

Preparation and purification of *Flavobacterium heparinum* chondroitinases AC and B by hydrophobic interaction chromatography

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Abstract

Flavobacterium heparinum is a soil bacterium that produces several mucopolysaccharidases such as heparinase, heparitinases I and II, and chondroitinases AC, B, C and ABC. The purpose of the present study was to optimize the preparation of *F. heparinum* chondroitinases, which are very useful tools for the identification and structural characterization of chondroitin and dermatan sulfates. We observed that during the routine procedure for cell disruption (ultrasound, 100 kHz, 5 min) some of the chondroitinase B activity was lost. Using milder conditions (2 min), most of the chondroitinase B and AC protein was solubilized and the enzyme activities were preserved. Tryptic soy broth without glucose was the best culture medium both for bacterial growth and enzyme induction. Chondroitinases AC and B were separated from each other and also from glucuronidases and sulfatases by hydrophobic interaction chromatography on HP Phenyl-Sepharose. A rapid method for screening of the column fractions was also developed based on the metachromatic shift of the color of dimethylmethylene blue.

Key words

- Chondroitinases
- Mucopolysaccharidases
- Dermatan sulfate
- Chondroitin sulfate

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Chondroitin and dermatan sulfates are the most common glycosaminoglycans in extracellular matrix proteoglycans (1). Chondroitin sulfate is a heteropolysaccharide made up largely of repeating disaccharide units, in which one sugar is *N*-acetyl-D-galactosamine and the other is D-glucuronic acid. The disaccharides can be sulfated at the 4 or 6 position of *N*-acetylgalactosamine. Dermatan sulfate is an isomer of chondroitin 4-sulfate in which some of the D-glucuronic

acid residues are replaced by L-iduronic acid.

Enzymatic methods have become increasingly important for the determination of the polysaccharide structure and are preferable to chemical methods. Two classes of enzymes act on glycosaminoglycans: prokaryotic lyases, which depolymerize glycosaminoglycans by an elimination mechanism (2) and eukaryotic enzymes, which act by hydrolysis (3,4).

Flavobacterium heparinum produces four

chondroitinases which form unsaturated disaccharides from dermatan and chondroitin sulfates, i.e., chondroitinases AC (5), B (6), C (7) and ABC (8). Using chondroitinases AC and C, we have shown that cartilage chondroitin sulfate contains both 4-sulfated and 6-sulfated disaccharide units in variable proportions, depending on the species and age (9,10) and using chondroitinases B and AC we demonstrated that dermatan sulfates of different origins contain glucuronic and iduronic acid in different proportions and at variable positions (11).

These chondroitinases were isolated by agarose gel electrophoresis (6,7) or by column chromatography procedures based on ion exchange (12), gel permeation (8), adsorption on hydroxyapatite (13), or affinity chromatography (14). However, agarose gel electrophoresis is very time consuming and provides pure enzymes in low yields whereas poor reproducibility was obtained for column chromatography, especially for hydroxyapatite and ion exchange media.

Here we describe improved conditions for bacterial growth and cell disruption, a reproducible scheme for purification of *F. heparinum* chondroitinases AC and B, as well as a rapid assay for chondroitinase activities based on decrease of metachromatic shift of the color of dimethylmethylene blue.

The bacteria were maintained and grown as previously described (7). For batch culture, three culture media were used, with or without the addition of chondroitin sulfate as an inducer (150 mg/l): meat peptone (10 g/l); tryptic soy broth (17 g/l casein peptone, 3 g/l soy peptone, 2.5 g/l glucose, 5 g/l NaCl, 2.5 g/l K_2HPO_4), and tryptic soy broth without glucose (17 g/l casein peptone, 3 g/l soy peptone, 5 g/l NaCl, 2.5 g/l K_2HPO_4). The presence of chondroitin sulfate or glucose did not significantly affect bacterial growth rate. However, *F. heparinum* grew to a higher cell density in tryptic soy broth than in meat peptone. Furthermore, in peptone with or without the inducer, high specific activities

for chondroitinases were obtained during the initial 6 h, when the cell densities were low, but decreased thereafter. In contrast, in tryptic soy broth, high specific activities were obtained after 24 h growth in the presence of the inducer, generating large amounts of chondroitinase-rich extracts.

For the preparation of chondroitinase-containing bacterial extracts, the cells grown in 1 liter glucose-free tryptic soy broth containing 150 mg of chondroitin sulfate were collected by centrifugation, suspended in 5 ml of 50 mM Tris-acetate buffer, pH 7.5, and disrupted by sonication. Our routine procedure for sonication was 100 kHz output, ten 30-s pulses, and 4°C (7). Nevertheless, we now report that under milder conditions (four 30-s pulses) most of the chondroitinases were solubilized and higher specific activities were obtained for chondroitinase B. Cell debris were removed by centrifugation at 100,000 g for 60 min at 4°C and the supernatant thus obtained was maintained at -20°C.

