CHARACTERIZATION OF SEMI-PURIFIED COLLAGENASE FRACTION FROM LOBSTER (HOMARUS AMERICANUS)

By

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ABSTRACT

A collagenolytic enzyme fraction was isolated from the hepatopancreas of the lobster (*Homarus Americanus*) and semi-purified by the successive steps of acetone precipitation, ammonium sulfate fractionation, ion exchange chromatography on Mono Q column, followed by gel filtration on a Superdex 75 column or by preparative isoelectric focusing using a Rotofor cell.

Semi-purified collagenase fractions from the lobster hepatopancreas was electrophoresed in polyacrylamide gels both in the presence or absence of SDS, and shown to have molecular weights ranging from 15,000-66,000. The enzymatically active peak 1 fraction from the isoelectric focusing step in the Rotofor cell migrated as a single band in 12 % polyacrylamide gel with few light protein bands. This represented the purest collagenase fraction as compared with the extracts recovered by: ammonium sulfate precipitation or ion-exchange chromatography.

The pH-activity data indicated that the collagenase fraction had two pH optima for the hydrolysis of native collagen, one at pH 4 and the other between pH 7-8. In this respect, the lobster enzyme differed from the commercial enzyme which was most active at pH 7.0. Furthermore the enzyme fraction from lobster hepatopancreas was most stable at pH 8.0 while commercial collagenase exhibited a much broader pH stability range of 6.0-9.0.

The temperature-activity data for the hydrolysis of native collagen indicated the lobster enzyme exhibited two temperature optima - a minor one at 25^0C and a more pronounced one between 40^0C and 50^0C, while commercial collagenase was most active at 40^0C. However, the lobster collagenase extract was more heat labile, losing about 20 % of its original activity after 30 min incubation at 35^0C, while the commercial enzyme retained virtually all its activity after 30 min incubation at 45^0C. The lobster protease fraction was recovered as the active form, and it was not apparent that it had a precursor form. Finally, the ability of various fractions from the various precipitation steps to hydrolyze native collagen, suggest the presence of multiple forms of the enzyme (isoenzymes) in the lobster hepatopancreas.

RESUME

Une fraction d'enzyme collagénolitique fut isolée de l'hépatopancréas de homard (*Homarus Americanus*) et semi purifiée par étapes successives: précipitation à l'acétone, fractionnement à l'ammonium sulfate, chromatographie d'échange d'ion sur une colonne Mono Q, puis gel filtration sur colonne Sephadex 75 ou par précipitation préparative par électrophorèse selon le point isoélectrique sur Rotofor cell.

Les fractions de collagénase semi-purifiées d'hépatopancréas de homard furent soumises à une électrophorèse en gel de polyacrylamide avec ou sans SDS, cela a permis de déterminer que le poids moléculaire variait de 15,000 à 66,000 Daltons. Le pic 1, enzymatiquement actif, obtenu par électrophorèse préparative sur Rotofor cell, migre en une seule bande dans un gel de 12 % de polyacrylamide avec quelques légères bandes de protéines. Cela représente la fraction de collagénase la plus pure quand on compare avec les extraits obtenus par précipitation avec de l'ammonium sulfate ou par chromatographie d'échange d'ion.

Les données de l'activité en fonction du pH indiquent que les fractions de collagénase ont deux valeurs de pH optimum pour l'hydrolyse du collagène natif, une à pH 4 et l'autre à pH 7-8. De plus l'enzyme extraite de homard était plus stable à pH 8 alors que la collagénase commerciale reste stable pour des valeurs de pH variant de 6.0 à 9.0.

L'activité enzymatique en fonction de la température montre que la

préparation enzymatique obtenue à partir du homard possède deux valeurs de températures optimum une à 25^{0}C et une plus prononcée entre 40^{0}C et 50^{0}C alors que la température optimum pour l'enzyme commerciale est de 40^{0}C. La préparation enzymatique de homard était plus thermosensible que l'enzyme commerciale car 20 % de l'activité originale est perdue pour la première après 30 min d'incubation à 35^{0}C alors que la seconde garde toute son activité initiale après 30 min d'incubation à 45^{0}C. La fraction protéase obtenue du homard fut reconue être la forme active et il ne parait pas exister de précurseur enzymatique. Finallement l'aptitude des différentes fractions, issues des diverses étapes de précipitations, à hydrolyser le collagéne natif suggère la présence de multiples formes de l'enzyme (isoenzymes) dans l'hépatopancréas de homard.

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LIST OF ABBREVIATIONS

APS	ammonium persulphate
Bis	N,N,-methylenelbisacrylamide
BSA	bovine serum albumin
Ca	calcium
Cl. histolyticum	*Clostridium histolyticum*
DFP	diisopropylphosphofluoridate
Dnp peptide	dinitrophenol-Pro-Leu-Gly-Leu-Ala-Gly-D-Arg
EDTA	ethylenediaminetetraacetate
FPLC	fast protein liquid chromatography
h	hour
IEF	isoelectric focusing
IEX	ion exchange chromatography
LMW	low molecular weight
$O.D._{280}$	optical density at 280 nm
Δ $O.D._{600}$	change in absorbance at 600 nm
PAGE	polyacrylamide gel electrophoresis
ppt	precipitation
SDS	sodium docdecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N,N-tetramethylenediamine
TES	tris (hydroxymethyl) methyl-2-aminoethane
Tris base	tris (hydroxymeythyl) aminomethane

CHAPTER I

INTRODUCTION

The term collagenases is used to describe those enzymes that are capable of specifically attacking the native collagen helix under non-denaturing conditions within the physiological pH range (Eisen *et al.*, 1970). Controlled degradation of collagen plays an important role in the physiological remodelling of animal tissues during growth and development as well as in repair and pathologic states (Jeffrey and Gross, 1970). Until 1962, most interest in collagenases centered around the enzyme elaborated by *Clostridium histolyticum* (Seifter *et al.*, 1959; Mandl, 1961). In that same year that Gross and Lapiere (1962) described the first vertebrate collagenase from bullfrog tadpole tissue culture media. Subsequent to this landmark contribution, a large number of collagenolytic enzymes were found in amphibia (Dresden and Gross, 1970; Mailman and Dresden, 1979), mammals (Eisen *et al.*, 1968; Jeffrey and Gross, 1970) as well as crustacean species (Eisen *et al.*, 1973; Baranowski *et al.*, 1984).

Collagenases obtained by tissue and cell culture have been detected from a wide range of animal species (Fullmer and Gibson, 1966; Jeffrey and Gross, 1967; Grillo *et al.*, 1968). A collagenolytic enzyme was also extracted directly from the hepatopancreas of the fiddler crab (*Uca pugilator)* in its active form by Eisen and Jeffrey (1969). Following this discovery, direct extraction of collagenolytic enzyme from rat uterus (Weeks *et al.*, 1976; Woessner, 1979), human leucocytes (Lazarus et al., 1972; Ohlsson and Olsson, 1973; Kobayashi and Nagai, 1978), synovial fluid

(Harris *et al.*, 1969), melanoma cells (Tana *et al.*, 1978) and prawn, *Macrobrachium rosenbergii*, (Baranowski *et al.*, 1984) were reported.

Collagenolytic enzymes have been extracted from hepatopancreas of crab and prawn, and characterized with respect to their specificity on native collagen, inhibition and sensitivity to pH and temperature (Eisen and Jeffrey, 1969; Eisen *et al.*, 1973; Grant *et al.*, 1983; Baranowski, 1984; Nip *et al.*, 1985). Even though these enzyme exhibit certain identical properties, they also show distinct differences from one specie to another.

Increasing concerns about the mushiness of sea foods, including economically important crustacean species such as crab and prawn, during storage at refrigerated temperatures have led to a variety of studies in microbiology and enzymology (Waters and Hale, 1981; Nip *et al.*, 1985; Angel *et al.*, 1985). The main cause of mushiness of freshwater prawn during ice storage has been attributed to the diffusion of collagenase activity from the autolyzing hepatopancreas to the muscle tissue (Lindner *et al.*, 1988).

Lobster is also a crustacean specie with collagen as a major structural component. The collagenase of lobster has not been characterized either in terms of isolation or in terms of its physicochemical properties. Thus, the aim of this work was to extract, purify and characterize collagenase of lobster with respect to its substrate specificity, response to pH and temperature and other physicochemical properties.

CHAPTER II

LITERATURE REVIEW

2.1. Definition of collagenase

The term "collagenase" was first used by Ssadikow in 1927 to describe the action of a pancreatic enzyme on heat-denatured collagen (c.f. Mandl, 1961). Later on, Weil and Kochlaty (1937) isolated a proteinase from *Clostridium histolyticum*, while Maschmann (1937) isolated a similar but different enzyme from *Clostridium perfringens* which digested gelatin, but had no effect on several other protein substrates. The first major study of the role of collagen-digesting enzymes in gas gangrene was made by Macfarlane and MacLennan (1945). They showed that an enzyme present in *Cl. perfringens* type A culture filtrates dissolved fresh Achilles' tendon and destroyed the collagen muscle framework, and they re-introduced the term "collagenase" to describe that enzyme. MacLennan and his co-workers also isolated, purified and characterized collagenases from 20 Clostridial strains (Maclennan *et al.*, 1953).

Collagen is widespread in the animal kingdom as important component of connective tissues. About 25% of vertebrate protein is collagen. Since denatured collagen is easily attacked by almost all the known proteases, the term collagenase is restricted to only those enzymes which hydrolyze undenatured collagen at

physiological pH. Thus, collagenase is defined as an enzyme capable of causing hydrolytic scission of peptide bonds located in the characteristic poly-L-proline type of helical regions when the substrate is in the undenatured state (Seifter and Harper, 1971).

2.2. The enzyme

At present, approximately 20 enzymes have been characterized and designated as collagenases. These enzymes are broadly divided into three groups.

One group is elaborated by microorganisms that in themselves do not contain collagen. Two sorts of functions for the collagenases of microorganisms are inferred. One is associated with mechanisms of invasion of a host. The collagenase of *Clostridia* are capable of weakening and destroying the connective tissue barriers of a host. The other function of collagenases of microorganisms could be digestive in the nutritional sense. The collagen of the host would be cleaved to smaller peptides by the collagenase, and associated peptidases could act to provide amino acids for nutritional purposes.

The second group of collagenases, the so-called tissue collagenases, are produced by multicellular organisms that have collagen as a major extracellular component of their tissues. The action of most tissue collagenases, however, appears to be more limited and directed to different ends. The collagenases such as those of the resorbing tail of the tadpole or the involuting postpartum uterus are exquisitely

controlled. They function in a program of remodelling of specialized tissues at a particular time of development or physiological expression. Other tissue collagenases are associated with the mechanism of repair of tissue, removing injured collagen and perhaps promoting regeneration or wound healing in an orderly manner. All tissue collagenases provide a means of conserving amino acids and, in this sense, serve a nutritional function as well.

The third group of collagenases serve a strictly nutritional (digestive) function in multicellular organisms. This is based on the discovery of an extractable collagenase from the hepatopancreas of the crab (Eisen and Jeffrey, 1969).

An example of collagenases from each group is shown in Table 1.

Bacterial collagenases appear to promote more extensive cleavage of collagen than do tissue collagenases. Clostridiopeptidase A may catalyze approximately 200 cleavage per α chain, but the collagenase of the tadpole tail may only catalyze one scission per α chain.

Almost all of the bacterial and tissue collagenases are inhibited by ethylene diamine tetraacetic acid (EDTA) and appear to require Ca^{2+} for activity except the collagenase extracted from crustacean (crab and prawn) which are inhibited by diisopropylfluorophosphate (DFP) and p-tosyllysylchloromethane, and therefore appear to be serine protease.

Table 1. Comparison of properties of selected collagenases

Source	Substrates and assay	Activators	Inhibited by*	Some properties
Clostridium histolyticum (extracellular)	Collagen; gelatin; synthetic peptides. Assay with collagen is viscometric; all other assays can be with ninhydrin	Ca dependent; Zn probably intrinsic	EDTA, cysteine	MW 105,000. Can yield inactive subunits & perhaps active ones. Not inhibited by DFP
Tadpole, *Rana catesbiana* (cell culture)	Collagen (viscosity; release of labelled peptides from ^{14}C-labelled collagen), Gelatin	Ca^{2+}	EDTA; cysteine; serum	Optimum pH 8-9
Hepatopancreas of the crab, *Uca pugilator*	Collagen (viscosity)		DFP; Phenyl-methylsulfonyl fluoride	Not inhibited by EDTA or cysteine

* Abbreviations: EDTA, ethylenediaminetetraacetate;
DFP, diisopropylphosphorofluoridate.
(Table adopted from Seifter and Harper, 1971)

2.3. The substrate

Collagen from a variety of sources, both vertebrate and invertebrate, have been studied as substrates for collagenase. Collagen is a long rod-like molecule consisting of three parallel polypeptide chains coiled about a common axis in a left handed poly-L-proline type of helix (Traub and Piez, 1971). Glycine occurs at every third residue of the main body chain and the amino acids, proline and hydroxyproline predominate (Gallop *et al.*, 1972). The entire structure is twisted in a rope-like fashion, termed coiled-coil, and has a molecular weight of approximately 300,000. The molecule is stabilized by interchain hydrogen bonding (Fietzek and Kuhn, 1976), and both intra- and intermolecular covalent crosslinks (Tanzer, 1973; Siegel, 1976).

Mammalian Achilles' tendon (cattle, horse, rabbit) is probably the most common native substrate available. It may not be as homogeneous as other collagens or procollagens, but in spite of these drawbacks it is preferred by most investigators. It is also the most resistant of all collagen substrates studied.

Mammalian skin collagen appears to be preferred by electron microscopists (Gross, 1953; Keech, 1955). Reticulin has been used in histochemical studies (Robb-Smith, 1945, 1953; Dresner and Schubert, 1955). These investigators found the susceptibility of reticulin to digestion by collagenase to be greater than that of other substrates. It is, however, difficult to purify.

Collagenases have also been shown to act on synthetic substrates (Grassmann *et al.*, 1959; Grassmann and Nordwig, 1960; Henry and Legler, 1959, 1960; Nagai *et*

al., 1960; Fujimoto, 1968). The main advantage lies in the homogeneity of the test solution, however they are not necessarily specific for collagenase attack only, with the exception of two of these synthetic substrates, dinitrophenol-Pro-Leu-Gly-Leu-Ala-Gly-D-Arg (Dnp peptide) produced by the Protein Research Foundation, Osaka, Japan (Masui *et al.*, 1977) and p-phenylazo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (Wuensch and Heidrich, 1963). These two synthetic substrates are the only ones commercially available.

2.4. Substrate preferences of different collagenases

Although all the known animal collagenases essentially attack at the same peptide bond locus, they appear to do so at different rates. For example, Horowitz *et al.* (1977) observed that collagenase extracted from human polymorphonuclear leucocytes preferentially attacked Type I collagen about 15 times more rapidly than it did Type III. Liotta *et al.* (1979) observed that collagenase from mouse fibrosarcoma failed to degrade Type I, but was significantly effective against Type IV collagen of basement membrane origin. There appears to be no information to suggest that animal collagenases attack Type IV collagen.

2.5. Differential susceptibility of different collagenases

Different collagen types appear to exhibit different susceptibilities to collagenolytic attack. It has been amply demonstrated by a number of investigators

that cartilage collagen both in solution and in the fibril form is more resistant to attack by a variety of collagenases than is Type I or Type III collagen (Harris and Cartwright, 1977). Another study by Liotta *et al.* (1979) on the relative susceptibilities of Types I, II, III, IV and AB collagens to cleavage by human skin and mouse tumour collagenases, revealed not only specificity of the tumour enzyme for Type IV collagen as a substrate, but also that both Type IV and AB are highly resistant to attack by human skin collagenase. Thus, there is again a marked difference in susceptibility of the native substrates to a particular collagenase.

2.6. The action of collagenase on collagen

The native collagen molecule, also called tropocollagen, is composed of three left-handed polypeptide helices coiled around each other to form a right-handed supercoil like a rope as shown in Fig. 1. Invariably, one third of the total residues is glycine and the molecule contains a high proportion of proline and hydroxyproline. The glycine residues are disposed regularly, at every third place along the chain in sequences of the type Gly-X-Y, where X and Y can be any amino acid except glycine. With the high proline and hydroxyproline content, this arrangement often results in Pro-X-Gly-Pro with the X-Gly bond as the cleavage site. Electron microscope studies by Gross (1953) and by Keech (1955) revealed that the action of collagenase resulted in a clear breakdown of fibril (Figs. 2 and 3).

