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PURIFICATION OF A SOLUBLE ADENOSINE 5′-PHOSPHOSULPHATE SULPHOHYDROLASE FROM BOVINE LIVER

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SUMMARY

Studies of the subcellular distribution of adenosine 5′-phosphosulphate (APS) sulphohydrolase have indicated a bimodal distribution of the enzyme between the lysosomal and supernatant fractions of the bovine liver cell. The supernatant enzyme has been purified 1200-fold by ammonium sulphate fractionation, DEAE-cellulose chromatography and fractionation on Sephadex G-100. Samples of the preparation gave a single homogeneous enzymically active component on polyacrylamide disc gel electrophoresis. The molecular weight was 68 000–69 000 by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and by gel filtration on Sephadex G-100. The enzyme was specific for APS and was free of 3′-phosphoadenosine 5′-phosphosulphate sulphohydrolase and 3′-nucleotidase activities.

INTRODUCTION

Adenosine 5′-phosphosulphate (APS) is the immediate biosynthetic precursor of "active sulphate" (3′-phosphoadenosine 5′-phosphosulphate (PAPS)) which is the main biological sulphate donor. The enzymes responsible for the synthesis of the two compounds are localized in the soluble fraction of the liver cell[1,2]. Recent studies[3] have indicated that a number of distinct enzymes capable of degrading "active sulphate" (*e.g.* PAPS sulphohydrolase[4–7], 3′-nucleotidase[8] and APS sulphohydrolase[9–11]) are also to be found in the cell supernatant. The several enzymes responsible for the synthesis and degradation of APS and PAPS might therefore play a significant part in the control of sulphate conjugation.

Since the original observation of APS desulphation by Spencer[9], studies of the

Abbreviations: APS, adenosine 5′-phosphosulphate; PAPS, 3′-phosphoadenosine 5′-phosphosulphate.

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enzyme responsible have been undertaken with partially purified preparations from rat liver[11] and from pig kidney cortex[12]. The present paper reports the subcellular distribution of APS desulphating activity in bovine liver and the purification of a highly specific and apparently homogeneous supernatant APS sulphohydrolase. The molecular weight and amino acid composition of the enzyme have also been determined.

MATERIALS AND METHODS

Bovine liver high-speed supernatant

Fresh bovine liver was homogenized in a convenient volume of cold 0.15 M KCl by means of a Waring Blendor operated at maximum speed for 25 s[13]. The concentration of the suspension was adjusted to 30% (w/v) with further KCl solution. An integrated field time of $3 \cdot 10^6 \times g_{av} \cdot$min was used to prepare the high-speed supernatant and was obtained by centrifuging at 19 000 rev./min using the No. 19 rotor and the Beckman L2 preparative ultracentrifuge.

Preparation of PAPS 3'-nucleotidase

The material precipitated from fresh bovine liver high-speed supernatant by 40% saturation with ammonium sulphate was resuspended and dialysed exhaustively against 0.01 M maleate–sodium hydroxide buffer at pH 6.8. The non-diffusible material was applied to a column of ECTEOLA-cellulose which had been pre-equilibrated with this buffer system. The PAPS 3'-nucleotidase activity passed through the column unretarded and was free of APS sulphohydrolase and PAPS sulphohydrolase.

Preparation of [^{35}S]PAPS

[^{35}S]PAPS was prepared by an enzymic method based on experimental conditions established in these laboratories[14], and purified according to the procedure of Banerjee and Roy[15] as modified by Bailey-Wood *et al.*[11].

Preparation of APS and [^{35}S]APS

APS was prepared by the method of Cherniak and Davidson[16] except that a double quantity of mono (trioctyl) ammonium AMP was employed and the sulphation was allowed to proceed at 4 °C in order to minimize the formation of products other than APS. The final product was stored in aqueous solution at pH 8.0 and −15 °C. It has been reported that APS is stable for at least 6 months if kept under these conditions[12].

[^{35}S]APS was obtained by the enzymic dephosphorylation of [^{35}S]PAPS. The following incubation mixture was employed for this purpose: 0.3 mM [^{35}S]PAPS (12.0 ml), 0.4 M sodium acetate–acetic acid buffer at pH 5.6, (4.0 ml), 0.08 M $CoCl_2$ (1.5 ml) and a suitable quantity of the PAPS 3'-nucleotidase preparation in a total volume of 24.0 ml. After incubation at 37 °C for 90 min the reaction was terminated by boiling for 2 min followed by cooling in ice. The precipitated protein was removed by centrifuging and the supernatant was adjusted to pH 7.0 with 2 M NH_4OH. The product was isolated according to the procedure of Cherniak and Davidson[16] and diluted to the required specific activity with unlabelled APS.

