Purification of Soluble Arylsulphatases from Ox Brain

By W. BLESZYNSKI AND L. M. DZIALOSZYNSKI

Biochemistry Department, Mikołaj Kopernik University, Toruń, Poland

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1. Two soluble arylsulphatases (A and B) have been extracted from ox brain by a modified Albers autolysis method and purified by acetone and ammonium sulphate precipitation and dialysis. 2. A 1600-fold purification was achieved with arylsulphatase A and 320-fold purification with arylsulphatase B. 3. The specific activity of arylsulphatase A was 266 000 4-nitrocatechol units/mg. of protein N, and that of arylsulphatase B was 64 600 units/mg. of protein N. 4. Arylsulphatase A seems to be electrophoretically homogeneous. 5. With 3 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate as substrate the optimum pH for the activity of arylsulphatase A was 4.7, and for arylsulphatase B it was 6.1 with a 60 mM solution of the same substrate.

Although many papers have been published on the subject of arylsulphatases (arylsulphate sulphohydrolases, EC 3.1.6.1), our knowledge of these enzymes is still inadequate. Possibly this is due to the lack of pure preparations, although there have been many attempts to extract and purify arylsulphatases from various sources.

Dodgson & Spencer (1952), working with visceral hump of *Patella vulgata*, obtained a 100-fold increase of arylsulphatase activity. The acetone-dried powder obtained by these authors had an activity of 3 680 000 4-hydroxyacetophenone units/g. (Dodgson & Spencer, 1953). Roy (1953a) demonstrated that the soluble arylsulphatase of ox liver is a mixture of two enzymes: arylsulphatases A and B. He separated and purified those two enzymes: arylsulphatase A, 1050-fold (Roy, 1953b), and arylsulphatase B, 7.6-fold (Roy, 1954). The final preparation of arylsulphatase A had an activity of 40 000 4-nitrocatechol units/mg. of protein N, and the activity of the arylsulphatase B preparation was 1187 such units/mg. of protein N.

Dodgson, Melville, Spencer & Williams (1954) achieved a 20-fold increase of arylsulphatase activity in an extract from a strain of *Alcaligenes metalcaligenes*, and Dodgson, Spencer & Williams (1955) further purified this preparation 15-fold. Arylsulphatase present in Clarase was purified 3.6-fold by Dzialoszynski (1956) and 8-fold by Dzialoszynski & Bleszynski (1961b). Ox liver also contains an insoluble arylsulphatase C. This enzyme was purified 40-fold by Roy (1956), and Dodgson, Rose & Spencer (1957) solubilized this enzyme from rat liver and increased its activity 3-fold in relation to protein content. A purified arylsulphatase B preparation from human liver

containing 18 750 4-nitrocatechol units/mg. of protein N was obtained by Dodgson & Wynn (1958). A very powerful preparation of arylsulphatase was obtained by Dodgson & Powell (1959) from the digestive gland of *Helix pomatia*. Its activity was 478 000 4-nitrocatechol units/mg. of protein N. This preparation was rather unstable and further attempts at purification led to total loss of activity. Takahashi (1960) purified 20-fold the arylsulphatase from liver of the marine gastropod *Charonia lampas*. Human brain was used as a source of arylsulphatase by Balasubramanian & Bachhawat (1963), who obtained a 300-fold purification. Nichol & Roy (1964) obtained a highly active preparation of arylsulphatase A from ox liver; the purification was 7000-fold. Wortman (1962) separated four arylsulphatases from corneal extracts by DEAE-cellulose chromatography.

The present paper describes the extraction and purification of the soluble arylsulphatases from ox brain, which is a fairly rich source of these enzymes (Dzialoszynski & Wenclewski, 1963).

EXPERIMENTAL

Ox brain. The starting material was ox brain obtained from the local slaughterhouse immediately after the death of the animals. The brains were freed from arachnoid and pia mater, washed, frozen and transported to the laboratory in the frozen state. Preliminary experiments showed that minced frozen brain did not lose arylsulphatase activity when stored at -5° for periods up to 48 hr.

Chemicals. The substrate, dipotassium 2-hydroxy-5-nitrophenyl sulphate, was prepared according to the method of Roy (1958). Other reagents were analytical grade. All solutions were made in redistilled water.

