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# METABOLISM OF D,L-CHLORO-PHENYLALANINES BY PHENYLALANINE AMINOTRANSFERASE ISOZYMES PURIFIED FROM BUSHBEAN SHOOTS*

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**Key Word Index**—*Phaseolus vulgaris*; Leguminosae; bushbean; metabolism; phenylalanine decarboxylase; phenylalanine aminotransferase; purification; substituted amino acids; D,L-chloro-phenylalanines.

**Abstract**—A series of mono-, di- and trichloro-D,L-phenylalanines was tested as substrates for both phenylalanine aminotransferase and phenylalanine decarboxylase partially purified from bushbean (*Phaseolus vulgaris* L.) seedling extracts by ammonium sulphate fractionation and Sephacryl S-300 gel filtration. While most of the D,L-chloro-phenylalanines were transaminated at rates of 35–100% of that observed with D,L-phenylalanine, no chloro-phenylalanine decarboxylase activity was observed. A transamination reaction is therefore likely to be the initial step in the conversion of chloro-phenylalanines to their corresponding chloro-phenylacetic acids via a reaction pathway similar to the known route for the metabolism of L-phenylalanine to phenylacetic acid. The highest specific activity of phenylalanine aminotransferase was found in both root and shoot tissues of bushbean at the 10-day stage of seedling growth. Partially purified extracts of these tissues were able to transaminate most of the mono- and dichloro-phenylalanines at *ca* 20–40% of the rate observed with D,L-phenylalanine, while the trichloro-phenylalanines (assayed at lower concentrations due to solubility) were transaminated at rates equal to those observed with D,L-phenylalanine. The 4-chloro derivative was the best substrate tested showing rates of transamination that were 25% higher than those observed with D,L-phenylalanine. Further purification of shoot fractions by DEAE-Sephacel chromatography resolved the phenylalanine aminotransferase activity into two peaks (enzymes I and II) which on further purification, were found to behave differently during hydrophobic chromatography and PAGE. These results indicated the presence of two isozymic forms of phenylalanine aminotransferase in bushbean shoots and both were found to catalyse transamination of the monochloro-phenylalanines examined in this study.

## INTRODUCTION

Studies have been made in this laboratory of the metabolic pathway in higher plants leading from L-phenylalanine to the natural auxin substance, phenylacetic acid (PAA) [1–5]. In addition to PAA, several synthetic chloro-phenylacetic acids have also been shown to be potent plant growth regulators [6] and certain acids are known to be powerful herbicides [7]. A series of 12 ring-substituted mono-, di- and trichloro-phenylalanines was therefore prepared [8] to test both the intrinsic physiological activity of these unnatural amino acids and their ability to serve as metabolic precursors for the corresponding chloro-phenylacetic acids.

In the biosynthetic pathway leading to PAA (Fig. 1), the initial metabolism of phenylalanine can involve either a transamination or decarboxylation reaction [1, 2, 4]. The aromatic aminotransferase from root tissue of bushbean (*Phaseolus vulgaris* L.) has been purified and thoroughly characterized [9–11] and the properties of plant aromatic aminotransferases have been reviewed in detail [12–14]. A tryptophan decarboxylase involved in the biosynthetic pathway leading to the formation of the auxin substance, 3-indoleacetic acid (IAA), has been partially purified and characterized in tomato, but the enzyme showed no activity with L-phenylalanine [15]. Phenylalanine can also be metabolized in higher plants to cinnamic acid through the action of phenylalanine ammonia lyase (PAL) [16, 17]. Although cinnamic acid is a key intermediate in the formation of phenolic acids and a wide range of secondary compounds, there is no evidence that it is an intermediate in the biosynthesis of PAA.

In this paper, we present results of preliminary experiments examining the substrate specificities of the two initial enzymes involved in the PAA biosynthetic pathways in bushbean seedlings, namely, phenylalanine aminotransferase and phenylalanine decarboxylase, to the series of mono-, di- and trichloro-phenylalanines. Changes in phenylalanine aminotransferase activity have been monitored in bushbean root and shoot tissues during the second week of seedling growth and the enzyme in 10-day-old shoots has been purified and resolved into two isozymes (enzymes I and II). The substrate specificity shown by each isozyme when tested with three monochloro-D,L-phenylalanines is reported.

## RESULTS AND DISCUSSION

A series of ring-substituted mono-, di- and trichloro-D,L-phenylalanines (Table 1) synthesized by an acet-

*Part 1 in the series: "Metabolism of chloro-phenylalanines by multispecific 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-phenylalanines to phenylacetic acid in higher plants. Mr. Rauthan was killed in the crash of the Air India Boeing 747 into the Atlantic Ocean on 23 June 1985.

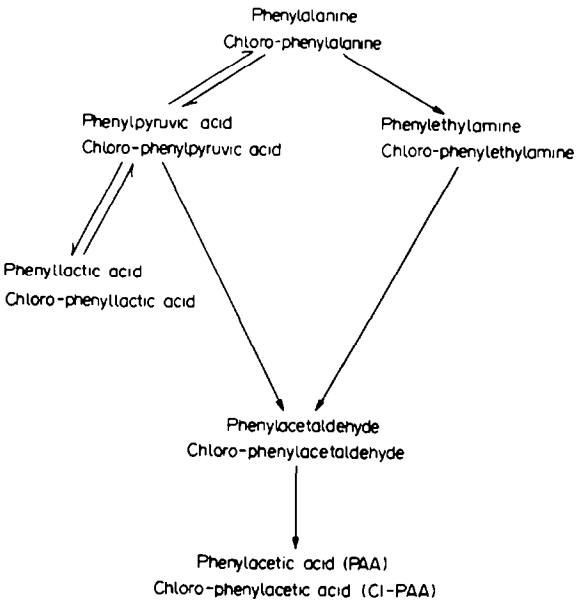
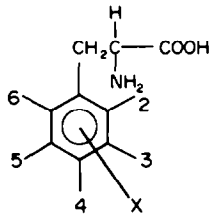



Fig. 1. Proposed biochemical pathways for the conversion of phenylalanine and chloro-phenylalanines to their corresponding phenylacetic acids.

