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PURIFICATION AND CHARACTERIZATION OF COLLAGENASES FROM THE SKELETAL MUSCLE OF WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*)

by

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FOREWORD

This thesis is presented in the traditional form, although, part of this thesis has been published. The first two sections consist of an introduction, the rationale, hypothesis, objectives and a comprehensive review of the literature on the topic. The next two sections constitute the body of the thesis, while the last chapter comprises the conclusions and recommendations.

While the work reported in this thesis is the responsibility of the candidate, it was supervised by Dr. B. K. Simpson, Department of Food Science and Agricultural Chemistry, MacDonald Campus of McGill University. The amino acid analysis of the fish collagenases was conducted by the Sheldon Biotechnology Centre, McGill University.

ABSTRACT

Collagenases were extracted from the skeletal muscle of winter flounder (*Pseudopleuronectes americanus*) with Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$. The crude extract in the active form was purified by ammonium sulfate fractionation, followed by a succession of column chromatographic steps which included ion-exchange, immobilized metal affinity and size-exclusion in the Fast Protein Liquid Chromatography (FPLC) system. The trypsin-like and chymotrypsin-like activities of the crude extract diminished with purification. A comparative study of the collagenase fraction from ion-exchange chromatography (IEX-1) and the commercial collagenase fraction from *Clostridium histolyticum* indicated that the two enzymes were similar with respect to their response to temperature but differed with respect to their response to pH. The enzymes differed slightly in terms of their thermal and pH stabilities. The winter flounder collagenase fraction from size-exclusion chromatography (SEC) had a higher optimum pH temperature than the IEX-1 fraction as well as the commercial collagenase. However, both SEC and the IEX-1 extracts had the same optimum pH. The collagenase fraction from SEC had a slightly lower thermal stability than the IEX-1 fraction and the commercial collagenase.

The higher catalytic efficiency (V_{max}/K_m') and the lower ΔG values for *C. histolyticum* collagenases showed that bacterial collagenases are better catalysts than winter flounder skeletal muscle collagenases for the PZ-peptide hydrolase reaction at 37° C and pH 7.1.

Zymography revealed the presence of two collagenase isoenzymes from the fish muscle, designated as WFC-1 and WFC-2 with molecular weights of 79,600 and 75,500, respectively. WFC-1 was separated from WFC-2 by electrophoretic blotting onto the

PVDF membrane. The amino acid composition of WFC-1 and WFC-2 were closely related.

The fish collagenases were inhibited by metal chelators, EDTA and 1,10-phenanthroline suggesting that these enzymes are metalloproteases. The enzyme activity in the presence of EDTA and 1,10-phenanthroline were recovered upon addition of low levels of calcium and zinc ions, respectively. Higher levels of these metal ions inhibited the isoenzymes. 2-Mercaptoethanol and dithiothreitol were also effective inhibitors.

RÉSUMÉ

Des collagénases ont été extraites du muscle squelettique de la plie rouge (*Pseudopleuronectes americanus*) en utilisant du tampon Tris-HCl, 7,4, contenant 5 mM de $CaCl_2$. L'extrait brut sous sa forme active a été purifié par fractionnement au sulfate d'ammonium, suivi par plusieurs étapes de chromatographies sur colonne comprenant les chromatographies échangeuse d'ions, d'affinité avec des métaux immobilisés et de tamisage moléculaire en utilisant un système FPLC. Les activités de type trypsine et chymotrypsine de l'extrait brut ont diminué avec la purification. Une étude comparative de la fraction collagénase obtenue par chromatographie échangeuse d'ions (IEX-1) et de la fraction collagénase commerciale de *C. histolyticum* a indiqué que les deux activités enzymatiques étaient similaires en terme de réponse à la température différaient en terme de réponse au pH. Les enzymes différaient légèrement en termes de stabilités à la température et au pH. La fraction collagénase de la plie rouge obtenue par chromatographie de tamisage moléculaire (SEC) avait une température optimale d'activité supérieure à celles mais de la fraction collagénase obtenue par IEX-1 et de la collagénase commerciale. Cependant, les fractions obtenues par SEC et IEX-1 avaient le même pH optimal. La fraction obtenue par SEC avait une stabilité à la température légèrement plus faible que celles de la fraction obtenue par IEX-1 et de la collagénase commerciale.

L'efficacité catalytique plus élevée et la plus faible valeur de ΔG pour les collagénases de *C. histolyticum* ont montré que les collagénases d'origine bactérienne étaient de meilleurs catalyseurs pour la réaction hydrolase du peptide PZ à 37°C et à pH 7,1, que les collagénases du muscle squelettique de la plie rouge.

La zymographie a révélé la présence de deux isoenzymes de la collagénase dans le muscle squelettique du poisson, désignées comme étant WFC-1 et WFC-2 avec des poids

moléculaires respectifs de 79.600 et 75.500. WFC-1 a été séparée de WFC-2 par transfert électrophorétique sur une membrane de PVDF. La composition en amino-acides de WFC-1 et WFC-2 étaient très proches.

Les collagénases du poisson ont été inhibées par des chélateurs de métaux, comme l'EDTA et la 1,10-phénanthroline, suggérant que ces enzymes étaient des métalloprotéases. L'activité enzymatique en présence d'EDTA et de 1,10-phenanthroline a été récupérée, respectivement, après l'addition d'ions calcium et d'ions zinc. L'addition de quantités plus élevées de ces ions métalliques a inhibé l'activité des isoenzymes. Le 2-mercaptoéhanol et le dithiothreitol étaient également des inhibiteurs de l'activité enzymatique.

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CONTRIBUTION TO KNOWLEDGE

1 Electrophoretic blotting had been used to isolate proteins for sequencing and/or amino acid analysis and to my knowledge this is the first time that this method was used to transfer winter flounder skeletal muscle collagenase isoenzymes from Tris-glycine gels onto the PVDF membrane for amino acid analysis.

2 The fish collagenases were shown to be metalloenzymes, i.e., inhibited by metal chelators in contrast to the reported crab collagenases which were serine proteases.

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LIST OF ABBREVIATIONS

BAPA	Benzoyl-DL-arginine p-nitroanilide
BIS	N,N'-methylene-bis-acrylamide
BTEE	Benzoyl-L-tyrosine ethyl ester
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FPLC	Fast protein liquid chromatography
SEC	Size-exclusion chromatography
IEX	Ion-exchange chromatography
IMAC	Immobilized metal affinity chromatography
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidine diflouride
PZ-peptide	ρ-phenylazobenzyloxycarbonyl-L-prolyl-leucyl-glycyl-prolyl-D-arginine
SDS	Sodium dodecyl sulfate
STI	Soybean trypsin inhibitor
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TLCK	n-α-ρ-tosyl-L-lysine chloromethylketone hydrochloride
TRIS	Tris (hydroxymethyl) aminomethane
WFC	Winter flounder collagenases

Chapter 1

INTRODUCTION

The term "collagenase" was first used in 1927 by Ssadikow to refer to a pancreatic enzyme which cleaved denatured collagen. However, this enzyme could have been any other protease capable of degrading denatured collagen. Collagenases are highly specific enzymes from prokaryotes and eukaryotes capable of cleaving the main body chain of native collagen at physiological pH and temperature (Harper, 1980). The group of collagenolytic enzymes which satisfies this definition was first isolated from several strains of *Clostridium histolyticum* in the 1930's and shown to digest native horse Achilles tendon at 37°C for 3 - 4 days (Mandl, 1961). In the following years, several microbial species were shown to produce collagenolytic enzymes. Since some of these microorganisms are pathogenic, investigators have suggested that collagenolytic enzymes maybe involved in microbial pathogenicity (Harper, 1980).

Vertebrate collagenase was first detected in tissue culture of bullfrog tadpoles (Gross and Lapiere, 1962). Further investigations in the following years led to the isolation of collagenolytic enzymes from mammals (Eisen *et al.*, 1968; Jeffrey and Gross, 1970; Werb and Reynolds, 1975; Gillet *et al.*, 1977), crustaceans (Eisen and Jeffrey, 1969, Eisen *et al.*, 1973; Baranowski *et al.*, 1984; Nip *et al.*, 1985) and fish (Yoshinaka *et al.*, 1978; Kim *et al.*, 1993). Vertebrate collagenases are ubiquitous enzymes which are closely associated with all types of collagen in a wide variety of tissues, frequently bound to collagen fibers, reticulum and basement membrane. Based on this association and their activity, it is assumed that these enzymes play a role in the metabolism of connective tissues (Montfort and Perez-Tamayo, 1975; Pardo and Perez-Tamayo, 1975). Furthermore, vertebrate collagenase is believed to play an important role in cell migration and collagen remodeling during tissue repair and regeneration (Agren *et al.*, 1992). On

the other hand, collagenolytic enzymes from the hepatopancreas of shellfish and digestive tract of teleosts probably play a nutritional role. Several fish and shellfish are carnivorous, thriving on animal tissues frequently containing collagen. Yoshinaka *et al.* (1978) demonstrated a correlation between collagenolytic activity and the diet of the fish, the enzyme activity was higher in the carnivores feeding on diets rich in collagen than omnivores and herbivores . In a similar manner, fiddler crabs scavenge on tissues usually containing collagen, thus collagenolytic activity in the digestive system of these organisms would facilitate breakdown and absorption of their diets.

The first hints of collagenase activity in fish probably dates back to the 1960's when women working in fish canneries showed signs of collagenolytic attack on their hands (Mandl, 1961). Collagenolytic enzymes from the fish skeletal tissues may had been responsible for this attack.

In the living organism, there is a control mechanism to regulate enzyme activity. Upon death of the animal, there is a breakdown of the control system such that biochemical activity continues unregulated causing changes in the tissues. In fish and fish products, texture is an important quality attribute that has to be maintained during storage. Several studies have shown that fish meat begins to soften after a day of chilled storage (Hatae *et al.*, 1985; Toyohara and Shimizu, 1988; Montero and Borderias, 1990a; Oka *et al.*, 1990; Toyohara *et al.*, 1990). It has been demonstrated that collagen, a major component of the skeletal muscle connective tissues, contributes to fish meat texture (Hatae *et al.*, 1986; Sato *et al.*, 1986). During ice/chilled storage of fish, degradation of collagen may occur which may eventually result in mushiness of the fish tissues. Softening of the fish flesh is a common problem which usually results in major losses to the fishing industry. Earlier studies attributed this particular type of spoilage to microbial activity, however, several investigations refuted this observation. Angel *et al.* (1985) showed that there was no correlation between the number of proteolytic bacteria (*Flavobacterium* and *Pseudomonas*) and textural changes in the prawn muscle leading them to suggest that

mushiness may have resulted from the activity of enzymes from the cephalothorax of the prawn. In a similar study, Lindner *et al.* (1988) also suggested that mushiness of prawn stored in ice was probably caused by the diffusion of collagenolytic and other proteolytic enzymes from the autolyzing hepatopancreas. In blue grenadier texture deterioration was traced to the structural breakdown of the fine collagenous fibrils which connect the muscle fibers to the myocommatal connective tissue (Bremner and Hallett, 1985). Similar structural degradation was detected in spotted trevalla (Bremner and Hallett, 1986). Endogenous collagenases and/or other proteases were also implicated in these fibrillar destruction (Bremner and Hallett, 1985; 1986). The role of other endogenous proteases in the softening of fish tissues during low temperature storage has also been suggested by several other workers. Several types of cathepsins isolated from the skeletal tissues have been implicated in the softening of the fish flesh (Reddi et al., 1972; Doke *et al.*, 1980; Makinodan *et al.*, 1985; Busconi *et al.*, 1989; Jiang *et al.*, 1992; Yamashita and Konagaya, 1992a, 1992b).

The fish industry is one of the important food industries in Canada and other countries. Despite the availability of the different post harvest handling techniques, the industry still encounters a number of problems, one of which is the softening/deterioration of the fish fresh texture during chilled storage. Texture deterioration lowers the general acceptability as well as the market value of the fish and fish products.

A clear understanding of the factors which contribute to the structural deterioration of the fish muscle is important for developing more effective preservative strategies. Even though the influence of pH, microbial activity and physical/mechanical factors in inducing textural deterioration in fish and shellfish have been thoroughly investigated, there is a paucity of information regarding the presence and role of collagenolytic enzymes in the skeletal muscles of fish. Texture deterioration of fish due to the breakdown of connective tissues by endogenous collagenolytic enzymes has been suggested by a number of investigators. Thus, there is a need to establish the presence of

collagenolytic enzymes in the skeletal muscles of fish and elucidate the role of these enzymes in the structural breakdown of fish tissues. Hence this study will be based on the hypothesis that there is/are collagenolytic enzyme(s) in skeletal muscles of winter flounder. If the above hypothesis is proven then proper post harvest handling and low temperature storage techniques can be devised to preserve the desirable texture of the fish.

Winter flounder, *Pseudopleuronectes americanus*, is a bottom dwelling fish, living from the tideline to one hundred feet, and found mainly in the Atlantic coast of North America. This fish species thrives on bottom dwelling crustaceans, molluscs and marine worms. Winter flounder which supports one of the most important commercial flounder fisheries in the United States are known as blackbacks when the size is 3½ pounds or less, and are marketed as lemon sole when over 3½ pounds (Faria, 1984). The fish tolerates low temperature well since it produces an "antifreeze" in the blood during winter. In this study, the fish samples were obtained from Moncton, New Brunswick, where the daily mean temperature during winter ranged from -2.4°C to -8.4°C and for the rest of the year from 2.1°C to 19.1°C (Canadian Climate Program, 1993). Cathepsins had been detected in the muscle of winter flounder (Reddi *et al.*, 1972), however, there is a dearth of data on the presence of collagenolytic enzyme(s) in the fish flesh and its (their) role in the postmortem fish flesh softening.

Objectives

The primary objective of this study was to isolate collagenolytic enzyme(s) from winter flounder (*Pseudopleuronectes americanus*) and to characterize the enzyme(s) with respect to their responses to pH, temperature, inhibitors, as well as their kinetic and thermodynamic properties. Determination of these properties would provide basic knowledge which could be useful in finding/evolving methods to prevent undesirable postmortem textural changes resulting from the action of these enzymes.

The specific objectives were:

(i) To isolate collagenolytic enzymes from the skeletal muscle of winter flounder, *P. americanus*;

(ii) To purify the collagenolytic enzymes so as to remove other enzymes and other contaminants which may influence their activities and properties;

(iii) To characterize the purified enzymes based on their response to pH, temperature and inhibitors;

(iv) To determine the kinetic and thermodynamic properties of the fish collagenases;

(v) To determine the amino acid composition of the fish collagenases.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

Microorganisms, have been implicated in the spoilage of fresh seafoods during postharvest handling and storage of fish/shellfish. These microorganisms utilize various substrates, e.g. protein, amino acids, etc. producing metabolites/products which cause undesirable changes in the flavor, odor and color of fish/shellfish. Proteolytic microorganisms, e.g., *Pseudomonas* and *Flavobacterium* were also thought to be involved in the textural deterioration of fish/shellfish during cold storage, however, studies showed that there was no correlation between the number of proteolytic bacteria and freshwater prawn muscle degradation (Angel *et al.*, 1985). Purging of freshwater prawn did not prevent the development of mushiness during ice-chilled storage (Nip *et al.*, 1985). Baranowski *et al.* (1984), Angel *et al.* (1985) and Lindner *et al.* (1988) suggested the possible role of collagenases and other proteases on the textural deterioration of shellfish during ice-chilled storage. Thus, the focus of this study was to establish the presence of collagenase(s) in the skeletal tissues of fish.

The integrity of the texture of fish skeletal muscle is influenced by a number of factors, mainly by the amount of connective tissues which give support and strength to the muscle, as well as the endogenous proteolytic enzymes which can cleave/attack these connective tissues, and myofibrillar proteins resulting to the gradual disintegration of the fish skeletal muscles, and finally mushiness. Collagen, a structural protein is one of the main connective tissues found in the muscles of vertebrates. The collagen content of mammals and birds are generally higher than those of fish. Terrestrial animals require more and stronger connective tissues to support and maintain their muscles, whereas,

bodies of aquatic species are supported by water (Hultin, 1985). A combination of these factors, i.e., lower collagen content and higher activity of endogenous proteolytic enzymes makes fish muscle more susceptible to texture deterioration than birds and mammals during processing and/or cold storage. Collagenases, i. e., tissue collagenases are highly specific enzymes that cleave peptide bonds in the triple-helical regions of collagen, otherwise collagen is very resistant to enzymatic attack (Stryer, 1988).

2.2 Collagen, structure and function

Connective tissues are present in all multicellular organisms, occurring in various forms, each having its physiological function(s). The three main roles of connective tissues are to give mechanical strength to organs, to provide a framework for movement and to promote the right environment for cell growth and proliferation (Bailey and Light, 1989).

The connective tissues in animals are classified as skeletal tissues and soft tissues. The soft connective tissues refer to the skin, tendons, blood vessels walls and the basement membrane. These connective tissues vary in composition but the major protein common to all is collagen.

Collagen belongs to a family of fibrous proteins present in all multicellular organisms. It is found in the skin, bone , tendon, cartilage, blood vessels, teeth and other organs of mammals, and serves to hold the cells together in discrete units (Stryer, 1988). Collagen constitutes about one-fourth of the total weight of mammals (dry weight) (Stryer, 1988) and about 30% of its total protein (Veis, 1970). It also has a directive role in developing tissues, i.e., tissues undergoing growth and remodeling (Stryer, 1988). In muscles, connective tissues comprise a small part yet they play a significant role in the physiological function of the muscle and in determining the eating quality of meat (Bailey and Light, 1989). In fish muscle, collagen exists mainly as fibrous sheets called

myocommata which surrounds the blocks of muscle tissue called myotomes (Kimura *et. al.*, 1988).

The term "collagen chains" refers to a set of homologous polypeptide chains (called α-chains), each of which exhibits a molecular weight of about 95,000 and each of which is characterized by the presence of the typical Gly-X-Y repeating sequence throughout the 90% or more of its entire length (Miller and Gay, 1982). The residues X and Y can be any amino acid, except glycine, frequently proline and hydroxyproline, respectively (Bailey and Etherington, 1980). The presence of these pyrrolidine residues at highly regular intervals induces the polypeptide chain to take up a polyproline II helix rather than the usual α-helix of globular proteins (Bailey and Etherington, 1980). Thus, proline is a major element in stabilizing this conformation. Hydroxylation of proline and other amino acids e.g., lysine has been observed in different types of collagen. The degree of hydroxylation varies among types of collagen, thereby influencing their properties. Nonhydroxylated collagen has been observed to be less stable than normal collagen, e.g., the melting temperature is 15°C lower (Berg and Prockop, 1973). Hydroxyproline, the imino acid which is almost confined to the Y - position is important in giving stability to triple helix at physiological temperatures (Berg and Prockop, 1973). Hydroxylysine another unusual amino acid appears to have a dual function. It is not only involved in the cross-linking mechanism (Bailey and Robins, 1973), but it also carries the carbohydrate groups of collagen (Spiro and Spiro, 1971). Vertebrate collagens contain significant but highly variable levels of covalently linked carbohydrate, thus they can be designated as glycoproteins (Miller, 1984). The carbohydrate units consist either of a galactose residue, α-D-galactopyranosyl hydroxylysine, or of the disaccharide, α-D-glucopyranosyl-(1→2-β-D-galactopyranosyl hydroxylysine (Spiro, 1967). The linkage to the protein is achieved through an acetal bond between the C-1 of the pyranose from the galactose and the hydroxy group of hydroxylysine (Miller, 1984).

Three collagen chains each in the form of a left-handed polyproline-type helix make up the collagen molecule. The rod-like collagen molecule is about 1.5 nm in diameter and 290 nm in length and is stabilized by interchain hydrogen bonds and by interactions of the radially extending amino acid residues with water molecules (Sikorski *et al.*, 1984). These collagen molecules aggregate into collagen fibrils which finally form into the collagen fibers (Fig. 1). Newly synthesized aggregates of collagen fibrils are stabilized by ionic and hydrophobic bonds, whereas, older aggregates are stabilized by covalent intermolecular cross-links (Sikorski *et al.*, 1984).

The ionic and hydrophobic bonds which maintain the precise alignment of collagen fibers are not responsible for the tensile strength of collagen (Bailey and Etherington, 1980). The high mechanical strength or stability of collagen fiber is due to the formation within the fibers of intra- and intermolecular cross-links (Bailey and Light, 1989). There are three distinct mechanisms involved in both the intermolecular and intramolecular cross-linking of collagen molecules. First, is the formation of the disulfide bonds which are confined to the cysteine-containing collagen, i.e., types III and IV. Second, is the formation of divalent bonds which link two collagen chains of the same or different molecules formed from lysine or hydroxylysine aldehydes. The third mechanism is the formation of more complex bonds which involve the linking of two α chains together from the simpler reducible divalent bonds (Bailey and Light, 1989).

2.3 Types of collagen

Differences in the chemistry of collagens from various tissues have been observed and the five types that had been identified and found ubiquitously distributed throughout the connective tissues of mammals are presented in Table 1. Type I collagen, the most common type, is a heteropolymer composed of two identical and one different chains while Type II and Type III are homopolymers having three identical chains (Bailey and

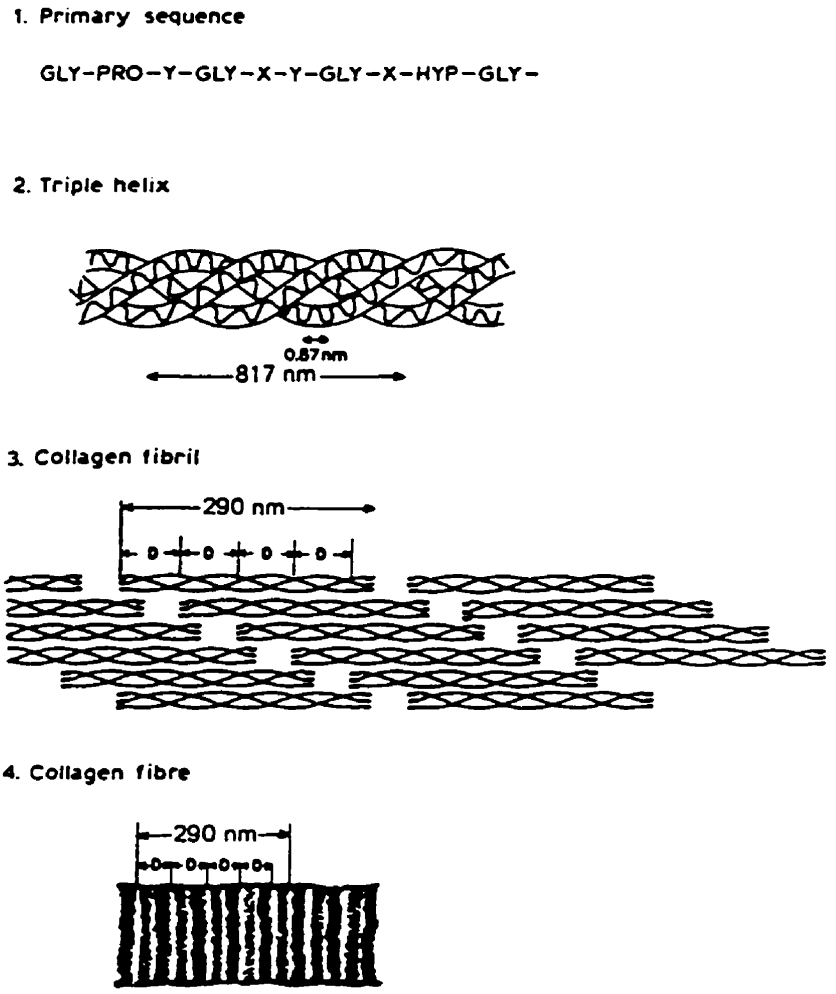


Fig. 1. Diagrammatic representation of the structure of the collagen fibre, depicting the typical repeating amino acid sequence, the triple helix of three polyproline type helices, and the quarter stagger-end overlap alignment of the molecules in the fibre (Bailey and Etherington, 1980).

Table 1. Isomorphic forms of collagen*

Type	Molecular composition	Tissue distribution	3-hydroxyproline content residues/1000	Hydroxylysine content residues/1000	Carbohydrate hylroxylysine % glycosylation
I	$[\alpha 1(I)]_2\alpha 2$	Tendon, bone dentine, dermis	1	6 - 8	20
II	$[\alpha II]_3$	Cartilage, vitreous homour, invertebral discs	2	20 - 25	50
III	$[\alpha\ III]_3$	Fetal dermis, cardiovascular system, synovial membrane, viscera, muscle	1	6 - 8.	15 - 20
IV	Possibly $[\alpha IV]_3$	Basement membrane, lens capsule and kidney glomeruli	10	60 - 70	80
	Two different α chains	Placental membrane, lung	5 - 10	60	-
V	$[\alpha]_2\alpha A$	Placental membrane, cardiovascular system, lung; minor component of most tissues	2 - 3	6 - 8	-

*Bailey and Etherington (1980)

Etherington, 1980). Type I collagen is concerned with mechanical support, whereas, type III collagen is present in the more flexible tissues (Miller *et al.*, 1976). Type IV collagen which is associated with membranes has been reported to have three identical chains (Kefalides, 1971), has several different α-chains, and a short collagenous section interspersed with glycoprotein (Spiro, 1972). The presence of more than one type of chain (Glanville *et al.*, 1979) and the presence of two more molecules have also been reported (Bailey *et al.*, 1979). Type V collagen, a basement membrane - associated collagen is generally believed to be made up of two identical and one chemically different chain (Bentz *et al.*, 1978). As mentioned earlier, the levels of carbohydrate in collagen varies for a given type of collagen from various sources, and for different types of collagen. Types I and III collagen chains have the lowest levels of carbohydrate (0.5 - 1.0%), whereas, types II, IV and V have more, from 6% in αI(II) to approximately 15% in αI(IV) (Miller, 1984).

Recently, other types of collagen referred to as types VI, VII, VIII, IX, X and XI were identified. Type VI collagen found in the extracellular matrix of vascular system, cartilage, cornea and perimysium of muscles has filaments which are not cross-linked by the usual collagen cross-links. Type VII collagen molecule which may occur in the endomysium is 1.5 times longer than the fibrous collagen. Types VIII, IX, X and XI which have been found in the epithelia, arteries and cartilage have the basic triple helical structure with greater or lesser proportions of non-triple helical peptides (Bailey and Light, 1989).

2.4 Collagen in fish, mammals and avian

In the animal kingdom, the main function of the muscle is locomotion, yet there are differences among muscles of the same animal and among different species. These differences are very obvious when fish muscles are compared with those of the mammals

and birds (Hultin, 1985). The observed differences are due to the following factors: firstly, fish do not require extensive and strong connective tissues to maintain and support their muscles since the fish body is supported by water; secondly, most fish of commercial importance are poikilothermic, and they live in cold water (Hultin, 1985). Thus, properties of their muscle proteins may differ from those of the warm blooded animals. Thirdly, due to the unique movement of fish the structural arrangement of their muscles differ from those of birds and land animals (Hultin, 1985).

The collagen content of red meat is generally 10 times higher than that of fish flesh (Sikorski *et al.*, 1984), although Jacqout (1961), had reported that connective tissues of cartilaginous fish species approximated that of land animals. For instance, catfish and dogfish had 7% and 11% of connective tissues, respectively. These values are close to those reported for mammals, like rabbit (15%), cattle (8 -20%), and white rat (7%). On the other hand, bony fishes had lower levels of connective tissue in the flesh, e.g., 2% for halibut, 3 - 5% for haddock and 5% for cod (Jacqout, 1961). Recent investigations have shown that the collagen content of the muscle in the dorsal part of the body varied in fish species, ranging from 1.6% to 12.4% of the total protein (Hatae *et al.*, 1986; Sato *et al.*, 1986). Furthermore, it has been observed that the firmness of raw fish meat increased at higher collagen content. Also different muscle parts of fish contain varying levels of collagen, e.g., carp white muscle contains 3.0 g collagen/100 g protein and the dark muscle contain 8.6 g collagen/100 g protein (Kimura *et al.*, 1979). Collagen content is also influenced by the season, i.e., period of maturation, period of starvation, etc. For instance, maturation and starvation have been related to increased collagen content in certain species of fish (Love *et al.*, 1976). However, the increase could be relative and may have partly resulted from other constituents being preferentially utilized (Thomson and Farragut, 1965). During period of starvation, fish albumin and myofibrillar proteins are degraded, however, the connective tissues are not. Thus during starvation, the proportion of collagen to total protein increases. However, the same reason could not

explain the thickening of the skin of herring during starvation (Hughes, 1963). In the case of cod, collagen content varies throughout the year (Love *et al.*, 1976: 1972b). Lavety and Love (1972), reported that cod myocommata increased in mechanical strength with starvation. In a similar study Love *et al.* (1976) showed that cod myocommata thickened during starvation. During this period the breaking strength of myocommata tissue is greater suggesting more intermolecular cross-linking in collagen, however, the new collagenous layer did not differ in molecular shape, amino acid composition, imino acid stabilization or intramolecular cross-linking (Love *et al.*, 1976). Similar observations have been noted during sexual maturation of fish. According to Sikorski *et al.* (1984) at this stage there is thickening of the connective tissue, then a thinning away in the following season of intensive feeding. Many reports have also shown that in herring the proportion of collagen to total protein increased during the period of maturation (Love, 1980).

Age also influences the collagen content of fish. Younger fish have a lower percentage of insoluble collagen than older fish (Ramshaw *et al.*, 1988). In trout, except for the very old ones, collagen content generally increases with age (Montero and Borderias, 1990b) and in cod, myocommata increased in thickness with fish growth (Love *et al.*, 1972b). Cross-linking of collagen in trout increased with size of the fish (Montero and Borderias, 1990b). In rockfish (*Sebastes* sp.), the high level of cross-linking could be due to the longevity of the fish (Bogason, 1984).

So far, only two types of collagen, i.e., Types I and V have been isolated from the skeletal muscle of various fish species. Type I collagen was found in eel, mackerel, saury salmon, carp (Kimura *et al.*, 1988), hake and trout (Montero *et al.*, 1990) while Type V collagen was isolated from the skeletal muscle of mackerel, carp, lizard fish, Japanese eel, sturgeon spotted shark and rainbow trout (Sato *et al.*, 1988).

Collagen in mammals, birds and fish show certain similarities, in terms of type and composition. Mammalian and fish collagens contain similar amino acids in approximately the same proportion, although quantitatively there is a wider range of composition among

the aquatic vertebrates, reflecting their evolutionary age and variation (Eastoe, 1957). Table 2 shows the amino acid composition of collagen from fish and mammalian sources. Fish collagens have lower hydroxyproline content than mammalian collagens. The lower

Table 2. Amino acid composition of fish myocommata collagen[1] (residues/1000 residue).

Amino acid	**Cod**	**Catfish**	**Hake**	**Bovine L. dorsi intramuscular connective tissue[2]**
3-hydroxyproline				
4-hydroxyproline	40.7	39.5	41.2	109
Aspartic acid	42.3	37.6	39.1	34
Threonine	25.8	25.4	30.2	17
Serine	62.9	64.2	48.3	36
Glutamic acid	82.2	72.2	81.7	83
Proline	87.6	79.2	91.5	113
Glycine	313.6	311.8	323.1	336
Alanine	106	92.7	118	107
Valine	26.1	25	24.4	25
Methionine	20.4	19.2	20.6	5
Isoleucine	18.6	14.5	15.6	12
Leucine	32.3	27.6	28.0	25
Tyrosine	6	3.6	4.3	3
Phenylalanine	14.5	14.4	15.1	14
Hydroxylysine	8.2	27.6	6.7	8
Lysine	39.6	48.8	38.4	23
Histidine	16.3	40.4	12.7	54
Arginine	59.1	56.5	61.3	45

[1]Yamaguchi *et al.*, (1976); [2]Mclain *et al.*, (1971)

hydroxyproline is accompanied by an increase in the threonine and serine content in the fish collagen. Thus, despite the reduced hydroxyproline in the fish collagen, the total number of the hydroxy group is similar to that of the mammalian collagen. Studies have shown that fish muscle collagen has a higher biological value than bovine muscle collagen, as indicated by the higher content of essential amino acids in the former.

Like mammalian collagen, however, fish collagen also contains carbohydrates. Most of these carbohydrates are linked o-glycosidically to hydroxylysine residues, mainly as glucosylgalactosylhydroxylysine residues (Sikorski *et al.*, 1984).

Collagen in fish is confined mainly in the muscles, the skin, fins and the skeleton, with the muscles having the lowest collagen concentrations. Being a major constituent of connective tissues, collagen is assumed to play an important role in the mechanical strength, integrity and rheological properties of the muscles and fillets, and the water-holding and gel-forming capacities of comminuted cooked products (Sikorski *et al.*, 1984).

The muscles of fish are divided into myotomes which are separated by thin membranes referred to as the myocommata. The myocommata is made up of connective tissues the amount of which increases with respect to the myotomes towards the tail region (Sikorski *et al.*, 1984). The myotomes comprise of muscle fibers surrounded by basement membranes which contain thin collagen fibrils.

2.5 Degradation of collagen

The degradation of native collagen is initiated by collagenase, a highly specific enzyme. The action of collagenase makes the collagen molecule an easy target for other proteolytic enzymes like chymotrypsin, trypsin, cathepsin, etc.

The native collagen triple helix is highly resistant to the action of most proteases, however, the connective tissue cells synthesize a number of proteases capable of acting on

collagen under physiological conditions (Bailey and Light, 1989). This group of proteases includes aspartic, cysteine, metallo- and serine proteases. Among these enzymes, collagenase, a zinc metalloendopeptidase plays a critical role. Collagenase cleaves all fibrous collagen, cleaving types I and III at a faster rate than type II. The action of this collagenase is conformationally dependent, acting on the collagen triple helix at a definite locus, between the Gly-Ile or Gly-Leu peptide bonds across all three peptide chains, but has little action on the same site on individual alpha chains. This collagenase has no effect on non-fibrous collagen types IV and V (Bailey and Light, 1989).

The rate controlling step in collagen degradation is the initial attack by collagenases (Harper, 1980). These enzymes seem to make only one initial specific scission at the end of the collagen molecule, i.e., Type I collagen (TC) and thus give rise to two reaction products, a larger peptide (75% of the substrate) which is referred to as TC_A, and a smaller peptide (25% of the substrate) referred to as TC_B (Fig. 2). A multienzyme degradation of the reaction products may follow to yield smaller peptides. The presence of cross-linkages prevents the release of shorter fragments from the fibers, and the activity of other enzymes on the non-helical regions becomes necessary to release these trapped fragments. Hence, collagen degradation requires the joint action of collagenase and at least another protease on the different sites of the collagen molecule.

2.6 Proteolysis in food myosystem and post harvest fish flesh softening

Upon death of animals the muscle becomes an isolated structure and is converted into meat. This conversion process is accompanied by a number of irreversible changes which include a drop in pH due to lactic acid formation, stiffening of the muscle or of rigor mortis due to the interaction of myosin heads and actin which becomes irreversible because of loss of ATP. After a certain period the meat becomes flaccid and eventually tender. The tenderization of meat/muscle food is associated with the dissociation of

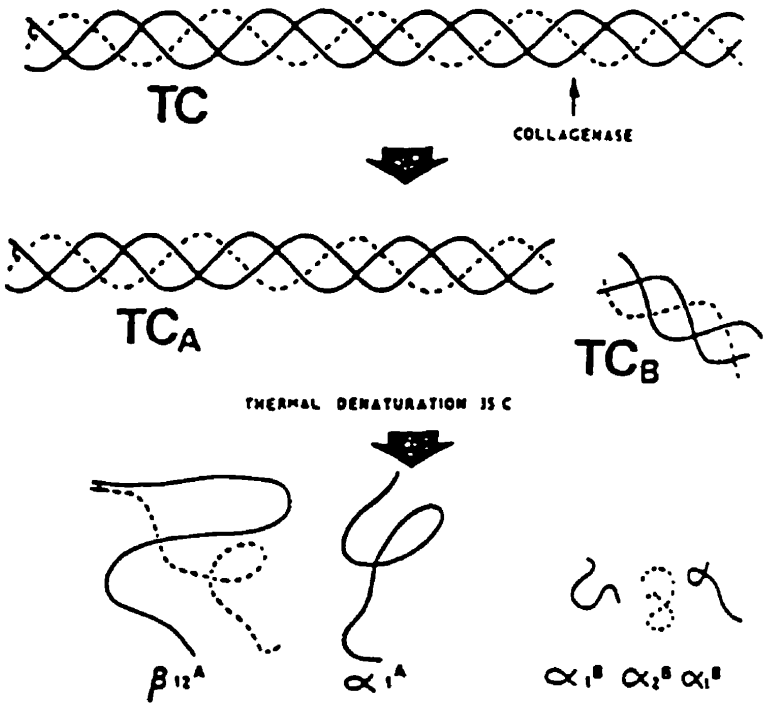


Fig. 2. Diagrammatic illustration showing cleavage of the type I collagen molecule (TC) by collagenase at 25°C. Two helical fragments, TC_A and TC_B, three quarters and one quarter the length of the original molecule, respectively, are produced (Woolley, 1984).

actomyosin complex, the loss of Z-disks, denaturation of collagen and disintegration of connectin (Robbins *et al.*, 1979; Koohmarie *et al.*, 1986; 1988). The resolution of rigor and consequent flesh softening/tenderization is due to a series of degradative enzymatic activity of neutral (cytoplasmic) and acid (lysosomal) proteases. The acid cathepsins cause partial breakdown of the actomyosin complex, its anchoring proteins and other intracellular proteins, and damage to the sarcolemma (Bailey and Light, 1989). The cathepsins also degrade the connective tissues surrounding the muscle cells.

The postmortem proteolysis in mammals and birds is desirable due to increased meat tenderness, an important quality attribute in mammals and birds. However, postmortem softening of raw fish flesh is not desirable, since it is associated with deterioration. While the process of fish flesh softening is very rapid, tenderization in mammals and birds occurs at much slower rates. The more tender texture and fast deterioration of the fish skeletal muscle could be influenced by the lower stability of its connective tissues, particularly collagen. Fish muscles have approximately one-tenth the collagen content and fewer collagen cross-links than those of the land animals (Bracho and Haard, 1990; Cepeda *et al.*, 1990). The lower stability of the fish collagens compared to mammalian collagens has been manifested in their lower shrinkage temperature, ease of gelatinization, solubility in various alkalis and acids and susceptibility to enzymes (Eastoe, 1957).

The connective tissue of the myocommata and the collagen fibers forming the endomysial reticulum are to a large extent responsible for the integrity of the muscles and the normally smooth and glossy appearance of the fish fillet (Sikorski *et al.*, 1984). Once the myotomes are disconnected from the myocommata, gaping occurs. Gaping is a phenomenon which occurs when the connective tissues fail to hold the muscle blocks (and fibers) together (Love *et al.*, 1969). Endogenous proteolytic enzyme activity may also play an important role in gaping. This phenomenon is characterized by the formation of slits and holes and possible disintegration of the fish flesh during filleting and skinning

operations. The species and size of fish influence gaping. Love *et al.* (1969) observed that roundfish were more susceptible to gaping than flatfish. After 20 h post-mortem, they found haddock to be most susceptible to gaping, followed by cod, saithe, redfish, halibut and sole. On the other hand, catfish and skate were resistant to gaping (Love *et al.*, 1969). Fillet thawed from smaller fish was observed to gape more than those from bigger fish with the same ultimate pH in the muscles. This observation could be explained by the fact that bigger fish have thicker myocommata which can resist greater strain (Love *et al.*, 1972a).

2.7 Major enzymes implicated in fish flesh softening

The activities of several enzymes have been demonstrated in the skeletal muscle of various fish species. In live fish the activities of these enzymes are regulated/controlled by naturally occurring inhibitors or other proteases. When the fish dies, endogenous enzymes remain active. With the enzyme regulatory mechanism broken down, endogenous enzyme degrade muscle tissues causing undesirable changes in the fish. Cathepsin activities in the fish muscles are approximately 10 times greater than in mammals and possibly contribute to the postmortem softening of the fish tissues (Seibert and Schmitt, 1964). In recent years lysosomal proteolytic enzymes particularly cathepsins isolated from seafoods, have been implicated in the texture deterioration of fish (Konagaya, 1985a; Makinodan *et al.*, 1985; Busconi *et al.*, 1989; Yamashita and Konagaya, 1992a; 1992b). Cathepsins D, E and L were shown to be present in the muscle of chum salmon, rainbow trout, carp, red sea bream and horse mackerel (Yamashita and Konagaya, 1992a). Cathepsins D and E are acidic proteases with optimum pH at 3.4 and 2.8, respectively, while cathepsin L exhibits optimum activity at pH at 5.7. These cathepsins have been shown to degrade myofibrillar proteins and eventually cause softening of the fish flesh. It has been suggested, however, that autolysis of fish muscle and its eventual degradation is mainly due to cathepsin L

activity, since the optimum pH of cathepsins D and E are far below the physiological pH of the fish (Yamashita and Konagaya, 1992a). It is worth noting that the activity of cathepsin D still persists at higher pH values although at much reduced rates and its role in texture deterioration could therefore not be readily discounted. It was observed in salmon, that during spawning the mature fish has very high cathepsin D activity and the fish tissues autolyze drastically (Konagaya, 1985a; 1985b). Cathepsin D found in the muscle of freshwater fish *Tilapia mosambica* (Doke *et al.*, 1980) and *Tilapia nilotica* (Jiang *et al.*, 1992) also participate in the textural degradation. Cathepsin D from the lysosomal extracts of *Tilapia nilotica* caused fragmentation of myofibrils isolated from the muscle of the same fish, at 4°C, pH 5.5 - 6.5 for three days. Maximum proteolysis by lysosomal enzymes was at pH 6.5. Cathepsin D from *T. mosambica* which has optimum pH at 2.8 - 3.8 with hemoglobin as substrate, also showed maximum degradation of endogenous sarcoplasmic proteins at pH 5 (Doke *et al.*, 1980). Cathepsin D from the skeletal muscle of winter flounder (Reddi *et al.*, 1972) and carp muscle (Makinodan *et al.*, 1982) were also implicated in the texture degradation of these fish species. A proteinase with characteristics similar to cathepsin E isolated from albacore (Groninger, 1964) and a trypsin-like serine proteinase in the muscle of white croaker (Busconi *et al.*, 1989) also acted on the skeletal myofibrils of these fishes.

The role of other enzymes aside from the proteases in the textural deterioration of fish has been suggested. A lipase isolated from rainbow trout muscles (Geromel and Montgomery, 1980) and phospholipase A from pollock muscles (Audley *et al.*, 1978) liberate fatty acids which can react with the proteins. According to Love and Elerian (1964) Anderson and Steinberg (1964), the liberated fatty acids may interact directly with muscle proteins causing denaturation and adversely changing the water holding capacity and texture of the meat.

Collagenases which have regulatory roles, i.e., tissue remodeling and repair in the living organisms may cause considerable postmortem degradation of the connective

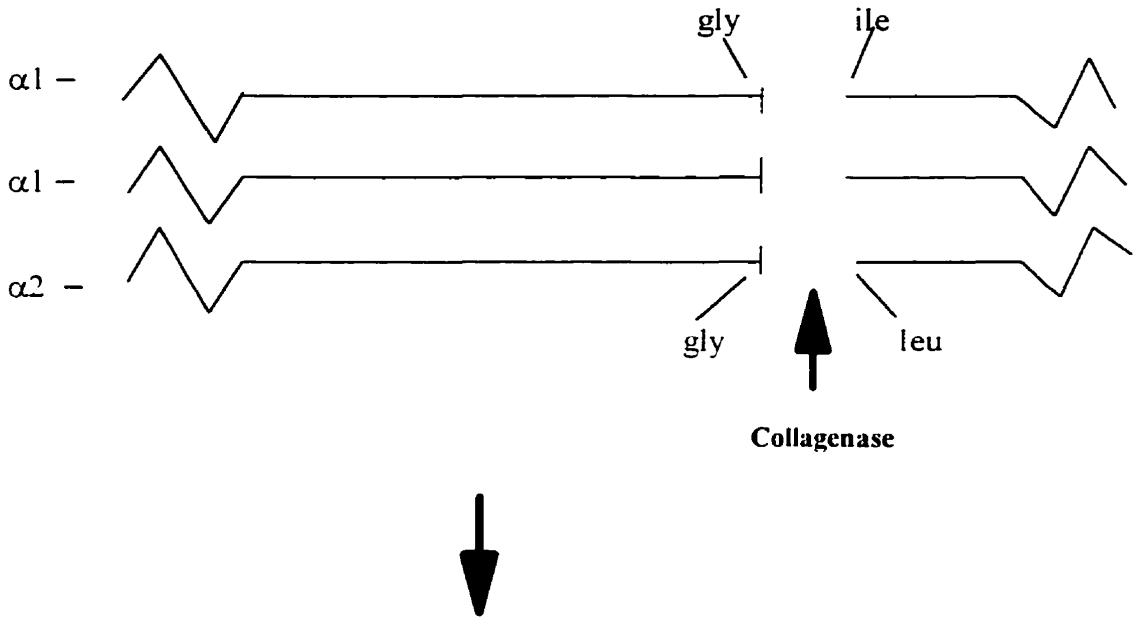
tissues. Several investigators have suggested the participation of collagenase(s) in the texture deterioration of fish/shellfish. The diffusion of collagenolytic and proteolytic enzymes from the hepatopancreas of prawn was suggested to cause mushiness of the skeletal tissues (Lindner *et al.*, 1988). In blue grenadier, the activity of endogenous collagenases and other proteases was implicated in the disintegration of the fibrils which attach the muscle fibers to the myocommatal connective tissue sheets of the fish (Bremner and Hallett, 1985). Similarly, degradation of the tissues in rainbow trout was traced to the degradation of nonhelical regions in type V collagen (Sato *et al.*, 1991).

