Studies on collagens and related proteases from the abalone muscle

クロアワビ閉殻筋コラーゲンおよび関連分解酵素に関する研究

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Contents

Introduction

People have been consuming fish and shellfish muscles as protein sources from ancient times because of their high protein contents. In case of Japanese people, they generally prefer raw fish and shellfish to cooked ones, so that texture is an important factor in consumer's acceptance. Raw meat texture is closely related to its collagen contents. The toughness of meat is increased with the increase of collagen content in fish (Hatae *et al.*, 1986; Sato *et al.*, 1986), whereas a high degree of intermolecular cross-linking of collagen is responsible for development of the tough texture of meat in livestock animals (Moller *et al.*, 1981; Light *et al.*, 1985).

A good correlation has also been observed between toughness and collagen content in invertebrate muscles. Gastropods such as abalone and turban shell unusually contain large amounts of collagen in the muscle. The turban shell *Batillus cornutus* has different muscle parts and showed the collagen contents of 8.2, 5.5, and 0.9% of wet tissue in the foot, opercular, and visceral muscle, respectively (Watabe *et al.*, 1986). The highest toughness was obtained with the foot muscle. Abalones, another gastropod, also show collagen content-dependent toughness in various muscle parts (Olaechea *et al.*, 1993). The toughness of the hard part of foot containing 4.1% collagen is higher than that of the middle part of adductor with 1.2% collagen for the kuro-awabi abalone *Haliotis discus* (Olaechea *et al.*, 1993).

Collagen is the most abundant structural protein in the animal body which accounts for about one third of the total protein mass in vertebrates. While locating in the extracellular matrix of connective tissues, collagen provides an insoluble scaffold for the provision of shape and form, for the attachment of macromolecules, glycoproteins, hydrated polymers and inorganic ions, and for cell attachment (Kalder, 1995). So far, 19 collagen types, referred to types I - XIX, have been characterized in vertebrates both at the protein and gene levels (Prockop and Kivirikko, 1995), and apparently separated into two main classes; fibril-forming and non-fibrillar collagens. The former group contains molecules with long continuous triple helices, which are the constituents of banded collagen fibrils. The non-fibrillar collagens are highly diverse in their supramolecular structure (Prockop and Kivirikko, 1995).

The common structural hallmark of the collagen molecule is triple helix in which three collagen polypeptides, called α-chains, are wound around one another in a rod-like superhelix (Figure 0-1). α-Chains are constructed from repeating Gly-X-Y triplets, where X and Y are occupied by any amino acids but are frequently by the imino acids, proline and hydroxyproline, respectively (Prockop and Kivirikko, 1995). Glycine in every third residue position of each chain is a prerequisite for folding of the three chains into a triple helix. The pyrrolidone ring of proline introduces a left-handed twist in the peptide backbone of each α-chain, placing glycine residues in each chain into the center of a triple helix. To accommodate glycine residues in different chains into the triple helix formed from three chains, adjacent chains are mutually staggered by one residue. The triple helix thus formed has a right-handed superhelix that repeats every 30 residues (Kalder, 1995).

Fibril-forming interstitial collagens (types I - III, V, and XI) are abundant in the connective tissues of tendon, skin, ligament, and bone. Fibril-forming collagens are similar in size and in that they contain large triple-helical domains with about 1,000 amino acids or about 330 triplets repeats per chain (Prockop and Kivirikko, 1995). For each collagen type the α-chains are identified with Arabic numerals followed by the Roman numeral for the type in parentheses, e.g., α1(I), α2(I), etc. Type I collagen is a major constituent of collagen fibrils found in a variety of tissues. It consists of two identical chains and one dissimilar chain, $[\alpha 1(I)]_2\alpha 2(I)$. Many of the other fibril-forming collagens have a more selective tissue distribution.

The biosynthesis of collagen follows a multistep process characterized by extensive cotranslational and posttranslational processing of individual chains. The individual collagen polypeptide chains are synthesized on membrane-bound ribosomes and translocated into the lumen of the endoplasmic reticulum (ER) as large, soluble precursors, called proα-chains, containing amino- and carboxyl-terminal extension propeptides (N- and C-propeptide, respectively) (Figure 0-2). In the lumen of the ER, proline and lysine residues at position Y are hydroxylated to 4-hydroxyproline and 5-hydroxylysine, respectively, and a few proline residues at position X are hydroxylated to 3-hydroxyproline (Prockop and Kivirikko, 1995). Some of the hydroxylysine residues are glycosylated with *O*-linked galactose or galactose conjugated with glucose.

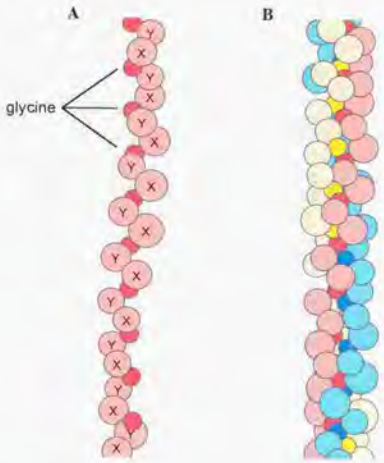

Figure 0-1. Schematic representation of the collagen molecule based on a repeating triplet of Gly-X-Y (Alberts *et al.*, 1994). (A) A model of part of a single collagen α-chain in which each amino acid is represented by a sphere. (B) A model of a part of a collagen molecule in which three α-chain, each shown in a different color, are folded into a triple helix with glycine residues in the center of the molecule.

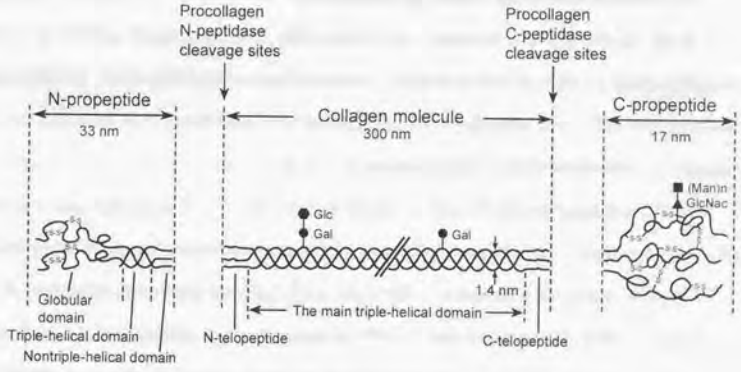

Fig. 0-2. Schematic representation of the structure of type I procollagen molecule (Prockop *et al.*, 1979; Kadler, 1995). Abbreviations used are Glc, glucose; Gal, galactose; Man, mannose; GlcNac, *N*-acetylglucosamine.

Glycosylation also occurs in C-propeptide or both in N- and C-propeptides providing asparagine-linked mannose-rich oligosaccharide (Kalder, 1995) (Figure 0-2).

The assembly of proα-chains into procollagen proceeds as follows: folding of the C-propeptides in individual chains, intrachain disulfide bonding in the three chains, interchain disulfide bond formation between C-propeptides, nucleation of the main triple-helical domain, and propagation of the triple helix in a C to N direction in a zipper-like action (Engel and Prockop, 1991).

Procollagen molecule is composed of N-propeptide, the main triple-helical domain, and C-propeptide with domain lengths about 17, 300, and 33 nm, respectively (Figure 0-2). Each proα-chain contains about 1,400 amino acid residues. N-propeptides of fibril-forming collagens exhibit a high degree of divergence both in length and in the domain structure. The N-propeptide consists of the following elements: a cysteine-rich globular domain, a short triple-helical domain, and a short globular domain ending in the amino-terminal telopeptide (N-telopeptide). The cysteine-rich globular domain is absent in proα2(I) chain (Kuivaniemi *et al.*, 1988) and proα1(II) chain which undergoes alternative splicing (Su *et al.*, 1989). It has been suggested that the N-propeptide plays a role in regulating the diameter of fibrils (Fleischmajer *et al.*, 1983). The C-propeptide of fibril-forming collagens shares the highest of sequence similarity both between different types and between different species (Vuorio and de Crombrugghe, 1990). The 243-247 amino acid C-propeptide is removed extracellularly by a specific procollagen C-peptidase, leaving a short telopeptide of 11-27 amino acids attached to the triple-helix. C-propeptide has a globular structure that is stabilized by intra- and interchain disulfide bridges and plays an important role in chain association (Lee and Bulleid, 1994). In addition, N-propeptide as well as C-propeptide shows an inhibitory effect on procollagen synthesis (Wiestner *et al.*, 1979; Wu *et al.*, 1986).

After procollagen secretion from the cell, the procollagen molecule is converted to collagen by cleavage of N- and C-propeptides with procollagen N-peptidase and procollagen C-peptidase, respectively.

The collagen molecules contain the main triple-helical domain and the short non-helical domain referred to telopeptide at each end. They are similar in that they all assemble into cross-striated fibrils with periodicity of 67 nm as shown by electron microscopy (Miller, 1985). Such

fibrils form by a quarter-staggered alignment of the rod-like molecule of fibril-forming collagens which have a length of 300 nm. The fibrils are further stabilized by formation of covalent intra- and intermolecular cross-links through specific lysine and hydroxylysine residues in strictly conserved positions (Eyre *et al.*, 1984).

Fibril-forming collagens are distributed not only to vertebrate, but also to all invertebrate phyla (Bairati and Garrone, 1985), where unusual collagens have been isolated, including long cuticle collagen from annelids (Gaill *et al.*, 1991, 1995). However, only very limited information is available on invertebrate collagens, since they are extraordinarily insoluble probably due to high degrees of cross-linking in the region of telopeptides, generally requiring an extensive pepsin treatment to produce acid-soluble collagens (Kimura *et al.*, 1969; Kimura, 1975). Kimura (1975) examined the physicochemical properties of pepsin-solubilized collagens from marine invertebrates including abalone. They were composed of three α-chains and the molecular weight of α-chain was about 95,000-100,000 which was similar to vertebrate counterparts. Collagens from gastropods such as turban shell and abalone were composed of a single types of α-chain whereas other invertebrate collagens were heterotrimer. In amino acid analysis, abalone pepsin-solubilized collagen has been shown to have less alanine and higher hydroxylysine contents than those of vertebrate type I collagens (Kimura and Matsuura, 1974). Furthermore, abalone collagen exhibited higher degrees of glycosylation *via* hydroxylysine than vertebrate collagens. Recently multiple molecular species have been demonstrated in the squid *Todarodes pacificus* and the prawn *Penaeus japonicus* collagens (Yoshinaka *et al.*, 1990; Mizuta *et al.*, 1992; Mizuta *et al.*, 1994a, b).

Recent molecular biological approaches on invertebrate collagens have revealed their primary structures from the sponge *Ephydatia mulleri* (Exposito and Garrone, 1990; Exposito *et al.*, 1993), sandworm *Arenicola marina* (Sicot *et al.* 1997), and sea urchins *Paracentrotus lividus* (D'Alessio *et al.*, 1989, 1990), *Strongylocentrotus purpuratus* (Exposito *et al.*, 1992a, b) and *Hemicentrotus pulcherrimus* (Tomita *et al.*, 1994). Invertebrate fibril-forming collagens are similar to those from vertebrates in terms of the domain structure of procollagens and their *in vivo* processing. The triple helical structures of invertebrate α-chains are also composed of characteristic triplet repeat sequences. However, certain invertebrate fibril-forming collagens

interrupt helical domains lacking one or two Gly-X-Y repeat (Exposito and Garrone, 1990; Exposito *et al.*, 1993; Sicot *et al*, 1997), unlike vertebrate counterparts. It is also noted that the sea urchin *Strongylocentrotus purpuratus* collagen has an unusual long N-propeptide in its 2α-chain (Exposito *et al.*, 1992b).

The turnover rate of this protein is very low in normal tissues, although its degradation is accelerated in the pathological remolding process such as embryonic development, tissue repair, inflammation, tumor invasion, and metastasis (Birkedal-Hansen, 1995). Collagenases are endopeptidases capable of cleaving the helical domain of native collagen molecules under physiological conditions (Harper, 1980). They have been isolated from various vertebrate tissues as well as microorganisms and both are zinc-containing metalloproteinases inhibited by chelating reagents. Bacterial collagenase requires the minimum sequence of Pro-X-Gly-Pro, where proline can be substituted by hydroxyproline (Peterkofsky, 1982). Cleavage occurs at between X and Gly. These sequences occur approximately half of triple-helical domain, so that collagen is degraded into oligopeptides (Oofusa and Yoshizato, 1994).

In vertebrates, matrix metalloproteinases (MMPs) play an important role in the remodeling of extracellular matrix. These enzymes comprise a family of eleven endopeptidases including interstitial collagenase, gelatinase, and stromelysin. MMP expression is tightly controlled by growth factors and cytokines. Their natural inhibitors, TIMPs (tissue inhibitors of metalloproteinases) are also involved in the expression of MMPs activities (Birkedal-Hansen, 1995). Interstitial collagenase (MMP-1) is responsible for the first step of the catabolism of collagen fibers (Gross *et al.*, 1974). MMP-1 cleaves type I, II, III collagen helices at a specific site between Gly-Ile or Gly-Leu bonds (residues 775 and 776) and generates a large N-terminal three-quarter fragment and a C-terminal one-quarter fragment (Birkedal-Hansen, 1987). These fragments spontaneously denature to gelatin that is then susceptible to further degradation by less specific proteases (Oofusa and Yoshizato, 1994). Other proteases such as plasminogen activator and cathepsin B are known to be involved in matrix remodeling (Tryggvason *et al.*, 1987)

On the other hand, collagenolytic serine proteases have been found in invertebrates. They were first isolated from the hepatopancreas of the fiddler crab *Uca pugilator* (Eisen *et al.*, 1973; Grant *et al.*, 1983) which are probably involved in food digestion rather than morphogenesis.

These enzymes were then purified from other crustaceans (Sakharov *et al.*, 1988; Klimova *et al.*, 1990; Lu *et al.*, 1990; Tsai *et al.*, 1991; Turkiewicz *et al.*, 1991; Wormhoudt *et al.*, 1992; Roy *et al.*, 1996), insect (Lecroisey *et al.*, 1979), and fungus (Hurion *et al.*, 1979). The amino acid sequences of these enzymes are highly similar to those of vertebrate pancreatic serine proteases (Grant *et al.*, 1980). These enzymes have been shown to display a catalytic function comparable to those of mammalian trypsin, chymotrypsin, and elastase.

As mentioned previously, foot muscle of gastropods such as abalone and turban shell contain unusually large amounts of collagen (Watabe *et al.*, 1986; Olaechea *et al.*, 1993). Abalone foot myofibrils are surrounded by very thick layers of collagen fibrils of about 1 μm which show cross-striation patterns with a typical periodicity of about 60 nm (Olaechea *et al.*, 1993). Although gastropod foot muscles are very solid, they have high degrees of morphological complexity and behavioral plasticity (Voltzow, 1990). Therefore, these biological features of gastropod foot muscles are likely to be at least partly related to the molecular properties of their abundant fibril-forming collagens. However, the precise molecular structures of gastropod fibril-forming procollagens and collagens have not yet been elucidated and their possible heterogeneity of α-chains has remained unclear.

Furthermore, collagen content in the muscle of kuro-awabi abalone varies seasonally, showing the lowest in July and August when abalone is most tasteful, and the highest in October to December during the spawning season. The collagen content of muscle is related to muscle toughness. The muscle has its lowest toughness in July and August (Olaechea *et al.*, 1993; Hatae *et al.*, 1995). In this season, free amino acids and oligopeptides are at the highest level, together with ATP and related compounds (Hatae *et al.*, 1995). Free and peptide-bound proline and hydroxyproline are also abundant in the summer muscle. These suggest that cellular metabolism in the abalone including collagen turnover is enhanced in this season, accumulating taste-forming compounds in the muscle. However, the mechanisms involved in collagen metabolism are not known.

The present study is concerned with 1) purification of abalone collagens and their properties; 2) molecular structures and gene expression of abalone collagens; 3) proteases related to abalone collagen metabolism.

In Chapter I, pepsin-solubilized collagens were prepared from foot and adductor muscles of abalone and examined for their properties. Adult abalone specimens of *Haliotis discus* (average body weight of 350 g including shells) were collected in February and July from the coastal area along the Pacific Ocean off the Miura Peninsula. Acid-soluble collagens were purified from foot and adductor muscles of abalone by limited pepsin digestion at enzyme-to-substrate weight ratios of 0.1% and 1% for collagens 0.1P and 1P, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of purified collagens showed single α-chain in a 5% polyacrylamide gel, whereas collagen 1P unexpectedly gave two bands of α-chain in a 12.5% gel, where the mobility of the upper α-chain band corresponded to that of collagen 0.1P. When these α-chain bands were subjected to N-terminal amino acid sequencing, the upper and lower α-chain bands of collagen 1P gave different sequences. N-Terminal amino acid sequence for the α-chain band from collagen 0.1P could not be determined. These results suggest that at least two types of α-chains exist in abalone collagen. Amino acid composition of collagen 0.1P was similar to that of vertebrate type I collagens except for less alanine content and higher hydroxylysine contents.

The collagen content of abalone muscle has been shown to decrease in summer and increase in winter. In order to assess possible seasonal changes of the molecule, acid-soluble collagens prepared by digesting with 0.1% pepsin were prepared from abalone collected in February and July. The two collagen preparations were similar in their SDS-PAGE patterns. Their amino acid compositions and peptide maps also resembled each other. Furthermore, differential scanning calorimetry (DSC) patterns showed that the transition temperature was almost similar between collagens in February and July.

Chapter II deals with the immunological properties of abalone collagen. Antisera were raised in rabbits against abalone collagen solubilized with 0.1% pepsin (collagen 0.1P) and its gelatin. To examine the specificity of these antisera, immunoblotting was performed on an Immobilone polyvinylidene difluoride (PVDF) membrane after SDS-PAGE of collagen and myofibrils

prepared from abalone muscle. A strong reactivity was observed with bands corresponding to α- and β-chains using anti-collagen antiserum, whereas this antiserum also reacted with paramyosin contained in myofibrils. In the case of anti-gelatin antiserum, the reactivity with collagen was weaker than that of anti-collagen antiserum, although the reactivity against paramyosin was considerably high. It seems that purified collagen which was used as an antigen still contained small amounts of paramyosin.

When the antiserum conventionally raised against abalone collagen 0.1P was reacted with proteins blotted to the membranes after electrophoresis, both collagens 0.1P and 1P exhibited two bands of α-chain, demonstrating that even collagen 0.1P contained small quantities of α-chain which corresponded to that of the faster-moving band found in collagen 1P.

The cross-reactivity with various vertebrate and invertebrate collagens was examined by immunoblotting with both antisera. Collagens used were prepared from the following tissues. Pepsin-solubilized type I collagens from the bullfrog *Rana catesbeiana*, chicken and rat, and acid soluble type I collagens from the carp *Cyprinus carpio*, chum salmon *Oncorhynchus keta* and calf were prepared from skin. Pepsin-solubilized type III and type V collagens were isolated from bovine placenta. Pepsin-solubilized collagens from invertebrates and a protochordate were isolated from the whole body of the sea anemone *Actina equina* and the jellyfish *Aurelia aurita*, and from the muscle of the ark shell *Scapharca broughtonii*, oyster *Crassostrea gigas*, turban shell *Batillus cornutus*, abalone *Haliotis discus*, squid *Todarodes pacificus*, octopus *Octopus ocellatus*, prawn *Penaeus japonicus*, and sea squirt *Halocynthia roretzi*.

Type I collagens are heterotrimers of two α1(I) and one α2(I) chains except for salmon collagen which has a subunit composition of α1(I) α2(I) α3(I). Anti-abalone collagen antiserum was reacted strongly with carp α1(I) chain and was not reactive with its α2(I) chain. The antiserum reacted with both α-chains of frog collagen. The cross-reactivity with α2(I) chain was observed with salmon, chicken, rat and calf, whereas α1(I) chain was not reactive. In the case of anti-gelatin antiserum, the high reactivity with type I collagens was observed for α2(I) chains. Type III and type V collagens from bovine placenta were not reactive with both antisera of abalone.

The cross-reaction of invertebrate and protochordate collagens was observed with both antisera. The reactivity was stronger with anti-collagen than anti-gelatin antiserum as in the case of vertebrate collagens. The strong reactivity was found with turban shell collagen, whereas the reactivity was weak with jellyfish and sea squirt collagens. In squid, octopus, and prawn collagens the highest reactivity was observed with the upper α-chains.

Chapter III describes structural properties of abalone collagens and their gene expression. A cDNA library was constructed from juvenile abalone foot muscle and screened with the anti-collagen antiserum. Two types of full-length cDNAs coding for collagen proα-chains, Hdcols (*Haliotis discus* collagens) 1α and 2α, were isolated and their nucleotide sequences were determined. These were composed of domain structures characteristic to fibril-forming collagens. N-Terminal amino acid sequences determined for the upper and lower α-chain bands of collagen IP were located at the boundaries between the N-telopeptide and main triple-helical domain of Hdcols 2α and 1α, respectively. In addition, several tryptic peptides prepared from the pepsin-solubilized collagen showed N-terminal amino acid sequences identical to those deduced from the two cDNA clones.

The N-propeptides of the two collagen proα-chains contained cysteine-rich globular domains. Hdcol 1α completely lacked a short Gly-X-Y triplet repeat sequence in its propeptide. Such unusual structure has never been reported before for any fibril-forming collagen. Their main triple-helical domains both consisted of 1,014 amino acids where a supposed glycine residue in the triplet at the 598th position from the N-terminus was replaced by alanine in Hdcol 1α and by serine in Hdcol 2α. Glycine residues were found in position X or Y of the main helical domain 4 and 13 times with Hdcols 1α and 2α, respectively. Such high abundancy of glycine has been demonstrated in invertebrate and lower vertebrate collagens. In both abalone proα-chains, a consensus sequence for the cross-linking site, Lys-Gly-His-Arg, was located at the 84th residue from the N-terminus of the main triple-helical domain. A similar tetrapeptide sequence, Lys-Gly-Typ(Asp)-Pro, was found at 83 amino acids upstream from the end of the main triple helix in each of two abalone proα-chains, while histidine, potentially essential to catalyze the cross-linking, was substituted by tryptophan in Hdcol 1α and by aspartic acid in Hdcol 2α.

Furthermore, both α-chains contain a lysine-mediated cross-linking site in each N- and C-

telopeptide. Both proα-chains of abalone collagens contained 6 cysteine residues in the C-propeptide, lacking 2 cysteine residues usually found in vertebrate collagens. Amino acid sequence identities between Hdcols 1α and 2α were 44.1% in the full length, 52.1% in the main triple-helical domain, 29.5% in C-propeptide, and 16.8% in N-propeptide.

Northern blot analysis demonstrated that the mRNA levels of Hdcols 1α and 2α in various tissues including muscles were similar to each other. The highest amount of mRNA was observed in mantle. Considerable amounts were also observed with foot muscle of adult and juvenile abalones and with adductor muscle of juvenile specimens.

To investigate seasonal changes of collagen mRNA levels in relation to seasonal change of collagen content, adult abalones were collected every month from August 1997 to July 1998 from the coastal area along the Pacific Ocean off the Miura Peninsula. Total RNAs were prepared from adductor and foot muscles and hepatopancreas. The seasonal changes of collagen mRNA levels were quite similar between Hdcols 1α and 2α in the three tissues. The highest mRNA levels were found in December and January, possibly related with the increase of collagen content. The collagen mRNA levels in adductor and foot muscles decreased in July when collagen content decreased. The differences in the mRNA levels between winter and summer were statistically significant. These results suggest that collagen transcripts levels are closely related to collagen contents.

