Immunological detection of translation products of type V/XI collagen α1 gene and their degradation products in red sea bream muscle

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ABSTRACT: We previously cloned cDNA of type V/XI collagen α1 chain (ColVa1) gene from cultured cells derived from red sea bream embryo. We raised an antibody against the deduced C-telopeptide of ColVa1 in order to detect the translation products of this cDNA and their degradation products in red sea bream muscle. To improve its specificity, the antibody was purified from rabbit antiserum by use of an affinity column cross-linked with recombinant C-terminal peptide of ColVa1 produced by Epicurian coli. The purified antibody recognized a band corresponding to the α chain of type V/XI collagen in western blot analysis of the extract of cultured cells. The antibody also recognized two bands in acid-soluble and pepsin-solubilized collagens, indicating that the translation products of the ColVa1 gene are present in muscle and that bands correspond to α and β chains of type V/XI collagen. A band corresponding to a molecular weight of approximately 65 k was detected in the NaOH extracts of muscle, suggesting that type V/XI collagen α1 chain is restrictedly digested in red sea bream muscle.

KEY WORDS: collagen degradation, red sea bream, type V/XI collagen, western blot analysis.

INTRODUCTION

Collagen plays a fundamental role in the structure and function of most connective tissues. Type V and type XI collagens are designated as minor fibril collagens because of their lower concentration in tissues. Whereas a small amount of type V collagen is widely codistributed with a large amount of type I collagen, type XI is present with type II collagen in various types of cartilage.1 These two collagens have been considered to share the same function in different tissues. However, it is reported that α1(XI), α1(V) and α2(V) chains,2 α1(XI) and α2(V) chains,3 and α1(XI) and α2(V) chains4 coexist in bovine bone, bovine vitreous, and human rhabdomyosarcoma cell line A204, respectively, suggesting that type V and XI subunits may form hetero-trimers. Thus, type V and type XI collagens are not regarded as distinct collagen types.

In fishes, types V and XI, type V/XI-like, and type V collagens have been biochemically detected and isolated in lamprey,5 shark6 and some teleosts,7-9 respectively. We have recently isolated the cDNA of type V/XI collagen α1 chain (ColVa1) gene from cultured cells established from eyed-period eggs of red sea bream Pagrus major (General Bank Data, accession No. AB045975).10 Whereas the deduced amino acid sequence of the ColVa1 resembles that of mammalian type XI collagen α1 chain, tissue distribution of ColVa1 gene mRNA resembles that of type V collagen based on reverse transcription–polymerase chain reaction (RT-PCR) analysis.

The collagen content in muscle contributes to firmness, which is an important factor in determination of the meat quality.11-13 A seasonal change is observed in collagen content, which is due to a change in the balance of collagen synthesis and breakdown.14 On the other hand, Sato et al. suggested that the cleavage of non-helical regions and/or cross-links of type V collagen causes muscle softening.15 It is important, therefore, to detect type V collagen and its degradation products in fish muscle. To detect the translation products of the ColVa1 gene and their degradation products...
in red sea bream muscle, we raised an antibody against the deduced C-telopeptide of ColVa1 and performed western blot analysis.

MATERIALS AND METHODS

Materials

Cultured red sea breams were obtained live from a commercial supplier. All materials used in this study were of the highest quality available commercially.