Belly burst in fish like capelin (Gildberg, 1978; Gildberg and Raa, 1979), herring and sprat (Marvik, 1974) is a phenomenon particularly observed when fish is caught during intense feeding period. This is characterized by connective tissue breakdown, a result of the activity of digestive enzymes which diffused into the surrounding tissue during storage (Gildberg, 1978) .

2.8 Collagenases, definition and characteristics

Collagenases (E.C. 3.4.24.7, for vertebrate collagenase) are enzymes that catalyze the hydrolytic cleavage of undenatured collagen (Seifter and Harper, 1970). A true collagenase should digest native collagen molecules (cleave the main body helix) at physiological pH and temperature below the thermal denaturation temperature of the substrate (Harper, 1980). According to the Enzyme Commission classification system, collagenases are highly specific for collagen (native or denatured) and are without activity on any other protein (Barman, 1969). If the collagen molecule is denatured it becomes susceptible to hydrolysis by other proteases.

True collagenases attack soluble collagen at one specific site (Gly-Ile; Gly-Leu), producing two characteristic products, the N-terminal 3/4 fragment and the C-terminal 1/4 fragment (Woolley, 1984) as shown in Fig. 3. Collagenase action on Type I collagen



α1(I): Pro-Gly-Pro-Gln-Gly- -Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp-

α2(I): Hyp-Gly-Pro-Gln-Gly- -Leu-Leu-Gly-Ala-Hyp-Gly-Phe-Leu-Gly-Leu-Hyp-

775 776

Fig. 3. Cleavage site of amino acid sequences in the region for chick skin α1(I) and skin calf α2(I) chains of collagen (Gross et al., 1980).

molecule results in cleavage of a Gly-Ile peptide bond of the α1(I) chain and a Gly-Leu bond in the α2(I) chain (Gross *et al.*, 1980). The α1(II) chains of cartilage collagen are cleaved at Gly-Ile bond, while α1(III) chains are attacked at Gly-Leu bond (Miller *et al.*, 1976; Gross *et al.*, 1980). It appears that the minimum specific sequence required for collagen cleavage is Gly-Ile-Ala or Gly-Leu-Ala (Woolley, 1984).

Collagenases from various sources exhibit slight differences in their physico-chemical properties. Microbial collagenases are stable over a wide pH range. For instance, *Vibrio* B-30 collagenase with optimum activity at 37°C and pH 7.6 is stable at pH 4.4 - 7.4 (Merkel and Dreisbach, 1978) and *Cytophaga* sp. with optimum pH of 7.5 is more stable at pH 6 - 8 (Sasagawa *et al.*, 1993). The same enzyme has optimum temperature of 30°C and stability at 15 - 40°C. Collagenase from *Bacillus alvei* has optimum pH of 4.0, 6.0 and 7.0 (Kawahara *et al.*, 1993). Pig synovial collagenase with optimum pH at 7.5 - 8.5 is stable at pH 6.5 - 9.0 (Tyler and Cawston, 1980) while rabbit tumor collagenase has an optimum pH range from 7.0 - 9.5 (McCroskery *et al.*, 1975).

Collagenolytic enzymes from fish and shellfish exhibit optimum pH similar to those of collagenases from microorganisms and mammals. A collagenolytic enzyme determined to be a serine protease from crab hepatopancreas has an optimum pH of approximately 8.0 and is stable at pH 6 - 9 (Eisen *et al.*, 1973). A collagenolytic enzyme from freshwater prawn has an optimum activity at pH of 6.5 - 7.5 and 37°C (Nip *et al.*, 1985), while collagenase from file fish was most active at pH 7.75 and at 55°C (Kim *et al.*, 1993).

A wide range of molecular weights have been observed in collagenases from different sources. Collagenases from *Clostridium histolyticum* have molecular weights ranging from 68 kDa - 125 kDa (Bond and Van Wart, 1984a). *V. anguillarum* collagenase has an estimated molecular weight of 35 kDa (Stenvag *et al.*, 1993) and *Vibrio* B-30 collagenase, a tetramer, has a molecular weight of 105 kDa (Merkel and Dreisbach, 1978). A 120 kDa collagenase was isolated from *Clostridium perfringens* (Matsushita *et al.*, 1994). Mammalian collagenases, mostly from tissue cultures, have

molecular weights as low as 33 kDa - 35 kDa for collagenase from rabbit tumors (McCroskery *et al.*, 1975), and as high as 92 kDa for Type IV collagenase (Ogata *et al.*, 1992).

Collagenolytic enzymes from shellfish have lower molecular weight than their mammalian and microbial counterparts. For instance, a collagenolytic protease from crab was found to be about 25 kDa in size (Eisen *et al.*, 1973). Collagenases from various sources also exhibited different isoelectric points (pI), e.g., pH 4.8 for *Cytophaga* sp. (Sasagawa *et al.*, 1993) and ~8.0 for rabbit synovial collagenase (Werb and Reynolds, 1975). Differences in properties could be due to differences in their chemical composition, e.g., amino acid composition.

2.9 Sources of collagenase

Microorganisms, e.g., *Clostridium histolyticum* (Gallop *et al.*, 1957; Debellis *et al.*, 1954) and *Clostridium perfringens* (Mandl, 1961) are among the first known producers of collagenolytic enzymes. Vertebrate collagenase was first observed in tissue culture of bullfrog tadpole in serum-free media (Gross and Lapiere, 1962). Mammals (Jeffrey and Gross, 1970; Eisen *et al.*, 1968), and fish/shellfish (Eisen and Jeffrey, 1969; Eisen *et al.*, 1973; Yoshinaka *et al.*, 1978; Grant *et al.*, 1980; Grant and Eisen, 1980; Welgus *et al.*, 1982; Grant *et al.*, 1983; Baranowski *et al.*, 1984; Nip *et al.*, 1985; Kim *et al.*, 1993) are also producers of the enzyme.

Collagenases from higher animals appear to be elaborated by special cells, e.g., lysosomes which are involved in the repair and remodeling processes, whereas, microbial collagenases are usually extracellular (Seifter and Harper, 1970). In general, the microorganisms producing collagenase are host-invasive, and presumably the enzymes contribute to their pathogenecity by allowing the microorganisms to penetrate connective tissue barriers (Seifter and Harper, 1970).

2.9.1 Types of collagenases

Collagenases from bacteria and vertebrates are metalloenzymes, i.e., they require metal ions specifically both zinc and calcium ions for optimum activity and stability. They are inhibited by chelating agents such as ethylenediaminetetraacetic acid (EDTA), and 1,10-phenanthroline. The presence of zinc as a catalytic metal has been established in collagenases from various sources. According to Seifter and Harper (1970) collagenases from microbial, amphibian and mammalian sources are metalloenzymes having an intrinsic metal component, most likely zinc atom at their active site. Extracellular collagenolytic protease from *Bacillus cereus* (Strain Soc 67) was shown to have 1 mole of Zn/mole enzyme (Makinen and Makinen, 1987). Collagenases from *Clostridium histolyticum* were found to have either slightly less or slightly more than 1 mole zinc ion/mole enzyme (Bond and Van Wart, 1984a). A 120-kDa collagenase from *Clostridium perfringens* is also a zinc protease (Matsushita et al., 1994). *Achromobacter* collagenase which showed a close similarity to *C. histolyticum* collagenase also has a metal ion at its active site (Lecroisey *et al.*, 1975). Human neutrophil collagenase has a catalytic zinc ion situated at the bottom of the active site cleft and it is pentacoordinated by 3 histidines and by hydroxamic acid oxygens of an inhibitor. A second non-exchangeable zinc ion and two calcium ions are also present and presumed to stabilize the catalytic domain (Bode et al., 1984). Rabbit bone collagenase also contains zinc on its active site (Swann *et al.*, 1981).

The influence of other ions, i.e., calcium (Seifter and Harper, 1970), cobalt (Yagisawa *et al.*, 1965) and magnesium (Takahashi and Seifter, 1970; Matsushita *et al.*, 1994) on the collagenolytic activity of the enzymes have also been investigated. Among these metals calcium ions have the most influence on the catalytic activity of collagenase. Calcium ions are required for both the binding of the enzyme to the collagen substrate and for full catalytic activity (Seifter and Harper, 1970). It appears that the calcium ion makes the enzyme conform around the putative zinc atom such that the latter is chelated with

two or three amino acid residues similar to that of the zinc in carboxypeptidase A. If calcium ion is insufficient, the protein conformation will vary, and possibly only one or two of the chelating positions of the zinc atom are occupied by amino acid residues of the enzyme, leaving two or three positions around the zinc atom available to bind with other substances. It can be assumed that one or two of these four positions normally bind(s) to the substrate.

Interaction of a multifunctional inhibitor, e.g., cysteine with the enzyme in the absence of calcium, results in stronger binding. Thus, the presence of calcium ions reduces the efficiency of collagenase inhibitors. Addition of calcium reversed the inhibitory action of EDTA on collagenase (Lecroisey *et al.*, 1975; Van Wart and Steinbrink, 1981). Calcium could be an inherent component of collagenase. Bond and Wart (1984a) observed that variable quantities of calcium ions co-purified with collagenases from *Clostridium histolyticum*. The amount of calcium bound to the collagenases varied from 2 atoms to about 7 atoms per molecule, increasing in number with increasing molecular weight of the enzyme (Bond and Van Wart, 1984a).

A second group of collagenases belong to the class of serine proteases. This group includes collagenases from crustaceans (Eisen *et al.*, 1973; Welgus *et al.*, 1982; Grant *et al.*, 1983; Baranowski *et al.*, 1984; Nip *et al.*, 1985), some fungi and arthropods (Keil, 1980). Unlike their mammalian counterpart this group of enzymes are not inhibited by metalloenzyme inhibitors but are inhibited by phenylmethylsulfonyl fluoride (PMSF), N-α-ρ-tosyl-L-lysine chloromethyl ketone (TLCK) and other serine protease inhibitors. The activity of these collagenases is also enhanced by calcium ions.

2.9.2 Latent collagenases

Collagenases from various sources may be secreted in the active or latent form. Latent collagenases refer to the precursors form of the enzyme in culture media of certain

cells or tissue explants or in tissue homogenates (Woolley, 1984). Collagenases isolated from the tissues of multicellular organisms are usually in their zymogen forms. This group of collagenases plays an important role in the remodeling of special tissues at specific times of development or physiological expression. The same group could be involved in the repair of damaged tissues, i.e., promote regeneration or healing of burns or wounds.

Two inactive forms of collagenases have been found from various sources, the procollagenase or zymogen form and the enzyme-inhibitor complex. The zymogen form of collagenase has been detected in tissue cultures of tadpoles (Harper *et al.*, 1971), bovine gengiva (Birkedal-Hansen *et al.*, 1976), rat uterus (Woessner, 1977), mouse bone (Gillet *et al.*, 1977; Sakamoto et al., 1978), human skin fibroblasts (Stricklin *et al.*, 1977; 1978) and pig synovium (Cawston and Tyler, 1979). Collagenase may be inhibited by serum proteins or other components and thus exists as an enzyme-inhibitor complex (Harper, 1980), as in the case of bovine gengival collagenase which exists in both inactive forms (Birkedal-Hansen *et al.*, 1976; Stricklin *et al.*, 1977).

Unlike the mammalian and amphibian collagenases, the enzymes isolated from fiddler crab (Eisen and Jeffrey, 1969; Eisen *et al.*, 1973) and freshwater prawn (Baranowski *et al.*, 1984; Nip *et al.*, 1985) were active. These digestive collagenolytic enzymes strictly play a nutritional function. There are speculations however, that during cold/ice storage, these group of enzymes may come in contact with the skeletal tissues of the shellfish/fish causing tissue softening or degradation (Lindner *et al.*, 1988).

2.9.3 Activation of latent collagenases

Activation of latent collagenases may be achieved by the use of several proteases. Incubation of bovine gengiva procollagenase with trypsin released the active enzyme accompanied by a decrease of molecular weight from 80,000 to 60,000 - 65,000 (Birkedal-Hansen *et al.*, 1976). A decrease in the molecular weight (~77,000) of a

precursor from the rat uterus when incubated with a naturally occurring activator, (serine protease) in the uterus was concomitant with the appearance of an active enzyme with a molecular weight of ~60,000 (Woessner, 1977). Other proteases observed to convert procollagenases to their active forms include plasmin, kalikrein, chymotrypsin, and cathepsin B (Eeckhout and Vaes, 1977).

Mercurials as well as chaotropic ions were also used to release the active enzymes. Maximum activation of latent pig synovial collagenase was achieved with the use of 4-aminophenylmercuric acetate and phenylmercuric chloride (Cawston and Tyler, 1979). Collagenase from wounded tissues of pigs (Agren *et al.*, 1992) and type IV collagenase from humans (Kleiner *et al.*, 1993) were also activated by aminophenylmercuric acetate.

Sodium thiocyanate had also been observed to activate complexes between alpha-2-macroglobulin and collagenase (Nagai, 1973; Abe and Nagai, 1972).

Procollagenases may also be activated by physical means, such as freezing and thawing as in the case of the latent collagenase in embryonic chick skin organ culture (Kawamoto and Nagai, 1976).

Spontaneous activation of the pig collagenase was also observed during purification (Cawston and Tyler, 1979).

2.9.4 Collagenolytic enzymes from fish/shellfish

Collagenolytic enzymes have been isolated from various fish/shellfish species including the fiddler crab, *Uca pugilator*, (Eisen *et al.*, 1973; Eisen and Jeffrey, 1969), freshwater prawn, *Macrobrachium rosenbergii*, (Nip *et al.*, 1985; Baranowski *et al.*, 1984), and filefish, *Novoden modestrus* (Kim *et al.*, 1993). The extraction procedures involved the use of solvents like 0.05 M Tris-HCl, pH 7.4, with or without $CaCl_2$ (Baranowski *et al.*, 1984; Nip *et al.*, 1985) and of organic solvents like acetone (Eisen *et al.*, 1973).

The collagenolytic enzyme from the hepatopancreas of fiddler crab, *Uca pugilator*, was first extracted by Eisen and Jeffrey in 1969 with 0.05M Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$. The activity of the enzyme on native reconstituted collagen at pH 7.5 and 37°C was linear with respect to time. The enzyme was also capable of reducing the initial specific viscosity of collagen solution by 55% in 3 h at pH 7.5 and 25°C without loss of optical rotation. This indicates that the enzyme can cleave the native collagen helix without producing denaturation (Eisen and Jeffrey, 1969). A similar observation was made by Eisen *et al.* (1973) on the purified crab hepatopancreas collagenase. The decrease in the viscosity of the collagen solution indicated changes, i.e., cleavage in the collagen structure which resulted to the production of fragments shorter than the α or β collagen chains, e.g., one-quarter length fragments. In a similar study, Eisen and Jeffrey (1969) observed that the action of the crude collagenolytic enzyme on the collagen molecule resulted to the production of three fragments which they identified as TC_{A75}, TC_{A70} and TC_{A67}, corresponding to 75%, 70%, and 67% of the molecular length from the N-terminal end of the collagen molecule, respectively.

A comparison of the amino acid composition of collagenase from fiddler crab hepatopancreas with microbial collagenases is shown in Table 3. The amino acid residues of collagenases are given as residues/molecule assuming a molecular weight of 25,500 for crab hepatopancreas collagenase (Eisen *et al.*, 1973), 68,000 for α1, 115,000 for β, 79,000 for γ1, 100,000 for δ, 110,000 for ε and 125,000 for ς collagenases from *C. histolyticum* (Bond and Van Wart, 1984a).

Crab hepatopancreas collagenase shows properties similar to microbial collagenase e.g., *C. histolyticum*, however, it also exhibit properties not observed in the latter, e.g., differences in pI, affinity to certain ligands, molecular weight, etc. Differences in certain properties between the crab collagenase and other collagenases could be traced to the differences in their amino acid composition.

Table 3. A comparison of the amino acid composition of fiddler crab hepatopancreas collagenase and clostridial collagenases.

Amino acid	Residue/molecule						
	Crab collagenase[1]	*Clostridium histolyticum* collagenases[2]					
		α_1	β	γ_1	δ	ε	ζ
Lysine	2	51	91	62	83	94	101
Histidine	5	6.1	17	13	19	21	22
Arginine	5	20	30	23	31	33	36
Aspartic acid	29						
Aspartic acid + Asparagine		90	156	98	131	144	160
Threonine	24	42	80	57	60	68	73
Serine	17	37	67	46	59	69	72
Glutamic acid	13						
Glutamic acid + Glutamine		59	98	69	95	104	115
Proline	12	16	27	18	32	38	47
Glycine	28	56	92	60	61	73	99
Alanine	21	38	66	47	43	46	57
Cysteine	8	2.2	3.5	2.4	4.1	4.5	4.5
Valine	20	31	52	32	38	42	62
Methionine	3	3.7	8.0	4.9	9.6	12	15
Isoleucine	17	32	50	31	38	38	56
Leucine	13	51	72	51	68	69	87
Tyrosine	8	43	70	52	68	75	78
Phenylalanine	8	30	51	37	36	39	34
Tryptophan	2						
Total	235	608	1030.5	703.3	875.7	969.5	1118.5

[1]Eisen *et al.*, (1973); [2]Bond and Van Wart (1984a)

The crab collagenolytic protease, both the crude extract and the purified enzyme also exhibit properties similar to other serine proteases. The enzyme demonstrated trypsinolytic and chymotrypsinolytic properties by degrading low molecular weight synthetic substrates specific for these proteases (Grant and Eisen, 1980; Grant *et al.*, 1983). Further degradation of the TC_{A75} fragment after cleavage of collagen molecule by the crude extract was attributed to the action of other proteases in the enzyme extract (Eisen and Jeffrey, 1969). The crab hepatopancreas collagenase is inhibited by leupeptin and Nα-p-tosyl-L-lysine chloromethylketone but not by chymostatin and N-benzyloxycarbonyl-L-phenylalanine chloromethylketone (Grant *et al.*, 1983). It is however, not inhibited by chelators like EDTA, 1,10-phenanthroline and cysteine which are known inhibitors of Zn-containing collagenases from mammalian and microbial sources.

The optimum pH for the enzyme is approximately 8.0 at 25°C with ^{14}C-labelled collagen fibrils as substrate. At pH values lower than 6.0, the enzyme loses activity which is irreversible at pH below 3.5 (Eisen *et al.*, 1973). The collagenolytic enzyme from fiddler crab is an acidic protease having an isoelectric point less than 3 (Grant *et al.*, 1983). The molecular weights of the crab collagenases ranged from 25,000 - 25,700 (Eisen *et al.*, 1973) and from 23,000 - 26,000 (Grant *et al.*, 1983), for crab collagenase I and collagenase II, respectively.

Collagenolytic activity in the hepatopancreas of the freshwater prawn, *Macrobrachium rosenbergii*, was first described by Baranowski *et al.*, (1984). Aside from collagenolytic activity, the crude enzyme extract showed slight trypsinolytic and α-chymotrypsinolytic activities, but no pepsinolytic activity. Similar to the crab collagenase, the crude prawn enzyme extract may contain mainly collagenase with smaller amounts of other enzymes or it can be an enzyme molecule containing multiple activities (Baranowski *et al.*, 1984).

A related study on freshwater prawn collagenase conducted by Nip *et al.*, (1985) showed the enzyme to be maximally active at 37°C and pH 6.5 to 7.5 with insoluble bovine collagen as substrate. The enzyme still retained its activity at 0°C. Exposure of prawn tissue to the enzyme at 0°C for 96 h caused increased hydrolysis of prawn collagen, thought to be responsible for the textural changes in prawn during ice-chilled storage.

A collagenolytic enzyme fraction was isolated from hepatopancreas of lobster, *Homarus americanus*, through the successive steps of acetone and butanol precipitation, followed by the suspension of the resulting powder in Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, ammonium sulfate precipitation, ion-exchange and gel filtration chromatography (Chen, 1991). The semi-purified enzyme had a molecular weight range from 15,000 - 66,000; two pH optima of 4 and between 7 - 8 for the hydrolysis of bovine insoluble collagen; two temperature optima one at 25°C and the second in the range of 40 - 50°C, and the enzyme fraction was most stable at pH 8.0.

Collagenase was also isolated with ammonium sulfate from the internal organs of filefish (*Novoden modestrus*) and purified by ion-exchange chromatography (DEAE-Sephadex A-50) followed by gel filtration chromatography on Sephadex G-150 (Kim *et al.*, 1993). The crude extract and the purified enzyme showed maximum activity at pH 8.0 and 7.75, respectively, for the hydrolysis of insoluble collagen. Both fractions had maximum activity at 55°C. Yoshinaka *et al.* (1978) also reported the presence of collagenases in the digestive organs of certain teleosts.

2.10 Inhibitors of collagenase

Collagenolytic enzymes from vertebrates and invertebrates are inhibited by various inhibitors, such as ethylene diaminetetraacetic acid (EDTA), 1,10-phenanthroline and cysteine. These compounds have been shown to be effective against collagenases from human skin (Woolley *et al.*, 1978), pig synovium (Cawston and Tyler, 1979), rabbit tumor

(McCroskery *et al.*, 1975), rabbit bone (Swann *et al.*, 1981) and microbial collagenases from *Bacillus cereus* (Makinen and Makinen, 1987), *Vibrio anguillarum* (Stenvag *et al.*, 1993), *Clostridium histolyticum* (Van Wart and Steinbrink, 1981) and *Vibrio* B-30 (Merkel and Dreisbach, 1978). These inhibitors however, have very minimal effects on the collagenolytic enzymes from crab hepatopancreas (Eisen and Jeffrey, 1969; Eisen *et al.*, 1973). Rather, the collagenolytic activity of the crab protease was effectively inhibited by serine protease inhibitors like diisopropylflourophosphate, phenylmethylsulfonyl fluoride (PMSF), N-α-ρ-tosyl-L-lysine chloromethyl ketone (TLCK) and soybean trypsin inhibitor (Eisen *et al.*, 1973; Eisen and Jeffrey, 1969), suggesting that these crustacean collagenases are serine proteases.

2.10.1 Chelating agents as inhibitors

There are two modes of action in which chelating agents can inhibit metalloenzymes: (1) the chelator binds to the enzyme forming an inactive complex; (2) the chelator detaches the functional metal ion producing an inactive apoenzyme (Vallee, 1960). If there is/are no irreversible conformational change(s) in the apoenzyme upon removal of the cation, adding back of the same cation to the reaction will restore enzyme activity (Whitaker, 1972).

Ethylenediaminetetraacetic acid (EDTA) forms strong complexes with divalent cations and cations of higher oxidation states like Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+} and Mo^{6+}. 1,10 phenanthroline also form stable complexes with certain cations, particularly Zn^{2+} and Fe^{2+}, through coordination of the cation with the two nitrogen atoms of the inhibitor. The effectiveness of these metal chelators as inhibitors depends upon the relative stabilities of the complex of the cation with the inhibitor and with the enzyme (Whitaker, 1972).

2.10.2 Thiol protease inhibitors

Thiol compounds, such as 2-mercaptoethanol, dithiothreitol and glutathione effectively inhibited collagenases from vertebrates (McCroskery *et al.*, 1975; Woolley *et al.*, 1978; Tyler and Cawston , 1980). Inhibition of enzymes by these compounds may result from the reaction between the inhibitor and an essential sulfhydryl, or a general reaction with the sulfhydryl groups which could lead to enzyme denaturation (Whitaker, 1972), i.e. disruption of disulfide bonds.

2.10.3 Naturally occurring inhibitors

Collagenase activity in vivo is regulated by a number of naturally occurring inhibitors. A low molecular weight cationic protein from the aorta and cartilage was found to inhibit collagenase from the mammary carcinomas and osteosarcoma (Kuettner *et al.*, 1977). α_2-Macroglobulin in blood serum inhibits collagenase (Eisen *et al.*, 1971; Werb *et al.*, 1974). The mechanism of enzyme inhibition by α_2-macroglobulin involves a two-stage process. The first stage consists of a proteolytic attack by the enzyme on α_2-macroglobulin, i.e., the cleavage of the sensitive region of the molecule. In the second stage, the cleaved α_2-macroglobulin undergoes a conformational change enabling it to trap the enzyme irreversibly. Thus access of the substrate to the active site of the enzyme is sterically hindered causing inhibition. This is more pronounced with larger substrate molecules but has negligible effect on smaller substrates since these are capable of diffusing in/out of the active site (Barret and Starkey, 1973). Thus being aware of the specificity of the enzyme, it can be assumed that a collagen region may exist in this inhibitor (Harper, 1980). Estradiol and progesterone inhibited the production or release of

collagenase present in involuting rat uterus (Ryan and Woessner, 1974). Serum anti-proteases and α_1-antitrypsin also inhibited human skin collagenase (Eisen *et al.*, 1971).

Human serum albumin capable of inhibiting vertebrate collagenase also inhibited crustacean hepatopancreas collagenase.

Collagenolytic activity is affected by inhibitors from microbial sources. Antipain and leupeptin which have been isolated from strains of *Streptomyces* and *Actinomyces* inhibit an acid collagenase from avian osteoclasts (Blair *et al.*, 1993). The same enzyme is also inhibited by pepstatin, a pentapeptide inhibitor isolated from *Steptomyces testaceus* and *Streptomyces argenteolus* (Blair *et al.*, 1993).

2.10.4 Inhibitory drugs against collagenase

Anti-inflammatory drugs against rheumatic diseases appear to inhibit the human leukocyte collagenase. Sanocricin, phenylbutazone, flufenamic acid, acetylsalysilic acid, and indomethacin were shown to be effective (Harper, 1980). Doxycycline was also found to inhibit neutrophil collagenase (Borsa *et al.*, 1993).

2.10.5 Inhibitory action of heavy metals

Collagenase from *B. cereus* (strain Soc. 67) is inhibited by Hg^{+}, Hg^{2+}, Pb^{2+}, Cu^{2+}, Fe^{2+}, Cr^{2+} and Ni^{2+}, the last two metals being more effective (Makinen and Makinen, 1987). Co^{2+} also caused slight inhibition of collagenase from *V. anguillarum* (Stenvag *et al.*, 1993). Gold(I) compounds inhibited human neutrophil collagenase (Mallya and Van Wart, 1989). High concentrations of Zn^{2+} inhibited microbial collagenase (Stenvag *et al.*, 1993; Matsushita *et al.*, 1994), rabbit bone collagenase (Swann *et al.*, 1981) and human neutrophil collagenase (Mallya and Van Wart, 1989). As discussed earlier, Zn^{2+} is an important component of metalloenzyme collagenase, however, excess levels of Zn^{2+} can

cause enzyme inhibition. The inhibitory action of high levels of Zn^{2+} is due to the formation of zinc monohydroxide, $[ZnOH]^{+}$, that bridges the Zn^{2+} to a side chain in the active site of the enzyme (Larsen and Auld, 1988). The inhibition is therefore competitive (Salvesen and Nagase, 1989). On the other hand, the inhibitory action of heavy metals is non-competitive, and can be due to the binding of the metal ion to a site different from the active site of the enzyme (Mallya and Van Wart, 1989).

2.11 Methods for preparing collagenases

Collagenases can be extracted directly from the tissues of multicellular organisms, e.g., crustaceans (Eisen and Jeffrey, 1969; Eisen *et al.*, 1973; Baranowski *et al.*, 1984; Nip *et al.*, 1985) and fish (Kim *et al.*, 1993). Collagenases from vertebrates are usually recovered from tissue cultures as in the case of mammalian collagenases (Eisen *et al.*, 1968; McCroskery *et al.*, 1975; Stricklin et al., 1977; Sakamoto *et al.*, 1978; Cawston and Tyler, 1979). Microorganisms as sources of collagenases are usually cultured/grown under conditions favorable for enzyme production.

2.11.1 Solvents for isolating collagenase

Buffers with pH near neutrality have generally been used to extract collagenase from various sources, since collagenases from microorganisms, vertebrates and invertebrates were observed to be stable at this pH range. Maintaining a stable pH is very important for the enzymes since biochemical processes can be severely affected by minute changes in the hydrogen ion concentrations (Stoll and Blanchard, 1990). Tris-HCl buffer, pH near neutrality, with calcium chloride was utilized to suspend cultures of human skin (Eisen *et al.*, 1968; Stricklin *et al.*, 1977; Woolley *et al.*, 1978), mouse bone tissue (Sakamoto *et al.*, 1978), pig synovium (Cawston and Tyler, 1979), rabbit tumors

(McCroskery *et al.*, 1975) and rheumatoid synovium (Woolley *et al.*, 1975). The same buffer was employed to extract collagenolytic enzymes from the hepatopancreas of shellfish (Eisen and Jeffrey, 1969; Baranowski *et al.*, 1984; Nip *et al.*, 1985). Incorporation of calcium chloride in the extraction medium has been recommended since calcium ions have been shown to activate as well as stabilize collagenolytic enzymes (Nordwig, 1971).

Organic solvents like acetone have also been utilized to extract collagenase from the hepatopancreas of fiddler crab (Eisen *et al.*, 1973).

2.11.2 Methods for the purification of collagenase

The use of a single method to purify collagenase from various sources is not possible due to variations in their chemistry (Woolley, 1984). Different purification schemes have been utilized attaining different levels of purity.

Suspension of tissue/cell cultures are either concentrated by means of ultrafiltration as in the case of collagenases from *Bacillus cereus* (Makinen and Makinen, 1987) and mouse bone (Sakamoto *et al.*, 1978) or lyophilized as in the case of rat skin collagenase and the residue resuspended in the appropriate buffer, i. e., Tris-HCl (pH 7.5) with calcium chloride (Tokoro *et al.*, 1972), before purification. Extraction of enzymes from higher eukaryotes requires break up or lysis of cells with blenders or homogenizers. The fiddler crab hepatopancreas was homogenized in an all- glass homogenizer in the presence of Tris-HCl buffer, pH 7.4 with calcium chloride to isolate collagenase. A similar extraction method was utilized by Baranowski *et al.* (1984) and Nip *et al.* (1985) to extract collagenolytic enzyme from freshwater prawn hepatopancreas.

Fractionation of crude extract with collagenolytic activity from vertebrates, invertebrates and microorganisms with ammonium sulfate preceding other purification steps had been reported for pig synovium (Cawston and Tyler, 1979), mouse bone

(Sakamoto *et al.*, 1978), rabbit tumors (McCroskery *et al.*, 1975), fiddler crab (Eisen *et al.*, 1973), *Bacillus alvei* (DC-1) (Kawahara *et al.*, 1993), *Bacillus cereus* (Strain Soc 67) (Makinen and Makinen, 1987), *Clostridium perfringens* (Matsushita *et al.*, 1994), and *Vibrio* B-30 (Merkel and Dreisbach, 1978).

Gel filtration (size-exclusion), ion-exchange chromatography in combination with other methods had been used to purify fiddler crab hepatopancreas collagenase (Eisen *et al.*, 1973) and vertebrate collagenases from rabbit tumors (McCroskery *et al.*, 1975), rabbit synovial fibroblast (Werb and Reynolds, 1975), rheumatoid synovium (Woolley *et al.*, 1975), mouse bone (Sakamoto *et al.*, 1978), human skin (Woolley *et al.*, 1978) and pig synovium (Cawston and Tyler, 1979), as well as bacterial collagenases from *B. cereus* (Makinen and Makinen, 1987), *C. perfringens* (Matsushita *et al.*, 1994), and *Vibrio anguillarum* (Stenvag *et al.*, 1993). Separation by gel filtration chromatography depends on the size or molecular weight of the biomolecules, i.e., smaller molecules which can enter the pores within the gel move at a slower rate than the larger molecules which cannot enter the pores. This method is applicable to all types of biomolecules and is easy to use. As long as the molecular weights lie within the fractionation range of the gel, effective separation can be attained. However, the loading capacity of this method is limited with respect to the sample volume. Resolution is also lower compared to gradient elution technique (Pharmacia, 1988).

Hydroxylapatite chromatography and affinity chromatography (cyanogen bromide activated Sepharose 4B coupled with ε-aminocaproyl-D-tryptophan) were used to further purify crab collagenase to homogeneity (Eisen *et al.*, 1973). A succession of purification steps which included hydroxylapatite chromatography followed by two successive affinity chromatography steps (Sephacryl S-200, and L-arginine- Affi-gel 202), reactive dye ligand chromatography, ion-exchange chromatography (DEAE-cellulose) and gel filtration chromatography (SP-sephadex) were effective in separating collagenases from the culture filtrate of *C. histolyticum* (Bond and Van Wart, 1984b).

Collagenase of high purity was obtained when pig synovial collagenase was further subjected to heparin-sepharose chromatography followed by zinc-chelate immobilized metal affinity chromatography (Cawston and Tyler, 1979).

Affinity chromatography using collagen linked to Sepharose was effective in separating procollagenase from the tissue culture of tibiae and calvaria from mice (Gillet *et al.*, 1977), but not effective in purifying rabbit synovial fibroblast collagenase (Werb and Reynolds, 1975). Cyanogen bromide collagen peptide CB7, linked to Sepharose effectively purified rabbit tumor collagenase (McCroskery *et al.*, 1975). Separation by affinity chromatography is based on the chemical structure or biological function of the biomolecules. In this method, a ligand which is covalently attached to a matrix is chosen specifically for the purification of a specific molecule or group of molecules because of the tendency of the two to bind. Molecules without affinity to the ligand are washed through the column and the adsorbed molecule(s) are eluted by decreasing the pH gradient or with the use of a competing agent for the ligand. This method is a concentrating step similar to ion-exchange chromatography and is highly specific, usually providing a high purity product in a single step. However, the ligand may not react with the matrix or lose its specificity once attached (Pharmacia, 1988).

In ion-exchange chromatography, separation of proteins is based on the differences of their charges. A column packed with an anion exchanger will bind the negatively charged molecule and will let the positively charged and neutral molecules pass through. Increasing salt gradient is utilized to elute the sample. This method of purification is fast and the recovery of biological activity and mass is high. However, this method requires desalting the sample before application into the column.

Ultrafiltration has been utilized to purify as well as concentrate collagenase activity (Werb and Reynolds, 1975; Woolley *et al.*, 1978). This method is time consuming, nevertheless, it provides superior recoveries in comparison to ammonium sulfate precipitation or lyophilization techniques (Woolley, 1984).

2.12 Assay of collagenase activity

Fixed time assays have been commonly used to measure collagenolytic enzyme activity. Methods include radioactive assays, viscometry, colorimetric methods and the use of collagen films or gels.

Specific substrates are available for collagenase activity determination. Collagen, a naturally occurring substrate has been widely utilized. Collagenase acts on collagen of different species with variable rates, reflecting in part differences in the primary and secondary structures of collagen (Seifter and Gallop, 1962).

Using the radioactive method, collagenase activity can be determined by monitoring the release of [^{14}C]amino acids or peptides from radioactive labeled collagen (Lim *et al.*, 1993). The release of [^{14}C]glycine containing peptides to measure collagenolytic activity was developed by Gross and Lapiere (1962). Radioactive collagen [(^{14}C)-glycine labeled guinea pig collagen] has been used for the assay of human skin collagenase (Eisen *et al.*, 1968). The amount of the soluble [^{14}C]glycine-containing peptides released by the reaction is measured. The same substrate was utilized by Eisen and Jeffrey (1969) to determine collagenolytic activity of the enzyme isolated from crustacean hepatopancreas. [^{14}C]glycine-labeled collagen was also used to evaluate activity of collagenase from various bacteria which included species of *Clostridium*, *Bacillus* and *Staphylococcus* (Waldvogel and Swartz, 1969). [^{14}C]acetylated collagen and [^{14}C]methylated collagen were used to assay *C. histolyticum* collagenase by Gisslow and McBride (1975) and Bond and Van Wart (1984a; 1984b), respectively. The use of radioactive labeled collagen is the most sensitive of the standard techniques, however, its application is limited by the difficulty and expense of preparing the radioactive substance (Levenson, 1976).

The viscometric assay to determine collagenase activity is based on the reduction in the viscosity of collagen solution as the enzyme action proceeds. This method was used to assay bacterial collagenase (Gallop *et al.*, 1957; Seifter and Gallop, 1962). Viscometric assay with the use of Ostwald viscometer to measure human skin collagenase activity was also utilized by Eisen et al. (1968). Human skin collagenase reduced the specific viscosity of a homogeneous mixture containing 0.1% purified acid-extracted calf-skin collagen in 0.05 M Tris-HCl, 0.2M NaCl, 0.0025M $CaCl_2$ (pH 7.5) to approximately 50 to 60% of the control values after 2 h at 28°C. No further change in viscosity was detected upon addition of fresh enzymes. Collagenase from the hepatopancreas of the fiddler crab, *Uca pugilator*, was also capable of reducing the initial specific viscosity of collagen solution in 3 h at 25°C (Eisen and Jeffrey, 1969).

Insoluble collagen is also used to assay collagenase activity. After a given reaction time, the amino acids and peptides liberated from collagen is measured by the colorimetric ninhydrin method (Moore and Stein, 1948; 1954; Rosen, 1957; Doi *et al.*, 1981). Insoluble bovine collagen was utilized by Nip *et al.* (1985) to determine collagenolytic activity of the enzyme from the hepatopancreas of freshwater prawn.

Determination of the amount of soluble hydroxyproline-containing fragments released from collagen is another method of measuring collagenase activity. This method was utilized by Morales *et al.* (1978) to assay activity of collagenase from graafian follicles of rats.

Several synthetic substrates have been utilized to assay for collagenase activity. The synthetic peptide 2,4-dinitrophenyl(DNP)-Pro-Leu-Gly-Ile-Ala-Gly-D-Arg is cleaved by collagenase at the Gly-Ile bond (Masui *et al.*, 1977). The products, i.e, DNP-peptide fragments released are extractable by organic solvents like ethyl acetate or ethyl acetate-n-butanol, and the degree of hydrolysis determined by reading the absorbance of the organic layer at 365 nm. The same substrate was utilized by Morales *et al.* (1978) to determine collagenase activity of enzyme from graafian rat follicles.

ρ-phenylazo-benzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide), another synthetic substrate, is cleaved between the Leu-Gly bond and the product, PZ-Pro-Leu is extracted with ethyl acetate and absorbance read at 320 nm (Wuensch and Heidrich, 1963). The degree of hydrolysis of the PZ-peptide can also be monitored by measuring the product formed with the use of high performance liquid chromatography (Chikuma *et al.*, 1985). PZ-peptide which has been used to determine *C. histolyticum* collagenase activity has an amino acid sequence similar to the (Gly-Pro-R)n tripeptide repeating pattern of collagen polypeptide chains (Lim *et al.*, 1993). The substrate has been widely utilized for animal proteases with collagenase-like specificity, isolated from granuloma tissue of rats (Ashwanikumar and Radhakrishnan, 1972; 1973), monkey kidney (Ashwanikumar and Radhakrishnan, 1975), bovine dental follicle (Hino and Nagatsu, 1976), chick embryos (Morales and Woessner, 1977) and rabbit serum (Nagelschmidt *et al.*, 1979). A limitation of this assay is that the chromogenic product has to be separated from the remaining substrate since the substrate has the same absorption spectra as the product (Chikuma *et al.*, 1985). Extraction of the product could be time consuming and laborious, although the assay time is shorter compared to viscometry and the use of labeled and unlabeled collagen. This assay system however, is less sensitive compared to methods which utilize soluble and radioactive labeled collagen, although, this synthetic substrate is cheaper and readily available.

Carbobenzoxy(CBZ)-Gly-Pro-Leu-Gly-Pro and CBZ-Pro-Leu-Gly-Pro are also synthetic substrates which are readily cleaved by *C. histolyticum collagenase* at the Leu-Gly bond (Nagai *et al.*, 1960). Dinitrophenylation followed by paper chromatography can be utilized to detect the hydrolysis products (Nagai *et al.*, 1960).

Barret *et al.* (1989), reported a fluorometric assay which detected the cleavage of (2,4-dinitrophenyl)-Pro-Leu-Gly-Pro-Trp-D-Lys at the Leu-Gly bond by clostridial collagenase. The substrate contains a fluorescent group and another group that quenches the fluorescence, such that the uncleaved substrate shows little or no flourescence. Upon

cleavage these two groups are separated, hence fluorescence appears (Barrett *et al.*, 1989). This substrate has the advantage of allowing both fixed time assay and continuous assay for collagenases. Thus for the first time continuous fluorometric assays for clostridial collagenase was conducted. The limitation for using this DNP-peptide however, is the high blank value seen with the crude enzyme samples containing high protein concentrations and thus significant amounts of unquenched tryptophan (Barrett *et al.*, 1989).

A continuous spectrophotometric assay for bacterial collagenase was developed by Van Wart and Steinbrink (1981) using 2-furanacryloyl-Leu-Gly-Pro-Ala (FALGPA) as substrate. Cleavage of FALGPA at the Leu-Gly bond upon addition of collagenase resulted to a decrease in absorbance at 345 nm. 2-furanacryloyl-Leu-Gly-Pro-Pro (FALGPP) can also be used for this continuous spectrophotometric assay (Bond and Van Wart, 1984a; 1984b). This assay is rapid, convenient and sensitive and should greatly facilitate detailed kinetic studies of collagenase, however, the substrate (FALGPA) is not expected to be a suitable substrate for mammalian and other vertebrate collagenases, since their substrate specificities may differ from that of bacterial collagenase (Van Wart and Steinbrink, 1981).

A collagen film for the assay of collagenase was developed by Levenson in 1976. A thin film of collagen in acetic acid solution is coated on a dried agar surface on a cover slip. An aliquot of the enzyme solution is placed on the surface of the film and incubated at 37°C in 100% humidity for a given time. The activity is determined by staining the surface of the film with Coomassie blue and clear areas of lysis detected against a dark blue background. The method was tested on bacterial and mouse bone collagenases. This assay has a sensitivity comparable to the radioactive method requiring only 5 to 60 min of incubation (Levenson, 1976). However, expressing the enzyme activity in terms of the standard units is one of the difficulties encountered since the products of digestion are not recovered.

2.13 Uses of collagenases

Collagenases, particularly those coming from microbial sources have shown potential applications in various areas.

The use of collagenases as investigative tools has been proven to be very useful to biochemists and biologists. Clostridial collagenase, a highly specific enzyme has been used in mapping out the repeating nature of amino acid sequence in collagen α-chains and to localize the aldehyde and carbohydrate functional groups of the tropocollagen molecule (Seifter and Harper, 1970). Harrington *et al.* (1959) used collagenase as a "probe" to measure the distribution of susceptible bonds and from the ratio of bonds cleaved in the slow and fast reactions distinguished the ordered and disordered regions of the collagen molecule. Keech (1954) also studied the unfolding and refolding of the collagen helix with the use of clostridiopeptidase A. Borstein (1967a; 1967b) and Butler (1970) successfully determined the amino acid sequence of α1-CB2 fragments from rat skin and tail tendon collagen and α1-CB5 fragment from rat skin collagen, respectively. The same enzyme was used by several investigators to determine the repeated sequences of collagen from other sources (Gallop *et al.*, 1957; von Hippel and Wong, 1963; Greenberg *et al.*, 1964; Manahan and Mandl, 1968). The enzyme was also utilized to locate specific structural features of collagen molecule such as hydroxylamine-sensitive linkages (Blumenfeld and Gallop, 1962) and enol cross-linkages (Rojkind *et al.*, 1966; 1968).

