PURIFICATION OF ARYLSULFATASE A FROM HUMAN URINE

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Summary: The purification of arylsulfatase A from human urine is described. The enzyme was isolated by ammonium sulfate precipitation, acetone fractionation, affinity chromatography, Sephadex G200 chromatography and preparative polyacrylamide gel electrophoresis. The enzyme migrates as a single band in both acid and alkaline polyacrylamide gel electrophoresis. The enzyme has a pH optimum of 4.75; the amino acid composition is presented.

A marked deficiency of arylsulfatase A activity has been observed in metachromatic leukodystrophy, a human lipid storage disease (1,2). Further studies on the precise nature of this defect have awaited the purification of the normal human enzyme. Arylsulfatase A from ox liver, ox brain and pig kidney have been significantly purified (3-5). Arylsulfatase A has been partially purified from human brain (6,7) and liver (8). Although human urine contains arylsulfatase A (9), the enzyme has not been significantly purified from this source. This communication presents a procedure for the purification of human arylsulfatase A from urine. The key steps in the procedure are delipidation and affinity chromatography.

METHODS

Step 1: 0-50% Ammonium Sulfate Precipitation. Step 1 was performed at 4°C. Urine was collected over a 24 hour period and then adjusted to pH 5.7 by adding 100 ml of 1.0 M NaAc, pH 5.7 to 900 ml of urine. This solution was filtered through glass fiber filter paper (Reeve Angel grade 934 AH) to remove cellular and other debris. 313 grams of enzyme grade ammonium sulfate (Schwarz-Mann) were slowly added to each liter of the filtrate. A copious precipitate formed during the next 2 hours and the mixture was then filtered through glass fiber filter paper. The precipitate was dialyzed exhaustively against 0.1 M NaAc buffer pH 5.7, and the retentate was centrifuged; the sediment was discarded and the supernatant used in step II.

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Step II: 33-67% Acetone Fraction. This fractionation was carried out at -20°C. Acetone (A.C.S. analytical reagent from Mallinckrodt Chemical Works) was slowly stirred into the soluble material from step I in the ratio of 1:2 (V:V), and a precipitate formed. The mixture was then centrifuged at 10,000 x g for thirty minutes. The supernatant was decanted and to it additional acetone was added, 1:1 (V:V). A second precipitate formed and the mixture was recentrifuged. The supernatant was now decanted and discarded, and the precipitate was resuspended in 0.1 M NaAc pH 5.7.

Step III: Affinity Chromatography. Psychosine sulfate (Pierce Chemical Company) was coupled to agarose (Sepharose 4B Pharmacia) (10) according to the method described for other ligands by Cuatrecasas (11). Approximately 50 ml of succinylated Sepharose 4B was suspended in 100 ml of 0.1 M sodium citrate (pH 6.0); 380 mg of psychosine sulfate and 4 grams of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were added and the suspension was stirred for twelve hours at room temperature. A portion of the affinity labeled Sepharose 4B was then thoroughly washed with 0.1 M NaAc pH 5.7 buffer and packed into a 9 x 250 mm column to which the final solution from Step II was applied. The column was then washed thoroughly with 0.1 M NaAc pH 5.7, which did not remove the enzyme. The enzyme activity could only be eluted with 0.1% Triton X-100 (W:V) in 0.1 M NaAc pH 5.7 buffer. Fractions with enzyme activity were pooled and dialyzed vs 0.1 M NaAc pH 5.7 buffer in order to reduce the concentration of Triton X-100. The retentate was then concentrated to 1.5 ml by filtration through a UM-2 Diaflo membrane (Amicon).

Step IV: Sephadex G200 Chromatography. A Sephadex G200 column (10 x 1500 mm) was equilibrated with 0.1 M NaAc, pH 5.7, buffer at 4°C. The final solution from Step III was applied to the column. The flow rate was maintained at 3 ml/hour and 3 ml fractions were collected. Those fractions with the greatest enzyme activity were then pooled and concentrated to .5 ml by filtration through a UM-2 Diaflo membrane.

Step V: Preparative Polyacrylamide Gel: A Canalco preparative polyacrylamide gel apparatus was then used to process the concentrate from Step IV.

