Purification and Characterization of Chick Corneal β-D-Glucuronyltransferase Involved in Chondroitin Sulfate Biosynthesis

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Received June 11, 2002; accepted July 23, 2002; published online July 24, 2002

β-D-Glucuronyltransferase, which transfers D-glucuronic acid (GlcA) from UDP-GlcA to *N*-acetyl-D-galactosamine (GalNAc) at the nonreducing end of chondro-pentasaccharide-PA (pyridylamino-), GalNAcβ1-(4GlcAβ1-3GalNAcβ1)$_2$-PA, was purified 339-fold with an 11.0% yield from 2-d-old chick corneas by chromatography on DEAE-Sepharose, WGA-agarose, heparin-Sepharose, and 1st and 2nd UDP-GlcA-agarose (in the presence of Gal) columns. The activity was detected by fluorescence of PA residues of the product. The purified enzyme has an optimum pH of 7.0 (Mes buffer), and much higher activity toward chondro-heptasaccharide-PA than toward the chondro-pentasaccharide-PA, but no activity toward *p*-nitrophenyl-β-GalNAc. The enzyme activity was almost completely inhibited by GalNAc (20 mM). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme fraction showed one band of 38 kDa with many other bands. The amino acid sequence was determined for the tryptic digests of the 38 kDa band protein. The sequences determined showed no homology to those of several β-glucuronyltransferases reported previously. It seems that the enzyme is involved in the elongation of chondroitin sulfate chains *in vivo*.

Key words β-glucuronyltransferase; β-*N*-acetylgalactosaminyltransferase; chick cornea; chondroitin sulfate

Proteoglycan (PG) and type I collagen constitute the major components of corneal stroma. Two families of small PG are present in the adult animal corneal stroma: one with chondroitin sulfate/dermatan sulfate (CS/DS) side chains, which has been termed decorin[1] and the other with keratan sulfate (KS) side chains, which has been termed lumican.[2] Furthermore, it has been reported that three types of KSPGs, lumican, keratocan, and mimecan, are present in bovine cornea.[3—5] In normal corneal stroma of many mammals and birds, the two families of PGs are present in almost the same amounts.[6—11] But the accumulation and biosynthesis of the two families of PGs in corneal stroma readily change with changes in conditions *in vivo* and *in vitro*: in corneal scar regions,[12—15] in culture of corneal cells on plastic dish[16—21] and during corneal development.[22—26] Because PGs help maintain the regular arrangement of collagen fibrils within the corneal stroma[12,27—29] and play an important role in corneal transparency,[30] these changes in biosynthesis of PGs should be associated with the change in the transparency. Therefore, to clarify how PGs are biosynthesized in corneas is important to knowing about the process of the corneal transparency and diseases.

Many glycosyltransferases, sulfotransferases and epimerases are involved in biosynthesis of glycosaminoglycan chains of PGs. The carbohydrate backbones of CS have a linear polymer structure composed of alternating D-glucuronic acid (GlcA) and *N*-acetyl-D-galactosamine (GalNAc) units, joined by β1—4 linkages. Thus, β-glucuronyltransferase (GlcA-T) and β-*N*-acetylgalactosaminyltransferase (GalNAc-T) are involved in the biosynthesis of CS backbones (elongation of CS chains). This GlcA-T is referred to as GlcA-T II and distinguished from GlcA-T I, which is involved in the completion of linkage region of glycosaminoglycan-protein (initiation of CS biosynthesis).[31] Kitagawa *et al.*[32] obtained the enriched fraction of GlcA-T II activity from fetal bovine serum by heparin-Sepharose chromatography and examined its properties. They reported that the pH optimum of the enzyme activity was between 5.5 and 6.0 and that the GlcA transfer rate increased with increasing chain length of acceptor substrates (chondro-oligosaccharides). Tsuchida *et al.*[33] also reported that both GlcA-T II and GalNAc-T II activities in the enzyme preparation from fetal bovine serum were coeluted at 160 kDa from a Superose 6 column and that a single protein could have GlcA-T and GalNAc-T activities, as heparan sulfate-polymerase (HS-POL) has both *N*-acetylglucosaminyltransferase (GlcNAc-T) and GlcA-T activities.[34] Furthermore, Sugumaran *et al.*[35] purified GlcA-T II from cartilage of 17-d-old chick embryos and reported that the molecular size of the enzyme was 80 kDa by its photoaffinity labelling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). cDNA of the GlcA-T II has not been cloned and its amino acid sequence has not been determined yet.

