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A Comparative Study Between a Chondroitinase B and a Chondroitinase AC from *Flavobacterium heparinum*

ISOLATION OF A CHONDROITINASE AC-SUSCEPTIBLE DODECASACCHARIDE FROM CHONDROITIN SULPHATE B

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A chondroitinase that degrades only chondroitin sulphate B was isolated from *Flavobacterium heparinum*, and separated from a constitutive chondroitinase AC also present in extracts of *F. heparinum*. The enzyme acts only on chondroitin sulphate B, producing oligo- and tetra-saccharides, plus an unsaturated 4-sulphated disaccharide (ΔDi-4S). The oligosaccharide fraction (mol.wt. 3000) is susceptible to chondroitinase AC, producing mainly ΔDi-4S. The chondroitinase B is distinguished from chondroitinase AC by several properties, such as the effect of certain metal ions, temperature for optimal activity, and susceptibility to increasing salt concentrations. The enzyme is induced in *F. heparinum* by all the chondroitin sulphates, as well as by the disaccharides prepared from the chondroitins. The mechanism of induction of the enzyme and the structure of chondroitin sulphate B are discussed in relation to these results.

Several mucopolysaccharidases from *Flavobacterium heparinum* have been characterized in the last few years. These include a constitutive system able to degrade chondroitin sulphates* A, C and hyaluronic acid to their constituents (Hoffman *et al.*, 1960; Yamagata *et al.*, 1968), as well as a series of inducible enzymes able to degrade heparin and heparitin sulphate to their basic constituents. The latter series of enzymes include a heparinase, two heparitinases, glycuronidases and specific sulphatases (Dietrich, 1969*a*,*b*; Dietrich *et al.*, 1973; Silva & Dietrich, 1974).

Hoffman *et al.* (1960) have observed that *F. heparinum* also degrades chondroitin sulphate B when it is grown in the presence of chondroitin sulphates, suggesting that a new chondroitinase is induced in the bacterium. Some properties of this enzyme were studied (Michelacci & Dietrich, 1973*a*), but there was no unequivocal evidence to support induction of a chondroitinase B and/or a chondroitinase ABC.

The present paper reports the isolation and characterization of an induced chondroitinase B from *F. heparinum*, as well as a comparison of this enzyme with a constitutive chondroitinase AC also present in the bacterium. The isolation of a chondroitinase B-resistant oligosaccharide from chondroitin sulphate B, and some characteristics of the induction process of the chondroitinase B, are also reported. Preliminary communications of parts of these findings have appeared (Michelacci & Dietrich, 1973*b*, 1974).

* These compounds are also known as: chondroitin sulphate A, chondroitin 4-sulphate; chondroitin sulphate C, chondroitin 6-sulphate; chondroitin sulphate B, dermatan sulphate.

Experimental

Materials

Chondroitin sulphates A, B and C, chondroitinase AC and chondroitinase ABC were purchased from Miles Laboratories (Elkhart, Ind., U.S.A.). Chondroitin sulphate B was also prepared from pig skin, essentially as described by Schiller *et al.* (1961). Heparitin sulphates A, B, C and D were prepared from a commercial sample supplied by the Upjohn Co., Kalamazoo, Mich., U.S.A., as previously described (Dietrich & Nader, 1974). Heparin was kindly given by Dr. J. T. Corell of the Upjohn Co., Agarose (Indubiose A 37) was purchased from L'Industrie Biologique Française, Gennevilliers, Seine, France.

Preparation of enzymes

Enzyme extracts from *F. heparinum* (A.T.C.C. 13125), previously grown in glucose, or in the presence of 150mg of chondroitin sulphates A and/or

B and/or C/litre, were prepared as previously reported (Dietrich, 1969*a*; Dietrich *et al.*, 1973). The 100000*g* supernatant extract was freeze-dried, and 200mg was resuspended in 2ml of 0.1M-EDA (ethylenediamine–acetate) buffer, pH8.0; any precipitate formed was removed by centrifugation. The supernatant was subjected to large-scale agarose-gel electrophoresis as previously described (Dietrich *et al.*, 1973) except that 0.1M-EDA buffer at pH8.0, instead of pH7.0, was used for the fractionation. Also the gel was subjected to 5V/cm for 48h.

Studies on the induction of the enzymes

For small-scale experiments on the induction of chondroitinase B, the following method was used. The cells were grown for 24h in trypticase soy broth containing 2.5g of glucose/litre, centrifuged and resuspended in a medium containing 100mg of trypticase soy broth/litre without glucose. A suspension of about 20mg (wet wt.) of cells/ml was prepared; 1ml portions were then immediately incubated at 30°C with 100μg of chondroitin sulphates, or other substances, for different periods of time. After incubation, the cell suspensions were harvested, and washed with cold (5°C) 0.1M-EDA buffer, pH8.0. The centrifuged cells were then resuspended in 100μl of the same buffer and incubated with different substrate, as described below. Control incubations with trypticase soy broth plus glucose were run throughout the series of experiments.

