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The minor anionic form of arylsulphatase B (arylsulphatase Bm) of monkey brain. Purification and phosphoprotein nature

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Abstract. The anionic form of arylsulphatase B (arylsulphatase Bm) was purified to apparent homogeneity from monkey brain through steps involving chromatography on diethylaminoethyl-cellulose, Blue-Sepharose, Biogel HTP and finally Biogel P-300 gel filtration. The molecular weight of the purified enzyme as deduced by gel filtration on Biogel P-300 and by sodium dodecylsulphate gel electrophoresis was ~ 30,000. *Escherichia coli* alkaline phosphatase treatment of arylsulphatase Bm resulted in the conversion of upto 84% of the enzyme into a less charged form of enzyme, that could not bind to diethylaminoethyl cellulose. Potassium phosphate an inhibitor of alkaline phosphatase prevented this conversion. Upon acid hydrolysis the purified enzyme yielded approximately 7·0 mol of inorganic phosphate per mol of protein. *Vibrio cholerae* neuraminidase treatment did not alter the charge on arylsulphatase Bm.

Keywords. Arylsulphatase Bm; monkey brain; purification; phosphoprotein.

Introduction

The importance of arylsulphatases A and B stems from their absence in the human genetic disorders metachromatic leukodystrophy and Maroteaux-Lamy syndrome (Austin *et al.,* 1963; Fluharty *et al.,* 1975). The physiological substrates for arylsulphatases A and B were identified as cerebroside sulphate and UDP-N acetyl galactosamine-4-sulphate respectively. A minor anionic form of arylsulphatase B termed arylsulphatase Bm which is found in the primate brain and which shows many properties of arylsulphatase B is of particular interest for the following reasons (i) It is found in a phosphorylated form only in the primate (human and monkey) brain but not in the brain of other species like rat, chicken or rabbit (J. Mathew and A. S. Balasubramanian, unpublished data), (ii) It is not found in the liver of primates (Stevens *et al.,* 1977; J. Mathew and A. S. Balasubramanian, unpublished data) and (iii)An anionic form of arylsulphatase B termed arylsulphatase B_1 found in transplantable tumours of athymic mice has been reported to be both phosphorylated and sialylated (Gasa *et al.,* 1981).

Arylsulphatase Bm has not been purified and characterized so far. We report here its purification from monkey brain and some of its characteristics which suggest that it is a phosphoprotein.

Abbreviations used: DEAE, Diethylaminoethyl; SDS, sodium dodecyl sulphate.

Materials and methods

Blue-Sepharose was prepared by coupling Cibacron Blue F3GA (Ciba Geigy) to Sepharose 6B (Ahmad *et al*., 1977). Biogel HTP and Biogel P-300 were from BioRad, USA, sodium metaperiodate from J. T. Baker, Phillipsburg, New Jersey, USA, and *Escherichia coli* alkaline phosphatase (Type III) from Sigma Chemicals, St. Louis, Missouri, USA. All other chemicals were obtained as described earlier (Lakshmi and Balasubramanian, 1980).

Purification of the enzyme

Unless otherwise mentioned all operations were done at 0-4°C. Monkey (*Macaca radiata*) brain (70g) kept frozen at – 18°C was thawed and homogenized with 5 volumes of 20 mM Tris-HCl, buffer pH 7·4 in a Waring blendor and centrifuged at 12000 g for 30 min. The supernatant was dialysed against 100 volumes of the same buffer for 24 h.

Diethyl aminoethyl (*DEAE*) *-Cellulose ion exchange chromatography*

The dialysed supernatant was loaded on a column packed with precycled DEAE-cellulose (26×4·5 cm), washed with 20 mM Tris-HCl buffer, pH 7·4 and eluted with a linear gradient of 0–0·2 M NaCl in the same buffer. Fractions of 25 ml were collected. By this method arylsulphatase B, Bm and A were completely separated from each other (Lakshmi and Balasubramanian, 1980). The active fractions of arylsulphatase Bm were pooled and dialysed against 100 volumes of 20 mM Tris-acetate buffer pH 6·5.

