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CHARACTERIZATION OF TWO MULTISPECIFIC ASPARTATE AMINOTRANSFERASE ISOZYMES PURIFIED FROM BUSHBEAN SHOOTS CAPABLE OF TRANSAMINATING AROMATIC AMINO ACIDS AND SEVERAL DL-CHLORO-PHENYLALANINES*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; bushbean; metabolism; enzyme characterization; multispecific aspartate aminotransferase; phenylalanine aminotransferase; isozymes; DL-chloro-phenylalanines.

Abstract—Two electrophoretically distinct isozymes of L-phenylalanine aminotransferase (Enz I, Enz II) purified from a total soluble shoot extract of bushbean have been characterized. The M_rs of Enz I and Enz II were 100000 and 110000, respectively. Both isozymes showed pH optima of 8.5. Enz I was able to use either 2-oxoglutarate (2-OG) or oxaloacetate (OAA) equally as a keto acid substrate when L-phenylalanine was the amino donor, while Enz II preferred 2-OG. Neither isozyme was able to use glyoxylate or pyruvate in the presence of L-phenylalanine. When tested with a range of protein amino acids, both Enz I and Enz II showed the highest rate of transamination with L-aspartate, indicating that both isozymes were L-aspartate aminotransferases capable of also showing L-aromatic aminotransferase activity. L-Phenylalanine aminotransferase activity relative to L-aspartate aminotransferase activity was found to be 0.6% for Enz I and 3.3% for Enz II. Lineweaver–Burk plots of kinetic data gave apparent K_m values (mM) for Enz I of 2.3 (L-Asp), 55.0 (L-Phe) and 9.0 (2-OG) and for Enz II, 2.8 (L-Asp), 320.0 (L-Phe) and 8.2 (2-OG). The values were confirmed by treatment of the data by Hill plots. When tested with a series of 12 ring-substituted DL-chloro-phenylalanines, Enz I was active only with the 3-chloro- and 4-chloro-compounds, while Enz II was active with all three monochloro-compounds as well as with the 2,4-, 2,6- and 3,4-dichlorophenylalanines. The activity of Enz II with 4-chlorophenylalanine was very high, 222% higher than that observed with DL-phenylalanine. Enz I was completely inhibited by 1.0 mM Ca^{2+} while Enz II was unaffected by this cation, which suggested different subcellular locations for each isozyme. Cell fractionation studies indicated, however, that both Enz I and Enz II were cytoplasmic. Different isozymes of this multispecific aspartate–aromatic aminotransferase were found in the chloroplasts and mitochondria of bushbean shoots.

INTRODUCTION

During studies of the metabolic pathways leading from L-phenylalanine to the natural auxin, phenylacetic acid (PAA) [1–4], we became interested in the possibility that synthetic ring-substituted chlorophenylalanines [5] might serve as metabolic precursors for the corresponding chlorophenylacetic acids [6]. Many of the latter compounds are known to be potent plant growth regulators and certain acids have been shown to be powerful herbicides [7, 8].

In the first paper of this series we reported that the initial metabolism of chloro-phenylalanines in bushbean (*Phaseolus vulgaris* L.) shoot extracts occurs via the activity of an aminotransferase rather than a decarboxylase [6], and suggested that this transamination reaction is likely to be the first step in a metabolic pathway leading to chlorophenylacetic acids. The enzyme potentially capable of catalysing this first reaction, L-phenylalanine aminotransferase, was therefore purified from 10-day old bushbean shoots and resolved into two electrophoretically distinct isozymes, Enzymes I and II (Enz I, Enz II), which were found to be capable of transaminating three monochloro-phenylalanines [6]. The present paper describes the detailed characterization of the L-phenylalanine aminotransferase isozymes I and II and an investigation of their subcellular location in bushbean shoots.

RESULTS AND DISCUSSION

The two isozymic forms of L-phenylalanine aminotransferase (Enz I and Enz II) purified from a total soluble extract of bushbean shoots by methods previously described [6], were characterized with respect to their M_rs, substrate specificities (particularly to a series of synthetic DL-chlorophenylalanines), their subcellular localization and a range of other physico-chemical properties.

*Part 2 in the series: Metabolism of chlorophenylalanines by multispecific aspartate-aromatic aminotransferases in crop and weed plants. This series is dedicated to the memory of a former colleague, Budhi Singh Rauthan, who first demonstrated in 1972 the *in vivo* conversion of L-phenylalanine to phenylacetic acid in higher plants. Mr. Rauthan was killed in the crash of the Air India Boeing 747 into the Atlantic Ocean, 23 June 1985.

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M_rs

The M_rs of Enz I and Enz II were estimated by gel filtration [9] on a Sephacryl S-300 column calibrated with marker proteins as described in the Experimental. There was a good linear correlation between the log of the M_r of the marker proteins and their V_e/V_o values. Assuming that L-phenylalanine aminotransferases I and II behave as globular proteins, their estimated M_rs are 100000 and 110000, respectively. These values are similar to those previously reported for aminotransferases from several plant sources [10–13]. Although Enz I and II were not resolved into their component subunits, plant aminotransferases in this M_r range have generally been found to be composed of two subunits, each half the M_r of the dimer (*ca* 50000 each) [14–16].

pH and temperature optima

The pH optima for Enz I and II were determined by examining phenylalanine aminotransferase activity at pH values ranging from 6.0 to 9.5, using a series of buffers to ensure that maximum buffering capacity was available at each pH tested (MES, pH 6–6.5; MOPS, 7–7.5; Tris, 8–8.5; CHES, 9–9.5). When assayed in the presence of 40 mM L-phenylalanine, 10 mM 2-oxoglutarate (2-OG) and 0.1 mM pyridoxal phosphate (PLP), the pH optimum for Enz I activity was found to be 8.5–9.0 while Enz II activity was optimal at pH 8.5. When the pH optima were examined in constant ionic strength Tris buffer (50 mM) over the same pH range, identical optima were observed. The pH optimum reported for a multispecific aromatic aminotransferase purified from bushbean roots was also 8.5 [13] and ranges of pH 8.0–8.9 are common for the optimal activity of plant aminotransferases [17].

The assay temperature for optimum activity of Enz I and II in the presence of 40 mM L-phenylalanine, 10 mM 2-OG and 0.1 mM PLP, was found to be 35°. Enzyme activity fell rather sharply on either side of this value. Aminotransferase activity was linear with time up to at least 2 hr under our assay conditions. Within the limits of enzyme concentration tested (2–20 μg of Enz I or Enz II), a linear relationship was observed between activity and amount of enzyme.

Substrate specificity

The keto acid substrate specificity of Enz I and II was determined by using 40 mM L-phenylalanine and 10 mM keto acid substrate. Of the four common keto acids utilized by plant aminotransferases, 2-oxoglutarate (2-OG) and oxaloacetate (OAA) were able to serve as amino group acceptors, but not glyoxylate or pyruvate. While Enz I could utilize 2-OG or OAA about equally when L-phenylalanine was the amino donor (2-OG, 47.6×10^{-3} units/mg protein; OAA, 48.8×10^{-3} units/mg protein), Enz II clearly preferred 2-OG under these conditions (2-OG, 133×10^{-3} units/mg protein; OAA, 85.2×10^{-3} units/mg protein). Since we were mainly concerned with phenylalanine and chlorophenylalanine transamination, 2-OG was chosen as the keto acid substrate for routine aminotransferase assays.

The substrate specificities of Enz I and II for a range of protein L-amino acids were next examined, with 2-OG supplied as the keto acid (Table 1). These results show that both isozymes were primarily L-aspartate aminotransferases and had relatively low activities (0.1–5.0%) with the aromatic amino acids, L-phenylalanine, L-tyrosine, and L-tryptophan. When oxaloacetate (OAA) was supplied as the keto acid, the isozymes were able to use L-glutamate as an amino donor almost as effectively as L-aspartate (Table 1). It should be noted that commercial sources of L-cysteine and L-asparagine were found to be contaminated with 1–2% aspartate while L-glutamine was similarly contaminated with glutamate. These substrate amino acids were therefore purified before testing by ion exchange chromatography on Dowex 1 (Cl) resin at pH 7.0, as described in ref. [18]. After purification they were found to be inactive as substrates when tested with Enz I and II.

