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Purification and Substrate Specificity of UDP-D-xylose:β-D-Glucoside α-1,3-D-Xylosyltransferase Involved in the Biosynthesis of the Xylα1-3Xylα1-3Glcβ1-*O*-Ser on Epidermal Growth Factor-like Domains

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A unique *O*-glycan structure, Xylα1-3Xylα1-3Glcβ1-*O*-Ser is found on the consensus sequence C-X-S-X-P-C (X denotes any amino acid) in epidermal growth factor (EGF)-like domains of plasma proteins such as clotting factor VII and IX. One of the enzymes involved in the biosynthesis of this trisaccharide, UDP-D-xylose:β-D-glucoside α1,3-D-xylosyltransferase has been identified in HepG2 cells (Omichi, K., Aoki, K., Minamida, S., and Hase, S. *Eur. J. Biochem.* 245, 143–146 [1997]). Here, we report that this enzyme activity can be detected in bovine liver and that the enzyme has been purified from the microsomal fraction. The enzyme was purified 6200-fold in terms of specific activity and ran as a single band on native-PAGE and isoelectric focusing gel electrophoresis. The best acceptor substrate of those tested was the EGF-like domain of bovine factor IX carrying β-glucoside at Ser53. The *K*m value for this substrate was 34 μM. Comparison of initial velocity with various acceptor substrates shows that this xylosyltransferase recognizes not only the glucose moiety to which xylose is transferred but also the tertiary structure of the EGF-like domain. With regard to the donor substrate, the enzyme does not recognize UDP-D-glucose but does recognize UDP-D-xylose.

Key words: EGF-like domain, glycosyltransferase, *O*-glucose, *O*-glycans, xylose.

Abbreviations: EGF, epidermal growth factor; FAB-MS, fast atom bombardment-mass spectrometry; Glc, D-glucose; Xyl, D-xylose; Glcβ-EGF, the EGF-like domain of bovine factor IX (45-87) carrying β-glucoside at Ser53; Glcβ-Ser, Glcβ1-*O*-Ser; Glcβ-R, 2-[(2-pyridyl)amino]ethyl β-glucoside.

The glucosyl-*O*-serine type modifications, XylGlc and Xyl_2Glc structures were first found at a specific serine residue in the epidermal growth factor (EGF)-like domain of bovine and human blood clotting factors VII and IX and protein Z (*1*, *2*). Further structural analysis of the trisaccharide, Xyl_2Glc, from bovine factor IX revealed the full structure to be Xylα1-3Xylα1-3Glcβ1-*O*-Ser (*3*). This structure is a unique form of post-translational modification in that the glucose residue is directly linked to a serine residue and that the xylose residue is attached to the glucose through an α-linkage. The same structure was later found on bovine thrombospondin (*4*). The *O*-glucose modification has also been found in fetal antigen-1(delta-like protein) (*5*) and Notch1 (*6*). The consensus amino acid sequence surrounding the sites of *O*-glucosylation was found to be C-X-S-X-P-C (X denotes any amino acid). The two cysteine resides are involved in the formation of disulfide bonds in the EGF-like domain and give a rigid structure (*2*, *7*). Little is known, however, about the biological function of this trisaccharide. A mutant of human blood clotting factor VII in which Ser 52 to which the trisaccharide is attached is replaced with an Ala residue had blood clotting activity 60% of wild-type (*8*) and a 2-fold increased association rate constant for binding to tissue factor (*9*). The reasons for these changes remain to be established but they suggest that this sugar chain might have some biological role. Unravelling the biosynthetic mechanism of this trisaccharide is essential for elucidation of its biological functions.

The whole process of biosynthesis of the *O*-glucose trisaccharide has not been elucidated. At least three enzymes, UDP-D-glucose: protein *O*-glucosyltransferase, UDP-D-xylose: β-D-glucoside α-1,3-D-xylosyltransferase and UDP-D-xylose:α-D-xyloside α-1,3-D-xylosyltransferase are involved in its biosynthesis. Two α-1,3-xylosyltransferases have been identified from the human hepatoma cell line HepG2 (*10*, *11*) and *O*-glucosyltransferase has been identified in Chinese hamster ovary cells (*12*). To understand the biochemical process of the biosynthesis of this trisaccharide, it is essential that these enzymes are purified in order that their properties, including substrate specificity, can be studied.

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We have attempted to purify the β-D-glucoside α-1,3-D-xylosyltransferase which transfers a xylose residue to glucose linked to a serine residue. Bovine liver was used as the enzyme source instead of HepG2 because factor IX bearing the trisaccharide is produced in liver (*13*). Here, the identification of the UDP-D-xylose:β-D-glucoside α-1,3-xylosyltransferase in bovine liver, the purification procedure used to obtain the enzyme from bovine liver and the substrate specificity and other properties of the purified enzyme will be described.