Assay of enzymes

A typical incubation mixture contained 0.1–50μg of enzyme protein, 100μg of chondroitin sulphates, or other substrates, and other additions as indicated, and 0.05M-EDA buffer, pH8.0, in a final volume of 20μl. After enzyme inactivation by heating, the reaction mixtures were spotted on to Whatman no. 1 paper and chromatographed in isobutyric acid–1M-NH_3 (5:3, v/v) for 48h. In some instances electrophoresis was also used as previously described (Dietrich, 1969*b*). The disaccharide products were detected and quantified by densitometry (in a Canalco model G computer microdensitometer) after staining with $AgNO_3$ as previously described (Dietrich *et al.*, 1973). The disaccharide products were characterized as described by Suzuki *et al.* (1968).

Preparation of chondroitin sulphate B products

Chondroitin sulphate B (100mg) was incubated with 400μg of chondroitinase B in 0.05M-EDA buffer, pH8.0, for 24h at 20°C in a final volume of 20ml. After the incubation, the mixture was concentrated and applied as a 40cm band on a Whatman 3MM paper and subjected to chromatography in isobutyric acid–1M-NH_3 (5:3, v/v) for 48h. The products were located with a u.v. lamp and were eluted with water. The eluted products were evaporated to dryness under vacuum, resuspended in a small volume of water and further purified by precipitation with methanol–acetone mixtures as described previously for the heparin-degradation products (Dietrich, 1968).

Other methods

Amino sugars were measured after acid hydrolysis (4M-HCl for 6h at 100°C) by a modified Elson–Morgan reaction (Rondle & Morgan, 1955). Sulphate was determined by densitometry after acid hydrolysis, and acetyl groups were determined by g.l.c. as previously described (Dietrich & Nader, 1974). Protein was measured by a modification of the method of Lowry *et al.* (Schacterle & Pollack, 1973). Agarose-gel electrophoresis was performed by a method previously described (Dietrich & Dietrich, 1972). Molecular-weight estimations were performed by the method of Hilborn & Anastassiadis (1971), except that gel slabs were used instead of gel cylinders (Dietrich & Nader, 1974).

Results

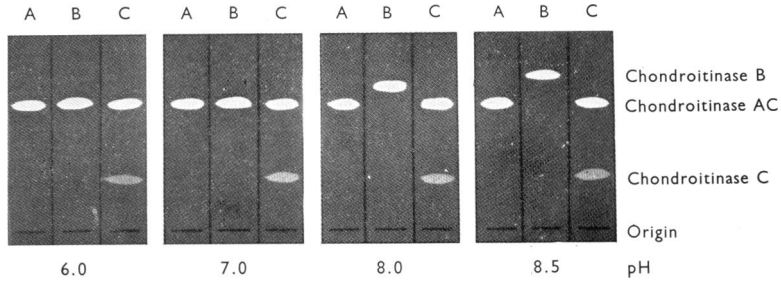
Induction of the chondroitin sulphate B-degrading enzyme by different mucopolysaccharides

The enzymic activities of crude extracts from *F. heparinum* grown in different mucopolysaccharides are shown in Table 1. A 100-fold enhancement in degradation of chondroitin sulphate B is observed when the cells are grown in chondroitin sulphates A, B or C, indicating the induction of a chondroitinase. An increased degradation of chondroitin sulphates A and C by the extracts prepared from cells grown in either one of the chondroitin sulphates is also observed. These results would suggest either the induction of a chondroitinase ABC by the chondroitin sulphates or the induction of a chondroitinase B together with an activation of the constitutive chondroitinase AC.

Fractionation of the constitutive and induced chondroitinases

Attempts to purify these two activities by conventional methods of protein fractionation, e.g. ion-exchange chromatography or salt precipitation, were unsuccessful. Initial experiments using agarose-gel electrophoresis in ammonium acetate buffer, pH7.0, succeeded in partially separating these two activities (Michelacci & Dietrich, 1973*b*). Nevertheless the fractions containing the induced chondroitinase activity were still heavily contaminated by the constitutive chondroitinase AC.