Polypeptide helix

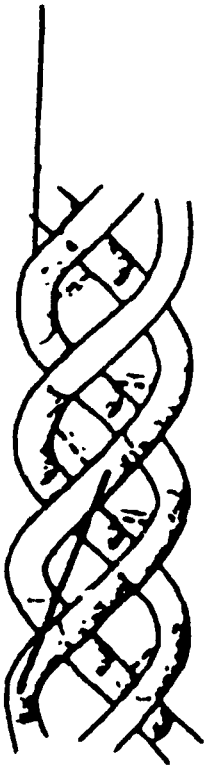


Fig 1. Segment of tropocollagen

triple lelix

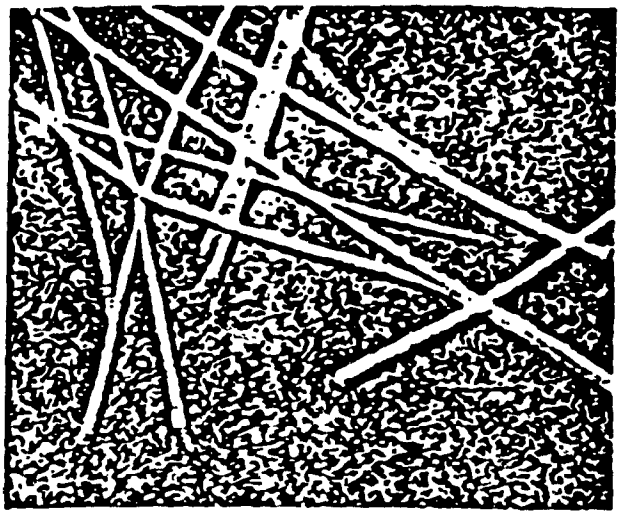


Fig 2. Electron micrograph of untreated collagen fibrils. (from Gross, J., 1953)

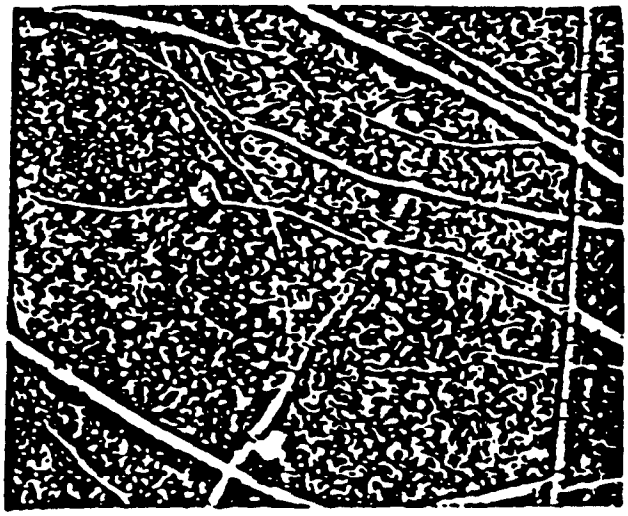


Fig 3. Electron micrograph showing the effect of Cl. histolyticum collagenase on collagen fibrils. (from Gross, J., 1953)

2.7. Applications of collagenases

Collagenases have been used in the laboratory, medical research and industry. In the laboratory, Clostridiopeptidase A has been used effectively to study the unfolding and refolding of the collagen helix (Keech, 1954). The enzyme has also been used for studying repeated sequences in collagen (Gallop *et al.*, 1957; Hipped and Wong, 1963) as well as helped to locate specific structural features of the collagen molecule such as the aldol cross-linkages (Rojkind *et al.*, 1966, 1968), and the hydroxylamine-sensitive linkages (Blumenfeld and Gallop, 1962).

Collagenases are useful as a specific means for identifying native collagen. Clostridiopeptidase A can digest native collagen *in situ* and thereby, by a subtractive procedure, help to locate the collagenous component in a tissue. Bacterial collagenase has been used as a tool in studies on the biosynthesis of collagen (Schrohenloher *et al.*, 1959). In studies relating to the biosynthesis of insulin, collagenase has been applied successfully for the preparation of islet cells of the pancreas (Grassmann *et al.*, 1962). Tadpole and bacterial collagenase have also been employed in structural studies of collagen (Bornstein, 1967).

Recently, collagenase was used to dissolve immune aggregates containing collagen. In immunological studies, it was used to remove collagen from a tissue before reaction with an anticollagen antibody (Steiner *et al.*, 1969; Sidney, 1972).

In medicine, collagenase has also been employed to study certain disease processes and the mechanism of wound-healing. In healing wounds, both the

granulation tissue and the epidermis were found to produce the enzyme to a greater extent than normal skin. Active remodelling of the new connective tissue might depend upon such enzyme activity.

Industrial applications of specific collagenolytic enzymes may be sought in the leather industry (bating and dehairing) and in food processing (meat tenderizing). Plant proteinases such as bromelin, ficin and papain are the main enzymes currently used as meat tenderizers in food processing (Bernholdt, 1975; Dransfield and Etherington, 1981). But these proteinases currently used for meat tenderization attack both connective tissue and myofibrillar proteins, thus they can bring about over-tenderization, leading to mushiness and undesirable texture softening. The ideal enzyme for degrading insoluble collagen in a meat system must not only demonstrate specificity for collagen, but must also function at (i) the relatively low pH of meat; (ii) the low temperatures at which meat is held during storage; or (iii) at the higher temperatures reached during cooking. Collagenases have been found to have the desired specificity. It has also been found that collagenases, especially those from marine microorganism, such as *Vibrio* B-30, offer high quality meat and meat products for consumers and, their use is encouraged for commercial application (Cronlund and Woychik, 1987; Miller *et al.*, 1989).

2.8. The preparation of collagenase

Several different strains of *Clostridium histolyticum* have been used for production of collagenases. Several investigators have prepared collagenases from

strain 47Q5 grown in the medium of Warren and Gray (Warren and Gray, 1961) as modified by Takahashi and Seifter (1972).

For tissue collagenases, tissues (e.g., tail and back skin) are allowed to metabolize under conditions of surviving cell tissue culture. A medium is employed that contains balanced salts, glucose, and antibiotics to limit bacterial contamination. The enzyme can be detected in the medium within two to three days of incubation (Nagai *et al.*, 1966; Harper and Gross, 1970; Seifter and Harper, 1970).

Collagenase has also been extracted directly from crustacean species in its active form, such as crab and fresh water prawn. The extraction of collagenase can either be achieved by the preparation of acetone powder (Eisen *et al.*, 1973) or by the use of 0.05 M tris-HCl buffer, pH 7.5 (Eisen and Jeffrey, 1969; Baranowski *et al.*, 1984).

2.9. Purification

Collagenases are commonly prepared by the successive steps of ammonium sulphate precipitation, gel filtration on Sephadex G-200 or G-150, and ion exchange chromatography using DEAE-Sephadex cellulose column (McCroskery *et al.*, 1975; Harris *et al.*, 1978; Vater *et al.*, 1978a, b; Woolley *et al.*, 1973, 1975, 1978).

2.10. Collagenase assay

In accord with the definition of collagenases, definitive assays ultimately must be based on the use of undenatured collagen as a substrate. Generally, physical

criteria for collagenolytic activity must be used; these represent either dissolution of insoluble collagen or a change in one of the physical properties of collagen in solution. Dissolution can be quantified by examining the supernatant fluid either with a biuret-phenol reagent or by ninhydrin reactivity. The action of collagenase on collagen in solution can be measured most conveniently by a decrease in viscosity as the enzyme digests the substrate, although change in optical rotatory activity could also be used.

2.10.1. Suspension method

This involves mixing the enzyme with a suspension of powdered "insoluble" collagen (such as that of bovine Achilles tendon) or reconstituted fibres prepared from acid-extracted collagen, in particular, of ichthyocol (Gallop *et al.*, 1957), and the progress of proteolysis is monitored by reacting the filtrates with either ninhydrin or Folin (phenol) reagents. The activity is then expressed in units of collagenase, which is defined as that amount causing the solubilization of 1 mg of suspended collagen under the conditions of the assay.

This method of assay is relatively easier to carry out and fairly reproducible.

2.10.2. Viscosity method

This is a variation of the viscosity method introduced by Gallop *et al.* (1957) as described in detail by Seifter and Gallop (1962). It is possible to use a substrate

in the form of a solution with this method. The principle of viscosity assay is based on the fact that solutions of collagen have extremely high viscosities which diminish rapidly as collagenolytic action proceeds. The modification employed usually utilizes guinea pig skin collagen (acid-extracted) dissolved to a concentration of 0.15% in buffer, pH 7.4, containing 0.05M tris and 0.4M NaCl. The reaction is conducted at 27^0C, and the drop in specific viscosity with time is recorded. This method has been established for dissolving substrate without denaturation or gelatinization while maintaining pH in the neutral range. It is also found that collagen dissolved in this manner is not subject to digestion by trypsin but was readily hydrolyzed by collagenase.

2.10.3. Assay based on release of ^{14}C-glycine-containing peptides

This assay, described by Gross and his colleagues (Gross and Nagai, 1965; Nagai *et al.*, 1966), is based on the release of radioactive breakdown products from a substrate of reconstituted [^{14}C] glycine-labelled guinea pig skin collagen fibrils. It involves the preparation of the radioisotopically labelled substrate from the tissues of an animal. Recently, Werb *et al.* (1977) used the procedure of Gisslow and McBride (1975) in which collagen is rendered radioactive by acetylation with ^{3}H or ^{14}C acetic anhydride.

Recently, a microassay based on hydroxyproline determination was reported by Morales *et al.* (1978).

2.11. Recent studies in crustacean species

Collagenolytic enzymes have been obtained by tissue and cell culture from a wide range of animal species. Collagenases with optimal activity at neutral pH have been detected in cultures of a number of amphibian and mammalian tissues (Gross and Lapiere, 1962; Walker *et al.*, 1964; Fullmer and Gibson, 1966; Beutner *et al.*, 1966; Jeffrey and Gross, 1967; Riley and Peacock, 1967; Grillo and Gross, 1967; Eisen, 1967; Bennick and Hunt, 1967; Fullmer and Lazarus, 1967; Grillo *et al.*, 1968). Some of these collagenases have been isolated from the culture medium and partially characterized (Gross and Nagai, 1965; Nagai *et al.*, 1966; Sakai and Gross, 1967; Evanson *et al.*, 1967; Eisen *et al.*, 1968; Evanson and Krane, 1968; Jeffrey and Gross, 1970).

Although collagenolytic enzymes have been studied extensively in human tissues, tadpole tailfin, rat uterus and rat skin, they have not received as much attention in crustacean species even though these animals are predacious scavengers that feed on animal tissues frequently containing collagen as a constituent protein. The major organ involved in the digestion of ingested protein in these organisms is the hepatopancreas. A collagenolytic enzyme was extracted directly from the hepatopancreas of the fiddler crab, *Uca pugilator*, in its active form by Eisen and Jeffrey (1969) and further purified to homogeneity using a variety of chromatographic procedures (Eisen *et al.*, 1973). The hepatopancreas collagenase acts both on native collagen fibrils and on collagen in solution and is capable of degrading the collagen molecule under conditions that do not denature the protein. The purified crab

hepatopancreas collagenase possesses specific collagenase activity as well as trypsin-like and chymotrypsin-like activities as an inherent part of the same molecule. Crab collagenase is not inhibited by EDTA and thus differs in this respect from other collagenases. It is inhibited by p-tosyllysylchloromethane and by soybean trypsin inhibitor, and inactivated at acid pH. The crab enzyme has also been shown by the determination of its complete covalent structure to be a serine protease (Grant *et al.*, 1980), homologous to the pancreatic serine proteases of vertebrates. This crab protease is a good general protease, displaying a broader peptide bond specificity than either trypsin or chymotrypsin on noncollagenous substrates (Grant and Eisen, 1980). The crab protease was the first example of a serine protease shown to exhibit significant collagenolytic activity; it is composed of 226 residues and is capable of degrading the native triple helix of collagen under physiological conditions. When aligned for optimal homology, crab collagenase displays 35% identity with bovine trypsin, 38% with bovine chymotrypsin B, and 32% with porcine elastase (Grant *et al.*, 1980). Similar collagenolytic serine proteases have now been reported from the dog pancreas (Takahashi and Seifter, 1974), the fungus *Entomophthora coronata* (Hurion *et al.*, 1979), and the insect *Hypoderma lineatum* (Lecroisey *et al.*, 1979). The first reported and the most extensively characterized of these serine proteases is protease I from the fiddler crab. Crab protease I successfully degrades all five types of native collagen, producing multiple cleavages in the triple helix of each native collagen at 25^0C. Unlike the crab protease, collagenases which have been identified in mammalian connective tissues belong to the class of metalloproteases, are specific

for collagen, and do not appear to attack other protein substrates (Harris and Krane, 1974; Seltzer *et al.*, 1977). Most of these collagenases, which include those derived from human skin fibroblasts (Stricklin *et al.*, 1977) and rabbit synovium (McCroskery *et al.*, 1975), can degrade collagen types I (skin, tendon, bone), II (cartilage), and III (skin, blood vessels, gastrointestinal tract) by initiating only a single cleavage in the native collagen molecule at a locus approximately three-fourths of the distance from the amino-terminal end (Gross, 1976). Compared with these vertebrate collagenases, the major early cleavage by crab protease I in the α1 polypeptide chain of collagen types I-III occurred at a 3/4 : 1/4 locus, resulting in fragments electrophoretically similar to the TC^A and TC^B products of mammalian collagenase action. The ability of the crab protease to degrade all native collagen types and to catalyze cleavages at multiple loci in the triple helix distinguishes its action from that of mammalian collagenases.

In addition to crab protease I, another collagenolytic serine protease designated as crab protease II, has also been purified and characterized from the hepatopancreas of crab (*Uca pugilator*) by Grant *et al.* (1983). Apart from its collagenolytic activity, crab protease II exhibits endopeptidase activity toward other polypeptides and small molecular weight synthetic substrates. The polypeptide bond specificity of this enzyme is similar to that of bovine trypsin as is its interaction with specific protease inhibitors. The amino-terminal sequence of this enzyme displays significant homology with other serine proteases, most notably with that of crayfish, and demonstrates that this enzyme is a member of the trypsin family of serine

endopeptidases. The relatively unique action of this protease with regard to both collagenous and noncollagenous substrates has important implications concerning the specificity and mechanism of collagen degradation.

Digestive enzymes were also isolated from prawn hepatopancreas by Lee *et al.* (1980). The determination of the action of suspected enzymes on collagen, a major structural component of prawn tissue, and on prawn tissue itself to explore the mushiness problem in *Macrobrachium rosenbergii* was carried out by Baranowski (1984). Of all the digestive enzymes, it was found that collagenase significantly degraded lyophilized prawn tissue. Textural changes of freshwater prawn during ice-chilled/ refrigerated storage are believed in part to be due to an autolytic process. A collagenolytic enzyme fraction, isolated from the hepatopancreas of the freshwater prawn, was most active at 37^{0}C and pH 6.5-7.5 and was also active at 0^{0}C. The cause of mushiness after storage on ice, which may be due to autolytic enzymes, microorganisms or a combination of both, has been investigated recently. Nip *et al.* (1985), Angel *et al.* (1985), and Waters and Hale (1981) reported the onset of mushiness after 4, 8 and 11 days of storage on ice, respectively, but no connection was found between development of mushiness on ice storage and total or proteolytic bacterial counts (Angel et al., 1986). Lindner *et al.* (1988) reported that collagenase induces mushiness when assisted by low amounts of proteolytic activity. The main cause of mushiness following ice storage, therefore, was attributed to diffusion of collagenolytic and proteolytic enzymes from the autolysing hepatopancreas (Lindner *et al.*, 1988).

2.12. Objectives of study

Collagenase has been extracted from the hepatopancreas of crab and prawn and characterized with respect to its specificity toward native collagen, inhibition and sensitivity to pH and temperature. Even though these enzymes exhibit certain identical properties, they also show distinct differences from one species to another. The similarities and differences observed in the properties of collagenases derived from a number of tissues from various species present an interesting problem in comparative enzymology. Collagenolytic enzymes have also been implicated in the post-mortem deterioration of fish and shellfish. It is, therefore, important to (i) determine whether lobster hepatopancreas contains collagenase enzyme(s), and (ii) evaluate the characteristics of the lobster enzyme(s) versus those of other collagenases derived from other sources. A better understanding of the properties and mechanisms of action of collagenolytic enzymes in marine organisms would enable food scientists and technologists to formulate effective process manipulations to control the deleterious effects elicited by these enzymes during postharvest handling and storage. Thus the objectives of present study were:

1. to investigate the presence of an active collagenase extract in the hepatopancreas of lobster (*Homarus Americanus*);
2. to isolate, purify and characterize collagenase from lobster hepatopancreas with respect to its pH, temperature optimum and stability;
3. to develop a method for purification of collagenase by the use of Fast Protein Liquid Chromatography (FPLC).

CHAPTER III

MATERIALS AND METHODS

Live lobsters (*Homarus Americanus*) used in this study were obtained from a local market. Immediately upon arrival in the laboratory, the animals were "butchered"to recover the hepatopancreas which was rapidly frozen in liquid N_2 and then stored at -80^{0}C until needed.

Insoluble bovine Achilles tendon collagen (Type I); collagenase (Type V); bovine serum albumin; ammonium sulphate; leucine and ninhydrin were purchased from Sigma Chemical Company.