PAGE of Enz I and Enz II preparations gave similar protein banding patterns to those reported in the previous paper of this series [6]. When identical gels were sliced and assayed for both L-phenylalanine and L-aspartate aminotransferase activities, only the major protein band in each

Table 1. L-Amino acid substrate specificity of the L-phenylalanine aminotransferase isozymes I and II purified from bushbean shoots

L-Amino acid tested*	Isozyme I % Relative†		Isozyme II % Relative†	
Asp	100.0		100.0	
Glu	87.5		81.0	
Ala	0		0	
Val	0		0	
Leu	0		0	
Ile	0		0	
Pro‡	0		0	
Phe	0.60	(100)	3.3	(100)
Try	0.13	(25)	5.1	(146)
Tyr	0.27	(55)	0.6	(17)
Met	0		0	
Cys§	0		0	
Gly	0		0	
Ser	0		0	
Thr	0		0	
Asn§	0		0	
Gln§	0		0	
Lys	0		0	
Arg	0		0	
His	0		0	

*All L-amino acids were supplied at 20 mM final concentration. 2-OG was supplied as the keto acid at 10 mM final concentration, except in the case of glutamic acid when OAA was the keto acid supplied at 10 mM final concentration. Amino acid products were measured by OPA-HPLC [6].

†Percentage activity is expressed relative to aspartate aminotransferase activity set at 100% (4 μ/mg protein). One unit (μ) of aminotransferase activity will convert 1 μmol of oxo acid substrate to 1 μmol of the corresponding amino acid. Values in parenthesis are percentage activity expressed relative to phenylalanine aminotransferase activity set at 100% (0.02 μ/mg protein).

‡Aminotransferase activity was measured by paper chromatography, as described in Ref. [6].

§Due to contamination with aspartic acid or glutamic acid, these amino acids were first purified by ion-exchange chromatography using Dowex 1 (Cl) resin at pH 7.0, before being tested as substrates.

isozyme preparation (at R_f 0.60 in Enz I fraction, and at R_f 0.70 in Enz II fraction) was shown to exhibit aminotransferase activity with both L-aspartate and L-phenylalanine, when 2-OG was supplied as the amino group acceptor. This confirms that only one protein band in each isozyme preparation was responsible for both the L-aspartate aminotransferase and L-phenylalanine aminotransferase activities.

The results of the gel electrophoresis study indicate that Enz I and II from bushbean shoots were both multispecific L-aspartate-L-aromatic aminotransferases, a finding similar to that reported in refs [12, 13] for a multispecific L-aspartate aminotransferase purified from bushbean roots and subsequently shown to be present in animals [19] and bacteria [20]. However, Enz I and II displayed distinct differences in their specificities for the three aromatic amino acids, as shown in Table 1, part B, where the results indicate that Enz I favoured L-phenylalanine as a substrate over L-tyrosine or L-tryptophan, while Enz II showed higher activities with L-tryptophan and L-tyrosine than with L-phenylalanine. These differences in substrate specificity between Enz I and Enz II with respect to L-phenylalanine and L-tryptophan may indicate a preferred role for each isozyme in the formation of the natural growth regulators, phenylacetic acid (PAA) and 3-indoleacetic acid (IAA), which are formed respectively, from L-phenylalanine and L-tryptophan via the arylpyruvate pathway for auxin synthesis in higher plants [1–4].

The substrate specificities of Enz I and II were next examined with the series of ring-substituted mono-, di- and trichlorophenylalanines. The results (Table 2) show that of the 12 chloro-phenylalanines tested, only the 3-chloro- and 4-chloro-compounds were active substrates for Enz I. Enz II showed a broader range of specificity, with six compounds being transaminated. All three monochlorophenylalanines and the 2,4-, 2,6- and 3,4-dichloro-compounds were active substrates with Enz II. These results obtained with the purified preparations of isozymes I and II contrast with previous data obtained with a Sephacryl S-300 purified extract of L-phenylalanine aminotransferase from bushbean shoots, which showed that 11 of the 12 chloro-phenylalanines tested could be transaminated [6]. A possible explanation for this difference in the range of chlorophenylalanine transamination is that in the relatively impure S-300 enzyme fraction, there may have been other aminotransferases which utilized some of the chlorophenylalanines as substrates.

An interesting finding in the present study (Table 2) was the relatively rapid rate of transamination obtained with 4-chlorophenylalanine when tested with Enz II. The other active substrates substituted at the 4-position (i.e. the 2,4- and 3,4-dichlorophenylalanines) did not show such high relative activity. This finding may indicate an important role for chloro-substitution at the 4-position in enzyme-substrate binding and this possibility will be examined in greater detail in the next paper of this series dealing with substrate structure/activity relationships of Enz I and II.

Kinetic studies

Kinetic studies were performed on the L-aspartate–2-OG and L-phenylalanine–2-OG aminotransferase reactions of Enz I and II, examining the effect of varying amino acid or keto acid substrate concentrations on reaction rates. The concentration of one substrate was varied in the presence of a fixed saturating concentration of the other substrate, at pH 8.5. The values for the apparent kinetic constants, K_m and V_{max} (Table 3) were obtained from Lineweaver–Burk plots derived from the initial velocity

Table 2. Substrate specificity of the bushbean multispecific aspartate-aromatic aminotransferase isozymes I and II for DL-phenylalanine and several ring-substituted DL-chlorophenylalanines

Isozyme I‡				Isozyme II§			
Amino acid substrate*	Conc. tested (mM)	Specific activity (U¶/mg protein) $\times 10^{-3}$	% Relative activity†	Amino acid substrate*	Conc. tested (mM)	Specific activity (U¶/mg protein) $\times 10^{-3}$	% Relative activity†
DL-Phe	32	13.8	100	DL-Phe	32	54.0	100
L-Phe	32	34.8	250	L-Phe	32	125.0	232
2-Cl-Phe	32	0	0	2-Cl-Phe	32	6.6	12
3-Cl-Phe	32	4.0	35	3-Cl-Phe	32	23.4	43
4-Cl-Phe	32	12.2	88	4-Cl-Phe	32	120.0	222
				D,L-Phe	16	28.2	100
				L-Phe	16	35.8	200
				2,4-Cl_2-Phe	16	22.2	79
				2,6-Cl_2-Phe	16	22.2	79
				3,4-Cl_2-Phe	16	5.4	20

*2-Oxo-glutarate (10 mM) was the keto acid supplied in all cases.

†Activity with DL-Phenylalanine was set as 100% since all the chlorophenylalanines were in the DL-form.

‡No aminotransferase activity was obtained with Isozyme I when supplied with any of the six dichlorophenylalanines or the three trichlorophenylalanines tested as amino donors in this study.

§No aminotransferase activity was obtained with Isozyme II when tested with 2,3-, 2,5- or 3,5-dichlorophenylalanines, or with any of the three trichlorophenylalanines.

¶One unit (U) of aminotransferase activity is defined as in Table 1.

data for each pseudo-first order reaction [21, 22].

The apparent K_m values for L-aspartate calculated for Enz I (2.29 mM) and Enz II (2.81 mM) are in the same concentration range as those reported for L-aspartate and other substrates of plant aminotransferases (e.g. L-aspartate, 4.14 mM [23]; L-aspartate, 7.25 mM [24]; L-alanine, 2.5 mM [10]; L-serine, 2.72 mM [25]; L-ornithine, 4.7 mM [26]. The K_m values for 2-OG could only be determined at saturating concentrations of L-aspartate, since solubility limits precluded measurements at saturating concentrations of L-phenylalanine (see discussion below). The K_m values for 2-OG were 1.65 mM for Enz I and 3.9 mM for Enz II. In the case of Enz II, the K_m value for the keto acid was higher than that calculated for L-aspartate. However, while the K_m value for the keto acid substrate is usually lower than the K_m for the amino acid substrate, there are exceptions to this generalization [27] (e.g. [26, 28]).

With L-phenylalanine, estimated apparent K_m values for Enz I (55 mM) and Enz II (320 mM) were quite high, although 'true' K_m values of 57–68 mM have been reported for the aromatic amino acids with a multispecific aminotransferase purified from bushbean roots [29]. Generally, the concentrations of substrate chosen to generate the reciprocal plot should be in the neighbourhood of the K_m. Due to the relatively poor solubility of L-phenylalanine, (limit *ca* 80 mM final conc. under assay conditions) this was clearly not possible with Enz II and only marginally feasible with Enz I. If the substrate concentrations chosen are very low relative to K_m, the Lineweaver–Burk plot will intercept both axes too close to the origin to allow V_{max} or K_m to be determined accurately. At very low substrate concentrations, the reaction is essentially first-order and there is no hint of saturation; V_{max} and K_m appear to be infinite [30].

These limitations and the high K_m for L-phenylalanine shown by Enz II, prompted a re-examination of the kinetic data using logarithmic Hill plots [30]. The values for n_H, the Hill coefficient, were all very close to unity, suggesting that there was no co-operativity and only one substrate binding site per subunit; two equivalent binding sites per dimer of enzyme. In addition, the $[S]_{0.5}$ values were in the same range as the corresponding apparent K_m values determined from Lineweaver–Burk plots (see Table 3).