Estimation of arylsulphatase A and B activities. Prelim-

Scheme 1. Purification of arylsulphatases A and B from ox-brain autolysate (from 300 g. of brain).

inary experiments showed that the method of Roy (1953a) could, with slight modifications, be successfully applied to the assay of arylsulphatases A and B in brain. Solutions of 3mm-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.125M-acetate buffer were used for the estimation of both arylsulphatase activities. For arylsulphatase A the pH was 4.8 and for arylsulphatase B the pH was 5.2. [It was found in other experiments (W. Bleszynski, unpublished work) that the optimum pH of arylsulphatase B shifts from 6.1 at 60mm-substrate to 5.2 at 3mm.] The actual tests were performed as follows: to 1.2ml. of 4mm-substrate solution, buffered to the required pH with 0.167M-acetate buffer, was added 0.4ml. of the enzyme solution to be tested and the mixture was incubated for 1hr. at 37°. The reaction was stopped by the addition of 8ml. of 20% (w/w) NaOH containing 0.2% of Na₂SO₃·7H₂O. The colour intensity of the liberated 4-nitrocatechol was measured on a Unicam SP.300 colorimeter with an Ilford 404 filter against a blank that contained the incubated substrate solution.

Protein N was estimated by the micro-Kjeldahl procedure as modified by Markham (1942). Precipitation of the protein was performed with uranyl acetate at boiling temperature and in the presence of K₂SO₄ (Dzialoszynski & Bleszynski, 1961a). The precipitated protein was filtered off and washed with 2% (w/w) uranyl acetate solution. Filter papers with the protein precipitate were dried and digested in conc. H₂SO₄. The NH₃ formed was distilled into H₃BO₃ solution and finally titrated with 0.01N-HCl.

Electrophoresis. Solutions of fractions A-3 and B-3 (see Scheme 1) were concentrated 40-fold by acetone precipitation and dissolved in a small amount of water. High-voltage electrophoresis was carried out at 15° on Whatman no. 1 paper in barbiturate buffer, pH 7.6 and I 0.005. The enzyme solutions were placed in the middle of the paper strip of dimensions 6cm. × 50cm. The current was 0.37 mA/cm. width and the voltage gradient was 57 v/cm. Electrophoresis was carried out for 70min. The paper strip was dried in air and a 4cm.-wide section was stained with bromophenol blue, as described by Dzialoszynski & Bleszynski (1961b), and sprayed with 1% (w/v) Na₂CO₃ solution in 80% (v/v) acetone; protein distribution was estimated photometrically with an automatic recorder. The remainder of the paper, 2cm. wide, was cut crosswise into 1cm.-long sections. Each section was shredded and placed in 1.6ml. of 3mm-dipotassium 2-hydroxy-5-nitrophenyl sulphate solution and incubated for 1hr. at 37°. For arylsulphatase A the pH of solution was 4.8 and for arylsulphatase B the pH was 5.2. Extinctions of the alkaline solutions were measured after centrifugation of the solutions to spin down small paper shreds.

Purification of enzymes. Preliminary experiments established that arylsulphatases A and B could best be extracted from brain tissue after autolysis. The butanol method of enzyme extraction (Morton, 1950, 1954, 1957) led to considerable losses in enzyme activity, and extracts prepared from acetone-dried powders of brain tissue also gave lower yields than those obtained after autolysis.

The following manipulations were carried out at about 0°. Brains were minced and 0.2M-ammonium acetate solution (100ml./100g.) was added. The mixture was homogenized, cooled to 0° and to each 100g. were added 28.2ml. of acetone and 12.8ml. of toluene-ethyl acetate mixture (1:1, v/v). After autolysis for 72hr. at 0° the autolysate was treated as outlined in Scheme 1.

RESULTS

The results of the purification of arylsulphatases A and B are summarized in Tables 1 and 2.

The effects of pH and substrate concentration on the reaction velocities of arylsulphatases A and B from ox brain are shown in Figs. 1 and 2.

The results of high-voltage electrophoretic examination of arylsulphatases A and B from ox brain are represented in Fig. 3.

Table 1. *Progress of the purification of arylsulphatase A of ox brain according to Scheme 1*

Enzyme activity was determined in 3mm-dipotassium 2-hydroxy-5-nitrophenyl sulphate, pH 4.8, incubation being for 1hr. at 37°. The average activities of five preparations, expressed as μ g. of released 4-nitrocatechol, are given.

Fraction	Total activity	Specific activity (μ g. of 4-nitrocatechol/mg. of protein N)	Increase of activity as compared with homogenate
Brain homogenate	745 000 $\pm$ 82 000	167 $\pm$ 18	—
Ac-1	1 279 200 $\pm$ 84 900	11 680 $\pm$ 1 820	69.9 $\times$
A-2	1 789 400 $\pm$ 84 900	128 440 $\pm$ 22 110	769 $\times$
A-2*	257 210	—	—
A-3	226 500	266 470	1596 $\times$

* Combined remainders of six A-2 fractions left after protein and activity estimations.

Table 2. *Progress of the purification of arylsulphatase B of ox brain according to Scheme 1*

Enzyme activity was determined in 3mm-dipotassium 2-hydroxy-5-nitrophenyl sulphate, pH 5.2, incubation being for 1hr. at 37°. The average activities of five preparations, expressed as μ g. of released 4-nitrocatechol, are given.

