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THE SULPHATASE OF OX LIVER

XX. THE PREPARATION OF SULPHATASES B1α AND B1β

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Summary

Sulphatases B1α and B1β (EC 3.1.6.1) have been prepared as apparantly homogeneous proteins by chromatography on ConA-Sepharose. Both have a mol. wt. of 56 000, and $E^{1\%}_{280nm}$ of 17 and a turnover number of 8600 min^{-1} with nitrocatechol sulphate as substrate. Their amino acid compositions are identical: like sulphatase A, the sulphatases B are rich in proline and yield glucosamine on hydrolysis. They are not altered by treatment with neuraminidase.

Both fractions show strong UDP-*N*-acetylgalactosamine 4-sulphatase activity, weak iduronate sulphatase activity, but no significant heparan *N*-sulphatase activity. It is suggested that the physiological activity of sulphatase B is that of the *N*-acetylgalactosamine 4-sulphatase which is lacking in the Maroteaux-Lamy Syndrome.

Introduction

The arylsulphatases (EC 3.1.6.1) have recently attracted considerable attention because of their medical importance and because it is evident that their specificities are quite different from that implied by their name. Sulphatase A is a cerebroside sulphatase [1], or better a galactoside 3-sulphatase, and a congenital defect in this enzyme is the cause of the metabolic disease metachromatic leucodystrophy which is characterised by an accumulation of cerebroside sulphate in the body. Sulphatase B is defective in the Maroteaux-Lamy Syndrome (Mucopolysaccharidosis VI) [2,3] and studies of the crude enzyme suggest that it might be a *N*-acetylgalactosamine 4-sulphatase [4]. Although sulphatases A have been obtained from several sources as homogenous proteins [5—7], correspondingly pure preparations of sulphatase B have been obtained

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only from autolysates of ox brain [8]. The purification of sulphatase B from ox liver is made difficult by it occurring in much lesser amounts than sulphatase A and by being present in several different forms which are separable by ion-exchange chromatography. The relationships between these different forms are by no means clear: they may be artefacts produced during the preparation, they may be intermediary forms produced during the biosynthesis of the enzyme [9] or they may truely be individual enzymes. Detailed information is certainly required in view of the suggestions [10], based on a study of multiple sulphatase deficiency, that sulphatases A and B may have a common subunit. Obviously this cannot be studied until the enzymes are available in a state of homogeneity and in some quantity.

The present paper describes the preparation of two forms of sulphatase B from ox liver.

Experimental

Determination of sulphatase B. Sulphatase B was determined as previously described [11] using 0.01 M 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) as substrate in 0.5 M sodium acetate/acetic acid buffer, pH 5.4, and measuring the amount of 4-nitrocatechol liberated during 5 min incubation. One unit of sulphatase B hydrolyses 1 μmol of nitrocatechol sulphate per min at 37° C.

Determination of protein. During the preparation of the enzyme, proteins were determined by the Lowry et al. method [12] using crystalline bovine serum albumin as standard. The concentrations of purified preparations of the enzyme were determined spectrophometrically using an $E^{1\%}_{280\,nm}$ of 17 (see below.

Purification of sulphatase B. Sulphatases B1α and B1β were purified to stage 6 of ref. 11 (these enzymes were called Bα and Bβ therein: see ref. 8) except that protein solutions were concentrated by ultrafiltration through Diaflo UM10 membranes (Amicon Corp., Lexington, U.S.A.). The enzymes from 72 kg of liver were exhaustively dialysed against a buffer (starting buffer) containing 0.02 M Tris, 0.1 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ adjusted to pH 7.4 with HCl.

These solutions were applied at room temperature to columns (20 × 1 cm) of ConA-Sepharose (Pharmacia, Stockholm) in starting buffer and eluted with the same buffer at a rate of 0.1 ml/min. After 40 ml of buffer had passed through the column elution was continued with a linear gradient formed from 75 ml of starting buffer and 75 ml of starting buffer containing 0.4 M methyl β-D-mannoside. The enzymes were eluted in rather sharp peaks after about 50 ml of this gradient had passed through the column, as shown in Fig. 1 for sulphatase B1α. The appropriate fractions were combined, concentrated by ultrafiltration and exhaustively dialysed against starting buffer or 0.1 M Tris · HCl, pH 7.4. The resulting preparations were stable for many months when stored at 5° C.

