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Short Communication

Identification by high-performance liquid chromatography of tyrosine ammonia-lyase activity in purified fractions of *Phaseolus vulgaris* phenylalanine ammonia-lyase

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ABSTRACT

Activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) were assessed at each stage of a three-step purification of PAL. Assays were performed by high-performance liquid chromatographic (HPLC) separation and ultraviolet detection of reaction products. Use of HPLC permitted assay of low activities of PAL and TAL for periods up to approximately four and two days, respectively. HPLC also facilitated the accurate quantitation of the product of the TAL reaction, *trans-p*-coumaric acid, which was observed to isomerize readily under experimental conditions. PAL and TAL were associated throughout the purification procedure, with TAL activity at 0.6–1.3% of PAL activity. It was concluded that, contrary to previous reports, TAL and PAL activities are mediated by the same enzyme, or else by chromatographically very similar enzymes.

INTRODUCTION

Most phenylalanine-metabolising enzymes also have activity towards tyrosine. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) from monocot plants is typical in this respect, but the same enzyme from dicotyledons is considered to be phenylalanine-specific [1], although the tobacco plant apparently has a separate tyrosine ammonia-lyase (TAL) [2]. The reactions catalysed by these enzymes are:

$$\text{L-phenylalanine} \xrightarrow{\text{PAL}} \textit{trans}\text{-cinnamic acid} + \text{ammonia}$$

$$\text{L-tyrosine} \xrightarrow{\text{TAL}} \textit{trans-p}\text{-coumaric acid} + \text{ammonia}$$

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We are interested in the use of enzymes for the detection and monitoring of abnormal blood phenylalanine levels in phenylketonuria. For this purpose, in order to avoid interference from high blood tyrosine concentrations, absolute specificity for phenylalanine is essential. PAL from dicotyledons might therefore be of use in assays for phenylalanine. First, though, it was necessary to test the enzyme rigorously for any cross-reactivity to tyrosine. French bean (*Phaseolus vulgaris*) was used as the PAL source as the enzyme from this plant had been purified previously [3–5] and its cDNA cloned [6].

EXPERIMENTAL

French bean cell suspension cultures were obtained from Professor D. H. Northcote (Department of Biochemistry, University of Cambridge, Cambridge, UK). PAL was purified from these cultures by a combination of methods [3–5], in a three-step process involving, successively, ammonium sulphate precipitation, anion-exchange chromatography and chromatofocusing. These three operations resulted in purification of PAL by factors of 3, 7 and 21 over the starting material, respectively. PAL and TAL activities were assayed after each stage.

Assay mixtures for PAL activity contained 50 m*M* Tris–HCl, pH 8.8, 4.0 m*M* L-phenylalanine and enzyme preparation. In assays for TAL activity, L-phenylalanine was replaced by 4.0 m*M* L-tyrosine. Controls for metabolism of products were formulated, containing 5 μM cinnamic acid or 0.5 μM *p*-coumaric acid in place of phenylalanine or tyrosine. Mixtures were incubated at 30°C. At intervals (up to 100 h), 0.3-ml aliquots were removed, combined with 15 μl of glacial acetic acid, and assayed for cinnamic acid or *p*-coumaric acid by high-performance liquid chromatography (HPLC). The HPLC mobile phase consisted of 1:500:500 (v/v/v) acetic acid–methanol–water [7] and the volume of sample injected was 100 μl. The column used was a μBondapak C_{18} radial compression cartridge 10 cm × 0.8 cm I.D. (Waters, Harrow, UK) and the flow-rate was 2.0 ml/min. Substrates and products were detected using a variable-wavelength UV monitor (Waters Model 480). Peaks were identified by coelution with standards and absorbance ratios (313 nm/333 nm). Enzyme units were calculated as μmol product formed per minute at 30°C.

Isomers of *p*-coumaric acid were separated by precipitation of *trans-p*-coumaric acid, but not the *cis* isomer, with 0.33 *M* hydrochloric acid [8].

RESULTS

In the HPLC system described, cinnamic acid eluted with a retention time of 8.0 min, whilst *p*-coumaric acid eluted as a double peak, with retention times of 3.7 min (peak A) and 4.1 min (peak B, Fig. 1). The relative proportions of the two components of this doublet varied with the conditions under which standard solutions were stored. Plots of peak B height *versus* peak A height for fixed amounts of standard *p*-coumaric acid solutions stored under different conditions showed that *p*-coumaric acid absorbed at 313 nm twice as much in the peak B form as in the peak A form (Fig. 2). From these data, *p*-coumaric acid in samples could be quantified (see Fig. 3, legend).

