Identification and Characterization of β-D-Galactosyl-transferase in Chick Corneas

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

Methods: The activity was assayed using pyridylaminated GlcNAcβ1-3Galβ1-4Glc as acceptor substrate.

Results: The β-galactosyltransferase did not bind to several ion exchange and affinity columns. In particular, it did not bind completely to an α-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 GlcNAcβ1-3Galβ1-4GlcNAc, as well as the pyridylaminated GlcNAcβ1-3Galβ1-4Glc, served as the acceptor substrate of the enzyme, but not p-nitrophenyl-β-GlcNAc. The crude extract from chick corneas contained a high activity of the β-galactosyltransferase, which transfers Gal to pyridylaminated 6-sulfo-GlcNAcβ1-3Gal, 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 β-galactosyltransferases 1 and 2.

Conclusions: β-Galactosyltransferase, which transfers Gal to 6-sulfo-GlcNAc end, was identified in chick corneas. The chick corneal β-galactosyltransferase(s) may be a novel one.

Key Words: β-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 stroma1-3 and play an important role in corneal transparency.4

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 β1-4 linkages to form so-called poly-N-acetyllactosamine. Thus, β-galactosyltransferase (Gal-T) and β-N-acetylgalcosaminyl-transferase (GlcNAc-T) are involved in the biosynthesis of KS backbones. This Gal-T is β1,4-galactosyltransferase (β4Gal-T), which transfers Gal to position 4 of the nonreducing GlcNAc end, and many isoforms of the enzyme are known: β4Gal-T 1-5 in humans.5,6 In addition, the human β4Gal-T 6 and 7, which transfer Gal to position 4 of nonreducing Glc and Xyl ends, respectively, have been reported.7-9 Of these, β4Gal-Ts, β4Gal-T 1 and β4Gal-T 2 show lactose synthase activity by forming a complex with α-lactalbumin.6,10 cDNA of two chicken β4Gal-Ts
(CKβ4Gal-Ts 1 and 2), which correspond to human βGal-Ts 1 and 2, respectively, have been cloned from a chicken hepatoma cDNA library.\(^1\) CKβ4Gal-Ts 1 and 2 are also responsive to α-lactalbumin like human βGal-Ts 1 and 2. Furthermore, Cai et al.\(^1\) have cloned the truncated cDNA of CKβ4Gal-T 1 from chick corneas, showing that CKβ4Gal-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 sulfates to the nonreducing GlcNAc end of oligosaccharides (but not internal GlcNAc residues) was found in chick corneas.\(^1\) If this GlcNAc-ST is involved in the biosynthesis of KS backbones, a βGal-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.\(^1\) This enzyme, which specifically transfers Gal to 6-sulfo-GlcNAc residues differs from β4Gal-T 1 in its substrate specificity, and is not responsive to α-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 α-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 CKβ4Gal-Ts 1 and 2. In addition, the Gal-T activity toward nonreducing 6-sulfo-GlcNAc ends was assayed during the purification. Although this activity was very high during the purification procedures, it was almost completely lost 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 hystolyticum*, Wako Pure Chemical, Osaka); β-galactosidase (from jack beans, Seikagaku); N-Glycanase (peptide-N-glycanase F) (recombinant in *Escherichia coli*, from *Flavobacterium menin-goosepticum*, Toyobo, Osaka); and trypsin (sequence grade, modified; from bovine pancreas, Promega, Tokyo). N-Acetyllactosamine dimer (L1L1) was a gift from Seikagaku. Galβ1-4GlcNAcβ1-3Galβ1-4Glc (lacto-N-neotetraose; LNnT) and 2-acetamide-2-deoxy-D-glucono-1, 5-lactone were purchased from Funakoshi, Tokyo; p-nitrophenyl-β-GlcNAc and p-nitrophenyl-β-(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 α-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.\(^1\) Briefly, LNnT and L1L1 were pyridylaminated according to Hase et al.\(^1\) LNnT-PA (4 mg) and L1L1-PA (4 mg) were purified by HPLC on TSK-gel ODS-120T (7.8 × 300 mm) and TSK-gel G2500 PWXL (7.8 × 300 mm) columns. The isolated LNnT-PA and L1L1-PA were degalactosylated with β-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, GlcNAcβ1-3Galβ1-4Glc-PA, 1,145 nmoles; and the degalactosylated L1L1, GlcNAcβ1-3Galβ1-4GlcNAc-PA, 1,010 nmoles. The preparation of 6-Sulfo-GlcNAcβ1-3Gal-PA was as follows. Bovine corneal keratan sulfate (22 mg), prepared as reported previously,\(^1\) was digested with 10 U keratanase (endo-β-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 × 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-GlcNAcβ1-3Gal-PA by comparison of its elution positions on TSK-gel ODS-120T and TSK-gel G2500 PWXL.
PWXL chromatographies with those of degalactosylated LNNt-PA and L1L1-PA, and by data of identification of the keratanase digests reported previously. The yield of 6-sulfo-GlcNAcβ1-3Gal-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 MnCl₂, 0.5 mM ATP, 10 mM UDP-Gal, 0.2 mM GlcNAcβ1-3Galβ1-4Glc-PA (or 6-sulfo-GlcNAcβ1-3Gal-PA) and enzyme in a final volume of 50 µL. The reaction mixture was incubated at 37°C for 3 hours, then centrifuged on a membrane filter (0.22 µm). The resultant filtrate was applied to a TSK-gel ODS-80TM column (4.6 × 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 florescence spectrometry (excitation wavelength, 320 nm; emission wavelength, 400 nm). The product was eluted earlier than the original substrate from the column. When p-nitrophenyl-β-GlcNAc and p-nitrophenyl-β-(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 Na₂HPO₄, 8.0 g NaCl, 0.2 g KH₂PO₄, 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 MgCl₂ 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 MgCl₂, 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 MgCl₂, 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 × 8.0 cm) equilibrated with buffer A. The column was then washed with 280 mL of buffer A, and the absorbed 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 × 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 × 8.0 cm) was equilibrated with 0.3 M ZnCl₂ 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 MnCl₂, 10 mM 2-mercaptoethanol, 20% glycerol, 100 mM NaCl, 10 mM GlnNAc, 100 mM Gln, 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 MnCl₂, 0.05% Triton X-100, 10 mM 2-mercaptoethanol, and 20% glycerol) containing 50 mM Gln, 5 mM GlnNAc, 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 GlnNAc 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. 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. 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 Sliver 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. Briefly, the membrane band was treated with poly(vinylpyrrolidone)-40 and digested with N-Glycanase, then digested with the trypsin. The tryptic 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.
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 GlcNAcβ1-3Galβ1-4Glc-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 bound fractions. GlcNAc-sulfotransferase and chondroitin-sulfotransferases 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 β4Gal-T 1,21 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 α-lactalbumin-agarose column equilibrated with the buffer containing 0.05% Triton X-100, 50 mM Glc, 5 mM MnCl₂, 0.5 mM ATP, and enzyme. The mixture was incubated at 37°C for 3 hours.