In this study, we have purified the β-GlcA-T from 2-d-old chick corneas and characterized it. The purified enzyme was different from the GlcA-T II reported previously[32—35] in its behavior on various chromatographic columns and other properties.

MATERIALS AND METHODS

Materials White Leghorn male chicks (2-d-old) were obtained from Hattori Youkei-en, Nagoya, Japan. The following enzymes were obtained from the commercial sources indicated: collagenase (from *Clostridium hystolyticum*; Wako Pure Chemical, Osaka, Japan), β-glucuronidase (from bovine liver; Seikagaku Corp., Tokyo, Japan), hyaluronidase (from sheep testis; Seikagaku Corp., Tokyo, Japan), *N*-glycanase (peptide-*N*-glycanase F) (recombinant in *E. coli*; from *Flavobacterium meningosepticum*; Toyobo, Osaka, Japan), and trypsin (sequence grade, modified; from bovine pancreas; Promega, Tokyo, Japan). Chondroitin (from whale cartilage) and *p*-nitrophenyl-β-*N*-acetyl-D-galactosamine were purchased from Seikagaku Corp., Tokyo, Japan, 2-acet-

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amide-2-deoxy-D-glucono-1,5-lactone was from Funakoshi, Tokyo, Japan and *N*-acetylglucosamine-PA was from Takara, Osaka, Japan. DEAE-Sepharose FF, Heparin-Sepharose CL-4B, Sephadex G-50, and Sephadex G-15 were purchased from Amersham Pharmacia Biotech, Tokyo, Japan; UDP-GlcA-agarose and UDP-hexanolamine-agarose were from Sigma, Tokyo, Japan; wheat germ agglutinin (WGA)-agarose was from Seikagaku Corp., Tokyo, Japan. The columns for HPLC, TSK-gel ODS-120T, TSK-gel ODS-80TM, and TSK-gel G2500 PWXL were purchased from Tosoh, Tokyo, Japan.

Preparation of Substrates for Enzymatic Assay Acceptor substrates for GlcA-T were prepared as described previously.[36] Briefly, chondroitin (50 mg) was digested with testis hyaluronidase and the resultant digest (chondro-oligosaccharides) was chromatographed on a Sephadex G-50 column (15×500 mm). Of peaks observed on the column, the highest peak was pyridylaminated according to Hase *et al.*[37] The chondro-oligosaccharide-PAs thus obtained were chromatographed on Sephadex G-15 column (9×500 mm) to remove excess reagents, then the excluded fractions were pooled and lyophilized. The lyophilized fraction (chondro-oligosaccharide-PAs having GlcA at the nonreducing end) was digested with 0.1 U bovine liver β-glucuronidase. The digest was chromatographed on a TSK-gel ODS-120T column (7.8×300 mm) and then on a TSK-gel G2500 PWXL column (7.8×300 mm). Two deglucuronated chondro-oligo-saccharide-PAs were obtained, and their amounts were determined from their fluorescence intensities (excitation wavelength, 320 nm; emission wavelength, 400 nm) using GlcNAc-PA as standard: chondro-pentasaccharide-PA, GalNAcβ1-(4GlcAβ1-3GalNAc)$_2$-PA, 1062 nmol, and chondro-heptasaccharide-PA, GalNAcβ1-(4GlcAβ1-3GalNAc)$_3$-PA, 970 nmol.

Acceptor substrate for β-GalNAc-T was also prepared by the method described above except that the β-glucuronidase digestion was omitted: chondro-hexasaccharide-PA, (4GlcAβ1-3GalNAc)$_3$-PA, 1026 nmol.

Assay of β-GlcA-T and β-GalNAc-T Activities The standard reaction mixture for assay of GlcA-T activity contained 50 mM 4-morpholine ethanesulfonic acid (Mes) buffer, pH 7.0, 5 mM $MnCl_2$, 0.5 mM ATP, 10 mM UDP-GlcA, 0.2 mM chondro-pentasaccharide-PA and enzyme in a final volume of 50 μl. The reaction mixture was incubated at 37 °C for 3 h, and 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. The reaction product was eluted with the same buffer at a flow rate of 1.0 ml/min for 40 min 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-β-GalNAc was used as acceptor, the product on the HPLC column was detected by the absorbance at 300 nm.

