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Detection of UDP-D-Xylose: α-D-Xyloside α1→3Xylosyltransferase Activity in Human Hepatoma Cell Line HepG2[1]

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We previously reported the detection of novel *O*-linked sugar chains classified as being of the glucosyl-*O*-serine type [Hase *et al.* (1988) *J. Biochem.* 104, 867-868]. The sugar chains are a disaccharide (Xylα1-3Glc) and a trisaccharide (Xylα1-3Xylα1-3Glc) linked to serine residues in epidermal growth factor-like domains of human and bovine blood coagulation factors. The structures of these sugar chains suggested the presence of an α1→3xylosyltransferase for their biosynthesis. We report here on the detection of α1→3xylosyltransferase activity which catalyzes the transfer of xylose to Xylα1-3Glc in the human hepatoma cell line HepG2. We employed pyridylaminated Xylα1-3Glc as a fluorescent acceptor and UDP-D-Xyl as a donor. The reaction product was purified by reversed-phase HPLC, and the structure of the transfer product isolated was confirmed to be pyridylaminated Xylα1-3Xylα1-3Glc by Smith degradation, mass spectrometry, and α- and β-xylosidase digestions. The apparent K_m value for pyridylaminated Xylα1-3Glc was 52 mM and for UDP-D-Xyl 0.28 mM. Optimum pH was 7.2. The enzyme was inactivated by addition of EDTA, and its activity was restored by addition of Mn^{2+} and Mg^{2+}. These results indicate the presence of a novel enzyme which is able to transfer xylose to Xylα1-3Glc, forming Xylα1-3Xylα1-3Glc in human cells.

Key words: α-1,3-xylosyltransferase, fluorogenic acceptor, human hepatoma HepG2 cell line, Xylα1-3Xylα1-3Glc.

Two glucosyl-*O*-serine-type sugar chains, Xyl-Glc and Xyl_2-Glc, were previously found in the first epidermal growth factor (EGF)-like domain of human and bovine blood coagulation factors VII and IX, and protein Z (*1, 2*). Detailed structural analysis of sugar chains from bovine factor IX showed the trisaccharide structure to be D-Xylpα1-3-D-Xylpα1-3-D-Glcpβ-1-*O*-Ser (*3*). These sugar chains have so far been reported mainly in blood coagulation factors. They have unique α-xyloside structures and glucose linked to the serine residue by β-linkage. Little is known, however, about their function except that a mutant of human blood coagulation factor VII defective in these glucosyl-*O*-serine-type sugar chains as a result of replacing Serine 52 with an alanine residue by site-directed mutagenesis showed only 60% of the blood coagulation activity of wild-type factor VII (*4*), suggesting that these sugar chains may serve as regulators of coagulation activity.

The structure of the trisaccharide (D-Xylpα1-3-D-Xylpα1-3-D-Glc, X_2G) suggests the presence of an α1→3xylosyltransferase which can transfer a xylose residue onto D-Xylpα1-3-D-Glc (XG), and which might be a key enzyme in the synthesis of X_2G. Thus, research into this biosynthetic enzyme represents an important aspect of studies on the function of the glucosyl-*O*-serine structure. As almost nothing has so far been reported about mammalian α-xylosyltransferase, we have tried to detect human α1→3xylosyltransferase activity that can use pyridylaminated (PA) XG as an acceptor. Here, we describe the detection of a novel α1→3xylosyltransferase that can form X_2G in the HepG2 cell line.

MATERIALS AND METHODS

Materials—Fetal calf serum and Dulbecco's Modified Eagle Medium were obtained from Gibco (Grand Island, NY) and used after heating at 56°C for 30 min. UDP-α-D-xylose and β-D-xylosidase (*Aspergillus niger*) were obtained from Sigma Chemical (St. Louis, MO), α-xylosidase (*Bacillus* sp. No. 693-1) from Seikagaku Kogyo (Tokyo), BCA Protein Assay Reagent from Pierce (Rockford, IL), Sepharose CL-6B and the calibration kit for gel filtration from Pharmacia LKB (Uppsala), Cosmosil $5C_{18}$-P, Lubrol PX, Triton X-100, and Nonidet P-40 from Nacalai Tesque (Kyoto), TSKgel Amide-80 from Tosoh (Tokyo), deoxycholate from Wako Pure Chem. (Osaka), CHAPS, CHAPSO, *n*-octyl β-D-glucoside, *n*-octyl β-D-thioglucoside, *n*-heptyl β-D-thioglucoside, MEGA-8, MEGA-9, and MEGA-10 from Dojin (Kumamoto). X_2G and XG were prepared as reported in a previous paper (*5*) and

