BBA 65823

ISOLATION OF A NOVEL SULPHATASE FROM RAT LIVER

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(Received June 7th, 1968)

SUMMARY

A lysosomal sulphatase active towards an oligosaccharide sulphate, has been isolated from rat liver and partially purified. The purified system still contained β-glucuronidase, β-*N*-acetylhexosaminidase and arylsulphatase activities. The substrate employed for the assay was isolated from the digestion products obtained from chick embryo chondroitin sulphate A following testicular hyaluronidase digestion. Specificity studies indicated that the system was only active towards relatively high molecular weight substrates, but not towards chondroitin sulphate A itself. Prior cleavage of the nonterminal glucuronosyl residue of the oligosaccharide was apparently essential in the overall hydrolytic process.

INTRODUCTION

It has become increasingly evident in recent years that lysosomal particles play a fundamental role in the degradation of mammalian mucopolysaccharides. Certainly a logical scheme may now be proposed to account for hyaluronic acid digestion solely by enzyme systems resident in the lysosome *viz*, hyaluronidase, β-glucuronidase and β-*N*-acetylhexosaminidase. In order for the chondroitin sulphates to undergo a similar degradation, it becomes necessary to postulate the existence of a hitherto unidentified mammalian carbohydrate sulphatase. Relatively little information concerning the *in vivo* fate of the ester sulphate grouping of the chondroitin sulphates is available. Both DOHLMAN[1] and DZIEWIATKOWSKI[2] have shown that inorganic [^{35}S]sulphate appears in urine following the administration of ^{35}S-labelled chondroitin sulphate A. More recently ARONSON AND DAVIDSON[3] have shown that about 1% of an administered dose of [^{35}S]chondroitin sulphate A (ref. 1) is taken up by rat liver lysosomes within 15 min of the administration and that after 4 days no further radio-activity was detectable in these particles. These results make it tempting to suggest that a full complement of enzymes capable of degrading the chondroitin

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sulphate A molecule resides in the lysosomes. Previous searches for a source of mammalian carbohydrate sulphatases[4] using a variety of substrates has proven unsuccessful, though it has been claimed[5] that a preparation of cerebroside sulphatase from pig kidney lysosomes was also capable of acting on chondroitin sulphates.

The present communication presents evidence for the existence of a carbohydrate sulphatase in lysosomes and indicates a possible mode of chondroitin sulphate A digestion in these particles.

MATERIALS AND EXPERIMENTAL PROCEDURE

Bovine liver β-glucuronidase, phenolphthalein β-glucuronide, *p*-nitrophenyl *N*-acetyl β-glucosaminide and *p*-nitrocatechol sulphate (2-hydroxy-5-nitro-phenylsulphate) were obtained from Sigma Chemical Company. Hyaluronidase was purchased from Worthington Biochemical Corporation.

Isolation and purification of enzyme

Except where otherwise stated, all manipulations were performed at 2°. Large scale isolation of rat liver lysosomes was performed by the technique of TRONET[6] as modified by C. DE DUVE (private communication). Lysosomes isolated from 185 g rat liver were suspended in 0.005 M Tris–0.1% Triton X-100 (pH 7.0), and the volume made up to 20 ml. The suspension was then incubated at 38° for 60 min after which time it was centrifuged at 50 000 × *g* for 30 min and the clear supernatant fluid retained (18 ml).

The lysosomal extract was made up to 50 ml with 0.005 M Tris (pH 7.0), treated with solid $(NH_4)_2SO_4$, and the material precipitating between 30 and 70% saturation was retained. The resulting precipitate was collected, dissolved in 0.005 M Tris (pH 7.0), and dialyzed for 16 h against the same buffer. The resulting solution (5 ml), was treated with 180 mg Cγ alumina gel (4.36% solid) and the suspension stirred for 30 min. The suspended gel was removed by centrifuging and the clear supernatant retained (4.3 ml). The amount of gel added was calculated at 20 mg wet weight gel/10 mg enzyme protein; any higher ratio of gel to protein led to a concomitant adsorption of enzyme activity.

The material resulting from the gel treatment was made up to 50 ml with 0.005 M Tris buffer (pH 7.0), and acetone precooled to − 15° was added slowly with stirring until a concentration of 40% was reached. The suspension was allowed to stand at 0° for 10 min, the precipitate collected by centrifuging then redissolved in buffer. Acetone was removed by dialysis against 0.005 M Tris (pH 7.0). The resulting solution (4.6 ml) was finally stored at − 20°. Under these conditions, enzyme activity was stable for several weeks.

