ISOLATION AND PARTIAL CHARACTERIZATION OF AN INDUCED CHONDROITINASE B FROM FLAVOBACTERIUM HEPARINUM

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Received December 26,1973

SUMMARY: A chondroitinase that degrades only chondroitin sulfate B was isolated from F. heparinum previously grown in the presence of chondroitin sulfates A, B or C. The chondroitinase B was separated from a constitutional chondroitinase AC also present in F. heparinum extracts. This enzyme acts only upon chondroitin sulfate B, producing oligo- and tetra- saccharides plus an unsaturated 4-sulfated disaccharide (Δ Di-4S). The oligosaccharide is in turn susceptible only to the chondroitinase AC, producing (Δ Di-4S) and other products. The action of these two enzymes confirms the hybrid nature of chondroitin sulfate B.

INTRODUCTION: A Flavobacterium capable of utilizing heparin as its only source of energy was isolated from the soil by Payza and Korn (1). Later it was shown that a series of enzymes able to degrade heparin to its basic constituents is induced in the bacteria when grown in the presence of heparin (2-4). This Flavobacterium also contains a constitutional enzymatic system which degrades hyaluronic acid, chondroitin sulfate A and chondroitin sulfate C (5). Yamagata and coworkers (6) have purified and characterized a chondroitinase AC which is able to degrade these three mucopolysaccharides. Linker and coworkers (5) have observed that F. heparinum is also able to degrade chondroitin sulfate B when it is grown in the presence of this mucopolysaccharide, thus suggesting that a new chondroitinase is induced in this bacterium. The induced enzyme responsible for

the degradation of chondroitin sulfate B was later suggested to be a chondroitinase ABC (7), similar to the one isolated from Proteus vulgaris (6).

The present paper reports the isolation of a chondroitinase B induced in F. heparinum by chondroitin sulfates A and/or B and/or C. A preliminary communication of these results has appeared (8).

MATERIALS AND METHODS: Materials - Chondroitin sulfates A, B, and C, chondroitinase AC (Chase AC) and chondroitinase ABC (Chase ABC) were purchased from Miles Laboratories (Elkhart, Indiana). Radioactive ^{35}S chondroitin sulfates A and B were prepared as previously described (7). Heparin, and heparitin sulfates were commercial samples from the UpJohn Co., Kalamazoo.

Preparation of enzymes - Enzyme extracts from F. heparinum (ATCC 13,125) previously grown in the presence of 150 mg/l of chondroitin sulfates A and/or B and/or C were prepared as previously reported (2, 7). The resulting freeze dried supernatant extract (200 mg) was ressuspended in 2 ml of 0.1 M ethylenediamine acetate pH 8.0 and the precipitate formed was removed by centrifugation. The supernatant was subjected to large scale agarose gel electrophoresis as previously described, except that 0.1 M ethylenediamine acetate at pH 8.0 instead of pH 7.0 was used for the fractionation.

Other methods - Radioactive and non radioactive products were detected and quantified after chromatography by methods previously described (2, 4, 7). Sulfate, hexosamine, uronic acid and protein determinations were performed by methods already referred (2, 4).

RESULTS: Mucopolysaccharidases from F. heparinum cells. The enzymatic activities of extracts obtained from F. heparinum cells previously grown in glucose or mucopolysaccharides are shown in Table 1. A 10 fold enhancement of degradation of chondroitin sulfate B is observed when the cells are previously grown either in chondroitin sulfate B or chondroitin sulfate A, indicating the induction of a new chondroitinase. Heparin does not induce this new activity. When the cells are previously grown in heparin, only heparinases are induced and no enhancement of degradation of the chondroitin sulfates is observed (Table 1). An enhancement of degradation of chondroitin sulfate A is also observed when the

TABLE I

Degradation of mucopolysaccharides by F. heparinum cells previously grown in the presence of chondroitin sulfates and heparin

CELLS PREVIOUSLY GROWN IN:	PRODUCTS FORMED FROM (c.p.m.)		
	Heparin*	Chondroitin sulfate B**	Chondroitin sulfate A**
Glucose	490	540	22,060
Heparin	8,640	680	23,070
Chondroitin sulfate B	820	5,620	44,740
Chondroitin sulfate A	570	5,300	48,580

* Glucosamine 2,6 disulfate formed from heparin

** Δ Di-4S formed from the chondroitin sulfates.

About 30,000 c.p.m. of mucopolysaccharide as indicated were incubated with 100 µg of (supernatant of 100.000 g) prepared from F. heparinum cells previously grown as indicated. The products formed were identified and quantified as described in Methods.

cells are grown in the presence of the chondroitin sulfates, suggesting either the induction of a Chase ABC or enhancement of the activity of the constitutional Chase AC.