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Abbreviations: EGF, epidermal growth factor; Glc, D-glucose; HPLC, high-performance liquid chromatography; PA-, pyridylamino; Xyl, D-xylose; XG, D-Xylpα1-3-D-Glc; X_2G, D-Xylpα1-3-D-Xylpα1-3-D-Glc.

pyridylaminated according to the reported procedure (*6*).

Preparation of Crude Enzyme Fraction from HepG2 Cells—HepG2 cells were cultured in a medium supplemented with 10% (v/v) fetal calf serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C until confluence. The following procedures were performed at 4°C. HepG2 cells (1×10^8 cells) were suspended for 15 min in 10 ml of 10 mM HEPES-KOH buffer (pH 7.9) containing 1.5 mM $MgCl_2$ and 10 mM KCl. Suspended cells were homogenized (300 rpm, 40 strokes) with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $700\times g$ for 5 min. Precipitates resuspended in 10 ml of the same buffer were again homogenized. The combined supernatant was centrifuged at $7,000\times g$ for 10 min. The supernatant was further centrifuged at $105,000\times g$ for 60 min, and the resulting pellet was suspended in 100 μl of 20 mM HEPES-NaOH buffer (pH 7.2) containing 150 mM NaCl and 20 mM $MgCl_2$. The suspended microsomal fraction was stored below −65°C until use.

Preparation of XG-PA and X_2G-PA from HepG2 Cells—Lyophilized HepG2 cells (10 mg of dried material) were heated with 1 ml of hydrazine at 100°C for 7 h followed by *N*-acetylation (400 μl of saturated sodium bicarbonate solution and 16 μl of acetic anhydride) and pyridylamination (200 μl of pyridylamination reagent and 700 μl of reducing reagent) as previously reported (*6*). Excess reagents were removed by gel filtration on an HW40-F column (1.5×60 cm) using 10 mM ammonium acetate buffer (pH 6.0) as an eluent. PA-oligosaccharides were purified by reversed-phase and size-fractionation HPLC (*7*).

Assay of α1→3Xylosyltransferase Activity—The reaction mixture (50 μl) containing 20 mM HEPES-NaOH buffer (pH 7.2), 350 μM XG-PA, 1.6 mM UDP-Xyl, 0.1% Triton X-100, 20 mM $MnCl_2$, 150 mM NaCl, and 10 μl of the enzyme solution were incubated at 37°C for 20 h. The reaction was stopped by heating at 100°C for 3 min or by adding 50 μl of 1 M acetic acid. Precipitates were removed by centrifugation, and an aliquot of the supernatant was analyzed by reversed-phase HPLC using a Cosmosil $5C_{18}$-P column (4.6×150 mm). HPLC was carried out by using 100 mM ammonium acetate buffer (pH 4.5) at a flow rate of 1.0 ml/min. One unit of α1→3xylosyltransferase activity was defined as 1 pmol of X_2G-PA produced per minute under the conditions used.

Mass Spectrometry—The molecular weight of a PA-oligosaccharide was analyzed with a Kompact MALDI III (Kratos) using 2,5-dihydrobenzoic acid as a matrix.