This preparation was still contaminated with β-glucuronidase, aryl sulphatase and *N*-acetylhexosaminidase activities, when assayed for these enzymes under the optimum conditions[7,8]. The purification procedure is summarized in Table I.

Enzyme assays

Standard assay procedures for desulphating activity were carried out by incubating 50 μl substrate, 100 μl enzyme and 10 μl β-glucuronidase (70 units) at 38° for 18 h. The β-glucuronidase was added as a suspension in 1.6 M acetate buffer

TABLE I

PURIFICATION OF LYSOSOMAL SULPHATASE SYSTEM*

(From 185 g liver)

Procedure	*Vol. (ml)*	*Protein (mg)*	*Specific activity (units/mg protein)*	*Yield (%)*	*Purification factor (-fold)*	*Aryl sulphatase**
Whole lysosome suspension	20	180.5	51.2	100	1	1
Soluble lysosome preparation	18	123.3	57.9	77	1.13	1.16
30–70% ammonium sulphate precipitate	5	11.5	113.1	14	2.21	1.84
Cγ alumina gel treatment	4.3	8.2	136.8	12	2.67	1.87
0–40% acetone precipitate	4.6	2.9	382.2	11	7.46	2.03

* Activity of fractions relative to that present in whole lysosome suspension.

(pH 3.7), containing 0.6 μmole $MgCl_2 \cdot 6\ H_2O$, 30 μmoles NaCl and 1 μl Triton X-100. At the end of the incubation period, reactions were arrested by immersing the reaction tubes in a boiling-water bath for 30 sec. Appropriate control determinations were run in which enzyme and substrate were separately incubated and only mixed immediately prior to denaturation. Precipitated protein was removed by centrifuging at 2500 × *g* for 10 min and 30 μl of the clear supernatant subjected to paper electrophoresis on Whatman No. 3 MM paper at 12 V/cm for 2 h in 0.05 M NaCl–HCl (pH 2.0). The criterion used for the demonstration of enzyme activity was the liberation of inorganic sulphate as a product, which was detected on dried electrophoresis strips either by scanning with a Packard Model 7201 Radiochromatogram Scanner or alternatively by cutting the strips into 1-cm wide pieces and counting these in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Specific activities are expressed as number of enzyme units per mg of protein, where 1 unit corresponds to 0.1% hydrolysis of total available substrates.

Protein concentrations were determined by absorption at 280 and 260 mμ.

β-Glucuronidase and β-*N*-acetylglucosaminidase were assayed according to LEVVY AND CONCHIE[7]. The method employed for the assay of arylsulphatase activity was that of DODGSON, SPENCER AND THOMAS[8].

Preparation of substrates

Twenty four 10-day chick embryos were each injected with 800 μC of carrier-free sulphate. After 4 additional days, the embryos were removed and the cartilage dissected out from the long bones. Protein polysaccharide was prepared from this material by the procedure of GERBER, FRANKLIN AND SCHUBERT[9]. The isolated chondromucoprotein was dissolved in 0.05 M acetate buffer (pH 5.0) and digested with testicular hyaluronidase employing 60 units of enzyme per mg of substrate. Digestion was carried out for 192 h with addition of fresh enzyme every 48 h; appropriate precautions were employed to prevent bacterial contamination. Portions (200 μl) of the digest were then streaked on 16 cm × 57 cm sheets of Whatman No. 3 MM chromatography paper and subjected to downward irrigation with isobutyric acid–1 M NH_4OH (100:60, by vol.) for 72 h. Areas of radioactivity were located with

the Packard Radiochromatogram Scanner, and the appropriate areas cut out and eluted with water (50 ml). The water eluates were then applied to a Dowex 1 (Cl^-) column (1 cm × 10 cm), washed with water (30 ml) and then with 1 M LiCl–0.01 M HCl until no more radio-activity appeared in the effluent (40 ml). The eluates were adjusted to pH 6.0 with 10% LiOH, freeze-dried, taken up in the minimal amount of water, and desalted on a column of G-10 Sephadex (3 cm × 104 cm). The Cl^--free eluate was again freeze-dried, taken up in water and finally stored at −20°. By this procedure it was possible to obtain material which was essentially homogeneous when subjected to chromatography as previously described.

A typical chromatographic pattern obtained from a hyaluronidase digest is presented in Fig. 1. In addition to ^{35}S-chondroitin sulphate A, Peaks I–IX were isolated by the procedure outlined above and each subsequently employed as a potential substrate.