Thus, it appears that the experimentally derived kinetic constants with respect to Enz I and II are reasonable and that Michaelis–Menten kinetics do hold for these determinations. Nevertheless, the high K_m values for L-phenylalanine shown by Enz I and especially Enz II, indicate that if these constants do indeed reflect true *in vivo* enzyme characteristics, then neither aminotransferase will ever encounter saturating substrate concentrations in the intact plant and thus neither will ever reach V_{max}. However, since these multispecific aminotransferases may take part in the catabolic pathway leading from L-phenylalanine to the growth-regulator, PAA [1–4] a pathway which produces only nanomolar amounts of this growth regulator in the lifetime of a normal plant [32, 33], the operation of Enz I or Enz II at maximum activity is probably not necessary and may, in fact, represent a rather coarse control mechanism for regulating the eventual amount of PAA produced, while still allowing a rapid turnover of L-aspartate by the same enzyme. Alternatively, the high apparent K_m values for L-phenylalanine shown by Enz I and especially Enz II, may reflect a degree of aggregation of L-phenylalanine molecules in solution at high concentrations due to their limited solubility in aqueous media. In this case, the enzyme may be 'seeing' a much lower concentration of free L-phenylalanine than that predicted from the volume of phenylalanine stock solution added to the reaction mixture. Indeed, such aggregate effects have been observed during NMR spectroscopy of DL-phenylalanine and the DL-chlorophenylalanines when carried out in D_2SO_4 solvent. This effect resulted in somewhat broader peaks than normally encountered in high resolution NMR spectroscopy [5].

Competition studies were performed to examine the effect of physiological concentrations of L-aspartate on the L-phenylalanine aminotransferase activity of Enz II. When assayed at an unsaturating concentration of L-phenylalanine (32 mM), the activity of Enz II was linearly inhibited by physiological concentrations of L-aspartate (Fig. 1a). The $[I]_{0.5}$ value [30] for L-aspartate was estimated to be 9.5 mM as shown in Fig. 1b. These findings support those of Forest and Wightman [29] who showed that the aromatic aminotransferase activity of a multispecific aspartate aminotransferase from bushbean roots was competitively inhibited by L-aspartate, indicating that both the aliphatic and aromatic amino acid substrates compete for the same active site on the enzyme [17]. Assuming a similar competitive inhibition for Enz II from bushbean shoots and using its known V_{max} value of 1.90 U/mg protein, the K_i for L-aspartate is *ca* 8.5 mM as estimated by a Dixon plot [30].

Table 3. Kinetic parameters of the aspartate-aromatic aminotransferase isozymes I and II from bushbean shoots

		Lineweaver–Burk Data*				Hill data					
		Isozyme I		Isozyme II		Isozyme I			Isozyme II		
Substrate tested	Substrate held constant	K_m (mM)	V_{max} (U†/mg. protein)	K_m (mM)	V_{max} (U†/mg. protein)	$S_{0.5}$	η_H	correl. coeff.	$S_{0.5}$	n_H	correl. coeff.
L-Phe (0–80 mM)	2-OG (10 mM)	55.0	0.090	320.0	1.90	56.3	0.8	0.995	260	1.1	0.998
L-Asp (0–10 mM)	2-OG (10 mM)	2.29	6.54	2.81	3.30	1.9	0.8	0.975	2.2	0.9	0.980
2-OG (0–4 mM)	L-Asp (20 mM)	1.65	6.68	3.9	6.67	1.15	1.1	0.978	3.5	1.04	0.990

*The correlation coefficients in all Lineweaver–Burk plots were 0.992 or better.

†One unit (U) of aminotransferase activity is defined as in Table 1.

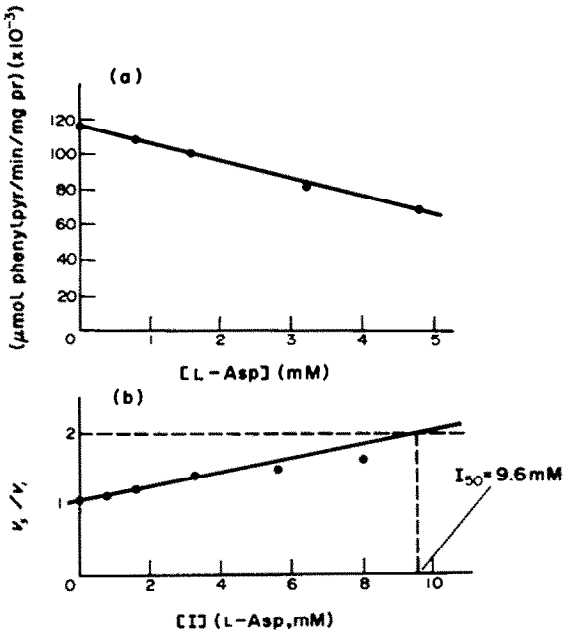

Fig. 1(a). Effect of increasing concentrations of L-aspartate on the L-phenylalanine: 2-OG aminotransferase activity of isozyme II. L-Phenylalanine and 2-OG were supplied at final concentrations of 32 mM and 10 mM, respectively. (b). Estimation of the $[I]_{0.5}$ value for the competitive inhibitor, L-aspartate, where V_s is the phenylalanine aminotransferase activity in the absence of inhibitor, and V_i is the enzyme activity in presence of a given concentration [I] of L-aspartate.

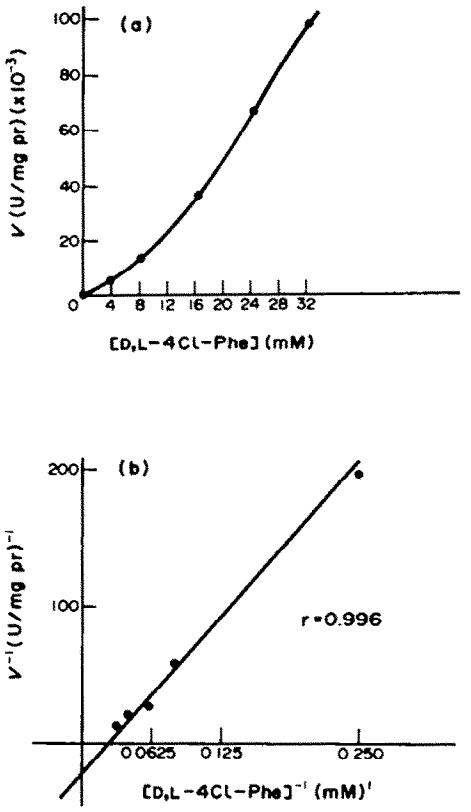

Fig. 2(a). Initial velocity plot of the aminotransferase activity of isozyme II as a function of DL-4-chloro-phenylalanine concentration in the presence of 10 mM 2-OG. (b). Lineweaver–Burk plot of the aminotransferase activity of isozyme II as a function of DL-4-chloro-phenylalanine concentration in the presence of 10 mM 2-OG.

A determination of the kinetic constants for the DL-chloro-phenylalanines transaminated by Enz II (see Table 2) was attempted. The initial velocity curves for each substrate when tested in the presence of 10 mM 2-OG were parabolic with no hint of saturation, but with apparent sigmoidal features. When the data was analysed by Lineweaver–Burk plots, in each case negative y and positive x intercepts were observed, suggesting that Enz II was indeed exhibiting sigmoidal kinetic behavior in the presence of the chlorophenylalanine [34]. Figure 2a gives a typical initial velocity curve for 4-chlorophenylalanine, while Fig. 2b shows the Lineweaver–Burk analysis of this data. These results suggest complex interactions between Enz II and the chlorophenylalanines [30]. There is, therefore, the possibility that Enz II can act as an allosteric enzyme, displaying co-operative effects between binding sites when a chlorophenylalanine is provided as the substrate. Why the kinetics of the DL-chlorophenylalanines (sigmoidal) differ from those of L-phenylalanine and L-aspartate (Michaelis–Menten) is not yet clear and will require further study. Competition experiments between L-phenylalanine and various DL-chlorophenylalanines should resolve this question but would require the availability of the corresponding chlorophenylpyruvic acid reaction products for an accurate determination of aminotransferase activity specific to the chlorophenylalanines. Since these compounds could not be purchased commercially, resolution of this interesting kinetic anomaly must await synthesis of the required chlorophenylpyruvates.