We had previously fractionated all the heparin-degrading enzymes in agarose with EDA buffer (Dietrich *et al.*, 1973). This buffer at different pH values in agarose was used to attempt the fractiona-



EXPLANATION OF PLATE 1

Fractionation of the chondroitinases by agarose-gel electrophoresis

Samples (20μg) of induced extracts in 5μl of EDA buffer were applied to 0.2cm thick agarose-gel slides (5cm× 7.5cm) prepared with 0.9% agarose in 0.1M-EDA buffer at the pH values indicated in the Plate, and submitted to electrophoresis (5V/cm) for 2h. Strips of cellulose acetate (1.5cm×7.5cm), previously immersed in chondroitin sulphates A, B and C (A, B and C in the Plate) were applied in the wet state over the agarose gel after the electrophoretic run and then incubated at room temperature (25°C) for 30min. After incubation the mucopolysaccharides in the gel were fixed with Cetavlon and stained with Toluidine Blue as previously described (Dietrich & Dietrich, 1972). The enzymes were located as white areas against a purple background (shaded area) in the slide.

(*Facing p.* 122)

Table 1. *Chondroitinase activities in F. heparinum cells grown in different chondroitin sulphates*

A 100μg portion of a 100000*g* supernatant from cells grown in the inducers indicated below was incubated with 100μg of each of the substrates for 4h at 30°C in 0.05M-EDA buffer in a final volume of 20μl. After incubation, the disaccharides formed were identified and quantified as described in Fig. 3.

Inducer	Products formed from: Chondroitin sulphate A* (μg)	Chondroitin sulphate B* (μg)	Chondroitin sulphate C† (μg)
Glucose	16.9	0.5	16.1
Chondroitin sulphate A	46.8	50.0	51.6
Chondroitin sulphate B	46.4	46.8	49.2
Chondroitin sulphate C	50.8	46.0	48.4

* ΔDi-4S formed from chondroitin sulphate A and chondroitin sulphate B.
† ΔDi-6S formed from chondroitin sulphate C.

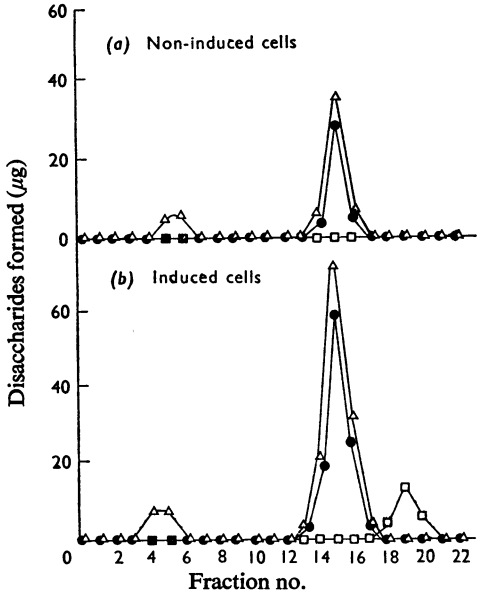


Fig. 1. *Large-scale fractionation of the chondroitinases from non-induced (a) and induced (b) cells by agarose-gel electrophoresis*

Chondroitin sulphates (100μg of each) were incubated with 10μl of the agarose-gel fractions for 18h at 30°C. The disaccharides formed were identified and quantified after chromatography as described in the Experimental section. ●, ΔDi-4S formed from chondroitin sulphate A; □, ΔDi-4S formed from chondroitin sulphate B; △, ΔDi-6S formed from chondroitin sulphate C.

tion of the chondroitinases. Plate 1 shows the results of these experiments. At pH8.0 and 8.5 the two chondroitinases separate from each other. The fast-moving enzyme degrades only chondroitin sulphate B, but had no activity towards the chondroitin sulphates A and C, indicating that the induced enzyme is a chondroitinase B. Extracts of non-induced cells subjected to gel electrophoresis in similar conditions show only the constitutive chondroitinase AC activity. Large-scale fractionations of extracts prepared from induced and non-induced cells by agarose-gel electrophoresis in EDA buffer, pH8.0, are shown in Fig. 1. Three enzymes that act on the chondroitin sulphates are separated in this fractionation. A fast-moving enzyme capable of degrading chondroitin sulphate B is present in significant amounts only in the extracts prepared from the induced cells. The chondroitinase AC is present in both extracts, although it has a higher activity in the one prepared from cells grown in the chondroitin sulphates. A third enzymic activity (fractions 4 and 5) is present in both induced and non-induced extracts. This enzyme degrades only chondroitin sulphate C and hyaluronic acid (Y. M. Michelacci & C. P. Dietrich, unpublished work). The degree of purification and yield of the two chondroitinases after agarose-gel electrophoresis fractionation are shown in Table 2. An 87-fold purification with 69% yield is obtained for the chondroitinase B in this single purification step. A 30-fold purification with a 60% yield is obtained for the chondroitinase AC after agarose-gel electrophoresis.