Enzymatic Hydrolysis of PA-Sugar Chains—A PA-sugar chain (300 pmol) was digested at 37°C for 24 h with 1.2 milliunits of α-xylosidase in 20 μl of 0.1 M phosphate buffer (pH 7.5), or with 1.2 milliunits of β-xylosidase in 20 μl of 0.05 M ammonium acetate buffer (pH 5.0). The enzymatic reaction was stopped by heating at 100°C for 3 min. The reaction mixture was analyzed by reversed-phase and size-fractionation HPLC. Reversed-phase HPLC was performed using a Cosmosil $5C_{18}$-P column (4.6×150 mm) and 50 mM ammonium acetate buffer (pH 4.5) as an eluent at a flow rate of 1.0 ml/min. Size-fractionation HPLC was performed using a TSKgel Amide-80 (7.5×75 mm) column and $CH_3CN : H_2O = 90 : 10$ (v/v) containing 3% CH_3COOH (pH 7.3) as an eluent at a flow rate of 1.0 ml/min.

Smith Degradation of the Transfer Product—A PA-sugar chain (400 pmol) was oxidized in 20 μl of 50 mM sodium acetate buffer (pH 4.0) containing 80 mM $NaIO_4$ at 4°C for 40 h in the dark (*8*). After oxidation, the reaction products were reduced by adding 100 μl of 0.1 M $NaBH_4$ at room temperature for 1 h in the dark. Excess sodium borohydride was decomposed by adding 10 μl of acetic acid. The reaction products were separated with a Cosmosil $5C_{18}$-P column (4.6×150 mm) using 50 mM ammonium acetate buffer (pH 4.5) containing 0.1% 1-butanol at a flow rate of 1.5 ml/min. The oxidation-reduction product purified by HPLC was hydrolyzed with 0.05 M H_2SO_4 at 80°C for 10 min. The hydrolysate of the oxidation-reduction product was neutralized with aqueous NaOH solution.

Solubilization of α1→3Xylosyltransferase from HepG2 Microsomal Fraction—A mixture (1.3 ml) containing 1.0 ml of the HepG2 microsomal fraction, 0.5% (w/v) Triton X-100, 20 mM HEPES-NaOH (pH 7.2), 20 mM $MgCl_2$, 150 mM NaCl, and 10% (v/v) glycerol was stirred gently at 4°C. After 1 h, the mixture was centrifuged at $105,000\times g$ for 1 h at 4°C, and the supernatant was collected.

Gel Chromatography on Sepharose CL-6B—Chromatography was performed on a Sepharose CL-6B column (1.3×142 cm) using 20 mM HEPES-NaOH (pH 7.2) containing 20 mM $MgCl_2$, 0.15 M NaCl, and 0.1% (w/v) Triton X-100 at a flow rate of 3.6 cm/h.

pH Dependence of Enzymatic Activity—A mixture containing the enzyme solution (0.8 unit/ml), 350 μM XG-PA, 1.6 mM UDP-Xyl, and 20 mM HEPES-NaOH buffer of various pH containing 150 mM NaCl, 20 mM $MnCl_2$, and 0.1% Triton X-100 was incubated at 37°C for 20 h. The amount of the transfer product in the reaction mixture was measured by HPLC.

Measurement of K_m Values—The apparent K_m values for UDP-Xyl and XG-PA were obtained from $[S]/v$ *vs.* $[S]$ plots. The enzymatic reaction was carried out at 37°C for 20 h in 20 mM HEPES-NaOH buffer, pH 7.2, containing 150 mM NaCl, 20 mM $MnCl_2$, and 0.1% Triton X-100. The concentration of UDP-Xyl was varied from 0.11 to 0.53 mM with 92 mM XG-PA to obtain K_m value for UDP-Xyl, and the concentration of XG-PA was from 19 to 96 mM with 0.5 mM UDP-Xyl for XG-PA.

RESULTS AND DISCUSSION

Detection of XG-PA and X_2G-PA in HepG2 Cells—HepG2 cells were selected as a potential source of enzymes participating in the biosynthesis of glucosyl-*O*-serine-type sugar chains, because glycoproteins possessing these sugar chains are synthesized in the liver (*2*). We first examined whether these sugar chains were present in HepG2 cells. Oligosaccharides were liberated by the hydrazinolysis-*N*-acetylation method from dried HepG2 cells and tagged with 2-aminopyridine. The PA-oligosaccharides thus obtained were fractionated by reversed-phase and size-fractionation HPLC. Peaks corresponding to X_2G-PA (1.1 pmol/mg dried material) and XG-PA (7.2 pmol/mg dried material) were detected, indicating the presence of the sugar chains and the enzyme(s) for their biosynthesis in the cells (data not shown).