GalNAc-T activity was assayed in the following reaction mixture: 50 mM 4-(2-hydroxyl)-1-piperzinyl ethanesulfonic acid (Hepes) buffer, pH 7.2, 5 mM $MnCl_2$, 100 mM GlcNAc, 0.5 mM ATP, 10 mM UDP-GalNAc, 0.2 mM chondro-hexasaccharide-PA and enzyme in 50 μl. The reaction mixture was incubated at 37 °C for 3 h. The reaction product was assayed as described above. The product was eluted later than the original substrate from the column.

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

Purification of β-Glucuronyltransferase All operations were performed at 4 °C. Protein in each fraction from the chromatographies described below was detected by monitoring absorbance at 280 nm.

Crude extract: Corneas were taken from 5992 eyeballs of 2-d-old male chicks, cut into sections and digested with 0.25% collagenase in phosphate-buffered saline, pH 7.2 (PBS: 1.15 g Na_2HPO_4, 8.0 g NaCl, 0.2 g KH_2PO_4, and 0.2 g KCl in 1000 ml of distilled water) at 37 °C for 1.5 h. The resultant cell suspension was centrifuged, and the pellet obtained was washed three times with PBS. The final resultant cell pellet was suspended in 7.0 ml of 10 mM Tris–HCl, pH 7.2 containing 20 mM $MgCl_2$ and homogenized with a Polytron-type microhomogenizer (Nition, Tokyo, Japan) in three 1-min bursts with 1-min intervals. The homogenate was centrifuged at 15000×***g*** for 10 min, and the resultant supernatant was removed. The precipitate was suspended 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 $MgCl_2$, 0.2% Triton X-100, 20 mM 2-mercaptoethanol, and 40% glycerol) and allowed to stand at 4 °C for 18 h. 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 $MgCl_2$, 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. GlcA-T was purified from this crude extract (35 ml) as described below.

DEAE-Sepharose FF Chromatography: The crude extract was applied at a flow rate of 2.0 ml/min to a DEAE-Sepharose FF column (2.6×8.0 cm) equilibrated with buffer A. The column was then washed with 360 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 min, then with 1 M NaCl in buffer A for 100 min. The GlcA-T activity passed through the column, and the pass-through fractions (100 ml) with the activity were pooled and concentrated to 32 ml by ultrafiltration using a YM-10 membrane (Millipore, Tokyo, Japan).

WGA-Agarose Chromatography: The concentrated DEAE-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 30 ml of the same buffer, and the adsorbed materials were eluted with 33 ml of buffer A containing 0.3 M GlcNAc and 0.15 M NaCl. The GlcA-T activity again passed through the column, and the pass-through fractions (24 ml) with the activity were pooled, concentrated to 8.0 ml by ultrafiltration as described above and dialyzed against buffer A.

Heparin-Sepharose Chromatography: The concentrated, dialyzed WGA-agarose fraction was applied at a flow rate of 0.5 ml/min to a heparin-Sepharose column (2.6×5.0 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—1.0 M of NaCl in buffer A for 60 min, then with 1.0 M NaCl in buffer A for 44 min. The fractions of each protein peak detected by absorbance at 280 nm (bar 1—4 in Fig. 1) were pooled and concentrated, respectively. Each concentrated fraction was assayed for the GlcA-T activity and the activity was detected in the first (bar 2 in Fig. 1) of three peaks adsorbed onto the column. The fraction with the activity (2.5 ml) was dialyzed against buffer B, which differed from buffer A in containing 10 mM $MnCl_2$ instead of 20 mM $MgCl_2$.

First UDP-GlcA-Agarose Chromatography: The concentrated, dialyzed heparin-Sepharose fraction was diluted 2-fold with buffer B from which Triton X-100 was omitted: thereby the concentration of Triton X-100 was decreased to 0.1%. This diluted fraction was applied at a flow rate of 0.2 ml/min to a UDP-GlcA-agarose column (1.0×5.5 cm) equilibrated with buffer B. The column was washed with buffer B containing 100 mM Gal for 80 min and the adsorbed materials were eluted with 15 ml of buffer B containing 1 M NaCl. Each tube was detected for protein content by absorbance at 280 nm. The large broad protein peak of pass-through fractions and the small peak of adsorbed fractions, both were concentrated and assayed for the GlcA-T activity. The activity was detected in the adsorbed fraction and the adsorbed fraction (2.2 ml) was dialyzed against buffer B.