Properties of the chondroitinases

The products of degradation of chondroitin A or B by the separated chondroitinase activities during prolonged incubations are shown in Fig. 2. The formation of di- and tetra-saccharides by the action of the chondroitinase B from chondroitin sulphate B is linear with time over the initial 10h period (Fig. 2*a*). Similarly, both tetra- and di-saccharides are formed

Table 2. *Purification and recovery of the chondroitinases*

A 36mg portion of the 100000*g* supernatant from extracts of *F. heparinum* was fractionated by agarose-gel electrophoresis in 0.1M-EDA buffer, pH8.0. The peaks containing each of the chondroitinases were pooled, and 10μl portions were incubated with 100μg of chondroitin sulphate B and chondroitin sulphate A for 4h at 30°C in a final volume of 20μl. The products formed were quantified as described in the Experimental section. A unit of enzyme activity was defined as the amount necessary to degrade 100μg of mucopolysaccharide/min.

Enzyme	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Chondroitinase AC				
Crude	62.13	1.72		
Agarose gel	37.35	51.87	30.1	60.1
Chondroitinase B				
Crude	16.98	0.47		
Agarose gel	11.70	41.05	87.3	68.9

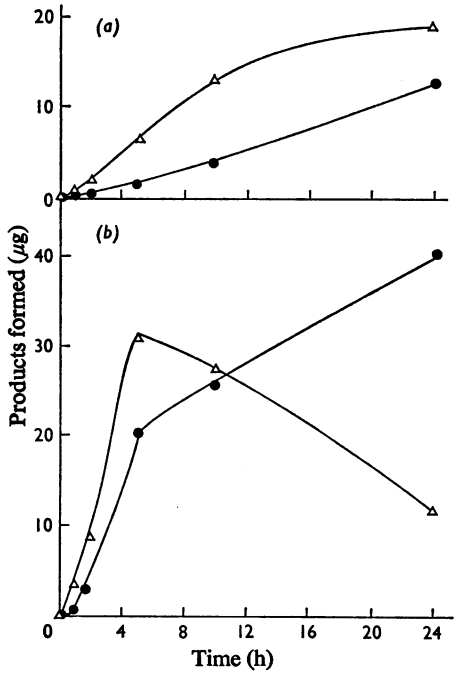


Fig. 2. *Formation of disaccharides from the chondroitin sulphates by the action of the chondroitinases at different times*

Chondroitin sulphates A and B (100μg of each) were incubated with 0.2μg of chondroitinase B (*a*) and chondroitinase AC (*b*) at 30°C in 0.05M-EDA buffer, pH8.0, for the times indicated. The ΔDi-4S (△) and tetrasaccharide (●) formed from chondroitin sulphate A, and ΔDi-4S (△) and tetrasaccharide (●) formed from chondroitin sulphate B, were identified and quantified as described in the Experimental section.

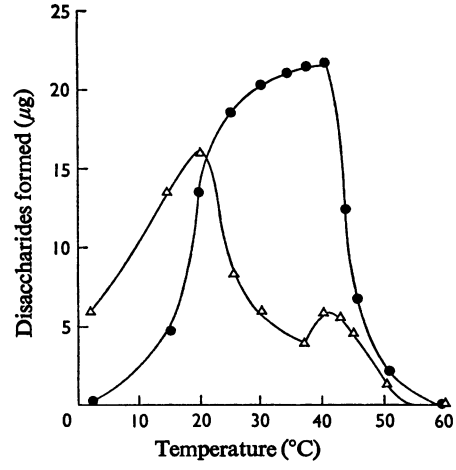


Fig. 3. *Effect of temperature on the chondroitinase activities*

The experiments were performed as described in Fig. 2, except that the mixtures were incubated for 4 h at the temperatures indicated. △, Chondroitinase B; ●, chondroitinase AC.

from chondroitin sulphate A by the action of the chondroitinase AC in short incubation periods. After 4h incubation, disaccharide is formed, possibly from the tetrasaccharide, by the action of the same enzyme.

The chondroitinase B has the highest activity at 20°C, whereas the chondroitinase AC has an optimal temperature for activity around 37–40°C (Fig. 3). Both chondroitinases have the highest activity at pH8.0 (Fig. 4). The chondroitinase B is more susceptible to increasing concentrations of NaCl compared with chondroitinase AC. Both enzymes are slightly activated by NaCl at 0.02M. The chondroitinase B activity is 50% inhibited at 0.1M-NaCl, whereas the chondroitinase AC activity is practically unaffected at this salt concentration (Fig. 5).