Table 1. Ring-substituted chloro-phenylalanines synthesized as described in the Experimental




| Monochloro- | Dichloro- | Trichloro- |
|---|---|---|
| 2- | 2,3- | 2,3,4- |
| 3- | 2,4- | 2,3,6- |
| 4- | 2,5- | 2,4,5- |
| | 2,6- | |
| | 3,4- | |
| | 3,5- | |

amidomalonate condensation with the appropriate chloro-benzyl halide followed by acid hydrolysis [8] formed the principal substrates examined in the enzyme investigations reported here. The ability of these chloro-phenylalanines to serve as substrates for the phenylalanine decarboxylase and phenylalanine aminotransferase in 10-day-old bushbean seedlings was examined in preliminary experiments. A total soluble extract ($S_{25}$) of whole plants was subjected to fractionation with ammonium sulphate and the 20–35% saturation step precipitated a protein fraction which contained all detectable phenylalanine decarboxylase activity. This protein fraction was tested for its ability to decarboxylate L- and D,L-phenylalanine and each of the D,L-chloro-phenylalanines. Appreciable activity was observed when 20 mM L-phenylalanine ($1.5 \times 10^{-3}$ U/mg protein) or D,L-phenylalanine ($8 \times 10^{-4}$ U/mg protein) was supplied as substrate (Table 2), an *in vitro* finding which agrees with previous *in vivo* results of Camirand *et al.* [4] in which tobacco shoots fed with L-[U-$^{14}$C]phenylalanine were shown to accumulate significant levels of [$^{14}$C]-$\beta$-phenylethylamine. Although L-phenylalanine decarboxylase activity was shown by the 20–35% ammonium sulphate protein fraction, no detectable decarboxylase activity was observed when any of the D,L-chloro-phenylalanines were supplied as substrates (Table 2). A similar inactivity of monochloro-phenylalanines was previously reported when the three analogues were tested as substrates for both mammalian (guinea pig kidney) and bacterial (*Streptococcus faecalis* R.) aromatic amino acid decarboxylases [18].

40–70% ammonium sulphate saturation of the bushbean total soluble extract yielded a protein fraction containing all detectable phenylalanine aminotransferase activity. When this preparation was tested for its ability to transaminate each of the D,L-chloro-phenylalanines, most of the compounds were found to support aminotransferase activity at rates from 35 to 100% of that observed with D,L-phenylalanine supplied at an equivalent concentration (Table 2). 4-Chlorophenylalanine was the best substrate tested among the mono- and dichloro-compounds, although both 2,3,4- and 2,4,5-trichloro-phenylalanines supported aminotransferase activity at a level equal to that sustained by the unsubstituted parent amino acid. When the aminotransferase activity for D,L-phenylalanine was set at 100%, the relative activity for L-phenylalanine was found to be only about 20% higher. Since in most cases aminotransferases are stereospecific for L-amino acid isomers [19], it was expected that the L-phenylalanine aminotransferase activity would be about twice that observed with D,L-phenylalanine. A check of the specific rotation of the D,L-phenylalanine and all of the synthetically prepared D,L-chloro-phenylalanines gave zero values in all cases, indicating that all compounds were truly racemic mixtures. The relatively high activity observed with D,L-phenylalanine therefore suggests that the enzyme preparation contained a racemase capable of converting some of the D-isomer to the physiologically active L-isomer. Alternatively, this stereo-selective conversion could have been accomplished via a D-amino acid oxidase which would oxidatively deaminate the D-phenylalanine isomer to yield the symmetrical phenylpyruvate. This keto acid could then be transaminated in a stereospecific reaction to give L-phenylalanine. Either explanation is plausible in view of the fact that the aminotransferase preparation was only partially purified by ammonium sulphate fractionation. It should be noted that any such racemase or D-amino acid oxidase activity may also have had similar effects on the ratio of L-isomer to D-isomer during metabolism of each D,L-chloro-phenylalanine in these preliminary experiments.

Thus, while significant transamination of the chloro-phenylalanines was obtained with the crude phenylalanine aminotransferase fraction from bushbean, no detectable decarboxylation of these amino acids was observed with the crude phenylalanine decarboxylase fraction. It may be inferred from these findings that the proposed route for the conversion of D,L-chloro-

Table 2. Substrate specificity of partially purified bushbean phenylalanine decarboxylase and phenylalanine aminotransferase for phenylalanine (Phe) isomers and a series of D,L-chloro-phenylalanines

| Substrate | Concentration tested (mM) | Sp. activity: Phenylalanine decarboxylase* Sp. activity ($10^{-3}$ U/mg protein) | Sp. activity: Phenylalanine decarboxylase* Rel. activity‡ (%) | Sp. activity: Phenylalanine aminotransferase† Sp. activity ($10^{-3}$ U/mg protein) | Sp. activity: Phenylalanine aminotransferase† Rel. activity‡ (%) |
|---|---|---|---|---|---|
| D,L-Phe | 20 | 0.80 | 100 | 0.340 | 100 |
| L-Phe | 20 | 1.50 | 188 | 0.400 | 118 |
| D,L-2Cl-Phe | 20 | 0 | 0 | 0.129 | 38 |
| D,L-3Cl-Phe | 20 | 0 | 0 | 0.136 | 40 |
| D,L-4Cl-Phe | 20 | 0 | 0 | 0.340 | 100 |
| D,L-2,3$Cl_2$-Phe | 20 | 0 | 0 | 0.126 | 37 |
| D,L-2,4$Cl_2$-Phe | 20 | 0 | 0 | 0.112 | 33 |
| D,L-2,5$Cl_2$-Phe | 20 | 0 | 0 | 0.119 | 35 |
| D,L-2,6$Cl_2$-Phe | 20 | 0 | 0 | 0.112 | 33 |
| D,L-3,4$Cl_2$-Phe | 20 | 0 | 0 | 0.109 | 32 |
| D,L-3,5$Cl_2$-Phe | 20 | 0 | 0 | 0.126 | 37 |
| D,L-Phe | 2 | 0.10 | 100 | 0.230 | 100 |
| D,L-2,3,4$Cl_3$-Phe | 2 | 0 | 0 | 0.250 | 109 |
| D,L-2,3,6$Cl_3$-Phe | 2 | 0 | 0 | 0 | 0 |
| D,L-2,4,5$Cl_3$-Phe | 2 | 0 | 0 | 0.240 | 104 |

*Phenylalanine decarboxylase activity measured in resolubilized and desalted 20–35% $(NH_4)_2SO_4$ protein fraction.
†Phenylalanine aminotransferase activity measured in resolubilized and desalted 35–75% $(NH_4)_2SO_4$ protein fraction.
‡Relative to the activity of D,L-Phe set at 100%, when assayed at an equivalent concentration.

phenylalanines to the corresponding chloro-phenylacetic acids will most likely occur in bushbean seedlings via a transamination reaction, rather than via decarboxylation as the initial enzymic step in the metabolic pathway (Fig. 1).

