Sulfated Glycopeptides, Containing Desmosine and Isodesmosine, Isolated from Porcine Aorta

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Aikawa, J., Munakata, H., Isemura, M. and Yosizawa, Z. Sulfated Glycopeptides, Containing Desmosine and Isodesmosine, Isolated from Porcine Aorta. Tohoku J. exp. Med., 1985, 145 (2), 175-183 —— Intima-media of porcine thoracic aorta was digested with pronase, after extraction of saline-soluble matters and fat. A crude sulfated glycopeptide fraction (CSGP) was precipitated with 90% (v/v) ethanol from the 80% ethanol-soluble fraction of the trichloroacetic acid (7%)-soluble fraction of the pronase digest. CSGP was fractionated by DEAE-Sephadex A-25 (Cl⁻ form) column chromatography. Of the resulting 9 fractions, 4 major fractions were further purified by gel filtration on Sephadex G-50, followed by affinity chromatography on concanavalin (Con) A-Sepharose 4B to a homogeneous state in electrophoresis on cellulose acetate membrane. All the purified fractions contained glucosamine, galactose, mannose, and sialic acid as the major sugars, and desmosine and isodesmosine as the unique constituents. The fractions with affinity for Con A (S1 and S2) contained much more mannose than those without affinity for this lectin (S3 and S4). The latter contained sulfate. The predominant amino acids in the former were glycine, aspartic acid (and/or asparagine), and serine, while those in the latter were glycine, proline, and alanine.

The presence of glycoproteins closely associated with the elastic structure of arterial tissues has been reported by many investigators (Barnes and Partridge 1968; Moczar and Robert 1970; Moczar et al. 1970; Robert et al. 1971; John and Thomas 1972; Saito and Yosizawa 1975; Saito et al. 1975; Gibson et al. 1982; Robert and Moczar 1982; Bressan et al. 1983; Moczar et al. 1983). The term “structural glycoprotein” has frequently been used to describe these glycoproteins (Robert and Moczar 1982). Structural glycoproteins (or glycopeptides) have not, hitherto, been known to contain desmosine and isodesmosine, which are the typical cross-linking components of elastin. On the other hand, some structural glycopeptides obtained from arterial tissues were reported to contain sulfate (Saito and Yosizawa 1975; Saito et al. 1975). Heifetz and Allen (1982) and Heifetz et
al. (1982) reported that sulfated glycoproteins are synthesized by vascular endothelial cells.

In the course of our studies on glycoconjugates in porcine thoracic aorta, we found a sulfated glycopeptide fraction containing desmosine and isodesmosine. In order to ascertain whether desmosine and isodesmosine are integral components of sulfated glycoprotein in arterial tissues, intima-media of porcine thoracic aorta was digested with pronase to separate sulfated glycoprotein from elastin components in the tissues. A sulfated glycopeptide fraction precipitated with 90% (v/v) ethanol from the 80% ethanol-soluble fraction contained desmosine and isodesmosine. This fraction was fractionated by ion-exchange column chromatography, and the resulting 4 major fractions were further purified to a homogeneous state. All the purified sialoglycopeptides contained desmosine and isodesmosine. Sulfate was also found in the two preparations.

This paper reports the isolation and characterization of sialoglycopeptides with and without sulfate, containing desmosine and isodesmosine, from intima-media of porcine thoracic aorta.

**MATERIALS AND METHODS**

**Materials.** Con A-Sepharose 4B, α-methyl D-glucoside and α-methyl D-mannoside were purchased from Sigma Chemical Co., St. Louis, U.S.A., Nakarai Chemicals Ltd., Kyoto and Seikagaku Kogyo Co., Tokyo, respectively. Desmosine and isodesmosine isolated from porcine ligamentum nuchae were generous gifts from Dr. Y. Nagai, Fukushima Medical College, Fukushima. Other materials were commercial products.

**Aortas.** Fresh normal thoracic aortas were obtained from 10 castrated pigs (male, 6 months old) immediately after sacrifice and stored at -20°C until use.