MATERIALS AND METHODS

Materials—Fresh bovine liver was purchased from Funakoshi (Tokyo, Japan). DEAE-Sephacel, Sephacryl S-200, Mono Q HR 5/5 (5.0 × 50 mm), PD-10 gel filtration columns and Ampholine PAG plates (pH 4.0–6.5) were obtained from GE healthcare Bio-Sciences (Poscataway, NJ, USA). *Caldocellum saccharolyticum* β-glucosidase and UDP-hexanolamine agarose were obtained from Sigma-Aldrich (St Louis, MO, USA), 2-[(2-pyridyl)-amino]ethanol and WakoSil-II5C_{18}-HG (6.0 × 150 mm) were from Wako Pure Chemicals (Osaka, Japan). α-Xylosidase from *Bacillus* sp. No.693-1 was purchased from Seikagaku Corp. (Tokyo, Japan). CarboPac PA-1 column (2.0 × 250 mm) was purchased from Dionex (Sunnyvale, CA, USA). Sep-pak C_{18} cartridge columns were from Waters (Milford, MA, USA). TSKgel Sugar AXI column (4.6 × 150 mm) was from Tosoh (Tokyo, Japan).

Preparation of Substrates—UDP-D-xylose (UDP-Xyl) was synthesized as described previously (*14*). UDP-D-glucose (UDP-Glc) was purchased from Nacalai Tesque (Kyoto, Japan). Glcβ1-*O*-Ser (Glcβ-Ser) and the EGF-like domain of bovine factor IX (45-87) carrying glucose at Ser53 with a β-linkage (Glcβ-EGF) was synthesized as described previously (*15*). Xylα1-3Glc was prepared as described elsewhere (*16*).

2-[(2-pyridyl)amino]ethyl β-glucoside (Glcβ-R) was enzymatically prepared as follows: *p*-Nitrophenyl β-glucopyranoside (1.1 g, Senn chemicals, Dielsdorf, Switzerland) and 2-[(2-pyridyl)amino]ethanol (2.0 g) were dissolved in 50 ml of water and the solution adjusted to pH 6.0 with sodium hydroxide. β-Glucosidase (9 U) was added to the mixture, and the solution incubated at 37°C for 20 h. The reaction was terminated by adding acetic acid (2.3 ml) followed by heating at 100°C for 15 min. The mixture was applied to a HW-40F gel-filtration column (35 × 1920 mm) equilibrated in 50 mM ammonium acetate buffer, pH 6.0, at a flow rate of 0.65 ml/min. Elution was monitored by measuring the absorbance at 320 nm and the fractions containing Glcβ-R collected. Glcβ-R was obtained with a 16% yield.

Reduced and alkylated Glcβ-EGF was prepared as follows: Glcβ-EGF (4.2 nmol) was dissolved in 50 μl of 2.5 M Tris-HCl, pH 8.0, containing 8 M urea. The peptide was reduced by adding 2.5 μmol of dithiothreitol followed by incubation at 50°C for 1 h. After cooling the solution to room temperature, the sample was alkylated with 2.5 μmol of iodoacetamide at room temperature for 30 min in the dark. The solution was applied to a PD-10 gel filtration column equilibrated in 0.1% trifluoroacetic acid in order to remove excess reagents. Elution was monitored by absorbance at 280 nm and the fraction containing the reduced and alkylated Glcβ-EGF collected. The reduced and alkylated Glcβ-EGF eluted somewhat later than the properly folded Glcβ-EGF on a WakoSil-II5C_{18}-HG column (6.0 × 150 mm) equilibrated in 0.1% trifluoroacetic acid ran at a flow rate of 1 ml/min with a linear gradient of acetonitrile from 0% to 80% over 30 min (data not shown). This result indicates that the conformation of the reduced and alkylated Glcβ-EGF is different from that of the native Glcβ-EGF.

Identification of the Enzyme Product—100 mg of Glcβ-R and 27 mg of UDP-Xyl were mixed with the microsome fraction of bovine liver (prepared as described later) in 10 ml of 20 mM HEPES-NaOH, pH 7.2, 20 mM $MgCl_2$, 150 mM NaCl and 0.1% Triton X-100 and incubated at 37°C for 24 h. The reaction was terminated by heating at 100°C for 5 min. The sample was applied onto an HW40-F column (17 × 1800 mm) equilibrated in 50 mM ammonium acetate, pH 6.0 and the product eluted with the same buffer. The fractions containing the enzyme product were collected and concentrated. The product was further purified on a Wakosil-II 5C18-HG column with isocratic elution using 50 mM ammonium acetate, pH 4.5.

Fast atom bombardment-mass spectrometry (FAB-MS): The molecular mass of the product was measured by FAB-MS using a JEOL JMS-HX100 equipped with a FAB ion source. The sample was mixed with glycerol and bombarded with a neutral atom beam accelerated at a potential of 10 keV.

