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PURIFICATION AND PROPERTIES OF ARYLSULPHATASE OF *ASPERGILLUS NIDULANS*

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SUMMARY

A method for the purification of arylsulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) of *Aspergillus nidulans* has been described. The enzyme elutes from DEAE-cellulose in two distinct fractions, designated as Fraction I and Fraction II. Fraction I was purified over 450-fold and Fraction II over 600-fold. The two fractions were characterized with respect to some of their physical and biochemical properties. They differ in their K_m values for *p*-nitrophenyl sulphate and nitrocatechol sulphate, sensitivity to some inhibitors, electrophoretic mobility and heat stability. In most other properties they are similar.

INTRODUCTION

The presence of arylsulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) activity in takadiastase from *Aspergillus oryzae* has been known for a long time[1]. ABBOT[2] has described the properties of unpurified sulphatase of takadiastase. HARADA AND SPENCER[3] found that, in a number of fungi including *Aspergillus nidulans*, the arylsulphatase activity of a culture depended upon the source of sulphur in the medium. In particular inorganic sulphate and sulphur-containing amino acids inhibited the formation of arylsulphatase. We have shown that the repression of arylsulphatase in *A. nidulans* is genetically regulated[4]. Upon derepression two distinct fractions of sulphatase are formed. In course of an investigation of the genetic control of sulphatase in *A. nidulans*, we purified the two fractions and examined their properties. We present here a method for purification and a comparative account of the properties of these two fractions of arylsulphatase in *A. nidulans*.

MATERIALS AND METHODS

Culture and medium. A green, biotin-requiring strain of *A. nidulans*, *Y bi*$_1$, obtained from G. Pontecorvo was used.

The minimal medium contained $NaNO_3$, 6 g; KH_2PO_4, 1.52 g; KCl, 0.52 g;

$MgSO_4 \cdot 7\ H_2O$, 0.52 g; and dextrose, 15 g per l of distilled water. The pH was adjusted to 6.5 with NaOH before sterilization. The medium was solidified with 1.5% agar when necessary. The *Y* bi_1 strain was maintained on minimal medium slants.

Assay of sulphatase. The activity of sulphatase was measured by incubating a mixture of 0.5 ml of *p*-nitrophenyl sulphate (2 mg/ml in 0.5 M Tris buffer, pH 7.4) with a suitable amount of the enzyme preparation at 37°. The reaction was stopped by adding 2 ml of 0.2 M NaOH and the final volume brought up to 3 ml. The absorbance of the liberated *p*-nitrophenol was measured at 410 mμ in a Bausch and Lomb colorimeter, using 0.5-cm cells.

An unit of sulphatase activity is arbitrarily defined as an increase in absorbance of 1 per h.

Starch-gel electrophoresis. The electrophoretic mobility of sulphatase in starch gel was determined according to the method of SMITHIES[5]. 13 g hydrolysed starch were digested in 100 ml of Veronal buffer by gentle heating. The hot slurry was deaerated by suction and was quickly poured in the form of 10 × 3.5 × 1.25 cm^3 slabs in perspex former.

6 units each of Fraction I and Fraction II were loaded on small 3 MM filter paper strips and the strips inserted in slots in the gel. The electrophoresis was carried out with 20 mA current and 160 V, at 2° for 16 h.

Staining of the gel. After the completion of the run, the gel was stained using a histochemical staining technique[6]. The gel was flooded with 40 ml of α-naphthyl sulphate (4 mg/ml in Tris buffer, pH 7.4) and incubated for 10 min at 37°. 20 ml of Fast Red Violet LB salt (10 mg/ml in water made alkaline with a few drops of 6% sodium tetraborate) were poured on the gel. The brown colour resulting from the combination of the Fast Red with the free α-naphthol radical tagged onto sulphatase showed its position in the gel after electrophoresis.

Determination of molecular weights. The molecular weights were determined from relative sedimentation velocity in a 5–20% sucrose gradient by the method of MARTIN AND AMES[7]. 0.1 ml of a mixture containing 20 units of Fraction I, 100 units of alkaline phosphatase and 10 mg of egg white lysozyme was layered on a 4.5-ml gradient. The second gradient was layered with a mixture of 20 units of Fraction II, alkaline phosphatase and lysozyme. A third gradient was layered with 20 units each of Fraction I and Fraction II, alkaline phosphatase and lysozyme. The three tubes were centrifuged at 37 000 rev./min in a Spinco, SW-39 rotor for 16 h at 4°. 2-drop fractions were collected in 0.5 ml of Tris buffer. The distribution of lysozyme in the gradient was determined by measuring the absorption of each fraction at 280 mμ. 0.1 ml from each fraction was assayed for phosphatase and the rest for sulphatase. Lysozyme (mol. wt. 14 000; $S = 1.87$ S) and alkaline phosphatase (mol. wt. 89 000; $S = 6.1$ S) served as reference markers.

