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STUDIES OF THE PARTICULATE AND SOLUBLE ASPARTATE AMINOTRANSFERASES IN GERMINATING PEA COTYLEDONS

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Abstract—The soluble and particulate aspartate aminotransferases (E.C. 2.6.1.1) present in germinating pea cotyledons were isolated by differential centrifugation and partially purified by ammonium sulphate fractionation and gel filtration. The activities of both enzymes varied during the first 5 days of germination, the soluble enzyme accounting for more than 90 per cent of the total extractable aspartate aminotransferase activity. Particulate enzyme activity was solubilized by various treatments including osmotic shock, sonication and by addition of deoxycholate. The general catalytic properties of both enzymes were compared and kinetic experiments suggested that both have a binary mode of action. The ability of various vitamin B_6 derivatives to reconstitute the soluble apoenzyme demonstrated that only pyridoxal-5′-phosphate and pyridoxamine-5′-phosphate were effective. These latter compounds were both tightly bound in the reconstituted enzyme as prolonged dialysis did not appreciably alter the specific enzyme activity. Studies of the rates of reconstitution of the soluble apoenzyme with these coenzymes showed that pyridoxal-5′-phosphate activated the enzyme more readily than equimolar amounts of pyridoxamine-5′-phosphate.

INTRODUCTION

THERE is now considerable information regarding the presence of cytoplasmic and mitochondrial aspartate aminotransferases (L-aspartate:2-oxoglutarate aminotransferase, E.C. 2.6.1.1) in mammalian tissues.[1–7] Both of these enzymes have been crystallized[8] and their physical and catalytic properties have been studied in detail.[2, 4–7, 9, 10]

Although aminotransferase reactions in plants have been known for many years,[11] there have been relatively few detailed studies of these plant enzymes. Partial purifications of plant aspartate aminotransferases have been reported[12–16] and the properties of this

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enzyme from cauliflower florets have been described by Davies and Ellis.[17] These studies have indicated that the plant enzyme has catalytic properties similar to those of the enzyme from mammalian sources. More recently, resolution studies[18] have confirmed that the enzyme from pea cotyledons, like the mammalian enzyme, requires pyridoxal or pyridoxamine-5′-phosphates as a coenzyme. The existence of distinct cytoplasmic and mitochondrial aspartate aminotransferases in plant tissues has not, so far, been demonstrated, although the presence of aminotransferase activities in subcellular fractions[19–22] and the multiplicity of aspartate aminotransferase when subjected to electrophoresis[15] suggest that several forms of these enzymes may occur in plant tissues. The present studies have been conducted in order to determine the possible existence of soluble and particulate aspartate aminotransferases in pea cotyledons during the first 5 days of germination.

RESULTS

Changes in Soluble and Particulate Aspartate Aminotransferase Activities during Germination

In preliminary studies, samples of germinating seeds were examined for possible changes in the levels of both soluble and particulate aspartate aminotransferase activity. Assays were performed on cotyledon extracts which had been subjected to differential centrifugation. The particulate fraction was further treated with 1 per cent deoxycholate before assay of enzyme activity. The results of this survey are summarized in Tables 1 and 2. The data are the average of triplicate assays on two separate samples of germinating cotyledons. Soluble and particulate aspartate aminotransferase activity fluctuated during the germination period examined, high levels of both enzymes being present in 3-and 4-day-old cotyledons. However the particulate fraction contained only 4–5 per cent of the total extractable aspartate aminotransferase activity during this 5-day germination period. These overall changes in enzyme activity suggest that both aspartate aminotransferases may possibly be synthesized or activated during the early stages of germination. The former possibility is likely as seeds which had imbibed 10^{-4} M solutions of chloramphenicol and cycloheximide had lower enzyme activity after 3 days of germination. Such treatments also gave marked inhibition of seedling growth. Treatment of pea seeds with gibberellic acid solutions (10^{-4} M) either

TABLE 1. CHANGES IN ACTIVITY OF THE SOLUBLE ENZYME DURING GERMINATION

Age of cotyledons (days)	Soluble enzyme activity	
	Units/150 g.f.wt.	Units/mg protein
1	208	0·032
2	315	0·051
3	510	0·102
4	560	0·108
5	540	0·088

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TABLE 2. CHANGES IN ACTIVITY OF THE PARTICULATE ENZYME DURING GERMINATION

Age of cotyledons (days)	Particulate enzyme activity	
	Units/150 g.f.wt.	Units/mg protein
1	10·5	0·020
2	12·5	0·026
3	22·0	0·080
4	20·5	0·110
5	16·5	0·052

during imbibition or watering germinating seeds with such solutions resulted in increases in epicotyl length but did not appreciably alter the levels of aspartate aminotransferase present in the cotyledons.