Second UDP-GlcA-Agarose Chromatography: The concentrated, dialyzed 1st UDP-GlcA-agarose fraction was rechromatographed on a UDP-GlcA-agarose column (1.0×5.5 cm) under the same conditions as described for the 1st chromatography. The adsorbed fraction with the activity (2.2 ml) was dialyzed against buffer B.

Assay of Protein Protein content was determined by the method of Bradford.[38] Protein assay reagents were obtained from Bio-Rad, Tokyo, Japan. 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.

Identification of β-GlcA in the GlcA-T Reaction Products To confirm the β-configuration of the GlcA incorporated into acceptor substrates, GlcA-T reaction was performed in the standard reaction mixture using the 2nd UDP-GlcA-agarose fraction (10 μl) as enzyme and chondro-pentasaccharide-PA or chondro-heptasaccharide-PA (10 nmol) as acceptor substrate at 37 °C for 18 h. Each mixture was, then, subjected to HPLC of TSK-gel ODS 80TM column as described above. Each reaction product was recovered and lyophilized. Each lyophilized product was dissolved in 100 μl of 50 mM sodium acetate, pH 5.0 containing 0.5 U of β-glucuronidase (bovine liver) and the solution was incubated at 37 °C for 20 h. After reaction, the solution was subjected to HPLC of the same TSK-gel ODS 80TM column and whether the fluorescent peak was shifted to the position of original acceptor substrate or not, was examined.

SDS-PAGE PAGE of proteins in SDS was performed on 10% polyacrylamide gel under reducing conditions according to the method of Laemmli.[39] Proteins (1—5 μg) in the samples were 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 min. The dissolved protein samples were subjected to gel electrophoresis, and the protein bands were detected with silver stain.

Amino Acid Sequencing of Peptides from Tryptic Digests of the Protein Bands Obtained by SDS-PAGE Second UDP-GlcA-agarose fraction (40 μg as protein) was subjected to SDS-PAGE as described above except that the precipitate was 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 min to be solubilized surely. Then, proteins in gel were electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P, 0.45 μm: Millipore, Tokyo, Japan) and visualized with Comassie Brilliant Blue. The band of the 38 kDa protein (see below) was excised, washed with 50% methanol to eliminate the dye, then digested with the modified trypsin according to the method of Aebersold *et al.*[40] Briefly, the membrane band was first 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 80-TM 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, Japan).

RESULTS

Purification of β-GlcA-T Table 1 summarizes the purification of the GlcA-T from 5992 chick corneas. The GlcA-T activity passed through the DEAE-Sepharose FF column,

Table 1. Purification of β-Glucunonyltransferase

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	3990	383	10.4	1	100
DEAE-Sepharose FF	3680	63.1	58.3	5.59	92.2
WGA-agarose	1460	17.3	84.3	8.08	36.6
Heparin-Sepharose	655	5.74	114	10.9	16.4
1st UDP-GlcA-agarose	680	0.583	1170	112	17.0
2nd UDP-GlcA-agarose	438	0.108	4040	389	11.0

One unit of activity is defined as the amount required to catalyze the transfer of 1 pmol/min of GlcA. The reaction mixture contained the following components in a final volume of 50 μl: 0.20 mM chondro-pentasaccharide-PA, 50 mM Mes buffer (pH 7.0), 5 mM $MnCl_2$, 0.5 mM ATP, 10 mM UDP-GlcA, and enzyme. The mixture was incubated at 37 °C for 3 h.

