Purification, characterization and specificity of chondroitin lyases and glycuronidase from *Flavobacterium heparinum*

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The chondroitin lyases from *Flavobacterium heparinum* (*Cytophaga heparinia*) have been widely used in depolymerization of glycosaminoglycan and proteoglycan chondroitin sulphates. Oligosaccharide products derived from chondroitin sulphate can be further degraded by glycuronidases and sulphatases obtained from the same organism. There has been no reported purification of these enzymes to homogeneity nor is there any information on their physical and kinetic characteristics. The absence of pure enzymes has resulted in a lack of understanding of the optimal conditions for their catalytic activity and their substrate specificity. This has limited the use of these enzymes as reagents for preparation of oligosaccharides for structure and activity studies. Reproducible schemes to purify a chondroitin AC lyase, a glycuronidase and chondroitin B lyase from *Flavobacterium heparinum* to apparent homogeneity are described. Chondroitin AC lyase (chondroitinase AC, EC 4.2.2.5), glycuronidase [chondro-(1 → 3)-glycuronidase, no EC number] and chondroitin B lyase (chondroitinase B, no EC number) have M_r values (assessed by SDS/PAGE) of 74000, 41800 and 55200 respectively, and isoelectric points (determined by isoelectric focusing) of 8.85, 9.28 and 9.05 respectively. Chondroitin lyase AC and B contain pyroglutamic acid at their N-termini precluding their analysis by Edman degradation. Deblocking with pyroglutamate aminopeptidase facilitated the determination of their N-terminal sequences. The kinetic properties of these enzymes have been determined as well as the optimum conditions for their catalytic activity. The specificity of the glycouronidase, determined using 17 different disaccharide substrates, shows that it only acts on unsulphated or 6-*O*-sulphated 1 → 3 linkages. The chondroitin lyases are both endolytic enzymes, and oligosaccharide mapping shows their expected specificity towards the chondroitin and dermatan sulphate polymers.

INTRODUCTION

Enzymes degrading glycosaminoglycans have become increasingly important in understanding the biological roles and structures of the glycosaminoglycans and proteoglycans, which are involved in the regulation of various cellular processes such as adhesion, differentiation, migration and proliferation [1–3]. Utilizing these enzymes, the design and preparation of glycosaminoglycan-based therapeutic agents also becomes possible [4,5]. Chondroitin sulphates are the most common type of glycosaminoglycan chains found in proteoglycans. They are sulphated linear polysaccharides with alternating 1 → 3, 1 → 4 linkages. The major classes are chondroitin sulphate A (chondroitin 4-sulphate), dermatan sulphate (chondroitin sulphate B) and chondroitin sulphate C (chondroitin 6-sulphate). The biological roles of chondroitin sulphate glycosaminoglycans are poorly understood and their exact chemical structures have not been determined [1]. Enzymic methods are preferable to chemical methods when determining polysaccharide structures. Enzymes by their nature are often very specific and act under mild conditions giving oligosaccharide products. Two classes of enzyme that act on glycosaminoglycans are polysaccharide lyases and hydrolases. Prokaryotic polysaccharide lyases depolymerize glycosaminoglycans through an eliminative mechanism [6] whereas enzymes from eukaryotic sources act through a hydrolytic mechanism.

Bacterial degradation of glycosaminoglycans has primarily been studied using enzymes produced from *Flavobacterium heparinum* [7–12]. Three heparin lyases from *F. heparinum* have recently been purified and characterized by our laboratory [13,14]. Chondroitin lyases catalysed the depolymerization of chondroitin sulphates into disaccharides and oligosaccharides with Δ4,5-unsaturated uronic acid residues at their non-reducing ends. These products are further degraded by hydrolases including glycuronidases and sulphatases. Sulphatases remove sulphate groups (either O-linked or N-linked) from the oligosaccharide products [15–19]. Glycuronidases hydrolyse the unsaturated disaccharide products of the lyase-catalysed depolymerization of chondroitin sulphate to a monosaccharide (sulphated *N*-acetylgalactosamine) and an α-keto acid [7,10,20]. There are no reports on the purification of chondroitin lyases and glycuronidases to homogeneity, thus there are no physical or reliable kinetic data available for these enzymes. The absence of pure enzymes with known properties has limited our understanding of their specificity and has prevented the full utilization of these enzymes in the study of chondroitin sulphate structure. We report here reproducible schemes for the purification of *flavobacterial* chondroitin AC lyase, glycuronidase and chondroitin B lyase to apparent homogeneity and the determination of their physical properties, kinetic properties, optimal catalytic conditions and specificity.

EXPERIMENTAL

Materials

Hydroxyapatite columns (1 cm × 30 cm with a 1 cm × 5 cm guard) were from Regis Technol (Morton Grove, IL, U.S.A.). Bio-Sil gel-permeation column was from Bio-Rad (Hercules, CA, U.S.A.). Sepharose S (Big Beads), quaternary ammonium ethyl (QAE) Sephadex and Superdex 200 were from Pharmacia (Piscataway, NJ, U.S.A.). Strong cation-exchange column (CBX-S) was from J. T. Baker (Phillipsberg, NJ, U.S.A.). Hydroxyapatite was from Asahi Optical Co. (Tokyo, Japan). Pressure filtration PM10 filters (10000) and cellufine sulphate were from

Abbreviations used: IEF, isoelectric focusing; CE, capillary electrophoresis.

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Amicon (Danvers, MA, U.S.A.). Chondroitin sulphate (70% of A and 30 of C) used in fermentation was from Sigma (St. Louis, MO, U.S.A.). Glycosaminoglycans and unsaturated disaccharides were from Seikagaku American (Rockville, MD, U.S.A.). N-Terminal acyl-amino-acid-releasing enzyme and pyroglutamate aminopeptidase were from Takara (Tokyo, Japan). All water used in reagents was deionized and distilled in glass.

Assays

The spectrophotometer was adjusted to the optimum temperature of the particular enzyme being assayed. A 700 μl quartz cuvette (path length 1 cm) containing substrate (500 μg of chondroitin sulphate C or dermatan sulphate or 100 μg of ΔUAp(1 → 3)GalNpAc6S (where ΔUAp is 4-deoxy-α-L-*threo*-hexo-4-enopyranosyluronic acid, GalNp is 2-deoxy-2-amino-D-galactopyranose, Ac is acetate and S is sulphate) in 50 mM sodium phosphate buffer (50 mM Tris/HCl buffer for chondroitin B lyase) was thermally equilibrated. A measured quantity of enzyme was added, bringing the final volume to 500 μl and the cuvette contents were gently mixed. The cuvette was then immediately returned to the spectrophotometer and the change in A_{232} was measured at 15 s intervals over 3 min. Enzyme activities were calculated using a molar absorption coefficient of 3800 $M^{-1} \cdot cm^{-1}$ for unsaturated oligosaccharide products formed by chondroitin AC or B lyase digestion or substrate converted during glycuronidase digestion. One unit of glycuronidase is defined as 1 μmol of unsaturated disaccharide cleaved/min. Protein concentration was measured [21] based on a BSA standard curve. Specific activities were calculated by dividing volumetric activity by protein concentration measurements.