Partial Purification of Soluble and Particulate Enzyme Activities

As 3-day-old cotyledons contained appreciable levels of both enzyme activities, extracts of these tissues were fractionated to increase specific enzyme activity. The soluble enzyme was purified over sixty-seven times when the procedure summarized in Table 3 was followed. The enzyme after Sephadex G-200 treatment was relatively stable as no appreciable losses of activity occurred when such preparations were stored overnight at 4° or for several months at −15° either in a frozen state or after lyophilization. In other fractionation studies chromatography on hydroxylapatite was substituted for the DEAE-cellulose step. Although such chromatography resulted in overall purifications of approximately 200-fold, the enzyme was extremely unstable, losing 60 per cent of its activity when stored at 4° for 24 hr. Similarly the soluble apoenzyme[18] could be purified in excess of 500-fold by DEAE-cellulose chromatography but such preparations were also extremely unstable.

TABLE 3. PARTIAL PURIFICATION OF THE SOLUBLE ASPARTATE AMINOTRANSFERASE

Purification step	Total units	Specific activity	Recovery (%)	Purification
Crude extract	455	0·04	100	—
40–60% $(NH_4)_2SO_4$	182	0·11	40	2·8
After DEAE-cellulose	127	0·92	27·9	23·0
After Sephadex G-200	125	2·70	27·4	67·5

When the particulate fraction was isolated from 3-day-old cotyledons and assayed for aspartate aminotransferase activity it was found that enzyme activity was only detected after transferring the fraction to a hypotonic medium (Table 4). Enzyme activity could be further increased by high-speed blending and by sonication (Table 4). The enzyme was, however, not completely solubilized by such treatments as much of the activity was sedimented when the preparations were centrifuged at 30,000 × *g* for 20 min. Further treatments with 1 per cent deoxycholate as described in the experimental section resulted in essentially complete solubilization of the particulate aspartate aminotransferase activity. Deoxycholate treated preparations were, therefore, used as sources of this enzyme for more detailed studies.

TABLE 4. EXTRACTION OF ENZYME ACTIVITY FROM THE PARTICULATE FRACTION*

Treatment of particulate fraction	Total aminotransferase activity (μmole OAA/min)
Suspended in isolation medium	Not detected
Suspended in 0·05 M Tris-HCl (pH 7·8)	0·037
Suspended in 0·05 M Tris-HCl (pH 7·8) followed by high-speed blending for 2 min	0·056
Suspended in 0·05 M Tris-HCl (pH 7·8) followed by sonication (0·96 A) for 4 min at 4° C	0·056

* Treatments represent alternatives and were not necessarily given in sequence. Enzyme activities are those present in the treated fraction and therefore represent solubilized activity in addition to that remaining in the particulate material.

The particulate enzyme was partially purified by $(NH_4)_2SO_4$ precipitation and gel filtration as shown in Table 5. This fractionation procedure resulted in a purification of approximately 27-fold over the deoxycholate treated material. The enzyme was, however, relatively unstable after such treatments as storing in 0·05 M Tris-HCl (pH 7·8) at $-15°$ for 1 week resulted in complete loss of activity.

TABLE 5. PARTIAL PURIFICATION OF THE PARTICULATE ASPARTATE AMINOTRANSFERASE

Purification step	Total units	Specific activity	Recovery (%)	Purification
Deoxycholate treated particulate suspension	46·5	0·04	100	—
40–60% $(NH_4)_2SO_4$	36·4	0·61	78·2	15·2
After Sephadex G-200	20·1	1·07	43·2	26·8

General Properties of the Soluble and Particulate Enzymes

Under the conditions employed in the spectrophotometric assay, the initial reaction velocities of both enzymes varied linearly with respect to protein concentration. Both enzymes also catalyzed freely reversible reactions as determined by spectrophotometric measurement of oxaloacetate. Furthermore production or disappearance of this keto acid was accompanied by consumption or production of glutamate as shown by paper chromatography of the reaction systems after incubation for 10 min at 37°.

