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

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Intima-media of porcine thoracic aorta were digested with pronase, after extraction of the saline-soluble matters and fat. A glycopeptide fraction was precipitated with 90% (vol/vol) ethanol from the 80% ethanol-soluble fraction of the trichloroacetic acid (7%)-soluble fraction of the pronase digest. The glycopeptide fraction was fractionated by affinity chromatography on concanavalin A (Con A)-Sepharose 4B, yielding 4 fractions (FA, FB, FC and FD). The most carbohydrate-rich fraction (FB) was further purified to a homogeneous state. The purified FB (FB-0.1) and all other fractions contained desmosine and isodesmosine. The major sugars in the fractions without or with low affinity for Con A (FA, FB, and FB-0.1) were glucosamine, galactose, mannose and sialic acid, while those in the fractions with high affinity for this lectin (FC and FD) were glucosamine, glucose and mannose. All the fractions contained glycine, aspartic acid (and/or asparagine), serine, proline, threonine, glutamic acid (and/or glutamine) and alanine as the major amino acids, amounting to approximately 80% of the total.

Glycoproteins (or glycopeptides) present in close association with the elastic structure of arterial tissues have been studied by many investigators [1-6]. The term "structural glycoprotein" has frequently been used to describe these glycoproteins [1]. Structural glycoproteins have not, hitherto, been known to contain desmosine and isodesmosine, which are the typical cross-linking components of elastin.

In the course of our studies on glycoconjugates in porcine thoracic aorta, we found glycopeptide fractions containing desmosine and isodesmosine. In order to ascertain whether desmosine and isodesmosine are integral components of structural glycoprotein in arterial tissues, intima-media of porcine thoracic aorta were digested with pronase to obtain glycopeptides with small peptide chains. A glycopeptide fraction precipitated with 90% (v/v) ethanol from the 80% ethanol-soluble fraction contained desmo-

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sine and isodesmosine. Even after the fractionation and purification of this fraction, the resulting glycopeptide fractions still contained these components.

This communication reports the isolation and characterization of glycopeptides, containing desmosine and isodesmosine, from intima-media of porcine thoracic aorta.

Materials and Methods

Materials

Fresh normal thoracic aortas were obtained from 2 castrated pigs (6 months old) immediately after sacrifice and stored at -20°C until use. Concanavalin A (Con A)-Sepharose 4B, α-methyl-D-glucoside and α-methyl-D-mannoside were purchased from Sigma Chemical Co., U.S.A., Nakarai Chemicals Ltd., Japan and Seikagaku Kogyo Co., Japan, respectively. Desmosine and isodesmosine isolated from porcine ligamentum nuchae were generous gifts from Dr. Y. Nagai, Japan. Other materials were commercial products.

Separation of GP-90EP

Thoracic aorta, stored at -20°C, was thawed and freed from adventitia. Intima-media thus obtained were digested with pronase, after extraction of saline-soluble matters and fat, followed by treatment of the pronase digest with cold trichloroacetic acid (TCA) (7%) as described previously [7]. Four volumes of ethanol containing 1% potassium acetate were then added to the TCA-soluble fraction with stirring.

The mixture was allowed to stand at 4°C overnight and then centrifuged in the cold at 8 000 rpm for 30 min. Ethanol containing 1% potassium acetate was added with stirring 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 (50 ml). The solution was dialyzed at 5°C against several changes of water (2 l each) for 1 week. The non-dialyzable fraction was lyophilized, yielding GP-90EP (glycopeptide fraction precipitated with 90% ethanol).

Affinity Chromatography of GP-90EP on Con A-Sepharose 4B

GP-90EP (11.5 mg) was dissolved in 0.5 ml of 0.1 M acetate buffer (pH 6.0) containing 1 mM $MgCl_2$, 1mM $CaCl_2$ and 1% n-butanol. The solution was loaded on a column (1.0 × 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), 20 mM α-methyl-D-glucoside in BS, 0.2 M α-methyl-D-glucoside in BS and 0.5 M α-methyl-D-mannoside in BS, in succession. Fractions of 3 ml were collected and the absorbance at 230 nm of each fraction was determined. 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 2 weeks. The non-dialyzable fractions were lyophilized.

