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Preliminary communication

Chromatofocusing of lysosomal hydrolases from bovine testis

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Chromatofocusing, a new separation method, was first described by Sluyterman *et al.*[1–3]. This method requires a specialized, anion-exchange medium with high, even-buffering capacity over a broad pH range, and an amphoteric buffer with similar characteristics. Suitable ion-exchange media and buffers are commercially available from Pharmacia Fine Chemicals (Piscataway, NJ 08854). For chromatofocusing, the ion-exchange column is equilibrated with a buffer of low ionic-strength and a pH higher than that of the amphoteric buffer. After application of a protein mixture, the column is eluted with the amphoteric buffer, establishing an internal pH gradient that results in desorption of the proteins in the order of their isoelectric points. The present investigation was carried out to determine the usefulness of chromatofocusing for the fractionation of lysosomal acid hydrolases.

A mixture of lysosomal enzymes was prepared from bovine testis as follows[4]: After homogenization of the tissue and three cycles of freezing and thawing, the insoluble material was removed by centrifugation, and the enzymes were precipitated with ammonium sulfate between 20 and 40% saturation. Further purification was accomplished by chromatography on concanavalin A–Sepharose. The material eluted with 0.5M methyl α-D-mannopyranoside in 0.1M citrate buffer (pH 6.0) was concentrated on a YM-10 membrane (Amicon Corporation, Lexington, MA 02173) under nitrogen and stored at −10°. Prior to chromatography, the enzyme preparation (protein content by Bio-Rad Assay, 3.6 mg/mL) was dialyzed overnight against 25mM imidazole–hydrochloric acid buffer, pH 7.4 (100 mL).

A column (1 × 20 cm) containing packed Polybuffer exchanger 94 (14 mL, Pharmacia Fine Chemicals) was prepared as recommended by the manufacturer[5]. The gel was equilibrated with 25 mM imidazole–hydrochloric acid buffer, pH 7.4 (~60 mL), and, immediately before sample application, Polybuffer 74 (4 mL), adjusted to pH 4.0 with 0.1M hydrochloric acid and diluted 1 : 10 with de-ionized water, was run into the gel. The dialyzed enzyme preparation (5 mL) was applied, and the column was eluted (4-mL fractions) with Polybuffer 74, pH 4.0 (200 mL), at a flow rate of 10 mL/h.

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The pH and protein content (Bio-Rad Assay) of the fractions were measured, and the activity of the following enzymes was determined: *N*-acetyl-α-D-glucosaminidase, *N*-acetyl-β-D-hexosaminidase, α-L-fucosidase, β-D-galactosidase, β-D-glucuronidase, α-L-iduronidase, and heparin *N*-sulfate sulfatase. These assays were carried out essentially as described by Hall *et al.*[6], except that heparin *N*-sulfate sulfatase activity was determined by a modification of the method of Thompson[7], and α-L-iduronidase activity according to the method of Rome *et al.*[8]. Substrates were obtained from Sigma Chemical Co. (St. Louis, MO 63178), except for *p*-nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside purchased from Aldrich Chemical Co. (Milwaukee, WI

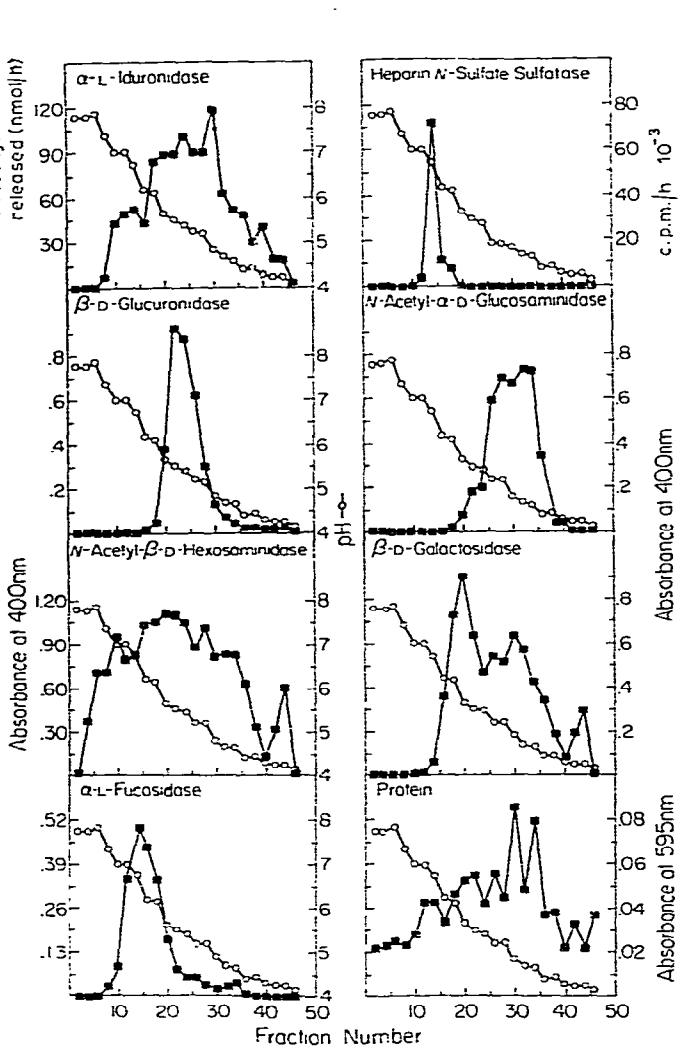

Fig. 1. Chromatofocusing of lysosomal hydrolases.