Buffers. 0.01 M Tris–HCl buffer, pH 7.4, contained 0.01 M NaCl and 2 mM $MgCl_2$. Veronal buffer at pH 8.6 was used for starch-gel electrophoresis.

Culture and derepression. A tenth of a ml of a suspension of the conidia of *A. nidulans* was spread on the surface of minimal agar slants in 3.5-cm-wide test tubes. The slants were incubated at 37°. After 5 days of incubation, conidia were harvested from five slants, by pouring 5 ml of 1/10 000 Tween-20 in each tube and scraping the conidia from the surface. The pooled suspension was shaken vigorously on a microid shaker for 15 min to break conidial chains. It was then filtered through

loosely packed sterile, non-absorbent cotton to remove the mycelial debris. The conidia were counted in a haemocytometer and their concentration adjusted to 10^7 conidia/ml.

4 l of liquid minimal medium supplemented with 4 ml of 0.1% biotin were dispensed in 500 -ml volumes in eight 2000-ml baffled flasks. Each flask was inoculated with 0.5 ml of the conidial suspension. The flasks were incubated on a gyrotory shaker at 37°. The culture grew in the form of mycelial pellets.

After 16 h of incubation the mycelial pellets were collected on a Buchner funnel, washed with 500 ml of liquid minimal medium free of inorganic sulphate and dried by suction. The mycelial pad was resuspended in 500 ml of sulphate free liquid medium and incubated on a shaker at 37° for 15 min. This procedure ensures removal of residual sulphate from the mycelium. The culture was filtered again and dried. The mycelial pad was suspended in 100 ml of sulphate-free liquid medium and dispersed by vigorous shaking. 6 l of sulphate-free liquid medium were distributed equally in ten 2000-ml baffled flasks. 10 ml of the mycelial suspension was added to each flask and the flasks were incubated on a gyrotory shaker for 20 h at 37°.

After 20 h of incubation the culture was harvested on a Buchner funnel and washed with 200 ml of 0.01 M Tris buffer at pH 7.4. About 200 g wet weight of mycelium was obtained.

Enzyme extraction. All operations were carried out at 4°, unless otherwise specified. 200 g wet weight of the mycelium was suspended in 1000 ml of 0.01 M Tris buffer at pH 7.4 and homogenized in six lots in a 300-ml Potter–Elvehjem homogenizer with an electrically operated motor. The homogenate was filtered through cheese cloth. The solid residue was resuspended in 400 ml of 0.01 M Tris buffer at pH 7.4, homogenized as before in two lots and filtered. The two filtrates were pooled. 1500 ml of this extract contained 50 000 units of sulphatase activity and 16 530 mg of protein.

Chemicals. All chemicals used were of analytical reagent quality unless otherwise stated. The hydrolysed starch was obtained from Connought Laboratories, New York, N.Y., U.S.A. *p*-Nitrophenyl sulphate, nitrocatechol sulphate and DEAE-cellulose were products of Sigma Chemical Company, St. Louis, Mo., U.S.A.

RESULTS

Purification of arylsulphatase

(I) $(NH_4)_2SO_4$ fractionation. 585 g of solid $(NH_4)_2SO_4$ were added slowly to 1500 ml of the light yellow culture extract with constant stirring (to a final saturation of 60%). The extract was stirred until the salt dissolved, stirred for an additional 15 min and then centrifuged at 12 000 × *g* for 15 min. The precipitate was discarded. Another 565.5 g of $(NH_4)_2SO_4$ were added to the supernatant as before. After centrifugation the supernatant was discarded and the precipitate dissolved in 0.01 M Tris buffer at pH 7.4, yielding 200 ml of a light-yellow solution. This 60–100% $(NH_4)_2SO_4$ fraction contained 63 000 units of enzyme activity and 11 620 mg of protein. Although the total enzyme activity had increased, the enzyme at this stage was unstable and lost activity with a half-life of about 24 h, when stored at 4°.

