Glycobiology vol. 4 no. 3 pp. 289–296, 1994

Action pattern of polysaccharide lyases on glycosaminoglycans

Kenneth A.Jandik, Kenan Gu and Robert J.Linhardt[1]

Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242, USA

[1]To whom correspondence should be addressed

The action pattern of polysaccharide lyases on glycosaminoglycan substrates was examined using viscosimetric measurements and gradient polyacrylamide gel electrophoresis (PAGE). Heparin lyase I (heparinase, EC 4.2.2.7) and heparin lyase II (no EC number) both acted on heparin in a random endolytic fashion. Heparin lyase II showed an ideal endolytic action pattern on heparan sulphate, while heparin lyase I decreased the molecular weight of heparan sulphate more slowly. Heparin lyase III (heparitinase, EC 4.2.2.8) acted endolytically only on heparan sulphate and did not cleave heparin. Chondroitin ABC lyase (chondroitinase ABC, EC 4.2.2.4) from *Proteus vulgaris* acted endolytically on chondroitin-6-sulphate (chondroitin sulphate C) and dermatan sulphate at nearly identical initial rates, but acted on chondroitin-4-sulphate (chondroitin sulphate A) at a reduced rate, decreasing its molecular weight much more slowly. Two chondroitin AC lyases (chondroitinase AC, both EC 4.2.2.5) were examined towards chondroitin-4- and -6-sulphates. The exolytic action of chondroitin AC lyase *A* from *Arthrobacter aurescens* on both chondroitin-4- and -6-sulphates was demonstrated viscosimetrically and confirmed using both gradient PAGE and gel permeation chromatography. Chondroitin AC lyase *F* from *Flavobacterium heparinum (Cytophagia heparinia)* acted endolytically on the same substrates. Chondroitin B lyase (chondroitinase B, no EC number) from *F.heparinum* acted endolytically on dermatan sulphate giving a nearly identical action pattern as observed for chondroitin ABC lyase acting on dermatan sulphate.

Key words: action pattern/chondroitin lyase/glycosaminoglycan/heparin lyase

Introduction

Polysaccharide lyases (EC 4.2.2.-) are eliminases that cleave acidic polysaccharides, such as glycosaminoglycans, at certain glycosidic linkages (Linker *et al.*, 1956; Linhardt *et al.*, 1986). These enzymes include the heparin and chondroitin sulphate lyases that are isolated from *Flavobacterium heparinum (Cytophagia heparinium), Arthrobacter aurescens* and *Proteus vulgaris* (Yamagata *et al.*, 1968). Glycosaminoglycans are polydisperse, sulphated co-polymers of hexosamine and uronic acid residues having average mol. wts of 10 000–30 000, and are isolated from animal tissues (Mathews and Cifonelli, 1977; Linhardt *et al.*, 1986). Bacterial polysaccharide lyases presumably serve a role in the initial catabolism of glycosaminoglycans (Linhardt *et al.*, 1986). Heparin and chondroitin sulphate lyases have found many applications including the determination of glycosaminoglycan structure (Linhardt *et al.*, 1991, 1992a), the preparation of new therapeutic agents from glycosaminoglycans (Linhardt *et al.*, 1982a, 1988), the analysis of glycosaminoglycans found in tissues and biological fluids (Al-Hakim and Linhardt, 1991a; Linhardt *et al.*, 1992b), and the removal of glycosaminoglycans from the circulation (Langer *et al.*, 1982).

Because of the increased use of these enzymes, more attention has been directed at understanding their specificity. Work in our laboratory has focused on the specificity displayed by three heparin lyases from *F.heparinum* towards heparin, heparan sulphate (Linhardt *et al.*, 1990; Desai *et al.*, 1993a) and defined oligosaccharide substrates (Rice and Linhardt, 1989; Desai *et al.*, 1993b). The specificity of chondroitin sulphate lyases from *F.heparinum, A.aurescens* and *P.vulgaris* has also been studied, and has recently been reviewed (Linhardt, 1993). The specificity of these lyases is clearly related to their eliminase mechanism (Linhardt *et al.*, 1986; Desai *et al.*, 1993a,b), by which certain glycosidic linkages between hexosamine and uronic acid are cleaved to form a Δ4,5 site of unsaturation in the uronic acid residue. This UV chromophore, formed in the non-reducing termini of the small oligosaccharide products, is useful for their detection.

Less attention has focused on the way in which lyases select the glycosidic linkages at which they act. Action patterns might range from pure exolytic to pure endolytic. Heparin lyase I (heparinase) acting on heparin demonstrates a random endolytic action pattern (Linhardt *et al.*, 1982b). The action of chondroitin AC lyase from *F.heparinum* on chondroitin-6-sulphate is also random endolytic, while chondroitin AC lyase from *A.aurescens* reportedly shows a mixed action pattern, initially endolytic followed by stepwise breakdown of chondroitin-6-sulphate substrate (Hiyama, 1976). This study follows the lyase-catalysed breakdown of glycosaminoglycans by using both UV and viscosimetric assays, and examines the composition of the product mixture as a function of time by gradient polyacrylamide gel electrophoresis (PAGE) and by gel permeation chromatography (GPC). For the first time, the action patterns of each lyase are determined on all of its polymeric substrates.

Results and discussion

A wide variety of polysaccharide lyases that act on glycosaminoglycans are now commercially available at very high levels of catalytic purity (containing no additional enzymes that could act either on substrate or products). Despite the availability and widespread use of these enzymes, no systematic study on the action pattern of these enzymes on their glycosaminoglycan substrates has been undertaken. Three heparin lyases I, II and III (Lohse and Linhardt, 1992) and a chondroitin AC lyase *F* (Gu *et al.*, 1993) were prepared from *F.heparinum* and purified to homogeneity in our laboratory. Studies on the corresponding commercially obtained heparin and chondroitin