Detection of α1→3Xylosyltransferase Activity—A fluorescent pyridylaminated sugar chain, XG-PA, was used as an acceptor. α1→3Xylosyltransferase activity was mea-

sured using HepG2 microsomal fraction, XG-PA and UDP-Xyl. A new peak appeared at the elution position of standard X_2G-PA (Fig. 1), which linearly increased during the course of the reaction (24 h) and with increase of the concentration of the microsomal fraction, indicating that it was a transfer product. Other peaks were already present at 0 time and remained constant thereafter.

Structure Determination of the Transfer Product—The transfer product was purified by reversed-phase HPLC, and its structure was determined. The molecular size of the transfer product was analyzed by matrix-assisted laser desorption mass spectrometry. A molecular ion $[M+H]^+$ was observed at $m/z=522$, which agreed exactly with the calculated value of 522 for Xyl_2Glc-PA. This suggested that 1 mol of xylose was transferred to XG-PA. The transfer product was hydrolyzed with α-xylosidase to PA-Glc (Fig. 2). An intermediate peak appearing at the elution position of XG-PA (Fig. 2) was observed by a controlled digestion of the transfer product for 6 h. The transfer product, however, was not hydrolyzed with β-xylosidase even after 24 h of digestion (data not shown). These results indicate that the xylose added was linked through α-linkage.

To analyze the linkage position of the xylose residue transferred, the transfer product was oxidized with $NaIO_4$ followed by reduction with $NaBH_4$. The scheme of the linkage position analysis is shown in Fig. 3. The oxidation-reduction product thus obtained was purified by reversed-phase HPLC. The acid hydrolysate of oxidation-reduction product was hydrolyzed with α-xylosidase, and PA-threose was detected (Fig. 4). These results indicate that the xylose residue added was linked to the xylose residue of XG-PA by α1-3 linkage.

Solubilization and Gel Chromatography of α1→3Xylosyltransferase—For solubilization of α1→3xylosyltransferase activity, Triton X-100 was the most efficient among the detergents tested (Triton X-100, Lubrol PX, Nonidet P-40, deoxy cholate, CHAPS, Tween 20, CHAPSO, *n*-octyl β-D-glucoside, *n*-octyl β-D-thioglucoside, *n*-heptyl β-D-thioglucoside, MEGA-8, MEGA-9, and MEGA-10). α1→3Xylosyltransferase (56 units) was solubilized from HepG2 microsomal fraction obtained from 1.5×10^9 cells, and separated by gel chromatography (Fig. 5). α1→3Xylosyltransferase activity was eluted at a molecular weight of about 230,000, and the recovery of the activity from the column was about 65%. The effects of divalent cations and EDTA on the enzymatic activity were examined using the gel-filtered enzyme preparation (Table I). The enzyme was inactivated by EDTA, and Mn^{2+} was the most suitable for the enzyme activity in the cations examined. Optimum pH was 7.2. The apparent K_m value was 52 mM for XG-PA and 0.28 mM for UDP-Xyl.

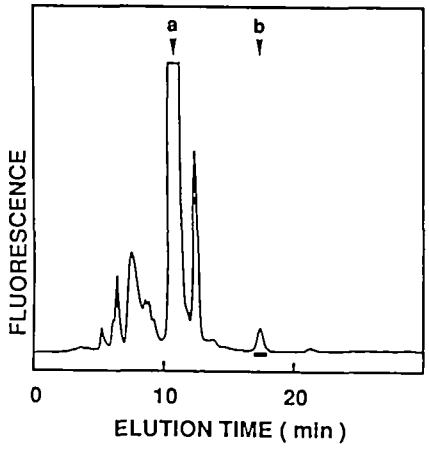

Fig. 1. **HPLC analysis of the products obtained by incubation of XG-PA, UDP-D-Xyl, and HepG2 microsomal fraction.** The 20-h incubation products were analyzed with a reversed-phase column (Cosmosil $5C_{18}$-P, 4.6×150 mm). The elution was monitored with a fluorescence detector. The wavelengths of excitation and emission were 320 and 400 nm, respectively. Arrowheads a and b indicate the elution positions of the standard XG-PA and X_2G-PA, respectively. The peak indicated by a bar was collected as the transfer product.