Amino acid analysis. Samples (approx. 2 mg) of sulphatases B1α and B1β were dialysed exhaustively against 0.14 mM $NaHCO_3$ prior to hydrolysis in vacuo for 22 h at 110° C in 6 M HCl in the presence of an equal weight of phenol; The hydrolysates were analysed under standard conditions using a Beckman 120C analyser. Considerable loss of protein occurred during dialysis under the

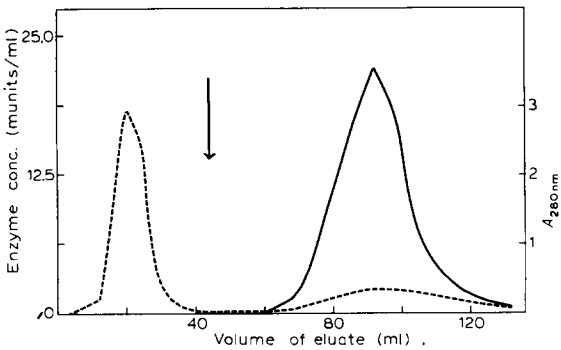


Fig. 1. The elution of sulphatase B1α from a column of ConA-Sepharose with a gradient of from 0 to 0.4 M methyl β-D-mannoside (150 ml, applied at arrow). Full experimental details are given in the text. ———, enzyme activity; - - - - - -, A_{280nm}.

above conditions: this was apparently a result of the low ionic strength because no loss occurred during dialysis at pH 7.4, $I = 0.1$.

Ultracentrifugation. Ultracentrifugation was carried out in a Spinco Model E ultracentrifuge fitted with schlieren or interference optics as appropriate. An-D, An-E and An-H rotors were used at 20° C.

Value of s and $\bar{s}$ were calculated using standard methods. Boundaries were analysed by the method of Baldwin [13]. Molecular weights were determined by the equilibrium method of Yphantis [14]. The value of $\bar{v}$ was taken to be 0.72, the same as that for sulphatase A [15]: the value calculated from the amino acid composition (Table I) was 0.73 and this would be lowered by the carbohydrate known to be present in the enzyme.

Treatment with neuraminidase. A mixture of sulphatases B1α and B1β (30 mg protein, specific activity 2 unit/mg) was treated in a total volume of 4 ml with 500 units of neuraminidase (from *Vibrio cholerae*, Behringwerke, G.F.R.) for 3 h at 37° C and pH 5.7. The reaction mixture was then exhaustively dialysed against an imidazole buffer, pH 6.5, $I = 0.1$ [11].

Results

An approximately 10^4-fold purification of sulphatase B was achieved with a total yield, from the initial extract, of about 10%. The final step, chromatography on ConA-Sepharose gave a 10-fold increase in specific activity in a yield of at least 80%. Sulphatases B1α and B1β were each obtained in a yield of about 0.07 mg/kg liver with specific activities of 130—140 units/mg. Unlike ox brain [8], ox liver contained only small amounts of sulphatase B2, the purification of which was not attempted, and no detectable sulphatase B3.

The concentrations of pure samples of sulphatases B1α and B1β were determined by differential refractometry, using a specific refractive increment of 0.00018 $l \cdot g^{-1}$. On this basis, the values of $E^{1\%}_{280nm}$ were 17 at both pH 7.4 and 9.0 for sulphatases B1α and B1β.

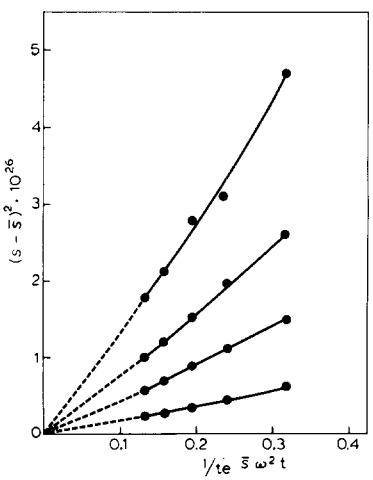
The samples of sulphatases B1α and B1β obtained by the above method were homogeneous with respect to sedimentation coefficient and molecular weight.

Both sedimented as single symmetrical boundaries and there was no significant difference between the values of s for the two fractions. Eight determinations, at protein concentrations between 0.1 and 0.5%, showed no significant variation of s with concentration and gave a mean $s_{20,w}$ of 4.5 S. Boundary analysis confirmed the homogeneity of the preparations with respect to s, as shown in Fig. 2 for sulphatase B1α .