Changes in phenylalanine aminotransferase activity in bushbean root and shoot tissues of different ages were next examined. The aim of this experiment was to determine the most suitable tissue from which to extract and purify this enzyme. Root and shoot tissues from light-grown 1-week-old plants were harvested at daily intervals between the time of hypocotyl hook opening (day 7) to full expansion of the first pair of leaves (day 12). The enzyme in each total soluble extract was isolated and partially purified by ammonium sulphate precipitation at the 40–70% saturation step. Results showed that both root and shoot tissues of 10-day-old seedlings possessed high phenylalanine aminotransferase activity and the enzyme also showed greatest specific activity at this stage of plant development (Fig. 2a and b).

The phenylalanine aminotransferases in such 10-day-old bushbean root and shoot tissues were then purified about 8-fold by 40–70% ammonium sulphate precipitation followed by Sephacryl S-300 gel filtration. The substrate specificity of each partially purified enzyme was tested with the series of chloro-phenylalanines and the results, presented in Table 3, showed that most of the monochloro- and dichloro-phenylalanines supported aminotransferase activity at a level of about 20–40% of that obtained with D,L-phenylalanine. The exceptions were 4-chlorophenylalanine which was 25% more active than

Table 3. Substrate specificity of Sephacryl S-300 purified phenylalanine aminotransferase preparations from bushbean root and shoot tissues for phenylalanine (Phe) isomers and a series of D,L-chloro-phenylalanines

| Substrate | Concentration tested (mM) | Rel. activity (%)*: Root | Rel. activity (%)*: Shoot |
|---|---|---|---|
| D,L-Phe | 32 | 100 | 100 |
| L-Phe | 32 | 147 | 103 |
| 2Cl-Phe | 32 | 19 | 17 |
| 3Cl-Phe | 32 | 19 | 40 |
| 4Cl-Phe | 32 | 46 | 124 |
| 2,3$Cl_2$-Phe | 16 | 0 | 17 |
| 2,4$Cl_2$-Phe | 16 | 37 | 42 |
| 2,5$Cl_2$-Phe | 16 | 17 | 30 |
| 2,6$Cl_2$-Phe | 16 | 23 | 40 |
| 3,4$Cl_2$-Phe | 16 | 28 | 27 |
| 3,5$Cl_2$-Phe | 16 | 27 | 23 |
| 2,3,4$Cl_3$-Phe | 2 | 107 | 82 |
| 2,3,6$Cl_3$-Phe | 1.6 | 0 | 0 |
| 2,4,5$Cl_3$-Phe | 2 | 113 | 92 |

*Relative to the activity of D,L-Phe set at 100%, when assayed at an equivalent concentration.

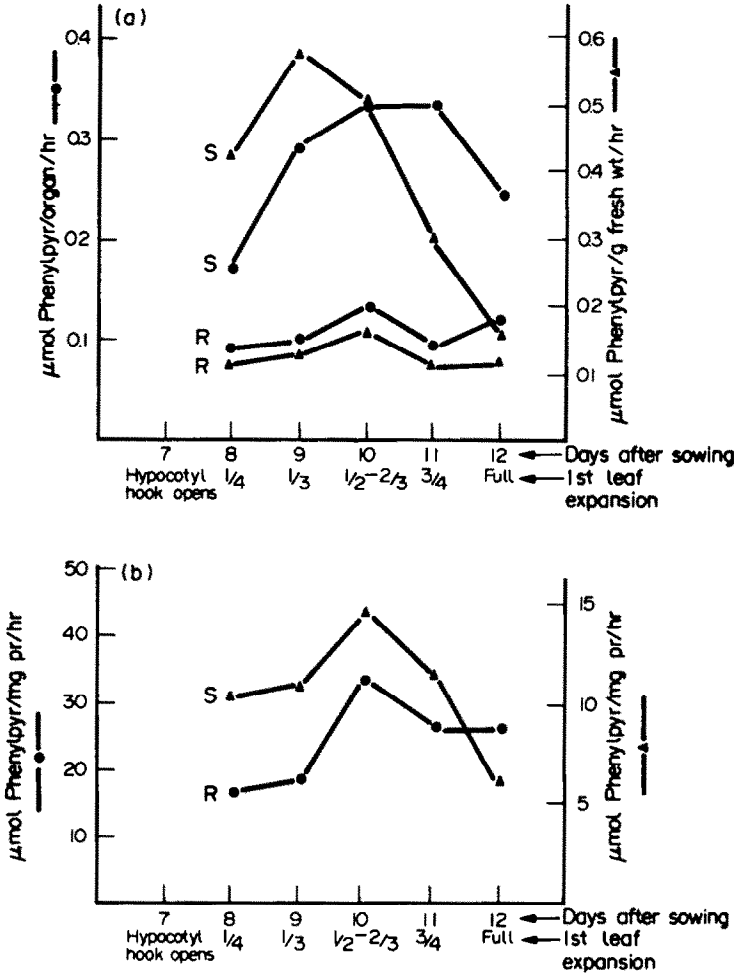



Fig. 2. Development of total activity (2a) and changes in specific activity (2b) of the phenylalanine aminotransferase in roots (R) and shoots (S) of bushbean seedlings during the second week of growth.

D,L-phenylalanine with the shoot system, and the 2,3-dichloro- derivative which was inactive with the root enzyme. Among the trichloro- derivatives, only the 2,3,4- and 2,4,5-trichloro-phenylalanines were actively transaminated by both root and shoot enzymes, showing activities of 80–113% of that observed with similar concentrations of D,L-phenylalanine. The 2,3,6-trichloro-phenylalanine, whose corresponding 2,3,6-trichloro-phenylacetic acid has been shown to be a very potent growth regulator [6], was not transaminated by the enzyme preparation from either tissue.