Acrylamide; N,N-methylene-bis-acrylamide; tris(hydroxymethyl)-aminomethane; Bio-Lyte 3/10 ampholyte, 40%; sodium dodecyl sulfate (SDS) and standard low mol. weight protein markers were purchased from Bio-Rad Laboratories.

All other reagents were of analytical grade and obtained from a variety of commercial sources as indicated at appropriate sections in the text.

3.1. Defatting of lobster hepatopancreas extract

Frozen hepatopancreas tissue was homogenized in cold acetone (-20^{0}C; 1/10

w/v) in a Waring Blendor for 5 min at low speed. The resulting homogenate was centrifuged at 12,000*g* for 30 min at 4^0C, and the acetone was removed by vacuum filtration. The pellets formed were suspended in 10 vol of cold butanol (-20^0C) followed by a similar quantity of cold acetone (Anachemia Co.). The preparations were dried in a vacuum desiccator over H_2SO_4 for 24 h and the resulting powdered product stored *in vacuo* at 4^0C in the presence of anhydrous $CaSO_4$.

3.2. Preparation of crude extract

All procedures were performed at 4^0C unless otherwise indicated. The defatted powder was suspended in cold tris-HCl buffer (0.05 M, pH 7.5) containing 5 mM $CaCl_2$ (1/10 w/v). The suspension was stirred for 1 h and the mixture was centrifuged in a Beckman refrigerated centrifuge (model J2-21) at 39,100xg and 4^0C for 20 min. The residue was re-extracted with an equal volume of the same buffer and centrifuged for 30 min as above. The precipitate was discarded and the two supernatants were combined, and then filtered through a Whatman No.1 filter paper. The clear filtrate was designated as fraction 1, and a small portion of this fraction 1 was analyzed for protein content and collagenase activity.

3.3. Ammonium sulphate fractionation

The fraction 1 was fractionated with solid ammonium sulphate to 70% saturation and stirred over night at 4^0C. The precipitate was collected by

centrifugation at 39,100xg for 30 min, and then re-suspended in a minimal amount of tris-HCl buffer solution (0.05 M, pH 7.5 containing 5 mM $CaCl_2$) and designated as fraction 2.

The redissolved precipitate (fraction 2) was then desalted by passing it through a PD-10 column (Pharmacia Co.). A portion of this fraction was also analyzed for protein content and collagenase activity.

3.4. Protein determination

The protein content in the samples were determined according to the method of Hartree (1972), using crystalline bovine serum albumin solution (BSA) as a standard.

Various dilution of protein samples were prepared in deionized water and 1 ml of each dilute protein solution was placed into each of two test tubes. The compositions of the solutions used are given in Table 2. Solution A (0.9 ml) was added to the samples in each tube and the reaction mixture was incubated in a water-bath at 50^0C for 10 min, then cooled to room temperature. Next, 0.1 ml of solution B were added to the cooled samples and the tubes and contents were thoroughly shaken. The reaction was allowed to proceed at room temperature for 10 min, then 3 ml of solution C added and the system was rapidly vortexed. The tubes and contents were incubated again in the water-bath at 50^0C for 10 min , cooled to room temperature, and the absorbance of the solutions in the tubes was measured spectrophometrically at 650 nm, using a Beckman DU-7500 Diode Array

Table 2. Solutions for protein determination

Solution A: 2 g potassium sodium tartrate and 100 g of sodium carbonate are dissolved in 500 ml 1 N NaOH, diluted to 1 liter with deionized water.
Solution B: 2 g potassium sodium tartrate and 1 g cupric sulphate are dissolved in 90 ml deionized water and 10 ml 1 N NaOH was added.
Solution C: 1 volume Folin & Ciocalteu's phenol reagent was diluted with 15 volume water, this solution was prepared fresh daily.

Spectrophotometer.

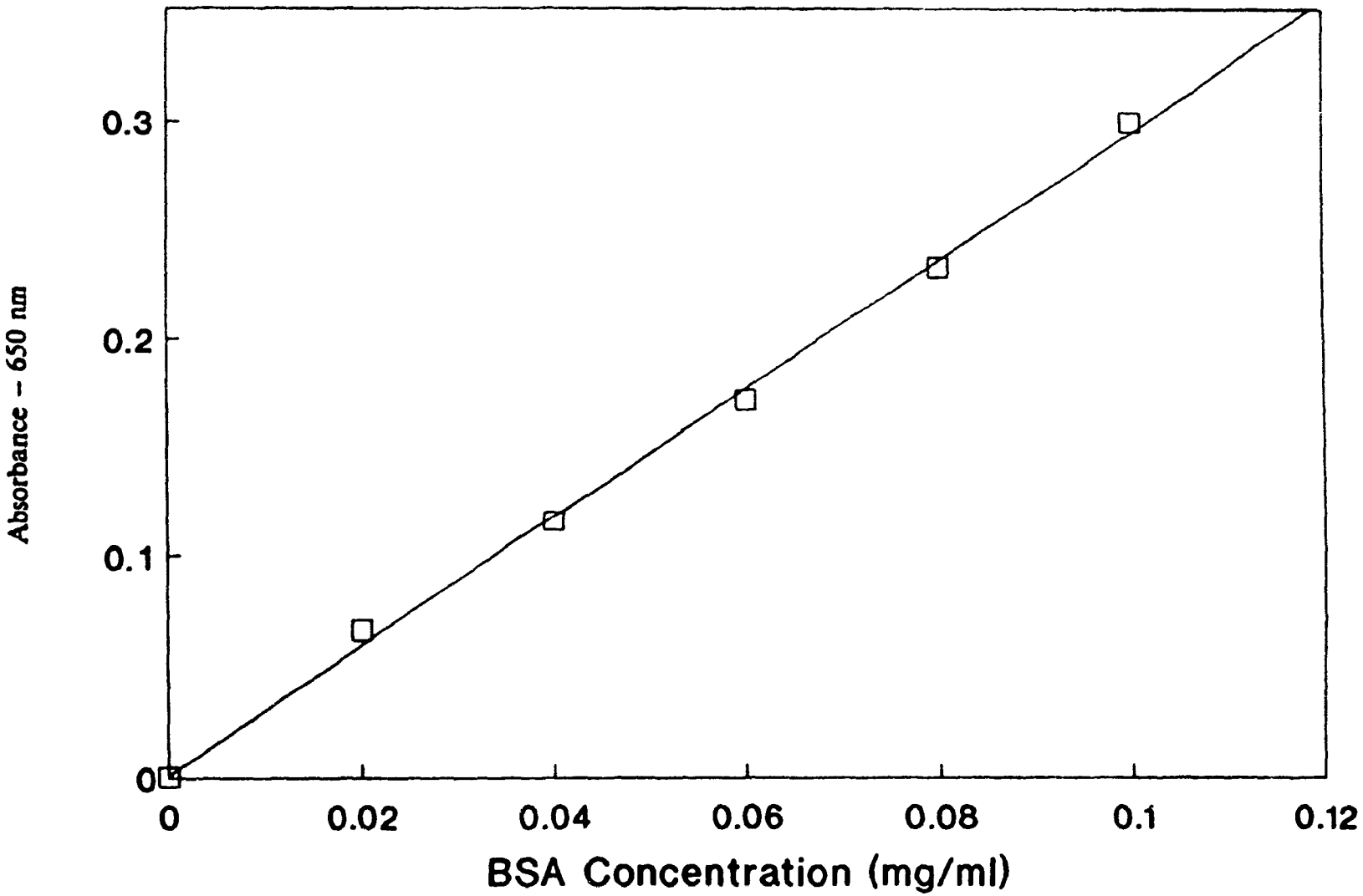
A protein standard curve was prepared by dissolving 10.0 mg BSA in deionized water in a 100 ml volumetric flask to give a final concentration of 0.1 mg BSA/ml. The range of protein concentration used to prepare the standard curve was 0.0 to 0.1 mg BSA/ml (Fig. 4). The protein sample determinations were carried out under the same conditions as for the protein standard.

3.5. Assay of collagenase activity

Collagenase activity was measured using a modified form of the procedure by Mandl *et al.*, (1953) as described in the "WorthingtonEnzyme Manual" (1972). In this assay, collagen (25 mg) was weighed into each of four test tubes, then 5 ml TES (Tris [hydroxymethyl] methyl-2-amino-ethane sulfonate) buffer were added and incubated at 37^0C for 10 min. The four tubes were divided into two sets. Set one (tubes 1 & 2) was the mixture for zero time and set two (tubes 3 & 4) was for 5 h incubation. Into each of tubes 1 and 3, 0.1 ml of the enzyme solution were added. Into each of tubes 2 and 4, 0.1 ml of TES buffer, in place of enzyme, were added to serve as blanks. Immediately after addition of TES buffer or enzyme solution, the contents of tubes 1 and 2 were filtered and 0.2 ml of filtrate transferred into duplicate test tubes each of which contained 1 ml of ninhydrin solution. The resulting mixture was boiled for 20 min, then cooled and 5 ml of a 1:1 mixture of deionized water and n-propanol were added. Following this, the reaction mixture was allowed to stand at room

Fig 4. Standard curve for protein determination

Standard curve for protein determination



temperature for 20 min and the activity measured spectrophotometrically at 600 nm. Tubes 3 and 4 were incubated at 37^{0}C for 5 h. At the end of the incubation the mixture was filtered and treated as described for zero time. A L-leucine standard curve was used to determine the micromoles of amino acid (equivalent to leucine) liberated by the action of the enzyme on collagen. A unit of enzyme activity was expressed as the number of micromoles of leucine produced per milligram collagenase in 5 h at 37^{0}C.

$$\text{units/mg} = \frac{\text{umoles amino acid produced in 5 hours}}{\text{mg enzyme used in assay}}$$

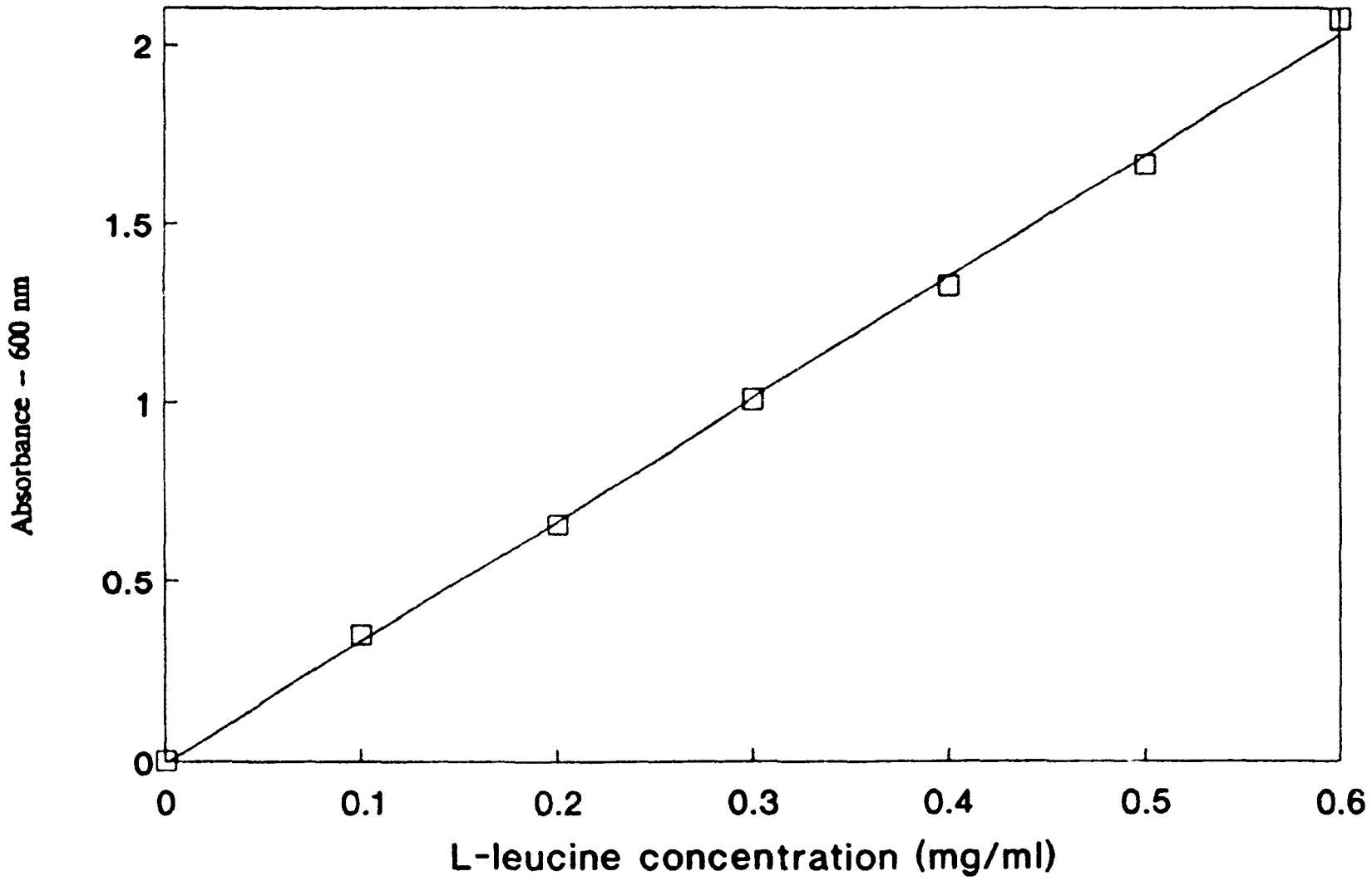
The L-leucine standard curve was prepared by dissolving 60.0 mg L-leucine (Sigma chemical Co.) in deionized water in a 100 ml volumetric flask to give a final concentration of 0.60 mg/ml. The L-leucine standard curve thus obtained is shown in Fig. 5. The L-leucine standard determination was carried out under the same conditions as those for the samples.

3.6. Liquid chromatography

Further purification of the ammonium sulfate extract (fraction 2) was performed using a Fast Protein Liquid Chromatography (FPLC). The FPLC system (Pharmacia Co.) used in the study was equipped with a controller LCC-500 Plus that has a programmable memory to allow (i) the control of chromatographic procedures;

Fig 5. Standard curve for collagenase determination

Standard curve of L-leucine



(ii) modification of separation parameters; and (iii) evaluation and print-out of data from the chromatography. The FPLC system is composed of two P-500 pumps. Peristaltic pump P-1 was used to pump the sample through the valve MV-7 which is designed for application of automatic injections. Valve MV-8 is motorized for use as an automated selection valve for columns, solvents and samples that are controlled by LCC-500 Plus without removal of any columns from the system. Other components are a UV-M monitor with HR flow cell for protein detection at 280 nm, a FRAC-100 fraction collector and a Model 1322 flatbed chart recorder (Bio-Rad Laboratories). Some of the advantages of the FPLC system include: (a) high recovery of biological activity, (b) compatibility with aqueous buffer and salts solution, (c) high speed, high resolution and high capacity and (d) analytical and preparative possibilities.

The samples and buffers were filtered and degassed through a 0.22 um filter (Millipore) under vacuum prior to their use in the FPLC system.

3.6.1. Ion-exchange chromatography on Mono Q

The partially purified extract (fraction 2) was separated on Mono Q HR 5/5 ion-exchange column using the FPLC system. The column was pre-equilibrated with tris-HCl buffer A (5 mM, pH 7.0) and the sample was solubilized in the same buffer. A gradient of buffer A and buffer B (5 mM tris-HCl, pH 7.0 containing 1.5 M sodium chloride) was used. The system was programmed to elute 2 ml of buffer A, perform a gradient (0-100%) of buffer B in a total volume of 23 ml, then buffer B

was run 100% in a volume of 2 ml, followed by 2 ml of buffer A only. The elution was performed at a flow rate of 1 ml/min. Peak fractions of 1 ml were collected, desalted by passing through a PD-10 column, concentrated by ultrafiltration using a CX-30 filter (Millipore) and lyophilized.

3.6.2. Gel filtration chromatography

The collagenase fraction obtained by ion-exchange chromatography on Mono Q column was subjected to Superdex 75 HR 10/30 gel filtration column (Pharmacia Co.) using the FPLC. The column was equilibrated with tris-HCl buffer (0.05 M, pH 7.5) containing 0.15 M NaCl and the sample was solubilized in the same buffer. Elution was performed at a flow rate of 0.3 ml/min and fractions of 1 ml/tube were collected, and filtered through PD-10 column prior to lyophilization.

3.6.3. Isoelectric focusing

The collagenase fraction obtained by ion-exchange chromatography on Mono Q column was also subjected to isoelectric focusing using Rotofor cell (Bio-Rad laboratory). The lyophilized peak 2 fraction from IEX was brought to a volume of 56 ml with 2% ampholyte (pH 3-10) and 3 M urea. Focusing proceeded for 4.5 h at 12 W at 4^0C. Twenty fractions were collected, and their pH values and optical densities at 280 nm were measured. Then, fractions were separately pooled according to protein concentration peaks and collagenase activities were determinated.