Equilibrium sedimentation showed the homogeneity of the preparations with respect to molecular weight, as shown in Fig. 3, again for sulphatase B1α. The molecular weights (mean ± S.D. for six determinations on three preparations) were 56 700 ± 3600 and 55 300 ± 1400 for sulphatase B1α and B1β respectively, both at pH 7.5, I = 0.1, in diethylbarbiturate buffer. These values are clearly indistinguishable, and a value of 56 000 was used for both fractions.

Chromatography on Sephadex G-100 gave considerably lower estimates for the molecular weight. In Tris · HCl buffer, pH 7.5, I = 0.1, both fractions were indistinguishable and their elution volumes were close to that of ovalbumin, corresponding to a mol. wt. of 47 000. In dilute solution the enzymes were quite strongly adsorbed to Sephadex G-100 and the elution profiles were quite asymmetric, with considerable tailing. The effect could be virtually completely eliminated by having bovine serum albumin (10 mg/ml) present in the solution applied to the column.

The amino acid compositions of sulphatases B1α and B1β were also indistinguishable (Table I) and differed only slightly from those of the sulphatase B of ox brain [9]. Small amounts of glucosamine, 2—3 residues/mol of protein, were



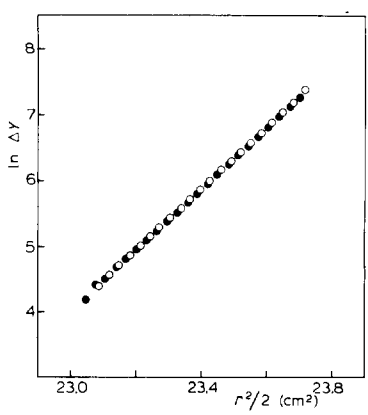


Fig. 2. The time dependence of the apparent distribution of the sedimentation coefficient of sulphatase B1α at pH 7.5 in diethylbarbiturate buffer, I = 0.1. AnE rotor, 3 cm cell, 52 000 rev./min, protein conc. 0.13%. The points show $(s - \bar{s})^2$ as a function of $1/te^{\bar{s}\omega 2t}$ at values of 0.2, 0.4, 0.6 and 0.8 for $g^*(s)/g^*(s)_{max}$. There was no difference between the leading and trailing sides of the pattern and the points are means.

Fig. 3. Fringe displacements in the equilibrium sedimentation of sulphatase B1α at pH 7.5 in diethylbarbiturate buffer, I = 0.1. •, ○; distribution after sedimenting for 10.5 and 11.5 h, respectively at 26 000 rev./min in an AnD rotor.

TABLE I

THE PARTIAL AMINO ACID COMPOSITION OF SULPHATASES B1α and B1β FROM OX LIVER

Values are given in duplicate and are based on a mol. wt. of 56 000. No corrections were made for possible destruction during hydrolysis so that the values for threonine and serine will be low.

	Residues/mol		
	B1α	B1β	Mean
Lys	23.0, 22.3	21.0, 20.4	22
His	18.9, 18.6	19.1, 17.2	18
Arg	31.3, 31.4	31.5, 29.9	31
Asx	52.5, 54.2	52.8, 54.0	53
Thr	32.9, 33.7	33.0, 33.6	33
Ser	27.9, 27.6	26.3, 27.0	27
Glx	44.2, 44.9	43.6, 44.7	44
Pro	44.2, 44.4	44.4, 44.6	44
Gly	45.2, 45.4	43.9, 45.7	45
Ala	32.1, 32.4	32.0, 33.0	32
Val	28.4, 28.8	28.1, 19.4	29
Met	5.5, 4.0	6.0, 4.3	5
Ileu	15.7, 16.5	16.0, 17.1	16
Leu	61.5, 63.2	61.9, 63.0	62
Tyr	22.3, 23.4	24.4, 23.0	23
Phe	19.8, 20.3	20.6, 20.0	20

consistently found in the hydrolysates of sulphatase B. The true content of the amino sugar must be about double this.

When a mixture of sulphatases B1α and B1β (stage 5 of ref. 11) was chromatographed on ConA-Sepharose as described above the enzyme was eluted in a diffuse peak (Fig. 4), quite different from that of the separated fractions (Fig. 1). Rechromatography of the leading and trailing portions of this peak, under the same conditions, gave much sharper peaks of enzyme activity (Fig. 4) and rechromatography of these under standard conditions [11] on CM-Sephadex showed that the leading and trailing portions of the original diffuse peak contained predominantly sulphatases B1α and B1β respectively (Fig. 4). These two forms of sulphatase B can therefore be separated by chromatography on ConA-Sepharose, although much less efficiently than by chromatography on CM-Sephadex, as can be seen by comparing the two elution patterns at the top of Fig. 4.