Assay of APS sulphohydrolase

The procedure adopted for the routine assay of APS sulphohydrolase was that described by Bailey-Wood *et al.*[11] with an APS concentration of 5.0 mM in 0.2 M sodium acetate–acetic acid buffer (pH 5.2). In this procedure liberated SO_4^{2-} is estimated after removal of unreacted APS. Two methods were employed for the determination of SO_4^{2-}, namely, the turbidimetric $BaSO_4$ method (Method B) of Dodgson[17] or a radioisotopic method in which ^{35}S-labelled APS was used as substrate. In the latter case samples (usually 50 μl) of the incubation mixture after removal of unchanged substrate were subjected to liquid scintillation counting in 10 ml of toluene –2-methoxyethanol (4:1, v/v) containing 2,5-diphenyloxazole (2.5 g/l). This was the more sensitive of the two methods and enabled a wide range of enzyme activities to be determined with relatively short enzyme/substrate incubation periods (usually 2–4 min). One unit of enzyme activity is defined as the amount which liberates 1 μmole of SO_4^{2-} from APS per min at pH 5.2 and 37 °C.

Assay of other enzymes

Other enzymes measured included rhodanese (EC 2.8.1.1)[18], glucose-6-phosphatase (EC 3.1.3.9)[19] and acid phosphatase (EC 3.1.3.2)[20] which were used as marker enzymes.

Determination of protein

Protein concentration was determined by the method of Lowry *et al.*[21] by reference to standard solutions of bovine serum albumin unless otherwise stated.

Cell fractionation procedure

The procedure was based on that of De Duve *et al.*[19] starting with 10 g of fresh liver. All pellets were resuspended in 0.25 M sucrose with the aid of a glass Dounce homogenizer, the nuclear pellet being diluted 1 to 5 (N fraction), the mitochondrial pellet 1 to 3 (M fraction), the lysosomal pellet 1 to 3 (L fraction), the microsomal pellet 1 to 4 (P fraction) whilst the soluble fraction was not diluted further (S fraction). Each fraction was assayed for APS sulphohydrolase and for appropriate marker enzymes and protein.

DEAE-cellulose chromatography and gel filtration

DEAE-cellulose (Whatman DE11 and DE52) was precycled according to the procedure recommended by the supplier and was washed extensively with buffer before use. Sephadex G-100 (Pharmacia) was equilibrated and packed at 4 °C in 0.1 M Tris–HCl buffer at pH 8.4 or pH 7.2. A 90 cm × 1.5 cm column (Pharmacia) was packed and eluted under a constant pressure of 18 cm of water and was used for molecular weight determinations after calibration with blue dextran 2000 (Pharmacia) and several protein standards[22].

Polyacrylamide disc gel electrophoresis

Polyacrylamide disc gel electrophoresis was carried out at ambient temperature in 7.5% (w/v) gels at pH 8.9 according to the procedure of Bailey-Wood[23]. Protein was visualized in the gels by staining with a 1% (w/v) solution of Amido-Schwartz dye in 7% (v/v) acetic acid for 1 h followed by electrolytic destaining. APS sulpho-

hydrolase activity was located by dispersing sections cut from unstained gels in cold 0.25 M sodium acetate–acetic acid buffer at pH 5.2 and assaying the supernatant solutions for enzyme activity.

Molecular weight determinations by polyacrylamide disc gel electrophoresis in sodium dodecyl sulphate were performed according to the procedure of Weber and Osborn[24].

Amino acid analysis

Samples of purified enzyme preparations (0.5 mg) were lyophilized and hydrolysed in 5.7 M HCl (2.0 ml) in a sealed glass tube at 110 °C for 48 h. Amino acids in hydrolysates were determined on a Locarte amino acid autoanalyzer using the buffer system of Spackman *et al.*[25]. Tryptophan[36] and cysteine[27] were determined by colourimetric methods.

