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The Purification and Properties of the L-Serine *O*-Sulphate Degrading System of Pig Liver

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1. The enzyme system from pig liver responsible for the $\alpha\beta$-elimination of L-serine *O*-sulphate was purified 1000-fold. 2. Isoelectric focusing produced two enzymically active fractions with isoelectric points at pH 5.6 and 5.9 respectively. 3. Osmometry and gel filtration showed both enzymes to possess molecular weights of approx. 54000. 4. The separate activities exhibited similar amino acid compositions.

Since the discovery of an enzyme system catalysing the $\alpha\beta$-elimination of L-serine *O*-sulphate (Dodgson, Lloyd & Tudball, 1961; Dodgson & Tudball, 1961) it has been shown to be widely distributed in Nature, occurring in most animal phyla examined (Dodgson & Tudball, 1961; Fowler, 1969) and also in some micro-organisms (Harada, 1964). The system is unusual, since even though it catalyses an $\alpha\beta$-elimination reaction, it does not require pyridoxal phosphate as an obligatory cofactor (Tudball, Thomas & Fowler, 1969). More recently it has been demonstrated that L-serine *O*-sulphate is capable of being utilized as a quasi-substrate by aspartate aminotransferase (John & Fasella, 1969), though clearly this latter activity is effected by a different mechanism, which involves pyridoxal phosphate.

Although the specific L-serine *O*-sulphate-degrading system has been extensively purified from rat liver (Thomas & Tudball, 1967; Tudball *et al.* 1969) the resulting preparation still yielded a number of different components when subjected to polyacrylamide-gel electrophoresis (Fowler, 1969). To provide unequivocal information on the nature of this novel system it was deemed necessary to obtain enzyme that had a much higher degree of purity than that previously available.

The present paper describes the preparation of homogeneous samples of two isoenzymes from pig liver, together with a description of some of their chemical and physical characteristics.

MATERIALS AND METHODS

Chemicals. The potassium salt of the *O*-sulphate ester of L-serine was prepared by the method of Tudball (1962). Cytochrome *c*, ovalbumin, serum albumin, bacitracin, trypsin, chymotrypsin, thyroglobulin and apoferritin were obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Haemoglobin was obtained from T. Gerrard and Co. Ltd., Littlehampton, Sussex, U.K. Ampholine was supplied by LKB-Produkter A.B., Stockholm, Sweden. Lactate dehydrogenase and NADH were obtained from Sigma (London) Chemical Co., London S.W.6., U.K.

Analytical methods. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), and from stage 4 onward in the enzyme purification by the procedure of Warburg & Christian (1941).

Assay of enzyme activities. L-Serine *O*-sulphate-degrading activity was assayed by the method of Thomas & Tudball (1967). For ease of assay during column-chromatographic procedures and for all experiments on the purified enzyme, liberated pyruvic acid was estimated by using a coupled enzyme assay technique employing lactate dehydrogenase. The assay mixture, contained in a 1ml quartz cell (path length 10mm), consisted of 0.1ml of suitably diluted enzyme solution, 0.1ml of aq. NADH (1mg/ml), 10μl of lactate dehydrogenase (10mg/ml), 0.2ml of 0.25M-L-serine *O*-sulphate and 0.6ml of 0.1M-tris buffer (5.0mM with respect to 2-mercaptoethanol) that had been adjusted to pH 7.0 with 2M-HCl. Enzyme activity at 25°C was then measured by following the decrease in E_{340} with a Unicam SP. 800 recording spectrophotometer equipped with a thermostatically controlled cell holder and scale-expansion accessory.

Alanine aminotransferase [L-alanine–2-oxoglutarate aminotransferase, EC 2.6.1.2] was measured by the method of Tonhazy, White & Umbreit (1950). Aspartate aminotransferase [L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1] was assayed as described by Velick & Vavra (1962).

Polyacrylamide-gel electrophoresis. This was performed with the Shandon analytical polyacrylamide-gel electrophoresis apparatus. Protein was shown by staining the gel with Amido Black (1%, w/v, in aq. 7% acetic acid).

Isoelectric focusing. The technique used was that of Vesterberg & Svensson (1966). Experiments were performed on a 110 or 440ml isoelectric-focusing column (L.K.B.).

Molecular-weight determinations. These were carried out by gel filtration on a column (1 cm × 75 cm) of Sephadex G-100, with standard protein preparations as marker molecules, and by osmometry with the Hewlett Packard 503 High-Speed Membrane Osmometer.

EXPERIMENTAL AND RESULTS

Purification of the enzyme. Unless otherwise stated all extraction and purification steps were carried out at 4°C. All centrifuging operations were performed at 0°C.