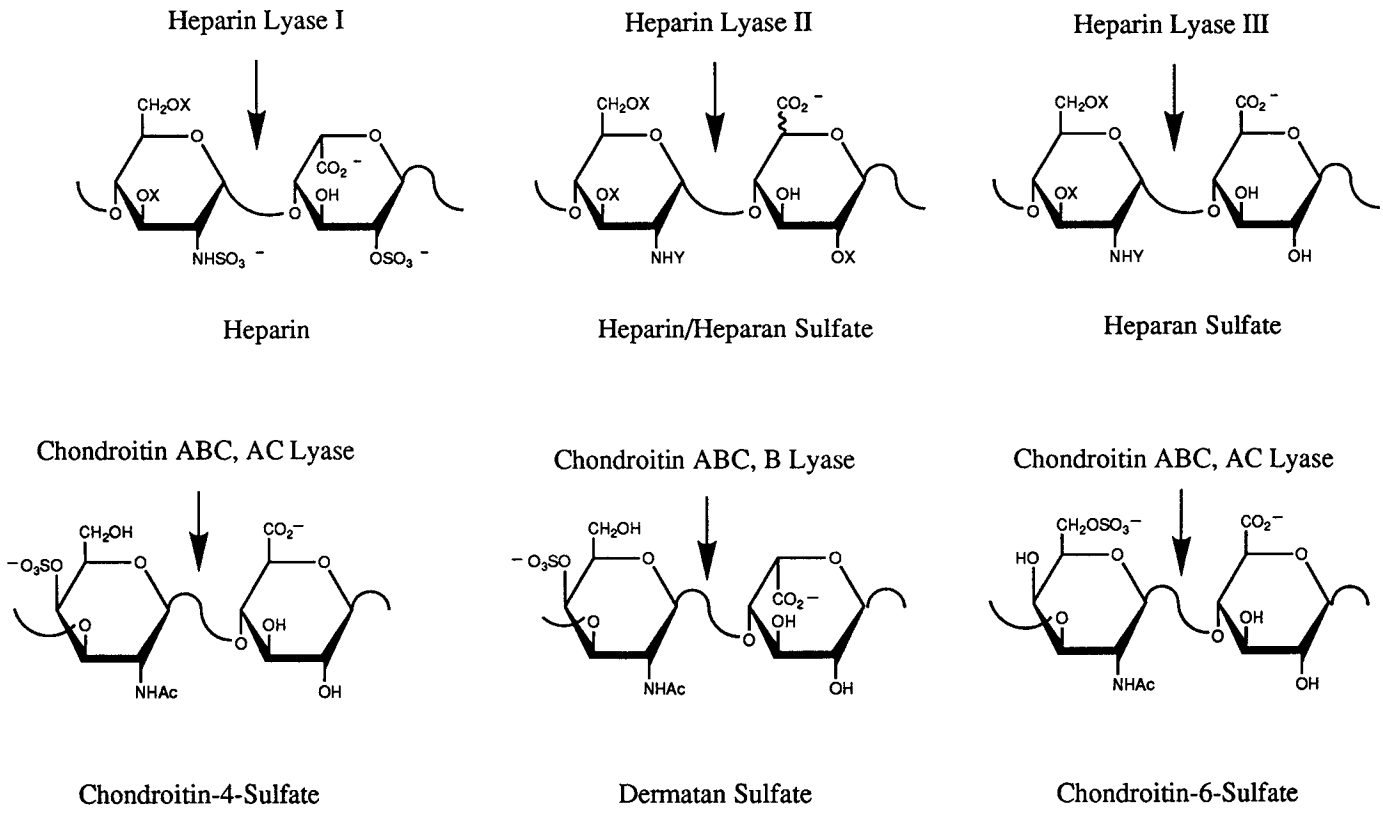


Fig. 1. The principal sites in glycosaminoglycans cleaved by the polysaccharide lyases.

lyases showed that they were identical and of comparable catalytic purity. Chondroitin AC lyase *A* from *A.aurescens*, chondroitin AC lyase *F* from *F.heparinum*, chondroitin B lyase from *F.heparinum* and chondroitin ABC lyase from *P.vulgaris*, obtained commercially, were of high catalytic purity and were used in this study without further purification. Figure 1 shows the linkages cleaved by each polysaccharide lyase and their occurrence in each polysaccharide substrate.

Disaccharide analysis of glycosaminoglycan substrates was performed by capillary electrophoresis (Al-Hakim and Linhardt, 1991b; Ampofo and Linhardt, 1991). Heparin fully degraded with heparin lyases I, II and III afforded 85% ΔUA2S (1→4) GlcNS6S (ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyroanosyluronic acid; GlcN, 2-deoxy-2-amino-D-glucopyranose; S, sulphate), 11% ΔUA (1→4)GlcNS6S, 4% other. Heparan sulphate similarly treated afforded 47% ΔUA (1→4)GlcNAc (Ac, acetate), 15% ΔUA (1→4)GlcNAc6S, 14% ΔUA2S (1→4)GlcNAc6S, 14% ΔUA2S (1→4)GlcNS6S, 5% ΔUA2S (1→4)GlcNAc, 5% other. Chondroitin-4-sulphate fully degraded with chondroitin ABC lyase afforded 60% ΔUA (1→3)GalNAc4S (GalN, 2-deoxy-2-amino-D-galactopyranose), 36% ΔUA (1→3)GalNAc6S, 3% ΔUA (1→3)GalNAc, 1% other. Chondroitin-6-sulphate similarly treated afforded 79% ΔUA (1→3)GalNAc6S, 18% ΔUA (1→3)GalNAc4S, 2% ΔUA (1→3)GalNAc, 1% other. Dermatan sulphate treated with chondroitin ABC lyase afforded 94% ΔUA (1→3)GalNAc4S, 5% ΔUA (1→3)GalNAc6S, 1% other. This dermatan sulphate contained 93% iduronic acid and 7% glucuronic acid as determined by enzymatic methods (Gu *et al.*, 1993) and ^{1}H-NMR (Linhardt *et al.*, 1991). These analyses confirmed the suitability of commercially available glycosaminoglycans for action pattern studies.

Studies were first performed to determine the initial velocity and final absorbance at 232 nm of each polysaccharide lyase on each of its substrates under the reaction conditions selected for the viscosimetric studies (Table I). From these measurements, heparin lyase I and II were shown to act on both heparin and heparan sulphate. As previously reported (Linhardt *et al.*, 1990; Lohse and Linhardt, 1992; Desai *et al.*, 1993a) heparin lyase I preferentially acts on heparin, while heparin lyase II prefers heparan sulphate as its substrate. Interestingly, while the final absorbance at 232 nm observed for heparin lyase II acting on heparin and heparan sulphate were similar, the initial velocities observed on these two substrates were very different (Table I). Heparin lyase III acts only on heparan sulphate (Linhardt *et al.*, 1990; Lohse and Linhardt, 1992; Desai *et al.*, 1993a). Similar studies on the chondroitin lyases showed that chondroitin AC lyases *A* and *F* give approximately the same conversion of substrate to products. Both chondroitin AC lyase *A* and *F* act at higher initial velocity and to a greater extent on chondroitin-4-sulphate than on chondroitin-6-sulphate (Table I). The chondroitin AC lyases both act on dermatan sulphate to such a limited extent that it is difficult to detect the products of these reactions (Gu *et al.*, 1993). Chondroitin ABC lyase acts on chondroitin-4-sulphate and chondroitin-6-sulphate and dermatan sulphate. Interestingly, the reaction velocity and extent of conversion were lowest on chondroitin-6-sulphate. Chondroitin B lyase acts on dermatan sulphate to a lesser extent than chondroitin ABC lyase, probably due to the presence of glucuronic acid (chondroitin-4-sulphate and chondroitin-6-sulphate sequences) within the dermatan sulphate being studied.