The substrate specificities of the chondroitinases are shown in Table 3. Chondroitinase AC acts on chondroitin sulphates A and C and hyaluronic acid, forming unsaturated 4,6- and non-sulphated di-

saccharides respectively, with no action on chondroitin sulphate B or other mucopolysaccharides. Chondroitinase B acts only on chondroitin sulphate B, forming 4-sulphated disaccharide (ΔDi-4S) as the main product. The effects of certain metal ions on the chondroitinase activities are shown in Table 4. Ca^{2+} and Mg^{2+} (at 10mM final concentration) inhibit chondroitinase AC, but have no effect on chondroitinase B, whereas Co^{2+} inhibits chondroitinase B. Fe^{3+} and Ba^{2+} inhibit both enzyme activities at 10mM, and K^+ has only a slight activating effect.

Products formed from the chondroitin sulphates by the action of the chondroitinases

The main products formed from chondroitin sulphates A and C by the action of chondroitinase AC are ΔDi-4S and ΔDi-6S respectively (80% yield), as shown in Table 5. The products formed by the action of chondroitinase B on chondroitin sulphate B are oligo-, tetra- and hexa-saccharides, besides ΔDi-4S. These products were prepared on a large scale and again incubated with chondroitinase B and chondroitinase AC. Fig. 6 shows the results of this experiment.

The tetra- and hexa-saccharides are still susceptible to the action of chondroitinase B, being slowly degraded to ΔDi-4S. These compounds were not degraded by chondroitinase AC. On the other hand, the oligosaccharide was completely resistant to the action of chondroitinase B, and was degraded only by chondroitinase AC, mainly to ΔDi-4S. The velocity of degradation of this oligosaccharide by chondroitinase AC was of the same order of magnitude as that for chondroitin sulphate A.

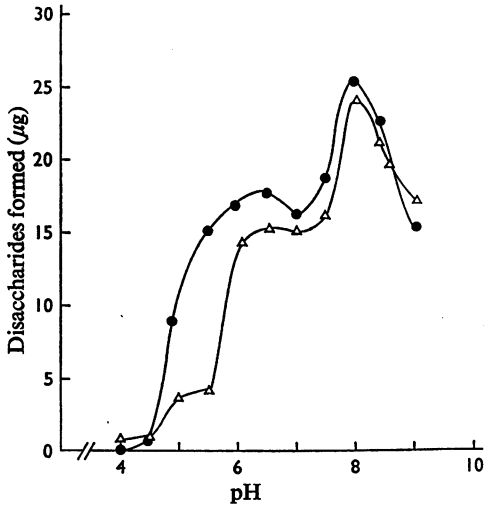


Fig. 4. *Effect of pH on the chondroitinase activities*

The experiments were performed as described in Fig. 2, except that the mixtures were incubated for 4h at the pH values indicated. △, Chondroitinase B; ●, chondroitinase AC.

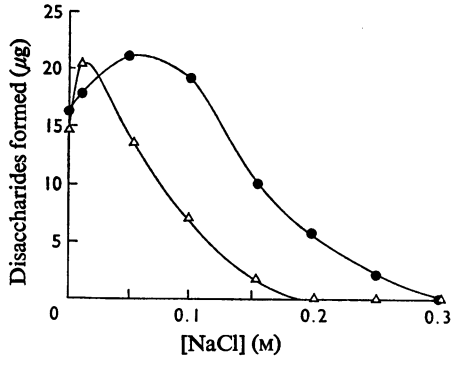


Fig. 5. *Effect of NaCl concentration on the chondroitinase activities*

The experiments were performed as described in Fig. 2, except that the mixtures were incubated for 4h in the presence of NaCl at the concentrations indicated. △, Chondroitinase B; ●, chondroitinase AC.

Table 3. *Substrate specificities of the chondroitinases*

A 100μg portion of each mucopolysaccharide was incubated with 0.2μg of chondroitinase AC or 0.2μg of chondroitinase B, for 4h in 0.05M-EDA buffer, pH8.0, at 37°C (chondroitinase AC) or 20°C (chondroitinase B). The disaccharide products were identified and quantified as described in the Experimental section.

Mucopolysaccharides Enzyme ...	Disaccharides formed (μg) Chondroitinase AC	Chondroitinase B
Chondroitin sulphate A	27	0.8
Chondroitin sulphate B	<0.2	20.0
Chondroitin sulphate C	40	<0.2
Hyaluronic acid	29	<0.2
Heparitin sulphates A, B, C and D	<0.2	<0.2
Heparin	<0.2	<0.2

Molecular weight and dispersity of the chondroitinase B-resistant oligosaccharide

Fig. 7 shows the migration of the oligosaccharide compared with chondroitin sulphate B on polyacrylamide-gel electrophoresis. The mol.wt. of this compound was shown to be 3000, compared with 19000 for chondroitin sulphate B. Surprisingly, the oligosaccharide was monodisperse, contrasting with the polydispersity of chondroitin sulphate B (Fig. 7) and other mucopolysaccharides (Dietrich & Nader, 1974). The yield of oligosaccharide from chondroitin sulphate B was 31%, and by knowing the molecular weight of both compounds, it was possible to calculate mol of oligosaccharide/mol of chondroitin sulphate B. Each mol of chondroitin sulphate B contains about 2 mol of oligosaccharide.