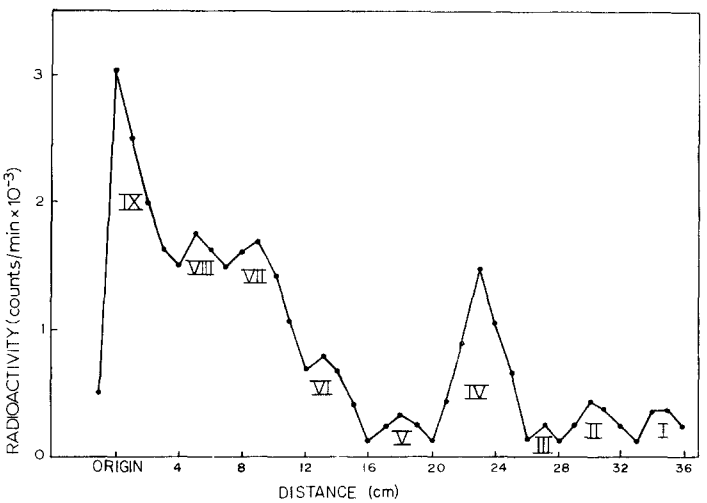

Fig. 1. Chromatographic pattern of sulfated oligosaccharides obtained after descending paper chromatography using isobutyric acid–1 M NH_4OH (100:60, by vol.) as solvent. The tracing is from the radiochromatogram scanner. Individual peaks were isolated as described in the text.

RESULTS

In initial experiments, Peaks I–IX were employed as substrates, using as enzyme sources, nuclear, mitochondrial, lysosomal, microsomal and soluble cell constituent fractions of rat liver, prepared according to TRONET[6]. Assays were conducted at pH values ranging from 3.7–8.0. Peak IX was the only material shown to undergo significant hydrolysis, and this only by the "lysosomal" pellet at pH 3.7. This material was thus routinely used for following desulphating activity. Approx. 20 000 counts/min of substrate were employed in a typical incubation mixture.

Localization of enzyme activity

The finding that enzyme activity was limited to the 250 000 × g·min pellet made it likely that it was associated with the lysosomal particles of the rat liver cell.

Lysosomes were isolated from three rats pre-treated with Triton WR-1339 by the technique previously described[6]. A portion of the lysosome pellet obtained after density gradient centrifugation was set aside for examination with the electron microscope and the remainder assayed for enzyme activity. Desulphating activity was found to be associated with particles which under the electron microscope had the typical appearance of Triton filled lysosomes (Fig. 2).

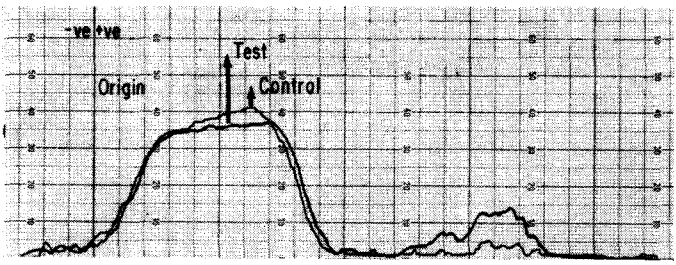

Fig. 2. Radiochromatogram scan of typical assay. The mobility of inorganic sulphate corresponds to the fast moving radioactive material. See text for details.

Demonstration of sulphatase activity

Experiments carried out with relatively impure enzyme preparations indicated that the product obtained as a result of enzyme action did not possess exactly the same mobility as marker inorganic [^{35}S]sulphate on paper electrophoresis. With the more purified enzyme fractions, however, a good correlation in the mobilities of the product and inorganic [^{35}S]sulphate was obtained (Fig. 3). Nevertheless, it was deemed necessary to obtain further confirmatory evidence for the nature of the product of enzyme action.

Three enzyme assays were performed, samples of the incubation mixtures subjected to paper electrophoresis in the usual way and areas of radioactivity located with the Packard Tri-Carb Liquid Scintillation Spctrometer. Those regions corresponding to the product were eluted with water, the combined eluates were made up to 40 ml and the resulting solution was divided into four 10-ml aliquots. Inorganic [^{35}S]sulphate was precipitated from two of the samples by adding 3 ml 1 M HCl, 3 ml 0.075 M Na_2SO_4 and 4 ml 10% $BaCl_2$. To the remaining samples 5 ml of 6 M HCl was added and hydrolysis carried out at 100° for 6 h. Inorganic [^{35}S]sulphate was then precipitated as before. Precipitated $Ba^{35}SO_4$ was collected in the centrifuge, washed with 3 × 30 ml water, 1 × 30 ml acetone and then dried at 100°. Activities were measured in the scintillation counter employing the system described by PATTERSON AND GREENE[10]. Before hydrolysis, the activity of the precipitated $Ba^{35}SO_4$ was 2325 counts/min and after hydrolysis it corresponded to 2262 counts/min. The product was thus concluded to be inorganic sulphate.