The depolymerization of each polysaccharide by polysaccharide lyase was followed by measuring both the change in

Table I.

Substrate[a]	Enzyme	Initial velocity (μmol/min)	Final absorbance (A_{232} nm)	Sites cleaved[b]	Action pattern
Heparin	Heparin lyase I	1.23	0.50	11	endo
	Heparin lyase II	0.36	0.35	8	endo
	Heparin lyase III	nr[c]	nr	0	nr
Heparan sulphate	Heparin lyase I	0.37	0.12	3	endo[d]
	Heparin lyase II	1.51	0.40	9	endo
	Heparin lyase III	1.04	0.77	18	endo
Chondroitin-4-sulphate	Chondroitin AC lyase (*A*)	0.94	0.85	20	exo
	Chondroitin AC lyase (*F*)	0.78	0.81	19	endo
	Chondroitin ABC lyase	0.98	0.85	20	endo[d]
Dermatan sulphate	Chondroitin ABC lyase	1.00	0.68	16	endo
	Chondroitin B lyase	1.00	0.36	8	endo
Chondroitin-6-sulphate	Chondroitin AC lyase (*A*)	0.47	0.60	14	exo
	Chondroitin AC lyase (*F*)	0.49	0.70	16	endo
	Chondroitin ABC lyase	0.65	0.73	17	endo

[a]The mol. wts of glycosaminoglycans used to calculate the values in this table are heparin 12 kDa, heparan sulphate 22 kDa, chondroitin-4-sulphate 25 kDa, chondroitin-6-sulphate 25 kDa, dermatan sulphate 25 kDa. Molecular weights were estimated by gradient PAGE (Edens *et al.*, 1992). A molar absorptivity of 5000 is used for the oligosaccharide products in 30 mM hydrochloric acid at 232 nm.

[b]Sites cleaved are determined based on the μmol of product formed (determined from absorbance) and give the relative sensitivity of each substrate towards each enzyme.

[c]nr is no reaction.

[d]The action pattern profile does not match the ideal pattern [computer-simulated pattern (Linhardt *et al.*, 1982b)] for random endolytic cleavage. This is probably due to the low frequency of cleavable sites in the substrate and their non-random distribution.

absorbance at 232 nm and the change in viscosity (Figure 2). With each enzyme-catalysed cleavage of a glycosidic linkage, an unsaturated uronic acid residue is formed at the non-reducing end of the product. Thus, the change in absorbance is proportional to the moles of product formed and the number of sites cleaved by the polysaccharide lyase. Viscosity affords a measure of the average molecular weight of glycosaminoglycans, such as heparin, that behave as Newtonian solutions at the concentrations studied (Liberti and Stivala, 1967; Roden *et al.*, 1972). The molecular weight measured by sedimentation of heparin has been shown to linearly correlate to its viscosity in solution (Lasker and Stivala, 1966). Thus, the change in viscosity measures the change in polysaccharide chain length. Computer simulation studies have been used to study the action pattern of heparin lyase I on heparin (Linhardt *et al.*, 1982b). Three theoretical plots of modelled weight average molecular weight (linearly proportional to viscosity) versus number of sites cleaved by heparin lyase I (linearly proportional to absorbance at 232 nm) were reported (Linhardt *et al.*, 1982b). A non-random exolytic action pattern, where the enzyme selects a single polysaccharide chain and removes one disaccharide unit at a time from the end of the chain, gave a linear relationship. A random exolytic action pattern, where the enzyme selects a polysaccharide chain, removes one disaccharide unit from the end of the chain, releases the chain and selects a new chain to repeat the process, gave a shallow concave curve (the rate of change in viscosity decreases throughout the reaction). A random endolytic action pattern, where the enzyme selects a polysaccharide chain, cuts any cleavable site within the chain, releases oligosaccharide product and the polysaccharide chain, and selects a new chain to repeat the process, gave a deep concave curve.

In the current study, the relative viscosity (*Cp*) was first plotted versus absorbance at 232 nm for all the polysaccharide lyases acting on a particular glycosaminoglycan substrate. Heparin lyase I acting on heparan sulphate, chondroitin ABC lyase and chondroitin AC lyase *A* acting on chondroitin-4-sulphate, and chondroitin AC lyase *A* acting on chondroitin-6-sulphate all show shallow concave curves. The remaining enzyme–substrate pairs were classified as exhibiting random endolytic action patterns by their steeply concave curves. These data can be more easily interpreted by grouping curves for a single enzyme acting on multiple substrates in each plot (Figure 3). To prepare these graphs, it is necessary to plot percent of initial viscosity versus percent of final absorbance because each substrate has a different molecular weight and hence a different relative viscosity. Heparin lyase I acting on heparin gave a steep, concave curve corresponding to its known random endolytic action pattern (Linhardt *et al.*, 1982b). Heparin lyase I gave a very different curve shape when acting on heparan sulphate. This curve was initially linear, followed by a concave portion beginning at ~70% of reaction completion (Figure 3a). The reduced number of heparin lyase I-cleavable sites within heparan sulphate (Table I) and their distribution within the polymer may be primarily responsible for the shape of this curve.

Heparin lyase II showed concave curves when acting on heparin and heparan sulphate (Figure 3b). Initial studies performed with this enzyme on these substrates had demonstrated that both the initial velocity and extent of reaction were very similar, suggesting an endolytic action pattern (Table I). The differences observed in the shape of these curves appear to be primarily the result of the distribution of heparin lyase II-cleavable sites in each polymer. Heparin lyase III acting on heparan sulphate gives a very steep concave curve, clearly demonstrating its random endolytic action pattern (Figure 3c). Chondroitin AC lyase *F* acts in a random endolytic fashion on both chondroitin-4- and -6-sulphates, while chondroitin AC

