

Purification and Specificity of β 1,2-Xylosyltransferase, an Enzyme That Contributes to the Allergenicity of Some Plant Proteins*

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The enzyme that transfers D-xylose from UDP-xylose to the β -linked mannose of plant N-linked oligosaccharides was purified about 51,000-fold to apparent homogeneity from soybean microsomes. On SDS gels, two proteins of 56 and 59 kDa were detected and both were labeled to the same extent by the photoaffinity label, 5-N₃-UDP-[³²P]xylose. Labeling of both proteins was inhibited by cold UDP-xylose, but not by UDP-glucose. The amount of 5-N₃-UDP-[³²P]xylose that bound to the two protein bands was greatly increased in the presence of oligosaccharide acceptors. The best acceptor for xylose transfer and for stimulation of UDP-xylose binding was GlcNAc₂Man₃GlcNAc₂-T, but GlcNAc₁Man₃GlcNAc₂, with the GlcNAc on the 3-branch, was also a good acceptor and a good stimulator. A number of other N-linked oligosaccharides were poor acceptors, especially those with galactose units at the nonreducing termini. Many of the properties of this enzyme have been described, and the product of the reaction of UDP-xylose and GlcNAc₂Man₃(GlcNAc)₂ was characterized as GlcNAc β 1,2Man α 1,6(GlcNAc β 1,2Man α 1,3)(Xyl β 1,2)Man β 1,4GlcNAc₂-T by chemical and NMR methods.

Immediate hypersensitivity reactions to foods occurs in 6–8% of children and about 1% of adults (1), and these reactions are mediated by the production of IgE antibodies to glycoproteins in these foods (2). The majority of allergies are to foods of plant origin, and a number of allergenic proteins have been identified from peanut, wheat, barley, rye, and soy (3–7). While linear amino acid sequences and conformational structures of proteins have been identified as IgE-binding epitopes (8), there is increasing evidence (9–11) that specific carbohydrate structures may also be important as allergens.

While there is some similarity in peptide sequence between the different allergenic proteins, a common characteristic is that most contain N-linked oligosaccharides having a β 1,2-linked xylose attached to the β -linked mannose of the core structure (12). Furthermore, in wheat and barley glycoproteins, xylose appears to be important for allergenicity, since those proteins containing a β 1,2-xylose were more reactive *in vivo* and *in vitro* than deglycosylated proteins (13). In addition, IgE from allergic patients recognized an endo-Lys C peptide containing the glycan, and this recognition was lost upon deglycosylation (14).

The enzyme that adds xylose to these N-linked glycoproteins is the plant β 1,2-xylosyltransferase. The identification of this enzyme in microsomes from *Phaseolus vulgaris* cotyledons (15), and from sycamore cells of *Acer pseudoplatanus* (16) has been reported, but there are no reports on the purification or properties of the enzyme. The substrate specificity for acceptor oligosaccharide was examined with particulate enzyme preparations from these two plant sources and in both cases, the enzymes acted on acceptors having a β 1,2-GlcNAc residue on the Man α 1,3-arm, but GlcNAc-Man₅(GlcNAc)₂ was not a good acceptor. Thus, it was postulated that in the processing pathway, xylose is added after the mannosidase II step (15). However, with the xylosyltransferase from the snail, *Lymnaea stagnalis*, the biantennary oligosaccharide containing a galactose in β 1,4 linkage to the GlcNAc on the Man α 1,3-arm was also an acceptor, although it was much less efficient (17). These studies suggested that xylose is added after the removal of the two mannoses on the Man α 1,6-arm, but before galactoses are added to the GlcNAc residues.

The xylose units on these N-linked oligosaccharides may play a critical role in allergenicity, and may also be important in regulating the structure of the oligosaccharide chains and the targeting of these proteins to various organelles such as storage bodies. Thus, it is important to purify this enzyme to study its properties and specificities in the absence of interfering activities and possible inhibitors. As reported here, we have purified the soybean xylosyltransferase about 51,000-fold to apparent homogeneity, and determined the substrate specificity and other properties of this enzyme.

EXPERIMENTAL PROCEDURES

Materials—UDP-[³H]xylose (7.5 Ci/mmol) and GDP-L-[³H]fucose (7.1 Ci/mmol) were purchased from American Radiolabeled Co. [²-³H]Mannose (15 Ci/mmol) and [⁶-³H]galactose (20 Ci/mmol) were from NEN-Life Science Products, and ³²P-inorganic phosphate was from ICN. Radioactive compounds were checked by chromatography before use. Various adsorbents were obtained from the following sources: DEAE-cellulose (DE-52) from Whatman Co. and UDP-hexanamine-Sepharose, iminodiacetic acid-Sepharose 6B, and Reactive Yellow 3-agarose from Sigma. The following materials were obtained from Bio-Rad: SDS, acrylamide, bisacrylamide, Coomassie Blue, protein assay reagent, and Bio-Gel P-4. All other chemicals were from reliable chemical sources and were of the best grade available.

Assay for Xylosyltransferase Activity—Reaction mixtures for the assay of β 1,2-xylosyltransferase contained the following components in a final volume of 25 μ l: 100 mM MES¹ buffer, pH 7.0, 10 mM MnCl₂, 0.2% Triton X-100, 5 mM ATP, 0.2 mM UDP-[³H]xylose (15,000 cpm), and 4 nmol of oligosaccharide acceptor (usually GlcNAc₂Man₃GlcNAc₂-tyrosinamide or the asialo-agalacto-oligosaccharide from transferrin).

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¹ The abbreviations used are: MES, 4-morpholineethanesulfonic acid; RP-HPLC, reverse phase-high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

The reaction was started by the addition of 5 μ l of the enzyme solution, and incubations were usually at 37 °C for 1 h. The reaction was stopped by the addition of 100 μ l of 1% acetic acid and the reaction mixtures were chromatographed on a column of Bio-Gel P-4, prepared by placing 10 ml of gel into a 10-ml plastic pipette having a total column volume of 13 ml. The columns were equilibrated with 1% acetic acid, and the product was separated from substrates by elution with 1% acetic acid containing 0.2 M NaCl. The xylosylated oligosaccharide product emerged within 2.5–7 ml of elution, whereas the UDP-xylose substrate eluted after 9 ml of elution fluid.

