Isolation and purification of decorin from bovine skeletal muscle and its structural changes under high pressure

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Summary

High hydrostatic pressure induces a weakening of intramuscular connective tissue, which is mainly composed of collagen. Decorin, a small proteoglycan, binds to and stabilizes collagen fibrils. It has been suggested that the weakening of intramuscular connective tissue may result from alteration of the decorin-collagen interaction due to structural changes of the decorin molecule. In the present study, decorin was isolated and purified from bovine skeletal muscle by successive steps of an extraction with 4 M guanidine hydrochloride, CsCl density gradient ultracentrifugation, DEAE-cellulose ion-exchange chromatography, and Sepharose CL-6B gel filtration. The isolated decorin possessed an average molecular mass of 100 kDa and contained a core protein mass of 48 kDa by SDS-PAGE. The structural changes in decorin from bovine skeletal muscle were investigated by measuring fluorescence spectra under high pressure. Fluorescence spectra of purified decorin indicated that the tertiary structure of decorin was altered reversibly under high pressure up to 400 MPa.

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Key words : Decorin, Collagen, High pressure, Meat tenderization, Proteoglycan

INTRODUCTION

The use of high-pressure technology in food processing has steadily increased over the past 10 years. Among products processed using high pressure, the number and variety of meat and meat products have risen dramatically worldwide (Simonin *et al*., 2012). High pressure is also used for tenderizing pre- and post-rigor meat or accelerating postmortem aging of meat (Macfarlane, 1973; Bouton *et al*., 1980; Suzuki *et al*., 1990; Ledward, 1995; Messens *et al*., 1997). High hydrostatic pressure affects actomyosin toughness and background toughness, leading to meat tenderization. For actomyosin toughness, the progress of the loss of structural continuity due to the rupture of I-filaments, loss of M-line materials, and cleavage of A-filaments has been observed in the myofibrils with increasing pressure applied to the muscle both in pre- and post-rigor states (Suzuki *et al*., 1991). The effect of high pressure on background toughness ascribed to connective tissue is gradually becoming clearer. Ichinoseki *et al*. (2006) reported that high pressure did not degrade collagen molecules but dissociated collagen fibrils. It has been suggested that a weakening of intramuscular connective tissue may result from structural changes to decorin, leading to alteration of the decorin-collagen interaction by high pressure.

Decorin belongs to a growing family of small leucine-rich proteoglycans, and is distributed in extracellular matrix of various tissue (Pearson *et al*., 1983; Day *et al*., 1987; Nomura *et al*., 1998) including skeletal muscle (Parthasarathy and Tanzer, 1987; Eggen *et al*., 1994; Nishiumi *et al*., 1997; Nishimura *et al*., 2003). Decorin has a core protein consisting of eight to 10 leucine-rich repeats at its central domain, and contains a negatively charged glycosaminoglycan chain that covalently links to a serine residue of the core protein (Hedbom and Heinegård, 1993; Schönherr *et al*., 1995; Vesentini *et al*., 2005). Decorin has a typical molecular mass of 90-140 kDa, and consists of a core protein with a molecular mass of 40-50 kDa (Wight *et al*., 1991). The decorin core protein model is arch-shaped structure, and the concave surface can accommodate one triple helix of collagen type I. Decorin proteoglycan is proposed to bind in the 0.6-D (1 D = 67 nm) gap occurring between staggered collagen molecules and could account for the proposed binding sites and functional role of decorin in regulating collagen fibrillogenesis (Weber *et al*., 1996). Komoda *et al*. (2013) found a change of the native structure of decorin molecules from bovine articular cartilage under high pressure at 200-400 MPa.

It is assumed that structural changes of decorin molecule are induced by high-pressure processing. In the present study, decorin was isolated and purified from bovine skeletal muscle by successive steps of an extraction with 4 M guanidine hydrochloride, CsCl density gradient ultracentrifugation, DEAE-cellulose ion-exchange chromatography, and Sepharose CL-6B gel filtration. The structural changes of purified decorin were then analyzed by fluorescence spectra under high pressure.

