We reported the presence of a new trisaccharide composed of two xylose and reducing terminal glucose residues linked to serine residues of bovine blood clotting factor VII and IX (Hase, S., Kawabata, S., Nishimura, H., Takeya, H., Sueyoshi, T., Miyata, T., Iwanaga, S., Takao, T., Shimonishi, Y., and Ikenaka, T. (1988) J. Biochem. (Tokyo) 104, 867–868). The present paper describes the detailed structural analysis of the trisaccharide. Glycopeptides were prepared from bovine factor IX by digestion with Pronase followed by purification by column chromatography. The trisaccharide was released from the protein by the β-elimination reaction with hydrazine, and the reducing end of the sugar chain was tagged with 2-aminopyridine. The fluorescent pyridylamino derivative of the trisaccharide was purified by gel filtration and reversed-phase high performance liquid chromatography. The glycopeptides and pyridylamino-trisaccharide thus obtained were subjected to methylation study, 500-MHz 1H nuclear magnetic resonance spectroscopy, and periodate oxidation. Glucose and xylose belong to the D series by high performance liquid chromatography on a chiral column. From the results, the structure of the trisaccharide is proposed as: D-Xylpa1-3-D-Xyl pa1-3-d-Glcpβ1-0-Ser-53. We have found recently a new trisaccharide chain linked to serine 53 and serine 52 located in the first epidermal growth factor-like domain of bovine factors IX and VII, respectively (6). The trisaccharide is composed of 2 mol of xylose and 1 mol of glucose and is linked through glucose to the serine residues of factors IX and VII (6). The biological significance of this sugar chain remains unknown.

As the first step in studying the function of the sugar chain in a molecular recognition of factor IX with endothelial cells or platelets, we analyzed the chemical structure of the trisaccharide.

**MATERIALS AND METHODS**

**RESULTS AND DISCUSSION**

**Determination of Absolute Configuration of the Component Sugars**—Glucose and xylose in the acid hydrolysate of fraction F4 were separated by paper chromatography. The perbenzoyl derivative of each sugar was analyzed by high performance liquid chromatography on a Chiralpak OT(+) column. Only perbenzoyl derivatives of D-xylose and D-glucose were detected (Fig. 1A and 1B, respectively). Xylose in fraction F3 also belongs to the D series (data not shown). Glucose in fractions 3 and 4 was oxidized by the glucose oxidase kit (Table I). L-Glucose, D-xylose, and L-xylose were not oxidized under the conditions used. These results indicated that glucose and xylose belong to the D series.

**Methylation Analysis**—To know the linkage points of the sugar residues in the trisaccharide, the PA-trisaccharide and fraction F4 were analyzed by methylation technique. Three peaks were obtained from the PA-trisaccharide and fraction F4 were analyzed by methylation technique. Three peaks were obtained from the PA-trisaccharide and fraction F4 were analyzed by methylation technique. Three peaks were obtained from the PA-trisaccharide and fraction F4 were analyzed by methylation technique. Three peaks were obtained from the PA-trisaccharide and fraction F4 were analyzed by methylation technique. Three peaks were obtained from the PA-trisaccharide and fraction F4 were analyzed by methylation technique. Three peaks were obtained from the PA-trisaccharide and fraction F4 were analyzed by methylation technique. Three peaks were obtained from the PA-trisaccharide and fraction F4 were analyzed by methylation technique.
At 4.480 ppm of GP-I is quite near the positions of the anomeric protons (4.43-4.46 ppm, 13C) of the xylose residue and one glucose residue remained intact as revealed by component analysis by gas-liquid chromatography, indicating one xylose residue was oxidized (data not shown). These results can be explained by the structure cited above.

**Determination of Anomeric Configurations**—Anomeric configurations of glycosidic linkages were determined by 500-MHz 1H NMR spectroscopy (Table III and Fig. 7). The resonance positions and the coupling constants (J-values, 4.0 Hz for 5.276 ppm and 3.4 Hz for 5.130 ppm) of the anomeric protons indicated that the anomeric configuration of the two xylose residues was α (21, 22). The resonance at 5.276 ppm was assigned to the anomeric proton of the nonreducing end xylose residue, since an α-anomeric proton of the nonreducing end sugar residue was oxidized (data not shown). These results can be explained by the structure cited above.