(II) Ethanol fractionation. 133.3 ml of absolute ethanol at −20° were added dropwise to the 200-ml frozen, $(NH_4)_2SO_4$ fraction to reach 40% saturation. The

$(NH_4)_2SO_4$ fraction melted after the addition of about 6–7 ml of ethanol and was stirred continuously during further addition of ethanol. The mixture was stirred for an additional 15 min and centrifuged at 12 000 × *g* for 15 min. The precipitate was discarded. To the supernatant another 333.3 ml of absolute ethanol at −20° were added as before to reach 70% saturation. The mixture was stirred for 15 min and centrifuged at 12 000 × *g* for 15 min. The supernatant was discarded. The precipitate was dissolved in 90 ml of 0.01 M Tris buffer at pH 7.4. It contained 40 500 units of sulphatase and 540 mg of protein.

About 14-fold purification was achieved by ethanol fractionation. This fraction was more stable than the $(NH_4)_2SO_4$ fraction and lost activity with a half-life of about 4 days, when stored at 4°. A repetition of the ethanol precipitation step greatly enhanced the stability of the enzyme.

60 ml of absolute ethanol at −20° were added to 90 ml of the 'first' ethanol fraction to reach 40% saturation. The precipitate was discarded. To the supernatant 150 ml of ethanol were added with constant stirring to reach 70% saturation. The precipitate was collected by centrifugation at 12 000 × *g* and dissolved in 44 ml of 0.01 M Tris buffer at pH 7.4. The supernatant was discarded. The 'second' 40–70% ethanol fraction contained 30 000 units of sulphatase activity and 140 mg of protein.

About 50% of the sulphatase activity of the $(NH_4)_2SO_4$ fraction was recovered in this fraction. The preparation was stable.

(III) Treatment with nucleases. The 'second' 40–70% ethanol fraction (44 ml) was dialysed overnight against 5000 ml of 0.01 M Tris buffer (pH 7.4) at 2°. 50 ml of the dialysed enzyme was incubated at 37° with 0.5 mg of deoxyribonuclease (10 μg/ml final concentration) and 0.5 mg of bovine pancreatic ribonuclease (10 μg/ml final

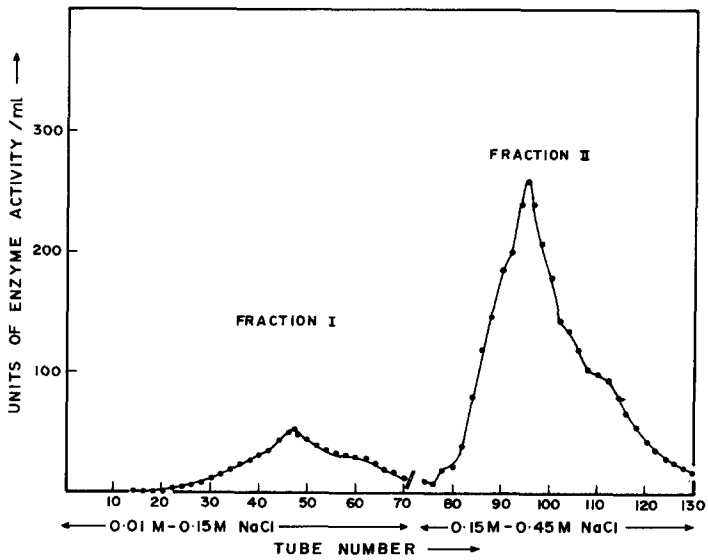

Fig. 1. DEAE-cellulose elution profile of sulphatase. The sulphatase activity elutes in two distinct fractions, one eluting between 0.01 and 0.15 M NaCl gradient and the other between 0.15 and 0.45 M NaCl gradient. The two fractions were designated as Fraction I and Fraction II, respectively.

concentration) for 1 h after which it was dialysed overnight against 5000 ml of 0.01 M Tris buffer (pH 7.4) at 2°.

(IV) Chromatography on DEAE-cellulose. 5 g of *N,N*-diethylamino ethyl cellulose soaked in water were washed extensively with 50 ml of 1 M NaCl, followed by 1 l of distilled water. The resin was packed in a 2-cm column to a height of 8 cm under pressure of 10 lb/inch^2. The column was equilibrated with 0.01 M Tris buffer (pH 7.4) for 1 h.