Fractionation of the constitutional and induced chondroitinases. In order to distinguish between the two possibilities mentioned above a fractionation of the induced extracts was performed. Fig. 1 shows the separation of two chondroitinases by means of agarose gel electrophoresis. The activities of these two chondroitinases upon hyaluronic acid and chondroitin sulfates A, B and C are shown in Fig. 2. One of the chondroitinases acts only upon chondroitin sulfate B with no action upon chondroitin sulfates A, C, hyaluronic acid or heparin. These results indicate that the enzyme is a chondroitinase B (Chase B). The second chondroitinase acts upon chondroitin sulfates A and C and hyal-

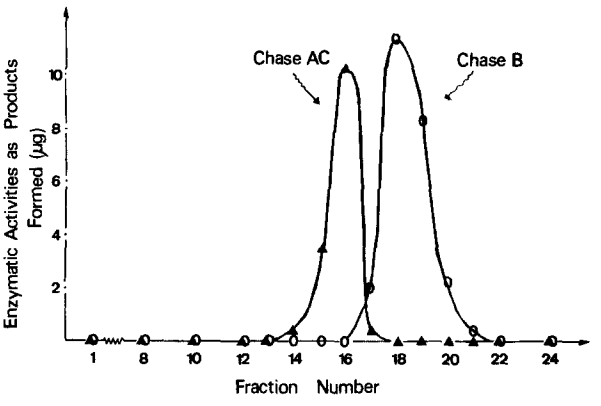

Figure 1. Fractionation of the chondroitinases by large scale agarose gel electrophoresis.

20 µl aliquots of each fraction were incubated with 100 µg of chondroitin sulfate B and/or chondroitin sulfate A at 30°C for 4 hours in 0.05 M ethylenediamine acetate pH 8.0 in a final volume of 30 µl. The incubation mixtures were then spotted in Whatman nº 1 paper and chromatographed in isobutyric acid - 1 M NH_3, 5/3 (V/V). The Δ Di-4S from chondroitin sulfate A (▲——▲) and from chondroitin sulfate B (O——O) were quantified by densitometry after silver nitrate staining.

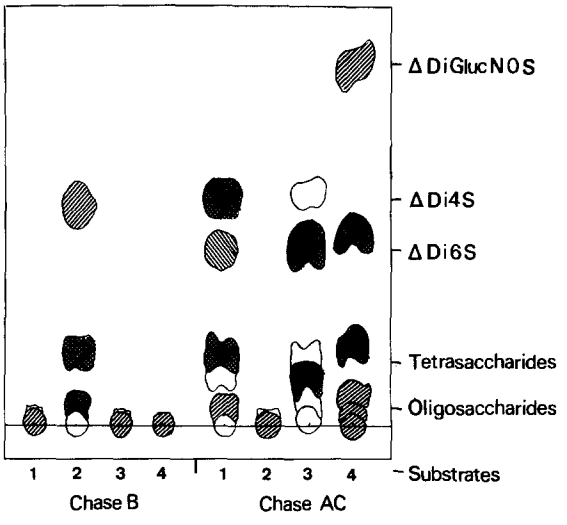

Figure 2. Chondroitinase B and chondroitinase AC activities upon different mucopolysaccharides.

The experiment was performed as described in Fig. 1, except that different mucopolysaccharides were used as substrates as indicated below and the time of incubation was 18 hours with 10 µl (less than 0.5 µg of protein) of enzyme. 1 - chondroitin sulfate A; 2 - chondroitin sulfate B; 3 - chondroitin sulfate C; 4 - hyaluronic acid.

uronic acid, indicating that it is a Chase AC similar to the one isolated by Yamagata et al. (6).

Products of the chondroitinases. The products obtained by the action of the Chase B are mainly oligosaccharides with only a relatively small amount of disaccharides (Fig. 2). This contrasts with the products formed by the Chase AC which are mainly 4-sulfated disaccharide (Δ Di 4S) for chondroitin sulfate A and 6-sulfated disaccharide (Δ Di 6S) for chondroitin sulfate C.

The compounds produced by Chase B from chondroitin sulfate B were prepared in large scale and incubated with the two Chases. Fig. 3 shows the results of such an incubation. The chondroitin

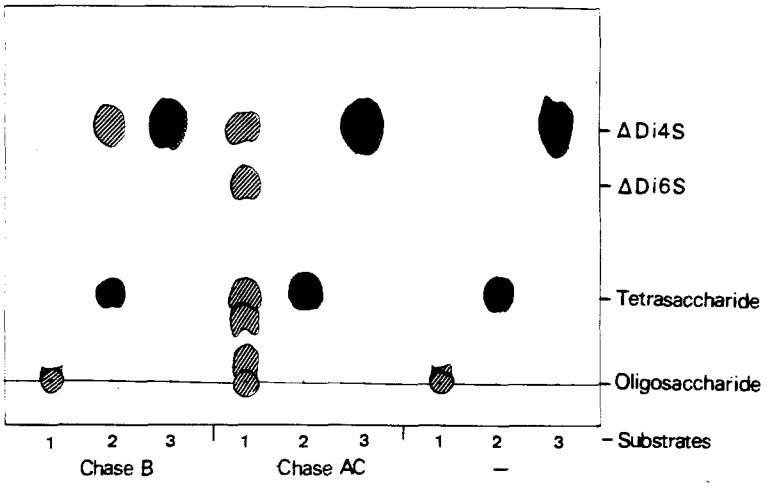

Figure 3. The action of chondroitinase B and chondroitinase AC upon chondroitin sulfate B degradation products.

