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Note

Isoelectric points of the polysaccharide-degrading enzymes in *Flavobacterium heparinum**

VICTOR C. YANG[†], LORI MORGAN[‡], MARY T. MCCARTY[§], AND ROBERT LANGER[†,‡,∥,¶]

[†]*Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (U.S.A.);* [‡]*Department of Surgery, Children's Hospital Medical Center, Boston, Massachusetts 02115 (U.S.A.),* [§]*Department of Anatomy and Cellular Biology, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, Massachusetts 02111 (U.S.A.); and* [∥] *Whitaker College of Health Sciences, Management and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (U.S.A.)*

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Flavobacterium heparinum contains a variety of important polysaccharide-degrading enzymes including heparinase, heparitinase, chondroitinases, hyaluronidase, sulfatases, and glycuronidases[1–3]. Because of their mode of reaction and substrate specificity, these enzymes have gained increased attention. They were used to identify glycosaminoglycans both quantitatively and qualitatively[4], to investigate microbial pathogenicity[5], to study the structures of proteoglycans[6], and to elucidate roles for glycosaminoglycans in cell morphology, cell attachment, cellular synthetic processes, and cytoskeletal organization[7,8]. Despite the importance of these enzymes, their isoelectric pH (pI) values, an important parameter that governs much of the art of separating and identifying the proteins, were almost unexplored. We report herein the isoelectric points of these proteins determined either by chromatofocusing, or by the newly developed method called "pH-Dependent Binding Analysis"[9]. This information will aid in selecting proper methods and experimental conditions for isolating and purifying each of these enzymes.

EXPERIMENTAL

Heparin (from porcine intestinal mucosa) was purchased from Hepar Industries (Franklin, Ohio 45005). Heparan monosulfate was generously supplied by Dr. Cifonelli of Wyler Children's Hospital, Chicago. The disaccharide trisulfate, *O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-(1→4)-2-deoxy-2-sulfamido-D-glucose 6-sulfate, was prepared from heparin by degradation with

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¶To whom reprint requests should be addressed.

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flavobacterial heparinase according to the procedure of Grant *et al.*[10], and was kindly supplied by Dr. Linhardt of the University of Iowa. Chondroitin 4- and 6-sulfates, dermatan sulfate, hyaluronic acid, and 2-hydroxy-5-nitrophenyl sulfate were obtained from Sigma Chemical Co. (St. Louis, MO 63178); and Polybuffer exchanger PBE 94, Polybuffer 96, QAE-Sephadex A-50, and SP-Sephadex C-50 from Phamarcia Fine Chemicals (Piscataway, NJ 08854). Other chemicals were reagent grade, and water was twice distilled.

The crude extract of *Flavobacterium heparinum* was prepared by the method described previously[11]. The cells were first disrupted by sonication, and then treated with protamine sulfate to remove nucleic acids. Heparinase, heparitinase, and chondroitinases were further purified by hydroxylapatite chromatography as described by Linker and Hovingh[1]. Chromatofocusing was performed on a PBE 94 column (1 × 10 cm) with Polybuffer 96 as the eluent. The column was equilibrated with 0.25M ethanolamine–acetic acid buffer, pH 9.4, and eluted with Polybuffer diluted 1:10 with de-ionized water and adjusted to pH 6.0 with acetic acid. The pH-Dependent Binding Analysis was developed on the basis of the pH-dependent binding affinity of proteins on ion-exchangers for estimating the pI values for the proteins. It was carried out[9] by equilibrating the protein and the ion-exchanger to an identical pH and ionic strength, mixing, and then measuring the amount of unbound protein in the supernatant solution as a function of pH. Heparinase, heparitinase, chondroitinase, and hyaluronidase activities were assayed by quenching the enzyme reaction at various times with 30mM HCl, then measuring the increase in u.v. absorption at 232 nm as described by Linker and Hovingh[1]. For the chondroitinase assay, the reaction was not quenched with acid because chondroitin sulfates and ampholytes coprecipitate under acidic conditions. Sulfatase activities were assayed, by the procedure of Ototani *et al.*[12] with 2-hydroxy-5-nitrophenyl sulfate as the substrate. Glycuronidase activities were monitored[13] by the rate of disappearance of absorption at 232 nm with the disaccharide trisulfate as the substrate. Proteins were monitored by the absorbance at 280 nm. Unless otherwise stated, all the pI measurements were carried out at 4°.

