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PURIFICATION AND PROPERTIES OF AN ISOENZYME OF ARYLSULPHATASE FROM *ASPERGILLUS ORYZAE*

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SUMMARY

The purification and some properties of an isoenzyme of arylsulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) from *Aspergillus oryzae* are described. Evidence is presented to support the hypothesis that this is the "classical" arylsulphatase of *A. oryzae*. Comparisons of the molecular weight (100 000) and Michaelis constant ($0.48 \cdot 10^{-4}$ M for *p*-nitrophenyl sulphate) are made with another recently reported isoenzyme. The essential dissimilarity of the two isoenzymes is described and discussed.

INTRODUCTION

Takadiastase is a commercial medicinal enzyme preparation made by growing *Aspergillus oryzae* on a heat-sterilised mash prepared from crushed wheat or rice. The mash is washed free of soluble mould products after a suitable period of growth. The washings are dried and the resultant solids finely ground. The activity of the powder is adjusted on the basis of its α-amylase (EC 3.2.1.1) content, so that the commercial powder is capable of hydrolysing 450 times its own mass of starch in 10 min at neutral pH. Amongst other enzymes, Takadiastase has been known for many years to possess arylsulphatases (aryl-sulphate sulphohydrolase, EC 3.1.6.1). Wheat and rice mashes have no arylsuphatase activity. Since those cereals contain only a low percentage of sulphur, it seems possible that the phenolic sulphate esterases are formed in response to this low sulphur level.

Cherayill[1] reported briefly the discovery of three isoenzymes of arylsulphatase in extracts of *A. oryzae* cultured as in the commercial manner. Benkovik *et al.*[2] have reported the purification and some properties of one of the isoenzymes grown in a similar fashion. This paper describes the purification in good yield of an isoenzyme physically and chemically distinct from that of Benkovik *et al.*[2]. Our evidence suggests that we have isolated the "classical" arylsulphatase of *A. oryzae*[3,4]. The optimum pH was 6.3, K_m was $0.48 \cdot 10^{-4}$ M using *p*-nitrophenyl sulphate. The molecular size of the

Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

isoenzyme, by gel chromatography, was in accordance with a molecular weight of 100 000.

MATERIALS AND METHODS

Arylsulphatase assay

The method used was adapted from Robinson *et al.*[4]. *p*-Nitrophenyl sulphate was prepared by the method of Dodgson and Spencer[8]. *p*-Nitrophenyl sulphate (30 mM, 0.5 ml) in acetate buffer (0.5 M) at pH 6.3 was incubated at 37 °C for 1 h with 0.5 ml of enzyme samples. The reaction was terminated and the nitrophenoxide colour developed with NaOH (1 M, 2 ml). The absorbance was determined at 400 nm. Controls with enzyme added after the NaOH were performed. In experiments to determine the effect of varying ionic strengths on the enzyme, it was found that the enzymic activity was unaltered in the range from 0.1 to 1.0 M acetate–acetic acid buffer.

Protein assay

The method of Lowry *et al.*[9] was used.

EXPERIMENTAL RESULTS

Takadiastase (Parke-Davies, Ltd) and "α-amylase type IVA" (Sigma, Ltd) were used as starting materials for the arylsulphatase. The specific activities of these two sources were identical.

Step A (salt fractionation)

12 g of the source material was stirred into 0.1 M sodium acetate–acetic acid buffer, pH 6.3 (300 ml) at 4 °C. $(NH_4)_2SO_4$ (195 g) was stirred in rapidly to effect 90% saturation[5]. Stirring at 4 °C was maintained for 30 min. The precipitate was recovered by centrifugation (20 000 × *g* for 10 min), suspended in 70% (w/v) aqueous $(NH_4)_2SO_4$ (100 ml) and stirred at room temperature for 6 h. The suspension was centrifuged as before and the pellet discarded. The supernatant, constituting the Step A preparation was dialysed against chilled distilled water (5 l, 2 changes) for 24 h and freeze-dried. Details of recoveries from this and subsequent stages are shown in Table I.

TABLE I

Purification step	*Arylsulphatase activity (units*)*	*Recovery (%)*	*Protein (mg)*	*Specific activity*
Crude material	8.33	100	12 000**	0.694
Step A	4.24	50.9	1 020***	4.16
Step B	2.92	35.0	103***	28.35
Step C	1.74	21.0	15***	116.0

* 1 unit is taken as the liberation of 1 μmole of *p*-nitrophenol per min under the standard assay conditions.

** Determined by weighing only and includes much insoluble material.

*** Determined by the protein assay.

Step B (Sephadex G-100 chromatography)

The Step A preparation was dissolved in 0.1 M acetate buffer, pH 6.3 (10 ml) and chromatographed on a column of Sephadex G-100 (265 ml volume). The arylsulphatase was found to be co-incident with the first protein peak (Fig. 1) monitored at 280 nm. The Step B preparation was then dialysed and lyophilised as in Step A.