Fermentation and enzyme recovery

F. heparinum (ATCC 13125) [22] obtained from Professor R. Langer (MIT, Cambridge, MA, U.S.A.) was stored at −70 °C in a defined medium containing heparin as the carbon source and DMSO [13,23]. Chondroitin sulphate-degrading strains of *F. heparinum* were screened using a modification of the agarose plate assay for heparin lyase activity [23,24]. Clear zones appearing around colonies grown on plates containing chondroitin sulphate indicated colonies that produced chondroitin lyase activity. Multiple large colonies isolated from these plates were grown in a 10-litre stirred tank fermenter in a modification of the defined medium [22] where chondroitin sulphate A and glucose were used as the carbon source [24]. A 180 *g* pellet (wet weight) was obtained from 10 litres of fermentation broth by centrifugation (15 min; 12000 *g*; 4 °C).

Purification of chondroitin AC lyase and glycuronidase

Recovery from cell pellet and QAE-Sephadex chromatography

The cell pellet obtained by fermentation was suspended in 500 ml of 10 mM sodium phosphate buffer at pH 6.8 and 4 °C. Cell disruption was achieved by sonication in 20 ml aliquots (100 W; 40% output; Pulsed mode; 9 min; 4 °C). The disrupted cells were centrifuged at 10000 *g* for 30 min at 4 °C. The 750 ml supernatant obtained contained 21 mg/ml protein. Nucleic acids were removed from the supernatant (750 ml at 21 mg/ml) by protamine sulphate (2% in the same buffer added to the supernatant in a ratio of 125 mg/g of protein) precipitation. After centrifugation at 10000 *g* at 4 °C for 30 min, the supernatant obtained was concentrated by pressure filtration with a PM10 membrane (M_r cut-off 10000) at 414 kPa and 4 °C. The concentrated solution (325 ml; 14.2 mg/ml protein) had activity towards chondroitin sulphate A and C and dermatan sulphate. A QAE-Sephadex chromatography step was performed at 4 °C using columns (2.5 cm × 20 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.8. Crude enzyme solution (20 ml) was applied (1 ml of swelled QAE beads/mg of protein) and the columns were washed with 1.5 vol. of the same buffer. The lyase activities passed through the column. The eluate (7.9 litres) was collected, concentrated by pressure filtration to 138 ml (containing 0.51 mg/ml protein), divided into 4 ml aliquots and stored at −70 °C.

Hydroxyapatite HPLC

Samples (4 ml) purified by QAE-Sephadex chromatography were injected on to a hydroxyapatite HPLC column equilibrated with 50 mM sodium phosphate buffer, pH 6.8. The column was washed with the same buffer, at 0.5 ml/min for 20 min. Bound proteins were eluted with a 60 ml linear gradient of 0.0–0.75 M NaCl in phosphate buffer, the eluate was monitored at 280 nm and 1 ml fractions were collected. The hydroxyapatite column was regenerated by washing with 5.0 ml of 1 M NaCl and re-equilibrated with 50 mM sodium phosphate buffer, pH 6.8. Fractions containing chondroitin AC lyase and chondroitin B lyase activity were individually pooled, dialysed against 100 vol. of 50 mM sodium phosphate buffer, pH 6.8, overnight at 4 °C, and concentrated 10-fold by pressure filtration. The two chondroitin lyase preparations were each divided into 1 ml aliquots and frozen at −70 °C.

Gel-permeation HPLC

The chondroitin AC lyase preparation (3 mg/ml protein) obtained from hydroxyapatite HPLC was applied to a Bio-Sil gel-permeation HPLC column (1 cm × 25 cm) that had been equilibrated with 200 mM sodium phosphate buffer, pH 6.8. The column was calibrated with the following standards: thyroglobulin, γ-globulin, ovalbumin, myoglobin and cyanocobalamin. The sample containing chondroitin AC lyase activity was injected (150 μg/50 μl) on to the column and eluted with 20 ml of 200 mM sodium phosphate buffer, pH 6.8, at a flow rate of 0.5 ml/min. The elution was monitored at 280 nm and fractions were collected and assayed for chondroitin AC lyase and glycuronidase activity. The active fractions were pooled and dialysed against 100 vol. of 50 mM sodium phosphate buffer (pH 6.8) overnight at 4 °C, concentrated 10-fold by pressure filtration, subdivided into 10 μl aliquots and stored at −70 °C.

Purification of chondroitin B lyase

Recovery from cell pellet and cation-exchange chromatography

The cells were harvested by centrifugation and the desired enzymes released from the periplasmic space by osmotic shock [25]. The osmolates obtained were subjected to centrifugation and the supernatant was applied to a cation-exchange column (5.0 cm × 30 cm; Sepharose S Big Beads) at a linear flow rate of 10 cm/min. The bound proteins were eluted at a flow rate of 5.1 cm/min with step gradients of 0 M NaCl, 0.25 M NaCl and 1.0 M NaCl, all in 10 mM sodium phosphate at pH 7.0.

Chromatography on cellufine sulphate

Chondroitin B lyase activity was eluted in the 0.25 M NaCl fraction which was further purified by diluting the chondroitin lyase-containing fraction 2-fold with 10 mM sodium phosphate and applying the material to a column (2.6 cm × 100 cm) containing cellufine sulphate and eluting at a flow rate of 1.88 cm/min with a linear (0.0–0.4 M) gradient of NaCl. Chondroitin AC

Table 1 Summary of purification of chondroitin lyases and glycuronidase

One unit of chondroitin AC lyase and chondroitin B lyase is defined as the amount of enzyme that causes 1 μmol of product to be formed/min. One unit of glycuronidase is defined as the amount of enzyme that catalyses the disappearance of 1 μmol of substrate/min. The slash separates activity towards chondroitin sulphate A from that towards chondroitin sulphate C. n.d., Not determined.