Due to its specificity, clostridial collagenase proved to be a useful tool in cell/tissue isolation and culture. The enzyme has been used for digestion to separate collagen from noncollagenous connective tissues (Oldroyd and Herring, 1967), isolation of acidic structural protein (Furthmayr and Timpl, 1970) and preparation of pure elastic fibers (Ross and Bornstein, 1969). The enzyme was utilized to isolate Langerhans islets

from guinea pig, or rabbit pancreas (Howell and Taylor, 1968). The use of collagenase has also been recommended for tissue disintegration and preparation of cell cultures (Cavanagh *et al.*, 1963). Grassman *et al.* (1962) used the enzyme for the preparation of islet cells of pancreas in studies related to insulin. Separation of myofibrils from the skeletal muscle (Perry, 1951) was achieved with the use of collagenase. The enzyme has also been used to confirm the collagenous characteristics of certain tissues like the Descemet's membrane (Dohlman and Balazs, 1955). Collagenase from *Clostridium perfringens* was also used as a "diagnostic test" to show the presence of collagen on the sheath of *Ascaris lumbricoides* (Dawson, 1960).

Due to the ability of the collagenase to digest collagen without affecting other proteins, it has become a useful tool in the medical field. The potential use of collagenolytic enzymic debridement in the treatment of burns, osteomyelitis, decubitus ulcers, etc., has been recognized and various preparations have been tested on experimental animals and patients (Mandl, 1961). Collagenase has been incorporated in ointments for the treatment of burns (Mandl, 1961) and debridement of ulcera and decubiti (Mazurek, 1971).

The use of collagenase in the leather industry during bating and dehairing and in the food industry as a meat tenderizer had been suggested by Mandl (1961). The presence of insoluble collagen contributes significantly to the toughness of meat (Bailey, 1972), hence a breakdown of this connective tissue could increase tenderness and improve the eating quality of meat and meat products. Collagenases could be of benefit in upgrading the coarser muscle(s) from older animals where collagen is more resistant to gelatinization during cooking (Etherington, 1991). The use of enzymes as meat tenderizers has been practiced for centuries. Proteases such as papain, bromelin and ficin are the major enzymes utilized as meat tenderizers in food processing (Bernholdt, 1975; Dransfield and Etherington, 1981). However, these enzymes often produce inferior products due to nonspecific proteolysis, i.e., degrading myofibrillar and stromal proteins somewhat

indiscriminately (Miller *et al.*, 1989) which may result to over-tenderization or mushiness of meat or meat products. According to Cronlund and Woychik (1987) an ideal enzyme for degrading insoluble collagen must have specificity to collagen and should remain active at low temperature and meat pH (5.5) during storage and at higher temperatures during cooking. Although the optimum pH and temperature for *Vibrio B-30* collagenase are at 7.6 and 37°C, respectively (Merkel and Dreisbach, 1978), it meets the above criteria and shows potential as meat tenderizer (Cronlund and Woychik, 1987; Miller *et al.*, 1989). Collagenase from *Clostridium histolyticum* and *Achromobacter iophagus* also solubilized collagen in restructured beef (Cronlund and Woychik, 1987). The safety aspects however, are not met by these microbial collagenases. Some microorganisms e.g., bacteria, molds and yeasts have been used as aids in food processing however, species of microorganisms which produce collagenases belong to genera which contain pathogenic species or strains. The presence of collagenases in some of these pathogenic strains may even contribute to the virulence of these microorganisms. Hence, collagenase from edible sources, e.g., fish/shellfish may turn out to be a potential for this particular purpose.

Effectiveness of fish collagenolytic enzyme as an aid in food processing was recently demonstrated by Kim *et al.* (1993) when they used collagenase from filefish to facilitate skinning of the same fish species.

Chapter 3

MATERIALS AND METHODS

3.1 Biological specimens

Live fish samples, of winter flounder (*Pseudopleuronectes americanus*) were provided by Dr. R. Morin (Department of Fisheries and Oceans, Gulf Fisheries Centre, Moncton, N. B.) and held at 4 - 7°C in the fish tanks in the laboratory until needed. The weights of the fish samples ranged from 64.8 - 89.5 g with an average length (standard) of 150 mm and width of 69.7 mm.

3.2 Chemicals

Tris (hydroxymethylaminomethane), acrylamide, glycine, riboflavin, sodium dodecyl sulfate, and sucrose were purchased from Bio-Rad (Mississauga, Ontario) calcium chloride dihydrate, *p*-phenylazo-benzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (PZ-peptide), ammonium sulfate, sodium phosphate (monobasic and dibasic), potassium sodium tartrate tetrahydrate, Folin-Ciocalteau reagent, glycerol, Coomassie blue (R-250), soybean trypsin inhibitor, n-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), ethylenediaminetetraacetic acid (EDTA, trisodium salt), 1,10-phenanthroline, 2-mercaptoethanol and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium sulfate, sodium chloride, sodium hydroxide, and trichloroacetic acid were purchased from BDH (Ville St.-Laurent, Quebec) and hydrochloric acid from Fisher (Montreal, Quebec). Sodium carbonate, sodium acetate, methanol, ethyl acetate, zinc chloride and acetic acid were purchased from Anachemia (Montreal, Quebec) ethanol

from ACP (St.-Leonard, Quebec) and copper sulfate pentahydrate from Aldrich Chemicals (Milwaukee, WI). Low molecular weight (LMW) calibration kit from Pharmacia (Baie d'Urfe', Quebec). Zymogram gels, Tris-glycine gels (10% and 6%), renaturing buffer, Tris-glycine transfer buffer, Tris-glycine running buffer, polyvinylidine diflouride (PVDF) membrane, Tris-glycine sodium dodecyl sulfate (SDS) running buffer, Tris-glycine SDS sample buffer, wide-range protein standard (Mark 12) and pre-stained markers (SeeBlue) from NOVEX Novel Experimental Technologies (San Diego, CA) (distributed by Helixx Technologies, Inc., Scarborough, Ontario).

3.3 Extraction of collagenolytic enzymes

Crude collagenase extract was prepared from the skeletal muscle of winter flounder based on the method of Baranowski *et al.* (1984). Live fish were killed by decapitation and the skeletal muscle was separated from the bones and skin. Extraction buffer (0.05M Tris-HCl, pH 7.4 containing 5mM $CaCl_2$ at 4°C) was added to the fish skeletal muscles in a ratio of 3:1(v/w). The mixture was immersed in an ice bath and homogenized using a polytron at a speed setting of 17,000 - 18,000 rpm for 5 min, and the fish muscle homogenate was centrifuged at 4°C and 12,000 xg for 30 min in a Beckman (Mississauga, Ontario) refrigerated centrifuge (Model J2-21). The residue was subjected to a second extraction, and the supernatants were combined, filtered through glasswool and the filtrate was centrifuged at 39,191 xg for 30 min at 4°C to obtain the crude extract. The crude extract was filtered through a 0.22 μm sterile filter, then stored at -80°C until needed.

3.4 Purification of collagenolytic enzymes

The crude extract was fractionated with solid ammonium sulfate and the fraction precipitating between 40 and 80% saturation was collected by centrifugation at 39,191 xg for 30 min at 4°C. The precipitate was resuspended in 0.05 M Tris-HCl buffer, pH 7.4, containing 5mM $CaCl_2$, desalted using a Pharmacia PD-10 column, then subjected to ion-exchange chromatography (IEX-1) on a Pharmacia MonoQ (HR5/5) column, using a Fast Protein Liquid Chromatography (FPLC) system, after preequilibrating the column with buffer A (0.05M Tris-HCL, pH 7.4). One milliliter of the enzyme solution was applied to the column per run and the applied sample was eluted using a gradient of 0 -100% buffer B (1.0M NaCl in buffer A) in a total volume of 20 ml at a flow rate of 1.0 ml/min. Fractions from IEX-1 with collagenolytic activity were pooled and concentrated using immersible CX-10 or ultrafree-15 ultrafilters (Millipore, Bedford, Massachusets) and subjected to a second ion-exchange chromatography (IEX-2) under the same conditions as in IEX-1. The fraction from IEX-2 with the higher specific activity was concentrated and subjected to immobilized metal affinity chromatography (IMAC) using Chelating Superose (HR 10/2, Pharmacia). Two hundred µl enzyme solution were loaded per run and were eluted using a pH gradient of 7.0 - 4.0 (acetate buffers, pH 7.0 and 4.0 with 0.5 M NaCl) in a total volume of 20 ml at a flow rate of 0.5 ml/min. The fractions from IMAC with collagenolytic activity were pooled, concentrated and subjected to size-exclusion chromatography using Superdex 75 (HR 10/30, Pharmacia). Two hundred µl enzyme solution were loaded per run and were eluted with 0.05 M Tris-HCl, pH 7.4 containing 5 mM $CaCl_2$ at a flow rate of 0.5 ml/min in a total volume of 30 ml.

3.4.1 Column chromatography

All column liquid chromatography steps were carried out using a Pharmacia FPLC system at 4°C. All solutions were filtered through a 0.22 μm filter (Millipore, Bedford, Massachusets) and degassed under vacuum before use.

3.4.1.1 Preparation of the ion-exchange chromatography column:(MonoQ HR5/5)

The column was washed with 5 ml of 0.05 M Tris-HCl buffer, pH 7.4 (buffer A), to remove the packing or storage solution followed by 10 ml 0.05 M Tris-HCl buffer, pH 7.4 containing 1 N NaCl (buffer B), then equilibrated with buffer A before sample application. Regular cleaning/washing of the column was conducted following the recommended procedure of Pharmacia.

One ml sample (~15 mg and ~8 mg protein for IEX-1 and IEX-2, respectively) was loaded to the column per run and the proteins were eluted using a gradient of 0 - 100% buffer B in a total volume of 20 ml at a flow rate of 1.0 ml/min. Two-ml fractions were collected per tube in a total of 10 tubes.

3.4.1.2 Preparation of the immobilized metal affinity column (IMAC) (Chelating Superose HR 10/2)

The column was washed with 10 ml ultra-pure water (Milli-Q grade), then equilibrated with 10 ml 0.05 M acetate buffer, pH 7.0 containing 0.5 M NaCl (buffer A). Twenty milliliters of 0.2 M $ZnCl_2$ were loaded into the column then it was equilibrated with 20 ml buffer A before sample application. After 4 runs the column was stripped of the Zn^{++} using 20 ml 0.05 M EDTA containing 0.5 M NaCl, followed by 10 ml 0.5 M

NaCl and 10 ml buffer A. The column was loaded with 20 ml 0.2 M $ZnCl_2$ then equilibrated with 20 ml buffer A before sample application.

Two hundred µl sample (~2.4 mg protein) were loaded to the column and the proteins were eluted using a pH gradient of 7.0 - 4.0 (acetate buffers, pH 7.0 and 4.0 with 0.5 M NaCl) in a total volume of 20 ml at a flow rate of 0.5 ml/min. Twenty 1-ml fractions were collected per run.

3.4.1.3 Preparation of the size-exclusion chromatography (SEC) column (Superdex 75 HR 10/30)

The column was washed with 50 ml 0.05 M Tris-HCl, pH 7.4 containing 5mM $CaCl_2$ then equilibrated with two column volumes of the same buffer before sample application. Cleaning/washing of the column was conducted regularly following the procedure recommended by Pharmacia.

Two hundred µl sample (~0.25 mg protein) were loaded to the column and proteins were eluted with 0.05 M Tris-HCl buffer, pH 7.4 containing 5 mM $CaCl_2$ at a flow rate of 0.5 ml/min, in a total volume of 30 ml. Forty-six 0.5-ml fractions were collected per run.

3.5 Assay for collagenase activity

3.5.1 Use of a synthetic substrate

Collagenase activity was determined using p-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (PZ-peptide) as substrate according to the method of Wuensch and Heidrich (1963). One-half ml (0.5) enzyme solution and 2.0 ml substrate (0.64 mM) were incubated in separate test tubes at 37°C for 5 min. After 5 min the enzyme and the

substrate were mixed followed by incubation at the same temperature. Immediately after 15 min incubation, 0.5 ml of the mixture was transferred into a test tube containing 1.0 ml 0.5% citric acid and 5.0 ml ethyl acetate followed by vigorous shaking for 15 sec (vortex at a speed setting of 6.5) to extract the product formed. The mixture was left to stand for 10 min to allow separation of the ethyl acetate layer containing the product PZ-Pro-Leu. Four ml of the ethyl acetate layer were transferred into 0.3 g anhydrous Na_2SO_4 to remove moisture which could interfere in the determination of the product which was measured at 320 nm in a Hitachi U-2000 spectrophotometer. The specific activity of the enzyme in units/mg enzyme was calculated as per the manufacturer's instructions (Sigma Chemical Co., 1993) as follows:

Units/mg enzyme = $A_{320\ nm}(2.5)/(0.5)(0.042)$(mg enzyme), where 0.042 is the millimolar extinction coefficient of PZ-pro-leucine at pH 7.1 at 37°C; 2.5 is the volume of the reaction mixture; 0.5 is the conversion factor for 5 ml reaction volume. A unit of enzyme activity is defined as the amount of collagenase which liberates 0.01 micromole of PZ-Pro-Leu per 5.0 ml of ethyl acetate in 15 min at 37°C.

3.5.2 Use of bovine Achilles tendon insoluble collagen

Assay of collagenase activity using the bovine insoluble collagen was conducted by modifying the method of Mandl *et al.* (1953). An aliquot of 0.5 ml of fish collagenase extract was incubated with 25 mg insoluble collagen in 5 ml 0.05M Tris-HCl buffer, pH 7.4 containing 5 mM $CaCl_2$ at 37°C, and the enzyme activity was stopped by acidifying the mixture with 2.5 ml 10% TCA after 12, 24, 36 and 48 h. The extent of collagen breakdown was determined using the colorimetric ninhydrin method of Moore and Stein (1948) . One milliliter aliquots from the mixture were transferred into eppendorf tubes and centrifuged at 13,000 rpm for 10 min using the Biofuge 13 (Baxter, Montreal, Quebec). Two hundred µl aliquots of the supernatant were transferred into tubes

containing 1.0 ml ninhydrin solution followed by heating for 20 min in a boiling water bath. The solution was cooled and diluted with 5 ml diluent (1:1 mixture of deionized water and n-propanol). After 15 min the product formed was read at 600 nm using the Beckman Diode Array DU 7500 spectrophotometer (Mississauga, Ontario).

3.6 Assay for chymotrypsin-like activity

Chymotrypsin-like activity was determined with benzoyl-L-tyrosine ethyl ester (BTEE) as substrate based on the method of Hummel (1959). One hundred microliters of the enzyme solution were mixed with 1.4 ml BTEE in 50% methanol and 1.5 ml 0.08 M Tris-HCl buffer, pH 7.8 with 0.1 M $CaCl_2$, and the change in absorbancy at 256 nm was measured at 10 sec intervals for 3 min. at 25°C in a Beckman Diode Array DU 7500 spectrophotometer (Mississauga, Ontario). One BTEE unit of activity was defined as $\Delta A_{256\,nm} \times 1000 \times 3.0/964$, where 964 is the molar extinction coefficient for N-benzoyl-DL-tyrosine.

3.7 Assay for trypsin-like activity

Trypsin-like activity was determined with benzoyl-DL-arginine *p*-nitroanilide (BAPA) as substrate following the method of Erlanger *et al.* (1961). Two hundred microliters of the fish enzyme were mixed with 2.80 ml of 1.0mM BAPA in 0.05M Tris-HCl, pH 8.2 containing 0.02M $CaCl_2.2H_2O$, and the increase in light absorption at 410nm was measured at 10 sec intervals for 3 min. at 25°C in a Beckman Diode Array DU 7500 spectrophotometer (Mississauga, Ontario). One BAPA unit of activity was defined as $\Delta A_{410\,nm} \times 1000 \times 3/8800$, where 8800 is the extinction coefficient of *p*-nitroaniline.

3.8 Protein determination

Protein was determined according to the method of Hartree (1972) using bovine serum albumin as a standard.

3.9 Electrophoreses and molecular weight determination

The active fraction from each purification step was subjected to electrophoreses in the presence or absence of sodium dodecyl sulfate (SDS) in polyacrylamide gels according to the methods of Laemmli (1970) and Davis (1964).

3.9.1 Native-polyacrylamide gel electrophoreses

The solutions and the mixing ratio used for preparing the polyacrylamide gels for electrophoresis under nondenaturing conditions are given in Table 4 and Table 5, respectively.

3.9.1.1 Preparation of gels and running of samples (disc gel)

Glass tubes (dimension: L = 12.5 cm; d_i = 5 mm; d_o = 7 mm) were cleaned and dried. The tubes were marked at the level (8 cm) to which acrylamide was to be poured and the opposite ends were sealed with parafilm. The tubes were placed vertically in a leveled tube gel preparation rack with the sealed end down. The separating gel was carefully transferred into each tube up to the marked level and the top was carefully layered with water to obtain a level surface. The gel was allowed to polymerize for at least 1 h. After polymerization, the water layer was removed and the spacer gel was placed on top of the separating gel and layered with water. The spacer gel was allowed to

Table 4. Reagents/solutions for the preparation of polyacrylamide gel (Native-PAGE)* for electrophoresis.

STOCK SOLUTIONS FOR GEL PREPARATION		SOLUTION E	
		Riboflavin	4 mg
SOLUTION A (pH 8.9)		Deionized H_2O to 100 ml	
1N HCl	48 ml	SOLUTION F	
[1]Tris	36.6 g		
[2]TEMED	0.23 ml	Sucrose	40 g
Deionized H_2O to 100 ml		Deionized H_2O to 100 ml	
SOLUTION B (pH 6.7)		SOLUTION G	
1N HCl	48 ml	Ammonium persulfate	0.24 g
Tris	5.98 g	Deionized H_2O	
TEMED	0.46 ml	100 ml	
Deionized H_2O to 100 ml			
		ELECTRODE BUFFER	
SOLUTION C			
		Tris	6.0 g
Acrylamide	28.0 g	Glycine	28.8 g
[3]BIS	0.735 g	Deionized H_2O to 1 l	
Deionized H_2O to 100 ml		(Dilute 1:10 with deionized H_2O before use)	
SOLUTION D			
		INDICATOR SOLUTION	
Acrylamide	10 g		
BIS	2.5 g	Bromo-phenol Blue	10 mg
Deionized H_2O to 100 ml		Deionized H_2O to 1 l solution	

*Native-PAGE: Polyacrylamide Gel Electrophoresis under non-denaturing conditions
[1]Tris - Tris (hydroxymethyl) aminomethane
[2]TEMED - N,N,N',N'-Tetramethylethylenediamine
[3]BIS - N,N'-methylene-bis-acrylamide
Source: Davis (1964)

Table 5. Mixing ratio of working solutions for polyacrylamide gel electrophoresis

SEPARATION GEL	SPACER GEL
1 part solution A	1 part solution B
2 parts solution C	2 parts solution D
1 part deionized water	1 part solution E
4 parts solution G	4 parts solution F

Source: Davis (1964)

photo polymerize for 45 min. The sample gel was prepared by mixing ~0.030 mg of protein with 0.100 ml of the spacer gel. Approximately 40 - 50 µl of this solution was then layered on top of the spacer gel. This corresponded to a protein content of ~12 - 15 µg per tube. The sample gel was photo polymerized for 30 min. After photo polymerization the parafilm seals were removed and the ends of the tubes were rinsed with water before inserting into the rubber grommets of the upper chamber of the electrophoretic apparatus. Both the upper and the lower chambers were filled with electrode buffer to completely cover the electrodes. Two milliliters of the indicator solution were added to the upper chamber and electrophoresis was ran at a constant current of 3 mA per tube. Electrophoresis was stopped when the tracking dye reached about 1 cm from the bottom of the tubes. The gels were removed from the tubes with the aid of water from a flexible needle passed between the gel and the inner wall of the tube.

3.9.1.2 Preparation of the gels and running of the samples (vertical slab).

The gel sandwiches were assembled using the 20 cm cell. The long rectangular plate was laid down first, then two spacers (1 mm thick) were placed along the edges of

the rectangular plate and the shorter plate was placed on top of the spacers so that it was flushed with one end of the long plate. The clamps were placed on the appropriate side of the glass next to the plate stack and the screws of the clamps were tightened and at the same time checking that the spacers were flushed against the sides of the clamp. The casting stand was then leveled and the silicone gaskets were placed in each casting slot. The assembled gel sandwiches were then aligned in the slot of the casting stand. The clamps were loosened and the plates and spacers were allowed to align at the surface of the alignment slot. After proper alignment the clamp screws were tightened and the cams in the casting stand were tightened by turning them 180° such that the handles of the cams were pointed downward. The comb was placed completely into the assembled sandwich and then the glass plate was marked ~ 1.5 cm below the teeth of the comb. The comb was removed and the separating gel was poured into the sandwich up to the marked level and immediately layered with deionized water. The gel was allowed to polymerize for at least an hour. After polymerization the water layer was removed and the comb was placed in the glass sandwich such that the teeth are tilted at 10° degree angle. The spacer gel solution was poured until the bottoms of the teeth were covered. The comb was then adjusted to its proper position and the sandwich completely filled with the spacer gel solution. The spacer gel solution was allowed to polymerize for 45 min. After polymerization, the comb was removed and the wells were rinsed with deionized water before loading the sample. The sample solutions were prepared by reconstituting the freeze-dried enzyme fractions from each purification step of known protein concentration in the spacer gel solution. The sample solutions were loaded into the wells. Sample loading were as follows, ~ 45 μg for the crude extract, ~45 μg for the ammonium sulfate fraction, ~25 μg for IEX-1, ~18 μg for IEX-2 and ~15 μg for IMAC and ~15 μg SEC. The sample gel solution was polymerized for 30 min.. After polymerization the glass sandwich was assembled into the protean II slab cell electrophoresis apparatus (Bio-Rad). The upper chamber was filled with the electrode buffer (~ 350 ml). The remaining

electrode solution (~1150 ml) was filled into the lower buffer chamber. Two ml of the indicator were added into the upper chamber. Electrophoresis was ran at a constant current of 3 mA per loaded well, with circulating tap water to keep the temperature constant during the run. Electrophoresis was stopped when the tracking dye reached to about 1 cm from the bottom of the glass gel sandwich. The gels were gently removed from the sandwich, fixed, stained and destained as described in the succeeding section.

3.9.1.3 Fixing, staining and destaining of gels

The solutions for fixing, staining and destaining of the gels are given in Table 6. The gels were fixed for about 6 h with 12.5% TCA then stained overnight with Coomassie blue (R-250). The gels were destained with several changes of the destaining solution until clear protein zones were detected. The gels were stored in 7% acetic acid.

3.9.2 SDS-Polyacrylamide gel electrophoresis

The stock solutions used for the preparation and running of SDS-polyacrylamide gel electrophoresis are given in Table 7. The formulations of the separating and the stacking gels are shown in Table 8.

3.9.2.1 Preparation of gels and running of samples

The gel sandwiches were assembled in the same manner as described for the native-PAGE. After degassing the separating gel solution for 15 min the initiators (ammonium persulfate and TEMED) were added and the mixture poured into the glass sandwich. The top of the separating gel was layered with water and the gel was allowed to polymerize for at least an hour. After polymerization the water layer was removed and

Table 6. Solutions for staining and destaining of polyacrylamide gels (Native-PAGE and SDS-PAGE).

SOLUTION	COMPOSITION	
[1]Fixative solution	Trichloroacetic acid	12.5 g
	Deionized H_2O	100 ml
[2]Staining solution	Coomassie blue (R-250)	0.1 g
	Isopropanol	25 ml
	Glacial acetic acid	10 ml
	Deionized H_2O to 100 ml solution	
[2]Destaining solution	Isopropanol	200 ml
	Glacial acetic acid	100 ml
	Deionized H_2O	700 ml
Storage solution	Glacial acetic acid	7 ml
	Deionized H_2O to 100 ml	

[1]Laemmli (1970)
[2]Bethesda Research Lab., Inc. (1981)

Table 7. Reagents used for the preparation of SDS-PAGE gel

STOCK SOLUTIONS FOR GEL PREPARATION	
A. ACRYLAMIDE/BIS (30% A, 2.67%C)	
Acrylamide	29.2 g
N' N'-bis methylene-acrylamide (Bis)	0.8 g
Deionized H_2O to 100 ml solution	
B. 1.5M Tris-HCl, pH 8.8	
Tris	18.15 g
Adjust to pH 8.8 with 1N HCl	
Deionized H_2O to 100 ml	
C. 0.5M Tris-HCl, pH 6.8	
Tris	6.0 g
Adjust to pH 6.8 with 5 - 10 N HCl	
Deionized H_2O to 100 ml solution	
D. 10 % SDS	
[1]SDS	10.0 g
Deionized H_2O to 100 ml solution	

SAMPLE BUFFER	
Deionized H_2O	4.0 ml
0.5M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.80 ml
10% (w/v) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
0.05% (w/v) bromophenol blue	0.2 ml
5X ELECTRODE BUFFER, pH 8.3	
Tris	15.0 g
Glycine	72.0 g
SDS	5.0 g
Deionized H_2O to 1 l solution	
*Dilute solution 1:5 with deionized H_2O for electrophoresis run	

[1]SDS - sodium dodecyl sulfate
Source: Laemmli (1970)

Table 8. Preparation of separating and stacking gels for SDS-PAGE

	SEPARATING GEL, 12% acrylamide	STACKING GEL, 4% acrylamide
Deionized H_2O	33.5 ml	6.1 ml
1.5M Tris-HCl, pH 6.8	25.0 ml	
0.5M Tris-HCl, pH 8.8		2.5 ml
10% SDS	1.0 ml	100 µl
Acrylamide/Bis (Degas ≥ 15 min)	40.0 ml	1.3 ml
10% ammonium persulfate	500 µl	50 µl
TEMED	50 µl	10 µl

Source: Laemmli (1970)

the comb was placed into the glass sandwich such that the teeth are tilted at 10° angle. The stacking gel solution was poured until the bottoms of the teeth were covered. The comb was adjusted to its proper position, and the sandwich completely filled with the stacking gel solution. The stacking gel was allowed to polymerize for 30 min. After polymerization the comb was removed and the wells were rinsed with deionized water. The gels were assembled into the protean II cell for electrophoresis and the electrode buffer was filled into the upper and lower chambers. The samples (freeze-dried) from each purification step and the protein standards were dissolved in the sample buffer and heated at 95°C for 4 min and 8 min, respectively prior to loading. The gels were pre-run at a constant voltage of 70 V for 30 min, then the samples and the standard were loaded and the run continued for 30 min using the same voltage. The amounts of the sample loaded were similar to that of Native-PAGE, ~ 45 µg for the crude extract, ~45 µg for the ammonium sulfate fraction, ~25 µg for IEX-1, ~18 µg for IEX-2 and ~15 µg for IMAC and ~15 µg SEC. Ten µl of the protein standard was loaded in one of the wells. The voltage was increased to 120 V and electrophoresis was continued for approximately

4 h or until the distance of the tracking dye was about 1 cm from the bottom of the glass gel sandwich.

3.9.2.2 Fixing, staining and destaining of gels

Fixing, staining and destaining of the gels were similar with that for the PAGE gels. The distance traveled by the tracking dye and the protein bands were measured and used to calculate the R_f values of the proteins. The protein standards used to estimate the molecular weight were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400) (Pharmacia). The gels were stored in 7% acetic acid.

3.9.3 Electrophoresis (SDS-PAGE) on 10% Tris-glycine gel and zymogram gel (10% Tris-glycine gel with 0.1% gelatin) (NOVEX)

Electrophoresis on the pre-cast 10% Tris-glycine gel and zymogram gel (NOVEX) were conducted simultaneously. The pre-cast gels were assembled in the XCell II Mini cell unit (electrophoresis and blotting unit from NOVEX). The upper chamber was filled with the running buffer until the wells were completely covered. The standard Mark 12 and/or SeeBlue markers were heated for 3 min at 100°C before loading. The enzyme solution (~117 μg protein) (SEC fraction) and the sample buffer were mixed at a ratio of 1:1 (v/v) and the mixture incubated at room temperature (~25°C) for 10 min. The wells in the Tris-glycine gel and the zymogram gel were loaded with ~ 8.35 μg protein. Ten μl of Mark 12 and SeeBlue were loaded in separate wells in the Tris-glycine gel. The lower chamber was then filled with the running buffer up to 2 cm level away from the top of the upper chamber. Electrophoresis was ran at a constant voltage of 125V for 1 h and 45 min or until the dye front reached the bottom of the gels.

3.9.3.1 Fixing, staining and destaining of the Tris-glycine gel

Fixing, staining and destaining of the Tris-glycine gels were similar to the gels in Native-PAGE with slight modification. Staining was conducted for about 2 h, followed by destaining until the clear protein zones were detected.

3.9.3.2 Renaturation, developing and staining of the zymogram gel

The proteins in the zymogram gel were renatured with 100 ml zymogram gel renaturing buffer for 30 min at room temperature (~25°C), with gentle shaking. After 30 min, the renaturing buffer was replaced with 100 ml developing buffer and the gel was incubated in water bath at 37°C for 30, min with gentle shaking. The developing buffer was replaced with 100 ml of the same buffer and the gel was incubated in a water bath at the same temperature, overnight. The developing buffer was drained and the gel was stained with Coomassie blue (0.5%) for about 10 min. The gel was then examined for reaction zones characterized by white areas against a dark blue background.

3.9.4 Electrophoretic blotting of protein from 10% Tris-glycine gel to the polyvinilidine diflouride (PVDF) membrane

3.9.4.1 Electrophoresis

Electrophoresis was conducted on the precast 10% Tris-glycine gel and the 6% Tris-glycine gel (NOVEX) following the method described in 3.9.3. Electrophoresis was immediately followed by blotting without fixing, staining and destaining of the gels.

3.9.4.2 Preparation of the PVDF membrane, filter paper and blotting pads

The PVDF membrane was pre-wetted in methanol for 30 sec, briefly rinsed with Milli-Q pure water then placed in the dish with 50 - 100 ml transfer buffer for 10 min together with the filter papers. The blotting pads were soaked in the transfer buffer until saturated ensuring that air bubbles were removed by squeezing the pads while they were submerged in the buffer.

3.9.4.3 Assembling the gel membrane sandwich

Two blotting pads were placed on the cathode core of the blot module, followed by a filter paper and the Tris-glycine gel from electrophoresis. The PVDF membrane was placed on top of the gel followed by another filter paper. The remaining blotting pads were then placed on top of the filter paper. The anode core was then placed on top of the cathode core and the blot module was slid into the X-cell unit and the system completely assembled. The transfer buffer was then poured into the blot module until the gel/membrane sandwich was covered. The lower chamber was then filled with appproximately 650 ml of Milli-Q pure water or until the water level reached to about 2 cm from the top of the lower buffer chamber. Blotting was ran at a constant voltage of 30 V for 1 h and 30 min. In cases wherein two gels were used the sandwich was prepared by placing two blotting pads on the cathode core followed by a filter paper, the first gel, PVDF membrane and another filter paper. A third blotting pad was placed on top of the filter paper followed by the second gel, another PVDF membrane a third filter paper and the remaining blotting pads. After completing the sandwich and assembling the module blotting was ran under the same conditions as described in the single gel sandwich system.

3.9.4.4 Staining and destaining of the PVDF membrane

After blotting the blot module was disassembled and the PVDF membrane was rinsed with deionized water, stained with 0.1% Coomassie blue in 40% ethanol and 10% acetic acid for 10 min and destained with 40% ethanol, 10% acetic acid until the bands were detected against a very light blue membrane. For amino acid analysis, after destaining the PVDF membrane was thoroughly rinsed with MilliQ pure water until ethanol and acetic acid were no longer detected by the disappearance of their characteristic odors. The protein bands, i.e., WFC-1 and WFC-2 were then subjected to amino acid analysis.

3.10 Amino acid analysis of winter flounder skeletal muscle collagenases, WFC-1 and WFC-2.

3.10.1 Acid hydrolysis

The bands corresponding to the WFC-1 and WFC-2 on the PVDF membrane were cut then dried in the Speed Vac in 6 x 50 mm KIMAX culture tube and hydrolyzed at 105 °C for 16 h using the vapor of constant boiling 12M HCl containing a small amount of phenol. Hydrochloric acid was evaporated under vacuum prior to analysis.

3.10.2 Amino acid analysis

Amino acids from acid hydrolysis were separated and quantitated on a high performance amino acid analyzer (System 6300, Beckman Instruments, Inc., Mississauga, Ontario) consisting of an automatic sample loading turntable, ion-exchange high-performance liquid chromatography (HPLC) and ninhydrin color reaction. The ninhydrin

derivatives were monitored by an on line spectrophotometer at 440 nm and 570 nm and the sum of peak areas was integrated by Hewlett Packard 3390A Reporting Integrator.

3.11 pH optimum and stability

The influence of pH on the activity of the fish collagenase fraction was determined according to the method of Simpson and Haard (1985). For the pH activity study, the enzyme solution (enzyme in 0.05M Tris-HCl buffer, pH 7.4 containing 5mM $CaCl_2$) and the substrate (0.64 mM PZ-peptide) which had been prepared in various buffer solutions of different pH values were incubated separately at 37°C for 5 min, mixed and incubated at the same temperature for activity determination. Composition of buffer solutions used were: 0.1M citrate-HCl, pH 2.0; 0.1M citrate-NaOH, pH 4.0, pH 5.0, 6.0, pH 6.5; 0.1M Tris-HCl, pH 7.5, pH 8.0; 0.1M glycine-NaOH, pH 10.0; 11.0, 12.0; 0.05M sodium phosphate, pH 7.1.

For pH stability studies, buffer ranges from pH 2 - 12 were used. For this study, the fish enzyme was incubated at various pH's with different buffer solutions in an ice bath for 30 min. The residual activity was assayed with the use of the PZ-peptide as substrate at 37°C and 320 nm.

3.12 Temperature optimum and stability

The influence of temperature on the activity of the fish collagenolytic enzyme was determined based on the method of Simpson *et al.* (1987). For the temperature optimum study, the PZ-peptide substrate (0.64 mM in phosphate buffer, pH 7.1) and the fish collagenases were incubated in separate test tubes at various temperatures (5 - 65°C) for 5 min before mixing and allowed to react for exactly 15 min at the same temperature.

Thermal stability studies were performed using temperature range of 0 - 65°C. For this study, the fish enzyme was incubated at various temperatures for 30 min, then rapidly cooled in ice for 5 min prior to the assay for residual activity at 37°C with the use of PZ-peptide as substrate (0.64 mM in phosphate buffer, pH 7.1).

3.13 Influence of various inhibitors on the collagenolytic activity of the fish skeletal enzymes

The sensitivity of fish collagenase fractions towards various inhibitors (soybean trypsin inhibitor [STI], n-α-p-tosyl-L-lysine chloromethylketone hydrochloride [TLCK], 2-mercaptoethanol, dithiothreitol [DTT], ethylene diamine tetraacetic acid [EDTA, trisodium salt] and 1,10-phenanthroline) was investigated as follows: Equal volumes of the inhibitor (0.5 mg/ml STI in 0.05 M Tris-HCL buffer, pH 7.4; 10 mM TLCK in 0.05 M Tris-HCl, pH 7.4; 100 mM 2-mercaptoethanol in deionized water; 20 mM DTT in deionized water; 100 mM EDTA in deionized water and 10 mM 1,10 phenanthroline in deionized water) and enzyme solution (~0.062 mg/ml in 0.05 M Tris-HCl buffer, pH 7.4 containing 5 mM $CaCl_2$) were mixed and incubated at 37°C for 30 min. Residual activity was determined with the use of PZ-peptide as substrate at 37°C and 320 nm. Control experiments were run simultaneously.

3.13.1 Influence of Zn and Ca ions on the collagenolytic activity of the fish enzyme

Equal volumes of $ZnCl_2$ (0.125 mM to 0.5 mM in deionized water; complete dissolution was accomplished by addition of dilute HCl) and the enzyme solution (~0.062 mg/ml) (SEC fraction) were mixed and incubated at 0°C (ice bath) for 1 h. The residual activity was determined using PZ-peptide as substrate at 37°C and 320 nm. Control experiment was ran simultaneously.

Furthermore, the fish enzyme was dialyzed against 3 changes of deionized water for 24 h (1:1000, v/v) at 4°C. After dialysis, collagenolytic activity was determined in the presence/absence of zinc ions (0.5 mM $ZnCl_2$) following the procedure above to determine if dialysis treatment influenced enzyme activity.

To determine the combined effect of various concentrations of zinc ions and 1,10-phenanthroline or EDTA, equal volumes of the enzyme (0.08 mg/ml), inhibitor (10 mM 1,10-phenanthroline or 100 mM EDTA) and zinc chloride solutions (0.5 mM to 40 mM in deionized water; complete dissolution was accomplished by addition of dilute HCl) were mixed and incubated at 37°C for 30 min. The residual activity of the enzyme was determined with the use of PZ-peptide as substrate at 37°C and 320 nm. Control experiments with and/or without inhibitor and zinc ions were ran simultaneously.

To determine the combined effect of various concentrations of calcium ions and EDTA, equal volumes of the enzyme (0.072 mg/ml), EDTA (100mM) and calcium chloride solution (5 - 20 mM) were mixed and incubated at 37°C for 30 min. The residual activity of the enzyme was determined with the use of PZ-peptide (0.64mM in phosphate buffer, pH 7.1) as substrate at 37°C and 320 nm. Control experiments with or without inhibitor were ran simultaneously.

3.14 Kinetic properties (K_m'and V_{max})

The apparent Michaelis-Menten constant (K_m') and the substrate turnover number (V_{max}) were calculated by least-squares analysis, from Lineweaver-Burk plots at 37°C and pH 7.1 with ρ-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg as substrate. The range of the substrate concentration used in all determinations were 0.1 - 1.6 mM.

3.15 Inhibition studies

For inhibition studies of winter flounder skeletal muscle collagenases, substrate concentrations ranging from 0.1 to 1.6 mM were used at different concentrations of inhibitors (EDTA, and 2-mercaptoethanol). Lineweaver-Burk plots were used to determine the type of inhibition caused by each inhibitor.

To determine the concentration of the inhibitor necessary for a 50% inhibition (I_{50}) a substrate concentration of 0.64 mM was used at different inhibitor concentrations. I_{50} was estimated from the plot V_o/V_i against inhibitor concentration.

The dissociation or binding constants K_i and αK_i were estimated from the secondary replots of the slopes (K_m'/V_{max}) and intercepts ($1/V_{max}$) against inhibitor concentrations from the primary reciprocal plots (Lineweaver-Burk plots) based on the method of Kuby (1991).

3.16 Thermodynamic properties (E_a, ΔH, ΔS and ΔG)

The Arrhenius energy of activation (E_a) were estimated from the temperature-activity data as described by Simpson and Haard (1984a; 1984b).

The E_a and V_{max} values were used to determine the thermodynamic parameters, change in enthalpy (ΔH), change in entropy (ΔS) and change in Gibbs free energy (ΔG) as described by Low *et al.* (1973).

Chapter 4

RESULTS AND DISCUSSION

4.1 Comparative studies of collagenases from winter flounder skeletal muscle and *C. histolyticum*

A comparative study of the characteristics, i.e., response to pH and temperature of the collagenases from the fish skeletal muscle (WFC) and commercial collagenases (*C. histolyticum*) was conducted.

4.1.1 Extraction of collagenase(s) from the fish skeletal muscle

The IEX collagenase fraction from the skeletal muscle of winter flounder was extracted with the use of 0.05 M Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$ following the scheme shown in Fig. 4. The crude extract was subjected to ammonium sulfate fractionation (40 - 80%) followed by ion-exchange chromatography. The partially purified enzyme was characterized with respect to its response to pH and temperature.

A summary of the purification process for the fish collagenase (WFC) is given in Table 9. An increase of the specific activity, as the purification proceeded, was observed in the IEX fraction as compared with the crude extract, and this corresponded to a purification fold of ~ 6 and a yield of 17%.

The crude fish enzyme also showed trypsin-like and chymotrypsin-like activities. A low trypsin-like activity (0.0098 units/mg) detected in the crude extract diminished with purification and was not detected in the IEX fraction. However, chymotrypsin-like activity was high for both the crude extract and the ammonium sulfate fraction but decreased abruptly after the IEX step. The effective removal of the

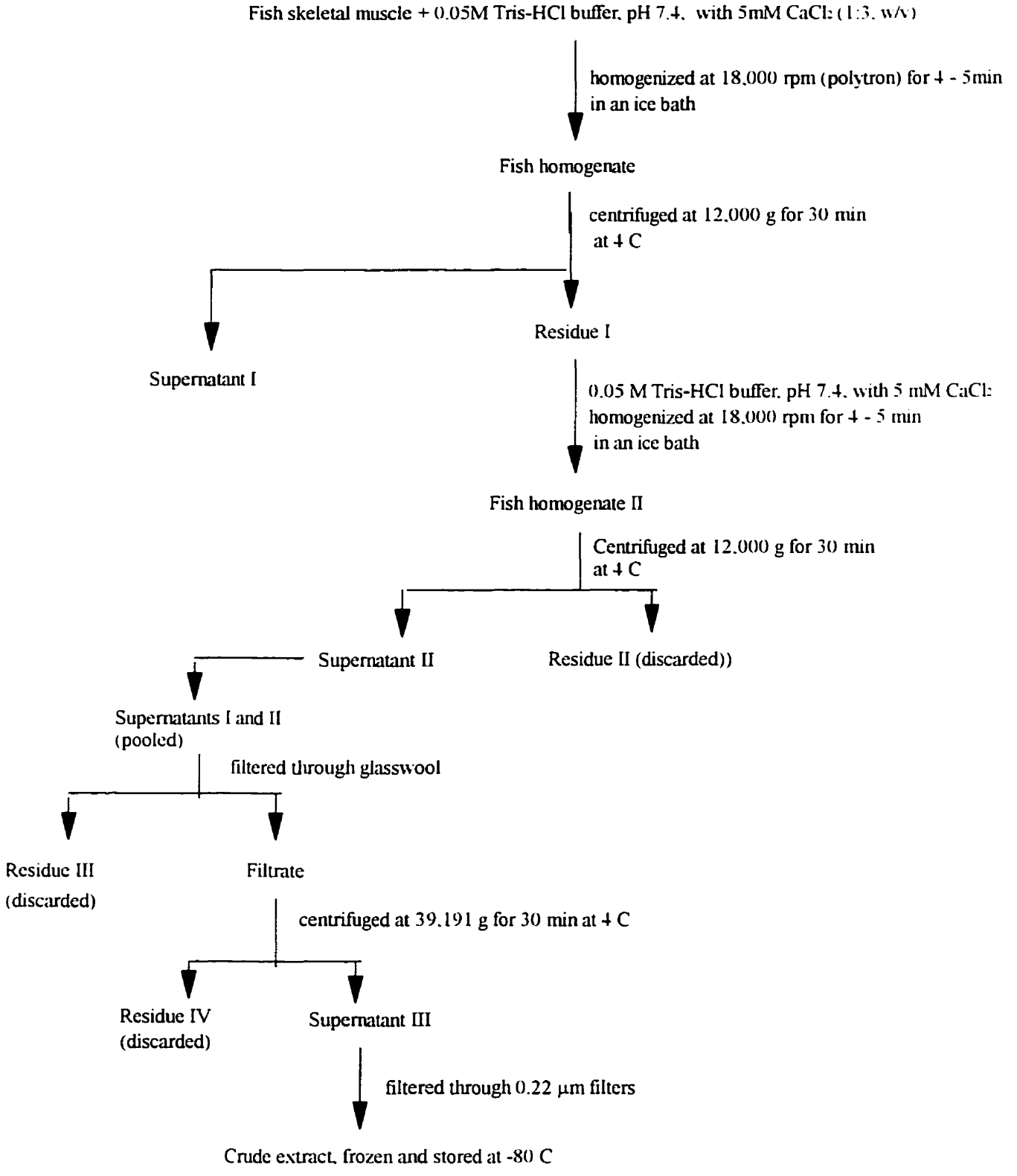


Fig. 4. Schematic diagram for the isolation of crude collagenase fraction from the skeletal muscle of winter flounder.

Table 9. Purification of collagenase from the skeletal muscle of winter flounder, *P. americanus*.

