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PURIFICATION AND SOME PROPERTIES OF ASPARTATE AMINOTRANSFERASE FROM WHEAT GERM

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SUMMARY

1. A six-step procedure is described for preparing highly purified aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) from wheat germ. An overall purification of 60-fold was achieved.

2. The purified enzyme was homogeneous, as shown by electrophoresis in starch gel and in polyacrylamide gel.

3. The apparent molecular weight measured by gel filtration was about 75 000.

4. The pH optimum was between 8.0 and 8.5.

5. The holoenzyme was readily resolved, and the apoenzyme was reactivated by pyridoxal 5'-phosphate or pyridoxamine 5'-phosphate.

6. The equilibrium constant was measured for the reaction: L-aspartate + α-oxoglutarate $\rightleftharpoons$ L-glutamate + oxaloacetate.

7. There was no evidence for metal-ion activation of this plant aminotransferase.

INTRODUCTION

Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) has been highly purified from pig or ox heart by several groups of workers (listed by MARINO *et al.*[1]). The coenzyme, pyridoxal 5'-phosphate, is tightly bound to the mammalian enzyme[2]. By contrast, the coenzyme is readily removed from the aspartate aminotransferase of wheat germ during extraction and dialysis[3].

The highly purified enzyme from pig heart contains no significant quantity of bound metal ions[2], and there is no evidence for the involvement of metal ions in the catalytic activity of this enzyme[4]. Aspartate aminotransferase from green beans, however, was reported to be activated by Fe^{2+} in the presence of pyridoxal 5'-phosphate[5].

The present paper reports the preparation of highly purified aspartate aminotransferase from commercial wheat germ. The iron content was measured at various

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stages of purification and the possibility of activation by Fe^{2+} was studied. The molecular weight, equilibrium constant, activation by pyridoxal 5'-phosphate and some other properties are also reported.

EXPERIMENTAL

Materials

DL-Aspartic acid, L-aspartic acid, L-glutamic acid, pyruvic acid, oxaloacetic acid, pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate were purchased from British Drug Houses (Poole, Dorset). 2-Oxoglutarate was from Halewood Chemicals (Stanwell, Middx.). Alumina Cγ gel was from Sigma Chemical Co. (London). Calcium phosphate gel was prepared and aged[6] for 3–4 weeks. Sephadex G-100 was from Pharmacia (Great Britain). DEAE-cellulose, DE 11, was from Reeve Angel and Co. Commercial aspartate aminotransferase from pig heart was from Sigma Chemical Co. (London). Other purified proteins for use as standards in the molecular weight determination were commercial samples[7].

$(NH_4)_2SO_4$ and all other chemicals were analytical grade commercial products. Glass-distilled water was used.

Source material of enzyme. Commercial wheat germ sold as a dietary supplement ("Bemax", Vitamins, London) was used throughout.

Methods

Purity of substrates. The purity of the amino acids was checked by high-voltage paper electrophoresis at appropriate pH values[8], and that of oxo acids by paper chromatography of the 2,4-dinitrophenylhydrazones[9]. The purity of pyruvic acid was also assayed using a pyruvate carboxylase preparation from yeast[10,25]. Pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate were examined by low-voltage paper electrophoresis[11].

Protein estimation. During the enzyme fractionation, protein concentration was determined colorimetrically[12]. Commercial freeze-dried whole serum of standardized protein content (Burroughs Wellcome and Co., London), appropriately diluted with 0.9% (w/v) aqueous NaCl, was used as protein standard.

Iron determination. Fe^{2+} was measured colorimetrically[13].

Molecular weight determination. A column of Sephadex G-100 (15 cm × 80 cm) was calibrated with proteins of appropriate molecular weight[7]. The void volume was measured using Blue Dextran.