Stage 1. Pig livers (5kg), which had previously been frozen and thawed, were minced and macerated in a one-gallon Waring Blendor with sufficient potassium chloride (1.2%, w/v) containing 5mM-disodium EDTA, to give a 25% (w/v) suspension. The resulting suspension was centrifuged at 3000*g* for 60min and the supernatant retained.

Stage 2. The supernatant from stage 1 was transferred to a stainless-steel bucket and immersed in a water bath maintained at 60°C. The contents of the bucket were stirred until the temperature had reached 55°C. After this temperature had been maintained for 10min the heat-treated material was rapidly cooled in an ice bath to 4°C before centrifuging for 30min at 3000*g*. The resulting supernatant was retained.

Stage 3. Sufficient ammonium sulphate was added, with mechanical stirring to the stage 2 supernatant, to give a 35% saturated solution. After standing for 3h the precipitate was removed by centrifuging at 3000*g* for 1h. Additional ammonium sulphate was added to the clear supernatant as before to give a 50% saturated solution and the whole was left overnight. The resulting brown precipitate was separated by centrifuging as above. The preparation could be stored in this solid form at −15°C for several months without appreciable loss of activity.

Stage 4. The precipitate from stage 3 was dissolved in the minimum volume (800ml) of 0.1M-sodium acetate–acetic acid buffer, pH6.0 (containing a final concentration of 5.0mM-2-mercaptoethanol). A portion (500ml) of this solution was then applied to a column (10cm × 100cm) of Sephadex G-200, previously equilibrated with the same buffer. The eluate was collected in 50ml fractions at a flow rate of 90–100ml/h. The fractions containing most of the enzyme activity (nos. 82–104) were pooled and active protein was precipitated by the addition of solid ammonium sulphate to yield a 70% saturated solution. Precipitated activity was stable for prolonged periods when stored at −15°C. Fig. 1 presents a typical separation obtained on a Sephadex G-200 column, and shows that most of the aspartate and alanine aminotransferase activities are separated from the L-serine *O*-sulphate-degrading activity.

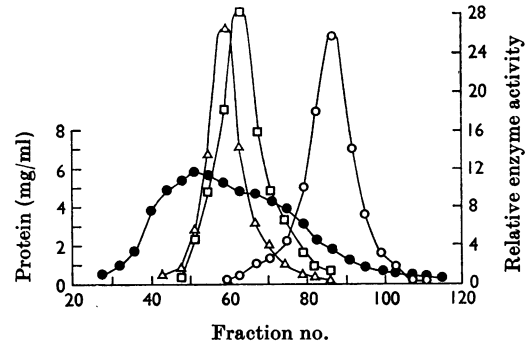

Fig. 1. Fractionation of stage 3 protein by gel filtration on Sephadex G-200. Protein (8.5g) dissolved in 0.1M-sodium acetate–acetic acid buffer, pH6.0 (containing a final concentration of 5.0mM-2-mercaptoethanol), was applied to a column (10cm × 100cm) equilibrated with the same buffer. ○, L-Serine *O*-sulphate degrading activity; △, alanine aminotransferase activity; □, aspartate aminotransferase activity; ●, protein concn. measured by the method of Lowry *et al.* (1951). Specific enzyme activities were obtained by the methods described in the text, but are expressed as relative specific enzyme activities for ease of presentation.

Stage 5. The protein from stage 4 was dissolved in the minimum amount (100ml) of 0.05M-sodium acetate–acetic acid buffer, pH6.0 (containing a final concentration of 5.0mM-2-mercaptoethanol) and dialysed for 24h against two changes of the same buffer (total volume 20litres). The diffusate was applied to a column (10cm × 30cm) of CM-Sephadex C-50 which had been allowed to equilibrate with the same buffer and the column was washed with buffer at a flow rate of 30ml/h until the eluate was free from protein. This washing serves to remove residual alanine aminotransferase activity. L-Serine *O*-sulphate-degrading activity was eluted with the above acetate buffer containing 0.5M-sodium chloride. Protein was precipitated with solid ammonium sulphate and stored at −15°C. Storage of the sample as a frozen aqueous solution resulted in a complete loss of activity.