RESULTS

Subcellular distribution of APS sulphohydrolase in bovine liver

Fig. 1 shows the intracellular distribution of APS sulphohydrolase in the bovine liver cell in relation to that of rhodanese, acid phosphatase and glucose-6-phosphatase which were employed as marker enzymes for the mitochondrial, lysosomal and microsomal fractions respectively. The results were plotted as relative specific activity on the basis of protein according to the procedure of De Duve *et al.*[19]. Contamination of the nuclear fraction with lysosomal and microsomal reference enzymes was found

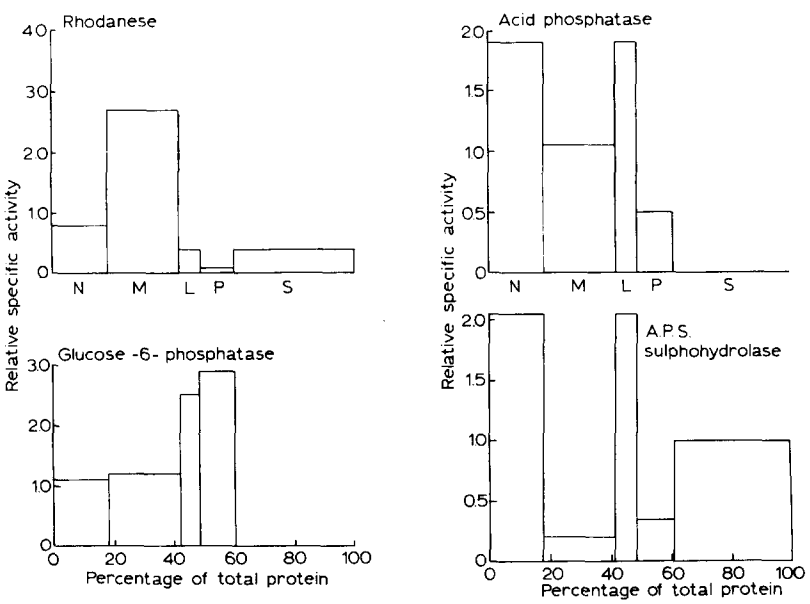

Fig. 1. Intracellular distribution of APS sulphohydrolase activity and various marker enzymes in the bovine liver cell. Fractionation procedure and enzyme assays were as described in the text. N, M, L, P and S represent the nuclear, mitochondrial, lysosomal, microsomal and cell sap fractions, respectively. The results are the average of five experiments.

to be more marked in bovine liver than in rat liver fractionations and in common with other workers[28] we were unable to obtain well resolved particulate fractions from bovine liver homogenates. The results indicate, however, a bimodal distribution of APS sulphohydrolase activity between the lysosomal and supernatant fractions similar to that observed by Bailey-Wood *et al.*[11] in rat liver. Comparison of the distribution pattern with that of acid phosphatase suggests that the considerable quantities of enzyme activity found in the supernatant fraction are present *per se* and not as a result of lysosomal rupture and/or leakage during the fractionation procedure.

Attempts were subsequently made to separate the APS sulphohydrolase activities from the supernatant and lysosomal fractions and to purify the enzyme from high-speed supernatant.

Purification of the high-speed supernatant enzyme

Stage 1. High-speed supernatant was prepared from bovine liver (5 kg on the preparative scale) as described above and was used immediately. Unless otherwise stated all purification procedures were performed at 4 °C and the degree of purification achieved at each stage is expressed relative to the specific activity of the high-speed supernatant preparation.

Stage 2. Sufficient solid $(NH_4)_2SO_4$ was slowly added to the supernatant to give 40% saturation (calculated from the nomogram of Dixon[29] with no correction for temperature) and after 2 h precipitated protein was separated and discarded. The concentration of $(NH_4)_2SO_4$ was then increased to 55% saturation, the precipitate was separated, redissolved in the minimum volume of 0.02 M citrate buffer (pH 6.6) and dialysed thoroughly against several changes of the same buffer.

Stage 3. The Stage 2 preparation was applied to a 50 cm × 6 cm column of fibrous DEAE-cellulose (Whatman DE11) which had been equilibrated with 0.02 M citrate buffer at pH 6.6. The column was then washed with the same buffer until the $A_{280\ nm}$ of the eluate fell below 0.1. Approx. 97% of the total protein was eluted under these conditions. The APS sulphohydrolase activity was then eluted with 0.06 M citrate buffer (pH 6.6). The purification factor at this stage of the preparation was typically 60-fold and the recovery of enzyme activity 40%.

