Purification and General Properties of Aspartate Aminotransferase of Ox Heart

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1. A five-step procedure for preparing highly purified aspartate aminotransferase from ox heart is described. 2. The homogeneity of the pure enzyme was established by criteria such as ultracentrifugation and electrophoresis in starch gel and in polyacrylamide gel. 3. The pure enzyme has an isoelectric point of about pH 5, and $E_{1\text{cm}}^{1\%}$ 14.40 at 278 m μ . 4. The molecular weight of the pure enzyme was determined as 96 000 by sedimentation equilibrium. 5. The pH optimum for the pure enzyme was about 8. It was determined by a new assay technique. 6. A difference in the electrophoretic migration rate between the enzyme from ox heart and brain and the enzyme from pig heart and brain suggests a species specificity rather than an organ specificity. 7. A new effect of deionization on the visible-absorption spectrum of the enzyme was observed.

Aspartate aminotransferase (L-aspartate-2-oxo-glutarate aminotransferase, EC 2.6.1.1) was first prepared from pig heart by Green, Leloir & Nocito (1945); further purification of the enzyme from the same source was obtained by other workers (O'Kane & Gunsalus, 1947; Lis, 1958; Jenkins, Yphantis & Sizer, 1959; Banks & Vernon, 1961). Marino & Scardi (1964) reported a purification procedure of aspartate aminotransferase from ox brain. Amino acid composition, sedimentation coefficient, electrophoretic migration rate on paper and absorption spectra of this enzyme were also determined and compared with those of aspartate aminotransferase of pig heart (Marino, Zito & Scardi, 1964). Some difference between the enzymes was observed, especially in the electrophoretic behaviour. To establish whether this difference was due to organ or species specificity or both, aspartate aminotransferase was also prepared from pig brain (G. Marino & V. Scardi, unpublished work) and ox heart. The purification procedure from this latter source, of which a preliminary communication has been given (Marino, Greco & Scardi, 1965a), proved to be a relatively easy method of obtaining, in fairly good yield, an aspartate aminotransferase in a state of high purity, as required for structural investigations.

The present paper reports the purification procedure and describes some properties of the purified enzyme; its amino acid composition, *N*- and *C*-

terminal residues, thiol groups and some other structural features are reported in the accompanying paper (Marino, Scardi & Zito, 1966).

EXPERIMENTAL

Materials

Chemicals. L-Aspartic acid and 2-oxoglutaric acid were purchased from L. Light and Co. Ltd., Colnbrook, Bucks. Disodium 2-oxo[5-¹⁴C]glutarate (7 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Crystalline bovine serum albumin was from Sigma Chemical Co., St Louis, Mo., U.S.A.

Alumina C_γ gel was obtained from Schugardt, Munich, Germany. Ion-exchange resins AG-1 (X4) and AG-501 (X8) were from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

Ammonium sulphate and all other chemicals were analytical-grade products obtained from several commercial sources.

Hydroxyapatite was prepared by the method of Tiselius, Hjertén & Levin (1956).

Source material of enzyme. Ox heart muscle was used as the source material. The hearts of freshly slaughtered animals were trimmed of auricles and visible fat and then stored at -15° until used.

Methods

Protein estimation. During the whole enzyme purification, protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

Enzyme assay. Aspartate-aminotransferase activity was

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assayed spectrophotometrically at $280\text{m}\mu$ by the rate of oxaloacetate formation, under the conditions described by Cammarata & Cohen (1951). A Beckman model DB spectrophotometer, equipped with a Sargent model SRL recorder and a constant-temperature (37°) circulating-water bath, was used for these assays.

All other spectrophotometric measurements were made in a Unicam SP. 500 spectrophotometer or in a Beckman model DK-1 recording spectrophotometer.