3.7. Determination of pH optimum and stability

The pH optimum of the "collagenase fraction" from different purification steps or commercial collagenase was determined by preparing the substrate (insoluble bovine achilles tendon collagen) in various buffer solutions and applying aliquots of the enzyme solution individually to the buffered substrate solutions. The reaction was allowed to proceed at 37^0C and enzyme activity was determined by measuring the release of leucine (after 5 h incubation) at 600 nm. The compositions of the buffer solutions used were: 0.05 M citrate-phosphate, pH 3.0; 0.05 M citrate-phosphate, pH 4.0; 0.05 M citrate-phosphate, pH 5.0; 0.05 M citrate-phosphate, pH 6.0; 0.05 M tris-HCl, pH 7.0; 0.05 M tris-HCl, pH 8.0; 0.05 M tris-HCl, pH 9.0. The final pH at each point was determined using appropriate blanks in the absence of enzyme protein.

The influence of pH on the stability of "collagenase fraction" or commercial collagenase was determined by pre-incubating the latter with the above buffer solutions (ratio of enzyme stock solution to buffer = 1:1 v/v) at 25^0C for 30 min. Residual enzyme activity was assayed as described previously.

3.8. Determination of temperature optimum and stability

The optimum temperature for the hydrolysis of native collagen by various "collagenase fractions" or commercial collagenase was determined by incubating mixtures of the enzyme and substrate at various temperatures (from 20-60^0C) at 5^0C intervals. After incubation at the various temperatures, collagenase activity was

measured as described in section 3.5.

To determine thermostability, the "collagenase fraction" or commercial collagenase was incubated at various temperatures for 30 min. After incubation, the enzyme solutions were rapidly cooled in an ice bath for 5 min prior to addition to the substrate and assay for residual activity.

3.9. Electrophoresis

3.9.1. SDS-polyacrylamide gel electrophoresis

Electrophoresis in the presence of sodium docdecyl sulfate (SDS) was carried out according to the method of Laemmli (1970) using a 12% acrylamide slab gels. A summary of the solutions used for the preparation of the polyacrylamide gels is shown in Table 3. The other solutions which were used in the electrophoretic analysis are presented in Table 4.

3.9.1.1. Preparation of gels and samples

Gels were prepared and polymerized according to the manufacturer's instructions using a vertical slab electrophoresis instrument, Protean II Slab Cell (Bi0-Rad Laboratories, 1414 Harbour Way South, Richmond, Ca. 94804).

Protein samples (1 mg) were dissolved in 1 ml of sample buffer, and heated at 95^0C for 4 min.

Table 3. Solutions for the preparation of polyacrylamide gels (SDS)

Solution gel	Composition	Mixing Ratios of Solution (ml)
Separation Gel		
(1)	29.2 g Acrylamide 0.8 g Bis[a] with Water to 100 ml	40.0
(2)	18.15 g Tris base[b] Adjust to pH 8.8 with 1 N HCl, dilute to 100 ml with water	25.0
(3)	10 g SDS to 100 ml with water	1.0
(4)	0.1 g APS[c] to 1 ml with water	0.5
(5)	TEMED[d]	0.05
(6)	Deionized water	33.5
Stacking Gel		
(1)	The same as above	1.3
(7)	6 g Tris base[b] Adjust to pH 6.8 with 1 N HCl, make to 100 ml with water	2.5
(3)	The same as above	0.1
(4)	The same as above	0.05
(5)	TEMED[d]	0.01
(6)	Deionized water	6.1

a. N,N,-methylenelbisacrylamide.
b. Tris (hydroxymethyl) aminomethane.
c. Ammonium persulphate prepared immediately prior to use.
d. N,N,N,N-tetramethylenediamine.

Table 4. Solutions for electrophoresis & staining of gels

Solution	Composition	
Sample buffer	0.05 M Tris-HCl (pH 6.8):	1.0 ml
	Glycerol:	0.8 ml
	10 % (w/v) SDS:	1.6 ml
	2 β-mercaptoethanol	0.4 ml
	0.1 % Bromophenol blue:	0.2 ml
	Deionized water:	4.0 ml
		8.0 ml
Electrode buffer	Tris (15 g), Glycine (72 g), deionized water to one liter.	
Fixative solution	Trichloroacetic acid (12.5 g) dilute to 100 ml with deionized water.	
Staining solution	0.1 % Commassie Brilliant Blue R-250 was dissolved in 30 % methanol and 10 % acetic acid.	
Destaining solution	Acetic acid, methanol and water in the ratio of 10: 30: 60 was mixed.	
Storage solution	30 % glycerol and 5 % acetic acid.	

3.9.1.2. Electrophoresis

The gel slabs were removed from the casting apparatus and positioned onto the central cooling core as described in the manufacturer's instructions manual (Bio-Rad). The composition of the buffer solution used for the electrophoresis is shown in Table 4. Approximately 1.5 L of the electrode buffer was poured into the lower chamber of the electrophoresis unit, and 400 ml of the same buffer poured into the upper chamber. A quantity (70 ul) of the prepared samples was applied to wells in gels. A duplicate gel slab was run using the same samples. Electrodes were connected to computer controlled electrophoresis power supply (Model 3000Xi, Bio-Rad) operated at constant current 16 mA/gel for the first 30 min and then at 24 mA/gel for the remainder of the electrophoresis. Electrophoresis was stopped when the tracking dye reached the bottom of the gel (~ 4.5 h).

3.9.1.3. Fixing, staining and destaining

The composition of the fixing, staining and destaining solutions are shown in Table 4. Gels were removed from the glass sandwich and the protein bands in the gels were fixed with 12.5 % trichloroacetic acid (TCA) for 10 min. The gels were stained overnight with staining solution and then destained for several days using the destaining solution until the background became clear. The gels were stored in a solution of 30 % glycerol and 10% acetic acid.

3.9.2. Polyacrylamide gel electrophoresis (without SDS)

For monitoring enzyme purification, polyacrylamide gel electrophoresis was also performed in the absence of SDS in 12 % acrylamide slab gels. The composition of polyacrylamide gel is given in Table 5.

Protein samples (1 mg) were dissolved in 1 ml of sample buffer. An aliquot of 70 ul of the prepared samples was applied to wells in gels. A duplicate gel slab was run using the same samples. Electrodes were connected to computer controlled electrophoresis power supply (Model 3000Xi, Bio-Rad) operated at constant current 15 mA/gel for the first 30 min and then at 22 mA/gel for the remainder of the electrophoresis. Electrophoresis was stopped when the tracking dye reached the bottom of the gel (~ 4 h). The protein fixing, stainining and destaining for PAGE were the same as SDS-PAGE.

Table 5. Solutions for the preparation of polyacrylamide gels

Solution gel	Composition	Mixing ratios of solution (ml)
Separation Gel		
(1)	29.2 g acrylamide 0.8 g bis[a] with water to 100 ml	40.0
(2)	18.15 g tris base[b] adjusted to pH 8.8 with 1 N HCl, dilute to 100 ml with water	25.0
(3)	0.1 g APS[c] to 1 ml with water	0.5
(4)	TEMED[d]	0.05
(5)	Deionized water	34.5
Stacking Gel		
(1)	The same as above	1.3
(6)	6 g Tris base[b] adjusted to pH 6.8 with 1 N HCl, make to 100 ml with water	2.5
(3)	The same as above	0.05
(4)	TEMED[d]	0.01
(5)	Deionized water	6.2
Sample buffer	0.05 M Tris-HCl (pH 6.8): Glycerol: 0.1 % Bromophenol blue: Deionized water:	1.0 ml 0.8 ml 0.2 ml 6.0 ml

a. N,N,-methylenelbisacrylamide.
b. Tris (hydroxymethyl) aminomethane.
c. Ammonium persulphate prepared immediately prior to use.
d. N,N,N,N-tetramethylenediamine.

CHAPTER IV

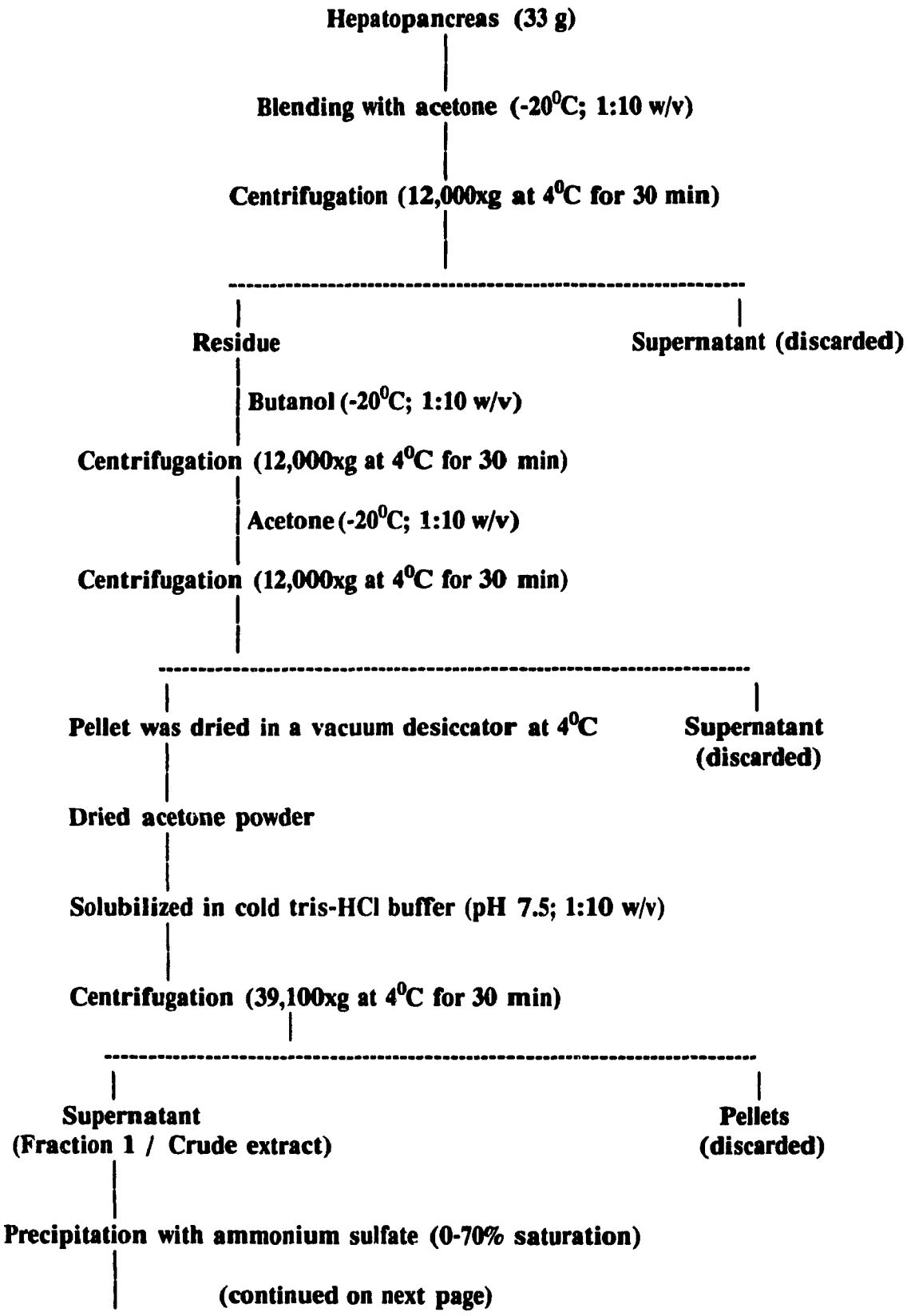
RESULTS AND DISCUSSION

4.1. Recovery of collagenase fraction

The scheme used to recover collagenase from the hepatopancreas of lobster is summarized in Fig. 6. The crude extract (fraction 1) recovered from the acetone powder (Fig. 6) appeared to be the active form of collagenase, based on its ability to hydrolyze native collagen substrate. This finding is similar to results obtained with collagenase from other crustacean species (Eisen *et al.*, 1973; Nip *et al.*, 1985). In this respect, the lobster enzyme differed from collagenases from amphibia (Harper *et al.*, 1971) or mammals (Vaes, 1972; Stricklin *et al.*, 1977, 1978) which were shown to occur in the zymogen form. It is also possible that the lobster enzyme existed as the zymogen which was converted to the active form during the recovery process.

The crude enzyme extract (fraction 1) was fractionated with solid ammonium sulphate and the precipitate formed up to 70% saturation with $(NH_4)_2SO_4$ was collected by centrifugation at 39,100xg and 4^0C for 30 min. The recovered precipitate was redissolved in extraction buffer and designated as "fraction 2" Next, fraction 2 was desalted by passing it through a Pharmacia PD-10 column.

The desalted semi-purified enzyme extract (fraction 2) was further purified by ion exchange chromatography on Mono Q, HR 5/5 column equilibrated in 0.005 M



(continued on next page)

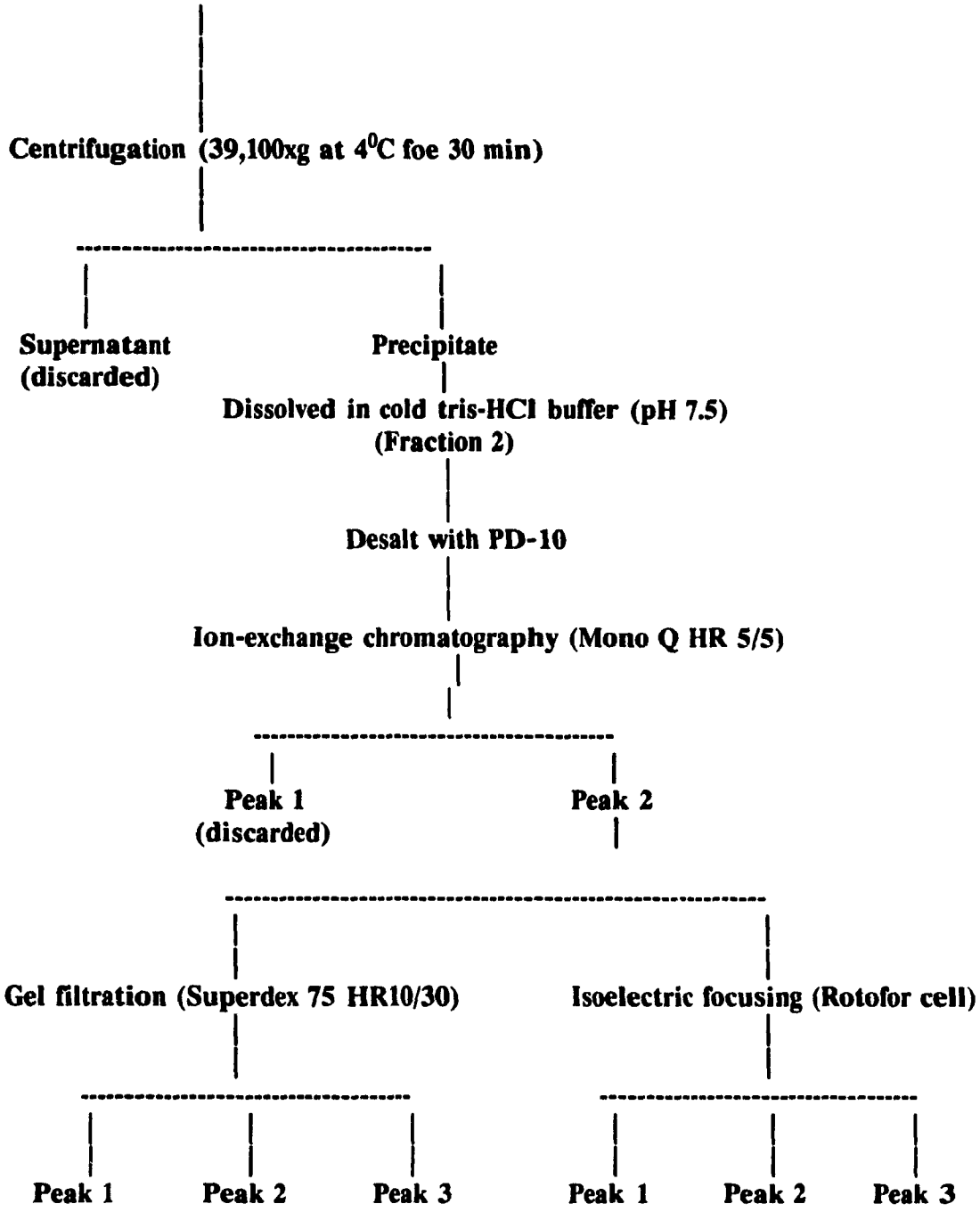


Fig 6. Scheme for the extraction and partial purification of collagenase(s) from lobster hepatopancreas

tris-HCl, pH 7.0 (starting buffer) using a FPLC system. The enzyme was eluted using a linear gradient established from the starting buffer to 1.5 M NaCl. Chromatography on the Mono Q column (Fig. 7) resolved at least 4 peaks, two of which had collagenolytic activity referred to as peaks 1 and 2. Since Peak 1 had significantly less protein than peak 2 and the two of them had identical specific activities, only peak 2 from various runs was pooled and then lyophilized for further examination. The pooled peak 2 fraction from the ion-exchange chromatography step was applied on to a gel filtration column (Superdex 75 HR 10/30 column) using a FPLC system, which separated it further into three peaks (Fig. 8).