Ammonium sulfate was added to 1 M final concentration to aliquots of crude extracts containing 2 mg of protein (0.5-1 ml). These samples were applied to a high performance (HP) Phenyl-Sepharose column (0.4 x 2.0 cm, 1-ml bed volume) previously equilibrated with 1 M $(NH_4)_2SO_4$ in 50 mM Tris-acetate buffer, pH 7.5. The enzymes were eluted in a stepwise fashion with 1-ml volumes containing decreasing concentrations of $(NH_4)_2SO_4$ in the same buffer (1.00, 1.00, 0.88, 0.75, 0.62, 0.50, 0.38, 0.25, 0.12 M), followed by 10 ml of ammonium sulfate-free buffer at a flow rate of 0.5 ml/min. One-milliliter fractions were collected and analyzed for protein and chondroitinase activities (Figure 1).

Total protein was estimated by the Coomassie blue method (15) and three enzyme assays were carried out to measure the chondroitinase activities as described below.

Dimethylmethylene blue. Samples containing enzyme (10 μ l of fractions eluted from columns or 2 μ l of *F. heparinum* crude

extracts) were incubated with 3 µg of chondroitin sulfate or dermatan sulfate in 96-well plates. After 1-4-h incubation at room temperature, 100 µl of dimethylmethylene blue reagent (16) was added and absorbance was measured at 620 nm. The dye showed a metachromatic shift from blue to purple in the presence of sulfated glycosaminoglycans. This assay permits the determination of chondroitinase activities in both crude extracts and purified enzymes. Chondroitinase AC- and chondroitinase B-containing fractions were pooled. For the determination of purification and recovery, mixtures (200 µl final volume) were prepared containing 200 µg of substrate (chondroitin sulfate or dermatan sulfate) and aliquots of either crude extracts (5 µl) or purified enzymes (100 µl). Incubations were carried out at the optimum temperature of the particular lyase being assayed (37°C for chondroitinase AC and 20°C for chondroitinase B). Aliquots (10 µl) were collected at different times (0 to 120 min), diluted to 100 µl with water and 1 ml of dimethylmethylene blue reagent was added. The changes in absorbance were measured at 525 nm immediately after dimethylmethylene blue addition, and compared to a standard curve of 0 to 10 µg of chondroitin sulfate.

Paper chromatography. The fractions were dialyzed against 50 mM Tris-acetate buffer, pH 7.5, to remove the ammonium sulfate. Aliquots (20 µl) of each dialyzed fraction were incubated with 50 µg of chondroitin sulfate or dermatan sulfate. After overnight incubation at 37°C (for chondroitin sulfate) or 20°C (for dermatan sulfate), the incubation mixtures were submitted to heat inactivation and to paper chromatography as described (7).

Absorbance at 232 nm. The activities of the purified enzymes were also measured by the increase in absorbance at 232 nm as a function of time. Samples (100 µl) were added to cuvettes containing 1 ml of 1 mg/ml chondroitin sulfate or dermatan sulfate in 50

mM Tris-acetate buffer, pH 7.5, under the appropriate temperature conditions. This method proved to be useful for measuring purified chondroitinases, but was ineffective for crude extracts due to the presence of glucuronidases which degraded the unsaturated disaccharides formed.

Figure 1 shows that chondroitin sulfate- and dermatan sulfate-degrading activities were separated by hydrophobic interaction chromatography (HIC) on HP Phenyl-Sepharose. The products formed were also analyzed by paper chromatography (Figure 1B). Fractions 5 to 9 contain chondroitinase AC, which degrades chondroitin sulfate producing unsaturated sulfated disaccharides (Δ Di6S and Δ Di4S) as main products. Fractions 8 and 9 also contain glucuronidase and sulfatase activity, which further degrade the disaccharides to *N*-acetylgalactosamine 6-sulfate (GalNAc6S) and non-sulfated disaccharide (Δ Di0S), respectively. The main products formed from dermatan sulfate were unsaturated 4-sulfated disaccharide (Δ Di4S) and oligosaccharides (fractions 10 to 12), indicating that chondroitinase B did not elute together with glucuronidase or sulfatase. A 2-fold purification with 57% yield was obtained for chondroitinase AC (fractions 5 to 7) and a 3-fold purification with 66% yield was obtained for chondroitinase B (fractions 10 to 12) in this single purification step. Both chondroitinase AC and B preparations were completely free from other mucopolysaccharidases and also from glucuronidases and sulfatases.

