

# Identification and Characterization of $\beta$ -D-Galactosyl-transferase in Chick Corneas

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**Purpose:** To purify  $\beta$ -galactosyltransferase, which is involved in the biosynthesis of keratan sulfate, from 2-day-old chick corneas.

**Methods:** The activity was assayed using pyridylaminated GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc as acceptor substrate.

**Results:** The  $\beta$ -galactosyltransferase did not bind to several ion exchange and affinity columns. In particular, it did not bind completely to an  $\alpha$ -lactalbumin-agarose column. The partially purified enzyme showed an optimum pH at 7.0 [(*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (HEPES) buffer]. Pyridylaminated GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc, as well as the pyridylaminated GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc, served as the acceptor substrate of the enzyme, but not *p*-nitrophenyl- $\beta$ -GlcNAc. The crude extract from chick corneas contained a high activity of the  $\beta$ -galactosyltransferase, which transfers Gal to pyridylaminated 6-sulfo-GlcNAc $\beta$ 1-3Gal, but the activity was almost all lost during the purification procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the enzyme fraction showed many bands. When each of the five main band proteins was partially analyzed for the amino acid sequence, none showed homology with the recently reported chick  $\beta$ -galactosyltransferases 1 and 2.

**Conclusions:**  $\beta$ -Galactosyltransferase, which transfers Gal to 6-sulfo-GlcNAc end, was identified in chick corneas. The chick corneal  $\beta$ -galactosyltransferase(s) may be a novel one. **Jpn J Ophthalmol** 2002;46:607–615 © 2002 Japanese Ophthalmological Society

**Key Words:**  $\beta$ -Galactosyltransferase, chick cornea, keratan sulfate, proteoglycans, sulfotransferase.

## Introduction

Two families of small proteoglycans (PGs) are present in the adult animal corneal stroma, one with chondroitin sulfate/dermatan sulfate (CS/DS) side chains and the other with keratan sulfate (KS) side chains. These PGs help maintain the regular arrangement of collagen fibrils within the corneal stroma<sup>1–3</sup> and play an important role in corneal transparency.<sup>4</sup>

Many glycosyltransferases, sulfotransferases, and epimerases are involved in the biosynthesis of the glycosaminoglycan chains of PG. The carbohydrate

backbone of KS has a linear polymer structure composed of alternating D-galactose (Gal) and *N*-acetyl-D-glucosamine (GlcNAc) units, joined by  $\beta$ 1-4 linkages to form so-called poly-*N*-acetylglucosamine. Thus,  $\beta$ -galactosyltransferase (Gal-T) and  $\beta$ -*N*-acetylglucosaminyl-transferase (GlcNAc-T) are involved in the biosynthesis of KS backbones. This Gal-T is  $\beta$ 1, 4-galactosyltransferase ( $\beta$ 4Gal-T), which transfers Gal to position 4 of the nonreducing GlcNAc end, and many isoforms of the enzyme are known:  $\beta$ 4Gal-T 1–5 in humans.<sup>5,6</sup> In addition, the human  $\beta$ 4Gal-T 6 and 7, which transfer Gal to position 4 of nonreducing Glc and Xyl ends, respectively, have been reported.<sup>7–9</sup> Of these,  $\beta$ 4Gal-Ts,  $\beta$ 4Gal-T 1 and  $\beta$ 4Gal-T 2 show lactose synthase activity by forming a complex with  $\alpha$ -lactalbumin.<sup>6,10</sup> cDNA of two chicken  $\beta$ 4Gal-Ts

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(CK $\beta$ 4Gal-Ts 1 and 2), which correspond to human  $\beta$ 4Gal-Ts 1 and 2, respectively, have been cloned from a chicken hepatoma cDNA library.<sup>11</sup> CK $\beta$ 4Gal-Ts 1 and 2 are also responsive to  $\alpha$ -lactalbumin like human  $\beta$ 4Gal-Ts 1 and 2. Furthermore, Cai et al<sup>12</sup> have cloned the truncated cDNA of CK $\beta$ 4Gal-T 1 from chick corneas, showing that CK $\beta$ 4Gal-T 1 is present in chick corneas. However, they have not examined the properties of the enzyme.

Recently, a GlcNAc-sulfotransferase (GlcNAc-ST) that transfers sulfate to the nonreducing GlcNAc end of oligosaccharides (but not internal GlcNAc residues) was found in chick corneas.<sup>13</sup> If this GlcNAc-ST is involved in the biosynthesis of KS backbones, a  $\beta$ Gal-T that transfers Gal to sulfated GlcNAc (6-sulfo-GlcNAc) nonreducing residues will be required for the elongation of KS chains. Recently, such an enzyme was identified in human colorectal mucosa.<sup>14</sup> This enzyme, which specifically transfers Gal to 6-sulfo-GlcNAc residues differs from  $\beta$ 4Gal-T 1 in its substrate specificity, and is not responsive to  $\alpha$ -lactalbumin.