Chapter IV deals with proteases related to abalone collagen metabolism. Gelatinolytic activities were examined for hemolymph and extracts from various tissues of abalone by gelatin zymogaphy. The active band at 45 kDa was commonly observed in all samples, whereas hemolymph showed a strong gelatinolytic band at 110 kDa. This 110 kDa active band disappeared in the absence of $CaCl_2$. The reactivity with abalone collagen fibrils at 37°C was observed in muscle extracts prepared at pH 4.0 and hepatopancreas extracts from acetone powder. The reactivity of muscle extracts prepared from abalone collected in February was higher than that from abalone collected in July. Collagenolytic activity of the muscle extract was inhibited by the inhibitors of metalloproteases such as EDTA, EGTA, and *1, 10*-phenanthroline at pH 7.5, and by the inhibitors of cysteine proteases such as *N*-ethylmaleimide (NEM) and E-64 at pH 4.0.

Collagen-film-degrading protease was isolated from the adductor muscle of abalone, by a series of chromatographic procedures involving DEAE-Toyopearl 650M ion exchange, TSK G3000SWG gel filtration, and Butyl-Toyopearl 650M hydrophobic columns. The reactivity with collagen was measured as the ability to solubilize the film containing pepsin-solubilized collagen from the same animal. Active fractions of various purification steps solubilized the film at 37°C within 60 min. The purified protein consisted of a single polypeptide chain and the molecular weight was estimated by SDS-PAGE to be 148,000. The properties of the 148 kDa protein was also examined. The solubilized proteins collected from the collagen film did not show any degradation products. It is suggested that the 148 kDa protein did not cleave the helical domain of the collagen molecule. While the activity of this protein was not observed in the absence of Ca^{2+}, it was recovered in the addition of 30-50 mM Mg^{2+}, 20-50 mM Ca^{2+}, and 20-50 mM Sr^{2+}. The level of the reactivity with collagen film was, however, very similar in winter and summer abalone specimens. This protein did not contain methionine and its N-terminal amino acid sequence differed from any sequences of known collagenases.

Chapter V is devoted to general discussion based on the results of the present and other related studies.

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Abbreviations

ATP: adenosine 5'-triphosphate
BSA: bovine serum albumin
bp: base pairs
CBB R-250: Coomassie Brilliant Blue R-250
cDNA: complementary deoxyribonucleic acid
C-propeptide: carboxyl-terminal propeptide
C-telopeptide: carboxyl-terminal telopeptide
dCTP: deoxycytidine 5'-triphosphate
DEAE: diethylaminoethyl
DIG: digoxigenin
DSC: differential scanning calorimetry
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
EGTA: ethylene glycol bis (β-aminoethyl ether)-*N, N, N', N'*-tetraacetcic acid
ER: endoplasmic reticulum
Hdcol 1α: *Haliotis discus* collagen proα-chain 1
Hdcol 2α: *Haliotis discus* collagen proα-chain 2
IPTG: isopropyl-β-*D*-thiogalactopyranoside
MMP: matrix metalloproteinase
mRNA: messenger ribonucleic acid
rRNA: ribosomal ribonucleic acid
NEM: *N*-ethylmaleimide
N-propeptide: amino-terminal propeptide
nt: nucleotide
N-telopeptide: amino-terminal telopeptide
PCR: polymerase chain reaction
PMSF: phenylmethanesulfonyl fluoride
PVDF: polyvinylidene difluoride
S. D.: standard deviation
SDS: sodium dodecyl sulfate
SDS-PAGE: SDS-polyacrylamide gel electrophoresis
SSC: standard saline citrate
STI: soybean trypsin inhibitor
TBS: Tris-buffered saline
TFA: trifluoroacetic acid
TIMP: tissue inhibitors of metalloproteinase
T_m: transition temperature
Tris: Tris (hydroxymethyl) aminomethane

Chapter I Purification of Abalone Collagens and Their Physicochemical Properties

Collagen is one of the most fundamental constituents of the extracellular matrix and plays mechanical and physiological roles in the animal body. Fibril-forming interstitial collagens are of the largest group in collagen family in vertebrates. However, information on invertebrate fibril-forming collagens is very limited. Kimura (1975) investigated physicochemical properties of pepsin-solubilized collagen from marine invertebrates and protochordate: intestine of the sandworm *Neanthes diversicolor*, mantles of the pearl oyster *Pinctada martensii* and the sea squirt *Halocynthia roretzi*, foot muscle of the abalone *Haliotis discus*, skins of the octopus *Octopus vulgaris* and the squid *Todarodes pacificus*, and subcuticular tissues of the crab *Portunus trituberculatus* and the lobster *Panulirus japonicus*. The molecular structures of these collagens were similar to those of vertebrate fibril-forming collagens in terms of the values of intrinsic viscosity and specific optical rotation. The molecular weights of these collagens were calculated to be 240,000-330,000 from the values of sedimentation constant. In addition, SDS-PAGE of these collagens showed that they were composed of α-chains whose molecular weights were 95,000-100,000. In histological studies, collagen fibrils of abalone, turban shell *Batillus cornutus*, and octopus showed cross-striation patterns with a typical periodicity of about 60 nm (Takema and Kimura, 1982; Watabe *et al.*, 1986; Olaechea *et al.*, 1993). These results indicate that fibril-forming interstitial collagens also exist in invertebrates.

Collagens from abalone, turban shell, and Chinese prawn *Penaeus chinensis* were homotrimer $(\alpha 1)_3$ (Kimura and Matsuura, 1974; Kimura and Tanaka, 1983; Minamisako and Kimura, 1989), while most of invertebrate collagens including the squid *Todarodes pacificus*, octopus *Octopus vulgaris*, starfish *Asterias amurensis*, and sea urchin *Asthenosoma ijimai* had the composition $(\alpha 1)_2\alpha 2$ (Kimura and Matsuura, 1974; Kimura *et al.*, 1993; Omura *et al.*, 1996), commonly found in various vertebrate collagens. The jellyfish *Stomolophus nomurai* collagen had three types of α-chains to form $(\alpha 1)(\alpha 2)(\alpha 3)$ (Miura and Kimura, 1985) which was similar to bonyfish type I collagens (Kimura *et al.*, 1987). Invertebrate interstitial collagens had high contents of hydroxylysine and glycosylated hydroxylysine except for starfish collagen. They

were classified into two groups by their chemical compositions, namely type I- and type V-like collagens. Type I-like collagens showed high contents of alanine, whereas type V-like collagens had low alanine contents together with considerably high contents of glycosylated hydroxylysine (Kimura, 1992). Collagens from abalone, turban shell, squid, octopus, and starfish belonged to type I-like collagen, while those from prawn, sea squirt, and jellyfish belonged to type V-like collagen (Kimura, 1992). The occurrence of two distinct types of collagens has been reported in the muscle of the squid *Todarodes pacificus* and kuruma prawn *Penaeus japonicus* (Yoshinaka *et al.*, 1990; Mizuta *et al.*, 1992; Mizuta *et al.*, 1994a, b). The major collagen from the squid was similar to those of type I in vertebrate, whereas a minor one resembled vertebrate type V collagens. The major collagen in prawn showed three molecular forms $(\alpha 1)_3$, $(\alpha 1)_2\,\alpha 2$, and $\alpha 1(\alpha 2)_2$ (Mizuta *et al.*, 1992).

On the other hand, collagen from the annelid *Alvinella pompejana* cuticle is the longest (2.4 μm) known so far (Gaill *et al.*, 1991, 1995). It is quite soluble in acidic solution, while most of invertebrate collagens are not solubilized without pepsin digestion. The molecular weight of cuticle collagen is 1,700,000 and composed of two A chains and one B chain (Gaill *et al.*, 1991). The amino acid composition of cuticle collagens showed a high degree (> 85%) of proline hydroxylation and the absence of hydroxylysine (Gaill *et al.*, 1991).

Thus, structural variety has been demonstrated in invertebrate collagens. However, the possible heterogeneity of α-chains for abalone collagen has remained unclear. The differences between collagen preparations from abalone collected in winter with the highest collagen content and in summer with the lowest content are also unknown. In this chapter, pepsin-solubilized collagens from abalone foot and adductor muscles were purified and examined for their partial amino acid sequences. Physicochemical properties were also investigated with the collagens prepared from abalone collected in February and July.

Section 1 Purification of collagens

The gastropod foot muscle contains large quantities of collagen which is almost insoluble in acidic solution, probably due to inter-molecular cross-links in its telopeptide. Pepsin digestion has been performed to solubilize acid-insoluble collagen by cleaving telopeptides (Rubin *et al.*, 1965). The resulting pepsin-solubilized collagen contained the main triple-helical domain and short non-helical domain at both ends. Kimura and Matsuura (1974) prepared pepsin-solubilized collagen from abalone muscle. Abalone collagen contained a single type of α-chain which forms $(\alpha 1)_3$ homotrimer, the molecular size of which was similar to those of fibril-forming vertebrate collagens.

In this section, pepsin-solubilized collagens were prepared from abalone foot and adductor muscles under two sets of condition. Collagens were also prepared from abalone collected in February and July in which collagen contents were high and low, respectively.

Materials and Methods

Materials

Adult abalone specimens of *Haliotis discus* (average body weight of 350 g including shells) were collected in February and July from the coastal area along the Pacific Ocean off the Miura Peninsula.

Preparation of pepsin-solubilized collagen

Pepsin-solubilized collagen was prepared from abalone foot and adductor muscles essentially according to Kimura and Kubota (1968) and all preparations were carried out at 4°C. The muscles were cut into small pieces and homogenized with distilled water using a Polytron homogenizer (Kinematica). The homogenate was stirred with 5 volumes of 0.6 M KCl for 24 h and centrifuged at 5,000 x g for 15 min. The precipitate was treated two more times in the same manner. The resulting precipitate was homogenized with 5 volumes of 0.45 M NaCl for 24 h under stirring and centrifuged at 5,000 x g for 15 min. The precipitate was treated with the same

procedures two more times and subsequently treated with 5 volumes of 0.1 M acetic acid for 48 h, followed by centrifugation at 10,000 x g for 30 min.

The acid insoluble fractions were subjected to limited proteolysis with pepsin (Sigma) at an enzyme-to-substrate weight ratio of 1 : 100 for 24 h (collagen 1P) or 1 : 1,000 for 48 h (collagen 0.1P). After centrifugation at 100,000 x g for 1 h, the solubilized collagen in the supernatant was precipitated by the addition of solid NaCl to a final concentration of 0.6 M. The collagen precipitate was suspended in 20 volumes of 50 mM Tris-HCl (pH 7.4) containing 1 M NaCl overnight and added solid NaCl to a final concentration of 2.8 M. After centrifugation at 15,000 x g for 1 h, the resulting precipitate was dissolved in and exhaustively dialyzed against 0.1 M acetic acid. Pepsin-solubilized collagen was obtained in the supernatant by centrifugation at 100,000 x g for 1 h.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970), using 5% or 7.5% polyacrylamide slab gels (30 : 0.8 by weight for acrylamide : bisacrylamide) containing 0.1% SDS and 12.5% polyacrylamide slab gels (30 : 0.135 by weight for acrylamide : bisacrylamide) containing 0.1% SDS. Samples were added with an equal volume of 20 mM Tris-HCl (pH 6.8) containing 2% SDS, 2% 2-mercaptoethanol, 40% glycerol, 4 mM EDTA, and 0.015% bromophenol blue (SDS-containing sample buffer). The sample mixtures were boiled for 2 min and subjected to SDS-PAGE. Gels were stained with 0.05% Coomassie Brilliant Blue (CBB) R-250 after electrophoresis and destained with a solution containing 25% methanol and 7% acetic acid. Standard molecular weight markers (Sigma) in SDS-PAGE were myosin heavy chain from rabbit muscle (205,000), β-galactosidase from *Escherichia coli* (116,000), phosphorylase b from rabbit muscle (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase from bovine erythrocytes (29,000).

Results

Pepsin-solubilized collagens were prepared from abalone foot and adductor muscles by limited pepsin digestion. Acid-insoluble collagen fibrils were digested with pepsin at 4°C at enzyme-to-substrate weight ratios of 0.1 and 1% for collagen 0.1P and 1P, respectively.

Figure 1-1 shows SDS-PAGE patterns of collagen 0.1P at various purification steps. Collagens were stained on metachromasy with CBB R-250 and appeared as pink-red color bands whereas other protein bands were stained blue-colored (Micko and Schlaepfer, 1978). No collagen band was seen in acid-insoluble fraction in which paramyosin was observed, although highly aggregated proteins were found at the top of the gel (Fig. 1-1, lane 1). The supernatant obtained after pepsin digestion contained both collagen α- and β-chains, together with non-collagenous proteins around 80 kDa and at the buffer front of the gel (Fig. 1-1, lane 2). Collagen was further purified by precipitation with NaCl at acidic and neutral pH. SDS-PAGE of purified abalone collagen revealed that α- and β-chains together with highly aggregated α-chain were stained on metachromasy where no low-molecular-weight contaminants were present (Fig. 1-1, lane 3). The molecular weight was estimated to be 123,000 for α-chain and 240,000 for β-chain. However, these values were probably overestimated since the electrophoretic mobilities of collagen are lower than other proteins (Hayashi, 1990).

Collagens 0.1P and 1P both showed single bands for α- and β-chains in a 5% polyacrylamide gel (Fig. 1-2). On the other hand, collagen 1P unexpectedly gave two bands of α-chain in a 12.5% gel, where the mobility of the upper α-chain band corresponded to that of collagen 0.1P α-chain. These two α-chains were observed with gels of more than 10%. It seems that a part of α-chain produced from acid-soluble abalone collagen with 0.1% pepsin digestion was further digested into another lower-molecular-weight α-chain by extensive treatment with 1% pepsin.

These results were obtained with collagen from abalone collected in February. However, no appreciable differences in SDS-PAGE patterns between collagens prepared from abalones collected in February and July.

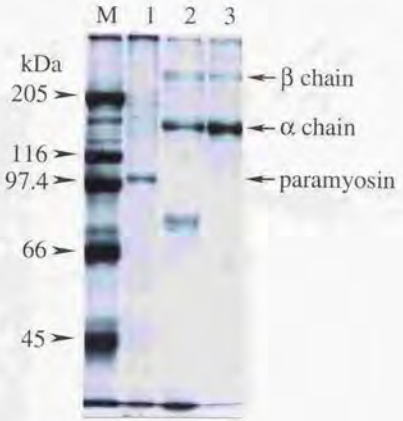


Fig. 1-1. SDS-PAGE patterns of abalone collagen at various purification steps. Acid-soluble collagens were prepared by digesting acid-insoluble fractions with 0.1% pepsin. Each fraction of 5 μg/lane was applied to the 7.5% polyacrylamide gel and stained with CBB R-250. Lane M contains molecular weight markers. Lane 1, acid-insoluble fraction; lane 2, supernatant after pepsin digestion; lane 3, pepsin-solubilized collagen. Arrows indicate collagen α- and β-chains, and paramyosin.

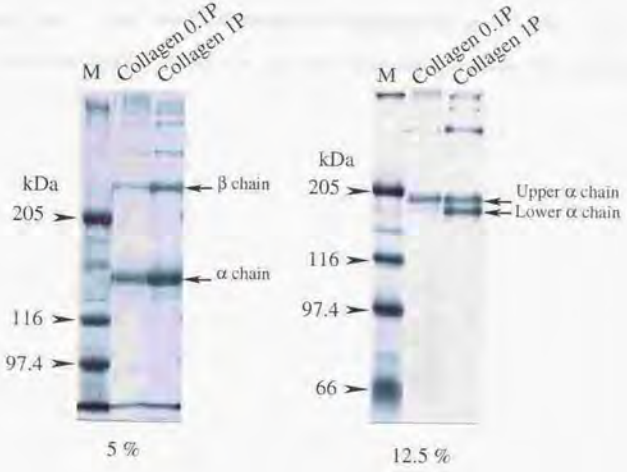


Fig. 1-2. SDS-PAGE patterns of pepsin-solubilized abalone collagens. Acid-soluble collagens were prepared by digesting acid-insoluble fraction with pepsin at an enzyme-to-substrate weight ratio of 0.1% (collagen 0.1P) or 1% (collagen 1P). Lane M contains molecular weight markers. Collagens of 2 μg/lane were applied to 5% and 12.5% polyacrylamide gels. Arrows indicate α- and β-chains in a 5% polyacryamide gel, and upper and lower α-chains in a 12.5% gel.

Summary

Pepsin-solubilized collagens were purified from foot and adductor muscles of abalone by limited pepsin digestion at enzyme-to-substrate weight ratios of 0.1% and 1% for collagens 0.1P and 1P, respectively. SDS-PAGE of purified collagens showed a single α-chain in a 5% polyacrylamide gel. On the other hand, collagen 1P unexpectedly gave two bands of α-chain in a 12.5% gel, where the mobility of the upper α-chain band corresponded to that of collagen 0.1P.

Section 2 Physicochemical properties of collagens

As described in the previous section, abalone acid-soluble collagens prepared by 1% pepsin digestion (collagen 1P) gave two α-chain bands in SDS-PAGE. In this section, N-terminal amino acid sequencing was performed to examine the heterogeneity of these α-chains for both intact pepsin-solubilized collagens and their tryptic peptide.

The collagen content of abalone muscle has been shown to decrease in summer and increase in winter (Olaechea *et al.*, 1993; Hatae *et al.*, 1995). In order to assess possible seasonal changes, amino acid composition, peptide mapping, and differential scanning calorimetry were performed for collagens prepared form abalone collected in February and July.

Materials and Methods

Materials

Acid-soluble collagens prepared as described in Section 1 of this chapter by digesting with 0.1% pepsin (collagen 0.1P) and 1% pepsin (collagen 1P) were used for the following experiments.

N-Terminal amino acid sequencing

The N-terminal amino acid sequences were determined by the method of Matsudaira (1987). The proteins in the SDS-PAGE gels were electrically transferred onto an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) using a blotting buffer containing 3-cyclohexlamino-1-propanesulfonic acid, 5% methanol and 0.1% SDS, and stained with CBB R-250. The α-chain bands were cut out and subjected to a Perkin Elmer Applied Biosystems model 476A protein sequencer.

Preparation of tryptic peptides

Collagen 0.1P prepared from abalone collected in July was digested with trypsin (Sigma) at an enzyme-to-protein weight ratio of 1 : 200 at 37°C for 6 h in 50 mM Tris-HCl (pH 8.0)

containing 2 M urea and 5 mM $CaCl_2$. The digest was subjected to HPLC equipped with a TSKgel ODS-80Ts reverse-phase column (4.6 x 150 mm, Tosoh). Peptides were eluted at a flow rate of 0.5 ml/min with a linear gradient for 50 min using solvent A containing 0.05% trifluoroacetic acid (TFA) in water and solvent B containing 0.02% TFA in 2-propanol : acetonitrile (7 : 3, v/v). The tryptic peptides eluted from the HPLC column were directly applied to the protein sequencer.

Amino acid composition analysis

Collagens solubilized with 0.1% pepsin for abalones collected in February and July were hydrolyzed in 6 N HCl at 110°C for 24 h. Amino acid analysis was performed with a Hitachi L-8500A amino acid analyzer.

Peptide mapping

Collagens solubilized with 0.1% pepsin for abalones collected in February and July were digested at 30°C with *Acromobacter* protease I (lysil endopeptidase) (Wako) at an enzyme-to-protein weight ratio of 1 : 6,000 in 5 mM Tris-HCl (pH 8.5) containing 4 M urea and 0.1% SDS. At certain time intervals, aliquots were taken from the reaction mixture and the digestion was stopped by adding phenylmethanesulfonyl fuloride (PMSF) at a final concentration of 1 mM. The digestion pattern was analyzed by SDS-PAGE.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed with a MicroCal model MC2 differential scanning microcalorimeter. Collagen 0.1P prepared from abalone collected in July and February were dialyzed against 0.1 M acetic acid. The sample cell was loaded with 1.2 ml of a collagen solution at a protein concentration of 0.9 mg/ml, whereas the reference cell was filled with 0.1 M acetic acid. DSC scans were performed at a rate of 45°C/h in the temperature range from 10 to 70°C under an excess N_2 pressure at about 220 kPa. DSC data were analyzed using a software package, Origin, developed by MicroCal.

Results

N-Terminal amino acid sequences of α-chains and those of tryptic peptides

The α-chain bands, two from collagen 1P and one from collagen 0.1P which were prepared from abalone collected in February as shown in the previous section, were subjected to N-terminal amino acid sequencing. The upper and lower α-chain bands of collagen 1P gave different sequences, although they both showed Gly-X-Y triplet repeat sequences and contained Gly-Pro-Arg-Gly in the middle part of the determined sequence (Fig. 1-3). It was impossible to determine the N-terminal amino acid sequence for the α-chain band from collagen 0.1P, possibly due to heterogeneity in the sequence. SDS-PAGE patterns and N-terminal amino acid sequence analyses implied the existence of at least two types of fibril-forming collagen in the abalone muscle.

In order to compare further the primary structure between the possible two types of abalone collagen, collagen 0.1P was subjected to tryptic digestion at 37°C for 6 h at an enzyme-to-collagen weight ratio of 1 : 200 and pH 8.0. Subsequently, the digests were applied to a reverse-phase column, yielding totally six peptides which could be determined for their N-terminal amino acid sequences (Fig. 1-4). As shown in Table 1-1, all the sequences were composed of Gly-X-Y triplet repeats and hydroxyproline was always found in position Y. However, all these sequences were completely different from each other and no conclusive data about heterogeneity of abalone collagens could be deduced from these analyses.

Amino acid composition of collagens

Table 1-2 shows the amino acid composition of collagen 0.1P prepared from abalone collected in February and July together with the data previously reported for foot muscle collagen from the same species of abalone (Kimura and Matsuura, 1974) and type I collagen from the carp *Cyprinus carpio* muscle (Kimura *et al.*, 1988). The amino acid compositions of collagens in February and July were quite similar to each other, showing a typical pattern of collagen, although the preparation in July was higher in glycine residues than that in February. The degree of proline hydroxylation related to thermal stability of collagen (Berg and Prockop, 1973; Zhu and Kimura, 1991) was almost similar between collagens in February and July. Abalone collagens

Upper α-chain QVGPIGPRGLSG

Lower α-chain YSPGAPGPRGPPGPP

Fig. 1-3. N-terminal amino acid sequences of α-chains of collagen solubilized with 1% pepsin digestions. P indicates hydroxyproline.

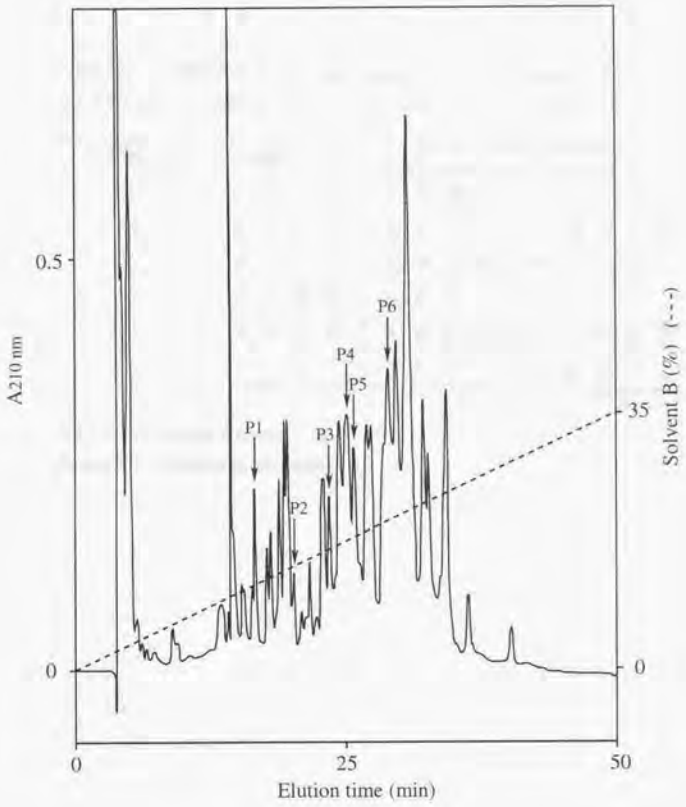


Fig. 1-4. The reverse-phase HPLC profile of tryptic digests from abalone collagen. Acid-soluble collagen prepared with 0.1% pepsin digestion was further digested with trypsin and applied to a TSK gel ODS-80Ts column (4.6 x 150 mm). Arrows indicate fractions subjected to N-terminal amino acid sequencing. Solvent A, 0.05% TFA; solvent B, 0.02% TFA containing 70% isopropanol and 30% acetonitrile.