For large scale preparation, a 10-ml HP Phenyl-Sepharose column was prepared (1.4 x 6.5 cm) and eluted with a) 1 M $(\text{NH}_4)_2\text{SO}_4$ in Tris-acetate buffer (40 ml), b) a linear gradient of 1 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in Tris-acetate buffer (80 ml), and c) Tris-acetate buffer (40 ml). Two-milliliter fractions were collected at a flow rate of 1 ml/min. The results obtained were similar to those obtained for the 1-ml column (Figure 2).

In conclusion, the chromatographic pro-

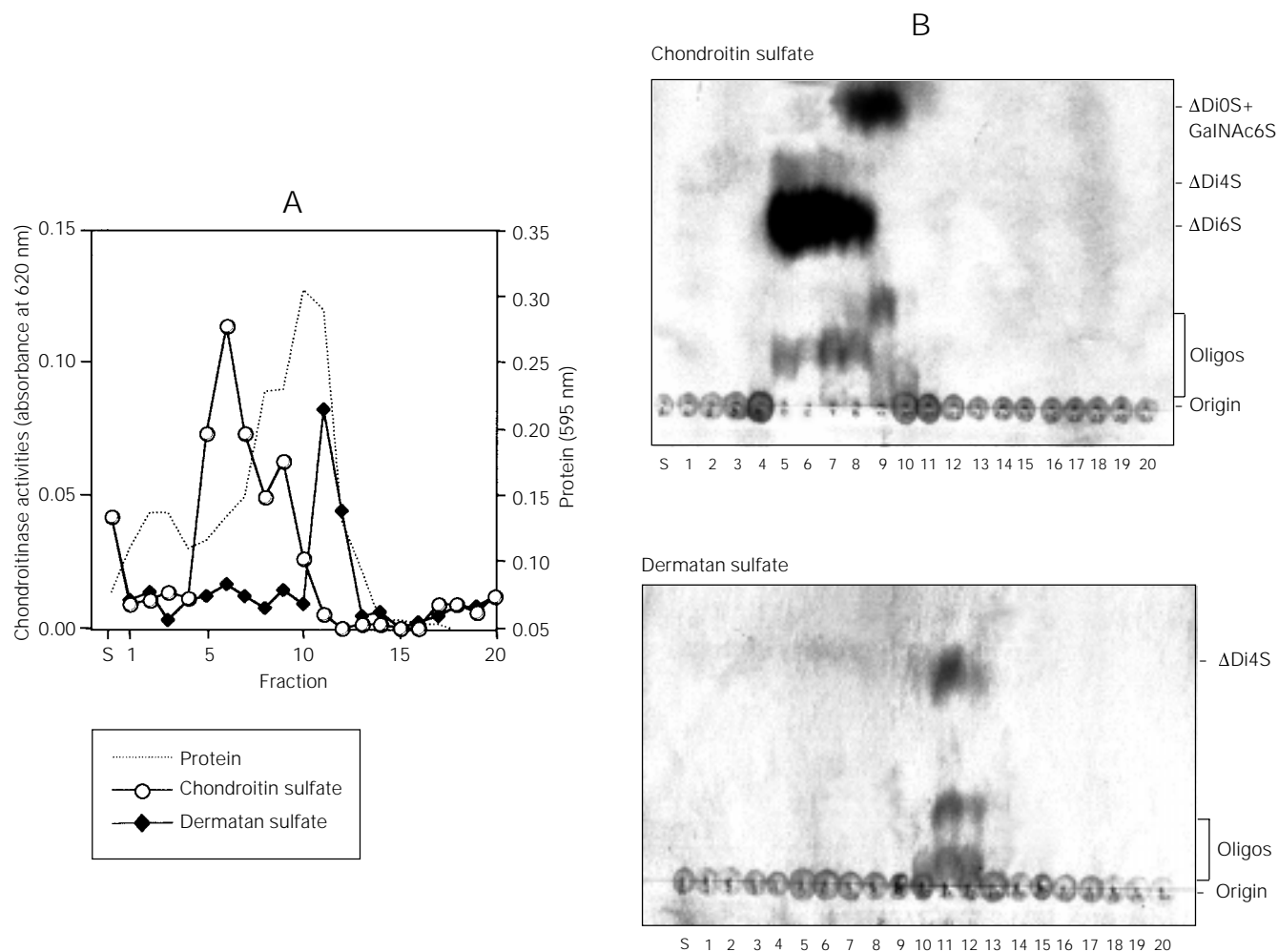


Figure 1 - Fractionation of *F. heparinum* chondroitinases by high performance (HP) Phenyl-Sepharose chromatography. An aliquot of *F. heparinum* extract containing 2 mg of protein, 1 M ammonium sulfate and 50 mM Tris-acetate buffer, pH 7.5, was applied to a 0.4 x 2-cm column of HP Phenyl-Sepharose previously equilibrated with 1 M ammonium sulfate and 50 mM Tris-acetate buffer, pH 7.5. The enzymes were eluted in a stepwise fashion with 1-ml solutions containing decreasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ in the same buffer (1.00, 1.00, 0.88, 0.75, 0.62, 0.50, 0.38, 0.25, 0.12 M), followed by 10 ml of ammonium sulfate-free buffer, at a flow rate of 0.5 ml/min. One-milliliter fractions were collected and analyzed for protein and chondroitinase activities. Total protein was estimated by the Coomassie blue method (15) and the chondroitinase activities were measured by two methods. A, Dimethylmethylene blue color shift: 10 μ l of each fraction was incubated with 3 μ g of chondroitin sulfate or dermatan sulfate in 96-well plates. After 4-h incubation at room temperature, 100 μ l of dimethylmethylene blue reagent (16) was added and the absorbance was measured at 620 nm. B, Paper chromatography: the fractions were dialyzed against 50 mM Tris-acetate buffer, pH 7.5, to remove the ammonium sulfate. Aliquots (20 μ l) of each dialyzed fraction were incubated with 50 μ g of chondroitin sulfate