Biochimica et Biophysica Acta, 481 (1977) 542—548

BBA 68079

AFFINITY CHROMATOGRAPHIC SEPARATION OF ARYLSULFATASE A AND B USING CIBACRON BLUE-SEPHAROSE

ATEEQ AHMAD *, AVADHESHA SUROLIA ** and B.K. BACHHAWAT **

Neurochemistry Laboratory, Department of Neurological Sciences, Christian Medical College Hospital, Vellore-632004 (India)

(Received September 10th, 1976)

Summary

A simple and rapid affinity chromatographic method is described using Cibacron Blue F3GA-Sepharose for the separation of arylsulfatases A and B (aryl-sulfate sulfohydrolase, EC 3.1.6.1)

Introduction

The arylsulfatase A and B (aryl-sulfate sulfohydrolase, EC 3.1.6.1) enzymes of vertebrates are acid hydrolases and are located in lysosomes. In recent years, these two enzymes have been increasingly employed as marker enzymes for lysosomes in cell fractionation studies [1], and histological work [2]. In these cases the combined activity of the two enzymes is measured. Both enzymes show high activity towards a commonly used chromagenic substrate, namely dipotassium 2-hydroxy-5-nitrophenyl sulfate (nitroatechol sulfate). Baum et al. [3] described a method for the independent assay of arylsulfatase A and B from human urine. This method has also been successfully applied to human tissues and its application led to the discovery of the relationship between metachromatic leukodystrophy and arylsulfatase A [4]. Unfortunately, this method in which activities are measured at a variety of pH values, substrate concentrations and inhibitor concentrations was not found to be practicable to measure the activity of either enzyme from rat tissues [5]. Rat arylsulfatase B in contrast to human arylsulfatase B, still exhibited appreciable activity when measured in the presence of pyrophosphate and chloride ions. Thus the method described by Baum et al. [3] was not useful for rat tissues and precise determination of arylsulfatase A and B requires prior seperation of the enzymes.

* Present address: Department of Pharmaceutics, Central Drug Research Institute, Lucknow 226001, India

** Present address: Indian Institute of Experimental Medicine, Calcutta-32, India.

Moreover, the literature on mammalian arylsulfatase enzymes cites a number of examples of marked species differences between enzymes [6,7]. Studies of Balasubramanian and Bachhawat [8] showed that arylsulfatase B from sheep brain has higher affinity for nitrocatechol sulfate compared to arylsulfatase A from the same source. This difference was striking since arylsulfatase B from ox liver and human brain are shown to have less affinity towards nitrocatechol sulfate in comparison to arylsulfatase A.

Most methods available in the literature, for the separation of arylsulfatase A and B are relatively complex, involving gradient elution from an ion-exchange column or gel filtration chromatography on Sephadex G-200 [9]. For this reason it was found necessary to develop a rapid and simple chromatographic method for the separation of arylsulfatase A and B from various animal tissues.

It has been observed that several proteins that normally enter the internal volume of Sephadex or Sepharose gel beads are totally excluded from the interval volume when chromatographed along with Dextran Blue, in the presence of low ionic strength solvents. A variety of proteins exhibit this behavior such as pyruvate kinase, glutathione reductase and phosphofructokinase [10]. It was found that Dextran Blue forms a complex with each of these proteins, and this complex is dissociable by salt. Earlier work from this laboratory showed that arylsulfatase B forms a complex with Dextran Blue at pH 6.0 in 0.01 M Tris/acetate buffer and this property was used in achieving purification of arylsulfatase B from sheep brain [8]. Recently Cibacron Blue F3GA, a chromophoric group present in Dextran Blue has been covalently attached to Sephadex and Sepharose gels. Several workers have used these dye gels in the purification of enzymes [11]. Easterday and Easterday [12] have utilized these resins for the affinity purification of kinases and dehydrogenases. This prompted us to synthesize Cibacron Blue-Sepharose 6B (Blue-Sepharose) and utilize it in the separation of arylsulfatase A and B from various tissues and different animal species.