Table 1-1. N-terminal amino acid sequences of tryptic peptides derived from abalone collagen solubilized with 0.1% pepsin digestion

Peptide	Sequence
P1	G Q P[1] G P P G P S
P2	G S G G I P G N S G T P G D P G
P3	G L S G T P G P P G P Q G R
P4	G L P G T P G M S G
P5	Q G D X[2] G I P G A A G P P G S
P6	G Y P G E A G P I G A P G N E

[1]P indicates hydroxyproline.

[2]X denotes an unidentified residue.

Table 1-2. Amino acid composition of collagen solubilized with 0.1% pepsin digestion from abalones collected in February and July in comparison with the data previously reported for abalone and type I collagen of carp

(residues/1,000 residues)

Amino acid	Abalone			Carp
	February	July	Foot muscle[1]	type I[2]
Hyp	81	79	81	84
Asx	60	59	59	47
Thr	20	20	17	26
Ser	63	61	55	35
Glx	103	100	96	72
Pro	88	87	102	110
Gly	342	358	334	337
Ala	87	86	87	119
Val	18	17	18	20
Met	8	9	12	13
Ile	12	11	13	10
Leu	27	25	28	22
Tyr	7	6	7	3
Phe	7	7	7	13
Hyl	11	10	14	7
Lys	8	8	8	25
His	1	1	1	5
Arg	57	56	61	52
Hyp (%)[3]	47.9	47.6	44.3	43.3
Hyl (%)[4]	58.0	55.6	63.6	21.9

[1] Kimura and Matsuura (1974).
[2] Kimura *et al.* (1988).
[3] The degree of proline hydroxylation.
[4] The degree of lysine hydroxylation.

showed a composition similar to that of type I collagen from carp muscle, although abalone collagens contained less alanine and higher degree of lysine hydroxylation than carp collagen as reported by Kimura and Matsuura (1974).

Peptide mapping

Collagens prepared from abalones collected in February and July were further digested with *Acromobacter* protease I (lysil endopeptidase). At various time intervals during digestion, portions were taken from the reaction mixture and analyzed by SDS-PAGE. As shown in Fig. 1-5, collagen samples were cleaved progressively with increase of the reaction time. Three major bands of 96, 55, and 45 kDa were observed in both collagens after digestion for 15 min. Two fragments of 40 and 20 kDa were produced with concomitant decrease of the 96 kDa fragment. After digestion for 60 min, the α-chains of both collagens disappeared. The digestion patterns of collagen together with SDS-PAGE patterns of collagen before digestion from abalone collected in February and July were similar to each other, indicating no apparent changes of their molecular species.

Differential scanning calorimetry (DSC)

Figure 1-6 shows the DSC scans in 0.1 M acetic acid for 0.1% pepsin-solubilized collagens prepared from abalones collected in February and July. Two endotherms having transition temperature (T_m) of 30.8 and 33.6°C were found for collagen from February. One prominent endotherm having T_m at 34.9°C was found for collagen from July, although the shape of this peak was not symmetrical but somewhat skew, implying overlap of a few peaks. The deconvolution analysis gave two endotherms having T_m at 28.8 and 34.2°C for collagen from February and three endotherms having T_m at 28.8, 35.0, and 41.9°C for collagen from July. The thermal denaturation of collagens were irreversible. In DSC analysis, the endothermal triple helix as found in collagen has been shown to be thermally denatured to random coil phase transition (Privalov, 1982). Similar thermal transition temperatures of the major endotherms in collagens from February and July together with the degree of proline hydroxylation suggest that molecular structures of both collagens closely resemble each other.

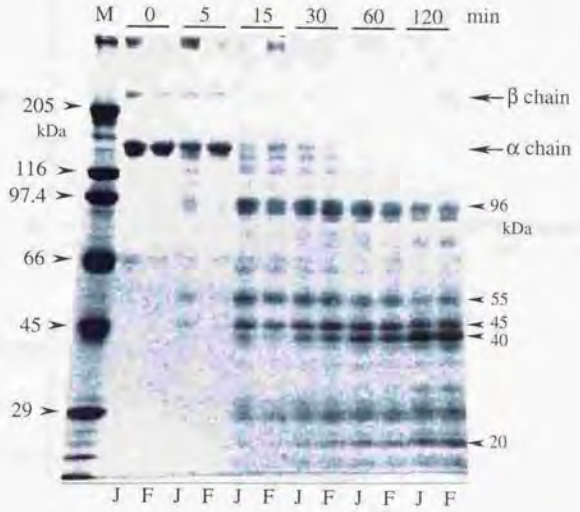


Fig. 1-5. Peptide maps of collagens from abalones collected in February and July. Abalone collagens solubilized with 0.1% pepsin were digested at 30°C for various time intervals with *Acromobacter* protease I (lysil endopeptidase) at an enzyme-to-protein weight ratio of 1 : 6,000 in 5 mM Tris-HCl (pH 8.5) containing 4 M urea and 0.1% SDS. Twenty μg of the reaction mixture were applied to a 7.5-20% gradient polyacrylamide slab gel containing 0.1% SDS. Arrows indicate α- and β-chains. Arrowheads indicate protein bands of interest. The numerals above the photograph represent different digestion periods. Abbreviations used are M, molecular weight markers; J, collagen prepared from abalone collected in July; F, collagen prepared from abalone collected in February.

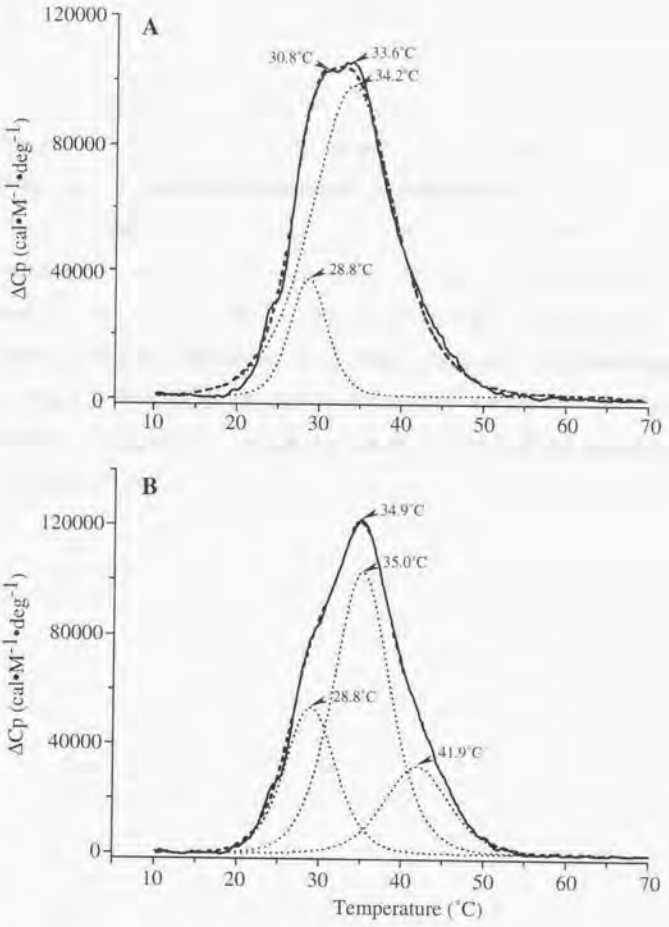


Fig. 1-6. DSC scans of 0.1% pepsin-solubilized collagen from abalone collected in February (A) and July (B), together with their computer-calculated differential endotherms by the deconvolution analysis. DSC scans were performed in 0.1 M acetic acid at protein concentrations of 0.89 (A) and 0.87 mg/ml (B). The observed DSC patterns (solid lines) were subjected to smoothing treatment (bold-faced dashed lines) and further to the convolution analysis (dotted lines) for differential endotherms. The scan rate was 45°C/h, and data were collected every 15 s. ΔC_P represents molar excess heat capacity. Arrows indicate transition temperatures.

Summary

The three α-chain bands, two from collagen 1P and one from collagen 0.1P, were subjected to N-terminal amino acid sequencing. The upper and lower α-chain bands of collagen 1P gave different sequences, although the N-terminal amino acid sequence for the α-chain band from collagen 0.1P could not be determined. SDS-PAGE patterns and N-terminal amino acid sequences suggest that at least two types of α-chains exist in abalone collagen. Amino acid composition of collagen 0.1P was similar to that of vertebrate type I collagen except for less alanine and higher hydroxylysine contents in the former. Amino acid composition and peptide maps of collagens in February and July resembled each other. Furthermore, DSC runs demonstrated that the transition temperatures for thermal unfolding were almost similar between collagens in February and July.

Section 3 Discussion

Abalone foot muscle contains large quantities of collagen, which is responsible for muscle toughness (Olaechea *et al.*, 1993; Hatae *et al.*, 1995). Collagen layers in abalone muscle surround myofibrils randomly running in the same muscle region (Olaechea *et al.*, 1993). In molluscan muscle, paramyosin is abundant which is located in the thick filaments of myofibrils (Castellani *et al.*, 1983). Acid-insoluble fractions containing both collagen and paramyosin were subjected to limited digestion with pepsin and collagen was further purified. SDS-PAGE patterns of collagen IP showed two α-chain bands in polyacrylamide gels of more than 10%. The heterogeneity of these α-chains was demonstrated by their N-terminal amino acid sequencing, although it had been considered that abalone collagen was composed of three α-chains of a single species (Kimura and Matsuura, 1974). In amino acid compositions, abalone collagens resembled vertebrate type I collagens. However, a high degree of lysine hydroxylation was characteristic of abalone collagens as has been demonstrated with collagens from marine invertebrates. In addition, they are rich in glycosylated hydroxylysine (Kimura, 1975; Miura and Kimura, 1985; Minamisako and Kimura, 1989; Omura *et al.*, 1996).

The denaturation temperature of collagen is closely related to the ambient temperature in poikilotherms (Takahashi and Yokoyama, 1954; Rigby, 1971). Animals living in high temperature environments possess thermally stable collagens with a high degree of proline hydroxylation which occurs with post-translational modification (Prockop and Kivirikko, 1995). Kimura *et al.* (1988) reported that the denaturation temperature of muscle type I collagen was higher by about 1°C than that of the skin counterpart from the same teleost in association with a higher degree of proline hydroxylation in the former. In the present study, the peptide map and thermal stability were not different between collagens from February and July. However, it seems still possible to consider that other differences such as that in the carbohydrate content may exist between them.

Chapter II Preparation of Anti-abalone Collagen and Anti-gelatin Antisera and Their Properties

Collagens represent a large family of structural extracellular matrix proteins with up to 19 groups identified so far in vertebrate (Prockop and Kivirikko, 1995). They all share a rod-like triple-helical segment as a typical structural element. Although most invertebrates contain collagens similar to either the fibril-forming or network-forming collagens of vertebrate, structural variability is indicated from recent studies on various invertebrate collagens (Bairati and Garrone, 1985). Antibodies are important tools for characterizing structural, biological, and pathological properties of collagens. The specific antibody is capable of distinguishing collagens and their submolecular species among various types.

Collagens possess a variety of different antigenic determinants owing to their large size. Human type I, II, and III collagens possess potent antigenic determinants in their telopeptide regions when rabbits are used for immunization (Becker *et al.*, 1975). Another conformation-dependent antigenic determinants locate in the triple helix of collagens (Hahn and Timpl, 1973). Antigenic reaction with these sites requires a correct chain composition and is allowed to conform identical types of collagens obtained from different animal species. A third class of antigenic structures, named central antigenic determinants, was identified in the central regions of unfolded α-chains (Timpl, 1982). Antibodies against these sites seem specific for unfolded chain constituents and show a broad interspecies cross-reaction (Furthmayr *et al.*, 1972).

Native and denatured collagens together with their small peptide fragments have been used as immunogens. The denatured collagens are mostly weaker as immunogens than native collagens (Timpl, 1982). However, the reactivity of the antibody raised against denatured collagen from the sea urchin *Hemicentrorus pulcherrimus* was higher than that of anti-native collagen antibody (Tomita *et al.*, 1994).

In this chapter, specific antisera against abalone collagen and gelatin were prepared and examined for their reactivity with abalone muscle proteins including collagen. Furthermore, the cross-reactivity against collagens from various animal species were examined.

Section 1 Preparation of anti-collagen and anti-gelatin Antisera

Abalone contains large amounts of collagen in the muscle. Pepsin-solubilized collagen from abalone resembled fibril-forming vertebrate collagens in the molecular size (Kimura, 1975). Pepsin-solubilized abalone collagen prepared in the previous section contained two types of α-chain and was similar to type I collagens in amino acid composition. Specific antisera against collagen are useful to distinguish various types of collagen as well as their submolecular species (Timpl, 1982). In this section, the specific antisera were raised against abalone collagen and gelatin, and examined for their specificities.

Materials and Methods

Materials

Adult abalone specimens as described in Section 1 of Chapter I were used for protein preparations.

Preparation of collagen and myofibrils

Acid-soluble collagen prepared by digesting with 0.1% pepsin (collagen 0.1P) as described in Section 1 of Chapter I was used as an antigen. To prepare abalone gelatin, a collagen 0.1P solution adjusted to pH 7.5 was boiled for 3 min.

Myofibrils were prepared according to Perry and Grey (1956) with some modifications as follows. Foot and adductor muscles were homogenized with a Polytron homogenizer in 10 volumes of 39 mM borate buffer (pH 6.9) containing 25 mM KCl and centrifuged at 600 x g for 10 min. The precipitate was suspended in 10 volumes of the same buffer. Large clots of myofibrils and connective tissues were removed by filtration through a gauze and fine myofibrils were collected by centrifugation at 600 x g for 10 min. Myofibrils thus obtained were suspended in the same buffer and used for immunoblotting. Protein concentrations were determined by the method of Lowry *et al.* (1951) as described in Section 1 of Chapter I.

Preparation of anti-collagen and anti-gelatin antisera

Specific antisera against abalone collagen and gelatin were raised in adult female rabbits. An antigen (10 mg) was dissolved in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 3.2 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and emulsified with an equal volume of complete Freund's adjuvant (DIFCO). Portions of the emulsion were injected subcutaneously into the back of rabbits. To the rabbits was then given 5-10 mg of antigen emulsified with an equal volume of incomplete Freund's adjuvant (DIFCO) after 4, 6, and 8 weeks. The booster injection (2.5 mg of antigen) was carried out after 12 weeks and the rabbits were bled two weeks later *via* ear and the jugular vein. The blood was incubated at 37°C for 1 h and kept overnight at 4°C. The serum collected by centrifugation at 500 x g for 5 min was kept frozen at -80°C until use.

SDS-PAGE

SDS-PAGE was carried out using 7.5% or 12.5% polyacrylamide slab gels as described in Section 1 of Chapter I.

Immunoblotting

Immunoblotting was performed essentially as described by Towbin *et al.* (1979). Proteins in SDS-PAGE gels were electrophoretically transferred to a PVDF membrane in a blotting buffer (pH 8.3) containing 25 mM Tris, 195 mM glycine, 10% methanol, and 0.02% SDS. The membrane was blocked with 2% BSA in 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl and then incubated with the anti-abalone collagen or gelatin antiserum at 1 : 500 or 1 : 20,000 dilution. Goat anti-rabbit IgG-horseradish peroxidase conjugate was used as the second antibody (1 : 5,000 dilution, Kirkegaard & Perry Laboratories). The binding of antibodies was visualized using 0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride and 0.005% H_2O_2.

Results

Antisera were raised against abalone pepsin-solubilized collagen prepared by digesting with 0.1% pepsin (collagen 0.1P) and its gelatin. SDS-PAGE patterns of abalone gelatin showed α- and β-chain bands but no bands for degradation products (data not shown).

Immunoblotting was performed on a PVDF membrane after SDS-PAGE for collagen and myofibrillar proteins of abalone to examine the specificity of these antisera. Figure 2-1 shows SDS-PAGE and immunoblotting patterns using the two antisera at 1 : 500 dilution. SDS-PAGE revealed that myofibrillar proteins contained myosin heavy chain, paramyosin, and actin. A strong reactivity was observed with bands corresponding to α- and β-chains using the anti-collagen antiserum, whereas this antiserum also reacted with paramyosin. The reactivity of the anti-gelatin antiserum with collagen was weaker than that of the anti-collagen antiserum, although the reactivity with paramyosin remained considerably high. It seems that purified collagen contained small quantities of paramyosin, which was hardly detected in gels stained with CBB R-250. Antigenicity of abalone paramyosin was much more stronger than that of collagen.

When the antiserum was reacted with abalone collagen preparations, both collagens 0.1P and 1P exhibited two bands of α-chain, demonstrating that even collagen 0.1P contained small quantities of α-chain which corresponded to that of the lower band found in collagen 1P (Fig. 2-2).

Summary

The antisera were raised against abalone collagen solubilized with 0.1% pepsin (collagen 0.1P) and its corresponding gelatin. The reactivity against collagen was higher with the anti-collagen than anti-gelatin antiserum. However, the reactivity against paramyosin was also observed with both antisera. The existence of two bands for α-chain in abalone collagen was demonstrated by immunoblotting.

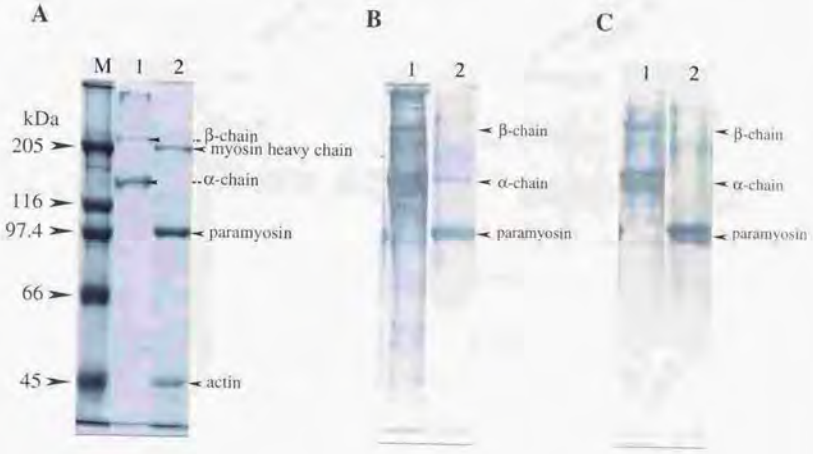


Fig. 2-1. SDS-PAGE patterns of pepsin-solubilized collagen and myofibrillar proteins from abalone (A) and their immunoblotting patterns using anti-abalone collagen (B) or anti-abalone gelatin antiserum (C). Collagen solubilized with 0.1% pepsin digestion (lane 1) and myofibrillar proteins (lane 2) were subjected to SDS-PAGE using 7.5% polyacrylamide gels and stained with CBB R-250 (A) or reacted with the two antisera at 1 : 500 dilution (B, C). Proteins applied were 2 μg/lane. Lane M contains molecular weight markers. Arrowheads indicate α- and β-chains of collagen, myosin heavy chain, paramyosin, and actin.

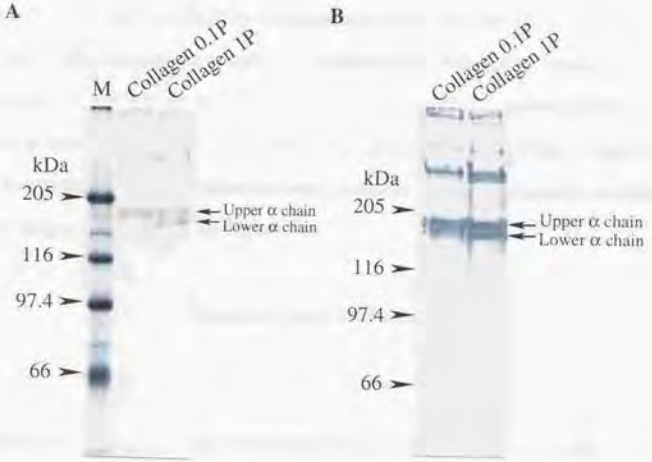


Fig. 2-2. SDS-PAGE (A) and immunoblotting (B) patterns of pepsin-solubilized abalone collagens. Acid-soluble collagens were prepared by digesting acid-insoluble collagen fibrils with pepsin at enzyme-to-substrate weight ratios of 0.1% (collagen 0.1P) and 1% (collagen 1P). Collagens were subjected to SDS-PAGE at 1 μg/lane using a 12.5% gel (A) and the membrane carrying blotted proteins was reacted with the anti-abalone collagen antiserum at 1 : 20,000 dilution (B). Lane M contains molecular weight markers. Arrows indicate upper and lower α-chains.

Section 2 Immunological properties of collagens

Collagen consists of a large family as structural extracellular matrix proteins in animal. Invertebrate collagens represent a large variety of their structures, so that the systematic classification as demonstrated in vertebrate collagen has not yet been performed. The specific antibody is capable of distinguishing proteins with various types and their submolecular species. In the previous section, the antisera were raised against abalone collagen and its gelatin, and their specificities were examined. In this section, the cross-reactivity against various collagens from vertebrates, invertebrates, and a protochordate were examined with both antisera to investigate the immunological properties of abalone collagen.

Materials and Methods

Collagens

Various collagens from vertebrate, invertebrate, and a protochordate were kindly supplied from Nippi Research Institute of Biomatrix (Tokyo): pepsin-solubilized type I collagens from the bullfrog *Rana catesbeiana*, chicken and rat, and acid soluble type I collagens from the carp *Cyprinus carpio*, chum salmon *Oncorhynchus keta* and calf. These type I collagens were isolated from skin. Pepsin-solubilized type III and type V collagens were isolated from bovine placenta. Invertebrate and protochordate collagens were prepared with pepsin digestion from the whole body of the sea anemone *Actina equina* and the jellyfish *Aurelia aurita*, and from the muscle of the ark shell *Scapharca broughtonii*, oyster *Crassostrea gigas*, turban shell *Batillus cornutus*, squid *Todarodes pacificus*, octopus *Octopus ocellatus*, prawn *Penaeus japonicus*, and sea squirt *Halocynthia roretzi*. Abalone collagen was prepared as described in Section 1 of Chapter I.

SDS-PAGE

SDS-PAGE was carried out using 5% polyacrylamide slab gels containing 0.1% SDS as described in Section 1 of Chapter I. Collagens applied were 5 μg/lane.

Immunoblotting

Immunoblotting was performed as described in Section 1 of this chapter using the anti-abalone collagen or anti-abalone gelatin antiserum at 1 : 1,000 dilution.

Results

The cross-reactivity of the two antisera against abalone collagen and gelatin was examined with various collagens by immunoblotting. SDS-PAGE patterns of vertebrate collagens, the subunit compositions of which have been elucidated (Kalder, 1995), are shown in Fig. 2-3 A. Most type I collagens were composed of a heterotrimer of two $\alpha1$(I) and one $\alpha2$(I) chains which corresponded to the upper and lower α-chain bands, respectively. On the other hand, salmon collagen from skin has a subunit composition of $\alpha1$(I) $\alpha2$(I) $\alpha3$(I) (Matsui *et al.*, 1991). The upper α-chain band of salmon collagen in the present study seemed to contain both $\alpha1$(I) and $\alpha3$(I) chains (Matsui *et al.*, 1991). Type III collagen was a homotrimer of three $\alpha1$(III) chains. The present type V collagen from calf had two $\alpha1$(V) and one $\alpha2$(V) chains, although it was contaminated with small amount of type I collagen. The electrophoretic mobility of type V collagen α-chain was slower than those of type I and III collagens as previously reported (Bailey and Sims, 1977).