Effect of inhibitors and pyridoxal-5-phosphate

The effects of various inhibitors on the activities of Enz I and II were tested in the presence of 40 mM L-phenylalanine, 10 mM 2-OG and 0.1 mM PLP. Aminooxyacetate (AOA) has been reported to be a potent inhibitor of plant aminotransferases and has been routinely used as a selective aminotransferase inhibitor [31], although it is now clear that AOA can also inhibit the activities of other pyridoxal-5-phosphate (PLP)- requiring enzymes such as decarboxylases. As expected, 0.5 mM AOA significantly inhibited the L-phenylalanine aminotransferase activity of both Enz I and II, and 1.0 mM AOA completely eliminated all activity.

Hydroxylamine binds with free carbonyl groups and thus acts as a PLP antagonist and aminotransferase inhibitor [35, 36]. At a concentration of 20 μM, hydroxylamine showed complete inhibition of the L-phenylalanine aminotransferase activity of both Enz I and II, strongly suggesting the requirement of the coenzyme PLP for activity. An absolute requirement for PLP could not be demonstrated since the activity of purified Enz I or II was insensitive to exogenously supplied 0.2 mM PLP (data not shown), which has also been reported by others [10, 13]. This is assumed to be due to the fact that the PLP of many plant aminotransferases seems to be more tightly bound than that of animal aminotransferases and

extensive treatments are usually required with such plant enzymes to resolve the apoenzyme from the holoenzyme [35]. However, precipitation of enzymes with ammonium sulphate can often result in at least partial removal of the PLP prosthetic group from the holoenzyme. It is believed that the sulphate anion strongly inhibits the binding of PLP to coenzymes by competing with the phosphate group of PLP for an anion-binding pocket on the apoenzyme [37]. In such cases, addition of PLP to the pellet resuspension medium is necessary to recover aminotransferase activity. This was verified in the present case. During purification of Enz I and II, if the protein pellet obtained after 40–70% ammonium sulphate fractionation of the total soluble extract was resuspended in buffer without PLP, there was a loss of about 50% of the phenylalanine aminotransferase activity [6]. While the effects of sulphydryl modifying agents on plant aminotransferases are known to be variable (see [35]), in this study iodoacetate had no effect on the L-phenylalanine aminotransferase activity of Enz I and II, even when present at 10 mM, suggesting that free sulphydryl groups are not essential for this activity. Such insensitivity to sulphydryl-modifying agents has previously been reported for an aromatic aminotransferase in mung bean seedlings [38, 39].

Table 4. Recovery of chlorophyll and marker enzymes from washed and Percoll-purified chloroplast fractions isolated from bushbean shoots. Values are expressed as a percentage of the total amount in the original extract and represent the average of three preparations

Characteristic marker	Washed chloroplasts	40% Percoll-purified chloroplasts
Chlorophyll		
mg	6.5	3.4
% Recovery	18.0	9.5
Cytochrome *c* oxidase		
Specific activity (ΔA_{550}/min/mg chl.)	0.023	nd
% Recovery	1.5	0
Catalase		
Specific activity (Units/mg chl.)	4.91	nd
% Recovery	2.7	0

nd = not detected.

Effect of cations

The activities of Enz I and II were examined in the presence of 40 mM phenylalanine, 10 mM 2-OG, 0.1 mM PLP and various mono-, di- and trivalent cations (Na^+, K^+, Mg^{2+}, Mn^{2+}, Cu^{2+} each 10 mM; Fe^{3+}, 3 mM; Ca^{2+}, 0.5 and 1 mM). No evidence of significant cation stimulation or inhibition of Enz II activity was observed. With Enz I, however, while activity was also unaffected by most of the cations tested, it was severely inhibited by Ca^{2+}; 1 mM Ca^{2+} caused complete loss of activity. These findings contrast with those of Forest [12] who reported that a multispecific aspartate aminotransferase from bushbean roots was stimulated 15–20% by the addition of Ca^{2+} or Mg^{2+}. The potent and specific inhibition of Enz I by Ca^{2+} suggested that this enzyme may not encounter mmol levels of Ca^{2+} *in vivo*, and therefore may be compartmentalized at a subcellular location quite different from Enz II. This possibility prompted a study of the subcellular localization of the L-phenylalanine aminotransferase isozymes I and II from bushbean shoots, which is reported below.

Subcellular localization studies

To determine the subcellular origin of Enz I and II, fractions enriched in purified cytoplasmic, chloroplast or mitochondrial components were prepared from 10-day old bushbean shoots. The L-phenylalanine aminotransferase activity in each enriched fraction was then characterized by ion exchange chromatography on identical columns of DEAE-Sephacel run at pH 6.8 and eluted with identical KCl gradients.

Results for the characterization of chloroplasts before and after purification by sedimentation through a 40% Percoll pad using the procedure of ref. [3], are given in Table 4. The values represent the average of three preparations and are expressed as percentage recovery of the total amount of chlorophyll or marker enzyme activity present in the crude filtrate. The washed chloroplast preparation (6.5 mg chlorophyll), obtained by pelleting the chloroplasts at 2000 *g*, showed an 18% recovery of total chlorophyll with low contamination by the mitochondria and peroxisome marker enzymes (% recovery of activity = 1.5 and 2.7% respectively). After sedimentation through 40% Percoll, 9.5% of the original chlorophyll was recovered. Contamination by mitochondria and peroxisomes was below the limits of detection for marker enzyme activities. To estimate the yield of intact chloroplasts, the ferricyanide test of ref. [40] was performed as described in the Experimental. The results indicated that the Percoll-purified chloroplasts were 93% intact cf washed chloroplasts, 59% intact. While Percoll purification resulted in the recovery of only 45% (3.1 mg) of the chlorophyll present in the washed chloroplast fraction, this represented 71% of the 'intact chlorophyll' contained in the washed chloroplasts. Thus, the Percoll-purified chloroplast fractions were enriched in intact chloroplasts and showed no biochemical evidence of contamination by mitochondria or peroxisomes. The characterization results obtained during this isolation of purified intact chloroplasts closely agreed with those reported in refs [41, 42] and indicate the reliability of the method.

Results for the characterization of mitochondria isolated by sedimentation through a discontinuous Percoll gradient are shown in Fig. 3. In the gradient, mitochondria were characterized by using cytochrome *c* oxidase as the marker enzyme. Catalase was used as a marker enzyme for peroxisomes, while chlorophyll was used to indicate the presence of chloroplast material. Mitochondrial cytochrome *c* oxidase was found to be concentrated mainly in the bottom 3 fractions of the Percoll gradient [associated with the 35% (v/v) Percoll layer], and was separated from fractions contaminated with chlorophyll and catalase by 1–2 gradient fractions. Since cytochrome *c* oxidase is a marker enzyme for the inner mitochondrial membrane, it was also used to estimate the percentage of intact mitochondria in each gradient fraction. A measurement of cytochrome *c* oxidase activity before and after release of the enzyme by treatment of the mitochondrial fraction with Triton X-100 detergent allowed a calculation of the

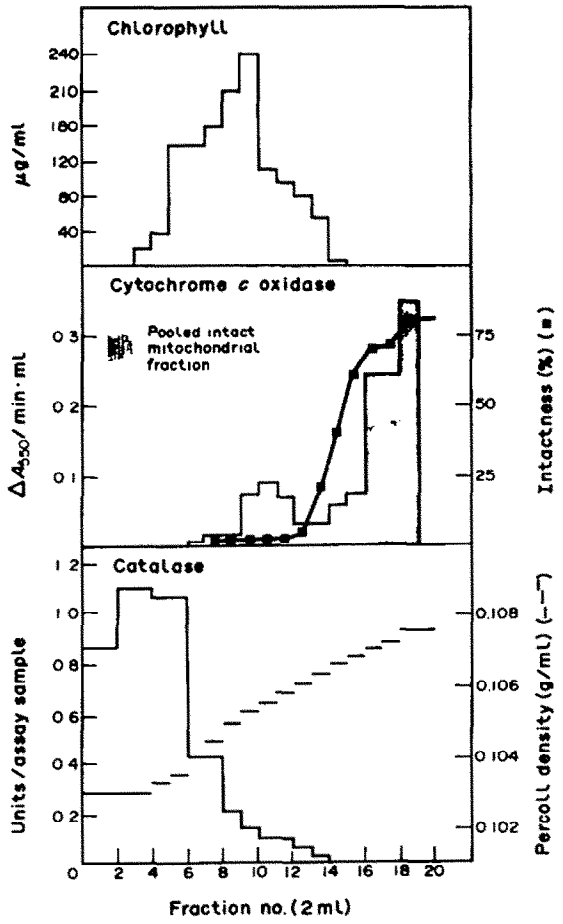

Fig. 3. Profile of discontinuous Percoll gradient showing the distribution of organelles as determined by marker enzymes. The gradient consisted of 10 ml bushbean shoot homogenate layered on to the following steps: 3 ml of 15%, 3 ml of 25%, 15 ml of 30% and 7 ml of 35% (v/v) Percoll. The gradient was centrifuged in a Beckman SW-27 rotor at 21 000 rpm for 45 min.

percentage of intact mitochondria. As shown in Fig. 3, fractions 17–19 of the discontinuous Percoll gradient were found to have mitochondria estimated to be 70–80% intact. These fractions were therefore pooled to give the enriched mitochondrial fraction.