When more purified enzyme preparations were used in the assays, the radiolabeled product could be separated from the radioactive substrate by ion-exchange chromatography on an Amberlite column (or a column of Dowex-1-Cl[−]) contained in a Pasteur pipette. In this case, the substrate, UDP-xylose, binds to the column whereas the product ([³H]xylose-oligosaccharide) emerges in the wash. Thus, the wash can be counted as a measure of radioactive xylose transferred to oligosaccharide. In these assays, it is important to be certain that the enzyme preparation is free of degradative enzymes that cleave UDP-xylose to free xylose.

Preparation of Oligosaccharide Substrates for the Enzyme—Asialoagalacto-oligosaccharide was prepared from human transferrin by the following procedure. Apo-transferrin was obtained from Sigma and was exhaustively digested with Pronase. Thus, 500 mg of transferrin were dissolved in 5 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM CaCl₂, and 2 mg/ml Pronase were added. The incubations were allowed to proceed for 24 h under a toluene atmosphere and another 2 mg/ml Pronase were added for an additional 24 h. At the end of this time, the reaction mixture was heated at 100 °C for 5 min, and the glycopeptides were purified on a 2 × 200-cm column of Bio-Gel P-4. The glycopeptide was then heated at 80 °C for 4 h in 2 N acetic acid to remove the sialic acid, and the asialo-glycopeptide was again purified on the Bio-Gel P-4 column. The asialo-glycopeptide was then incubated with 0.2 units of jack bean β -galactosidase to remove the terminal galactose residues. This final product was purified on the Bio-Gel P-4 column, and its concentration was determined by the anthrone method, using mannose as a standard.

Tyrosinamide biantennary oligosaccharides, with or without a core fucose, were prepared from bovine fetuin and porcine fibrinogen as described previously (18, 19). A biantennary oligosaccharide (Fig. 4, structure 1) was converted into various xylosyltransferase acceptor substrates as follows: Oligosaccharide 1 (1 μ mol) was treated with 16.5 milliunits of bovine testis β -galactosidase (EC 3.2.1.23) from Boehringer Mannheim in 100 μ l of 100 mM phosphate-citrate buffer, pH 4.3, at 37 °C for 4 h. The reaction was stopped by boiling for 5 min. The partially degalactosylated biantennary structure was reacted with 500 milliunits (2 μ l) of hexosaminidase (EC 3.2.1.52) from V-labs, for 12 h at 37 °C. The four product peaks were purified on a semi-preparative RP-HPLC C-18 column run at 50 °C, and the column was eluted at 3 ml/min with 14% acetonitrile in 0.1% acetic acid. Peaks were detected by monitoring at 280 nm. The second and third peaks were treated individually with 3 milliunits of β -galactosidase in 10 μ l of 100 mM phosphate-citrate buffer, pH 4.3, for 24 h at 37 °C. The final products were purified on a polymeric PRP-1 RP-HPLC column, which was eluted with 13% acetonitrile in 0.1% acetic acid at 2 ml/min. Peaks were detected at 280 nm. Fractions were collected and freeze-dried. The overall yield was about 30%. The identity of each oligosaccharide product was established by NMR and electrospray mass spectrometry as described previously (18, 19).

Large scale synthesis of xylosyltransferase product was performed by incubating oligosaccharide 4 (200 nmol) with 1.9 μ mol of UDP-xylose and 1 milliunits of xylosyltransferase in 665 μ l of buffer (100 mM MES buffer, pH 7.0, 10 mM MnCl₂, 0.25% Triton X-100, and 5 mM ATP) for 24 h at 37 °C. The reaction progress was monitored by RP-HPLC, which demonstrated a single product that eluted 1 min earlier than the substrate. At completion, the product peak was isolated from analytical RP-HPLC and characterized by proton NMR.

Preparation of 5-N₃-UDP-[³²P]xylose Substrate Analog—5-N₃-UDP-[³²P]xylose was prepared from N₃-UDP-[³²P]glucuronic acid using the purified UDP-glucuronic acid carboxylase as described previously (20). This procedure involved the synthesis of 5-N₃-UDP-[³²P]glucose from glucose-1-³²P and N₃-UTP by previously published methods (21), conversion of this 5-N₃-UDP-[³²P]glucose to 5-N₃-UDP-[³²P]glucuronic acid by the UDP-glucose dehydrogenase, and then decarboxylation of the N₃-UDP-[³²P]glucuronic acid to 5-N₃-UDP-[³²P]xylose using the partially purified UDP-glucuronic acid carboxylase from wheat germ (20).

Preparation of Microsomes from Soybean Cells—Soybean cells were routinely grown for 7 days at 28 °C in suspension culture in 500-ml

TABLE I
Solubilization of xylosyltransferase by detergents

Detergent tested	Xylose transferred to acceptor ^a by	
	Particulate suspension ^a	High speed supernatant
	cpm	
None	1971	325
Triton X-100, 0.5%	2481	1189
Triton X-100, 1%	2689	2188
Triton X-100, 2%	2695	2297
Taurocholate, 1%	1570	1574
Deoxycholate, 1%	316	197
CPC, ^b 1%	1341	64
Octylglucoside, 1%	2395	210
CHAPS, 1%	2168	541
SDS, 1%	144	27

^a In each case, the amount of protein was adjusted to a common value (10 mg/ml), so that each enzyme solution could be directly compared.

^b CPC, cetylpyridinium chloride.