Since the buffer system affects both the direct spectrophotometric measurement of oxaloacetate formed during transamination (Banks, Lawrence, Thain & Vernon, 1963) and the indirect method (Karmen, 1955) based on the reduction of oxaloacetate by NADH_2 under the influence of malate dehydrogenase (EC 1.1.1.37), a simple isotope method was used for assaying aspartate-aminotransferase activity as a function of pH. This method is a slight modification of the assay technique by Marino, Greco & Scardi (1965b), which consists in measuring the activity of the labelled glutamate formed by incubation of the enzyme with L-aspartate and 2-oxo[5- ^{14}C]glutamate, the labelled glutamate being isolated from the incubation mixture by paper chromatography after conversion of oxo acids into their corresponding 2,4-dinitrophenylhydrazones.

For each pH value, a reaction tube was prepared containing, in 0.2 ml., $10\text{ }\mu\text{moles}$ of the appropriate buffer, $2\text{ }\mu\text{moles}$ of L-aspartate and $0.08\text{ }\mu\text{mole}$ of 2-oxo[5- ^{14}C]glutamate ($0.05\text{ }\mu\text{C}$). Reactions at 37° were started by the addition of $5\text{ }\mu\text{l.}$ of enzyme ($0.1\text{ }\mu\text{g.}$) and stopped after 10 min. by adding 0.2 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2N-HCl. After 15 min., $20\text{ }\mu\text{l.}$ portions were taken from each tube and spotted on a Whatman no. 1 paper sheet, and then chromatographed overnight with descending butan-1-ol-acetic acid-water (12:3:1, by vol.), together with unlabelled glutamate ($10\text{ }\mu\text{l.}$ of a 0.02M solution) as a marker. The position of the radioactive glutamate formed during transamination was located by reference to the marker separately stained with ninhydrin. From the unstained chromatogram squares ($5\text{ cm.} \times 5\text{ cm.}$) were cut out and placed in standard counting vials containing liquid scintillator. The latter and counting procedure were those described by Grippo, Iaccarino, Rossi & Scarano (1965). A Packard Tri-Carb model 314EX liquid-scintillation spectrometer was used at approx. 60% counting efficiency.

Under the above conditions the velocity of glutamate formation is linear with time for at least the first 20 min. of reaction, and no transamination occurs in the absence of enzyme (as shown by controls from which enzyme was omitted). Paper blanks give scintillation counting values practically equal to background (about 80 counts/min.). Although the measured radioactivity is obviously proportional to the amount of glutamate formed by enzymic transamination, no attempt was made to convert counts/min. into μmoles of glutamate. Therefore enzyme activity as a function of pH was not expressed as μmoles of glutamate formed/min. but simply as arbitrary units representing the radioactivity (corrected for background) of the glutamate spot isolated as described above.

Purification of the enzyme

(1) *Heat treatment.* Ox heart muscle was passed once through a meat grinder; then the minced muscle was dispersed in a Waring Blendor containing 1.5 vol. of 0.05M-

potassium maleate buffer, pH 6.0, containing EDTA (5 mM). The resulting homogenate was heated at 75° for 20 min., with the addition of 2-oxoglutarate at 60° , according to the procedure of Jenkins *et al.* (1959), and then cooled to 0° and centrifuged.

(2) *First ammonium sulphate fractionation.* To the supernatant from step (1), kept at 4° , $(\text{NH}_4)_2\text{SO}_4$ (31.3 g./100 ml.) was slowly added under continuous stirring. After 15 min. the precipitate formed was centrifuged off and further $(\text{NH}_4)_2\text{SO}_4$ (17.6 g./100 ml.) was added to the supernatant. The precipitate formed was collected by centrifugation and dissolved in the minimum volume of 0.3M-maleate buffer, pH 6.0, and then dialysed for 24 hr. against repeated changes (5 l.) of deionized water.

(3) *Alumina C_γ treatment.* The dialysed solution from step (2), clarified by centrifugation, was treated at 4° with alumina C_γ gel (0.2 mg. of gel/mg. of protein). The mixture was allowed to stand for 20 min. and then centrifuged and the gel discarded. This treatment was repeated at least four times, each time with the same amount of gel calculated initially.

(4) *Second ammonium sulphate fractionation.* 2-Oxoglutarate was added to the clear supernatant from step (3) to give 1 mM concentration; then the enzyme was precipitated with solid $(\text{NH}_4)_2\text{SO}_4$ in the fraction between 55% and 65% saturation. The precipitated enzyme was dissolved in the minimum volume of 0.3M-maleate buffer, pH 6.0, and dialysed overnight against deionized water.

