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PARTIAL PURIFICATION AND SOME PROPERTIES OF ARYLSULPHATASES FROM THE GUT OF THE GIANT AFRICAN SNAIL, *ACHATINA ACHATINA*

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Abstract—1. Three protein peaks showing arylsulphatase activity have been isolated from the gut of the giant African snail *Achatina achatina* by ion-exchange chromatography on DEAE-cellulose. The two absorbed peaks A_1 and A_2 were eluted with a linear gradient formed from 300 cm^3 of 50 mM Tris–HCl and 300 cm^3 of 100 mM NaCl in 50 mM Tris–HCl pH 7.4. The unabsorbed fraction UA was later resolved into two fractions UA_1 and UA_2 on Sephadex G-200.

2. Partial purification of the isolated fractions was achieved by ammonium sulphate precipitation and further chromatography on Sephadex G-200. Some kinetic properties of these enzymes have been measured and their apparent molecular weights determined by gel filtration.

3. The partially purified preparations remained stable at room temperature and when incubated for 30 min at 55°C lost only about 15% activity.

4. Neither the crude extract nor the isolated fractions, showed steroid sulphatase activity. The two absorbed fractions A_1 and A_2, but not the unabsorbed fraction, were active towards *p*-nitrophenyl sulphate (NPS). Chloride ions did not appear to affect the rate of hydrolysis of NPS. Phosphate ions nearly completely inhibited the hydrolysis of dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) by all fractions.

5. The existence of multiple forms of the enzyme is discussed in relation to its possible involvement in the various metabolic cycles of the organism.

INTRODUCTION

There has been little detailed investigation of the arylsulphatase (arylsulphate sulphohydrolase EC 3.1.6.1) of invertebrates although such enzymes are known to be widespread. Only the arylsulphatases of a few molluscs—the European limpet *Patella vulgata* (Wortman & Schneider, 1960), the Roman snail *Helix pomatia* (Dodgson & Powell, 1959; Jarrige, 1963) and the Japanese marine gastropod *Charonia lampas* (Suzuki *et al.*, 1959)—have been purified to a slight extent. No suitable preparations of these enzymes have been available for detailed kinetic studies. Available preparations so far have proved extremely labile; they are extensively denatured by even gently agitating the reaction mixture in which the assay is taking place.

The arylsulphatase of *P. vulgata* was reportedly separated into three fractions by chromatography on DEAE-cellulose (Wortman & Schneider, 1960), while Suzuki *et al.* (1959) reported two arylsulphatases differing in their electrophoretic mobility and pH optima from the mucous gland but not from the hepatopancreas (digestive gland) of *C. lampas*. Neither Dodgson & Powell (1959) nor Jarrige (1963) reported any evidence of the occurrence of more than one arylsulphatase from *H. pomatia*. Agogbua & Wynn (1975) reported the separation of the arylsulphatase of *P. vulgata* into two fractions by affinity chromatography on a column of nitroquinol sulphate–sepharose matrix. These authors did not obtain a similar result, working under the same conditions, with *H. pomatia*.

Studies by Shimony & Nigrelli (1972) have identified two soluble arylsulphatases similar in properties to arylsulphatases A and B of mammalian tissues in the mantle tissues of the barnacles *Balanus eburneus*. These authors did not, however, separate these activities but chose the "modifier" method of Baum *et al.* (1959) for the assay of their relative activities. This assay technique has been criticized by Worwood *et al.* (1973). In view of the probable relationship between the arylsulphatase activity and the cyclic formation and hardening of the exoskeleton of the barnacles reported by these authors, we decided to investigate the arylsulphatase of the giant African snail *Achatina achatina* about which nothing is known. We report in this paper a preliminary account of the fractionation and properties of the arylsulphatases from the gut of this animal.

MATERIALS AND METHODS

Enzyme

The species of *A. achatina* used in these studies was obtained from a local market near Benin City in Bendel State of Nigeria where they grow wild in the bushes of the region. After removing the shells by cracking and peeling off, the gut was carefully dissected out along with the buccal plate so that the gut contents remained intact. The guts from 7 to 8 snails were pooled and weighed. Variation of arylsulphatase activity with individual snail was therefore, not accounted for. The weighed material was diced into small pieces before being homogenized in four volumes of chilled 0.1 M sodium acetate–acetic acid buffer, pH 5.0, using a Sorvall Omni Mixer 17220 (Sorvall Inc. Newton Conn. U.S.A.).