Blue Sepharose affinity chromatography

The dialysed fraction of arylsulphatase Bm from DEAE-cellulose column was loaded on a Blue-Sepharose column (8·2×2·8 cm) equilibrated with 20 mM Tris-acetate buffer pH 6·5, washed with the same buffer and with 20 mM Tris-HCl buffer pH 7·4.The enzyme was eluted with 20 mM Tris-HCl buffer pH 7·4/0·25 M NaCl. Fractions of 5 ml were collected and the active fractions were pooled.

Hydroxyapatite chromatography

The pooled fractions of arylsulphatase Bm from the previous step was dialysed against 10 mM Tris-acetate buffer pH 6·5 and loaded on a Biogel HTP column (6·5×2·0 cm) equilibrated with the same buffer. The column was initially washed with the loading buffer, then with 0·1 M potassium phosphate buffer pH 7·5 and the enzyme eluted with 0·25 M potassium phosphate buffer pH 7·5. The eluate (60 ml) was dialysed for 2 h against 120 volumes of 20 mM Tris-HCl buffer pH 7·4 with one change. The enzyme concentrated against Aquacide II to 1·5 ml was redialysed exhaustively against the same buffer to remove the phosphate completely.

Gel filtration on Biogel P-300

The concentrated enzyme from the previous step was layered on a Biogel P-300 column (26·5×2·1 cm) equilibrated with 20mM Tris-HCl buffer pH 7·4/0·15 M NaCl. Fractions of 2 ml were collected at a flow rate of 8 ml/h. The active fractions were pooled, concentrated against Aquacide II and dialysed against 20 mM Tris-HCl buffer pH 7·4.

Polyacrylamide gel electrophoresis

The concentrated eluate (12 μg) from Biogel P-300 column was subjected to Polyacrylamide gel electrophoresis under non-denaturing conditions in the Davis system (Davis, 1964) as described earlier (Lakshmi and Balasubramanian, 1980). The protein was stained with Coommassie Brilliant Blue G250 according to the method of Holbrook and Leaver (1976).

Molecular weight determination by sodium dodecyl sulphate (*SDS*) *gel electrophoresis and gel filtration on Biogel P-300*

Twenty μg of the purified enzyme containing 2% (w/v) SDS with 5% (v/v) 2-mercaptoethanol was boiled for 2 min and subjected to gel electrophoresis according to the method of Laemmli (1970). The electrophoretic mobility was compared against standards of known molecular weights—human IgG (150,000), bovine serum albumin (62,000), ovalbumin (43,000) and soybean trypsin inhibitor (21,000).

The molecular weight of the enzyme was also determined by gel filtration on Biogel P-300 by comparing the Ve/Vo values with those of the standard marker proteins used in the SDS gel electrophoresis.

Treatment with E. coli alkaline phosphatase

E. coli alkaline phosphatase treatment of the Bm enzyme (obtained after the Blue-Sepharose chromatography step) was carried out as follows. The incubation mixture in a final volume of 2·0 ml contained the enzyme protein, 10 mM $MgCl_2$,100 mM Tris-acetate buffer pH 8·0 and *E. coli* alkaline phosphatase (2 units/mg protein). After incubation for 3 h or 5 h at 37°C, the mixture was dialysed against 100 volumes of 20 mM Tris-HCl buffer pH 7·4 with 4 changes and loaded on a DEAE-cellulose column (9·8 cm×0·8 cm) equilibrated with the same buffer. The column was washed, eluted with 0·2 M NaCl in the buffer and the fractions were assayed using nitrocatechol sulphate as substrate. In the case of the purified Bm enzyme, the incubation mixture contained 17·4μg protein, 1 unit of alkaline phosphatase, 10 mM $MgCl_2$ and 100 mM Tris-acetate buffer pH 8·0 in a final volume of 10 ml. After an incubation period of 1 h, it was dialysed and passed through a DEAE-cellulose column (7·5 cm×0·8 cm) as before. The fractions were assayed for activity using 4-methyl umbelliferyl sulphate as substrate.

Treatment with Vibrio cholerae neuraminidase

The Blue-Sepharose eluate of arylsulphatase Bm (1.36 mg protein) was incubated with *V. cholerae* neuraminidase (0·1 unit) in the presence of 5 mM $CaCl_2$ and 100 mM sodium-acetate buffer pH 5·5 for 1 h at 37°C. The incubation mixture after dialysis against 20 mM Tris-HCl buffer pH 7·4 was passed through a DEAE-cellulose column and eluted as described for the phosphatase treatment.