Induction of the chondroitinase B by the chondroitin sulphates and their degradation products

The time-courses of the induction of the chondroitinase B by several compounds are shown in Fig. 8. Chondroitin sulphates A, B and C are equally potent inducers of the enzyme in *F. heparinum*. The highest specific activity of the enzyme is obtained only after long induction periods (24h). The chondroitin sulphate B di- and tetra-saccharide, on the other hand, elicit a high enzyme activity after a short period of incubation. By using ΔDi-4S as the inducer, the maximum specific activity is reached after 1h of in-

Table 4. *Effect of ions on the chondroitinase activities*

This experiment was performed as described in Fig. 2, except that different ions (at 10mM) were added as indicated and the mixtures were incubated for 4h.

	Enzyme activity (% of control)	
Ion	Chondroitinase B	Chondroitinase AC
None	100	100
Na^+	82	100
K^+	110	108
Ca^{2+}	105	65
Ba^{2+}	18	24
Mg^{2+}	108	49
Mn^{2+}	29	35
Co^{2+}	10	106
Fe^{3+}	1	1

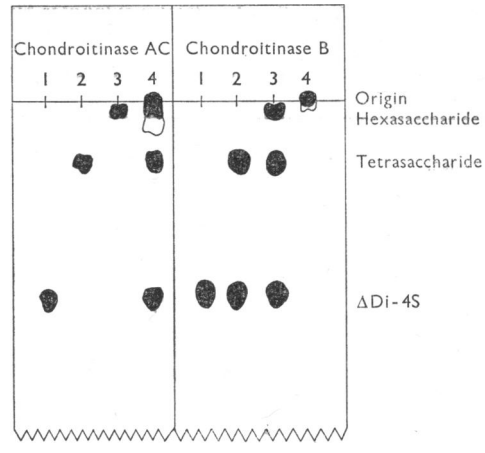


Fig. 6. *Action of chondroitinase B and chondroitinase AC on the chondroitin sulphate B degradation products*

About 100μg of each of the degradation products, prepared from chondroitin sulphate B by the action of chondroitinase B, was incubated with 0.5μg of chondroitinase B and/or chondroitinase AC for 18h at 30°C in 0.05M-EDA buffer, pH8.0. The mixtures were submitted to chromatography and the paper was stained with the $AgNO_3$ reagent. 4, Oligosaccharide; 3, hexasaccharide; 2, tetrasaccharide; 1, disaccharide.

Table 5. *Products formed from the chondroitin sulphates by the action of the chondroitinases*

Chondroitin sulphates A and C (1 mg of each) were incubated with 4μg of chondroitinase AC for 24h at 37°C in 0.05M-EDA buffer, pH8.0, in a final volume of 100μl. Also, 1 mg of chondroitin sulphate B was incubated with 4μg of chondroitinase B for 24h at 20°C in 0.05M-EDA buffer, pH8.0. After incubation the mixtures were spotted on Whatman no. 1 paper as 5cm bands and chromatographed. The products formed were located with the aid of a u.v. lamp, and eluted with water. Hexosamine determinations were then performed on the eluates. The relative concentrations of the products are expressed as a percentage of the total hexosamine.

		Hexosamine (% of total)		
	Enzyme ...	Chondroitinase AC		Chondroitinase B
Product	Substrate ...	Chondroitin sulphate A	Chondroitin sulphate C	Chondroitin sulphate B
Oligosaccharide		0.3	2.5	31.0
'Hexasaccharide'				10.0
'Tetrasaccharide'		1.2	15.0	23.0
ΔDi-6S		20.0	80.0	
ΔDi-4S		78.5	2.5	36.0

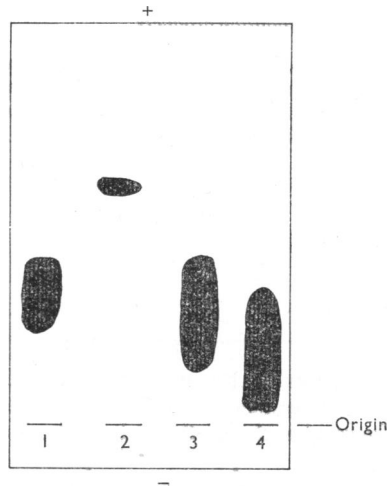


Fig. 7. *Polyacrylamide-gel electrophoresis of chondroitin sulphate B oligosaccharide*

1, Chondroitin sulphate B; 2, chondroitin sulphate B oligosaccharide; 3, chondroitin sulphate A; 4, chondroitin sulphate C.