Figure 2-3 B, C shows immunoblotting patterns of vertebrate collagens. Anti-abalone collagen antiserum strongly reacted with carp $\alpha1$(I) chains and was not reactive with $\alpha2$(I) counterparts. The antiserum reacted with both α-chains of frog collagen. On the other hand, the cross-reactivity against $\alpha2$(I) chains was clearly observed with collagens from salmon, chicken, rat, and calf, whereas $\alpha1$(I) chains were not reactive. No reactivity was observed with the anti-abalone collagen antiserum against bovine type III and V collagens. The anti-gelatin antiserum gave different immunoblotting patterns from those with the anti-collagen antiserum for type I collagens from carp and frog, and which was highly reactive with $\alpha2$(I) chains as demonstrated in type I collagens from chicken, rat, and calf. The antiserum reacted with not only salmon $\alpha2$(I) chain but also $\alpha1$(I) or $\alpha3$(I) chains, although the reactivity against the latter α-chains was weak. The reactivities with the anti-gelatin antiserum against vertebrate collagens were weaker than those

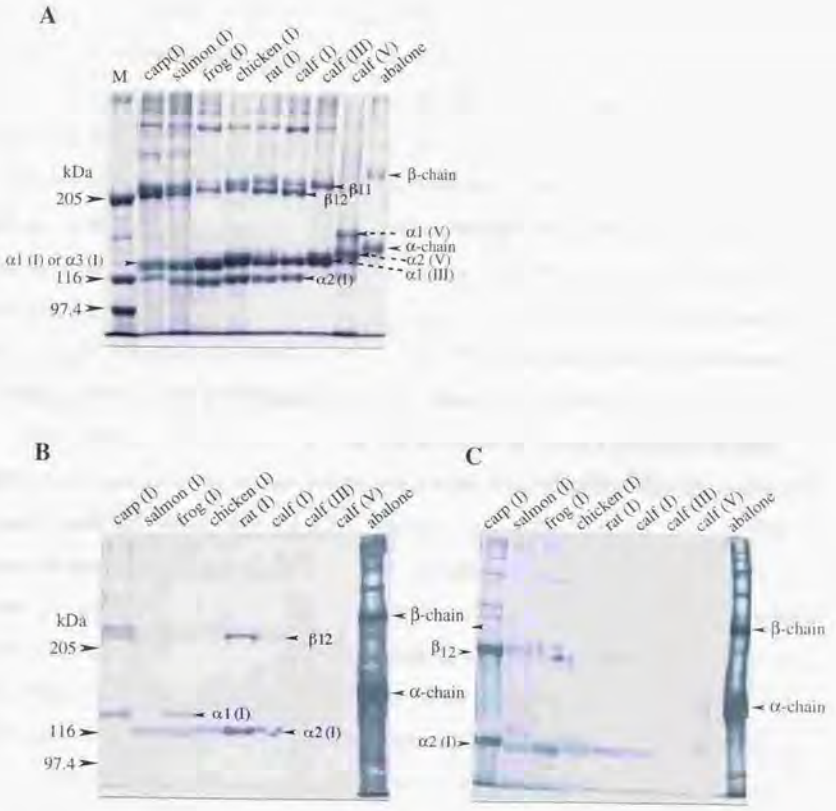


Fig. 2-3. SDS-PAGE (A) and immunoblotting (B, C) patterns of various vertebrate collagens together with abalone preparation. Collagens were subjected to SDS-PAGE at 5 μg/lane in a 5% gel (A) and the membranes carrying blotted proteins were reacted with the anti-abalone collagen (B) or anti-abalone gelatin antiserum (C) at 1 : 1,000 dilution. Lane M indicates molecular weight markers. Type I collagens were isolated from skin of carp, salmon, frog, chicken, rat, and calf. Type III and type V collagens were isolated from bovine placenta. These collagens were solubilized with pepsin except for type I collagens from carp, salmon, and calf. Abalone collagen was prepared from muscle. Arrows indicate α- and β-chains. β_{11} and β_{12} indicate the dimer of α-chain composed of α1 chain and that composed of α1 and α2 chains, respectively.

with the anti-collagen antiserum. However, type III and type V collagens from bovine placenta were not reactive with the anti-gelatin antiserum as in the case of the anti-collagen antiserum.

Figure 2-4 A shows SDS-PAGE patterns of pepsin-solubilized collagens from invertebrates and a protochordate in which various sizes of α-chain bands were found together with β-chains and highly aggregated α-chains. Single α-chain bands were observed with collagens from abalone, turban shell, and the jellyfish *Aurelia aurita*, although Miura and Kimura (1985) reported that the jellyfish *Stomolophus nomurai* collagen contained three types of α-chains. Ark shell collagen had two α-chains and other collagens contained not less than three α-chains. Two distinct types of collagens have been reported for squid the *Todarodes pacificus* and the prawn *Penaeus japonicus* muscles (Yoshinaka *et al.*, 1990; Mizuta *et al.*, 1994a).

Both antisera exhibited cross-reactivities against invertebrate and protochordate collagens. However, the reactivity was stronger with the anti-collagen than anti-gelatin antiserum as observed against vertebrate collagens (Fig. 2-4 B, C). The strong reactivity was observed against turban shell collagen as well as against abalone collagen. A higher reactivity with both antisera was observed against the upper α-chain bands from squid, octopus, and prawn which were considered to be minor types of α-chain (Mimamisako and Kimura, 1989; Yoshinaka *et al.*, 1990; Mizuta *et al.*, 1994a). On the other hand, the α-chain of the fastest mobility from squid did not react with both antisera. The reactivities with the anti-collagen antiserum were very weak against collagens from jellyfish, ark shell, oyster and sea squirt, whereas certain α-chains of collagens from ark shell, oyster, and sea squirt marked with asterisks in Fig. 2-4 C exhibited higher reactivity with the anti-gelatin antiserum than that with the anti-collagen antiserum.

Summary

The anti-collagen and anti-gelatin antisera exhibited cross-reaction against collagens from vertebrates, invertebrates, and a protochordate. The two antisera recognized all type I collagens, whereas these had no reactivity against vertebrate type III and type V collagens. While the anti-collagen antiserum was reactive with the α1(I) chain from carp and with both α1(I) and α2(I) chains from frog, the anti-gelatin antiserum highly reacted with α2(I) than α1(I) chains from the

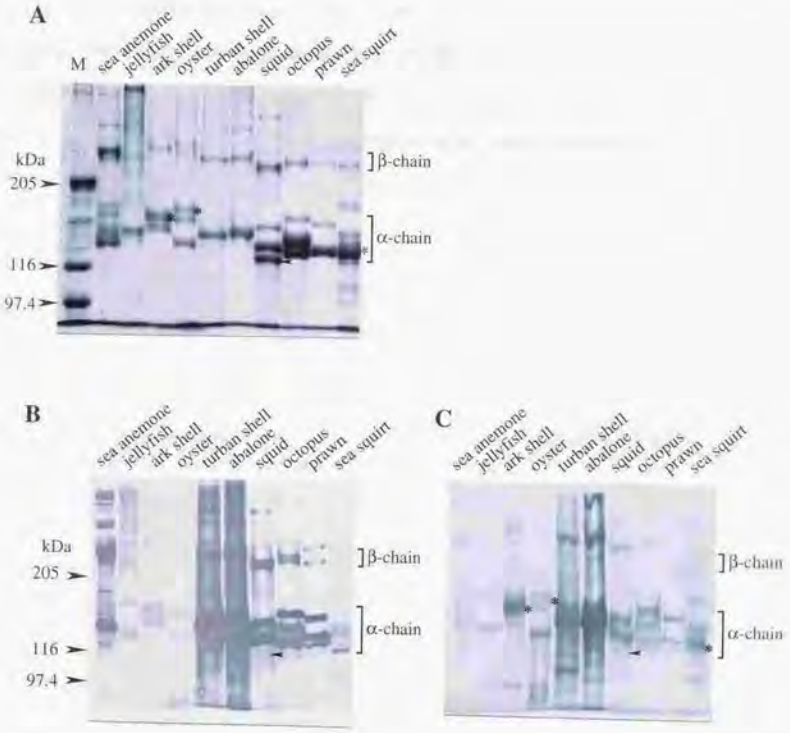


Fig. 2-4. SDS-PAGE (A) and immunoblotting (B, C) patterns of various invertebrate and protochordate collagens. Collagens were subjected to SDS-PAGE at 5 μg/lane in a 5% gel (A) and the membranes carrying blotted proteins were reacted with the anti-abalone collagen (B) or anti-abalone gelatin antiserum (C) at 1 : 1,000 dilution. Lane M indicates molecular weight markers. Pepsin-solubilized collagens were isolated from the whole body of sea anemone and jellyfish, and from muscle of ark shell, oyster, turban shell, abalone, squid, octopus, prawn, and sea squirt. Arrows indicate non-reactive α-chain bands of squid collagen. Asterisks show the α-chain bands reacted stronger with anti-gelatin antiserum than anti-collagen antiserum.

two animals. Both antisera reacted with only α2(I) chains from chicken, rat, and calf. In the case of invertebrate and protochordate collagens, the cross-reaction was also observed with the two antisera. While the reactivity of the anti-collagen antiserum against turban shell collagen was the strongest, this antiserum was also highly reactive with collagens from squid, octopus, and prawn, in which the highest reactivities were observed in the upper α-chain bands.

Section 3 Discussion

Collagen has relatively poor immunogenecity and small quantities of contaminating noncollagenous proteins (0.1% or less) result in the production of antibody against these impurities together with that against collagen (Timpl, 1982). Antisera raised against abalone collagen and gelatin were reactive not only with collagen but also with paramyosin. Further purification of antigen or antisera seems necessary to obtain higher specificity.

Specificity of the anti-gelatin antiserum to abalone collagen was considered to be lower than that of the anti-collagen antiserum, since conformation-dependent antigenitic determinants disappeared due to heat treatment.

Immunoblotting of abalone collagens 0.1P and 1P with the antiserum raised against collagen 0.1P demonstrated that the two collagen preparations contained two types of α-chains. The lower α-chain band was accumulated after extended pepsin digestion, thus it is likely that the lower α-chain band was produced by proteolytic digestion from the upper α-chain band.

The two antisera raised against abalone collagen and gelatin exhibited cross-reaction with collagens from vertebrates, invertebrates, and a protochordate, although the reactivity with vertebrate type III and type V collagens was not observed. Abalone collagen had immunochemical properties similar to those of vertebrate type I collagens which are abundant in all related phyla. It is interesting that the anti-collagen antiserum was reactive with the α1(I) chain from carp and with both α1(I) and α2(I) chains from frog, while type I collagens from salmon and higher vertebrates showed their reactivity for α2(I) chains. Furthermore, the anti-gelatin antiserum was highly reactive for α2(I) chains irrespective of animal species. It is considered that the gene for proα2(I) has evolved prior to those for proα1(I) and proα1(II) in higher vertebrates (Miller, 1985). Abalone collagen α-chains were similar to α2(I) chain rather than α1(I) chain of higher vertebrates. SDS-PAGE patterns of invertebrate and protochordate collagens were very complicated due to their high diversity of subunit composition. It is also interesting that minor α-chains of squid and prawn exhibited the immunochemical properties similar to those of abalone collagen.

Chapter III cDNA Cloning of Abalone Collagens and Their Gene Expression

Fibril-forming collagens provide the fibrous scaffold maintaining the integrity of tendons and ligaments in vertebrate, and have a similar molecular size containing a long uninterrupted main triple-helical domain with about 1,000 amino acid residues (Kadler, 1995). Genomic and/or cDNA sequences encoding collagen α-chains so far reported are mostly for human and chicken. Fibril-forming collagens are also present in invertebrates. However, structural variety has been indicated from recent studies on various invertebrate collagens including long cuticle collagen from annelids (Bairati and Garrone, 1985; Gaill *et al.*, 1991, 1995).

Protein sequences are now available for fibril-forming collagens from several invertebrate sources including the sponge *Ephydatia mulleri* (Exposito and Garrone, 1990; Exposito *et al.*, 1993), worms *Rifitia pachyptila* (Mann *et al.*, 1992) and *Arenicola marina* (Sicot *et al.* 1997), and sea urchins *Paracentrotus lividus* (D'Alessio *et al.*, 1989, 1990), *Strongylocentrotus purpuratus* (Exposito *et al.*, 1992a, b) and *Hemicentrotus pulcherrimus* (Tomita *et al.*, 1994). These collagens are similar to vertebrate counterparts in their domain structure and molecular size. The gene encoding collagen proα-chain from sponge has been cloned and sequenced (Exposito and Garrone, 1990; Exposito *et al.*, 1993). The exon-intron structure of this collagen gene is similar to that of vertebrate collagen genes, suggesting the formation of the ancestral fibril-forming collagen gene by multiple duplication of a 54 bp exon unit encoding six Gly-X-Y repeats. However, certain invertebrate fibril-forming collagens interrupt the main triple-helical domains lacking one or two Gly-X-Y repeat (Exposito and Garrone, 1990; Exposito *et al.*, 1993; Sicot *et al.* 1997), unlike vertebrate counterparts.

Foot muscle of abalone contains unusually large amounts of fibril-forming collagen which shows cross-striation patterns with a typical periodicity of about 60 nm (Olaechea *et al.*, 1993). Collagen contents in the muscle change seasonally, showing the lowest in summer and the highest in winter (Olaechea *et al.*, 1993; Hatae *et al.*, 1995). Pepsin-solubilized abalone collagen resembled vertebrate type I collagen in size and amino acid composition, whereas abalone collagen has less alanine and higher hydroxylysine contents (Kimura and Matsuura, 1974; see Table 1-2).

As described in Chapters I and II, the two types of α-chain were observed in pepsin-solubilized preparations from abalone foot muscle. However, the precise molecular structures of gastropod fibril-forming procollagens and collagens have not yet been elucidated.

In this chapter, cDNA clones encoding collagen α-chains were isolated from abalone foot muscle and determined for their deduced amino acid sequences. Furthermore, tissue distribution and seasonal changes of their transcripts were performed by northern blot analysis.

Section 1 cDNA cloning of collagens

Collagens are the major structural proteins in extracellular matrices of multicellular animals. cDNA sequences of collagen α-chains have been reported for both vertebrate and invertebrate. Collagen mRNAs have been isolated mostly from cultured cells in the case of higher vertebrates, since it is difficult to isolate mRNAs *in vivo* due to the presence of large quantities of connective tissues surrounding the cells. In addition, accumulated collagen mRNA levels are quite low due to the low turnover rate of collagens (Sandell, 1995). Conventional approaches for preparing full-sized cDNAs are also difficult for collagen mRNAs due to their large size (>5 kbp) and high GC content (Kohno *et al.*, 1984).

In this section, a cDNA library was constructed from juvenile abalone which is faster growing than adult specimens. cDNAs encoding collagen proα-chain were isolated from the library by screening with the anti-abalone collagen antiserum.

Materials and Methods

Materials

Juvenile specimens (average body weight of 1.4 g including shells) were grown at the Kanagawa Prefectural Fisheries Experimental Station. The foot muscle was dissected, immediately frozen with liquid nitrogen, and stored at -80°C until use. The anti-abalone collagen antiserum prepared in Section 1 of Chapter II was used.

Construction of a cDNA library

Total RNA was prepared from 3 g of foot muscles of juvenile abalones of ten individuals according to the manufacturer's protocol with an RNA extraction solution (Isogen, Nippon Gene). Poly(A)$^+$ RNAs were isolated with an oligo(dT) cellulose spin column (Pharmacia), and their corresponding cDNAs were synthesized using SuperScript™ lambda system for cDNA synthesis and lambda cloning (GIBCO BRL). The cDNAs synthesized were tailed with *Not* I and *Sal* I,

size-fractionated by gel filtration, and ligated into the λZipLox vector (GIBCO BRL) digested with *Not* I and *Sal* I.

cDNA cloning

The cDNA library was screened using the anti-abalone collagen antiserum by the method of Sambrook *et al.* (1989). Briefly, *Escherichia coli* (Y1090) infected with the λZipLox cDNA library was cultured on an agar plate at 42°C for 4 h, and a nitrocellulose filter containing 10 mM isopropyl-β-D-thiogalactopyranoside was overlaid to the plate and incubated at 37°C for 4 h. The nitrocellulose filter replica was screened with the anti-collagen antiserum. Positive plaques were purified, and the inserts were excised in the form of pZL 1 plasmid vectors according to the manufacture's protocol. The plasmid DNAs were purified by an alkaline lysis method (Sambrook *et al.* 1989) and used for further analysis.

To obtain clones encoding the N-terminal portion of proα-chain, the 5' portion of cDNA clones was amplified by PCR and the amplified products were randomly labeled with digoxigenin (DIG) using DIG DNA labeling kit (Boehringer Mannheim). The cDNA library was screened with these DIG-labeled probes according to the manufacturer's protocol using DIG DNA detection kit (Boehringer Mannheim). The cDNA library was cultured on an agar plate at 37°C for 10 h and overlaid with Hybond N⁺ nylon membranes (Amersham). Then the membranes were denatured in 0.5 M NaOH containing 1.5 M NaCl and subsequently neutralized with 0.5 M Tris-HCl (pH 7.5) containing 1.5 M NaCl followed by baking for 20 min at 120°C. Hybridization was carried out at 68°C for 12 h with a 1 : 600 dilution of the DNA probes in 5 x SSC (standard saline citrate, 1 x concentration = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 1.0% blocking reagent, 0.1% N-lauroylsarcosine, and 0.02% SDS. Positive clones were detected with alkaline phosphatase-conjugated anti-DIG and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium.

Various restriction fragments of cDNA clones encoding abalone collagen proα-chains were subcloned into plasmid vector pBluescript II. Sequencing was performed for both strands on subclones using Dye Deoxy™ terminator cycle sequencing kits with a DNA sequencer model 373S (Perkin Elmer Applied Biosystems).

Results

A cDNA library was constructed from juvenile abalone foot muscle. Screening of 1.6×10^6 plaques using the anti-collagen antiserum yielded 200 positive clones, and two distinct clones encoding partial length of collagen proα-chains, Hdcols (*Haliotis discus* collagens) 1α and 2α, were isolated. The size of cDNA was approximately 4 kbp for Hdcol 1α and 3 kbp for Hdcol 2α, both including regions encoding a part of the main triple-helical domain and C-propeptide as well as the 3'-untranslated regions (Fig. 3-1).

To obtain the full-length cDNAs, the library was again screened using DIG-labeled probes (probes 1 and 2) which were prepared from the cDNA clones isolated as described above. The second screening yielded additional 16 and 14 positive clones with Hdcol 1α and 2α cDNA probes, respectively. The largest clones from the screening contained the initiation and termination codons together with putative polyadenylation signals for both types. Figure 3-2 shows the restriction maps of Hdcol 1α and 2α cDNAs which facilitated the subsequent sequence determination.

Hdcol 1α cDNA contained 4,790 nucleotide (nt) with 5' non-coding 182 nt, 3' non-coding 471 nt, and coding 4,137 nt for 1,378 amino acids (Fig. 3-3). Hdcol 2α cDNA was slightly larger than Hdcol 1α, containing 4,966 nt with 5' non-coding 205 nt, 3' non-coding 441 nt, and coding 4,320 nt for 1,439 amino acids (Fig. 3-4). The 3' non-coding region of Hdcol 1α cDNA showed a putative polyadenylation signal, AATAAA, at 11 nt upstream a poly (A) tail (Fig. 3-3). On the other hand, Hdcol 2α cDNA contained four putative polyadenylation signals; one was adjacent to a poly (A) tail, and others found at 14, 55 and 330 nt upstream the poly (A) tail (Fig. 3-4). The nucleotide sequence identities between Hdcols 1α and 2α were 52.0% in the full length, 44.7% in the 5' non-coding, 50.3% in the 3' non-coding, and 52.6% in the coding region.

Summary

A cDNA library was constructed from juvenile abalone foot muscle. Screening with the anti-collagen antiserum yielded 200 positive clones, and two distinct clones encoding collagen proα-

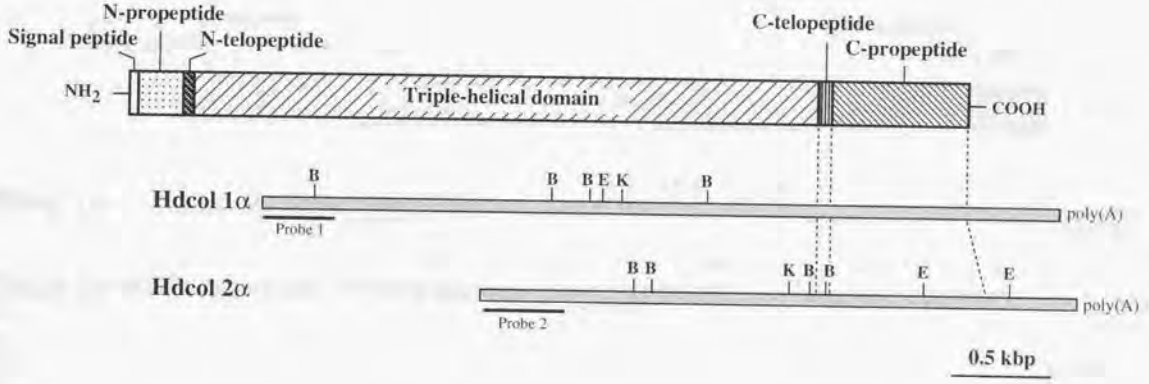


Fig. 3-1. Restriction maps of cDNA clones encoding partial length of abalone collagen proα-chains. The clones were isolated from the library by screening with the anti-abalone collagen antiserum. Bars under the clones indicate locations of probes 1 and 2 used for further screening of the library for the two types of proα-chains, Hdcol 1α and 2α, respectively. Restriction enzymes are B, *Bam*HI; E, *Eco*RV; K, *Kpn*I.

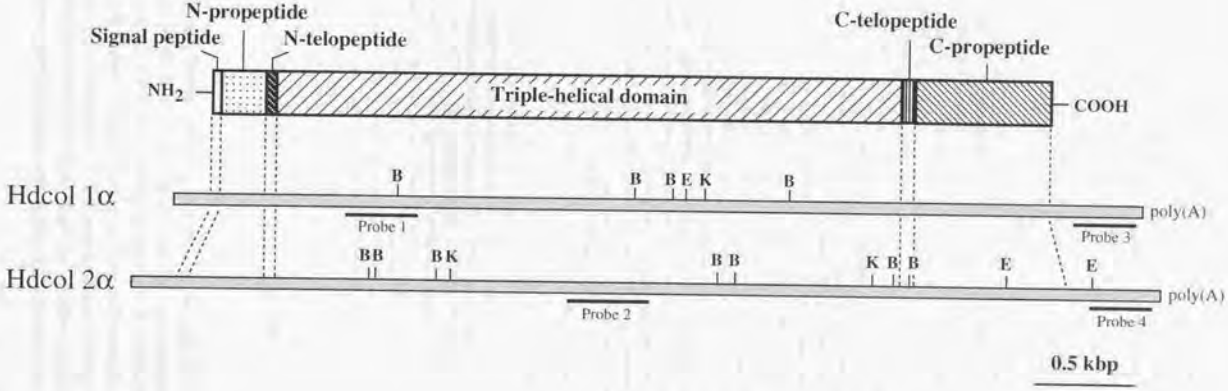


Fig. 3-2. Restriction maps of cDNA clones encoding the full-length of abalone collagen proα-chains. Bars under the clones indicate locations of probes used in screening of the library (probes 1 and 2) and northern blot analysis (probes 3 and 4), respectively. Restriction enzymes are B, *Bam*HI; E, *Eco*RV; K, *Kpn*I.