Purification step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)
Chondroitin AC lyase				
Cell homogenate	1000	36/30	$3.6 \times 10^{-2}/3.0 \times 10^{-2}$	1/1
Protamine Precipitate	930	73.4/83.1	$7.9 \times 10^{-2}/8.9 \times 10^{-2}$	2.2/3
QAE chromatography	72	n.d./83.3	n.d./1.2	n.d./40
Hydroxyapatite HPLC	2.8	45.7/35.4	16.3/12.6	453/420
Gel-permeation HPLC	0.3	20.4/33.2	68/111	1889/3700
Glycuronidase				
Cell homogenate	1000	46	4.6×10^{-2}	1
Protamine Precipitate	930	n.d.	n.d.	n.d.
QAE chromatography	72	4.2	5.8×10^{-2}	1.3
Hydroxyapatite HPLC	2.8	10.6	3.8	82.6
Gel-permeation HPLC	0.3	8.8	29.4	639
Chondroitin B Lyase				
Osmolate	1000	589	0.59	1
Cation exchange	113	533	4.7	8
Cellufine sulphate	11.4	242	21.2	36
Hydroxyapatite	2.3	153	65.6	111
Strong cation exchange	0.95	158	167	283
Gel-permeation chromatography	0.34	108	278	471

lyase was primarily eluted at 0.27–0.3 M NaCl and chondroitin B lyase at 0.23–0.26 M NaCl.

Hydroxyapatite chromatography

Chondroitin B lyase was diluted two-fold with 10 mM sodium phosphate and applied to a hydroxyapatite column (2.6 cm × 30 cm). The bound proteins were eluted with 0.25 M NaCl followed by a linear gradient of 0.25–1.0 M NaCl in 25 mM sodium phosphate at pH 7.0. Chondroitin B lyase was eluted at 0.25 M NaCl.

Strong cation-exchange chromatography

The chondroitin B lyase fraction was diluted two-fold in 0.01 M sodium phosphate and applied to a CBX-S strong cation-exchange column (1.6 cm × 10 cm). The bound material was eluted at a flow rate of 1.0 cm/min with a 0.125–0.325 M linear gradient of NaCl in 25 mM sodium phosphate at pH 7.0. Chondroitin B lyase was eluted in a protein peak at 0.175–0.225 M NaCl.

Gel-permeation chromatography

The chondroitin B lyase obtained on strong cation-exchange chromatography contained a minor contaminating protein of M_r 20000. This protein was removed by loading the chondroitin B lyase on to a Superdex 200 column (1.0 cm × 30 cm), eluting with 0.05 M sodium phosphate, pH 7.2, at a linear flow rate of 1.25 cm/min and collecting the chondroitin B lyase-containing fractions.

Characterization of chondroitin AC lyase, glycuronidase and chondroitin B lyase

Assessment of purity by electrophoresis

Discontinuous SDS/PAGE was by a modification of a previously described procedure [26]. The gels were fixed with 12% (w/v) trichloroacetic acid, rinsed with water, stained with Coomassie Blue and destained. Isoelectric focusing (IEF)/PAGE was carried out in a 40% ampholyte buffer (pH 3–10) on a horizontal electrophoresis cell with 3 M phosphoric acid (anolyte) and 1 M NaOH (catholyte). The gel was prefocused at 7 W for 15 min, samples were loaded and electrophoresis was at 7 W constant power for 90 min. The gel was fixed in 12.5% (w/v) trichloroacetic acid, blotted, rinsed with water, dried overnight, stained with Coomassie Blue R-250 and destained.

Amino acid compositional and N-terminal analysis

Amino acid compositional analysis was performed on 7–10 μg of purified enzyme. N-Terminal sequence analysis of chondroitin AC lyase and glycuronidase was by direct sequencing after electrotransfer poly(vinylidine difluoride) membrane [24]. N-Terminal sequencing was also performed after treating purified enzymes with N-terminal acyl-amino-acid-releasing enzyme [27] and after treatment of chondroitin AC and B lyases with pyroglutamate aminopeptidase [28].

Effect of pH on activity

Six buffer systems were selected to cover a pH range from 4.5 to 9.0: sodium succinate (4.5–6.5), sodium citrate (4.5–6.5), Mes, sodium salt (5.5–7.0), Bistris/propane/HCl (6.0–8.0), Tris/HCl (7.0–9.0) and sodium phosphate (5.0–9.0). The assay was performed in these six buffers by adding 2 μl of the enzyme solution containing 172 ng of protein and 487 μl of a buffer at a specific pH into a cuvette; the reaction was then initiated by adding 20 μl of chondroitin sulphate C (25 mg/ml) solution as substrate. Glycuronidase activity was assayed by adding 2 μl of the enzyme solution containing 400 ng of protein and 487 μl of a buffer at a specific pH into a cuvette. The reaction was initiated by adding 20 μl of ΔUAp(1 → 3)GalNpAc6S (5 mg/ml) solution as substrate. After gentle shaking to ensure proper mixing, A_{232} was measured every 15 s for 3 min. Chondroitin B lyase used 50 mM succininc acid, Tris/HCl or Bistris/propane/HCl buffers in the

presence of 25 mM dermatan sulphate and 1.25 μg/ml enzyme. The reaction velocities were calculated and plotted against pH to generate a curve from which the pH optima were established.

Temperature for optimum activity

Temperature for optimum activity was determined for chondroitin lyase AC and glycuronidase at their optimum pH in 50 mM sodium phosphate buffer in 10° increments at temperatures between 10 and 60 °C.

Temperature stability optima

Assay stock solutions were prepared at the enzyme pH optima in 50 mM sodium phosphate buffer, placed in water baths at 40 °C (chondroitin AC lyase) or 37 °C (glycuronidase and chondroitin B lyase). Small aliquots were removed at various time intervals (1–12 h) to measure enzyme activity remaining.

Determination of kinetic constants

Michaelis–Menten constants were determined using the optimized conditions. The final absorbance value for total depolymerization was divided by ten to find a value that represented 10% reaction completion. The purified enzyme preparations were diluted so that 10% of total depolymerization would be reached only at the end of a 3 min assay. The reaction velocities at specific molar concentrations for each lyase and their substrates were used for kinetic analysis using EZ-FIT [29]. Substrate solutions were prepared from 25 mg/ml chondroitin sulphate C, dermatan sulphate and 5 mg/ml ΔUAp(1 → 3)GalNpAc6S stock solutions. These constants were determined at 40 °C for chondroitin AC lyase in 50 mM sodium phosphate buffer, pH 6.8, at 37 °C in 50 mM sodium phosphate buffer pH 6.5 for glycuronidase, and at 30 °C for chondroitin B lyase in 50 mM Tris/HCl pH 8.0, buffer.