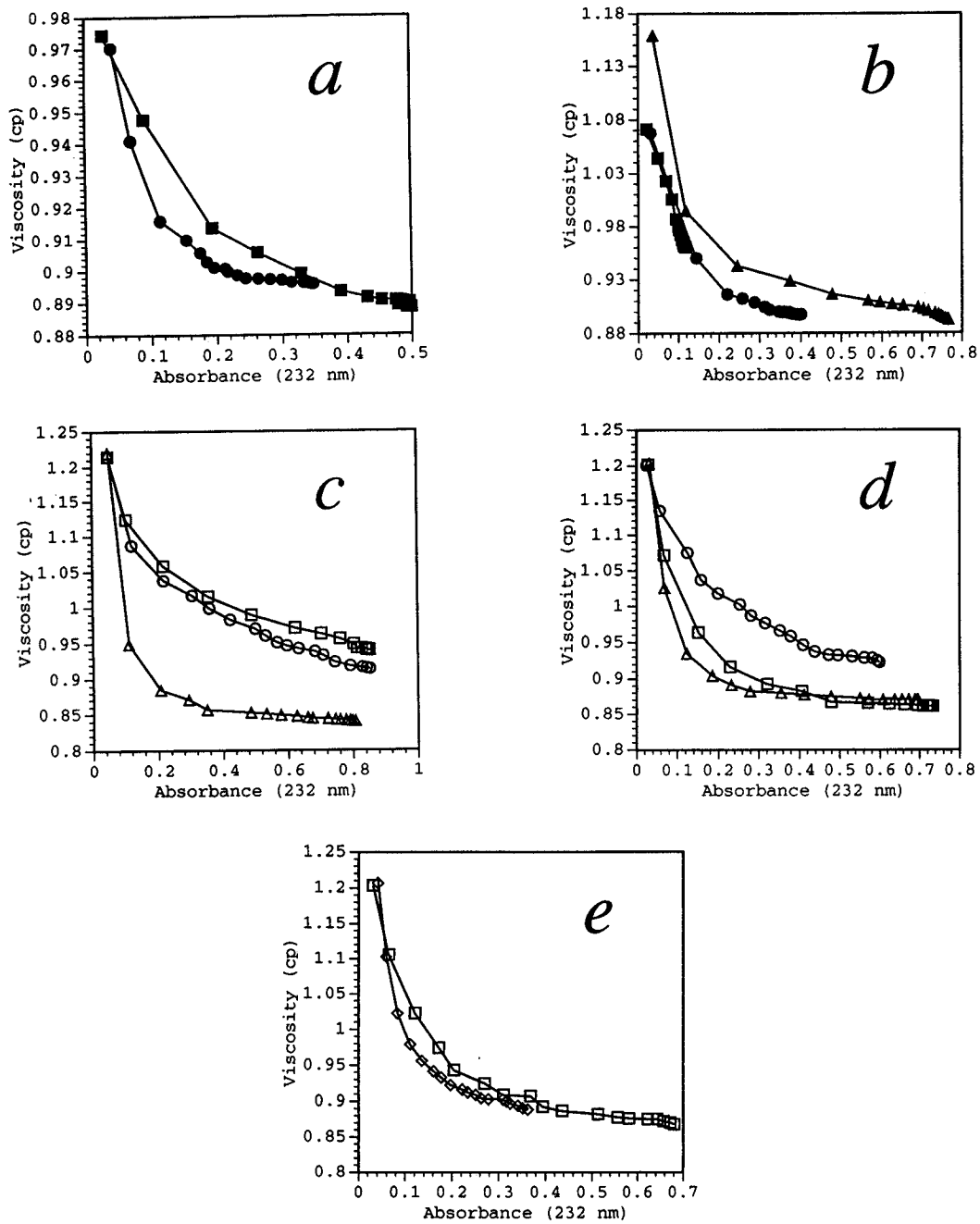


Fig. 2. Viscosity (*Cp*) is plotted as a function of absorbance at 232 nm throughout the time course of the polysaccharide lyase-catalysed depolymerization of various glycosaminoglycans. (**a**) Heparin treated with heparin lyase I (■) and heparin lyase II (●). (**b**) Heparan sulphate treated with heparin lyase I (■), II (●) and III (▲). (**c**) Chondroitin-4-sulphate treated with chondroitin ABC (□), AC*A* (○) and AC*F* (△) lyase. (**d**) Chondroitin-6-sulphate treated with chondroitin ABC (□), AC*A* (○) and AC*F* (△) lyase. (**e**) Dermatan sulphate treated with chondroitin ABC (□) and B (◇) lyase.

lyase *A* (following an initial rapid drop in viscosity) acts in a non-random exolytic fashion on the same substrates (Figure 3d and e). Chondroitin ABC lyase gives nearly identical curves when acting on chondroitin-6-sulphate and dermatan sulphate, but a somewhat shallower curve on chondroitin-4-sulphate (Figure 3f). This enzyme probably acts with a random endolytic action pattern on all three substrates. The shallowness of the curve for chondroitin-4-sulphate again may be attributable to the reduced number of chondroitin ABC lyase-cleavable sites present in this substrate (Table I). Chondroitin B lyase clearly gives a random endolytic action pattern when acting on dermatan sulphate (Figure 3g).

Aliquots taken from the viscometer were analysed together by gradient PAGE. Gradient PAGE of glycosaminoglycans has been used to analyse the molecular weight of these glycosaminoglycans (Lohse and Linhardt, 1992). The gradient PAGE analysis of chondroitin AC lyase *A* and *F* acting on chondroitin-6-sulphate is shown in Figure 4. These gels were scanned and digitized to obtain the average molecular weights of polysaccharide and oligosaccharide present in each lane (Figure 5). Heparin partially depolymerized with heparin lyase I was used to calibrate the gel because the structures (and, hence, the molecular weight) of the oligosaccharides corresponding to many of the intense bands observed in the ladder