Each of the subcellular fractions enriched in cytoplasmic, chloroplast or mitochondrial components was then subjected to ion-exchange chromatography on DEAE-Sephacel at pH 6.8. The L-phenylalanine aminotransferase in each subcellular fraction was eluted from the column by a 0–250 mM KCl gradient. The protein elution profile and graph showing associated L-phenylalanine aminotransferase activity for each subcellular fraction are shown in Figs 3–4. The cytoplasmic fraction (Fig. 4) was found to contain two peaks of L-phenylalanine aminotransferase activity; peak 1 eluted at *ca* 0.11 M KCl and peak 2 at *ca* 0.15 M KCl. This elution pattern very strongly resembles that reported in our previous paper [6] in which two isozymes of L-phenylalanine aminotransferase (Enz I and II) were isolated from a total soluble extract of bushbean shoots by gradient elution from a DEAE-Sephacel column. Isozymes I and II eluted at *ca* 0.11 M and 0.15 M KCl, respectively, when the column was run under conditions identical to those used here. In contrast, the chloroplast fraction showed only one peak of L-phenylalanine aminotransferase activity which eluted at *ca* 0.18 M KCl (Fig. 5), and only a single peak of activity was obtained from the mitochondrial fraction which eluted at *ca* 0.20 M KCl (Fig. 6). The aminotransferase peak fractions from each subcellular preparation were pooled and each combined fraction assayed for total activity (Table 5). The estimates of total L-phenylalanine aminotransferase activity in each subcellular preparation were based on recovery of organelle markers and the percentage intactness of the isolated organelles.

In addition to the total activity values, Table 5 also lists the DEAE-Sephacel elution conditions at pH 6.8, the estimated percentage distribution of total L-phenylalanine aminotransferase activity in each subcellular fraction and the ratio of L-phenylalanine/L-aspartate aminotransferase

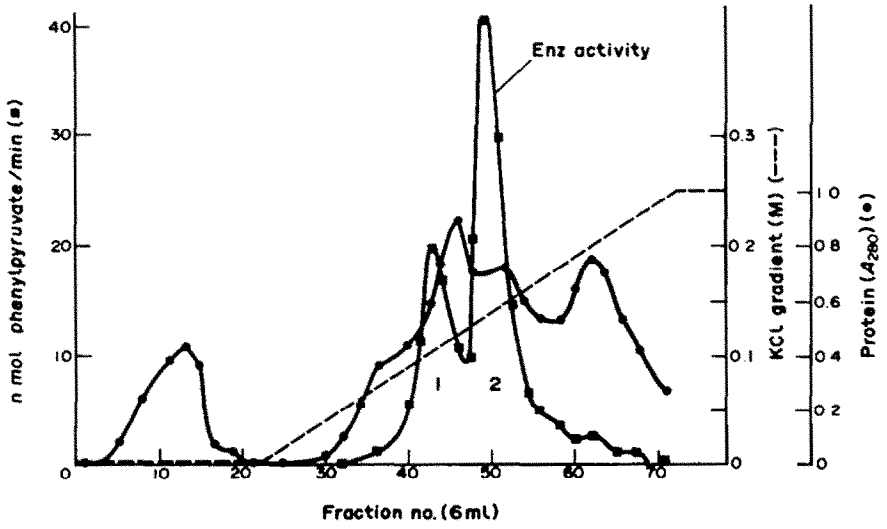

Fig. 4. Protein elution profile and related L-phenylalanine aminotransferase activity obtained from DEAE-Sephacel chromatography at pH 6.8 of an enriched cytoplasmic fraction isolated from a bushbean shoot homogenate.

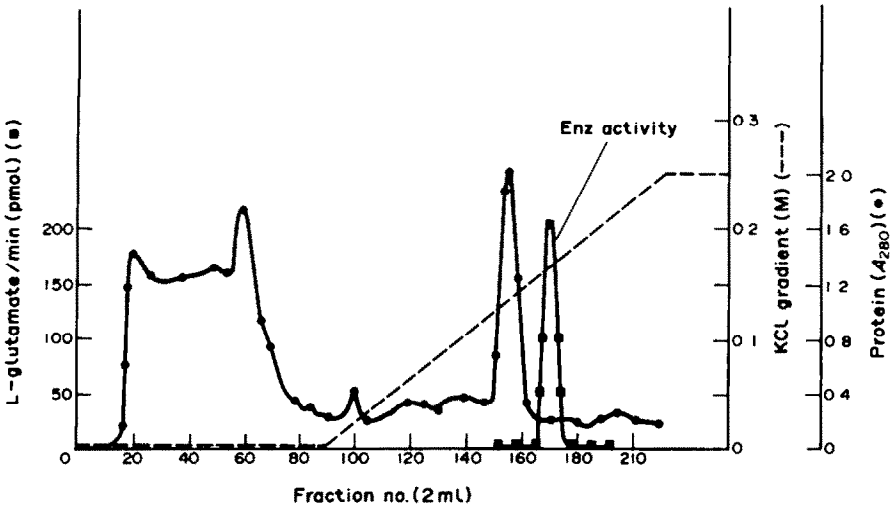

Fig. 5. Protein elution profile and related L-phenylalanine aminotransferase activity obtained from DEAE-Sephacel chromatography at pH 6.8 of an enriched chloroplast fraction isolated from a bushbean leaf homogenate.

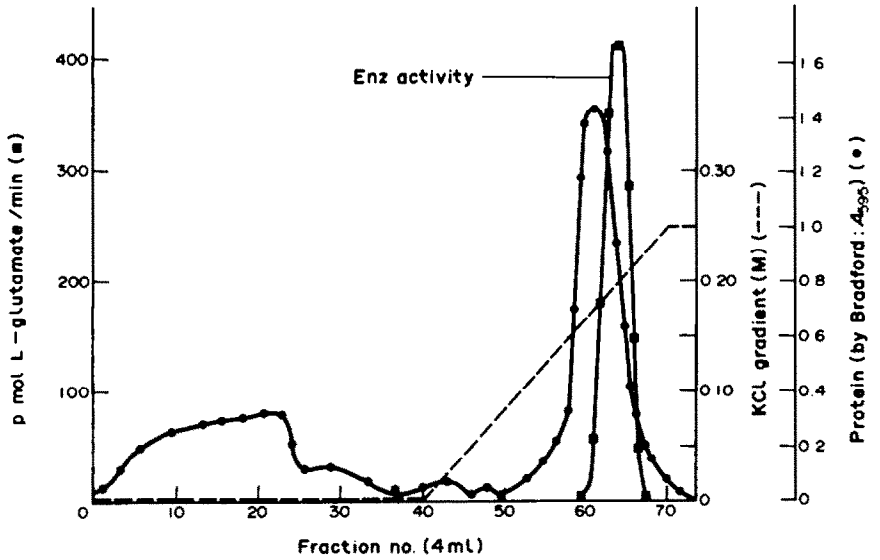

Fig. 6. Protein elution profile and related L-phenylalanine aminotransferase activity obtained from DEAE-Sephacel chromatography at pH 6.8 of an enriched mitochondrial fraction isolated from a bushbean shoot homogenate.

activity for Enzymes 1–4 from the subcellular fractions compared to the ratios from Enz I and II purified from a total soluble shoot extract. While all six aminotransferases exhibited a marked preference for L-aspartate, showing only 0.6–4.0% relative activity with L-phenylalanine, the activity ratios were identical for Enz I and cytoplasmic Enzyme 1, and almost identical for Enz II and cytoplasmic Enzyme 2. In addition, the ion-exchange elution conditions for Enzymes 1 and 2 corresponded with those for Enz I and II, respectively, as discussed above. Based on the estimated percentage distribution of total L-phenylalanine aminotransferase activity (i.e. Enz I plus Enz II activities), it appears that more than 95% of this total activity is accounted for by the combined activity of Enzymes 1 and 2 from the cytosolic fraction. The combined activities of Enzyme 3 (chloroplastic) and Enzyme 4 (mitochondrial) on the other hand accounted for only about 4% of the total activity.