DEAE-Sephadex A-25 (Cl^- form) Column Chromatography of FB

An aqueous solution (2.5 mg in 0.5 ml) of FB was loaded on a column (1.0 × 10 cm) of DEAE-Sephadex A-25 (Cl^- form). Stepwise elution was performed with 60 ml each of water, then 0.1, 0.2, 0.3, 0.4 and 2.0 M NaCl solutions. Fractions of 3 ml were collected. The absorbance at 230 nm and the hexose content of each fraction were determined. The fraction eluted with 0.1 M NaCl was dialyzed against water and the non-dialyzable fraction was lyophilized, yielding FB-0.1 (0.1 M NaCl eluate from FB).

Gel Filtration of FB-0.1 on Sephadex G-50

FB-0.1 (1.5 mg) was dissolved in 0.2 ml of 0.5 M NaCl or 0.5 M NaCl containing 7 M urea (pH 7.5) or 0.5 M NaCl containing 7 M urea and 50 mM dithiothreitol (pH 7.5). The solution was loaded on a column (1.5 × 153 cm) of Sephadex G-50 (medium). Elution was performed with the same solvent and fractions of 3 ml were collected. The absorbance at 230 nm and the hexose content of each fraction were determined.

Electrophoresis

Electrophoresis on cellulose acetate membranes (Separax) was performed as described previously [8].

Determination of Constituents

Hexose, hexosamine, sialic acid and sulfate were determined by the methods reported previously [8]. Neutral sugars were determined by GLC as follows: a sample was hydrolyzed with 1 M HCl at 100°C for 4 h. After addition of *meso*inositol as an internal standard, the hydrolysate was reduced with sodium borohydride and then acetylated with acetic anhydride. GLC was performed on a Shimadzu GC-4B gas chromatograph, using a glass column (0.3 × 300 cm) packed with 3% ethylene succinate cyanoethyl silicons polymer on Chromosorb W at 200°C. Using a Hitachi 034 liquid chromatograph, the molar ratio of glucosamine and galactosamine was determined on an acid hydrolysate (4 M HCl, 100°C, 6 h). Amino acids were analyzed with a Hitachi 835 amino acid analyzer on an acid hydrolysate (6 M HCl, 110°C, 24 h). Desmosine and isodesmosine were determined by this amino acid analysis, referring to the peaks of the authentic compounds examined under the same conditions.

Results

Separation of GP-90EP

The wet tissues (44 g) of intima-media of porcine thoracic aortas were treated with the procedures described in Materials and Methods, yielding 74 mg of GP-90EP.

Table 1. Chemical composition of the glycopeptide fractions (μg/mg sample). The numbers in parentheses are molar ratios with respect to glucosamine.

	FA	FB	FC	FD	FB-0.1
Mannose	34.2(0.47)	152.3(0.68)	40.7(0.23)	51.9(0.41)	155.7(0.67)
Galactose	107.5(1.48)	156.8(0.70)	ND	ND	165.1(0.71)
Glucose	ND	ND	148.5(0.84)	70.8(0.56)	ND
Fucose	5.1(0.07)	23.1(0.10)	ND	ND	27.4(0.12)
Glucosamine	72.7(1.00)	224.0(1.00)	176.8(1.00)	126.5(1.00)	232.5(1.00)
Galactosamine	7.4(0.10)	15.6(0.07)	ND	ND	16.2(0.07)
Sialic acid[a]	27.6(0.38)	67.2(0.30)	5.3(0.03)	ND	74.4(0.32)
Sulfate	26.9(0.37)	ND	ND	ND	ND
Amino acids	661.8	243.0	560.0	683.5	234.4

ND = not detectable
[a]Expressed as *N*-acetylneuraminic acid.

Table 2. Amino acid composition of the glycopeptide fractions (residues/100 amino acid residues).