Stage 6. The stage 5 precipitate was dissolved in 0.01M-tris–hydrochloric acid buffer, pH7.0 (containing a final concentration of 5.0mM-2-mercaptoethanol), and dialysed for 24h against two changes of the same buffer (total volume 10 litres). The diffusate (50ml) was then applied to a column (20cm × 5cm) of DEAE-Sephadex A-50 (medium grade) previously equilibrated with the same buffer. The column was eluted with buffer at a flow rate of 30ml/h until the eluate was free from protein, and

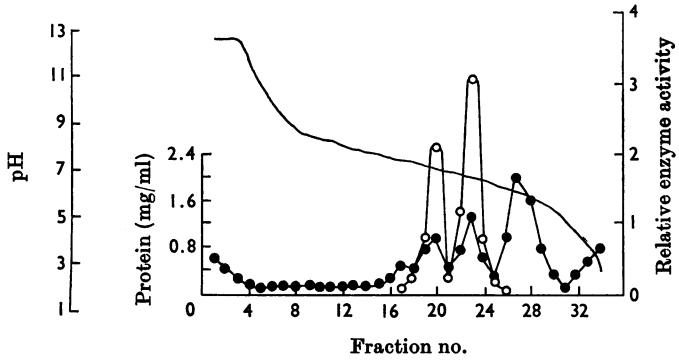

Fig. 2. Isoelectric focusing of stage 7 protein. The protein solution (40mg dissolved in 5ml of 1% glycine) was injected into a sucrose gradient containing 2% Ampholine, pH range 5–8, held in a 110ml column. Separation was allowed to proceed for 30h with a power input of 1.5W. ○, L-Serine O-sulphate-degrading activity; ●, protein concn. measured at E_{280}; ——, pH gradient. Specific enzyme activities were obtained by the methods described in the text, but are expressed as relative specific enzyme activities for ease of presentation.

the desired enzyme activity eluted with the starting buffer containing 0.05M-sodium chloride. Active enzyme was again precipitated with solid ammonium sulphate. The precipitate lost about 5% of its activity after 1 week when stored at −15°C.

Stage 7. The stage 6 preparation was again dissolved in the stage 6 buffer and dialysed as previously described. The resulting diffusate was reapplied to a column (10cm×2cm) of DEAE-Sephadex A-50 and the column washed with starting buffer as before. Enzyme activity was eluted with a linear 0–0.1M-sodium chloride gradient. The eluate was collected in 10ml fractions, and the active fractions (nos. 18–20) were pooled and again precipitated with solid ammonium sulphate. At this stage the preparation yielded a single component when subjected to polyacrylamide-gel electrophoresis in the range pH5.2–9.8.

Stage 8. Preliminary isoelectric-focusing experiments with 1% Ampholine between pH3 and 10 showed the enzyme activity occurred within the pH range 5.5–6.5.

A portion (40mg dissolved in 1ml of stage 7 starting buffer) of the enzyme from stage 7 was dialysed for 24h against two changes (total volume 6 litres) of an aq. 1% glycine solution (containing a final concentration of 5.0mM-2-mercaptoethanol). The enzyme solution (5ml) containing the appropriate concentration of sucrose was injected into the middle of the sucrose gradient containing 2% Ampholine, pH range 5–8, which was used to fill a 110ml separating column. As a protective measure a final concentration of 5.0mM-2-mercaptoethanol was also incorporated into the gradient. The separation was allowed to proceed for 30h, the power input being kept at 1.5W as far as possible. At the end of this period the potential was 1200V and the current 0.75mA. Fractions (3ml) were then collected and portions of each fraction were taken for the determination of protein, enzyme activity and pH. Fig. 2 presents the separation pattern observed in a typical experiment. Three distinct protein-containing components were observed, two of which were enzymically active. The fractions corresponding to the active protein peaks were separately pooled and the Ampholine removed by gel filtration on a column (43cm×2.5cm) of Sephadex G-50 (Vesterberg, 1969). Development was effected with 0.01M-tris–hydrochloric acid buffer, pH7.0 (containing a final concentration of 5.0mM-2-mercaptoethanol). Refocusing of the combined peaks as previously described resulted in the separation of two active peaks with isoelectric points corresponding to the active peaks obtained from the stage 8 preparation. When the individual peaks were subjected to the same separation procedure, then in each case a single active peak of protein was obtained. The active fractions possessed isoelectric points at pH5.9 and 5.6 and have been designated the α-enzyme and β-enzyme respectively. Identical separations were also obtained when 100mg of the stage 8 preparation was applied to a 440ml column. After the removal of Ampholine from the refocused α and β peaks as previously described, the individual peaks were concentrated by using Aquacide (Calbiochem, Los Angeles, Calif., U.S.A.) packed around a dialysis bag until the protein concentration was 5–8mg/ml. A mixture of the α and β peaks did not separate when subjected to polyacrylamide gel electrophoresis in the range pH5.2–9.8. When stored at 4°C, about 10% of enzyme activity was lost after