Purification step	Total protein mg	Total activity units	Specific activity units/mg	Yield %	Purification fold
Crude extract	371.26 ± 37.61	1417.44 ± 141.06	3.82 ± 0.59	100	1.00
Ammonium sulfate fraction 40 - 80%	184.58 ± 16.30	1089.31 ± 118.13	5.90 ± 1.12	76.85 ± 2.05	1.54 ± 0.19
IEX fraction	10.98 ± 0.40	243.45 ± 33.12	22.18 ± 3.81	17.18 ± 2.19	5.81 ± 0.77

60 ml of the crude extract (equivalent to 10 grams of the fish skeletal muscle) were treated as described in the Materials and Methods; data presented are average values of triplicate results for 3 experiments. (Teruel and Simpson, 1995)

chymotrypsin-like activity as purification of the fish muscle enzyme progressed is summarized in Fig. 5. The absence of the trypsin-like and chymotrypsin-like activities from the active collagenolytic enzyme fraction suggests that these enzymes were present together with the collagenolytic enzyme in the crude extract, and not due to the action of the fish muscle collagenolytic enzyme, itself, in contrast to that reported for crab hepatopancreas collagenase (Eisen *et al.*, 1973).

Insoluble collagen had been used as substrate for shellfish enzyme with collagenase-like activities. The fish enzyme showed activity against bovine insoluble collagen. WFC hydrolyzed insoluble bovine tendon collagen after 12 h incubation at 37°C (Fig. 6). Very little to no activity was observed at incubation times lower than 12 h, however, collagenase-like activity increased with incubation time and maximum activity was observed after 48 h incubation period.

4.2 Influence of pH on the activity and stability of collagenases from fish (IEX fraction) and *C. histolyticum*

The partially purified fish muscle collagenase after IEX had an optimum pH of 7.5 for PZ-peptide hydrolase reaction, while the collagenase from *C. histolyticum* had an optimum pH of 8.0 for the same reaction (Fig. 7). This optimum pH value for WFC is similar to those reported for collagenases from various shellfish, e.g., fresh water prawn hepatopancreas collagenase, pH 6.5 - 7.5 with insoluble collagen as substrate (Nip *et al.*, 1985), and fiddler crab hepatopancreas collagenase, ~ pH 8.0 with soluble collagen as substrate (Eisen *et al.*, 1973).

The fish collagenase was relatively stable within the pH range of 6.0 - 8.0, whereas, the bacterial collagenase was most stable within the pH range of 7.0 - 9.0 (Fig. 8). Both enzymes lost their collagenolytic activities at pH < 4.0, and in this respect are similar to crab collagenase which showed loss of activity at below pH 6.0 and complete inactivation

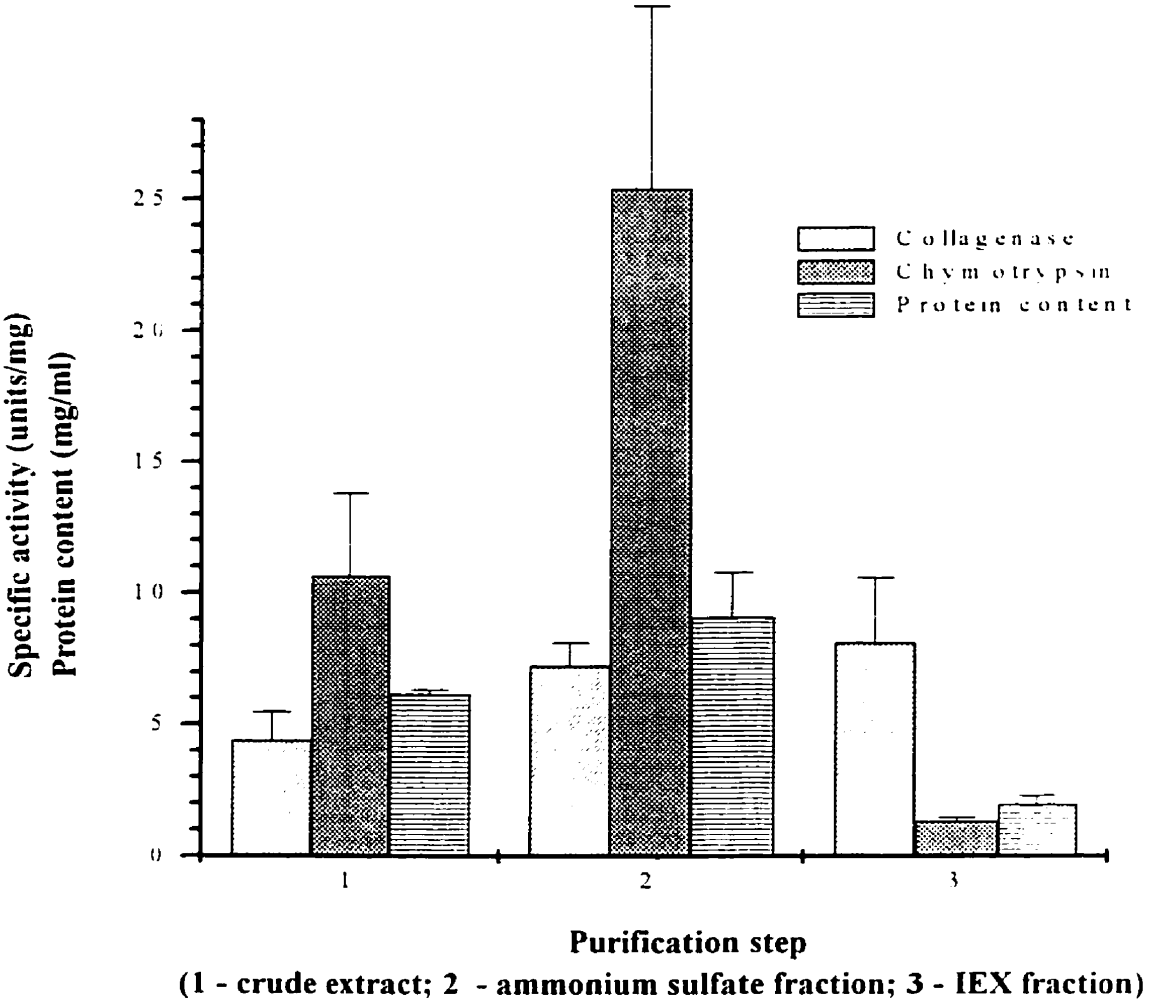


Fig. 5. Effect of purification steps on the activities of fish skeletal muscle enzymes (Values presented are means of triplicate results for 3 experiments) (Teruel and Simpson, 1995)

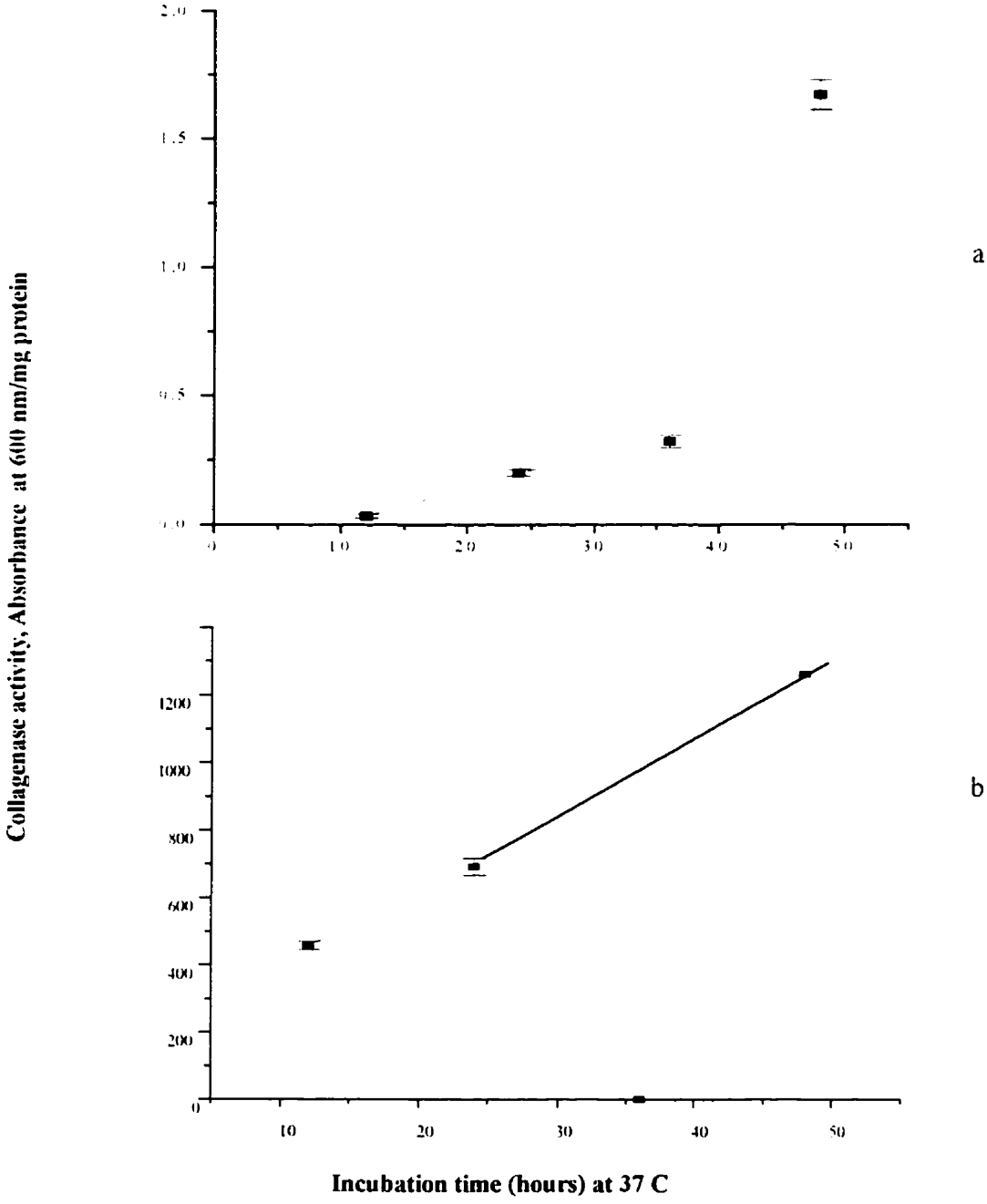


Fig. 6. Hydrolysis of collagen (insoluble, bovine Achilles tendon) by the fish collagenases (a) and *C. histolyticum* collagenases (b). (Values presented are means of 3 replicates for 1 experiment) (Teruel and Simpson, 1995)

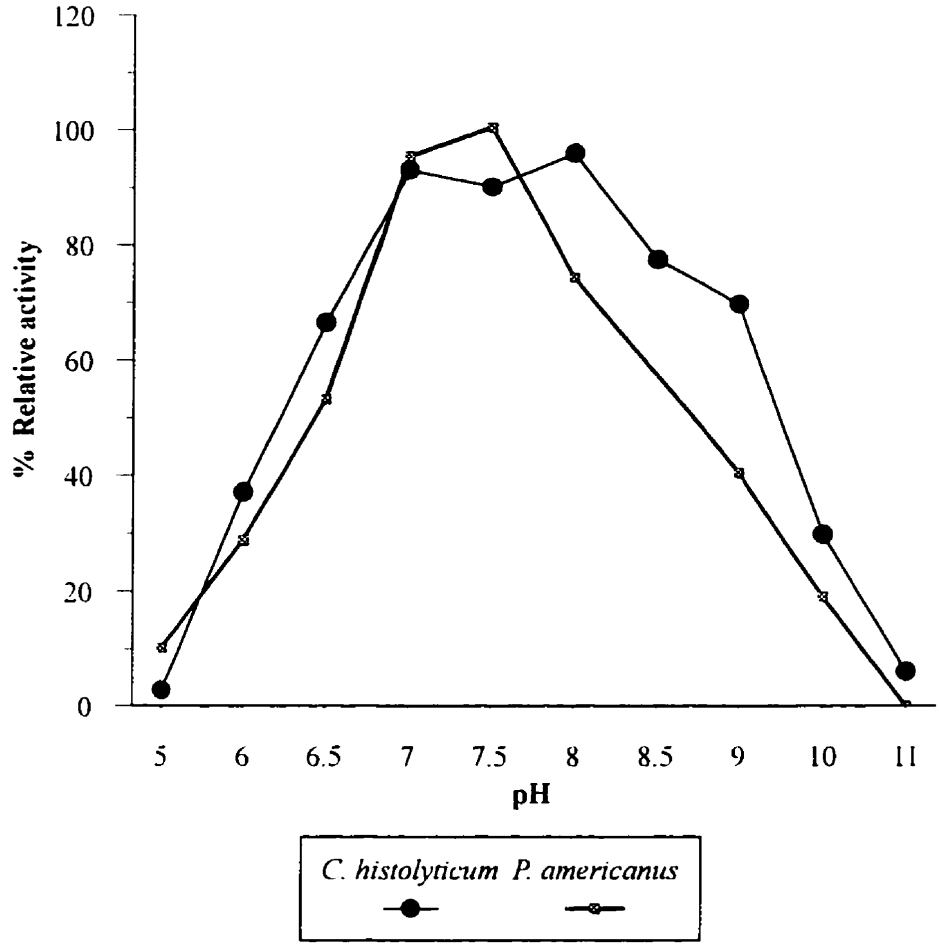


Fig. 7 . pH optima of collagenases from *C. histolyticum* and *P. americanus* (IEX fraction).
(Values presented are means of 3 replicates for 1 experiment)
(Teruel and Simpson, 1995)

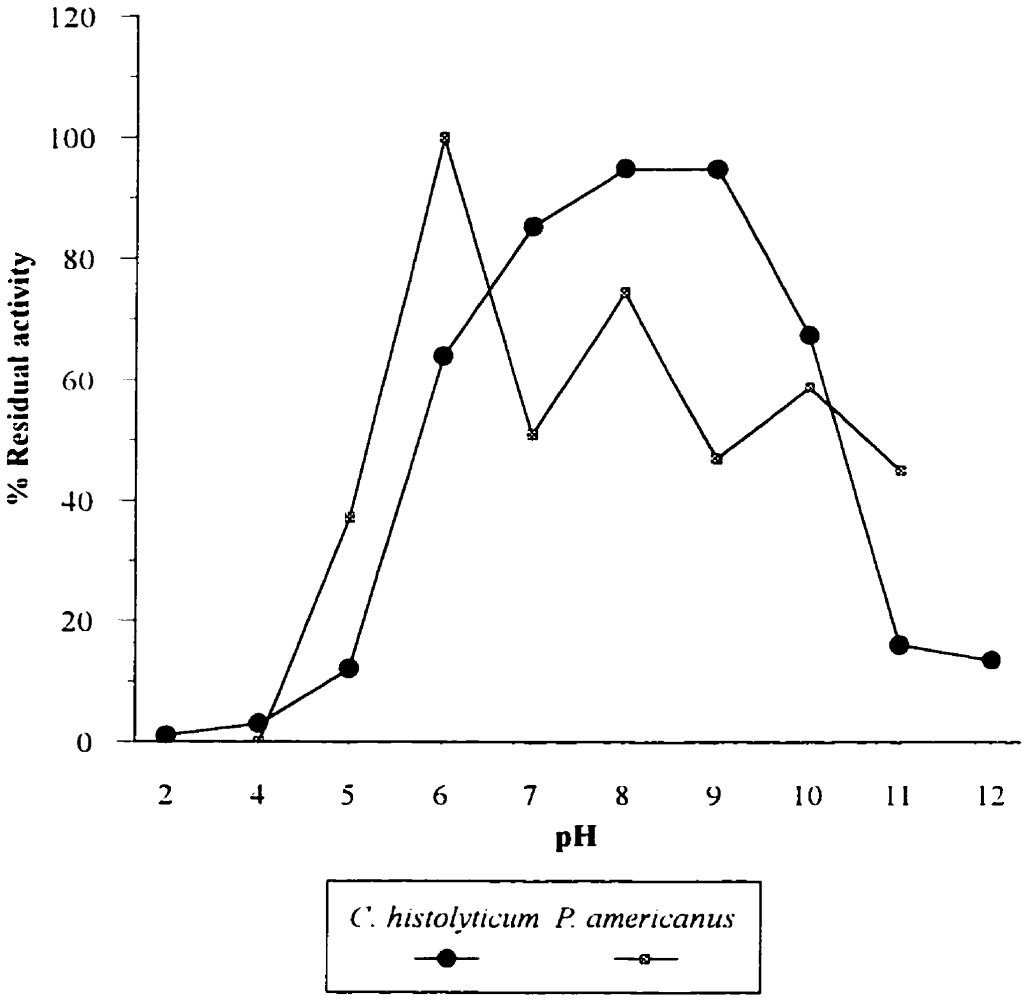


Fig. 8. pH stability of collagenases from *C. histolyticum* and *P. americanus* (IEX fraction).
(Values presented are means of 3 replicates for 1 experiment)
(Teruel and Simpson, 1995)

at below pH 3.5 (Eisen *et al.*, 1973).

4.3 Influence of temperature on the activity and stability of collagenases from fish (IEX fraction) and *C. histolyticum*

The partially purified fish collagenolytic enzyme exhibited a temperature optimum of 40°C for the hydrolysis of PZ-peptide, similar to collagenase from *C. histolyticum* (Fig. 9). This value is similar to that reported for freshwater prawn collagenase (37°C - Nip *et al.*, 1985) and for lobster collagenase (40 - 50°C; Chen, 1991) and Vibrio B-30 collagenase (37°C - Merkel and Dreisbach, 1978). However, it is higher than that reported for collagenase from *Cytophaga* sp. (30°C - Sasagawa et al., 1993) but lower than that for filefish (55°C - Kim *et al.*, 1993).

The collagenolytic enzyme from fish muscle and collagenase from *C. histolyticum* were stable at 0 - 45°C (Fig. 10). At 50°C, the residual activity of bacterial collagenase dropped to 65.50%, and at 65°C complete loss of activity was observed. Collagenase from *Cytophaga* sp. was stable from pH 15 - 40°C (Sasagawa *et al.*, 1993). However, in the case of fish collagenolytic enzyme the loss of activity was only ~31% at 65°C. The higher stability of the partially purified enzyme from fish tissue may be due to the presence of other molecules in the crude extract which contributed to the stabilization of some enzymes (Stauffer, 1989).

4.4 Further purification of the fish skeletal muscle collagenase(s) fraction

Several methods were used to further purify the collagenase from the skeletal muscle of fish and this included isoelectric focusing (IEF) with the use of a rotofor from BioRad and elution of the collagenase fractions from the PVDF membrane (NOVEX) and column chromatography in the FPLC system (Pharmacia Baie' D'Urfe, Quebec).

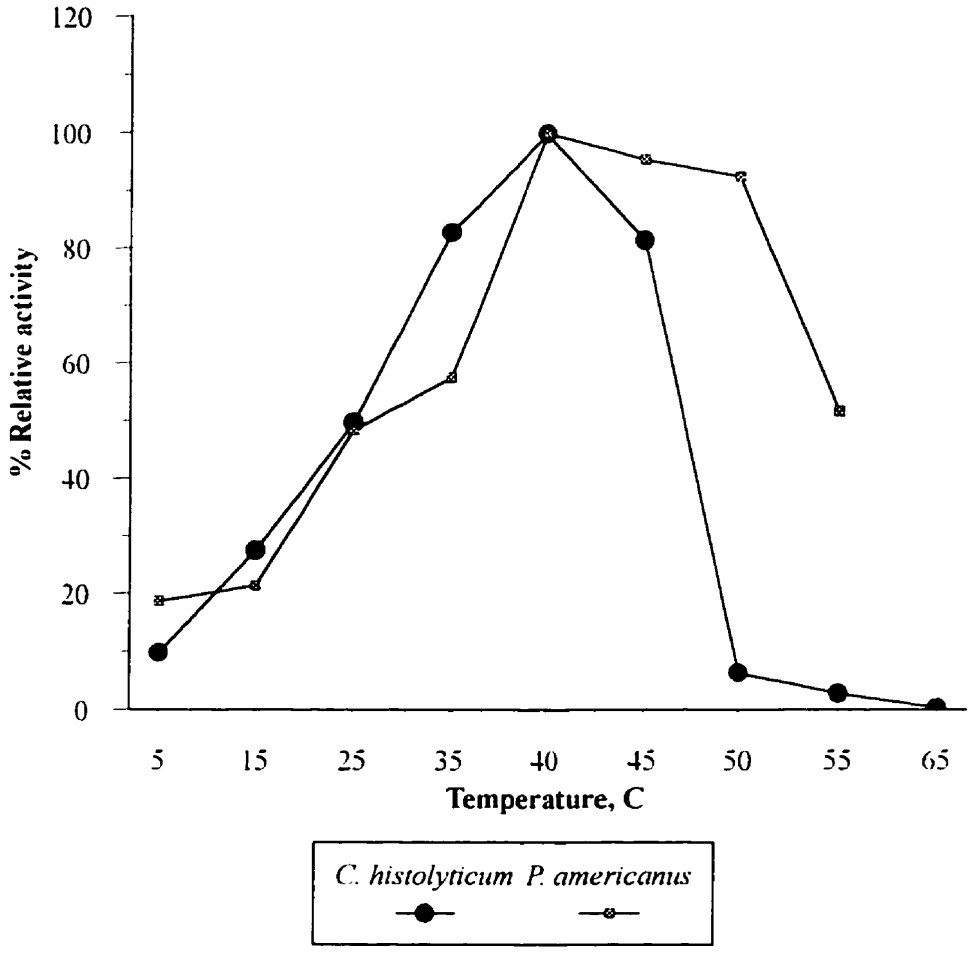


Fig. 9. Temperature optima of collagenases from *C. histolyticum* and *P. americanus* (IEX fraction).
(Values presented are means of 3 replicates for 1 experiment)
(Teruel and Simpson, 1995)

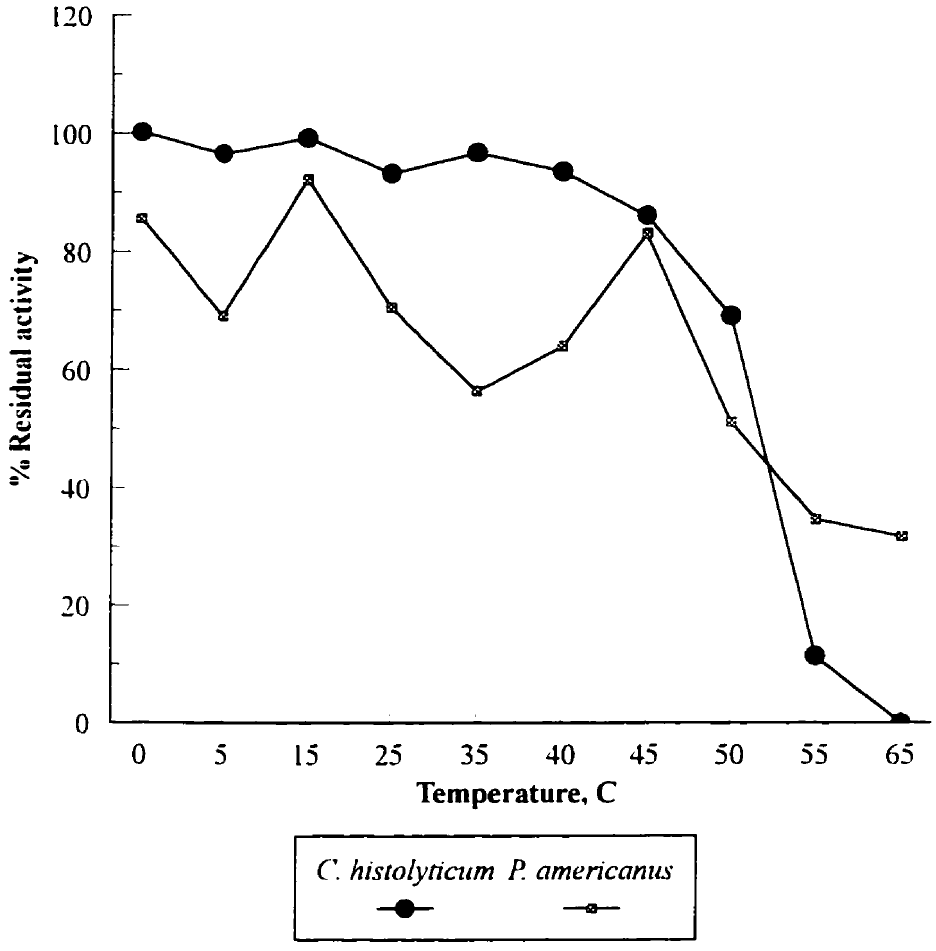


Fig. 10. Temperature stability of collagenases from *C. histolyticum* and *P. americanus* (IEX fraction).
(Values presented are means of 3 replicates for 1 experiment)
(Teruel and Simpson, 1995)

Isoelectric focusing is a fast method of protein purification, however, the removal of ampholytes which interfere with protein determination and probably lower the specific activity of the enzyme was a problem. Protein precipitation was also observed during the run and this could also be responsible for the loss of collagenase activity. Modification of the IEF runs such as incorporation of glycerol in sample preparation and/or prerunning of the system with ampholytes without the enzyme and addition of the enzyme after establishing the pH gradient were made. Precipitation was eliminated, however, recovery of the enzyme activity was still very low. Elution of the collagenase bands from the PVDF membrane was 100%, however, recovery of collagenase activity was very low despite renaturation of the enzyme after elution. Furthermore, the distance between the two isoenzymes was very small such that cutting them from the PVDF membrane without staining was a problem. Among the methods used, column chromatography with the use of the FPLC system was found to be efficient in the purification of the fish skeletal muscle enzymes.

4.4.1 Further purification of the enzymes by immobilized metal affinity chromatography (IMAC) and size-exclusion chromatoraphy (SEC)

The scheme used to further purify the fish skeletal muscle collagenases is given in Fig. 11. The crude extract was subjected to ammonium sulfate precipitation (40 -80%) as a preliminary purification of the enzyme solution followed by a series of column chromatography, namely, ion-exchange, immobilized metal affinity and size-exclusion in the FPLC system. Increasing specific activity was observed with purification (Table 10). The increase in the specific activity of the fish skeletal muscle collagenases could be due to the removal of proteins or other molecules which may be inhibitory on collagenases. The degree of the removal of these impurities is shown in the chromatograms from column chromatography and the electropherograms. In Table 10, the value for IEX-1 fraction

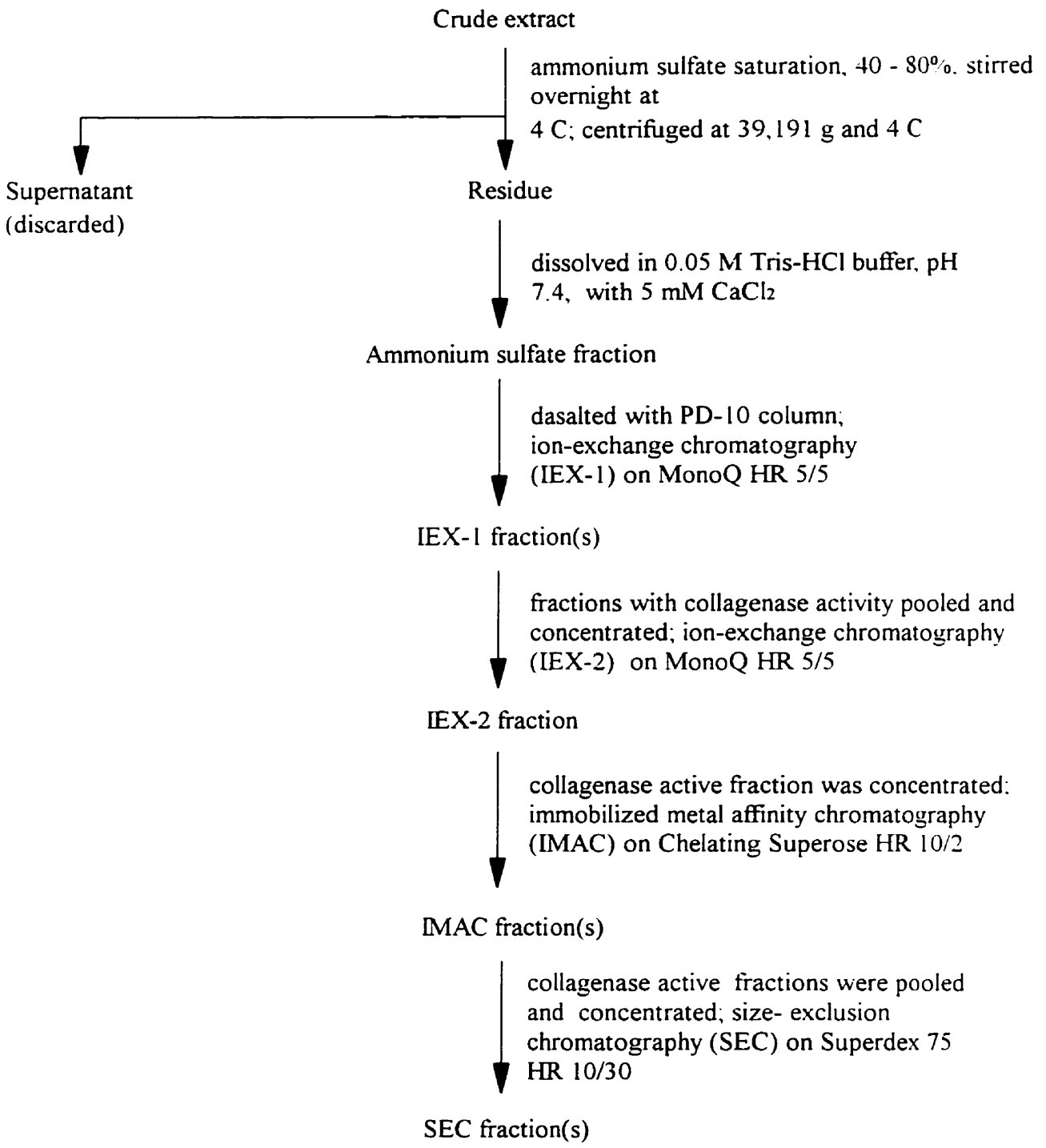


Fig. 11. Purification scheme for winter flounder skeletal muscle collagenases.

Table 10. Purification scheme for winter flounder skeletal muscle collagenases.

Purification scheme	Protein content mg/ml	Total protein mg	Specific activity units/mg	Total activity units	Yield %	Purification fold
Crude extract	4.72 ± 0.2	3397.32 ± 145.08	2.19 ± 0.16	7424.07 ± 233.00	100	1.00
Ammonium sulfate ppt. (40 -80%)	5.56 ± 0.98	1335.79 ± 33.81	4.64 ± 1.12	5934.00 ± 409.02	79.84 ± 3.00	2.08 ± 0.36
IEX-1	1.32 ± 0.02	253.44 ± 3.84	10.11 ± 2.63	2552.18 ± 627.72	34.14 ± 7.38	4.54 ± 0.86
IEX-2	0.86 ± 0.01	46.69 ± 0.29	19.40 ± 3.98	904.36 ± 179.93	12.12 ± 2.05	8.76 ± 1.16
IMAC	0.15 ± 0.01	6.00 ± 0.40	74.20 ± 10.52	440.96 ± 33.47	5.94 ± 0.27	33.67 ± 2.31
SEC	0.04 ± 0.003	1.56 ± 0.12	192.29 ± 10.28	302.33 ± 40.00	4.05 ± 0.42	87.84 ± 1.82

720 ml of the crude extract (equivalent to 120 g of the skeletal muscle) were treated as described in the Materials and Methods; data presented are average values of triplicate results for two experiments.

was obtained by pooling fractions 3 and 4, whereas values presented in Table 9 was obtained only from fraction 3. The pooled fractions from IEX-1 were further purified up to the SEC level.

4.4.1.1 Ammonium sulfate fractionation

The fish collagenase was recovered from the residue obtained from 40 - 80% saturation with ammonium sulfate. The specific activity recovered was about two times higher than that of the crude extract corresponding to a yield of 79.84% (Table 10).

4.4.1.2 Ion-exchange chromatography (IEX-1 and IEX-2)

The ion-exchange column, MonoQ is a strong anion exchanger in which the charged group on the gel $-CH_2N^+(CH_3)_3$, is totally independent of pH in the range of 2 - 12. Therefore the sample loading capacity does not change with pH due to loss of charge. The charged group also provides an ionic interaction in which a simple mechanism does not depend on hydrogen bonding or weak electrostatic interactions. Thus, negatively charged components of the sample interact with the charged group and is eluted from the column with the use of a high salt concentration (Pharmacia, 1985).

Winter flounder skeletal muscle collagenases were adsorbed by the anion exchange column as shown in Figs. 12 and 13, for IEX-1 and IEX-2, respectively, suggesting that the enzyme could be an acidic protein similar to the fiddler crab hepatopancreas collagenolytic protease (Eisen *et al.*, 1973), but differs from the basic human skin collagenase, which has a higher affinity for the cation exchange column (phosphocellulose/carboxymethylcellulose) (Stricklin *et al.*, 1977), and pig synovial collagenase which passed unretarded through the DEAE-cellulose column (Cawston and Tyler, 1979). The collagenase fractions were eluted with increasing concentrations of

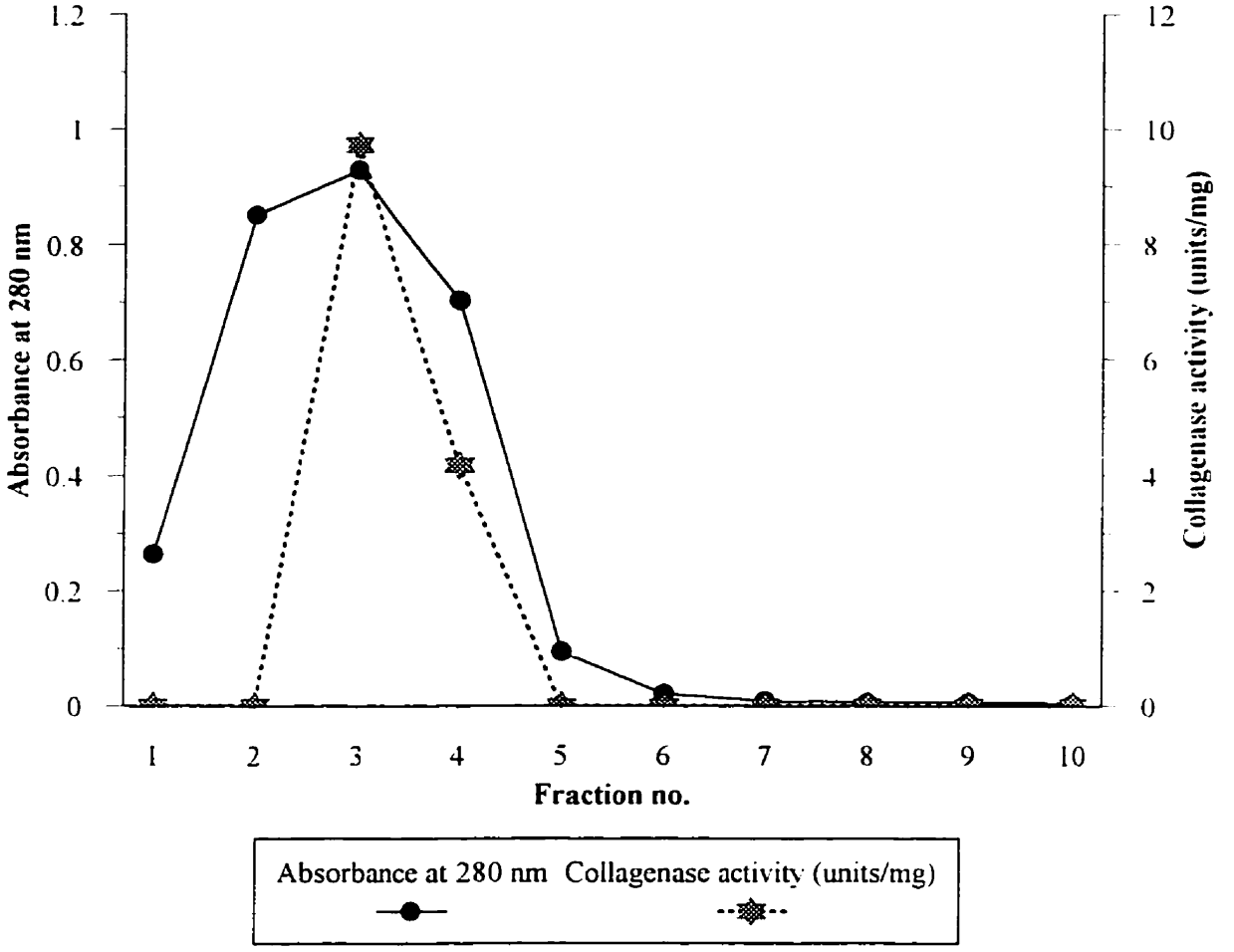


Fig. 12. Elution profile of fish collagenases on MonoQ HR5/5 (ion-exchange chromatography 1) using the FPLC system.

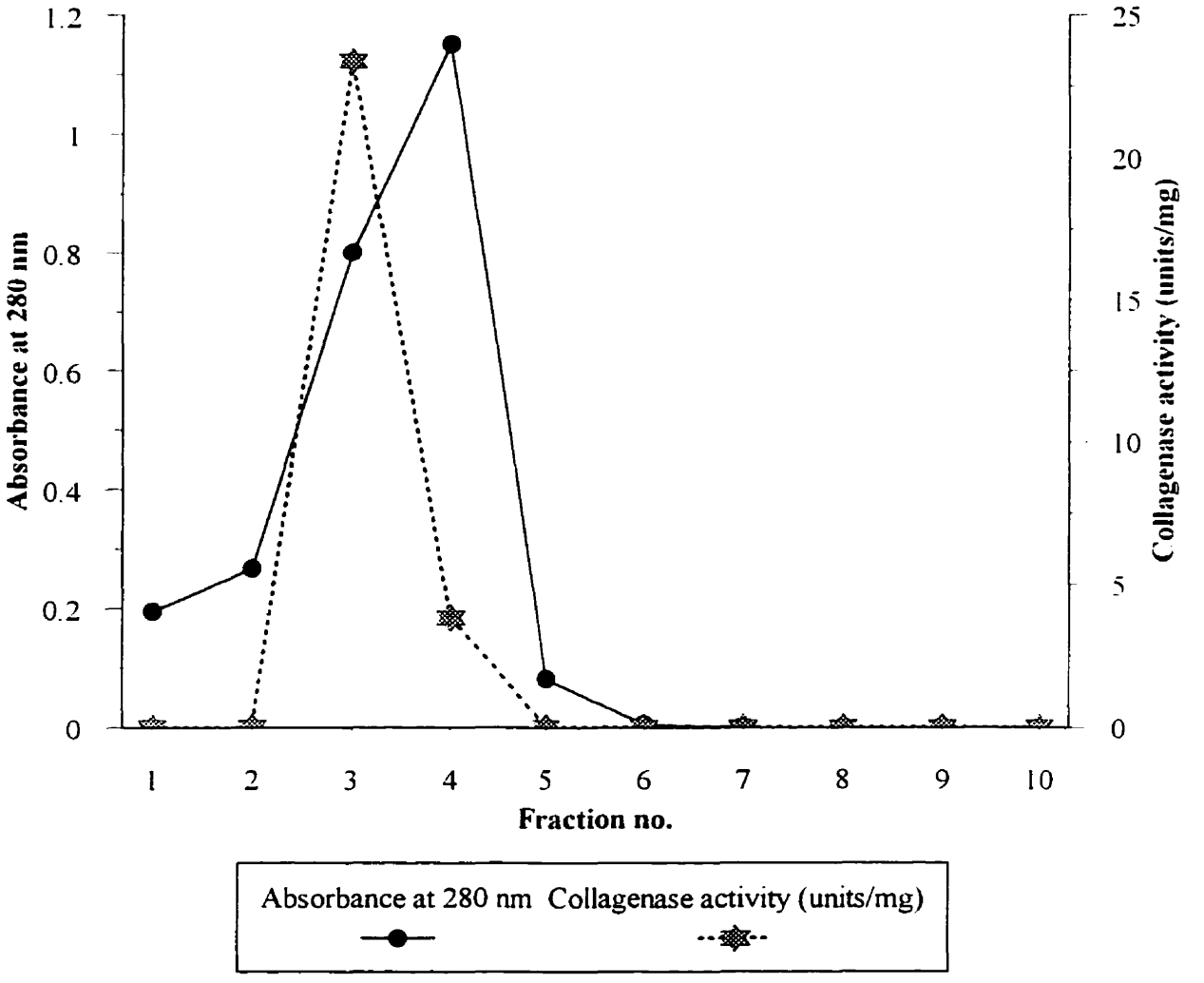


Fig. 13. Elution profile of fish collagenases on MonoQ HR5/5 (ion-exchange chromatography 2) using the FPLC system.

NaCl.

Ten 2-ml fractions were collected per run for IEX-1. Fractions 3 and 4 which showed collagenase acivity were pooled, concentrated and subjected to IEX-2. Ten 2-ml fractions were collected per run for IEX-2. Fractions 3 and 4 showed collagenase activity, but only fraction 3 was considered for further purification since it had a much higher specific activity compared to fraction 4.

4.4.1.3 Immobilized metal affininity chromatography (IMAC)

The chelating Superose affinity column composed of iminodiacetic acid covalently attached to the agarose-based gel is stable over a pH range of 2 - 13. Protein purification by IMAC is based on the affinity of some proteins for certain metal ions, and thus a gel loaded with strongly fixed Zn^{++} and Cu^{++} ions with excess binding capacity may interact with the surface exposed imidazole and thiol groups of proteins (Porath *et al.*, 1975). Elution of the bound protein is effected by a gradient or step-wise decrease in the pH, by a competitive elution, or by the use of a chelating agent like EDTA. In this particular study, Zn^{++} was loaded onto the column before sample application. The protein loaded was eluted with a decreasing pH gradient.

The chromatogram from IMAC showed two peaks and the first peak to be eluted had collagenase activity, whereas, the second peak had no collagenase activity (Fig. 14). The fish enzyme had a low affinity for the Zn-chelate affinity column (chelating superose, HR10/2) in contrast to the pig synovial collagenase which was strongly adsorbed by the column (Cawston and Tyler, 1979). This further shows that the enzyme may differ from mammalian collagenases in terms of amino acid composition. The weak affinity of the fish enzyme to this column suggests low levels of histidine and cysteine, not necessarily their absence, or the conformations of the isoenzymes were such that these residues were not readily accessible (i.e., buried within the molecule) for binding. Despite the low affinity of

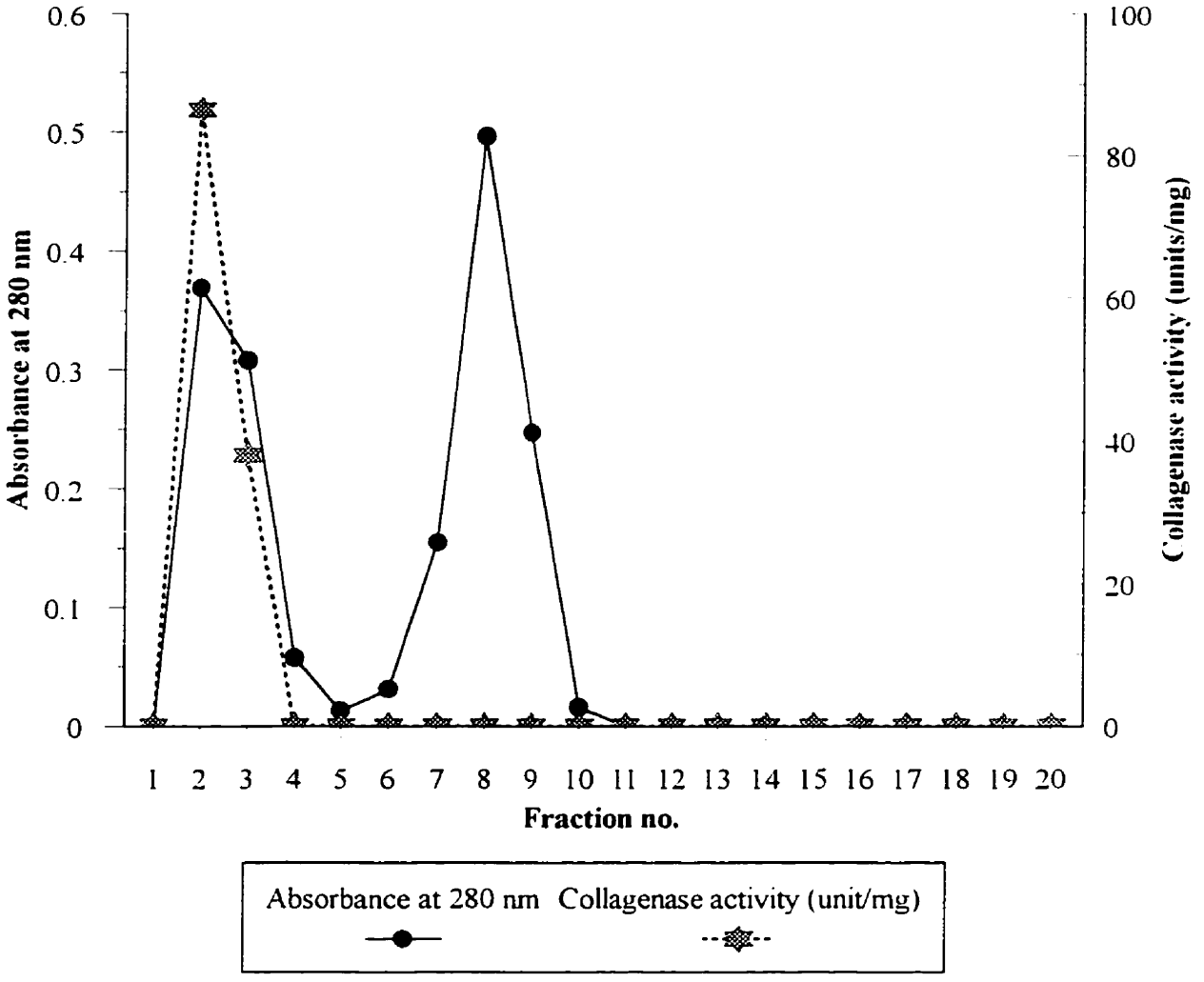


Fig. 14. Elution profile of fish collagenases on chelating Superose (HR10/2) (immobilized metal affinity chromatography) using the FPLC system.

the fish collagenases to the chelating Superose, increased purity of the enzyme was attained as a result of the removal of the protein component which had a stronger affinity to the column. The purification fold increased 34 times compared to the crude extract.