In this study, we attempted to purify Gal-T from chick corneas. However, the Gal-T activity did not bind to many of the ion exchange and affinity columns used. Notably, it did not bind completely to an  $\alpha$ -lactalbumin-agarose column. The partially purified enzyme fraction showed many bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The tryptic peptides of five main band proteins were analyzed, and none was found to show amino acid sequence homology with CK $\beta$ 4Gal-Ts 1 and 2. In addition, the Gal-T activity toward non-reducing 6-sulfo-GlcNAc ends was assayed during the purification. Although this activity was very high in the crude extract, it was almost completely lost during the purification procedures.

## Materials and Methods

### Materials

White Leghorn male chicks (2 days old) were obtained from Hattori Youkei-en, Nagoya. The following enzymes were obtained from the commercial sources indicated: keratanase (from *Pseudomonas* sp., a gift from Seikagaku, Tokyo); collagenase (from *Clostridium histolyticum*, Wako Pure Chemical, Osaka);  $\beta$ -galactosidase (from jack beans, Seikagaku); N-Glycanase (peptide-N-glycanase F) (recombinant in *Escherichia coli*, from *Flavobacterium meningosepticum*, Toyobo, Osaka); and trypsin (sequence grade, modified; from bovine pancreas, Promega, Tokyo). N-Acetyllactosamine dimer (L1L1) was a gift from Seikagaku. Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc

(lacto-N-neotetraose; LNnT) and 2-acetamide-2-deoxy-D-glucono-1, 5-lactone were purchased from Funakoshi, Tokyo; *p*-nitrophenyl- $\beta$ -GlcNAc and *p*-nitrophenyl- $\beta$ -(6-sulfo-)GlcNAc were purchased from Seikagaku; and pyridylaminated N-acetylglucosamine (GlcNAc-PA) was purchased from Takara, Osaka. Sephadex G-50 (superfine), heparin-Sepharose, CM-Sepharose FF, Chelating-Sepharose, Q-Sepharose, and Superdex 200 HR 10/30 were purchased from Amersham Pharmacia Biotech, Tokyo; WGA-agarose was purchased from Seikagaku; and UDP-hexanolamine-agarose, GlcNAc-agarose, and  $\alpha$ -lactalbumin-agarose were purchased from Sigma, Tokyo. The columns for high-performance liquid chromatography (HPLC), TSK-gel ODS-120T, TSK-gel ODS-80TM, and TSK-gel G2500PWXL (size exclusion column), were purchased from Tosoh, Tokyo.

### Preparation of Substrates for Enzymatic Assay

Acceptor substrates for Gal-T were prepared as described previously.<sup>15</sup> Briefly, LNnT and L1L1 were pyridylaminated according to Hase et al.<sup>16</sup> LNnT-PA (4 mg) and L1L1-PA (4 mg) were purified by HPLC on TSK-gel ODS-120T (7.8  $\times$  300 mm) and TSK-gel G2500 PWXL (7.8  $\times$  300 mm) columns. The isolated LNnT-PA and L1L1-PA were degalactosylated with  $\beta$ -galactosidase, and the degalactosylated LNnT-PA and L1L1-PA were isolated by HPLC on a TSK-gel G2500 PWXL column. The amounts of two PA-sugars were determined from their fluorescence intensities (excitation wavelength, 320 nm; emission wavelength, 400 nm) using GlcNAc-PA as standard: the degalactosylated LNnT-PA, GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA, 1,145 nmoles; and the degalactosylated L1L1, GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc-PA, 1,010 nmoles. The preparation of 6-Sulfo-GlcNAc $\beta$ 1-3Gal-PA was as follows. Bovine corneal keratan sulfate (22 mg), prepared as reported previously,<sup>17</sup> was digested with 10 U keratanase (endo- $\beta$ -galactosidase) in 0.05 M Tris-HCl, pH 7.5 at 37°C for 20 hours. The digest was chromatographed on a Sephadex G-50 column (15  $\times$  500 mm) equilibrated with 0.1 mM ammonium acetate, and the eluted sugars were detected by monitoring absorbance at 214 nm. The fractions of the highest peak were pooled and lyophilized. The lyophilized fraction was pyridylaminated as described above, and the resultant PA-sugars were separated mutually by HPLC on TSK-gel ODS-120T and TSK-gel G2500 PWXL columns. The PA-sugar recovered in the highest yield was used for experiments described below. This sugar was determined to be 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA by comparison of its elution positions on TSK-gel ODS-120T and TSK-gel G2500

PWXL chromatographies with those of degalactosylated LNT-PA and L1L1-PA, and by data of identification of the keratanase digests reported previously.<sup>17</sup> The yield of 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA was 1,199 nmoles.

### Assay of Gal-T Activity

The standard reaction mixture for assay of Gal-T activity contained 50 mM 2-morpholinoethanesulfonic acid (MES) buffer, pH 6.8, 5 mM MnCl<sub>2</sub>, 0.5 mM ATP, 10 mM UDP-Gal, 0.2 mM GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA (or 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA) and enzyme in a final volume of 50  $\mu$ L. The reaction mixture was incubated at 37°C for 3 hours, then centrifuged on a membrane filter (0.22  $\mu$ m). The resultant filtrate was applied to a TSK-gel ODS-80TM column (4.6  $\times$  250 mm) equilibrated with 50 mM ammonium acetate, pH 4.0, and the reaction product was eluted with the same buffer at a flow rate of 1.0 mL/min for 60 minutes and detected by fluorescence spectrometry (excitation wavelength, 320 nm; emission wavelength, 400 nm). The product was eluted earlier than the original substrate from the column. When *p*-nitrophenyl- $\beta$ -GlcNAc and *p*-nitrophenyl- $\beta$ -(6-sulfo-)GlcNAc were used as acceptors, the product on the HPLC column was detected by the absorbance at 300 nm.

