (pH 7.6).

10 min. The proteases most active at pH 7.4 were more stable than those active at pH 3. Proteases from the inedible portion could probably survive a mild blanching operation.

Effect of pH on Stability of Proteases from the Inedible Portion

The inedible portion was adjusted to pH 1.8 with 1N HCl and held on ice for various lengths of time before 1N NaOH was added to readjust the pH to the original level. Activity on the muscle homogenate for a 20 min incubation period was then determined at pH 3 and 7.4. Ten min at pH 1.8 were sufficient to completely inactivate proteases from the inedible portion.

These results are in agreement with those reported by Grajcer (1972) who found digestive enzymes in the heads of Gulf shrimp to be very unstable at pH 1.8. He recommended pH 1.8 for 6-24 hr at room temperature. For Pacific shrimp, refrigeration temperatures and less time at pH 1.8 may be sufficient for protease inactivation.

Stabilization by adjustment of pH in combination with refrigerated brine may be the most effective means of storing whole shrimp. Refrigerated sea water has been used to hold shrimp (Collins, 1960). Barnett et al. (1971) reported that CO_2 could extend the usefulness of seawater for preserving whole fish. The pH of chilled brine saturated with CO_2 was reduced from 7.5 to 4.0. The lower pH inhibited bacterial growth. Groninger (1972) reported that the keeping time of

shrimp increased roughly from 2-3 days to 6-7 days when CO_2 saturated brine was used as the holding medium. Although bacteria are inhibited by pH 4, endogenous proteases are still active. An even longer shelf life may be obtained if brine buffered to pH 1.8 is used as the holding medium since endogenous proteases are unstable and inactive under these conditions. A brief holding period in a low pH solution, which also contains a bacteriacidal agent, may be an effective way to extend shelf life.

The control of autolysis is also important for the proper utilization of shrimp scrap. Koury et al. (1971) found that autolysis reduced the yield of fish protein concentrate made by the isopropanol extraction method and suggested that autolysis rates should be closely controlled. The same conditions should apply to shrimp. When raw shrimp scrap is incorporated into animal feeds, autolysis can lead to a physically undesirable product and degradation products can promote microbial spoilage. In the case of a pelleted fish food, extensive liquification due to proteolytic enzymes may yield a product which permanently sticks together upon freezing.

Stabilization by pH adjustment has certain advantages over heat stabilization. The nutritive value of pH stabilized shrimp scrap should be higher than a heat-stabilized product. Pigment losses upon heating reduce the quality of meals used to promote color development in hatchery fish (Rousseau, 1960).

Stabilization of scrap may be of value when heat is to be used in the preparation of a meal. Steel (1971) found that once shrimp scrap was ground in preparation for meal production, immediate drying was necessary to prevent production of putrid odors which formed shortly after grinding. Spoilage was attributed to the combined action of bacteria and digestive enzymes.

Work is needed to evaluate the effectiveness of pH stabilization under conditions of commercial utilization. A possible disadvantage concerns the residual levels of NaCl if HCl and NaOH were used for pH adjustment. Although less than 0.5% NaCl was formed in this experiment, the amount would be higher when undiluted scraps are processed. The product should also be evaluated against other meals in feeding tests to determine nutritive value.

Effect of NaCl on Autolysis

Autolysis was measured for reaction mixtures which contained different levels of NaCl. NaCl had a strong inhibitory effect on autolysis at pH 3 (Figure 17) and almost no degradation occurred at 10% NaCl. NaCl also had an inhibitory effect on autolysis at pH 7.4 although the effect was slight at low salt concentrations (Figure 18).

Preliminary investigations suggested that the substrate rather than the enzyme was affected by low levels of NaCl at pH 3. NaCl at

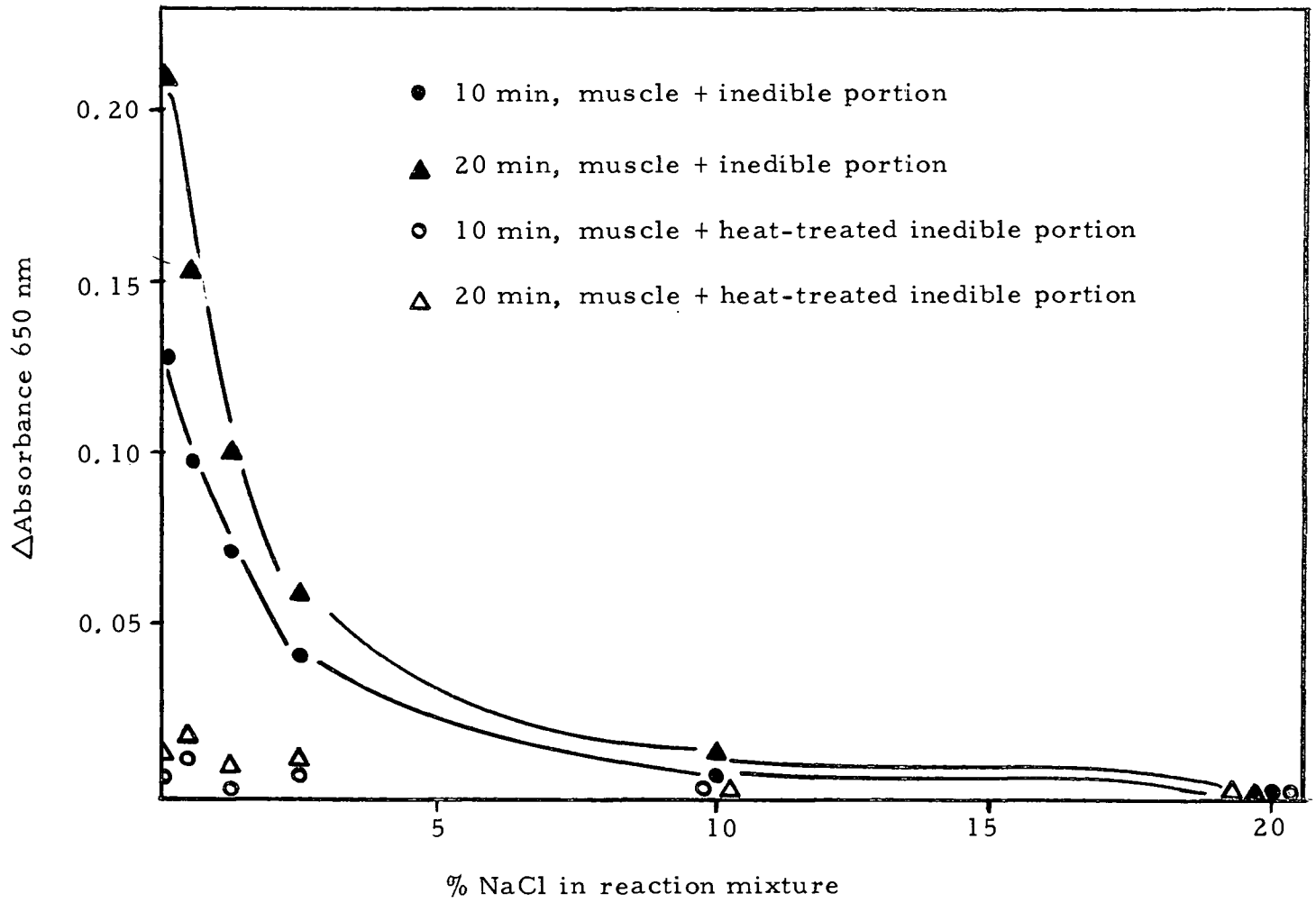


Figure 17. Effect of NaCl on autolysis at pH 3.