Table 1. *Purification of the* L-*serine* O-*sulphate-degrading system from pig liver*

Fractionation scheme	Vol. (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Sp. activity (units/mg of protein)	Purification
25% (w/v) Homogenate	18000	1084370	—	—	—	
Supernatant	16900	590900	419440	100	0.71	1
55°C-treated extract	15100	170000	331330	79	1.9	2.6
$(NH_4)_2SO_4$ precipitate (35–50% saturation)	800	54400	239560	57	4.4	6.1
Gel filtration on Sephadex G-200 (fractions 82–104)	100	8530	167780	40	20.0	28.2
CM-Sephadex	50	1910	117450	28	61.4	86.5
DEAE-Sephadex	50	540	109020	26	223	314
DEAE-Sephadex (gradient separation, fractions 18–20)	30	285	83300	20	293	412
Isoelectric focusing	5	40	11730	—		
Peak α	9	5.4	3880	—	718	1010
Peak β	9	7.4	5150	—	695	980
Peaks $\alpha+\beta$	18	12.8	9030	15.1	705	990

1 week. Complete loss of activity occurred if enzyme-containing solution was frozen and thawed.

Table 1 shows the results obtained from a typical purification schedule.

Properties of α and β enzymes. Both systems exhibited optimum activity with a final substrate concentration of 0.05 M and at pH 7.0 in the presence of 0.1 M-tris–hydrochloric acid buffer (containing a final concentration of 5.0 mM-2-mercaptoethanol). The K_m for both systems calculated from the double-reciprocal plot of Lineweaver & Burk (1934) was 2.6×10^{-2} M; this compares favourably with the value of 2.3×10^{-2} M quoted by Thomas & Tudball (1967). The catalytic-centre activity for the α and β enzymes was 2377 and 2317 mol of substrate transformed/min per mol of enzyme at 37°C, assuming one catalytic centre.

Determination of molecular weight. As a prelude to the determination of the molecular weights of the α and β enzymes it was necessary to establish the relationship between the dry weight of the purified enzyme preparations and their extinction at 280nm.

Enzyme preparations from which Ampholine had been removed were extensively dialysed for 48h against ten changes of water (total volume 20 litres). The extinction of a solution of enzyme containing approx. 4mg of protein/ml was measured at 280nm. A known volume (2ml) of this solution was then dried *in vacuo* to constant weight. An aqueous solution of both α and β enzyme containing 1mg of protein/ml gave an extinction of 0.78 at 280nm. This value was subsequently employed in experiments designed to estimate the molecular weights of the systems by osmotic-pressure measurements.

From the position of elution of enzyme activity on the Sephadex G-200 column in relation to that observed for alanine and aspartate aminotransferases it could be deduced that the molecular weight of the enzyme system was less than 100000. To provide a more accurate assessment of the molecular weight both α and β enzymes were subjected to gel filtration on a column (1cm × 75cm) of Sephadex G-100. Before use the column was equilibrated with 0.1 M-tris–hydrochloric acid buffer, pH 7.0 (containing a final concentration of 5.0 mM-2-mercaptoethanol). The column was calibrated employing standard preparations of haemoglobin, cytochrome *c*, ovalbumin, serum albumin, bacitracin, trypsin, chymotrypsin, thyroglobulin and apoferritin. The void volume of the column was calculated by using Blue Dextran and the inclusion volume by using pyridoxal 5′-phosphate and sodium chloride. About 1cm of Sephadex G-25 was added to the top of the column to facilitate layering of samples. Samples of protein (0.5ml of a solution containing 5mg/ml) in tris buffer were applied to the column and the eluate was collected in 0.5ml fractions. The molecular weights were computed from a plot of volume of eluate against log of molecular weight. The molecular weights of the α and β enzymes were 52000 and 50000 respectively, and the molecular weight of a mixture of α and β enzymes was 50000.

Confirmation of the values obtained for the molecular weight by gel filtration was achieved when the two enzyme preparations were subjected to high-speed membrane osmometry. Preparations of the α and β enzymes from which the Ampholine had been removed were concentrated as described above to yield solutions with concentrations approx. 10mg/ml. The concentrated solutions were dialysed for 16h against 5 litres of 0.01 M-tris–hydrochloric acid buffer, pH 7.0 (containing a final concentration

of 5.0mM-2-mercaptoethanol). The resulting buffer was used as the solvent in the osmotic-pressure determinations. The osmotic pressure was measured at four protein concentrations (8, 6, 4 and 2 mg/ml respectively) for both enzymes. Values of π/c were plotted against c and values for the molecular weights were calculated from the expression:

$$\text{Mol.wt.} = \frac{RT}{\pi/c \ (c \rightarrow 0)}$$

where R is the gas constant per mole, T is the absolute temp., c is the concn. in g/ml and π is the osmotic pressure. The molecular weights of the α and β enzymes were 53800 and 54500 respectively.