Fig. 3-3. The nucleotide sequence of the full-length cDNA encoding abalone collagen proα-chain Hdcol 1α. Boxes are for the initiation codon, ATG, for the termination codon, TAA, and for the putative polyadenylation signal, AATAAA. The poly A tail is underlined. The large box indicates the region of the cDNA clone obtained by screening with the anti-abalone collagen antiserum. The regions shown with red and blue letters indicate the location of probes 1 and 3 used in the second screening of the library and northern blot analysis, respectively.

Fig. 3-4. The nucleotide sequence of the full-length cDNA encoding abalone collagen proα-chain Hdeol 2α. Boxes are for the initiation codon, ATG, for the termination codon, TGA, and for the putative polyadenylation signals, AATAAA. The poly A tail is underlined. The large box indicates the region of the cDNA clone obtained by screening with the anti-abalone collagen antiserum. The regions shown with red and blue letters indicate the location of probes 2 and 4 used in the second screening of the library and northern blot analysis, respectively.

chains, Hdcols 1α and 2α, were isolated. To obtain the full-length cDNAs, the library was again screened using DIG-labeled probes which were prepared from the cDNA clones isolated by using the specific antiserum. Finally two types of full-length cDNAs coding for collagen proα-chains were isolated and their nucleotide sequences were determined. Hdcol 1α cDNA contained 4,790 nt with 5' non-coding 182 nt, 3' non-coding 471 nt, and coding 4,137 nt for 1,378 amino acids. Hdcol 2α cDNA contained 4,966 nt with 5' non-coding 205 nt, 3' non-coding 441 nt, and coding 4,320 nt for 1,439 amino acids. The nucleotide sequence identities between Hdcols 1α and 2α were 52.0% in the full length.

Section 2 The primary structure of collagens

Fibril-forming collagens in vertebrate have a similar molecular size and structure containing a long uninterrupted main triple-helical domain with about 1,000 amino acid residues (Prockop and Kivirikko, 1995). Genomic and/or cDNA sequences encoding different collagen α-chains have been reported, mostly for human and chicken. cDNA sequences of frog α2(I) and α1(II) chains (Su *et al.*, 1991; Asahina *et al.*, 1994), zebrafish α1(II) chain (Yan *et al.*, 1995), and rainbow trout α1(I), α2(I), and α3(I) chains (Saito *et al.*, 1998) are only available for lower vertebrates. Protein sequences are also available for several invertebrates from sponge (Exposito and Garrone, 1990; Exposito *et al.*, 1993), worms (Mann *et al.*, 1992; Sicot *et al.* 1997), and sea urchins (D'Alessio *et al.*, 1989, 1990; Exposito *et al.*, 1992a, b; Tomita *et al.*, 1994). Invertebrate fibril-forming collagens are similar to those from vertebrates in terms of the domain structure of procollagens and their *in vivo* processing. However, certain invertebrate fibril-forming collagens interrupt helical domains lacking one or two Gly-X-Y repeat (Exposito and Garrone, 1990; Exposito *et al.*, 1993; Sicot *et al.* 1997), unlike vertebrate counterparts. It is also noted that the sea urchin *Strongylocentrotus purpuratus* collagen has an unusual long N-propeptide in its 2α-chain (Exposito *et al.*, 1992b).

In the previous section, two distinct cDNA clones encoding abalone collagen proα-chain were isolated from the foot muscle cDNA library. The objective of this section was to disclose their structural properties from the deduced amino acid sequences.

Materials and Methods

Data

Nucleotide sequences of two types of cDNAs encoding collagen proα-chains obtained in Section 1 of this chapter were used for analysis.

Results

Figure 3-5 shows the deduced amino acid sequences from the two cDNAs encoding abalone procollagens. These were composed of domain structures characteristic to fibril-forming collagens. In the N-terminus of each proα-chain was found a signal peptide tentatively assigned to have 18 amino acids rich in hydrophobic groups, where two cysteine residues were observed only in Hdcol 2α. The N-propeptide, which forms a globular domain (Prockop and Kivirikko, 1995), contained 10 and 6 cysteine residues for Hdcols 1α and 2α, respectively. Ten cysteine residues found in Hdcol 1α were similarly spaced as demonstrated in human proα1(I), proα1(II), proα1(III), and proα2(V) chains (Tromp *et al*, 1988; Ala-Kokko *et al*., 1989; Su *et al*., 1989; Woodbery *et al*., 1989). Following this domain, a short triple-helical domain consisting of 13 Gly-X-Y repeats was found in Hdcol 2α, in contrast to Hdcol 1α which contained no corresponding domain. Such structure of N-propeptide in Hdcol 1α has never been reported before, and thus this protein is very unusual. The site connecting the N-propeptide and N-telopeptide was likely between Ala-Gln for Hdcol 1α and between Asp-Gln for Hdcol 2α, based on comparisons to the sequences of procollagens previously reported (Sandell and Boyd, 1990). One lysine residue which is possibly involved in intermolecular cross-linking was found in the supposed N-telopeptide region of abalone proα-chain.

The main triple-helical domain consisted of 1,014 amino acids for both Hdcols 1α and 2α, showing a regular Gly-X-Y triplet repeat in the most part. One exception was found at the 598th position from the N-terminus of this domain, where glycine was replaced by serine in Hdcol 1α and by alanine in Hdcol 2α. N-terminal amino acid sequences determined for the upper and lower α-chain bands of collagen 1P (see Fig. 1-3) were located at the boundaries between the N-telopeptide and main triple-helical domain of Hdcols 2α and 1α, respectively (Fig. 3-5).

Tryptic peptides (see Table 1-1) which were isolated from collagen 0.1P were all located at the main triple-helical domain in either of two types of proα-chain: P2 and P4 - P6 in Hdcol 1α and P1 and P3 in Hdcol 2α (Fig. 3-5). An unidentified residue in the sequence of P5 was determined to be lysine according to the cDNA nucleotide sequence of Hdcol 1α, which is likely to be hydroxylated and subsequently glycosylated. Glycine residues were found in position X or

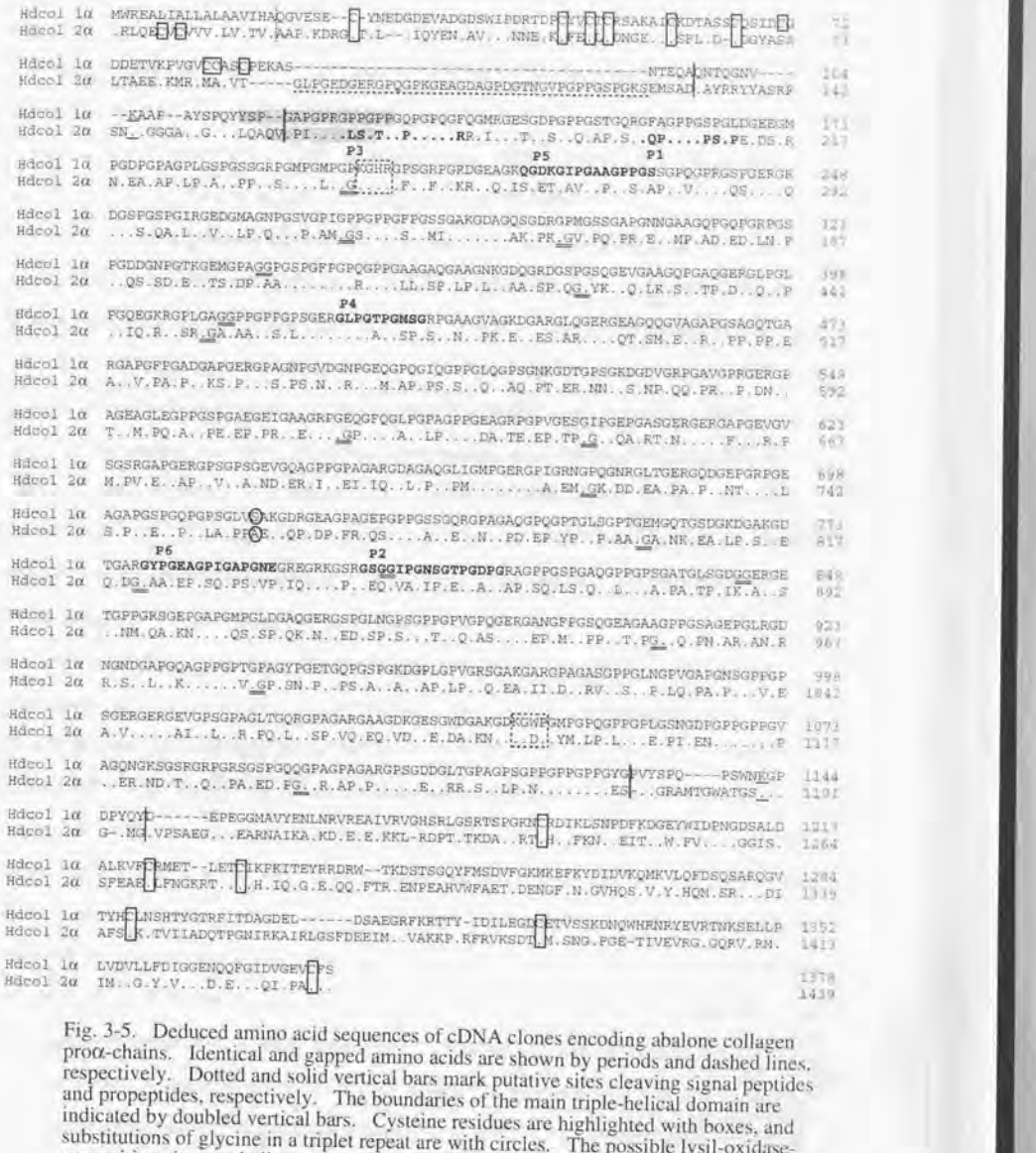


Fig. 3-5. Deduced amino acid sequences of cDNA clones encoding abalone collagen proα-chains. Identical and gapped amino acids are shown by periods and dashed lines, respectively. Dotted and solid vertical bars mark putative sites cleaving signal peptides and propeptides, respectively. The boundaries of the main triple-helical domain are indicated by doubled vertical bars. Cysteine residues are highlighted with boxes, and substitutions of glycine in a triplet repeat are with circles. The possible lysil-oxidase-recognition sites are indicated with dotted boxes. The putative cross-linking sites in telopeptides are underlined and Gly-Gly sequences are double underlined. The dashed underline indicates Gly-X-Y triplets in N-propeptide. Boldfaced letters indicate coding sequences identical to those determined by N-terminal amino acid sequencing for tryptic peptides, P1 - P6, from adult abalone muscle collagen (see Table 1-1). Amino acid sequences directly determined for intact α-chains of adult abalone muscle collagen are meshed (see Fig. 1-3).

Y of the main helical domain 4 and 13 times with Hdcols 1α and 2α, respectively. Such high abundancy of glycine has been demonstrated in invertebrate and lower vertebrate collagens (D'Alessio *et al.*, 1990; Su *et al.*, 1991; Exposito *et al.*, 1992a, b; Mann *et al.*, 1992; Exposito *et al.*, 1993; Asahina *et al.*, 1994; Sicot *et al*, 1997; Saito *et al.*, 1998).

In both abalone proα-chains, a consensus sequence for the cross-linking site (Eyer *et al.*, 1984), Lys-Gly-His-Arg, was located at the 84th residue the N-terminus of the main triple-helical domain, the position of which was the same as that of the tube worm *Rifitia pachyptila* (Mann *et al.*, 1992). A similar tetrapeptide sequence, Lys-Gly-Typ(Asp)-Pro, was found at 83 amino acids upstream the end of the main triple helix in each of two abalone proα-chains, while histidine, potentially essential to catalyze the cross-linking, was substituted by tryptophan in Hdcol 1α and by aspartic acid in Hdcol 2α. A tetrapeptide, Lys-Gly-Trp-Pro, has been also found in worms in a similar position (Mann *et al.*, 1992; Sicot *et al*, 1997). Several Gly-Pro-Pro triplets possibly involved in the stabilization of α-chain trimer were also observed in the C-terminal region of the main triple-helical domain.

Non-helical C-propeptides of the two abalone proα-chains were rich in cysteine as the case for N-propeptides, while cleavage sites between C-telopeptide and C-propeptide were predicted at 18 and 20 amino acids downstream the last Gly-X-Y triplet for Hdcols 1α and 2α, respectively (Dion *et al.*, 1987). In this assumption, the mature α-chains contain a lysine-mediated cross-linking site in the C-telopeptide. As shown in Fig. 3-6, six cysteine residues and their positions were comparable to those for fibril-forming collagens reported so far (Bernard *et al.*, 1983, 1988; Kuinaniemi *et al.*, 1988; Ala-Kokko *et al.*, 1989; Kimura *et al.*, 1989; Su *et al.*, 1989; Woodbery *et al.*, 1989; Exposito and Garrone, 1990; Takahara *et al.*, 1991; Exposito *et al.*, 1992a, b; Sicot *et al*, 1997). Each of the two abalone C-propeptides lacked two cysteine residues, which are common to vertebrate fibril-forming collagens. The missing two cysteine residues are both between the first and second ones in the abalone sequences. The sandworm *Arenicola marina* proα-chain also lacks these two cysteine residues (Sicot *et al*, 1997). A carbohydrate-bound asparagine residue is usually found in fibril-forming vertebrate collagens near the fourth or sixth cysteine in total 8 residues, forming a tripeptide of Asn-Ile-Thr, Asn-Phe-Thr, or Asn-Val-Thr (Dion *et al.*, 1987). Invertebrate collagens from sponge, worm, and sea urchins also contain

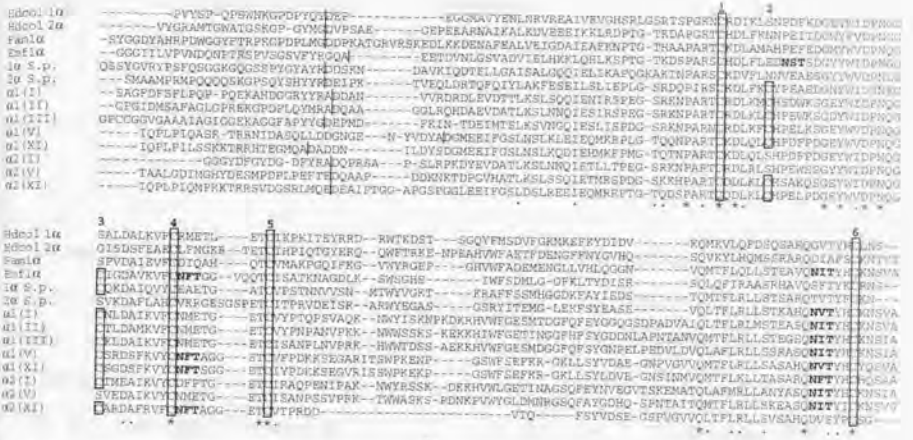


Fig. 3-6. Comparison of the amino acid sequences of the C-terminal non-helical domain of abalone Hdcols 1α and 2α with those of other fibril-forming collagen proα-chains. The sequences are follows: Fam1α, 1α-chain from *Arenicola marina* (Sicot *et al*, 1997); Emf1α, 1α-chain from *Ephydatia mulleri* (Exposito and Garrone., 1990); 1α S. p., 1α-chain from *Strongylocentrotus purpuratus* (Exposito *et al*., 1992a); 2α S. p., 2α-chain from *Strongylocentrotus purpuratus* (Exposito *et al*., 1992b); α1(I), proα1(I) chain from human (Bernard *et al*., 1983); α1(II), proα1(II) chain from human (Su *et al*., 1989); α1(III), proα1(III) chain from human (Ala-Kokko *et al*., 1989); α1(V), proα1(V) chain from human (Takahara *et al*., 1991); α1(XI), proα1(XI) chain from human (Bernard *et al*., 1988); α2(I), proα2(I) from human (Kuivaniemi *et al*., 1988); α2(V), proα2(V) chain from human (Woodbery *et al*., 1989); α2(XI), proα2(XI) chain from human (Kimura *et al*., 1989). The conserved cysteine residues are boxed and indicated by numbers 1-8. The vertical bars mark the putative C-propeptide cleavage sites and boldfaced letters indicate the potential glycosilation sites. Gapped amino acids are shown by dashed lines. Asterisks and dots indicate conserved and similar residues, respectively.

such supposed carbohydrate-bound asparagine (D'Alessio *et al.*, 1989, 1990; Exposito and Garrone, 1990; Exposito *et al.*, 1992a, b; Tomita *et al.*, 1994; Sicot *et al.* 1997). However, this asparagine was not contained in both abalone C-propeptides.

Amino acid sequence identities between Hdcols 1α and 2α were 44.1% in the full length, 52.1% in the main triple-helical domain, 29.5% in C-propeptide, and 16.8% in N-propeptide.

Summary

Abalone collagen proα-chains, Hdcols 1α and 2α, consisted of 1,378 and 1,439 amino acids, respectively, showing the primary structure typical to those of fibril-forming collagens. The two N-terminal amino acid sequences of the abalone pepsin-solubilized collagen preparation corresponded to either of the two sequences deduced from the cDNA clones. In addition, several tryptic peptides prepared from the pepsin-solubilized collagen showed N-terminal amino acid sequences identical to those deduced from the two cDNA clones. The N-propeptides of the two collagen proα-chains contained cysteine-rich globular domains. Hdcol 1α completely lacked a short Gly-X-Y triplet repeat sequence in its N-propeptide. Such unusual structure has never been reported before for any fibril-forming collagen. Their main triple-helical domains both consisted of 1,014 amino acids where a supposed glycine residue in the triplet at the 598th position from the N-terminus was replaced by alanine in Hdcol 1α and by serine in Hdcol 2α. Both proα-chains of abalone collagens contained 6 cysteine residues in the C-propeptide, lacking 2 cysteine residues usually found in vertebrate collagens.

Section 3 Seasonal changes of collagen mRNA levels

In Section 1 of this chapter, two distinct cDNA clones encoding abalone collagen proα-chain were isolated. The deduced amino acid sequences of Hdcols 1α and 2α showed the primary structures typical to those of fibril-forming collagens. The two N-terminal amino acid sequences of the abalone pepsin-solubilized collagen corresponded to either of the two sequences deduced from the cDNA clones.

In this section, collagen mRNA levels were examined in various tissues of adult and juvenile abalone specimens by northern blot analysis. In addition, seasonal changes of collagen mRNA levels were examined in relation to those of collagen contents in abalone muscle.

Materials and Methods

Materials

Three or four individuals of adult abalone specimens were collected every month from August 1997 to July 1998 from the coastal area along the Pacific Ocean off the Miura Peninsula. The total weight including shell was 333 ± 52 g as the mean value with standard deviations. Juvenile specimens were obtained as described in Section 1 of this chapter. These specimens were used for total RNA extraction as described below.

Dot blot analysis

One ng unlabeled plasmid DNA encoding each of abalone collagen proα-chains, Hdcols 1α and 2α, was spotted onto a Hybond N^+ nylon membrane. Then the membranes were denatured in 0.5 M NaOH containing 1.5 M NaCl and subsequently neutralized in 0.5 M Tris-HCl (pH 7.5) containing 1.5 M NaCl followed by baking for 30 min at 80°C. The membranes were prehybridized at 65°C for 20 h in 0.5 M Na_2HPO_4 (pH 7.2) containing 1 mM EDTA and 7% SDS (Church and Gilbert, 1984). Hybridization was carried out at 65°C for 20 h in the same solution containing ^{32}P -labeled DNA probes which were randomly primed in the presence of [α-^{32}P]dCTP using a random primer DNA labeling kit (Takara). The membranes were washed at 65°C with

several buffer changes of decreasing SSC concentrations from 2 x to 0.5 x and autoradiographed on X-ray films with intensifying screens at -80°C.

Northern blot analysis

The following procedures were performed essentially according to Sambrook *et al.* (1989). Total RNAs were isolated from several tissues, including foot and adductor muscle from both adult and juvenile abalones together with mantle, gill and hepatopancreas from adult specimens, using RNA extraction solution, Isogen. The concentration of RNA was determined spectrophotometrically by reading A_{260} for each sample. Ten µg of total RNAs were denatured at 65°C for 15 min in 50% formamide and subjected to electrophoresis on 0.9% agarose gels in 20 mM 3-(*N*-morpholino) propanesulfonic acid (pH 7.0) containing 2.2 M formamide, 5 mM sodium acetate, and 1 mM EDTA, then transferred to Hybond N$^+$ nylon membranes in 20 x SSC. The membranes bearing RNA blots were hybridized with DNA probes under the same condition as in the dot blot analysis. The hybridized membranes were scanned using a Fujix Bas 1000 computerized densitometer scanner and quantified using a recommended scanning program. The quantified mRNA levels of collagen mRNA were statistically analyzed using Student's *t*-test.

Results

Specificity of probes

The specificity of each probe was examined by dot blot analysis. The DNA probes about 300 bp in length were derived from the 3'-untranslated region by PCR amplification (see Figs. 3-2 - 3-4). Although double-stranded DNA is known to be less stable than DNA-RNA hybrid (Sambrook *et al.*, 1989), the cross-reactivity against heterologous probe was not observed (Fig. 3-7). Therefore, the two probes were considered to be highly specific to the corresponding two types of mRNA.

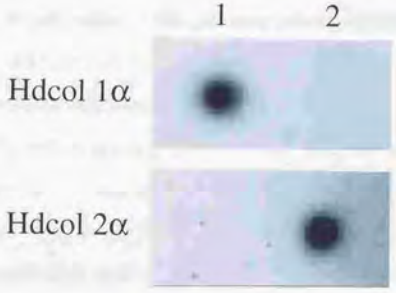


Fig. 3-7. Specificity of probes for northern hybridization from the two types of cDNA clones encoding abalone collagen proα-chains. Nylon membranes incorporating Hdcol 1α (dot 1) and Hdcol 2α (dot 2) cDNA clones were hybridized with ^{32}P-labeled oligonucleotide probes specific to Hdcols 1α and 2α mRNAs, respectively (see Fig. 3-2 - 3-4).

Collagen mRNA levels in various tissues

As shown in Fig. 3-8, northern blot analysis was carried out to determine expression levels of Hdcol 1α and 2α mRNAs with probes 3 and 4 specific to respective proα-chains (see Fig. 3-2). Total RNAs were prepared from foot and adductor muscles of adult and juvenile abalones as well as from mantle, gill, and hepatopancreas of adult specimens. Total RNAs of 10 μg each from various tissues were run on the gels together with two larger rRNAs (23S and 16S) from *E. coli* as molecular weight markers. The gels were stained with ethidium bromide to confirm that the same amount of RNAs was applied to each lane (Fig. 3-8 B), while blotted membranes were subsequently hybridized with either of the two specific probes. Figure 3-8 A shows mRNA levels of Hdcols 1α and 2α having similar patterns in tissue distribution. The highest levels of mRNA were observed in mantle for both Hdcols 1α and 2α, which secrete collagens as well as shell frame proteins (Sudo *et al.*, 1997). Considerable amounts were also observed with foot muscle of adult and juvenile abalones and with adductor muscle of juvenile specimens. However, hybridization signals in the adductor muscle of adult specimens were even weaker than those in hepatopancreas. Only traces of collagen transcripts were found in the gill.

Seasonal changes of collagen mRNA levels

To investigate seasonal changes of collagen mRNA levels, adult abalones were collected every month and total RNAs were prepared from various tissues. The seasonal changes in accumulated mRNAs encoding abalone collagen proα-chains in adductor and foot muscles and hepatopancreas are shown in Figs. 3-9, 3-10, and 3-11, respectively. Probes specific to abalone collagen proα-chains were hybridized to collagen mRNAs of about 4.9 kbp in length in three tissues. However, low molecular weight counterparts which reacted with the probes were also observed in adductor and foot muscles. It is noted that considerable individual differences were observed in every month. Levels of collagen mRNAs were standardized with that from foot muscle of one specimen collected in August.