51 ml of the dialysed, nuclease treated fraction was applied to a DEAE-cellulose column at a rate of 1 ml/min. The column was washed with 50 ml of 0.01 M Tris buffer at pH 7.4 and 100 ml of a linear salt gradient, 0.01 to 0.15 M NaCl in 0.01 M Tris buffer was applied to the column. The flow rate was adjusted to 20 ml/h. 2-ml fractions were collected. After the elution was over, the column was washed with an additional 20 ml of 0.15 M NaCl in 0.01 M Tris buffer. A 100 ml of a second salt gradient, 0.15 to 0.45 M NaCl in 0.01 M Tris buffer, was applied to the column and the elution of sulphatase was continued at the same flow rate.

Fig. 1 shows the elution profile of sulphatase activity from DEAE-cellulose.

TABLE I

SUMMARY OF THE PURIFICATION OF ARYLSULPHATASE FROM *A. nidulans*

Fraction	*Vol. (ml)*	*Total enzyme (units)*	*Total protein (mg)*	*Specific enzyme activity (units/mg protein)*
1. Crude extract	1500	50 000	16 530	3.3
2. 60–100% $(NH_4)_2SO_4$ precipitation (pH 7.4)	200	63 000	11 620	5.42
3. 40–70% first ethanol precipitation	90	40 500	540	74
4. 40–70% second ethanol precipitation	44	30 000	140	214
5. Deoxyribonuclease and ribonuclease treated and dialyzed fraction	51	23 000	110	209
6. DEAE-cellulose column				
Fraction I	6	252	0.54	466.6
Fraction II	6	1 170	1.80	629.9

The sulphatase activity elutes in two distinct fractions, one eluting between 0.01 to 0.15 M NaCl and the other between 0.15 and 0.45 M NaCl. The two fractions were designated as Fraction I and Fraction II, respectively. About 80% of the enzyme applied was eluted from the column.

The contents of three tubes in each of the peak regions of Fraction I and of Fraction II were pooled. The resulting 6 ml each of Fraction I and Fraction II were stored at 2°. These peak fractions were used for further characterization. The purification of sulphatase is summarized in Table I.

Properties of arylsulphatase

(I) Substrate specificity. Among the various aromatic sulphates tested, phenyl sulphate, *p*-nitrophenyl sulphate and nitrocatechol sulphate are good substrates for Fractions I and II. Both fractions show some activity towards phenolphthalein disulphate. No enzyme activity is, however, detected when indoxyl sulphate or choline

TABLE II

SUBSTRATE SPECIFICITY: COMPARISON OF THE HYDROLYSIS OF SOME SULPHATE ESTERS BY FRACTION I AND FRACTION II OF SULPHATASE

The rate of hydrolysis is expressed relative to *p*-nitrophenyl sulphate. The reactions were carried out at 37° with 2 mg/ml of all sulphate esters and 5 units of enzyme.

Substrate	*Relative rate of hydrolysis*	
	Fraction I	*Fraction II*
p-Nitrophenyl sulphate	1.0	1.0
Phenyl sulphate	0.9	0.95
O-Nitrocatechol sulphate	0.8	0.75
Phenolphthalein disulphate	0.05	0.05
Indoxyl sulphate	0.01	0.01
Choline sulphate	0.01	0.01

sulphate are used as substrates. Table II presents a comparison of the hydrolysis of some sulphate esters by Fraction I and Fraction II.

The Michaelis constants of Fraction I and Fraction II were determined with *p*-nitrophenyl sulphate and nitrocatechol sulphate as substrates. The values of K_m for the two substrates were determined directly from the Woolf plots (Figs. 2 and 3). The K_m values of Fractions I and II of sulphatase for *p*-nitrophenyl sulphate are $3.5 \cdot 10^{-5}$ M and $1.0 \cdot 10^{-4}$ M, respectively. The K_m values of the two fractions for nitrocatechol sulphate are $9.5 \cdot 10^{-4}$ M and $5.0 \cdot 10^{-4}$ M, respectively.

(II) pH optima. The pH optima for the two fractions of sulphatase were determined with a series of buffers, ranging in pH from 5.32 to 9.95. Both fractions show optimal activity in a broad range of pH from 6.5 to 8.7. The enzyme activity falls sharply above pH 9.2.