One unit of enzyme activity is defined as the amount required to catalyze the transfer of 1 pmol of Gal/min.

### Purification of Gal-T

All operations were performed at 4°C. Protein in each fraction from the chromatography described below was detected by monitoring absorbance at 280 nm.

**Crude extract.** Corneas were taken from 5992 eyeballs of 2-day-old male chicks, cut into sections and digested with 0.25% collagenase in phosphate-buffered saline (PBS), pH 7.2 (PBS: 1.15 g Na<sub>2</sub>HPO<sub>4</sub>, 8.0 g NaCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, and 0.2 g KCl in 1,000 mL of distilled water) at 37°C for 1.5 hours. The resultant cell suspension was centrifuged, and the pellet obtained was washed three times with PBS. The final cell pellet was suspended in 7.0 mL of 10 mM Tris-HCl, pH 7.2, containing 20 mM MgCl<sub>2</sub> and homogenized with a Polytron-type microhomogenizer (Niti-on, Tokyo) in three 1-minute bursts with 1-minute intervals. The homogenate was centrifuged at 15,000g for 10 minutes, and the resultant supernatant was removed. The precipitate was suspended again in 7 mL of the same Tris-HCl buffer and homogenized as described above. The second homogenate was centrifuged, and the second supernatant was removed. The second

precipitate was suspended in 14 mL of buffer A' (10 mM Tris-HCl, pH 7.2, 20 mM MgCl<sub>2</sub>, 0.2% Triton X-100, 20 mM 2-mercaptoethanol, and 40% glycerol) and allowed to stand at 4°C for 18 hours. The suspension was centrifuged, and the third supernatant was removed. The third precipitate was suspended again in 10 mL of buffer A (10 mM Tris-HCl, pH 7.2, 20 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, and 20% glycerol) and centrifuged to recover any enzyme remaining in the precipitate. The resultant supernatant was combined as crude extract with all of the other supernatant fractions obtained above. Gal-T was purified from this crude extract (38 mL) as described below.

**CM-Sepharose FF chromatography.** The crude extract was applied at a flow rate of 2.0 mL/min to a CM-Sepharose FF column (2.6  $\times$  8.0 cm) equilibrated with buffer A. The column was then washed with 280 mL of buffer A, and the adsorbed materials were eluted with a linear gradient of 0–0.7 M NaCl in buffer A for 140 minutes, then with 1 M NaCl in buffer A for 60 minutes. The Gal-T activity passed through the column, and the pass-through fractions (72 mL) with the activity were pooled and concentrated to 38 mL by ultrafiltration using a YM-10 membrane (Millipore, Tokyo).

**WGA-agarose chromatography.** The concentrated CM-Sepharose fraction was applied at a flow rate of 0.5 mL/min to a WGA-agarose column (2.6  $\times$  4.0 cm) equilibrated with buffer A containing 0.15 M NaCl. The column was then washed with 135 mL of the same buffer, and the adsorbed materials were eluted with 140 mL of buffer A containing 0.3 M GlcNAc and 0.15 M NaCl. The Gal-T activity again passed through the column, and the pass-through fractions (80 mL) with the activity were pooled, concentrated to 29 mL by ultrafiltration as described above and dialyzed against buffer A.

**Chelating-Sepharose chromatography.** A Chelating-Sepharose column (2.6  $\times$  8.0 cm) was equilibrated with 0.3 M ZnCl<sub>2</sub> and then with buffer A containing 0.3 M NaCl. The concentrated WGA-agarose fraction was applied at a flow rate of 1.0 mL/min to this column. The column was then washed with 72 mL of buffer A containing 0.2 M glycine and 0.3 M NaCl, and the adsorbed materials were eluted with 150 mL of buffer A containing 0.05 M EDTA and 0.3 M NaCl. The Gal-T activity passed through the column, and the pass-through fractions (22 mL) with the activity were pooled, concentrated to 7.5 mL by ultrafiltration as described above and dialyzed against buffer A.

**Q-Sepharose FF chromatography.** The concentrated Chelating-Sepharose fraction was applied at a flow rate of 1.0 mL/min to a Q-Sepharose FF column (2.6 × 4.0 cm) equilibrated with buffer A. The column was then washed with 40 mL of buffer A, and the adsorbed materials were eluted with a linear gradient of 0–0.5 M of NaCl in buffer A for 100 minutes, then with 20 mL of 1 M NaCl. A major part (72%) of the applied activity passed through the column, and a minor part (38%) was eluted with 0–0.1 M NaCl. The pass-through fractions (34 mL) with the activity were pooled and concentrated to 3.5 mL by ultrafiltration as described above.