The average mRNA levels in each month expressed as a percentage of the maximum value are shown in Fig. 3-12, together with collagen contents of adductor muscles which were determined for abalone collected from the same area and reared at the Kanagawa Prefectural Fisheries

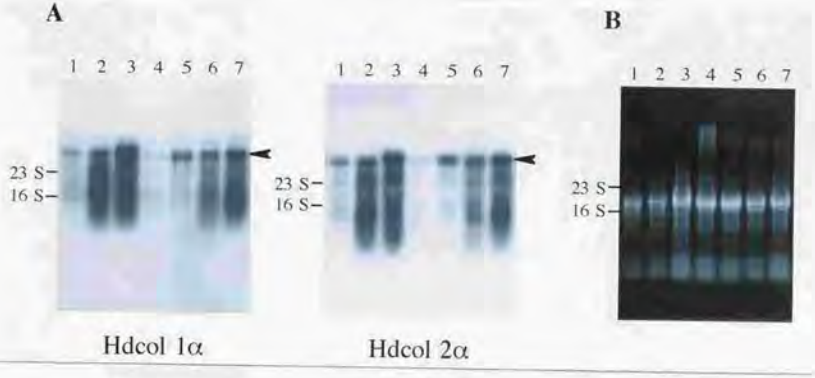


Fig. 3-8. Northern blot analysis of mRNAs encoding abalone collagen proα-chains in various tissues from adult and juvenile specimens. Total RNAs were isolated from various tissues of adult (lanes 1 - 5) and juvenile (lanes 6 and 7) abalone, including adductor muscle (lanes 1 and 6), foot muscle (lanes 2 and 7), mantle (lane 3), gill (lane 4) and hepatopancreas (lane 5), and subjected to electrophoresis on a 0.9 % agarose gel (B). Subsequently, RNAs were blotted onto nylon membranes and hybridized with probes derived from the 3'-untranslated region of Hdcol 1α and 2α cDNAs (A) (see Figs. 3-2 - 3-4). Arrowheads indicate collagen proα-chain mRNA, whereas two larger rRNAs (23S and 16S) from *Escherichia coli* are shown as molecular weight markers.

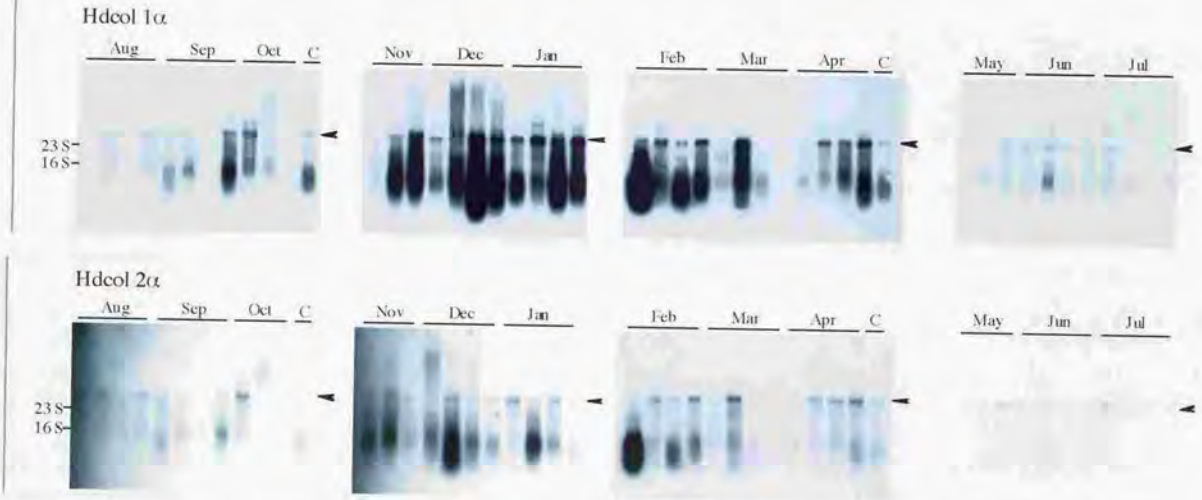


Fig. 3-9. Northern blot analysis of mRNAs encoding abalone collagen proα-chains in adductor muscle of abalone collected from August 1997 to July 1998. Three or four individuals were analyzed each month. The letter "C" indicates total RNA prepared from foot muscle of one specimen collected in August to compare intensities of the blots in various membranes. Refer to the legend of Fig. 3-8 for further details.

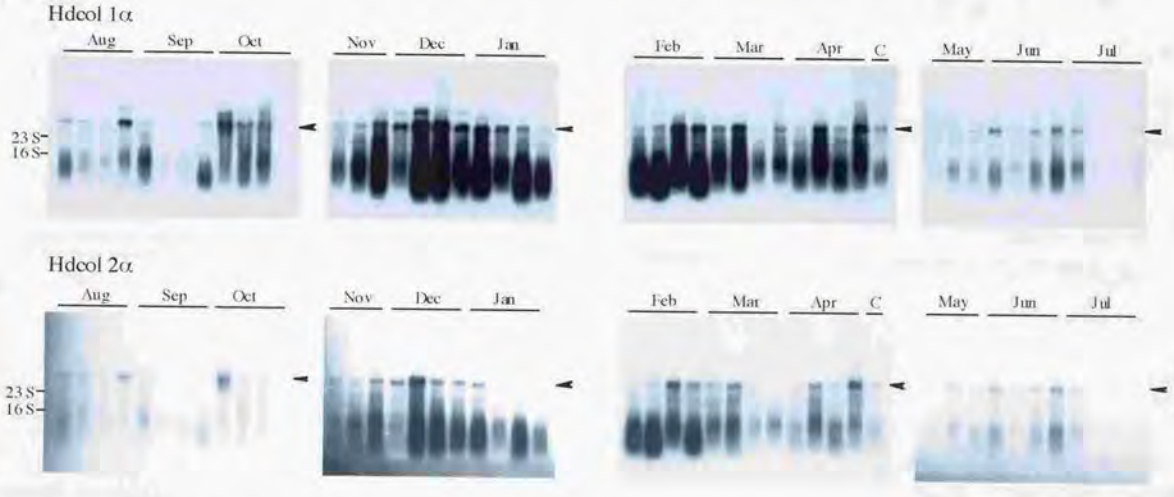


Fig. 3-10. Northern blot analysis of mRNAs encoding abalone collagen proα-chains in foot muscle of abalone collected from August 1997 to July 1998. Three or four individuals were analyzed each month. The letter "C" indicates total RNA prepared from foot muscle of one specimen collected in August to compare intensities of the blots in various membranes. Refer to the legend of Fig. 3-8 for further details.

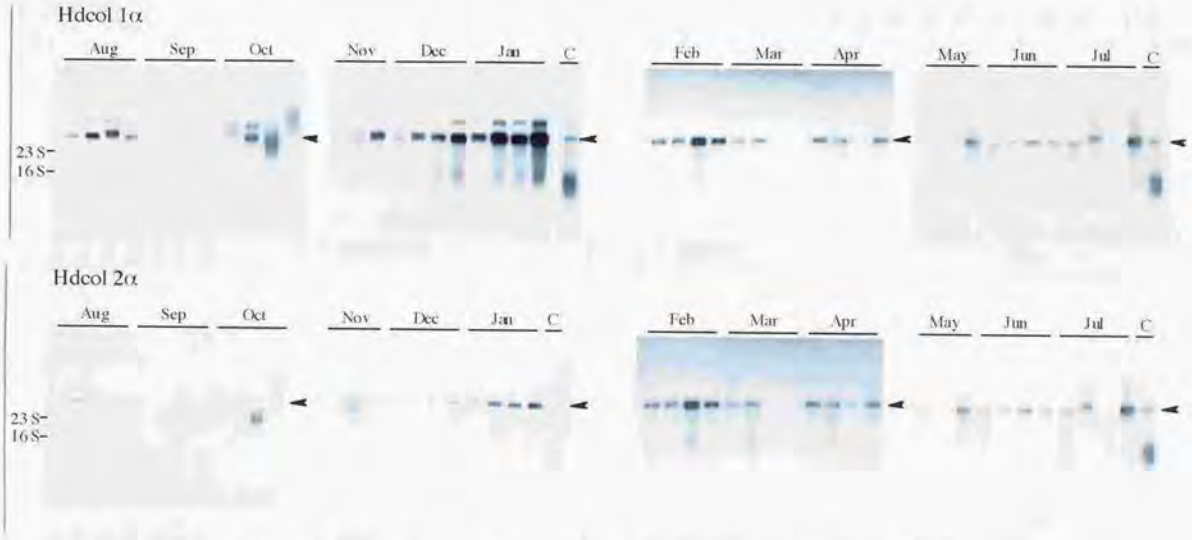


Fig. 3-11. Northern blot analysis of mRNAs encoding abalone collagen proα-chains in hepatopancreas of abalone collected from August 1997 to July 1998. Three or four individuals were analyzed each month. The letter "C" indicates total RNA prepared from foot muscle of one specimen collected in August to compare intensities of the blots in various membranes. Refer to the legend of Fig. 3-8 for further details.

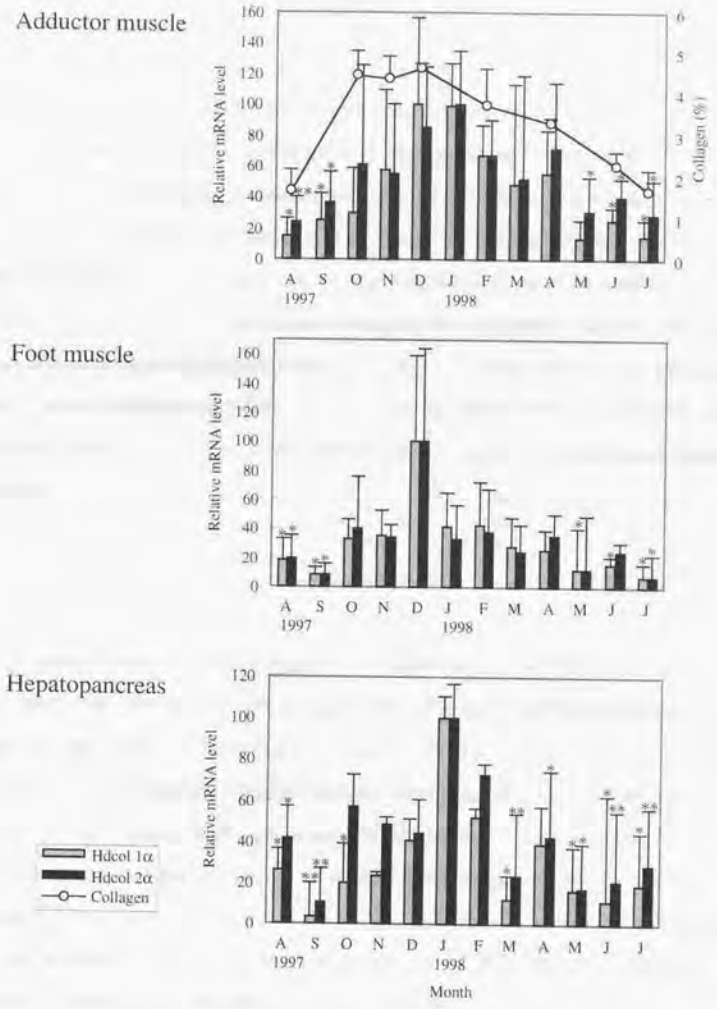


Fig. 3-12. Seasonal changes of collagen mRNA levels in comparison with those of collagen contents in adductor muscle. RNA levels were quantified with a Fujix Bas 1000 computerized densitometer scanner. Collagen contents of adductor muscles represent the percentage of total weight (g/100 g wet tissue) (Hatae *et al.*, 1995). * ($p < 0.05$) and ** ($p < 0.01$) indicate the levels of significance in differences from the highest mRNA level. Bars represent mean ± S. D. from three or four individuals.

Experimental Station from October 1991 to August 1992 (Hatae *et al.*, 1995). The seasonal changes of collagen mRNA levels were quite similar between Hdcols 1α and 2α. Adductor and foot muscles showed the highest mRNA levels in December and January and the lowest in July, in good agreement with changes in the collagen content of adductor muscle which has been shown to decrease in July and August and increase from October to December (Hatae *et al.*, 1995). In the case of hepatopancreas, the highest and the lowest mRNA levels were observed in January and September, respectively, showing a pattern similar to those of muscular tissues. The mRNA levels in summer were significantly lower ($p < 0.05$ or 0.01) than those in December and January which showed the highest mRNA levels. However, the mRNA levels of muscular tissues were unexpectedly low from September to November when collagen contents have been reported to increase.

Summary

Northern blot analysis demonstrated that the mRNA levels of Hdcols 1α and 2α in various tissues including muscles were similar to each other. The highest amount of mRNA was observed in the mantle of adult and juvenile abalones followed by their foot muscles and adductor muscle of juvenile specimens. Seasonal changes of collagen mRNA levels were investigated with adductor and foot muscles and hepatopancreas in which Hdcols 1α and 2α showed similar patterns. The highest mRNA levels of the two collagens were observed in December and January in good agreement with the increase of collagen content. The mRNA levels of adductor and foot muscles decreased in July when collagen content decreased. These results indicate that collagen transcription levels are closely related to collagen contents.

Section 4 Discussion

Screening the cDNA library with the anti-abalone collagen antiserum resulted in isolation of two distinct abalone cDNAs which encoded fibril-forming proα-chains, Hdcols 1α and 2α. N-propeptides of fibril-forming collagens exhibit a high degree of divergence both in length and in the domain structure (Vuorio and de Crombrugghe, 1990). Both abalone proα-chains contained a cysteine-rich globular domain in their N-propeptides, whereas Hdcol 1α was devoid of a short helical domain unlike common fibril-forming collagens. It has been suggested that the N-propeptide plays a role in regulating the diameter of fibrils (Fleischmajer *et al.*, 1983). Vertebrate type V collagens, the N-propeptide of which is not processed intracellularly, form very thin fibers. In addition, N-propeptide, as well as C-propeptide, has an inhibitory effect on procollagen synthesis (Wiestner *et al.*, 1983; Wu *et al.*, 1986), although the participation of the short helical domain in such inhibitory effect has remained unknown. It seems interesting to examine any possible effects on procollagen synthesis for Hdcol 1α N-propeptide which lacks the short helical domain.

The main triple-helical domain of each abalone proα-chain consisted of 1,014 amino acid residues. The same residue number has been reported in human α1(I), α2(I), α1(II), α1(V), α2(V), α1(XI), and α2(XI) chains (Bernard *et al.*, 1983, 1988; Kuinaniemi *et al.*, 1988; Tromp *et al*, 1988; Kimura *et al.*, 1989; Su *et al.*, 1989; Woodbery *et al.*, 1989; Yoshioka and Ramirez, 1990; Takahara *et al.*, 1991; Vuoristo *et al.*, 1995). One substitution of glycine at the first position in a triplet repeat sequence was found in the main triple-helical domain, which was one of the characteristic features of abalone collagens. Such imperfection has been reported in invertebrate fibril-forming collagens including sponge and worms (Exposito and Garrone 1990; Mann *et al.*, 1992; Exposito *et al.*, 1993; Sicot *et al*, 1997). Since substitutions of glycine by other amino acid at triplet sites cause severe diseases in the case of vertebrate fibril-forming collagens (Kuivaniemi *et al.*, 1991), it seems very interesting to reveal functional significance of such events in invertebrate. In vertebrate collagens, four lysine-associated intermolecular cross-linking sites are evident, comprising two locations each in N- and C-telopeptide and another two in carbohydrate-bound tetrapeptides of the main helical domain (Eyer *et al.*, 1984).

Corresponding sites were also found in abalone proα-chains, possibly being involved in cross-linking.

In vertebrate fibril-forming collagens, C-propeptide plays a crucial role in directing procollagen assembly which is stabilized by intra- and interchain disulfide bonds (Vuorio and de Crombrugghe, 1990). The vertebrate α-chains, which form homotrimer as well as heterotrimers in one collagen molecule, have 8 cysteine residues, while those forming only heterotrimers have 7 cysteine residues (Vuorio and de Crombrugghe, 1990). However, invertebrate proα-chains have been claimed to form homotrimer, although they contain 7 and 6 cysteine residues in their C-propeptide for the sea urchin *Paracentrotus lividus* and the sandworm *Arenicola marina*, respectively (D'Alessio *et al*., 1989; Sicot *et al*, 1997).

Northern hybridization demonstrated that accumulated mRNA levels of Hdcols 1α and 2α were quite similar in tissue distribution of adult and juvenile specimens, suggesting that the two α-chains possibly form heterotrimer. More detailed investigation is needed in order to disclose trimer formation of abalone proα-chains.

Seasonal changes of collagen mRNA levels were investigated for both muscular tissues and hepatopancreas. The spawning season is from October to November for kuro-awabi abalone around Kanagawa Prefecture where the present samples were harvested. The mRNA levels of both abalone collagens increased in December and January soon after spawning season. However, the accumulated mRNA levels were unexpectedly low from September to November when collagen contents has been reported to increase (Hatae *et al*., 1995). The water temperature in the coastal area off the Miura Peninsula was higher by 0.5-1.1°C from October to December in 1997 when the present samples were collected (personal communication from the Kanagawa Prefectural Fisheries Experimental Station) than that from October to December in 1991 when collagen contents were determined (Hatae *et al*., 1995). The warm water temperature in 1997 might have delayed the increase of collagen mRNA levels.

Collagen mRNA levels in abalone hepatopancreas changed seasonally as well as those in muscular tissues. An extraordinarily large growth of the gonad (as much as 20%) in spawning season (Webber, 1970) has been reported for abalone and thus may have some effect on the

collagen metabolism. It remains ambiguous whether or not the decreases of collagen content and mRNA levels in summer are related to the energy usage for gonad growth.

Chapter IV Proteases Related to Abalone Collagen Metabolism

Matrix metalloproteinases (MMPs) play an important role in the remodeling of extracellular matrix in vertebrate. These enzymes consist of a family of eleven zinc-dependent endopeptidases including interstitial collagenase, gelatinase, and stromelysin (Birkedal-Hansen, 1995). Interstitial collagenase (MMP-1) is responsible for the first step of the catabolism of collagen fibers (Gross *et al.*, 1974). Other proteases such as plasminogen activator and lysosomal cysteine protease are known to be involved in matrix remodeling (Birkedal-Hansen, 1995). Cathepsin L participates in the extensive proteolysis of the salmon muscle, cleaving native collagen at the non-helical domain (Yamashita and Konagaya, 1991).

Collagenolytic serine proteases have been also found in invertebrates. They were first isolated from the hepatopancreas of the fiddler crab *Uca pugilator* (Eisen *et al.*, 1973) which are probably involved in food digestion rather than morphogenesis.

Collagen content in the abalone muscle varies seasonally, showing the lowest content in July and August when abalone is considered to be most tasteful (Olaechea *et al.*, 1993; Hatae *et al.*, 1995). On the other hand, the highest collagen content is observed from October to December during the spawning season. As described in Chapter III, the highest collagen mRNA levels were demonstrated for both muscular tissues and hepatopancreas in December and January, while the lowest levels were shown in summer. These results suggest that the increase of collagen contents from autumn to winter is associated with enhancement of collagen synthesis. On the other hand, free amino acids and oligopeptides have been reported to be at the highest level, together with ATP and related compounds in summer (Hatae *et al.*, 1995). Free and peptide-bound proline and hydroxyproline have been also reported to be abundant in the summer muscle (Hatae *et al.*, 1995). These suggest that cellular metabolism in abalone including collagen turnover is enhanced in this season, accumulating taste-forming compounds in the muscle. However, the mechanisms involved in changes of collagen metabolism are not known.

In this chapter, proteases possibly related to abalone collagen metabolism were examined in various tissues. A collagen-film-degrading protease was purified from the adductor muscle.

Section 1 Gelatin-degrading proteases

A family of MMP initiate and extend extracellular matrix degradation in vertebrate. Activities of most MMPs have been detected by gelatin zymography (Woessner, 1991). Other classes of proteases such as serine or cysteine proteases also participate in collagen degradation. Therefore, gels containing gelatin as a substrate are most often used in initial studies (Fisher and Werb, 1995). In this section, the distribution of gelatinolytic activities in abalone tissues were examined by zymographic analysis. The reactivity with abalone collagen fibrils was also investigated.

Materials and Methods

Materials

Adult abalone specimens of *Haliotis discus* (average body weight of 350 g including shells) were collected in February and July from the coastal area along the Pacific Ocean off the Miura Peninsula.

Preparation of crude extracts from various tissues

All procedures were performed at 4°C unless otherwise noted. Hemolymph was drawn from the muscle part of live specimens with a syringe fit with a 22-gauge needle. Hemolymph samples were centrifuged at 2,000 x g for 20 min and plasma was stored at -20°C until use. Crude extracts were prepared from intestine, hepatopancreas, and adductor muscle. Intestine was dissected out and connective tissues were teased free of the intestinal wall. The intestine tract was homogenized with 5 volumes of 50 mM Tris-HCl (pH 7.5) and centrifuged at 11,000 x g for 10 min. The supernatant was used as the intestine extract. Acetone powder of hepatopancreas was prepared and homogenized with 100 volumes of 50 mM Tris-HCl (pH 7.5). The suspension was stirred gently for 30 min and then centrifuged at 11,000 x g for 20 min. The resultant supernatant was used as the hepatopancreas extract. Muscle extracts were prepared with solutions at neutral and acidic pH. The adductor muscle (10 g) was homogenized with 5 volumes of 50 mM sodium acetate (pH 4.0) containing 5 mM $CaCl_2$ and 0.5 mM dithiothreitol (DTT), or 50 mM Tris-HCl

(pH 7.5) containing 5 mM $CaCl_2$ and 0.5 mM DTT using a Polytron homogenizer. The homogenate was allowed to stand under stirring at 0°C for 5 min and centrifuged at 6,000 x g for 20 min. The supernatant was used as the water-soluble fraction. The precipitate was homogenized with 5 volumes of 50 mM sodium acetate (pH 4.0) containing 0.5% (v/v) Triton X-100, 5 mM $CaCl_2$ and 0.5 mM DTT, or 50 mM Tris-HCl (pH 7.5) containing 0.5% (v/v) Triton X-100, 0.15 M NaCl, 5 mM $CaCl_2$ and 0.5 mM DTT. After the homogenate was stirred at 0°C for 1 h and subsequently centrifuged at 6,000 x g for 20 min, the resultant supernatant was used as the membrane-bound fraction.

Gelatin zymography

Gelatin zymography was carried out essentially by the method of Yamagata *et al.* (1988). Briefly, 10% SDS-polyacrylamide slab gels containing 0.5 mg/ml gelatin from bovine skin, were prepared. Samples were dissolved in the SDS-containing sample buffer (see Section 1 of Chapter I) without 2-mercaptoethanol for SDS-PAGE. After electrophoresis, SDS was removed by washing the gels with 2.5% Triton X-100 for 1 h and then the gels were incubated for 16 h at 37°C in 50 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl and 0.02% NaN_3 with or without 10 mM $CaCl_2$. The incubated gels were stained with 0.05% CBB R-250.

Analysis of the reactivity with abalone collagen fibrils

Collagens solubilized with 0.1% pepsin for abalones collected in February and July as described in Section 1 of Chapter I were used as substrate. Abalone collagen (1 mg/ml) was dialyzed against 50 mM sodium acetate (pH 4.0) containing 5 mM $CaCl_2$ and 0.5 mM DTT, or 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ and 0.5 mM DTT. The reaction mixture containing 100 µl of collagen fibrils and 200 µl of crude extract was incubated at 25°C or 37°C. The reaction was stopped by the addition of an equal volume of SDS-containing sample buffer followed by boiling for 2 min before SDS-PAGE.