TABLE I. **Effects of divalent cations and EDTA on enzyme activity.** A mixture containing the enzyme solution (0.8 unit/ml), 350 μM XG-PA, 1.6 mM UDP-Xyl, and 20 mM HEPES-NaOH buffer, pH 7.2, containing 150 mM NaCl, 20 mM bivalent cation, or EDTA, and 0.1% Triton X-100 was incubated at 37°C for 20 h. The amount of the transfer product in the reaction mixture was measured by HPLC.

Divalent cation	Activity[a]
$MnCl_2$	1.0
$MgCl_2$	0.52
$CaCl_2$	0.23
$CoCl_2$	0.23
EDTA	0.00

[a] The activity with 20 mM Mn^{2+} was taken as unity.

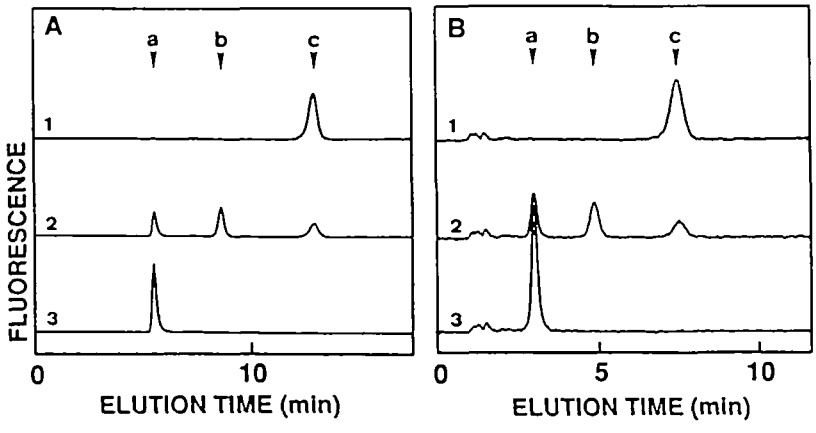

Fig. 2. **HPLC analysis of α-xylosidase digestion of the transfer product.** The transfer product was digested with α-xylosidase, and the digest was analyzed by reversed-phase HPLC (A) and size-fractionation HPLC (B) after (1) 0 h, (2) 6 h, and (3) 24 h. Fluorescence detection was as described in the legend to Fig. 1. Arrowheads a, b, and c indicate the elution positions of PA-Glc, XG-PA, and X_2G-PA, respectively.