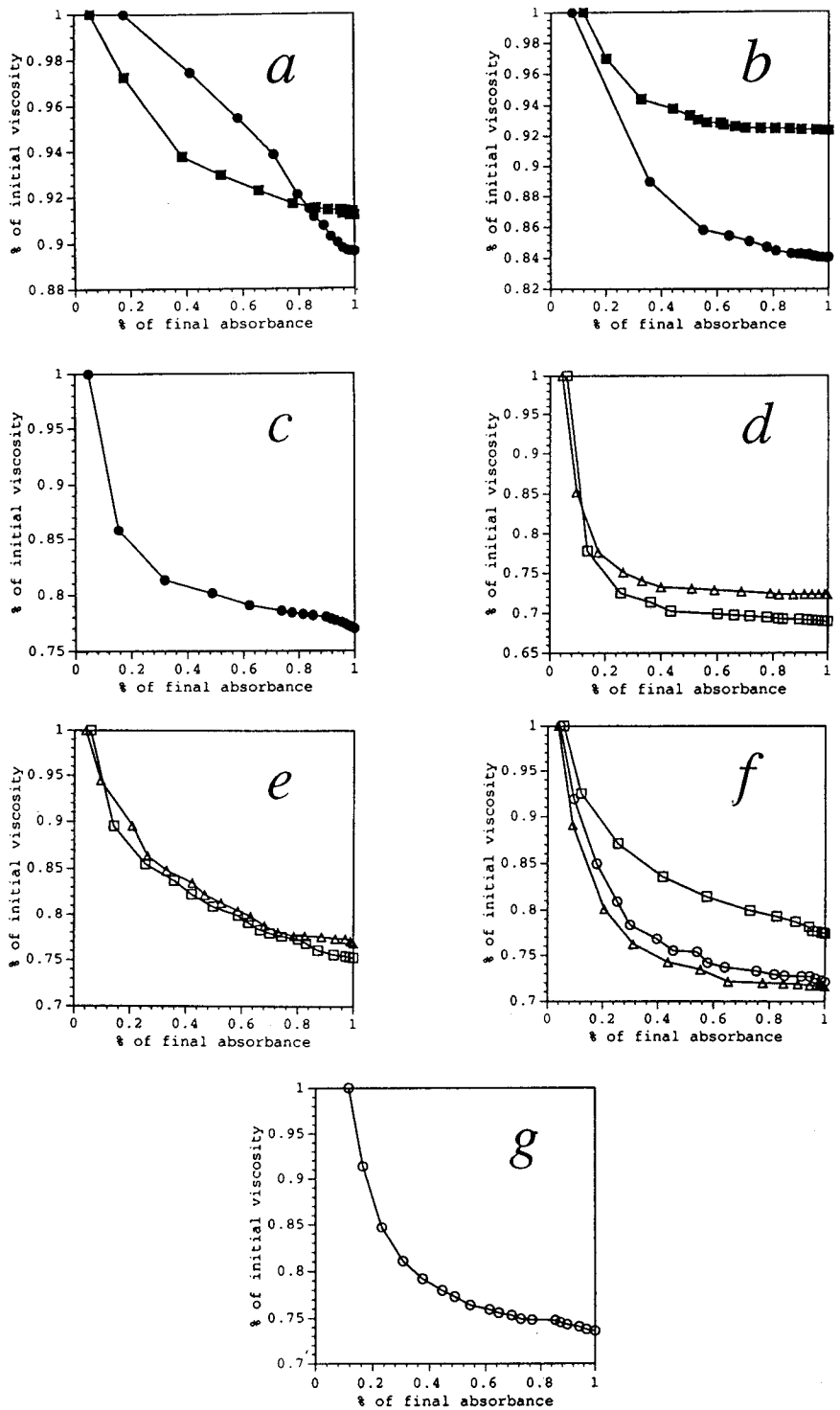


Fig. 3. Percent of initial viscosity plotted as a funtion of percent of final absorbance. (**a**) Heparin lyase I acting on heparin (■) and heparan sulphate (●). (**b**) Heparin lyase II acting on heparin (■) and heparan sulphate (●). (**c**) Heparin lyase III acting on heparan sulphate (●). (**d**) Chondroitin AC lyase *F* acting on chondroitin-4- (□) and -6- (△) sulphate. (**e**) Chondroitin AC lyase *A* acting on chondroitin-4- (□) and -6- (△) sulphate. (**f**) Chondroitin ABC lyase acting on chondroitin-4- (□) and -6- (△) sulphate and dermatan sulphate (○). (**g**) Chondroitin B lyase acting on dermatan sulphate (○).

have been characterized spectroscopically (Edens *et al.*, 1992). The rapid initial drop in molecular weight of chondroitin-6-sulphate associated with chondroitin AC lyase *F* confirmed its action pattern as random endolytic, while chondroitin AC lyase *A* acting on chondroitin-6-sulphate showed a considerably slower decrease in molecular weight, again suggesting an exolytic action pattern.

Gel permeation chromatography was used to further confirm the differences in the action patterns of the chondroitin AC lyases on chondroitin-6-sulphate. Aliquots of each reaction having the same absorbance at 232 nm (corresponding to 20% reaction completion) were analysed using a Sephadex G-25 column (Figure 6). The most pronounced difference between the products formed by chondroitin AC lyases *F* and *A* is the