The activity of both aminotransferases was affected by pH as shown in Fig. 1. The soluble enzyme displayed maximal activity at pH 8·0 when assayed spectrophotometrically and by the aspartate dependent conversion of ^{14}C-α-ketoglutarate to ^{14}C-glutamate. Activity of the particulate enzyme was maximal over a broader range of pH (Fig. 1) with high levels of activity being detected between pH values of 6·8 and 8·5. The effects of pH on activity of the soluble and particulate enzymes are therefore very similar to those reported by Morino and Wada[23] for the cytoplasmic and mitochondrial enzymes from bovine liver.

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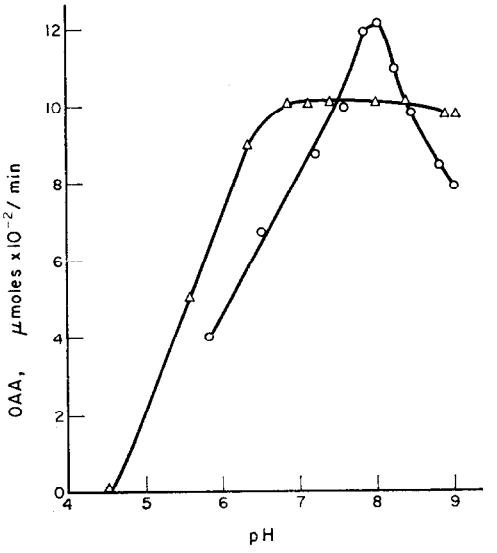

FIG. 1. THE EFFECT OF pH ON THE INITIAL REACTION VELOCITIES OF THE SOLUBLE AND PARTICULATE ENZYMES.

Aliquots containing 0·3 mg protein and 0·14 mg protein of the soluble and particulate enzymes respectively, were assayed in the presence of Tris-acetate buffer. ○—○ soluble enzyme; △—△, particulate enzyme.

The production of oxaloacetate from aspartate and the reverse reaction catalyzed by the soluble enzyme were found to have absolute requirements for α-ketoglutarate and glutamate respectively. The requirement for α-ketoglutarate could not be replaced by pyruvate, hydroxypyruvate or glyoxylate. Similarly the formation of glutamate did not occur in the presence of D-aspartate, L-asparagine, L-serine, L-leucine, L-glutamine, γ-aminobutyrate, L-alanine or L-glycine. These data clearly suggest that the soluble aspartate aminotransferase has a high degree of substrate specificity.

Both enzymes are inhibited by concentrations of oxaloacetate above saturation (Fig. 2). The soluble enzyme was also inhibited by concentrations of α-ketoglutarate above saturation

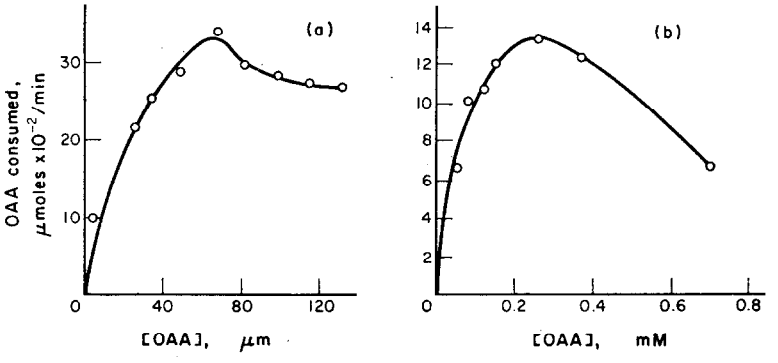

FIG. 2. INITIAL REACTION VELOCITIES OF THE SOLUBLE AND PARTICULATE ENZYMES AS A FUNCTION OF OXALOACETATE CONCENTRATION.

Activities of the soluble (a) and the particulate (b) enzymes were assayed at pH 8·0.

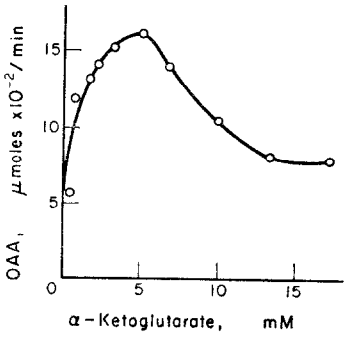

FIG. 3. EFFECT OF VARYING THE α-KETOGLUTARATE CONCENTRATION ON THE INITIAL REACTION VELOCITIES OF THE SOLUBLE ENZYME.