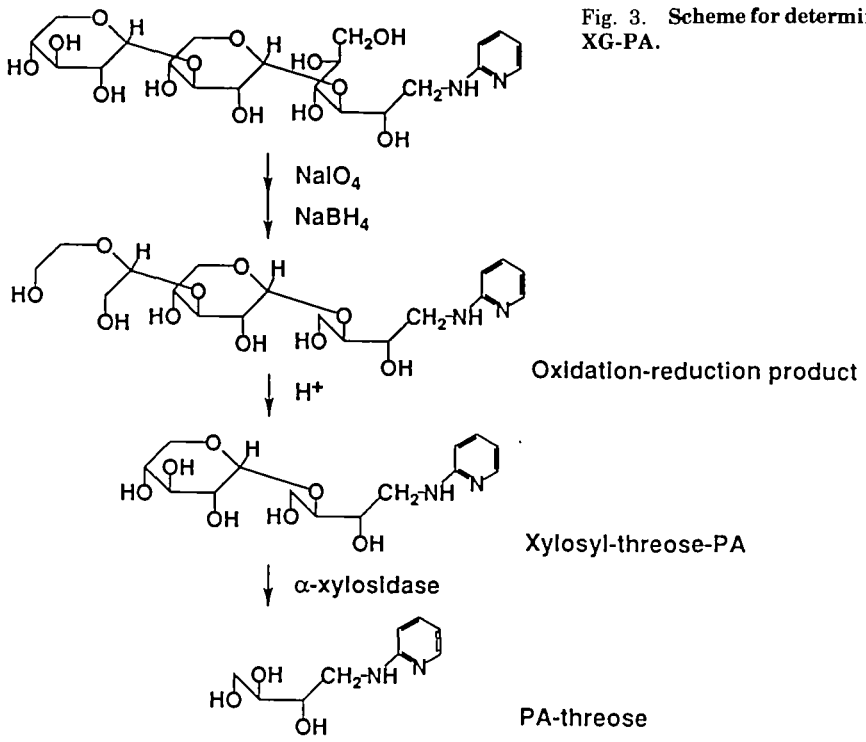

Fig. 3. **Scheme for determining the linkage position of the xylose added to XG-PA.**

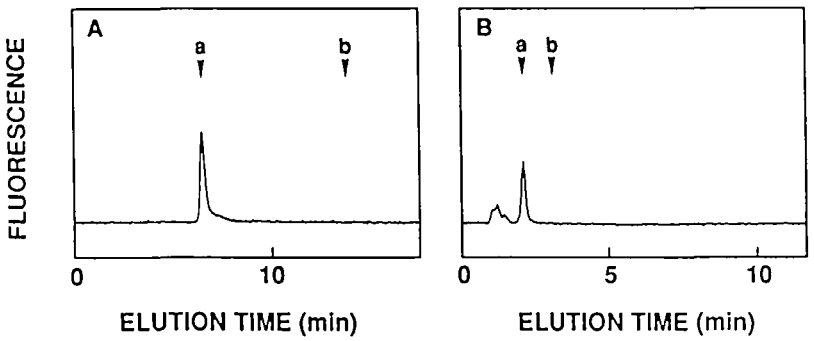

Fig. 4. **HPLC analysis of the acid hydrolysate of the oxidation-reduction product after incubation with α-xylosidase.** The acid hydrolysate of the oxidation-reduction product of the transfer product was incubated with α-xylosidase and the digest was analyzed by reversed-phase HPLC (A) and by size-fractionation HPLC (B). Arrows a and b indicate the elution position of PA-threose (*9*) and the acid hydrolysate of the oxidation-reduction product, respectively.

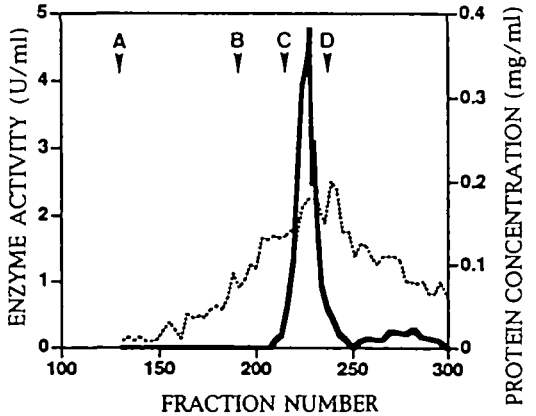

Fig. 5. **Gel filtration of solubilized α1→3xylosyltransferase from HepG2 microsomal fraction.** α1→3Xylosyltransferase solubilized with 0.5% (w/v) Triton X-100 was applied to a Sepharose CL-6B column (1.3×142 cm) as described in "MATERIALS AND METHODS." Fractions of 0.52 ml were collected and assayed for α1→3xylosyltransferase. Solid line indicates the enzyme activity and dotted line protein concentration. Arrowheads A, B, C, and D indicate the elution positions of Blue Dextran 2000, thyroglobulin, ferritin, and aldolase, respectively.

The only α-xylosyltransferase hitherto reported is UDP-D-xylose:β-D-glucoside α1→6xylosyltransferase, which participates in biosynthesis of xyloglucan in suspension-cultured soybean cells (*10*). Our results indicate the presence of a new type of enzyme, UDP-D-xylose:α-D-xyloside α1→3xylosyltransferase, which participates in the biosynthesis of the Glc-*O*-Ser-type sugar chains. Purification of this enzyme is necessary for further study to clarify the possibility that the enzyme can also transfer a xylose residue to the glucose residue. Reports on the detection of XG and X_2G in human urine suggest that the sugar chains are metabolized in human cells (*11, 12*).