The lyophilized peak 2 fraction from ion exchange chromatography step was also subjected to isoelectric focusing using a Rotofor cell. A mixture containing 25 mg of freezed-dried peak 2 fraction from IEX, 2 % Bio-Lyte ampholyte (pH 3-10) and 3 M urea was focused on the Rotofor cell for 4.5 h at 12 W at 4^0C. The starting voltage was 520 V and the final voltage was 824 V. Twenty fractions were collected, and their pH values and optical densities at 280 nm measured (Figs. 9 & 10). A 3.02 to 10.13 pH gradient was formed with a linear region from 4.21 to 10.03 in fractions 3-19. Three protein concentration peaks, all displaying activities, were detected as shown in Fig. 10. Since peak 1 had relative higher specific activity and protein content than peaks 2 and 3, only peak 1 was pooled and desalted by passing it through PD-10 column, which was further electrophoresed in 12 % gels as shown in Fig. 12. The protein contents as well as collagenase activities of the various extracts recovered at various steps during the purification process are summarized

Fig 7. **The profile of collagenase purification by ion-exchange chromatography (Mono Q HR 5/5 column) using the FPLC system**

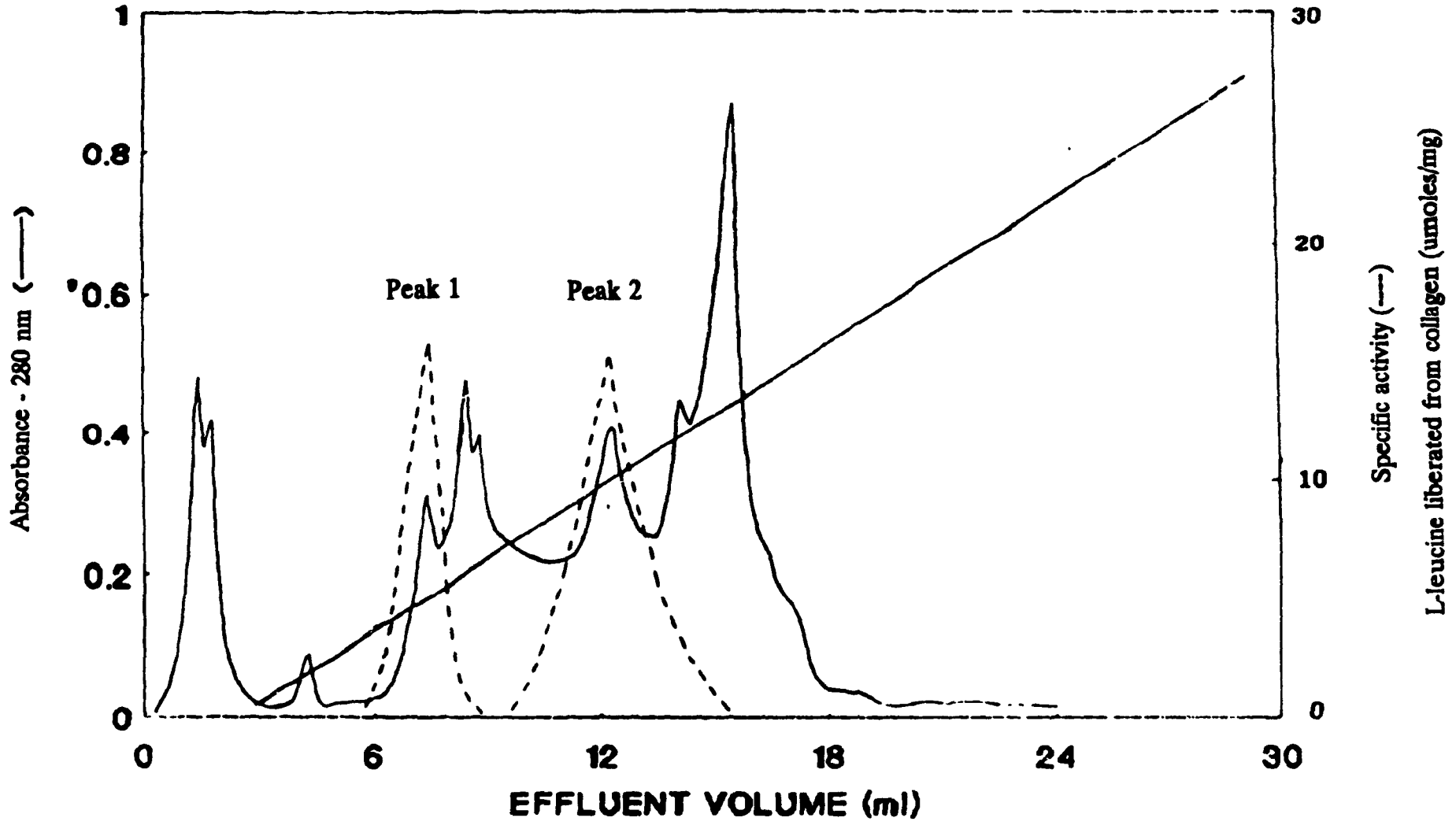

Absorbance - 280 nm (——)
Specific activity (---)
L-leucine liberated from collagen (umoles/mg)
Peak 1
Peak 2
EFFLUENT VOLUME (ml)
0
0.2
0.4
0.6
0.8
1
0
6
12
18
24
30
0
10
20
30

Fig 8. **The profile of collagenase purification by gel filtration chromatography (Superdex 75 HR 10/30 column) after ion exchange chromatography using the FPLC system**

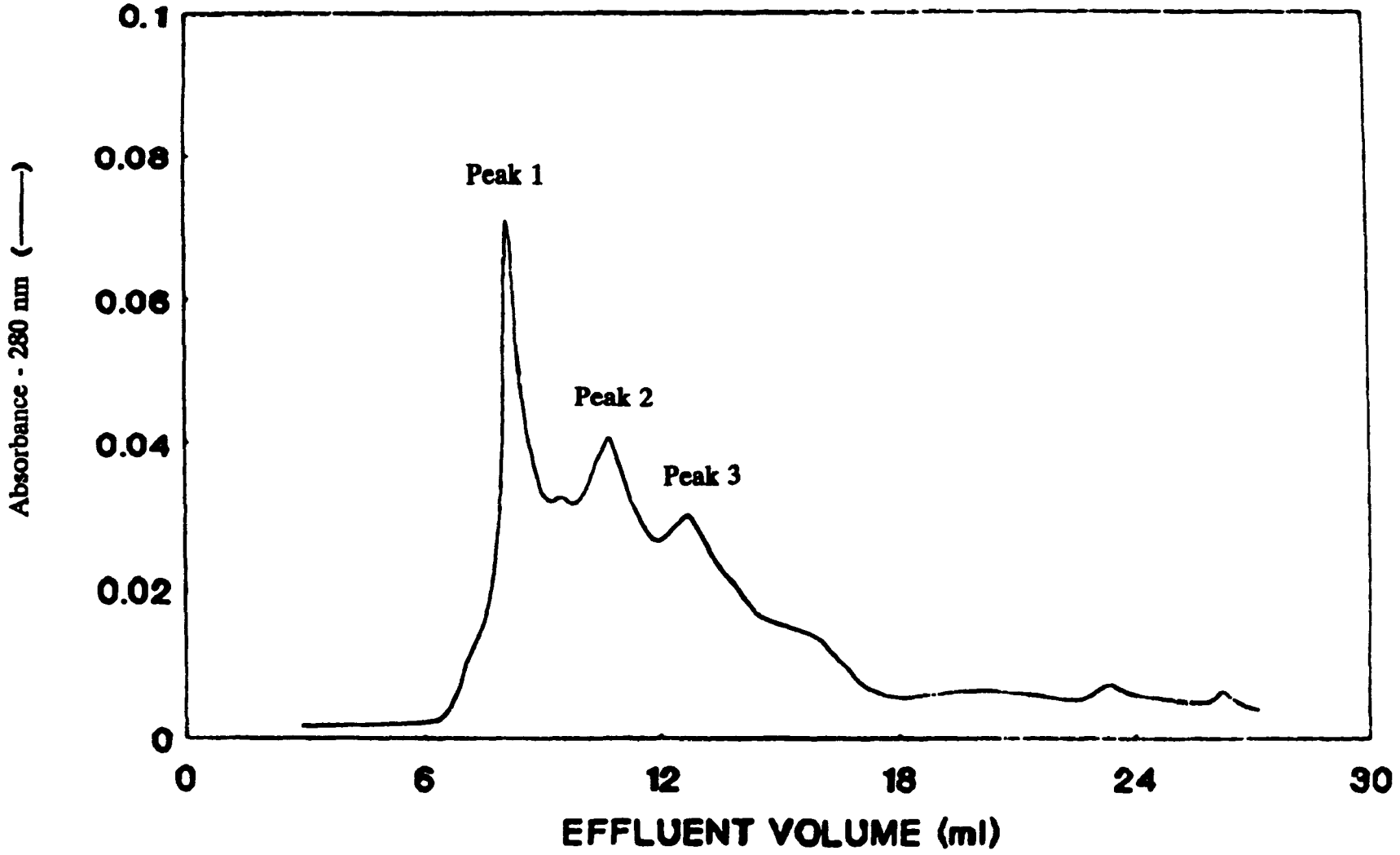

0.1
0.08
0.06
0.04
0.02
0
Absorbance - 280 nm (——)
Peak 1
Peak 2
Peak 3
0
6
12
18
24
30
EFFLUENT VOLUME (ml)

Fig 9. pH of Rotofor fractions

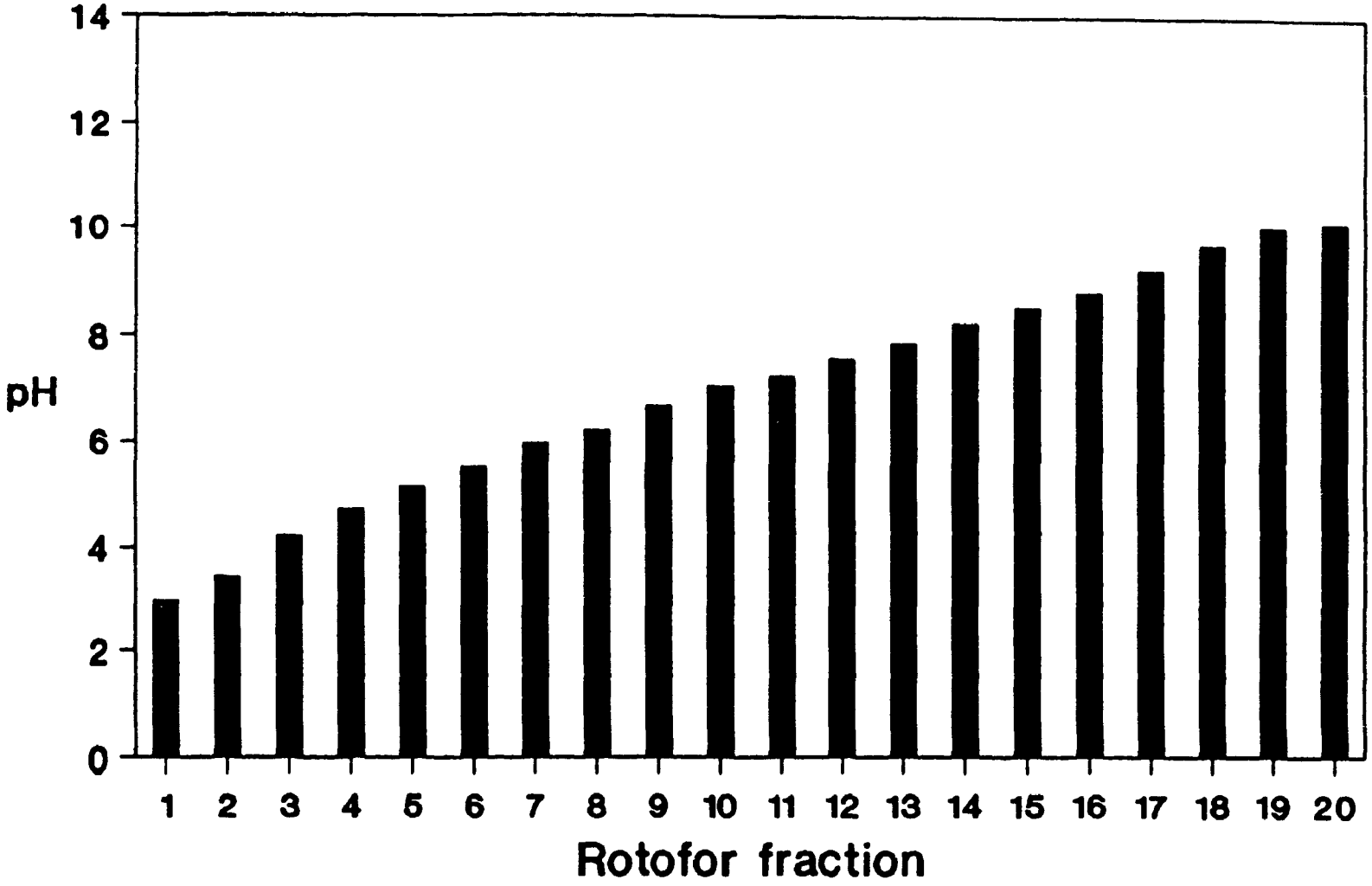
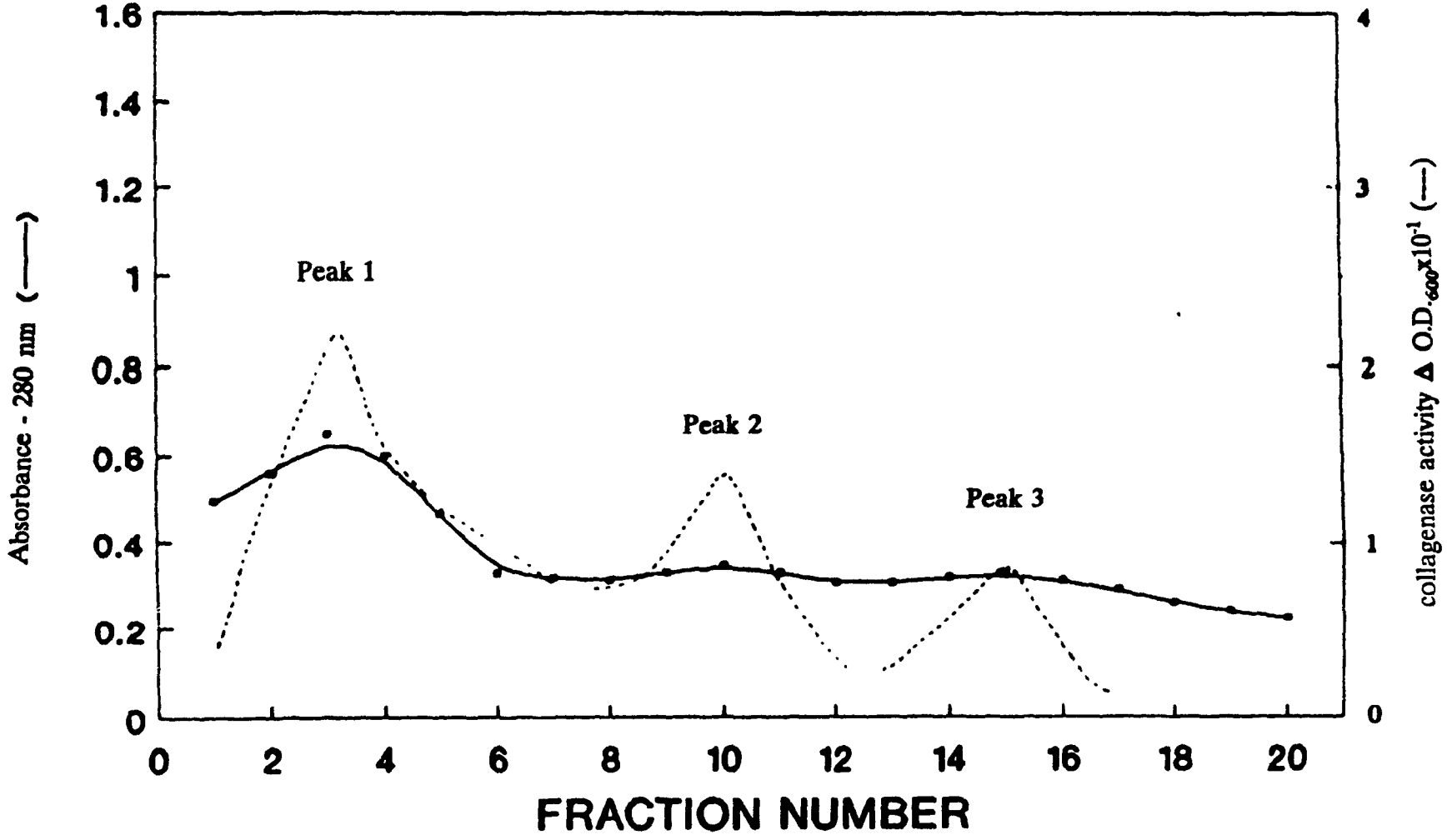

14
12
10
8
6
4
2
0
pH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Rotofor fraction

Fig 10. **A graph showing the $O.D._{280}$ and Δ $O.D._{600}$ (activity) of the fractions from isoelectric focusing**


Absorbance - 280 nm (——)
collagenase activity Δ O.D.600x10-1 (——)
Peak 1
Peak 2
Peak 3
FRACTION NUMBER
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
20

purification scheme -- Table 6.