**Separation of a crude sulfated glycopeptide fraction (CSGP).** Thoracic aortas, stored at -20°C were thawed and freed from adventitia. Intima-media (63 g) was digested with pronase (total 1.3 g), after extraction of saline-soluble matters and fat, followed by treatment of the pronase digest with cold trichloroacetic acid (TCA) (7%) as described previously (Aikawa et al. 1984 a). Four volumes of ethanol containing 1% potassium acetate were added to the TCA-soluble fraction with stirring. The mixture was stood at 4°C overnight and then centrifuged in the cold at 8,000 rpm for 30 min. Ethanol containing 1% potassium acetate was added to the supernatant to give a final concentration of 90% (v/v). The precipitate was collected by centrifugation in the cold at 8,000 rpm for 30 min, and then dissolved in water (200 ml). The solution was dialyzed at 5°C against several changes of water (3 l each) for 1 week. The non-dialyzable fraction was lyophilized, yielding CSGP.

**DEAE-Sephadex A-25 (Cl- form) column chromatography.** An aqueous solution (337 mg in 30 ml) of CSGP was loaded on a column (3.3 x 30 cm) of DEAE-Sephadex A-25 (Cl- form). Stepwise elution was performed as described previously (Aikawa et al. 1984 b). The fractions indicated by bars in Fig.1 were separately combined and dialyzed at 5°C against several changes of water (3 l each) for 1 week. The non-dialyzable fractions were lyophilized, yielding DH2O, Dla, D1b, D2, D3, D4, D5, D6 and D7.

**Gel filtration on Sephadex G-50.** Of the above fractions, the major 4 fractions, Dla (14.4 mg), D1b (11.7 mg), D2 (12.1 mg) and D3 (12.5 mg), were separately dissolved in 0.5 ml of water. Each solution was loaded on a column (1.4 x 210 cm) of Sephadex G-50 pre-equilibrated with 0.1 M acetic acid-pyridine buffer (pH 5.0), followed by elution with the same buffer. Fractions of 4.1 ml were collected. The hexose content in each fraction was determined. The main peak-fractions were combined and dialyzed as described above.
The non-dialyzable fractions were lyophilized, yielding D1aS, DabS, D2S and D3S. Gel filtration was repeated once more by the same procedures.

**Affinity chromatography on Con A-Sepharose 4B.** The purified preparations of D1aS (1.6 mg), D1bS (1.2 mg), D2S (3.6 mg) and D3S (2.3 mg) were separately dissolved in 0.5 ml of 0.1 M acetate buffer (pH 6.0) containing 1 mM MgCl₂, 1 mM CaCl₂, and 1% n-butanol. Each solution was loaded on a column (1.0 x 10 cm) of Con A-Sepharose 4B pre-equilibrated with the same buffer. Elution was performed with 60 ml each of the above buffer containing 0.1 M NaCl (buffered saline) (BS) (a), 20 mM α-methyl D-glucoside in BS (b), 0.2 M α-methyl D-glucoside in BS (c) and 0.5 M α-methyl D-mannoside in BS (d), in succession. The fractions of 3 ml were collected and the absorbance at 230 nm of each fraction was determined. The major fractions indicated by bars in Fig. 2 were combined and dialyzed as above. The non-dialyzable fractions were lyophilized, yielding N1, S2, N3 and S4.

**Electrophoresis.** Electrophoresis on cellulose acetate membrane (Separax) was performed as described previously (Aikawa et al. 1984 b).

**Determination of constituents.** Total hexose, total hexosamine, and sialic acid were determined by the methods reported previously (Munakata and Yosizawa 1980). Neutral sugars, the molar ratio of glucosamine and galactosamine, amino acids, and sulfate were determined by the methods reported previously (Aikawa et al. 1984 b). Desmosine and isodesmosine were determined by the amino acid analysis, referring to the peaks of the authentic compounds examined under the same condition.