Enzyme assay. Aspartate aminotransferase activity was assayed colorimetrically[14]. 0.1 ml of aqueous enzyme with 0.1 ml aqueous pyridoxal 5'-phosphate (1 μg) and 0.8 ml of 0.05 M Tris–HCl (pH 8.5) were preincubated at 30° for 10 min. The reaction was then started by addition of 0.5 ml of substrate mixture, containing 100 μmoles of DL-aspartate and 1 μmole of α-oxoglutarate in 0.3 M borate buffer (pH 8.5). Enzyme action was stopped after 10 min at 30° by addition of 0.5 ml saturated aniline citrate solution, which also converted the oxaloacetate formed into pyruvate. The pyruvate was finally measured as the 2,4-dinitrophenylhydrazone[14].

Zone electrophoresis. Starch-gel electrophoresis was carried out horizontally[15]. The gel was prepared in pH 8.4 buffer (0.076 M Tris–0.005 M citric acid) The final pH

of the gel was 8.0. The electrode vessels contained 0.3 M borate buffer (pH 8.4). Protein bands were detected in the gel with Amino Black 10B and aspartate aminotransferase activity was detected using the method of BOYDE AND LATNER[16]. Electrophoretic mobility values were not corrected for electro-endosmotic flow. Disc electrophoresis in polyacrylamide gel was carried out as described by ORNSTEIN AND DAVIES[17]

Purification

(1) Extraction. The wheat germ was extracted, at room temperature, 3 times with 1.5 vol. of ethyl ether and dried at room temperature. 50 g of this defatted wheat germ were ground to a fine powder with a pestle and mortar, and suspended in 200 ml of distilled water in a stoppered bottle. The suspension was slowly agitated by rotating the bottle on a roller mill for 3 h at room temperature. The mixture was centrifuged at 0° for 30 min at 10 000 rev./min. All further stages in the purification were carried out in a cold room at 4°.

(2) pH fractionation. The original supernatant was adjusted to pH 5.7 with 2 M acetic acid, and the mixture was again centrifuged as above for 30 min.

(3) $(NH_4)_2SO_4$ fractionation. The supernatant from Step 2 was adjusted to pH 7.0 with 2 M NaOH and treated with a saturated aqueous solution of $(NH_4)_2SO_4$ (pH 7.0). The enzyme was precipitated between 33 and 66% saturation with $(NH_4)_2SO_4$. This fraction was dialysed against several changes of 10 l 1% (w/v) KCl for 15 h.

(4) Treatment with calcium phosphate gel. The dialysed enzyme fraction (20–25 mg protein/ml) was treated with calcium phosphate gel (1 ml of gel equivalent to 62 mg dry wt., per 20 mg total protein). The mixture was stirred well and allowed to stand for 20 min. The suspension was then centrifuged for 30 min at 5000 rev./min. The supernatant retained most of the enzyme activity but lost about one-half of the protein content (Table I).

(5) Treatment with alumina Cγ gel. The supernatant after Step 4 was treated with alumina Cγ gel (580 mg wet wt. equivalent to 46 mg dry wt., per 50 mg total protein). After being mixed and allowed to stand for 20 min, the mixture was centrifuged. The supernatant retained only a small percentage of the enzyme activity. The

TABLE I

PURIFICATION OF WHEAT GERM ASPARTATE AMINOTRANSFERASE

Summary of a typical fractionation of aspartate aminotransferase from 50 g defatted wheat germ (= 46 g dry wt.). 1 unit of enzyme activity is defined as μmoles oxaloacetate (measured as pyruvate) formed per 1 min at 30° in borate buffer (pH 8.4).