The present paper describes a simple and rapid method for the separation of arylsulfatase A and B from different sources. This involves the chromatography of these enzymes on Blue-Sepharose.

Materials

Bovine serum albumin and Nitrocatechol sulfate were obtained from Sigma Chemical Company, U.S.A. Sepharose 6B and Dextran Blue were purchased from Pharmacia, Uppsala, Sweden. The Cibacron Blue F3GA dye was a gift from Ciba-Geigy, Switzerland. All other reagents used were of analytical grade.

Methods

Protein estimation

Protein was estimated according to the method of Lowry et al. [13] using crystalline bovine serum albumin as standard.

Preparation of epichlorohydrin cross-linked Sepharose 6B

Epichlorohydrin cross-linked desulphated Sepharose 6B was prepared

according to the procedure of Porath et al. [14]. 20 g of Sepharose 6B gel was treated with 18 ml of 1 M NaOH and 2 ml of epichlorohydrin at room temperature in presence of 40 mg of sodium borohydride. The suspension was heated to 60° C with stirring and the reaction was stopped, after 2 h. The gel was washed free of alkali with distilled water.

Coupling of Cibacron Blue-F3GA to Sepharose 6B

Blue-Sepharose 6B was synthesized by coupling Cibacron Blue-F3GA to epichlorohydrin cross-linked Sepharose 6B essentially according to the procedure described by Bohme et al. [15].

A solution of 200 mg of Cibacron Blue-F3GA in 50 ml of water was added dropwise with vigorous stirring to a 10 g suspension of epichlorohydrin cross-linked Sepharose 6B in 350 ml of water at a temperature of 60° C. After stirring for 30 min 45 g of NaCl was added and the stirring was continued for 1 h. After that the mixture was heated to 80° C and it was treated with 4 g of Na_2CO_3 and kept for 2 h with stirring at this temperature. After cooling to room temperature the resin was filtered by suction on a Buchner funnel and washed repeatedly at pH 4.5 and 9 with 0.02 M acetate buffer, pH 4.5, in 2 M NaCl and then with 0.02 M bicarbonate buffer pH 9.0 in 2 M NaCl. After washing at pH 9, gel was washed with water. The dye substitution was determined by hydrolyzing the gel in 6 M HCl at 40° C for 30 min, reading the absorbance at 650 nm and comparing the value to a standard curve prepared with pure dye. The dye concentration was 0.8 μmol/ml of packed gel.

Preparation of soluble lysosomal enzymes

Soluble lysosomal fraction from rat kidney, rat liver was prepared essentially according to the method described earlier [16]. The crude mitochondrial fraction which also contained lysosomes was suspended in a volume of 0.02 M Tris · HCl pH 7.4, equal to half the weight of the original wet tissue. The lysosomes were ruptured by repeated freezing and thawing and then dialyzed against 0.01 M Tris acetate buffer, pH 6.0 for 8 h. After dialysis it was centrifuged at 100 000 × *g* for 1 h and supernatant was collected and used for the separation of arylsulfatase A and B. The protein concentration of this crude lysosomal enzyme extract was 4.8 mg/ml.

Preparation of pH 5.0 supernatant enzymes

9 g of rat brain was subjected to repeated freezing and thawing and homogenized with 18 ml of 0.2 M sodium acetate buffer, pH 5.0. It was centrifuged for 12 000 × *g* for 30 min, supernatant was collected and kept for dialysis against 0.01 M Tris/acetate buffer, pH 6.0. After dialysis insoluble particles were removed by centrifugation at 12 000 × *g* for 30 min. The clear supernatant (8.12 mg protein/ml) was used in the study.

Enzyme assay

Mostly arylsulfatase A and B assays have been performed in a total of 0.1 ml containing 10 mM nitrocatechol sulfate, 0.2 M sodium acetate buffer, pH 5.5 and enzyme at 37° C. The time of incubation for the assay of these two enzymes was 10 min and the reaction was stopped by the addition of 2.9 ml

of 0.11 M NaOH. The nitrocatechol released was read at 500 nm using a colorimeter. One unit of enzyme has been defined as the amount of enzyme required to produce 1 μmol of nitrocatechol from nitrocatechol sulfate per min under the assay condition. The method of Baum et al. [3] was used for the independent assay of acrylsulfatases A and B from human urine.