(III) Effect of inhibitors on sulphatase. The effect of some inhibitors on the activity of Fraction I and Fraction II is shown in Table III. Both fractions are

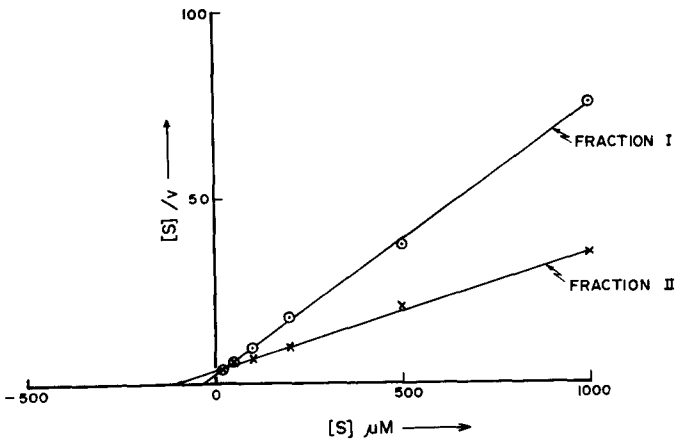

Fig. 2. Effect of substrate concentration on the rate of enzymic hydrolysis of *p*-nitrophenyl sulphate. The reaction was carried out at 37° in 0.2 M Tris at pH 7.4.

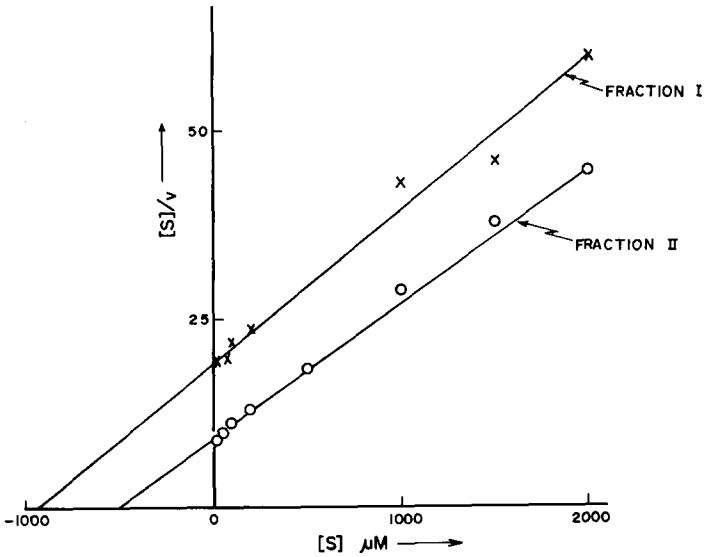

Fig. 3. Effect of substrate concentration on the rate of enzymic hydrolysis of nitrocatechol sulphate. The reaction was carried out at 37° in 0.2 M Tris at pH 7.4.

strongly inhibited by KCN and sulphite. Fraction II is not susceptible to iodoacetic acid whereas Fraction I is inhibited almost 70%. Phosphate and sulphate cause only 20% inhibition of sulphatase activity. In general Fraction I is more sensitive to the inhibitors than Fraction II.

(IV) Effect of temperature on the rate of substrate hydrolysis. Fig. 4 shows a plot of per cent of maximum rate of enzyme activity against temperature. In the temper-

TABLE III

SHOWING THE EFFECT OF SOME INHITORS ON THE ACTIVITY OF FRACTION I AND FRACTION II OF SULPHATASE

The incubation mixture of 1 ml at pH 7.4 contained 5 units of Fraction I or Fraction II, 10 mmoles of *p*-nitrophenyl sulphate and the inhibitor. The enzyme reaction was stopped after 10 min of incubation at 37° by adding 0.2 M NaOH and the absorbance measured at 410 mμ.

Inhibitor	*Concentration (mM)*	*Relative enzyme activity*	
		Fraction I	*Fraction II*
None	—	100	100
Na_2SO_4	10	82.6	80
Na_2SO_3	10	8.7	37.1
$Na_2S_2O_3$	10	73.9	97.1
Na_2S	10	91.4	94.2
Na_2HPO_4	10	77.2	91.4
Methionine	10	60.8	97.1
Sodium arsenite	10	78.2	100
Sodium arsenate	10	69.5	100
NaF	10	69.5	97.1
Iodoacetic acid	10	30.4	97.1
KCN	1	Negligible	7.1