Specificity of chondroitin AC lyase, glycuronidase and chondroitin B lyase

Capillary electrophoresis (CE) with UV detection at 232 nm was used in this study [30–32]. Purified chondroitin AC and B lyase were incubated with different glycosaminoglycans as substrates including chondroitin sulphate A, C, dermatan sulphate, hyaluronic acid, heparan sulphate and heparin. The 17 glycosaminoglycan-derived disaccharides with unsaturated uronic acid at their non-reducing ends and phenolphthalein glucuronide were used to examine the glycuronidase specificity. Enzyme was added to substrate solution under optimized assay conditions and incubated for 12 h. Aliquots were analysed by CE throughout the incubation [30]. The depolymerized freeze-dried samples of chondroitin sulphate and dermatan sulphate were reconstituted at 1 mg/ml. The final UV absorbance value of the enzymic digestion mixture was measured at 232 nm in 30 mM HCl solution (ε5500 $M^{-1} \cdot cm^{-1}$) [6]. The M_r of the disaccharide resulting from the major repeating unit of the polysaccharide chain (see Scheme 1) was used to calculate the mol of product formed. The percentage of linkages cleaved was calculated on the basis of the expected UV absorbance from the known amount of polymeric substrate (100 μg) assuming complete depolymerization of substrates to disaccharides. The viscosity at each time point was determined using a capillary viscometer [33].

The enzymic digestion mixtures were analysed by linear-gradient PAGE. The linear gradient resolving gels (32 cm × 16 cm × 0.75 cm; 12–22% total acrylamide) were prepared as previously described [34]. Polysaccharide samples (40 μl) and marker samples (10 μl containing trace quantities of Bromophenol Blue and Phenol Red) in 50% (w/v) sucrose were loaded on each gel. Samples on a single gel were subjected to electrophoresis at a constant voltage of 400 V for 8 h at 10–15 °C and visualized by staining with Alcian Blue followed by silver.

RESULTS

F. heparinum cells were lysed by sonication to release enzyme activity. Protamine sulphate was added to cell homogenates to remove nucleic acids and residual chondroitin sulphate. Anion-exchange chromatography on QAE-Sephadex was then used to eliminate most of the contaminating protein (Table 1). After concentration, the crude enzyme sample was divided into aliquots and stored at −70 °C. Hydroxyapatite HPLC using a linear NaCl gradient in sodium phosphate buffer gave two peaks that contained enzyme activity capable of digesting dermatan sulphate and chondroitin sulphate (Figure 1a). The first peak, eluted in

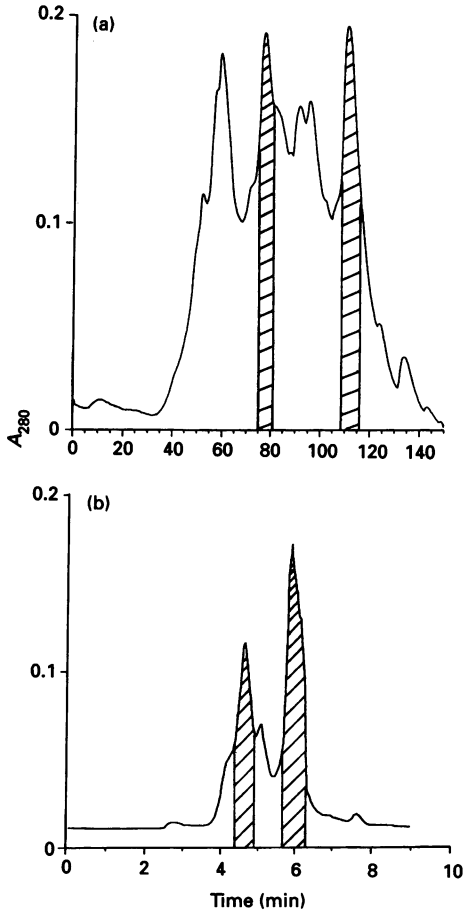


Figure 1 Purification of chondroitin AC lyase and chondro(1→3)glycuronidase from *F. heparinum*

Protein isolated from *F. heparinum* by sonification, protamine precipitation and fractionated by QAE-Sephadex was further purified by (**a**) hydroxyapatite HPLC. The two peaks indicated by hatch marks had lyase activity. The first hatch-marked peak showed activity towards dermatan sulphate and the second showed activity towards chondroitin sulphates A and C and towards ΔUAp(1 → 3)GalNpAc6S. This second hatch-marked peak was further purified (**b**) by gel-permeation HPLC to separate chondroitin AC lyase from glycuronidase. The first hatch-marked peak had activity towards chondroitin sulphate (4.3–4.8 min) and the second (5.7–6.2 min) had activity towards ΔUAp(1 → 3)GalNpAc6S, corresponding to chondroitin AC lyase and the chondro-(1 → 3)-glycuronidase respectively.

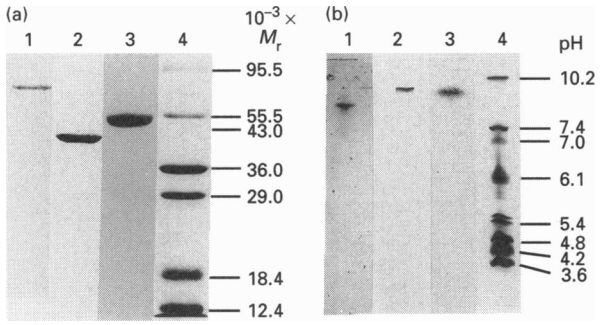


Figure 2 Gel electrophoretic analysis of purified enzymes

(**a**) SDS/PAGE of chondroitin AC lyase (lane 1), 1 → 3 glycuronidase (lane 2), chondroitin B lyase (lane 3) and M_r standards (lane 4). M_r standards are cytochrome *c* (12400), β-lactoglobulin (18400), carbonic anhydrase (29000), lactate dehydrogenase (36000), ovalbumin (43999), glutamate dehydrogenase (55000) and phosphorylase *b* (95000). (**b**) IEF/PAGE of chondroitin AC lyase (lane 1), 1 → 3 glycuronidase (lane 2), chondroitin B lyase (lane 3) and pI markers (lane 4). pI markers are amylogulcosidase (3.6), glucose oxidase (4.2), ovalbumin (4.8), β-lactoglobulin (5.4), carbonic anhydrase (6.1), myoglobin (minor band 7.0 and major band 7.4) and cytochrome *c* (10.2).

488 nM NaCl, only contained activity towards dermatan sulphate. Chondroitin B lyase was purified 172-fold over the cell homogenate, but SDS/PAGE showed that this fraction had more than five major bands. Because of the low yield and purity at this step, further purification of chondroitin B lyase was not carried out using this scheme. The second peak was eluted in 700 mM NaCl and showed activity towards both chondroitin sulphate A and C and glycuronidase but no activity towards dermatan sulphate. SDS/PAGE showed only two major bands in this fraction corresponding to proteins of substantially different M_r. Gel-permeation HPLC was used to purify this second fraction further (Figure 1b) affording both chondroitin AC lyase and glycuronidase of apparent homogeneity, as demonstrated by single bands on SDS/PAGE (Figure 2a) and IEF/PAGE (Figure 2b).