Treatment of a mixture of sulphatases B1α and B1β with neuraminidase as described in the experimental section did not alter either the proportions of the two enzymes in the mixture nor their elution volumes from CM-Sephadex.

Carbohydrate-sulphatase activities of sulphatase B

Purified samples of sulphatases B1α and B1β, prepared as above, show carbohydrate-sulphatase activities. The results are summarised in Table II. Both fractions show a pronounced UDP-*N*-acetylgalactosamine 4-sulphatase activity (personal communication from Fluharty, A.L.). Although neither shows significant heparan *N*-sulphatase activity [16] (personal communication from Neufeld, E.F. and Hall, C.), both show small amounts of iduronate sulphatase activity with *O*-(α-L-idopyranosyluronic acid 2-sulphate)-(1 → 4)-2,5-anhydro-D-[^{3}H-1]-

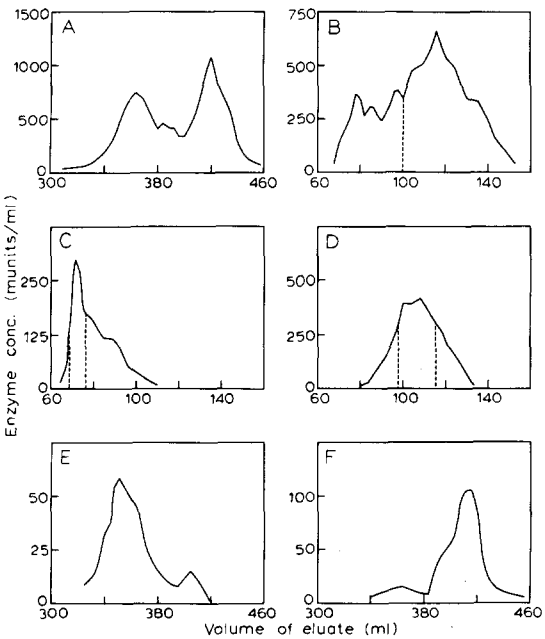


Fig. 4. The chromatography of sulphatases B1α and B1β. A. The separation of sulphatases B1α and B1β on CM-Sephadex as detailed in ref. 11. B. Partial resolution of the mixture on ConA-Sepharose as described in the text. C and D. Rechromatography on ConA-Sepharose of the leading and trailing sides respectively of the elution profile shown in B. E and F. Chromatography on CM-Sephadex (as in A) of the peak tubes from the elution profiles shown in C and D, respectively.

TABLE II

CARBOHYDRATE-SULPHATASE ACTIVITIES OF SULPHATASE B

The activities were determined by the methods named in the text but are converted to standard units to allow comparison. Two values for arylsulphatase activity are given: the first was obtained as described in the text, the second by a similar method but using 30 min incubation at pH 5.8. The major part of the discrepancy between the two sets of results can be accounted for by the different conditions, thus showing that little activity was lost during transport of the enzyme from Canberra to laboratories in the U.S.A.

Type of activity	Time of incubation	Sulphatase activity (μmol · min^{-1} · mg^{-1})	
		B1α	B1β
Arylsulphatase (1)	5 min	17.0	17.5
Arylsulphatase (2)	30 min	6.0	6.3
N-Acetylgalactosamine 4-sulphatase	2 h	0.048	0.051
Iduronate sulphatase	24 h	0.00010	0.00013
Heparan *N*-sulphatase	4 h	0	0

mannitol 6-sulphate as substrate [17] (personal communication from Neufeld, E.F. and Liebaers, I.).

Discussion

Sulphatases B1α and B1β have been isolated from ox liver in a yield of about 0.07 mg/kg of liver: this low yield reflects the low concentration in the tissue

[18] rather than loss during preparation. Both enzymes are homogeneous with respect to sedimentation coefficient and molecular weight. The latter, determined by equilibrium sedimentation, is 56 000, similar to that of the sulphatases B of ox brain [8] but considerably greater than the value of 45 000 previously reported [11] for the liver enzymes. This lower value was determined by gel filtration and it has been confirmed in the present work. The sulphatases B of ox brain [8] and human liver [19] also gave lower molecular weights by gel filtration. The reason for the discrepancy probably lies in the adsorption of sulphatase B to Sephadex giving falsely high elution volumes. Some properties of the sulphatase B of ox liver are summarised in Table III along with corresponding values for ox liver sulphatase A, and for the sulphatase B of ox brain [8] and human liver [19]. Some of the parameters of the latter enzyme are surprisingly different from those of the other sulphatases B.