Inhibition studies

To study the effect of inhibitors the arylsulfatase assay was carried out in a total volume of 0.1 ml containing nitrocatechol sulfate — 0.2 M sodium acetate buffer, pH 5.5/0.25 mM $AgNO_3$ or 1 M NaCl — and enzyme. The enzyme activity was assayed as described above. The effect of Dextran Blue and Blue dye were studied on the purified sheep brain arylsulfatase A and arylsulfatase B from rat brain which was obtained after Blue-Sepharose chromatography. The sheep brain arylsulfatase A was purified according to the method of Balasubramanian and Bachhawat [17].

Polyacrylamide gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed according to the procedure of Davis [18] with 7.5% in 0.04 M barbitone buffer, pH 8.6, for 3 h using 3 mA/tube in a Canalco instrument. To detect enzymatic activity, the gels were immersed for 15 min in 0.4 M sodium acetate buffer, pH 5.0 at 37° C, and then for 30 min at 37° C in a 0.02 M solution of nitrocatechol sulfate in the same buffer. The red band of nitrocatechol appeared within a few seconds upon addition of 1 M NaOH.

Separation of arylsulfatases A and B on Blue-Sepharose column

The Blue-Sepharose resin was packed in a column (0.8 × 11 cm) at 4° C and equilibrated with 10 mM Tris/acetate buffer, pH 6.0. 5 ml of 'pH 5 supernatant enzyme' (40.6 mg protein) dialysed against 10 mM Tris/acetate buffer pH 6.0 was applied on the column at a flow rate of 10 ml/h and 2 ml fractions were collected. The column was washed with 35 ml of Tris/acetate buffer, pH 6.0. Each fraction was monitored for protein absorbance at 280 nm and assayed for arylsulfatase. The enzyme eluted with 10 mm Tris · HCl buffer, pH 7.4. The elution profile of arylsulphatase A and B is shown in Fig. 1.

The human urine was dialysed against 2 l of 10 mM Tris/acetate buffer, pH 6.0, for 8 h with three changes. The dialysed 35 ml of urine (14 mg protein) containing arylsulfatase A and B was applied to a Blue-Sepharose column (0.8 × 11 cm) at 4° C. All other conditions were the same as described above. The chicken brain arylsulfatase A was purified up to the Con-A-Sepharose chromatographic step as reported elsewhere [19]. This enzyme is unstable at pH 6.0 and loses its activity if stored. Therefore, enzyme was dialysed against 1 mM Tris · HCl buffer, pH 7.4 for 8 h, and after dialysis 1 M Tris acetate buffer, pH 6.0, was added to bring the final molarity of this buffer to 10 mM in the enzyme solution. The 10 ml of enzyme solution (23 mg protein) was immediately applied to the Blue-Sepharose column (0.8 × 11 cm) at 4° C which was equilibrated with 10 mM Tris/acetate buffer, pH 6.0. Elution conditions were the same as described above.