Since phenylalanine aminotransferase is the most likely initial enzyme in the proposed metabolic pathway for the conversion of chloro-phenylalanines to their corresponding chloro-phenylacetic acids (Fig. 1), and since future experiments will examine the metabolism of the series of chloro-phenylalanines in excised shoots of bushbean and other crop plants, it was decided to purify the phenylalanine aminotransferase in bushbean shoots for full characterization of the enzyme particularly with respect to its substrate specificity to chloro-phenylalanines. The procedure initially involved precipitation of the enzyme by 30–70% ammonium sulphate fractionation, gel filtration of the protein fraction on Sephacryl S-300 followed by ion exchange chromatography at pH 6.8 on DEAE-Sephacel. At this stage, phenylalanine aminotransferase was resolved into two peaks of activity (Fig. 3); one enzyme eluting at *ca* 0.105 M KCl (enzyme I) and a second enzyme eluting at *ca* 0.15 M KCl (enzyme II). Fractions enriched in enzymes I or II were pooled separately and each total fraction was then subjected to hydroxylapatite chromatography. Enzyme I eluted between 15 and 24 mM Na phosphate (Fig. 4) and peak fractions were pooled to give a purified enzyme I preparation. Enzyme II eluted between 33 and 40 mM Na phosphate (Fig. 5) and peak fractions were combined and then re-chromatographed on a column of DEAE-Sephacel using buffer 6 at pH 8.10 (Fig. 6). A sharp peak of enzyme II activity was eluted and active fractions were pooled to give a purified enzyme II preparation.

Table 4 summarizes the purification steps, percentage recovery and specific activity of each enzyme. Overall, a purification of 480-fold and a recovery of 65% were achieved. Enzyme II was purified about 650-fold with a recovery of 50%; it was found to be the more active of the two phenylalanine aminotransferases (relative total activity enz I:enz II 1:4). Each purified preparation was subjected to PAGE as described in the Experimental.

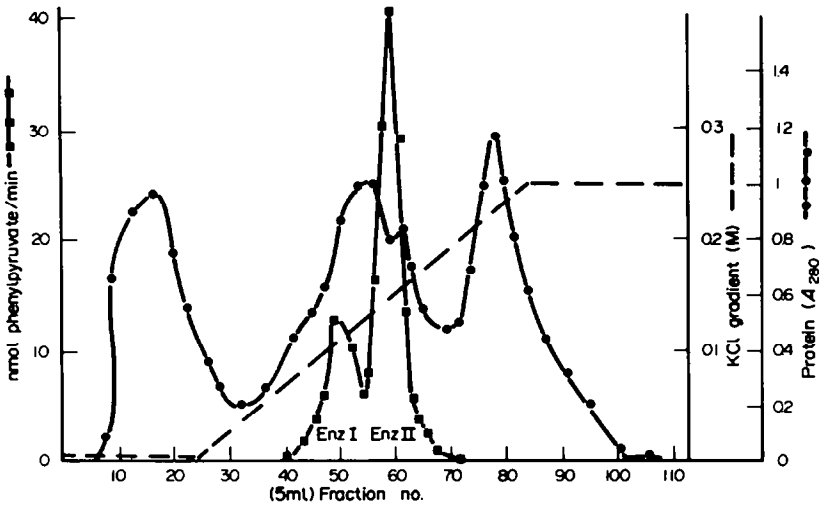



Fig. 3. Protein elution profile and related phenylalanine aminotransferase activity obtained from DEAE-Sephacel chromatography at pH 6.8 of a partially purified aminotransferase preparation from bushbean shoots.

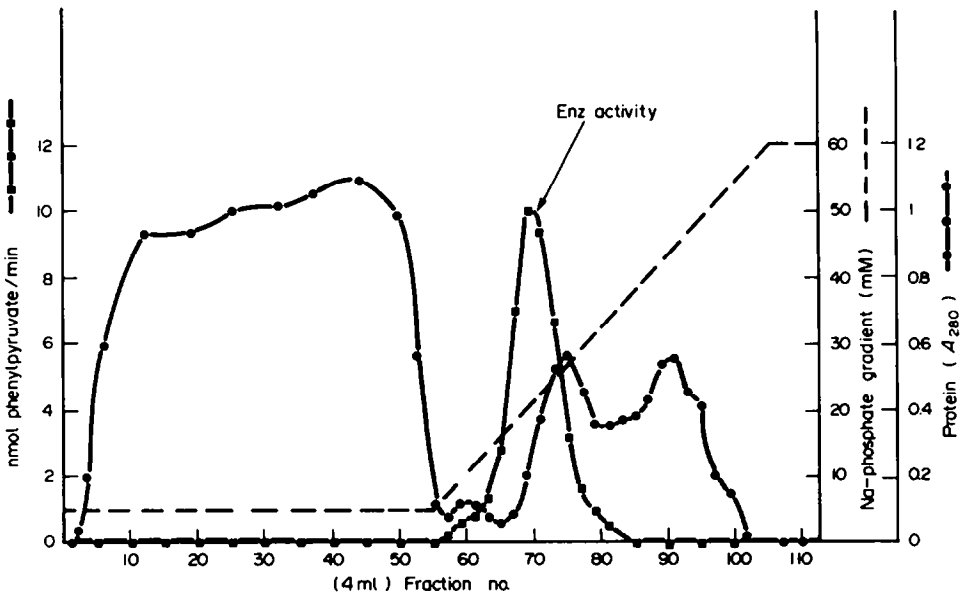



Fig. 4. Protein elution profile and related phenylalanine aminotransferase activity obtained from hydroxylapatite chromatography at pH 7.5 of enzyme I aminotransferase preparation from bushbean shoots.

Figure 7 shows the position of the protein bands in each preparation and the relative positions of phenylalanine aminotransferase activity in identical gels assayed for enzyme activity. The enzyme I preparation contained three bands of protein, but only the band at $R_f$ 0.58 showed phenylalanine aminotransferase activity. The enzyme II preparation showed six bands of protein, but phenylalanine aminotransferase activity was only associated with the major protein band at $R_f$ 0.72. Thus, the two purified preparations of enzymes I and II were shown to be free of other phenylalanine aminotransferase activity and were therefore considered to be of adequate purity for further characterization studies.