(Fig. 3) but no such inhibition could be observed for the particulate enzyme even at concentrations of α-ketoglutarate as high as $6{\cdot}7 \times 10^{-3}$ M. Various metabolic inhibitors including isonicotinic acid hydrazide and sulphydryl group reagents also inhibited the activity of both enzymes as shown in Tables 6 and 7. As isonicotinic acid hydrazide is known to inhibit

TABLE 6. THE EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF THE SOLUBLE ENZYME

Inhibitor	Concentration (M)	Inhibition (%)
KCN	$1{\cdot}7 \times 10^{-2}$	100
Isonicotinic acid hydrazide	$3{\cdot}3 \times 10^{-3}$	100
Iodoacetate	$3{\cdot}3 \times 10^{-3}$	40
Iodoacetamide	$1{\cdot}7 \times 10^{-2}$	100
p-Chloromercuribenzoate	$2{\cdot}7 \times 10^{-4}$	23

Assay conditions: enzyme (0·4 mg protein) was incubated with the inhibitor and 100 μmoles of Tris-HCl at 37°C for 10 min. Then 20 μmoles of L-aspartate were added and the mixture incubated for a further 5 min. 10 μmoles of α-ketoglutarate were then added to initiate the reaction. Final volume, 3 ml; final pH, 8·0.

TABLE 7. THE EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF THE PARTICULATE ENZYME

Inhibitor	Concentration (M)	Inhibition (%)
KCN	$3{\cdot}3 \times 10^{-3}$	100
Isonicotinic acid hydrazide	$3{\cdot}3 \times 10^{-3}$	100
Iodoacetate	$3{\cdot}3 \times 10^{-3}$	40
Iodoacetamide	$3{\cdot}3 \times 10^{-3}$	40
p-Chloromercuribenzoate	$1{\cdot}9 \times 10^{-4}$	40

Assay conditions: aliquots of the enzyme (0·05 mg protein) were pre-incubated with the inhibitors and 100 μmoles of Tris-HCl at 37°C for 10 min. 40 μmoles of L-aspartate were then added and the mixture incubated for a further 10 min at 37°C. 10 μmoles of α-ketoglutarate were then added to initiate the reaction. Final volume, 3 ml; final pH, 8·0.

enzyme catalyzed reactions which involve pyridoxal-5′-phosphate,[24] it is conceivable that both the soluble and particulate aminotransferases are holoenzymes containing tightly bound pyridoxal-5′-phosphate. The inhibition caused by sulphydryl group reagents suggests that both enzymes contain free sulphydryl groups which are essential for enzyme activity.

Although the role of metal ions in enzymic transamination is still not clear, there have been several reports of activation of various aminotransferases by cations.[16, 25] Table 8 shows that addition of calcium, magnesium and manganese chlorides to the standard reaction system increased the rate of oxaloacetate production by both enzymes. This stimulation of oxaloacetate production was also accompanied by increases in the rate of glutamate formation as shown by the isotopic assay method. As the effect of these ions may be due to a non-enzymic reaction, further experiments with boiled enzyme preparations were conducted. In the presence of $CaCl_2$ and $MgCl_2$ no production of oxaloacetate could be detected. However in the presence of $MnCl_2$ some increase in absorbance at 280 nm was observed but this was considerably lower than the readings obtained with the unboiled enzyme preparations.

TABLE 8. STIMULATION OF SOLUBLE AND PARTICULATE ENZYME ACTIVITY BY DIVALENT CATIONS

Addition of cation*	Enzyme activity, μmole OAA/min	
	Soluble enzyme	Particulate enzyme
None	0·14	0·05
Ca^{2+}	0·20	0·08
Mg^{2+}	0·20	0·06
Mn^{2+}	0·67	0·14

* As the chloride, final concentration $3{\cdot}3 \times 10^{-3}$ M. Sodium, potassium and ammonium chlorides had no effect on the production of OAA at this concentration.

Kinetics and Possible Mechanism of Action of the Soluble and Particulate Enzymes

The apparent Michaelis constants for the substrates attacked by both enzymes were determined according to the method of Lineweaver and Burk[26] (Table 9). In agreement with data reported for the aspartate aminotransferases from mammalian tissues[23] and cauliflower florets[17], the soluble and particulate enzymes from pea cotyledons both displayed higher apparent affinities for the keto acids. Furthermore both enzymes had a greater affinity for oxaloacetate than for α-ketoglutarate.