Twenty 1-ml fractions were collected per run and fractions 2 and 3 showed collagenase activity. These two fractions were pooled, concentrated and loaded onto the SEC column for further purification.

4.4.1.4 Size-exclusion chromatography (SEC)

Superdex 75 composed of dextran and agarose is stable over pH 3 -12. Separation of the proteins in SEC is based on differences in the molecular weight/size of the molecules and molecules are eluted in order of decreasing size.

Forty-six 0.5-ml fractions were collected every run and fractions 6, 7 and 8 showed collagenase activity (Fig. 15). Result of electrophoresis suggested that these 3 fractions had the same size (Fig. 16), thus they were pooled and used for characterization. Further increase in the purity level of the fish collagenase was achieved by SEC and this corresponded to a purfication fold of 88.

4.5 Electrophoresis

The electrophoretic patterns of winter flounder skeletal muscle enzyme fractions from every purification step are shown in Figs. 17 and 18 in Native-PAGE (Davis, 1964) and SDS-PAGE (Laemmli, 1970), respectively. Impurities were removed with purification as shown in the gels. The pooled fractions from SEC showed a single band on the native-PAGE 7% acrylamide gel. However, in the SDS-PAGE 12% acrylamide gel the same fraction showed 1 dominant band with traces of other proteins. This dominant band had an R_f value which corresponded to a molecular weight of approximately 73,000

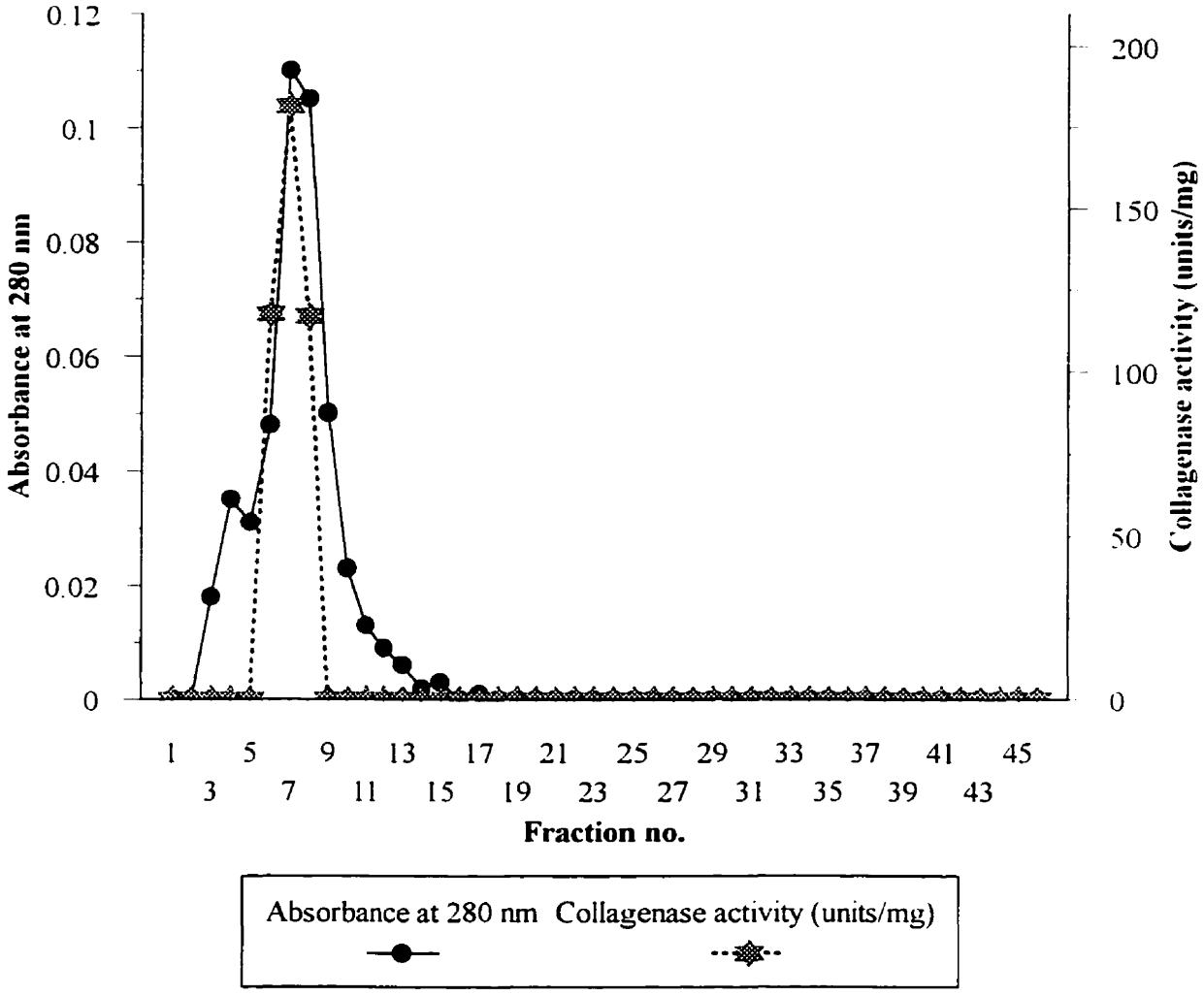


Fig. 15. Elution profile of fish collagenases on Superdex 75 (size- exclusion chromatography) using the FPLC system.

Fig. 16. Electrophoresis (Native-PAGE) of fractions from SEC with collagenase activity on 7% polyacrylamide gel (gel 1 - fraction 6; gel 2 - fraction 7; gel 3 - fraction 8).

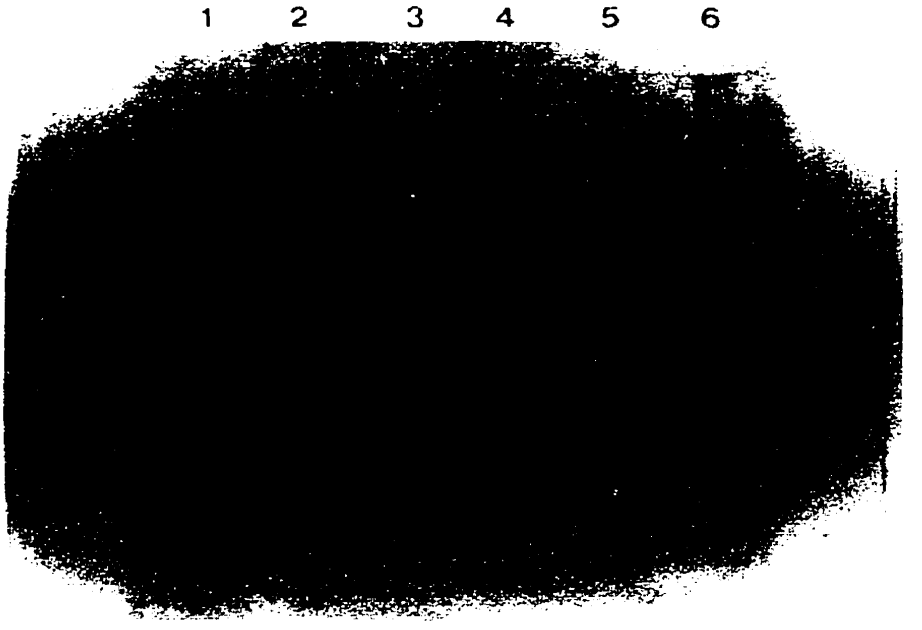


Fig. 17. Electrophoresis (Native-PAGE) of winter flounder skeletal muscle enzymes from each purification step on 7% polyacrylamide gel (lane 1 - SEC fraction; lane 2 - IMAC fraction; lane 3 - IEX-2 fraction; lane 4 - IEX-1 fraction; lane 5 - ammonium sulfate fraction; lane 6 - crude extract).

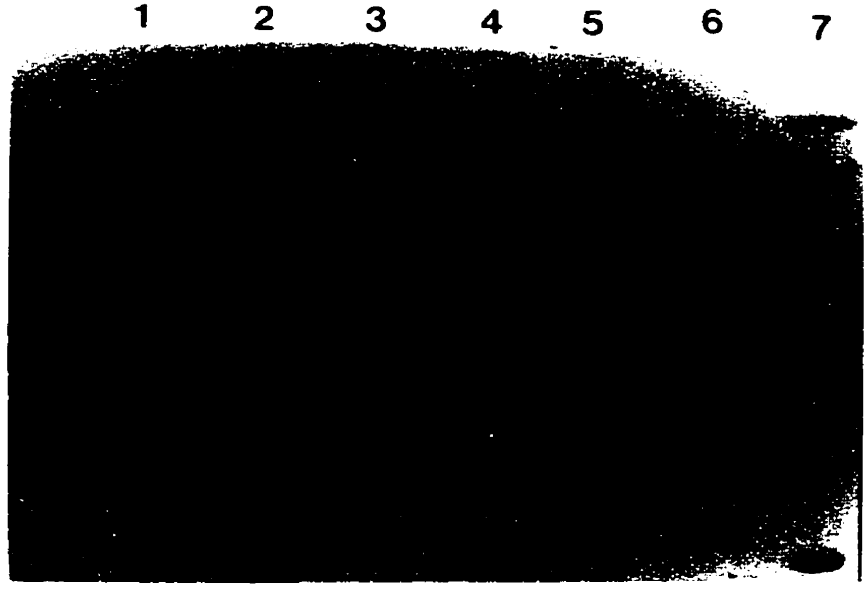


Fig. 18. Electrophoresis (SDS-PAGE) of winter flounder skeletal muscle enzymes from each purification step on 12% polyacrylamide gel (lane 1 - crude extract; lane 2 - ammonium sulfate fraction; lane 3 - IEX-1 fraction; lane 4 - IEX-2 fraction; lane 5 - IMAC fraction; lane 6 - SEC fraction; lane 7 - Pharmacia LMW standard: phosphorylase B - 94,000; bovine serum albumin - 67,000; ovalbumin - 43,000; carbonic anhydrase - 30,000; trypsin inhibitor - 20,100; α-lactalbumin - 14,400).

based on the Pharmacia LMW standard (Table 11). The results of the electrophoresis on the 12% acrylamide gel were not conclusive regarding the identity of the band(s) corresponding to collagenase(s). To ascertain the identity of the band(s) corresponding to collagenase(s), electrophoresis was conducted simultaneously on the precast zymogram gel and the 10% Tris-glycine gel (NOVEX) following the scheme in Fig. 19. Zymogram gels are 10% Tris-glycine gels with 0.1% gelatin used to detect and characterize collagenases and other proteases that can utilize gelatin as substrate (NOVEX, 1994-95 catalogue). These gels had been used to demonstrate/detect the presence of Collagenase IV, a matrix metalloproteinase (MMP) (Brown *et al.*, 1990; Kleiner *et al.*, 1993). Two adjacent reaction zones were detected in the zymogram gels (Fig. 20), suggesting that two collagenase isoenzymes are present in the winter flounder skeletal muscle. These isoenzymes were designated as WFC-1 and WFC-2 corresponding to the 1st and 2nd reaction zones, respectively. These reaction zones corresponded to the two adjacent bands detected in the 10% Tris-glycine gel (Fig. 21) and the PVDF membrane (Fig. 22). The estimated molecular weights of WFC-1 and WFC-2 were 79,600 and 75,500, respectively (Table 12) based on the wide range molecular standards from NOVEX. The concentration of WFC-1 in the fish skeletal muscle was very low in comparison to WCF-2 as demonstrated in the 10% Tris-glycine gel and the PVDF membrane, i.e., the band corresponding to WFC-1 was lighter and narrower than WFC-2. However, WFC-1 seemed to have a higher activity than WFC-2 based on their zymographic activity. The area of the reaction zones observed on the zymogram gel for WFC-1 and WFC-2 were almost the same.

After separation of collagenase isoenzymes, electrophoretic blotting from the 6% Tris-glycine gel to the PVDF membrane was conducted to facilitate the isolation of WFC-1 from WFC-2 for amino acid analysis. A slight increase in the distance between WFC-1 and WFC-2 was observed with the use of 6% Tris-glycine gel.

Table 11. Relative mobilities (R_f) and molecular weights of protein standards and fish muscle collagenases on 12% polyacrylamide gel.

Component	R_f	Molecular weight
Standards		
Phosphorylase B	0.1674	94,000
Bovine serum albumin	0.2594	67,000
Ovalbumin	0.4017	43,000
Carbonic anhydrase	0.5858	30,000
Trypsin inhibitor (soybean)	0.7866	20,100
α-lactalbumin	0.9456	14,400
Sample		
WFC (SEC fraction)	0.2267	73,107

R_f values were calculated from SDS-polyacrylamide gel (12%) electrophoregrams. (R_f = distance protein has migrated from origin/distance from origin to reference point)

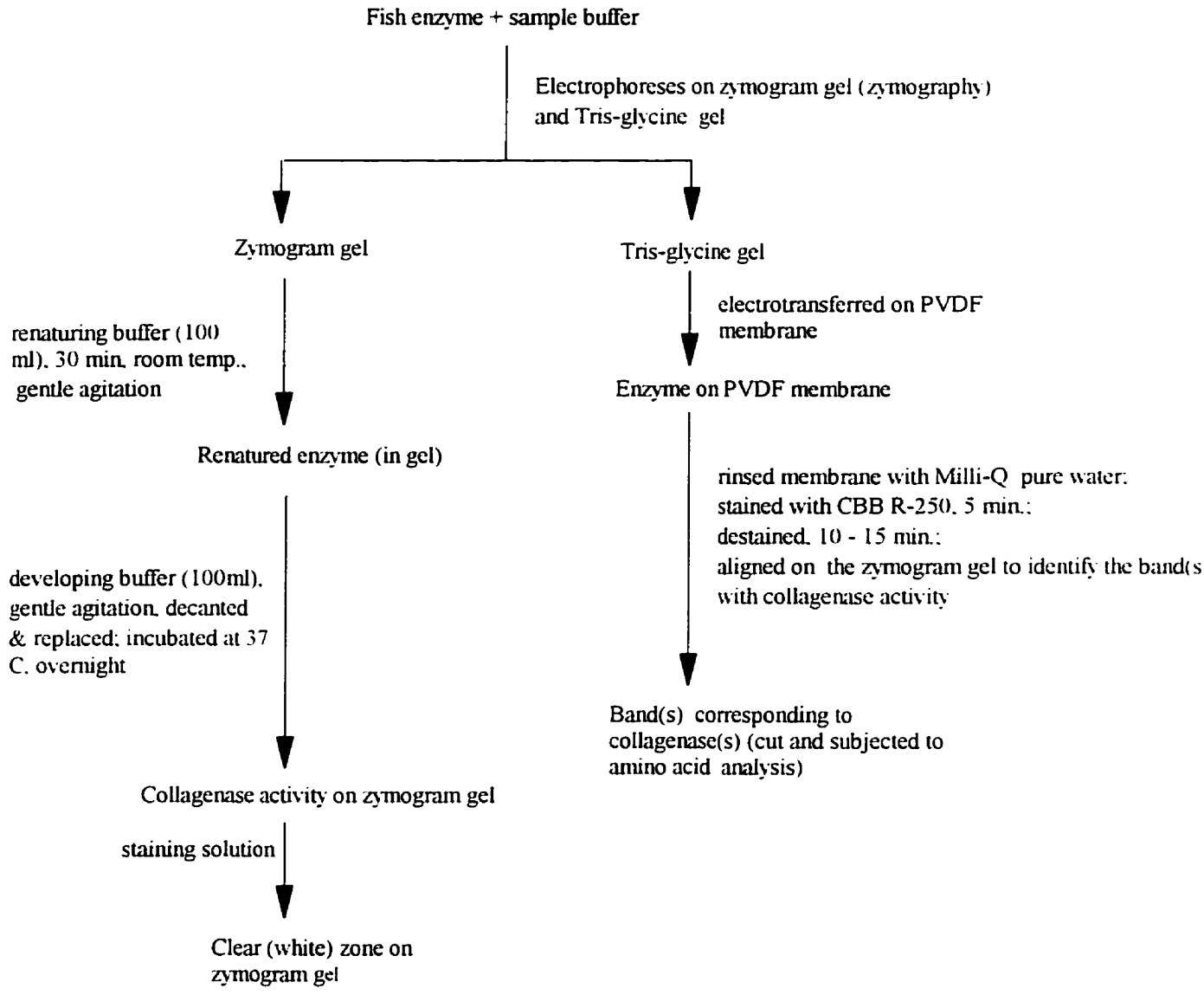


Fig. 19. Schematic diagram for the detection of collagenase with the use of a zymogram gel and electrophoretic blotting from the Tris-glycine gel to the PVDF membrane

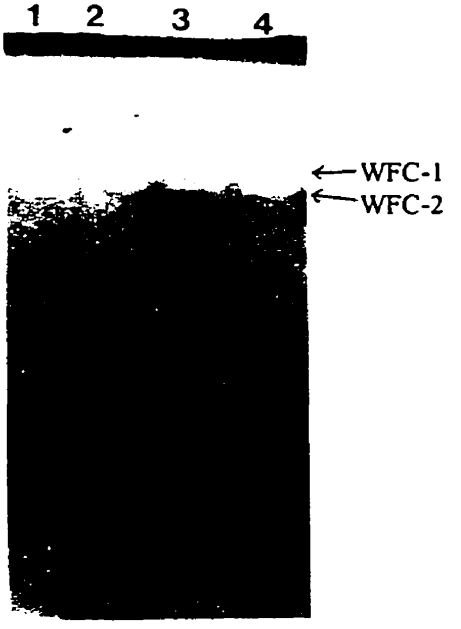


Fig. 20. Zymogram gel showing the reaction zones of winter flounder skeletal muscle collagenases (SEC fraction), WFC-1 (first reaction zone from the top of gel) and WFC-2 (reaction zone just below WFC-1) (lanes 1 to 4 - winter flounder skeletal muscle collagenases).

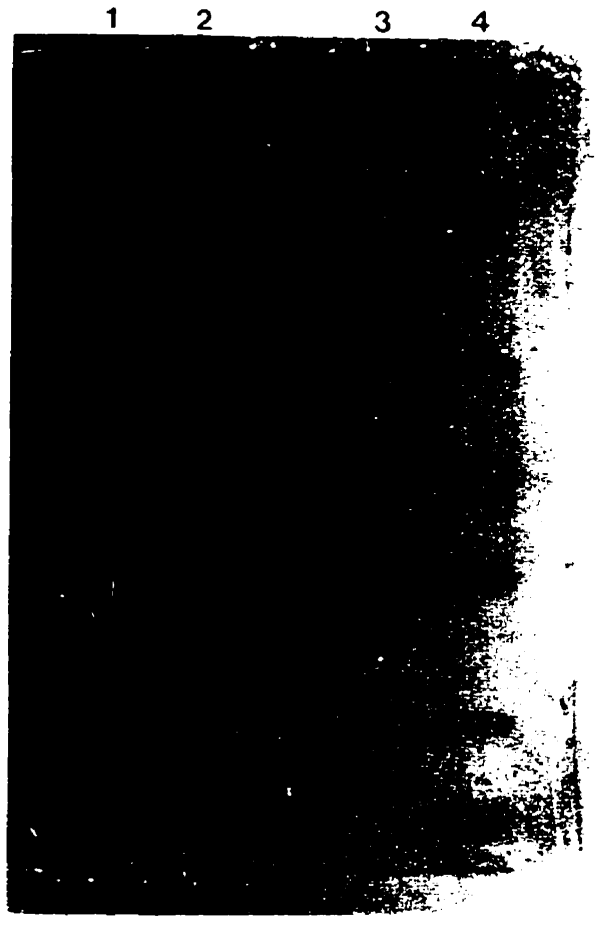


Fig. 21. Electrophoresis (SDS-PAGE) of winter flounder skeletal muscle collagenases (SEC fraction) on 10% Tris-glycine gel (lane 1 - Mark 12 wide range protein standard, NOVEX: myosin - 200,000; β galactosidase - 116,300; phosphorylase B - 97,400; bovine serum albumin - 66,300; glutamic dehydrogenase - 55,400; lactase dehydrogenase - 36,500; carbonic anhydrase - 31,000; trypsin inhibitor - 21,500; lysozyme - 14,400; aprotinin - 6,000; insulin B - 3,500; insulin A - 2,500; lanes 2 and 3 - winter flounder skeletal muscle collagenases; lane 4 - Seeblue prestained markers, NOVEX: myosin - 250,000; bovine serum albumin - 98,000; glutamic dehydrogenase - 64,000; alcohol dehydrogenase - 50,000; carbonic anhydrase - 36,000; myoglobin - 30,000; lysozyme - 16,000; aprotinin - 6,000; insulin β chain - 4,000).

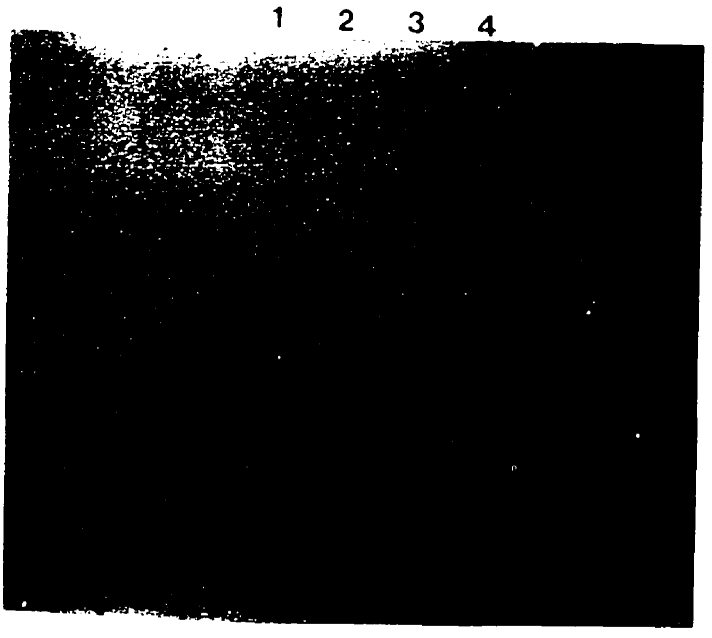


Fig. 22. PVDF membrane showing the winter flounder skeletal muscle collagenases (SEC fraction) (lane 1 - Mark 12 standard, NOVEX; lanes 2 and 3 - winter flounder skeletal muscle collagenases; lane 4 - Seeblue markers, NOVEX).

Table 12. Relative mobilities (R_f) and molecular weights of protein standards and fish muscle collagenases on 10% Tris-glycine gel

Component	R_f	Molecular weight
Standards		
β galactosidase	0.3165	116,300
Phosphorylase B	0.3597	97,400
Bovine serum albumin	0.4604	66,300
Glutamic dehydrogenase	0.5540	55,400
Lactase dehydrogenase	0.7410	36,500
Carbonic anhydrase	0.8417	31,000
Sample		
WFC-1	0.4316	79,600
WFC-2	0.4532	75,500

R_f values were calculated from SDS-polyacrylamide gel electrophoregrams on 10% Tris-glycine gel (NOVEX). (R_f = distance protein has migrated from origin/distance from origin to reference point)

4.6 pH optimum and stability

Collagenases from various sources have been shown to exhibit a wide range of pH optima, e.g., pH 6.5 - 7.5 for prawn hepatopancreas collagenase (Nip *et al.*, 1985), and ~pH 8.0 for fiddler crab hepatopancreas collagenase (Eisen *et al.*, 1973), whereas mammalian collagenase from rheumatoid synovium (Woolley *et al.*, 1975) and rabbit tumors (Mckroskery *et al.*, 1975) had pH optima ranging from 7.5 - 8.5 and 7.0 - 9.5, respectively. Microbial collagenases on the other had pH optima of 7.5 for *Cytophaga* sp. (Sasagawa *et al.*, 1993), and a slightly higher value, pH, 7.6 for collagenase from *Vibrio* B-30 (Merkel and Dreisbach 1978). The profile of the pH activity of the fish skeletal muscle collagenases (SEC fraction) is shown in Fig. 23. The fish collagenase fraction from SEC is most active at pH 7.5 and 37°C with PZ-peptide as substrate similar to the fraction from IEX and similar to the reported values as shown above.

The fish collagenase from SEC fraction was stable over a wide pH range from pH 6 - 11 (Fig. 24) with PZ-peptide as substrate at 37°C. At pH 5.0 the enzyme lost about 60% of its activity and at pH 4.0 complete loss of activity was observed. In this respect, the fish enzyme is similar to the crab collagenase which had a low activity at pH 6.0, was almost abolished at below pH 5.0 and was completely inactivated at below pH 3.5 (Eisen *et al.*, 1973). Collagenases from *C. histolyticum* were also unstable at pH below 6.0 (Bond and Van Wart, 1984b).

Upon death, the pH of fish skeletal muscle drops due to the accumulation of lactic acid. For most fishes the ultimate pH is between 6 to 7 (Davis, 1995), the pH values at which the fish collagenases remain active. Thus it is possible that these endogenous collagenases participate in the structural breakdown of the skeletal tissues of the fish by attacking the connective tissues, i.e., collagen and exposing the other parts of the tissues to attack by other endogenous proteases.

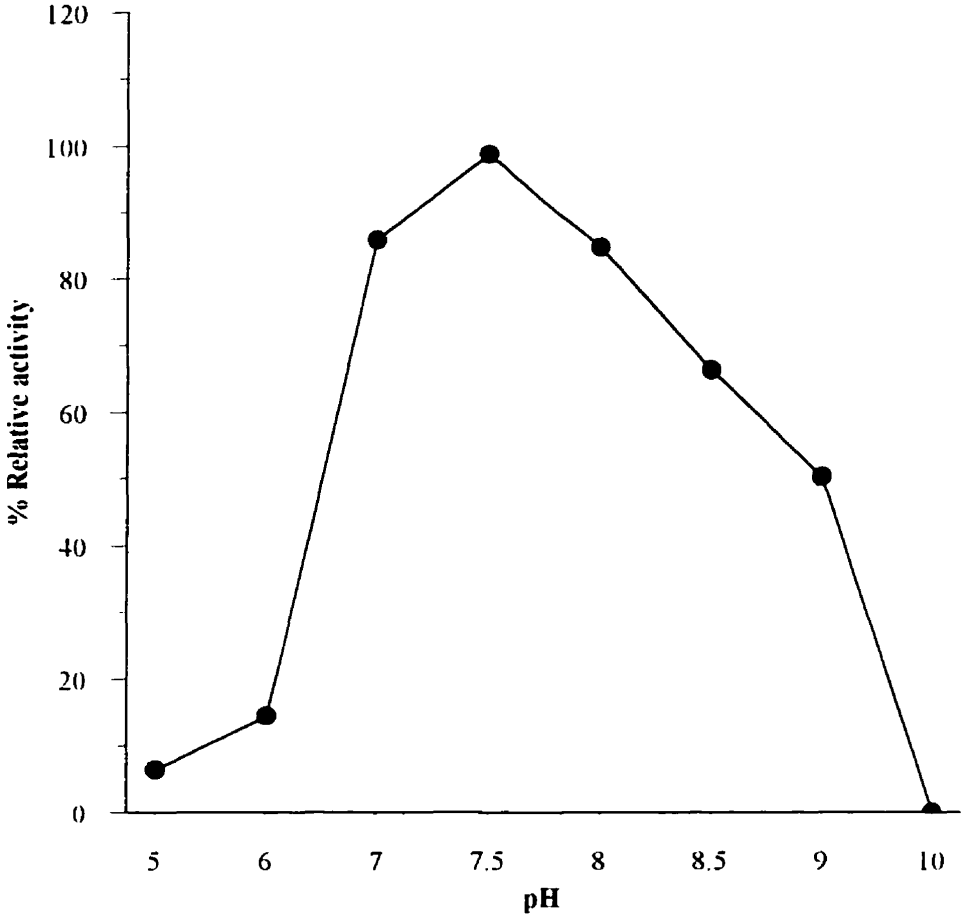


Fig. 23. pH optimum of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment).

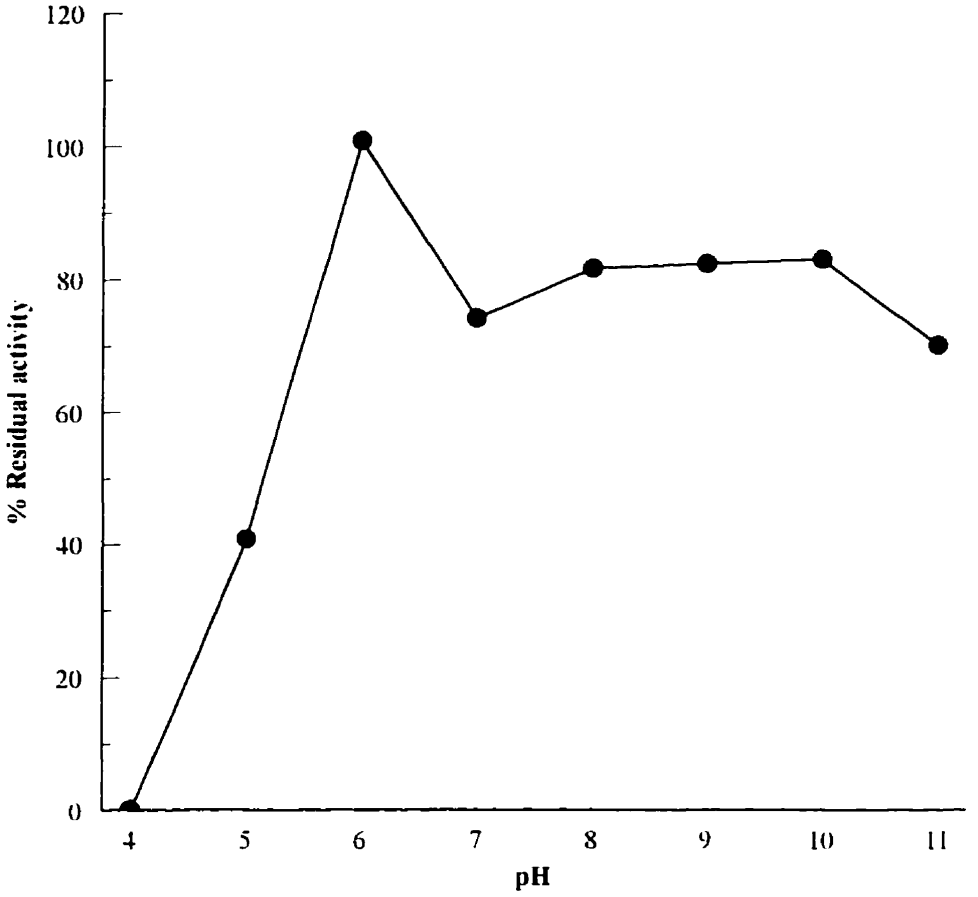


Fig. 24. pH stability of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment).

4.7 Temperature optimum and stability

The temperature optimum for fish collagenases (SEC fraction; 0.120 mg/ml) was 45°C at pH 7.1 with PZ-peptide as substrate (0.64mM) (Fig. 25). This value was similar to the optimum temperature of the crude extract in the preliminary studies and within the range reported for lobster collagenase (40 - 50°C; Chen, 1991), however, it is slightly higher than the reported values for collagenase from freshwater prawn (37°C - Nip *et al.*, 1985), *Vibrio* B-30 (37°C - Merkel and Dreisbach, 1978) and *Cytophaga* sp. (30°C - Sasagawa *et al.*, 1993) but lower than that value reported for filefish (55°C - Kim *et al.*, 1993).

The fish collagenase fraction after SEC was stable from 0 to 40°C (Fig. 26). This range is comparable to the value reported for collagenase from *Cytophaga* sp. (15 - 40°C - Sasagawa *et al.*, 1993). At 50°C, there was a loss of ~64% activity and at 55°C there was complete loss of enzyme activity.

4.8 Influence of inhibitors on WFC activity

Fig. 27 shows the influence of various inhibitors on the collagenase activity of the fish enzyme. Trypsin inhibitor (soybean) and TLCK failed to inhibit the activity of the fish enzyme. TLCK belong to the group of chloromethylketones which are known irreversible inhibitors of serine proteases (Shaw *et al.*, 1965). Inhibition of the enzyme by TLCK is due to the binding of the proteinase with the inhibitor in a substrate - like manner followed by the alkylation of the active-site histidine by the chloromethyl moiety (Salvesen and Nagase, 1989). Soybean trypsin inhibitor on the other hand forms a very tight yet reversible complex with proteinases (Salvesen and Nagase, 1989). Ethylene diamine tetraacetic acid (trisodium salt) and 2-mercaptoethanol lowered the collagenase activity of the fish enzyme by 45% and 50%, respectively. Dithiothreitol and 1,10 phenanthroline

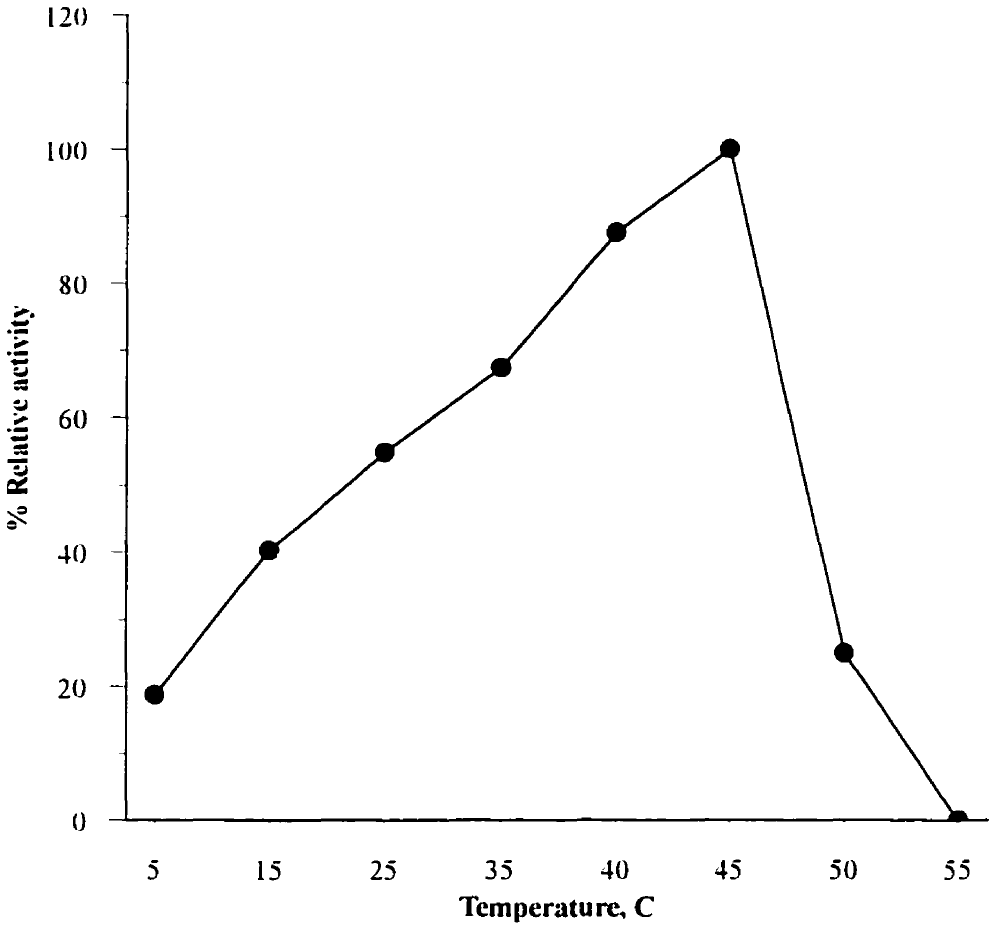


Fig. 25. Temperature optimum of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment).

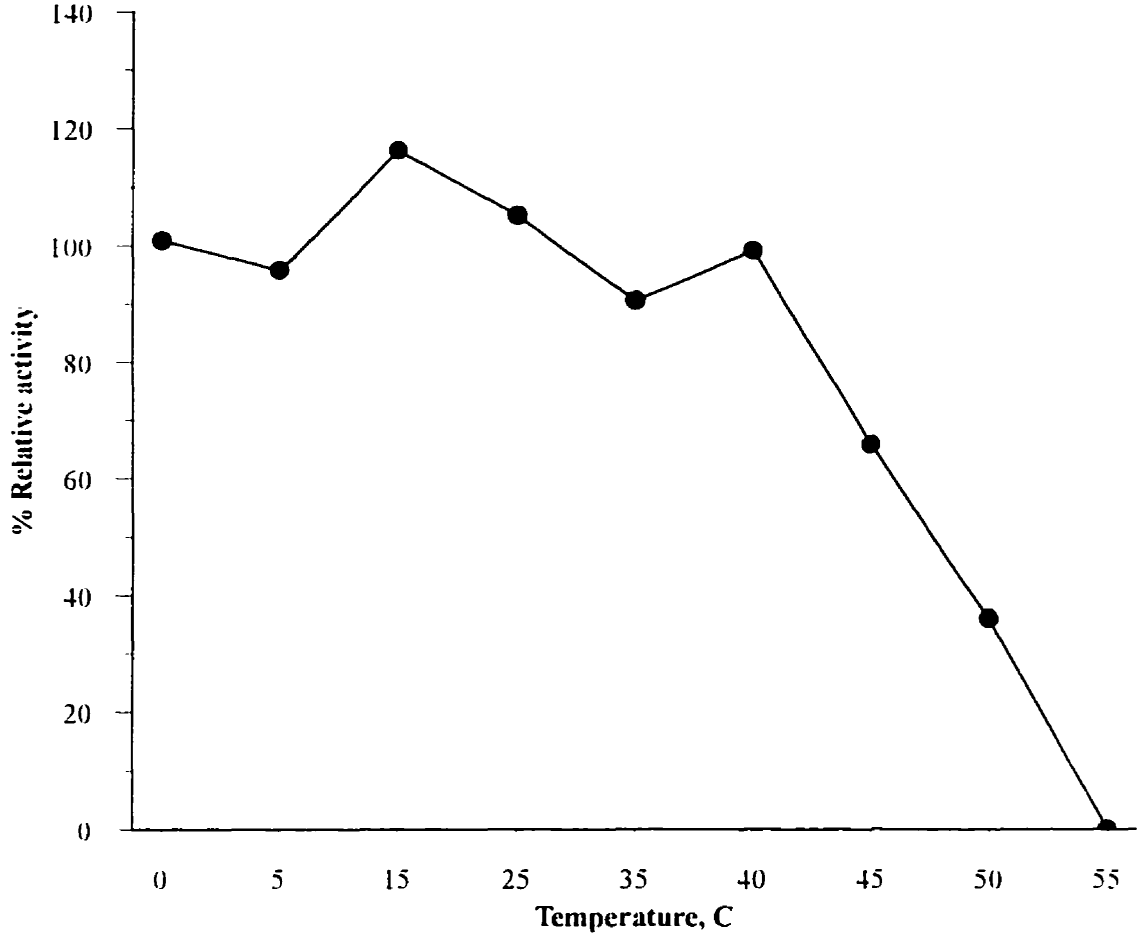
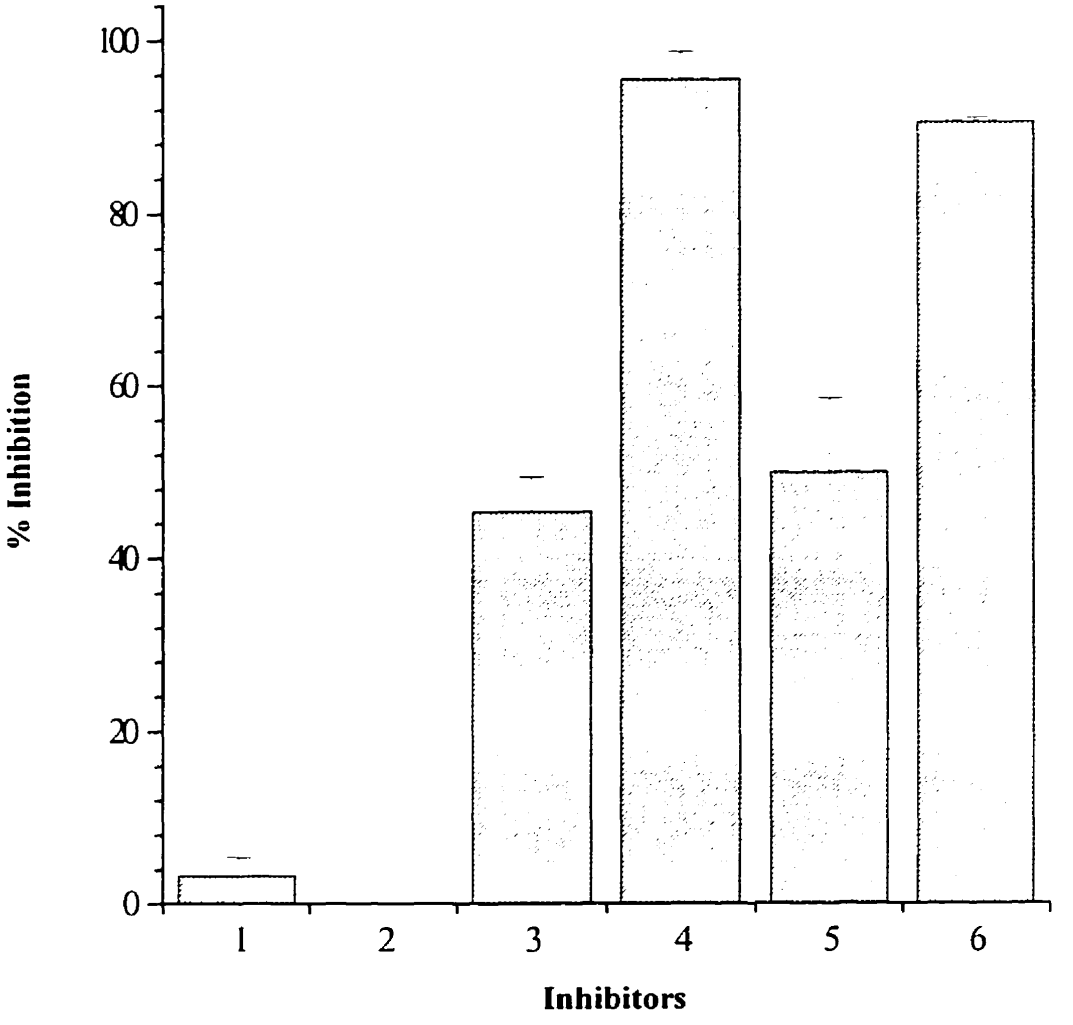


Fig. 26. Temperature stability of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment).