Step	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg of protein)*	*Total yield (%)*
(1) Original supernatant.	3640	291	0.08	100
(2) After pH fractionation	3030	273	0.09	93
(3) After $(NH_4)_2SO_4$ fractionation	1200	216	0.18	74
(4) Supernatant from calcium phosphate gel	600	180	0.30	62
(5) Eluate from alumina Cγ gel	250	113	0.45	39
(6) Eluate from DEAE-cellulose	150	105	0.70	36
(7) Rechromatography on DEAE-cellulose	16	75	4.80	26

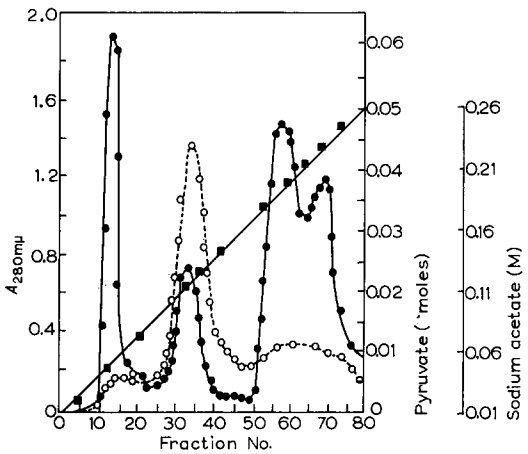


Fig. 1. Rechromatography of wheat germ aspartate aminotransferase on DEAE-cellulose column. Each fraction contained 5 ml. A gradient of 0.01–0.5 M acetate was applied. ●—●, $A_{280\ m\mu}$; ■—■, sodium acetate concentration; ○— — —○, μmoles of pyruvate formed in 1 min at 30°. Fractions 30–40 were pooled.

gel was washed with water and the enzyme eluted with 3 × 20 ml 0.2 M phosphate buffer (pH 8.0).

(6) Chromatography on DEAE-cellulose. The solution from Step 5 was concentrated by freeze-drying and dissolved in 5 ml 0.05 M phosphate buffer (pH 7.4). After dialysis for 8 h against 1 vol. of the same buffer, the solution was applied to a DEAE-cellulose column (2.5 cm × 35 cm). The enzyme was eluted with an NaCl gradient of 0.05–0.5 M. 5-ml fractions were collected, assayed for enzyme activity and the absorbances read at 280 mμ. The active fractions (No. 15–30), eluted with 50 mM NaCl, were pooled and freeze-dried.

(7) Rechromatography on DEAE-cellulose. The enzyme from Step 6 was dissolved in 5 ml of 0.01 M acetate buffer (pH 5.6), dialysed against the same buffer for 8 h and applied to a DEAE-cellulose column (2.5 cm × 40 cm) equilibrated with the buffer. It was eluted with a linear gradient of 0.01–0.5 M acetate buffer of pH 5.6 (Fig. 1). The active fractions were pooled and concentrated by freeze-drying. A summary of the purification is given in Table I.

RESULTS AND DISCUSSION

Purity

Aspartate aminotransferase prepared from wheat germ as described had a high state of purity, although the specific activity is only about 5% of that of similar enzymes obtained from pig and ox heart. This is not due to the method of assay but may be a species difference. An overall purification of 60-fold had been achieved (Table I). This purified enzyme migrates as a single protein band, corresponding to a single band of enzyme activity, in disc and starch-gel electrophoresis. In this latter analysis the homogeneity of the enzyme was tested at different pH values. Mobilities over the range pH 3.1–8.5 indicate an isoelectric point of about

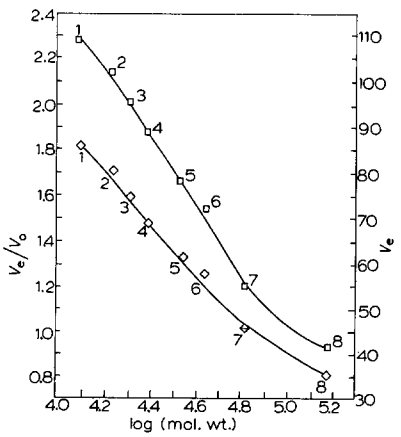
pH 5.0 for the enzyme, comparable to that reported for the ox heart enzyme[1]. However, the migration rate of the wheat germ enzyme was markedly greater than that of the ox heart enzyme.