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MATERIALS AND METHODS

Isolation and purification of decorin

Longissimus dorsi muscles were dissected from each carcass of two 3-month-old Holstein steers, trimmed to remove all visible external fat and epimysium, and then stored at -30℃. Decorin was extracted and purified according to the method of Nishiumi *et al*. (1997) with slight modifications. The muscles were minced finely, homogenized briefly in a Waring blender (Masuda, Japan) with 4 volumes of a solution containing 4 M guanidine hydrochloride, 0.1 M 6-aminohexanoic acid, 1 mM benzamidine hydrochloride, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride in 1 M sodium acetate buffer (pH 6.0), and extracted for 72 h at 4℃ with gentle stirring. The supernatant was collected by centrifugation at 24,900 x g for 1 h at 4℃. To separate decorin from other proteins of the muscle, direct dissociative CsCl density gradient ultracentrifugation was employed. The density of the guanidine hydrochloride extract was adjusted with CsCl to about 1.37 g/mL, and ultracentrifuged (CP-80WX; Hitachi, Japan) at 156,000 x g for 48 h at 4℃ in 40 PA tubes (Hitachi) on an angle rotor (P50-AT2; Hitachi). After ultracentrifugation, the tubes were divided into four fractions (D1-D4; D1=bottom) according to the CsCl density. The D2 fraction (density=1.43 g/mL) was subjected to further purification. The fraction was extensively dialyzed against 20 mM tris-acetate buffer (pH 7.0) containing 7 M urea and applied to a DEAE-cellulose column (2.5 x 25 cm; Sigma, U. S.A.) equilibrated with the same buffer at 4℃ . After washing the column with the same buffer, decorin was eluted with a linear gradient of 0-0.5 M NaCl in 7 M urea and 20 mM tris-acetate buffer (pH 7.0) at a rate of 15 mL/h (one fraction, 10 mL). The content of uronic acid in each fraction was determined (Bitter and Muir, 1962), and fractions containing decorin were pooled. Further purification was performed by Sepharose CL-6B gel filtration. The pooled fraction containing decorin was concentrated to about 10 mL by ultrafiltration, and then applied to a Sepharose CL-6B column (1.5 x 120 cm; Sigma) equilibrated with 420 mL of 50 mM tris-acetate buffer (pH 7.0) containing 4 M guanidine hydrochloride. Decorin was eluted with the same buffer at a rate of 10 mL/h (one fraction, 5 mL) at 4℃. The content of uronic acid in each fraction was measured and subjected to SDS-PAGE analysis.

SDS-PAGE analysis

SDS-PAGE was carried out on 7.5% polyacrylamide slab gels with a 3.75% stacking gel, according to the method of Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250 or silver-staining for proteins or Alcian blue for glycosaminoglycans (Fisher *et al*.,1983).

Measuring fluorescence spectra

Fluorescence spectra were obtained using a spectrometer (F-2500; Hitachi) fitted with a high-pressure vessel (PCI-400; Syn Corporation, Japan) and pump (TP-500; Syn Corporation). Purified decorin (1.4 mg/mL) in 50 mM tris-acetate buffer (pH 7.0) was subjected to a range of pressures (0.1-400 MPa), and fluorescence spectra were measured after pressure was achieved. After 400 MPa compression, the decorin solution was decompressed continually. Fluorescence spectra of decorin were measured during pressurization and depressurization with excitation at 280 nm. Changes in the center of the spectral mass (ν) were calculated in accordance with the method of Ruan *et al*. (1998):

$$\nu = \Sigma \nu_i \mathrm{xFi} \ / \ \Sigma \mathrm{Fi}$$

where ν_i is the wavenumber and Fi is the fluorescence intensity at ν_i.

RESULTS AND DISCUSSION

Isolation and purification of decorin

The density and uronic acid content of each fraction after CsCl density gradient ultracentrifugation were shown in Table 1. As the target, decorin was contained in the D2 fraction, in which a small size proteoglycan (about 100 kDa) was shown by SDS-PAGE (Fig. 1).