(1 - 10 mM TLCK; 2 - 0.5 mg/ml Trypsin inhibitor (soybean); 3 - 100 mM EDTA;
4 - 10 mM 1,10-Phenanthroline; 5 - 100 mM 2-Mercaptoethanol; 6 - 20 mM Dithiothreitol)

Fig. 27. Influence of various inhibitors on the activity of winter flounder skeletal muscle collagenases (SEC fraction, 0.062 mg/ml; PZ-peptide,0.64 mM).
(Values presented are means of 3 replicates for 1 experiment)

were the most effective inhibitors, decreasing the collagenase activity of the fish enzyme by 90% and 96%, respectively.

The fish enzyme differs from the crab hepatopancreas collagenase enzymes which were serine proteases, i.e., inhibited by serine protease inhibitors, but showed similarities with mammalian collagenases (known metalloproteases) from human skin (Woolley *et al.*, 1978), pig synovium (Tyler and Cawston, 1980), and rabbit tumors (McCroskery *et al.*, 1975) which were inhibited by both metalloenzyme and thiol protease inhibitors like EDTA and dithiothreitol, respectively.

4.8.1 Inhibitory action of 1,10-phenanthroline

The inhibitory action of 1,10-phenanthroline is directed towards the active site of the enzyme. It is an irreversible inhibitor of metal proteinases (Mihalyi, 1978). This inhibitor forms stable complexes with certain metal ions like zinc and iron through the coordination of the metal ion with the 2 nitrogen atoms of the inhibitor (Whitaker, 1972). In fish collagenase the action of 1,10-phenanthroline is directed towards zinc ion as will be shown and discussed in section 4.9. Increasing degree of inhibition with increasing levels of 1,10-phenanthroline was observed (Fig. 28) and 50% inhibition of the fish collagenase was effected at a concentration of 1.84 mM, the I_{50} for the inhibitor (Table 13). A plot of the relationship between V_o/V_i and the inhibitor concentration is shown in Fig. 29.

4.8.2 Inhibitory action of EDTA

Similar to 1,10-phenanthroline, EDTA is a metal chelator which can bind or form complexes with the metal component of metalloenzymes. Studies have shown that calcium and zinc ions are inherent components of collagenases from various sources (Seifter and Harper, 1970; Swann et al., 1981; Bode et al., 1984; Bond and Van Wart,

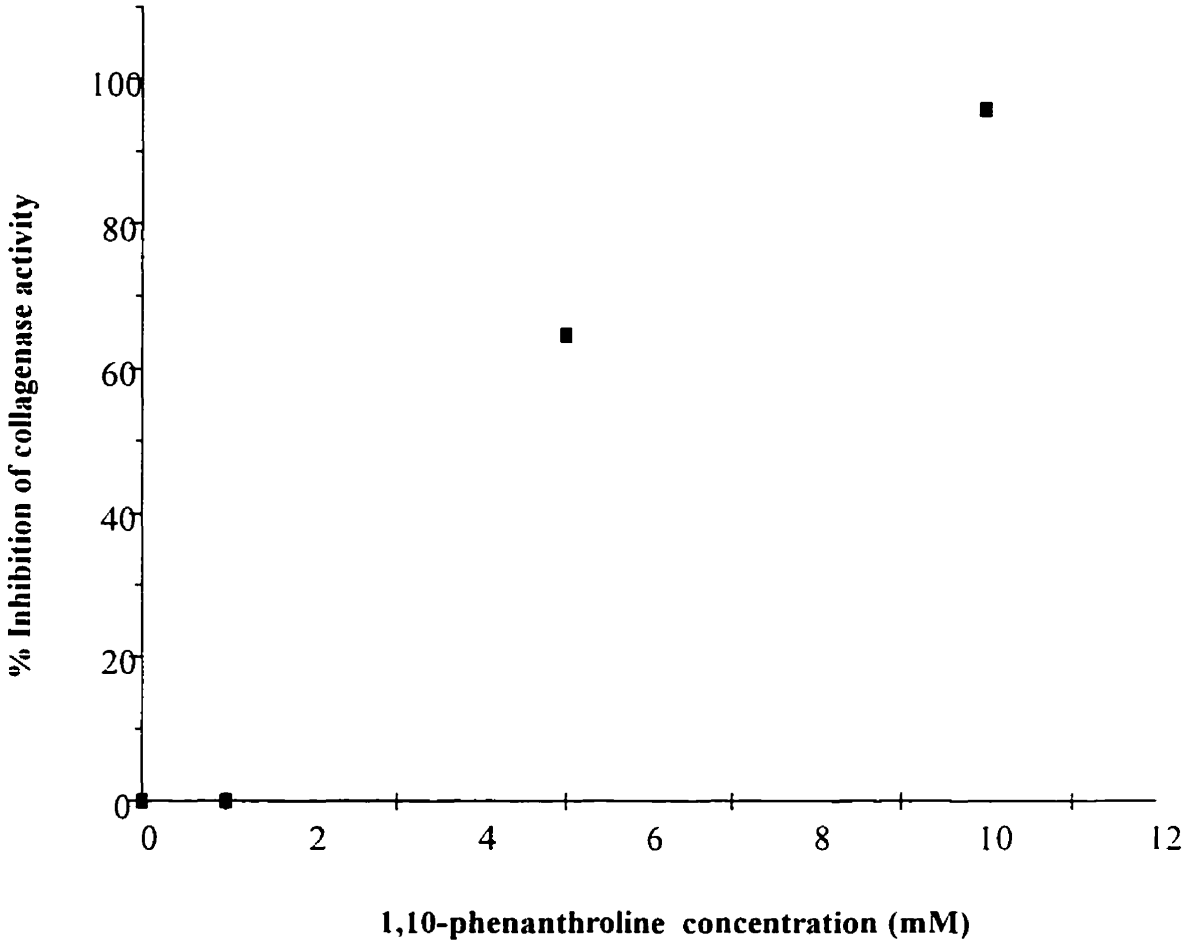


Fig. 28. Influence of various concentrations of 1,10-phenanthroline on the activity of winter flounder skeletal muscle collagenases (SEC fraction, 0.08 mg/ml; PZ-peptide, 0.64 mM at pH 7.1 and 37 C). (Values presented are means of 3 replicates for 1 experiment)

Table 13. I_{50} values of various inhibitors on winter flounder skeletal muscle collagenases with the use of paraphenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg as substrate.

Inhibitor	I_{50} (mM)
1,10-phenanthroline	1.84 ± 0
EDTA	151.47 ± 21.35
2-mercaptoethanol	118 ± 2.66
Dithiothreitol	5.22 ± 1.11

Data presented are means of 3 replicates for 1 experiment.

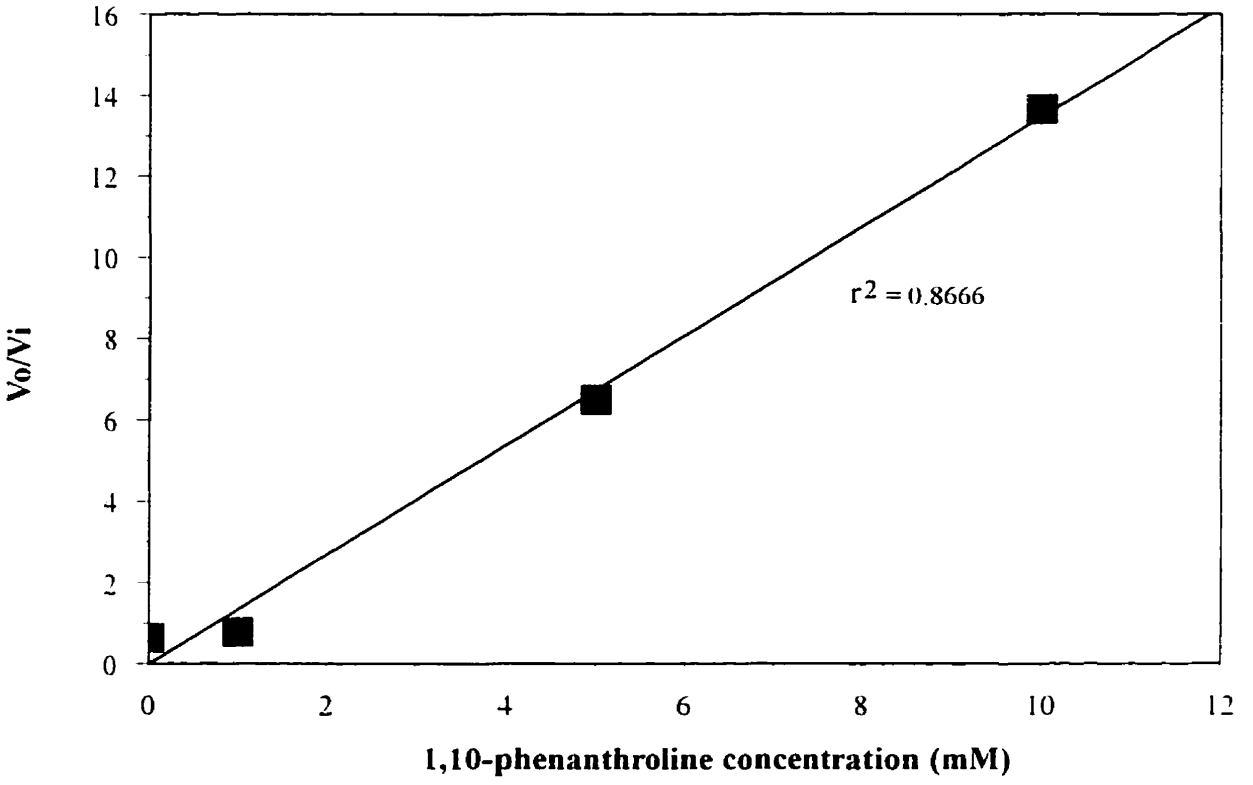


Fig. 29. A plot of Vo/Vi against various 1,10-phenanthroline concentrations. (Data used to plot the figure are average values of 3 replicates for 1 experiment using regression analysis.)

1984a; Matsushita et al., 1984; Makinen and Makinen, 1987). It has been observed that freezing of fish collagenase in the presence of calcium ions increased enzyme stability, suggesting that calcium could be an important component of the fish collagenase. The presence of calcium ions was observed to be important in maintaining the stability of the fish collagenase. The fish collagenase (SEC fraction) frozen without calcium ions lost its activity after about a month of storage at -80°C. SEC fraction in 0.5 mM Tris-HCl, pH 7.4 with 5 mM $CaCl_2$ was still active after 11 months of storage at the same temperature, although there was a decrease of activity to about 50% (specific activity decreased from ~218 units/mg to 99 units/mg). The action of EDTA could be directed towards calcium and not zinc since the addition of zinc in the presence of EDTA did not restore collagenase activity as discussed in section 4.9. Studies have shown that addition of calcium ions reversed the inhibitory action of EDTA on collagenase (Lecroisey *et al.*, 1975; Van Wart and Steinbrink, 1981). Increasing levels of EDTA resulted in increasing degree of inhibition (Fig. 30). Fifty percent inhibition was achieved at a concentration of ~151 mM EDTA, the I_{50} value for EDTA determined from the relationship shown in Fig. 31. A mixed type of inhibition on fish skeletal muscle collagenases was exhibited by EDTA as shown by the Lineweaver-Burk reciprocal plot (Fig. 32). A mixed type of inhibitor affects both K_m' and V_{max} (Kuby, 1991). V_{max} values decreased in the presence of EDTA, however the K_m' increased (Table 14). The difference between the V_{max} for both EDTA concentrations (100 mM and 200 mM) was low suggesting possible saturation of the enzyme with EDTA such that further increases in the inhibitor concentration did not have an adverse effect on the V_{max}. The inhibitor constants, K_i and αK_i (Table 15), were estimated from the secondary replots of the slopes and intercepts from the Lineweaver-Burk plots against inhibitor concentration as shown in Figs. 33 and 34, respectively, following the method of Kuby (1991). The Lineweaver-Burk reciprocal plots if extended downwards will intersect at points above the X-axis. In this type of mixed inhibition, the K_i is less than αK_i (Dixon and Webb, 1979), suggesting that the affinity of EDTA to the

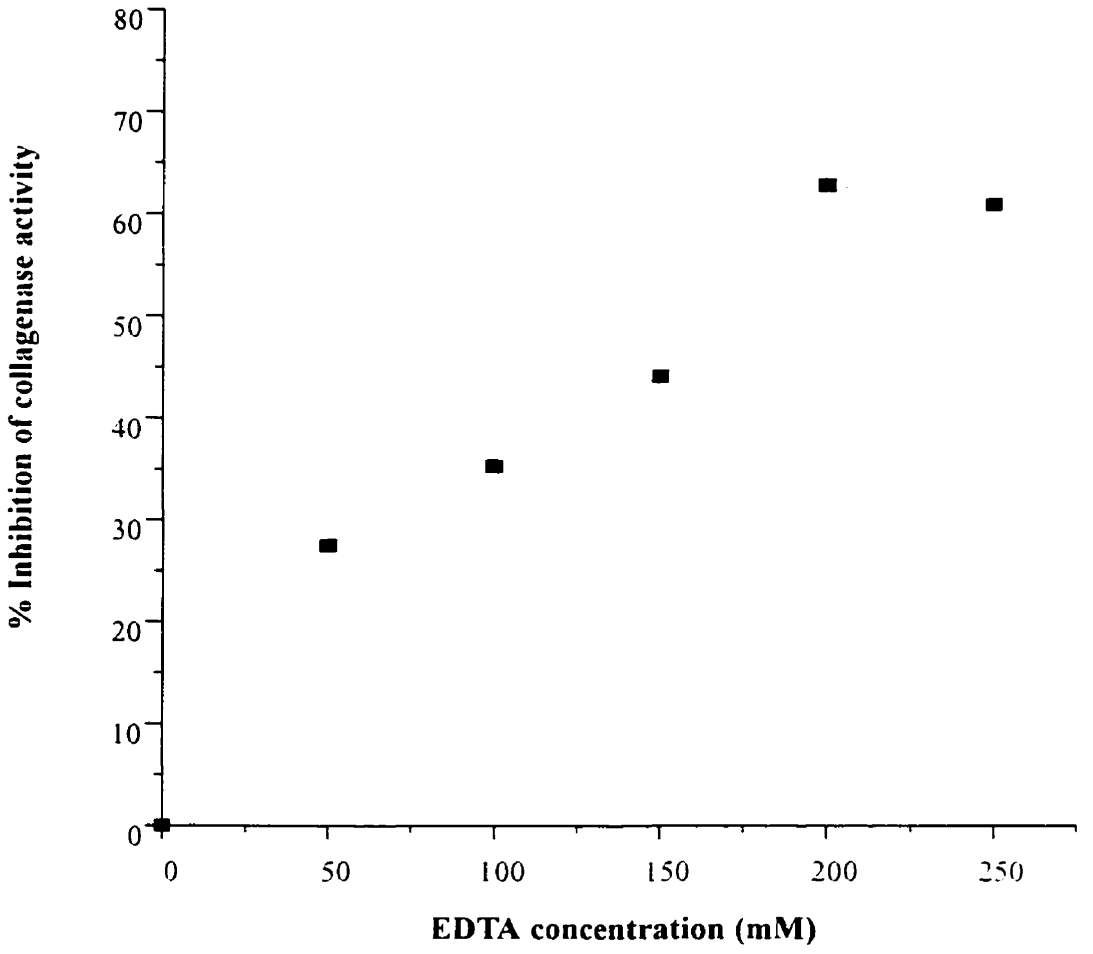


Fig. 30. Influence of various EDTA concentrations on the activity of skeletal muscle collagenases (SEC fraction, 0.08mg/ml; PZ-peptide, 0.64 mM at pH 7.1 and 37 C).
(Values presented are means of 3 replicates for 1 experiment)

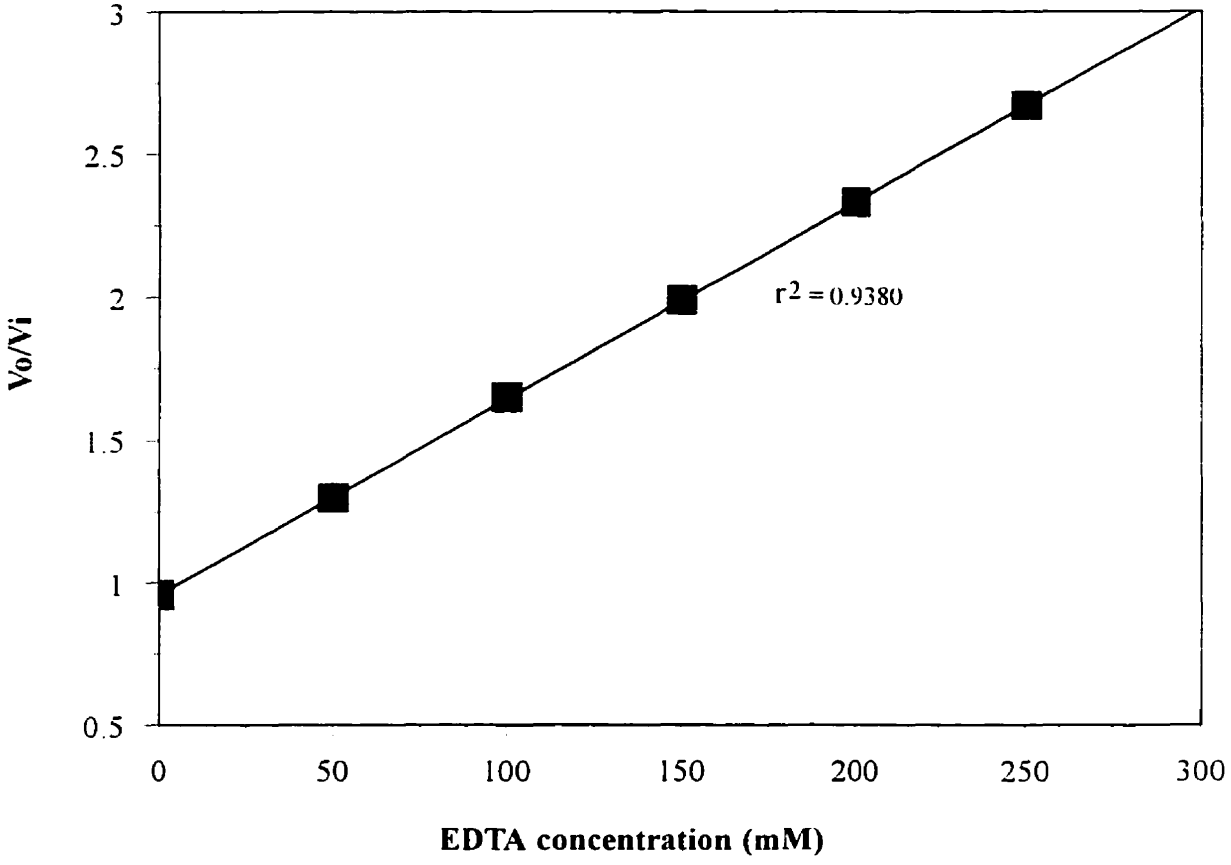


Fig 31. A plot of Vo/Vi against various EDTA concentrations. (Data used to plot the figure are average values of 3 replicates for 1 experiment using regression analysis.)

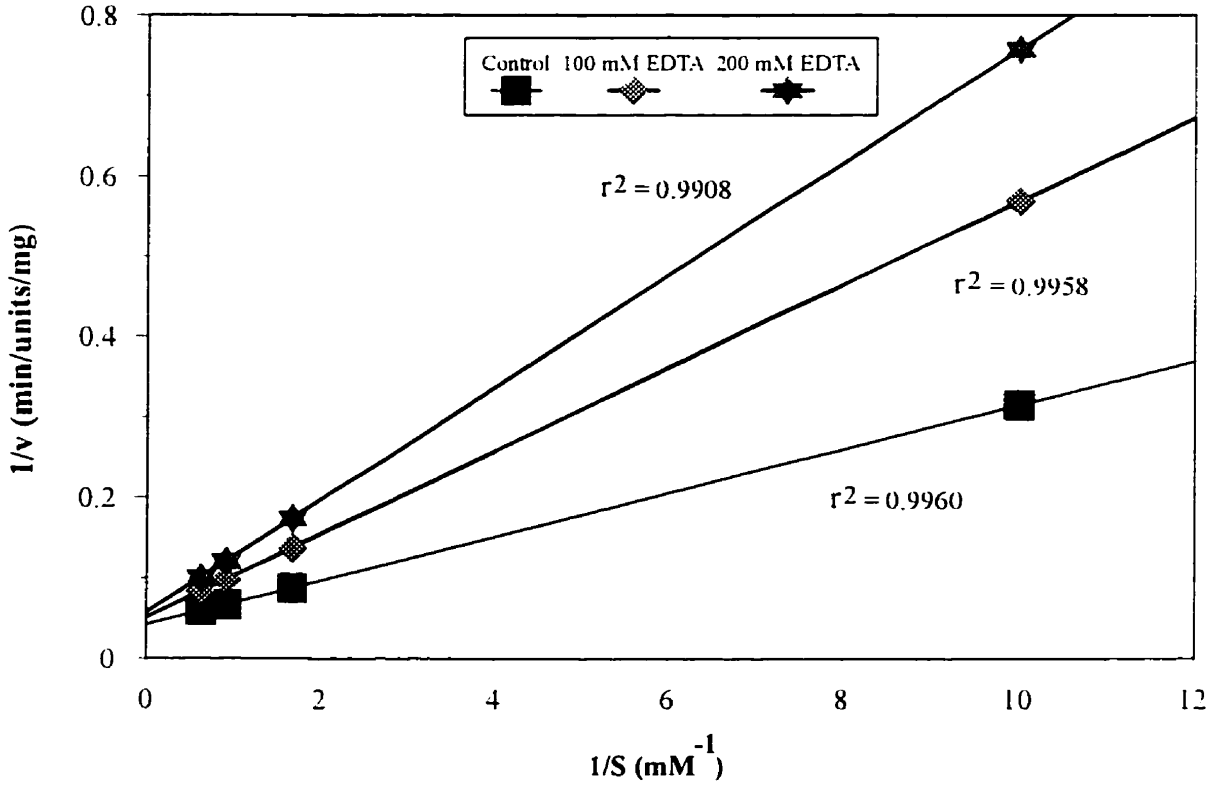


Fig. 32. Lineweaver -Burk reciprocal plot for WFC for the determination of the type of inhibition caused by EDTA with PZ as substrate (0.10 - 1.60 mM) at pH 7.1 and 37 C.
(Data used to plot the figure are average values of 3 replicates for 2 experiments for the control and 100 mM EDTA, and 3 replicates for 1 experiment for 200 mM EDTA using regression analysis.)

Table 14. Influence of inhibitors on the kinetic parameters, K_m and V_{max} of winter flounder muscle collagenases (WFC) with ρ-phenylazobenzyloxycarbonyl-pro-leu-gly-pro-D-arg as substrate.

Inhibitor	K_m, (mM)	V_{max} (units/min)
None (control)	0.65±0.10	23.79±5.70
100 mM EDTA	1.03±0.09	19.91±2.07
200 mM EDTA	1.24±0.11	17.72±1.53
50 mM 2-mercaptoethanol	0.51±0.04	11.66±1.28
100 mM 2-mercaptoethanol	0.48±0.02	9.96±1.18

Data are average values of 3 replicates for 1 set of experiment for 200 mM EDTA and 50 mM 2-mercaptoethanol; average values of 3 replicates for 2 experiments for the control, 100 mM EDTA and 100 mM 2-mercaptoethanol.

Table 15. Inhibitor constant values (K_i and αK_i) for EDTA and 2-mercaptoethanol with PZ-peptide as substrate.

Inhibitor	K_i (mM)	αK_i (mM)
EDTA	129.77	588.43
2-mercaptoethanol	139.54	80.45

The data presented were obtained from secondary replots of slopes and intercepts of the primary plots (Lineweaver-Burk reciprocal plots) vs inhibitor concentration to estimate the K_i and the αK_i, respectively, using regression analysis.

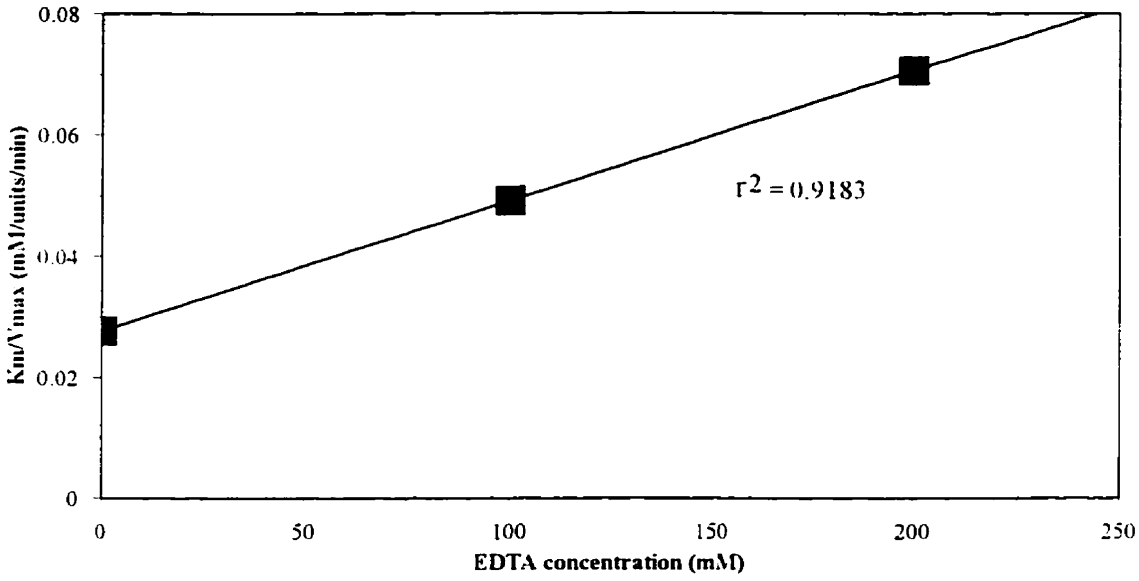


Fig. 33. Secondary replot of slopes from the primary reciprocal plots (Lineweaver-Burk) for EDTA as inhibitor.

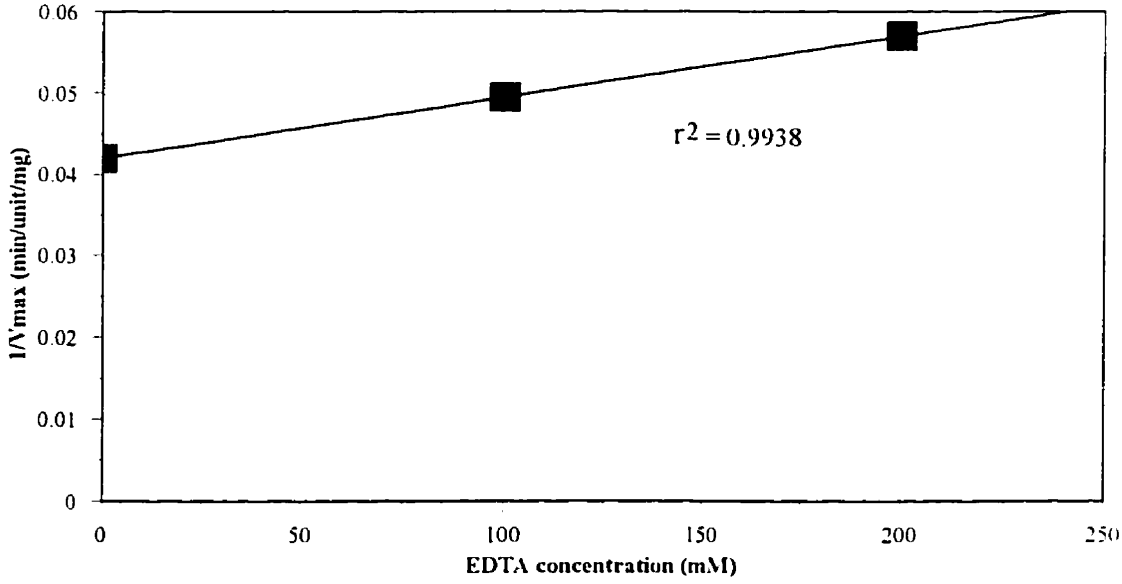


Fig. 34. Secondary replot of intercepts from the primary reciprocal plots (Lineweaver-Burk) for EDTA as inhibitor.

the enzyme alone is greater than its affinity to the enzyme bound to the substrate, i.e., the ES complex.

4.8.3 Inhibitory action of 2-mercaptoethanol

The inhibitory effect of 2-mercaptoethanol suggests the binding of cysteinyl and histidyl residues (Whitaker, 1972). 2-Mercaptoethanol is a thiol-protease inhibitor, however, it has been shown to bind metalloenzyme collagenases from bacteria (Harper, 1972; Seifter *et al.*, 1959) and other sources (McCroskery *et al.*, 1975; Woolley *et al.*, 1978; Tyler and Cawston, 1980). It had been suggested that the inhibitory action of 2-mercaptoethanol and similar compounds, e.g., cysteine and 2,3-mercaptopropanol for *C. histolyticum* collagenase is directed towards zinc since such enzymes were shown to be deficient in free disulfide groups or low in amino acids such as cystine and cysteine (Harper, 1972; Seifter *et al.*, 1959). On the other hand, fish skeletal muscle collagenases were shown to contain higher levels of cysteine (31 and 15 residues per molecule, for WFC-1 and WFC-2, respectively) than *C. histolyticum* collagenases (range of 2.2 - 4.5 residues per molecule [Bond and Van Wart, 1984a]). The inhibitory effect of 2-mercaptoethanol and similar compounds, e.g., dithiotreitol, could be directed towards two different sites, first, towards zinc suggested to be located in the active site, and second, the S-containing amino acid residues of these fish skeletal muscle collagenases. Increasing levels of 2-mercaptoethanol resulted in increased inhibition of the fish collagenase (Fig. 35). 2-Mercaptoethanol had an I_{50} of ~119 mM with the PZ-peptide as substrate as determined from Fig. 36. Similar to EDTA, 2-mercaptoethanol exhibited a mixed-type inhibition (Fig. 37). Both the V_{max} and K_m' decreased in the presence of 2-mercaptoethanol (Table 14). The inhibitor constants, K_i and αK_i, were estimated from the secondary replots of the slopes and intercepts from the Lineweaver-Burk plots as shown in Figs. 38 and 39, respectively. The mode of action of 2-mercaptoethanol differs from

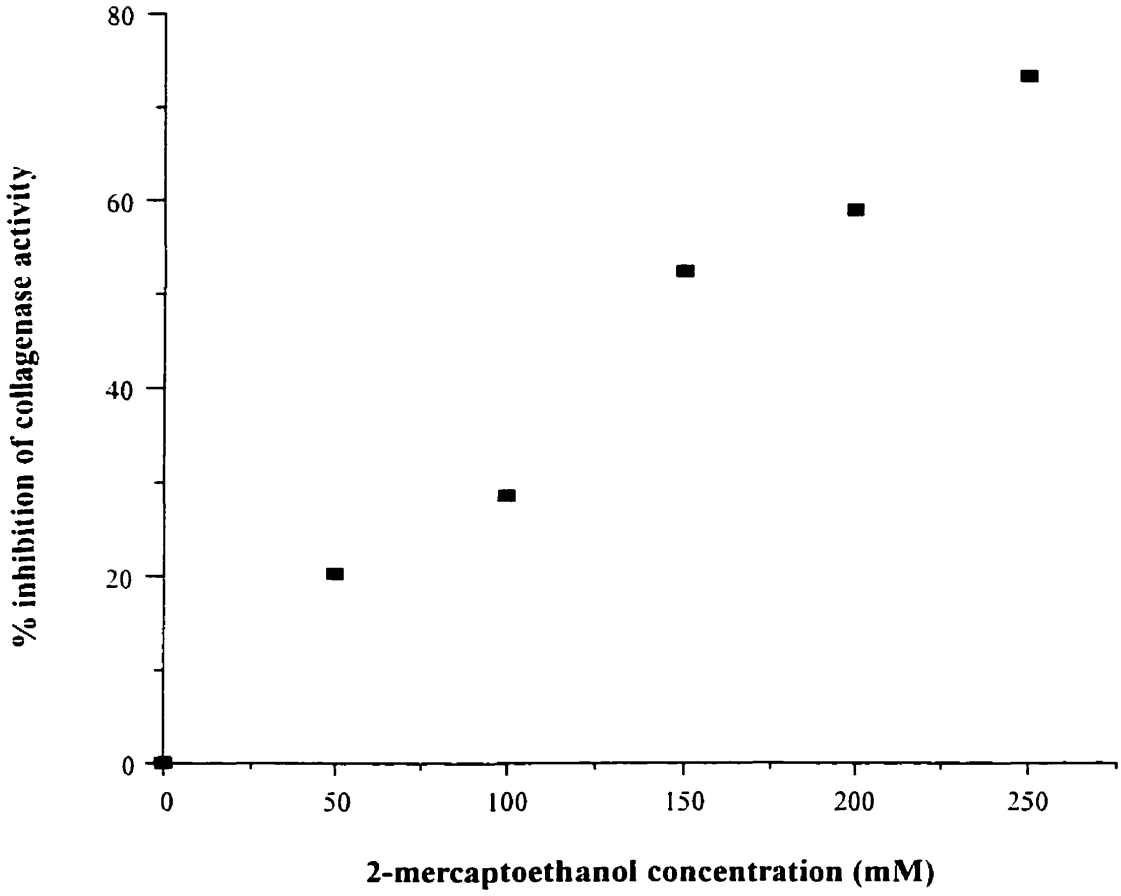


Fig. 35. Influence of various 2-mercaptoethanol concentrations on the activity of skeletal muscle collagenases (SEC fraction, 0.08 mg/ml; PZ-peptide, 0.64 mM at pH 7.1 and 37C).
(Values presented are means of 3 replicates for 1 experiment)

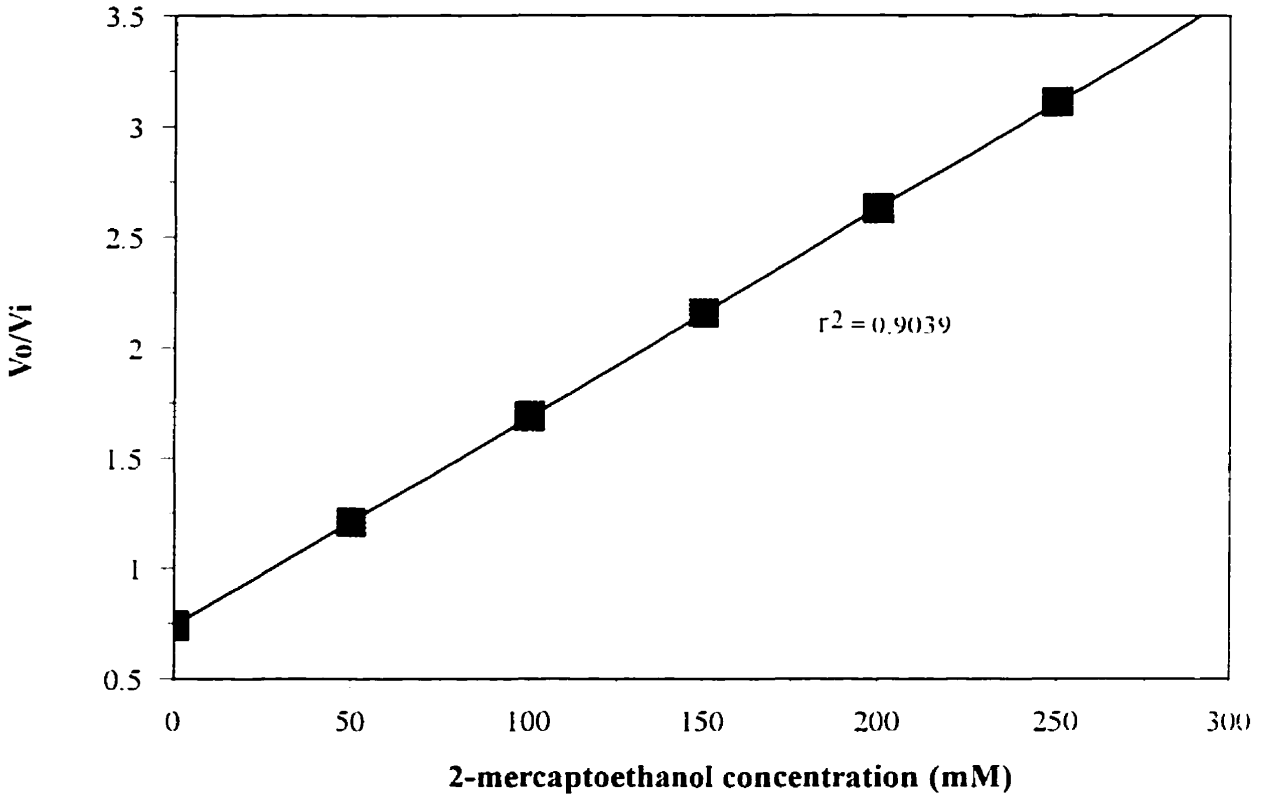


Fig. 36. A plot of Vo/Vi against various 2-mercaptoethanol concentrations. (Data used to plot the figure are average values of 3 replicates for 1 experiment using regression analysis.)

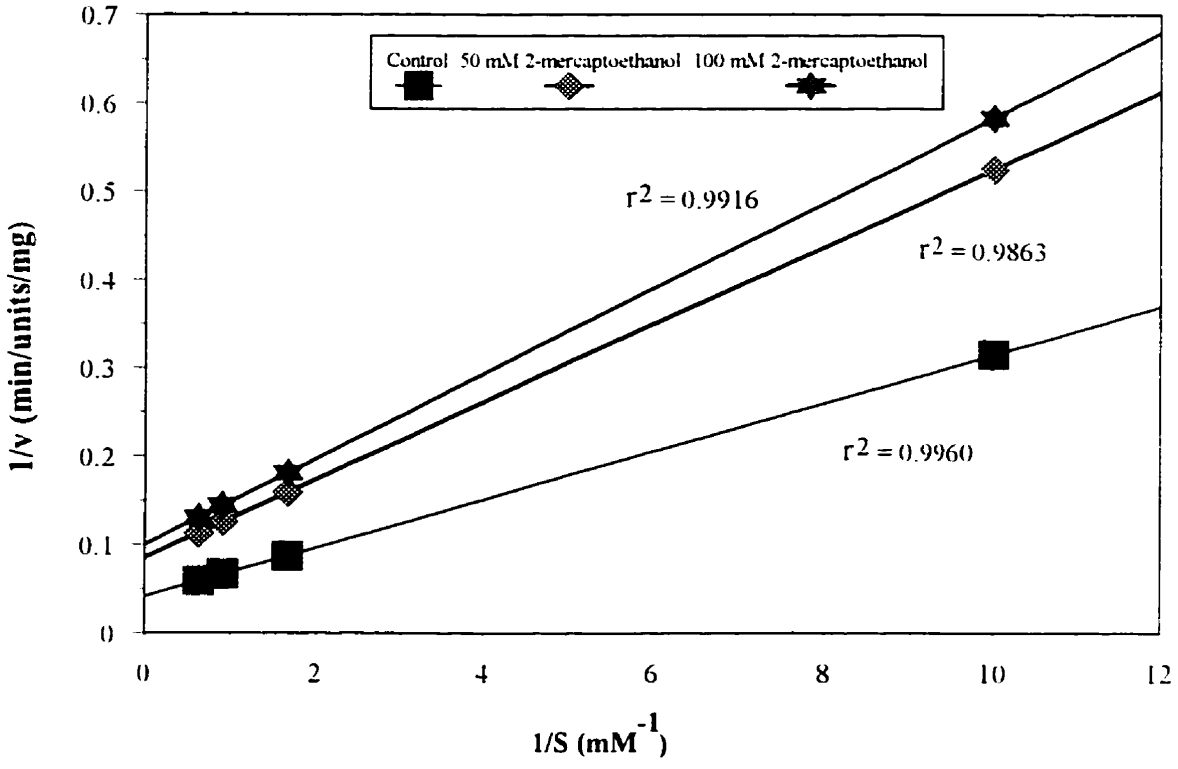


Fig. 37. Lineweaver -Burk reciprocal plot for WFC for the determination of the type of inhibition caused by 2-mercaptoethanol with PZ as substrate (0.10 - 1.60 mM) at pH 7.1 and 37 C.
(Data used to plot the figure are average values of 3 replicates for 2 experiments for the control and 100 mM 2-mercaptoethanol, and 3 replicates for 1 experiment for 50 mM 2-mercaptoethanol using regression analysis.)

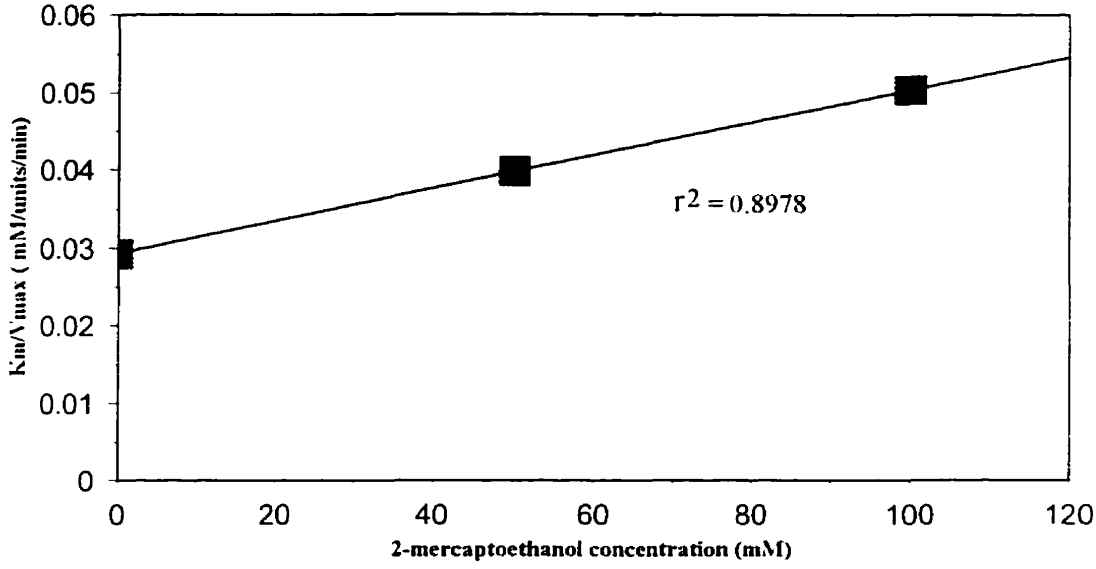


Fig. 38. Secondary replot of slopes from the primary reciprocal plots (Lineweaver-Burk) for 2-mercaptoethanol as inhibitor.

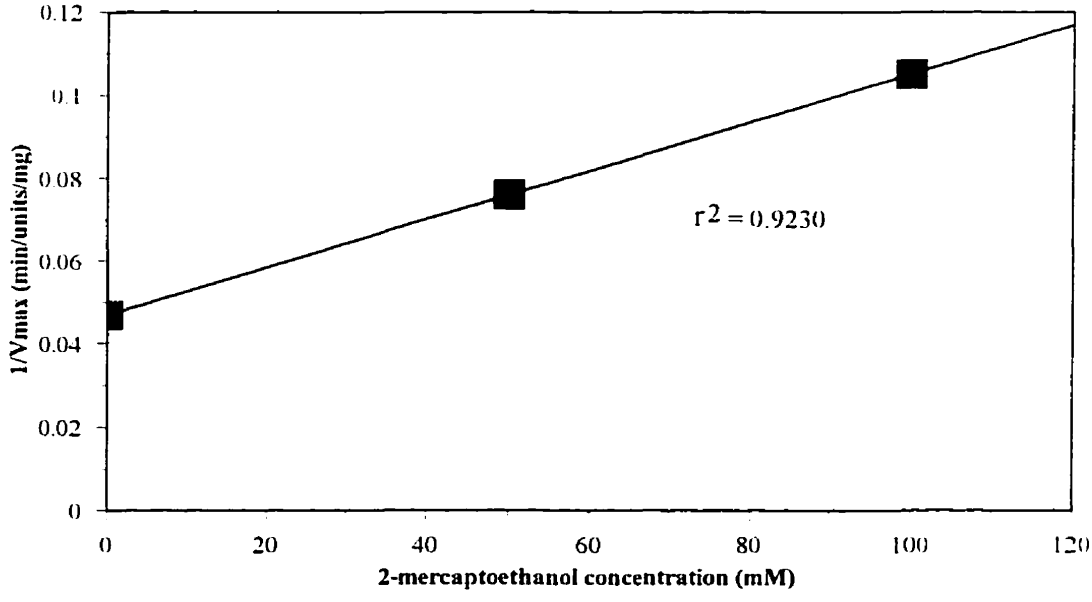


Fig. 39. Secondary replot of intercepts from the primary reciprocal plots (Lineweaver-Burk) for 2-mercaptoethanol as inhibitor.

that of EDTA. Extension of the plots in the Lineweaver-Burk reciprocal plots downwards will show that the lines will intersect below the X-axis. This type of inhibition with K_i values greater than αK_i (Dixon and Webb, 1979) manifested by 2-mercaptoethanol (Table 15), suggests that the affinity of 2-mercaptoethanol to the enzyme alone is lower than its affinity to the enzyme- substrate (ES) complex.