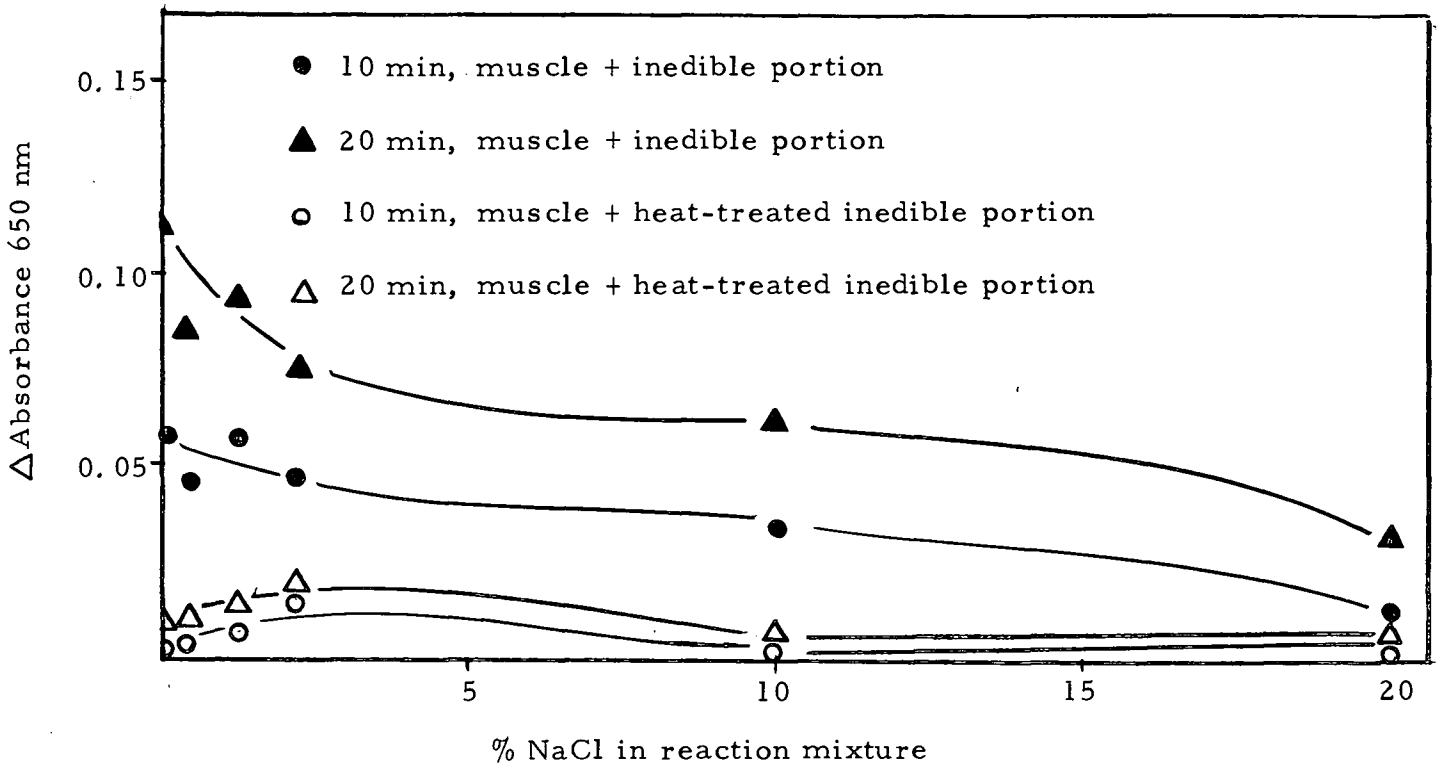


Figure 18. Effect of NaCl on autolysis at pH 7.4.

3% or less did not inhibit proteases from the inedible portion when hemoglobin was used as substrate at pH 3.5. The addition of even slight quantities of NaCl to muscle at pH 3 resulted in precipitation of protein. To evaluate the effects of NaCl upon solubility of the substrate, reaction mixtures in which water replaced enzyme were centrifuged at 30,900 x G for 30 min and the supernatants were analyzed for protein level. Supernatants from mixtures with NaCl had much less protein than the control mixture which contained no NaCl (Table 4). Insoluble proteins are less susceptible to hydrolysis.

Table 4. Effect of NaCl on solubility of proteins in reaction mixtures containing only muscle homogenate. Protein concentration of supernatants from centrifuged (30,900 x G for 30 min) homogenates of varying NaCl concentrations.

% NaCl	Protein concentration (mg/ml) pH 3.	pH 7.4
20	0.18	5.65
10	0.42	7.35
2.5	0.64	7.43
0 (control)	7.43	2.54

NaCl had a salting-in effect upon muscle proteases at pH 7.4 (Table 4). These results suggest that substrate availability may not be the reason for inhibition of autolysis at pH 7.4.

Salts in tissue become concentrated upon freezing and cold storage. Results indicate that variations in pH and salt concentration

can lead to widely different protein solubility patterns.

In turn, protein solubility is a factor which determines availability of substrate for proteolysis. The data illustrate the complexity of post-mortem conditions that may exist in shrimp and indicate that NaCl may have value in controlling shrimp proteases. NaCl may promote the usefulness of CO_2 saturated solutions for holding shrimp at pH 4 (Groninger, 1972).

Activity of Proteases from the Inedible Portion Upon Heat-Denatured Muscle Protein

A flask containing muscle homogenate was placed in a boiling water bath for 15 min. The homogenate was cooled and then dispersed with the Tissumizer. Activity of the inedible portion on the heat-denatured proteins of the muscle was determined at pH 3 and 7.4 and compared to native muscle proteins. Reactions were also conducted with heat-treated muscle homogenate in which substrate concentration was increased from 12.5 ml to 17.5 ml per assay mixture. Results are presented in Table 5.

For 10 min incubation, activity on heat-denatured muscle proteins was slightly less than on native muscle proteins at both pH 3 and 7.4. After 20 min incubation, the difference became more pronounced. Mixtures with additional heat-treated muscle showed increased hydrolysis after 20 min incubation although autolysis was still less than

Table 5. Activity of proteases from the inedible portion on heat-denatured proteins from the muscle.

Description	Δ Absorbance 650 nm	
	10 min incubation	20 min incubation
pH 3, 12.5 ml of muscle homogenate per reaction mixture	0.114	0.207
pH 3, 12.5 ml of heat-treated muscle homogenate per reaction mixture	0.095	0.146
pH 3, 17.5 ml of heat-treated muscle homogenate per reaction mixture	0.095	0.165
pH 7.4, 12.5 ml of muscle homogenate per reaction mixture	0.047	0.101
pH 7.4, 12.5 ml of heat-treated muscle homogenate per reaction mixture	0.043	0.058
pH 7.4, 17.5 ml of heat-treated muscle homogenate per reaction mixture	0.045	0.079

mixtures with native proteins. These results suggested that substrate was limiting in mixtures with heat-treated muscle proteins. Native and heat-denatured homogenates were centrifuged at 30, 900 x G for 30 min and the supernatants were analyzed for protein. Supernatants from heat-treated homogenates contained less protein than their native counterparts and the difference was greatest at pH 3 (Table 6). The ratio of soluble to insoluble protein was different when protein concentration was the same for mixtures containing native and heat-treated homogenate.