When the substrate specificities of enzymes I and II were examined with the three monochlorophenylalanines, enzyme I showed aminotransferase activity with only the 3-chloro- and 4-chloro-analogues, whereas enzyme II was active with all three compounds (Table 5). Enzyme II, in fact, showed a very high rate of activity with the 4-chloro-analogue (*ca* 225%) relative to that observed with an equivalent concentration of D,L-phenylalanine. It would appear that monochloro-substitution in any ring position does not prevent binding of the phenylalanine derivative to the active site of enzyme II, whereas *ortho*-substitution excludes the derivative from functioning as a substrate for enzyme I. Whether, in

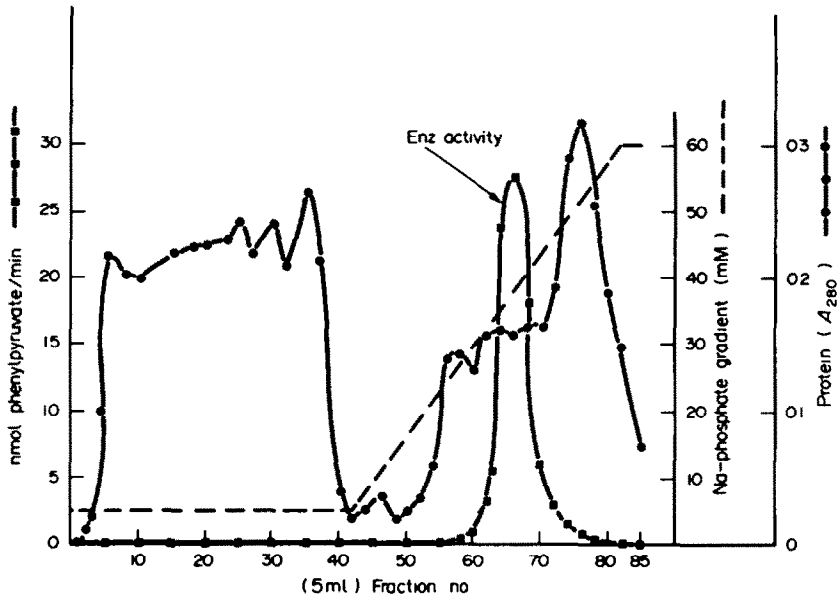



Fig. 5. Protein elution profile and related phenylalanine aminotransferase activity obtained from hydroxylapatite chromatography at pH 7.5 of enzyme II aminotransferase preparation from bushbean shoots.

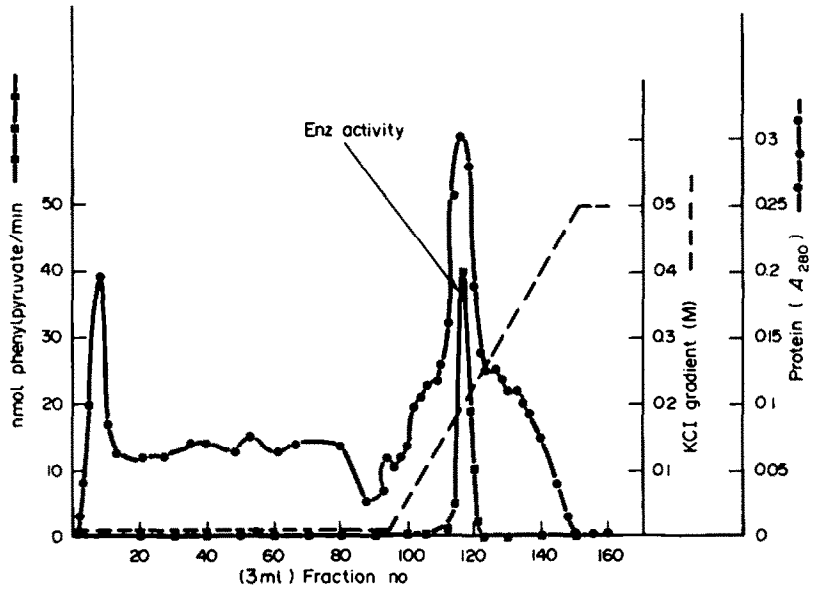



Fig. 6. Protein elution profile and related phenylalanine aminotransferase activity obtained from a second DEAE-Sephacel chromatography at pH 8.1 of enzyme II aminotransferase purified from bushbean shoots.

fact, these chloro-phenylalanines react with the same binding site on enzymes I and II as that occupied by L-phenylalanine is not yet known. Substrate competition experiments should resolve this question. The most interesting finding in this experiment, however, was the high activity exhibited by *para*-chlorophenylalanine with both isozymes, but especially with isozyme II. The significance of this positional effect of the ring-substituent on phenylalanine aminotransferase activity is under further investigation and will be considered in the next paper of this series.

Thus, it appears that there are two isozymic forms of phenylalanine aminotransferase in bushbean shoots and both are capable of catalysing the transamination of monochloro-phenylalanines. This conclusion is supported by the fact that enzymes I and II behaved quite differently during identical ion exchange and hydroxylapatite (hydrophobic) chromatography and during polyacrylamide gel electrophoresis. The two enzymes also exhibited differences in their substrate specificity to the monochloro-phenylalanines. To our knowledge, this is the first time that multiple forms of this enzyme have been

Table 4. Purification of phenylalanine aminotransferase isozymes I and II from 10-day-old bushbean shoots

| Purification step | Protein (mg) | Sp. activity ($10^{-3}$ units*/mg protein) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|
| 1. Total soluble extraction | 300 | 0.200 | 100 | — |
| 2. 40–70% $(NH_4)_2SO_4$ precipitation | 1870 | 0.305 | 95 | 1.5 |
| 3. S-300 gel filtration | 380 | 1.47 | 93 | 7.5 |
| 4. DEAE-Sephacel chromatography at pH 6.8 isozyme I | 78 | | | |
| isozyme II | 115 | | | |
| | 193 | 3.37 | 108 | 20 |
| 5. Hydroxylapatite chromatography isozyme I | 1.5 | | | |
| 6. Hydroxylapatite chromatography isozyme II | 8.5 | | | |
| | 10.0 | 53.0 | 88 | 265 |
| 7. DEAE-Sephacel chromatography at pH 8.1 isozyme II | 2.5 | | | |
| Plus step (5) | 1.5 | | | |
| | 4.0 | 95.5 | 65 | 480 |

*1 Unit of enzyme activity will convert 1 μmol Phe to 1 μmol phenylpyruvate per min at 35°, pH 8.5.