**Results**

Separation of a crude sulfated glycopeptide fraction (CSGP)

The wet tissues (222 g) of porcine thoracic aortas were defatted as described previously (Aikawa et al. 1984 a), yielding the defatted tissues (63.9 g). CSGP (0.37 g) was obtained from the defatted tissues (63 g) after digestion with pronase, followed by treatment with cold TCA and ethanol-fractionation, in succession, as described in MATERIALS AND METHODS.

**DEAE-Sephadex A-25 (Cl⁻ form) column chromatography of CSGP**

CSGP was fractionated by DEAE-Sephadex A-25 (Cl⁻ form) column chromatography as described in MATERIALS AND METHODS. The fractions indicated by bars in Fig. 1 yielded DH₂O (5 mg), D1a (25.3 mg), D1b (35.0 mg), D2 (37.8 mg), D3 (34.4 mg), D4 (13.4 mg), D5 (4.5 mg), D6 (2.0 mg) and D7 (2.2 mg).

**Gel filtration on Sephadex G-50**

D1a, D1b, D2 and D3 were separately gel filtered as described in MATERIALS AND METHODS, yielding D1aS (7.3 mg), D1bS (6.0 mg), D2S (4.5 mg) and D3S (8.4 mg), respectively. These fractions were purified again by the same procedures.

**Affinity chromatography on Con A-Sepharose 4B**

The purified D1aS, D1bS, D2S and D3S were separately subjected to affinity chromatography on a column of Con A-Sepharose 4B as described in MATERIALS AND METHODS. The major fractions of D1aS and D1bS were eluted.
with 20 mM α-methyl D-glucoside, while those of D2S and D3S were recovered from the breakthrough fractions. The major fractions indicated by bars in Fig. 2 were collected. The yields of S1, S2, S3 and S4 were 1.1, 0.8, 2.6 and 1.8 mg, respectively.

Electrophoresis of S1, S2, S3 and S4 on Separax

Electrophoresis was performed as described previously (Aikawa et al. 1984 b). S1, S2, S3 and S4 gave single band each at pH 3.0 and pH 8.6 (Fig. 3).

Characterization of S1, S2, S3 and S4

Aqueous solutions of S1, S2, S3 and S4 showed the UV-absorption at 273 nm and the fluorescence with excitation at 345 nm and emission at 415 nm. The chemical compositions of these fractions are shown in Tables 1 and 2. Glucosamine, galactose, mannose and sialic acid were the major sugars in all the fractions. Small amounts of fucose were found in S1 and S3. Small amounts of galactosamine and glucose were also found in S1 and S3, respectively. The most acidic fraction (S4) contained large proportions of sialic acid, sulfate and galactosamine as compared with other fractions. S3 contained a considerable amount of sulfate. The proportions of mannose in the fractions with Uffinity for
Con A (S1 and S2) were higher than those in the fractions without affinity for this lectin (S3 and S4). The latter contained much more amino acids than the former. S1 and S2 contained much more desmosine than isodesmosine. This relation was reversed in S3 and S4. The predominant amino acids in S1 and S2 were glycine.

Fig. 2. Affinity chromatography on Con A-Sepharose 4 B of D1aS(A), D1bS(B), D2S(C), and D3S(D). The purified D1aS (1.6 mg), D1bS (1.2 mg), D2S (3.6 mg) and D3S (2.3 mg) were subjected to affinity chromatography as described in the text. The major fractions indicated by bars were combined and treated as described in legend for Fig. 1. a, b, c and d, see text.