RESULTS AND DISCUSSION

The results of chromatofocusing of heparinase, chondroitinases, and hyaluronidase are shown in Fig. 1. Heparinase activity was eluted with the protein peak emerging at a pI of 8.3 (Fig. 1A). This result is consistent with that determined by isoelectric focusing[11] and by the pH-Dependent Binding Analysis[9]. As shown in Fig. 1B, chondroitinase A, chondroitinase C, and hyaluronidase activities occurred coincidently at a pI of 8.7. It has been reported[3] that chondroitinase isolated from *Flavobacterium heparinum* is chondroitinase AC acting on both chondroitin 4- and 6-sulfates[14,15]. Based upon the analysis of the amount of products formed from chondroitin 4-sulfate and chondroitin 6-sulfate by action of the enzyme, it appears that the enzyme is specific for chondroitin 6-sulfate[14]. Several authors[3,14,15] have

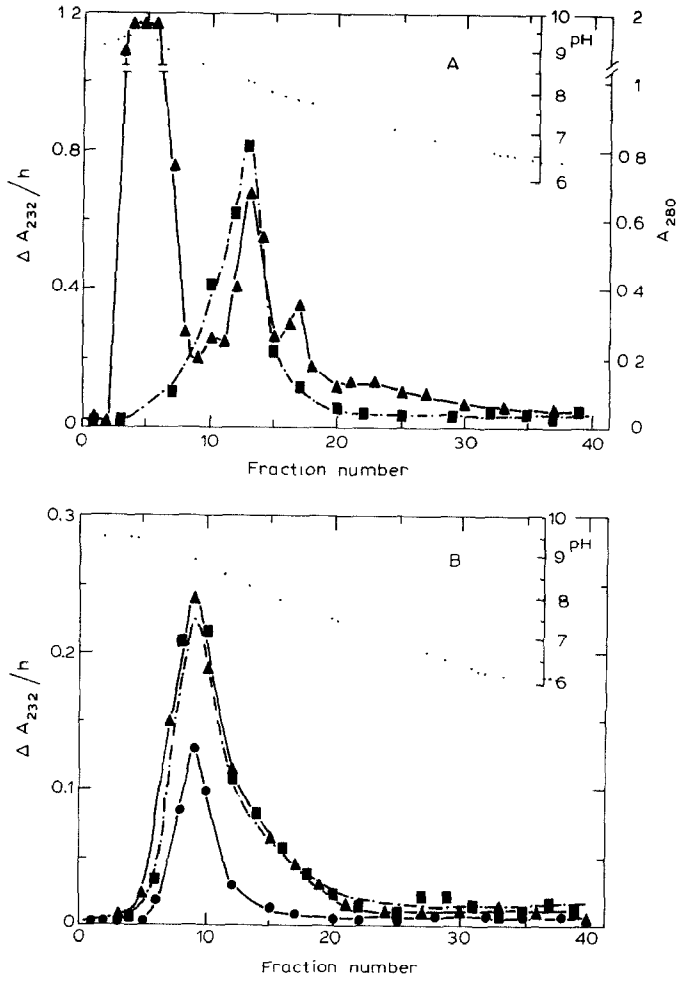

Fig. 1. Chromatofocusing of: (A) Heparinase, and (B) chondroitinases and hyaluronidase. Protein solutions obtained from hydroxylapatite chromatography[1] were concentrated ~30 fold by ultrafiltration through a YM 30 membrane. A sample (3 mL) was applied to the PBE 94 column and eluted with Polybuffer 96-acetic acid (pH 6.0) as described in the Experimental section, 2-mL fractions were collected. (· · · · · ·) pH. (A) (—▲—▲—) Absorbance at 280 nm (protein) and (—■—■—) absorbance at 232 nm (heparinase activity/h). The protein peak (▲—▲) observed at the void volume of the column (fractions 3–8) was composed of protein aggregates formed from the effect of concentration polarization during the ultrafiltration process. (B) Change in adsorbance/h at 232 nm: (—●—●—) Chondroitinase A activity/h, (—▲—▲—), chondroitinase C activity/h, (—■— · — · —■) hyaluronidase activity/h.