(5) *Chromatography on hydroxyapatite.* Volumes (10–12 ml.) of enzyme solution in 0.02M-potassium phosphate buffer, pH 6.8, containing 250–300 mg. of protein and prepared by addition of the 2M-buffer to the dialysed solution from step (4), were added to the top of hydroxyapatite columns ($15\text{ cm.} \times 1.5\text{ cm.}$) previously equilibrated overnight against 0.02M-potassium phosphate buffer, pH 6.8. The elution was carried out with stepwise-increasing concentrations of the potassium phosphate buffer (0.02M–0.04M–0.06M–2.0M). The resolved yellow band of the enzyme was eluted with the 0.06M-buffer, and only the fractions containing more than 1 mg. of protein/ml. were saved and combined. The enzyme was concentrated by precipitation with solid $(\text{NH}_4)_2\text{SO}_4$ (75% saturation).

The results obtained by this purification procedure are very reproducible. A summary of a typical purification from 5.0 kg. of ox heart muscle is given in Table 1.

Preparation of the apoenzyme. This was performed by combining the resolution procedures of Wada & Snell (1962) and of Scardi, Scotto, Iaccarino & Scarano (1963). Portions containing approx. 20 mg. of holoenzyme were incubated at 37° for 20 min. with 1 m-mole of L-aspartate in a final volume of 2 ml. After cooling, 3 ml. of a saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added and the pH adjusted to 3.8 with 4N-acetic acid. The mixture was left overnight at 0° and then centrifuged. The precipitate, washed with saturated $(\text{NH}_4)_2\text{SO}_4$, was dissolved in 1 ml. of 0.2M- NaHCO_3 . The resulting enzyme solution, which does not show any absorption maximum in the range 300–500 $\text{m}\mu$, was 98% resolved, the degree of resolution being calculated as described by Scardi *et al.* (1963).

Deionization of enzyme. The deionization column was made up as described by Timasheff, Dintzis, Kirkwood & Coleman (1957), with resin AG-1 (X_4 ; OH^- form) and mixed-bed resin AG-501 (X_8). The deionization was conducted in the cold (2°).

Table 1. *Purification of ox-heart aspartate aminotransferase*

Summary of the effects of the purification steps in the preparation of aspartate aminotransferase from 5.0 kg. of ox heart muscle, as described in the text. One unit of enzyme activity is defined as that amount of enzyme that catalyses the formation of 1 μ mole of oxaloacetate/min. at 37°, and in 0.05M-phosphate buffer, pH7.4, under the conditions described by Cammarata & Cohen (1951). The specific activity of the crude homogenate before heating (i.e. step 1) was 0.20.

Step	Total protein (mg.)	Total activity (units)	Specific activity (units/mg.)	Total yield (%)
(1) Heat treatment	15695	34560	2.2	100
(2) First (NH ₄) ₂ SO ₄ fractionation	3070	32640	10.6	94
(3) Alumina C _γ treatment	640	25990	38.8	72
(4) Second (NH ₄) ₂ SO ₄ fractionation	485	24600	49.5	70
(5) Chromatography on hydroxyapatite	165	16400	98.0	47

Fig. 1. Sedimentation patterns of ox-heart aspartate aminotransferase. The rotor speed was 59780 rev./min., the temperature 19.9° and the phase-plate angle 55°. The initial enzyme concentration was 6.73 mg./ml. in water. The photographs were taken at 8 min. intervals.

Characterization of the pure enzyme

Zone electrophoresis. Horizontal starch-gel electrophoresis was carried out essentially as described by Smithies (1955), and disk electrophoresis in polyacrylamide gel as described by Ornstein & Davis (1961). The gels were stained with Amido Black 10B.

Specific extinction. $E_{1\text{cm}}^{1\%}$ at 278 μ was established by correlation of extinction with the dry weight of the dialysed or deionized enzyme. Dry weight was measured on a Cahn Gram electrobalance, by using the 10mg. range, permitting accuracy to 1 μ g.