	FA	FB	FC	FD	FB-0.1
Asx[a]	4.7	14.7	11.4	8.7	14.1
Hyp	ND	ND	ND	0.9	ND
Thr	4.3	10.1	6.3	5.2	10.1
Ser	5.5	10.8	15.4	18.3	10.6
Glx[b]	7.2	8.6	12.1	13.2	8.6
Pro	18.5	10.5	6.8	5.0	10.4
Gly	21.4	20.6	17.4	20.0	20.5
Ala	18.6	7.3	8.6	8.6	7.8
Val	4.0	5.6	4.8	2.8	5.7
1/2 Cys	1.0	0.8	0.8	0.5	0.7
Met	0.3	0.6	0.3	0.4	0.5
Ile	0.8	1.5	2.6	1.7	1.6
Leu	2.2	1.6	2.8	2.0	1.6
Tyr	2.1	1.0	1.8	1.7	1.1
Phe	0.9	1.0	1.3	1.1	1.2
ID[c]	2.7	0.4	0.4	0.3	0.5
D[d]	2.2	1.2	1.9	4.9	1.5
Lys	1.8	1.4	1.7	1.6	1.2
His	0.7	1.3	2.0	2.3	1.0
Arg	1.1	1.0	1.6	0.8	1.3

[a]Asp (and/or Asn).
[b]Glu (and/or Gln).
[c]Isodesmosine.
[d]Desmosine.
ND = not detectable.

Affinity Chromatography of GP-90EP on Con A-Sepharose 4B

GP-90EP (11.5 mg) was fractionated by affinity chromatography on Con A-Sepharose 4B as described in Materials and Methods (Fig. 1). The yields of FA, FB, FC and FD were 6.94, 2.72, 0.28 and 0.50 mg, respectively. The chemical compositions of these fractions are shown in Tables 1 and 2.

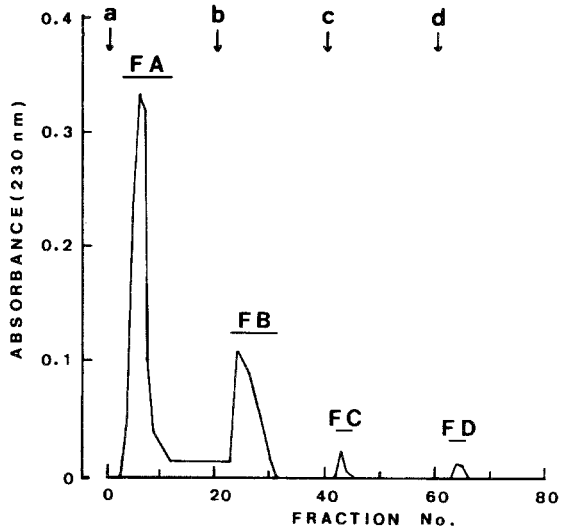

Figure 1. Affinity chromatography of GP-90EP on Con A-Sepharose 4B. Affinity chromatography was performed as described in the text.

a, b, c and d; start of the elution with 0.1 M acetate buffer (pH 6.0) containing a) 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% n-butanol and 0.1 M NaCl (buffered saline) (BS), b) 20 mM α-methyl-D-glucoside in BS, c) 0.2 M α-methyl-D-glucoside in BS, d) 0.5 M α-methyl-D-mannoside in BS.

DEAE-Sephadex A-25 (Cl^- form) Column Chromatography of FB

Since FB was richest in carbohydrate among the above four fractions (Table 1), FB was further purified by DEAE-Sephadex A-25 (Cl^- form) column chromatography as described in Materials and Methods. The fraction eluted with 0.1 M NaCl contained approximately 95% of the total hexose eluted from the column. The yield of FB-0.1 was 1.9 mg from 2.5 mg of FB.

Gel Filtration of FB-0.1 on Sephadex G-50

In order to check the homogeneity of FB-0.1, this fraction was gel filtered on Sephadex G-50 with three different solvents as described in Materials and Methods. All the gel filtration profiles showed symmetrical elution patterns (data not shown), indicating the

homogeneity of FB-0.1 on gel filtration on Sephadex G-50. The K_{av} of the sample was 0.38 with 0.5 M NaCl.

Electrophoresis of FB-0.1 on Separax

Electrophoresis of FB-0.1 was performed on Separax in formic acid- pyridine buffer (pH 3.0) at 1 mA/cm for 25 min (A) and in 0.06 M veronal buffer (pH 8.6) at 1 mA/cm for 25 min (B) as described previously [8]. The substances were located by staining with alcian blue (0.05% in 90% ethanol containing 1% acetic acid). FB-0.1 migrated as a single band in each case with $R_{hyaluronate}$ (flow rate relative to hyaluronate) 0.42 (A) and 0.35 (B).