α-Xylosidase digestion: The reaction product (600 pmol) dissolved in 20 μl of 100 mm sodium phosphate, pH 7.5, was digested with 0.05 mU of α-xylosidase at 37°C for 12 h. The reaction was terminated by heating at 100°C for 3 min. The reaction mixture was analyzed on a Wakosil 5C_{18}-HG column with isocratic elution in 50 mm ammonium acetate, pH 4.5, containing 0.05% (v/v) 1-butanol at a flow rate of 2 ml/min.

Smith degradation: The purified product (600 pmol) dissolved in 40 μl of 50 mM sodium acetate buffer, pH 4.0, containing 80 mM sodium periodate was incubated at 4°C for 45 h (*17*). 200 μl of 100 mM sodium borohydride solution was added and allowed to react for 1 h at ambient temperature in the dark. Acetic acid (20 μl) was added to the mixture to degrade the residual reducing reagent and then 135 μl of 1 M sodium hydroxide added to neutralize the solution. The product was purified by reversed-phase HPLC as described earlier. The purified product was mixed with 0.05 M sulfuric acid and allowed to stand for 10 min at 80°C after which the acid hydrolysis was stopped by neutralization with sodium hydroxide.

Assay Procedure for β-D-Glucoside α-1,3-D-Xylosyltransferase Activity—The reaction mixture of total volume 25 μl contained 5 μl of the enzyme solution, 20 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 20 mM $MnCl_2$, 1 mM Glcβ-R and 1.3 mM UDP-xylose. The reaction was performed at 37°C for 12 h and terminated by heating at 100°C for 3 min. The reaction products were analysed by a WakoSil-II 5C_{18}-HG reversed-phase column with isocratic elution in

50 mM ammonium acetate buffer, pH 4.5, at a flow rate of 2 ml/min. The substrate and product were detected by their fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm). This assay method was used throughout the purification procedures. One unit of the enzyme activity was defined as the amount of the enzyme that transferred 1 nmol of xylose from UDP-Xyl to Glcβ-R per hour under the conditions described previously.

Purification of β-D-Glucoside α-1,3-D-Xylosyltransferase—All steps of the purification were performed at 4°C. The buffers used were as follows. Buffer A, 20 mM HEPES-NaOH, pH 7.4, containing 250 mM sucrose and 5 mm $MgCl_2$. Buffer B, 20 mM HEPES-NaOH, pH 7.2, containing 20 mM $MgCl_2$, 150 mM NaCl, 10% (v/v) glycerol and 0.5% Triton X-100. Buffer C, 20 mm HEPES-NaOH, pH 7.2, containing 10 mM $MgCl_2$ and 0.1% Triton X-100. Buffer D, 20 mm HEPES-NaOH, pH 7.2, containing 10 mM $MgCl_2$, 0.1% Triton X-100 and 150 mM NaCl. Buffer E, 10 mm sodium phosphate buffer, pH 6.5, containing 10 mM $MgCl_2$ and 0.1% Triton X-100. Buffer F, 20 mm HEPES-NaOH, pH 7.2, containing 5 mM $MnCl_2$ and 0.1% Triton X-100. Buffer G, 20 mm HEPES-NaOH, pH 7.2, containing 5 mM $MgCl_2$ and 0.1% Triton X-100.

Step 1. Preparation of the microsomal fraction: Bovine liver (1 kg) was homogenized in 9 l of Buffer A. The homogenate was centrifuged at 10,000×*g* for 25 min and the supernatant further centrifuged at 105,000×*g* for 1 h. The pellets were collected and the proteins solubilized by stirring in Buffer B for 30 min. The supernatant obtained after centrifugation (105,000×*g* for 1 h) was used as the microsomal fraction.

Step 2. DEAE-Sephacel chromatography: The dialysed microsomal fraction was applied to a DEAE-Sephacel column (77 × 350 mm) equilibrated with Buffer C. The column was washed with 6 l of the same buffer and then elution performed with a linear gradient using 2 l of Buffer C and 2 l of Buffer C containing 0.5 M NaCl. The enzyme-containing fractions were pooled and concentrated to 25 ml using an Amicon YM-30 membrane.

Step 3. Sephacryl S-200 gel filtration: The enzyme fraction obtained in Step 2 was applied to a Sephacryl S-200 column (25 × 1640 mm) equilibrated with Buffer D. The enzyme was eluted with the same buffer. The fractions with enzyme activity were concentrated to 15 ml with an Amicon YM-30 membrane. The apparent molecular mass of protein was deduced from its elution volume compared with those of standard proteins. The standard proteins used were bovine pancreas chymotrypsinogen A (25 kDa), hen egg ovalbumin (43 kDa), bovine serum albumin (67 kDa) and rabbit muscle aldolase (158 kDa).