Table 6. Effect of thermal denaturation on solubility of proteins in the muscle homogenate. Protein concentration of supernatants from native and heat-denatured homogenates centrifuged at 30, 900 x G for 30 min.

	Protein concentration in mg/ml	
	Supernatant from native homogenates	Supernatant from heat-treated homogenates
pH 3	11.74	2.66
pH 7.4	3.90	3.07

Denaturation can have both an inhibitory and activating effect on autolysis. Heat unfolds polypeptide chains and an unfolded structure is more available for hydrolysis. Heat denaturation also reduces solubility of proteins and insolubility impairs hydrolysis. Here, the most important effect was solubility since a net reduction in autolysis was observed.

These results may help explain results of a study conducted by Flores and Crawford (1973). They found tyrosine levels to increase in whole ice stored Pacific shrimp during the first two days followed by a decrease throughout the remainder of the storage period. They suggested that the losses through enzymatic browning may only partially explain the loss in tyrosine levels since tyrosine levels have been shown to increase throughout the storage period of finfish (Bradley and Bailey, 1940; Sigurdsson, 1947). Muscle proteins become denatured upon storage and lose solubility (Umemoto and Kanna, 1970). The differences between shrimp and fin fish regarding post-mortem autolysis could be reflected in the different effects denaturation has upon availability of substrate. In fin fish, unfolding of polypeptide chains may be most important while solubility could be the dominant factor in shrimp. The net result could be a difference in the accumulation of tyrosine.

Electrophoresis of Autolytic Mixtures

Figure 19 illustrates the electrophoretic profile of soluble proteins from autolytic mixtures (pH 7.4) incubated for different times at 50°C. The profile of the mixture which contained only muscle (Figure 19, B, D, F and H) showed the disappearance of two major bands as incubation times increased. These bands may represent proteins which served as substrate for cathepsins in the muscle.

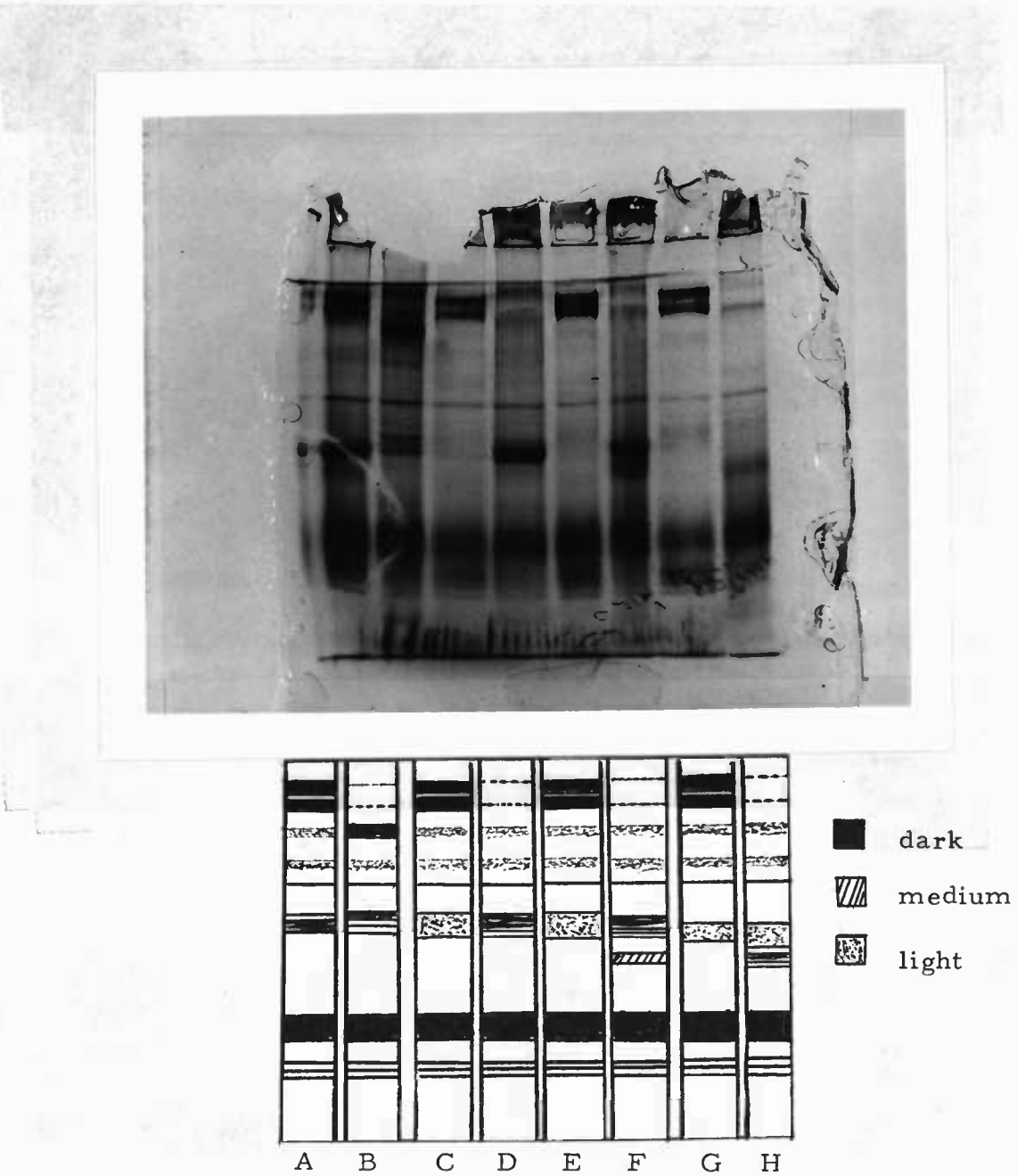


Figure 19. Electrophoretic pattern of soluble proteins from autolytic mixtures (pH 7.4) at various times of incubation (50°C). Samples taken at 0 min were brought to 50°C.
A - Inedible portion + muscle, 0 min; B - muscle, 0 min; C - Inedible portion + muscle, 10 min; D - Muscle, 10 min; E - Inedible portion + muscle, 20 min; F - Muscle, 20 min; G - Inedible portion + muscle, 2 hr; H - Muscle, 2 hr.

The profile of the mixture which contained both muscle and inedible portion (Figure 19, A, C, E, and G) showed the disappearance of one major band as incubation times increased. Moreover, this band appeared to originate from muscle proteins and its disappearance was more rapid than when the inedible portion was absent. Proteins of this band may serve as substrate for proteases in both the muscle and inedible portion.

For the 0 min samples, the mixture with muscle, only, contained a major band which did not appear in the electrophoretic profile of the mixture which contained both muscle and inedible portion. Proteins of this band may have been degraded by proteases of the inedible portion during preparation for electrophoresis at 0^{o}C.

No differences were found in the electrophoretic pattern of soluble proteins from autolytic mixtures at pH 3. The amount of soluble protein was higher at pH 3 than 7.4 and the darker bands may have masked changes which took place.

Additional work is needed to fully evaluate protein changes which occur as the result of autolysis.

Characterization of Low pH Active Proteases from the Hepatopancreas

Blending

Tissue and cold distilled water were blended for various lengths of time with the Tissumizer blender and activity of the homogenates was determined. Time of blending had no effect on activity (Table 7). A 60 sec blending time was selected.