Treatment with sodium metaperiodate

The Bm enzyme (DEAE-cellulose fraction, 1·7 mg protein) was concentrated by Aquacide II and dialyzed against 10 mM phosphate buffer, pH 6·0. It was incubated with 10 mM sodium metaperiodate for 2 h at 4°C in the dark. Ethylene glycol (0·1 ml) was added to arrest the reaction and dialysed against 20 mM Tris- HCl buffer pH 7·4. Incubation of the enzyme with periodate for periods longer than 2 h resulted in considerable loss of activity (about 80% loss for 6 h). The mixture was passed through a DEAE-cellulose column and eluted as described for phosphatase treatment.

Acid hydrolysis of purified arylsulphatase Bm

The homogeneous arylsulphatase Bm (29 μg) was hydrolysed with 6 NHCl at 110°C for 8 h in a sealed pyrex hydrolysis tube. After hydrolysis, the sample was dried by repeated evaporation under vacuum over KOH to remove HCl completely and the phosphate liberated was quantitated by the method of Chen *et al.* (1956).

Enzyme assays

The incubation mixture for arylsulphatase Bm contained 7·5 mM nitrocatechol sulphate, 0·2 M sodium-acetate buffer, pH 5·5 and the enzyme in a total volume of 0·2 ml. One unit of enzyme activity corresponds to 1 μmol of nitrocatechol released/h (Lakshmi and Balasubramanian, 1980).

In experiments where 4-methyl umbelliferyl sulphate was used as the substrate, the incubation mixture contained 2·5 mM 4-methyl umbelliferyl sulphate, 0·2 M sodium-acetate buffer, pH 5·5 and the enzyme in a total volume of 0·2 ml. After incubation at 37°C for 4 h, the reaction was stopped by the addition of 1·0 ml of 0·4 M glycine-NaOH buffer, pH 10·5 and the fluorescence of 4-methyl umbelliferone was measured in a Hitachi 204-A fluorescence spectrometer (Stevens *et al.,* 1977).

Protein was measured according to Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Results and discussion

Purification of arylsulphatase Bm

Table 1 shows the purification data of a typical batch of arylsulphatase Bm from monkey brain. The enzyme at the final step of gel filtration on Biogel P-300 was 87 fold

Table 1. Purification of arylsulphatase Bm from monkey brain

Purification step	Total units*	Specific activity (units/mg protein)	Purification fold	Recovery (%)
DEAE-cellulose	83	0·94	—	100
Blue-Sepharose	44	7·0	7	53
Biogel-HTP	12	40·0	42	14
Biogel P-300	10	82·37	87	12

* 1 unit = 1 *μmol* nitrocatechol released/h.

purified with an overall recovery of 11·5% over the DEAE-cellulose fraction. The enzyme moved as a single diffuse band on Polyacrylamide gel electrophoresis under non-denaturing conditions and as a sharp band under denaturing conditions in the presence of SDS. The final yield varied with different batches of purification. This was mainly due to the varying recoveries obtained at the step of Blue-Sepharose chromatography. It should also be mentioned that the final purified enzyme was very labile. Storage of the enzyme at 4°C for 24 h resulted in 90% loss of activity. Addition of 2-mercapto-ethanol or bovine serum albumin did not stabilize the enzyme.

Molecular weight determination

The molecular weight of the purified enzyme by SDS Polyacrylamide gel electrophoresis and by gel filtration on Biogel P-300 was found to be 30,000 and 33,000 respectively. Arylsulphatase B (concentrated DEAE-cellulose fraction) was also found to have a molecular weight comparable to that of arylsulphatase Bm on Biogel P-300 gel filtration.

Treatment with E. coli alkaline phosphatase and V. cholerae neuraminidase

The elution profile of the arylsulphatase Bm enzyme (obtained after Blue-Sepharose chromatography) subjected to *E. coli* alkaline phosphatase treatment is shown in figure 1A. About 76% and 84% of the enzyme was converted to a less acidic presumably dephosphorylated form after 3 h and 5 h of incubation respectively. The near absence of any dephosphorylated enzyme in the control experiments upto 5 h of incubation was suggestive of the absence of any endogenous phosphatase activity. When *E. coli* phosphatase treatment was done in the presence of 100 mM potassium phosphate buffer, pH 8·0 (an inhibitor of alkaline phosphatase) there was no conversion of arylsulphatase Bm into the less charged form (not shown in figure).