After standing at 4°C for 10 min the homogenate was spun at 24,000 *g* for 20 min with a Sorvall RC5 refrigerated

high-speed centrifuge. Acetone precipitation was carried out on the supernatant as described by Dodgson *et al.* (1957). The resulting acetone dried powder was stored at 4°C and served as source of enzyme in the subsequent procedures that follow.

Substrates

Dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) was prepared by the method of Roy (1953) as modified by Dodgson & Spencer (1956).

p-Nitrophenyl sulphate (NPS) was prepared by direct sulphatation of *p*-nitrophenol with chlorosulphonic acid as described by Dodgson & Spencer (1957*a*).

Steroid sulphate (epiandrosterone sulphate) was a generous gift from Dr A. B. Roy of the Department of Physical Biochemistry, Australian National University, Canberra, Australia. The sample was used as supplied without further purification.

Methylene blue was a Sigma Chemical Company product. All other reagents used were of analytical grade.

Assay procedure

Arylsulphatase activity was measured by spectrophotometric estimation of the anionic form of the phenol liberated during enzymic hydrolysis. To 0.5 cm^3 of enzyme solution, adjusted to pH 6.1 and preincubated at 37°C for 3 min, was added 0.5 cm^3 of 10 mM NCS in 0.2 M acetate buffer at the same pH, similarly preincubated at 37°C. Unless otherwise stated incubation was usually for 1 hr. The reaction was terminated by the addition of 4.0 cm^3 of 0.2 N-NaOH and the liberated 4-nitrocatechol was read at 515 nm (ϵ 515 = 10900) against a reagent blank, prepared by incubating enzyme and substrate separately and mixing after the addition of the NaOH to the enzyme solution. Enzyme solutions were diluted so that the absorbance of the liberated 4-nitrocatechol did not exceed 0.8. A similar procedure was used when NPS was the substrate except that the substrate concentration was 15 mM in 0.2 M acetate buffer pH 6.8 and the resulting nitrophenol was read at 400 nm.

Test for steroid sulphatase activity was carried out according to the procedure described by Roy (1956) using the methylene blue method.

One unit of enzyme activity is defined as the amount of enzyme which will liberate 1 μmol of 4-nitrocatechol/min under the above experimental conditions.

Protein determination

The protein content of crude extracts was estimated by the method of Kalckar (1947). The method of Lowry *et al.* (1951) was found unsuitable for such extracts as the colour developed after the addition of Folin reagent quickly faded. For partially purified samples the method of Lowry *et al.* (1951) was however adopted with bovine serum albumin as standard.

EXPERIMENTAL AND RESULTS

Stage 1. *Purification procedure* (*Table* 1)

Acetone precipitation was carried out on the crude extract as described by Dodgson *et al.* (1957). About 5.0 g of the acetone powder was suspended in 30 cm^3 of 50 mM Tris-HCl buffer pH 7.4 and dialysed against two changes of 2 l of the same buffer overnight, at room temperature. The dialysate was clarified by centrifugation.

Stage 2. *DEAE-cellulose chromatography* (*Fig.* 1)

The dialysate from stage 1 (about 45 cm^3 and containing 24.4 mg/cm^3 protein) was applied on a column (46 cm × 2.5 cm) of DEAE-cellulose (DE-52) previously equilibrated with 50 mM Tris–HCl buffer pH 7.4. The column was washed with the starting buffer to remove all unabsorbed materials and the effluent was continuously monitored at 280 nm. Fractions of volume 8.0 cm^3 were collected. When no more materials absorbing at 280 nm were washed from the column, a linear salt gradient formed from 300 cm^3 each of 50 mM Tris–HCl and 0.5 M NaCl in 50 mM Tris–HCl pH 7.4 was applied to the column to elute bound proteins. The eluted fractions were assayed for arylsulphatase activity and the absorbance read at 280 nm for protein determination.