Step 4. Mono Q chromatography: The enzyme solution obtained from Step 3 was applied to a Mono Q HR 5/5 column (5.0 × 50 mm) which was attached to an FPLC system (GE healthcare Bio-Sciences) and which was equilibrated with Buffer E at a flow rate of 0.5 ml/min. After injecting a sample, the column was washed with the same buffer. The enzyme was eluted with a linear gradient of NaCl from 0 mM to 500 mM over 160 min. The active fractions were desalted and concentrated to 1.5 ml using an Amicon YM-30 membrane.

Step 5. UDP-hexanolamine agarose affinity chromatography: The enzyme fraction obtained from Step 4 was applied to a UDP-hexanolamine agarose column (9.0 × 530 mm) equilibrated with Buffer F. The column was washed with Buffer F for one column volume then the enzyme eluted with Buffer G. Flow rate was 80 μl/min. The enzyme fractions were collected and used for further analyses.

Protein Assay—The amount of protein was determined using BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions with bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis—Native-PAGE was performed on a 5% polyacrylamide gel, pH 7.5, at 4°C according to the method of Davis (*18*). The proteins electrophoresed were visualized using a silver staining kit (2D silver stain II DAIICHI; Daiichi pure chemicals, Tokyo, Japan). One lane was cut into 2 mm pieces and each slice homogenized with a pellet mixer. The protein was extracted with 40 μl of buffer (50 mM HEPES-NaOH, pH 7.2, 375 mM NaCl, 0.25% Triton X-100 and 10 mM $MnCl_2$) and a part of the extract was assayed for α-xylosyltransferase activity.

Isoelectric focusing gel electrophoresis of the enzyme was carried out on an Ampholine PAG plate gel pI 4-6 (*19*). After electrophoresis one lane was cut into 2 mm pieces and each slice was homogenized with a pellet mixer. The enzyme activity of the proteins extracted was assayed as described earlier. The other lane was stained with Coomassie brilliant blue G-250.

Measurement of the Initial Velocity of the β-D-Glucoside α-1,3-D-Xylosyltransferase against Various Acceptor Substrates—The reaction mixture of total volume 25 μl containing the enzyme solution, 20 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 20 mM $MnCl_2$, 100 μM of acceptor substrate and 130 μM UDP-Xyl, was incubated at 37°C. The reaction was stopped by heating at 100°C for 5 min.

Use of Glc, Xylα1-3Glc, or Glcβ-Ser as acceptor substrates: The reaction mixture was analysed on a CarboPac PA-1 column with 70 mM NaOH at a flow rate of 1 ml/min. The saccharides were quantitated using a pulsed-amperometric detector.

Use of Glcβ-EGF and reduced and alkylated Glcβ-EGF as acceptor substrates: The products were loaded onto a Sep-pak C_{18} column and held for 30 min. The column was washed with 8 ml of water and the glycopeptides eluted with 2 ml of 80% methanol. The lyophilized samples were heated with 200 μl of anhydrous hydrazine at 60°C for 50 h to release oligosaccharide from the peptide (*20*). Excess hydrazine was removed by repeated coevaporation with toluene four times *in vacuo*. The sample was pyridylaminated as reported previously (*20*). The unreacted 2-aminopyridine was coevaporated with 30 μl trietylamine, 30 μl methanol and 40 μl toluene three times with a stream of nitrogen under reduced pressure to remove the excess reagents. The sample was coevaporated further with 20 μl methanol and 40 μl toluene as described earlier. The sample was dissolved in 150 μl of water and the solution applied to a HW-40F gel filtration column (10 × 490 mm) equilibrated in 10 mM ammonium acetate buffer, pH 6.0, at a flow rate of 0.1 ml/min to

remove residual reagents. The pyridylaminated (PA-) sugar chains were monitored by measuring their fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm). The fractions containing the PA-sugar chains were collected. The PA-sugar chains were separated on a TSKgel Sugar AXI column (4.6 × 150 mm) at a flow rate of 0.3 ml/min at 74°C. The elution buffer was 0.8 M boronic acid, which was adjusted to pH 9.0 with potassium hydroxide, containing 10% acetonitrile. The PA-sugar chains were quantified by their fluorescence.

Use of Xylα1-3Glcβ-R as acceptor substrate: The reaction mixture was analysed by size-fractionation HPLC performed on a Shodex Asahipak NH2P-50 column (6.0 × 100 mm) at a flow rate of 0.6 ml/min at 25°C. Eluent A and B were used. Eluent A was acetonitrile: water: acetic acid (800:200:3, v/v/v) titrated to pH 7.0 with 12.5% aqueous ammonia. Eluent B was acetonitrile: water: acetic acid (200:800:3, v/v/v) titrated to pH 7.0 with 12.5% aqueous ammonia. The column was equilibrated with Eluent A. After injecting a sample, a linear gradient was ran from 0% to 50% Eluent B (1:1, v/v) over 60 min. The oligosaccharides were monitored by measuring the fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

Determination of K_m and V_{max} Values—The apparent K_m and V_{max} values of the β-D-glucoside α-1,3-D-xylosyltransferase for Glcβ-R and Glcβ-EGF were determined by assay with various concentrations of Glcβ-R (0.05–5 mM) and Glcβ-EGF (10–60 μM) in the presence of 1.3 mM UDP-Xyl.