Stage 4. The Stage 3 preparation was dialysed against several changes of 0.05 M Tris–HCl (pH 7.2) and applied to a 12 cm × 4 cm column of microgranular DEAE-cellulose (Whatman DE52) which had been equilibrated with this buffer system. After washing with starting buffer elution was continued with a convex NaCl concentration gradient (0 to 0.12 M NaCl, 10 column volumes) obtained by using a constant volume mixing vessel. Fractions (15 ml) were collected and assayed for protein and enzyme and appropriate ones were pooled and concentrated by precipitating the protein with $(NH_4)_2SO_4$ (80% saturation) and redissolving the separated material in a minimum volume of 0.02 M Tris–HCl (pH 7.2). A typical elution profile is shown in Fig. 2.

Stage 5. The concentrated Stage 4 preparation was subjected to gel filtration on a 90 cm × 2.5 cm column of Sephadex G-100 employing upward flow elution with 0.02 M Tris–HCl at pH 7.2. Fractions (5 ml) were collected and assayed for protein and enzyme and the active fractions were pooled and concentrated by the method described above. A typical elution profile is shown in Fig. 3A.

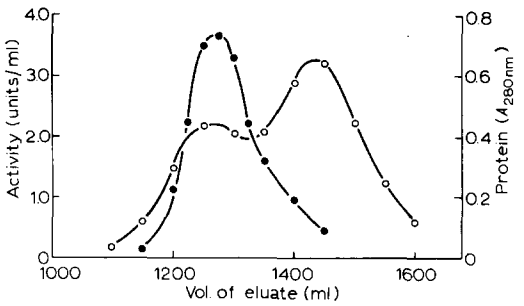

Fig. 2. Fractionation of Stage 3 protein by chromatography on DEAE-cellulose (Whatman DE52). Elution was performed with a convex NaCl concentration gradient (0 to 0.12 M NaCl in 0.05 M Tris–HCl, pH 7.2, 10 column volumes). APS sulphohydrolase activity (●) was assayed as described in the text, protein concentration (○) was measured as $A_{280\ nm}$.

Stage 6. The concentrated Stage 5 preparation was again subjected to gel filtration on Sephadex G-100 under the conditions described above. Fractions containing the peak of APS sulphohydrolase activity were pooled and stored in the frozen state. The purification achieved with respect to the high-speed supernatant was 1200-fold with a recovery of about 5% of the enzyme activity. The symmetry of the elution profile for the second fractionation on Sephadex G-100 (Fig. 3B) and the constant specific activity of the active fractions suggested that an examination of the purity of the preparation might be appropriate at this stage.

A summary of the purification procedure is shown in Table I. The various stages were tested for PAPS-degrading activity according to the procedure of Bailey-Wood *et al.*[11]. This activity had virtually disappeared at Stage 3 and was totally absent from all subsequent stages. In addition no ATPase or ADPase activity could be detected in the final preparation.

Polyacrylamide disc gel electrophoresis

Samples of the Stage 6 enzyme preparation (20–200 μg of protein) were sub-

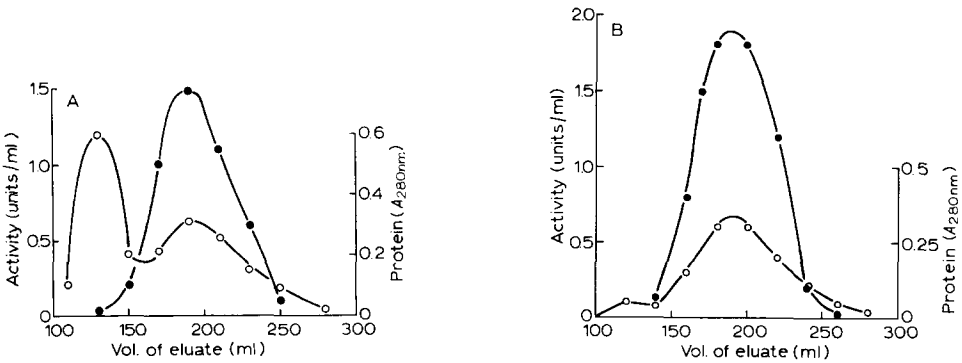

Fig. 3. (A) Fractionation of Stage 4 protein by gel filtration on a column (90 cm × 2.5 cm) of Sephadex G-100 employing upward flow elution with 0.02 M Tris–HCl buffer at pH 7.2. APS sulphohydrolase activity (●) was assayed as described in the text, protein (○) was measured as $A_{280\ nm}$. (B) Re-chromatography of Stage 5 protein on Sephadex G-100. APS sulphohydrolase activity (●) was assayed as described in the text, protein (○) was measured as $A_{280\ nm}$.