Figure 1 shows the elution profile and arylsulphatase activity distribution. Three distinct arylsulphatase peaks, one (UA) occurring in the unabsorbed region and two peaks A_1 and A_2, eluted with increasing ionic gradient, are clearly indicated. These peaks of arylsulphatase activity were reproduced in sub-

Table 1. Fractionation scheme for arylsulphatase from the gut of *A. achatina*

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	% Total activity
Acetone extract	8640	4525	1.9	100
DEAE-cellulose:				
Unabsorbed fraction (UA)	954	110	8.7	11
Absorbed fraction I (A_1)	947	37	25.6	11
Absorbed fraction II (A_2)	1512	126	12	18
Dialysed 40–60% satd. $(NH_4)_2SO_4$ ppt:				
UA	795	29	27.4	9
A_1	811	13	62.4	9
A_1	1210	34	35.6	14
Sephadex G-200 chromatography:				
UA_1	282	6	47.0	3
UA_2	466	11	42.4	5
A_1	771	2	385.5	9
A_2	751	6	125.2	9

Extraction and details of each step are as described in the main text. One unit of activity is defined as the amount of enzyme which releases 1 μmol of nitrocatechol/min.

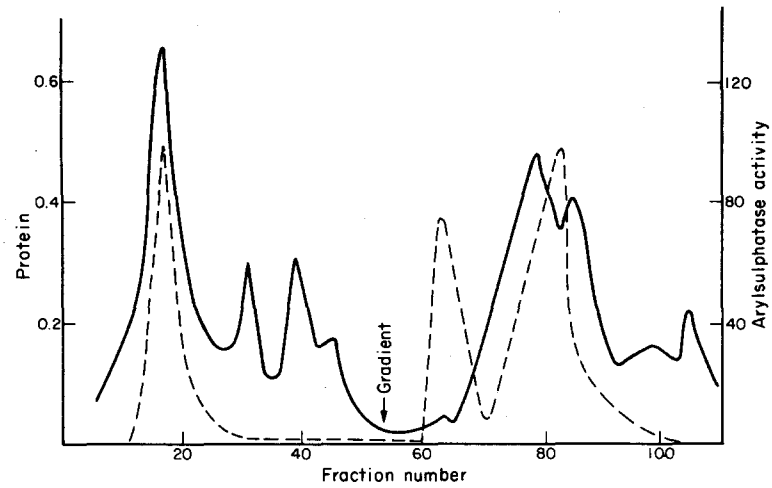

Fig. 1. Ion-exchange chromatography on DEAE-cellulose (DE-52) of acetone precipitate of *A. achatina* gut extract. The column was equilibrated with 50 mM Tris–HCl buffer (pH 7.4) and elution was by salt gradient formed from 300 cm^3 of each of the starting buffer and 0.1 M NaCl in 50 mM Tris–HCl (pH 7.4). Arylsulphatase activity (------) was determined spectrophotometrically with 4-nitroacetechol sulphate (NCS) as substrate. Protein (——) was measured by extinction at 280 nm.

sequent repeat fractionations on DEAE-cellulose showing that they represent multiple forms of the same enzyme. The purification achieved by this procedure for each enzyme peak is illustrated in Table 1.

Stage 3. *Ammonium sulphate precipitation and dialysis*

Fractions from stage 2 above showing up to 30% activity of the maximum were pooled for each arylsulphatase peak and subjected to ammonium sulphate precipitation. Materials precipitating between 40% and 60% saturation were collected by centrifugation and dissolved in 10 cm^3 of 50 mM Tris–HCl buffer pH 7.4. The materials were dialysed against the same buffer and stored at 4°C until used.

Stage 4. *Sephadex G-200 gel filtration*

Portions (3.0 cm^3) of the dialysate from each fraction from stage 3 above were applied to a column (48 × 2.5 cm) of Sephadex G-200 equilibrated in 50 mM Tris–HCl pH 7.4 and calibrated with standard marker proteins–ribonuclease A (14,000), ovalbumin (45,000), bovine serum albumin (65,000), creatine phosphokinase (83,000) and aldolase (150,000). The column was eluted with the starting buffer until the eluate contained no material absorbing at 280 nm. Fraction volumes of 6.0 cm^3 were collected, assayed for arylsulphatase activity and protein content. Fractions exhibiting arylsulphatase activity greater than 30% of the maximum were pooled and used for other tests.