RESULTS

Identification of β-D-Glucoside α-1,3-D-Xylosyltransferase Activity in Bovine Liver—Bovine liver was chosen as an enzyme source because the enzyme activity was detected in the microsomal fraction of HepG2 cells and because the clotting factor IX bearing Xylα1-3Xylα1-3Glcβ structure is specifically expressed in liver (*13*). β-D-Glucoside α-1,3-D-xylosyltransferase activity in bovine liver was examined according to the method used to detect the enzyme activity in the microsomal fraction of HepG2 cells (*10*). When Glcβ-R and UDP-Xyl were incubated with the microsomal fraction of bovine liver, a new peak appeared on reversed-phase HPLC chromatograms (Fig. 1A, peak A). This peak increased linearly in a time- and enzyme concentration-dependent manner (data not shown). α-Xylosidase digestion of the product gave a peak with a retention time identical to that of Glcβ-R [Fig. 1B(a)], demonstrating that the xylose residue was transferred to Glcβ-R with an α-linkage. The enzyme product was subject to periodate oxidation and completely disappeared [Fig. 1B(b)]. This degradation product was hydrolysed under mild acid conditions and gave Glcβ-R [Fig. 1B(c)]. The major peak in Fig. 1B(b) turned out to be Xyl-Glcβ-R oxidized and reduced in the Xyl residue. These results demonstrate the C3-substitution of the Glc residue. In addition, the observed $[M+H]^+$ molecular mass of the product (peak A) was 433.2 (the calculated $[M+H]^+$ molecular mass of Xyl-Glcβ-R is 433.2.). These results show that the enzyme product was Xylα1-3Glcβ-R (R denotes 2-[(2-pyridyl)amino]ethyl) and that β-D-glucoside α-1,3-D-xylosyltransferase activity exists in the microsomal fraction of bovine liver.

Purification of β-D-Glucoside α-1,3-D-Xylosyltransferase from Bovine Liver—β-D-Glucoside α-1,3-D-xylosyltransferase was purified from the microsomal fraction of bovine liver (Fig. 2 and Table 1). The majority of proteins were separated from the fraction with the enzyme activity by DEAE-Sephacel, Sephacryl S-200 and Mono Q chromatography (Fig. 2A, B and C). Two peaks of transferase activity were detected in the Sephacryl S-200 gel filtration step (Fig. 2B). Apparent molecular masses of the enzyme in the two peaks were 124 k and 52 k, respectively. Because both peaks gave the same elution

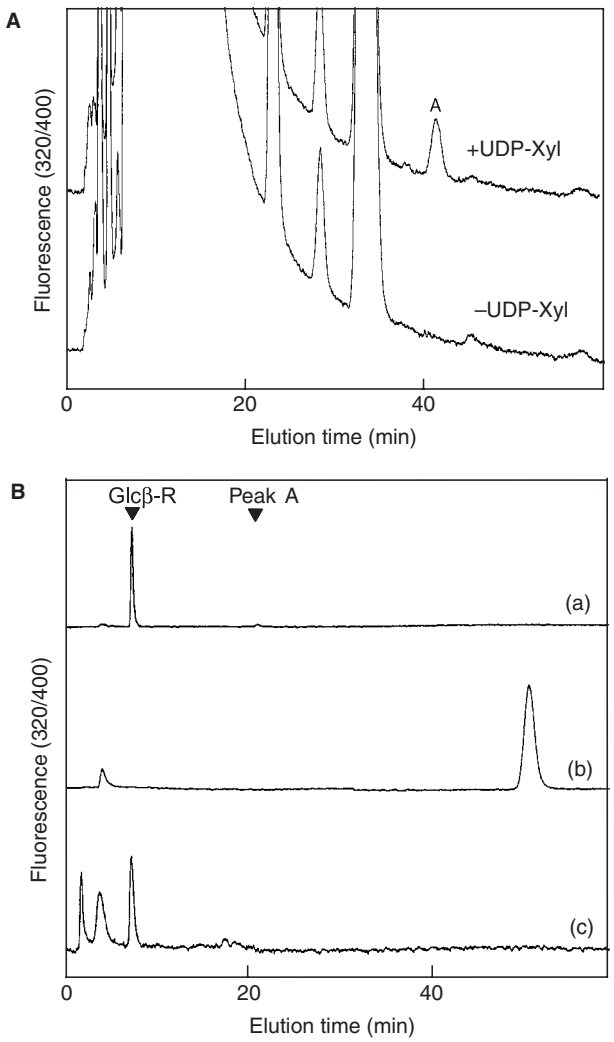

Fig. 1. **Detection of β-glucoside α1-3xylosyltransferase activity in bovine liver.** (A) The microsomal fraction of bovine liver incubated with Glcβ-R in the presence (upper panel) or absence (lower panel) of UDP-Xyl. Peak A in the upper panel was further analysed. (B) (a) Digestion of peak A with α-xylosidase. (b) Periodate oxidation followed by borohydride reduction product of peak A. (c) Hydrolysate of the oxidation–reduction product in (b) resulting from mild acid hydrolysis. Arrowheads indicate the elution positions of Glcβ-R and peak A.