Stability

Storage at −10° for a few months partially inactivated the purified enzyme. Repeated freezing and thawing also inactivated the preparation. The dialysed $(NH_4)_2SO_4$ fraction, after freeze-drying, was very stable. No appreciable inactivation occurred over a period of 2 years when stored at −10°.

Molecular weight

Gel filtration on Sephadex G-100 gave the apparent molecular weight of the purified enzyme as 75 000 ± 5 000 (six experiments in duplicate). This value is different from the molecular weight of pig heart aspartate aminotransferase, reported to be 110 000 ± 6 000 by ultracentrifugal analysis[18]. The apparent molecular weight of ox heart enzyme by light-scattering measurements is 117 000 ± 4 000 and by ultracentrifugal analysis is 96 000 (see ref. 1). Measurements were made on pig heart enzyme to exclude the possibility that a different molecular weight might be due to use of a different method. In the present study, using the same Sephadex G-100 columns (Fig. 2), aspartate aminotransferase from pig heart gave an apparent molecular weight of 100 000–105 000, in good agreement with the published value obtained by other methods. Therefore, the apparent molecular weight of wheat germ aspartate aminotransferase seems significantly lower than that of the enzyme from ox or pig heart. However, a molecular weight of 78 600 has been reported recently for the pig heart enzyme[19].



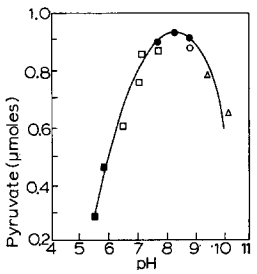


Fig. 2. ◇, plot of protein elution volume (V_e) against log (mol. wt.) for proteins; □, plot of the ratio of the protein elution volume (V_e) to the column void volume (V_0) against log (mol. wt.) of proteins on a Sephadex G-100 column. Experimental details are given in the text. 1, cytochrome *c*; 2, myoglobin; 3, trypsin; 4, α-chymotrypsinogen; 5, pepsin; 6, ovalbumin; 7, serum albumin; 8, γ-globulin.

Fig. 3. Effect of pH on the activity of wheat germ aspartate aminotransferase. The enzyme activity is expressed in μmoles of pyruvate formed per 1 min at 30°, The method of assay is described in the text. The 0.05-M buffers were: ■, acetate; □, phosphate; ●, Tris–HCl; ○, boric acid–borax; △, carbonate–bicarbonate.

Resolution of holoenzyme

The enzyme was partially resolved in the original extract and in the $(NH_4)_2SO_4$ fraction after dialysis[3]. The purified enzyme was found to be almost completely resolved. This ready loss of coenzyme is in marked contrast to the tight binding of coenzyme to the heart enzyme[2].

Reactivation of apoenzyme

In routine assays enzyme was preincubated with 1.0 μg of pyridoxal 5'-phosphate for at least 10 min to activate the enzyme maximally before adding the substrate mixture. The apoenzyme could be activated maximally with similar concentrations of pyridoxamine 5'-phosphate but the time of incubation required was a little longer (15 min). It was thus established that (a) complete activation occurred within 15 min, and (b) the level of activity obtained was independent of using either pyridoxal 5'-phosphate or pyridoxamine 5'-phosphate as cofactor. Smaller amounts of pyridoxal 5'-phosphate (100 mμg) were sufficient to activate the enzyme maximally but the required time of incubation was increased to 60 min.

Optimum pH

With purified enzyme the optimum pH was found to be 8.0–8.5 (Fig. 3). The use of maleate buffers was avoided, since irreversible inhibition of the enzyme occurs at pH 8.0. This is in contrast to the use of maleate buffer to protect the mammalian enzyme during purification[18].