Substrate specificity

As previously stated, with the exception of Peak IX, other sulphated degradation products did not undergo desulphation by rat liver preparations. Owing to the very small amounts of material initially available, we were unable to repeat the specificity experiments using the most pure enzyme fraction, employing Peaks I–VI

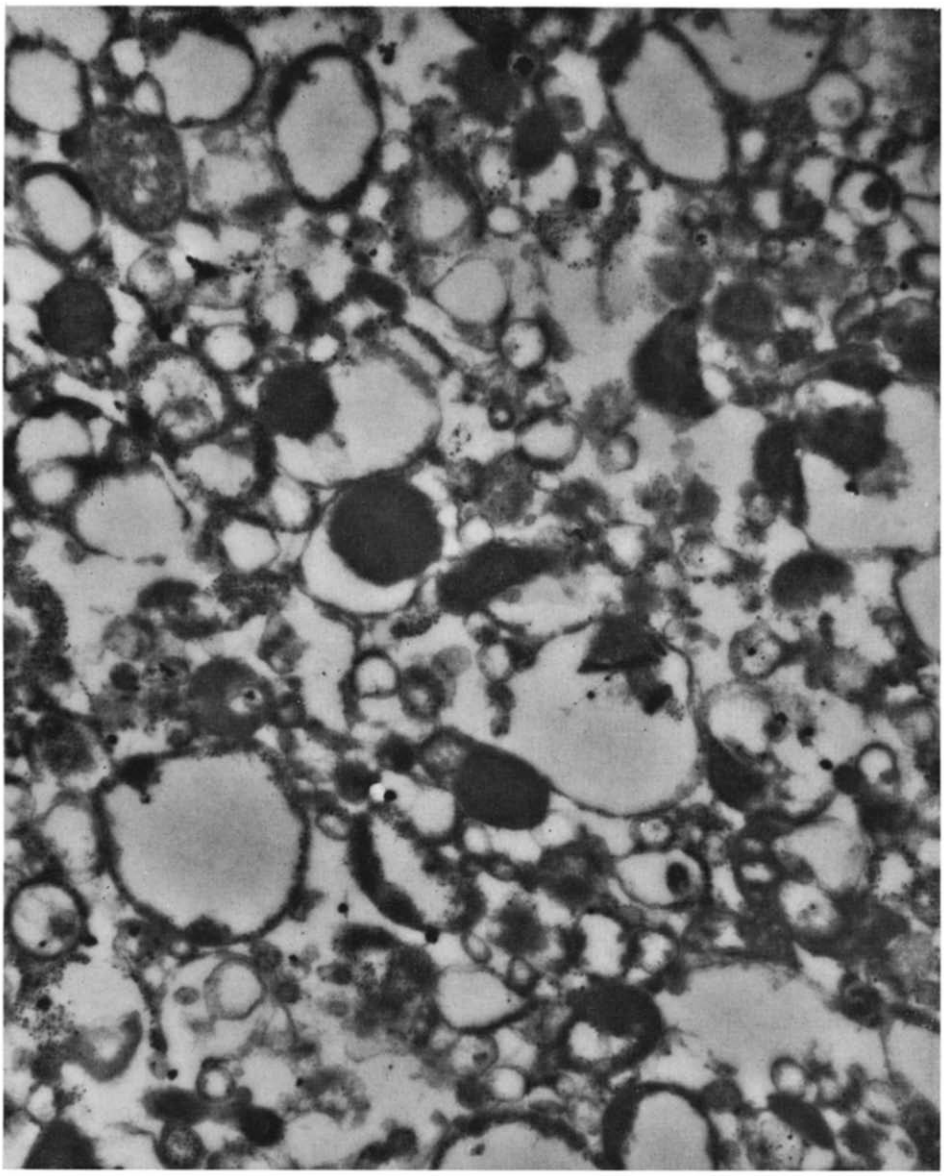

Fig. 3. Electron micrograph of lysosomal pellet obtained after density gradient separation according to the procedure of TRONET[6]. Final magnification is 31 880 ×.

as substrates. It was possible, however, to test the ability of Peaks VII–VIII and a preparation of [^{35}S]chondroitin sulphate A to act as substrates. The radioactivities of substrates employed were comparable to the activity of Peak IX normally used in assay procedure. No activity was observed using Peaks VII and VIII and [^{35}S]-chondroitin sulphate A as substrates.