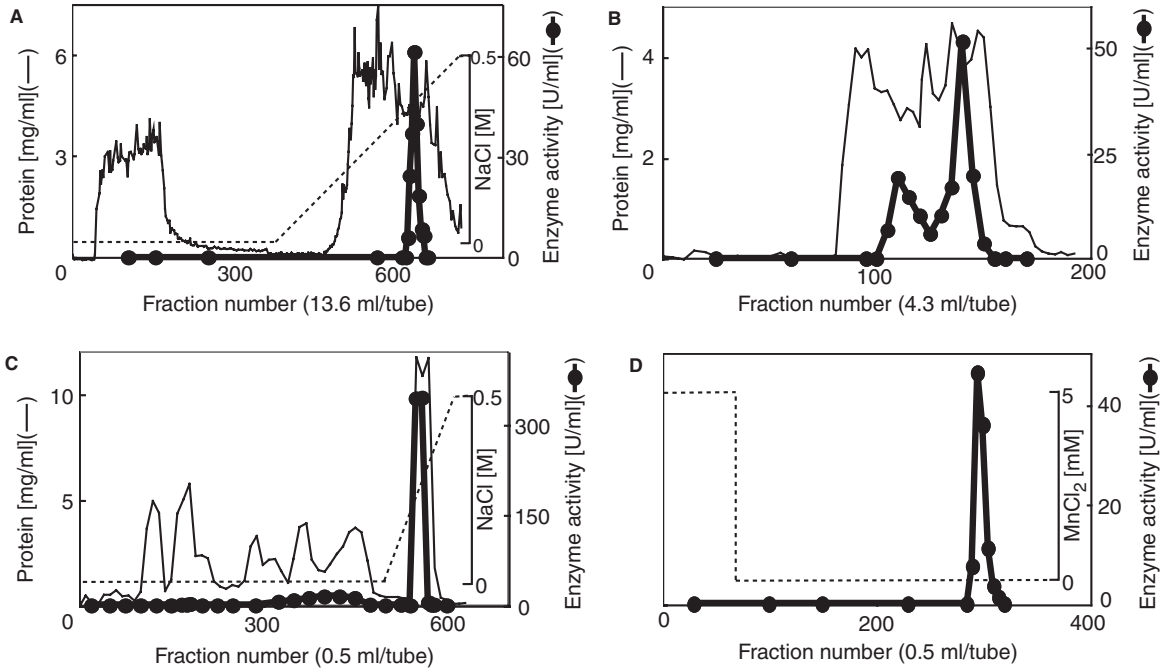

Fig. 2. **Purification of β-D-glucoside α-1,3-D-xylosyltransferase from bovine liver.** Chromatography was performed as described in 'Materials and methods'. β-D-Glucoside α-1,3-D-xylosyltransferase activity was measured with Glcβ-R and UDP-Xyl as substrates. (A) DEAE-Sephacel chromatography of the microsomal fraction of bovine liver. (B) Sephacryl S-200 gel filtration of the active fractions (fractions 650-674) in (A). (C) MonoQ chromatography of the active fractions (fractions 130-145) in (B). (D) UDP-hexanolamine agarose chromatography of the active fractions (fractions 540–575) in (C). The protein content of each fraction in (D) was not determined.

Table 1. **Summary of purification of β-D-glucoside α-1,3-D-xylosyltransferase from bovine liver.**

Step	Protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)	Purification (-fold)
Microsomal fraction	9100	6600	0.73	100	1
DEAE-Sephacel	1100	6100	5.5	91	7.6
Sephacryl S-200	410	6800	17	103	23
Mono Q	22	1100	50	17	69
UDP-hexanolamine	0.014	63	4500	0.95	6200

pattern in subsequent chromatography (data not shown), it is likely that the earlier peak is a dimer of the protein in the later peak. The more abundant later peak was used for further purification. The total activity in this peak increased from the previous step (Fig. 2B and Table 1). A possible explanation for this observation is that a α-xylosidase and/or a β-glucosidase which degraded the products were removed in this step. UDP-hexanolamine agarose affinity column chromatography was the most effective step for purification of this enzyme (Fig. 2D and Table 1). This chromatography step resulted in 90-fold purification with 6% recovery of the enzyme activity. Starting with 1 kg of bovine liver, β-D-glucoside α-1,3-D-xylosyltransferase was purified 6200-fold with a final recovery of 0.95% (Table 1).