**TABLE 1. Chemical composition* of S1, S2, S3, and S4**

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<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
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<td>Fucose</td>
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<td>9.4 (0.07)†</td>
<td>ND‡</td>
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<td>Mannose</td>
<td>173.5 (0.76)†</td>
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<td>Galactose</td>
<td>168.9 (0.74)†</td>
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<td>47.0 (0.98)</td>
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<tr>
<td>Glucose</td>
<td>ND‡</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>Glucosamine</td>
<td>227.6 (1.00)</td>
<td>248.0 (1.00)</td>
<td>146.5 (1.00)</td>
<td>47.7 (1.00)</td>
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<tr>
<td>Galactosamine</td>
<td>15.9 (0.07)</td>
<td>ND</td>
<td>T§</td>
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<td>Sialic acid</td>
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<td>143.8 (0.58)</td>
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<tr>
<td>Sulfate</td>
<td>ND</td>
<td>ND</td>
<td>9.1 (0.12)</td>
<td>17.8 (0.70)</td>
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<td>Amino acids</td>
<td>199.0</td>
<td>123.5</td>
<td>421.3</td>
<td>653.3</td>
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* Expressed as µg/mg of the sample. † Molar ratio to glucosamine. ‡ Not detectable. § Trace. || Expressed as N-acetylneuraminic acid.
aspartic acid (and/or asparagine) and serine, while those in S3 and S4 were glycine, proline and alanine.

**DISCUSSION**

In order to ascertain whether desmosine and isodesmosine are integral components of sulfated glycoprotein in arterial tissues, intima-media of porcine thoracic aorta was digested with pronase. A crude sulfated glycopeptide fraction (CSGP) was precipitated with 90% (v/v) ethanol from the 80% ethanol-soluble fraction of the TCA (7%)-soluble fraction of the pronase digest. CSGP contained desmosine and isodesmosine, which are the typical crosslinking components of elastin, besides the constituents of sulfated glycopeptide. CSGP was then fractionated into 9 fractions by DEAE-Sephadex A-25 (Cl- form) column chromatography. Of these fractions, 4 major fractions were subjected to gel filtration on Sephadex G-50, followed by affinity chromatography on Con A-Sepharose 4B. The purified preparations were homogeneous in electrophoresis at pH 3.0 and pH 8.6. Analytical data indicated that all the purified preparations are sialoglycopeptides containing desmosine and isodesmosine. In addition, S3 and S4 contained sul-

<table>
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<th>Table 2. Amino acid composition* of S1, S2, S3, and S4</th>
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<td>Asx†</td>
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* Expressed as residues/100 amino acid residues. † Asp (and/or Asn). ‡ Not detectable. § Glu (and/or Gln). ‖ Isodesmosine. ¶ Desmosine.
fate. Since S1 and S2 contained large proportions of mannose and aspartic acid (and/or asparagine) and had affinity for Con A, it is conceivable that these glycopeptides may have N-glycosidic biantennary complex type carbohydrate chains.

The presence of structural glycoproteins (or glycopeptides) in arterial tissues has been reported by many investigators (Barnes and Partridge 1968; Moczar and Robert 1970; Moczar et al. 1970; Robert et al. 1971; John and Thomas 1972; Saito and Yosizawa 1975; Saito et al. 1975; Gibson et al. 1982; Robert and Moczar 1982; Bressan et al. 1983; Moczar et al. 1983). Of these preparations
some of the acidic glycopeptides were suggested to contain sulfate (Saito and Yosizawa 1975; Saito et al. 1975). Desmosine and isodesmosine were, however, not found in any of these preparations. Although Heifetz and Allen (1982) and Heifetz et al. (1982) reported that sulfated glycoproteins are synthesized by vascular endothelial cells, no detailed chemical composition of the synthesized sulfated glycoproteins was described in their papers. It is therefore, indicated, that all the present preparations are novel sialoglycopeptides containing desmosine and isodesmosine. Specifically, S3 and S4 are unique sulfated glycopeptides containing desmosine and isodesmosine.

Since desmosine and isodesmosine are indicated to be integral components of the present sialoglycopeptides with and without sulfate, it is conceivable that these types of structural glycoproteins may contribute to a certain extent to maintain elastic structure of intima-media of porcine thoracic aorta.

Acknowledgments

We thank Dr. Y. Nagai for supply of materials. We are also indebted to Dr. Y. Kuboki, School of Dentistry, Tokyo Medical and Dental University, Tokyo, for identification of desmosine and isodesmosine. We thank Professor K. Tada, Department of Pediatrics, for sending J.A. to work in Department of Biochemistry. This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References