Ultracentrifugal analysis. Measurements of sedimentation velocity of holoenzyme and apoenzyme were carried out at 59780 rev./min. in a Spinco model E analytical ultracentrifuge equipped with a phase-plate optical system and by using a KEL-F centrepiece. Photographs were taken at 8 min. intervals. In each analysis the solution of the enzyme was about 6 mg./ml. All enzyme solutions were dialysed thoroughly against deionized water. A Leitz micro-comparator was used in the calculation of the sedimentation coefficients.

For the sedimentation equilibrium the standard method of Archibald (1947) at a speed of 4059 rev./min. was used. The enzyme was 5 mg./ml. in tris-HCl buffer, pH7.2 and 10-l. Photographs were taken at 16 min. intervals. The synthetic-boundary run was made with the same enzyme solution in a cup cell at a speed of 5227 rev./min.

Light-scattering. This was measured in a Brice Phoenix apparatus with a quadrangular cell. The enzyme solutions were clarified by filtration through G5 sintered-glass disks under N₂ pressure. The enzyme concentrations were determined after the measurements by using the extinction coefficient of the ultraviolet protein band. Refractive-index increment was determined with a Phoenix differential refractometer.

RESULTS AND DISCUSSION

Purity. Aspartate aminotransferase prepared as described above has a purity degree never attained before by starting from pig heart, the usual source of the enzyme, and by using any of the procedures referred to above. This is due to the inclusion of the treatment with alumina C_γ gel, which represents the most effective purification step, in a procedure similar in many respects to that of Jenkins *et al.* (1959).

The purified enzyme sediments as a single symmetrical peak ($S_{20,w}$ 5.3 s) in the ultracentrifuge (Fig. 1) and migrates as a single band in disk and starch-gel electrophoresis. In this latter analysis the homogeneity of the enzyme has been tested at different pH values. Table 2 summarizes the

electrophoretic migration rates under standard conditions. The results indicate an isoelectric point of about 5.0 for the enzyme.

Fig. 2 shows the sedimentation pattern of the apoenzyme, which shows no heterogeneity and has a sedimentation coefficient ($S_{20,w}$ 5.2s) very close to that of the holoenzyme, thus indicating that the removal of the coenzyme causes no change in the quaternary structure of the enzyme protein.

The electrophoretic migration rate of ox-heart aspartate aminotransferase is markedly less than that of the pig-heart enzyme, the ratio being about 0.6. The same ratio was observed for aspartate aminotransferases prepared from ox brain and pig heart (Marino *et al.* 1964), whereas the enzyme prepared from pig brain shows the same migration rate as that from pig heart (G. Marino & V. Scardi,

unpublished work). These differences therefore seem to be dependent on the animal species rather than on the organ used as an enzyme source.

On the other hand, practically no difference exists between the sedimentation coefficients of aspartate aminotransferase from ox heart and pig heart; the latter shows the same $S_{20,w}$ value as the enzyme from ox brain (Marino *et al.* 1964).

Stability. Repeated freezing and thawing (e.g. 70 times) does not inactivate the purified enzyme, whereas prolonged storage at -15° partially inactivates it.

Molecular weight. The weight-average molecular weight determined by light-scattering measurements at various enzyme concentrations was $117\,000 \pm 4000$, the refractive-index increment being 0.190. Values of Hc/τ were independent of concentration, c , in the range 3.53–0.98 mg. of protein/ml. A molecular weight of 117 000 is in good agreement with that of the pig-heart enzyme as reported by

Table 2. *Electrophoretic mobilities of ox-heart aspartate aminotransferase at different pH values*

All experiments were carried out in the cold room at 4° at a constant current of 1.5 mA/cm. width of starch gel. The pH values reported are those measured electrometrically at 20° .

pH	Buffer (10.1)	Distance moved from origin in 14 hr. (mm.)
3.2	Formate	–12
4.5	Acetate	–4
5.3	Acetate	+1
6.2	Phosphate	+3
7.5	Phosphate	+10

Fig. 2. Sedimentation pattern of ox-heart apo-(aspartate aminotransferase). The rotor speed was 59780 rev./min., the temperature 19.5° and the phase-plate angle 55° . The initial apoenzyme concentration was 5.9 mg./ml. in water. The photograph was taken 40 min. after reaching full speed. Since the apoenzyme is somewhat unstable when exposed to u.v. light, the cell was exposed to u.v. light only at the time of photography.