Effects of inhibitors on the reactivity of the muscle extract with abalone collagen fibrils

To examine the effects of inhibitors on the reactivity of muscle extracts with abalone collagen fibrils, following protease inhibitors were added to the reaction mixture; EDTA (10 and 50 mM), EGTA (10 and 50 mM), *1, 10*-phenanthroline (1 and 10 mM), and PMSF (1 mM), leupeptin (20 μM), chymostatin (20 μM), soybean trypsin inhibitor (STI) (100 μg/ml), *N*-ethylmaleimide (NEM) (10 mM), E-64 (10 μg/ml), and DTT (1 mM). Incubation was carried out for 12 h at 37°C, pH 4.0 and 7.5, and then the reaction mixtures were analyzed by SDS-PAGE.

Results

Gelatin zymography

Figure 4-1A showed the zymograms for tissue extracts and hemolymph from abalone collected in July. An active band at 45 kDa was found in all samples. The activity of the membrane-bound fraction was higher than that of the water-soluble fraction, indicating the existence of the membrane-bound type gelatinase in the muscle. Hemolymph showed a highly active band at 110 kDa. Many active bands were found with hepatopancreas extract including those of high molecular weight compounds more than 200 kDa. When the gel was incubated in the absence of $CaCl_2$, the active band at 110 kDa in hemolymph was completely inhibited, whereas the common active band at 45 kDa was not affected (Fig. 4-1B). The bands which corresponded to active bands were not found in SDS-PAGE patterns (Fig. 4-1C). The muscle extracts prepared at pH 4.0 also showed an active band at 45 kDa, although their activity was lower than that of extracts prepared at pH 7.5 (data not shown).

The reactivity with abalone collagen fibrils

Muscle extracts at acidic pH were prepared from abalone collected in February and July, whereas other extracts and hemolymph were prepared from abalone collected in July. The degraded products of collagen by tissue extracts and hemolymph were analyzed by SDS-PAGE. No degraded product was found in all reaction mixtures incubated at 25°C for 96 h. However,

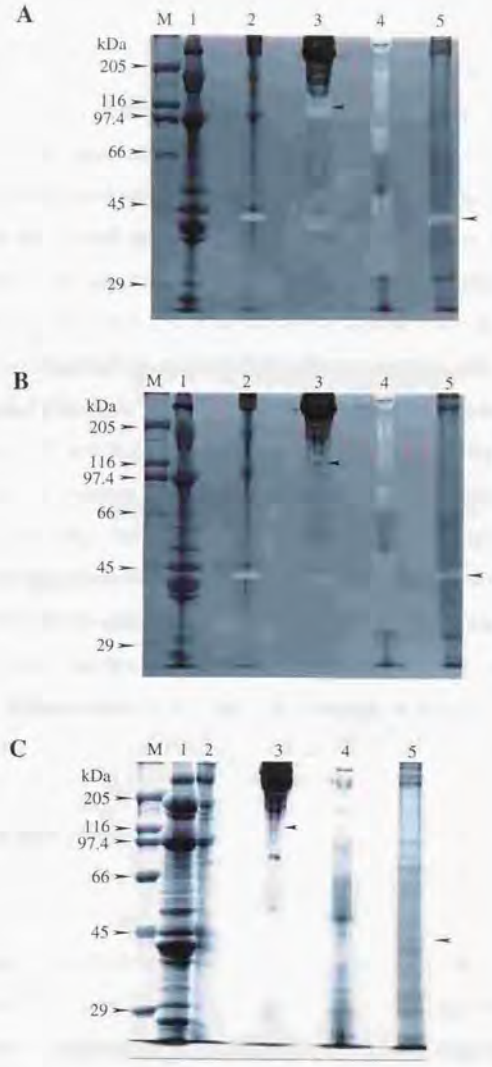


Fig. 4-1. Gelatin zymography (A and B) and SDS-PAGE (C) of crude extracts and hemolymph from abalone. Crude extracts were prepared from adductor muscle (lanes 1 and 2), hepatopancreas (lane 4), and intestine (lane 5). Hemolymph was collected from one live specimen (lane 3). The gels were incubated with (A) or without (B) 10 mM $CaCl_2$. Lane M contains molecular weight markers. Lanes 1 and 2 show water-soluble and membrane-bound fractions prepared at pH 7.5 from the muscle, respectively. Arrowheads indicate active bands at 45 and 110 kDa.

degraded products were observed in the muscle extracts prepared at pH 4.0 and hepatopancreas extracts from acetone powder when incubation was carried out at 37°C (Figs. 4-2 - 4-4).

Both water-soluble and membrane-bound fractions prepared at pH 4.0 from the muscle of abalone collected in February hydrolyzed collagen fibrils at 37°C and at either pH 4.0 or 7.5 (Fig. 4-2). Digestion patterns with both muscle extracts were similar to each other, although these were different between pH 4.0 and 7.5 where incubation was carried out. However, the patterns were different from those with vertebrate collagenase which produces a large N-terminal three-quarter fragment and a C-terminal one-quarter fragment (Birkedal-Hansen, 1987). Together with α-chain bands, degraded products of 120 and 100 kDa were found after incubation for 6 h at pH 4.0, while fragments of 122 and 70 kDa were produced after incubation for 6 h at pH 7.5.

On the other hand, the reactivity of muscle extracts prepared from abalone collected in July was hardly observed (Fig. 4-3). The reactivity of muscle extracts with collagen fibrils was similar between collagen preparations from February and July (data not shown). On the other hand, the reactivity with abalone collagen of hepatopancreas extracts prepared from abalone collected in July was higher than that of muscle extracts, which was probably due to the presence of digestive enzymes in hepatopancreas, resulting in disappearance of α-chain band after incubation for 24 h (Fig. 4-4).

Effects of inhibitors on the reactivity of the muscle extracts with abalone collagen fibrils

The following results shown in Figs. 4-5 and 4-6 were obtained with abalone collected in February. Figure 4-5 shows the effects of metalloprotease inhibitors on the reactivity of water-soluble fraction prepared from the muscle at pH 4.0 with abalone collagen fibrils for 12 h at 37°C and at either pH 4.0 or 7.5. Low concentrations of metalloprotease inhibitors (10 mM EDTA, 10 mM EGTA, and 1 mM *1, 10*-phenanthroline) did not inhibit the collagen degradation at pH 4.0, whereas they completely inhibited at pH 7.5. Higher concentrations of these reagents (50 mM EDTA, 50 mM EGTA, and 10 mM *1, 10*-phenanthroline) inhibited the collagenolytic activity even at pH 4.0.

The degradation of collagen fibrils was inhibited by PMSF (an inhibitor of serine protease), and by NEM and E-64 (inhibitors of cysteine protease) at pH 4.0 (Fig. 4-6A). Although the

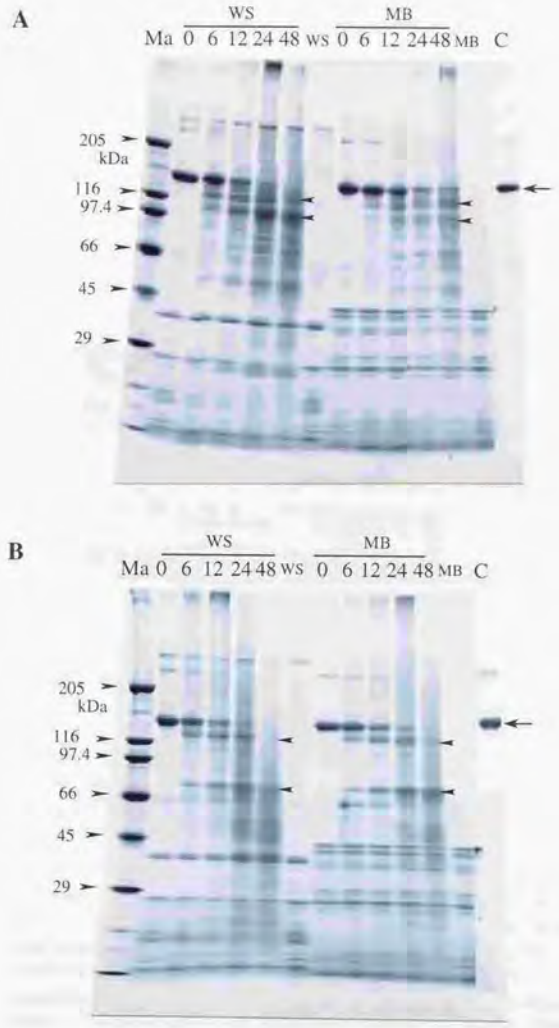


Fig. 4-2. The reactivity with abalone collagen of muscle extracts prepared at pH 4.0 from abalone collected in February. Water-soluble and membrane-bound fractions prepared from muscle were incubated with collagen at 37°C at either pH 4.0 (A) or 7.5 (B). Abbreviations used are M, molecular weight markers; WS, water-soluble fraction; MB, membrane-bound fraction; C, collagen. Arrows show collagen α-chain. Arrowheads indicate protein bands of interest. Numerals above the photographs indicate incubation time (h).

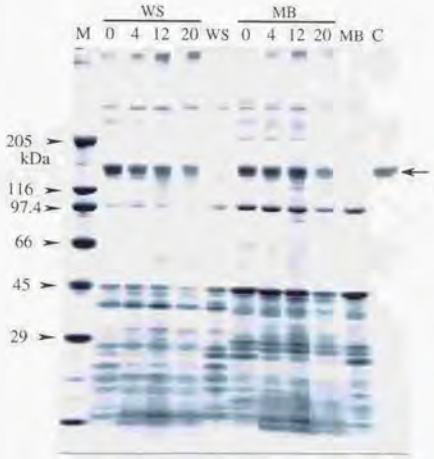


Fig. 4-3. The reactivity with abalone collagen of muscle extracts prepared at pH 4.0 from abalone collected in July. Water-soluble and membrane-bound fractions prepared from muscle were incubated with collagen at 37°C and pH 4.0. Abbreviations used are M, molecular weight markers; WS, water-soluble fraction; MB, membrane-bound fraction; C, collagen. An arrow shows collagen α-chain. Numerals above the photograph indicate incubation time (h).

Fig. 4-4. The reactivity with abalone collagen of hepatopancreas extracts from abalone collected in July. Hepatopancreas extracts from acetone powder were incubated with collagen at 37°C and pH 7.5. Abbreviations used are M, molecular weight markers; Hp, hepatopancreas extract; C, collagen. An arrow indicates collagen α-chain. Numerals above the photograph indicate incubation time (h).

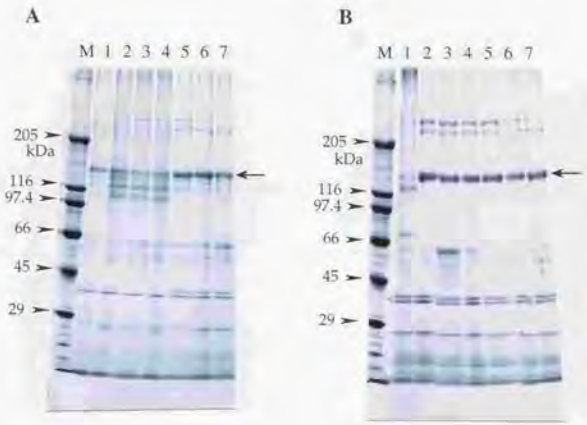


Fig. 4-5. Effects of metalloprotease inhibitors on the reactivity with abalone collagen of muscle extracts prepared from abalone collected in February. Water-soluble fraction prepared at pH 4.0 from the muscle was incubated with collagen for 12 h at 37°C and at either pH 4.0 (A) or pH 7.5 (B) in the absence (lane 1) or presence of the following metalloprotease inhibitors: lane 2, 10 mM EDTA; lane 3, 10 mM EGTA; lane 4, 1 mM *1, 10*-phenanthroline; lane 5, 50 mM EDTA; lane 6, 50 mM EGTA; lane 7, 50 mM *1, 10*-phenanthroline. Lane M contains molecular weight markers. Arrows indicate collagen α-chain.

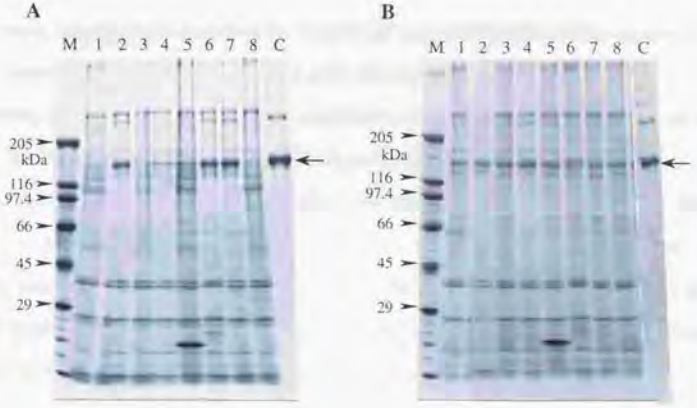


Fig. 4-6. Effects of protease inhibitors on the reactivity with abalone collagen of muscle extract prepared from abalone collected in February. Water-soluble fraction prepared at pH 4.0 from the muscle was incubated with collagen for 12 h at 37°C and at either pH 4.0 (A) or pH 7.5 (B) in the absence (lane 1) or presence of the following inhibitors: lane 2, 1 mM PMSF; lane 3, 20 μM leupeptin; lane 4, 20 μM chymostatin; lane 5, 100 μg / ml STI; lane 6, 10 mM NEM; lane 7, 10 μg / ml E-64; lane 8, 1 mM DTT. Abbreviations used are M, molecular weight markers; C, abalone collagen. Arrows indicate collagen α-chain.

collagenolytic activity was lower at pH 7.5 than at pH 4.0, PMSF and chymostatin (an inhibitor of chymotrypsin) inhibited the degradation of collagen fibrils at pH 7.5 (Fig. 4-6B). These results indicate that the muscle extracts contain metallo-, cysteine, and serine proteases.

Summary

Gelatinolytic activities were examined for hemolymph and extracts from various tissues of abalone by gelatin zymogaphy. An active band at 45 kDa was commonly observed in all samples, whereas hemolymph showed a strong gelatinolytic band at 110 kDa. In the absence of $CaCl_2$, the 110 kDa active band disappeared. The degradation of collagen fibrils was demonstrated with muscle extracts prepared at pH 4.0 and hepatopancreas extracts at 37°C. The reactivity of muscle extracts prepared from abalone collected in February was higher than that from abalone collected in July. Collagenolytic activity of the muscle extract was inhibited by the inhibitors of metalloproteases such as EDTA, EGTA, and *1, 10*-phenanthroline at pH 7.5, while it was inhibited by inhibitors for cysteine proteases such as NEM and E-64 at pH 4.0.

Section 2 Purification of collagen-film-degrading protease

As described in the previous section, gelatinolytic activity was found in various tissue extracts from abalone including those of muscle. Collagen degradation at 37°C was demonstrated with a crude muscle enzyme solution extracted at pH 4.0. The activity was inhibited by the inhibitors of metalloproteases at pH 7.5, indicating the existence of MMP-like protease(s) in abalone muscle. The objective of this section was to isolate protease(s) from abalone muscle which has the reactivity with abalone collagen at neutral pH.

Materials and Methods

Materials

Adult abalone specimens of *Haliotis discus* (average body weight of 350 g including shells) were collected in February and July from the coastal area along the Pacific Ocean off the Miura Peninsula.

Isolation of a collagen-film-degrading protease

All procedures were performed at 4°C unless otherwise noted. The adductor muscle (70 g) was homogenized with 5 volumes of 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ and 0.5 mM DTT using a Polytron homogenizer. The homogenate was allowed to stand under stirring at 0°C for 5 min and centrifuged at 5,000 x g for 15 min. To the supernatant was added solid ammonium sulfate to provide 60% saturation. The precipitate formed was dissolved in and dialyzed against 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ and 0.5 mM DTT (buffer A), and dialyzed against the same buffer. The dialysate was applied to a DEAE-Toyopearl 650M column (1.5 x 7 cm) equilibrated with buffer A and proteins were eluted with a linear gradient using 200 ml each of buffer A and the same buffer containing 0.3 M NaCl. Fractions solubilizing collagen film (refer to the following section for assay) were pooled, dialyzed against 10 mM sodium phosphate (pH 6.8) containing 0.1 M NaCl and 0.5 mM DTT (buffer B), and applied to a TSK G3000SWG column (2.15 x 60 cm) equilibrated with buffer B. Active fractions were

pooled and dialyzed against 10 mM Tris-HCl (pH 7.5) containing 0.8 M ammonium sulfate, 10 mM $CaCl_2$, and 0.5 mM DTT (buffer C). Fractions showing the activity were applied to a Butyl-Toyopearl 650M column (1.5 x 7 cm) equilibrated with buffer C. Absorbed proteins were eluted with a linear gradient from 0.8 to 0 M ammonium sulfate in totally 300 ml of buffer C.

The purity of proteins was checked by SDS-PAGE using 7.5 - 20% polyacrylamide slab gels.

Measurements of reactivity with collagen film

The reactivity was measured at 37°C as the activity for solubilizing the film prepared from abalone collagen as follows (Birkedal-Hansen and Taylor, 1982). Pepsin-solubilized abalone collagen were prepared from foot and adductor muscles with 0.1% pepsin treatment as described in Section 1 of Chapter I. After dilution with an appropriate amount of 0.1 M acetic acid, the pepsin-solubilized collagen was spread on a petridish of 9 cm in diameter at a protein concentration of 25 $\mu g/cm^2$ and air-dried at 4°C. Fractions containing 5 µl in various purification steps were spotted on the film and incubated at 37°C. After incubation for a certain period, the film was rinsed with distilled water and stained with 0.05% CBB R-250. The diameter of solubilized area on the film by spotting was measured and defined as the activity.

Molecular weight determinations

Molecular weight markers for SDS-PAGE were as described in Section 1 of Chapter I. Molecular weight determination was also performed by gel filtration on a TSK G3000SWG column using the following standard molecular weight markers (Sigma): blue dextran (2,000,000), β-amylase from sweet potato (200,000), alcohol dehydrogenase from yeast (150,000), bovine serum albumin (66,000), carbonic anhydrase from bovine erythrocytes (29,000), and cytochrome *c* from horse heart (12,400).

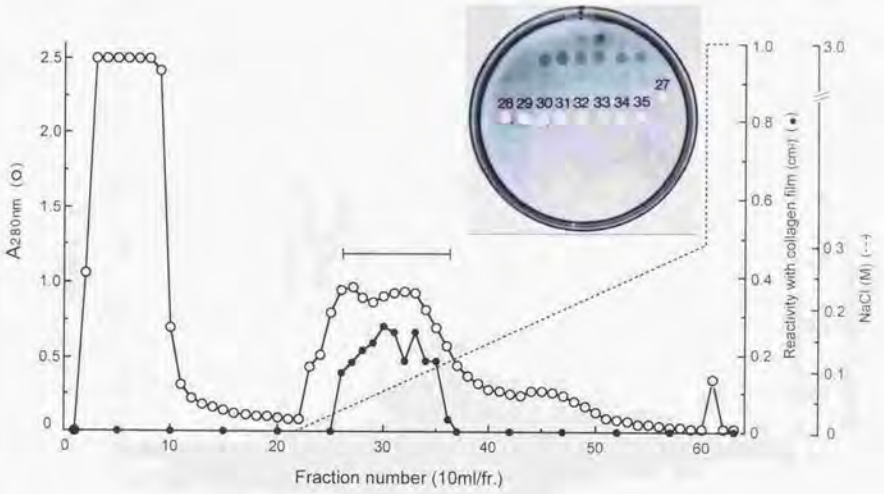
Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951) as described in Section 1 of Chapter I.

Results

The following results obtained were essentially the same between abalones collected in July and February. To crude extract from abalone muscle was added ammonium sulfate to make 60% saturation, then the precipitate formed was dissolved in buffer A and subjected to DEAE ion-exchange chromatography. The elution profile by DEAE-Toyopearl 650M ion exchange chromatography is shown in Fig. 4-7. Besides the large quantities of unabsorbed proteins, those absorbed to the column were eluted around 0.1 M NaCl. Fractions each containing 5 µl were spotted on the collagen film. After incubation at 37°C for 30 min, the film was stained with CBB R-250. As shown in Fig. 4-7, fractions with no activity showed no change of the film which turned blue after CBB R-250 staining. On the other hand, fractions from 27 to 35 eluting at 0.1 M NaCl solubilized the film, showing colorless spots. The activity curve was roughly consistent with the protein elution pattern. The crude extract from abalone adductor muscle showed no solubilization at 20°C for 4 h. Thus, the incubation temperature for measuring the reactivity was set at 37°C thereafter.

Figure 4-8 shows the elution profile of gel filtration chromatography. After large peaks eluting near the void volume, the small peak around fraction 54 was followed. The active fractions solubilized the film within 60 min at 37°C. The molecular weight at the activity peak was approximately 127,000. SDS-PAGE patterns showed that this chromatography was effective in removing about 230 kDa and 155 kDa proteins eluting around fraction 38 and 42, respectively. Active fractions were pooled and applied to Butyl-Toyopearl 650M hydrophobic chromatography. Fractions from 28 to 35 eluting at 0.2 M ammonium sulfate, solubilized the collagen film (Fig. 4-9). SDS-PAGE of these fractions showed a single band having a molecular weight of approximately 148,000. These procedures yielded 774 µg of the purified 148 kDa protein from 70 g of adductor muscle.



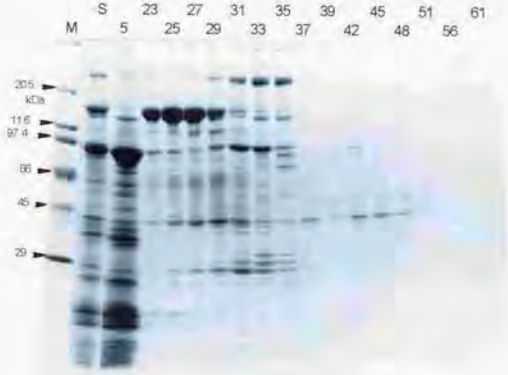


Fig. 4-7. The elution profile on DEAE-Toyopearl 650M ion exchange column chromatography for collagen-film degrading protein from abalone adductor muscle. The photograph below the elution pattern shows SDS-PAGE of the crude extract applied (S) and selected fractions from the column. Lane M contains molecular weight makers. Numerals above the photograph correspond to fraction numbers shown in the elution profile. An inset shows collagenolytic activity of each fraction eluted. Fractions each containing 5 µl were spotted on the film, incubated at 37°C for 30 min, and stained with CBB R-250.