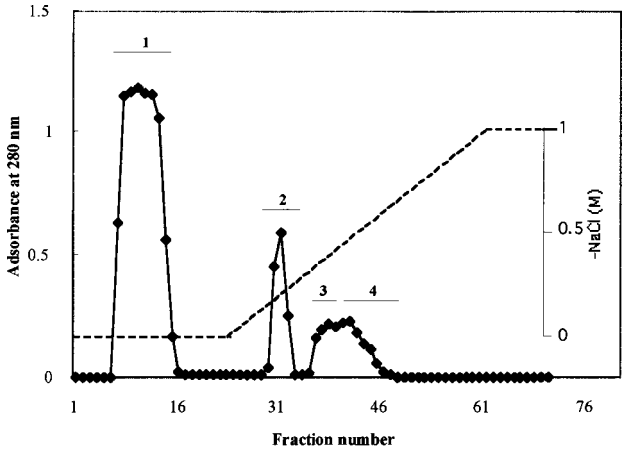

Fig. 1. Heparin-Sepharose Column Chromatography

The concentrated WGA-agarose fraction was applied to a heparin-Sepharose column (2.6×5.0 cm) equilibrated with buffer A. The column was washed with 40 ml of buffer A and the bound proteins were eluted with a linear gradient of 0—1.0 M NaCl in buffer A. Protein in each fraction was detected by monitoring absorbance at 280 nm (closed diamonds). The fractions indicated by horizontal bars 1 to 4 were pooled and concentrated, respectively.

while the major portion (83%; 14500 units) of the GalNAc-T activity bound to the column. This result suggests that the GlcA-T protein is different from the GalNAc-T protein, although Tsuchida *et al.*[33] stated that a single protein could have GlcA-T and GalNAc-T activities (as described above). The major portion of the GlcA-T activity was recovered with a small loss after DEAE-Sepharose FF chromatography, and some increase in the specific activity was found. The GlcA-T activity was not found in the bound fractions of DEAE-Sepharose chromatography. The GlcA-T activity also passed through a WGA-agarose column, and no GlcA-T activity was found in the fractions bound to the column. Much GlcA-T activity was lost in this step, although some increase in the specific activity was found, and GlcNAc-sulfotransferase, which bound to the WGA-agarose column, was separated from GlcA-T. GlcA-T thus showed a little different chromatographic behavior from the enzymes reported previously, which bound to WGA-agarose columns.[33,35] After the WGA-agarose chromatography, UDP-hexanolamine-agarose chromatography of the enzyme fraction was attempted but the GlcA-T passed through the column. Next, the enzyme fraction was applied to a heparin-Sepharose column (Fig. 1). The GlcA-T activity was bound to this column and found in the first of three peaks eluted with NaCl. The GlcA-T activity (bar 2 in Fig. 1) was eluted with a low concentration of NaCl (0.1—0.25 M), and the peaks (bars 3, 4 in Fig. 1) eluted with a higher concentration of NaCl contained β-*N*-acetylgalactosaminidase activity but not GlcA-T activity. The UDP-GlcA-agarose chromatography in the presence of Gal was the most important step for the purification of GlcA-T. The specific activity was greatly increased by the 1st and 2nd UDP-GlcA-agarose chromatographies. In a separate experiment, the GlcA-T was not bound to a UDP-GlcA-agarose column in the buffer without Gal. In the presence of Gal (analogue with GalNAc end of acceptor substrate), a complex of GlcA-T protein, Gal and UDP-GlcA-agarose may be formed like an intermediate complex in an actual enzyme reaction. The GalNAc-T activity (2950 units) which was found with GlcA-T activity in pass-through fraction of the DEAE-Sepharose

Table 2. Effects of Various Additions on the Activity of β-Glucuronyltransferase

Addition	Relative activity (%)
Control	100
GlcNAc (20 mM)	77.1
GalNAc (20 mM)	4.09
2-Acetamide-2-deoxy-D-glucono-1,5-lactone (5 mM)	0.13

The reaction mixture contained the following components in a final volume of 50 μl: 0.20 mM chondro-pentasaccharide-PA, 50 mM Mes (pH 7.0), 5 mM $MnCl_2$, 0.5 mM ATP, 10 mM UDP-GlcA, the added substance, and enzyme. The mixture was incubated at 37 °C for 5 h. The table is a composite of two separate experiments and each value is an average of two data; standard errors are <5% of the means.

