The Isolation and Primary Structure of a 22-kDa Extracellular Matrix Protein from Bovine Skin*

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The primary structure of a 22-kDa protein which was isolated during the purification of bovine skin dermatan sulfate proteoglycan is described. The uronate-rich fraction from DEAE-Sepharose chromatography of a 7.8 M urea extract of bovine fetal skin was subjected to gel filtration on Sepharose CL-6B in 4 M guanidine HCl. A prominent component of mass 22 kDa was separated from the proteoglycan and further purified on octyl-Sepharose. The primary structure of this component was determined and found to contain three repeat regions. Each of the three sections contains a similar pattern of loops disulfide bonds. A six-amino acid consensus sequence, Asp-Arg-Glx-Trp-Asn/Gln/Lys-Phe/Tyr, is found in each loop. This domain may be involved in associations of the molecule with other extracellular matrix components.

Connective tissue consists of a variety of high molecular weight structures: collagens, proteoglycans, fibronectin, laminin, elastin, etc. The protein sequences of some of these molecules have been described. Some repeating structures are fundamental building blocks which interact with other extracellular matrix components (for example: the hyaluronic acid binding region of aggregating proteoglycans (1) or the type IV collagen globular domains (2)). Other repeating sequences are specific for the macromolecules in question (for example, the segments which contain Ser-Gly sequences in proteoglycans (3) or the repeating Gly-X-Y triplets of collagen helical domains (4)).

In addition to these large molecules, there are smaller structural macromolecules which can be found in the extracellular matrix. These are typified by link protein, which has a clearly defined function in the stabilization of cartilage proteoglycan aggregates (5), and chondrocalcin (derived from type II collagen (6)) which has been implicated in the calcification of cartilaginous tissues. However, SDS-PAGE shows that there are many other components of connective tissue of less than 50 kDa. Most of these have not been characterized in detail. On a molar basis, these molecules are present in significant concentrations. It is possible that these constituents play a substantial part in the structural arrangement and stabilization of the matrix, as well as in the process of intercellular signalling.

In the course of isolation of dermatan sulfate proteoglycans (DS-PG) from bovine skin, a small (22-kDa) protein was observed to co-purify with the proteoglycan (7). In experiments designed to assess the effect of DS-PGs on cellular adhesion, it was observed that this protein impedes the inhibitory effects of DS-PG on cellular adhesion to fibronectin substrata. This publication describes the isolation and primary structure of this new matrix protein. The structure appears to be unrelated to other known protein sequences and contains a short triple repeat sequence.

MATERIALS, METHODS, AND RESULTS

Dermatan sulfate proteoglycans are isolated from fetal skin by urea extraction of the tissue followed by DEAE ion exchange chromatography (7). When the uronate-rich fraction eluted from the ion exchange column (Fig. M1) was chromatographed on Sepharose CL-6B under dissociative conditions (4 M guanidine HCl), a low molecular weight component was consistently observed (Fig. M2). This component of skin could be further purified by adsorption to octyl-Sepharose and elution by a gradient of guanidine HCl (Fig. M3). SDS-PAGE analysis of this protein resulted in a single band of mass 22 kDa. On reversed-phase HPLC, using the conditions described in the miniprint section, the preparation gave a single predominant peak and some minor peaks which represented <5% of the total UV absorbance.

There are no hexosamines in the amino acid composition of the 22-kDa protein, indicating that this is not a glycoprotein. It is remarkably rich in tyrosine (Table I). There is a low level of lysine and histidine and a moderately high level of cysteine (analyzed as S-carboxymethylcysteine after reduction and carboxymethylation). Reaction of the 22-kDa protein with iodoacetic acid under dissociative conditions, followed by amino acid analysis, indicated that there are no free cysteine residues as revealed by an absence of S-carboxymethylcysteine.

RESULTS AND DISCUSSION

Dermatan sulfate proteoglycans are isolated from fetal skin by urea extraction of the tissue followed by DEAE ion exchange chromatography (7). When the uronate-rich fraction eluted from the ion exchange column (Fig. M1) was chromatographed on Sepharose CL-6B under dissociative conditions (4 M guanidine HCl), a low molecular weight component was consistently observed (Fig. M2). This component of skin could be further purified by adsorption to octyl-Sepharose and elution by a gradient of guanidine HCl (Fig. M3). SDS-PAGE analysis of this protein resulted in a single band of mass 22 kDa. On reversed-phase HPLC, using the conditions described in the miniprint section, the preparation gave a single predominant peak and some minor peaks which represented <5% of the total UV absorbance.