A second scheme was used to purify chondroitin B lyase to homogeneity. Osmotic shock was used to release 70% of the chondroitin B lyase from the cell pellet. The specific activity of the osmolate was increased nine-fold by cation-exchange chromatography. Chromatography on cellufine sulphate gave a separation of chondroitin B lyase from chondroitin AC lyase (Figure 3a). Hydroxylapatite chromatography followed by strong cation-exchange chromatography (Figures 3b and 3c) resulted in a nearly homogeneous preparation of chondroitin B lyase. A minor protein, of M_r 20000, contaminating this preparation was removed by gel-permeation chromatography affording a homogeneous preparation of chondroitin B lyase (Figures 2a and 2b).

The results of the purification of chondroitin AC lyase, glycuronidase and chondroitin B lyase by these schemes are summarized in Table 1.

Characterization of chondroitin AC lyase, glycuronidase and chondroitin B lyase purity and physical properties

Discontinuous SDS/PAGE illustrated that chondroitin AC lyase, glycuronidase and chondroitin B lyase were apparently homogeneous and their M_r values were estimated to be 74000, 41800 and 55200 respectively (Figure 2a). The pI values of chondroitin AC lyase, glycuronidase and chondroitin B lyase determined by IEF (Figure 2b) were 8.85, 9.28 and 9.05 respectively.

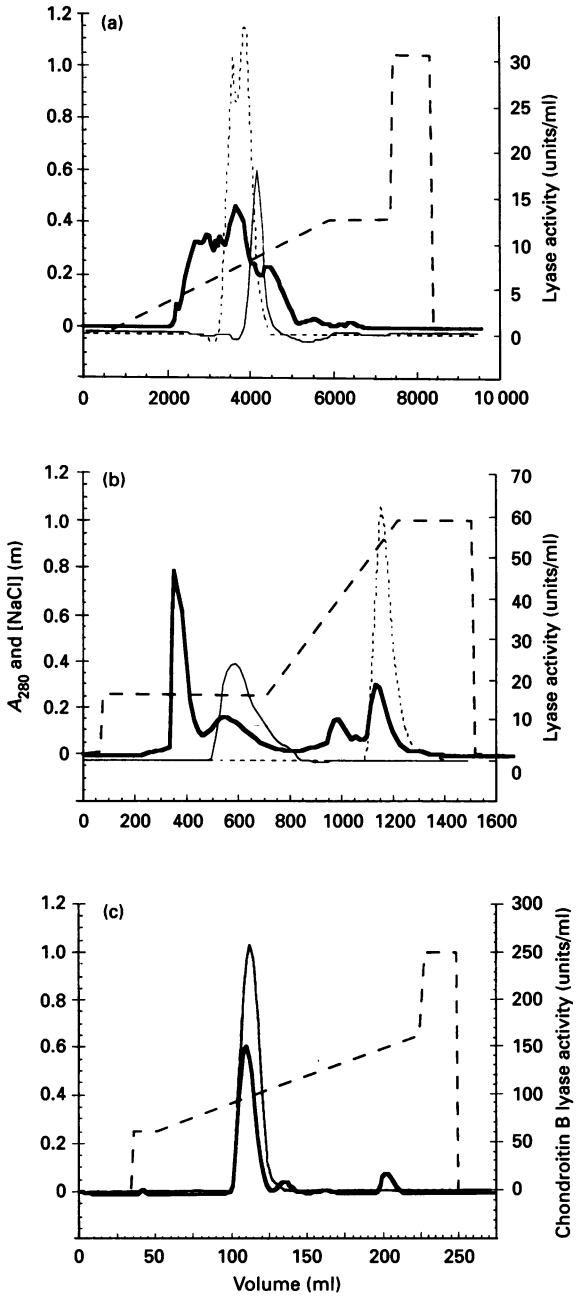


Figure 3 Purification of chondroitin B lyase from *F. heparinum*

Protein isolated from *F. heparinum* by osmotic shock and cation-exchange chromatography was further purified by (**a**) cellufine sulphate chromatography (column 2.6 cm × 100 cm; linear flow rate, $Q = 1.88$ cm-min^{-1}). Chondroitin AC lyase (....) and chondroitin B lyase (——) were eluted in a linear gradient. Chondroitin lyase activity purified by cellufine sulphate chromatography was further purified by (**b**) hydroxyapatite chromatography (column 2.6 cm × 30 cm; $Q = 1.88$ cm-min^{-1}). Chondroitin B lyase (——) was eluted in the 0.25 M NaCl step gradient and chondroitin AC lyase (....) in the gradient at 0.85–0.95 M NaCl. Chondroitin B lyase isolated from hydroxyapatite chromatography was further purified by (**c**) strong cation-exchange chromatography using CBX-S resin (column 1.6 cm × 100 cm; $Q = 1.0$ cm-min^{-1}); chondroitin B lyase (——) was eluted at 0.35–0.45 M NaCl. ——, protein; – – – –, NaCl gradient.

The amino acid composition of these three enzymes was analysed and the results are presented in Table 2. No N-terminal amino acid was detected suggesting that their N-termini were blocked. Treatment with N-terminal acyl-amino-acid-releasing

Table 2 Amino acid composition of chondroitin lyase AC, glycuronidase and chondroitin B lyase

The values are calculated assuming that the M_r of chondroitin AC lyase is 74000, that of glycuronidase is 41800 and that of chondroitin B lyase is 55200. nd, Not determined.

	Composition		
Amino acid	Chondroitin AC lyase	Glycuronidase	Chondroitin B lyase
Cys	nd	nd	13
Asx	71	41	65
Thr	37	25	20
Ser	47	25	23
Glx	59	32	38
Pro	25	16	20
Gly	62	24	46
Ala	48	30	42
Val	36	15	31
Met	nd	nd	5
Ile	34	14	39
Leu	55	30	35
Tyr	31	21	23
Phe	23	16	26
His	13	9	13
Lys	53	33	36
Trp	nd	nd	nd
Arg	25	9	24
Total	619	340	499