4.8.4 Inhibitory action of dithiothreitol (DTT)

Similar to 2-mercaptoethanol, dithiothreitol is a thiol protease inhibitor. In like manner, its inhibitory action towards the fish collagenase could be directed towards the S-containing amino acids and the zinc ions. However, DTT is a more effective inhibitor for the fish collagenase than 2-mercaptoethanol based on the I_{50} values. Increasing degree of inhibition was observed as the levels of DTT increased (Fig. 40). Fifty percent inhibition was effected at ~5 mM DTT, the I_{50} value for the inhibitor, while for 2-mercaptoethanol the I_{50} value was 118 mM. A plot of V_o/V_i against DTT concentration is shown in Fig. 41.

4.9 Influence of Zn and Ca ions on the collagenase activity of the fish enzyme

Based on the results in section 4.8 studies were conducted to determine if zinc ions influenced the collagenase activity.

An attempt to remove Zn (a component of metalloenzyme collagenases) was conducted by dialysis. However, this method had a negligible effect on the activity of the fish enzyme (Fig. 42), suggesting that Zn if present, was strongly bound to the fish enzyme. Incorporation of Zn^{++} in the reaction mixture failed to enhance the collagenase activity of the fish enzyme, instead inhibition was observed (Fig. 43). Increasing concentrations of Zn^{++} resulted in a decrease in the activity of the fish

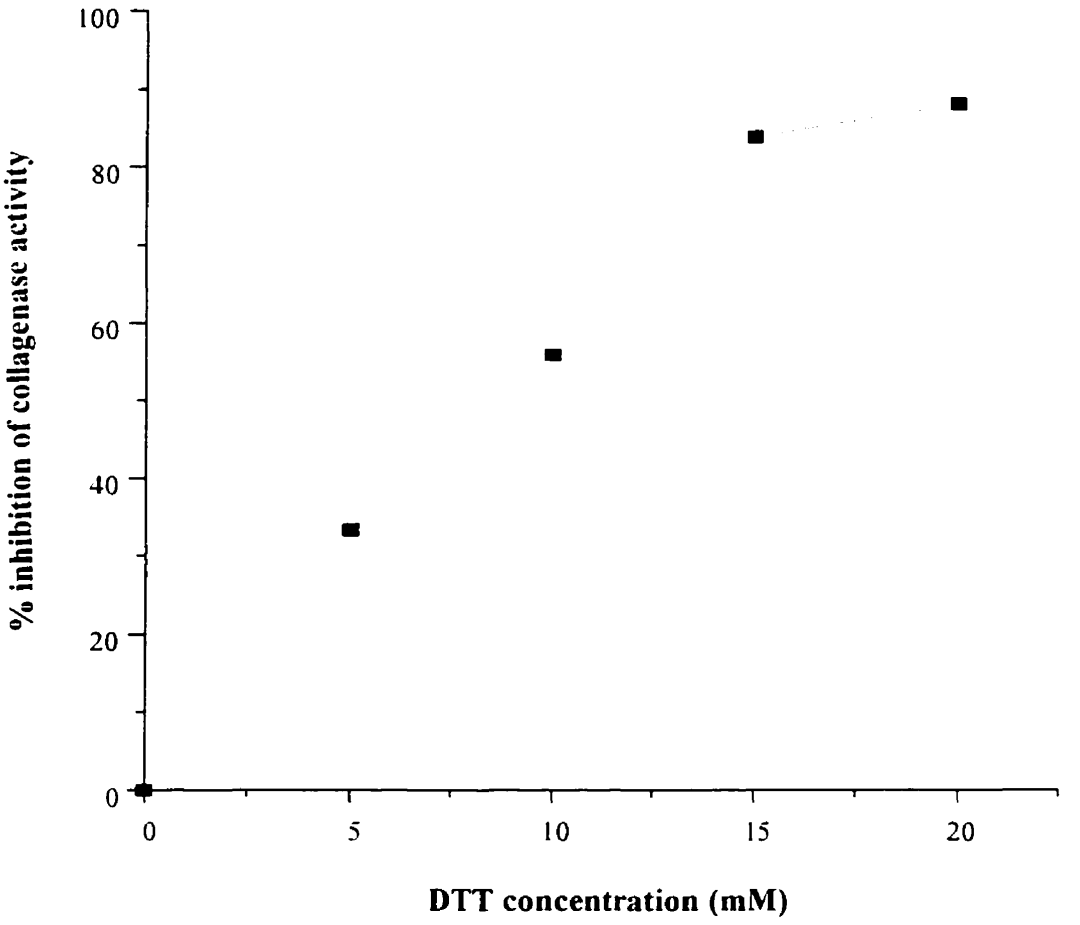


Fig. 40. Influence of various dithiotreitol (DTT) concentrations on the activity of skeletal muscle collagenases (SEC fraction, 0.08 mg/ml; PZ-peptide, 0.64 mM at pH 7.1 and 37 C).
(Values presented are means of 3 replicates for 1 experiment)

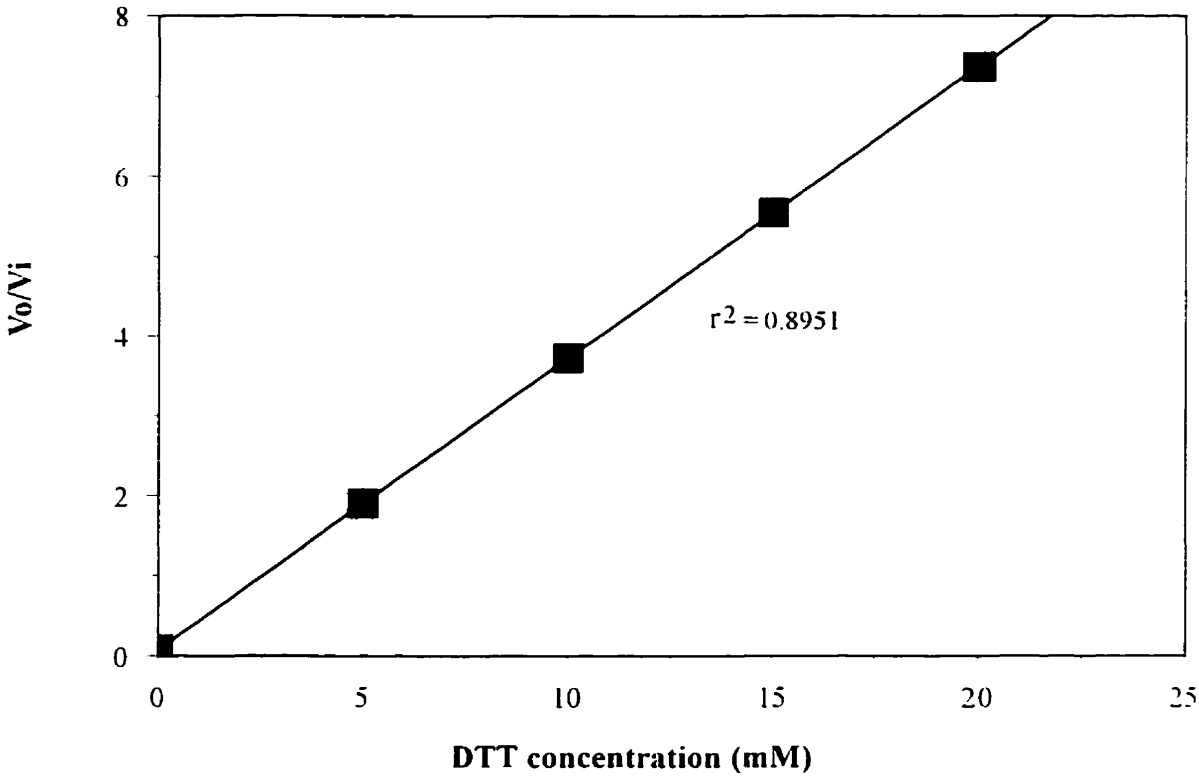


Fig. 41. A plot of Vo/Vi against various dithiothreitol (DTT) concentrations. (Data used to plot the figure are average values of 3 replicates for 1 experiment using regression analysis.)

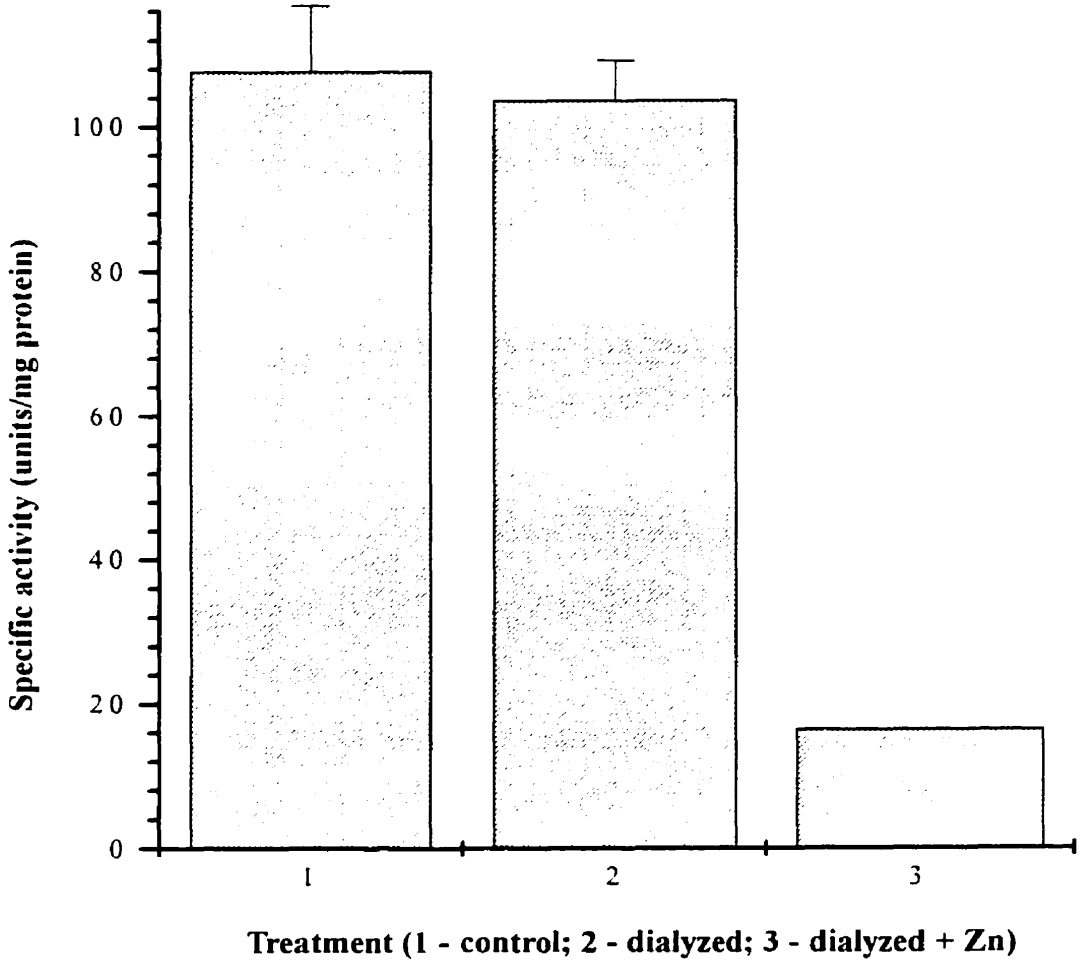


Fig. 42. Influence of dialysis and addition of Zn ion on the activity of winter flounder skeletal muscle collagenases (SEC fraction, 0.062 mg/ml; PZ-peptide, 0.64 mM)). (Values presented are means of 3 replicates for 1 experiment)

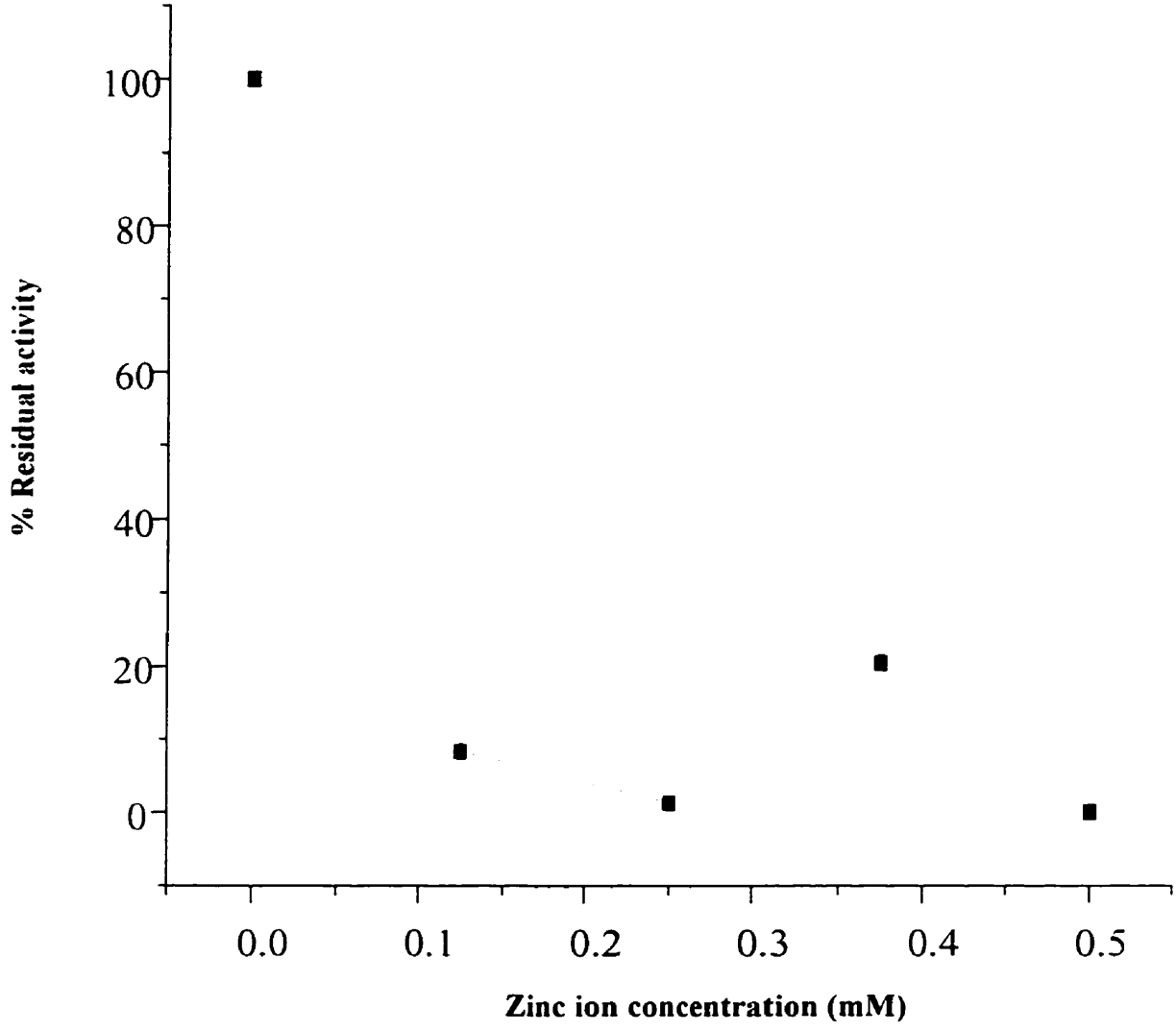


Fig.43. Influence of various zinc ion concentrations on the activity of skeletal muscle collagenases (SEC fraction, 0.08 mg/ml; PZ-peptide, 0.64 mM at pH 7.1 and 37 C).
(Values presented are means of 3 replicates for 1 experiment)

enzyme, suggesting that although zinc is an important component of collagenase, excess zinc ions in solution could cause inhibition. The inhibitory effect of zinc had been observed in collagenases from rabbit bone (Swann *et al.*, 1981) and *Clostridium perfringens* (Matsushita *et al.*, 1994). This inhibitory action of excess zinc ions is due to the formation of zinc monohydroxide that bridges the catalytic ion to the side chain in the active site of the enzyme (Larsen and Auld, 1988).

Low levels of zinc ions reversed the inhibitory activity of 1,10-phenanthroline against the fish collagenases and maximum reactivation was obtained at 1.0 mM Zn^{++}, beyond which the degree of reactivation decreased (Fig. 44). The influence of Zn^{++} in reversing the inhibitory action of 1,10-phenanthroline was observed in rabbit bone collagenase (Swann *et al.*, 1981) and bacterial collagenases (Van Wart and Steinbrink, 1981; Makinen and Makinen, 1987). As stated earlier metal chelators can either form complexes with and/or remove the essential cation from the apoenzyme, subsequently inactivating the enzyme. If the removal of the cation does not cause irreversible conformational changes in the apoenzyme, addition of the cation back to the enzyme will restore its activity (Whitaker,1972). Swann et al. (1981) suggested that Zn^{++} apparently reactivates collagenase, not by removal of chelator but by maintaining a sufficient concentration of the free Zn^{++} to ensure that the dissociation equilibrium favors the Zn^{++}-containing holoenzyme rather than the inactive apoenzyme. In the case of the fish collagenases addition of low levels of Zn^{++} re-activated the enzyme, suggesting that inhibition by 1,10-phenanthroline did not alter the conformation of the apoenzyme. Thus inhibition of the fish enzyme by this chelator may have resulted from the removal of the metal ion, i.e. Zn^{++}, which could be an important component of the fish collagenase enzyme. The use of 1,10-phenanthroline as a chelating agent is usually diagnostic for a Zn^{++} metallo-proteinase (Salvesen and Nagase, 1989).

Low levels of Zn^{++} failed to reactivate the fish collagenase enzyme fraction in the presence of EDTA (Fig. 45). Instead, a higher degree of inhibition was observed in the

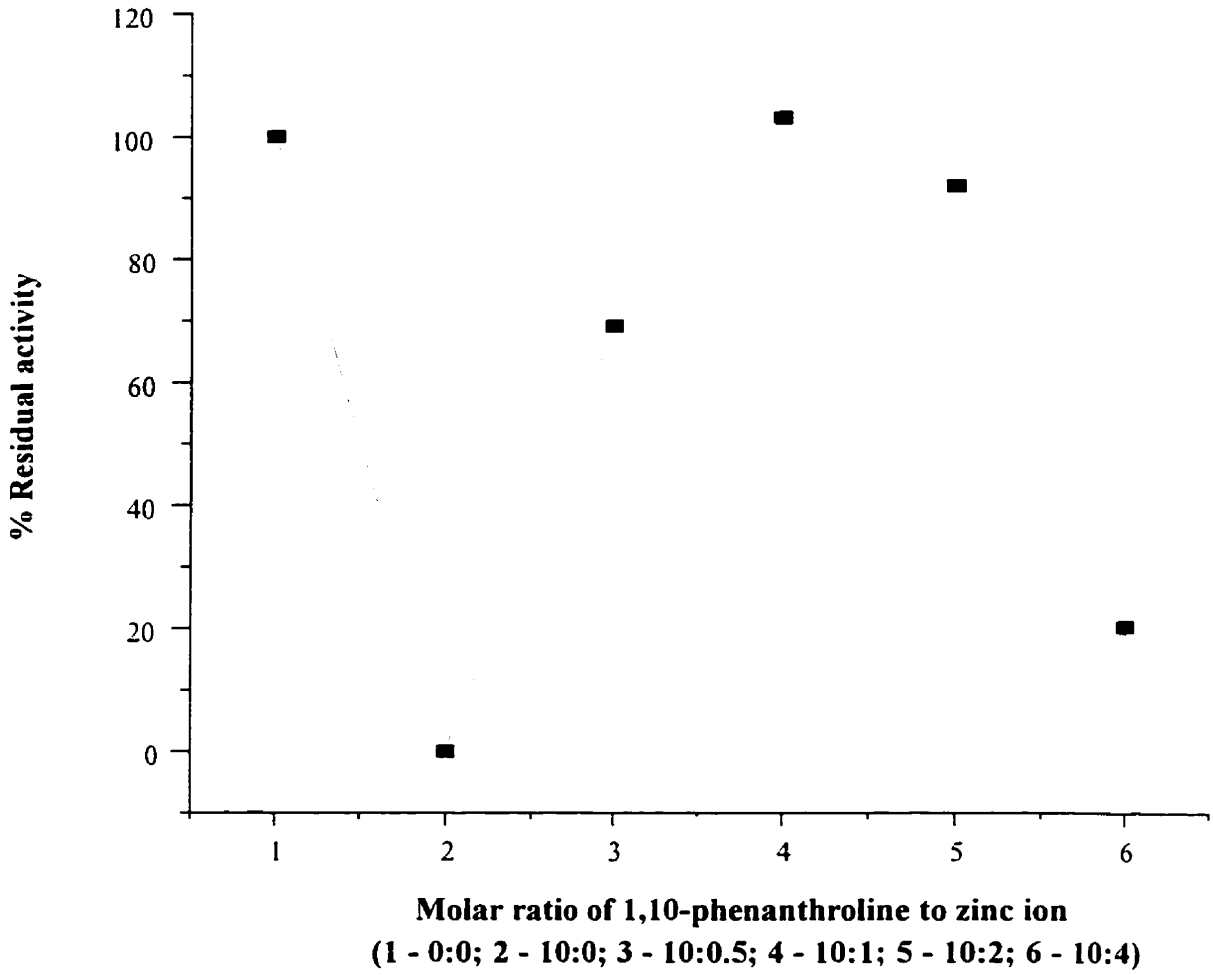


Fig. 44. Inhibition of winter flounder skeletal muscle collagenases (SEC fraction, 0.08 mg/ml) by 1,10-phenanthroline (10.0 mM) and their reactivation by various concentrations of zinc ions (0.5, 1.0, 2.0 and 4.0 mM) (PZ-peptide, 0.64 mM at pH 7.1 and 37 C).
(Values presented are means of 3 replicates for 1 experiment)

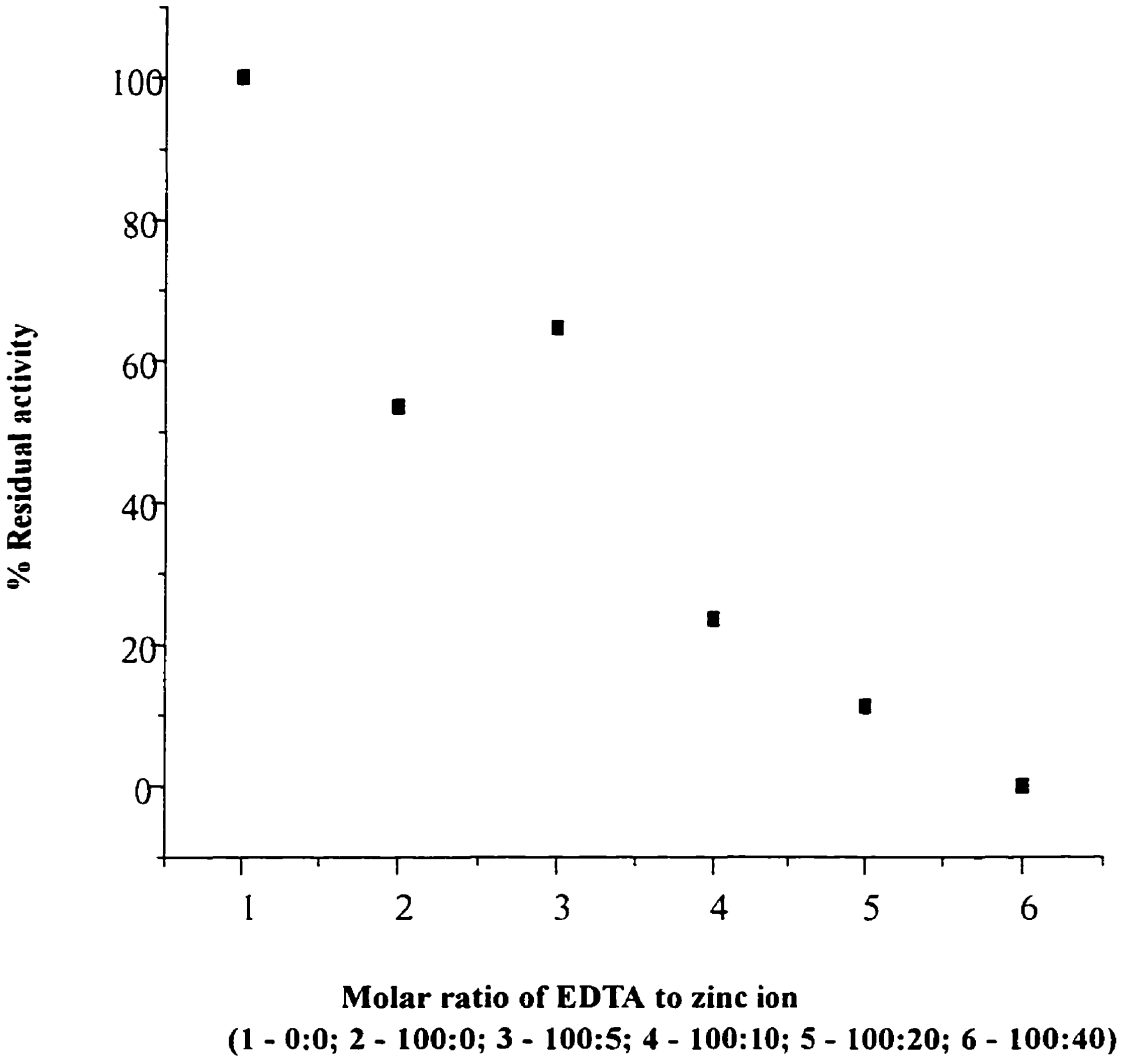


Fig. 45. Influence of various concentrations of zinc ions (5, 10, 20 and 40 mM) on the inhibitory action of EDTA (100 mM) on winter flounder skeletal muscle collagenases (SEC fraction, 0.08 mg/ml; PZ-peptide, 0.64 mM at pH 7.1 and 37 C) (Values presented are means of 3 replicates for 1 experiment)

presence of both EDTA and Zn^{++}, suggesting that the mechanism of the fish enzyme inhibition by EDTA differs from that of 1,10-phenanthroline. The same result was observed for bacterial collagenases (Van Wart and Steinbrink, 1981; Makinen and Makinen, 1987), in contrast to the behavior of rabbit bone collagenase (Swann *et al.*, 1981). The inhibitory action of EDTA could be directed towards calcium as mentioned in section 4.8.2. Studies have shown that addition of calcium ions reversed the inhibitory action of EDTA on collagenase (Lecroisey *et al.*, 1975; Van Wart and Steinbrink, 1981). Similarly, addition of calcium ions in the fish collagenase-EDTA mixture recovered enzyme activity (Fig. 46). Addition of 5 mM calcium ions (making a total of 10 mM calcium ions in the mixture) was sufficient to restore enzyme activity to almost 100%. However, further increase in the calcium concentration failed to restore collagenase activity. Excess amount of calcium ions over EDTA levels was suggested to restore enzyme activity, however, in the case of fish collagenases calcium concentrations beyond 10 mM but lower than EDTA concentration seemed to inhibit enzyme activity. The inhibitory action of calcium ions could be directed towards the transformation site, i.e., carboxyl groups in the active site of the enzyme, similar to the action of hydrogen ions when the enzyme is exposed to acidic conditions (Chapman and Burke, 1990).

4.10 Kinetic and thermodynamic properties of fish collagenases (WFC-1 and WFC-2) and *C. histolyticum* collagenases

The kinetic and thermodynamic properties of the fish collagenases and *C. histolyticum* collagenases are presented in Table 16. The K_m' for the hydrolysis of p-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg to PZ-Pro-Leu by the fish skeletal muscle collagenase (0.65 mM) is similar to that of *C. histolyticum* collagenases (0.66 mM). The turnover number (V_{max}) however, for the same substrate was higher for *C. histolyticum* than for the fish muscle collagenases. The catalytic efficiency which is the

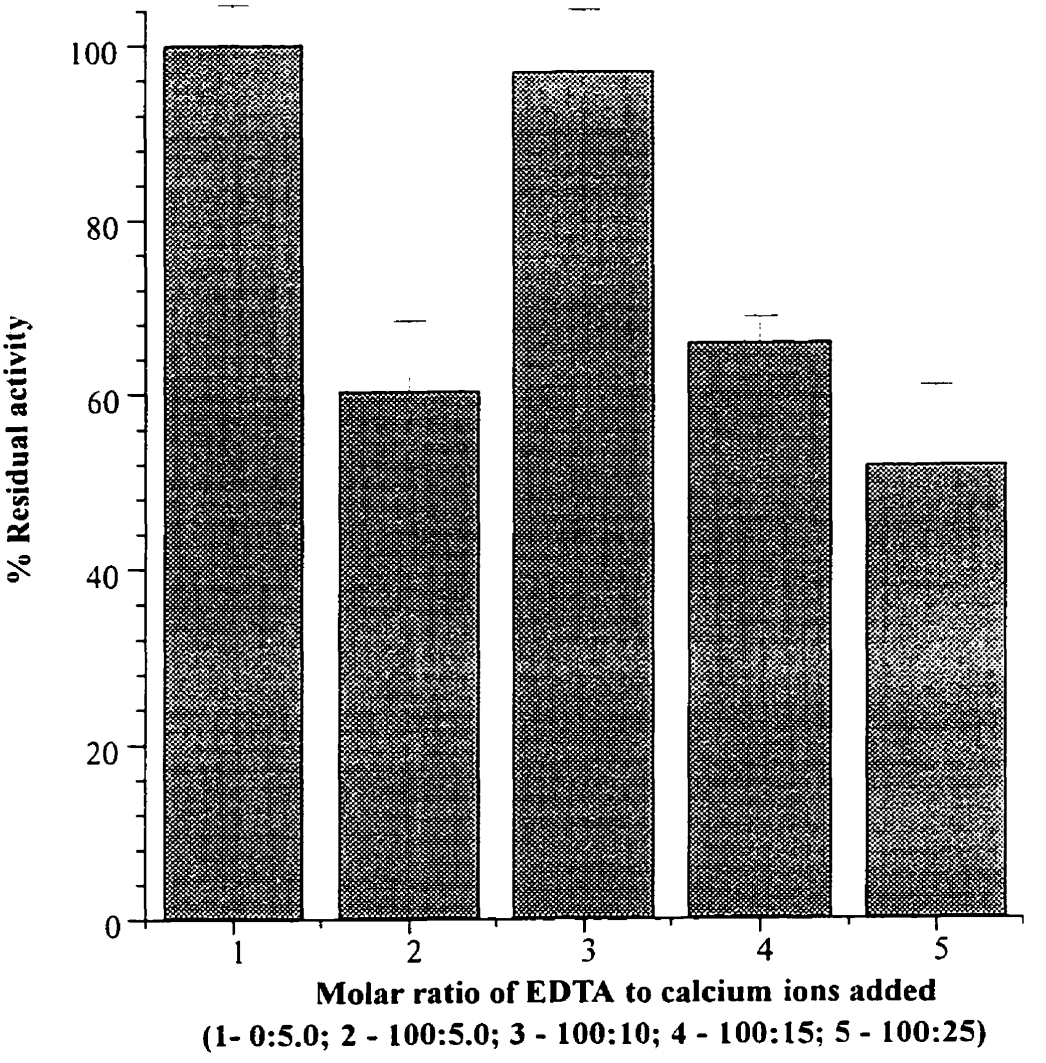


Fig. 46. Influence of various concentrations of calcium ions (5, 10, 15 and 25 mM) on the inhibitory action of EDTA (100 mM) on winter flounder skeletal muscle collagenases (SEC fraction, 0.072 mg/ml; PZ-peptide, 0.64 mM at pH 7.1 and 37 C)
(Values presented are means of 3 replicates for 1 experiment)

Table 16. Kinetics and thermodynamic properties of winter flounder muscle collagenases (WFC) (SEC fraction) and *Clostridium histolyticum* collagenases with PZ-pro-leu-gly-pro-D-arg as substrate.

Parameter	WFC	*C. histolyticum* collagenase
K_m' (mM)	0.65±0.10	0.66±0.10
V_{max} (units/min)	23.79±5.7	175.35±10.29
V_{max}/K_m' (units/min/mM)	36.60±2.73	265.68±56
E_a (kcal/mole)*	7.23±0.53 ($3.02 \times 10^4 \pm 0.22 \times 10^4$)	11.98±0.76 ($5.01 \times 10^4 \pm 0.32 \times 10^4$)
ΔH (kcal/mole)**	6.61±0.53 ($2.89 \times 10^4 \pm 0.22 \times 10^4$)	11.36±0.76 ($4.75 \times 10^4 \pm 0.32 \times 10^4$)
ΔS (e. u.)**	-39.64±1.73	-20.27±2.47
ΔG (kcal/mole)**	18.90 ± 0 ($7.91 \times 10^4 \pm 0$)	17.64 ± 0 ($7.38 \times 10^4 \pm 0$)

*E_a values were estimated from temperature activity- profiles from 5 - 35°C **Estimated at 37°C. Values in parenthesis are equivalent values in joules (J)/mole. $\Delta G = \Delta H - T\Delta S$; $\Delta H = E_a - RT$; $\Delta S = 4.576(\log K - 10.753 - \log T + E_a/4.576T)$. The data presented for WFC for kinetic properties are average values of triplicate determinations for 2 experiments and for the thermodynamic properties the average values of triplicate determinations for 1 experiment. For *C. histolyticum*, the data presented for kinetic and thermodynamic properties are average values of triplicate determinations for 1 experiment. The protein concentrations of the stock solutions used in this study were 0.120 mg/ml for WFC and 0.010 mg/ml for *C. histolyticum*.

ratio of V_{max} to K_m' (Whitaker, 1972) of the collagenases was higher for *C. histolyticum* collagenases than for the fish muscle collagenases, estimated to be 265.68 units/mg/min/mM and 36.60 units/mg/min./mM, respectively. Based on the catalytic efficiencies, *C. histolyticum* collagenases appear to be more suited for the hydrolysis of PZ-peptide than the fish muscle collagenases at 37°C and pH 7.1.

The free energy of activation (ΔG) for the fish muscle collagenases (18.90 kcal/mole) was higher than that of the *C. histolyticum* collagenases (17.64 kcal/mole), suggesting that the bacterial enzyme is a more efficient catalyst than the fish enzyme for the hydrolysis of PZ-peptide at 37°C and pH 7.1. In biological reactions, reactants are formed into products after overcoming an "energy barrier" or ΔG. Enzymes catalyze these reactions by reducing the energy barrier, thus it follows that for a given set of reactions, the enzyme most capable of reducing the energy barrier will be the most efficient catalyst (Simpson and Haard, 1987). Cold-adapted enzymes from poikilothermic species have been shown to be more efficient catalysts at cold assay temperatures than their homologs from the homoiothermic organism, as well as other warm-temperature adapted poikilothermic organisms (Simpson and Haard, 1987). The average daily mean habitat temperature of winter flounder is ~5.5°C and pressure of ~981 kPa (depth of 100 m). The difference in the conditions of the habitat, i.e., temperature and pressure of the bacteria and the fish may have influenced the efficiency of their enzymes in the breakdown of PZ-peptide. The fish collagenase could be a more efficient enzyme at lower temperatures than the bacterial collagenase, however the affinity of the former for PZ-peptide probably decreased as temperature increased. It has been shown that the affinity of enzymes from poikilotherms for substrate decreased (K_m' increased) as temperature increased as in the case of lactase dehydrogenase (Hochacka and Somero, 1968; Somero, 1969; Baldwin and Aleksiuk, 1973). Increased pressure had been observed to increase K_m' of acetylcholine esterase (Hochachka, 1974). Also, PZ-peptide could be a more suitable

substrate for microbial collagenases inspite the fact that it has been used for the assay of collagenases from various sources, including vertebrates.

The Arrhenius energies of activation (E_a) estimated for the hydrolysis of the subtrate were 7.23 kcal/mole and 11.98 kcal/mole for the fish muscle collagenases and *C. histolyticum* collagenases, respectively. Based on the E_a values alone, one would predict that the fish collagenases are more effective catalysts than *C. histolyticum* collagenases. However, in this particular study the use of E_a alone is not reliable since this parameter is a valid index for comparison of the abilities of the different enzymes to reduce the energy barrier to a given reaction only if the change in entropy (ΔS) is the same in all cases (Low *et al.*, 1973). The large differences between the values for change in entropy (ΔS) reduced the differences in the observed free energy of activation (ΔG) which can be predicted on the basis of E_a. E_a is directly related to ΔH which in turn is directly related to ΔG. The ΔS for bacterial collagenase is about 2x higher than for WFC. Similarly, the change in enthalpy (ΔH) is about 2x higher for bacterial collagenase than for WFC. The differences in the ΔS and ΔH values between collagenases from fish and bacteria could have a structural basis. According to Low *et al.* (1973), the most probable basis for the differences in the ΔS and ΔH values among variants of the same enzyme lies in the internal structure of the protein. Lower values of ΔS for trypsin from fish suggested that these enzymes have relatively more flexible structure than their bovine counterpart (Simpson *et al.*, 1989). In like manner, the lower ΔS value for WFC suggests that the fish collagenases have a relatively more flexible structure than the bacterial enzyme. An enzyme with a flexible sructure can conform to the structure of the substrate for proper binding thereby facilitating rates of reaction. However, there could be other factors which influenced the lower efficiency of the fish collagenases in comparison with the bacterial collagenases for the PZ-peptide hydrolase reaction at pH 7.1 and 37°C.

4.11 Amino acid composition of fish skeletal muscle collagenases, WFC-1 and WFC-2

The amino acid composition of the fish collagenase isoenzymes is shown in Table 17. The collagenase isoenzymes, WFC-1 and WFC-2 were both acidic proteins having more acidic than basic amino acid residues. WFC-1 had 1.56 acidic amino acid residues to every basic amino acid residue, whereas, WFC-2 had 2.63 acidic amino acid residues to every basic amino acid residue per enzyme molecule. Both isoenzymes were rich in glycine, alanine, threonine, serine, proline, valine and leucine. The methionine content of WFC-2 was higher than that of WFC-1. WFC-1 and WFC-2 had considerably higher levels of cysteine, in contrast to the *C. histolyticum* collagenases which had very low levels of the same amino acid.

With the use of the amino acid composition data, the degree of relatedness between the enzymes can be determined by evaluating their difference index (DI) (Price and Stevens, 1982). Two proteins with similar amino acid composition will have a DI of zero, whereas, proteins with no amino acid in common will have a DI of 100. The difference index was calculated by taking the difference in the fractional contents of each amino acid, obtaining the sum of the absolute values of those differences and multiplying that sum by 50 (Metzger, 1968). The similarity between the two collagenase isoenzymes, WFC-1 and WFC-2 was assessed (Table 18). A difference index of 17.28 suggested a close relationship between these two isoenzymes. The winter flounder skeletal muscle collagenases were further compared with collagenases from crustacean and microorganism. WFC-1 and WFC-2 had a closer similarity to the crab hepatopancreas collagenase than the *C. histolyticum* collagenase (α fraction) based on the DI values (Table 19). This closer similarity in the amino acid composition of the crab and fish collagenase explains their similar behavior towards the anion-exchange column.

The data on amino acid composition was also used to calculate for the average

Table 17. Amino acid composition of winter flounder muscle collagenases, WFC-1 and WFC-2.

Amino acid	No. of residues*	
	WFC-1	WFC-2
Aspartic acid/asparagine	48	72
Threonine	31	60
Serine	73	87
Glutamic acid/glutamine	68	70
Proline	33	31
Glycine	153	159
Alanine	190	106
Cysteine	31	15
Valine	40	38
Methionine	6	57
Isoleucine	21	13
Leucine	35	53
Tyrosine	15	9
Phenylalanine	22	15
Histidine	23	16
Lysine	31	24
Arginine	20	14
Tryptophan	-	-

*Calculation of the number of residues was based on the molecular weights of 79.6 and 75.5 kDa for WFC-1 and WFC-2, respectively. Tryptophan was not determined. The molecular weights of the amino acids used for calculation was based on the values of Feng et al. (1991).

Table 18. Evaluation of difference index for collagenases from winter flounder muscle.

Amino acid	Moles per 10^5 g enzyme		Mole fraction		|Δ|*
	WFC-1	WFC-2	WFC-1	WFC-2	
Aspartic acid/asparagine	60	95	0.0572	0.0861	0.0289
Threonine	39	79	0.0369	0.0717	0.0348
Serine	92	115	0.0869	0.1040	0.0171
Glutamic acid/glutamine	85	93	0.0810	0.0837	0.0027
Proline	41	41	0.0393	0.0370	0.0023
Glycine	192	210	0.1821	0.1901	0.0079
Alanine	239	140	0.2262	0.1267	0.0995
Cysteine	39	20	0.0369	0.0179	0.019
Valine	50	50	0.0476	0.0454	0.0022
Methionine	8	75	0.0071	0.0681	0.0610
Isoleucine	26	17	0.0250	0.0155	0.0095
Leucine	44	70	0.0417	0.0634	0.0217
Tyrosine	19	12	0.0179	0.0108	0.0071
Phenylalanine	28	20	0.0262	0.0179	0.0083
Histidine	29	21	0.0274	0.0191	0.0083
Lysine	39	32	0.0369	0.0287	0.0082
Arginine	25	18	0.0238	0.0167	0.0071
Total	1055	1108			0.3456

*Absolute value of the difference between the mole fraction of WFC-1 and mole fraction of WFC-2.

Difference Index (DI) = 50 x 0.3456 = 17.28

Table 19. Difference index for collagenases from various sources.

Pair of collagenases	Difference Index (DI)
WFC-1 & WFC-2	17.28
WFC-1 & Crab collagenase	27.62
WFC-2 & crab collagenase	24.68
WFC-1 & *C. histolyticum* collagenase, α	34.52
WFC-2 & *C. histolyticum* collagenase, α	30.32

hydrophobicity ($H\phi_{ave}$) indices of the collagenase isoenzymes from the fish skeletal muscle (Table 20) using the procedure of Bigelow (1967). Hydrophobic interactions are known to play an important role in the thermal stability of proteins, their strength increasing with temperature up to 75°C (Scheraga *et al.*, 1962; Brandt, 1964), and possibly up to 110°C (Primalov *et al.*, 1986; Baldwin, 1986). Based on the known amino acid composition of the proteins, hydrophobic indices can be computed and compared with the thermal stability of the protein. According to Bigelow (1967) a positive correlation exists between average hydrophobicity and thermal stability of proteins. Proteins from thermophilic species have been shown to have higher average hydrophobicity indices than the mesophilic species based on their amino acid composition (Bigelow, 1967; Hazel and Prosser, 1974). Based on the the $H\phi_{ave}$ values (782.80 cal and 736 cal for WFC-1 and WFC-2, respectively), it may be predicted that the thermal stability of the fish collagenases would be slightly higher for WFC-1 higher than for WFC-2. The fish collagenases had lower average hydrophobicities in comparison with the collagenases from fiddler crab and *C. histolyticum* as shown in Table 20, suggesting that the collagenases from the latter two species could potentially have higher thermal stabilities than the fish collagenases. Results of the thermal stability studies however, showed only slight differences in the thermal stabilities of the collagenases from *C. histolyticum* and winter flounder (SEC fraction). Thus other factors, e.g., the amount of aliphatic residues and S-containing amino acids probably influenced the thermal stability of the the fish collagenases. The total amount of the aliphatic residues such as alanine, valine, leucine and isoleucine has been suggested to contribute to the thermal stability of proteins. Ikai (1980) showed that proteins from thermophilic sources have higher aliphatic indices than ordinary proteins, particularly for those with molecular weights less than 100,000. The aliphatic indices of collagenases from *C. histolyticum* and winter flounder are given in Table 21. Similar to the results of the $H\phi_{ave}$, the aliphatic index values further show the slight differences in the relative contributions to thermal stability of WFC-1 and WFC-2. Collagenases from *C.*

Table 20. $H\phi_{ave}$ of collagenases from winter flounder skeletal muscle (WFC-1 and WFC-2), crab hepatopancreas and *C. histolyticum*.