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No NH₂-terminal sequence could be determined for the intact protein. Treatment of the reduced and carboxymethylated protein with pyroglutamate aminopeptidase followed by NH₂-terminal sequence analysis resulted in the characterization of a 20-residue, tyrosine-rich region at the NH₂ terminus. The NH₂-terminal sequence could be followed for 41 residues (Table M1, Miniprint). The derivation of the sequence shown in Fig. 1 is further discussed in the Miniprint. The amino acid composition of the protein agrees with that based on an average from two time courses with hydrolysis times derived from the sequence (Table I). The found molecular weight (21,990) agrees well with that found by SDS-PAGE.

Disulfide bond positions were derived after digestion of the native protein. Initially, an unsuccessful effort was made to tryptically digest the native molecule into separate, disulfide-bonded domains. A single, large fragment was obtained which had multiple NH₂-terminal groups corresponding to peptides from both the NH₂-terminal and COOH-terminal ends of the intact molecule. No specific disulfide bonded fragments were obtained. Digestion of the native molecule with chymotrypsin and separation of the products by gel filtration and reversed-phase HPLC (Fig. M9) followed by analysis of the resulting peptides by NH₂-terminal sequencing enabled us to find a set of fragments which had more than one NH₂-terminal group (see Miniprint). One disulfide bond was clearly between Cys and Cys, and another was clearly between Cys and Cys. Two disulfide bonds were assigned to Cys, Cys, Cys, and Cys. As two of these cysteine residues were adjacent to each other, it was not possible to unequivocally determine whether the disulfide bonds were Cys-Cys and Cys-Cys or whether they were Cys-Cys and Cys-Cys. When sequencing through these disulfide bonds, an additional PTH peak corresponding to cysteine was observed, indicating that the disulfide bonds were in the latter positions (see Miniprint). While a disulfide bond between Cys and Cys was not detected directly, as there were no free cysteines in the protein, it was assigned by default.

The five disulfide bonds define three loops (Fig. 1) and explain the fact that tryptic digestion of the intact protein only releases a few fragments. The short, basic segment immediately prior to the last two disulfide bonds is apparently protected from proteolytic cleavage by the close juxtaposition of disulfide bonds. If this region is sterically prevented from being cleaved by trypsin, the found disulfide bond pattern would hold all the cysteine-containing fragments together.

The 22-kDa protein does not show any significant homology with any published data in the Protein Identification Resource sequence database (issue 15) and neither does a back-translated DNA sequence show any significant homology with any data in the GenBank DNA sequence library. The sequence of the 22-kDa protein is composed of four domains: 1)
a domain consisting of the tyrosine-rich NH2-terminal 16 residues; 2 and 3) two domains which display considerable similarity to each other; and 4) a domain consisting of the remainder of the molecule which possesses slight similarity to domains 2 and 3. Domains 2, 3, and 4 all include a 6-residue repeating segment which has a consensus sequence of Asp-Arg-Glx-Trp-Asn/Gln/Lys-Phe/Tyr. These similarities are shown in Fig. 2.

Analysis for regions of hydrophobicity by the method of Kyte and Doolittle (13) (Fig. 3) indicates that the protein is generally very hydrophilic, apart from regions of strong hydrophobicity around residue 40 and around residue 90. Strongly hydrophilic regions are evident around residue 50 and around residue 162.

A comparison of the probabilities for \( \alpha \)-helix and \( \beta \)-sheet, derived by the method of Chou and Fasman (14) (Fig. 3), indicates that there are no overall strong tendencies toward either secondary structure. We have not obtained CD spectra for the protein, so we are unable to confirm these indications.

Preliminary indications are that this protein may be involved in binding to DS-PGs. It displays a striking tendency to co-purify with DS-PG except under dissociative conditions. Its exact function is not clear, but it may well be involved in the modulation of the DS-PG-mediated inhibition of cellular attachment to fibronectin (10). This might be achieved by its binding to DS-PG, cell membranes via a specific receptor, or both. It remains to be determined as to whether the short repeating domain represents another connective tissue subdomain such as the Arg-Gly-Asp-Ser structures that are found in collagen, fibronectin, and laminin.