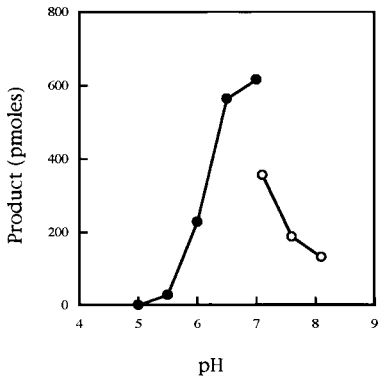

Fig. 2. Effects of pH on the Activity of the β-GlcA-T

The reaction mixture contained the following constituents in a volume of 50 μl: 50 mM buffer indicated, 5 mM $MnCl_2$, 0.5 mM ATP, 10 mM UDP-GlcA, 0.2 mM GlcNAcβ1-3Galβ1-4Glc-PA, and enzyme. The mixture was incubated at 37 °C for 5 h. The buffers were Mes (closed circles) and Hepes (open circles).

chromatography as described above, was not detected in both pass-through and bound fractions of the 2nd UDP-GlcA-agarose chromatography. But which purification step the GalNAc-T activity was lost in is unknown because the GalNAc-T activity was not assayed during the steps after the DEAE-Sepharose chromatography. The 2nd UDP-GlcA-agarose fraction was examined for its properties.

Properties of the Purified β-Glucuronyltransferase Figure 2 shows the effects of pH on the activity of GlcA-T. Optimum pH was 7.0. This is different from that of GlcA-T of fetal bovine serum: pH 5.5—6.0.[32] The activity at pH 7.0 in the Mes buffer was markedly higher than that in the Hepes buffer. Thus, the Mes buffer of pH 7.0 was used for the experiments described below. Table 2 shows the effects of various additions on the activity. GalNAc (20 mM) strongly inhibited the GlcA-T reaction. The GlcA-T protein may have a high affinity to GalNAc, but, because GalNAc does not serve as an acceptor substrate (see below), it may inhibit the reaction. GlcNAc (20 mM) inhibited the reaction to a lesser extent. 2-Acetamide-2-deoxy-D-glucono-1,5-lactone (5 mM) which is a strong inhibitor of β-*N*-acetylhexosaminidase,[41] completely inhibited the GlcA-T reaction. This compound could have a higher affinity to GlcA-T than GalNAc. Table 3 shows the acceptor substrate specificity of the GlcA-T. The activity toward chondro-heptasaccharide-PA, GalNAcβ1-(4GlcAβ1-3GalNAcβ1)$_3$-PA was 10-fold higher than that toward chondro-pentasaccharide-PA, GalNAcβ1-(4GlcAβ1-3GalNAc)$_2$-PA. Kitagawa *et al.*[32] have also reported that the GlcA transfer rates roughly increased with increasing chain length. *p*-Nitrophenyl-β-GalNAc did not serve as an accep-

Table 3. Acceptor Substrate Specificity of β-Glucuronyltransferase

Acceptor (0.2 mM)	Relative activity (%)
GalNAcβ1-(4GlcAβ1-3GalNAcβ1)$_3$-PA	1110
GalNAcβ1-(4GlcAβ1-3GalNAcβ1)$_2$-PA	100
p-Nitrophenyl-β-GalNAc	—[a]

The reaction mixture contained the following components in a final volume of 50 μl: 0.2 mM substrate indicated above, 50 mM Mes (pH 7.0), 5 mM $MnCl_2$, 0.5 mM ATP, 10 mM UDP-GlcA, and enzyme. The mixture was incubated at 37 °C for 5 h. The table is composite of two separate experiments and each value is an average of two data; standard errors are <5% of the means. *a*) Not detected.

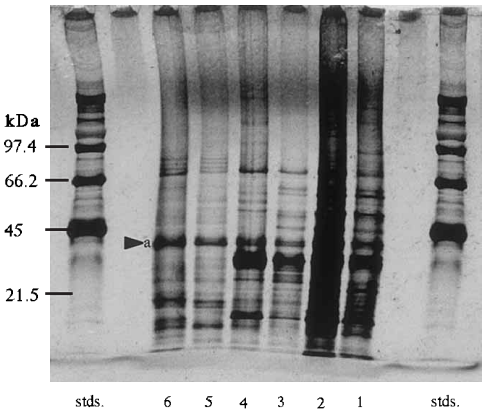

Fig. 3. SDS-PAGE of Enzyme Preparation from Each Purification Step

Lane 1, crude extract; lane 2, DEAE-Sepharose; lane 3, pass-through fraction of UDP-GlcA-agarose (without Gal) in a separate experiment; lane 4, heparin-Sepharose; lane 5, 1st UDP-GlcA-agarose (with Gal); lane 6, 2nd UDP-GlcA-agarose (with Gal). Stds, standards. Proteins were stained with silver nitrate. Band (a) on lane 6 indicated by arrowhead was analyzed for amino acid sequence.

tor. How the GlcA-T protein recognizes the oligosaccharide and sterically binds to it is unknown. The GlcA-T could recognize not only the non-reducing GalNAc but also several internal GlcA and GalNAc residues, interact with those residues and present the non-reducing GalNAc to its own active site.