also reported the presence of copurification of both chondroitinase and hyaluronidase activities throughout all their preparations of these enzymes from *F. heparinum*. The results shown in Fig. 1 confirm their finding. The coincidence of these enzyme activities may therefore be rationalized in terms of the presence of a single protein, possibly chondroitinase AC, which acts on chondroitin 4- and 6-sulfates as well as hyaluronic acid. No chondroitinase B activity was detected in our

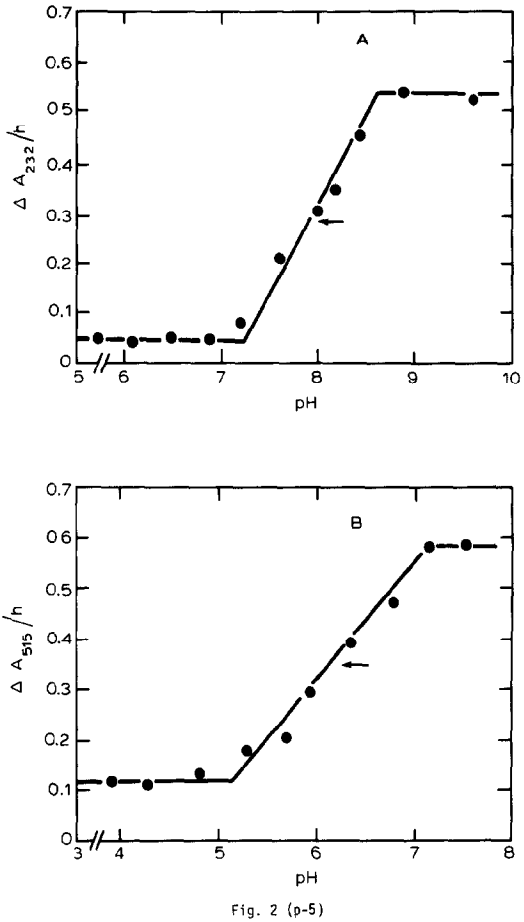

Fig. 2. The pH-dpendent adsorption profile of: (A) Heparitinase, and (B) sulfatases. The pH -Dependent Binding Analysis was performed according to the procedure of Yang and Langer[9]. Data points of this and Fig. 3 were best fitted by lines following a least-squares procedure. The arrow indicates the isoelectric point.

system using dermatan sulfate as the substrate. Although the pI values of heparinase and chondroitinases are very close, they are not attributed to the same protein because heparinase shows no activity on chondroitin sulfates, nor do chondroitinases on heparin[12,15].

The isoelectric points of heparitinase, sulfatases, and glycuronidases were determined by the method of pH-Dependent Binding Analysis, partially because of the simplicity of this method and partially to conserve costly substrates, such as heparan sulfate, and substrates such as disaccharide trisulfate which are not commercially available. Fig. 2 shows the pH-adsorption profiles of heparitinase and sulfatases. The isoelectric point of heparitinase, as determined by the pH at the midpoint of the inflection[9], was 7.9 (Fig. 2A). In the separation of heparitinase and heparinase by cation-exchange chromatography on phosphocellulose, Linker and

Hoving[1] indicated that heparitinase was eluted from the column with a buffer of lower ionic-strength. The different affinities of these two enzymes for the ion-exchanger are due to differences in their charge, and can be accounted for in terms of the difference in their isoelectric points. Heparitinase (pI 7.9) possesses a pI lower than that of heparinase (pI 8.5) (ref. 9) and thus its affinity for phosphocellulose at the pH at which the chromatography is carried out[1] is lower.