Collectively, the data in Table 5 strongly suggest that the aspartate-phenylalanine aminotransferase Enz I and II are both located in the cytoplasm and are responsible for most of the L-phenylalanine aminotransferase activity in bushbean shoots. A slight possibility exists, however, that one of these isozymes may originate in the peroxisomes, since these organelles are known to be very fragile and easily broken during isotonic organelle isolation. Low levels of L-aspartate: 2-OG aminotransferase in spinach

Table 5. Comparison of some properties of the aspartate-aromatic aminotransferase isozymes I and II (Enz I and II) purified from a total soluble shoot extract with the L-phenylalanine aminotransferases (Enzymes 1–4) found in enriched subcellular fractions from bushbean shoots

Enzyme source and designation	Conc. of KCl required for elution from DEAE-Sephacel at pH 6.8 (mM)	Total activity in fraction (U¶ × 10^{-3})	% Distribution of total activity†	% Relative Phe/Asp activity
Total soluble extract		600*	100	
Enz I	108			0.60
Enz II	150			3.50
Cytoplasmic fraction		580	96.6	
Enzyme 1	110			0.60
Enzyme 2	150			4.10
Chloroplast fraction				
Enzyme 3	175	12.7‡	2.1	1.2
Mitochondrial fraction				
Enzyme 4	200	13.0§	2.2	1.0

*Reported in Ref. [6].
†Total activity = Activity of total soluble extract (600 mU) set at 100%.
‡Based on 8% recovery of 'intact chlorophyll'.
§Based on 64% recovery of total cytochrome *c* oxidase activity in mitochondria which were 75% intact.
¶One unit (U) of aminotransferase activity is defined as in Table 1.

leaf peroxisomes have been reported and it was calculated that the peroxisomal isozyme accounted for only 3% of the total L-aspartate aminotransferase activity in such leaves [43]. Such findings, coupled with the high dilution factor involved in the release of peroxisome contents into the large volume of a cytoplasmic extract, make it very unlikely that the high L-aspartate aminotransferase activity associated with both Enz I and II could be peroxisomal in origin. While different chromatographic behavior on identical ion-exchange columns is only one way to demonstrate the presence of different isozymes or subforms of aminotransferases [27], it appears from differences in their chromatographic properties and in their ratios for L-phenylalanine/L-aspartate aminotransferase activity that both chloroplasts and mitochondria in bushbean contain their own isozymic form of aspartate-aromatic aminotransferase (Enzymes 3 and 4). The present demonstration of several subcellular isozymes of this enzyme in bushbean shoots is in agreement with earlier results where four isozymes of L-aspartate aminotransferase in spinach leaves [43] and six isozymes of the enzyme in cotyledons of cucumber seedlings were found [56].

In summary, the two isozymic forms of L-aspartate-L-aromatic aminotransferase (Enz I and II) purified from a total soluble extract of bushbean shoots have been shown to possess many properties similar or identical to those of other multispecific L-aspartate aminotransferases isolated from plants and other organisms [13, 17, 19, 20, 27, 35]. These two isozymes appear to be cytoplasmic in origin and account for at least 95% of the total activity of this multispecific aminotransferase in bushbean shoots. Two other isozymic forms of the aminotransferase (Enz 3 and 4) have been isolated from organelle-enriched fractions and were shown to originate from the chloroplasts and mitochondria, respectively. The significance of the presence of two major isozymes of this aminotransferase in the cytoplasm of bushbean shoots is not yet clear, and is complicated by differences in their sensitivity to added Ca^{2+} and in their substrate specificity to the DL-chlorophenylalanines. Although certain of these synthetic DL-chlorophenylalanines were transaminated by Enz II at rates comparable to those obtained with DL-phenylalanine, the highest activity was observed when 4-chlorophenylalanine was supplied as a substrate. This finding has prompted an examination of the structural features of a range of *para*-substituted aromatic amino acids which appear to lead to high aminotransferase activity. The results of this study and their implication on the nature of the enzyme–substrate reaction will be reported in the next paper of this series.

EXPERIMENTAL

Plant materials and chemicals. Bushbean seeds (*Phaseolus vulgaris* L. cv Pencil Pod Black Wax) were germinated in moist vermiculite in a growth cabinet at 25/22° day/night temperatures and the seedlings exposed to 16 hr daily photoperiods of 4600–4800 $\mu W/cm^2$. At the 10-day stage of seedling growth (when the first pair of leaves were about half fully expanded), the young shoots were harvested for enzyme extraction. The series of ring-substituted DL-chlorophenylalanines used in this study were synthesized and fully characterized as reported in ref. [5]. Sephacryl S-300, DEAE-Sephacel, Percoll density gradient medium and a high-M_r Gel Filtration Calibration Kit were purchased from Pharmacia. All other chemicals used in these investigations were purchased from Sigma or Aldrich Chemical Companies and were of analytical grade.

Enzyme purification. Details of the resolution and purification of the L-phenylalanine aminotransferase isozymes I and II from a total soluble extract of bushbean shoots are described in the previous paper of this series [6]. The main steps involved were: 40–70% $(NH_4)_2SO_4$ fractionation, Sephacryl S-300 gel filtration, DEAE-Sephacel ion exchange chromatography and Biogel HTP hydroxylapatite chromatography, with an additional purification step using a second DEAE-Sephacel column for Enz II.

Assay of aminotransferase activity of the purified Enz I and II. Experiments to determine the enzymic characteristics of L-

phenylalanine aminotransferase Enz I and II were carried out in a standard assay mixture consisting of 50 mM Tris–HCl buffer, pH 8.5, containing in most instances the following additional components: 0.1 mM EDTA, 2 mM dithiothreitol (DTT), 0.1 mM pyridoxal phosphate (PLP) and either Enz I (100 μl, 2.5 μg protein) or Enz II (50 μl, 5.8 μg protein) in a final vol of 0.75 ml. L-Amino acid and keto acid substrates were added at the final concentrations indicated in the Results section. Assays were normally carried out for 60 min at 35° since the reactions tested were found to be linear for at least 2 hr. When the product amino acid was to be determined, control systems contained all components of the reaction mixture except the substrate amino acid. The experimental and control reactions were stopped by the addition of 100 μl of 30% sulphonyl salicylic acid (SSA), the product amino acid isolated and its quantity measured by HPLC after pre-column derivatization with o-phthalaldehyde, as previously described [6]. When the product aromatic keto acid, phenylpyruvate, was to be determined, the reaction was terminated by the addition of 1.0 ml of 1.75 M NaOH; the soln was mixed and the enol form of the phenylpyruvate was measured directly by the method of ref. [45]. Using this method, the control system contained all components of the reaction mixture, but the NaOH was added before the keto acid at time zero. This control was then incubated along with the experimental systems for the required time period. One unit (U) of aminotransferase activity will convert 1 μmol of L-phenylalanine to 1 μmol of phenylpyruvate (or 1 μmol 2-OG to 1 μmol of L-glutamate) at pH 8.5, 35°.

Determination of protein. Protein was estimated by the method of Bradford [46] using bovine γ-globulin as a standard, except in the case of column monitoring during enzyme purification when the optical method was used [47].

M_r determinations. The M_r's of L-phenylalanine aminotransferase Enz I and II were determined by gel filtration [9] on Sephacryl S-300. A column (90 × 2.5 cm) of Sephacryl S-300 was prepared according to the manufacturer's instructions and equilibrated with 50 mM Tris–HCl buffer, pH 7.5, containing 0.1 mM EDTA, 12 mM 2-mercaptoethanol and 1 mM DTT. The column was calibrated and a standard curve of log M_r vs V_e/V_0 for the calibration proteins was used to estimate the M_r's of the aminotransferases, where V_e = elution vol. of protein in question and V_0 = void vol. of column $\simeq V_e$ of Blue Dextran 2000.

Kinetic studies. The apparent K_m and V_{max} values were determined from Lineweaver–Burk plots, $1/v$ vs $1/[S]$; [21, 44]. The kinetic data were supported by subjecting the data to graphical analysis by Hill plots: log $(v/V_{max} - v)$ vs log $[S]$; [30].

Subcellular localization studies. Fractions enriched in cytoplasmic, chloroplast or mitochondrial components were compared chromatographically to determine the subcellular origin of phenylalanine aminotransferase isozymes I and II.