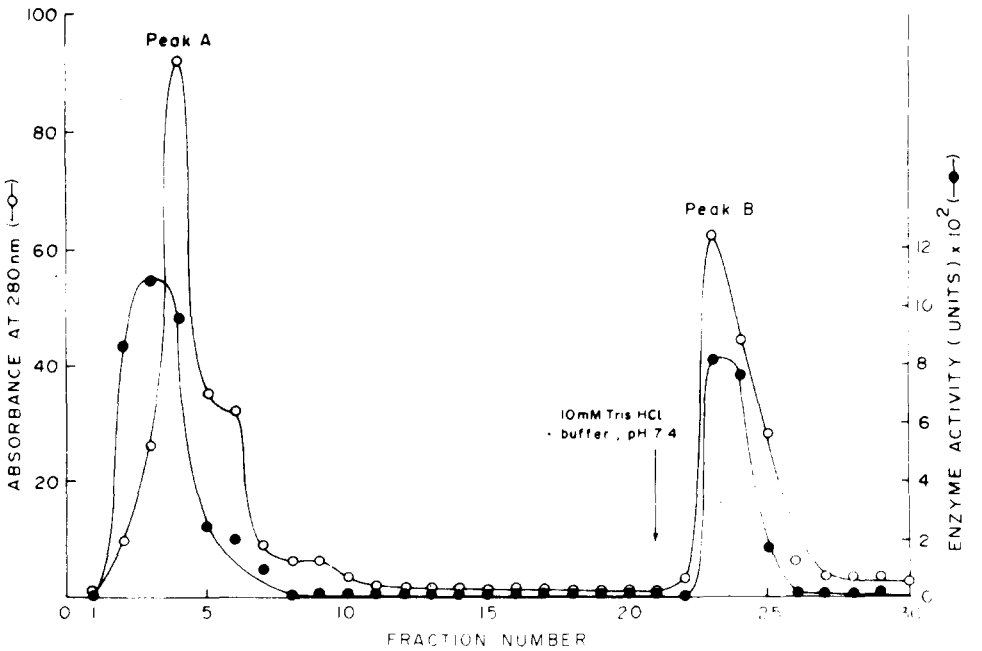

Fig. 1. Chromatography of arylsulfatases A and B from rat brain on Blue Sepharose 6B column. The "pH 5.0 supernatant" was dialysed against 10 mM Tris/acetate buffer, pH 6.0, and applied to the column. All other experimental details are described in Methods. ○———○, absorbance at 280 nm; ●———●, enzyme activity.

Results

Our studies showed that the Blue-Sepharose can be used for the separation of arylsulfatase A and B from human urine, rat liver, rat brain and sheep brain. Using solubilised lysosomal enzyme fraction from rat kidney and rat liver we observed that this procedure can be applied for the separation of arylsulfatase A and B of these tissues also. In Fig. 1, elution profile of arylsulfatase A and B from rat brain is shown. The enzymes eluted in Peak A and B were found to be arylsulfatase A and B respectively as judged by inhibition studies. It is known that chloride ions do not affect arylsulfatase A activity and inhibit arylsulfatase B activity whereas Ag^+ ions (0.25 mM) inhibit the former and do not affect latter enzyme. The differential effect of Ag^+ and chloride ions is well known [20]. The Peak A enzyme was completely inhibited by Ag^+ ions and there was no effect of chloride ions on this enzyme. The arylsulfatase A in peak A is completely free of arylsulfatase B since it was completely inhibited by Ag^+ ions. In the case of Peak B enzyme Ag^+ ions did not have any effect on the activity of the enzyme, though chloride ions which are known to inhibit only the B enzyme showed nearly complete inhibition (87%) of enzyme activity. This demonstrated that peak B enzyme is arulsulfatase B and is free of arylsulfatase A. Polyacrylamide gel electrophoresis of Peak A and Peak B proteins showed the presence of one single enzymatic band corresponding to arylsulfatase A and B respectively.

The rat brain arylsulfatase B was noncompetitively inhibited by Dextran

Blue and Cibacron Blue dye. The K_i values obtained for the enzyme were $3.32 \cdot 10^{-4}$ M and $4.5 \cdot 10^{-4}$ M with Dextran Blue and blue dye respectively. Neither Dextran Blue nor blue dye had any effect on the enzymatic activity of sheep brain arylsulfatase A.

Since chicken brain arylsulfatase A differs from other arylsulfatases of A type in its electrophoretic mobility and has isoelectric point near neutrality [21] it was retained on the Blue-Sepharose column and was eluted by 0.01 M Tris · HCl buffer pH 7.4. This chromatography resulted in 2.2-fold purification with 70% recovery of the enzyme.