Inhibition

During assays for enzyme activity β-glucuronidase (70 units) was routinely added, even though β-glucuronidase activity was always detectable in the enzyme preparations. In order to examine the effect of inhibiting β-glucuronidase activity, the specific inhibitor saccharodilactone was employed.

Pre-incubation of the substrate with glucuronidase resulted in the liberation of free glucuronic acid as evidenced by paper chromatography of the incubation mixture. Addition of saccharodilactone (0.001 M final concentration) to a sample treated in this manner, followed by incubation with partially purified sulphatase resulted in 6.8% hydrolysis. This value is comparable to that obtained when saccharodilactone was not present throughout but significantly greater than that found when the inhibitor was present during the initial incubation. Results are summarized in Table II. It should be noted that arylsulphatase activity was also unaffected by the presence of saccharodilactone.

TABLE II

EFFECT OF SACCHARODILACTONE ON SULPHATASE ACTIVITY

Assays were carried out as described in the text utilizing isolated Peak IX as substrate and the 0–40% acetone fraction as enzyme. In Expt. I, saccharodilactone was absent and 70 units of β-glucuronidase were added; in Expt. II, the substrate was pre-incubated with 70 units of β-glucuronidase for 1 h at 37° at which point saccharodilactone (0.001 M final concentration) and sulphatase were added; Expt. III is identical to Expt. II except that the saccharodilactone was added at the start of the incubation; Expt. IV is a control containing no sulphatase.

Expt.	*Hydrolysis (%)*	*Inhibition (%)*
I	7.0	—
II	6.8	3
III	1.5	80
IV	0.1	—

Since Peak IX was the only substrate which was found to be active in the lysosomal system, it was of interest to assay for desulphating activity in the presence of hyalurondiase. The usual incubation mixtures were set up with and without 5 μl of a homogeneous lysosomal hyaluronidase. No desulphating activity was detectable in the presence of hyaluronidase.

DISCUSSION

The present communication records the first demonstration of a mammalian carbohydrate sulphatase. In common with the bacterial chondro-sulphatase, degradation of the polysaccharide chain of the chondroitin sulphates seems to be a prerequisite for sulphatase action[11,4]. The two systems probably differ in their substrate specificity however, since the chondrosulphatase of *Proteus vulgaris* is capable of acting on *N*-acetyl-chondrosine 6-*O*-sulphate[11] whereas the mammalian system was inactive against the disaccharide sulphate ester from chondroitin sulphate A or tetra- and hexasaccharides; the identity of Peak IX, the only degradation product of chondroitin sulphate A acting as a substrate has been placed minimally at the

octasaccharide level (unpublished results). The identity of the true substrates for both the mammalian and bacterial systems is very difficult to establish owing to the problems associated with the isolation of even small amounts of pure high molecular weight materials. However, the failure to demonstrate sulphatase action in the presence of hyaluronidase suggests that the tetra- and hexasaccharides which would be thereby produced from Peak IX are inactive as substrates. One positive observation concerning the nature of the substrate for the mammalian sulphatase may be made on the basis of experiments performed on β-glucuronidase inhibition. From these results it is concluded that the sulphatase acts on a terminal nonreducing *N*-acetylhexosamine 4-*O*-sulphate residue. Since the bacterial chondrosulphatase is also capable of hydrolysing 6-*O*-sulphated residues it is tempting to suggest that the mammalian system has a similar specificity and as such would also be involved in chondroitin 6-sulphate catabolism.

During the purification of the enzyme system only a 7.5-fold increase in specific activity was obtained from purified lysosomes, but it is worthwhile mentioning that close to 1000 × purification results from the mere isolation of lysosomes themselves.

Relatively small amounts of enzyme are in fact present in the liver cell. The most active preparation obtained was only capable of bringing about 7% liberation of ^{35}S as inorganic sulphate. If we consider that an octasaccharide represents the minimal molecular weight of substrate this amount of cleavage would then approximate 30% hydrolysis of available substrate.

In the absence of more data on the system, it is not possible at this stage to define the exact physiological significance of the present findings. It is logical to conclude, however, that these results taken in conjunction with the results of ARONSON AND DAVIDSON[12] suggests that the system is in part responsible for the *in vivo* turnover of the ester sulphate grouping of chondroitin sulphate A.

ACKNOWLEDGEMENTS

This work was supported by Grants A 2903 and A 4315 from the National Institutes of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

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