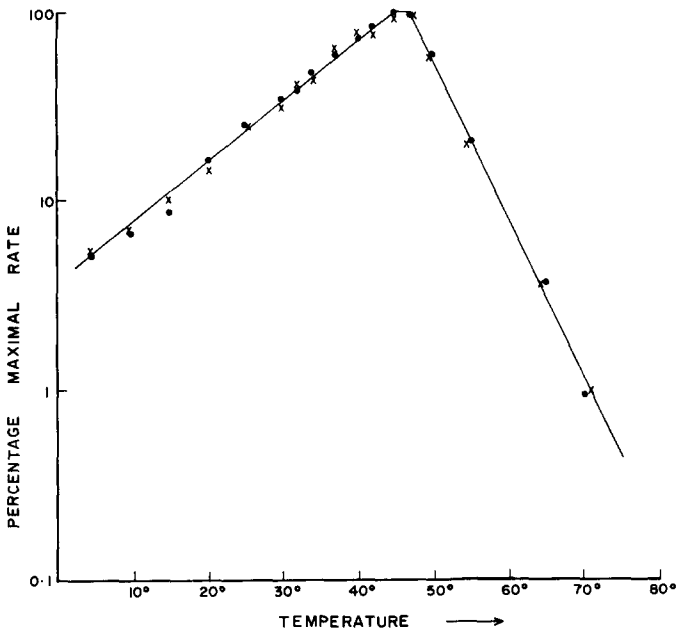

Fig. 4. Effect of temperature on the rate of hydrolysis of *p*-nitrophenyl sulphate. The reaction was carried out at temperatures ranging from 5° to 70°. ●—●, Fraction I; ×—×, Fraction II.

ature range from 5° to 45°, the reaction exhibited a normal Q_{10} of about 2 for both fractions of sulphatase. The rate of reaction has a sharp maximum and quickly falls off above 45°. The residual activity observed above 60° might simply reflect the limited sulphatase activity before the reaction mixture attained a thermal equilibrium. The profiles for Fraction I and Fraction II are similar.

The activation energy was calculated from the Arrhenius plots for Fraction I and Fraction II. The activation energy is about 9000 cal/mole for both fractions.

(V) Thermal stability. Both fractions of sulphatase are stable upto 55°. The heat inactivation kinetics for Fraction I and Fraction II were studied at 70°. Inactivation of both fractions is exponential. Fraction II shows typical single component inactivation kinetics whereas Fraction I gives a biphasic inactivation curve. The initial rate of inactivation for Fraction I is the same as that for Fraction II but changes after 70% inactivation to a rate which is 1/3 that of the initial rate (Fig. 5).

(VI) Electrophoretic mobility. Electrophoretic mobility of the two sulphatase fractions was determined on starch gel at pH 8.6 according to the method of SMITHIES[5] and stained with a histochemical dye (see MATERIALS AND METHODS). The electrophoretic mobilities of Fraction I and Fraction II at pH 8.6 were $6.3 \cdot 10^{-4}$ $cm^2 \cdot h^{-1} \cdot V^{-1}$ and $1.2 \cdot 10^{-3}$ $cm^2 \cdot h^{-1} \cdot V^{-1}$, respectively.

The isoelectric points for the two fractions were determined by carrying out electrophoresis in starch gels in buffers at different pH values ranging from pH 3.0 to pH 8.6. The sulphatase fractions migrated towards the cathode at pH 3.5 and towards the anode at pH 4.0. The isoelectric points for both fractions therefore lie between pH 3.5 and pH 4.0.