Fig. 3. Ultraviolet-absorption spectrum of ox-heart aspartate aminotransferase in distilled water. The enzyme concentration was 0.585 mg./ml.

Fig. 4. Effect of deionization on visible-absorption spectrum of ox-heart aspartate aminotransferase. ----, Spectrum of the enzyme thoroughly dialysed against distilled water; —, spectrum of the enzyme after deionization as described in the text. The enzyme concentration was 10.0 mg./ml. The pH value of the dialysed enzyme solution was 6.2; it remained practically unchanged after deionization.

Jenkins *et al.* (1959), who found a value of $110\,000 \pm 11\,000$ by ultracentrifugal analysis. However, four sedimentation equilibrium runs gave molecular weights of 93 000, 100 000, 95 000 and 96 000 at the top meniscus. The bottom-meniscus values were consistently about 10% greater than the top ones, and the water-silicone meniscus was somewhat thickened during the run. This indicates the presence of small fractions of aggregates, which are probably responsible for the higher values of the weight-average molecular weight found by light-scattering measurements. The molecular weight of the enzyme was therefore assumed to be 96 000, and this value was used for all the calculations.

Absorption spectrum. The protein band of the purified enzyme is centred at $278\text{m}\mu$ (Fig. 3). The extinction coefficient for a solution containing 1 mg./ml. is 1.440 ($E_{1\text{cm}}^{1\%}$ 14.40); this value is about the same as for the apoenzyme ($E_{1\text{cm}}^{1\%}$ 14.12). The E_{280}/E_{250} ratio is 2.0; the same or slightly higher values were found in several enzyme preparations.

In addition to the well-known indicator behaviour of aspartate aminotransferase, another striking feature for this enzyme has been observed, as reported by Marino *et al.* (1965a); after prolonged dialysis against deionized water, the enzyme solution, which has a pH of 6.2, shows two symmetrical absorption bands at 425 and $365\text{m}\mu$, with $E_{1\text{cm}}^{1\%}$ values 0.470 and 0.325 respectively, whereas

Fig. 5. Effect of pH on the activity of ox-heart aspartate aminotransferase. The enzyme activity is expressed in arbitrary units representing the radioactivity (counts/min.) of the glutamate formed under standard conditions by transamination between L-aspartate and 2-oxo[5- ^{14}C]glutarate, and isolated by paper chromatography as described in the text. The buffers were: ■, acetate; □, phosphate; ●, tris-HCl; ○, boric acid-borax; ▲, carbonate-bicarbonate. All buffers were 0.05 M and the cation was Na^+ . The pH values reported are those measured electrometrically at 20° . Enzyme activity, assayed in three distinct sets of experiments with three different preparations of the pure enzyme, fell within the limits indicated by vertical lines.

after deionization the peak at $425\text{m}\mu$ disappears with concurrent increase of the one at $365\text{m}\mu$ ($E_{1\text{cm}}^{1\%}$ 0.890) (Fig. 4). This effect, which resembles what happens after an increase in pH from 6.2 to 7.7, can be partially reversed by the addition of neutral salts, such as sodium chloride. The apparent pH value of the deionized protein solution is unchanged as measured electrometrically.

Optimum pH. Fig. 5 shows the effect of pH on ox-heart aspartate-aminotransferase activity. This latter is expressed in arbitrary units representing the radioactivity (counts/min.) of glutamate formed by transamination between L-aspartate and labelled 2-oxoglutarate under standard conditions. The assay technique used in these experiments is satisfactory in the whole pH range of enzyme stability. Optimum activity is observed between pH 7.5 and 8.0; 50% of the maximum activity is observed at pH 5.5 and 9.2.

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