**α-Lactalbumin-agarose chromatography.** The concentrated Q-Sepharose fraction (3.5 mL) was diluted with 3.5 mL of 10 mM Tris-HCl, pH 7.2 containing 10 mM MnCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 20% glycerol, 100 mM NaCl, 10 mM GlcNAc, 100 mM Glc, and 10 mM UDP. This diluted Q-Sepharose fraction was applied at a flow rate of 0.5 mL/min to an α-lactalbumin-agarose column (1.0 × 12.0 cm) equilibrated with buffer B (10 mM Tris-HCl, pH 7.2, 20 mM MnCl<sub>2</sub>, 0.05% Triton X-100, 10 mM 2-mercaptoethanol, and 20% glycerol) containing 50 mM Glc, 5 mM GlcNAc, 5 mM UDP, and 50 mM NaCl. The column was washed with 50 mL of buffer B containing the same components, and the adsorbed materials were eluted with 50 mL of buffer B containing 50 mM NaCl, then with 44 mL of buffer B containing 1 M NaCl. The Gal-T activity again passed through the column. The pass-through fractions (28 mL) with the activity were pooled and concentrated to 2.4 mL by ultrafiltration as described above.

**1st Superdex 200 HR chromatography.** The concentrated α-lactalbumin-agarose fraction was applied at a flow rate of 0.25 mL/min to a Superdex 200 HR column (1.0 × 30 cm) equilibrated with buffer A containing 5 mM GlcNAc and 150 mM NaCl. The adsorbed materials were eluted with 30 mL of the same buffer, and the protein amount in each fraction (0.5 mL) was assayed by the method of Bradford (1976; see below). The fractions (4.5 mL) with the activity were pooled and advanced to the next step without concentration.

**2nd Superdex 200 HR chromatography.** The pooled 1st Superdex 200 fraction was applied to the same Superdex 200 column under the same conditions. The adsorbed materials were eluted with the same buffer, and the protein amount in each fraction was assayed by the method of Bradford. Each fraction (0.5 mL) containing the Gal-T activity (Figure 2), was subjected separately to the experiments described above.

### *Assay of Protein*

Protein was determined by the method of Bradford.<sup>18</sup> Protein assay reagents were obtained from Bio-Rad (Tokyo). When the protein concentration in a sample was too low to be determined directly, the sample was concentrated as follows. The protein was precipitated with 5% trichloroacetic acid (TCA), washed twice with a small amount of acetone, and dissolved in 50 μL of 0.3 M NaOH. This solution was used for the protein determination.

### *SDS-PAGE*

Electrophoresis of proteins was performed on 10% polyacrylamide gel containing SDS under reducing conditions according to the method of Laemmli.<sup>19</sup> Protein (3 μg) in each of fractions 31 to 37 of 2nd Superdex 200 chromatography was precipitated with 5% TCA. The resultant precipitates were washed twice with a small amount of acetone, then dissolved in the sample buffer (0.1% SDS, 20% glycerol, 50 mM Tris-HCl, pH 7.4, 100 mM dithiothreitol, and 0.02% bromothymol blue) by incubation at 70°C for 30 minutes. The dissolved protein samples were subjected to gel electrophoresis, and the protein bands were detected with silver stain (Bio-Rad Silver Stain Plus).

### *Amino Acid Sequencing of Peptides from Tryptic Digests of the Protein Bands Obtained by SDS-PAGE*

Fractions 32 to 34 of the 2nd Superdex 200 chromatography (200 μg as a total amount of protein) were combined and precipitated with 5% TCA. The resultant precipitate was washed twice with a small amount of acetone and then dissolved in the buffer (1.0% SDS, 20% glycerol, 50 mM Tris-HCl, pH 7.4, 100 mM dithiothreitol, and 0.02% bromothymol blue) by incubation at 70°C for 60 minutes. The dissolved protein sample was subjected to SDS-PAGE. Proteins in the gel were then electrotransferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P, 0.45 μm; Millipore, Tokyo) and visualized with Coomassie Brilliant Blue. Bands (a) to (e) (Figure 2) were excised and washed with 50% methanol to eliminate the dye, then the protein on the membrane band was digested with trypsin (sequence grade) according to the method of Aebersold et al.<sup>20</sup> Briefly, the membrane band was treated with poly(vinylpyrrolidone)-40 and digested with N-Glycanase, then digested with the trypsin. The trypsin digest was subjected to HPLC on TSK-gel 80TM column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of 0–100% of acetonitrile.

**Table 1.** Partial Purification of  $\beta$ -Galactosyltransferase from Chick Corneas

Steps	Total Protein (mg)	GlcNAc-Gal-Glc-PA		6-Sulfo-GlcNAc-Gal-PA	
		Total Activity (units*)	Specific Activity (units/mg protein)	Total Activity (units*)	Specific Activity (units/mg protein)
Crude extract	380	$3.38 \times 10^4$	88.9	$7.76 \times 10^4$	204
CM-Sepharose	231	$2.93 \times 10^4$	127	– <sup>†</sup>	–
WGA-agarose	182	$2.39 \times 10^4$	132	–	–
Chelating-Sepharose	17.9	$2.48 \times 10^3$	139	$7.22 \times 10^2$	40.3
Q-Sepharose	4.16	$1.12 \times 10^3$	267	–	–
$\alpha$ -Lactalbumin agarose	3.18	$4.48 \times 10^2$	141	–	–
1st Superdex 200	1.27	$2.02 \times 10^2$	158	–	–
2nd Superdex 200 (fraction 33)	0.13	$1.35 \times 10^2$	1038	$3.53 \times 10$	272

\*One unit of activity is defined as the amount required to catalyze the transfer of 1 pmol of Gal/min. The reaction mixture contained the following components in a final volume of 50  $\mu$ L: 0.2 mM GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA, 50 mM MES buffer (pH 6.8), 10 mM UDP-Gal, 5 mM MnCl<sub>2</sub>, 0.5 mM ATP, and enzyme. The mixture was incubated at 37°C for 3 hours.