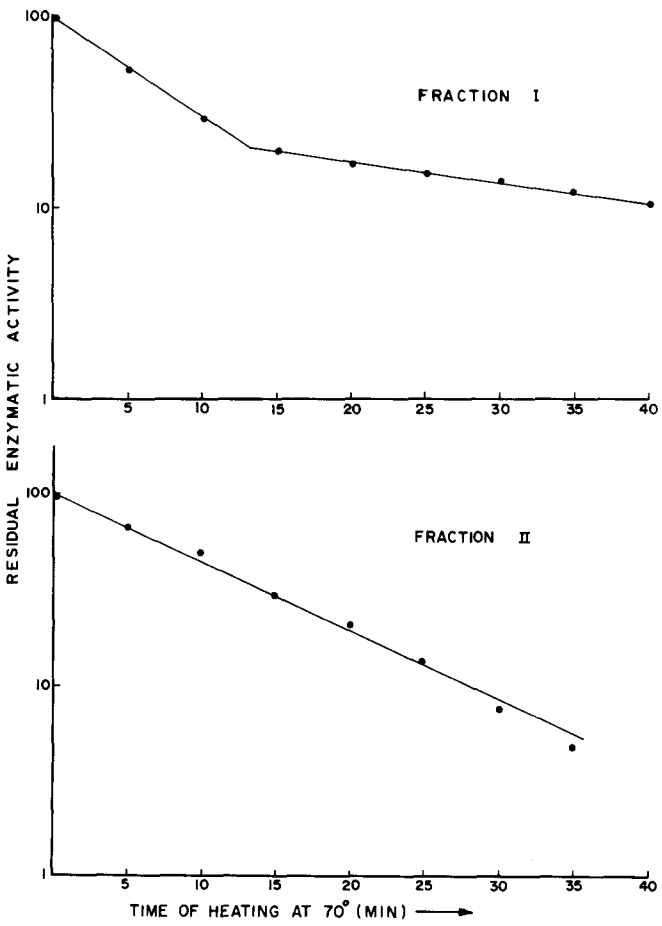

Fig. 5. Heat inactivation kinetics of sulphatase. The enzyme preparation was incubated at 70° in 0.01 M Tris (pH 7.4) at a concentration of 5 units/ml. At the times indicated, samples were removed and immediately assayed as described in MATERIALS AND METHODS.

(VII) Molecular weight. The molecular weights of the two fractions were determined by the method of MARTIN AND AMES[7] (see MATERIALS AND METHODS). The sedimentation of Fraction I and Fraction II of sulphatase, alkaline phosphatase and lysozyme is shown in Figs. 6 and 7. The sedimentation coefficients and the molecular weights for the two fractions of sulphatase were obtained by using the following three equations. Lysozyme (mol. wt. = 14 000; s = 1.87 S) and alkaline phosphatase (mol. wt. = 89 000; s = 6.1 S) served as reference markers.

$$R = \frac{\text{distance travelled by sulphatase from meniscus}}{\text{distance travelled by the standard from meniscus}} \quad (1)$$

$$R = \frac{s_1}{s_2} \quad (2)$$

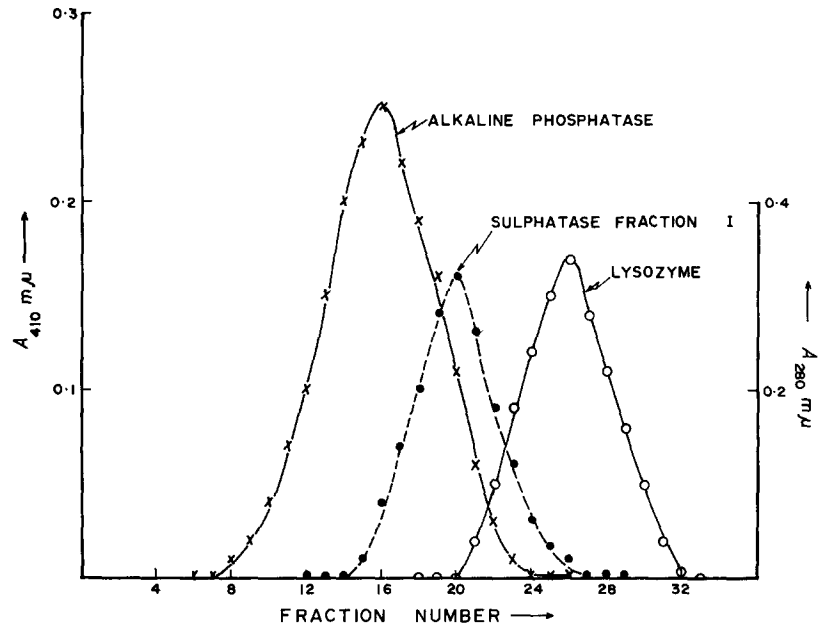

Fig. 6. Distribution of Fraction I of sulphatase, alkaline phosphatase and lysozyme following centrifugation in a 5–20% sucrose concentration gradient. 0.1 ml of a mixture of 20 units of Fraction I, 100 units of alkaline phosphatase and 10 mg of egg white lysozyme was layered on the sucrose gradient and centrifuged for 16 h at 37 000 rev./min at 4° in the SW-39 rotor of the Spinco Model L centrifuge.