Acknowledgment—The excellent technical assistance of Carmen Nathalia Young is very much appreciated.

REFERENCES
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Supplementary Material


Materials

Quantitative HCl was either from Research Plus Laboratories or Sigma grade D, C4H8O2N2. gramine was from Gallenkamp-Behring and Hyamine hydroxide was from Alfa, Acheson, selenium was from Merck, and acetic acid was from B.D.H. 42.55 U.S. Protein 22-kDa Protein was from Pharmacia. Endoproteinase Lys-C, Arg-C, and Glu-C (VIII proteases) were from Boehringer Mannheim. 2 lg/mL chymotrypsin, TPCK-treated trypsin, and pyroglaucine amiopeptidase were from Sigma. Lysozyme was from BMB.

Methods

Amino acid analyses were performed by post-column derivatization of peptides and peptide hydrolysates using PTC (4). The instrument was an Applied Biosystems 420 A derivatization unit with on-line analysis of derivatized products on a 130 A micromeritics HPLC. The program used for sequencing on the Applied Biosystems 431 A gas- phase sequencer was Normal-1, as described in the manufacturer's literature, action described elsewhere. PTH amino acid analysis was on an Applied Biosystems 202A on-line HPLC using a 2.3 x 1.6 mm diam. column as supplied by the manufacturer.

Isolation of the 22 kDa protein

A 0.1 M MgO-containing fraction was isolated from bovine skin essentially as described for cartilage by Rosenberg et al. (9). Bovine skin from- stead and early stage connective tissue from bovine (175 g) was sliced and extracted in 0.1 M NaCl, 0.05 M EDTA, 10 mM Tris, pH 6.5, containing 5 mM beta-mercaptoethanol, PMSF and leupeptin (0.27 mM). The extract was filtered through cheesecloth and batch-adsorbed with fast-flow CM-Sepharose to remove collagen. The CM-Sepharose with adsorbed collagen was recovered by centrifugation at 5 C.

The supernatant was subject to fast-flow DEAE-Sepharose on a column (1.5 x 30 cm) equilibrated in 0.5 M NaCl, 0.1 M Tris, 0.05 M EDTA, 10 mM Tris, pH 6.5 in a flow rate of 86 ml/h. After the A280 of the eluate was reduced in less than 0.2 (approximately 1 liter later), the DEAE-Pogs, together with the 22 kDa protein, were eluted with a linear gradient of 0.1 to 1 M NaCl (total volume, 4 liters). 2 ml fractions were collected and monitored for A280 and for n-butyryl phenylalanine for reaction against factor VII (Fig. 1). The absorbance-rich fraction from figure M1 (primarily DE-PIG) was concentrated on an Amicon YM-10 filter and further purified against 500 ml of 0.1 M NaCl, 0.13 M sodium acetate, pH 6.5. The concentrated and dried material was suspended by 10 mi dialysis on a column (35 x 2.5 cm) of Sephrose CL-4B equilibrated for the 4 M sodium HCI-containing buffer at a flow rate of 10 ml/hr. Fractions were monitored for A280 and vitamin K, as well as by SDS-PAGE (4-20% acrylamide gradient gel). This is shown in figure M2.

The material eluting from the Sephrose CL-4B column with a Kav of 0.81 was concentrated to 30 ml and the concentration adjusted to bring the glycine content to 3 M. The material was then applied to a column of oxy-Sepharose (2.7 x 25 cm), equilibrated in 3 M guanidine HCl, 0.13 M sodium acetate, pH 6.5 at 25°C. The protein was allowed to bind to the column for two hours and then eluted with 3 column volumes of buffer before a gradient of 0-1 M NaCl was applied. Fractions (900 ml) were collected as a flow rate of 63 ml/hr and monitored for A280 and by SDS-PAGE (4-20% acrylamide gradient gel). This is shown in figure M3.