In order to examine the possible mechanism of action of the soluble and particulate enzymes the reaction kinetics were examined as described by Alberty.[27] When initial reaction velocities are plotted versus varying concentrations of one substrate at fixed concentrations of the second substrate two distinct types of Lineweaver–Burk plots can result. Firstly, in reactions which involve simultaneous substrate binding (ternary mechanism) the Lineweaver–Burk plots will give a series of straight lines which tend to converge. Secondly, in reactions which involve successive binding of substrates (binary mechanism) the

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TABLE 9. APPARENT MICHAELIS CONSTANTS FOR THE SOLUBLE AND PARTICULATE ENZYMES

Substrate concentration not varied (M)	Substrate concentration varied	k_m (M)
(a) Soluble enzyme		
L-Aspartate $6{\cdot}7 \times 10^{-3}$	α-Ketoglutarate	$6{\cdot}7 \times 10^{-4}$
L-Glutamate $6{\cdot}7 \times 10^{-3}$	Oxaloacetate	$4{\cdot}8 \times 10^{-5}$
α-Ketoglutarate $3{\cdot}3 \times 10^{-3}$	L-Aspartate	$8{\cdot}7 \times 10^{-4}$
Oxaloacetate $3{\cdot}3 \times 10^{-4}$	L-Glutamate	$4{\cdot}4 \times 10^{-3}$
(b) Particulate enzyme		
L-Aspartate $13{\cdot}4 \times 10^{-3}$	α-Ketoglutarate	$7{\cdot}3 \times 10^{-4}$
L-Glutamate $13{\cdot}4 \times 10^{-3}$	Oxaloacetate	$4{\cdot}5 \times 10^{-5}$
α-Ketoglutarate $3{\cdot}3 \times 10^{-3}$	L-Aspartate	$1{\cdot}6 \times 10^{-3}$
Oxaloacetate $0{\cdot}33 \times 10^{-3}$	L-Glutamate	$6{\cdot}4 \times 10^{-3}$

Assay conditions (a) soluble enzyme (0·3–0·4 mg protein) was preincubated with the amino acid and 100 μmoles of Tris-HCl for 5 min at 37°; (b) particulate enzyme (0·08 mg protein) was preincubated with 100 μmoles of Tris-HCl and the amino acid at 37° for 10 min. The keto acid was then added in both cases to initiate the reaction. Final volume, 3 ml; final pH, 8·0.

Lineweaver–Burk plots will give a series of parallel lines. Experiments were therefore conducted with both enzymes using suitable combinations of the four substrates in order to determine whether the reaction had a ternary or binary mechanism. A large number of determinations were made using different samples of the partially purified enzymes. In all cases the Lineweaver–Burk plots were linear and parallel. Representative data from these experiments are given in Figs. 4 and 5.

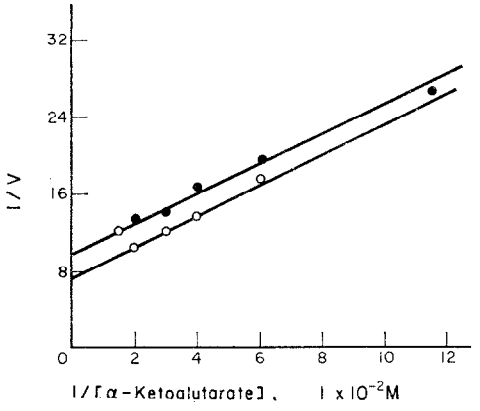

FIG. 4. LINEWEAVER–BURK PLOT OF INITIAL REACTION VELOCITIES OF THE SOLUBLE ENZYME VS. α-KETOGLUTARATE CONCENTRATION AT TWO FIXED CONCENTRATIONS OF ASPARTATE.

Aliquots of the soluble enzyme (0·1 mg protein) were preincubated for 10 min at 37° with 100 μmoles Tris-HCl (pH 8·0) and L-aspartate (o—o, $13{\cdot}3 \times 10^{-3}$ M; ●—●, $6{\cdot}5 \times 10^{-3}$ M). Various concentrations of α-ketoglutarate were then added to initiate the reaction.

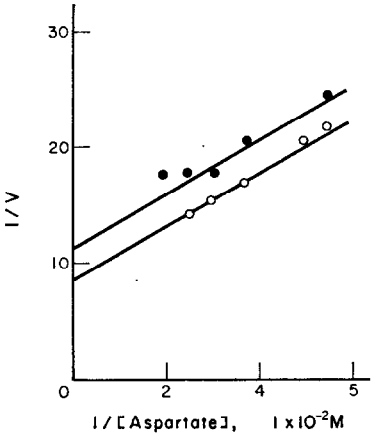

FIG. 5. LINEWEAVER–BURK PLOTS OF INITIAL REACTION VELOCITIES OF THE PARTICULATE ENZYME VS. ASPARTATE CONCENTRATION AT TWO FIXED CONCENTRATIONS OF α-KETOGLUTARATE.