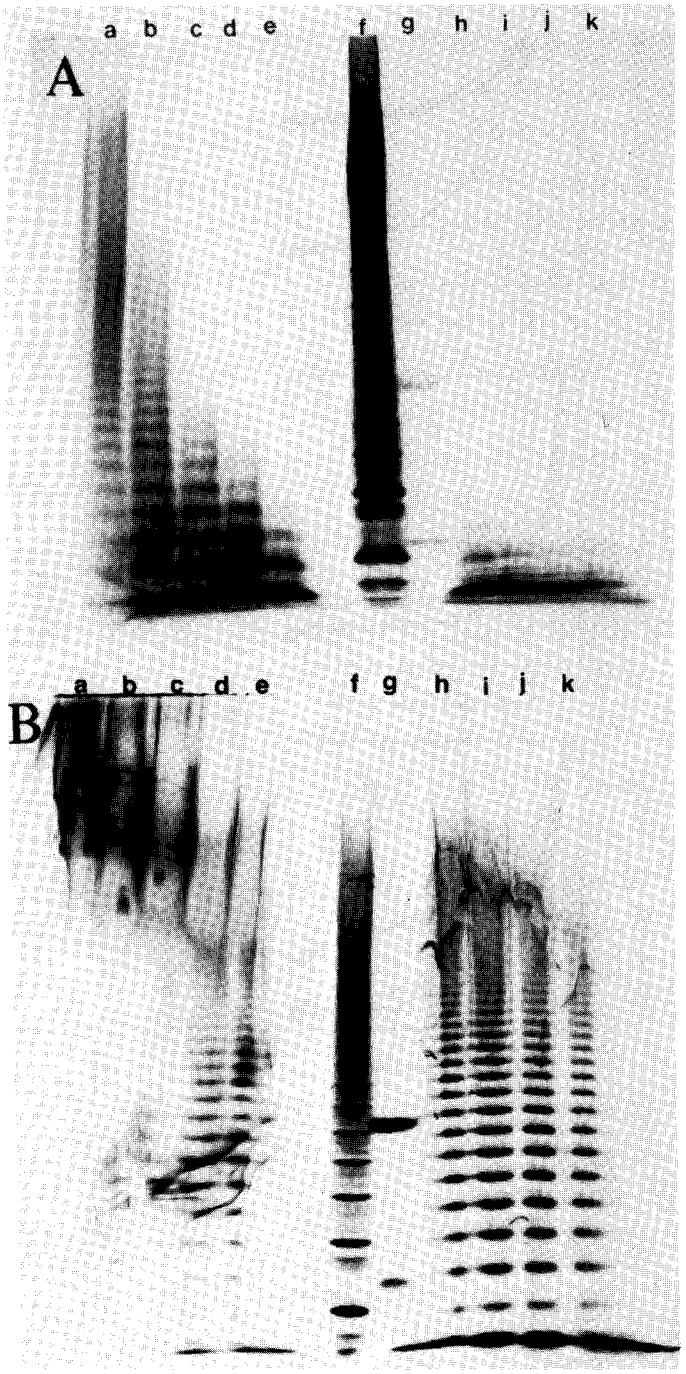


Fig. 4. Gradient PAGE analysis of chondroitin AC lyase *F* and *A* acting on chondroitin-6-sulphate. **Panel A** shows samples taken from the viscometer containing chondroitin AC lyase *F* and chondroitin-6-sulphate loaded in lanes a–e and h–k of the gel. The samples corresponded to 6, 23, 37, 57, 78, 87,94, 99 and 100% reaction completion. A set of standards prepared from bovine lung heparin partially depolymerized with heparin lyase I is used in lane f to calibrate the gel (Edens *et al.*, 1992). Bromophenol blue (upper band) and phenol red (lower band) were used as tracking dyes and are shown in lane g. **Panel B** shows the same analysis of samples from the viscometer containing chondroitin AC lyase *A* and chondroitin-6-sulphate. The samples corresponded to 6, 23, 40, 51, 62, 72, 82, 93 and 100% reaction completion. The standards and tracking dyes are in lane f and g, respectively.

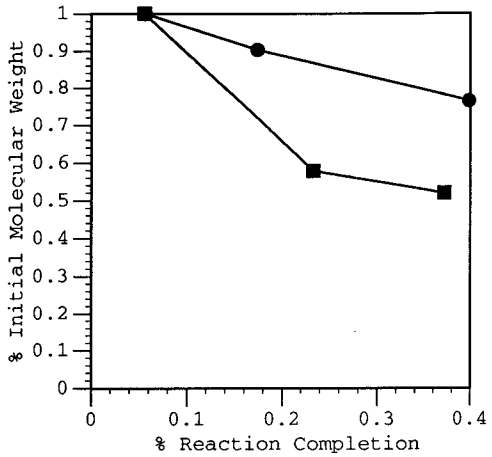
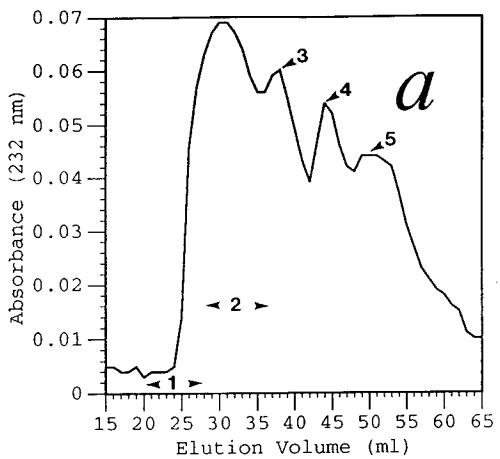


Fig. 5. Percent of initial molecular weight determined by gradient PAGE plotted as a function of percent reaction completion as determined by absorbance at 232 nm. Chondroitin-6-sulphate treated with chondroitin AC lyase *F* (■) and chondroitin AC lyase *A* (●) is shown.



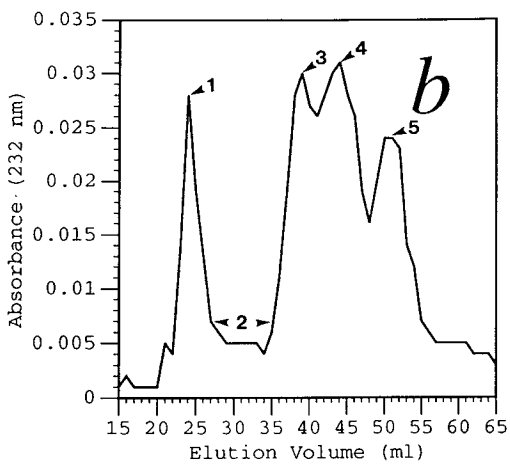


Fig. 6. GPC analysis of chondroitin-6-sulphate partially depolymerized (20% by absorbance 232 nm) with (**a**) chondroitin AC lyase *F* and (**b**) chondroitin AC lyase *A*. Five major components labelled 1–5 are observed in each chromatogram corresponding to oligosaccharides of degree of polymerization (dp) >12, oligosaccharides of dp 6–12, tetrasaccharides, monosulphated disaccharides and unsulphated disaccharides, respectively.

Table II. Mole percent of products

Product	Chondroitin AC lyase *F*	Chondroitin AC lyase *A*
Unsulphated disaccharide[a]	11	14
Monosulphated disaccharides[a]	23	36
Tetrasaccharides[b]	26	26
Oligosaccharides dp 6–12[b]	29	6
Oligosaccharides dp > 12[b]	11	18

[a]The elution positions of the unsulphated and monosulphated disaccharide were determined using disaccharide standards of known structure. On complete depolymerization, chondroitin-6-sulphate affords only 2 mol % unsulphated disaccharide, as determined by capillary electrophoresis. While the amount of unsulphated disaccharide may be overestimated because of the presence of buffer salts eluting near this peak, the large amount of this disaccharide observed at 20% depolymerization suggests that unsulphated disaccharide may be selectively released by these enzymes.
[b]The elution position of tetrasaccharide and higher oligosaccharides was determined using a chondroitin-6-sulphate partially depolymerized with chondroitin ABC lyase and counting peaks from the identified monosulphated disaccharide. The oligosaccharides of degree of polymerization (dp) > 12 afforded using chondroitin AC lyase *A* are particularly large, voiding on the Sephadex G25 column (Figure 6b) and migrating at the top of the gradient PAGE gel (Figure 4b); thus, the wt % of these products is much higher than the 11 and 18 mol % given in this table.

diminution of hexasaccharide to dodecasaccharide products in the chondroitin AC lyase *A* product mixture (6%) when compared to the chondroitin AC lyase C product mixture (29%) (Table II). These results again confirm the exolytic action pattern of chondroitin AC lyase *A*. The presence of nearly equal quantitites of tetrasaccharides in the product mixture of both chondroitin AC lyases suggests that either two or four saccharide residues are removed by chondroitin AC lyase *A* when it acts exolytically on chondroitin-6-sulphate.