Preparation of peptide fragments

Cysteine bromide cleavage was performed in 70% formic acid. The concentration of cysteine bromide was 5 mg/ml. Cleavage was under nitrogen in the dark and was performed for 1 hour (85). Protein-synthetic enzymes (Endoproteinase Lys-C, Arg-C, and Glu-C) Cysteine bromide cleavage was performed as described in the manufacturer's literature. Digestions were for 32 hours in 37°C. Substrate concentration was 8.5 mg/ml

Reversed Phase HPLC separation of peptides

The HPLC system was a Perkin Elmer series 410 with an L C 335 dye-array detector. Samples were dissolved in 0.1 M ammonium acetate, pH 7 and applied in a total volume of 300 and 200 ml. A Vydac 218 reversed-phase column (4.6 x 250 mm) was used, equilibrated in water (0.05%TFA) at a flow rate of 1 ml/min. Elution was achieved with a 0-50% gradient of acetonitrile (0.1% TFA) for 45 min at a flow rate of 1 ml/min (Fig. M4). Spectral information enabled us to identify peptide which contained try and tye tye.

Peptide Nomenclature

Peptides are referred to by the first three letters of their sequence. A letter preceding this refers to the digestion procedure followed C- c. chymotrypsin, L- Lys-C; E, endoproteinase glo-C (as used by Boehringer Mannheim).

Peptide alignment

Dimerization of the reduced and carbamoylated peptides with endoproteinase Lys-C and subsequent separation of the products by RP-HPLC was perfomed by BF. Alignment of the sequence of the reduced and carbamoylated peptides with cyanogen bromide also produced a limited number of detectable peptides (figure M5). Sequence analysis of the crude product from the digestion indicated that peptides with N-terminals of K, P, Pro, CMC, and Arg could be expected. After removal of the CNBr, the products could be divided into 4 M guanidine HCl-stable and 4 M guanidine HCl-susceptible groups of peptides. The major peptides in the 4 M guanidine HCl-stable group were presented to be the N-terminals of the intact protein. Further digestion of this peptide with chymotrypsin, endoproteinase Arg-C or V8 protease revealed some details of its sequence and provided overlap among the N-terminal sequences determined digestion (figure endoproteinase M6, miniprint).

The test of the sequence (figure 1 and table M1) was determined by aligning peptide sequence onto the CNBr and endoproteinase Lys-C products after digestion of the reduced and carbamoylated peptides with cyanogen bromide (not shown). V8 protease (figure M7) or endoproteinase Arg-C (Figure M8). The CNBr-stable sequence was not determined directly, but was inferred to be Val by the existence of peptides ending in Glu-Phe-Ala-Asn-Val in all cleavage methods.

Peptides defining disulfide bonds

The 22 kDa protein (0.1 mg) was digested with TLCK-capped chymotrypsin at a molar ratio of 1:50 enzyme:substrate. The digest was then separated into two fractions on a Sephadex G-200 column (1 x 210 mm) equilibrated with 4 M guanidine HCl, buffered with Tris (20 mM) pH 6.5. Fractions (1 ml) were analyzed on a gradient of acetonitrile/0.1% TFA (50-70% over 50 minutes). Peaks eluted from the column were analyzed for their N-terminal sequences (figure M9).

Of the peptides which were found, the following had two or more equimolar N-termini and therefore defined disulfide bonds:

1. SLYANGTPQGPOQQFGQYRPTV
   Cys10-Cys59
2. ALREIFYGQVQVAVR
   Cys37-Cys59
3. QTVQDSITYGWGLEDVNSGELMV
   Cys72-Cys115 and Cys88-Cys114
4. MTDSYKPYSANF
   Cys121-Cys178
5. MKTDSYKPYSANF
   Cys121-Cys178

In disulfide-linked peptides, the release of a cysteine residue was deemed as cycle 4. As the only disulfide bond that this could come from was between Cys 88 and Cys 114, the other disulfide bond must be Cys72-Cys115.

Figure M1. DEAE-Sephacel column chromatography of bovine fetal skin extract.

A 2.5 M extract of bovine fetal skin was applied to a 1000 ml DEAE- Sephacel (fast flow) column as described in the methods. The column was eluted with a salt gradient of 0-2.5 M NaCl (0-250 ml). Peptide was eluted at salt gradients of 0.3 M NaCl (dashed line). 2 ml fractions were collected and analyzed for A280 (closed circles) and vitamin K content (open circles).

Figure M2. Separation of the 22 kDa protein on a Sephacell CL-4B column.