When the gel-filtration and osmotic-pressure experiments were repeated in the presence of 8M-urea, no substantial alteration in the molecular weights was observed, though a complete and irreversible inactivation of enzyme activity resulted.

Amino acid composition. Further comparison between the α and β enzymes was achieved by the elucidation of their respective amino acid compositions. Samples (2mg) of both enzymes were hydrolysed in 0.5ml of constant-boiling hydrochloric acid *in vacuo* for 12, 24 and 36h at 110°C. Excess of hydrochloric acid was removed by repeated freeze-drying. For the subsequent determination of the amino acid composition, dried samples were dissolved in 2ml of water and 1ml samples were analysed by using the Technicon Amino Acid Analyser with a column (0.62cm × 110cm) packed with Dowex 50 Chromabead resin. Table 2 presents the amino acid composition of the α and β enzymes. The number of amino acid residues/mol was computed by using a value of 52000 for the molecular weight of both enzymes. There are only slight differences in the amino acid compositions of the respective enzymes, which could account for the observed differences in isoelectric points.

Table 2. *Amino acid compositions of α and β enzymes*

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

Amino acid	Composition (no. of residues/mol of enzyme)	
	α enzyme	β enzyme
Cys*	5	5
Asp	29	38
Thr†	21	20
Ser†	24	23
Glu	43	35
Pro	18	17
Gly	35	34
Ala	33	32
Val	28	26
Met	13	13
Ile	22	26
Leu	34	32
Tyr	10	10
Phe	17	18
Lys	28	28
His	14	12
Arg	19	24
Try‡	7	6

* Least estimate.

† Values extrapolated to zero time.

‡ Estimated by the method of Goodwin & Morton (1946).

DISCUSSION

The evidence presented in this paper indicates that the pig liver system responsible for the $\alpha\beta$-elimination of L-serine O-sulphate may be separated to yield two proteins possessing essentially similar catalytic properties, molecular weights and amino acid compositions. It seems reasonable to assume in addition that they are not further composed of hydrogen-bonded subunits, since no change in the molecular weight was observed when 8M-urea was employed in the gel-filtration and osmotic-pressure experiments.

No physiological role has yet been assigned to the enzyme, principally due to the doubt surrounding the possible natural occurrence of the substrate normally used for its assay, i.e. L-serine O-sulphate. Whereas we are not able at this juncture to exclude definitely L-serine O-sulphate as the natural substrate it is worth emphasizing that all attempts to sulphate either serine or its derivatives biologically have met with failure (Spencer, 1960; Dodgson *et al.* 1961). However, the recent demonstration (John & Fasella, 1969) that the $\alpha\beta$-elimination of L-serine O-sulphate can also be catalysed by a glutamate-utilizing system may very well suggest that the enzyme under investigation is in reality concerned in some way with glutamate metabolism. An extension of investigations in this direction in these laboratories has indicated that L-serine O-sulphate acts as a quasi-substrate in the alanine aminotransferase and aspartate decarboxylase systems and in addition acts as a good inhibitor for glutamate dehydrogenase. X-ray examination of crystals of L-serine O-sulphate illustrates that it possesses a conformation similar to that exhibited by glutamate (N. Tudball, J. P. G. Richards & H. Davies, unpublished work). Other evidence suggests that the two compounds also adopt similar conformations in solution, which undoubtedly accounts at least in part for the ability of systems that normally

act on glutamate to bind L-serine *O*-sulphate also and in some cases to bring about some molecular transformation. It has not yet proved possible to effect any chemical change in the glutamate molecule by utilizing the system which seems to be specific towards L-serine *O*-sulphate, though glutamate, as would be expected, acts as an efficient competitive inhibitor. Perhaps this unsuspected relationship between glutamate and L-serine *O*-sulphate should not be forgotten, therefore, when considering the physiological role of this novel enzyme.

Fowler (1969) reported a value of 315000 for the molecular weight of the rat liver enzyme, which probably indicates it is composed of subunits. It is not inconceivable in the case of the pig enzyme that dissociation of a high-molecular-weight system occurred during the preparation, though attempts made to effect a re-association of the α and β enzymes to yield a similar complex proved unsuccessful.

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