<sup>†</sup>Not determined.

The amino acid sequences of the separated peptides were analyzed with a model 491A protein sequencer (PE Applied Biosystems, Urayasu).

## Results

### *Purification of Gal-T from Chick Corneas*

Table 1 summarizes the purification of the Gal-T from 5924 chick corneas. When the activity was assayed using GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA as acceptor substrate (see the columns of GlcNAc-Gal-Glc-PA in Table 1), the Gal-T passed through both CM-Sepharose and WGA-agarose columns with a slight loss of activity, and no Gal-T activity was detected in the bound fractions. GlcNAc-sulfotransferase and chondroitin-sulfotransferases were eliminated from the Gal-T fraction after WGA-agarose chromatography. Gal-T also passed through a Zn-Chelating-Sepharose column, but the major part of the activity was lost in this step. The  $\beta$ -N-acetylgalactosaminyltransferase and  $\beta$ -glucuronyltransferase activities were lost completely in both pass-through and bound fractions and, as a result, were both eliminated from the Gal-T fraction. In separate experiments, we tried many affinity chromatographies of the fraction obtained by WGA-agarose chromatography. The Gal-T passed through heparin-Sepharose, UDP-hexanolamine-agarose, GlcNAc-agarose, and  $\alpha$ -lactalbumin-agarose (with buffer containing 0.1% Triton X-100 and 50 mM Glc) columns. When the pass-through fraction obtained from these affinity chromatographies was applied to a TSK-gel AF-blue Toyopearl or ConA-Sepharose column, the Gal-T activity was completely lost in both pass-through and bound fractions on both chromatographies. Because it was considered that Gal-T

might require an activator to express its activity, the activity was assayed with various combinations of pass-through fraction, bound fraction, phosphatidylinositol and sphingomyelin, but in no case was it detected. It might have bound to the AF-blue and ConA columns, but it could be very unstable in the purified state.

Thus, the pass-through fraction of Chelating-Sepharose chromatography described above was used for the purification described below. A portion of the pass-through fraction bound to a Q-Sepharose column: 1,120 units (72%) of activity were detected in the pass-through fraction and 441 units (38%) in the bound fraction. Because it was considered that the Gal-T might become more labile with increasing purification, 5 mM GlcNAc, a known stabilizer for  $\beta$ 4Gal-T 1,<sup>21</sup> was added to the enzyme fraction and buffer from the next step. The pass-through fraction from the Q-Sepharose column was applied to an  $\alpha$ -lactalbumin-agarose column equilibrated with the buffer containing 0.05% Triton X-100, 50 mM Glc, 5 mM GlcNAc, 5 mM UDP, and 50 mM NaCl as described under Materials and Methods. Because Triton X-100 might inhibit the binding of Gal-T protein to  $\alpha$ -lactalbumin, its concentration was reduced from 0.1% to 0.05%. In addition, because UDP might stabilize the complex of the Gal-T with  $\alpha$ -lactalbumin and Glc as donor substrate (UDP-Gal) analogue, 5 mM UDP was added to the buffer. Nevertheless, the Gal-T activity passed through the  $\alpha$ -lactalbumin-agarose column. Finally, the pass-through fraction was serially twice chromatographed on a Superdex 200 column. After the 2nd Superdex 200 chromatography, each fraction containing the activity was used separately for experiments described below. The activity of the 2nd Superdex 200 fraction in Table 1 is that of fraction 33 on the chromatography.

### Gal-T Activity Toward 6-Sulfo-GlcNAc $\beta$ 1-3Gal-PA

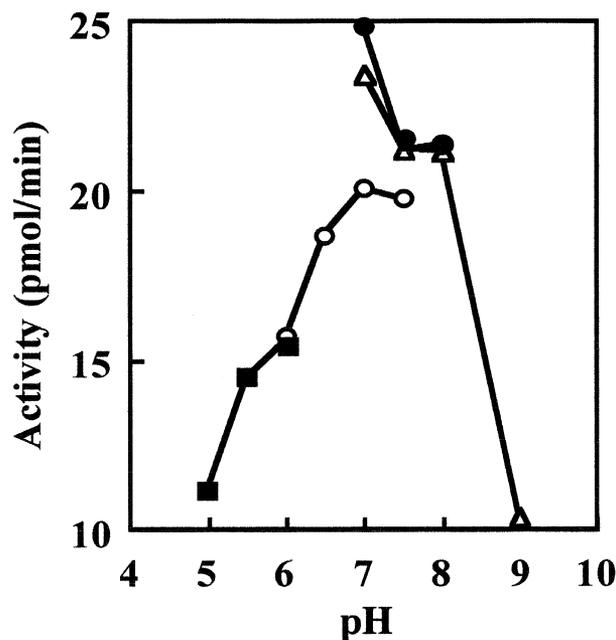
As described above, a  $\beta$ Gal-T that is capable of transferring Gal to sulfated GlcNAc (6-sulfo-GlcNAc) ends is required to biosynthesize KS chains in concert with GlcNAc-ST. Thus, the Gal-T activity toward 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA was assayed in some steps of the purification (see the columns of 6-sulfo-GlcNAc-Gal-PA in Table 1). The activity in the crude extract was very high, being 2-fold higher than that toward GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA. The major part of the activity was lost during the purification. It appears that the activity is more labile than that toward GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA, judged from the values of these activities after the 2nd Superdex 200 chromatography in comparison with those in crude extract (Table 1).