Table 1. Distribution of glycosaminoglycan chains uronic acid on CsCl density gradient ultracentrifugation of 4 M guanidine hydrochloride extract from bovine skeletal muscle.

fraction	density (g/mL)	GAG uronic acid (mg/100g meat)
D1(bottom)	1.51	28.9
D2	1.43	3.0
D3	1.37	2.2
D4	1.33	2.6

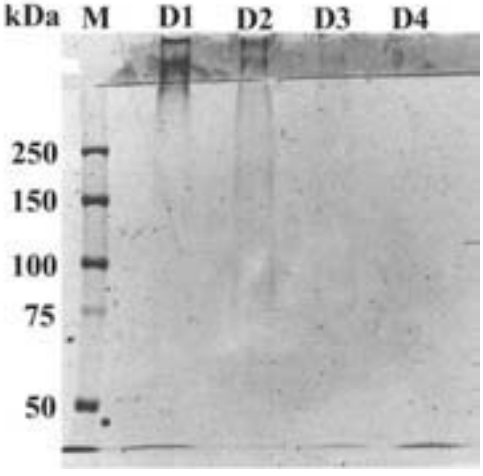

Fig 1. SDS-PAGE of D1 to D4 fractions after CsCl density gradient ultracentrifugation.
Decorin was contained in the D2 fraction, in which a small size proteoglycan (about 100 kDa). The gel was stained with Alcian blue. M, molecular weight marker.

The D2 fraction was then applied to DEAE-cellulose ion-exchange chromatography for further purification. Uronic acid-containing materials were eluted as four peaks at fraction numbers (NaCl concentrations) of 17 (0.17 M), 19 (0.20 M) 25 (0.26 M), 32 (0.33 M), respectively (Fig. 2). Among them, fractions 30-40 at 0.30-0.41 M NaCl contained decorin with a molecular mass of 100 kDa by SDS-PAGE (Fig. 3), and were subjected to Sepharose CL-6B gel filtration chromatography. A large proportion of the uronic-acid containing materials were recovered in fractions 22-30, shown as the decorin fractions in Fig. 4.

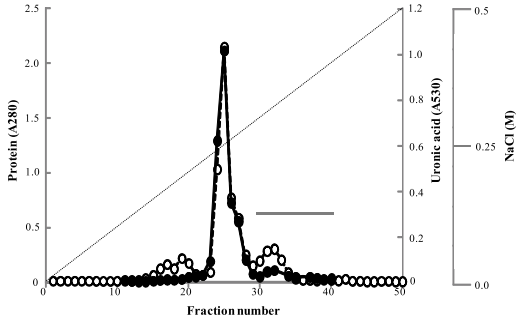

Fig 2. DEAE-cellulose ion-exchange chromatograph of decorin from bovine skeletal muscle.
Proteoglycans were eluted with liner gradient of 0-0.5 M NaCl, shown by dotted line. The absorbance at 280 nm (○) and the uronic acid content of each fraction (●) were determined. Fractions 30-40, indicated by the bar, were combined for further purification.

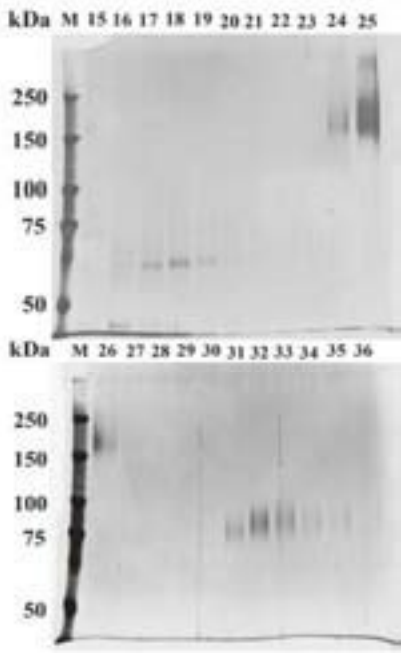

Fig 3. SDS-PAGE after application of DEAE-cellulose ion-exchange chromatography.
The gels were stained with silver-staining. M, molecular weight marker; 15-36, fraction number.

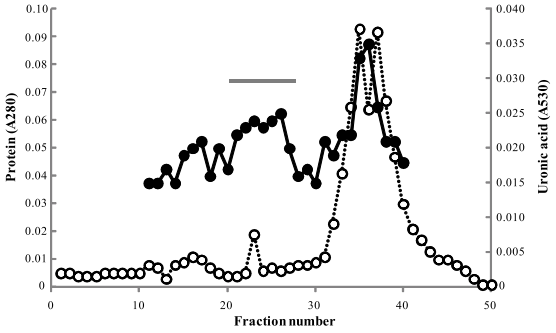

Fig 4. Sepharose CL-6B gel filtration chromatograph of decorin from bovine skeletal muscle.
The absorbance at 280 nm (○) and the uronic acid content of each fraction (●) were determined. Decorin was contained in fractions 22-30, indicated by the bar.