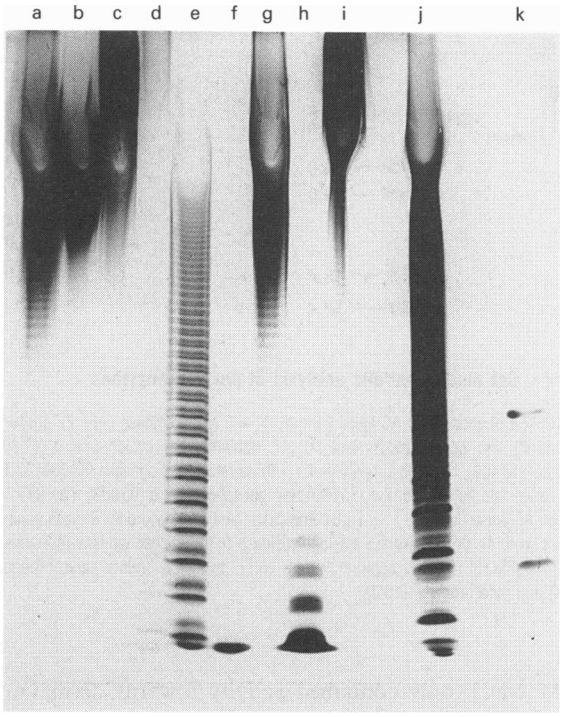


Figure 4 Gradient PAGE analysis of untreated and enzyme-treated chondroitin sulphate and dermatan sulphates

Lanes a, b and c contain untreated chondroitin sulphate A, dermatan sulphate B and chondroitin sulphate C respectively. Lanes d, e and f contain the same three polymers treated with chondroitin AC lyase. Lanes g, h and i contain the same three polymers treated with chondroitin B lyase. Lanes j and k contain oligosaccharide markers prepared from heparin [5] and tracking dyes Bromophenol Blue and Phenol Red (migrating fastest) respectively. The percentage of $1 \rightarrow 4$ glycosidic linkages cleaved in the enzyme reactions are consistent with the products afforded in the oligosaccharide maps shown in lanes d–i.

enzyme [27] did not expose their N-termini. Treatment of chondroitin AC lyase and chondroitin B lyase with pyroglutamate aminidase followed by N-terminal amino acid analysis produced sequences QQTGTA and QVVASN respectively.

Characterization of optimum catalytic activity for chondroitin AC lyase, glycuronidase and chondroitin B lyase

Chondroitin AC lyase demonstrated a relatively broad pH activity optimum (pH 6–7.5) on chondroitin sulphate C with a maximum at pH 6.8 in 50 mM sodium phosphate buffer. The temperature optimum for this enzyme activity was 40 °C. At this temperature an activity decrease of 50 % was observed after 6 h. Glycuronidase displayed an activity optimum at pH 6.5 and 37 °C; when incubated at 37 °C it showed an activity decrease of 50 % after 3.2 h. Chondroitin B lyase displayed a broad optimum between pH 6.8 and 8.0. At pH 8.0 in 50 mM Tris/HCl buffer, excellent activity was observed at 30 °C. At 37 °C this enzyme retained 40 % of its activity after 7 days in this buffer.

The Michaelis–Menten constants of chondroitin AC lyase, glycuronidase and chondroitin B lyase were determined from the reaction velocities measured at less than 10 % substrate consumption using optimum reaction conditions (Table 3).

Characterization of substrate specificity

Chondroitin AC lyase acts primarily on chondroitin sulphates A and C and to a lesser degree on dermatan sulphate and hyaluronic acid (Table 4). In addition to forming higher oligosaccharide products, observed on gradient PAGE (Figure 4), ΔUAp(1 → 3)GalNpAc4S and ΔUAp(1 → 3)GalNpAc6S are formed (CE data not shown) from chondroitin A and C

Table 3 Kinetic constants of chondroitin AC lyase, chondroitin B lyase and glycuronidase

Enzyme	Substrate	$V_{max.}$ (μmol/min per mg of protein)	K_m (app.) (μM)	$k_{cat.}/K_m$ $(s \cdot \mu M)^{-1}$
Chondroitin AC lyase	Chondroitin sulphate C	121 ± 8.0	9.3 ± 1.4	16.0
Glycuronidase	ΔUA(1 → 3)GalNAc6S	117 ± 10.6	163 ± 43.5	0.5
Chondroitin B lyase	Dermatin sulphate	209 ± 8.3	7.4 ± 2.0	25.9

Table 4 Action of chondroitin lyases on glycosaminoglycan substrates

Initial rate was measured at substrate saturation using 10 m-unit/mg of substrate [33]. nd, Not determined.

	Chondroitin sulphate A		Dermatan sulphate		Chondroitin sulphate C	
Enzyme	1 → 4 Glycosidic linkages cleaved (%)	Initial rate (μmol/min)	1 → 4 Glycosidic linkages cleaved (%)	Initial rate (μmol/min)	1 → 4 Glycosidic linkages cleaved (%)	Initial rate (μmol/min)
Chondroitin AC lyase	86	0.78	15	nd	84	0.49
Chondroitin B lyase	< 1	nd	45	0.36	3	nd

respectively. No disaccharide product is observed by CE analysis when chondroitin AC lyase acts on dermatan sulphate. Chondroitin B lyase acts primarily on dermatan sulphate and to a lesser extent on chondroitin A and C (Figure 4, Table 4). CE analysis of chondroitin B lyase-treated dermatan sulphate (results not shown) afforded primarily ΔUAp(1 → 3)GalNpAc4S product. No disaccharide products were observed when this enzyme was used to treat chondroitin sulphate A or C. The actions of both enzymes on their various polysaccharide substrates were followed kinetically using viscosometric measurements [33]. Both chondroitin lyases acted endolytically on all substrates. Glycuronidase specificity was examined by CE using a collection of 17 disaccharide standards (see [35] for structures) containing both 1 → 3 and 1 → 4 linked disaccharides. Only ΔUAp(1 → 3)-GalNpAc, ΔUAp(1 → 3)GalNpAc6S and ΔUAp(1 → 3)GlcNpAc were substrates for this glycuronidase.