Sulphatase B, like sulphatase A [15], contains rather large amounts of proline (Table I). Relative to sulphatase A, it contains a higher proportion of basic amino acids, accounting for its higher isoelectric point [11], and of tyrosine, accounting for its higher $E^{1\%}_{280\,nm}$. Sulphatase B is, like sulphatase A [20], a glycoprotein yielding glucosamine on hydrolysis: unfortunately, scarcity of material has prevented a complete analysis of its carbohydrate content. However, the fact that the ion-exchange chromatographic behaviour of neither sulphatase B1α nor B1β is altered by treatment with neuraminidase suggests that terminal sialic acid residues are lacking. As these two sulphatases are partially resolved by chromatography on ConA-Sepharose it is possible, but by no means certain, that they may differ in their carbohydrate content.

The data in Table II show that both sulphatases B1α and B1β have pronounced UDP-*N*-acetylgalactosamine 4-sulphatase activity. The ratio of this activity to the arylsulphatase activity is similar to that of a purified preparation of sulphatase B from human placenta (personal communication from Fluharty, A.L.)

TABLE III

SOME PROPERTIES OF SULPHATASES A AND B OF OX LIVER

Sulphatases B1α and B1β do not differ in the parameters listed. The turnover numbers were determined at 37°C with nitrocatechol sulphate as substrate: sulphatase A, pH 5.0, 0.1 M NaCl; sulphatase B, pH 5.4, 0.5 M acetate. The values for the enzymes from ox brain and human liver are given for comparison and were taken from refs. 8 and 19, respectively.

	Sulphatase			
	A ox liver	B ox liver	B ox brain	B human liver
$s^0_{20,w}$ (S)	6.4	4.5	4.4	2.5
Mol.wt. (equilibrium sedimentation)	107 000	56 000	60 000	60 000
(gel filtration	—	47 000	45 000	50 000
pI	3.4	8.3 *	—	—
$E^{1\%}_{280\,nm}$	7.0	17	17	0.98
Turnover no. (min^{-1})	22 600	8 600	5 800	5 600 **

* Isoelectric focussing [11].

** Calculated from data in ref. 19.

and the findings therefore support the suggestion [4] that UDP-*N*-acetylgalactosamine 4-sulphatase and sulphatase B activities are due to a single protein. The former must be related to the physiological activity of sulphatase B. The iduronate sulphatase activities are slight but may nevertheless be significant: this could only be decided after further, and more concentrated, preparations of sulphatase B became available. The view that sulphatase B and iduronate sulphatase are separate entities is supported by the fact that these activities are deficient in two distinct inborn errors of metabolism, the Maroteaux-Lamy Syndrome [2,3] and the Hunter Syndrome [16] respectively. As stated above, sulphatases B1α and B1β showed no significant heparan *N*-sulphatase activity.

No light is shed by this work on the nature of the multiple forms of sulphatase B. As reported, this method of preparation yields only sulphatases B1α and B1β whereas a similar preparation from an autolysate of ox brain gives at least seven fractions [8]. This could mean that changes have occurred during autolysis but Eto et al. [21] have noted that the sulphatase B of human brain is more heterogeneous than that of human liver so that the multiplicity might be a characteristic of the tissue rather than of the method of preparation. On the other hand, the recent studies of Agogbua and Wynn [19] have detected only one sulphatase B in human liver under conditions where autolysis might have been expected to be severe. Attempts to prepare sulphatase B from autolysates of liver were not successful and, whatever the result, interpretation would have been difficult because it could not be assumed that autolysis would follow the same course in brain and in liver. The possibility should not be discounted that the different sulphatases B may be different enzymes and that their arylsulphatase activities might simply reflect some quite different type of sulphatase activity. They might, in fact, be the as yet unstudied sulphatases at present known only as enzymes which are defective in certain mucopolysaccharidoses [22]. If this be the case, then a tissue specificity is not unexpected.

Acknowledgements

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