Table 7. Effect of blending time on activity of proteases in the hepatopancreas.

	Blending time (sec)		
	30	60	90
$\Delta A^{275\ nm}_{1\ cm}$/10 min/ml	0.446	0.440	0.424

Extraction

Tissue of the hepatopancreas was homogenized with various salt, buffer, and detergent solutions. The homogenates were centrifuged at 30,900 x G for 30 min and activity on the supernatants was determined. The data are presented in Table 8. Maximum specific activity and highest recovery were obtained by extracting with 0.6 M NaCl. A further study to determine optimum NaCl concentration indicated best results with 0.6 M NaCl (Table 9).

Table 8. Extraction of proteases from hepatopancreas with various solutions.

Solution	Activity $\Delta A^{275\ nm}_{1\ cm}$/10 min/ml	Protein concentration (mg/ml)	Specific activity (activity per mg protein)	Percentage recovery
Homogenate	0.412			
Aqueous extract	0.298	1.80	0.166	72
Acetate (pH 4.5, 0.2 M)	0.128	1.06	0.121	31
Phosphate (pH 7.5, 0.2 M)	0.304	2.48	0.123	74
0.05 M NaCl	0.312	1.90	0.164	76
0.2 M NaCl	0.334	2.00	0.167	81
0.6 M NaCl	0.380	1.80	0.210	92
Triton X-100 (1%)	0.344	2.83	0.122	84

Table 9. Effect of NaCl concentration on extraction of proteases from the hepatopancreas.

Solution	Activity ($\Delta A^{275\ nm}_{1\ cm}$/10 min/ml)	Protein concentration (mg/ml)	Specific activity (activity per mg protein)
0.2 M NaCl	0.366	1.42	0.258
0.4 M NaCl	0.410	1.47	0.279
0.6 M NaCl	0.460	1.53	0.300
0.8 M NaCl	0.418	1.53	0.273
1.0 M NaCl	0.410	1.60	0.256
1.4 M NaCl	0.362	1.55	0.233

pH Stability

To determine the buffer pH that would provide the best enzyme stability for column chromatography, the stability of proteases in the NaCl extract under different conditions of pH were determined. HCl or NaOH was added to the NaCl extract to vary pH and water was manipulated to give constant volume. Samples were stored in the cold room for 18 hr. pH was then readjusted to that of the original extract and activity was determined.

Results are presented in Table 10. The natural pH of the NaCl extract was most effective in preserving activity. Low pH was most harmful as would be expected from earlier findings regarding low pH inactivation of proteases from the inedible portion. Potassium

phosphate buffer (0.005 M, pH 6.5) was selected for use in chromatography.

Table 10. Stability of proteases in a 0.6 M NaCl extract of the hepatopancreas as a function of pH. 18 hr incubation on ice.

pH	Activity (% of control)
6.3 (control)	100
3.5	51
4.5	48
5.5	67
7.5	90
8.5	76

Effect of Time on Stability

A NaCl extract of the tissue was prepared and activity was determined after 0, 5, 10 and 25 hr of incubation on ice. Results are presented in Table 11. Proteolytic enzymes were highly unstable and only 63% of the activity remained after 5 hr.

Table 11. Effect of time on stability of proteases in a 0.6 M NaCl extract of the hepatopancreas.

Time at 5°C (hr)	Activity (% of control)
0 (control)	100
5	63
10	46
25	37

Ammonium Sulfate Fractionation

Ammonium sulfate fractionation was evaluated as a means of concentrating activity from the NaCl extract of the hepatopancreas. Solid $(NH_4)_2SO_4$ was added to the NaCl extract and the precipitate was recovered by centrifuging. Additional $(NH_4)_2SO_4$ was added to the supernatant and the procedure was repeated in a step-wise fashion. The precipitates were dissolved in a minimum amount of 0.005 M phosphate buffer pH 6.5 and dialyzed against the same buffer for 6 hr at 4°C.

The results indicated (Table 12) that active material was precipitated between 0.4 and 0.6 saturation with an increase in specific activity. Specific activity of the NaCl extract was considerably less than that of extracts which were prepared from fewer numbers of shrimp. A reduction in specific activity might be attributed to a combination of greater time needed to dissect and handle larger numbers of shrimp and to the instability of the proteases with respect to time.

In addition, it was found necessary to either freeze or immediately subject the $(NH_4)_2SO_4$ fraction to column chromatography. The enzyme preparation was sensitive to autolysis under concentrated conditions and although $(NH_4)_2SO_4$ fractionation was effective in concentrating activity, little or no purification resulted unless work was conducted as rapidly as possible.

Table 12. Ammonium sulfate precipitation of proteolytic activity from a 0.6 M NaCl extract of the hepatopancreas.

Saturation of ammonium sulfate	Activity ($\Delta A^{275\ nm}_{1\ cm}$/10 min/fraction)	Protein concentration (mg/fraction)	Specific activity (activity/mg protein)	Percentage recovery
NaCl extract	49.8	498	0.100	100
0 to 0.20	1.34	15.3	0.088	2.7
0.20 to 0.40	2.30	31.1	0.074	4.6
0.40 to 0.60	20.2	97.6	0.207	40.6
0.60 to 0.80	7.2	43.9	0.164	14.5
0.80 to 1.00	0	93.6	0	0

Dialysis

Several problems were encountered when using conventional techniques of dialysis. In addition to loss of activity as a result of dialysis time, dialysis tubing would occasionally break. The latter suggested the presence of cellulases although no attempt was made to confirm their existence.

A Bio-Fiber 50 Beaker (Bio-Rad Laboratories) was used as a means for rapid dialysis of the $(NH_4)_2SO_4$ fraction. The device consisted of an enclosed chamber (100 ml) into which hundreds of small hollow cellulose tubes entered and exited at the same end. The tubes gave a dialyzing surface of 1000 cm^2 and had a molecular exclusion limit of 5, 000. The solution to be dialyzed was placed in the chamber and came into contact with the outside of the hollow tubes while the dialyzing solution flowed inside the tubes.

Using refractive index as a measure of salt concentration, 30 min of dialysis were sufficient to remove excess salt.

Gel Filtration

Gel filtration of an undialyzed $(NH_4)_2SO_4$ fraction on Sephadex G-150 separated hemoglobin digesting activity into two distinct fractions (Figure 20). Similar results were obtained in preliminary experiments with Sephadex G-100 except separation was not as well