The standard Ornstein-Davis alkaline buffers were used (12,13). A 2.5% stacking gel (1 cm) and a 5% resolving gel (2 cm) were employed. The material from Step IV was applied to the stacking gel and electrophoresis was performed at 3 mA; the system was maintained at 4°C. Eluting buffer (a 1:3 dilution of lower/gel buffer) was pumped at a rate of 50 ml/hour and 5 ml fractions were collected.

Enzyme Assay: Arylsulfatase A activity was assayed as described by Baum (14).

Protein Determinations: Proteins were determined by the method of Lowry (15).

Purity: Polyacrylamide gel electrophoresis was employed as the criterion for assessing purity. Electrophoresis was performed as described by Davis (12); 1.5 cm stacking gel (2.5% acrylamide) and a 4 cm resolving gel (5% acrylamide) were used. The gels were routinely run at pH 8.9. The purity of the product of Step V was also verified by gel electrophoresis at pH 2.9 in the presence of 8 molar urea.

Protein Hydrolysis and Amino Acid Analysis: 0.1 mg of the pure protein was dialyzed vs .005 M NH_4HCO_3, buffer pH 8.6, lyophilized and then hydrolyzed in 1 ml of 6 N HCl at 108°-110° for twenty-four hours under vacuum. The HCL was evaporated and the amino acid analysis was determined on a Beckman automatic amino acid analyzer (Model 121) using two columns.

RESULTS

Table 1 shows the course of a typical purification of arylsulfatase A. The ammonium sulfate fractionation was effective as both a purification and concentration step. The polyacrylamide gel electrophoresis (PAGE) of the 0-50% $(NH_4)_2SO_4$ fraction is shown in Figure 1 (1).

Although acetone fractionation did not have a profound effect on specific activity, it was necessary for further purification of the enzyme. The acetone treatment reduced neutral and polar lipid concentrations below the limit of detection of thin layer chromatography and decreased the amount of phosphorus associated with the protein to < 3% (16). The PAGE of the 33-67% acetone fraction is shown in Figure I (2).

The affinity chromatography step revealed that arylsulfatase A binds firmly

TABLE 1

PURIFICATION OF ARYLSULFATASE A FROM HUMAN URINE[1]

Fraction	Total Activity[2] Units	Recovery Percent of Total Activity	Protein mgm	Specific Activity units/mgm protein
Urine	12.6[3]		704	.02
Ammonium sulfate 0-50%	15.1	119	53	.29
Acetone 33-67%	14.5	115	35	.41
Affinity chromatography	10.1	80	11	.90
G200 chromatography	8.1	64	2.8	2.9
Preparative polyacrylamide Gel	1.6	13	.45	3.5

1 The starting material was 10.8 liters of urine.

2 Enzyme was assayed for 5 minutes. Units are given as μmoles of PNC liberated/min. at 37°C.

3 This is a falsely low value for activity probably due to the presence of inhibitors of arylsulfatase A activity in urine.

to the pseudo-natural substrate resembling sulfatide. Standard methods of elution were attempted: salt (0.1-4.0 molar), buffers of pH 2 through 10, and the substrate p-nitro catechol sulfate (Sigma) (PNCS) (0.01-0.1 M). None of these procedures were very effective at eluting the enzyme from the affinity column. Dilute NaOH (pH 10, 10^{-4}M) however, removed about 10% of the activity. Buffers of higher salt concentration at pH 10 did not elute any enzyme. In order to effect complete liberation of the enzyme from the column, a non-ionic detergent, Triton X-100, was employed. This solution quantitatively removed the enzymatic activity. Several proteins in addition to arylsulfatase A were bound to the affinity column and eluted by Triton. The PAGE is shown in Figure I (3).

An example of the PAGE of the fractions with enzyme activity obtained by chromatography on Sephadex G200, is shown in Figure I (4). G200 chromatography was performed at both pH 8 and 5.7. The K_{AV} at both pH's is 0.30. The PAGE