Erlenmeyer flasks containing 100 ml of B-5 medium (22). This medium has the following composition (mg/liter): KNO₃, 3000; (NH₄)₂SO₄, 134; MgSO₄·7H₂O, 500; CaCl₂·7H₂O, 150; NaH₂PO₄·H₂O, 150; MnSO₄·H₂O, 100; KI, 0.75; H₃BO₃, 3; ZnSO₄·7H₂O, 2; CuSO₄, 0.025; Na₂MoO₄·2H₂O, 0.25; CoCl₂·6H₂O, 0.025; EDTA-ferric sodium salt, 28; myoinositol, 100; thiamine-HCl, 10; nicotinic acid, 1; pyridoxine-HCl, 1; sucrose, 20,000; 2,4-dichlorophenoxyacetic acid, 0.5. The final solution was adjusted to pH 5.5. Cells were harvested by filtration at the mid-log phase of growth and washed well with water and then with Buffer A (25 mM HEPES, pH 7.2, containing 0.25 M sucrose, 0.5 M dithiothreitol, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride). About 100 g of washed cells were suspended in 300 ml of Buffer A and disrupted by sonic oscillation (three 5-min periods of sonication at 80% output, with 10 min of cooling on ice between each sonication). The suspension was centrifuged at 3000 × g for 15 min to remove unbroken cells, nuclei, and mitochondria, and the supernatant liquid was then centrifuged at 100,000 × g to isolate the microsomal fraction. The microsomal pellet was resuspended in Buffer B (25 mM HEPES, pH 7.2, containing 10% glycerol, 0.1 M NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride).

RESULTS

Purification of the Xylosyltransferase—The microsomal fraction from soybean cells transferred xylose from UDP-xylose to GlcNAc₂Man₃(GlcNAc)₂-tyrosinamide (or peptide). To purify this enzymatic activity, it was necessary to solubilize the xylosyltransferase from the microsomes. A variety of detergents were tested at different concentrations for their ability to solubilize the enzyme and maintain its stability. The results of these experiments are shown in Table I. In each case, the enzyme was mixed with detergent, and the mixture was stirred on ice for 15 min and then centrifuged at high speed for 30 min to pellet the membranes. The enzymatic activity in the supernatant liquid was then measured in the usual transferase assay. As seen in Table I, the activity was best solubilized from the membranes by treatment with either 1% or 2% Triton X-100 in Buffer B. Thus, at either concentration of Triton X-100, at least 80% of the enzymatic activity was recovered in the supernatant liquid, and the enzyme appeared to be fairly stable under these conditions.

As a result of these experiments, a large scale preparation of microsomes was done from 3 kg of soybean cells as described under "Experimental Procedures." The microsomes (2900 mg of protein in 250 ml of buffer) were treated with 1% Triton X-100 for 30 min, and the mixture was then centrifuged at 100,000 × g for 1 h. The pellet was resuspended in Buffer B containing 1% Triton X-100, and centrifuged again at 100,000 × g for 30 min. The two supernatant fractions were pooled for further purification steps. Eighty to ninety percent of the xylosyltransferase activity was recovered in the pooled supernatant fractions after this treatment, whereas in control membranes that were stirred on ice for 30 min in buffer but without detergent, more

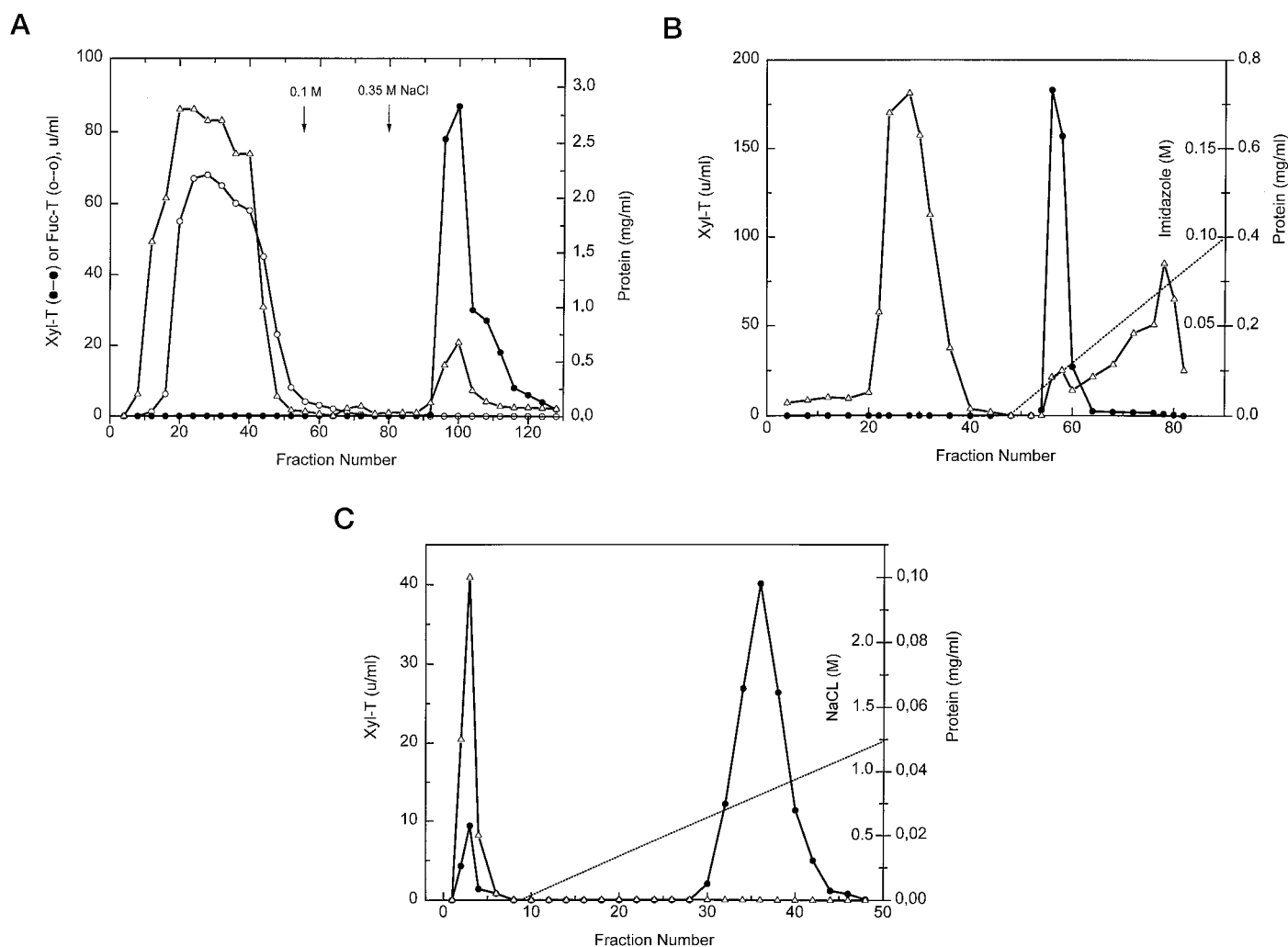


FIG. 1. Steps in the purification of the soybean xylosyltransferase. A, separation on a column of cellulose-phosphate; B, chromatography on iminodiacetic acid-Sepharose; C, purification on a column of UDP-hexanolamine-Sepharose. Each chromatographic procedure was done as described in the section on enzyme purification. Various fractions from each column were assayed for protein (Δ), for xylosyltransferase ($\bullet$), and for fucosyltransferase ($\circ$).

than 85% of the activity was pelleted when centrifuged at $100,000 \times g$ for 1 h. In many cases, the supernatant liquids were used immediately in further purification steps, but they could be stored at -80°C for 1 month with little loss of xylosyltransferase activity.