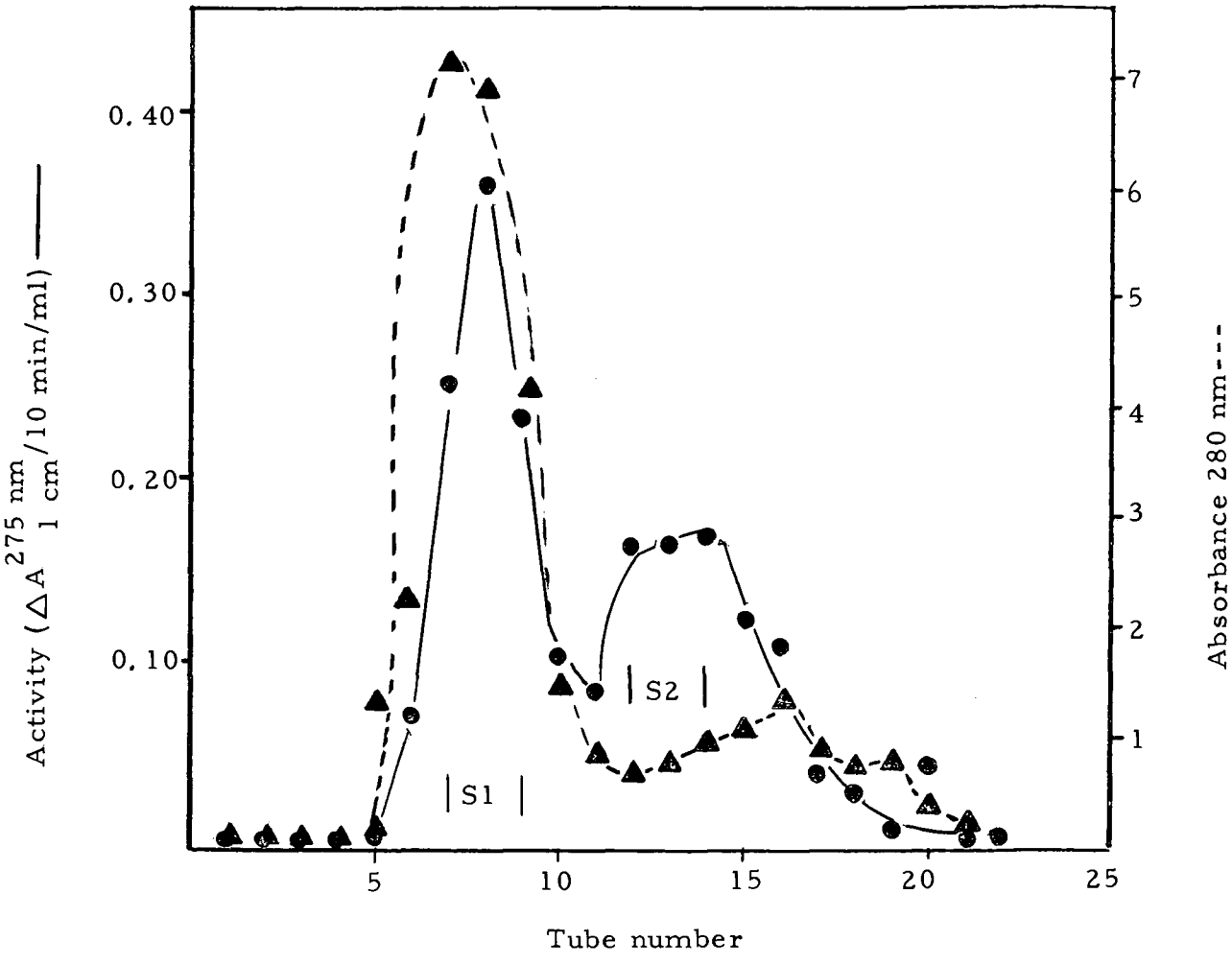


Figure 20. Elution profile of hemoglobin digesting proteases on Sephadex G-150. Load, 12 ml (109 mg protein). Equilibrated and eluted with 0.005 M potassium phosphate, pH 6.5. Flow rate of 15 ml per hr at 4°C. 10 ml per tube. ●-●, activity; ▲-▲ , absorbance 280 nm.

defined. Tubes 7, 8, and 9 were combined and designated as fraction S1 while tubes 12, 13, and 14 were combined and designated as fraction S2. The fractions were frozen and held (-30°F) for inhibitor studies.

Fractions S1 and S2 were purified 1.5 and 3.4-fold, respectively (Table 13). The two fractions were effectively separated by gel filtration although purification was not great. The results suggested the existence of at least two hemoglobin digesting proteases which differed in molecular weight.

Ion-Exchange Chromatography

Chromatography of a dialyzed $(NH_4)_2SO_4$ fraction on DEAE-cellulose separated activity into at least two and possibly three fractions (Figure 21). Tubes 14, 15, and 16 were combined and designated as D1 while tubes 46 through 52 were combined and designated as D2. The fractions were frozen and held (-30°F) for inhibitor studies.

Fractions D1 and D2 were purified 3.2 and 12-fold, respectively (Table 14). D1 was eluted before the gradient was started, although it did not coincide with the first protein peak. Different gradients were evaluated in an attempt to effect a better separation (Table 15) but only two distinct peaks of activity were obtained and purification did not exceed 3.2-fold for D1. The data further supported the existence of at least two different low pH active proteases.

Table 13. Data summary on purification of hemoglobin digesting proteases with Sephadex G-150.

Purification step	Volume (ml)	Total Activity ($\Delta A^{275\ nm}_{1\ cm}$/10 min)	Total protein (mg)	Specific[a] Activity ($\Delta A^{275\ nm}_{1\ cm}$/10 min/mg)	Yield (%)	Purification
1. NaCl extract	51	27.6	301	0.092		
2. $(NH_4)_2SO_4$ 40-60%	12	13.8	109	0.127	50	1.4
3. Sephadex G-150						
S1	30	8.4	62.8	0.140	30	1.5
S2	30	5.0	17.3	0.310	18	3.4

[a]Most active tube of each fraction.