Aliquots of the particulate enzyme (0·1 mg protein) were preincubated for 10 min at 37° with 100 μmoles of Tris-HCl (pH 8·0) and α-ketoglutarate (o—o, $6{\cdot}7 \times 10^{-3}$ M; ●—●, $1{\cdot}7 \times 10^{-3}$ M). Various concentrations of L-aspartate were then added to initiate the reaction.

Reconstitution of the Soluble Apoenzyme

Earlier studies by Wong and Cossins[18] showed that the soluble aspartate aminotransferase had absolute requirements for either pyridoxal-5′-phosphate or pyridoxamine-5′-phosphate after the partially purified enzyme was subjected to treatments that are effective in resolving the enzyme from mammalian sources. Before such treatments, activity of the plant enzyme was only slightly stimulated by additions of pyridoxal-5′-phosphate. In the present work coenzyme requirements were examined in more detail using various other derivatives of vitamin B_6. In agreement with earlier experiments the apoenzyme displayed enzyme activity in the presence of pyridoxal-5′-phosphate and pyridoxamine-5′-phosphate. However, no production of oxaloacetate could be detected when the apoenzyme was preincubated with pyridoxal-HCl, pyridoxine phosphate or pyridoxamine-HCl. Binding of pyridoxal-5′-phosphate and pyridoxamine-5′-phosphate to the active site was of a firm nature, as prolonged dialysis against distilled water at 4° for periods up to 70 hr did not appreciably alter the specific enzyme activity. However, samples of the apoenzyme that were preincubated with the other vitamin B_6 derivatives and then dialyzed against distilled water for 70 hr at 4° were readily activated on addition of either pyridoxal-5′-phosphate or pyridoxamine-5′-phosphate. The latter compounds were, however, apparently bound to the apoenzyme at different rates when these coenzymes were added in small amounts. For example in experiments with 0·14 mg of apoenzyme protein and 0·04 μmole of the authentic coenzymes, maximal enzyme activity was achieved after 10 min preincubation at 37° with pyridoxal-5′-phosphate but such incubations with pyridoxamine-5′-phosphate had to be extended to 50 min before maximal activity was observed. In other experiments using 0·14 mg of apoenzyme protein and 0·2 μmole of pyridoxal and pyridoxamine-5′-phosphates, maximal enzyme activities were reached within 10 min at 37°.

The reconstituted enzyme was sufficiently stable to allow at least two further resolution and reconstitution treatments without appreciable loss of enzyme activity. Furthermore the reconstituted soluble enzyme was, like the partially purified soluble holoenzyme, inhibited

by iodoacetate and *p*-CMB and was stimulated by the chlorides of manganese, calcium and magnesium.

DISCUSSION

The present studies have shown that aspartate aminotransferase activity is readily detected in both the soluble and particulate fractions of germinating pea cotyledons. Solubilization of the particulate enzyme activity by means of osmotic shock, sonication and treatment with deoxycholate clearly suggests that this activity is membrane bound. Although the exact nature of the particulate fraction was not fully determined in the present work, preliminary examination of this material by electron microscopy after negative staining with phosphotungstic acid revealed the presence of intact mitochondria. However, the particulate material was heterogenous when subjected to centrifugation in a discontinuous sucrose density gradient.[28] After such treatment four distinct bands of material were observed, two of which contained detectable aspartate aminotransferase activity. Experiments are now being conducted in order to examine the enzyme activities of these fractions in more detail.

The general catalytic properties of the soluble and particulate enzymes are in many cases sufficiently different to indicate that these are distinct proteins with aspartate aminotransferase activity. Both enzymes have different degrees of stability after partial purification. The soluble enzyme was very stable but the particulate enzyme lost activity under similar conditions. Both enzymes displayed a high degree of substrate specificity but the range of pH over which the reaction could be detected was different (Fig. 1). The cytoplasmic and mitochondrial enzymes of bovine liver also have different pH optima.[23]

On the basis of the Michaelis constants (Table 9), the decreasing order of affinity for the substrates of both enzymes was found to be oxaloacetate, α-ketoglutarate, aspartate and glutamate. This order is therefore similar to that displayed by both enzymes from bovine liver.[8] However, when comparing the apparent affinities for each substrate (Table 9) it is clear that the two enzymes differ in their affinities for aspartate and glutamate. The soluble enzyme has a higher apparent affinity for the two amino acids than the particulate enzyme.