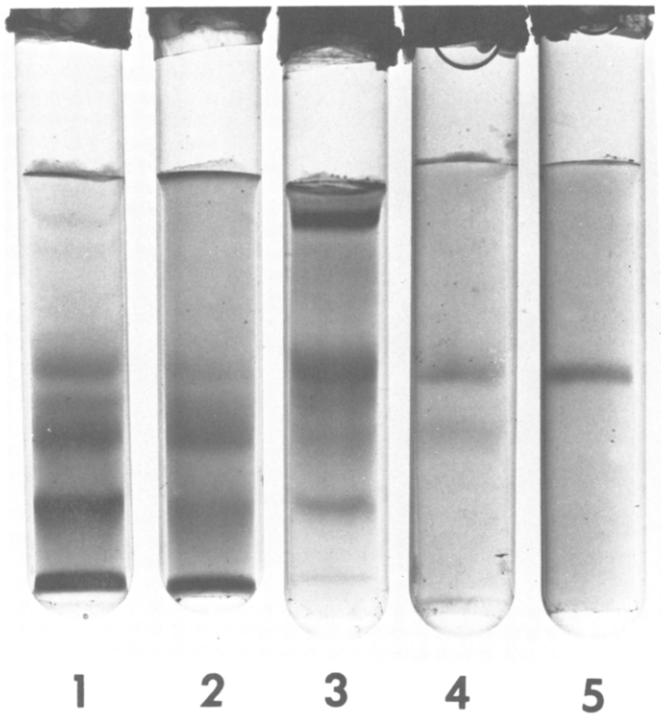

FIGURE I - Analytical polyacrylamide gels of arylsulfatase A at five stages of purification. Following: (1) ammonium sulfate precipitation, (2) acetone fractionation, (3) affinity chromatography, (4) Sephadex G200 chromatography, and (5) preparative polyacrylamide gel electrophoresis.

of the fractions with enzyme activity obtained by preparative polyacrylamide gel is shown in Figure I (5). The pH optimum was determined in 0.166 M sodium acetate buffer at 37°C. Each assay tube contained 0.5 μgm of enzyme protein and 1 μmole of PNCS in a final volume of 0.3 ml. The pH optimum is 4.75. The amino acid composition is shown in Table 2.

DISCUSSION

In the current report, arylsulfatase A from human urine has apparently been completely purified, as judged from its migration as a single band on PAGE at pH 8.9 and 2.9.

The necessity for early acetone fractionation suggests that several proteins in the company of a small amount of lipids, perhaps representing a membrane, are joined to arylsulfatase A. Apparently mild delipidation disrupts this association sufficiently to permit further purification of the enzyme.

TABLE 2

AMINO ACID ANALYSIS OF ARYLSULFATASE A[1]

	RESIDUES/ MONOMER UNIT		RESIDUES/ MONOMER UNIT
LYSINE	50	ALANINE	62
HISTIDINE	39	VALINE	50
ARGININE	37	METHIONINE	12
ASPARTIC ACID	74	ISOLEUCINE	25
THREONINE	37	LEUCINE	50
GLUTAMIC ACID	112	TYROSINE	12
SERINE	74	PHENYLALANINE	25
PROLINE	149	GLYCINE	124

[1] Values are given as residues/monomer unit assuming a monomer molecular weight of 110,000.

Surprisingly, several proteins that did not have arylsulfatase A activity also bound tightly to the affinity column or to the arylsulfatase A itself. Ox liver arylsulfatase A has been shown to be a tetramer at low pH and a monomer at high pH (17). Since arylsulfatase A activity eluted with a K_{AV} of 0.30 from Sephadex G200 at both pH 5.7 and pH 8.0, this implies that in this pH range, at least, one did not have the same tetramer $\rightleftharpoons$ monomer equilibrium.

The pH optimum of the pure enzyme of 4.75 is similar to other reports on less pure preparations. The amino acid composition does not reveal any unusual features except for a high proline content. The latter was also observed by Nichol and Roy in ox liver arylsulfatase A (18).

Two methods of purifying human arylsulfatase A from brain have been reported. One procedure involved zinc acetate precipitation and ammonium sulfate fractionation of the soluble subcellular fraction of brain; this preparation (320 fold purification) had a pH optimum of 4.5 using PNCS as substrate (6). In the other method, the same fraction of brain was chromatographed on DEAE cellulose and Sephadex G150. This arylsulfatase A preparation had a specific activity of 10.95

μmoles/mgm prot/hr with PNCS as substrate (7). No criteria of protein purity wei provided in either of these papers. Neuwelt et al have recently described the partial purification of arylsulfatase A from human liver. PAGE of their preparation demonstrated 3 protein bands (8).

We conclude that our preparation represents the first homogeneous preparation of human arylsulfatase A.

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