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REFERENCES

1. Hase, S., Kawabata, S., Nishimura, H., Takeya, H., Sueyoshi, T., Miyata, T., Iwanaga, S., Takao, T., Shimonishi, Y., and Ikenaka, T. (1988) A new trisaccharide sugar chain linked to a serine residue in bovine blood coagulation factors VII and IX. *J.*

Biochem. **104**, 867-868
2. Nishimura, H., Kawabata, S., Kisiel, W., Hase, S., Ikenaka, T., Takao, T., Shimonishi, Y., and Iwanaga, S. (1989) Identification of a disaccharide (Xyl-Glc) and a trisaccharide (Xyl_2-Glc) O-glycosidically linked to a serine residue in the first epidermal growth factor-like domain of human factors VII and IX and protein Z and bovine protein Z. *J. Biol. Chem.* **264**, 20320-20325
3. Hase, S., Nishimura, H., Kawabata, S., Iwanaga, S., and Ikenaka, T. (1990) The structure of $(Xylose)_2$Glucose-O-Serine 53 found in the first epidermal growth factor-like domain of bovine blood clotting factor IX. *J. Biol. Chem.* **265**, 1858-1861
4. Bjoern, S., Foster, D.C., Thim, L., Wieberg, F.C., Christensen, M., Komiyama, Y., Pedersen, A.H., and Kisiel, W. (1991) Human plasma and recombinant factor VII. Characterization of *O*-glycosylations at serine residues 52 and 60 and effects of site-directed mutagenesis of serine 52 to alanine. *J. Biol. Chem.* **266**, 11051-11057
5. Fukase, K., Hase, S., Ikenaka, T., and Kusumoto, S. (1992) Synthesis of new serine-linked oligosaccharides in blood clotting factors VII and IX and protein Z. The synthesis of *O*-α-D-xylopyranosyl-(1→3)-D-glucopyranose, *O*-α-D-xylopyranosyl-(1→3)-*O*-α-D-xylopyranosyl-(1→3)-D-glucopyranose, and their conjugates with serine. *Bull. Chem. Soc. Jpn.* **65**, 436-445
6. Kuraya, N. and Hase, S. (1992) Release of *O*-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions. *J. Biochem.* **112**, 122-126
7. Hase, S., Koyama, S., Daiyasu, H., Takemoto, H., Hara, S., Kobayashi, Y., Kyogoku, Y., and Ikenaka, T. (1986) Structure of a sugar chain of a protease inhibitor isolated from barbados pride (*Caesalpinia pulcherrima Sw.*) seeds. *J. Biochem.* **100**, 1-10
8. Irimura, T., Tsuji, T., Tagami, S., Yamamoto, K., and Osawa, T. (1981) Structure of a complex-type sugar chain of human glycophorin A. *Biochemistry* **20**, 560-566
9. Omichi, K. and Hase, S. (1994) Linkage position analysis of pyridylamino-disaccharides by HPLC of fluorogenic Smith degradation products. *J. Biochem.* **115**, 429-434
10. Hayashi, T. and Matsuda, K. (1981) Biosynthesis of xyloglucan in suspention-cultured soybean cells. Occurrence and some properties of xyloglucan 4-β-D-glucosyltransferase and 6-α-D-xylosyltransferase. *J. Biol. Chem.* **256**, 11117-11122
11. Lundblad, A. and Svensson, S. (1973) Isolation and characterization of 3-*O*-α-D-xylopyranosyl-D-glucose and 2-*O*-α-L-fucopyranosyl-D-glucose from normal human urine. *Biochemistry* **12**, 306-309
12. Toyota, J., Omichi, K., Fukase, K., Kusumoto, S., and Hase, S. (1994) Identification of trisaccharide Xylα1-3Xylα1-3Glc in human urine. *Biosci. Biotech. Biochem.* **58**, 567-569