The pattern of distribution of enzyme activity is as shown in Fig. 2a–c for fractions A_1, A_2 and UA respectively. Fractions A_1 and A_2 eluted as single enzyme proteins while fraction UA was resolved into two separate arylsulphatase activities labelled UA_1 and UA_2. When the two fractions were combined and rechromatographed, they were separated into two fractions indicating that they are two distinct arylsulphatase fractions.

Physical and kinetic properties of the partially purified enzymes

Various physical and kinetic parameters measured by conventional techniques are given in Table 2. The almost complete inhibition of the hydrolysis of NCS by the various fractions observed in the presence of 0.1 M phosphate, identifies these enzyme fractions closely with the soluble arylsulphatases of mammalian tissues (Dodgson & Spencer, 1957*a*).

Heat stability of the fractions

One characteristic which appeared common to the arylsulphatases of the molluscs that had been studied is the finding that the enzymes were usually more labile that most arylsulphatases from other sources. They were extensively denatured even by slightly agitating their assay mixtures (Dodgson & Powell, 1959). Our experience so far with our preparations from *A. achatina* shows that they are remarkably stable even on incubation at 55°C. Our investigation shows that the enzymes display maximum activity in the region of 35°–40°C. At 55°C however, loss of activity of about 10–20% of initial activity was usually observed.

Table 2. Physical and kinetic properties of the various arylsulphatase fractions of *A. achatina* gut

Fractions	UA_1	UA_2	A_1	A_2
Molecular weight by gel filtration	110,000 ± 900	50,000 ± 500	93,000 ± 300	110,000 ± 900
pH optimum (NCS as substrate)	6.0	5.8	6.5	6.3
Apparent Michaelis constant (mM)	0.29	0.44	1.0	1.05

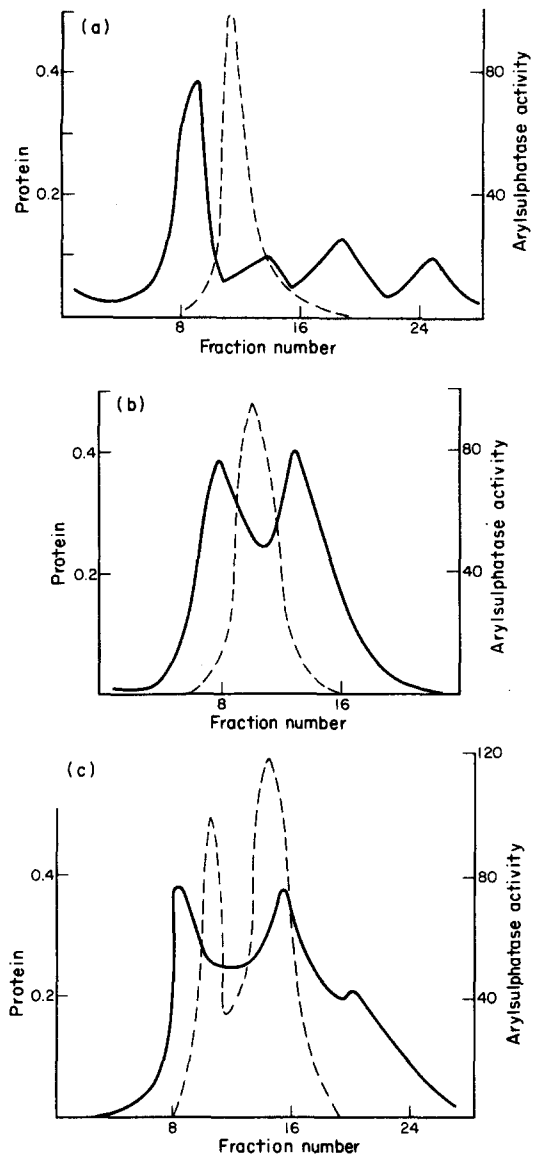

Fig. 2(a,b,c). Gel-filtration on Sephadex G-200 of 40–60% saturated $(NH_4)_2SO_4$ ppt. of fraction UA, A_1 and A_2 from DEAE-cellulose eluates. Arylsulphatase activity (------) measured as described in Fig. 1. Protein (——) measured by extinction at 280 nm.