Characterization of Glycopeptide Fractions

Aqueous solutions of FA, FB, FC, FD and FB-0.1 showed a UV-absorption at 273 nm and fluorescence with excitation at 345 nm and emission at 415 nm. The chemical compositions of these fractions are shown in Tables 1 and 2.

Discussion

In order to separate glycopeptide from elastin, intima-media of porcine thoracic aorta were digested with pronase. A glycopeptide fraction was precipitated with 90% (v/v) ethanol from the 80% ethanol-soluble fraction of a TCA (7%)-soluble fraction of the pronase digest. This glycopeptide fraction contained desmosine and isodesmosine, which are the typical cross-linking components of elastin. Since highly purified elastin did not contain carbohydrate (unpublished data), the glycopeptide fraction was subjected to affinity chromatography on Con A-Sepharose 4B to eliminate elastin fragment(s) containing the cross-linking components, if any, in this fraction. Desmosine and isodesmosine were found not only in the fractions without affinity and with low affinity for Con A (FA and FB), but also in the fractions with high affinity for this lectin (FC and FD). FB was further purified by DEAE-Sephadex A-25 (Cl^- form) column chromatography to a homogeneous state in gel filtration and electrophoresis. The analytical data showed that the purified glycopeptide (FB-0.1) was very similar to FB (Tables 1 and 2). These observations indicated that desmosine and isodesmosine were the integral components of the present glycopeptides.

The carbohydrate compositions of the glycopeptide fractions without affinity and with low affinity for Con A differed significantly from those with high affinity for this lectin. Although galactose was a major monosaccharide constituent in the former, it was not detected in the latter. The relation was reversed in the glucose content. Since the galactosamine content of these glycopeptide fractions was low, it is suggested that these glycopeptides had *N*-glycosidic-type carbohydrate chains. Judging from the carbohydrate compositions and the affinity for Con A, it is conceivable that FB-0.1 may have biantennary complex type carbohydrate chains, while FC and FD may have unusual carbohydrate chains.

The proportions of the constituent amino acids in these fractions differed from each other. All the fractions, however, contained glycine, aspartic acid (and/or asparagine),

serine, proline, threonine, glutamic acid (and/or glutamine) and alanine as the major amino acids, amounting to approximately 80% of the total. Although the present glycopeptides contained desmosine and isodesmosine, their amino acid compositions differed significantly from those of elastin [9-11].

The presence of structural glycoproteins (or glycopeptides) in arterial tissues has been reported by many investigators [1-6]. Their chemical compositions also differed significantly from those of the present glycopeptides. It is indicated therefore that the present preparations are novel glycopeptides containing desmosine and isodesmosine in intima- media of porcine thoracic aorta.

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References

1 Robert L, Moczar M (1982) in Methods in Enzymology, Vol 82, eds. Cunningham LW, Frederiksen DW, Academic Press, New York, p 839-52.
2 Saito H, Yosizawa Z (1975) J Biochem 77:919-30.
3 Saito H, Ototani N, Yosizawa Z (1975) J Biochem 77:931-38.
4 Gibson MA, Grant ME, Jackson DS (1982) Connect Tissue Res 10:145-60.
5 Moczar M, Phan-Dinh-Tuy B, Moczar E, Robert L (1983) Biochem J 211: 257-65.
6 Bressan GM, Castellani I, Colombatti A, Volpin D (1983) J Biol Chem 258:13262-67.
7 Aikawa J, Munakata H, Isemura M, Ototani N, Yosizawa Z (1984) Tohoku J Exp Med, in press.
8 Munakata H, Yosizawa Z (1980) J Biochem 87:1559-65.
9 Robert B, Szigeti M, Derouette J-C, Robert L, Bouissou H, Fabre M-T (1971) Eur J Biochem 21:507-16.
10 John R, Thomas J (1972) Biochem J 127:261-69.
11 Paz MA, Keith DA, Gallop PM (1982) in Methods in Enzymology Vol 82, eds. Cunningham LW, Frederiksen DW, Academic Press, New York, p 517- 87.