The purified enzyme gave a single band on native-PAGE (Fig. 3A) and enzyme activity was associated with the protein band. By isoelectric focusing gel electrophoresis, the purified xylosyltransferase migrated as one major component which coincided with the xylosyltransferase activity (Fig. 3B). These results demonstrate that β-D-glucoside α-1,3-D-xylosyltransferase was purified to homogeneity.

The optimum pH of enzyme activity was around pH 7 (Fig. 4A). The effect of divalent cations on the enzyme activity was studied (Fig. 4B) and this showed that Mn^{2+} activated the enzyme, while Co^{2+}, Cu^{2+}, Fe^{2+} and Ni^{2+} inhibited enzyme activity. EDTA also inhibited activity.

Substrate Specificity of the Purified β-D-Glucoside α-1,3-D-Xylosyltransferase—Acceptor substrate specificity: The acceptor substrate specificity of the purified xylosyltransferase was examined with the following substrates; Glc, Glcβ-R, Glcβ-Ser, Glcβ-EGF, reduced and alkylated Glcβ-EGF, Xylα1-3Glc and Xylα1-3Glcβ-R. UDP-Xyl was used as a donor substrate. The initial velocities for these substrates are listed in Table 2. The purified enzyme transferred a xylose residue to Glc and Glcβ-R to some extent. The enzyme activity with Glcβ-EGF was 92- and 16-fold higher than those with Glc and Glcβ-R, respectively. The initial velocity for reduced and alkylated Glcβ-EGF was 12-times lower than that for Glcβ-EGF. These results show that the enzyme recognizes a

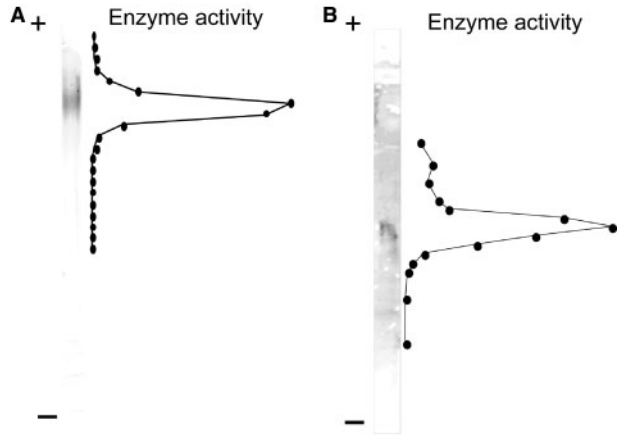

Fig. 3. **Native-PAGE and isoelectric focusing gel electrophoresis of purified β-D-glucoside α-1,3-D-xylosyltransferase.** (A) Native-PAGE of the purified enzyme. The enzyme activity of gel slices was measured using Glcβ-R and UDP-Xyl as substrates. (B) Isoelectric focusing gel electrophoresis of the purified enzyme. The enzyme activity of each gel slice was measured.

properly folded EGF-like domain. Glcβ-Ser was not a good acceptor substrate, indicating that the enzyme requires peptide portion for proper recognition rather than just a glucose residue attached to a Ser. A Lineweaver–Burk plot analysis of the xylosyltransferase gave an estimate of apparent K_m values for Glcβ-EGF and Glcβ-R of 34 μM and 4.2 mM, respectively. Apparent V_{max} values for Glcβ-EGF and Glcβ-R were calculated as 0.53 and 2.0 nmol h^{-1} mg^{-1} protein, respectively. These results are consistent with the result of the initial velocities against Glcβ-EGF and Glcβ-R. The transfer of a xylose residue to Xylα1-3Glc and Xylα1-3Glcβ-R by this enzyme was not detected under the conditions used (Table 2). This clearly shows that the purified β-D-glucoside α-1,3-D-xylosyltransferase is different from the α-D-xyloside α-1,3-D-xylosyltransferase (*10*, *11*).

Donor substrate specificity: The initial velocities of the enzyme for UDP-Xyl and UDP-Glc were 85 and <0.01 nmol h^{-1} mg^{-1} protein, respectively, when Glcβ-R was used as an acceptor substrate. The structural difference between UDP-Xyl and UDP-Glc is found at the C-5 position, suggesting that the hydroxymethyl group at the C-5 position inhibits recognition of donor substrate by the enzyme.

DISCUSSION

β-D-glucoside α-1,3-D-xylosyltransferase involved in the biosynthesis of Xylα1-3Xylα1-3Glcβ-*O*-Ser has been purified from bovine liver for the first time. Thus far, several α-xylosyltransferases have been reported including this enzyme (*10*), α-D-xyloside α-1,3-D-xylosyltransferase involved in the biosynthesis of Xylα1-3Xylα1-3Glcβ-*O*-Ser (*11*), β-D-glucoside α-1,6-D-xylosyltransferase involved in xyloglucan synthesis (*21*, *22*) and α-L-fucoside α-1,3-D-xylosyltransferase involved in pectin synthesis (*23*). However, this is the first report of purification of a α-xylosyltransferase.