SDS-PAGE of purchased decorin from bovine cartilage and isolated decorin from bovine skeletal muscle revealed one band with an average mass of 100 kDa (Fig. 5 lanes A and B), which was stained with Alcian blue. After chondroitinase ABC treatment, a molecular mass of 48 kDa was confirmed, which is the core protein derived from decorin (data not shown). These results suggested that decorin was purified from bovine skeletal muscle. However, further experiments such as electrophoretic separation of the GAG chains and N-terminal amino acid sequencing of the core protein were required.

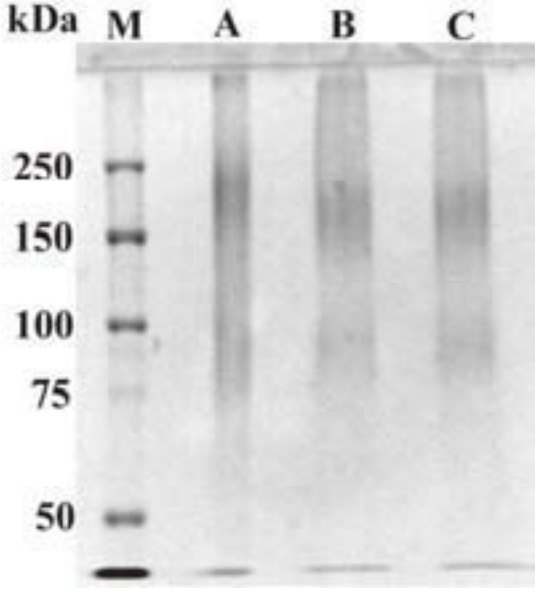

Fig 5. SDS-PAGE of purchased decorin from bovine cartilage (A), purified decorin from bovine skeletal muscle (B) and purified decorin from bovine skeletal muscle after high pressure up to 400 MPa (C). The gel was stained with Alcian blue.

Fluorescence spectra

Changes in fluorescence spectra of purified decorin under high pressure were shown in Figs. 6 and 7. Fluorescence spectroscopy was used to analyze the environment of the peripheral tyrosine and tryptophan residues in native and denatured decorin (Krishnan *et al*., 1999). The fluorescence intensity of the decorin gradually decreased and the peak emission wavelength shifted from 337 nm to 342 nm with increasing pressure level (Fig. 6). A reduction of intensity indicated that the environment of the peripheral tyrosine and tryptophan residues were more polar due to pressure-induced changes in the tertiary structure of decorin. The results of shifting peak emission wavelength suggested that, in native decorin, the tyrosine and tryptophan residues were partially buried, and, in denatured decorin, were exposed to a polar environment. Additionally, fluorescence intensity and peak emission wavelength were reversed nearly to native structure of decorin with decreasing pressure level (Fig. 7).

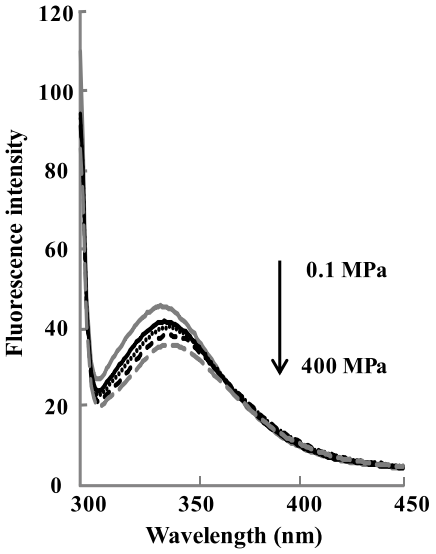

Fig 6. Changes in fluorescence spectra of decorin under high pressure between 0.1 and 400 MPa (compression).
The arrow indicated that the fluorescence intensity was decreased with increasing pressure level from 0.1 to 400 MPa. The peak emission wavelength was shifted from 337 nm to 342 nm with increasing pressure level (red shift). 0.1 MPa, (——); 100 MPa, (——); 200 MPa, (·····); 300 MPa, (– – –); and 400 MPa, (— —).