The solubilized enzyme was then divided into two equal portions, and each was placed on a 2.5×30 -cm column of cellulose phosphate that had been equilibrated with Buffer C (25 mM HEPES buffer, pH 7.2, containing 10% glycerol, 0.15% Triton X-100, and 0.5 mM dithiothreitol). After applying the enzyme solution, the column was washed with Buffer C, followed by Buffer C containing 100 mM NaCl until no more protein was eluted. As shown in Fig. 1A, the column was then washed with Buffer C, containing 350 mM NaCl and the xylosyltransferase activity was eluted at this salt concentration. Fractions were assayed for transferase activity and active fractions were pooled. This step gave a 20-fold increase in specific activity with an 65% recovery of total activity (Table II).

The active fractions from the phosphocellulose column (about 250 ml) were pooled and concentrated to about 30 ml. Sodium chloride was added to this solution to a final concentration of 0.5 M, and the mixture was loaded onto a 1.5×15 -cm column of iminodiacetic acid-Sepharose 6B that had been equilibrated with Buffer D (25 mM HEPES buffer, pH 7.2, containing 10% glycerol, 0.15% Triton X-100, 0.5 mM dithiothreitol, and 0.5 M

TABLE II
Summary of the soybean cell xylosyltransferase purifications

Step	Total protein	Total units ^a	Specific activity	Purification factor	Recovery
	mg	units/mg		-fold	%
Microsomal fraction	2894	25,127	8.7	1	100
Phosphocellulose	84.2	16,306	193	22	64.9
Iminodiacetic acid-Sepharose	6.5	13,272	2041	234	52.8
Yellow 3-agarose	2.1	8105	3859	443	32.2
UDP-hexanolamine	0.006 ^b	2672	445,333	51,187	10.6

^a A unit is defined as the amount of enzyme which transfers 1 nmol of xylose to acceptor per hour.

^b This amount of protein was estimated by comparing the staining of the xylosyltransferase on SDS gels to staining of known amounts of bovine serum albumin.

NaCl). The column was washed with Buffer D until no more protein emerged, and the xylosyltransferase was eluted with a linear gradient of imidazole (0–0.15 M) in a total volume of 600 ml of Buffer D (Fig. 1B). This column gave about a 10-fold purification with a recovery of about 85%. Active fractions were pooled, concentrated to about 25 ml on an Amicon filtration apparatus, and dialyzed against Buffer C, containing 15% glycerol.

The dialyzed enzyme from the iminodiacetic acid-Sepharose

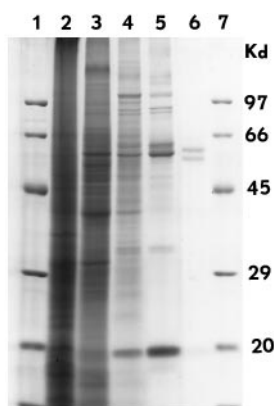


FIG. 2. SDS-PAGE analysis of the protein profiles at various stages of purification. Lanes are as follows: lanes 1 and 7, protein standards; lane 2, microsome fraction (400 μ g of protein); lane 3, active fraction from phosphocellulose (250 μ g of protein); lane 4, active fraction from iminodiacetic acid-Sepharose (34 μ g of protein); lane 5, active fraction from Reactive Yellow 3 Sepharose (21 μ g of protein); lane 6, active fraction from UDP-hexanolamine Sepharose (1.5 μ g of protein).

was placed on a 2.5×15 -cm column of Reactive Yellow 3-Sepharose, that had been equilibrated with Buffer C but contained 15% glycerol. The column was washed with Buffer C until no more protein emerged, and bound protein was then eluted with a linear gradient of 0–0.25 M NaCl in Buffer C, containing 15% glycerol. Fractions of 10 ml were collected, and active fractions were pooled and concentrated to 5 ml on an Amicon filtration apparatus. To decrease the concentration of NaCl, 15 ml of Buffer C containing 15% glycerol were added to the concentrated enzyme solution and it was again concentrated to 5 ml on the Amicon apparatus.

The concentrated enzyme fraction from the Reactive Yellow 3 column was applied to a 1×15 -cm column of UDP-hexanolamine-Sepharose that had been equilibrated with Buffer C containing 20% glycerol. The column was first washed with 100 ml of this buffer and then was eluted with 300 ml of a linear gradient of 0–1.5 M NaCl in the same buffer (Fig. 1C). This affinity step increased the specific activity by a factor of about 115-fold with a recovery of about 30%.

Using these purification steps, the xylosyltransferase was purified about 51,000-fold from the solubilized enzyme fraction with a final recovery of about 10%. The summary of the purification procedure is presented in Table II. Fig. 2 shows the protein profiles at various steps in the purification procedure as determined by SDS-PAGE. At the final step of purification, two major protein bands of 56 and 59 kDa were detected on SDS gels. To determine which band was really the xylosyltransferase, the enzyme fraction from UDP-hexanolamine was incubated with 5- N_3 -UDP-[^{32} P]xylose in 50 mM Tris buffer, pH 7.2, at room temperature for 60 s, and then the mixture was irradiated for 60 s with a hand-held UV lamp (254 nm UVP-11) to activate the azido group. The incubation mixture was then adjusted to 5% with trichloroacetic acid to precipitate the protein, and the protein pellets were suspended in a denaturing gel-loading buffer and subjected to SDS-PAGE.