53233); heparin *N*-[^{35}S]sulfate from Amersham Corp. (Arlington Heights, IL 60005); and (4-methylumbelliferyl α-L-idopyranosyluronic) acid from Research Products International Corp. (Elk Grove, IL 60007). All assay mixtures were incubated at 37° for 3 h except that for *N*-acetyl-α-D-glucosaminidase, which was incubated for 48 h owing to the low activity of this enzyme. Between 5 and 10 μg of protein in a 50-μL sample was used for all assays.

The results of chromatofocusing of the bovine testis preparation are shown in Fig. 1. Heparin *N*-sulfate sulfatase, α-L-fucosidase, and β-D-glucuronidase were eluted from the gel as single peaks having pI's of 6.7, 6.8–6.2, and 5.5, respectively. The elution profiles of the other five enzymes show more than one peak of activity. *N*-Acetyl-α-D-glucosaminidase activity was eluted as a broad peak with indication of two components of approximately equal activity. α-L-Iduronidase gave a heterogeneous pattern partly overlapping with that of β-D-glucuronidase; however, the peaks with the highest and lowest pI's (6.7 and 4.6) were essentially free of this enzyme. β-D-Galactosidase and *N*-acetyl-β-D-hexosaminidase were eluted broadly in three or more peaks. This was expected, since both enzymes have been reported to exist in several isoenzymic forms[9]. With the exception of the pI of heparin *N*-sulfate sulfatase (pH 6.7), which was 2 pH units above that reported for the enzyme from rat spleen, the pI's observed in this study were within 0.5 pH units of previously reported values (Table I).

The chromatofocusing procedure afforded a high recovery of protein (86%), and recovery of enzymic activities ranged from 19% for heparin *N*-sulfate sulfatase to 91% for *N*-acetyl-α-D-glucosaminidase; however, five of the seven enzymes were in the range of 49 to 66% (Table I). This is in contrast to the poor yields obtained for several of these enzymes in our previous fractionations on Cibacron Blue–Sepharose[16].

The purification of β-D-glucuronidase, heparin *N*-sulfate sulfatase, and *N*-acetyl-α-D-glucosaminidase was 1.6-, 1.7-, and 2.6-fold, respectively (calculated for the entire pool of each enzyme). The specific activity of the other four enzymes remained essentially unchanged, and, in these cases, the separation from other proteins was apparently offset by the loss of enzyme activity. However, further improvements of the chromatofocusing procedure are obviously possible, *e.g.*, fractionation over a narrower pH range should give better resolution of some of the enzymes, and, if the substantial loss of heparin *N*-sulfate sulfatase activity can be avoided, chromatofocusing should be especially useful for the purification of this enzyme.

Although a direct comparison between pI values determined by chromatofocusing and other methods has not yet been carried out, it is apparent that the new method will be valuable for such measurements. The results of the present study also demonstrate clearly that chromatofocusing will be a powerful tool, analytical as well as preparative, in continuing investigations of the lysosomal hydrolases.

TABLE I

pI VALUES FROM CHROMATOFOCUSING AND COMPARISON WITH PUBLISHED VALUES FROM ISOELECTRIC FOCUSING

Enzyme	*Activity applied*[a] *(units)*	*Activity recovered (units)*	*Recovery (%)*	*pI Values*	
				From chromatofocusing	*From isoelectric focusing (published)*
α-L-Fucosidase	11.6	6.5	56	6.7–6.1	6.2–5.2[b]
β-D-Galactosidase	74.7	36.9	49	6.1–4.4	4.4[c]
N-Acetyl-α-D-glucosaminidase	0.6	0.55	91	5.2–4.5	5.7–5.1[d]
β-D-Glucuronidase	32.7	18.1	55	5.6–5.2	6.0–5.8[e]
N-Acetyl-β-D-hexosaminidase	157.6	86.9	55	7.8–4.4	4.7[f]
Heparin *N*-sulfate sulfatase[g]	117.0	22.5	19	6.7	4.7[h]
α-L-Iduronidase[i]	3.4	2.2	66	6.7–4.6	[j]

[a] A unit is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol/h. [b] Human placenta[10]. [c] Human liver[11]. [d] Human placenta[12]. [e] Rat liver[13]. [f] Human plasma[14]. [g] A unit is defined as the amount of enzyme that releases 1000 c.p.m. of $^{35}SO_4$/h. [h] Rat spleen[15]. [i] A unit is defined as the amount of enzyme that releases 1 μmol of 4-methylumbelliferone/h. [j] No published value.

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