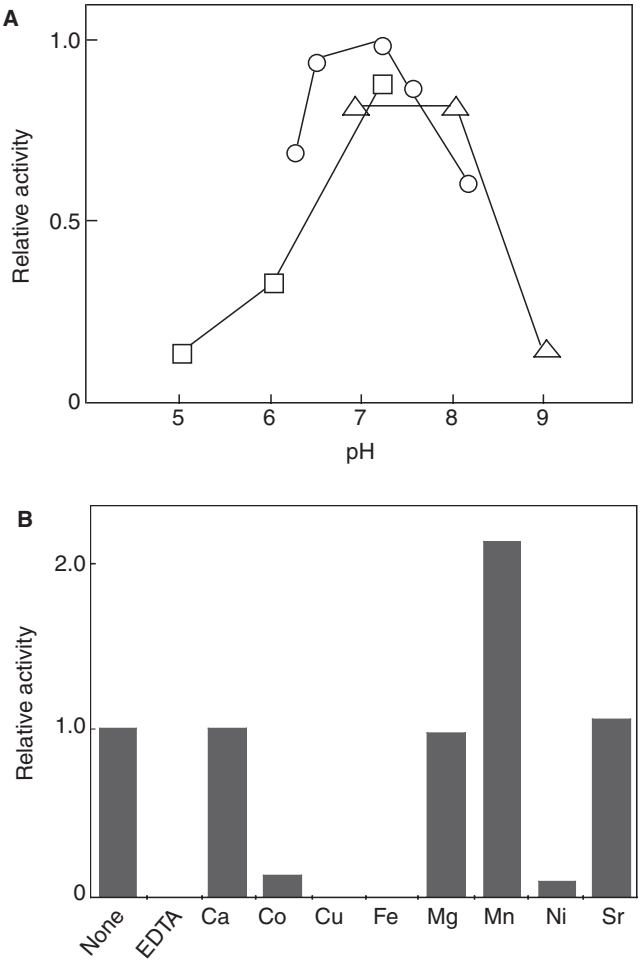

Fig. 4. **pH and divalent cation dependence of purified β-D-glucoside α-1,3-D-xylosyltransferase.** Enzyme activity was measured using Glcβ-R and UDP-Xyl as substrates. (A) pH dependence of the purified enzyme. The buffers used were 50 mM Mes-NaOH (open square), HEPES-NaOH (open circle), and Tris-HCl (open triangle). The value obtained at pH 7.0 was taken as 1.0. (B) Divalent cation dependence of the purified enzyme. Enzyme activity was measured in the presence of 20 mM of various divalent cations as chloride salt.

Table 2. **Initial velocity of purified β-D-glucoside α-1,3-D-xylosyltransferase against various acceptor substrates. The assay methods are described in 'Materials and methods'.**

Acceptor substrate	Initial velocity (nmol h^{-1} mg^{-1} protein)
Glc	12
Glcβ-R	70
Glcβ-Ser	6.9
Glcβ-EGF	1100
the reduced and alkylated Glcβ-EGF	90
Xylα1-3Glc	<0.5
Xylα1-3Glcβ-R	<0.2

Differentiation between β-D-glucoside α-1,3-D-xylosyltransferase and α-D-xyloside α-1,3-D-xylosyltransferase was demonstrated in terms of fractionation and pH dependence (*10*). In addition, this study shows that the β-D-glucoside α-1,3-D-xylosyltransferase was different from α-D-xyloside α-1,3-D-xylosyltransferase with regard to substrate specificity (Table 2).

The β-D-glucoside α-1,3-D-xylosyltransferase was shown to have strict substrate specificity (Table 2). This enzyme recognizes not only the glucose portion but also the tertiary structure of the EGF-like domain even though it transfers xylose to a glucose residue and not to peptide. The consensus peptide sequence for modification by Xylα1-3Xylα1-3Glcβ trisaccharide was identified as C-X-S-X-P-C in which the two cysteine residues are involved in formation of disulfide bonds in the EGF-like domain (*2*). The tertiary structure around this consensus sequence must be recognized by β-D-glucoside α-1,3-D-xylosyltransferase as is the case for peptide *O*-glucosyltransferase (*12*). Fringe, which transfers *N*-acetylglucosamine to fucose on the EGF-like domain, also appears to recognize both a sugar moiety (fucose) and the peptide portion of some EGF-like domains (*24*). Strict substrate specificity of β-D-glucoside α-1,3-D-xylosyltransferase may need for some biological functions as the case for Fringe. Unusual carbohydrate modifications are often related to a specific biological event (*25*), therefore, the unique glucosyl-*O*-serine type modifications may have some biological role. Molecular cloning of the gene encoding this enzyme based on the structural information of the enzyme purified in this study will allow us to provide a tool for clarifying the function of this unique modification.

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