The radiolabeled proteins were then located by autoradiography as shown in Fig. 3. Lanes 1–3 of Fig. 3A show that both the 56- and 59-kDa proteins became labeled in a concentration-dependent manner when they were exposed to increasing amounts of the UDP-xylose photoaffinity probe and then exposed to UV light. On the other hand, the incubation mixture that was not exposed to UV light did not show any labeled protein bands (data not shown). Lanes 4 and 5 of Fig. 3A show that when labeling was done in the presence of 10 or 100 μ M unlabeled UDP-xylose, the amount of radioactivity associated

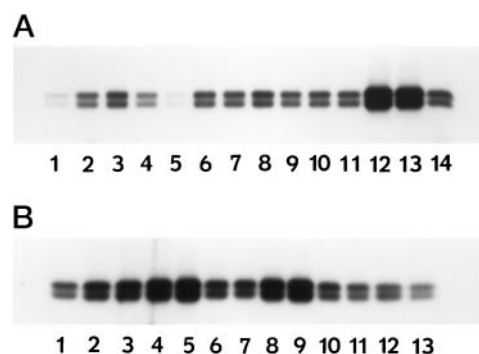


FIG. 3. Photolabeling studies of xylosyltransferase with 5- N_3 -UDP-[^{32} P]xylose. Photolabeling was done as described under "Results." In A, labeling with 5- N_3 -UDP-[^{32} P]xylose is concentration-dependent (lanes 1–3) and is inhibited by unlabeled UDP-xylose but not by other nucleoside-diphosphate sugars. Thus, lanes are as follows: lanes 1–3, 1, 2.5, and 5 μ l of the UDP-xylose probe; lanes 4 and 5, 5 μ l of the UDP-xylose probe with 10 and 100 μ M unlabeled UDP-xylose; lanes 6 and 7, 5 μ l of UDP-xylose probe with 10 and 100 μ M UDP-galactose; lanes 8 and 9, 5 μ l of UDP-xylose probe with 10 and 100 μ M UDP-glucose; lanes 10 and 11, 5 μ l of UDP-xylose probe with 10 and 100 μ M UDP-glucuronic acid; lanes 12–14, 5 μ l of UDP-xylose probe with 80 μ M oligosaccharide 4 (lane 12), oligosaccharide 9 (lane 13), or oligosaccharide 8 (lane 14). In B, the effects of various acceptor oligosaccharides on labeling of the enzyme by 5- N_3 -UDP-[^{32} P]xylose is shown as follows. All lanes contained 5 μ l of the UDP-xylose probe. Lane 1, no acceptor; lanes 2–5, 4, 10, 30, and 80 μ M oligosaccharide 4; lanes 6–9, 4, 10, 30, and 80 μ M oligosaccharide 9; lane 10, 80 μ M oligosaccharide 8; lane 11, 80 μ M core oligosaccharide 7; lane 12, 80 μ M Man $_9$ (GlcNAc) $_2$ -T; lane 13, 80 μ M oligosaccharide 1.

with both the 56- and 59-kDa bands was greatly diminished, indicating that labeling is specific for UDP-xylose. Furthermore, incubation of enzyme and UDP-xylose probe in the presence of other nucleoside diphosphate sugars, such as UDP-galactose (lanes 6 and 7), UDP-glucose (lanes 8 and 9), or UDP-glucuronic acid (lanes 10 and 11) did not result in significant inhibition of labeling.

Interestingly enough, addition of oligosaccharide 4 (lane 12) or oligosaccharide 9 (lane 13) to incubations with 5- N_3 -UDP-[^{32} P]xylose resulted in a dramatic increase in the labeling of both protein bands, but oligosaccharide 8, having a single GlcNAc on the mannose-linked α 1,6 to the β -linked mannose, did not stimulate labeling. Since the native enzyme has a molecular mass of about 50 kDa by gel filtration (see below), and two protein bands of 56 and 59 kDa are detected by photolabeling on SDS gels, it seems likely that the 56-kDa band arises by limited proteolysis of the 59-kDa band. Sequencing and cloning studies should indicate the relationship of these two xylosyltransferase activities.

Since the oligosaccharides caused such a great increase in labeling of xylosyltransferase by 5- N_3 -UDP-[^{32} P]xylose, it was of interest to compare their stimulation as a function of oligosaccharide concentration. Thus, as shown in Fig. 3B, various amounts of each oligosaccharide were added to incubations of enzyme plus 5 μ l of the 5- N_3 -UDP-[^{32} P]xylose. It can be seen from lanes 2–5 that addition of oligosaccharide 4, at 4, 10, 30, and 80 μ M, gave a concentration-dependent increase in labeling, as compared with lane 1, which is without acceptor oligosaccharide. Lanes 6–9 show that oligosaccharide 9, with a single GlcNAc on the mannose-linked α 1,3 to the β -linked mannose, also gave a concentration-dependent increase in labeling, but this oligosaccharide was significantly less effective in stimulating labeling than was oligosaccharide 4. On the other hand, oligosaccharide 7 (core structure, *i.e.* Man $_9$ (GlcNAc) $_2$) or oligosaccharide 1 with two terminal galactoses did not cause any stimulation in labeling (even at 80 μ M). These data are in agreement with studies shown in Table III on the ability of the

TABLE III
Specificity of plant β 1,2-xylosyltransferase for oligosaccharide acceptor

Oligosaccharide acceptor	Activity ^a with	
	Purified enzyme	Particulate enzyme
	<i>cpm xylose transferred</i>	
No acceptor	35	98
No. 1 (see Fig. 4)	26	59
No. 10 (see Fig. 4)	3212	2387
No. 4 (see Fig. 4)	3578	3648
GlcNAc-Man ₃ -5'-isomer (no. 8)	69	
GlcNAc-Man ₃ -5-isomer (no. 9)	1194	
No. 11 (see Fig. 4)	68	56
No. 7 (see Fig. 4)	57	371
Man ₃ (GlcNAc) ₂	48	262
Transferrin		106
Asialo-agalacto-transferrin peptide	3215	2449

^a Incorporation of radioactivity from UDP-[³H]xylose into acceptor oligosaccharide was measured by gel filtration on Bio-Gel P-4.

various oligosaccharides to act as xylose acceptors. The results presented here suggest that the acceptor oligosaccharide binds to the enzyme first, and this binding then enhances the binding of the UDP-xylose photoprobe.

