Immunological detection of type V/XI collagen α1 gene in red seabream muscle

KEN TOUHATA,* YUKI TOKUDA, MORIHIKO SAKAGUCHI, AND HARUHIKO TOYOHARA
Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo, Kyoto 606-8502, Japan, * corresponding author: (touhata@kais.kyoto-u.ac.jp)

SUMMARY: We have already isolated a cDNA of type V/XI collagen α1 chain (ColVa1) from cultured cells derived from red seabream embryo. In order to detect the translation and degradation products of this cDNA in red seabream muscle, we raised an antibody against the deduced C-telopeptide of ColVa1. For improvement in specificity, we purified the antibody from rabbit antiserum by an affinity column cross-linked with the recombinant peptide of C-terminal of ColVa1 produced by E. coli. A positive band corresponding to a chain of type V/XI collagen was detected for the extract of cultured embryo cells in Western blot analysis. In addition, two positive bands, which correspond to α chain and β chain of type V/XI collagen, were recognized in acid soluble collagen fraction of red seabream muscle, indicating that the translation product of ColVa1 is present in muscle. A position of the positive bands reveals that the antibody recognizes specifically type V/XI collagen. A 65k band was detected for the NaOH extract fraction of muscle, suggesting that type V/XI collagen α1 chain was restrictedly cleaved in turnover process of muscle.

Key Words: type V/XI collagen, red seabream, Western blot analysis, collagen degradation

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 from their lower concentration in tissues. Since recent findings indicate that types V and XI subunits form hetero-trimers,1, 2 type V and type XI collagens are not regarded as distinct collagen types. In fishes, type V and XI, type V/XI-like, and type V collagens have been biochemically detected and isolated in lamprey,3 shark,4 and some teleosts5-7 respectively. We have recently isolated the cDNA of type V/XI collagen α1 chain (ColVa1) from cultured cells established from eyed-period eggs of red seabream, Pagrus major.8 While the deduced amino acid sequence of it resembles that of mammalian type XI collagen α1 chain, tissue distribution of ColVa1 mRNA resembles that of type V collagen based on reverse transcribed polymerase chain reaction (RT-PCR) analysis.

Muscle firmness, which is an important factor for determination of the quality, is considered to depend on collagen content.9-11 We previously reported that the seasonal change in collagen content is due to the change in a balance of collagen synthesis and breakdown.12 In addition, Sato et al. suggested that the cleavage of nonhelical regions and/or cross-links of type V collagen cause muscle softening.13 Thus, it is important to detect type V collagen and its degradation products in fish muscle. To detect the translation and degradation products of ColVa1 in red seabream muscle, we raised an antibody against the deduced C-telopeptide of ColVa1 and performed Western blot analysis.

MATERIALS AND METHODS

Materials

Cultured red seabream were obtained alive from a commercial supplier. All materials used in this study were of the highest quality available from commercial vendors.

Purification of antibody

A rabbit antiserum against the peptide of the deduced C-telopeptide of ColVa1 added by a cysteine residue at N-terminal (Cys-IQFLFFMQQSARKTRRQAEM-OG\(^{157}\)) was produced by Sawady Technology. For improvement in specificity, the antibody was purified by an affinity column cross-linked with the recombinant peptide of C-terminal of ColVa1 as described below. Briefly, we designed a set of oligonucleotide primers (5'-dGGGAATTCAGGAAGCTCAGGCTCAGGC) (5'-dGGGTGACGAGCGCTCAGCTCGC) based on DNA sequence of ColVa1 and performed PCR using these primers with KOD DNA
polymerase (Toyobo) according to modified manufacture’s protocol. After 30 cycles of PCR (30 sec at 96 C, 30 sec at 62 C, and 60 sec at 74 C), the product was separated by electrophoresis and subcloned into glutathione S-transferase (GST) gene fusion vector, pGEX-6P-1 (Amersham Pharmacia Biotech Inc.). E. coli (BL21) which was transformed with the fusion protein vector (pGEX-CVteC) were cultured in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Purification of GST fusion protein by using glutathione sepharose 4B (Amersham Pharmacia Biotech Inc.) and cleavage by PreScission Protease (Amersham Pharmacia Biotech Inc.) were performed according to manufacture’s protocol. The specific antibody was purified by AF-Amino Toyopearl (Toso) cross-linked with the recombinant peptide by m-maleimidobenzoyl-N-hydoxysuccinimide ester (Pierce).

Western blot analysis

Whole cell extracts from E. coli which were cultured in the presence of IPTG and purified recombinant peptide were separated by sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%) in the presence of 2-mercaptoethanol, and then electrophoretically transferred to a Clear Blot Membrane-p (Atto). Non specific staining was blocked by incubating with 5% skim milk in phosphate buffered saline (PBS). The membrane was then incubated with a 1/1000 dilution of the antiserum for 30 min at room temperature, followed by sequential 60 min incubation with alkaline phosphatase conjugated anti rabbit IgG antibody (New England Biolabs). Alkaline phosphatase activity was developed with 173 mM nitrobluetetrazorium 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.