DISCUSSION

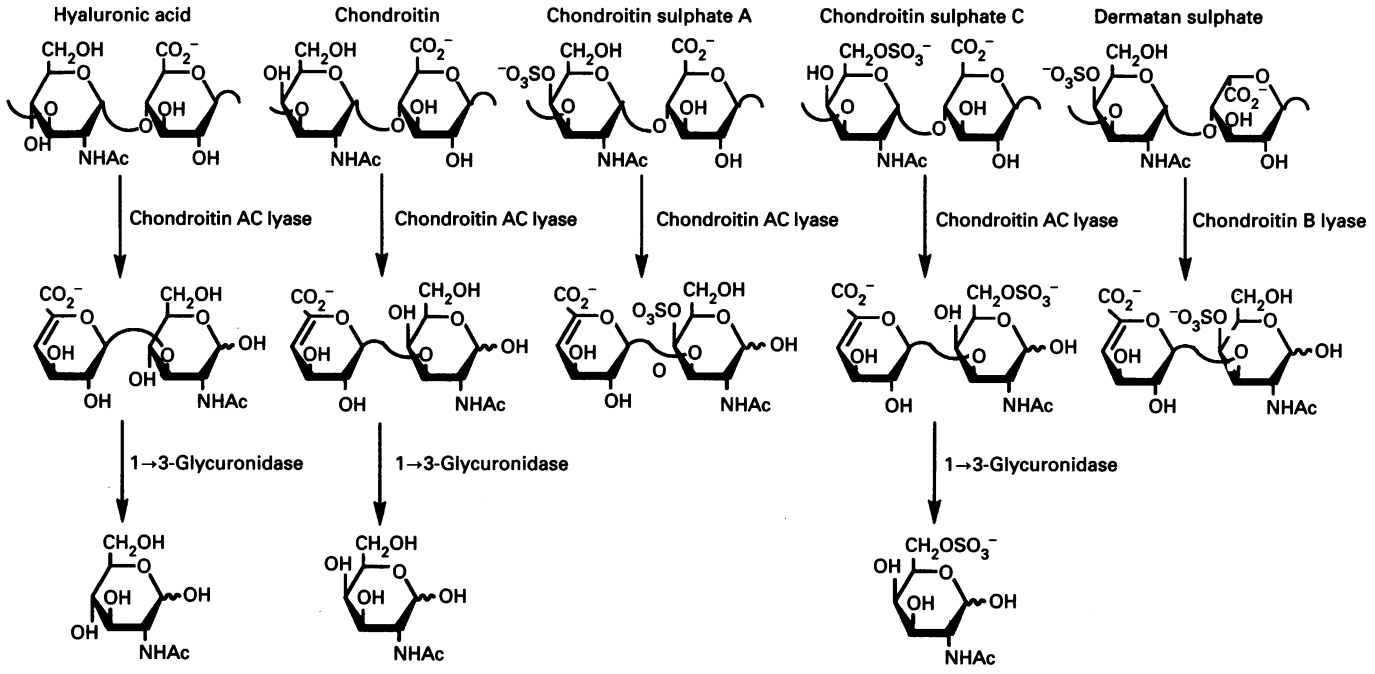
F. heparinum [36], a Gram-negative soil bacterium also known as *Cytophaga heparinia* [37], produces a variety of enzymes that degrade glycosaminoglycans. This organism is induced when utilizing glycosaminoglycans as the source of carbon, nitrogen and sulphur, producing a culture greatly enriched in chondroitin and heparin lyases, glycuronidases, sulphoesterases and a sulphamidase [38]. Chondroitin lyases are also produced by other bacteria such as *Arthrobacter*, *Bacteroides* and *Pseudomonas*. Chondroitin AC lyase isolated from *Arthrobacter aurescens* differs from the chondroitin AC lyase isolated in this study in its M_r (76000), pI (5.46), amino acid composition, K_m (360 μM) and exolytic action pattern [33,39].

Linker and co-workers [7] first prepared an inducible chondroitin AC lyase activity from *F. heparinum* by $(NH_4)_2SO_4$ precipitation. The products of this enzyme appeared to be sulphated and unsulphated unsaturated disaccharides as assayed by paper chromatography. Suzuki [38] and Yamagata et al. [40] further purified chondroitin AC lyase, using phosphocellulose and Sephadex G-200 chromatography, affording two peaks that showed activity towards both chondroitin sulphates A and C. The first peak also had activity towards dermatan sulphate and sulphoesterase and glycuronidase activities. The second peak was further purified and the chondroitin AC lyase obtained was partially characterized. Chondroitin sulphate A, chondroitin sulphate C, chondroitin and hyaluronic acid were good substrates of this enzyme (pH optimum 6.6) while dermatan sulphate was an inhibitor (K_i 10^{-5} M) and heparan sulphate and heparin were not substrates. Michelacci and Deitrich [41] also separated chondroitin AC lyase from crude extract using agarose-gel electrophoresis that had a temperature optimum around 37–40 °C and a pH optimum at 8.0. No evidence of homogeneity or physical properties such as M_r and pI were reported by either group.

Hoffman and co-workers [42] observed that *F. heparinum* was able to degrade dermatan sulphate. Michelacci and Dietrich [11] isolated a chondroitin lyase from induced *F. heparinum* that only degraded dermatan sulphate. A one-step purification of this chondroitin B lyase by agarose-gel electrophoresis afforded an enzyme with a temperature optimum of 20 °C acting on dermatan sulphate to give ΔUAp(1 → 3)GalNpAc4S (Figure 4) together with tetrasaccharide and hexasaccharide products. Otatani and Yosizawa [43] also reported a method of purifying chondroitin B lyase using affinity chromatography. No proof of homogeneity or physical properties such as M_r and pI have been described for chondroitin B lyase.

Flavobacterial glycuronidases are specific for the glycuronide products of polysaccharide lyases, containing an unsaturated uronic acid at their non-reducing end. These bacterial enzymes differ from their mammalian counterparts, the glucuronidases and iduronidases, which act to remove non-reducing terminal glucuronic acid and iduronic acid from various glycoconjugates. The products afforded by the action of the flavobacterial glycuronidases are an α-keto acid and an amino sugar. The two types of flavobacterial glycuronidases [10,44] are chondro-glycuronidases, which hydrolyse 1 → 3-linked unsaturated disaccharides derived from chondroitin sulphate, dermatan sulphate and hyaluronic acid, and heparo-glycuronidases, which hydrolyse 1 → 4-linked disaccharides, derived from heparin and heparan sulphate [20]. Chondro-glycuronidase activity was first reported in crude extracts of chondroitin sulphate C-induced *F. heparinum* [7,40]. Hovingh and Linker [10] partially purified this enzyme and found it acted well on ΔUAp(1 → 3)GalNpAc6S and ΔUAp(1 → 3)GalNpA, had some activity towards ΔUAp2S-(1 → 4)GlcNpAc prepared from heparan sulphate, but no activity towards ΔUAp(1 → 4)GlcNpAc6S. Surprisingly, the chondro-glycuronidase did not act on ΔUAp(1 → 3)GalNpAc4S obtained from chondroitin sulphate. Although this enzyme appeared to be primarily a 1 → 3-glycuronidase, it apparently also showed activity towards a 1 → 4-glycuronide. The heparo-glycuronidase reportedly hydrolysed only 1 → 4-glycuronides [10]. Neither glycuronidase from *F. heparinum* has been purified to homogeneity and no physical properties have been reported.