Amino acid	WFC-1		WFC-2		Crab hepatopancreas collagenase[1]		C. histolyticum collagenase, α_1 [2]		C. histolyticum collagenase, ζ[2]	
	No. of residues	$H\phi_{ave}$ (kcal)*	No. of residues	$H\phi_{ave}$ (kcal)*	No. of residues	$H\phi_{ave}$ (kcal)*	No. of residues	$H\phi_{ave}$ (kcal)*	No. of residues	$H\phi_{ave}$ (kcal)*
Aspartic acid/asparagine	48	0	72	0	29	0	90	0	160	0
Threonine	31	13.95	60	27.00	24	10.80	42	18.90	73	32.85
Serine	73	0	87	0	17	0	37	0	72	0
Glutamic acid/glutamine	68	0	70	0	13	0	59	0	115	0
Proline	33	85.80	31	80.60	12	31.20	16	41.60	47	122.20
Glycine	153	0	159	0	28	0	56	0	99	0
Alanine	190	142.50	106	79.50	21	15.75	38	28.50	57	42.75
Cysteine	31	31.00	15	15.00	8	80.00	2.2	22.00	4.5	45.00
Valine	40	68.00	38	64.60	20	34.00	31	52.70	62	105.40
Methionine	6	78.00	57	74.10	3	3.90	3.7	48.10	15	19.50
Isoleucine	21	61.95	13	38.35	17	50.15	32	94.40	56	165.20
Leucine	35	84.00	53	127.20	13	31.20	51	122.40	87	208.80
Tyrosine	15	42.75	9	25.65	8	22.80	43	122.55	78	222.30
Phenylalanine	22	58.30	15	39.75	8	21.20	30	79.50	34	90.10
Histidine	23	0	16	0	5	0	6.1	0	22	0
Lysine	31	46.50	24	36.00	2	30.00	51	76.50	101	151.50
Arginine	20	15.00	14	10.50	2	37.50	20	15.00	36	27.00
Total	840	657.55	839	618.25	235	235.75	608	659.06	1118.5	1192.10
$H\phi_{ave}$(kcal)/residue		0.78		0.74		1.00		1.08		1.07

*Calculated by multiplying the $H\phi_{ave}$/aa (Tanford, 1962) by the total no. of residues.
[1]Eisen et al., (1973); [2]Bond and Van Wart (1984a)

Table 21. Aliphatic indices of collagenases from winter flounder (WFC-1 & WFC-2) and *C. histolyticum* (α & ζ).

Enzymes	Aliphatic indices
WFC-1	62.44
WFC-2	56.46
C. histolyticum, α.	74.27
C. histolyticum, ζ.	71.04

Aliphatic index = $x_A + ax_V + b(x_I + x_L)$, where x_A, x_V, x_I and x_L are mole % of alanine, valine, isoleucine and leucine, respectively; a = 2.9 and b = 3.9 (Ikai, 1980).

histolyticum have higher aliphatic indices than collagenases from winter flounder, suggesting higher thermal stability of the former. Enzymes of thermophilic and mesophilic origins have aliphatic indices ranging from 66 - 105 and 63 - 101, respectively, with the isoenzymes from the former having higher aliphatic indices than the latter (Ikai, 1980).

A third factor, the presence of disulfide bonds probably contributed to the thermal stability of the winter flounder collagenases. The presence of disulfide bonds may stabilize the native state of proteins by preventing unfolding (Anfinsen and Scheraga, 1975; Thornton, 1981; Wetzel *et al.*, 1988) or by lowering the conformational entropy and therefore destabilization of the unfolded state (Wetzel, 1987). The higher amount of S-containing amino acid residues in fish collagenases (WFC-1 and WFC-2) than in *C. histolyticum* collagenases could be responsible for the slight differences in the thermal stability of the enzymes from both sources, i.e., the higher levels of these amino acid residues in the fish enzymes counterbalanced their lower average hydrophobicities and aliphatic indices.

Chapter 5

CONCLUSION AND SUGGESTIONS FOR FUTURE WORK

Two isoenzymes designated as WFC-1 and WFC-2 were isolated from the skeletal muscles of winter flounder (*Pseudopleuronectes americanus*) by the successive steps of ammonium sulfate precipitation (40 - 80% saturation), ion-exchange chromatography (IEX-1 and IEX-2), immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC) using an FPLC system. Isoelectric focusing with a rotofor unit was ineffective as a purification method for fish collagenase isoenzymes. Detection of the presence of the two isoenzymes was accomplished by electrophoresis on the zymogram gel (zymography) and separation of WFC-1 and WFC-2 was achieved by electrophoretic blotting from the Tris-glycine gel onto the PVDF membrane. The molecular weights of the fish isoenzymes were 79,600 and 75,500 for WFC-1 and WFC-2, respectively.

The fish collagenases hydrolyzed the synthetic substrate PZ-peptide as well as insoluble bovine collagen. The crude extract also exhibited trypsin-like and chymotrypsin-like activities which diminished with purification, suggesting that these proteolytic activities were not integral parts of the fish collagenases.

A comparison of the characteristics of the collagenase fraction from IEX-1 and the commercial collagenase from *C. histolyticum* was conducted. The pH studies for the hydrolysis of PZ-peptide showed that the fish collagenase fraction was most active at pH 7.5, while the *C. histolyticum* collagenase fraction was most active at pH 8.0. The fish collagenase fraction was most stable at pH 6.0 - 8.0 and the bacterial collagenase was most stable at pH 7.0 - 9.0.

The influence of temperature on the activity and stability of the enzymes showed that both collagenases from fish and *C. histolyticum* had a maximum activity at 40°C,

however, there was a difference in their thermal stability. At 65°C the collagenase from *C. histolyticum* completely lost its activity, while the fish collagenase fraction had an ~31% loss of activity.

Although separation of collagenase isoenzymes, WFC-1 and WFC-2 was achieved by electrophoretic transfer of the fraction from SEC, this latter fraction was used for further studies on the fish skeletal muscle enzyme.

The result of inhibition studies suggested that the winter flounder skeletal muscle collagenase isoenzymes were metalloenzymes, probably containing zinc in their active sites similar to the vertebrate and bacterial collagenases. 1,10-Phenanthroline inhibited the winter flounder collagenases, however, addition of low levels of zinc ions reactivated the fish collagenases. Nevertheless, the chemical reversion of 1,10-phenanthroline inhibition by zinc ion does not suggest that the inhibitor belongs to the class of reversible inhibitors. 1,10-Phenanthroline is an active site directed irreversible inhibitor. At 10 mM 1,10-phenanthroline, increasing levels of zinc ions beyond 2 mM resulted in a decrease in the recovery of collagenase activity. Zinc ions were ineffective in reactivating the winter flounder skeletal muscle collagenases in the presence of EDTA. However, addition of low levels of calcium ions in the presence of EDTA, restored collagenase activity, suggesting that the mode of inhibition of EDTA differs from that of 1,10-phenanthroline. The action of EDTA could be directed towards calcium ions which may also be part of the prosthetic group of the fish collagenases. Calcium ions have been shown to be important components of collagenases from various sources. Although zinc ions were thought to be present in the winter flounder collagenases, addition of zinc ions in the absence of metalloenzyme inhibitors caused enzyme inhibition. 2-Mercaptoethanol and dithiothreitol inhibited winter flounder skeletal muscle collagenases. Similar to EDTA, 2-mercaptoethanol also manifested mixed inhibition. The action of 2-mercaptoethanol and dithiothreitol could be directed towards zinc ions as well as the S-containing amino acid

residues of the isoenzymes. Serine protease inhibitors, TLCK and STI were ineffective against fish collagenases.

The pH optimum of collagenases from various sources range between pH 7 and 9, with loss of activity below pH 6. The optimum pH of the fish collagenase fraction after SEC was 7.5 for PZ-peptide hydrolase reaction. The SEC fraction was stable at pH 6 - 11.

The fish collagenase fraction from SEC was most active at 45°C for the hydrolysis of PZ-peptide and was stable from 0 to 40°C. Complete loss of enzyme activity was observed at 55°C.

The kinetic and thermodynamic properties showed that *C. histolyticum* collagenases are better catalysts than collagenases from winter flounder skeletal muscle for the hydrolysis of PZ-peptide at 37°C and pH 7.1, the former having a higher catalytic efficiency and lower ΔG value than the latter.

The amino acid composition shows that the winter flounder muscle collagenases, WFC-1 and WFC-2 had higher amounts of acidic amino acid residues than basic residues, were both rich in glycine, alanine, threonine, serine, proline, valine and leucine. However, WFC-1 had a lower methionine content than WFC-2. Evaluation of DI values showed that WFC-1 and WFC-2 were closely related in terms of amino acid composition. A comparison of DI values suggested that both isoenzymes had a closer similarity to the crab hepatopancreas collagenase than the *C. histolyticum* collagenases. The $H\phi_{ave}$ and aliphatic index values suggested that the relative contributions of these parameters to the thermal resistance of WFC-1 are slightly higher than WFC-2. The higher $H\phi_{ave}$ and aliphatic index values of *C. histolyticum* collagenases than those of fish collagenases implied that the former would be more thermostable than the latter. Results of temperature studies however, showed slight differences in thermal stability of the isoenzymes from both sources. The presence of cysteine in the fish collagenase isoenzymes probably made up for the lower values of $H\phi_{ave}$ and aliphatic indices of these

isoenzymes. Unlike *C. histolyticum* collagenases, fish collagenase isoenzymes were relatively high in cysteine.

The determination of the presence of the metal ions, i.e., zinc and calcium in WFC-1 and WFC-2 is suggested based on their responses to the various inhibitors. In addition, the determination of the location(s) as well as the number of these ions in the respective sites are also recommended.

The activity of fish collagenases on the collagen isolated from the fish and the mechanism of connective tissue breakdown in the fish skeletal muscles can be studied so proper measures can be recommended to minimize, if not completely prevent, fish tissue softening during cold/ice storage. Among the inhibitors studied, EDTA being an acceptable food additive can be used to control collagenase activity in the fish. In addition $ZnCl_2$ and various salts of calcium, e.g., calcium sulfate, calcium carbonate and calcium phosphate may turn out to be effective.

The isolation and characterization of collagenases was the focus of this study and their roles in the textural quality of the fish had been suggested. Based on the characteristics of the fish collagenases, it is also worth speculating regarding its use as an aid in food processing, particularly as a meat tenderizer. Meat from land animals generally contain higher amounts of collagen, thus meat toughness is the problem rather than softening. One of the criteria of a good tenderizer is for it to remain active at low temperature and meat pH (5.5) during storage and at higher temperature during cooking. Although the optimum pH of the fish collagenases was 7.5 the enzyme was still active at pH 5.5 and at temperatures of up to 50°C. The fish collagenases are also safe processing aids compared to collagenases from bacteria. Microorganisms which produce collagenases usually belong to genera which contain pathogenic species or strains. The presence of these enzymes may even contribute to the virulence of these microbial strains.

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APPENDIX 1

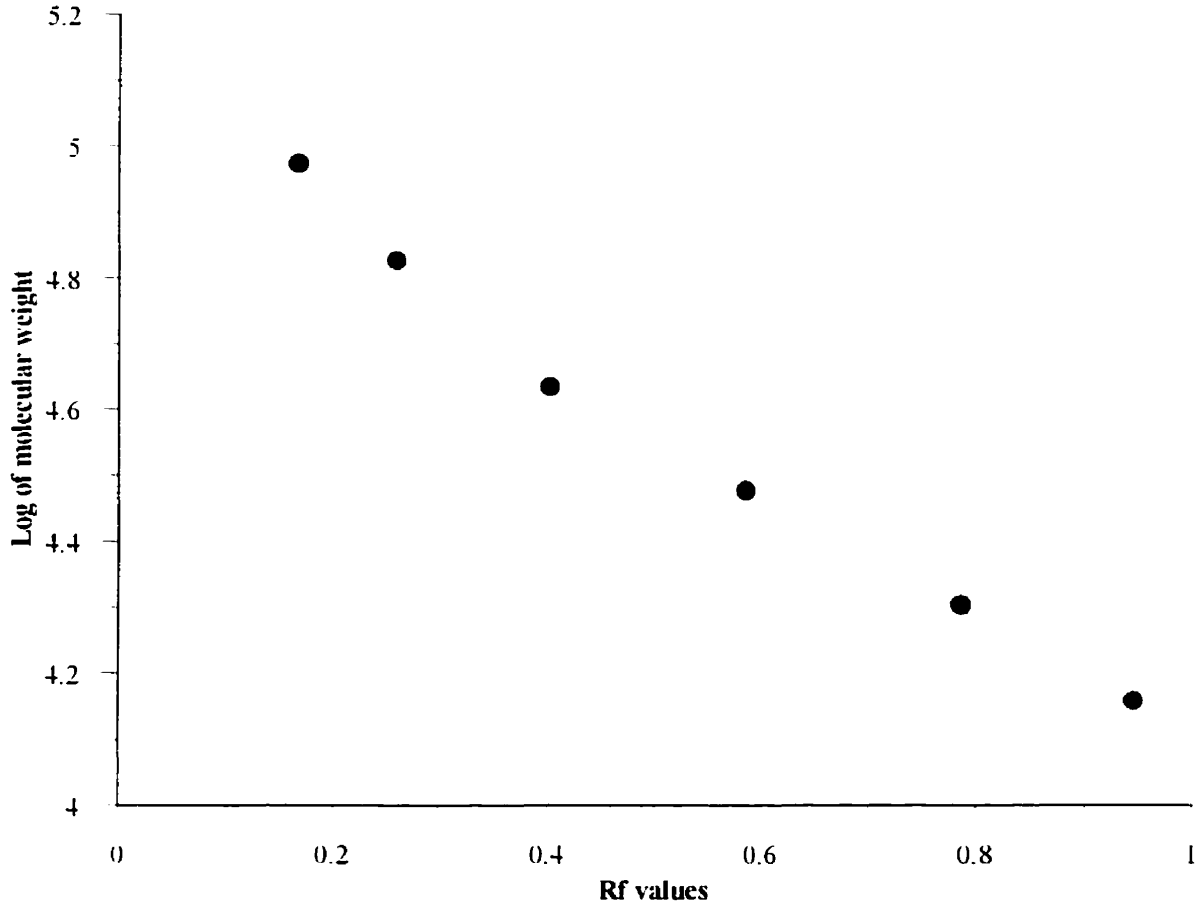


Fig. . Standard curve for the determination of molecular weight (Standard; phosphorylase B-94,000; bovine serum albumin-67,000; ovalbumin-43,000; carbonic anhydrase-30,000; trypsin inhibitor (soybean) -20,100; alpha-lactalbumin-14,4000).

APPENDIX 2

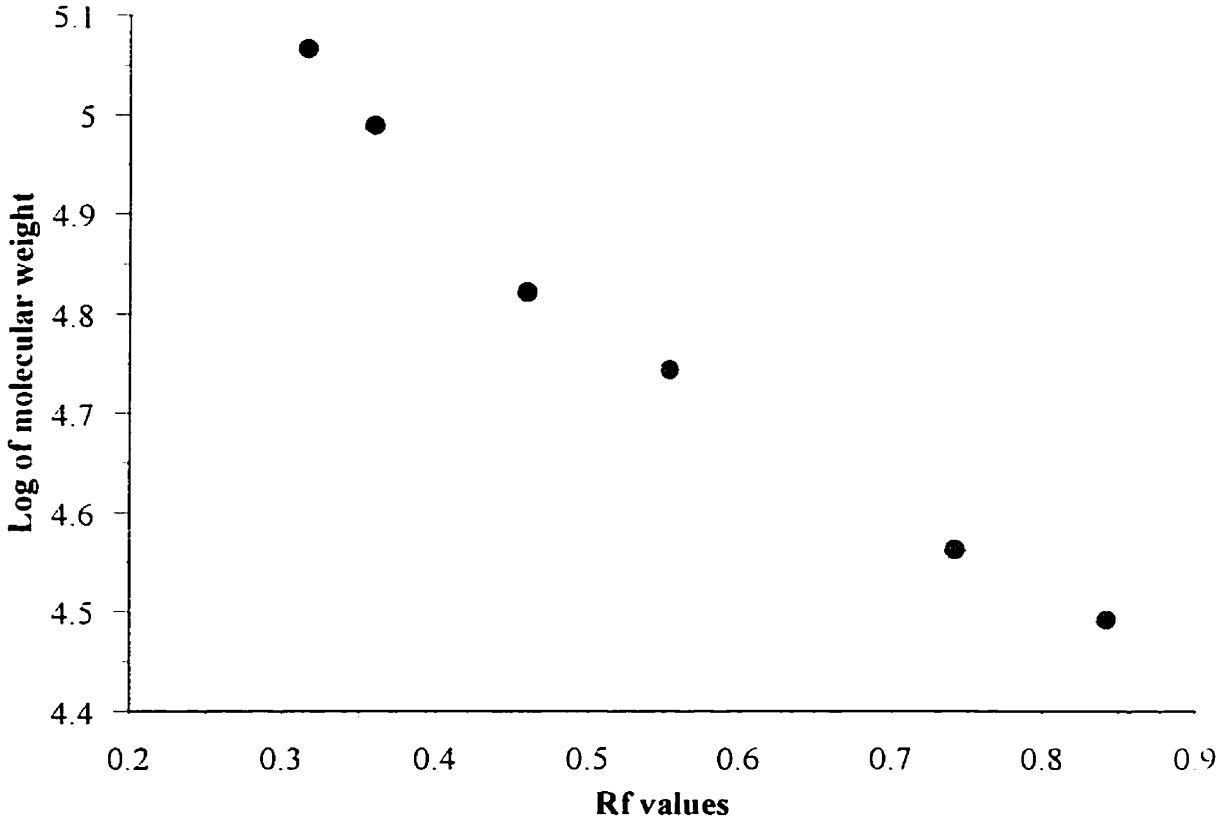


Fig. . Standard curve for the determination of molecular weight (Standard: beta galactosidase-116,300; phosphorylase B-97,400; bovine serum albumin-66,300; glutamic dehydrogenase-55,400; lactase dehydrogenase-36,500; carbonic anhydrase-31,000).

APPENDIX 3

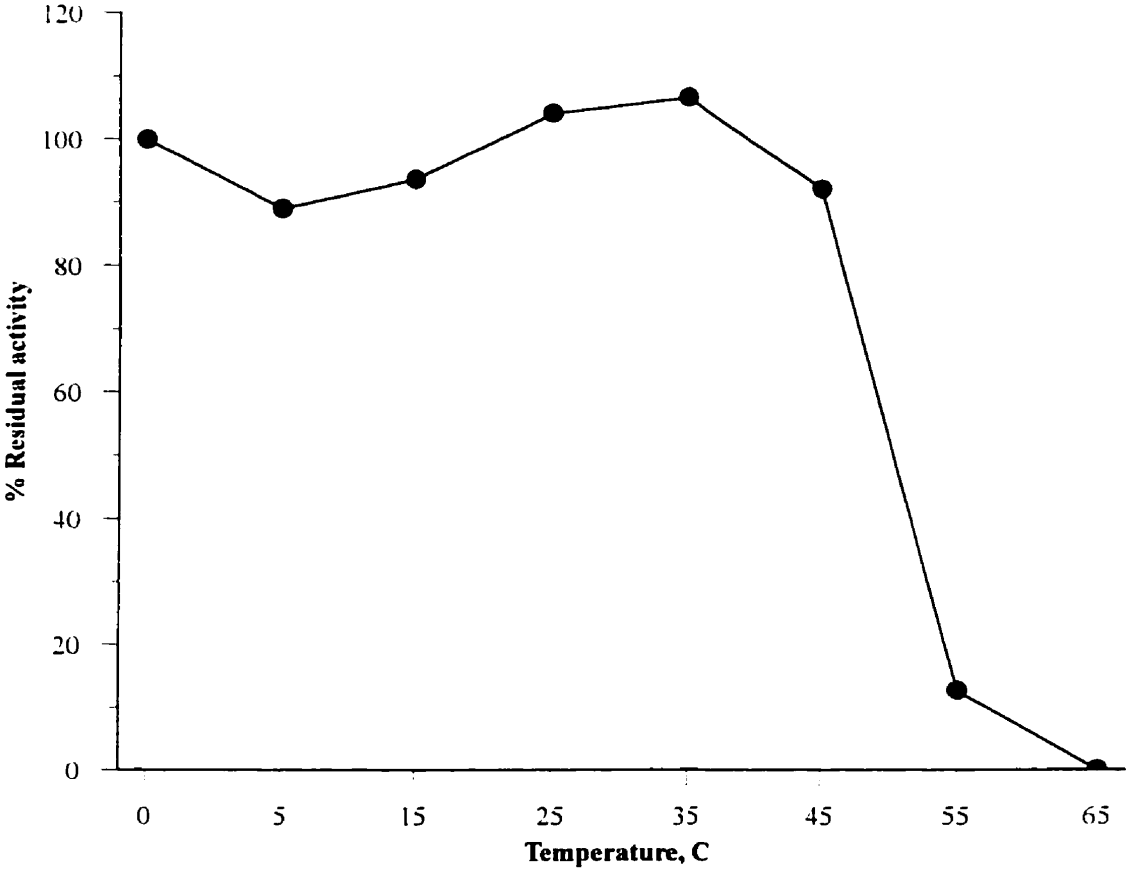


Fig. . Temperature stability of collagenolytic enzymes in the crude enzyme extract from the skeletal muscle of winter flounder. (Values presented are means of 3 replicates for 1 experiment)

Temperature, C	% Residual activity
0	99.97±16.75
5	88.91±18.72
15	93.62±9.53
25	104.05±4.27
35	106.54±26.90
45	91.93±6.59
55	12.75 ±2.01
65	0

APPENDIX 4

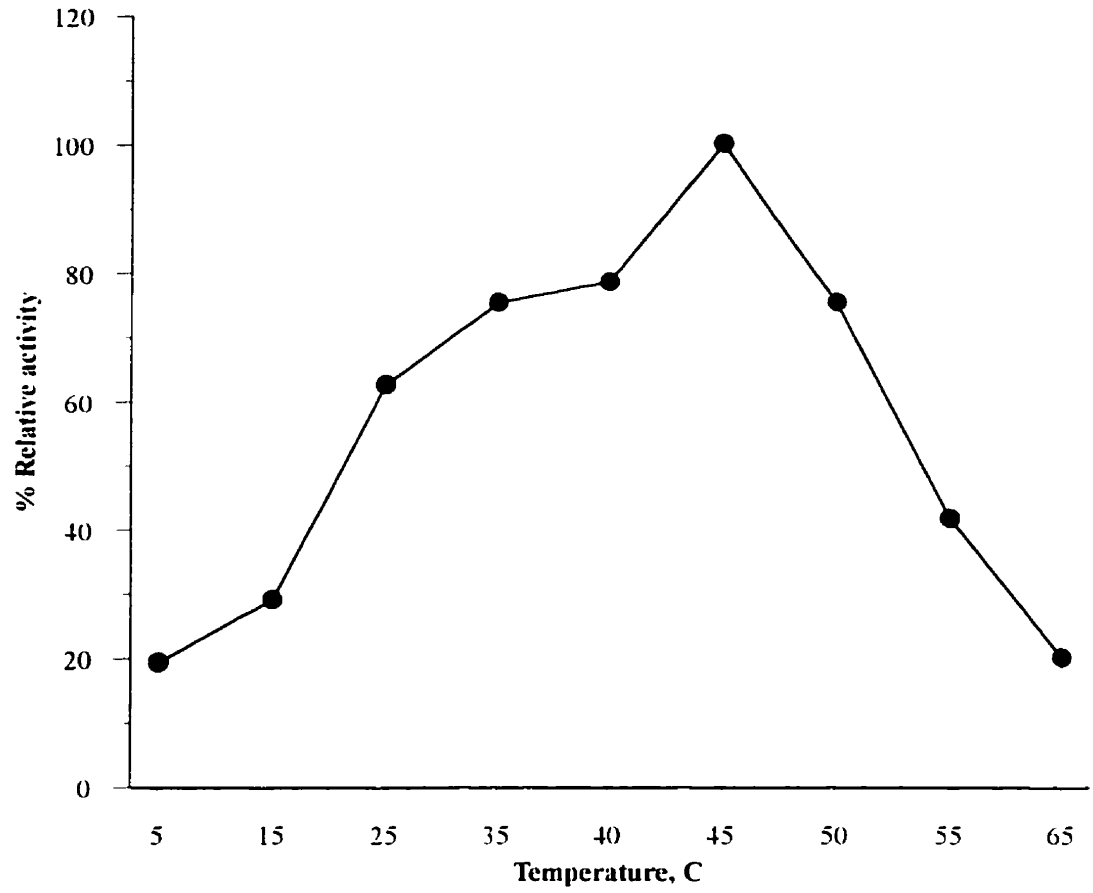


Fig. . Temperature optimum of collagenolytic enzymes in the crude enzyme extract from the skeletal muscle of winter flounder. (Values presented are means of 3 replicates for 1 experiment)

Temperature, C	% Relative activity
5	19.54± 2.69
15	29.24 ±11.69
25	62.70 ± 7.11
35	75.48± 7.84
40	78.62 ±14.44
45	100 ±14.18
50	75.37 ±12.65
55	41.80 ±12.31
65	20.2 ±2.24

APPENDIX 5

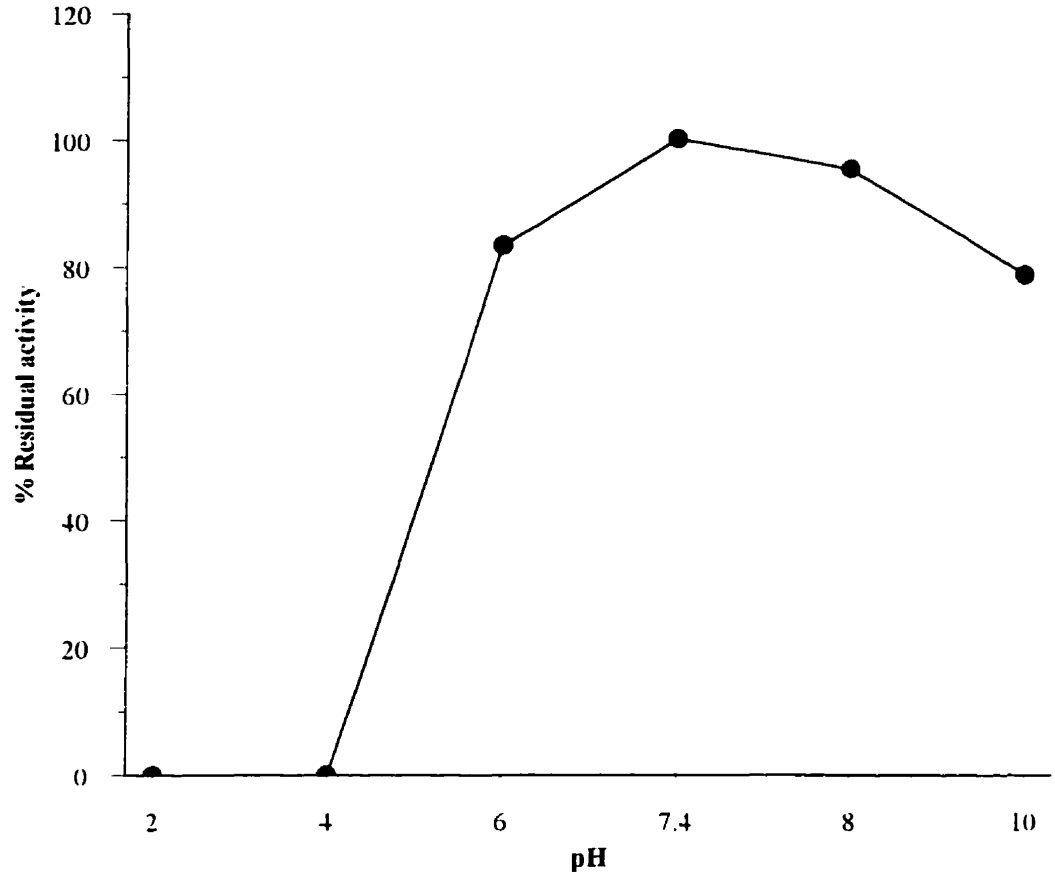


Fig. . pH stability of collagenolytic enzymes in the crude extract from the skeletal muscle of winter flounder. (Values presented are means of 3 replicates for 1 experiment)

pH	% Residual activity
2	0
4	0
6	83.35±4.51
7.4	100±1.84
8	95.23±4.86
10	78.68±8.27

APPENDIX 6

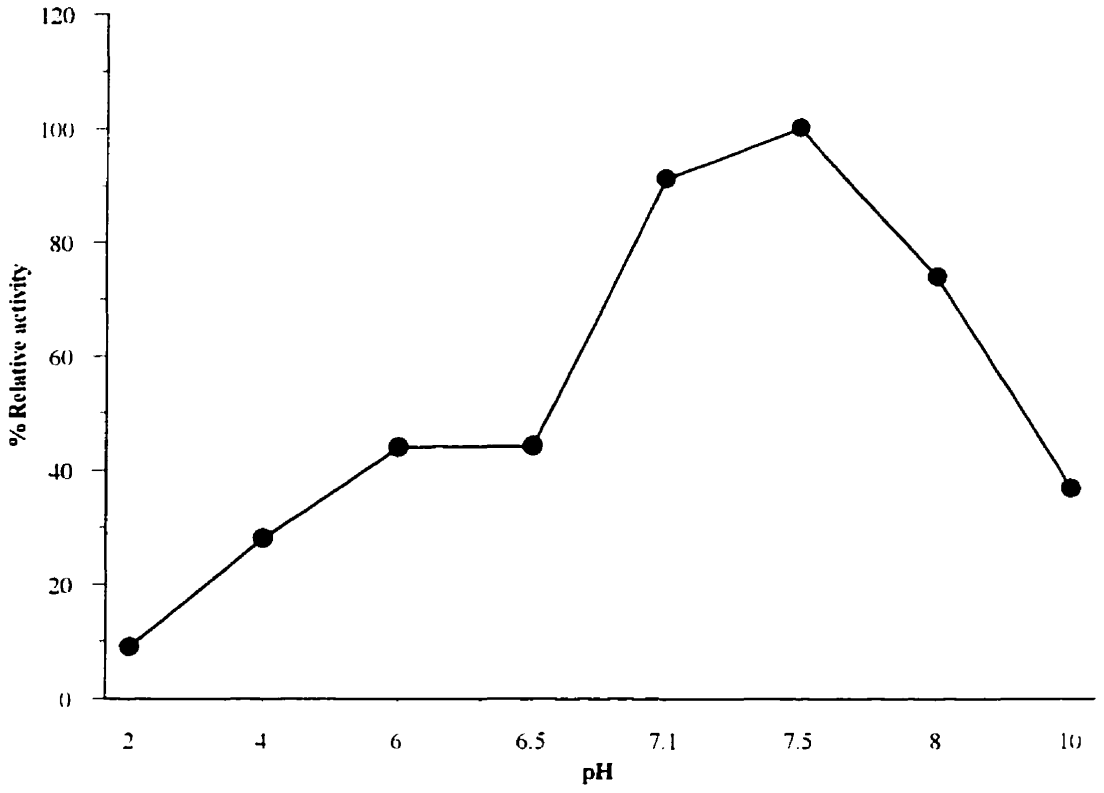


Fig. . pH optimum of collagenolytic enzymes in the crude enzyme extract from the skeletal muscle of winter flounder. (Values presented are means of 3 replicates for 1 experiment)

pH	% Relative activity
2	9.09±3.63
4	28.16±7.45
6	44.08±1.64
6.5	44.26 ±17.07
7.1	91.23±4.36
7.5	100.02±5.60
8	73.92±5.36
10	36.89±10.14

APPENDIX 7

Collagenase and chymotrypsin-like activities of winter flounder skeletal muscle enzymes (Values presented are means of triplicate results for 3 experiments)

Purification step	Collagenase activity (units/mg)	Chymotrypsin-like activity (units/mg)	Protein content (mg/ml)
Crude extract	4.37±1.09	10.62±3.18	6.14±0.16
Ammonium sulfate (40 - 80%)	7.23±0.85	25.36±6.95	9.061.70
IEX-1	8.09±2.45	1.30±0.16	1.94±0.34

APPENDIX 8

Hydrolysis of collagen (bovine Achilles tendon) by collagenases from *P. americanus* and *C. histolyticum* (Values presented are means of 3 replicates for 1 experiment)

Incubation time (h)	Collagenase activity (A_{600}nm/mg)	
	P. americanus	*C. histolyticum*
12	0.032±0.01	457.24±12.42
24	0.20±0.01	691.14±26.28
36	0.32±0.02	-
48	1.66±0.06	1260.50± 0

APPENDIX 9

pH optima of collagenases from *P. americanus* (IEX fraction) and *C. histolyticum* (Values presented are means of 3 replicates for 1 experiment)

pH	% Relative activity	
	P. americanus	*C. histolyticum*
5.0	10.16±3.66	2.85±1.32
6.0	28.78±3.82	37.10±1.12
6.5	53.19±1.21	66.54±1.78
7.0	95.51±4.02	93.12±1.15
7.5	100.48±8.91	90.14±1.81
8.0	74.32±4.92	96.00±5.66
8.5	-	77.43±2.60
9.0	40.43±7.8	69.68±0.27
10.0	19.04±1.78	29.78±2.74
11.0	0	6.02±1.08

pH stability of collagenases from *P. americanus* (IEX fraction) and *C. histolyticum* (Values presented are means of 3 replicates for 1 experiment)

pH	% Residual activity	
	P. americanus	*C. histolyticum*
2.0	-	1.00±0.35
4.0	0	2.95±1.12
5.0	37.25±2.77	12.09±2.09
6.0	100.00±4.80	63.94±0.94
7.0	50.98±7.34	85.30±1.37
8.0	74.51±10.00	94.85±3.22
9.0	47.06±4.80	94.74±2.70
10.0	58.82±0	67.34±1.62
11.0	45.09±7.34	16.04±1.54
12.0	-	13.52±1.18

APPENDIX 10

Temperature optima of collagenases from *P. americanus* (IEX fraction) and *C. histolyticum* (Values presented are means of 3 replicates for 1 experiment)

Temperature °C	% Relative activity	
	P. americanus	*C. histolyticum*
5	18.79±0.98	9.87±1.62
15	21.43±9.72	27.56±1.11
25	48.41±5.76	49.58±4.49
35	57.45±2.54	82.79±2.64
40	100.00±21.24	99.88±1.80
45	95.36±3.36	81.35±2.95
50	92.44±14.19	6.40±1.88
55	51.54±5.83	2.77±2.18
65	45.09±7.34	0.36±0

Temperature stability of collagenases from *P. americanus* (IEX fraction) and *C. histolyticum* (Values presented are means of 3 replicates for 1 experiment)

Temperature °C	% Residual activity	
	P. americanus	*C. histolyticum*
0	85.53±6.03	100.14±4.86
5	69.08±7.36	96.52±2.77
15	92.27±12.25	99.18±2.96
25	70.50±7.74	93.20±4.14
35	56.37±2.84	96.67±5.32
40	63.96±1.32	93.51±3.01
45	83.09±4.51	86.10±3.60
50	51.16±2.92	69.02±4.46
55	34.63±3.81	11.43±0.66
65	31.75±0.79	0

APPENDIX 11

pH optimum of winter flounder skeletal muscle collagenases (SEC fraction)
(Values presented are means of 3 replicates for 1 experiment)

pH	% Relative activity
5.0	6.47±0.59
6.0	14.51±0.55
7.0	85.88±3.46
7.5	98.80±7.23
8.0	84.70±13.62
8.5	66.28±3.64
9.0	50.19±6.18
10.0	0

pH stability of winter flounder skeletal muscle collagenases (SEC fraction)
(Values presented are means of 3 replicates for 1 experiment)

pH	% Residual activity
4.0	0
5.0	40.81±7.26
6.0	100.84±11.00
7.0	74.15±8.39
8.0	81.64±8.66
9.0	82.31±6.74
10.0	82.99±6.74
11.0	70.08±0

APPENDIX 12

Temperature optimum of winter flounder skeletal muscle collagenases (SEC fraction)
(Values presented are means of 3 replicates for 1 experiment)

Temperature, °C	% Relative activity
5	18.75±4.17
15	40.28±14.27
25	54.86±11.58
35	67.36±10.94
40	87.50±3.40
45	100.00±6.44
50	25.00±12.27
55	0

Temperature stability of winter flounder skeletal muscle collagenases (SEC fraction)
(Values presented are means of 3 replicates for 1 experiment)

Temperature, °C	% Residual activity
0	100.86±16.86
5	95.73±11.90
15	116.24±12.09
25	105.13±20.19
35	90.60±13.62
40	99.15±15.71
45	65.81±11.53
50	35.90±8.37
55	0

APPENDIX 13

Influence of various inhibitors on the activity of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

Inhibitor	% Inhibition
10mM TLCK	3.24±2.2
0.5 mg/ml STI	0
100mM EDTA	45.46±4.01
10mM 1,10-Phenanthroline	95.57±3.16
100mM 2-Mercaptoethanol	50.00±8.55
20mM DTT	90.56±0.42

Influence dialysis and addition of zinc ions on the activity of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

Treatment	Specific activity (units/mg)
Control	107.73±8.98
Dialyzed	103.57±5.5
Dialyzed + zinc ions	16.43±0

Influence of various concentrations of Zn ions on the activity of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

Zn ion concentrations (mM)	% Resisual activity
0	100
0.125	8.33±1.85
0.250	1.23±0.87
0.375	20.37±2.72
0.500	0

Influence of various concentrations of 1,10-Phenanthroline on the activity of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

1,10-Phenanthroline concentration (mM)	% Inhibition
0	0
1.0	0
5.0	64.60±0
10.0	95.57±3.1

Inhibition of winter flounder skeletal muscle collagenases by 1,10-Phenanthroline and their reactivation by various concentrations of zinc ions (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

Molar ratio of 1,10-Phenanthroline to Zinc ions	% Residual activity
0:0	100±0
10:0	0
10:0.5	69.05±3.31
10:1.0	102.86±6.31
10:2.0	91.67±6.73
10:4.0	20.17±3.45

Influence of various concentrations of EDTA on the activity of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

EDTA concentration (mM)	% Inhibition
0	0
50	27.48±7.33
100	35.30±4.16
150	44.12±0
200	62.74±3.67
250	60.78±3.67

Influence of various concentrations of Zn ions on the inhibitory action of EDTA on winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

Molar ratio of EDTA to Zn ions	% Residual activity
0:0	100±0
100:0	53.45±2.97
100:5	64.56±8.37
100:10	23.42±1.27
100:20	11.26±1.35
100:40	0

Influence of various concentrations of Ca ions on the inhibitory action of EDTA on winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

Molar ratio of EDTA to Ca ions	% Inhibition
0:5	100±4.56
100:5	60.21±8.04
100:10	96.77±6.97
100:15	65.59±3.04
100:25	51.61±9.13

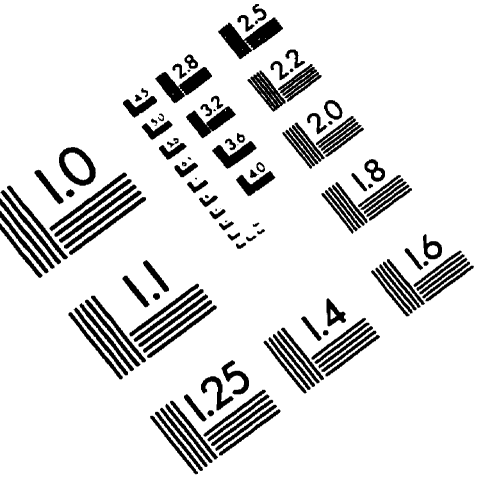
Influence of various concentrations of 2-Mercaptoethanol on the activity of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

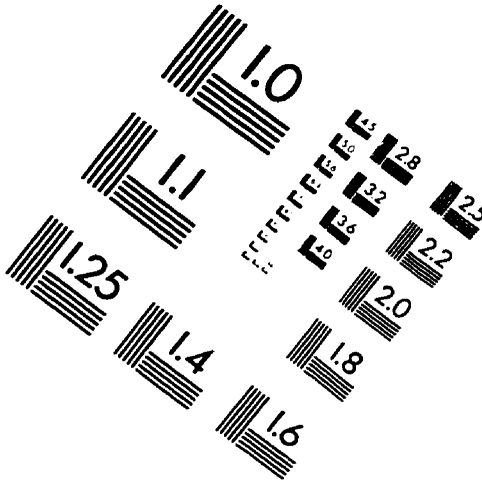
2-Mercaptoethanol concentration (mM)	% Inhibition
0	0
50	20.24±6.07
100	28.57±10.51
150	52.38±6.73
200	58.92±5.36
250	73.22±5.36

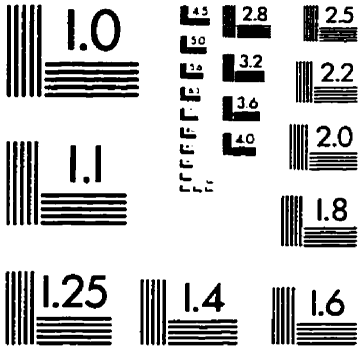
Influence of various concentrations of DTT on the activity of winter flounder skeletal muscle collagenases (SEC fraction) (Values presented are means of 3 replicates for 1 experiment)

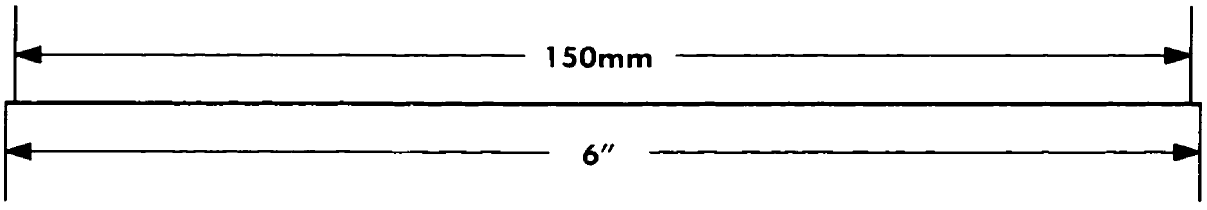
DTT concentration (mM)	% Inhibition
0	0
5	33.33±5.48
10	55.91±6.08
15	83.87±9.47
20	88.17±12.45

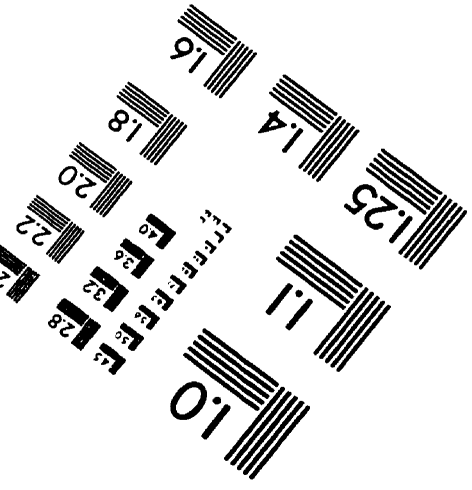
IMAGE EVALUATION
TEST TARGET (QA-3)

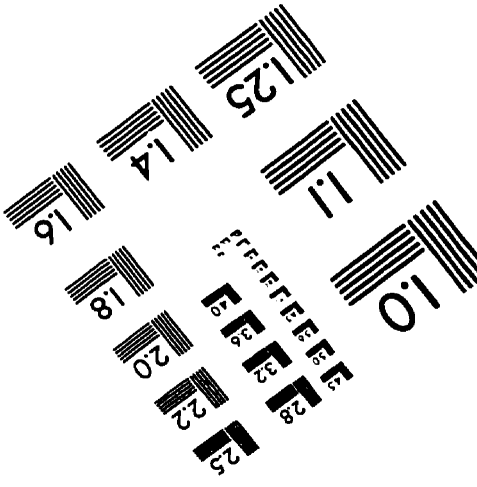












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