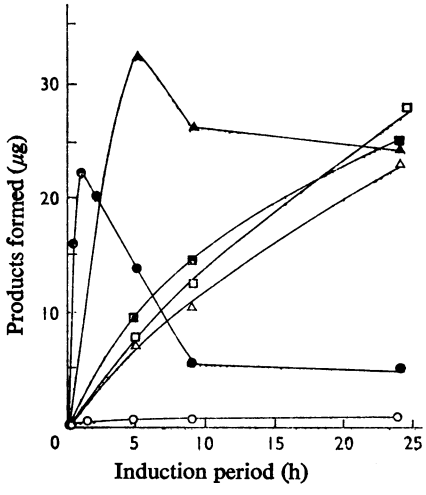


Fig. 8. *Induction of the chondroitinase B by chondroitin sulphates and chondroitin sulphate B degradation products*

F. heparinum cells (0.1 mg dry wt.) grown in glucose were incubated in 100 μl of induction media for different times and in the presence of 50 μg of each of following: chondroitin sulphate B (■), chondroitin sulphate A (△), chondroitin sulphate C (□), chondroitin sulphate B tetrasaccharide (▲), chondroitin sulphate B ΔDi-4S (●), and glucose (○). After incubation the cells were harvested, washed once with 100 μl of 0.1M-EDA buffer, pH 8.0, resuspended in 10 μl of the same buffer and incubated with 100 μg of chondroitin sulphate B for 5 h in a final volume of 20 μl. The disaccharide products were identified and quantified as described in the Experimental section.

duction, but falls rapidly. With the tetrasaccharide, the maximum specific activity is reached after 5h, and then decreases slowly. The induction of chondroitinase B is inhibited by antibiotics that interfere with protein synthesis and DNA replication, as shown in Table 6.

Product identification

Table 7 shows the results of the chemical analysis of the products formed from chondroitin sulphate B. All the products contain about 1 mol of acetyl groups, uronic acid and sulphate/mol of galactosamine. The tetrasaccharide was characterized as such by the fact that it releases only ΔDi-4S after chondroitinase B degradation, contains about 50% of galactosamine resistant to borohydride reduction, and has half of the reducing power of the disaccharide. The hexasaccharide has 70% of its galactosamine resistant to borohydride reduction, and releases ΔDi-4S and tetrasaccharide after partial chondroitinase B degradation. The oligosaccharide does not migrate in isobutyric acid–NH_3 for 48 h, contains about equimolar amounts of sulphate, galactosamine, acetyl groups and uronic acid, and is degraded by the chondroitinase AC to ΔDi-4S. On the basis of its mol.wt. (3000) and its monodispersity, it can be concluded that this compound is probably a dodecasaccharide, containing 4-sulphated disaccharide repeating units.

Discussion

The results reported in the present paper show that a chondroitinase B is induced in *F. heparinum* when the cells are grown in the presence of one of the chondroitin sulphates A, B or C. These mucopolysaccharides are equally efficient in inducing chondroitinase B. The degradation products of chondroitin sulphate B are also equally efficient as inducers of the enzyme. The unsaturated 4-sulphated disaccharide is able to promote almost immediate induction of the chondroitinase B (1 h), whereas the tetrasaccharide requires 5 h for full induction, and the chondroitin sulphates more than 10 h, under the conditions of the

Table 6. *Effect of antibiotics on the induction of the chondroitinase B*

The conditions for this experiment were the same as described in Fig. 8, except that antibiotics were used in a final concentration of 50 μg/ml and chondroitin sulphate B was used as the inducer.

Antibiotic	Induction (%)
None	100
Penicillin G	100
Chloramphenicol	<2
Tetracycline	<2
Mytomicin C	<2

Table 7. *Analytical data for the chondroitin sulphate B degradation products*

Compound	Molar proportions				Reducing sugar (mol/mol of hexosamine)	Borohydride reduction (% of hexosamine remaining after reduction)
	Hexosamine*	Uronic acid	Total sulphate	Acetyl groups		
Chondroitin sulphate B	1	—	1.01	1.17	—	100
Oligosaccharide	1	1.3	0.98	1.15	0.20	88
Hexasaccharide	1	1.3	1.25	0.98	0.23	70
Tetrasaccharide	1	1.4	1.02	1.14	0.40	50
ΔDi-4S	1	1.3	0.95	0.90	0.82	1

* Only galactosamine was detected after acid hydrolysis and chromatography.