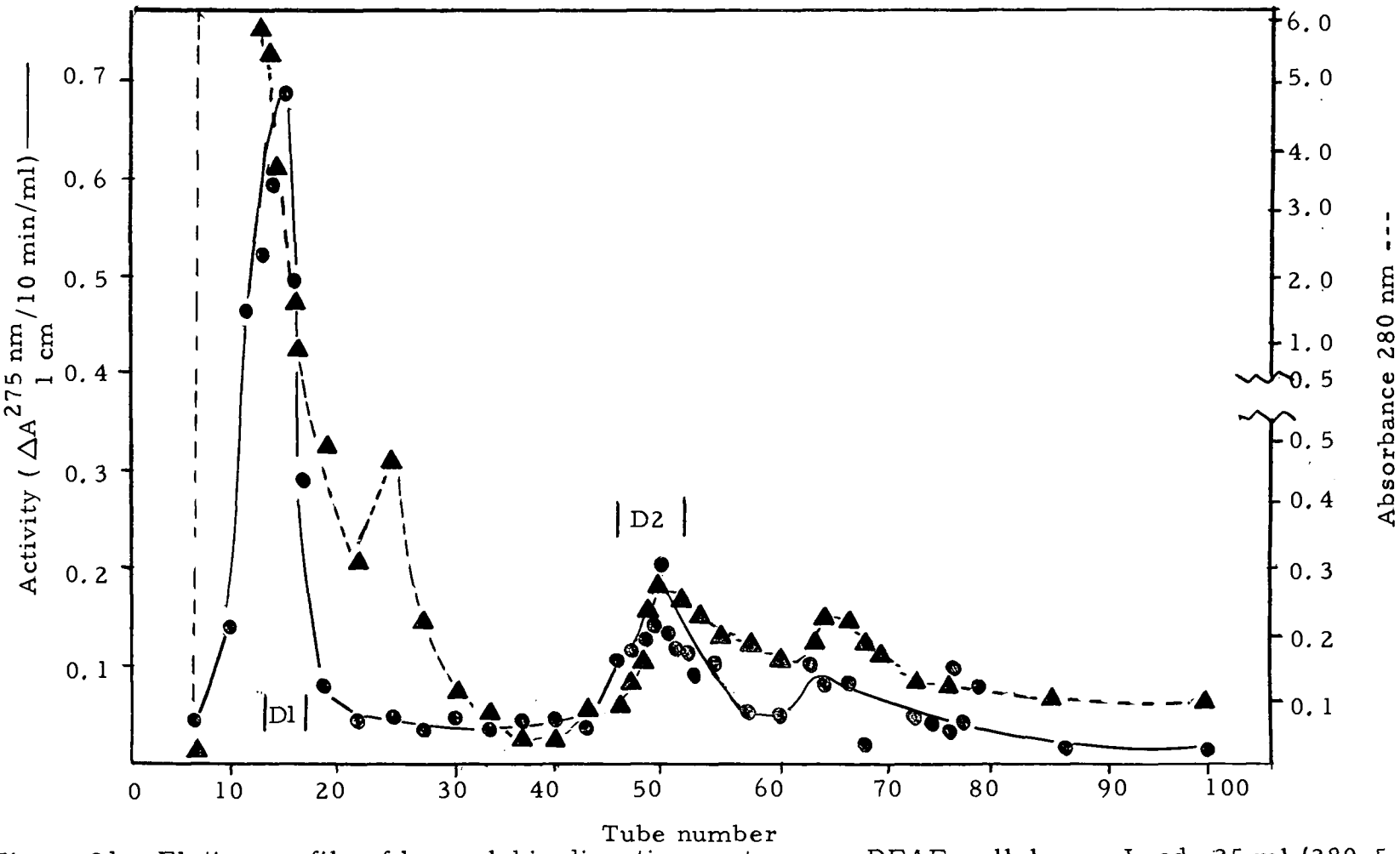


Figure 21. Elution profile of hemoglobin digesting protease on DEAE-cellulose. Load, 25 ml (280.5 mg protein). Linear gradient elution (800 ml, 0.15 M to 0.6 M NaCl in 0.005 M potassium phosphate buffer, pH 6.5. Flow rate of 120 ml/hr at 4°C. 10 ml per tube. ●-●, activity; ▲-▲ , absorbance 280 nm. Gradient started on tube no. 23.

Table 14. Data summary on purification of hemoglobin digesting proteases with DEAE-cellulose.

Purification step	Volume (ml)	Total activity ($\Delta A^{275\ nm}_{1\ cm}$/10 min)	Total protein (mg)	Specific[a] activity ($\Delta A^{275\ nm}_{1\ cm}$/10 min/mg)	Yield (%)	Purification
1. NaCl extract	241	203.4	1561.7	0.130		
2. $(NH_4)_2SO_4$						
40-60%	25	60	280.5	0.213	29	1.6
3. DEAE-cellulose						
D1	30	14.4	45	0.410	7	3.2
D2	70	9.9	9.3	1.550	5	12

[a]Most active tube of each fraction.

Table 15. Effect of different gradients on the elution profile of hemoglobin digesting proteases on DEAE-cellulose.

Gradient	Elution volume (ml)	Number of peaks	Fraction number	Purification
0 to 0.7 M NaCl	700	1	48-54	3.1
0.15 to 0.6 M NaCl	800	1	14-16	3.2
		2	46-52	12
0.1 to 0.6 M NaCl	800	1	14-16	2
		2	59-62	6

Inhibitor Studies

Phenylmethylsulphonylfluoride (PMSF), p-chloromercuribenzoate (pCMB), ethylenediaminetetraacetic acid (EDTA) and KCN were incubated 15 min on ice with various fractions from column chromatography before activity on hemoglobin was determined. In some instances, assay incubation times of greater than 10 min were employed to provide activity which could be more accurately measured. PMSF was dispersed in a Triton-X 100 solution (1% w/v) and pCMB was dissolved in 0.1N NaOH. Suitable controls were run in every case.

Results are presented in Table 16. EDTA activated every fraction, PMSF had little effect, pCMB inhibited every fraction while KCN activated or had little effect on the various fractions.

All data were consistent in indicating that -SH groups are important for enzyme activity. In this regard, these shrimp proteases

Table 16. Effect of different inhibitors on activity of a crude extract and various fractions from column chromatography.[a]

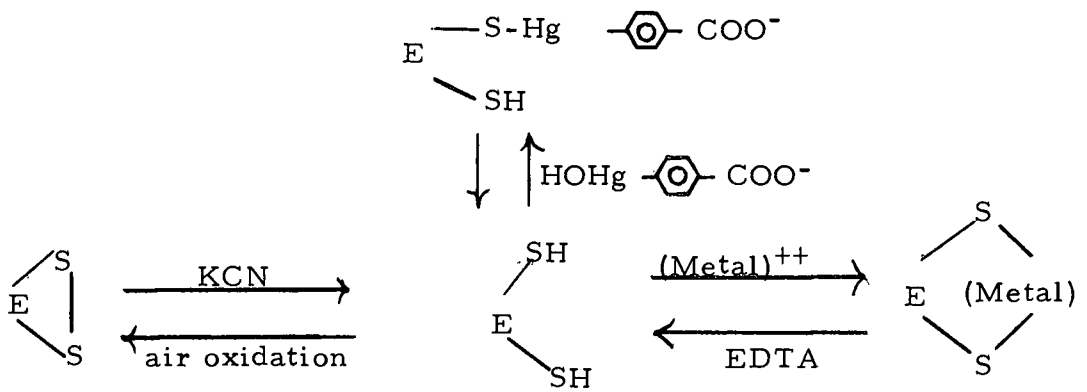
Inhibitor[b]	Concentration[c] (M)	NaCl Extract (%)	S1 (%)	S2 (%)	D1 (%)	D2 (%)
EDTA	10^{-2}	132	172	171	159	208
PMSF	10^{-2}	98	85	98	126	95
PCMB	10^{-2}	0	36	55	0	---
	10^{-3}	52	0	17	---	58
	10^{-4}	94	77	100	---	85
KCN	10^{-2}	102	175	143	115	110

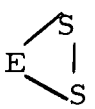
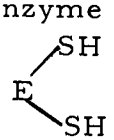
[a] Activity is represented as % of the control which contained no inhibitor.

[b] Abbreviations used: PMSF, phenylmethylsulphonylfluoride; PCMB, p-chloromercuribenzoate; EDTA, ethylenediaminetetraacetic acid.

[c] Concentration refers to amount of inhibitor present in the preincubated enzyme solution.

are similar to mammalian cathepsins A, B, and C (Mycek, 1970; Misaka and Tappel, 1971). A possible mechanism for enzyme inhibition and activation, which is similar to one proposed by Misaka and Tappel (1971) for cathepsin B, is illustrated below:



The nonactivated enzyme is expressed by  and the activated form is given by  EDTA is known to activate thiol groups by converting the metal complex back to the activated form. KCN is a mild reducing agent and can reduce the inactive air oxidized form. pCMB inactivates by reversibly forming a mercaptide with the sulfhydryl groups. A detailed investigation of inhibition should be conducted on highly purified fractions to accurately elucidate the mechanism of activation and inhibition of these enzymes.

It is interesting to note that fractions S1 and S2 were inhibited more by 10^{-3} M pCMB than a more concentrated solution. A similar phenomenon was observed by Misaka and Tappel (1971) when they

studied the inhibitory effects of pCMB on cathepsin A from rat liver lysosomes. They explained the phenomenon by the existence of two kinds of -SH groups in the molecule. One group would be sensitive to low concentrations of pCMB and react readily to form the mercaptide while the other group would not react unless an excess of pCMB was present or the molecule had changed form. Activity of the former would be less than that of the latter. Their explanation was supported by Misaka and Nakanishi (1965) who found that lipoamide dehydrogenase of baker's yeast contained two functional -SH groups. Activity varied depending upon which of the two -SH groups was blocked.