### Properties of Gal-T

Figure 1 shows the effects of pH on the activity of the Gal-T. Optimum pH was 7.0 and the highest activity was detected in the (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (HEPES) buffer of pH 7.0. Thus, HEPES buffer of pH 7.0 was used for the experiments described below. Table 2 shows the effects of various additions on the activity of the Gal-T. GlcNAc (20 mM) and 2-acetamide-2-deoxy-D-glucono-1,5-lactone (5 mM), which is known as an inhibitor of the *N*-acetyl- $\beta$ -hexosaminidase reaction,<sup>22</sup> inhibited the Gal-T reaction slightly. A high concentration (100 mM) of GlcNAc completely inhibited the reaction (data not shown). Table 3 shows acceptor substrate specificity of the Gal-T. The activity toward GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA was 58.9% of that toward GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA, suggesting that the Gal-T recognizes not only the nonreducing GlcNAc end but also the third internal sugar from the nonreducing end. Low activity toward 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA was detected. Whether the enzyme associated with this activity is different from the Gal-T with the activity toward GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA, remains to be clarified (see below). *p*-Nitrophenyl- $\beta$ -GlcNAc did not serve as substrate, suggesting that CKGal-Ts 1 and 2 are not present in the 2nd Superdex 200 fraction (fraction 33).<sup>6</sup> *p*-Nitrophenyl- $\beta$ -(6-sulfo-)GlcNAc also did not serve as substrate, suggesting that the Gal-T, which transfers Gal to 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA, also recognizes not only the nonreducing 6-sulfo-GlcNAc residues but also internal sugar residues for a maximal reaction.

### Identification of $\beta$ -Gal in the Reaction Products

To confirm the  $\beta$ -configuration of the Gal transferred to two acceptor substrates, GlcNAc $\beta$ 1-3Gal $\beta$ 1-



**Figure 1.** Effects of pH on the activity of  $\beta$ -Gal-T. The effects of pH on the transfer of Gal to GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA by the enzyme (3  $\mu$ g: Fraction 34 of the 2nd Superdex 200 chromatography) were examined under the standard assay conditions with the buffers indicated. The buffers (50 mM) were sodium acetate (■), HEPES (●), MES (○), and Tris-HCl (△).

4Glc-PA and 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA, the sensitivity of the reaction products to digestion with  $\beta$ -galactosidase was tested. After  $\beta$ -galactosidase digestion, each digest was applied to a TSK-gel ODS-80TM column. The elution position of each digest (oligosaccharide-PA) was the same as that of the corresponding original acceptor substrate (data not shown).

**Table 2.** Effects of Various Additions on the Activity of  $\beta$ -Galactosyltransferase\*

Addition	Concentration	Relative Activity (%)
Control	—	100
<i>N</i> -Acetylglucosamine	20 mM	86.5
<i>N</i> -Acetylgalactosamine	20 mM	92.9
2-Acetamide-2-deoxy-D-glucono-1,5-lactone	5 mM	81.7

\*The reaction mixture contained the following components in a final volume of 50  $\mu$ L: 0.2 mM GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA, 10 mM UDP-Gal, 50 mM HEPES, 5 mM ATP, the added substance, and enzyme (3  $\mu$ g: Fraction 33 of the 2nd Superdex 200 chromatography). The mixture was incubated at 37°C for 5 hours. Activity is expressed as percent of  $\beta$ -Gal-transfer observed in the absence of inhibitor (control).

**Table 3.** Acceptor Substrate Specificity of  $\beta$ -Galactosyltransferase\*

Acceptor (0.2 mM)	Relative Activity (%)
GlcNAc $\beta$ 1-3 Gal $\beta$ 1-4 Glc-PA	100
GlcNAc $\beta$ 1-3 Gal $\beta$ 1-4 GlcNAc-PA	58.9
6-Sulfo-GlcNAc $\beta$ 1-3 Gal-PA	24.5
<i>p</i> -Nitrophenyl- $\beta$ -GlcNAc	— <sup>†</sup>
<i>p</i> -Nitrophenyl- $\beta$ -(6-sulfo-)-GlcNAc	—

\*The reaction mixture contained the following components in a final volume of 50  $\mu$ L: 0.2mM substrate indicated above, 50 mM HEPES (pH 7.0), 5 mM MnCl<sub>2</sub>, 0.5 mM ATP, 10 mM UDP-Gal and enzyme (3  $\mu$ g: Fraction 33 of the 2nd Superdex 200 chromatography). The mixture was incubated at 37°C for 5 hours.