The experiment was performed as described in Fig. 1, except that different substrates were used (as indicated below) with 10 µl (less than 0.5 µg of protein) of enzymes. 1 - chondroitin sulfate B; 2 - chondroitin sulfate B oligosaccharide; 3 - chondroitin sulfate B tetrasaccharide; 4 - Δ Di 4S.

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sulfate B "tetrasaccharide" was degraded only by Chase B to Δ Di 4S, and was completely resistant to the action of the Chase AC.

The rate of this reaction was much lower than that using chondroitin sulfate B as substrate.

The oligosaccharide which remains in the origin of the chromatogram was degraded only by the Chase AC to "tetrasaccharide", Δ Di 4S and other products. It was completely resistant to the action of the Chase B.

Other distinguishing properties of the chondroitinases. Fig. 4 shows the effect of increasing concentrations of NaCl upon the

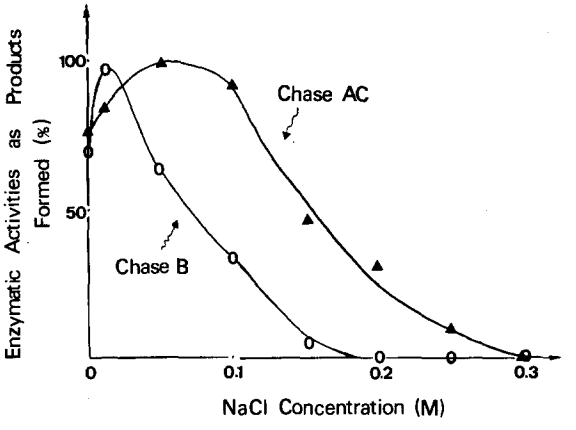

Figure 4. Effect of NaCl concentration upon the chondroitinase activities.

The experiment was performed as described in Fig. 1. O——O Δ Di 4S formed by the action of the Chase B upon chondroitin sulfate B. ▲——▲ Δ Di 4S formed from chondroitin sulfate A by the action of the chondroitinase AC.

activities of the Chase B and Chase AC. At 0.1 M NaCl concentration the Chase B shows a 70% inhibition whereas the Chase AC is practically unaffected by this salt concentration. Table II shows the effect of ions upon the Chase B and Chase AC. Chase B is strongly inhibited by Co^{2+} ions whereas Chase AC is not inhibited by this ion. Conversely, only Chase AC is inhibited by Ca^{2+} and Mg^{2+}. Fe^{3+}, Ba^{2+}, Mn^{2+} inhibit the activity of both enzymes.

TABLE II

Effect of ions upon the chondroitinases

ION	CHONDROITINASE ACTIVITY %	
10^{-2} M	CHASE AC	CHASE B
none	100	100
Na^{+}	100	82
K^{+}	108	110
Ca^{2+}	65	105
Ba^{2+}	24	18
Mg^{2+}	49	108
Mn^{2+}	35	29
Co^{2+}	106	10
Fe^{3+}	1	1

The experiments were performed as described in Fig. 3, except that 10^{-2} M ions were added.

DISCUSSION: The results reported in this paper strongly indicate that a chondroitinase B is induced in F. heparinum when the cells are grown in the presence of chondroitin sulfates A and/or B and/or C. Induced heparinase and chondroitinase systems seem to be completely independent from each other. The question whether a chondroitinase ABC is concomitantly induced by the chondroitin sulfates as we have previously suggested (7) is unanswered in the present paper. Different methods of isolation were used for the characterization of the Chase B now reported. Thus, it is possible that the activity of the chondroitinase ABC was lost during the purification procedure. The production of oligosaccharides from chondroitin sulfate B by the action of the Chase B and their susceptibility only to Chase AC confirms the earlier suggestion (9) of a hybrid nature for chondroitin sulfate B.

This new Chase B will possibly permit to calculate the percent amounts of the linkages between the two types of disaccharide repeating units within the molecule.

ACKNOWLEDGEMENTS: Aided by grants from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), CNPq (Conselho Nacional de Pesquisas), and FINEPE (Financiadora de Projetos). The authors want to express their gratitude to Dr. S.M.C. Dietrich for help in the preparation of the manuscript.

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