Table 5. Substrate specificity of purified phenylalanine aminotransferase isozymes I and II for phenylalanine isomers and D,L-monochloro-phenylalanines

| Substrate* | Concentration tested (mM) | Enzyme I† Sp. activity ($10^{-3}$ U/mg protein) | Enzyme I† Rel. activity (%)‡ | Enzyme II† Sp. activity ($10^{-3}$ U/mg protein) | Enzyme II† Rel. activity (%)‡ |
|---|---|---|---|---|---|
| D,L-Phe | 32 | 13.8 | 100 | 54.0 | 100 |
| L-Phe | 32 | 34.8 | 250 | 125.0 | 232 |
| D,L-2Cl-Phe | 32 | 0 | 0 | 6.6 | 12 |
| D,L-3Cl-Phe | 32 | 4.0 | 35 | 23.4 | 43 |
| D,L-4Cl-Phe | 32 | 12.2 | 88 | 120.0 | 222 |

*2-OG (10 mM) was the keto acid supplied in all cases.
†Purified as summarized in Table 4.
‡Relative to the activity of D,L-Phe set at 100%, when assayed at an equivalent concentration.

reported in bushbean. Previous work on a multispecific aromatic aminotransferase purified from roots of bushbean seedlings [10] showed that there is only one form of the enzyme in this organ and that it occurs in the cytosol.

There is the possibility that one of the two isozymic forms of phenylalanine aminotransferase purified in the present study is compartmentalized *in vivo*. Data on the subcellular localization and complete characterization of the phenylalanine aminotransferases I and II from bushbean shoots will be presented in the next paper of this series.

## EXPERIMENTAL

*Plant material and chemicals.* Bushbean seeds (*Phaseolus vulgaris* L. cv. Pencil Pod Black Wax) were soaked in distilled water at room temp. overnight, sown in moist vermiculite and germinated in a growth cabinet at 25/22° day/night temp. with 16 hr daily photoperiods of 4600–4800 μW/cm². The time of planting was taken as day 0. Seedlings were harvested at the desired age and the whole plant, shoot or root tissue was collected for enzyme extraction. Root material was freed of vermiculite with several rinses of distilled $H_2O$.

The ring-substituted mono-, di- and trichloro-D,L-phenylalanines examined as substrates in this study (Table 1) were synthesized by an acetamidomalonate condensation with the appropriate chloro-benzyl halide, followed by acid hydrolysis [8]. The chloro-phenylalanines were characterized by mp, IR, $^1H$ NMR, GC-MS and CI-MS. Sephadex G-25 (fine), Sephacryl S-300 and DEAE-Sephacel were purchased from Pharmacia while Biogel HTP (hydroxylapatite) was purchased from Bio-Rad Laboratories. All other chemicals used in these investigations were purchased from Sigma or Aldrich Chemical Companies and were of analytical grade.

*Enzyme assays.* Phenylalanine aminotransferase activity was determined by measuring the production of glutamate (method A or B) or phenylpyruvate (method C), the amino acid and keto acid products of an aminotransferase reaction involving L-phenylalanine (L-Phe) and 2-oxoglutarate (2-OG). Reactions were carried out in 50 mM Tris–HCl, pH 8.5, containing final concentrations of 10–40 mM L- or D,L-Phe or a D,L-chloro-phenylalanine (except for the trichlorophenylalanines which were much less soluble), 10 mM 2-OG, 0.1 mM pyridoxal

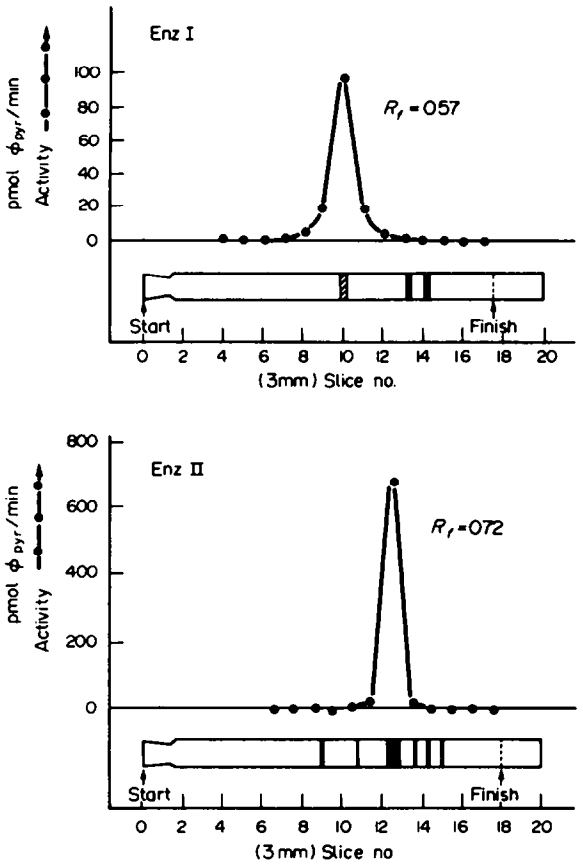



Fig. 7. Position of protein bands and profiles of phenylalanine aminotransferase activity in disc-polyacrylamide gel of purified enzymes I and II preparations after electrophoresis as described in Experimental.

phosphate (PLP), 0.1 mM EDTA, 2 mM DTT and 50–100 μl of enzyme, in a final vol. of 0.75 ml. In methods A and B, control reactions contained all the above components except the amino acid substrate, which was replaced by an equal vol. of Tris buffer. In method C, the control reaction contained all reagents, but the NaOH was added before the 2-OG at time zero and this control was incubated with the experimental reactions for the time period specified below. Due to solubility difficulties, all chloro-phenylalanine stock solns were prepared in 50 mM Tris–HCl, pH 8.5, containing 12.5% DMSO. Final concn of DMSO in the assay reaction mixtures was always 10% or less and for comparative purposes, equivalent complete reaction mixtures containing L-Phe or D,L-Phe plus DMSO were run simultaneously. The presence of DMSO was found to have no deleterious effect on L- or D,L-phenylalanine aminotransferase activity. After 1 hr incubation, the reactions were stopped and the products measured by method A, method B or method C.