**Scheme I. Proposed structure for the trisaccharide bound to the serine 53 residue of bovine factor IX.**

3 linkage and all sugar residues were the pyranose type. These results, together with the previous results (6), indicated the following structure for the trisaccharide: Xylp1-3Xylp1-3Glc-0-Ser. To confirm this, fractions F3 and F4 were subjected to periodate oxidation. One xylose residue and one glucose residue remained intact as revealed by component analysis by gas-liquid chromatography, indicating one xylose residue was oxidized (data not shown). These results can be explained by the structure cited above.

Acknowledgements—We wish to express our thanks to Dr. K. Lee (Osaka University) for operation of the NMR spectrometer and K. Fukuda (Osaka University) for operation of the mass spectrometer.

**REFERENCES**


Continued on next page.
Materials

1.tube number

Preparation of Calcium from Bovine Factor IX.

The purified factor IX was isolated in 2-4 M ammonium acetate by precipitation with 5% calcium chloride (pH 7.4). The precipitate was then dissolved in 2 M ammonium acetate, pH 7.4, at a concentration of 10-12 mg/100 ml. The solution was then dialyzed against 2 M ammonium acetate, pH 7.4, at 4°C and frozen at -20°C prior to use.

The lyophilized factor IX was reconstituted in 0.1 M phosphate buffer, pH 7.4, and used at a concentration of 1-2 mg/ml for all experiments.

Methods

1. Gel filtration: The factor IX solution was passed over a column of Sepharose 6B (Pharmacia) equilibrated with 0.1 M phosphate buffer, pH 7.4, and the factor IX was eluted with the same buffer.

2. Electrophoresis: The factor IX solution was subjected to electrophoresis on a 7.5% slab gel, and the factor IX was detected by staining with Coomassie Brilliant Blue R-250.

3. Immunoblotting: The factor IX solution was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The factor IX was detected by immunoblotting using the factor IX-specific antibody.

4. Western blotting: The factor IX solution was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The factor IX was detected by Western blotting using the factor IX-specific antibody.

5. Mass spectrometry: The factor IX solution was subjected to mass spectrometry to determine the molecular weight and amino acid sequence of the factor IX.

Results and Discussion

Preparation and Properties of Isolated Factor IX.

The factor IX solution was mixed with 2.5 M calcium chloride and dialyzed against 0.1 M phosphate buffer, pH 7.4, at 4°C. The factor IX was then subjected to gel filtration on a Sepharose 6B column, and the factor IX was collected and used for further experiments.

The purified factor IX was then subjected to electrophoresis on a 7.5% slab gel, and the factor IX was detected by staining with Coomassie Brilliant Blue R-250.

The factor IX solution was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The factor IX was detected by immunoblotting using the factor IX-specific antibody.

The factor IX solution was subjected to Western blotting using the factor IX-specific antibody.

The factor IX solution was subjected to mass spectrometry to determine the molecular weight and amino acid sequence of the factor IX.

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The factor IX solution was subjected to Western blotting using the factor IX-specific antibody.

The factor IX solution was subjected to mass spectrometry to determine the molecular weight and amino acid sequence of the factor IX.
FIG. 5. O-Linked Sugar Chain in Bovine Clotting Factor IX

The tri- or tetrasaccharides liberated from bovine factor IXa were pyridylated, and the resulting products were analyzed by liquid chromatography. The chromatogram obtained is shown in the ordinate.

FIG. 6. A mass spectrum of peak C in Fig. 5.

FIG. 7. (A) TMS spectra of GP-I, PA-trisaccharides, and PA-nucleoside. (B) TMS spectrum of GP-II B, PA-trisaccharides, and PA-nucleoside.
The structure of \((\text{xylose})_2\text{glucose-O-serine}\) 53 found in the first epidermal growth factor-like domain of bovine blood clotting factor IX.
S Hase, H Nishimura, S Kawabata, S Iwanaga and T Ikenaka


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