In agreement with earlier studies on aspartate aminotransferase from plant[17] and animal tissues,[29] the enzymes from pea cotyledons were both inhibited by high concentrations of oxaloacetate (Fig. 2). In addition the soluble enzyme was also inhibited by high concentrations of α-ketoglutarate (Fig. 3). This latter keto acid did not inhibit the particulate enzyme at a comparable concentration. Velick and Vavra[29] have suggested that these keto acids form abortive complexes with the cytoplasmic enzyme from pig heart. Both enzymes also appear to have a binary mechanism of action (Figs. 4 and 5). The detailed studies of Davies and Ellis[17] on the aspartate aminotransferase from cauliflower florets and by Turano *et al.*[30] on the alanine aminotransferase from cotton seeds have also given data consistent with a binary mechanism for transamination in plants. Similar kinetic studies of the cytoplasmic aspartate aminotransferases from mammalian tissues[3, 4, 29, 31, 32] are consistent with a binary mechanism in which amino group transfer occurs via the intermediary formation of a pyridoxamine phosphate–enzyme complex. Further support for operation of this type

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of mechanism in the present plant enzyme comes from the observation that the resolved soluble enzyme can be reconstituted with either pyridoxal-5′-phosphate or pyridoxamine-5′-phosphate. This suggests that the enzyme can exist in aminic and aldehydic forms which are presumably interconvertible as addition of either coenzyme led to production of oxaloacetate from aspartate. Direct evidence for interconversion of the pyridoxal and pyridoxamine forms of pig heart aspartate aminotransferase has come from the spectrophotometric studies of Lis *et al.*[33] However, similar studies with the soluble enzyme from pea cotyledons were inconclusive, possibly due to the relatively low specific enzyme activities of these preparations even after gel filtration. In addition the coenzyme content of these plant preparations may limit application of spectrophotometry in elucidation of this property.

Present investigations of these plant aspartate aminotransferases are designed to determine the coenzyme contents of the soluble and particulate enzymes.

MATERIALS AND METHODS

Plant Material

Seeds of *Pisum sativum* L. var. Homesteader, were soaked in distilled water for 24 hr at room temperature and then surface sterilized by rapid washing in 0·1 per cent (w/v) mercuric chloride solution followed by thorough rinsing in distilled water. The seeds were then germinated in moist vermiculite in darkness at 28° for periods up to 5 days. Under these growth conditions the radicles emerged approximately 24 hr after planting the seeds in vermiculite.

Chemicals

All general chemicals were of the highest purity available commercially. $(NH_4)_2SO_4$ (special enzyme grade) was supplied by Mann Research Laboratories, New York and Sephadex gels were purchased from Pharmacia Canada Ltd., Montreal. Sodium α-ketoglutarate-5-^{14}C was supplied by the Radiochemical Centre, Amersham, England and cycloheximide was purchased from Sigma Chemical Company, St. Louis, U.S.A. Solutions were in all cases prepared in glass redistilled demineralized water.

Extraction of Soluble and Particulate Aspartate Aminotransferases

All procedures were carried out at 4°. The testas and embryos were removed from the germinating seeds and samples of the cotyledons (300 g fresh weight) were ground in a hand mill with 600 ml of a grinding medium containing 0·3 M mannitol, 0·1 per cent bovine serum albumin, 0·05 per cent cysteine and 0·001 M EDTA at pH 7·2 (Ref. 34). The homogenate was ground manually for a further 30 sec in a mortar and then passed through fine cheesecloth to remove cellular debris. The homogenate was centrifuged for 15 min at $1500 \times g$ and the supernatant again centrifuged for 15 min at $10,000 \times g$. Enzyme activity in this supernatant is designated as soluble aspartate aminotransferase. Material sedimented at $10,000 \times g$ was suspended in a medium containing 0·3 M mannitol, 0·1 per cent bovine serum albumin and 0·001 M EDTA at pH 7·2 and again sedimented by centrifugation for 15 min at $10,000 \times g$. The material sedimented was then suspended in the wash medium or in 0·05 M tris-HCl buffer (pH 7·8). Enzyme activity in this fraction is designated as particulate aspartate aminotransferase.