Peaks A and B were presumed to represent *cis*/*trans* isomers of *p*-coumaric acid. This was confirmed by using hydrochloric acid to selectively

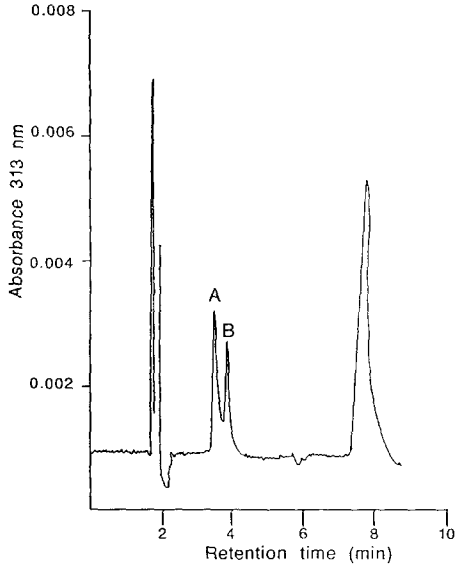

Fig. 1. HPLC of *p*-coumaric acid and cinnamic acid. *p*-Coumaric acid (100 pmol) eluted as double peaks at 3.7 min (A) and 4.1 min (B). Cinnamic acid (1 nmol) eluted at 8.0 min.

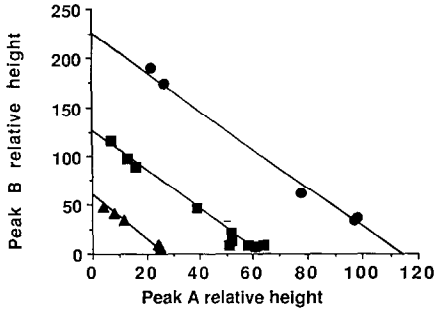

Fig. 2. *p*-Coumaric acid standard solutions analysed by HPLC and detected at 313 nm. Peak B height was plotted *versus* peak A height for varied amounts of *p*-coumaric acid. Extrapolation of plots to each axis gives estimates of peak heights which would be obtained if *p*-coumaric acid was wholly in the peak A or peak B form. Absorbance at 313 nm of the peak B form is shown to be 2.0 times greater than absorbance of the peak A form. Key: ● = 100 pmol, ■ = 50 pmol and ▲ = 25 pmol *p*-coumaric acid.

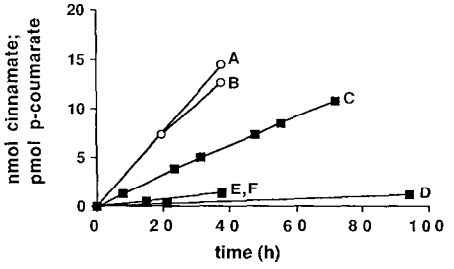

Fig. 4. Illustrative time courses for *p*-coumaric acid or cinnamic acid production from tyrosine or phenylalanine by respective ammonia-lyases. The scale indicates amount of cinnamic acid or *p*-coumaric acid detected in HPLC assays. Key: ○ = *p*-coumaric acid; ■ = cinnamic acid. Lines: A and B: tyrosine assays of chromatofocusing fractions; C and D: phenylalanine assays of DEAE ion-exchange fractions; E and F: phenylalanine assay of chromatofocussing fractions (corresponding to tyrosine assay lines A and B).

precipitate *trans-p*-coumaric acid. When this technique was used, 98% of peak B was removed but peak A was reduced by only 30%. Therefore peak B was probably *trans-* and peak A *cis-p*-coumaric acid.

Use of HPLC permitted assay of PAL with linear response over periods up to 56 h, and within 90% of linearity up to 94 h. TAL activity was linear up to a maximum of 38 h (Fig. 4).