An important finding of the inhibition study concerns the activating effect of EDTA. Low pH active proteases should be prepared in the presence of EDTA.

pH Optimum

The effect of pH upon activity of D1 was determined by methods described previously. Results are illustrated in Figure 22. Activity was relatively uniform between pH 3 and 4. The pH profile was similar to that obtained from a crude homogenate of the hepatopancreas (Figure 23).

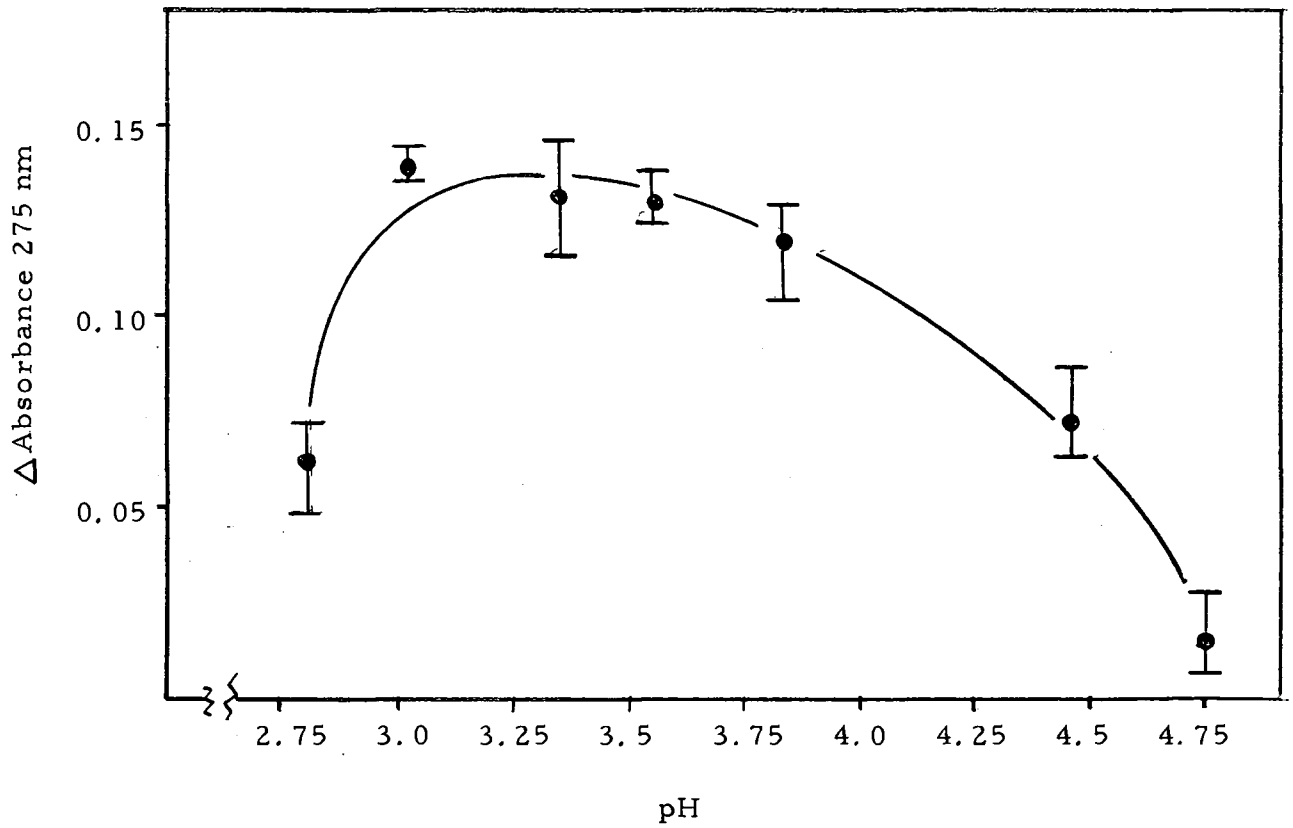


Figure 22. Activity of proteases from D1 on hemoglobin as a function of pH. Triplicates, I--range, ●--mean.

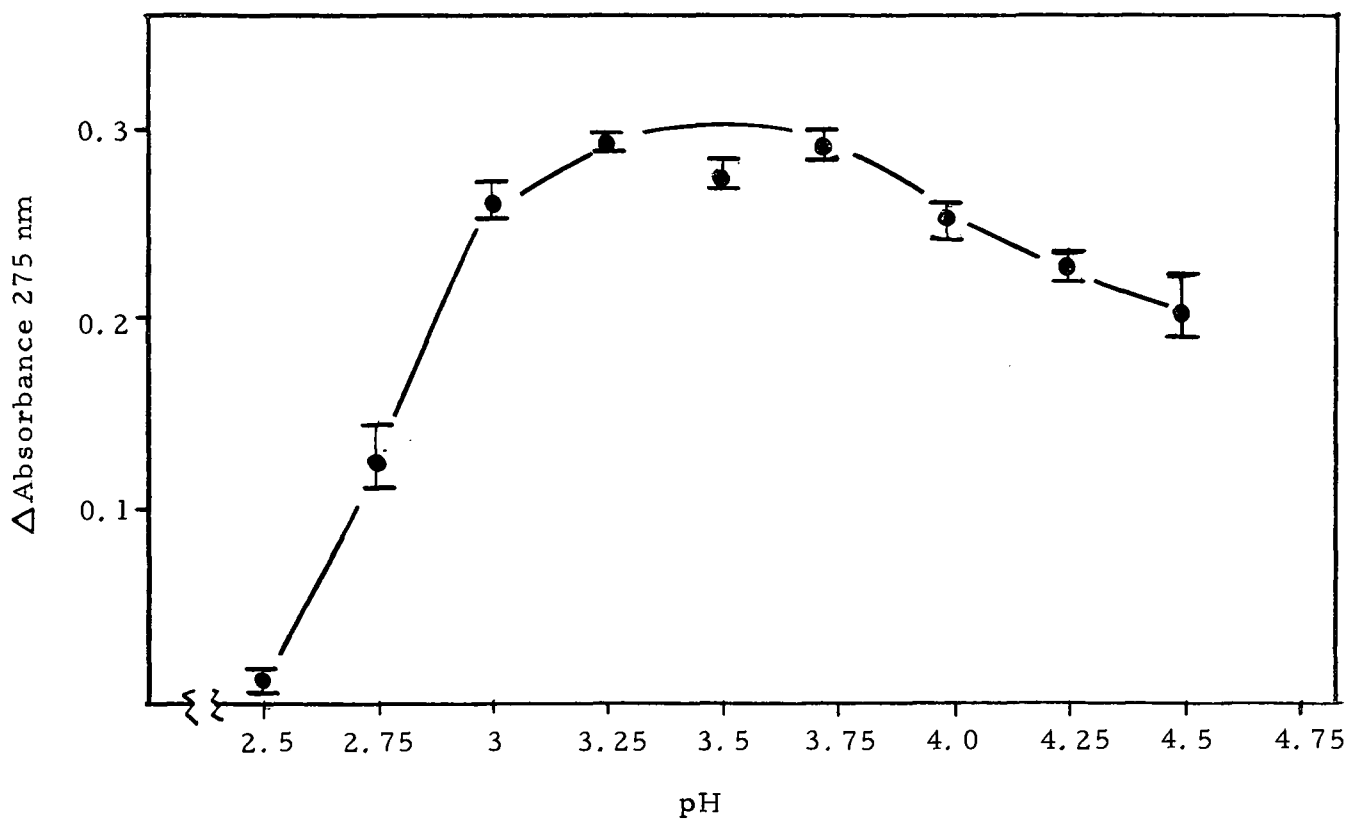


Figure 23. Activity of proteases from a homogenate of the hepatopancreas on hemoglobin as a function of pH. Triplicates, I--range, ●--mean.