Stability of the Purified Enzyme—The most purified preparation of xylosyltransferase was examined for its stability under various conditions. The enzyme was stable for up to 90 days, when dissolved in 25 mM HEPES buffer, pH 7.0, containing 10% glycerol, 0.2% Triton X-100, and 1 mM dithiothreitol, and kept either in an ice bucket at 4 °C or at -20 °C (data not shown). After a month of storage under these conditions and at either temperature, at least 80% of the original enzyme activity still remained. Thus, storage of the purified enzyme in the above buffer solution does not appear to be a problem. The stability of the enzyme is, however, sensitive to changes in pH of the storage solution. Thus, above pH 7.5 and below pH 6.5, the stability of the enzyme rapidly declines.

Synthesis of Oligosaccharide Acceptor Substrates—Exoglycosidase trimming was used to convert biantennary substrates (see Fig. 4 for structures of oligosaccharides) into substructures that could serve as xylosyl acceptors. Treatment of substrate 1 with β -galactosidase resulted in its complete conversion into substrate 4, and subsequent treatment with β -hexosaminidase resulted in the formation of oligosaccharide 7. Likewise, β -galactosidase and β -hexosaminidase digestions were used to prepare oligosaccharides 10 and 11, starting with a core fucosylated biantennary oligosaccharide obtained from porcine fibrinogen.

Partial removal of galactose was accomplished by monitoring the β -galactosidase reaction by RP-HPLC, using an RP-HPLC C-18 column and eluting with 12% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min. Fluorescence was detected at an excitation of 275 nm and an emission of 305 nm. Oligosaccharide 1 resolved as a single peak (Fig. 5A) that was converted into four peaks at an intermediate time point in the β -galactosidase reaction (Fig. 5B). The numbers assigned to these peaks tentatively corresponded to the structures shown in Fig. 4. Hexosaminidase digestion was then used to convert oligosaccharide 2 into 5, 3 into 6, and 4 into 7A (Fig. 5C). As expected, oligosaccharide 1 resisted digestion by β -hexosaminidase, confirming that it was the biantennary structure presented in Fig. 4 (structure 1). Oligosaccharides 5 and 6 were isolated from the mixture and then treated again with β -galactosidase to prepare structures 8 and 9 (Fig. 5, D and E).

Electrospray mass spectrometry revealed that oligosaccharides 8 and 9 possessed identical masses of m/z 1375, consistent with the $M + 1$ of the structures proposed in Fig. 4. Proton NMR analysis of oligosaccharides 8 and 9 also revealed anomeric and *N*-acetyl resonances associated with the three GlcNAcs as well as the anomeric proton for Man 4 and 4'. However,

the chemical shift of each resonance was nearly identical for both isomers, resulting in an ambiguous identification of each isomer.

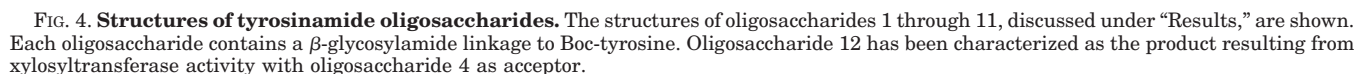
Isomers 8 and 9 (1 nmol) were treated for 24 h at 37 °C with 20 milliunits of an α -mannosidase (EC 3.2.1.24 from New England Biolabs) that cleaves α 1,2- and α 1,3-mannose linkages. Reactions were done in 50 mM sodium citrate buffer, pH 6.0, containing 5 mM CaCl₂ and 1 μ g of bovine serum albumin. The reactions were monitored by RP-HPLC with fluorescence detection as described above, which demonstrated a shift in the retention time for oligosaccharide 8 but not 9. This result established that oligosaccharide 8 possessed a terminal α 1,3-linked mannose residue, whereas the terminal α 1,6-linked mannose residue on oligosaccharide 9 was not removed by this enzyme. These data lead to the assignments shown in Fig. 4.

Properties of the Purified Xylosyltransferase—The incorporation of xylose from UDP-[³H]xylose into GlcNAc₂Man₃-(GlcNAc)₂-tyrosinamide by the purified enzyme was linear with time of incubation and with the amount of protein added to the incubation mixtures (data not shown). The pH optimum for enzyme activity was determined using HEPES buffer at a variety of different pH values. The pH profile showed a fairly symmetrical and sharp peak with optimum activity at pH 7.0 and considerably diminished activity above pH 7.5 and below pH 6.5 (data not shown). In terms of enzyme stability, we also found that the enzymatic activity is most stable when maintained at pH values around 7.0.

Gel filtration studies on Sephadex G-100 were done with the partially purified enzyme to estimate the molecular weight of the native enzyme. In these studies, the enzyme, eluted from phosphocellulose in 25 mM HEPES buffer, pH 7.0, containing 10% glycerol, 0.15% CHAPS, 0.05% deoxycholate, 1 mM dithiothreitol and 0.2 M NaCl, was concentrated to 1 ml and applied to a 2 $\times$ 100-cm column of Sephadex G-100. The column was eluted with the same buffer, and enzymatic activity emerged in the area, suggesting a molecular mass of 49 kDa. Thus, the xylosyltransferase appears to be a monomer with a molecular mass of 55–59 kDa.

Surprisingly, the purified xylosyltransferase did not show a requirement for a divalent cation. Thus, only a slight stimulation in the activity was observed when 1 mM MnCl₂ or MgCl₂ was added to the enzyme, or when the enzyme was dialyzed in the presence of EDTA, and activity was then measured in the presence of 1 mM MgCl₂ (11% stimulation) or 1 mM CaCl₂ (15% stimulation). There was also no stimulation in activity by various other divalent cations such as Ca²⁺, Co²⁺ or Fe²⁺, but Pb²⁺, Hg²⁺, and Fe³⁺ inhibited at 1 mM concentrations. Most glycosyltransferases require the presence of a divalent cation, but such a requirement does not appear to apply to the xylosyltransferase. Perhaps this enzyme has a bound metal ion that is not removed by dialysis against EDTA. Future studies with the purified enzyme will determine whether there is a bound metal ion at the catalytic site of this enzyme.