Substrate specificity

Preliminary experiments show that fractions A_1 and A_2 are active to NPS as substrate while UA_1 and UA_2 show negligible activity to this substrate.

None of the fractions tested showed significant steroid sulphatase activity when epiandosterone sulphate was used as substrate.

DISCUSSION

Our long-term objective in these studies is to clarify the role of the arylsulphatases in the general metabolism of *Achatina achatina*, and to see if the levels of activity of any arylsulphatase fraction isolated can be correlated with the formation and hardening of the adhesive material with which the organism covers itself during hibernation. A high level of arylsulphatase activity has been reported in the gut content of the molluscs and Leon *et al.* (1960) have indicated that a correlation exists between the high level of activity and the herbivorous habit of the animals. A digestive role for this enzyme is strongly suggested by these observations. Because of the diverse nature of the tropical foliage on which the *Achatina* feeds, it is reasonable to expect that if the arylsulphatase plays a digestive role, the existence in the gut of arylsulphatases with differing specificities towards the myriads of possible natural substrates from the diet of the snail is possible.

The results described above show the separation of four arylsulphatase fractions from the gut of the snail *Achatina achatina*. This finding confirms the observation by Wortman & Schneider (1960), of the existence of multiple arylsulphatases in the gut of *Patella vulgata*. The inability of earlier workers—Dodgson & Powell (1959); Jarrige (1963)—to detect multiple forms of the enzyme could be attributed to differences in the methods of separation and assay conditions. Our finding in a preliminary experiment that the crude extract could not be resolved on Sephadex G-200 probably due to overlapping molecular sizes and to possible protein-protein interactions could explain the failure of these groups of workers to establish the existence of more than one arylsulphatase. It is possible that different tissues of the molluscs contain more than one arylsulphatase fraction as might be shown with improved separation techniques such as those described in this work.

It is not possible to classify these preparations as arylsulphatases A and B, since their pH optima and K_m values appear to differ from those reported for the classical arylsulphatase A and B of mammalian tissues. We have chosen to call these preparations absorbed fraction one (A_1) absorbed fraction two (A_2) unabsorbed fraction one (UA_1) and unabsorbed fraction two (UA_2) to indicate their behaviour on a column of DEAE-cellulose and Sephadex G-200 equilibrated with 50 mM Tris–HCl pH 7.4. The general properties of these fractions (Table 2) show their resemblance to the type II arylsulphatases (Dodgson & Spencer, 1957*b*). All fractions are nearly completely inhibited by phosphate ions but are not affected by chloride ions. The absorbed fractions A_1 and A_2 show considerable activity towards NPS whereas UA_1 and UA_2 show very little activity towards this substrate making them more like the arylsulphatase B of human liver.

In contrast to the arylsulphatases of other molluscs studied (Wortman & Schneider, 1960; Dodgson & Powell, 1959; Jarrige, 1963; Suzuki *et al.*, 1959), enzyme preparations described here show remarkable stability even on incubation at 55°C. The heat stability may be a natural adaptation to the tropical conditions in which these organisms thrive. By the preparation technique described here, it has been possible to obtain relatively pure, stable enzyme samples which will now open the way for more detailed studies of these enzymes, hitherto neglected for want of suitable enzyme preparations. Separate studies are now being undertaken in our laboratory on the distribution of these enzymes in the various tissues of *Achatina achatina* as well as identification of possible

metabolic functions of the fractions. The possibility that one of these arylsulphatase fractions may show sulpho-transferase activity is being investigated. Similar suggestions had been made for the arylsulphatase from the mucous gland of *Charonia lampas* (Suzuki *et al.*, 1959).

From these results obtained in this study, neither the crude preparation nor the isolated fractions of *A. achatina* arylsulphatase showed any activity towards epiandrosterone sulphate. This contrasts with the findings of Roy (1956) who worked on extracts from *Patella vulgata*.

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