Compared with the crude extract, the ammonium sulphate fraction showed a 88% recovery of enzyme activity and a purification of 1.9 fold, and this corresponded to a protein content of about 420 mg/50 g of raw material.

The specific activities of the peaks 1 and 2 fractions from ion exchange steps were 17.5 and 16.2 units/mg respectively, and corresponded to a recovery of 14% and 60% of the total activity.

4.2. Electrophoresis

The extracts recovered at various steps in the purification scheme were electrophoresed in polyacrylamide gels under various conditions.

Polyacrylamide gel electrophoresis patterns in the presence of SDS showed that the fractions after $(NH_4)_2SO_4$ fractionation, or ion exchange chromatography had relatively fewer bands than the crude extract (Figs. 11 & 12). These bands ranged in molecular weights from 15,000-66,000.

Examinations of enzymatically active peak 1 fraction from Rotofor cell by electrophoresis in 12 % polyacrylamide at pH 8.3 demonstrated only one sharp protein band and few light bands as compared with peak 2 fraction from IEX step (Fig. 12).

Table 6. Purification scheme for collagenase fraction from lobster hepatopancreas

Step	Total protein (mg)	Total activity (units)	Specific activity[a] (units/mg)	Yield (%)	Purification fold
Crude	590.3	1771	3.00	100	1.0
$(NH4)_2SO_4$ ppt	274.2	1566	5.71	88	1.9
Ion exchange					
peak 1	14.3	250	17.45	14	5.8
peak 2	66.1	1068	16.16	60	5.4

33 g of lobster hepatopancreas were treated as described in the materials and methods section: data presented in Table 6 are representative of four other trials.

[a] Specific activity is defined as micromoles of amino acid (equivalent to L-leucine) liberated per milligram enzyme in 5 h.

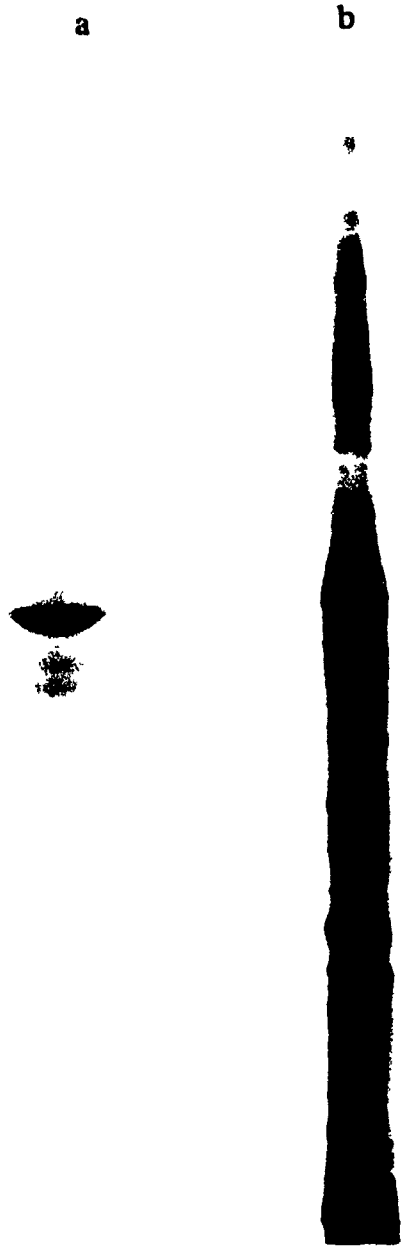
Fig 11. **SDS-Polyacrylamide gel electrophoresis patterns of various collagenase fractions**

(a) Crude extract (b) $(NH_4)_2SO_4$ fraction

(c) Commercial collagenase (d) Protein standard (LMW)

(e) IEX peak 2, FPLC

Fig 12. Polyacrylamide gel electrophoresis patterns of collagenase fractions after IEX and isoelectric focusing



(a) IEF peak 1 (Rotofor) after IEX

(b) IEX peak 2 (FPLC)

4.3. Properties of collagenase fractions

4.3.1. pH optima and stability

4.3.1.1. With "fraction 2" (i.e., ammonium sulfate fraction)

The pH-activity profile (Fig. 13) for the hydrolysis of insoluble collagen by partially purified collagenase fraction (i.e., the ammonium sulfate fraction / fraction 2) indicated that this "collagenase fraction" had two pH optima, a major one at pH 4.0 and a minor one at pH 7.0, while the commercial enzyme was most active at pH 7.0. The two pH optima suggests that there was probably more than one collagenolytic enzyme present in this fraction.

The collagenase "fraction 2" was also most stable at pH 8.0 while the commercial enzyme exhibited a much broader pH stability range from 6.0-9.0, as shown in Fig. 14. Both the commercial enzyme and the collagenase "fraction 2" were quite unstable at acid pH. For example, Fig. 14 shows that about 50% of collagenase "fraction 2" activity and 60% of commercial collagenase activity were lost after 30 min incubation at pH 4.0. This finding is similar to observations made by Eisen *et al.* (1973), who reported that collagenase from crab hepatopancreas was most active at pH 8.0 but was very unstable below pH 5.0.

Fig 13. **pH optima of ammonium sulfate collagenase fraction and commercial collagenase on native collagen as substrate**

Fig 14. **pH stability of ammonium sulfate collagenase fraction and commercial collagenase**

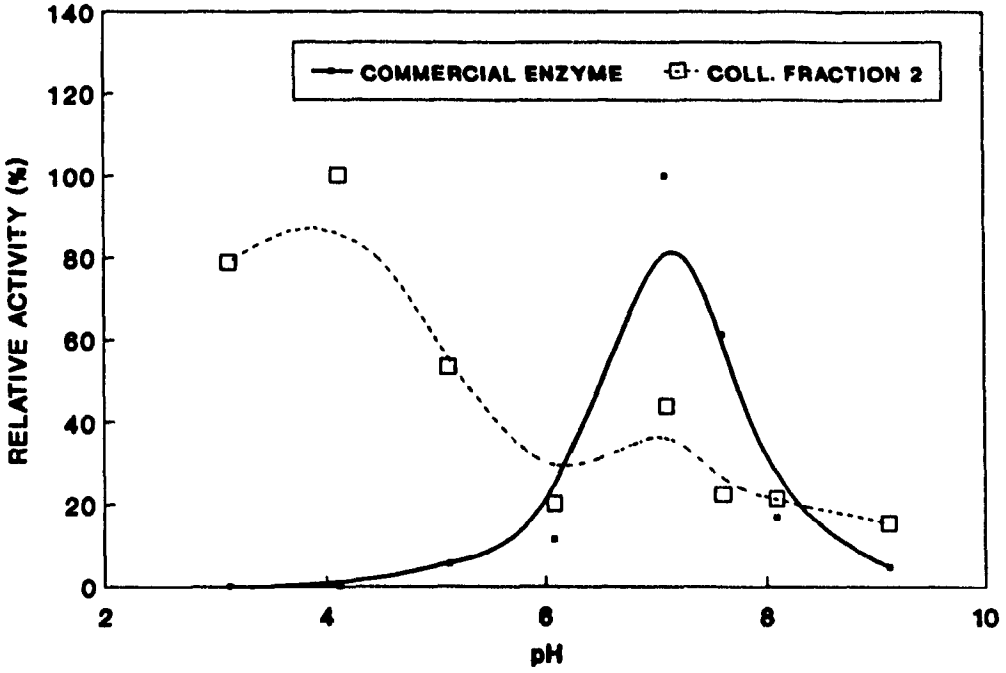

COMMERCIAL ENZYME
COLL. FRACTION 2
RELATIVE ACTIVITY (%)
140
120
100
80
60
40
20
0
2
4
6
8
10
pH

Fig. 13

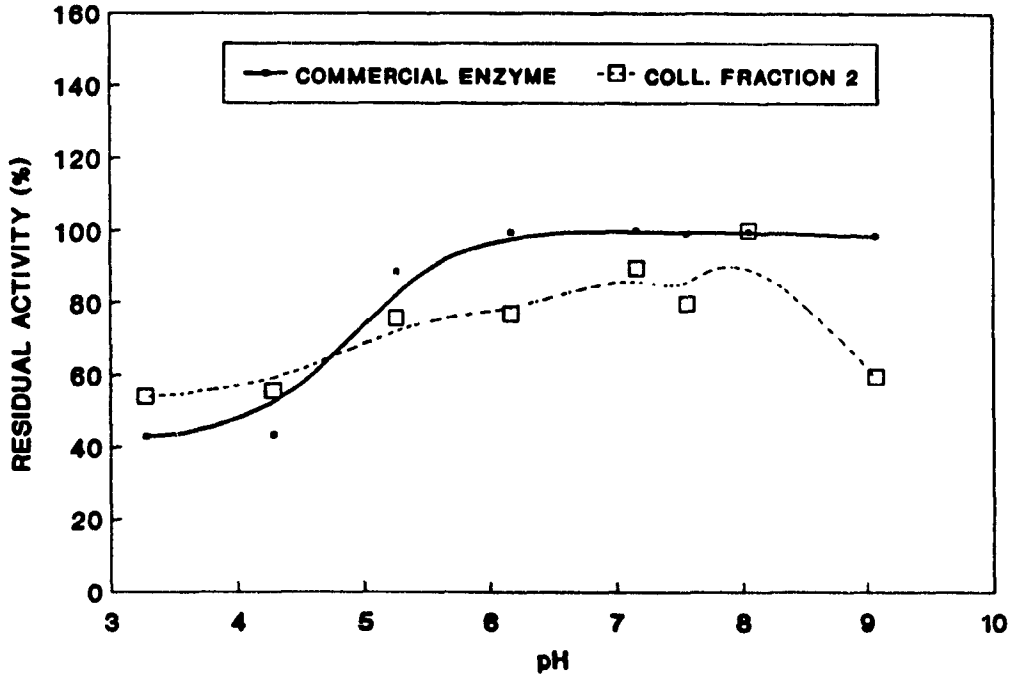

COMMERCIAL ENZYME
COLL. FRACTION 2
RESIDUAL ACTIVITY (%)
160
140
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
pH

Fig. 14

4.3.1.2. With the IEX extract (peak 2)

The pH-activity profile of the semi-purified peak 2 fraction after ion exchange chromatography (Fig. 15) showed a similar pattern to that of the $(NH_4)_2SO_4$ fraction (fraction 2), with two pH optima at 4 and 7-8, respectively. In this respect, the lobster enzyme was similar to the crab collagenase with a pH optimum at 8 (Eisen *et al.*, 1973) and collagenase A-α from *Cl. histolyticum* with a pH optimum between 7 and 9 (Kono, 1968), but none of collagenases with a acid pH optima has been found in animal, human or bacteria. In this respect, the collagenase fraction from lobster differed from other collagenolytic enzymes thus far characterized. However, unlike the $(NH_4)_2SO_4$ fraction (i.e., collagenase "fraction 2"), the peak 2 fraction from the IEX step showed remarkable stability throughout the pH range (3-9), retaining 90-100% of its original activity (Fig 16).

4.3.2. Temperature optimum and thermostability

4.3.2.1. With $(NH_4)_2SO_4$ fraction

The influence of temperature on the activity of the enzymes is presented in Fig. 17 which shows that the commercial collagenase had a temperature optimum at 40^{0}C while the lobster collagenase "fraction2"exhibited two temperature optima: one at 25^{0}C and a broader one between 40^{0}C and 50^{0}C. This observation supports the

Fig 15. **pH optima of IEX peak 2 collagenase fraction vs commercial collagenase on native collagen as substrate**

Fig 16. **pH stability of IEX peak 2 collagenase fraction vs commercial collagenase**

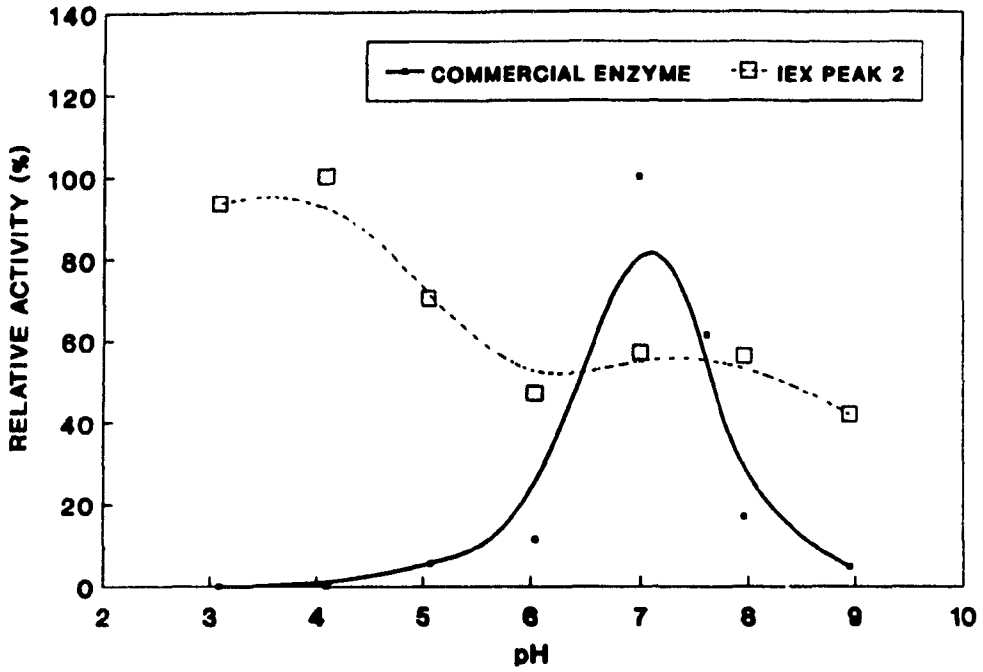

COMMERCIAL ENZYME
IEX PEAK 2
RELATIVE ACTIVITY (%)
140
120
100
80
60
40
20
0
2
3
4
5
6
7
8
9
10
pH

Fig. 15

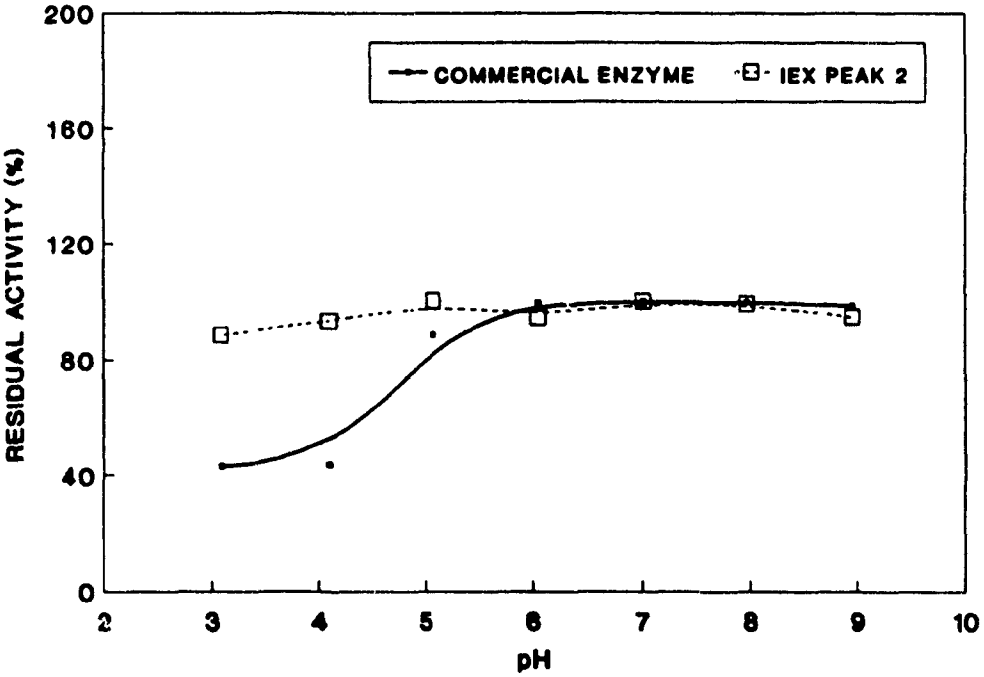

COMMERCIAL ENZYME
IEX PEAK 2
RESIDUAL ACTIVITY (%)
200
160
120
80
40
0
2
3
4
5
6
7
8
9
10
pH

Fig. 16

Fig 17. Temperature optima of ammonium sulfate collagenase fraction and commercial collagenase on collagen as substrate