Table 1. Partial Purification of β-Galactosyltransferase from Chick Corneas

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units*)</th>
<th>Specific Activity (units/mg protein)</th>
<th>Total Activity (units*)</th>
<th>Specific Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>380</td>
<td>3.38 × 10⁴</td>
<td>88.9</td>
<td>7.76 × 10⁴</td>
<td>204</td>
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<tr>
<td>CM-Sepharose</td>
<td>231</td>
<td>2.93 × 10⁴</td>
<td>127</td>
<td>1.30 × 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>WGA-agarose</td>
<td>182</td>
<td>2.39 × 10⁴</td>
<td>132</td>
<td>1.30 × 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>Chelating-Sepharose</td>
<td>17.9</td>
<td>2.48 × 10³</td>
<td>139</td>
<td>7.22 × 10²</td>
<td>40.3</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>4.16</td>
<td>1.12 × 10³</td>
<td>267</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-Lactalbumin agarose</td>
<td>3.18</td>
<td>4.48 × 10²</td>
<td>141</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1st Superdex 200</td>
<td>1.27</td>
<td>2.02 × 10²</td>
<td>158</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2nd Superdex 200</td>
<td>0.13</td>
<td>1.35 × 10²</td>
<td>1038</td>
<td>3.53 × 10³</td>
<td>272</td>
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</table>

*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 μL: 0.2 mM GlcNAcβ1-3Galβ1-4Glc-PA, 50 mM MES buffer (pH 6.8), 10 mM UDP-Gal, 5 mM MnCl₂, 0.5 mM ATP, and enzyme. The mixture was incubated at 37°C for 3 hours.

†Not determined.
Gal-T Activity Toward 6-Sulfo-GlcNAcβ1-3Gal-PA

As described above, a βGal-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-GlcNAcβ1-3Gal-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 GlcNAcβ1-3Galβ1-4Glc-PA. The major part of the activity was lost during the purification. It appears that the activity is more labile than that toward GlcNAcβ1-3Galβ1-4Glc-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-β-hexosaminidase reaction, 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 GlcNAcβ1-3Galβ1-4GlcNAc-PA was 58.9% of that toward GlcNAcβ1-3Galβ1-4Glc-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-GlcNAcβ1-3Gal-PA was detected. Whether the enzyme associated with this activity is different from the Gal-T with the activity toward GlcNAcβ1-3Galβ1-4Glc-PA, remains to be clarified (see below). p-Nitrophenyl-β-GlcNAc did not serve as substrate, suggesting that CKGal-Ts 1 and 2 are not present in the 2nd Superdex 200 fraction (fraction 33).6 p-Nitrophenyl-β-(6-sulfo-)GlcNAc also did not serve as substrate, suggesting that the Gal-T, which transfers Gal to 6-sulfo-GlcNAcβ1-3Gal-PA, also recognizes not only the nonreducing 6-sulfo-GlcNAc residues but also internal sugar residues for a maximal reaction.