Step C (DEAE-cellulose chromatography)

Ion-exchange chromatography was performed using a column 15 cm × 1 cm of DEAE-cellulose (Whatman DE-52). Pre-swollen DE-52, cleared of fines, was washed with 0.2 M Tris–HCl buffer, pH 7.5, until no change in pH was detected. It was then packed into a glass column. Pre-equilibration was carried out with a solution of the same Tris buffer at 50 mM concentration. The Step B preparation was taken up in 5 ml of the 50 mM Tris buffer and dialysed for 5 h against the same buffer. It was then added to the top of the column and given 1 h equilibration before elution was begun. This was performed using a linear gradient 50 to 200 mM Tris–HCl, pH 7.5, superimposed on a linear gradient 0–1 M NaCl. The gradients were developed on a 3-channel pump[6]; the total volume was 125 ml. Arylsulphatase was recovered after about 30 ml elution (Fig. 2). The recovered arylsulphatase was found to denature when dialysed

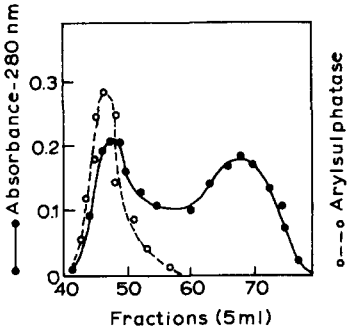


Fig. 1. The elution of the Step A preparation from Sephadex G-100. Absorbance at 280 nm was monitored automatically. The arylsulphatase activity is expressed in arbitrary units. Other conditions as described in the text.

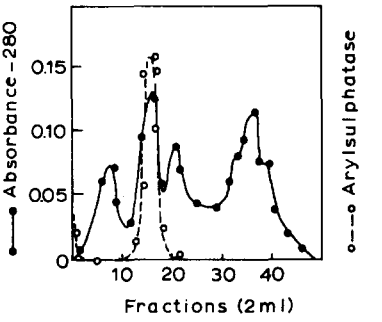


Fig. 2. The elution of the Step B preparation from DEAE-cellulose. Absorbance at 280 nm was monitored automatically. The arylsulphatase activity is expressed in arbitrary units. Other conditions were as described in the text.

against water and was therefore dialysed against 2% (w/v) aqueous $(NH_4)_2CO_3$ as a stabiliser. After freeze–drying (during which the $(NH_4)_2CO_3$ was lost by sublimation), this constituted the Step C preparation. The Step C preparation was homogeneous save for minor contaminants when tested by polyacrylamide gel electrophoresis and end-group analysis. DE-52 chromatography of Takadiastase showed only one position of arylsulphatase elution, although a small percentage of the applied activity was not bound by the cellulose. This unbound fraction was also detected in chromatography of the Step A preparation.

Polyacrylamide gel electrophoresis and end-group analysis

Polyacrylamide gels of 7.5% cross-linkage were cast in glass tubes (60 mm × 5

mm) in Tris–HCl buffer pH 7.5, using ammonium persulphate polymerisation and the general procedures of Davis[7]. The reservoir buffer was Tris–glycine, pH 9.5. Samples for electrophoresis were added to the top of the gel in 30% (w/v) sucrose in pH 9.5 buffer. Duplicate gel samples were either stained using amido black or were frozen rapidly, sliced and assayed for arylsulphatase. The single major protein band of the Step C preparation showed concurrent arylsulphatase activity. Takadiastase, Step A and Step B preparations, appeared to possess only a single arylsulphatase. However, polyacrylamide gel electrophoresis has problems regarding arylsulphatase work[12] and it is possible that low levels of arylsulphatase would not be detected.

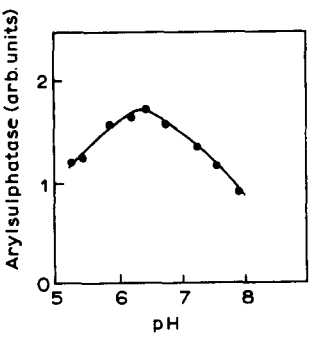
As a further check, the dinitrophenyl derivative of the N-terminal amino acid of the polypeptide was prepared[11]. Step C enzyme (2–3 mg) was dissolved in water (0.2 ml) containing $NaHCO_3$ (10 mg). 1-Fluoro-2,4-dinitrobenzene (FDNB) was added (1 ml of a 1% w/v ethanolic solution). After 2 h the suspension was brought to pH 4 with HCl. Unreacted FDNB was removed by successive washes with water saturated diethyl ether until the washings were colourless. The aqueous phase was dried *in vacuo* after which 6 M HCl (3 ml) was added. This mixture was sealed and autoclaved at 120 °C for 4 h. DNP-amino acids were extracted with ether and dried. Thin-layer and paper chromatography were carried out on the extracted DNP-amino acids with *tert*-amyl alcohol saturated with water. A single DNP-amino acid was observed and on the basis of its R_F with respect to standard DNP-amino acids (B.D.H. Ltd) it was ascribed to phenylalanine. A similarly treated sample of Takadiastase showed a complete range of DNP-amino acids.