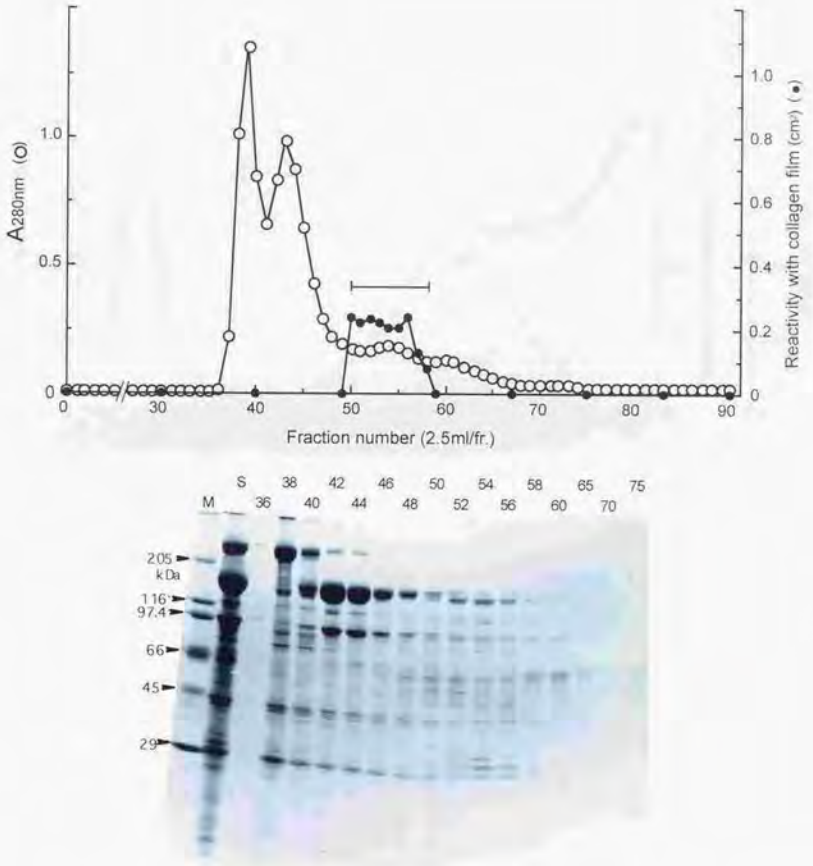
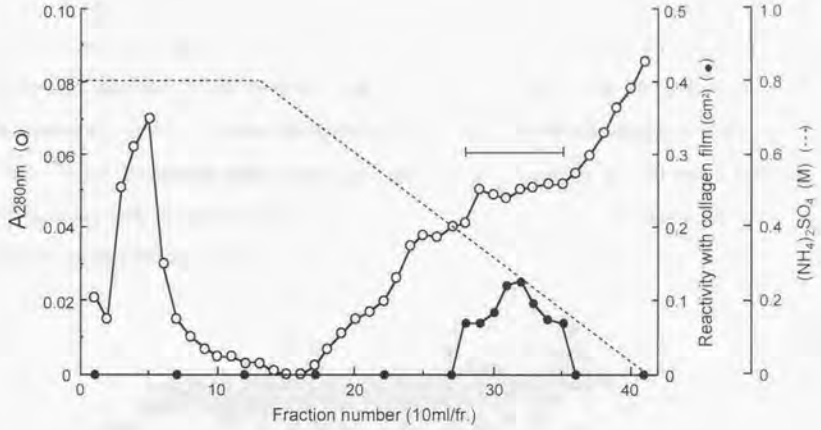


Fig. 4-8. The elution profile on TSK G3000SWG gel filtration column chromatography for collagen-film degrading protein from abalone adductor muscle. The active fraction from the DEAE-Toyopearl 650M column shown in Fig. 4-7 was applied. Refer to the legend of Fig. 4-7 for further details.



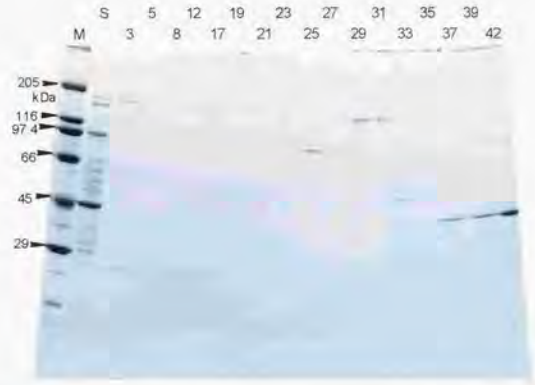


Fig. 4-9. The elution profile on Butyl-Toyopearl 650M hydrophobic column chromatography for collagen-film degrading protein from abalone adductor muscle. The active fraction from the TSK G3000SWG column shown in Fig. 4-8 was applied. Refer to the legend of Fig. 4-7 for further details.

Summary

A 148 kDa protein was isolated from the adductor muscle of the abalone, by a series of chromatographic procedures involving DEAE-Toyopearl 650M ion exchange, TSK G3000SWG gel filtration, and Butyl-Toyopearl 650M hydrophobic columns. Reactivity with collagen was measured as the ability to solubilize the film containing pepsin-solubilized collagen from the same animal. Active fractions of various purification steps solubilized the film at 37°C within 60 min. The purified protein consisted of a single polypeptide chain and the molecular weight was estimated by SDS-PAGE to be 148,000.

Section 3 Properties of collagen-film-degrading protease

A collagen-film-degrading protease, the 148 kDa protein, was isolated from abalone adductor muscle. This protein had the ability to solubilize the collagen film at 37°C within 60 min. In this section, the degradation products from collagen film were analyzed by SDS-PAGE and the effects of metal ions on the activity were investigated for the 148 kDa protein. Furthermore, amino acid composition and N-terminal amino acid sequence for the 148 kDa protein were determined.

Materials and Methods

Analysis of degradation products from the collagen film

For analyzing degradation products from the collagen film by SDS-PAGE, the purified protein was spotted on the collagen film. After incubation at 37°C for 60 min, solubilized proteins were collected and added with an equal volume of the SDS-containing sample buffer. The samples were boiled for 2 min and subjected to SDS-PAGE.

Effects of metal ions on the reactivity of the 148 kDa protein with collagen film

To examine the effects of metal ions, the purified 148 kDa protein was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.5 mM DTT, and then added with various metal ions at final concentrations of 1 to 50 mM. After the reaction mixture was allowed to stand at 4°C for 1 h, 5 µl of each sample was spotted on the film and incubated at 37°C for 1 h. Measurements of the reactivity with collagen film are described in Section 2 of this chapter.

Amino acid composition and N-terminal amino acid sequence of the 148 kDa protein

Amino acid composition and N-terminal amino acid sequence of the purified protein were determined as described in Section 2 of Chapter I. The protein homology search was performed by using the SWISS-PROT database coordinated with the Inherit program (Applied Biosystems).

Results

Analysis of degradation products from the collagen film

The purified active protein was spotted on the collagen film. After incubation at 37°C, solubilized proteins were collected and subjected to SDS-PAGE (Fig. 4-10). No degradation product was detected and no change in the mobility of collagen band was observed. It is suggested, therefore, that this protein did not cleave the helical domain of the collagen molecule.

Effects of metal ions on the reactivity of the 148 kDa protein with collagen film

The 148 kDa protein was purified in the presence of 5-10 mM $CaCl_2$. After the 148 kDa protein was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.5 mM DTT, the dialysate did not solubilize the collagen film with prolonged incubation for 2 h (Fig. 4-11A). Various metal ions were added in the Ca^{2+}-free sample at final concentrations of 1 to 50 mM. Alkaline-earth metal ions such as Mg^{2+}, Ca^{2+}, and Sr^{2+} were effective on the reactivation of the 148 kDa protein at concentrations of 30-50 mM, 20-50 mM, and 20-50 mM, respectively (Fig. 4-11B).

Amino acid composition and N-terminal amino acid sequence of the 148 kDa protein

The amino acid composition of the 148 kDa protein is shown in Table 4-1. Methionine was not present, whereas glutamic acid plus glutamine and leucine were both contained more than 100 residues in total 1,000 residues.

Figure 4-12 shows N-terminal amino acid sequence of the 148 kDa protein for 17 residues. No further analysis was possible due to the limited amount of the protein on the membrane. A homology search for this sequence was conducted using the SWISS-PROT database, revealing that similar proteins are not available.

Summary

The properties of a collagen-film-degrading protease, the 148 kDa protein, were examined. The solubilized proteins collected from the collagen film did not show any degradation products of

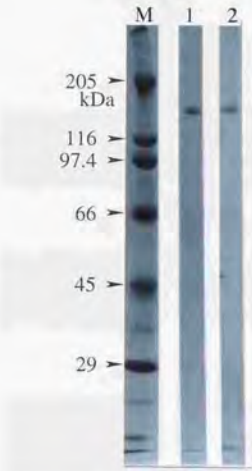


Fig.4-10. SDS-PAGE patterns of degradation products from collagen film treated with the 148 kDa protein. Lane 1, degradation products of collagen film; lane 2, collagen control.

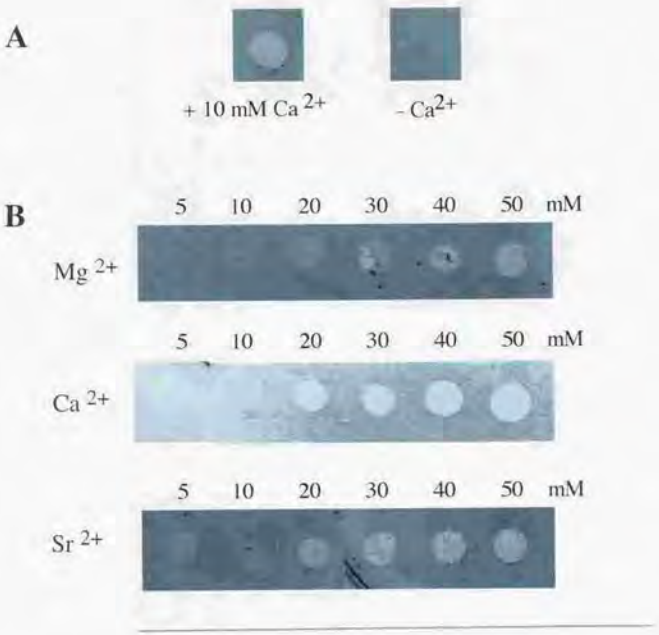


Fig. 4-11. Effects of metal ions on the reactivity of 148 kDa protein with collagen film. (A) The reactivity with collagen film in the presence or absence of 10 mM Ca^{2+}. (B) The reactivation of the Ca^{2+}-free 148 kDa protein. $MgCl_2$, $CaCl_2$, and $SrCl_2$ were added at final concentrations of 5-50 mM. The reaction mixture was left for 30 min at 4°C and then the collagen film spotted with each sample was incubated at 37°C for 1 h.

Table 4-1. Amino acid composition of the 148 kDa protein from abalone adductor muscle

(residues / 1,000 residues)

Amino acid	Residues
Asx	86
Thr	66
Ser	75
Glx	131
Pro	54
Gly	88
Ala	79
Val	71
Ile	42
Leu	103
Tyr	30
Phe	39
Lys	58
His	21
Arg	57

1 17
PVTQARVLTLNRGENRE

Fig. 4-12. The N-terminal amino acid sequence of the 148 kDa protein from abalone adductor muscle.

collagen. It is suggested that the 148 kDa protein did not cleave the helical domain of the collagen molecule. While this protein showed no activity in the absence of Ca^{2+}, the activity was recovered by the addition of 30-50 mM Mg^{2+}, 20-50 mM Ca^{2+}, and 20-50 mM Sr^{2+}. The N-terminal amino acid sequence of this protein differed from any sequences of known collagenases.

Section 4 Discussion

Collgenases are endopeptidases capable of cleaving the helical domain of native collagen molecules under physiological conditions. They have been isolated from various vertebrate tissues (Harper, 1980) as well as from microorganisms (Peterkofsky, 1982; Sasagawa *et al.*, 1993). These are zinc metalloenzymes that also require calcium for the full activity. Gelatinolytic activities were found in various extracts and hemolymph from abalone in this study. An active band from hemolymph at 110 kDa was sensitive to Ca^{2+} like MMPs. Collagen degradation at 37°C was demonstrated with muscle extracts prepared at pH 4.0 and hepatopancreas extracts from acetone powder. The effects of various inhibitors on the activity indicated that cysteine protease and metalloproteases participated in the degradation of collagen fibrils at acidic and neutral pH, respectively. Cysteine proteases such as cathepsins B and L are stable at pH 4.0 - 6.5 (Kominami and Bando, 1993). These proteases degrade collagen during tumor invasion and metastasis (Birkedal-Hansen, 1995), and extensively salmon muscle (Yamashita and Konagaya, 1991).

A certain group of collagenases are classified into serine proteases. It was first isolated from the midgut of the fiddler crab *Uca pugilator* (Eisen *et al.*, 1973; Grant *et al.*, 1983) and later obtained from various organisms (Hurion *et al.*, 1979; Lecroisey *et al*, 1979; Sakharov *et al.*, 1988; Klimova *et al.*, 1990; Lu *et al.*, 1990; Tsai *et al.*, 1991; Turkiewicz *et al.*, 1991; Wormhoudt *et al.*, 1992; Roy *et al.*, 1996). These enzymes hydrolyze not only the helical domain of native collagen but also unrelated proteins. These are homologous proteins to the pancreatic serine proteases of vertebrates. The molecular weight has been reported to be 105,000 for collagenase from *Clostlidium histolyticum* (Peterkofsky, 1982), 60,000 for collagenase from human skin (Woolley *et al.*, 1978), and 23,000 for collagenolytic serine protease from fiddlar crab (Grant *et al.*, 1980). The molecular weight of the collagen-film-degrading protein from adductor muscle in this study was estimated to be 148,000 which was larger than any known collagenolytic enzymes.

The 148 kDa protein easily solubilized the film within 60 min at 37°C and required alkaline-earth metal ions such as Mg^{2+}, Ca^{2+}, and Sr^{2+} for the activity (see Figs. 4-7 and 4-11). When the

solubilized protein was collected and subjected to SDS-PAGE, no change in the mobility of the collagen band was observed (see Fig. 4-10). In order to know how the 148 kDa protein degrades abalone collagen, the enzyme was incubated at 37°C in a test tube containing the pepsin-solubilized abalone collagen. When the reaction mixture was subjected to SDS-PAGE, neither degraded products nor changes in the mobility of the collagen band were detected (data not shown). Azocoll, generally used as a synthetic substrate for collagenolytic activity (Yoshida and Noda, 1965; Matsushita *et al*., 1994), was not sensitive substrate for this protein (data not shown). These results indicate that this protein can not hydrolyze the helical domain of the pepsin-solubilized abalone collagen, but possibly does telopeptides, non-helical domains at the end of the collagen molecule. Invertebrate collagens are extremely insoluble so that they need to be digested with pepsin to be solubilized (Kimura, 1975). It seems that pepsin-solubilized abalone collagen prepared in this study may still have parts of telopeptides even after limited pepsin digestion (see Fig. 1-3). The solubilized proteins collected from the film was electrically transferred onto PVDF membrane after separation by SDS-PAGE. The blotted α-chain band was subjected to N-terminal amino acid sequence analysis and compared to that of pepsin-solubilized collagen. The glycine residue was predominant at any cycles for both preparations, giving no information on the cleavage site for the solubilized product (data not shown). Further investigation is needed to clarify such ambiguity.

Measurements of the reactivity with collagen fibrils or collagen film were set at 37°C which was much higher than the ambient temperature, 12 - 25°C, for this abalone in the collection area. The pepsin-solubilized abalone collagen fibrils were degraded as found by SDS-PAGE, when treated with trypsin at 37°C, but not at 25°C (data not shown). Taken together, incubation at 37°C probably gave rise to gelatinization of the abalone collagen. Gelatinized α-chains would further be digested with the muscle crude extracts or the 148 kDa protein, and subsequently could be solubilized.

The 148 kDa protein of abalone consisted of a single polypeptide chain and differed from any known collagenolytic enzymes at the level of the primary structure. The level of the reactivity with collagen film was, however, very similar in winter and summer abalone specimens. Since the ambient temperature seasonally varies, the enzymatic activity may also change seasonally *in*

vivo depending on environmental temperature. On the other hand, the reactivity of muscle extracts prepared from abalone collected in February was higher than that from abalone collected in July. As described in Section 3 of Chapter III, the highest mRNA levels of the two collagen proα-chains were observed in December and January while the mRNA levels decreased in July, possibly related with the change of collagen content. Collagenolysis might be active in February when the levels of collagen mRNAs start to decrease. Another proteases which cleave native collagen molecules might be involved in the collagen metabolism. Such lines of investigation are also needed.

Chapter V General Discussion

In this study, attempts were made to disclose the mechanisms involved in collagen metabolism of the abalone muscle which shows seasonal changes of toughness in association with alterations of collagen content. First, abalone collagens were purified and examined for their properties. Second, the primary structures of abalone collagens were determined by cDNA cloning and seasonal changes in their gene expression were investigated. Third, proteases possibly related to abalone collagen metabolism were screened and one enzyme which degraded abalone collagen film was isolated.

Collagen is the most abundant structural protein in animals. Fibril-forming collagens form the most homogenous subfamily of collagens. Fibril-forming collagens isolated from various marine invertebrates with limited pepsin digestion show structural similarity to vertebrate counterparts (Kimura, 1975).

Pepsin-solubilized collagens were prepared in this study from abalone foot and adductor muscles to examine their properties. Collagen preparation solubilized with 1% pepsin contained two types of α-chains which were different in their N-terminal amino acid sequences. Amino acid composition of abalone collagen was similar to those of vertebrate type I collagens except for less alanine content and higher hydroxylysine contents as reported by Kimura and Matsuura (1974).

Immunological properties of abalone collagen were investigated using anti-collagen and anti-gelatin antisera. Both antisera exhibited cross-reaction against vertebrate type I collagens, although the reactivity was not observed against type III and type V collagens. It is interesting that both antisera were reactive with the α2(I) chain from higher vertebrates. It is considered that the gene for proα2(I) has evolved prior to those for proα1(I) and proα1(II) in higher vertebrates (Miller, 1985). Abalone collagen seems to be in a group of α2(I) chain rather than α1(I) chain of higher vertebrates.

Two types of full-length cDNAs coding for collagen proα-chains, Hdcols 1α and 2α, were isolated from foot muscle cDNA library. These were composed of domain structures characteristic to fibril-forming collagens. The two N-terminal amino acid sequences of the

abalone pepsin-solubilized collagen preparation corresponded to either of the two sequences deduced from the cDNA clones. It is noted that Hdcol 1α completely lacked a short Gly-X-Y triplet repeat sequence in N-propeptide which has never been reported before for any fibril-forming collagen. A substitution of glycine residues in the main triple-helical domain was observed in the two proα-chains. Such imperfection has been reported in invertebrate fibril-forming collagens including sponge and worms (Exposito and Garrone, 1990; Mann *et al.*, 1992; Exposito *et al.*, 1993; Sicot *et al.* 1997), whereas substitutions of glycine by other amino acids at triplet sites cause severe diseases in the case of vertebrate fibril-forming collagens. These substitutions produce a kink in the triple helix and cause assembly of abnormally branched collagen fibrils (Kuivaniemi *et al.*, 1991).

The cross-linking of invertebrate collagens results in their low solubility, while it is supposed to participate in the fibril strength for assembly of the collagen molecules containing one or two interruptions in the main triple-helical domain. Abalone collagens may form unique molecular structure, so that rotary shadowing technique is probably useful to examine the molecular shape.

Non-fibrillar collagens which are composed of short triple-helical domains are widely distributed in invertebrates rather than fibril-forming collagens (Yoshizato, 1991). It seems that fibril-forming collagens have evolved posterior to non-fibrillar collagens. Interruptions of the main triple-helical domain found in some invertebrate fibril-forming collagens are reminiscent of non-fibrillar collagens (Obara *et al.*, 1998). In addition, fibril-forming collagens in invertebrates are closely related with type V and type XI collagens by phylogenetic analysis using the sequences of C-propeptide (Obara *et al.*, 1998). However, abalone collagens purified from the muscle were similar to type I collagen in their amino acid composition and immunological properties. Some invertebrate collagens are also classified into type I collagens from their chemical compositions (Kimura, 1992). Further study on the primary structures of other invertebrate collagens can provide useful information in the view of comparative biochemistry.

Northern hybridization demonstrated that the accumulated mRNA levels of Hdcols 1α and 2α were quite similar in various tissues, suggesting that the two α-chains possibly form heterotrimers. More detailed investigation such as microscopic observation by

immunohistochemical technique using specific antisera against respective α-chains is needed to comprehend trimer formation of abalone proα-chains.

Seasonal changes of collagen mRNA levels were investigated which might be related to seasonal changes of collagen content. The highest mRNA levels were observed with specimens collected in December and January for each of adductor and foot muscles and hepatopancreas. The mRNA levels of muscular tissues decreased in July when collagen content decreased. These results suggest that the level of collagen synthesis increased in winter and decreased in summer. It still remains ambiguous whether the decrease in collagen content and mRNA levels is related to the energy usage for gonad growth in summer.

However, it is likely that collagenolytic proteases are also involved in collagen metabolism, since free and peptide-bound proline and hydroxyproline are abundant in the summer muscle (Hatae *et al.*, 1995). Gelatinolytic activities were detected in various tissues by gelatin zymography in the present study. Collagen degradation at 37°C was demonstrated by muscle extracts prepared at pH 4.0, although abalone collagen is probably denatured at this temperature.

The 148 kDa protein, a collagen-film-degrading protease, was isolated from adductor muscle by a series of chromatographic procedures. The purified protein solubilized the collagen film at 37°C within 60 min and required alkaline-earth metal ions such as Mg^{2+}, Ca^{2+}, and Sr^{2+} for activity. The N-terminal amino acid sequence of this protein differed from any sequences of known collagenases. The 148 kDa protein did not hydrolyze the main triple-helical domain of native collagen molecule, but possibly hydrolyzed the non-helical domain. It seems that other types of proteases which cleave the main triple-helical domain may participate in the initial step of collagen catabolism. Further investigation focusing such proteases is needed. The elucidation of possible factors concerning collagen metabolism will provide methods to process acceptable abalone meat all around the year.

Abalone foot or pedal tissue consists of two distinct regions, the columellar muscle and tarsic ones (Voltzow, 1990). The columellar region consists primarily of muscle fiber bundles that are oriented parallel to the long axis of the muscle in at least two directions. On the other hand, the tarsic region consists of bundles of muscle fibers that branch and change directions as they extend from their origins to the insertions. They form a complex three-dimensional network of

interconnecting contractile fibers. Both the columellar muscle and tarsic regions contain the thick connective-tissue sheaths surrounding the muscle fibers. Although gastropod foot muscles are very solid, they have high degrees of morphological complexity and behavioral plasticity (Voltzow, 1990). These characteristics of abalone foot muscle seem to be correlated with their adaptation for locomotion over a variety of muscle wave propagation. In the present study, the author found two types of fibril-forming collagen in abalone foot muscle. More information about the fine structure and precise orientation of the collagen sheaths in relation to the existence of the two types of collagen α-chain molecule is necessary to understand how the connective tissue transmits the forces of muscle cells contraction in abalone foot muscle.

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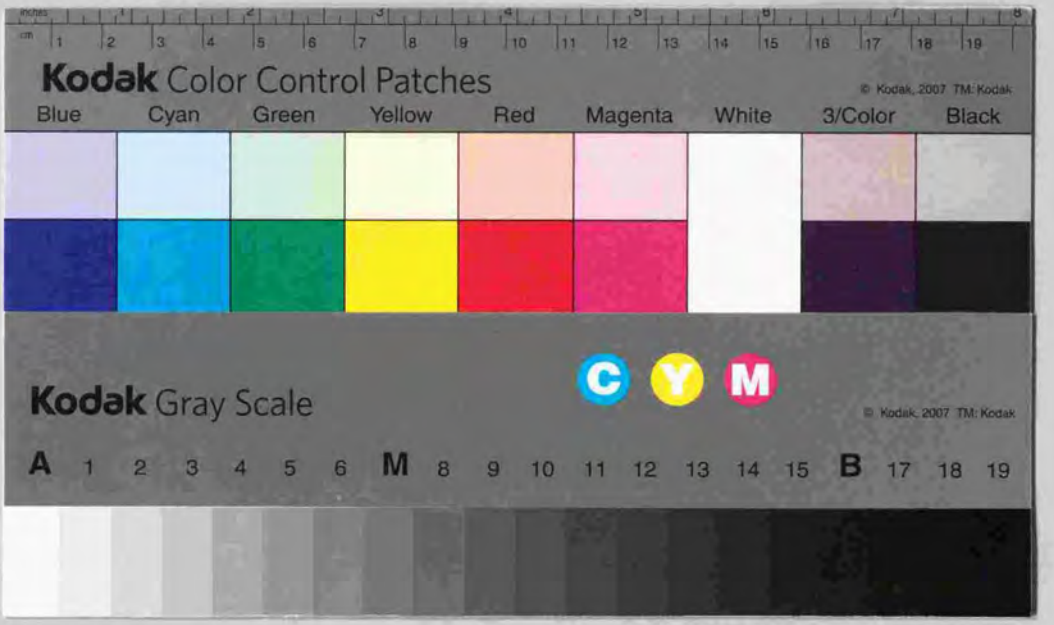
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