Partial Purification of the Soluble Aspartate Aminotransferase

As preliminary experiments demonstrated that the bulk of the tissue aspartate aminotransferase activity was associated with the $10,000 \times g$ supernatant this enzyme was routinely extracted by grinding 250 g samples of the cotyledons with 500 ml of 0·05 M tris-HCl (pH 7·8) in a Waring blendor for 1 min followed by centrifugation at $10,000 \times g$ for 20 min. The soluble enzyme was then partially purified by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose as previously described.[18] Following chromatography on DEAE-cellulose, fractions containing enzyme activity were dialyzed against distilled water, lyophilized and dissolved in approximately 10 ml of distilled water. 3 ml of this solution, containing approximately 85 mg of protein, were then applied to a $2·8 \times 50$ cm column of Sephadex G-200 which had previously been equilibrated with 0·005 M Tris-HCl (pH 7·8). The column was eluted with 0·005 M Tris-HCl (pH 7·8) at a flow rate of 20 ml/hr. Fractions of 3 ml were collected. Enzyme activity was located in fractions 51–64.

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Partial Purification of the Particulate Aspartate Aminotransferase

Enzyme activity in the particulate material was solubilized by various treatments including high speed blending for 2–4 min at 4°, by sonication in a Raytheon Sonic Oscillator operated at 0·96 A for 4 min and by osmotic shock treatment in 0·005 M Tris-HCl (pH 7·8). Further solubilization was achieved by suspending the washed particulate material in 0·05 M Tris-HCl containing 1 per cent (w/v) deoxycholate at pH 8·0. After centrifugation for 20 min at 30,000 × *g* the supernatant was fractionated with $(NH_4)_2SO_4$ as described for the soluble enzyme. Protein precipitating between 40 and 60 per cent of saturation was recovered and dissolved in 15 ml of 0·05 M Tris-HCl (pH 7·8). 12 ml of this solution were then applied to a 2·8 × 50 cm column of Sephadex G-200 which had previously been equilibrated with this buffer. The column was eluted with 0·05 M Tris-HCl (pH 7·8) at a flow rate of 50 ml/hr. Fractions of 3 ml were collected. Enzyme activity was located in fractions 45–58.

Assay of Enzyme Activity

Aspartate aminotransferase activity was assayed spectrophotometrically at 280 nm using a slight modification of the method described by Jenkins *et al.*[35] Assay of the soluble enzyme activity involved preincubation of the enzyme (up to 2 mg protein) with 20 μmoles of L-aspartate and 100 μmoles of Tris-HCl (pH 8·0) for 5 min at 37°. α-Ketoglutarate (10 μmoles) was then added to initiate the reaction. Assay of the particulate enzyme activity involved preincubation of the enzyme (up to 0·5 mg protein) with 40 μmoles of L-aspartate and 100 μmoles of Tris-HCl (pH 8·0) for 10 min at 37°. α-Ketoglutarate (10 μmoles) was then added to initiate the reaction. In both cases changes in absorbance of the reaction mixtures (total volume 3 ml) were followed at 280 nm in a Beckman double-beam spectrophotometer (model DB-G) equipped with a potentiometric recorder. The reference cuvette contained all the reactants with the exception of α-ketoglutarate which was replaced by distilled water. Changes in absorbance were followed for periods up to 5 min at 37°. Initial reaction velocities were calculated from the tangent drawn to the reaction time course curve. One unit of enzyme activity is defined as the amount required to catalyze the production or consumption of 1 μmole of oxaloacetate under the defined experimental conditions.

Aspartate aminotransferase activity was also assayed by measuring the aspartate dependent production of ^{14}C-labelled glutamate from ^{14}C-α-ketoglutarate. The reaction system contained the components described for the spectrophotometric assay except that 9 μmoles of α-ketoglutarate (containing 0·3 μc of ^{14}C) were added to initiate the reaction. After incubation at 37° for 30 sec the reaction was terminated by addition of 4 ml of 4 N HCl. The reaction system was then fractionated using Dowex 50W × 8 (H^+ form) as described earlier.[36, 37] The amino acid fraction obtained in this way was further separated by descending paper chromatography.[38] Radioactive areas on the chromatograms were carefully cut out and placed directly into scintillation vials containing 9·6 g of 2,5-biphenyloxazole (PPO) and 0·4 g of 1,4-bis,2,5-phenyloxazolyl (POPOP) in an 800 ml mixture of dioxane:anisole:1,2-dimethoxyethane (6:1:1 by volume). Samples were assayed for radioactivity using a Nuclear Chicago liquid scintillation counter (Model Unilux II) with a counting efficiency of approximately 68 per cent. In all cases the counts were corrected for background and for the small amounts of glutamate labelling occurring in the absence of aspartate. Protein was assayed colorimetrically.[39]

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