or dermatan sulfate. After overnight incubation at 37°C (for chondroitin sulfate) or 20°C (for dermatan sulfate), the incubation mixtures were submitted to heat inactivation and paper chromatography as previously described (7). Δ DiOS: Unsaturated non-sulfated disaccharide; GalNAc6S: N-acetylgalactosamine 6-sulfate; Δ Di4S: unsaturated 4-sulfated disaccharide; Δ Di6S: unsaturated 6-sulfated disaccharide; Oligos: oligosaccharides.

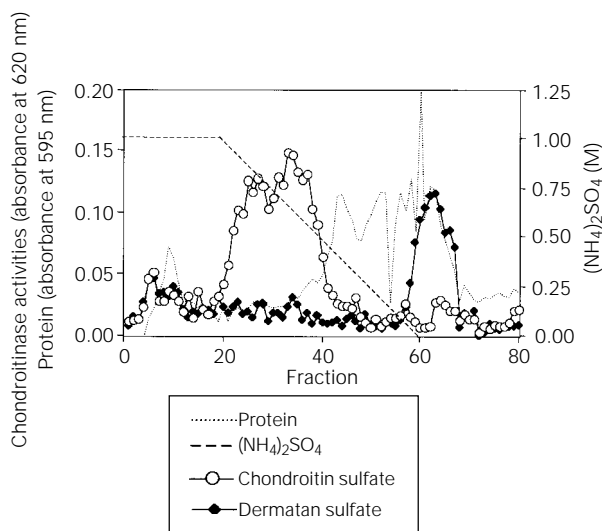


Figure 2 - Large scale preparation of *F. heparinum* chondroitinases by high performance (HP) Phenyl-Sepharose chromatography. An *F. heparinum* extract containing 20 mg of protein, 1 M ammonium sulfate and 50 mM Tris-acetate buffer, pH 7.5, was applied to a 1.4 x 6.5-cm column of HP Phenyl-Sepharose previously equilibrated with 1 M ammonium sulfate and 50 mM Tris-acetate buffer, pH 7.5. The enzymes were eluted with 1 M (NH₄)₂SO₄ in Tris-acetate buffer (40 ml), a linear descending gradient of 1 to 0 M (NH₄)₂SO₄ in Tris-acetate buffer (80 ml) and Tris-acetate buffer (40 ml). Two-milliliter fractions were collected at a flow rate of 1 ml/min. Total protein and chondroitinase activities were estimated as described in Figure 1 (Method 1).

cedure described here based on hydrophobic interaction chromatography on HP Phenyl-Sepharose eluted with decreasing concentrations of ammonium sulfate is suitable for both analytical scale and large scale preparation of *Flavobacterium heparinum* chondroitinase AC and chondroitinase B. The chondroitinases were not inhibited by ammonium sulfate and the fractions eluted from

the columns could be rapidly assayed by the decrease in the dimethylmethylene blue color shift upon depolymerization of chondroitin sulfate or dermatan sulfate. Prior to the identification of the products by paper chromatography, the ammonium sulfate had to be removed from the enzyme-containing solutions by dialysis.

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