In conclusion, these studies confirm the results of Hiyama (1976), demonstrating that the action patterns of chondroitin AC lyase *A* and *F* on chondroitin sulphate are primarily non-random exolytic and random endolytic, respectively. These studies also confirm the results of Linhardt *et al.* (1982b), showing that heparin lyase I acts on heparin with a random endolytic action pattern. Heparin lyase II acts on both heparin and heparan sulphate with a random endolytic action pattern, as does heparin lyase III acting on heparan sulphate. Chondroitin B lyase acting on dermatan sulphate and chondroitin ABC lyase acting on chondroitin-6-sulphate or dermatan sulphate also show random endolytic action patterns. Some ambiguities were encountered when examining the action pattern of chondroitin ABC lyase on chondroitin sulphate A and heparin I lyase on heparan sulphate. Both showed shallow concave curves in plots of absorbance at 232 nm versus viscosity. These could be interpreted as: (i) a non-random exolytic action pattern; (ii) the result of the limited number of cleavable sites in these polysaccharide substrates; or (iii) a non-random distribution of cleavable sites within these polysaccharide substrates. Further studies will be required to fully understand the action patterns of these enzyme–substrate pairs.

Experimental procedures

Materials

Heparin lyases I, II and III (from *F.heparinum*) were purified to apparent homogeneity as previously reported (Lohse and Linhardt, 1992). Chondroitin AC lyase (*F*) (from *F.heparinum*) was purified to apparent homogeneity by our laboratory (Gu *et al.*, 1993) and was also obtained from Seikagaku America (Rockville, MD). Chondroitin ABC lyase (from *P.vulgaris*), chondroitin AC lyase (*A*) (from *A.aurescens*), chondroitinase B (from *F.heparinum*) and chondroitin disaccharide standards were purchased from Seikagaku. Heparin from porcine intestinal mucosa and chondroitin-4-sulphate (chondroitin sulphate A) from bovine trachea were purchased from Celsus Laboratories, Inc. (Cincinnati, OH). Chondroitin-6-sulphate (chondroitin sulphate C) from shark cartilage was purchased from Seikagaku. Heparan sulphate from bovine kidney was purchased from Grampian Enzymes (Aberdeen, UK). Dermatan sulphate (chondroitin sulphate B) from porcine intestinal mucosa was a gift from Dr Erwin Coyne of Loyola University Medical Center (Maywood, IL). Sephadex G-25 (fine) was from Sigma Chemical Company (St Louis, MO). The reagents used in electrophoresis were acrylamide and *N,N,N′,N′*-tetramethylenediamine (TEMED) and Alcian blue from Boehringer Mannheim (Indianapolis, IN), bis-*N′,N′*-methylenebisacrylamide from International Biotechnologies Inc. (New Haven, CT) and ammonium persulphate from MCB Manufacturing Chemists (Cincinnati, OH). All inorganics were reagent grade or better.

All spectrophotometric measurements were made with a Shimadzu model UV160 spectrophotometer from Shimadzu Corporation (Kyoto, Japan). All viscometry measurements were made with a Cannon–Ubbelohde semi-micro viscometer from Cannon Instrument Company (State College, PA). Gradient PAGE analysis was performed on a 20 cm vertical slab gel with Protean™ II from Bio-Rad (Richmond, CA). Gels were scanned using a computer-controlled Kodak Megaplus on-line video camera running image grabber software on a Macintosh II computer and data processed using Pixels tools software from Perceptics.

Lyase digestion of glycosaminoglycans and viscosity and UV assays for activity

A few small-scale digestions of sample were initially performed to ensure that the digestion reached completion within 150 min. Once the ratio of substrate and enzyme was established, all the remaining reactions were performed within the capillary viscometer.

The viscometer was first cleaned, rinsed with water, methanol then rinsed thoroughly with distilled, deionized water and air dried. The viscometer was placed vertically in a holder within a water bath at a temperature regulated at 30 ± 0.1°C. Assay solution was prepared that contained 8.33 mg/ml glycosaminoglycan substrate in a 250 mM sodium acetate, 2.5 mM calcium acetate and 1 mM Tris–acetate buffer solution at pH 7.0. Assay solution was added to the viscometer and allowed to equilibrate for 20 min in a 30°C water bath. Sufficient enzyme was then added (without diluting the substrate >1%) so that there was ~10 mU enzyme/mg substrate. The precise amount of enzyme used was adjusted based on the preliminary small-scale experiments (described above). The digestion reaction immediately begins once the enzyme is added to the assay solution. The viscosity is measured as the time required for the level of the solution to fall between two marks above and below a bulb, divided by the time required for distilled water to fall between the same marks. This value is then multiplied by the viscosity of water at 30°C (0.7975). Measurements were accurate to within 0.1 s. The viscosity was measured ~15 times over 3 h or until the reaction was complete. At the midpoint of each viscosity measurement, two 25 μl aliquots were removed from the viscometer and immediately frozen on dry ice. One aliquot was used for gradient PAGE analysis and the second was added to 2 ml of 30 mM hydrochloric acid and the absorbance at 232 nm was measured. At the end of the digestion, any remaining assay solution was frozen on dry ice and stored at −70°C until needed.

Gradient PAGE analysis

Lyase-treated glycosaminoglycans were analysed using gradient PAGE (Edens *et al.*, 1992; Linhardt *et al.*, 1992b). Each sample (10 μl of 8.33 mg/ml) was removed from the freezer, heated at 100°C for 2 min and combined with an equal volume of 50% (w/w) sucrose containing trace amounts of bromophenol blue and phenol red, and loaded into a stacking gel of 5% (total acrylamide) and fractionated with a 12–22% linear resolving gel. Electrophoresis was performed at 400 V for 5–6 h. Bromophenol blue and phenol red were used as indicators

and, throughout the run, the heat was dissipated using refrigerated water recirculation at 4°C. The gel was stained and fixed with Alcian blue in acetic acid (Al-Hakim and Linhardt, 1991a). The molecular weight was determined from a scan of the polyacrylamide gel using standards prepared from partially depolymerized bovine lung heparin (Edens *et al.*, 1992).

Gel permeation chromatography analysis

Samples of chondroitin-6-sulphate treated with chondroitin AC lyases *A* and *F* were removed from the viscometer (having the same absorbance at 232 nm in 30 mM hydrochloric acid) and heated at 100°C for 2 min to terminate the enzymatic reaction. Each sample (100 μg/500 μl) was loaded onto a 1.5 × 50 cm Sephadex G-25 column equilibrated with 200 mM sodium chloride; 1 ml fractions were collected and their absorbance measured at 232 nm. Chondroitin-6-sulphate completely depolymerized with chondroitin ABC lyase was used to calibrate the column, and unsulphated and monosulphated disaccharide standards were used to confirm the assignment of peaks.