Discussion

The Blue-Sepharose chromatography described here for the separation of arylsulfatase A and B is simple and rapid and may prove useful in the separation of arylsulfatase A and B in biological fluids and biopsy tissues even in small amounts. The capacity of Blue-Sepharose to bind arylsulfatase B, ease in regeneration of Blue-Sepharose in columns by washing with 1 M NaCl and the potential selectivity for this enzyme suggest that this technique is very useful for the large scale purification of arylsulfatase B from different tissues and different species.

It is to be noted here that the ratio of arylsulfatase B to arylsulfatase A is low and is due to the isolation procedure which is different from other reports [7,9].

The retention of arylsulfatase B on the Blue-Sepharose column may be due to either or both of two possible mechanisms:

(a) The enzyme may possess a "nucleotide fold", similar to that of kinases and dehydrogenases. UDP-*N*-acetyl galactosamine-4-sulfate resembles the natural substrate for arylsulfatase B [22]. It is conceivable that Cibacron Blue dye might have an affinity for the UDP-binding site of the enzyme.

(b) It is possible that arylsulfatase B binds to the dye through ionic forces. This postulate is supported by the elution of the enzyme by a change in pH from 6.0 to 7.4. Moreover, most arylsulfatase enzymes of the A type (which have isoelectric points between pH 3 and 4) do not bind to the dye, and atypical arylsulfatase A from chicken brain (which has isoelectric point in the neutral range), does bind to Cibacron Blue; this further suggests the role of ionic forces in the enzyme-dye binding.

Acknowledgements

This work was supported by a grant from Department of Atomic Energy, Government of India. AA and AS are the Senior Research Fellows of the Council of Scientific and Industrial Research, India.

References

1 Ragab, H., Beck, C., Dillard, C. and Tappel, A.L. (1967) Biochim. Biophys. Acta 148, 501–505
2 Goldfischer, S. (1965) J. Histochem. 30, 227–237
3 Baum, H., Dodgson, K.S. and Spencer, B. (1959) Clin. Chim. Acta 4, 453–455

4 Austin, J.H., Balasubraminan, A.S. Pattabiraman, T.N., Saraswathi, S., Basu, D.K. and Bachhawat, B.K. (1963) J. Neurochem. 10, 805—816
5 Worwood, M., Dodgson, K.S., Hook, G.E. and Rose, F.A. (1973) Biochem. J., 134, 183—190
6 Roy, A.B. (1958) Biochem. J., 68, 519—528
7 Farooqui, A.A. and Bachhawat, B.K. (1971) J. Neurochem. 18, 635—646
8 Balasubramanian, K.A. and Bachhawat, B.K. (1976) J. Neurochem. 27, 485—492
9 Hook, G.E.R., Dodgson, K.S., Rose, F.A. and Worwood, M. (1973) Biochem. J. 134, 191—195
10 Thompson, S.T., Cass, K.H. and Stellwagen, E. (1975) Proc. Natl. Acad. Sci. U.S. 72, 669—672
11 Ryan, L.D. and Vestling, C.S. (1974) Arch. Biochem. Biophys. 160, 279—284
12 Easterday, R.L. and Easterday, I.M. (1974) Adv. Exp. Med. Biol. 42, 123—133
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Porath, J., Janson, J.C. and Lass, T. (1971) J. Chromatogr. 60, 167—177
15 Bohme, H.J., Kopperschlager, G., Schulz, J. and Hoffmann, E. (1972) J. Chromatogr., 69, 209—214
16 Bishayee, S. and Bachhawat, B.K. (1974) Neurobiology 4, 48—56
17 Balasubramanian, K.A. and Bachhawat, B.K. (1975) Biochim. Biophys. Acta 403, 113—121
18 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
19 Ahmad, A., Surolia, A. and Bachhawat, B.K. (1977) Ind. J. Biochem, in the press
20 Dubois, G. and Baumann, N. (1973) Biochem. Biophys. Res. Commun. 50, 1129—1135
21 Ahmad, A. (1976) Ph.D. Thesis, Aligarh. Muslim University, Aligarh, India
22 Fluharty, A.L., Stevens, R.L., Fung, D., Peak, S. and Kihara, H. (1975) Biochem. Biophys. Res. Commun. 64, 955—962