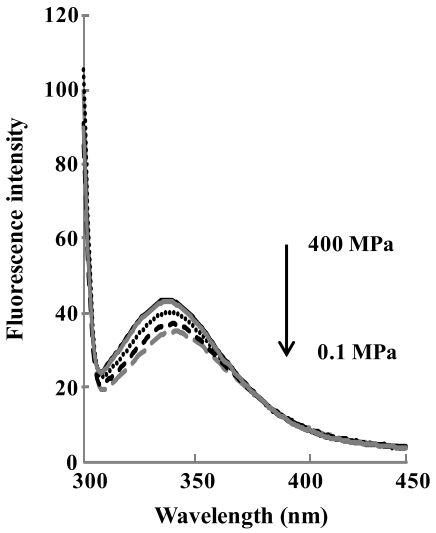

Fig 7. Changes in fluorescence spectra of decorin under high pressure between 400 and 0.1 MPa (decompression).
The arrow indicated that the fluorescence intensity was increased with decreasing pressure level from 400 to 0.1 MPa. The peak emission wavelength was shifted from 342 nm to 338 nm with decreasing pressure level (blue shift). 0.1 MPa, (——); 100 MPa, (——); 200 MPa, (·····); 300 MPa, (– – –); and 400 MPa, (— —).

Changes in the environment of the peripheral tyrosine and tryptophan residues leads to changes in the center of spectral mass [(ν) value], and making it possible to follow denaturing processes of protein caused by various treatments including high pressure. Change in the (ν) value of decorin was shown in Fig. 8. The (ν) value decreased gradually with increasing pressure during compression. Furthermore, the (ν) value after decompression returned nearly to the initial value. The (ν) values indicated that the structural changes in decorin induced by high pressure at 400 MPa were reversible.

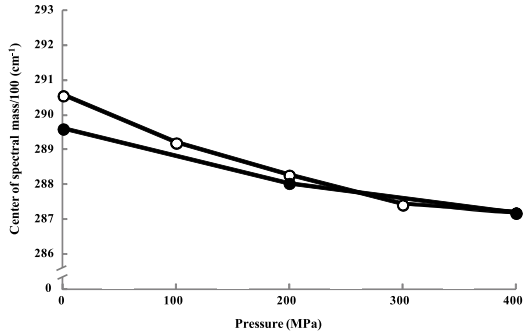

Fig 8. Changes in the center of spectral mass of decorin as a function of pressure.
compression (○); decompression (●).

Decorin after measuring of fluorescence spectra was subjected to SDS-PAGE analysis (Fig. 5 lane C). SDS-PAGE revealed that decorin from bovine skeletal muscle was not degraded under high pressure up to 400 MPa.

Therefore, the present results indicate that decorin from bovine skeletal muscle is not degraded, however; the tertiary structure of decorin is altered reversibly under high pressure up to 400 MPa.

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ウシ骨格筋由来デコリンの単離・精製ならびに高圧下における構造変化

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要　約

高圧処理は主にコラーゲンから構成される筋肉内結合組織の脆弱化を引き起こす。デコリンは小型プロテオグリカンの一つであり、アーチ型構造のコアタンパク質と1本のグリコサミノグリカン鎖を持つ。デコリンの役割の一つとしてコラーゲン細線維に結合し、その構造を束ねるようにして安定化させることが知られている。当研究室では、結合組織の脆弱化は高圧処理がデコリン分子の構造に何らかの影響を及ぼし、デコリンとコラーゲンの相互作用が変化することで生じると推測している。本研究ではまず、ウシ肩ロース肉から4 M グアニジン塩酸溶液によりプロテオグリカンを抽出した後、塩化セシウム密度勾配遠心分離によりプロテオグリカンを単離した。その後、DEAE- セルロースイオン交換クロマトグラフィーと Sepharose CL-6B ゲルろ過クロマトグラフィーにより精製した。デコリンは通常、約48 kDa のコアタンパク質を持ち、グリコサミノグリカン鎖と合わせると全体の分子質量は約100 kDa になる。 SDS-PAGE の結果からこれらの特徴を有していたため、単離・精製の過程を経て得られたものをウシ骨格筋由来精製デコリン試料とし、高圧下での蛍光スペクトル分析に用いた。精製デコリンの高圧下での蛍光スペクトルは400 MPa までの圧力で可逆的な三次構造の変化を引き起こすことを示した。

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キーワード：デコリン、コラーゲン、高圧、食肉軟化、プロテオグリカン

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