A. Preparation of cytoplasmic fraction. A fraction enriched in soluble cytoplasmic components was prepared by differential centrifugation. Bushbean shoots (50 g fr. wt) were chopped into fine pieces and then ground in a mortar for 3–5 min in 200 ml of an ice-cold grinding medium modified from ref. [49] containing 400 mM sucrose, 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 10 mM KCl, 10 mM EDTA, 0.2 mM PLP, 8 mM 2-OG, 2 mM DTT, 12 mM 2-mercaptoethanol and 5% glycerol. A small amount of acid-washed sand was added to act as an abrasive. The homogenate was filtered through 4 layers of cheesecloth and 2 layers of 20 μm nylon mesh, the residue discarded and the filtrate centrifuged at 2000 g for 10 min in a Sorvall RC-2B refrigerated centrifuge using an SS-34 fixed angle rotor. The pellet, containing chloroplasts, nuclei and other cell debris, was discarded. The supernatant was centrifuged at 25 000 g for 10 min, yielding a pellet of mitochondria (discarded) and a supernatant which was re-centrifuged at 100 000 g for 120 min in a Beckman L5-50 ultracentrifuge equipped with an SW-27 rotor. The resulting microsomal pellet was discarded and the supernatant, a fraction enriched in soluble cytoplasmic components, was then subjected to a purification protocol (previously reported in ref. [6]) designed to isolate and purify the L-phenylalanine aminotransferase.

B. Preparation of chloroplast fraction. Intact chloroplasts were isolated from the primary leaves of 10-day old bushbean seedlings following the method of ref. [3], which is a modification of that reported in ref. [41]. *ca* 60 g of leaf tissue were harvested and placed on ice for 30 min. The tissue was then cut into small pieces with scissors and placed into 300 ml of ice-cold extraction buffer at pH 8.0 containing 330 mM sorbitol, 50 mM tricine, 4 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM $MgCl_2$ and 0.1% bovine serum albumin. After grinding for 1 min in a Polytron PT 20, the crude homogenate was filtered through 4 layers of cheesecloth and then squeezed through 6 layers of 20 μm nylon mesh. The filtrate was centrifuged at 2000 g for 1 min in a Sorvall RC-2B refrigerated centrifuge using an SS-34 fixed-angle rotor to rapidly sediment the chloroplast fraction. The pelleted chloroplasts were gently resuspended in extraction medium (30 ml) using a small piece of nylon mesh attached to a glass rod. The washed fraction was then divided into 4 aliquots and each was underlayed with 15 ml of Percoll medium containing 40% (v/v) Percoll, 330 mM sorbitol, 50 mM tricine, pH 8.2 and 0.1% BSA. The intact chloroplasts were then pelleted by centrifugation at 2000 g for 1 min in the Sorvall centrifuge equipped with an HB-4 swinging bucket rotor. After centrifugation, the supernatant containing broken chloroplasts was removed along with the Percoll by aspiration. The remaining chloroplast pellets were resuspended in *ca* 10 ml of extraction buffer without BSA and characterized as described below. After characterization, the intact chloroplasts were sedimented by centrifugation at 2000 g for 5 min and then gently resuspended in the enzyme isolation medium specified in Section E. below.

C. Preparation of the mitochondrial fraction. Mitochondria were prepared from the 10-day old bushbean shoots using the method of ref. [50] with some modifications. Epicotyls (15–18 g fr. wt) were finely chopped with a razor blade, placed in ice-cold grinding medium (75 ml) containing 350 mM Sorbitol, 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM $MgCl_2$, 10 mM DTT, 1% polyvinylpyrrolidone (PVP)-10, and 0.1% bovine serum albumin and ground for 3–5 min with a pestle and mortar. The homogenate was filtered through two layers of cheesecloth and 2 layers of 20 μm nylon mesh wetted with 20 ml cold grinding medium and then centrifuged at 1500 g for 10 min in a Sorvall RC-2B refrigerated centrifuge using an SS-34 fixed angle rotor. The pellet containing whole cells, chloroplasts and cellular debris was discarded. 10 ml of the supernatant (equivalent to 2.5–3.0 g fresh weight) were layered on top of a discontinuous Percoll gradient consisting of the following steps: 3 ml of 15%, 3 ml of 25%, 15 ml of 30% and 7 ml of 35% Percoll. Each step also contained 0.25 M sucrose, 1 mM KH_2PO_4, 10 mM HEPES, pH 7.5 and 0.1% bovine serum albumin. Six gradients were prepared for each experiment. The gradients were spun in a Beckman L5-50 ultracentrifuge equipped with an SW-27 swinging-bucket rotor at 21 000 rpm for 45 min. After centrifugation, each gradient was fractionated from the bottom into 2 ml fractions using an Isco Model 640 Density Gradient Fractionator and the fractions were characterized as described below. Percoll density was determined as described in the Percoll Handbook supplied by Pharmacia Fine Chemicals. The mitochondrial band sedimented in the 35% Percoll layer. The fractions enriched with purified intact mitochondria were combined, diluted with 5 volumes of washing medium containing 330 mM mannitol, 50 mM HEPES, pH 7.5, 1 mM EDTA and 0.1% bovine serum albumin, and the mito-

chondria pelleted by centrifugation at 25 000 *g* for 5 min using the Sorvall centrifuge. The pellets were then gently resuspended in the enzyme isolation medium specified in Section E below.

D. Characterization of organelles.

(i) *Markers used to identify organelle fractions. Chloroplasts* (intact or broken): Chlorophyll was estimated in 96% ethanol extracts using the extinction coefficient of ref. [51] for absorption at 654 nm where:

$$\text{concentration of chlorophyll } (\mu g/ml) = \frac{1000 \times A_{654}}{39.8}$$

Mitochondria: Cytochrome oxidase activity (EC 1.9.3.1) was measured spectrophotometrically by the decrease in A at 550 nm on an LKB Ultraspec interfaced with an Apple II computer using the 'Reaction Rate' program. The method was based on that reported in ref. [52]. Reactions were run at room temp. Thirty ml of a soln of cytochrome *c* (17 μM) in 100 mM Pi buffer, pH 7.0 were reduced in an Erlenmeyer flask by adding 100 μl of a freshly prepared soln of sodium dithionite (1.2 M). The soln was shaken vigorously for 5 min to remove the excess dithionite. The ratio of A_{550}/A_{565} of the cytochrome *c* soln was always *ca* 12, indicating that the cytochrome *c* was in its reduced form. A 0.2 ml aliquot of the organelle preparation was added to 0.8 ml of the buffered cytochrome *c* solution, the reactants mixed by inverting the cuvette and the rate of change in the A_{550} recorded.

Peroxisomes: Catalase activity (EC 1.11.1.6) was determined by the decrease in *A* at 240 nm as described by Luck [53]. 100 μl of 30% H_2O_2 were added to 65 ml of 50 mM Pi buffer, pH 7.0. 50 μl of the organelle preparation was added to 3 ml of this buffered H_2O_2 soln, the reactants were mixed by inversion and the rate of change in the A_{240} was recorded on a LKB Ultraspec. The control contained the organelle sample in H_2O_2-free phosphate buffer. One catalase unit is the amount of enzyme which liberates half the peroxide oxygen from a H_2O_2 solution in 100 sec at 25° [54].

(ii) *Measurement of the intactness of organelles. Chloroplast* intactness was estimated by the ferricyanide test described in ref. [40]. Ferricyanide-dependent O_2 evolution was measured in 4 ml of buffered medium containing 330 mM sorbitol, 50 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES), pH 7.6, 2 mM EDTA, 1 mM $MgCl_2$, 1.5 mM potassium ferricyanide [$K_3Fe(CN)_6$], 10 mM DL-glyceraldehyde and 5 mM NH_4Cl. Chloroplasts (200 μg chlorophyll, *ca* 0.6 ml) were added before and after osmotic shock and the rate of oxygen evolution followed polarigraphically with a Clarke-type oxygen electrode (YSI, Model 5533, Yellow Springs, OH, USA) at 25° in light (250 W/m^2 at the level of the cuvette) calibrated against air-saturated water, the O_2 concentration of which was taken as 250 μM. A comparison of the ferricyanide-dependent O_2 evolution rate by chloroplasts kept in isotonic medium vs the rate shown by osmotically-shocked chloroplasts gave an estimation of the percentage of intact organelles, calculated by the following equation:

$$\%\,\text{intactness} = 100 - \left[\frac{\text{rate of ferricyanide-dependent } O_2 \text{ evolution by 'isotonic' chloroplasts} \times 100}{\text{rate of ferricyanide-dependent } O_2 \text{ evolution by 'osmoticall-shocked' chloroplasts}}\right]$$

Mitochondrial membrane integrity was assessed by comparing cytochrome *c* oxidase activity in mitochondria before and after Triton X-100 treatment, as described in ref. [55]. Cytochrome oxidase activity was assayed as described in Section D (i) except that the assay medium also contained 330 mM sorbitol to keep it isotonic. Mitochondria were disrupted by adding Triton X-100 to a final concentration of 0.02%.