Acknowledgements

This work is supported by the National Institutes of Health grant GM 38060. The authors thank Dr Azra Pervin for performing the disaccharide compositional analysis on the glycosaminoglycans studied.

Abbreviations

Ac, acetate; dp, degree of polymerization; GalN, 2-deoxy-2-amino-D-galactopyranose; GlcN, 2-deoxy-2-amino-D-glucopyranose; GPC, gel permeation chromatography; PAGE, polyacrylamide gel electrophoresis; S, sulphate; TEMED, *N,N,N',N'*-tetramethylenediamine.

References

Al-Hakim,A. and Linhardt,R.J. (1991a) Electrophoresis and detection of nanogram quantities of exogenous and endogenous glycosaminoglycans in biological fluids. *Appl. Theor. Electrophor.*, **1**, 305–312.

Al-Hakim,A. and Linhardt,R.J. (1991b) Capillary electrophoresis for the analysis of chondroitin sulfate and dermatan sulfate-derived disaccharides. *Anal. Biochem.*, **195**, 68–73.

Ampofo,S.A. and Linhardt,R.J. (1991) Disaccharide compositional analysis of heparin and heparan sulfate using capillary zone electrophoresis. *Anal. Biochem.*, **199**, 249–255.

Desai,U.R., Wang,H.M. and Linhardt,R.J. (1993a) Specificity studies on the heparin lyases from *Flavobacterium heparinum*. *Biochemistry*, **32**, 8140–8145.

Desai,U.R., Wang,H.M. and Linhardt,R.J. (1993b) Substrate specificity of the heparin lyases from *Flavobacterium heparinum*. *Arch. Biochem. Biophys.*, **306**, 461–468.

Edens,R.E., Al-Hakim,A., Weiler,J.M., Rethwisch,D.G., Fareed,J. and Linhardt,R.J. (1992) Gradient polyacrylamide gel electrophoresis for determination of the molecular weight of heparin and low molecular weight heparin derivatives. *J. Pharm. Sci.*, **81**, 823–827.

Gu,K., Liu,J., Pervin,A. and Linhardt,R.J. (1993) Comparison of the activity of two chondroitin AC lyases on dermatan sulfate. *Carbohydr. Res.*, **244**, 369–377.

Hiyama,K. (1976) Action of chondroitinases. II. Numerical calculation of degree of multiple attack. *J. Biochem.*, **80**, 1209–1214.

Langer,R., Linhardt,R.J., Cooney,C.L., Klein,M., Tapper,D., Hoffberg,S.M. and Larsen,A. (1982) An enzymatic system for removing heparin in extracorporeal therapy. *Science*, **217**, 261–263.

Lasker,S. and Stivala,S. (1966) Physiochemical studies of fractionated bovine heparin. I. Some dilute solution properties. *Arch. Biochem. Biophys.*, **115**, 360–372.

Liberti,P.A. and Stivala,S.S. (1967) Physical chemical studies of fractionated bovine heparin. II. Viscosity as a function of ionic strength. *Arch. Biochem. Biophys.*, **119**, 510–518.

Linhardt,R.J. (1993) In Varki,A. (ed.), *Current Protocols in Molecular Biology, Analysis of Glycoconjugates*. Chapter 17, in press.

Linhardt,R.J., Grant,A., Cooney,C.L. and Langer,R. (1982a) Differential anticoagulant activity of heparin fragments prepared using microbial heparinase. *J. Biol. Chem.*, **257**, 7310–7313.

Linhardt,R.J., Fitzgerald,G.L., Cooney,C.L. and Langer,R. (1982b) Mode of action of heparin lyase on heparin. *Biochim. Biophys. Acta*, **702**, 197–203.

Linhardt,R.J., Galliher,P.M. and Cooney,C.L. (1986) Polysaccharide lyases. *Appl. Biochem. Biotechnol.*, **12**, 135–176.

Linhardt,R.J., Rice,K.G., Kim,Y.S., Engelken,J. and Weiler,J. (1988) Homogeneous, structurally defined heparin–oligosaccharides with low anticoagulant activity inhibit the generation of the amplification pathway C3 convertase *in vitro*. *J. Biol. Chem.*, **263**, 13090–13096.

Linhardt,R.J., Turnbull,J.E., Wang,H.M., Loganathan,D. and Gallagher,J.T. (1990) Examination of the substrate specificity of heparin and heparan sulfate lyases. *Biochemistry*, **29**, 2611–2617.

Linhardt,R.J., Al-Hakim,A., Liu,J., Hoppensteadt,D., Fareed,J., Mascellani, G. and Bianchini,P. (1991) Structural features of dermatan sulfates and their relationship to anticoagulant and antithrombotic activities. *Biochem. Pharmacol.*, **42**, 1609–1619.

Linhardt,R.J., Wang,H.M., Loganathan,D. and Bae,J.H. (1992a) Search for the heparin antithrombin III binding site precursor. *J. Biol. Chem.*, **267**, 2380–2387.

Linhardt,R.J., Ampofo,S.A., Fareed,J., Hoppensteadt,D., Mulliken,J.B. and Folkman,J. (1992b) Isolation and characterization of a human heparin. *Biochemistry*, **31**, 12441–12445.

Linker,A., Meyer,K. and Hoffman,P. (1956) The production of unsaturated uronides by bacterial hyaluronidases. *J. Biol. Chem.*, **219**, 13–25.

Lohse,D.L. and Linhardt,R.J. (1992) Purification and characterization of heparin lyases from *Flavobacterium heparinum*. *J. Biol. Chem.*, **267**, 24347–24355.

Mathews,M.B. and Cifonelli,J.A. (1977) *Acidic Reference Standards Data*. NIAMD, Contract no. N01-AM-5-2205.

Rice,K.G. and Linhardt,R.J. (1989) Study of defined oligosaccharide substrates of heparin and heparan monosulfate lyases. *Carbohydr. Res.*, **190**, 219–233.

Roden,L., Baker,J.R., Cifonelli,J.A. and Mathews,M.B. (1972) Isolation and characterization of connective tissue polysaccharides. *Methods Enzymol.*, **28**, 73–140.

Yamagata,T., Saito,H., Habuchi,O. and Suzuki,S. (1968) Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.*, **243**, 1523–1535.

Received on October 22, 1993; revised on December 23, 1993; accepted on January 2, 1994