experiment described in Fig. 8. Since a low activity of the chondroitinase B is present in non-induced cells, it is suggested that the actual inducer is the unsaturated disaccharide, which can be formed from the chondroitin sulphate B by low activity of the chondroitinase B. The different times needed for full induction would be accounted for by the slow formation of disaccharides in the non-induced cells. This hypothesis would also corroborate previous results obtained for the induction of the heparinase (Dietrich, 1969*c*) and other systems (Jacob & Monod, 1961; Reese *et al.*, 1969), in which the disaccharides are the real inducers of the enzyme. If the disaccharides are the actual inducers of chondroitinase B, it would also be easy to explain why two different mucopolysaccharides, chondroitin sulphate A and chondroitin sulphate B, are able to induce the same enzyme. The unsaturated disaccharide formed from these two mucopolysaccharides by the action of the chondroitinases is the same, namely ΔDi-4S. The chondroitinases act as eliminases by producing an unsaturation between C-4 and C-5 of the uronic acid moiety, thus removing the asymmetry of C-5 and consequently eliminating the differences between iduronic acid and glucuronic acid that differentiate chondroitin sulphate A from chondroitin sulphate B. The position of the sulphate in the uronic acid moiety of the disaccharides does not seem to be important for the induction process, since ΔDi-6S and chondroitin sulphate C are also inducers of the enzyme.

The production of an oligosaccharide from chondroitin sulphate B by the action of chondroitinase B, which is susceptible to chondroitinase AC, shows unequivocally that chondroitin sulphate B is a hybrid structure containing two types of uronic acids, glucuronic acid and iduronic acid, as previously described (Franson *et al.*, 1970). It also shows that the glucuronic acid-containing disaccharides are segregated in a specific region of the chondroitin sulphate B molecule, forming an oligosaccharide, and not alternated throughout the molecule. The pig skin chondroitin sulphate B used in these experiments contains about 2 mol of oligosaccharide/mol of chondroitin sulphate B. It is possible that the chondroitin sulphate B isolated from other sources might differ in the content and arrangement of the glucuronic acid-containing oligosaccharides in the polysaccharide, and the chondroitinase B reported in the present paper will help in the investigation of their structures.

We have previously reported the isolation, from ox lung tissue, of a heparitin sulphate (heparitin sulphate C) with a homogeneous molecular weight (Dietrich & Nader, 1974). The glucuronic acid-containing oligosaccharide reported in the present paper is, to our knowledge, the second example of a polysaccharide with such a property. The implications of these findings remain to be seen.

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References

Dietrich, C. P. (1968) *Biochem. J.* **108**, 647–653
Dietrich, C. P. (1969*a*) *Biochemistry* **8**, 2089–2094
Dietrich, C. P. (1969*b*) *Biochem. J.* **111**, 91–95
Dietrich, C. P. (1969*c*) *Biochemistry* **8**, 3342–3347
Dietrich, C. P. & Dietrich, S. M. C. (1972) *Anal. Biochem.* **46**, 209–215
Dietrich, C. P. & Nader, H. B. (1974) *Biochim. Biophys. Acta* **345**, 34–44
Dietrich, C. P., Silva, M. E. & Michelacci, Y. M. (1973) *J. Biol. Chem.* **248**, 6408–6415
Franson, L. A., Anseth, A., Antonopoulos, C. A. & Gardell, S. (1970) *Carbohydr. Res.* **15**, 73–89
Hilborn, J. C. & Anastassiadis, P. A. (1971) *Anal. Biochem.* **39**, 88–92
Hoffman, P., Linker, A., Lippman, V. & Meyer, K. (1960) *J. Biol. Chem.* **235**, 3066–3069
Jacob, F. & Monod, J. (1961) *J. Mol. Biol.* **3**, 318–356
Michelacci, Y. M. & Dietrich, C. P. (1973*a*) *Biochimie* **55**, 893–898
Michelacci, Y. M. & Dietrich, C. P. (1973*b*) *Global Impacts Appl. Microbiol. Proc. Conf. 4th*, 17

Michelacci, Y. M. & Dietrich, C. P. (1974) *Biochem. Biophys. Res. Commun.* **56**, 973–980

Reese, E. T., Lola, J. E. & Parrish, F. W. (1969) *J. Bacteriol.* **100**, 1151–1154

Rondle, C. J. M. & Morgan, W. T. J. (1955) *Biochem. J.* **61**, 586–589

Schacterle, G. R. & Pollack, R. L. (1973) *Anal. Biochem.* **51**, 654–655

Schiller, S., Slover, G. A. & Dorfman, A. (1961) *J. Biol. Chem.* **236**, 983–987

Silva, M. E. & Dietrich, C. P. (1974) *Biochem. Biophys. Res. Commun.* **56**, 965–972

Suzuki, S., Saito, H., Yamagata, T., Anno, K., Seno, K., Kawai, Y. & Furuhashi, H. (1968) *J. Biol. Chem.* **243**, 1543–1550

Yamagata, T., Saito, H., Habucchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535