E. Isolation and partial purification of the phenylalanine aminotransferase activity from Percoll-purified organelles. The pellets of Percoll-purified intact chloroplasts (Section B, above) and mitochondria (combined yield of 3 experiments as described in Section C above) were each resuspended in *ca* 90 ml of enzyme isolation medium consisting of 50 mM Tris–HCl buffer, pH 7.5 containing 0.2 mM PLP, 12 mM 2-mercaptoethanol, 0.1 mm EDTA and 0.1% Tergitol NPX. The suspensions were stirred on ice for 1 hr and then centrifuged at 25 000 *g* for 1 hr to sediment the membrane fractions. The supernatant fractions containing the soluble stromal or matrix proteins were dialysed overnight at 4° against 50 vols of 50 mM Tris–HCl, pH 7.5 containing 0.2 mM PLP, 2 mM DTT, 12 mM 2-mercaptoethanol and 0.1 mM EDTA (Buffer A). The chloroplast and mitochondrial dialysates were each applied to a DEAE-Sephacel column (1.6 × 25 cm) pre-equilibrated to pH 6.8 with Buffer B (Buffer A without PLP) and phenylalanine aminotransferase was eluted with a linear gradient from 0–250 mM KCl in Buffer B, as previously described [6].

F. PAGE of purified preparations of Enzymes I and II. Canalco equipment (Model 1200R) was used for PAGE. The gels were prepared in glass columns using Tris–glycine buffer at pH 8.3. Electrophoresis was carried out on 5–50 μg protein samples of Enz I and II preparations; the running conditions and the protein staining procedures employed were those described in ref. [6]. L-Aspartate and L-phenylalanine aminotransferase activities were located on the gels as follows: gels were sliced into 3 mm sections and each slice was assayed for aminotransferase activity by incubating for 90 min at 35° in 0.75 ml of 50 mM Tris–HCl, pH 8.5, containing 40 mM L-aspartate or L-phenylalanine, 5 mM 2-OG, 0.1 mM PLP, 0.1 mM EDTA and 2 mM DTT. Aspartate aminotransferase reactions were stopped by the addition of 100 μl 30% sulphonyl salicylic acid and the glutamate present was measured by OPA-HPLC as previously described [6]. Phenylalanine aminotransferase reactions were stopped by the addition of 1 ml 1.75 N NaOH and the phenylpyruvate present was measured by the method of ref. [45].

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REFERENCES

1. Wightman, F. (1973) *Biochem. Soc. Symp.* **38**, 247.
2. Schneider, E. A. and Wightman, F. (1978) in *Phytohormones and Related Compounds—A Comprehensive Treatise*, Vol. 1 (Letham, D. S., Goodwin, P. B. and Higgins, T. J. V., eds) pp. 29–105. Elsevier/North Holland Biochemical Press, Amsterdam.
3. Fregeau, J. A. and Wightman, F. (1983) *Plant Sci. Letters* **32**, 23.
4. Camirand, A., Phipps, J. and Wightman, F. (1983) *Can. J. Botany* **61**, 2302.
5. Taylor, D. C., Wightman, R. H. and Wightman, F. (1987) *Bioorg. Chem.* (in press).
6. Taylor, D. C. and Wightman, F. (1987) *Phytochemistry* (in press).
7. Pybus, M. B., Smith, M. S., Wain, R. L. and Wightman, F. (1959) *Ann. Appl. Biol.* **47**, 593.
8. Pybus, M. B., Wain, R. L. and Wightman, F. (1958) *Nature* **182**, 1094.

9. Andrews, P. (1970) *Meth. Biochem. Anal.* **18**, 1.
10. Gazeau-Reyjal, M. and Crouzet, J. (1976) *Phytochemistry* **15**, 1619.
11. Ireland, R. J. and Joy, K. W. (1983) *Arch. Biochem. Biophys.* **223**, 291.
12. Forest, J. C. (1971) Ph.D. Thesis, Carleton University, Ottawa, Canada.
13. Forest, J. C. and Wightman, F. (1972) *Can. J. Biochem.* **50**, 813.
14. Hart, G. E. and Langston, P. J. (1977) *Theor. Appl. Genet.* **50**, 47.
15. Gottilieb, L. D. (1973) *Biochem. Genet.* **9**, 97.
16. Reynolds, P. H. S., Boland, M. J. and Farnden, K. J. F. (1981) *Arch. Biochem. Biophys.* **209**, 524.
17. Wightman, F. and Forest, J. C. (1978) *Phytochemistry* **17**, 1455.
18. Prusiner, S. and Milner, L. (1970) *Anal. Biochem.* **37**, 429.
19. Shrawder, E. and Martinez-Carrion, M. (1972) *J. Biol. Chem.* **247**, 2486.
20. Mavrides, C. and Orr, W. (1975) *J. Biol. Chem.* **250**, 4128.
21. Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658.
22. Lehninger, A. L. (1975) *Biochemistry* 2nd edition, pp. 183–216. Worth, New York.
23. Reed, R. E. and Hess, J. L. (1975) *J. Biol. Chem.* **250**, 4456.
24. Davies, D. D. and Ellis, R. J. (1961) *Biochem. J.* **78**, 623.
25. Rehfeld, D. R. and Tolbert, N. E. (1972) *J. Biol. Chem.* **247**, 4803.
26. Lu, Ti-Shen, and Mazelis, M. (1975) *Plant Physiol.* **55**, 502.
27. Givan, C. V. (1980) in *The Biochemistry of Plants*, Vol. 5, *Amino Acids and Derivatives*, (Miflin, B. J., ed.) pp. 329–357. Academic Press, New York.
28. Carpe, A. I. and Smith, I. K. (1974) *Biochim. Biophys. Acta* **370**, 96.
29. Forest, J. C. and Wightman, F. (1973) *Can. J. Biochem.* **51**, 332.
30. Segel, I. H. (1975) *Enzyme Kinetics* pp. 46 and 346–75. Wiley, New York.
31. Miflin, B. J. and Lea, P. J. (1977) *Ann. Rev. Plant Physiol.* **28**, 299.
32. Schneider, E. A., Kazakoff, C. W. and Wightman, F. (1985) *Planta* **165**, 232.
33. Schneider, E. A. and Wightman, F. (1986) *Physiol. Plant* **68**, 396.
34. Bardsley, W. G. and Childs, R. E. (1975) *Biochem. J.* **149**, 313.
35. Ireland, R. J. and Joy, K. W. (1985) in *Transaminases* (Christen, P. and Metzler, D. E., eds) pp. 376–384. Wiley, New York.
36. Brock, B. L. W., Wilkinson, D. A. and King, J. (1970) *Can. J. Biochem.* **48**, 486.
37. Scrimgeour, K. G. (1977) *Chemistry and Control of Enzyme Reactions* p. 228. Academic Press, New York.
38. Gamborg, O. L. (1965) *Can. J. Biochem.* **43**, 723.
39. Gamborg, O. L. and Wetter, L. R. (1963) *Can. J. Biochem. Physiol.* **41**, 1733.
40. Lilley, R. McC., Fitzgerald, M. P., Rienits, K. G. and Walker, D. A. (1975) *New Phytol.* **75**, 1.
41. Mills, W. R. and Joy, K. W. (1980) *Planta* **148**, 75.
42. Fregeau, J. A. (1982) Ph.D. Thesis, Carleton University. Ottawa, Canada.
43. Huang, A. H. C., Liu, K. D. F. and Youle, R. J. (1976) *Plant Physiol.* **58**, 110.
44. Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*. Butterworth, London.
45. Scandurra, R. and Cannella, C. (1969) *Anal. Biochem.* **27**, 253.
46. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
47. Kalckar, H. M. (1947) *J. Biol. Chem.* **167**, 461.
48. Goodwin, T. W. and Mercer, E. I. (1983) *Introduction to Plant Biochemistry* pp. 49–53. Pergamon Press, New York.
49. Hanks, J. F., Tolbert, N. E. and Schubert, K. R. (1981) *Plant Physiol.* **68**, 65.
50. Hrubec, T. C., Robinson, J. M. and Donaldson, R. P. (1985) *Plant Physiol.* **77**, 1010.
51. Wintermans, J. F. G. M. and DeMots, A. (1965) *Biochim. Biophys. Acta* **100**, 448.
52. Cooperstein, S. J. and Lazarow, A. (1951) *J. Biol. Chem.* **189**, 665.
53. Luck, H. (1965) Catalase in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd edition pp. 885–894. Academic Press, New York.
54. Bergmeyer, H. U. (1955) *Biochem. Z.* **327**, 255.
55. Jackson, C., Dench, J. E., Hall, D. O. and Moore, A. L. (1979) *Plant Physiol.* **64**, 150.
56. Liu, K. D. F. and Huang, A. H. C. (1977) *Plant Physiol.* **59**, 777.
57. Gibson, R. A., Schneider, E. A. and Wightman, F. (1972) *J. Exp. Botany* **23**, 381.