Substrate Specificity and Kinetic Constants of the Transferase—The specificity for acceptor substrate was determined using a variety of oligosaccharide substrates that varied in the degree of processing, or in terminal glycosylation. The structures of these oligosaccharides are shown in Fig. 4, and their ability to serve as acceptors is indicated in Table III. Xylosyltransferase activity was measured with both the solubilized enzyme preparation, and with the purified enzyme. The data demonstrate that the best acceptors are structures having two GlcNAc residues attached to the core mannose oligosaccharide, *i.e.* structures 4 and 10 of Fig. 4. Thus, the presence of L-fucose on the innermost GlcNAc has little effect on xylose transfer. However, the addition of galactose to the non-reducing terminal GlcNAc residues strongly inhibits activity (*i.e.* oligosaccha-



The substrate specificity of the xylosyltransferase for the nucleoside diphosphate sugar donor was also examined, using a variety of radiolabeled sugar nucleotides, as shown in Table IV. Each of these substrates was adjusted to have the same specific activity, and compared with each other at that activity. It can be seen that the purified enzyme was very specific for UDP-xylose, and no activity was detected with other uridine diphosphate sugars such as UDP-glucose. Furthermore, addition of unlabeled UDP-glucose, even at 5 mM concentrations, did not inhibit the incorporation of xylose into oligosaccharide, indicating that UDP-glucose is not recognized by this enzyme. With the solubilized enzyme preparation, both UDP-xylose and GDP-fucose were active as sugar donors for transfer to oligosaccharide, and some activity was also seen with UDP-GlcNAc. Purification and properties of these other transferases will be described in future publications.²

transfer of xylose to the β -linked mannose was examined as shown in Fig. 6. In this experiment, oligosaccharide 4 was used as the acceptor, since it was the most active acceptor. The data show that the reaction rate was proportional to the amount of acceptor added to concentrations of about 200 μM , and the K_m for acceptor oligosaccharide was determined to be about 75 μM . The other active acceptor, oligosaccharide 10, was also examined at various concentrations, and the K_m for this substrate was calculated to be about 150 μM (Fig. 6). The donor substrate in the reaction, *i.e.* UDP-xylose, was also tested at various concentrations, and the K_m for UDP-xylose was estimated to be about 80 μM (data not shown).

² Y. Zeng, G. Bannon, A. Elbein, manuscript in preparation.

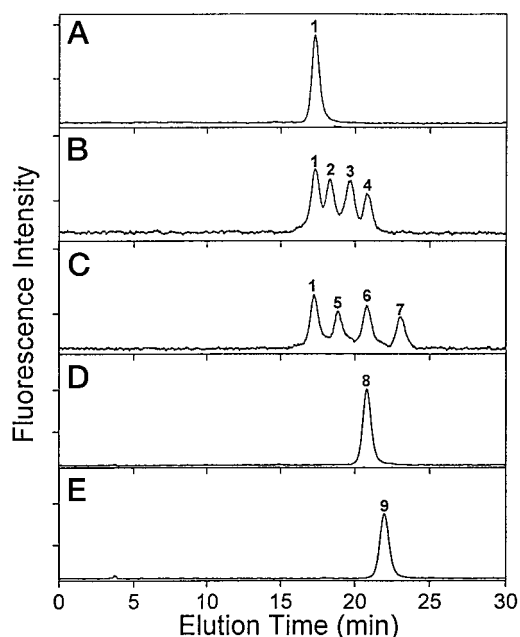


FIG. 5. RP-HPLC analysis of oligosaccharide trimming reactions. Asymmetric trimming of tyrosinamide biantennary oligosaccharide was monitored by RP-HPLC using fluorescence detection. Partial removal of galactose from oligosaccharide 1 (A) resulted in the formation of peaks 2, 3, and 4, as shown in B. Subsequent treatment with hexosaminidase removed terminal GlcNAc residues to form peaks 5, 6, and 7 (C). β -Galactosidase treatment of peaks 5 and 6 resulted in the formation of isomers 8 and 9 (D and E). The proposed structure of each peak is shown in Fig. 4.

TABLE IV
Specificity of nucleoside diphosphate sugar donor for xylosyltransferase

Enzyme (particulate fraction or purified xylosyltransferase (about 20 ng of protein) from UDP-hexanolamine-Sepharose) was incubated with radioactive sugar nucleotide (20,000 cpm; 200 μ M) and asialo-agalactotransferrin glycopeptide for 1 h at 37 °C. The reaction mixture was then separated on a Bio-Gel P-4 column, and radioactivity in oligosaccharide was determined.

Sugar nucleotide donor	Particulate enzyme	Purified enzyme
	cpm incorporated into oligosaccharide	
GDP-mannose	75	59
GDP-fucose	1033	142
UDP-xylose	1479	2597
UDP-glucose	109	82
UDP-galactose	71	93
UDP-GalNAc	72	63
UDP-GlcNAc	246	128

sessed the xylose linked to the 2-position of β -linked mannose (17). On this basis, the oligosaccharide product was assigned to that of structure 12 of Fig. 4.

The product was also susceptible to digestion by β -hexosaminidase, which gave a radioactive product (P1) that migrated more slowly on a long calibrated column (200 cm) of Bio-Gel P-4 than the original oligosaccharide by an amount indicative of the loss of 2 GlcNAc residues. That oligosaccharide was also susceptible to digestion by jack bean α -mannosidase, and this digestion gave a second product (P2) that moved more slowly on Bio-Gel P-4, suggesting the loss of 2 hexose residues. These data confirm that the product is indeed structure 12 of Fig. 4.