Preparation and purification of antibody

The peptide of the deduced C-telopeptide of ColVa1 with an added cysteine residue at the N-terminus (Fig. 1) was synthesized, coupled with keyhole limpet hemocyanin, then injected into rabbits by Sawady Technology (Tokyo, Japan). To improve its specificity, the antibody was purified from rabbit antisera by use of an affinity column cross-linked with recombinant C-terminal peptide of ColVa1 (Fig. 1) as described below. Briefly, we designed a set of oligonucleotide primers (5’-dGGGAATTCAGGAAGCTCAGGCTCAGGC, 2824–2844) (5’-dGGGTCGACGGCCCTGCATCTCA GCCGTGCC, 5961–5980) based on the DNA sequence of the ColVa1 gene and performed the PCR using these primers with KOD DNA polymerase (Toyobo, Osaka, Japan) by a modified version of the manufacturer’s protocol. After 30 cycles of PCR (30 s at 96°C, 30 s at 62°C, and 60 s at 74°C), the product was separated by electrophoresis and subcloned into the EcoRI/SalI site of a glutathione S-transferase (GST) gene fusion vector, pGEX-6P-1 (Amersham Biosciences Corp., Piscataway, NJ, USA), to produce an overexpression vector (pGEX-CVteC). The plasmid was transfected into Escherichia coli, BL21. The transformed bacteria were inoculated in Luria-Bertani (LB) broth (50 mL) containing 100 μg/mL ampicillin. The saturated culture was diluted with 10 volumes of LB broth, grown for 1 h at 37°C, then protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. Purification of GST fusion protein by using glutathione sepharose 4B (Amersham Biosciences Corp.) and cleavage by PreScission Protease (Amersham Biosciences Corp.) were performed according to the manufacturer’s protocol. The specific antibody was purified by the use of AF-Amino Toyopearl (Toso, Japan) cross-linked with the recombinant peptide. Briefly, approximately 1 mg recombinant peptide was cross-linked with approximately 200 μL AF-Amino Toyopearl (Toso, Japan) cross-linked with the recombinant peptide. Briefly, approximately 1 mg recombinant peptide was cross-linked with approximately 200 μL AF-Amino Toyopearl (Toso, Japan) cross-linked with the recombinant peptide. Briefly, approximately 1 mg recombinant peptide was cross-linked with approximately 200 μL AF-Amino Toyopearl through m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Biotechnology Inc., Rockford, IL, USA) according to the manufacturer’s protocol. Toyopearl (100 μL) was transferred to a column, to which 200 μL antiserum diluted with the same volume of phosphate-buffered saline (PBS: 0.8% (w/v) NaCl, 0.02% KCl, 0.29% Na2HPO4·12H2O, 0.02% KH2PO4) was then applied. The column was washed three times with 20 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl and 0.1% (w/v) polyoxyethylene sorbitan monolaurate and three times with distilled water. The antibody was eluted with 100 mM glycine-HCl buffer (pH 2.2) and immediately neutralized by addition of 2 M Tris solution.

Western blot analysis

All samples described below were diluted by 2× sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer in the presence of 2-mercaptoethanol18 and heated to 100°C for 3 min. Whole-cell extracts of E. coli, which had been cultured in the presence IPTG and purified recombinant peptide, were separated by SDS-PAGE (12.5%) and then electrophoretically transferred to a Clear Blot Membrane-p (Atto, Tokyo, Japan). Non-specific staining was blocked by incubating the membrane with 5% skim milk in PBS. The membrane was then incubated with a 1/1000 dilution of the antiserum for 30 min at room temperature, then for a sequential 60 min incubation with alkaline phosphatase conjugated with anti rabbit IgG antibody (New England Biolabs,
Alkaline phosphatase activity was developed with 173 mM nitroblue tetrazolium and 115.3 mM 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 50 mM MgCl₂. Confluent cells established from eyed-period eggs of red sea bream on 150-mm tissue culture dishes (Asahi Techno Glass Corp., Tokyo, Japan) were washed with PBS, detached with a scraper, pelleted by centrifugation at 900 g for 1 min, then dissolved in 250 μL SDS-PAGE sample buffer. The sample (12.5 μL) was separated by SDS-PAGE (7.5%) and transferred to a membrane as described above. Development of the blot for the secondary antibody was performed as described above.

Acid-soluble collagen (ASC) and pepsin-solubilized collagen (PSC) were prepared from red sea bream muscle by the method of Sato et al. and separated by SDS-PAGE (7.5%). To detect the type V/VI collagen degradation product, NaOH extracts from muscle was prepared. Briefly, to remove the native collagen, fresh dorsal muscle was homogenized with nine volumes of ice-cold 0.1 N NaOH, then centrifuged at 10 000 g for 10 min at 4°C. The supernatant (10 μL) was immediately separated by SDS-PAGE (7.5%). Western blot analyses of ASC, PSC and the NaOH extract were performed as described above.

**Immunocytochemistry**

Red sea bream cells were cultured on slide glasses as described previously. The glasses were air-dried quickly, fixed in acetone-methanol (2:3) for 1 min, and then incubated with 5% skim milk in PBS for 30 min. Endogenous peroxidase activity was blocked by treatment for 10 min at room temperature with 0.3% H₂O₂ and 0.1% NaN₃ dissolved in distilled water. The glasses were reacted with the 15 μg/mL solution of purified anti-ColVa1 telopeptide antibody for 60 min at room temperature. After washing the glasses three times with PBS containing 0.1% Triton X-100, peroxidase activity was developed by the method of Tokuda et al.