In our first purification scheme, chondroitin AC lyase and glycuronidase activities were obtained. Chondroitin AC lyase activity on chondroitin sulphate A and C was purified by 1889-fold and 3700-fold respectively (Table 1). Although the difference in the enrichment of the activity towards the two substrates is difficult to explain, it is possible that some contaminant, interacting preferentially with one substrate, might be removed in the course of purification. Glycuronidase was purified by 639-fold (Figure 1, Table 1). Using a second purification method, chon-



Scheme 1 Specificity of chondroitin AC lyase, chondroitin B lyase and 1 → 3 glycuronidase

Chondroitin AC lyase cleaves the 1 → 4 linkages in hyaluronic acid, chondroitin, chondroitin sulphate A and chondroitin sulphate C. Chondroitin B lyase cleaves the 1 → 4 linkages in dermatan sulphate. Chondro-(1 → 3)-glycuronidase completely cleaves the 1 → 3 linkages of unsulphated disaccharides containing *N*-acetylglucosamine, and unsulphated and 6-*O*-sulphated disaccharides containing *N*-acetylgalactosamine.

droitin B lyase was purified by 471-fold (Figure 3, Table 1). All enzymes are homogeneous by SDS/PAGE and IEF/PAGE (Figure 2). Their M_r values estimated by SDS/PAGE and gel-permeation HPLC are similar, suggesting that no subunits are present. Their isoelectric points are higher than 7 consistent with them binding and utilizing negatively charged substrates. The pI values of the related heparin lyases from *F. heparinum* are similar [13,14]. In contrast, chondroitin AC lyase from *A. aurescens* is an acidic protein having a pI of 5.46 [39].

Chondroitin AC lyase, glycuronidase and chondroitin B lyase have blocked N-termini similar to that reported for the heparin lyases [13,14]. The DNA-deduced sequence of the related heparin lyase I suggested that its N-terminus was a blocked glutamine, a very unusual finding for a prokaryotic protein [45]. Based on this information, chondroitin AC lyase and chondroitin B lyase were each treated with pyroglutamate aminopeptidase [28]. Successful Edman analysis confirmed that both chondroitin lyases were capped with an N-Terminal pyroglutamic acid, which might spontaneously arise from the lactamization of an N-terminal glutamine residue, increasing the hydrophobicity of the N-termini of these proteins.

Chondroitin AC lyase and chondroitin B lyase demonstrated broad activity optima at pH 6.8 and pH 7.5 respectively. These pH optima were expected since, like heparin lyases (pH optima 6.9–7.6 [13,14]), chondroitin AC and B lyases are probably periplasmic enzymes and thus should act on substrates at or near the pH (6.5–8) optimum for *F. heparinum* growth. The temperature optimum of chondroitin AC lyase from *F. heparinum* (40 °C) is similar to the related heparin lyases (35–40 °C) [13,14] but lower than chondroitin AC lyase from *A. aurescens* (50 °C) [46,47]. The stability of chondroitin AC lyase from *F. heparinum* is slightly less than that of the chondroitin AC lyase from *A. aurescens* [46]. Chondroitin B lyase is more stable than the AC lyase.

The apparent Michaelis–Menten constants for chondroitin AC lyase, glycuronidase and chondroitin B lyase are listed in Table 3. Hiyama and Okada [47] reported an apparent K_m value of 69 μM for chondroitin AC lyase on chondroitin sulphate C at 40 °C but in 50 mM sodium acetate buffer (pH 6.0). Using the same substrate the chondroitin AC lyase from *A. aurescens* has a K_m of 360 μM [47], 40-fold greater than the K_m of 9.3 μM for chondroitin AC lyase from *F. heparinum* observed in this study. Thus the flavobacterial chondroitin AC lyase has a stronger avidity for the substrate than the *Arthrobacter* enzyme, possibly the result of the lower pI of the latter. Different action patterns of the two chondroitin AC lyases may be the result of the weaker binding of substrate to the *Arthrobacter* enzyme. Chondroitin AC lyase from *A. aurescens* has a non-random exolytic action pattern [33] suggesting that it prefers binding to the less anionic ends of the chondroitin sulphate chain. Both the $V_{max.}$ and K_m of chondroitin B lyase are about twice that of chondroitin AC lyase resulting in a catalytic efficiency ($k_{cat.}/K_m$) nearly the same as that of chondroitin AC lyase (Table 3). The K_m of chondroitin lyases is approximately one order of magnitude lower than that of the heparin lyases [12–14]. Thus the chondroitin lyases may bind chondroitin sulphate and dermatan sulphate more tightly than the heparin lyases bind heparin and heparan sulphate. The $V_{max.}$ of chondroitin lyases is similar to those measured for heparin lyases I and III but an order of magnitude greater than the $V_{max.}$ of heparin lyase II [12–14].

Substrate specificity of chondroitin AC lyase, glycuronidase and chondroitin B lyase was investigated using gradient PAGE (Figure 4) and CE analysis [24,35]. Chondroitin AC lyase only acted on chondroitin sulphate A and C and hyaluronic acid and

to a limited extent on dermatan sulphate [48], contrary to previous reports [38,40] (Scheme 1, Table 4). The chondroitin AC lyase acted at a higher rate on chondroitin sulphate A and C than on hyaluronic acid, particularly in the initial stage of the reaction but the final product concentration was approximately the same on each substrate. Chondroitin B lyase acted on dermatan sulphate and to a very limited extent on chondroitin sulphate A and C (Scheme 1, Table 4). Both chondroitin AC and B lyases exhibit random endolytic action patterns [33]. CE analysis shows that chondroitin AC and B lyases, acting on chondroitin sulphate A or C and dermatan sulphate, afford the expected disaccharide products (results not shown). CE analysis of chondroitin AC and chondroitin B lyases acting on dermatan sulphate and on chondroitin sulphate A or C respectively afforded little or no disaccharide products. This suggests that the susceptible linkages (i.e. glucuronic acid residues in dermatan sulphate and iduronic acid residues in chondroitin sulphate A and C) are widely spaced throughout these polysaccharides and when cleaved give rise to larger oligosaccharide products, as observed by gradient PAGE analysis (Figure 4).

Seventeen glycosaminoglycan-derived disaccharides with unsaturated uronic acid at their non-reducing ends were used to examine the glycuronidase specificity. The glycuronidase acted on the unsulphated disaccharides and a 6-*O*-sulphated disaccharide (Scheme 1). The glycuronidase did not act on the 2-*O*- and 4-*O*-monosuphated disaccharides nor did it act on any of the disulphated or trisulphated disaccharides or disaccharides containing $1 \rightarrow 4$ glycosidic linkages. This glycuronidase did not act on phenolphthalein glucuronide (a substrate for β-glucuronidase from bovine liver [49]) to liberate free phenolphthalein.

In conclusion, chondroitin AC lyase, chondro-$(1 \rightarrow 3)$-glycuronidase and chondroitin B lyase have been purified to apparent homogeneity from *F. heparinum*. The physical and kinetic properties and specificity of these enzymes have been established and their high purity and specificity suggest their utility in studying the structure of glycosaminoglycans.

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