<sup>†</sup>Not detected.

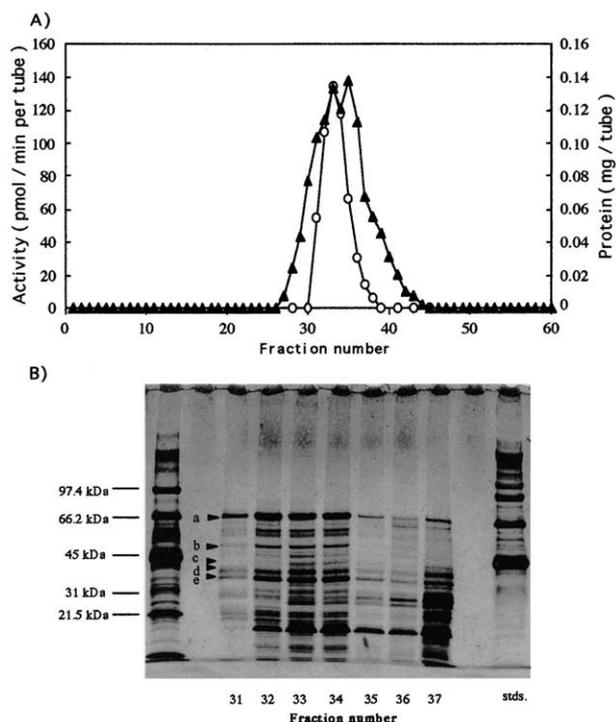
This shows that Gal was transferred to the acceptors through a  $\beta$ -linkage.

### SDS-PAGE of the Enzyme Fraction

Figure 2 shows the profile of the 2nd Superdex 200 chromatography and SDS-PAGE of the fractions from the chromatography. Each fraction showing Gal-T activity (Figure 2A) was subjected to SDS-PAGE (Figure 2B). Many bands were found on all the lanes of fractions 31 to 37. Five bands (a) to (e) at the positions indicated by arrowheads were analyzed for the amino acid sequences: (a) 66 kDa; (b) 48 kDa; (c) 42 kDa; (d) 38 kDa; and (e) 36 kDa.

### Amino Acid Sequences of Five Protein Bands of (a) to (e)

The molecular sizes of CK $\beta$ 4Gal-Ts 1 and 2 are 41 and 43 kDa, respectively<sup>11</sup>; that of  $\beta$ Gal-T from mouse testis, 46 kDa<sup>23</sup>; that of human  $\beta$ Gal-T 7, 37 kDa<sup>9</sup>; those of human  $\beta$ Gal-T 1 to 6, between 37 kDa and 46 kDa.<sup>6</sup> Thus, the proteins of bands (b) to (e) and (a) which were stained very intensely on the gel (Figure 2B), were analyzed for the amino acid sequences as described under Materials and Methods. Because the bands between bands (b) and (c) were not found in fractions 31 and 35, where the Gal-T activity was detected (Figure 2), they were not analyzed. The proteins of bands (a) to (e) were digested with trypsin on the PVDF membrane and peptides were isolated by HPLC. Each peptide was then analyzed for amino acid sequence. None of the peptides from the five bands showed sequence homology with CK $\beta$ 4Gal-Ts 1 and 2,<sup>11</sup> bovine  $\beta$ 4Gal-T,<sup>24</sup> murine  $\beta$ 4Gal-Ts,<sup>23,25</sup> human  $\beta$ 4Gal-Ts 1 to 7,<sup>6,9,10,26-28</sup> human  $\beta$ 3Gal-T family,<sup>5,26</sup> and murine  $\alpha$ 3Gal-T.<sup>29</sup> One peptide (FDIEVPI) from the band (d) showed some



**Figure 2.** 2nd Superdex 200 chromatography (A) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of individual fractions from the chromatography (B). (A) The pooled fraction from the 1st Superdex 200 chromatography was applied to the same Superdex 200 column and eluted with 20 mM GlcNAc/150 mM NaCl/buffer B as described under Materials and Methods. Fractions were assayed for  $\beta$ Gal-T activity ( $\circ$ ) and protein ( $\Delta$ ). Activity at ordinate is expressed as that in each fraction tube (0.5 mL). (B) SDS-PAGE of individual fractions from the chromatography. The bands (a) to (e) from the fractions 32 to 34 at the position indicated by arrowheads were analyzed for the sequences of amino acid. stds: standards.

homology (71.4%) with one part (FDIKVPL) of goat GlcNAc-6-sulfatase protein<sup>30</sup> and one peptide (KAPYQAPRSP) from the band (e) showed some homology (66.7%) with one part (WAPPQSPRRP) of human  $\beta$ GalNAc-T protein.<sup>31</sup>