Identification of β-GlcA in the Reaction Products To confirm the β-configuration of the GlcA transferred to acceptor substrates, the sensitivity of the GlcA-T reaction products obtained using chondro-heptasaccharide-PA and chondro-pentasaccharide-PA as acceptor to digestion with β-glucuronidase was tested as described under Materials and Methods. After β-glucuronidase digestion, each digest was applied to a TSK-gel ODS-80TM column. The fluorescent peak (oligosaccharide-PA) was found only at the same position as that of the corresponding original acceptor substrate (data not shown). This result shows that GlcA was transferred to each acceptor through a β-linkage.

SDS-PAGE of the Purified Enzyme Fraction Figure 3 shows SDS-PAGE of the GlcA-T fractions from different purification steps. The 2nd UDP-GlcA-agarose fraction showed one band 'a' of 38 kDa and many other bands (lane 6 in Fig. 3). Because many glycosyltransferases have a molecular size around 40 kDa, the band of 38 kDa may be the GlcA-T (see Discussion).

DISCUSSION

In this study, the GlcA-T was purified 389-fold with relatively high yield (11.0%) from chick corneas. This comparatively low purification of the enzyme occurred because the enzyme did not bind to several ion exchanger columns and affinity columns: DEAE-Sepharose, CM-Sepharose, GlcNAc-agarose, UDP-hexanolamine-agarose, and WGA-agarose. The enzyme did bind to heparin-Sepharose (Fig. 1). The elution profile of this chromatography showed three peaks eluted with a gradient of NaCl. Peak 2 contained the GlcA-T activity and Peaks 3 and 4 contained the exo-β-galactosaminidase activity. The latter enzyme had an optimum pH of 4.0, a low activity at pH 6.8 and no activity at pH 7.0 (the activity was assayed using chondro-pentasaccharide-PA as substrate). The reaction product of chondro-pentasaccharide-PA with this galactosaminidase was eluted from the TSK-gel ODS-80TM column at almost the same position as the product formed by the GlcA-T. Thus, while the reaction by the GlcA-T fractions before the step of heparin-Sepharose chromatography should be slightly interfered by the contaminant galactosaminidase, the peak of product on the HPLC column should contain a small amount of the galactosaminidase reaction product. Therefore, although the galactosaminidase has no activity at pH 7.0, the values of the activity in Table 1 until the third step of purification may be a little inaccurate. Peak 2 (GlcA-T fraction) in Fig. 1 showed no galactosaminidase activity when assayed using chondro-pentasaccharide-PA as substrate without UDP-GlcA. The GlcA-T bound to UDP-GlcA-agarose in the presence of Gal. The GlcA-T did not bind to the column in the absence of Gal, unlike the GlcAT-P involved in the biosynthesis of the HNK-1 carbohydrate epitope.[42] The purification of the GlcA-T was increased markedly by the 1st and 2nd UDP-GlcA-agarose chromatographies.

But, even after the 2nd UDP-GlcA-agarose chromatography, SDS-PAGE of the purified enzyme fraction showed many bands on the gel (Fig. 3): one strongly stained band of 38 kDa, bands below 21.5 kDa including four strongly stained bands, and bands over 66.2 kDa. Which band is GlcA-T is unknown at present. But deducing from the reports described below, the band of 38 kDa may be the GlcA-T. The molecular size of GlcA-T I involved in the biosynthesis of the glycosaminoglycan-protein linkage region is estimated as 37 kDa from the amino acid sequence deduced by nucleotide sequencing of the cloned cDNA[43] and as 43 kDa by Superdex 200 chromatography of the bacterial-expressed protein.[44] The molecular sizes of human and rat GlcA-Ts involved in the biosynthesis of the HNK-1 epitope[45—47] all are estimated as 37 kDa by the amino acid sequence deduced from nucleotide sequence of the cDNAs. In addition, the molecular sizes of many glycosyltransferases are around 40 kDa.[48,49] Furthermore, the molecular size of bacterial chondroitin synthase, which has both GlcA-T and GalNAc-T activities[50] is 80 kDa: this value is reasonable when it is considered that the synthase consists of GlcA-T and GalNAc-T protein parts, each of which could have a size of about 40 kDa.