Fig 18. Thermostability of ammonium sulfate collagenase fraction and commercial collagenase

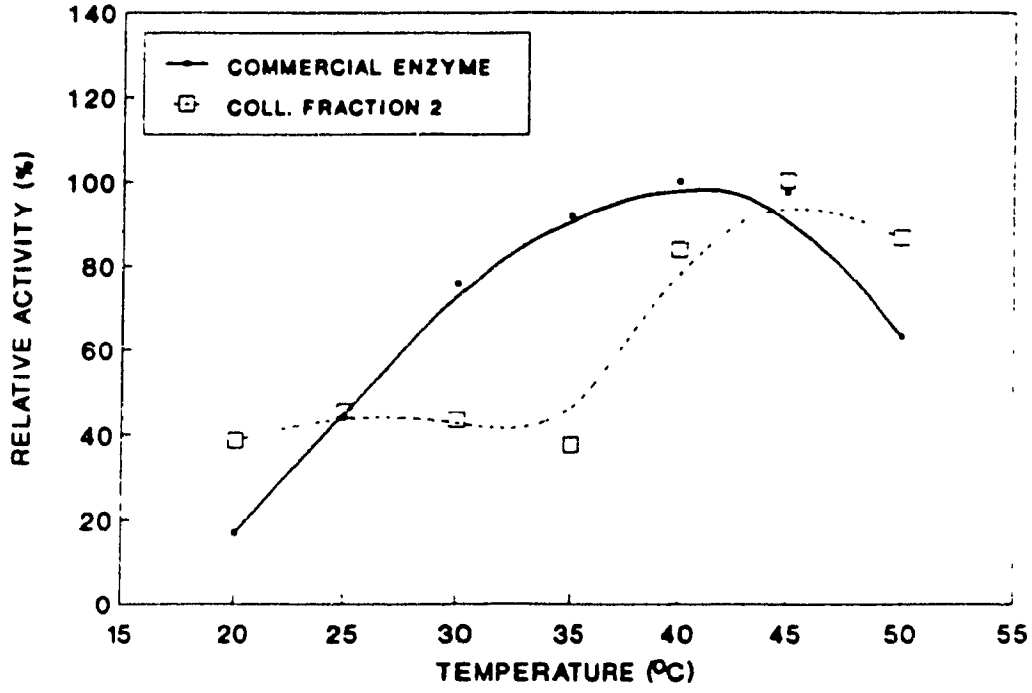

COMMERCIAL ENZYME
COLL. FRACTION 2
RELATIVE ACTIVITY (%)
140
120
100
80
60
40
20
0
15
20
25
30
35
40
45
50
55
TEMPERATURE (°C)

Fig. 17

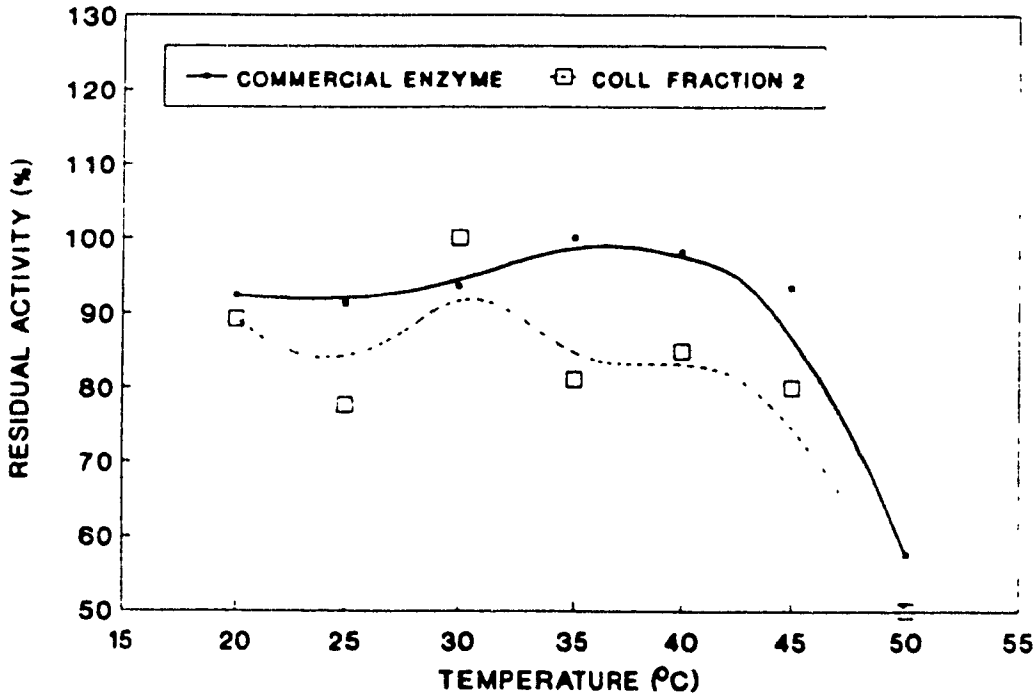

COMMERCIAL ENZYME
COLL FRACTION 2
RESIDUAL ACTIVITY (%)
130
120
110
100
90
80
70
60
50
15
20
25
30
35
40
45
50
55
TEMPERATURE (°C)

Fig. 18

finding made in the pH-activity study, and also suggests the presence of at least two active collagenolytic enzymes in the collagenase "fraction 2".

The thermostability data, summarized in Fig. 18, also show 2 peaks (at 30^0C and 35-45^0C) and supports the suggestion of the possibility of two forms of collagenolytic enzymes in the collagenase "fraction 2". Thus one form of the collagenolytic enzyme appeared to be more heat labile than the other, with the former exhibiting an optimum stability at 30^0C while the latter appeared to be relatively stable up to a temperature range of 35-45^0C.

The commercial enzyme was relatively more heat stable, retaining 90-100% of its original activity even after 30 min incubation at 45^0C.

4.3.2.2. With IEX peak 2 fraction

Unlike the collagenase fraction from the ammonium sulfate step, the semi-purified fraction after the IEX step (i.e., the peak 2 fraction) had one distinct temperature optimum between 50^0C and 55^0C (Fig. 19). This suggest that further purification by IEX either elimited (i) the active enzyme responsible for second optimum peak, or (ii) destroyed the activity of this other enzyme component. The IEX extract appeared to be relatively more heat stable and lose relatively less activity at higher temperatures as compared with that of the $(NH_4)_2SO_4$ extract (Fig. 20). A possible explanation for the observed differences in sensitivity to temperature could be the presence of other components in the $(NH_4)_2SO_4$ fraction that acted co-operatively

Fig 19. Temperature optima of IEX peak 2 collagenase fraction vs commercial collagenase on native collagen as substrate

Fig 20. Thermostability of IEX peak 2 collagenase fraction vs commercial collagenase

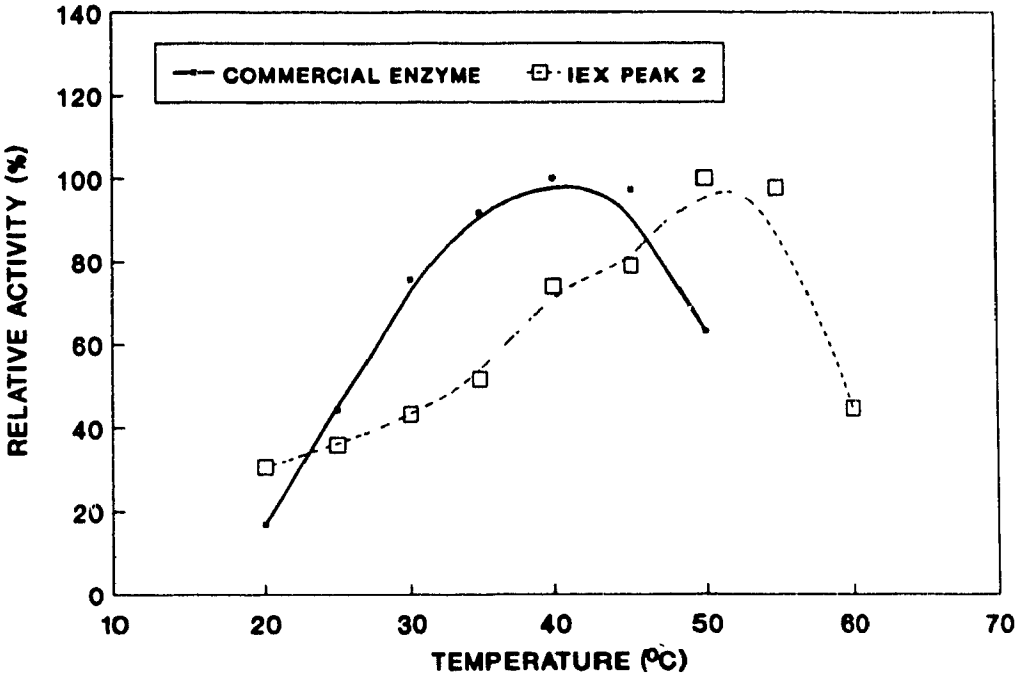

COMMERCIAL ENZYME
IEX PEAK 2
RELATIVE ACTIVITY (%)
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
TEMPERATURE (°C)

Fig. 19

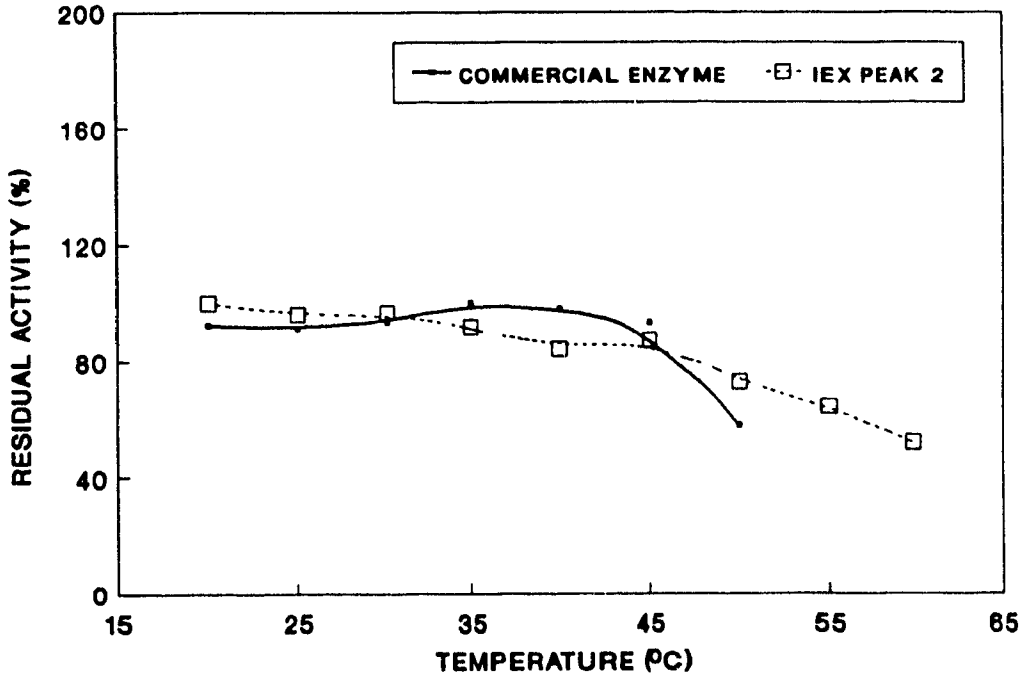

COMMERCIAL ENZYME
IEX PEAK 2
RESIDUAL ACTIVITY (%)
200
160
120
80
40
0
15
25
35
45
55
65
TEMPERATURE (°C)

Fig. 20

with increased temperature to decrease the activity of this extract to a greater extent. It is clear from the electrophoregrams Fig. 11 that the $(NH_4)_2SO_4$ fraction was less pure (i.e., had more protein bands) than the IEX extract. Possibly some of these protein/polypeptides or other components could act as inhibitors of the enzyme. The inference from this reasoning is that if those components were present, they were removed by the IEX step. The thermal stability of the IEX extract was similar to the commercial enzyme - retaining 90-100 % of its original activity.

CHAPTER V

CONCLUSIONS & SUGGESTIONS FOR FURTHER STUDY

Based on the findings presented in the preceding section, the following conclusions can be made:

(1) Collagenolytic enzyme(s) are present in the hepatopancreas of the American lobster (*Homarus Americanus*).

(2) These enzymes can be recovered by the successive steps of acetone precipitation, ammonium sulfate fractionation and liquid chromatographic techniques.

(3) The active enzyme fractions from the lobster hepatopancreas was most active at pH 4.0 and between pH 7-8, and most stable at pH 8.0.

(4) The lobster enzyme was relatively more heat labile than commercial collagenase.

(5) The active fraction from the lobster hepatopancreas exhibited multiple bands with molecular weights ranging from 15,000-66,000 in SDS-polyacrylamide gels.

(6) Various fractions from the chromatographic steps exhibited activity toward native collagen suggesting the presence of isoenzyme forms of the collagenase enzyme.

There appears to exist at least two active collagenolytic enzymes (possible more) in lobster hepatopancreas. Further work needs to be done to isolate/purify the active enzymes in the various fractions to homogeneity using the isoelectric focusing approach. After these enzymes have been purified, further characterization with respect to the kinetic and thermodynamic properties, as well as response to

inhibitors, etc., would be very useful in fully understanding the postharvest deterioration phenomenon. The additional information gained from those studies would also be useful in rationalizing process/handling manipulations for controlling the problem of texture deterioration.

REFERENCES

Angel, S., Juven, B. J., Weinberg, Z. G., Lindner, P. and Eisenbery, E. 1986. Effects of radurization and refrigerated storage on quality and shelf-life of freshwater prawns, *Macrobrachium rosenbergii*. J. Food Prot. 49: 142-145.

Angel, S., Weinberg, Z. G., Juven, B. J. and Lindner, P. 1985. Quality changes in the fresh water prawn, *Macrobrachium rosenbergii* during storage on ice. J. Food Tech. 20: 553-560.

Baranowski, E. S., Nip, W. K. and Moy, J. H. 1984. Partial characterization of a crude enzyme extract from the freshwater prawn, *Macrobrachium rosenbergii*. J. Food Sci. 49: 1494-1495.

Bennick, A. and Hunt, A. M. 1967. Collagenolytic activity in oral tissues. Arch. Oral. Biol. 12: 1-9.

Bernholdt, H. F. 1975. Meat and other proteinaceous foods. In "Enzymes in Food Processing" (2nd ed). Gerald Reed (Ed.), p. 473-492, Academic Press, New York.

Beutner, E. H., Triftshouse, C. and Hazen, S. P. 1966. Collagenase activity of gingival tissue from patients with periodontal diseases. Proc. Soc. Exptl. Biol. Med. 121: 1082-1085.

Blumenfeld, O. O. and Gallop, P. M. 1962. The participation of aspartyl residues in the hydroxylamine- or hydrazine- sensitive bonds of collagen. Biochem. 1: 947-959.

Bornstein, P. 1967. Comparative sequence studies of rat skin and tendon collagen. I. Evidence for incomplete hydroxylation of individual prolyl residues in the normal proteins. Biochem. 6: 3082-3093.

Cronlund, A. L. and Woychik, J. H. 1987. Solubilization of collagen in restructured beef with collagenases and α-amylase. J. Food Sci. 52 (4): 857-860.

Dransfield, E. and Etherington, D. 1981. Enzymes and Food Processing. (Birch, G.G., Blankebrough, N. and Parker, K.J. (eds)), p. 177-178. Applied Science Publishers, London.

Dresden, M. H. and Gross, J. 1970. The collagenolytic enzyme of the regenerating limb of the newt triturus viridescens. Dev. Biol. 22: 129-137.

Dresner, E. and Schubert, M. 1955. The comparative susceptibility to collagenase and trypsin of collagen, soluble collagens and renal basement membrane. J. Histochem. Cytochem. 3: 360-368.

Eisen, A. Z. 1967. Collagenolytic activity in normal and diseased human skin. J. Clin. Invest. 46: 1052.

Eisen, A. Z., Eugene, A. B. and Jeffrey, J. J. 1970. Special review article: Animal and human collagenases. J. Invest. Derm. 55: 359-373.

Eisen, A. Z., Henderson, K. D., Jeffrey, J. J. and Bradshaw, R. A. 1973. A collagenolytic protease from the hepatopancreas of the fiddler crab, *Uca pugilator*. Purification and properties. Biochem. 12: 1814-1822.

Eisen, A. Z. and Jeffrey, J. J. 1969. An extractable collagenase from crustacean hepatopancreas. Biochim. Biophys. Acta 191: 517-526.

Eisen, A. Z., Jeffrey, J. J. and Gross, J. 1968. Human skin collagenase isolation and mechanism of attack on the collagen molecule. Biochim. Biophys. Acta 151: 637-645.