The sequence in figure M1 were pooled, concentrated and applied to a Sephacell CL-4B column (100 ml) as described in the text. Fractions were collected and analyzed for A280 (closed circles), vitamin K content (open circles) and by SDS-PAGE (4-20% gradient) as indicated.
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Figure M3. Purification of the 22 kDa protein on an Oxyt.- Sepharose column.
The material with a molecular weight of 22 kDa shown in figure M2 was pooled, concentrated, and applied to an Oxyt.-Sepharose column (150 ml) at 23°C as described in the text. The sample was allowed to bind for two hours before the column was washed with three column volumes of 2 M guanidine HCl and then eluted with a gradient of guanidine HCl (0.4 M and a total volume of 500 ml). Fractions were monitored by A235 and SDS-PAGE.

Figure M4. Reversed-phase HPLC of reduced and carboxymethylated 22 kDa digested with endoproteinase Lys-C.

5 ml of reduced and carboxymethylated 22 kDa was digested with enzyme as described in the text. The digest was diluted in 4 M guanidine HCl, 50 mM Tris-HCl, pH 8.5 (5 ml) and 200 ml was applied to a Vydac C-18 column (0.46 x 25 cm) equilibrated in aqueous 0.1% TFA. The column was developed with a linear gradient of acetonitrile: 0.1% TFA (0-70% over 45 minutes, flow rate 1 ml/min) and the column eluate monitored at 220 nm. The N- and C-terminal sequences of the fragments were determined and are shown adjacent to the peaks.

Figure M5. Gel filtration analysis of reduced and carboxymethylated 22 kDa cleaved by cyanogen bromide.

10 ml of reduced and carboxymethylated 22 kDa was incubated with cyanogen bromide (7 mg/ml) in 1 ml of 70% formic acid. The digest was dried and was then dissolved in 4 M guanidine HCl, 50 mM Tris-HCl, pH 8.5 (5 ml) and 200 ml was applied to a Sephadex G-25 column (1 x 50 cm) equilibrated in the same buffer. The flow rate was 5 ml/min.

Figure M6. Reversed-phase HPLC of reduced and carboxymethylated 22 kDa digested with endoproteinase Arg-C.

5 ml of reduced and carboxymethylated 22 kDa was digested with enzyme as described in the text. The digest was diluted in 4 M guanidine HCl, 50 mM Tris-HCl, pH 8.5 (5 ml) and 200 ml was applied to a Vydac C-18 column (0.46 x 25 cm) equilibrated in aqueous 0.1% TFA. The column was developed with a linear gradient of acetonitrile: 0.1% TFA (0-70% over 45 minutes, flow rate 1 ml/min) and the column eluate monitored at 220 nm. The N- and C-terminal sequences of the fragments were determined and are shown adjacent to the peaks.

Figure M7. Reversed-phase HPLC of reduced and carboxymethylated 22 kDa digested with endoproteinase Glu-C.

5 ml of reduced and carboxymethylated 22 kDa was digested with enzyme as described in the text. The digest was diluted in 4 M guanidine HCl, 50 mM Tris-HCl, pH 8.5 (5 ml) and 300 ml was applied to a Vydac C-18 column (0.46 x 25 cm) equilibrated in aqueous 0.1% TFA. The column was developed with a linear gradient of acetonitrile: 0.1% TFA (0-70% over 45 minutes, flow rate 1 ml/min) and the column eluate monitored at 220 nm. The N- and C-terminal sequences of the fragments were determined and are shown adjacent to the peaks.

Figure M8. Reversed-phase HPLC of reduced and carboxymethylated 22 kDa digested with cyanotryptase.

10 ml of native 22 kDa was digested with enzyme as described in the text. The digest was diluted in 4 M guanidine HCl, 50 mM Tris-HCl, pH 8.5 (5 ml) and 200 ml was applied to a Vydac C-18 column (0.46 x 25 cm) equilibrated in aqueous 0.1% TFA. The column was developed with a linear gradient of acetonitrile: 0.1% TFA (0-70% over 45 minutes, flow rate 1 ml/min) and the column eluate monitored at 220 nm. The N- and C-terminal sequences of the fragments were determined and are shown adjacent to the peaks (right frame).
Table 1. Yields, in parts, of amino acids determined in peptide

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The table above shows the yields, in parts, of amino acids determined in peptide digestion, with CNBr peptides from digestion with CNBr, and K peptides from digestion with CNBr. The yields are expressed as percentages, E(1) and E(2), and the results are reported in the text.