Identification of β-Gal in the Reaction Products

To confirm the β-configuration of the Gal transferred to two acceptor substrates, GlcNAcβ1-3Galβ1-4Glc-PA and 6-sulfo-GlcNAcβ1-3Gal-PA, the sensitivity of the reaction products to digestion with β-galactosidase was tested. After β-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 β-Galactosyltransferase*

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Relative Activity (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>20 mM</td>
<td>86.5</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>20 mM</td>
<td>92.9</td>
</tr>
<tr>
<td>2-Acetamide-2-deoxy-D-glucono-1,5-lactone</td>
<td>5 mM</td>
<td>81.7</td>
</tr>
</tbody>
</table>

*The reaction mixture contained the following components in a final volume of 50 μL: 0.2 mM GlcNAcβ1-3Galβ1-4Glc-PA, 10 mM UDP-Gal, 50 mM HEPES, 5 mM ATP, the added substance, and enzyme (3 μg: Fraction 33 of the 2nd Superdex 200 chromatography). The mixture was incubated at 37°C for 5 hours. Activity is expressed as percent of β-Gal-transfer observed in the absence of inhibitor (control).
This shows that Gal was transferred to the acceptors through a β-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 CKβ4Gal-Ts 1 and 2 are 41 and 43 kDa, respectively; that of βGal-T from mouse testis, 46 kDa; that of human βGal-T 7, 37 kDa; those of human βGal-T 1 to 6, between 37 kDa and 46 kDa. 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 CKβ4Gal-Ts 1 and 2, bovine β4Gal-T, murine β4Gal-Ts, human β4Gal-Ts 1 to 7, human β3Gal-T family, and murine α3Gal-T. One peptide (FDIEVPI) from the band (d) showed some homology (71.4%) with one part (FDIKVPL) of goat GlcNAc-6-sulfatase protein and one peptide (KAPYQAPRSP) from the band (e) showed some homology (66.7%) with one part (WAPPQSPRRP) of human βGalNAc-T protein.

**Discussion**

Shaper et al cloned cDNAs of two Gal-Ts (CKβ4Gal-T 1 and CKβ4Gal-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 had isolated a truncated cDNA of βGal-T from a chicken corneal cDNA library and found that its sequence showed high homology with both human β4Gal-T 1 and bovine milk β4Gal-T. It was
subsequently found that the sequence of the βGal-T cDNA of Cai et al.\textsuperscript{12} was identical to that of the CKβ4Gal-T 1 cDNA. Therefore, we attempted to purify βGal-T from chick corneas. However, the βGal-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 α-lactalbumin-agarose column, suggesting that CKβ4Gal-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 CKβ4Gal-T 1 activity was lost at this step, while the activities of the other βGal-Ts remained intact and could pass through an α-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 α-lactalbumin-agarose column, and therefore the CKβ4Gal-T activity should be absent in the WGA-agarose fraction before the Chelating-Sepharose chromatography or present only at a low level. The possibility also cannot be excluded that the bound fraction of Q-Sepharose chromatography might contain CKβ4Gal-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 CKβ4Gal-T 1. Because the Gal-T in the 2nd Superdex 200 fraction showed no activity toward p-nitrophenyl-β-GlcNAc, it might be β3Gal-T.\textsuperscript{14} However, none of the peptides from the bands (a) to (e) on SDS-PAGE showed homology with the human β3Gal-Ts.\textsuperscript{5,26}

The βGal-T that transfers Gal to the 6-sulfo-GlcNAc nonreducing end (6-sulfo-GlcNAcβ1-3Gal-PA) was found to be present in chick cornea. Although this activity was much higher in the crude extract than that toward GlcNAcβ1-3Galβ1-4Glc-PA (Table 1), a major part of the activity was lost during the purification. This βGal-T may become more labile with increasing purification. Whether a single enzyme has Gal-T activity toward both 6-sulfo-GlcNAcβ1-3Gal-PA and GlcNAcβ1-3Galβ1-4Glc-PA is unknown. Seko et al.\textsuperscript{14} reported that the GlcNAc-6-sulfate-specific β1,4 Gal-T was identified in human colorectal mucosa. They separated this Gal-T from β3Gal-T and β4Gal-T (probably β4Gal-T 1) by UDP-hexanolamine-Sepharose and asialo-agalacto-ovomucin-Sepharose chromatographies. These three βGal-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 β4Gal-T of Seko et al.\textsuperscript{14} showed activity toward a monosaccharide, 6-sulfo-GlcNAc, while our enzyme showed no activity toward p-nitrophenyl-β-(6-sulfo-)GlcNAc. Nevertheless, our enzymes might be the chick orthologues of the human β4Gal-T of Seko et al.\textsuperscript{14} Some activity toward 6-sulfo-GlcNAcβ1-3Gal-PA was also found in the bound fraction of Q-Sepharose chromatography: the ratio of the activities toward GlcNAcβ1-3Galβ1-4Glc-PA and toward 6-sulfo-GlcNAcβ1-3Gal-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 β4Gal-Ts and β3Gal-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 βGalNAc-transferase. Band (d) might contain a novel 6-sulfo-GlcNAc-specific CKβ-Gal-T; and band (e), a novel CKβ4Gal-T, if the enzyme with the activity toward 6-sulfo-GlcNAc end is different from the βGal-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 βGal-T.

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