Fraction	Total activity	Specific activity (μ g. of 4-nitrocatechol/mg. of protein N)	Increase of activity as compared with homogenate
Brain homogenate	897 400 $\pm$ 78 600	201 $\pm$ 19	—
Ac-1	1 407 900 $\pm$ 108 800	13 320 $\pm$ 2 170	66.3 $\times$
B-2	1 051 500 $\pm$ 108 800	43 340 $\pm$ 1 350	216 $\times$
B-2*	1 583 300	39 580	197 $\times$
B-3	1 330 000	64 560	321 $\times$

* Combined remainders of six B-2 fractions left after protein and activity estimations.

Fig. 1. Effect of pH on the reaction velocity of arylsulphatases A (fraction A-3) (○) and B (fraction B-3) (●) from ox brain. The substrate (dipotassium 2-hydroxy-5-nitrophenyl sulphate) concentration for arylsulphatase A was 3 mM and that for arylsulphatase B was 60 mM.

Fig. 2. Effect of varying substrate concentration on the reaction velocity of arylsulphatases A (○) and B (●) from ox brain. Measurements were made at the optimum pH values: arylsulphatase A, pH 4.7; arylsulphatase B, pH 6.1.

DISCUSSION

The described method of purification of soluble arylsulphatases from ox brain is simple and leads to a high degree of purification with small losses of enzyme activity. The apparent loss of activity between fraction Ac-1 and A-2 (Table 1) was caused by the separation of arylsulphatase B. With this method 1600-fold purification of aryl-

Fig. 3. Results of electrophoretic investigation of arylsulphatases A (fraction A-3) (a) and B (fraction B-3) (b) from ox brain. Electrophoresis was carried out for 70 min. at a voltage gradient of 57 v/cm. in veronal buffer, pH 7.6 and I 0.005. Hatching indicates protein distribution; the histogram indicates enzyme activity (as extinction of liberated 4-nitrocatechol).

sulphatase A and 320-fold purification of arylsulphatase B were achieved.

The arylsulphatase A was 6.7-fold more active than that described by Roy (1953b) from ox liver, but about 30-fold less active than that prepared from ox liver by Nichol & Roy (1964). It was 3.6-fold more potent than the preparation obtained by Baum & Dodgson (1958) from human liver. Arylsulphatase A from human brain (Balasubramanian & Bachhawat, 1963) had a specific activity of 75 800 4-nitrocatechol units/mg. of protein N. The specific activity of arylsulphatase A was 266 500 and that of arylsulphatase B was 64 600 4-nitrocatechol units/mg. of protein N, the most active preparation so far described.

Examination of the purest arylsulphatase A and B fractions by high-voltage electrophoresis (Fig. 3) showed that the arylsulphatase A fraction was an almost homogeneous material, but that the arylsulphatase B fraction was contaminated by some proteins with negligible sulphatase activity. In pH 7.6 buffer arylsulphatase A was almost stationary, whereas arylsulphatase B moved quickly towards the cathode. High-voltage electrophoresis could not be used for further purification of the enzymes owing to great losses of enzyme activity caused by the unavoidable increase of temperature in the apparatus used and presumably by adsorption of the enzyme on to the chromatography paper (Dzialiszynski & Bleszynski, 1964).

The influence of pH and substrate concentration on the activity of our preparations under the conditions described by Roy (1953b, 1954) showed them to be similar to arylsulphatases A and B from ox liver (Roy, 1953a,b, 1954).

The curve showing the pH-dependence of the reaction velocity of the arylsulphatase B fraction from ox brain is regular and very much like that of arylsulphatase B from ox liver (Roy, 1954) and the same enzyme from human liver (Dodgson & Wynn, 1958). It had a peak at pH 6.1 in 60 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (Fig. 1). The rapid fall of the curve on the alkaline side is caused, according to Dodgson & Wynn (1958), by the inhibitory effect of OH^- ions, which compete with the substrate for active centres of enzyme.

With the arylsulphatase A fraction from ox brain, the curve illustrating the pH-dependence of reaction velocity had an optimum at pH 4.7 in 3 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (Fig. 1), a value that is usually given for purified preparations of arylsulphatase A (Baum, Dodgson & Spencer, 1958; Roy, 1953b).

The influence of substrate concentration was measured at the optimum pH for both arylsulphatase fractions from ox brain. With the arylsulphatase B fraction, at low substrate concentrations, the relationship between rate of reaction and substrate concentration does not quite follow the typical Michaelis-Menten curve. With the arylsulphatase A fraction, on the other hand, the curve is similar to that obtained by Roy (1953b), Baum & Dodgson (1958) and Baum *et al.* (1958) for arylsulphatase A from liver.

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