Molecular size determination

The molecular size of the arylsulphatase was determined by the method of Andrews[10] with Sephadex G-100, calibrated with blue dextran, bovine serum albumin, ovalbumin, horse-heart cytochrome *c* and methyl green. The arylsulphatase eluted at a point consistent with its having the molecular size of a protein of molecular weight of 100 000 assuming it to be spherical and of normal density.

Optimum pH

The pH activity profile (Fig. 3) was determined using a modification of the



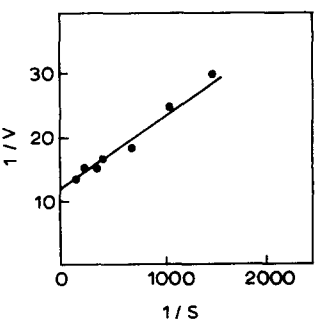


Fig. 3. The activity of the arylsulphatase Step B preparation *versus* pH. Tris–maleate buffers were used (0.2 M) with *p*-nitrophenyl sulphate at 15 mM concentration.

Fig. 4. Lineweaver–Burk plot of the enzymic activity (v) and substrate concentration (s). v is expressed in μmoles of *p*-nitrophenol liberated ($\times$ 100); s is expressed in moles/l. Other conditions are described in the text.

arylsulphatase assay with saturating substrate (15 mM). Tris–maleate buffers (0.2 M) were used. The enzyme was a sample of Step B preparation at an incubation concentration of 1 mg/ml. A broad optimum at pH 6.3 was indicated. The pH optimum was measured also in the same buffers in NaCl at 0.2, 0.4 and 0.8 M to test the effect of ionic strength; no variation was observed. Cl^- exerted no activating effect.

Michaelis constant, K_m

The arylsulphatase assay was performed under conditions of varying substrate concentration to give data for the Lineweaver–Burk plot (Fig. 4). A 1% (w/v) sample of the Step B preparation was used. The graph indicated K_m as being 0.48 mM. No deviations were observed over the range of substrate covered. This isoenzyme does not appear to suffer from substrate inhibition using *p*-nitrophenyl sulphate.

DISCUSSION

On the basis of the concomitant position of arylsulphatase elution with the protein peak elution in DEAE-cellulose chromatography, we believed that the Step C preparation was mainly pure arylsulphatase. This was given further weight by finding only one major protein band on an amido black stained polyacrylamide gel after electrophoresis since on a parallel gel, arylsulphatase activity was observed at the corresponding position. The observation of a single DNP-amino acid from the Step C preparation gave further confirmation.

Our inability to detect evidence of the presence of other arylsulphatase isoenzymes in commercial *A. oryzae* preparations is perhaps at first sight rather strange. However, the observation that a small percentage of arylsulphatase activity from the Step B preparation was not bound by DEAE-cellulose may explain this. Also as polyacrylamide gel electrophoresis causes some reduction in arylsulphatase activity, our inability to detect other isoenzymes in Takadiastase may be explicable. It appears certain that Takadiastase is a much richer source of one isoenzyme of arylsulphatase than of others. The relative proportions of the three isoenzymes is known to be dependent on the level of, *e.g.* molybdenum, in the growth medium[1] and this may in part explain the difference in isoenzyme levels in our source material compared with those in ref. 1 and ref. 2.

There is, however, another possibility. The *A. oryzae* used for the production of Takadiastase may not be the same as that used in other studies. We have observed that not all *A. oryzae* strains produce arylsulphatase (unpublished observations). Strains of *A. oryzae* are difficult to type; mixed strains of *A. oryzae* and *Aspergillus flavus* are common. The wide variation in the sources of *A. oryzae* strains may in part be responsible for the varying enzyme complement.

The molecular size of the isoenzyme suggests a molecular weight of 100 000. That of Benkovik *et al.*[2] had a molecular weight of 65 000. The two isoenzymes have markedly different pH optima; 6.3 and 4.8, respectively. The two isoenzymes have the same K_m for *p*-nitrophenyl sulphate, however. It might therefore be possible to speculate that *A. oryzae* possesses multiple arylsulphatase isoenzymes to accomodate variations in external pH.

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