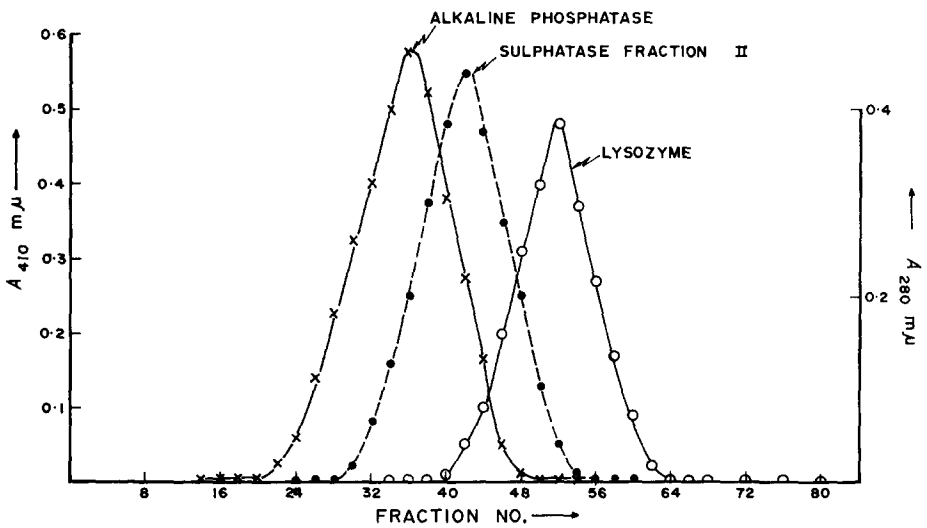

Fig. 7. Distribution of Fraction II of sulphatase, alkaline phosphatase and lysozyme following centrifugation in a 5–20% sucrose concentration gradient. 0.1 ml of a mixture of 20 units of Fraction II, 100 units of alkaline phosphatase and 10 mg of egg white lysozyme was layered on the sucrose gracient and centrifuged for 16 h at 37 000 rev./min at 4° in the SW-39 rotor of the Spinco Model L centrifuge.

TABLE IV

SOME PROPERTIES OF FRACTION I AND FRACTION II OF SULPHATASE OF *A. nidulans*

	Fraction I	*Fraction II*
Molecular weight	44 000	44 000
K_m (*p*-nitrophenyl sulphate)	$3.5 \cdot 10^{-5}$ M	$1.0 \cdot 10^{-4}$ M
K_m (nitrocatechol sulphate)	$9.5 \cdot 10^{-4}$ M	$5.0 \cdot 10^{-4}$ M
Activation energy	9 kcal/mole	9 kcal/mole
Electrophoretic mobility at pH 8.6	$6.3 \cdot 10^{-4}$ cm$^2 \cdot$h$^{-1} \cdot$V^{-1}	$1.2 \cdot 10^{-3}$ cm$^2 \cdot$h$^{-1} \cdot$V^{-1}
Isoelectric point	3.5–4.0	3.5–4.0

where s_1 and s_2 are the sedimentation coefficients of sulphatase and the standard, respectively.

$$\frac{s_1}{s_2} = \left(\frac{MW_1}{MW_2}\right)^{2/3} \quad (3)$$

where MW_1 and MW_2 are the molecular weights of sulphatase and the standard, respectively.

The average molecular weights of Fraction I and Fraction II are about 44 000. The physicochemical properties of the two fractions of sulphatase are summarized in Table IV.

DISCUSSION

The two fractions of arylsulphatase of *A. nidulans* are very similar in most of their physicochemical characteristics, molecular weight, substrate specificity, activation energy and isoelectric point. On the basis of their substrate specificity and sensitivity to sulphate, phosphate and cyanide both fractions would be classified as type I sulphatases in the classification proposed by ROY[8]. Nevertheless the two fractions differ in certain ways. They have contrasting affinities for *p*-nitrophenyl sulphate and nitrocatechol sulphate, their surface charges as evident from chromatographic and electrophoretic behaviour are different and Fraction I is more sensitive to Na_2SO_3 and iodoacetate than Fraction II. The question arises whether the two fractions are different proteins or one is a modified form of the other. The observed differences could be due to a configurational change or a chemical modification of the same protein. We have obtained some genetic evidence (to be published elsewhere) that the two fractions are in fact, related. A confirmation of this hypothesis, however, requires further work on the structure and the genetic control of the two fractions.

From its heat inactivation kinetics Fraction II appears to be homogeneous while Fraction I is heterogeneous. The strong inhibition of either fraction by KCN suggests that a heavy metal is involved in catalytic activity.

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