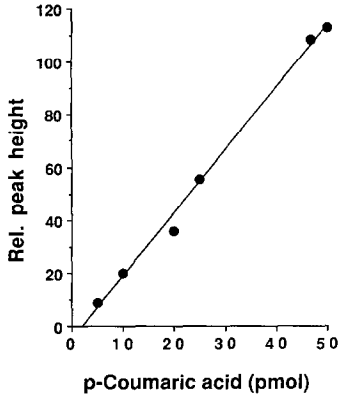

Fig. 3. Standard curve for *p*-coumaric acid (in peak B form). Data points are derived from intercepts on the peak B axis of plots similar to Fig. 2, or, for individual samples, by multiplying peak A height × 2 and adding to peak B height. Peak-height data from unknown samples were treated similarly before reading off values for *p*-coumaric acid from the standard curve. The response was linear to 100 pmol *p*-coumaric acid, but is shown up to 50 pmol only for clarity.

There was evidence of PAL activity at all stages of PAL purification. In crude preparations, *p*-coumaric acid was metabolised further, as evidenced by its rapid (within 2 h) disappearance (shown by HPLC) from control mixtures. The *p*-coumaric acid-degrading activity was removed by ammonium sulphate fractionation. The degree of cross-reactivity (TAL activity/PAL activity) was estimated after this and subsequent

TABLE I

RELATIVE ACTIVITIES OF PAL AND TAL DURING PAL PURIFICATION

Purification step	TAL activity (as % PAL activity)
Ammonium sulphate 40–55% fractionation	1.3
Ion-exchange fractions (average of three fractions, range 0.6–0.7)	0.6[a]
Chromatofocusing: fraction eluting at pH	
5.2–5.1	1.0
5.1–4.9	1.2
4.9–4.8	1.3

[a] The low level of TAL here necessitated a longer incubation period of 68 h. This may have resulted in a lower estimate of relative TAL activity.

purification steps (Table I). The two activities were associated throughout the purification procedure.

The production of *p*-coumaric acid could not be accounted for by chemical breakdown of tyrosine, even in chromatofocusing fractions, which had the lowest activity of samples tested. Incubation at 30°C of 5 m*M* tyrosine in an assay mixture lacking enzyme preparation did not lead to the production of *p*-coumaric acid after 24 h (detection limit 3–5 pmol *p*-coumaric acid). The reactions illustrated in Fig. 4 produced 7 pmol within 20 h. Other chromatofocusing fractions, assayed at a single time point (64 h) produced 41 pmol in this period (15 pmol per 24 h assuming a linear reaction rate).

DISCUSSION

The HPLC assay of PAL allowed low levels of PAL activity to be measured over periods up to four days. HPLC was particularly useful for quantifying *p*-coumaric acid accurately (in contrast to spectrophotometric methods, see ref. 1). The two *p*-coumaric acid peaks observed are probably *cis/trans* isomers. The *trans* isomer absorbs at 313 nm twice as much as the *cis* isomer. A previous separation of *p*-coumaric acid from TAL assay mixtures by HPLC [7] showed only a single peak for the metabolite. In the present work, it was found impossible to maintain *p*-coumaric acid as a single isomer (note that none of the points in Fig. 2 lie on the axes). The peak noted before [7] may have been due to a combination of isomers, which absorbing in the UV to differing extents, were not quantified accurately.

Activity towards tyrosine at 1.0–1.3% of levels of activity towards phenylalanine was found at the beginning and the end of the purification procedure. The failure to separate the two activities and the almost constant ratio between them suggest that they are mediated by the same enzyme, or by enzymes with very similar chromatographic characteristics. Bolwell *et al.* [3] isolated four isoenzymes of PAL from *P. vulgaris* which eluted from chromatofocusing columns over the pH range 5.4–4.9. Here, we found consistent TAL activity in PAL fractions eluting in the pH range 5.2–4.8.

The present work casts doubt on the claim that PAL from dicotyledons is phenylalanine-specific. More detailed work on the enzyme from other such plants might also reveal activity towards tyrosine. Nevertheless, this enzyme, among phenylalanine-metabolising enzymes, has one of the lowest activities towards tyrosine. TAL activity is present in cereals at greater than 5% of the level of PAL activity and at higher relative activities in fungi [1]. Phenylalanine aminotransferases typically have activity to other aromatic amino acids [9] and all phenylalanine dehyhdrogenases studied so far have activity to at least one other amino acid [10,11]. *Phaseolus* PAL may therefore be the most useful of these enzymes for use in clinical assays for phenylalanine, as interference from tyrosine would be minimal.

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