### Discussion

Shaper et al<sup>11</sup> cloned cDNAs of two Gal-Ts (CK $\beta$ 4Gal-T 1 and CK $\beta$ 4Gal-T 2) by screening a chicken hepatoma cDNA library and showed that both enzymes are expressed in chicken brain, kidney, liver, lung, spleen, and pancreas. Before this, Cai et al<sup>12</sup> had isolated a truncated cDNA of  $\beta$ Gal-T from a chicken corneal cDNA library and found that its sequence showed high homology with both human  $\beta$ 4Gal-T 1 and bovine milk  $\beta$ 4Gal-T. It was

subsequently found that the sequence of the  $\beta$ Gal-T cDNA of Cai et al<sup>12</sup> was identical to that of the CK $\beta$ 4Gal-T 1 cDNA. Therefore, we attempted to purify  $\beta$ Gal-T from chick corneas. However, the  $\beta$ Gal-T activity passed through many affinity columns. While the total activity decreased markedly during the purification procedures, the specific activity hardly increased during each step (Table 1). In particular, it passed through an  $\alpha$ -lactalbumin-agarose column, suggesting that CK $\beta$ 4Gal-T 1 is absent in chick cornea. Because the major part of the Gal-T activity was lost after the Chelating-Sepharose chromatography (Table 1), it is possible that only the CK $\beta$ 4Gal-T 1 activity was lost at this step, while the activities of the other  $\beta$ Gal-Ts remained intact and could pass through an  $\alpha$ -lactalbumin-agarose column. This possibility, however, is unlikely. In a separate experiment, the WGA-agarose fraction (the Gal-T activity was hardly lost during the WGA-agarose chromatography) also passed through an  $\alpha$ -lactalbumin-agarose column, and therefore the CK $\beta$ 4Gal-T activity should be absent in the WGA-agarose fraction or present only at a low level. The possibility also cannot be excluded that the bound fraction of Q-Sepharose chromatography might contain CK $\beta$ 4Gal-T 1, because some Gal-T activity is bound to the Q-Sepharose column. What the Gal-T activity found in the 2nd Superdex 200 fraction is remains to be clarified, if this Gal-T is not CK $\beta$ 4Gal-T 1. Because the Gal-T in the 2nd Superdex 200 fraction showed no activity toward *p*-nitrophenyl- $\beta$ -GlcNAc, it might be  $\beta$ 3Gal-T.<sup>14</sup> However, none of the peptides from the bands (a) to (e) on SDS-PAGE showed homology with the human  $\beta$ 3Gal-Ts.<sup>5,26</sup>

The  $\beta$ Gal-T that transfers Gal to the 6-sulfo-GlcNAc nonreducing end (6-sulfo-GlcNAc $\beta$ 1-3Gal-PA) was found to be present in chick cornea. Although this activity was much higher in the crude extract than that toward GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA (Table 1), a major part of the activity was lost during the purification. This  $\beta$ Gal-T may become more labile with increasing purification. Whether a single enzyme has Gal-T activity toward both 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA and GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA is unknown. Seko et al<sup>14</sup> reported that the GlcNAc-6-sulfate-specific  $\beta$ 1,4Gal-T was identified in human colorectal mucosa. They separated this Gal-T from  $\beta$ 3Gal-T and  $\beta$ 4Gal-T (probably  $\beta$ 4Gal-T 1) by UDP-hexanolamine-Sepharose and asialo-agalacto-ovomucin-Sepharose chromatographies. These three  $\beta$ Gal-Ts all bound to UDP-hexanolamine-Sepharose, while our Gal-T activities toward nonreducing GlcNAc and 6-sulfo-GlcNAc

residues both passed through the same column. Furthermore, the GlcNAc-6-sulfate-specific  $\beta$ 4Gal-T of Seko et al<sup>14</sup> showed activity toward a monosaccharide, 6-sulfo-GlcNAc, while our enzyme showed no activity toward *p*-nitrophenyl- $\beta$ -(6-sulfo-)GlcNAc. Nevertheless, our enzymes might be the chick orthologues of the human  $\beta$ 4Gal-T of Seko et al.<sup>14</sup> Some activity toward 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA was also found in the bound fraction of Q-Sepharose chromatography: the ratio of the activities toward GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc-PA and toward 6-sulfo-GlcNAc $\beta$ 1-3Gal-PA in the bound fraction was almost the same as that in the pass-through fraction. Therefore, it appears that the Gal-Ts in the bound fraction are identical to those in the pass-through fraction. The proteins of bands (a) to (e) obtained by SDS-PAGE showed no homology with any of the  $\beta$ 4Gal-Ts and  $\beta$ 3Gal-Ts reported previously. One peptide from the band (d) showed some homology with goat GlcNAc-6-sulfatase; and one from band (e) showed some homology with human  $\beta$ GalNAc-transferase. Band (d) might contain a novel 6-sulfo-GlcNAc-specific CK $\beta$ -Gal-T; and band (e), a novel CK $\beta$ 4Gal-T, if the enzyme with the activity toward 6-sulfo-GlcNAc end is different from the  $\beta$ Gal-T with the activity toward the GlcNAc end. Cloning of the cDNAs of bands (c), (d), and (e) is now in progress on the basis of partial amino acid sequences of the proteins in these bands. When these cDNAs have been cloned, their expression will clarify which band contains the novel 6-sulfo-GlcNAc-specific  $\beta$ Gal-T.

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