*Method A.* The aminotransferase reaction was stopped by the addition of 100 μl of 30% sulphonyl salicylic acid solution (SSA). Reaction mixtures were centrifuged to pellet the precipitated protein; an aliquot of the supernatant was removed, adjusted to pH 4–5 with 1 M KOH and made up to 2 ml with 10% HPLC-grade MeOH in double distilled $H_2O$. The glutamate in this sample was measured as follows: the sample was derivatized with σ-phthalaldehyde (OPA) and 2-mercaptoethanol by a procedure similar to that reported by Jones *et al.* [20] yielding thio-substituted isoindole amino acid derivatives which were then separated by chromatography on a Waters Resolve $C_{18}$ column (15 cm × 3.9 mm i.d., 5 μm particle size) equipped with a Corasil $C_{18}$ guard column, and maintained at 40°. The derivatives were detected with a fluorescence detector. The flow rate was 1.5 ml/min (except for the first 2 min of the program when the OPA and amino acids passed through the guard column at a flow rate of 0.1 ml/min). The programmed gradient for separation of the OPA-glutamate derivative was from 100% solvent A (MeOH–THF–$H_2O$, 1:1:48, containing 0.05 M NaOAc and 0.05 M $Na_2HPO_4$ and the pH adjusted to 7.0 with HOAc) to 100% solvent B (MeOH–$H_2O$, 4:1) over 4.5 min, followed by isocratic elution at 100% solvent B for 4.0 min. The column was regenerated by a gradual return to 100% solvent A over 4.0 min followed by a 6.0 min isocratic period at 100% solvent A. The amount of glutamate present ($R_t \sim 5.8$ min) was calculated with reference to the peak area given by a known standard amount of OPA-glutamate. One unit (U) of enzyme activity will convert 1 μmol of phenylalanine (or chloro-phenylalanine) to 1 μmol of product (glutamate or phenylpyruvate) per min at 35°, pH 8.5.

*Method B.* The reaction was terminated by the addition of an equal vol. of hot MeOH, the precipitated protein pelleted by centrifugation and an aliquot (200–400 μl) of the MeOH supernatant applied to a 10 cm band on the starting line of a large sheet (46 × 57 cm) of Whatman no. 1 chromatography paper. Chromatograms were developed in the descending manner for 24 hr in *n*-BuOH–HOAc–$H_2O$ (90:10:29), and the glutamate present determined by the method of Atfield and Morris [21] using a modified cadmium acetate–ninhydrin colorimetric reagent.

*Method C.* The reaction was terminated by the addition of 1.0 ml 1.75 M NaOH and any phenylpyruvate present was measured directly by the method of ref. [22]. The $A_{318}$ was measured and the amount of phenylpyruvate determined from a standard curve. This was the method of choice for monitoring enzyme activity in eluant fractions from CC.

Phenylalanine decarboxylase activity was determined by measuring the production of phenylethylamine, or chloro-phenylethylamine when a chloro-phenylalanine was provided as the substrate. Reactions were carried out in 50 mM Tris–HCl, pH 8.5, containing final concentrations of 10–20 mM L- or D,L-Phe (or a D,L-chloro-phenylalanine), 0.1 mM pyridoxal phosphate and 400 μl enzyme in a final vol. of 1.0 ml. Control reactions were minus the substrate amino acid. After a 2 hr incubation period at 35°, the reactions were stopped by the addition of 1.0 ml hot MeOH. Centrifugation pelleted the precipated protein and two 500 μl aliquots of the MeOH supernatant were streaked in 10 cm bands on a 46 × 57 cm sheet of Whatman no. 1 chromatography paper. Chromatograms were developed in the descending manner for 12 hr in *n*-BuOH–HOAc–$H_2O$ (120:30:50) and any phenylethylamine (or chloro-phenylethylamine) present was determined by the cadmium–ninhydrin colorimetric assay of ref. [23]. [$R_f$ values: Phe (or chloro-Phe) *ca* 0.55; phenylethylamine (or chloro-phenylethylamine) *ca* 0.75].

*Determination of protein.* Protein was estimated by the method of Bradford [24] using bovine γ-globulin as standard. For monitoring protein profiles from CC, the optical method ($A_{280}$) was used [25].

*Preparation of enzyme extracts.* All steps in the preparation of bushbean extracts for the isolation of total soluble phenylalanine aminotransferase and phenylalanine decarboxylase were carried out at 4°. Ten-day-old seedlings were harvested and washed, cotyledons removed and the root and shoot tissues (60 g) ground in a Waring blender at high speed for 1 min using 120 ml 50 mM Tris–HCl buffer, pH 7.5, containing 12 mM 2-mercaptoethanol,

0.1 mM EDTA, 1 mM DTT and 0.1 mM PLP (buffer 1). The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 1000 *g* for 20 min to remove cell debris. The supernatant was then treated with Tergitol NPX detergent to a final concn of 0.1% to facilitate bursting of the organelles. After stirring for 30 min, the soln was centrifuged at 25000 *g* for 30 min; the pellet (which did not contain any detectable Phe-aminotransferase or Phe-decarboxylase activity) was discarded and the supernatant ($S_{25}$) used as a crude extract to measure total soluble protein and the total soluble activities of phenylalanine aminotransferase and phenylalanine decarboxylase. Solid $(NH_4)_2SO_4$ was then added to bring the extract up to 20% salt satn and after stirring for 1 hr, the material that precipitated was collected by centrifugation at 35000 *g* for 15 min and discarded. The supernatant was then brought to 35% $(NH_4)_2SO_4$ satn, stirred for 1 hr and centrifuged; the supernatant was saved and the pellet of precipitated protein containing Phe-decarboxylase activity was resuspended in 5 ml of buffer 1. This protein fraction was then desalted by passing the extract through a Sephadex G-25 column (1 × 15 cm, void vol. 6.5 ml) using buffer 1 as the elution buffer. The main protein fraction was collected (6.5–12.0 ml) and samples were assayed directly for Phe or chloro-Phe decarboxylase activity as described above.

The supernatant remaining after the 35% $(NH_4)_2SO_4$ centrifugation step was then brought to 75% $(NH_4)_2SO_4$ saturation and stirred for 1 hr. The precipitated protein was sedimented by centrifugation and the pellet, containing Phe-aminotransferase activity, was resuspended and desalted as described above. Samples were assayed for Phe or chloro-Phe aminotransferase activity by method B.

*Examination of the substrate specificity of partially purified phenylalanine aminotransferase from bushbean root and shoot tissue for a series of chloro-phenylalanines.* Ten-day-old light grown bushbean seedlings were harvested, separated into root (25 g) and shoot (epicotyl) (30 g) tissue and total soluble extracts ($S_{25}$) of each tissue were prepared in buffer 1 as described above. Each extract was then brought up to 40% $(NH_4)_2SO_4$ satn, stirred for 1 hr and centrifuged at 35000 *g* for 15 min. The pellet of precipitated protein was discarded. The supernatant was brought up to 70% $(NH_4)_2SO_4$ satn, stirred for 1 hr, centrifuged at 35000 *g* for 15 min and the precipitated protein containing all the soluble phenylalanine aminotransferase was resuspended in 5–10 ml of buffer 1. Two columns (90 × 2.5 cm) of Sephacryl S-300 were 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 (buffer 2). The concentrated aminotransferase fractions [from the 40–70% $(NH_4)_2SO_4$ precipitation step] from root and shoot tissue were each applied to a S-300 column and eluted with buffer 2 at a flow rate of 30 ml/hr. The column effluent was monitored at $A_{280}$ and 5 ml fractions were collected. Fractions were assayed for aminotransferase activity by method C, using L-phenylalanine and 2-OG as substrates. All fractions showing activity were pooled to give the S-300 phenylalanine aminotransferase fraction from bushbean root or shoot tissue. The chloro-phenylalanines were then individually tested as substrates for both the root and shoot enzymes by method A.