The isoelectric point of sulfatase was determined to be pH 6.3 (Fig. 2B). It has been reported that three distinctive sulfatases, including sulfamidase, monosaccharide sulfoesterase, and disaccharide sulfoesterase, are present in the extract of *F. heparinum*[2]. Owing to the non-specificity of the assay currently used, it is not certain which sulfatase the pI 6.3 represents. In fractionating the heparin-degrading enzymes at pH 7.0 by agarose-gel electrophoresis, Dietrich *et al.*[2] showed that sulfamidase and monosaccharide sulfoesterase remained at the point of application on the agarose slide, while disaccharide sulfoesterase migrated fast towards the negative electrode. They also indicated that the two first-named enzymes were relatively insoluble at pH 7.0 in 0.1M ethylenediamineacetate buffer[2]. Since a protein is least soluble, and normally fails to move in the electric field at its isoelectric point, the aforementioned results suggest that sulfamidase and monosaccharide sulfoesterase may have isoelectric points in the vicinity of pH 7. The sulfatase having pI 6.3 may, therefore, represent one of these two enzymes. For a microheterogeneous protein (*i.e.*, the protein exists in several isomeric forms with different isoelectric points), the pH-Dependent Binding Analysis is not capable of resolving the individual pI values when the isoelectric points differ by less than one pH unit[9]. In this case, an inflection covering a broad range of pH would be observed. Fig. 2B indeed shows such a characteristic. The inflection covers nearly two pH units, as compared to less than one pH unit usually observed for proteins devoid

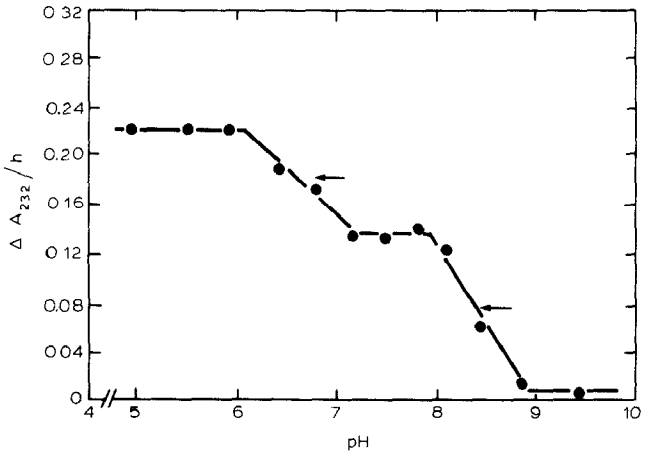

Fig. 3. The pH-dependent adsorption profile of glycxuronidases. The pH-Dependent Binding Analysis was performed according to the procedure of Yang and Longer[9] The arrows indicate the isoelectric point.

of microheterogeneity. Hence, it is possible that sulfamidase and monosaccharide sulfoesterase having different isoelectric points are present and unresolvable by the present method, and the pI value of 6.3 only represents the "weight average" of the pI values of these two enzymes. It is also likely that both sulfatases possess a similar isoelectric pH since gel electrophoresis on 0.9% agarose, which presumably separates proteins primarily on the basis of their charges, has failed in resolving these two sulfatases[2]. However, the pI of disaccharide sulfoesterase was not obtained in this study. This may be due to a low content of the enzyme in the cell extract, to a low sensitivity of the assay method used, or even to the denaturation of the enzyme.

The pH-adsorption profile of glycuronidase is shown in Fig. 3. It contains three plateaus connected by two clearly separate inflections. The two pI values, as obtained from the pH value at the midpoint of each inflection, were 6.6 and 8.3. Dietrich *et al.*[2] showed that two bands, one slow-moving and one fast-moving, which corresponded to the activities of glycuronidase I and II, respectively, moved toward the negative pole in the electric field. The pI 6.6 value may represent Glycuronidase I, while the pI 8.3 value may be related to Glycuronidase II. However, it is not clear why Glycuronidase I migrated toward the negative electrode at pH 7.0 if the enzyme indeed possesses a pI of 6.6 and would be negatively charged at pH 7. One possibility is that the buffer (0.1M ethylenediamineacetate buffer at pH 7) used for agarose-gel electrophoresis changes the electrophoretic mobility of the enzyme, since Dietrich *et al.*[2] showed that different buffer systems alter selectively the degree of migration of some proteins.

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