DISCUSSION

We have purified the plant enzyme that adds a β 1,2-linked xylose to the β -linked mannose on the *N*-linked oligosaccha-

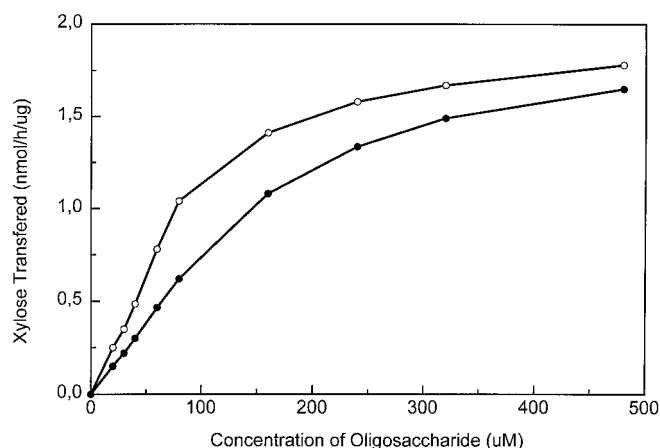


FIG. 6. Effect of oligosaccharide substrate concentration on the incorporation of xylose from UDP-xylose into xylosylated oligosaccharide. The effect of increasing concentrations of oligosaccharide 4, i.e. GlcNAc₂Man₃(GlcNAc)₂-T (○), or oligosaccharide 10, i.e. GlcNAc₂Man₃GlcNAc(Fuc)GlcNAc-T (●), on the transfer of xylose to that acceptor was examined. In these experiments, about 200 ng of enzyme were used in each incubation. Xylose incorporation was determined as described under "Experimental Procedures."

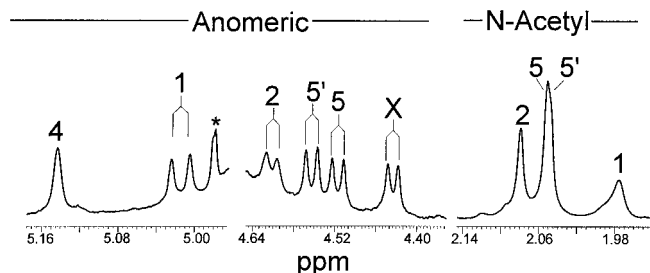


FIG. 7. NMR characterization of the oligosaccharide product produced by the xylosyltransferase. The structural reporter group regions of the 500-MHz proton NMR spectra of the xylosyltransferase product (oligosaccharide 12 of Fig. 4) are shown. The symbol X identifies the new anomeric resonance corresponding to the β -xylosyl residue leading to the proposed structure 12 (Fig. 4).

rides of storage glycoproteins. While some of the properties of a β 1,2-xylosyltransferase have been reported with enzyme systems from the snail, *L. stagnalis* (17), as well as the plant enzymes from *P. vulgaris* (15) and from *A. pseudoplatanus* (16), this paper represents the first report of the purification of this enzyme. We purified the β 1,2-xylosyltransferase from the microsomal fraction of soybean cells approximately 51,000-fold to apparent homogeneity with a recovery of about 10%.

The most purified enzyme fraction still gave two major protein bands of 56 and 59 kDa on SDS gels, but both of these proteins appear to be the active xylosyltransferase since both bands were specifically radiolabeled with the photoaffinity label, N₃-UDP-[³²P]xylose. Since these two proteins are present in the same ratio throughout the purification, and the labeling with the UDP-xylose probe and stimulation of this labeling by oligosaccharide acceptor are identical, it seems most likely that they are closely related. Our hypothesis at this stage is that the 56-kDa protein results from limited proteolysis of the 59-kDa protein. If that proves to be the case, it will be of considerable interest and importance to determine whether the proteolysis step is specific and whether it has some physiological significance. It is, of course, also possible that these two proteins differ in the extent of post-translational modification such as glycosylation, or that they represent two distinct xylosyltransferases. However, treatment of the proteins with peptide *N*-glycosidase A did not cause any alteration in their migration on SDS gels. Once we have amino acid sequence and can prepare

oligonucleotides, we will be able to study the two proteins in more detail and determine the basis for their difference.

Based on the various results in this study, and especially on the oligosaccharide acceptor specificity, it seems likely that the β 1,2-xylosyltransferase is a medial or trans-Golgi enzyme that recognizes a GlcNAc on the α 1,3-mannose of the trimannose structure, but also prefers a GlcNAc on the α 1,6-mannose as well. These data indicate that this enzyme acts in the processing pathway after the action of mannosidase II.

The oligosaccharide specificity reported here differs somewhat from the acceptor specificities reported for the other xylosyltransferases. For example, the enzyme from sycamore cells showed better activity with the trimannose structure with a GlcNAc on the 3-branched mannose (*i.e.* structure 9 in Fig. 4) than it did with the di-GlcNAc oligosaccharide (*i.e.* structure 4 of Fig. 4). In addition, reasonable activity was also observed with the GlcNAc-Man₅-oligosaccharide (16). On the other hand, the snail enzyme was more like the soybean enzyme and showed best activity with the di-GlcNAc structure but reasonable activity with the GlcNAc β 1,2Man α 1,3(Man α 1,6)Man β 1,4-GlcNAc-GlcNAc (17).

One of the questions that will be of considerable interest and importance to address with the purified enzyme is whether the protein structure of the acceptor glycoprotein, *i.e.* the storage glycoproteins, is a factor in the addition of xylose to these proteins. Specifically, in addition to recognizing a specific oligosaccharide structure for the addition of xylose, does the β 1,2-xylosyltransferase also recognize specific proteins that are destined to be localized in storage bodies? There is precedence for glycosyltransferases recognizing both the oligosaccharide acceptor and the protein to be glycosylated. Thus, the signals for targeting lysosomal enzymes to the lysosomes are the presence of phosphates on several mannose residues of the high mannose oligosaccharides. These phosphates are added by the transfer of GlcNAc-1-P from UDP-GlcNAc via a GlcNAc-1-P phosphotransferase. This enzyme recognizes not only high mannose oligosaccharides but specifically oriented lysine residues on those proteins that are destined to become lysosomal enzymes (23). A similar situation exists with respect to glycosylation of some of the glycoprotein hormones, where the GalNAc transferase recognizes both the carbohydrate acceptor and some specific region(s) of the glycoprotein hormone (24). Stud-

ies will be initiated with various storage glycoproteins and aglycoproteins to determine the role of protein structure in the activity and specificity of the xylosyltransferase. Finally, the isolation and characterization of an enzyme that contributes to the allergenicity of plant proteins will be a valuable tool for studies directed at determining the biochemical characteristics that predispose some proteins to be allergens.

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