Then, as described under Experimental Procedures, we attempted to digest the band protein of 38 kDa with trypsin, and to determine the amino acid sequences of four peptides obtained. None of the sequences showed homology with the GlcA-Ts reported previously.[34,43,45—47,50] Because GlcA-T I[43] and the GlcA-T involved in the biosynthesis of HNK-

1[46,47,50] transfer GlcA to a nonreducing Gal residue of a sugar chain, their substrate specificities are different from that of GlcA-T II. Heparan sulfate-polymerase[34] has both GlcA-T and α-GlcNAc-T activities, and this GlcA-T transfers GlcA to a nonreducing GlcNAc residue. Bacterial chondroitin synthase also has both GlcA-T and GalNAc-T activities, but this enzyme shows a high homology (87%) to the hyaluronic acid synthase of the same bacterium.[50] In view of these reports, the lack of amino acid sequence homology between our GlcA-T and the other GlcA-Ts is not unreasonable, if our GlcA-T is GlcA-T II. Judged from acceptor substrate specificity of our GlcA-T in Table 3, our GlcA-T could be GlcA-T II involved in elongation of chondroitin sulfate chain, although we have not determined which carbon of nonreducing GalNAc of the substrates GlcA was transferred to by our GlcA-T.

Our GlcA-T is similar to bovine serum GlcA-T reported by Tsuchida *et al.*[33] and Kitagawa *et al.*[32] on the substrate specificity. But, as described above, Tsuchida *et al.*[33] have reported that both GlcA-T and GalNAc-T activities in the enzyme preparation were coeluted at 160 kDa on gel filtration of a Superose 6 column and that a single protein may have GlcA-T and GalNAc-T activities. The molecular size of 160 kDa is much higher than 38 kDa which we suppose as the molecular size of our GlcA-T. Recently Kitagawa *et al.*[51] cloned and reported cDNA of a human chondroitin synthase which has both GlcA-T and GalNAc-T activities. Molecular size of this enzyme is 91728 Da from the deduced amino acid sequence and it showed no homology with any GlcA-Ts whose amino acid sequences have been reported so far. Also, four peptides from our 38 kDa protein described above showed no homology with this chondroitin synthase. Bovine serum GlcA-T described above may be a part of bovine analogue of the chondroitin synthase unlike our GlcA-T. On the other hand, very recently Uyama *et al.*[52] have reported that cDNA of human chondroitin GalNAc-T, the amino acid sequence of which showed 27% identity to human chondroitin synthase described above, has been cloned. This enzyme has the GalNAc-T activity only and the deduced molecular size of the protein is 61350 Da. The GalNAc-T can transfer GalNAc to chondroitin, and $(GlcA\beta 1\text{-}3GalNAc)_3$. Because the GalNAc-T of 61 kDa was found, it cannot be excluded that one of minor band proteins over 66.2 kDa on lane 6 in Fig. 3 may be GlcA-T. Cloning of 38 kDa protein cDNA is in process on the basis of amino acid sequences of four peptides obtained.

Much higher activity of GalNAc-T (17450 units) was found in the crude extract than that of GlcA-T (3990 units). But when the bound fraction (14500 units) of DEAE-Sepharose chromatography was chromatographed on TSK-gel AF-blue Toyopearl 650 column (Tosoh, Tokyo, Japan), no GalNAc-T activity was found in either pass-through or bound fraction. GalNAc-T could form a complex with GlcA-T *in vivo* (in Golgi membrane), and GalNAc-T alone (not associated with GlcA-T) could be unstable *in vitro*.

Acknowledgments This work was supported by a Grant-in aid for Scientific Research No. 11671762 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Ministry of Education, Culture, Sports, Science and Technology of Japan (High-Tech Research Center Project), by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Scientific Frontier Research Project), and by a Grant-in-aid for Specially Promoted Research from the Research Institute, Meijo University.

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