*Purification of phenylalanine aminotransferase from bushbean shoots.* Purification of the phenylalanine aminotransferase in bushbean shoots was achieved by the procedure described below. All operations were carried out at 4°. Shoots (65 g) from 10-day-old seedlings were ground in 180 ml 50 mM Tris–HCl, pH 7.5, containing 0.1 mM EDTA, 2 mM DTT, 12-mM 2-mercaptoethanol, 8 mM 2-OG, 0.2 mM PLP and 5% glycerol (buffer 3). Extraction and partial purification of the aminotransferase up to the end of the Sephacryl S-300 chromatographic step were carried out as described in the previous section. All fractions from the S-300 column showing phenylalanine aminotransferase activity were pooled to give the 'S-300 fraction' which was then further purified by DEAE-Sephacel and hydroxylapatite chromatography.

A suspension of DEAE-Sephacel in 50 mM Tris–HCl, pH 6.8, containing 0.1 mM EDTA, 2 mM DTT and 12 mM 2-mercaptoethanol (buffer 4) was packed in a column (1.6 × 30 cm) to a bed height of 25 cm and equilibrated with buffer 4. The 'S-300 fraction' was applied to the column at a rate of *ca* 65 ml/hr. After the protein had been absorbed, unbound protein was washed through the column with 80 ml of buffer 4. Bound protein was then eluted with a linear gradient from 0 to 250 mM KCl in buffer 4. The total gradient vol. was 300 ml and the flow rate was 30 ml/hr. 5 ml fractions were collected overnight. Assay of the eluted fractions indicated that 2 separate peaks of phenylalanine aminotransferase activity had been resolved (Fig. 3).

DEAE-Sephacel peak I fractions were pooled and to adjust conductivity, the soln was diluted 3-fold with 5 mM NaPi buffer, pH 7.5, containing 0.1 M NaCl, 1 mM DDT, 10 μM PLP, 12 mM 2-mercaptoethanol and 5% glycerol (buffer 5). This diluted peak I fraction was applied at a rate of *ca* 50 ml/hr to a hydroxylapatite (Biogel HTP) column (1.0 × 30 cm) pre-equilibrated with buffer 5 and any unbound protein was washed away with 35 ml of buffer 5. The phenylalanine aminotransferase was eluted with a linear gradient from 5 to 60 mM NaPi, pH 7.5, containing 0.1 M NaCl, 1 mM DTT, 10 μM PLP, 12 mM 2-mercaptoethanol and 5% glycerol. The total gradient vol. was 200 ml and the flow rate was 30 ml/hr. 4 ml fractions were collected, assayed and a single peak of activity was observed (Fig. 4). Active fractions were pooled to give the 'hydroxylapatite peak I' fraction (enzyme I) which was then subjected to PAGE as described in the next section.

DEAE-Sephacel peak II fractions were also pooled, diluted 4-fold with buffer 5, applied to a hydroxylapatite column and eluted under the same gradient conditions described above for the DEAE-Sephacel peak I enzyme, except that 5 ml fractions were collected. Again a single peak of phenylalanine aminotransferase activity was observed (Fig. 5) and the active fractions were pooled to give the 'hydroxylapatite peak II' fraction. This was diluted 6-fold with 50 mM Tris–HCl, pH 8.1, containing 0.1 mM EDTA, 2 mM DTT and 12 mM 2-mercaptoethanol (buffer 6) and applied at *ca* 50 ml/hr to a second DEAE-Sephacel column (1.0 × 30 cm) pre-equilibrated to pH 8.1 with buffer 6. After washing the column with 40 ml buffer 6, the phenylalanine aminotransferase was eluted with a linear gradient from 0 to 500 mM KCl in buffer 6. The total gradient vol. was 180 ml and the elution rate was 25 ml/hr; 3 ml fractions were collected. Fractions comprising the single peak of aminotransferase activity (Fig. 7) were pooled (enzyme II) and subjected to PAGE. Further details of the purification are presented in Table 4.

*PAGE.* The gels were prepared in glass columns (0.5 × 8 cm) and the procedure followed was that of Davis [26] using Tris–glycine buffer, pH 8.3. Bromophenol Blue was used as the tracking dye. Electrophoresis was carried out on 5–50 μg protein samples using an initial current of 3 mA per gel column until the tracking dye ran into the small pore ('running') gel (*ca* 40 min) at which time the current was increased to 4 mA/gel, for a further 80 min. Protein bands were detected by staining with 0.25% Coomassie Brilliant Blue R-250 in MeOH–HOAc–$H_2O$ (9:2:9) for 30 min as reported by Weber and Osborne [27]. The gels were destained in MeOH–HOAc–$H_2O$ (4:1:15).

Phenylalanine aminotransferase activity was located on the gels as follows: gels were sliced into 3 mm sections and each slice was assayed for aminotransferase activity by incubating at 35° for 90 min in 0.75 ml of 50 mM Tris–HCl, pH 8.5, containing 40 mM L-phenylalanine, 5 mM 2-OG, 0.1 mM PLP, 0.1 mM EDTA and

2 mM DTT. Reactions were stopped by the addition of 1.75 N NaOH and the enzyme activity measured by method C. Figure 7 shows the relative position of protein bands and the region of aminotransferase activity in gels of purified phenylalanine aminotransferase enzyme I and II.

The purified enzyme fractions were individually concd by filtration through an Amicon Ultrafiltration (Model 52) system using a PM-30 membrane under 30 psi $N_2$ gas with about 93% recovery of activity in both cases. The concentrates of enzyme I and enzyme II were then diluted to 50% with glycerol containing 20 $\mu$M PLP and stored at $-22°$. The enzyme preparations were found to be very stable in this form and maintained 90–97% of their activity over several months.

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