About 21% of the purified arylsulphatase Bm was converted into an unbound dephosphorylated form on DEAE-cellulose chromatography after treatment with *E. coli* alkaline phosphatase for 1 h. Longer incubation periods was not possible because the purified Bm enzyme lost activity rapidly at 37°C.

Unlike phosphatase, neuraminidase treatment did not result in the conversion of the arylsulphatase Bm into a less acidic form unbound to DEAE-cellulose column (figure

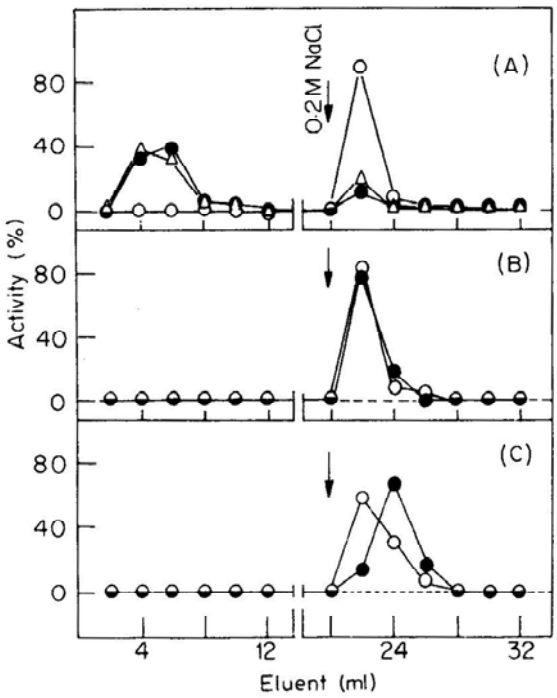

Figure 1. Profile of arylsulphatase Bm on DEAE-cellulose column chromatography when subjected to (A) Phosphatase (B) neuraminidase and (C) periodate treatment. **(A)** Blue Sepharose eluted arylsulphatase Bm incubated with alkaline phosphatase for 3 h (Δ) and 5 h (●); without alkaline phosphatase for 5 h (O). **(B)** Blue Sepharose eluted arylsulphatase Bm incubated with (●) and without (O) neuraminidase for 1 h. **(C)** DEAE-cellulose eluted arylsulphatase Bm incubated with (●) and without (O) sodium metaperiodate at 4°C for 2 h. Details of procedure are given under Materials and methods.

1B). Sodium metaperiodate treatment failed to bring about any significant change in the elution profile of the enzyme on DEAE-cellulose (figure 1C).

The dephosphorylated arylsulphatase Bm showed the same molecular weight as the phosphorylated enzyme on Biogel P-300 gel filtration.

Phosphate content of the purified enzyme

The homogenous enzyme upon acid hydrolysis yielded 7·01 mol of inorganic phosphorus/mol protein. This quantitative estimation of phosphorus confirmed the phosphorylated state of arylsulphatase Bm.

The present studies indicate that arylsulphatase Bm of brain differs from arylsulphatase B_1 of transplantable lung tumours (Gasa *et al.,* 1981) in its molecular weight and in its non-susceptibility to neuraminidase action. Although the phosphorylated nature of purified arylsulphatase Bm has been suggested by the present experiments, the nature of the phosphate residues remains to be determined. Periodate treatment which is known to result in the oxidation of carbohydrate residues failed to

alter the elution profile of arylsulphatase Bm. However this experiment does not completely exclude the presence of sugar phosphate residues (such as mannose-6-phosphate) because the sugar phosphate may still remain attached to the enzyme after periodate treatment. Serine, threonine or tyrosine phosphate residues are known to be present in a number of phosphoproteins. Gasa and Makita (1983) have recently shown the presence of phosphorylated residues on both the protein and carbohydrate moieties in arylsulphatase B_1. Attempts to label the Bm enzyme with ^{32}P and analyze the phosphorylated residues are under way now.

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