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE FOR APS SULPHOHYDROLASE FROM BOVINE LIVER HIGH-SPEED SUPERNATANT

Stage	*Procedure*	*Vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Recovery (%)*	*Spec. activity (units/mg protein)*	*Purification*
1	High-speed supernatant	7300	420 000	2117	100	0.005	1
2	$(NH_4)_2SO_4$ precipitate (40–55% saturation)	1000	107 000	1200	60	0.011	2.2
3	DEAE-cellulose DE11	1000	2 700	840	40	0.31	60
4	DEAE-cellulose DE52	100	210	392	19	1.9	373
5	Sephadex G-100	55	33	149	7	4.5	900
6	Sephadex G-100 rechromatography	30	15	90	5	6.0	1200

jected to polyacrylamide disc gel electrophoresis as described above. The single band of protein detected by staining with Amido-Schwartz dye was coincident with the position of APS sulphohydrolase activity. In addition polyacrylamide disc gel electrophoresis in sodium dodecyl sulphate resulted in a single homogeneous band when these gels were stained with Coomassie blue[24].

Amino acid composition of APS sulphohydrolase

The amino acid composition of the Stage 6 enzyme is shown in Table II. It is possible that the apparent deficiency in methionine may be due to loss by oxidation during hydrolysis. There were only slight variations in the amino acid composition of subsequent enzyme preparations and this provides further evidence of homogeneity in the final preparation.

Molecular weight

The molecular weight of APS sulphohydrolase was estimated by gel filtration of the enzyme, at various concentrations, on a calibrated column of Sephadex G-100. The elution volume of the enzyme was determined by assaying small fractions (2 ml)

TABLE II

AMINO ACID COMPOSITION OF APS SULPHOHYDROLASE

Experimental details are given in the text. Results are average value of two separate enzyme preparations.

Amino acid	*No. of residues/molecule of enzyme*	*Amino acid*	*No. of residues/molecule of enzyme*
Cys	7	Met	—
Asp	66	Ile	32
Thr	29	Leu	60
Ser	37	Tyr	16
Glu	92	Phe	22
Pro	32	His	25
Gly	61	Lys	54
Ala	62	Arg	30
Val	34	Try	3

for enzyme activity and for protein concentration ($A_{280\ nm}$). A value of 69 000±3000 (average of three determinations) was obtained by this method.

Polyacrylamide disc gel electrophoresis in sodium dodecyl sulphate according to the procedure of Weber and Osborn[24] gave a value of 68 000 ± 2000. This value represents the molecular weight of denatured and reduced enzyme and is the average of four determinations.

DISCUSSION

The present work indicates that the subcellular distribution of APS sulphohydrolase activity in bovine liver is similar to the bimodal distribution of the enzyme observed in rat liver by Bailey-Wood *et al.*[11]. Furthermore it has been established that the desulphation of APS by high-speed supernatant preparations of bovine liver is the result of the action of a single homogeneous APS sulphohydrolase which is devoid of activity towards PAPS. The molecular weight of the purified sulphohydrolase has been determined as 68 000–69 000 and it seems reasonable to assume that the enzyme is not composed of subunits linked either by hydrogen bonds or by disulphide cross-linkages since similar values for molecular weight were obtained by gel filtration and by polyacrylamide disc gel electrophoresis in sodium dodecyl sulphate. Amino acid analysis of the purified enzyme has revealed a composition typical of a globular protein except for an apparent deficiency in methionine. It is perhaps of interest that the high proline content which is a feature of arylsulphohydrolase preparations (EC 3.1.6.1)[30,31] is not observed in APS sulphohydrolase.

The procedures described in this paper permit the isolation of moderate quantities of APS sulphohydrolase in an apparently homogeneous form and the previously reported[11] substrate specificity of this enzyme has been confirmed by the present work. The basis of this substrate specificity is currently being studied.

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