Equilibrium constant

The apparent equilibrium constant K_e for the reaction: L-aspartate + α-oxoglutarate $\leftrightharpoons$ L-glutamate + oxaloacetate, was measured in 0.2 M phosphate buffer (pH 8.0) at 30°.

$$K_e = \frac{[\text{L-aspartate}]\,[\alpha\text{-oxoglutarate}]}{[\text{L-glutamate}]\,[\text{oxaloacetate}]}$$

An amount of activated enzyme, sufficient to bring the system to chemical equilibrium within a few minutes, was added to equimolar amounts of the substrates dissolved in 0.2 M phosphate buffer (pH 8.0). The amounts of products were followed by one of three methods: (a) determination of oxaloacetate in portions of the reaction mixture by direct readings at 270 mμ in the Unicam SP 500 using 0.3 M borate buffer (pH 8.4)[4] or (b) readings at 270 mμ using an automatic Gilford unit attached to a Unicam SP 500 or (c) determination of L-glutamate and L-aspartate as the cadmium–ninhydrin complex spectrophotometrically after high-voltage paper electrophoresis (Locarte and Co., London) on Whatman 3MM paper[20]. Fifteen determinations of K_e were made over a range of concentrations of L-aspartate and α-oxoglutarate from 1.25–20.0 mM. The mean value for K_e was 5.5 $\pm$ 0.3 showing excellent agreement among the three assay methods. By comparison, a mean value of 6.5 was obtained for pig heart enzyme[4] at 25°.

Attempted activation by Fe^{2+}

Possible effects of added Fe^{2+} were tested using the purified enzyme and

$(NH_4)_2SO_4$ fractions. The purified enzyme, which contained no detectable iron (less than 1 μg of Fe^{2+} per mg of protein), was dialysed against chelating agents for 16–18 h at 4°. The slight loss of activity of about 5% on dialysis against 0.1% (w/v) aqueous EDTA, *o*-phenanthroline hydrate, methyl salicylate, or 5 mM 8-hydroxyquinoline in 0.05 M Tris–HCl buffer (pH 8.2), was the same as with dialysis in the absence of chelating agents. No increase in activity was observed after addition of Fe^{2+} (10 μg) in the presence of pyridoxal 5'-phosphate (1 μg per assay). The $(NH_4)_2SO_4$ fraction contained 2 μg of Fe^{2+} per mg of protein. This fraction was dialysed as above. Most of the Fe^{2+} was then lost with about 5% loss in enzyme activity. Addition of Fe^{2+} (10 μg) was without effect on the activity of this treated enzyme when assayed with adequate coenzyme. In the absence of coenzyme, but with added Fe^{2+}, no increase in activity was observed. Thus there was no evidence for the involvement of a metal cation in the catalytic activity of aspartate aminotransferase from wheat germ. This is in contrast with the results of PATWARDHAN[5] using purified aspartate aminotransferase from Indian green beans.

A critical assessment of this latter work reveals that "activation" of the enzyme from green beans may be attributed to nonenzymic decarboxylation of oxoloacetate to pyruvate by Fe^{2+}. This process will tend to drive the reaction in the direction of oxaloacetate production from L-aspartate, *i.e.*, cause an apparent increase in transamination. The difference in activity on incubation of the enzyme from green beans with pyridoxal 5'-phosphate or with pyridoxal 5'-phosphate and Fe^{2+}, was 70 units of pyruvate produced. The chemical decarboxylation of oxaloacetate by Fe^{2+} in the absence of enzyme was 50 units (ref. 5). This apparent activation of enzyme by 20 units, when the enzyme has a full activity of 900 units, can scarcely be regarded as good evidence for metal ion activation. Apparent activation of heart muscle aspartate aminotransferase by Mg^{2+} (ref. 21) was later attributed to Mg^{2+}-catalysed nonenzymic decarboxylation of oxaloacetate[22].

Our findings that aspartate aminotransferase does not require Fe^{2+} as a cofactor are compatible with the hypothesis that the enzymic protein has the same electromeric function as the metal ions in nonenzymic transamination and other such reactions catalysed by pyridoxal 5'-phosphate[23,24]. This evidence does not exclude the possibility that other pyridoxal 5'-phosphate-dependant enzymes may utilize metal ion cofactors.

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