Evanson, J. M., Jeffrey, J. J. and Krane, S. M. 1967. A collagenolytic enzyme from rheumatoid synovium in tissue culture. Clin. Res. 15: 293.

Evanson, J. M. and Krane, S. M. 1968. Studies on collagenase from rheumatoid synovium in tissue culture. J. Clin. Invest. 47: 2639-2651.

Fietzek, P. P. and Kuhn, K. 1976. The primary structure of collagen. Int. Rev. Connect. Tissue Res. 7: 1-60.

Fujimoto, D. 1968. Correlation between pronase susceptibility and hydroxy proline content of collagen fiber. Biochim. Biophys. Acta 154: 183-189.

Fullmer, H. M. and Gibson, W. 1966. Collagenolytic activity in gingivae of man. Nature 209: 728-729.

Fullmer, H. M. and Lazarus, G. S. 1967. Collagenase in human, goat and rat bone. Israel J. Med. Sci. 3: 758-761.

Gallop, P. M., Blumenfeld, O. O. and Seifter, S. 1972. Structure and metabolism of connective tissue proteins. Ann. Rev. Biochem. 41: 617-672.

Gallop, P. M., Seifter, S. and Meilman, E. 1957. Studies on collagen. I. The partial purification, assay and mode of activation of bacterial collagenase. J. Biol. Chem. 227: 891-906.

Gisslow, M. T. and McBride, B. C. 1975. A rapid sensitive collagenase assay. Anal.

Biochem. 68: 70-78.

Grant, G. A. and Eisen, A. Z. 1980. Substrate specificity of the collagenolytic serine protease from *Uca pugilator*: studies with noncollagenous substrates. Biochem. 19: 6089-6095.

Grant, G. A., Henderson, K. O., Eisen, A. Z. and Bradshaw, R. A. 1980. Amino acid sequence of a collagenolytic protease from the hepatopancreas of the fiddler crab, *Uca pugilator*. Biochem. 19: 4653-4659.

Grant, G. A., Sacchettini, J. C. and Welgus, H. G. 1983. A collagenolytic serine protease with trypsin-like specificity from the fiddler crab *Uca pugilator*. Biochem. 22: 354-358.

Grassmann, W., Hormann, H. and Nordwig, A. 1962. Degradation of collagen with collagenase: Structure of the nonpolar regions of the collagen fibers. in "Collagen" (N. Ramanathan, ed.), P. 263-278. Interscience publishers. A division of John Wiley & Sons. New York. London.

Grassmann, W., Hormann, H., Nordwig, A. and Wunsch, E. 1959. Zur spezifitat der kollagenase. Hoppe-Seyler's Z. Physiol. Chem. 316: 287-289.

Grassmann, W. and Nordwig, A. 1960. Quantitativer kolorimetrischer test auf kollagenase. Hoppe-Seyler's Z. Physiol. Chem. 322: 267-272.

Grillo, H. C., Lapiere, C. M., Dresden, M. H. and Gross, J. 1968. Collagenolytic activity in regenerating forelimbs of the adult newt (*Triturus viridescens*). Dev. Biol. 17: 571-583.

Grillo, H. C. and Gross, J. 1967. Collagenolytic activity during mammalian wound

repair. Dev. Biol. 15: 300-317.

Gross, J. 1976. Aspects of the animal collagenases. In "Biochemistry of Collagen" (Ramachandran, G.N. and Reddi, A.H., Eds.) pp 275-317, Plenum Press, New York.

Gross, J. 1953. Evaluation of structural and chemical changes in connective tissue. Ann. N. Y. Acad. Sci. 56: 674-683.

Gross, J. and Lapiere, C. M. 1962. Collagenolytic activity in amphibian tissues: A tissue culture assay. Proc. Natl. Acad. Sci. USA 48: 1014-1022.

Gross, J. and Nagai, Y. 1965. Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. Proc. Natl. Acad. Sci. U.S. 54: 1197-1204.

Harper, E., Bloch, K. J. and Gross, J. 1971. The zymogen of tadpole collagenase. Biochem. 10: 3035-3041.

Harper, E. and Gross, J. 1970. Separation of collagenase and peptidase activities of tadpole tissues in culture. Biochim. Biophys. Acta 198: 286-292.

Harris, E. D. Jr. and Cartwright, E. C. 1977. Mammalian collagenases. In "Proteinases in Mammalian Cells and Tissues", Vol. 2 (Barrett, A. J. ed.) p. 249-283. North-Holland publishing company. Amsterdam. New York. Oxford.

Harris, E. D. Jr., Dibona, D. R. and Krane, S. M. 1969. Collagenase in human synovial fluid. J. Clin. Invest. 48: 2104-2113.

Harris, E. D.,Jr., and Krane, S. M. 1974. Collagenases. N. Engl. J. Med. 291, 557-563, 605-608, 652-661.

Harris, E. D. Jr., Mainardi, C. L. and Vater, C. A. 1978. Rheumatoid synovial collagenase: secretion from cells as an inactive complex with inhibitor. Clin. Res. 26, PT.1, 515A.

Hartree, E. P. 1972. Determination of protein: A modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48: 422-427.

Henry, H. and Legler, G. 1960. Die hydrolyse synthetischer und naturlicher substrate durch kollagenase aus *Clostridium histolyticum*. Hoppe-Seyler's Z. Physiol. Chem. 321: 184-200.

Henry, H. and Legler, G. 1959. Uber die spezifitat der kollagenase aus *Clostridium histolyticum* und ihre anwendung zur ermittlung der primarstruktur des collagens. Hoppe-Seyler's Z. Physiol. Chem. 315: 288-290.

Hipped, P. H. Von and Wong, K. 1963. The collagen = gelatin phase transition. II. Shape of the melting curves and effect of chain length. Biochem. 2: 1399-1413.

Horowitz, A. L., Hance, A. J. and Crystal, R. G. 1977. Granulocyte collagenase: Selective digestion of type I relative to type III collagen. Proc. Natl. Acad. Sci. USA 74: 897-901.

Hurion, N., Fromentin, H. and Keil, B. 1979. Specificity of the collagenolytic enzyme from the fungus *Entomophenora coronata*: comparison with the bacterial collagenase from *Achromobacter iophagus*. Arch. Biochem. Biophys. 192: 438-445.

Jeffrey, J. J. and Gross, J. 1967. Isolation and characterization of a mammalian collagenolytic enzyme. Fed. Proc. 26: 670.

Jeffrey, J.J. and Gross, J. 1970. Collagenase from rat uterus. Isolation and partial characterization. Biochem. 9: 268-273.

Keech, M. K. 1955. Human skin collagen from different age groups before and after collagenase digestion. Ann. Rheumatic Diseases 14: 19-50.

Keech, M. K. 1954. The effect of collagenase on human skin collagen. Comparison of different age-groups and of cases with and without "collagen disease". Yale J. Biol. Med. 26(4): 295-306.

Kobayashi, S. and Nagai, Y. 1978. Human leucocyte neutral proteases, with special reference to collagen metabolism. J. Biochem. 84: 559-567.

Kono, T. 1968. Purification and partial characterization of collagenolytic enzymes from *Clostridium histolyticum*. Biochem. 7: 1106-1114.

Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T_4. Nature 227: 680-685.

Lazarus, G. S., Daniels, J. R., Lian, J. and Burleigh, M. C. 1972. Role of granulocyte collagenase in collagen degradation. Am. J. Pathol. 68: 565-578.

Lecroisey, A., Boulard, C. and Keil, B. 1979. Chemical and enzymatic characterization of the collagenase from the insect *Hypoderma lineatum*. Eur. J. Biochem. 101: 385-393.

Lee, P. G., Blake, N. J. and Rodrick, G. E. 1980. A quantitative analysis of digestive enzymes for the freshwater prawn *Macrobachium rosenbergii*. Proc. World Maricul. Soc. 11: 392-402.

Lindner, P., Angel, S., Weinberg, Z. G. and Granit, R. 1988. Factors inducing mushiness in stored prawns. Food chemistry 29: 119-132.

Liotta, L. A., Abe, S., Robey, P. G. and Martin, G. R. 1979. Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. Proc. Natl. Acad. Sci. USA 76 (5): 2268-2272.

Macfarlane, R. G. and MacLennan, J. D. 1945. The toxaemia of gas-cangrene. The Lancet 249: 328-331.

MacLennan, J. D., Mandl, I. and Howes, E. L. 1953. Bacterial digestion of collagen. J. Clin. Inv. 32: 1317-1323.

Mailman, M. L. and Dresden, M. H. 1979. Enervation effects on newt limb regeneration: Collagen and collagenase. Dev. Biol. 71 (1): 60-70.

Mandl, I. 1961. Collagenases and elastases. Adv. Enzymol. 23: 163-264.

Mandl, I., MacLennan, J. D. and Howes, E. L. 1953. Isolation and characterization of proteinase and collagenase from *Cl. histolyticum*. J. Clin. Invest. 32: 1323- 1329.

Maschmann, E. 1937. Uber bakterienproteasen II. Biochem. J. 295: 1-10.

Masui, Y., Takemoto, T., Sakakibara, S., Hori, H. and Nagai, Y. 1977. Synthetic substrates for vertebrate collagenase. Biochem. Med. 17: 215-221.

McCroskery, P. A., Richards, J. F. and Harris, E. D. Jr. 1975. Purification and characterization of a collagenase extracted from rabbit tumours. Biochem. J. 152: 131-142.

Miller, A. J., Strange, E. D. and Whiting, R. C. 1989. Improved tenderness of restructured beef steaks by a microbial collagenase derived from *Vibrio* B-30. J. Food Sci. 54 (4): 855-857.

Morales, T. I., Woessner, J. F., Howell, D. S., Marsh, J. M. and Lemaire, W. J. 1978. A microassay for the direct demonstration of collagenolytic activity in graafian follicles of the rat. Biochim. Biophys. Acta 524: 428-434.

Nagai, Y., Lapiere, C. and Gross, J. 1966. Tadpole collagenase, preparation and purification. Biochem. 5: 3123-3130.

Nagai, Y., Sakakibara, S., Noda, H. and Akabori, S. 1960. Hydrolysis of synthetic peptides by collagenase. Biochim. Biophys. Acta 37: 567-569.

Nip, W. K., Moy, J. H. and Tzang, Y. Y. 1985. Effect of purging on quality changes of ice-chilled freshwater prawn, *Macrobrachium rosenbergii*. J. Food Technol. 20: 9-15.

Ohlsson, K. and Olsson, I. 1973. The neutral proteases of human granulocytes, isolation and partial characterization of two granulocyte collagenases. Eur. J. Biochem. 36: 473-481.

Riley, W. B., Jr. and Peacock, E. E., Jr. 1967. Identification, distribution, and significance of a collagenolytic enzyme in human tissues. Pro. Soc. Exptl. Biol. Med. 124: 207-210.

Robb-Smith, A. H. T. 1953. Significance of collagenase. In "Nature and Structure of Collagen" (J. T. Randall, ed.), p. 14-26. Academic Press, New York.

Robb-Smith, A. H. T. 1945. Tissue changes induced by *Cl. welchll* type A filtrates.

Lancet 249: 362-368.

Rojkind, M., Blumenfeld, O. O. and Gallop, P. M. 1966. Localization and partial characterization of an aldehydic component in tropocollagen. J. Biol. Chem. 241: 1530-1536.

Rojkind, M., Rhi, L. and Aguirre, M. 1968. Biosynthesis of the intramolecular cross-links in rat skin collagen. J. Biol. Chem. 243: 2266-2272.

Sakai, T. and Gross, J. 1967. Some properties of the products of reaction of tadpole collagenase with collagen. Biochem. 6: 518-528.

Schrohenloher, R. E., Ogle, J. D. and Logan, M. A. 1959. Two tripeptides from an enzymatic digest of collagen. J. Biol. Chem. 234: 58-61.

Seifter, S. and Gallop, P. M. 1962. Collagenase from *Clostridium histolyticum*. Methods in Enzymology 5: 659-665.

Seifter, S., Gallop, P.M., Klein, L. and Meilman, E. 1959. Studies on collagen II. Properties of purified collagenase and its inhibition. J. Biol. Chem. 234: 285-293.

Seifter, S. and Harper, E. 1971. The collagenase. In "The Enzymes" Volume III (Boyer, P. D. ed.), p.649-693. Academic Press, Inc. (London) Ltd.

Seifter, S. and Harper, E. 1970. Collagenases. Methods in Enzymology 19: 613-635.

Seltzer, J. L., Jeffrey, J. J. and Eisen, A. Z. 1977. Evidence for mammalian collagenases as zinc ion metalloenzymes. Biochim. Biophys. Acta 485: 179-187.

Sidney, U. 1972. The use of bacterial collagenase as a tool in studies on the biosynthesis of collagen. In "Collagenase' (Ines Mandl, ed.) p. 57-60. New York.

Siegel, R. C. 1976. Collagen cross-linking, synthesis of collagen cross-links *in vitro* with highly purified lysyl oxidase. J. Biol. Chem. 251: 5786-5792.

Steiner, D. F., Clark, I. L., Nolan, C., Rubenstein, A. H., Margoliash, E., Aten, B. and Oyer, P. E. 1969. Proinsulin and the biosynthesis of insulin. Recent Progr. Hormone Res. 25: 207-272.

Stricklin, G. P., Bauer, E. A., Jeffrey, J. J. and Eisen, A. Z. 1977. Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ cultures. Biochemistry 16: 1607-1615.

Stricklin, G. P., Eisen, A. Z., Bauer, E. A. and Jeffrey, J. J. 1978. Human skin fibroblast collagenase: Chemical properties of precursor and active forms. Biochem. 17: 2331-2337.

Takahashi, S. and Seifter, S. 1974. An enzyme with collagenolytic activity from dog pancreatic juice. Isr. J. Chem. 12: 557-571.

Takahashi, S. and Seifter, S. 1972. New culture conditions for *Clostridium histolyticum* leading to production of collagenase of high specific activity. J. Appl. Bacteriol. 35: 647-657.

Tana, N., Hashimoto, K., Kanzaki, T. and Ohyama, H. 1978. Collagenolytic activities of cultured human malignant melanoma cells. J. Biochem. 84: 1171-1176.

Tanzer, M. L. 1973. Cross-linking of collagen. Science 180: 561-566.

Traub, W. and Piez, K. A. 1971. The chemistry and structure of collagen. Adv. Protein Chem. 25: 243-352.

Vaes, G. 1972. The release of collagenase as an inactive proenzyme by bone explants in culture. Biochem. J. 126: 275-289.

Vater, C. A., Mainardi, C. L. and Harris, E. D. Jr. 1978a. Binding of latent rheumatoid synovial collagenase to collagen fibrils. Biochim. Biophys. Acta 539: 238-247.

Vater, C. A., Mainardi, C. L. and Harris, E. D. Jr. 1978b. Activation in vitro of rheumatoid synovial collagenase from cell cultures. J. Clin. Invest. 62: 987-992.

Walker, D. G., Lapiere, C. M. and Gross, J. 1964. A collagenolytic factor in rat bone promoted by parathyroid extract. Biochem. Biophys. Res. Commun. 15: 397-402.

Warren, G. H. and Gray, J. 1961. A simplified culture medium for the production of collagenase. Nature 192: 755-756.

Waters M. E. and Hale, M. B. 1981. Quality changes during iced storage of whole freshwater prawns (*Macrobrachium rosenbergii*). Proc. of the 6th Annual Tropical and Subtropical Fisheries Technological Conference of the Americas. San Antonio, TX, 116-127.

Weeks, J. G., Halme, J. and Woessner, J. F. 1976. Extraction of collagenase from the involuting rat uterus. Biochim. Biophys. Acta 445 (1): 205-214.

Weil, L. and Kocholaty, W. 1937. Studies on the proteinase of *Clostridium histolyticum*. Biochem. J. 31: 1255-1267.

Werb, Z., Mainardi, C. L., Vater, C. A. and Harris, E. D. 1977. Endogenous activation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. N. Eng. J. Med. 296 (18): 1017-1023.

Woessner, J. F. 1979. Total, latent and active collagenase during the course of post-partum involution of the rat uterus. Biochem. J. 180: 95-102.

Woolley, D. E., Glanville, R. W., Crossley, M. J. and Evanson, J. M. 1975. Purification of rheumatoid synovial collagenase and its action on soluble and insoluble collagen. Eur. J. Biochem. 54: 611-622.

Woolley, D. E., Glanville, R. W., Crossley, M. J. and Evanson, J. M. 1973. Differences in the physical properties of collagenase isolated from rheumatoid synovium and human skin. Biochem. Biophys. Res. Commun. 51: 729-734.

Woolley, D. E., Glanville, R. W., Roberts, D. R. and Evanson, J. M. 1978. Purification, characterization and inhibition of human skin collagenase. Biochem. J. 169: 265-276.

Worthington Enzyme Manual. 1972. Worthington Biochemical Corporation. Freehold, New Jersey, U. S. A. 07728.

Wuensch, E. and Heidrich, H. G. 1963. Zur quantitativen bestimmung der kollagenase. Hoppe-Seyler's Z. Physiol. Chem. 333: 149-151.