The confluent culture of cells established from eyed-period eggs of red seabream was washed with PBS, detached with a scraper, pelleted by centrifugation at 3,000 rpm for 1 min, and then dissolved in SDS-PAGE sample buffer. The sample was separated by SDS-PAGE (7.5%) in the presence of 2-mercaptoethanol and transferred to a membrane as described above. The blocked membrane by skim milk was then incubated with a 5 µg/ml of purified anti-ColVa1 telopeptide antibody for 90 min at room temperature. Development of the blot for the secondary antibody was described above.

Acid soluble collagen (ASC) was prepared from red seabream muscle by the method of Sato et al. To detect the type V/XI collagen degradation products, muscle was extracted by 9 volume of 0.1N NaOH. ASC and the NaOH extract were separated by SDS-PAGE (7.5%) in the presence of 2-mercaptoethanol and Western blot analysis was performed as described above.

Immunocytochemistry

Red seabream cells were cultured on slide glasses. 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 for 10 min at room temperature by treatment with 0.3% H₂O₂ and 0.1% NaN₃ dissolved in distilled water. The glasses were reacted with the 15 µg/ml purified antibody for 60 min at room temperature. Development of the peroxidase activity was performed according to the method of Tokuda et al.

RESULTS AND DISCUSSION

Purification of anti-ColVa1 telopeptide antibody

The antiserum cross-reacted various non-specific signals on the acid soluble collagen derived from muscle in Western blot analysis (data not shown). Thus, we have purified the antibody from the serum by an affinity column coupled with the recombinant peptide. Fusion protein vector, pGEX-CVteC, was transfected into E. coli and the production of recombinant proteins was induced by the addition of IPTG. As shown in Fig. 1A, the whole-cell extract from the bacteria carrying the control mock plasmid, pGEX-6P-1, produced GST of molecular weight about 26k as a major band (lane 1). On the other hand, that carrying pGEX-CVteC produced the recombinant fusion protein of molecular weight about 47k as a major band (lane 2). SDS-PAGE analysis indicated a major band corresponding to the purified recombinant peptide of molecular weight about 23k (lane 3). This peptide was stained metachromatically with Coomassie Brilliant Blue R-250, which is known to be specific for collagen. Molecular weight of it assessed by SDS-PAGE analysis (23k) is larger than that calculated from deduced amino acid sequence (19k).

We performed Western blot analysis by using the antiserum. The antiserum recognized the recombinant fusion protein (Fig. 1B, lane 2) and recombinant peptide (lane 3). Because non-immune serum also recognized the bands observed in lane 1 of Fig. 1B.
(data not shown), they were due to the non-specific cross-reaction. This recombinant peptide was cross-linked with AF-Amino Toyopearl, and then used as bait to purify the antibody from the serum.

Western blot analysis on acid soluble collagen and NaOH extract from muscle

The antibody detected two bands in ASC (Fig. 3A), indicating that translation products of ColVa1 actually present in red seabream muscle. The dense band showed slower mobility than that of type I collagen α chain and faster mobility than that of type I collagen β chain. The faint band showed slower mobility than type I collagen β chain. Thus, the dense and the faint bands correspond to α chain and β chain of type V/XI collagen respectively, because similar observation is given in acid soluble type V collagen of rainbow trout.16 In the previous study, deduced amino acid sequence of ColVa1 showed high identity (68-75%) with those of mammalian α chains (V) and α chains (XI), and RT-PCR indicates that ColVa1 expressed in muscle and skin of red seabream. These facts reveal that translation products of ColVa1 in muscle detected in the present study corresponds to so called type V collagen biochemically detected in various fish tissues.3-7, 16 In Western blot analyses, the antibody detected neither chains of type I collagen as shown in Fig.3A.

A single band, corresponding to about 65k (Fig. 3B), was detected on Western blot analysis on NaOH extract of muscle. Woessner et al.17 reported that human type V collagen α chain is digested by matrix metalloproteinase-9 (MMP-9, gelatinase B) at
C terminal of glycine$^{997}$ in GPGG−VVGP$^{1001}$. Similar sequence, GPGG−VVGP$^{981}$, locates at the same position in triple helix of red seabream α1 (V/XI) chain. Molecular weight of ColVa1 peptide from valine$^{978}$ to glycine$^{1573}$ (C-terminal of deduced C-telopeptide) is estimated about 55k on the basis of deduced amino acid sequence. However, accurate molecular weight of it is slightly larger due to hydroxylation of proline and lysine residues. In addition, the peptide contained triple helix of collagen migrates slower than the expected molecular weight as shown in Fig. 1. On the other hand, Saito et al.$^{18}$ reported that recombinant rainbow trout MMP-2 digests pepsin-treated human type V collagen and to generate some fragments which had electrophoretic mobilities similar to those digested by human MMP-9. Although it is unknown whether MMP-2 digests native type V/XI collagen, together with these facts, this degradation product is maybe restrictedly digested by MMP-2 or MMP-9 in turnover process of red seabream muscle. To confirm this possibility, we are now trying to determine the N-terminal sequence of degradation product.

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REFERENCES