**RESULTS AND DISCUSSION**

**Purification of anti-ColVa1 telopeptide antibody**

The antiserum against the deduced C-telopeptide of ColVa1, which specifically cross-reacted with the synthesized peptide in an enzyme-linked immunosorbent assay (ELISA), demonstrated various non-specific signals on ASC derived from muscle in western blot analysis (data not shown). We, therefore, have purified the antibody from the serum by the use of an affinity column coupled with the recombinant peptide prepared as described below. Overexpression vector, pGEX-CVteC, was transfected into E. coli carrying the control plasmid pGEX-6P-1; lane 2, whole cell extracts of E. coli carrying the overexpression vector pGEX-CVteC; lane 3, the purified recombinant peptide. Lines indicate molecular weight of the marker proteins. The arrowhead, closed triangles and open triangles indicate glutathione S-transferase (GST, 26 k), recombinant GST-fusion protein (47 k) and purified recombinant protein (23 k), respectively.
Type V/XI collagen in muscle

SDS-PAGE (23 k) was larger than that calculated from the deduced amino acid sequence (19 k).

Western blot analysis was performed in order to detect the recombinant peptide by using the antiserum. The antiserum recognized the recombinant fusion protein corresponding to 47 k (Fig. 2b, lane 2) and recombinant peptide corresponding to 23 k (lane 3). Bands observed in lane 1 of Fig. 2b were due to the non-specific cross-reaction, because non-immune serum also recognized these bands (data not shown). The purified recombinant peptide was cross-linked with AF-Amino Toyopearl and used as bait to purify the antibody from the serum.

Western blot analysis and immunocytochemical analysis of cultured cells

Western blot analysis of whole-cell extract of cultured red sea bream cells indicated that purified anti-ColVa1 telopeptide antibody detected a single band at the distinct positions of type I collagen α and β chains (Fig. 3a). Figure 3b shows a micrograph of cultured red sea bream cells stained with anti-ColVa1 telopeptide antibody. Treatment with only second antibody as a negative control gave no signal (data not shown). These findings indicate that the translation product of the ColVa1 gene is present in the extracellular matrix of cultured red sea bream cells.

Western blot analysis of acid-soluble and pepsin-solubilized collagen from muscle

The antibody recognized two proteins in ASC (Fig. 4b), revealing that translation products of the ColVa1 gene are present in red sea bream muscle. The dense band showed slower mobility than that of type I collagen α chain and faster mobility than...
type I collagen β chain. The faint band showed slower mobility than the type I collagen β chain. The dense and faint bands corresponded to the α and β chains of type V/XI collagen, respectively, because a similar observation has been made with acid-soluble type V collagen of rainbow trout.21

Western blot analysis of PSC also demonstrated two bands, corresponding to the α and β chains of type V/XI collagen, as judged from their slower mobility than the corresponding type I collagen chains (Fig. 4b). These findings on ASC and PSC indicate that the translation product of the ColVa1 gene in red sea bream muscle detected in the present study corresponds to the so-called type V collagen that has been detected biochemically in various teleost tissues.5–9,21 The bands of PSC showed higher mobility than the corresponding bands of ASC. Judged from the marked difference in mobility between ASC and PSC, pepsin may act mainly on the N-terminal non-helical region (N-telopeptide) of type V/XI collagen α chain. In no case did the antibody detect either chain of type I collagen, as shown in Fig. 4b.

Detection of degradation product of type V/XI collagen α1 chain in NaOH extracts from muscle

Western blot analysis of NaOH extracts of muscle revealed a single band, corresponding to about 65 k (Fig. 4c). NaOH extracts were prepared from the muscle immediately after death, and it is reported that the NaOH extract contains non-collagenous protein,18 suggesting that the 65 k band corresponded to the degradation product of ColVa1. It is reported that human type V collagen α1 chain is digested by matrix metalloproteinase-9 (MMP-9, gelatinase B) at the carboxyl side of glycine597 in GPPG–VVGP-1001.22 A similar sequence, GPPG–VVGP-981, is located at the same position in the triple helix of ColVa1 (Fig. 1). The molecular weight of the ColVa1 peptide from valine978 to glycine1573, which is cleaved by C-telopeptidase, is estimated at approximately 55 k on the basis of the deduced amino acid sequence. Its actual molecular weight, however, is larger, due to hydroxylation of proline and lysine residues, and/or glycosylation of hydroxy lysine residues. In addition, the peptide containing the triple helical region of collagen migrates more slowly than would be expected from the predicted molecular weight as shown in Fig. 2. However, recombinant rainbow trout MMP-2 was reported to digest pepsin-treated human type V collagen and to generate fragments with electrophoretic mobilities similar to those produced by digestion with human MMP-9.23 It is unknown whether MMP-2 digests native type V/XI collagen. These facts suggest that the degradation product of the ColVa1 may be produced by digestion by MMP-2 or MMP-9. To confirm this possibility, we are currently attempting to determine its N-terminal amino acid sequence.

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REFERENCES