SUMMARY AND CONCLUSIONS

The distribution of proteolytic enzymes in shrimp processing waste was determined using casein and hemoglobin as substrates. The effects of these enzymes upon muscle proteins were studied under different conditions and proteases active at low pH were partially characterized using column chromatography.

A crude homogenate of the inedible portion of shrimp showed maximum activity on casein at pH 6.25 although activity was relatively uniform between pH 5.75 and 7.5. Hemoglobin digestion was greatest at pH 3-3.65. Reports on proteolytic enzymes active near pH 3 are rare although serine proteases which digest casein near neutrality are common in Crustacea.

Hand-cleaned shrimp from two catches yielded 63-64% waste. There was no difference between the two catches regarding hepatopancreas, muscle, inedible portion, and total body weights (t-test, $p > 0.1$). The foreguts from one catch were slightly larger than foreguts from the other catch due to the presence of food (t-test, $p < 0.1$). Using hemoglobin and casein as substrates at pH 3.5 and 6.25, respectively, total and specific activities were determined on shrimp parts for both catches. Results were similar, regardless of the substrate. Specific activity was greatest in the foregut followed by the hepatopancreas. Very low specific activity was found in the inedible

portion with the digestive organs removed. The distribution of total activity differed between the two catches and a dependence upon feeding status was suggested. The catch without food in the foreguts had greatest total activity in the hepatopancreas followed by the foregut and remainder of the inedible portion. For the catch which had been feeding, total activity was greatest in the foregut followed by the hepatopancreas and remainder of the inedible portion. Negligible casein and hemoglobin digestion occurred when muscle was used as the enzyme source.

Activity of endogenous proteases on shrimp proteins from the muscle and inedible portion showed a major maximum at pH 3 and minor maximum at pH 7-9. Incubation times of 10 and 20 min were employed at 37°C. The results of the use of heat-treated inedible portion indicated that it was the major source of proteases. Activity in the muscle was detected when incubation times were extended to 2 hr. The protein concentration of reaction mixtures was changed by varying the amount of muscle homogenate. Substrate became limiting in reaction mixtures when the naturally occurring ratio (1:1.85) of muscle to inedible portion was approached at pH 3 and 7.4. Activity in autolytic mixtures was linearly proportional to the amount of inedible portion at pH 3 for 10 min of incubation. The relationship between activity and concentration of inedible portion, although proportional, was not linear at pH 3 for 20 min incubation or at pH 7.4 for 10 or 20

min incubation.

Maximum autolytic activity occurred at 50°C for pH 3 and at 55°C for pH 7.4. A possible role for these enzymes in loosening shell from meat was suggested since water precooks near these optimum temperatures have been used to condition fresh shrimp for automatic peeling.

Proteases in the inedible portion were unstable to heat at 65°C although 30 min were required for complete inactivation. Enzymes active at pH 7.4 were more stable to heat than those active at 3.

Proteolytic enzymes in shrimp scrap were very unstable at pH 1.8 and 10 min on ice were required for complete inactivation. These results suggested that holding whole shrimp in a solution buffered to pH 1.8 for a short period of time might be useful for controlling autolysis. pH stabilization of shrimp scrap was also suggested as a means for expanding the usefulness of this by-product.

NaCl at a concentration of 10% completely inhibited autolysis at pH 3. NaCl in low concentrations did not affect activity of shrimp proteases on hemoglobin at pH 3.5. NaCl was found to greatly reduce the solubility of shrimp proteins at pH 3 and this was suggested as an explanation for autolytic inhibition. NaCl also inhibited autolysis at pH 7.4 although the effect was less dramatic than at pH 3. NaCl increased solubility of shrimp proteins at pH 7.4.

Activity of proteases from the inedible portion on muscle proteins (pH 3 and 7.4) was less when these proteins were heat-denatured. Further investigations showed that substrate was limiting when heat-denatured shrimp proteins were used as substrate and heat denaturation had reduced protein solubility. Denaturation can increase the availability of proteins for hydrolysis by unfolding polypeptides and may also reduce availability by decreasing solubility. The latter may be most important with shrimp.

Changes occurred in the electrophoretic profile of soluble proteins (pH 7.4) in a muscle mixture which was incubated at 50°C. These changes may have been the result of cathepsins present in the muscle. Changes also occurred when incubation mixtures contained inedible portion and these changes were more rapid than when inedible portion was absent. This suggested that soluble muscle proteins had served as substrate for proteases in the inedible portion. The lack of noticeable changes in the electrophoretic profile of soluble proteins from mixtures at pH 3 was attributed to a masking effect due to the relatively large amount of soluble protein.

Low pH active proteases from the hepatopancreas were determined using hemoglobin as substrate at pH 3.5. These enzymes were most effectively extracted from the hepatopancreas with 0.6 M NaCl. Although enzymes were most stable at the natural pH of extraction, they were still unstable and only 63% of the activity of a NaCl extract

remained after 5 hr on ice. Ammonium sulfate fractionation between 0.4 and 0.6 saturation resulted in a concentration of activity with an increase in specific activity. Proteases were particularly unstable in the concentrated form after fractionation with $(NH_4)_2SO_4$; the precipitated enzyme needs to be frozen or subjected to column chromatography immediately after redissolving in buffer. Problems were encountered with classical means of dialysis since a reduction in activity occurred as the result of long dialysis times. In addition, breakage of dialysis tubing occurred possibly due to the presence of cellulases in the $(NH_4)_2SO_4$ fraction. A rapid means of dialysis with a Bio-Fiber 50 Beaker gave satisfactory results.

Gel filtration of the undialyzed $(NH_4)_2SO_4$ fraction on Sephadex G-150 separated hemoglobin digesting activity into two distinct fractions which were designated S1 and S2. This provided evidence for the existence of at least two enzymes with different molecular weights. Fractions S1 and S2 were purified 1.5 and 3.4-fold, respectively, and although purification was not high, separation was effective.

Chromatography of the dialyzed $(NH_4)_2SO_4$ fraction on DEAE-cellulose separated activity into two fractions which were designated D1 and D2. This further provided evidence for the existence of at least two different enzymes. Fractions D1 and D2 were purified 3.2 and 12-fold, respectively. Variation of eluting conditions revealed a maximum number of two activity peaks.

Inhibitor studies were conducted on a NaCl extract of the hepatopancreas and fractions S1, S2, D1 and D2 from column chromatography. The fractions were unaffected by PMSF. pCMB greatly inhibited all fractions, EDTA greatly activated all fractions, and KCN slightly activated or had little effect upon the proteases. The results indicated that -SH groups are important for enzyme activity. Furthermore, low pH active proteases should be purified and analyzed in the presence of EDTA.

D1, the major peak from ion-exchange chromatography, showed greatest activity on hemoglobin between pH 3 and 4. This pH profile was identical to that obtained with a crude homogenate of the hepatopancreas.

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