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AN AFFINITY MATRIX FOR THE ISOLATION OF L-PHENYLALANINE AMMONIA-LYASE

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Key Word Index *Phaseolus vulgaris*; Leguminosae; affinity chromatography; phenylalanine ammonia-lyase.

Abstract An affinity matrix for L-phenylalanine ammonia-lyase has been synthesized by coupling a protected 2-aminooxy-3-(4'-hydroxyphenyl)propionic acid derivative to epoxy-activated Sepharose 6B through the phenolic hydroxyl group. This avidly and specifically binds bean phenylalanine ammonia-lyase from partially purified preparations; active enzyme, albeit in relatively low yield, is released by incubation with 2-aminooxy-3-phenylpropionic acid. Alternatively, pure phenylalanine ammonia-lyase subunits may be released by elution with sodium dodecyl sulphate or urea.

INTRODUCTION

The enzyme L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the first reaction in the biosynthesis from phenylalanine of a wide variety of plant phenylpropanoid natural products, and the enzyme from bean (*Phaseolus vulgaris*) has recently been the subject of much attention at both the protein and molecular genetic levels [1–4]. This work has required a suitable affinity matrix for use in the purification of the enzyme in relation to the development of antibody probes [1, 2], for confirmation of the subunit M_r [2], and for measurement of the levels of active-site-intact subunits in studies on specific inactivation of the enzyme [4]. Previous attempts at the affinity purification of phenylalanine ammonia-lyase [5, 6] have utilized phenylalanine coupled to solid supports through the carboxyl- or amino-groups with limited success, these immobilized ligands functioning more as hydrophobic matrices. L-2-Aminooxy-3-phenylpropionic acid (AOPP) has been shown to be a very potent inhibitor of PAL [7] and its binding characteristics to the bean enzyme have been studied in detail [8]. Attempts to utilize this compound, coupled through the carboxylic group, as an affinity probe have also met with limited success due in part to the apparent instability of the linkage [9]. We now describe a new affinity matrix in which ethyl 2-(*tert*-butoxycarbonylaminooxy)-3-(4'-hydroxyphenyl)propionic acid has been coupled through the phenolic group to epoxy-activated Sepharose and the blocking groups subsequently removed. PAL binds avidly to this matrix and with careful procedures can be purified to homogeneity with respect to enzyme subunits.

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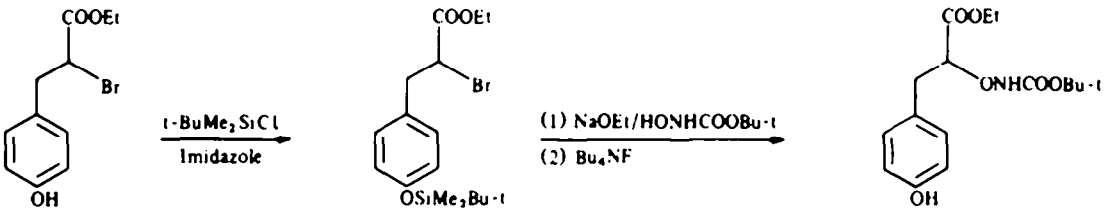
RESULTS AND DISCUSSION

Synthesis of the ligand–Sepharose matrix

Scheme 1 shows the stages involved in the synthesis of ethyl-2-(*tert*-butoxycarbonylaminooxy)-3-(4'-hydroxyphenyl)propionate (3). Protection of the phenolic hydroxyl of 1 was necessary to prevent reaction with the bromine of another molecule of 1 during the substitution with the protected hydroxylamine. Reaction of 3 with epoxy-activated Sepharose was very rapid and most efficient at pH 12 (Fig. 1). 50% of the ligand coupled within 1 hr of mixing followed by a further 12% over the next 17 hr. Although several treatments for rapid removal of the protecting groups using mineral acids were attempted, the most effective at preserving the integrity of the matrix was the prolonged incubation with weak trifluoroacetic acid. The presence of the ligand could be detected on the gel resuspended in 60% sucrose by a characteristic peak of absorbance at 270 nm when compared with unsubstituted ethanolamine-blocked gel (Fig. 1).

Purification of PAL

Enzyme activity was rapidly bound when partially purified (ammonium sulphate and Sephacryl S300) bean PAL preparations [1, 2] were applied to the column. Following extensive washing with 1 M NaCl, which removed all non-specifically bound protein, the enzyme remained tightly bound. Activity could not be eluted using high (pH 11.0) or low (pH 4.0) buffers. Elution with saturated phenylalanine at 37°, which removes AOPP from the active site, when the enzyme is in solution, failed to release the enzyme. However, the presence of active enzyme on the column was, perhaps surprisingly, revealed



1 2 3

Scheme 1. The stages involved in the synthesis of ethyl 2-(*tert*-butoxy-carbonylaminoxy)-3-(4'-hydroxyphenyl)propionate (3). Protection of the phenolic hydroxyl of 1 was necessary to prevent reaction with the bromine of another molecule of 1 during the substitution with the protected hydroxylamine. Reaction of 3 with epoxy-activated Sepharose 6B was carried out in the presence of 0.1 M Na_2CO_3, pH 12.0 containing 10% (v/v) dimethyl formamide at 40° for 18 hr (see Fig. 1).

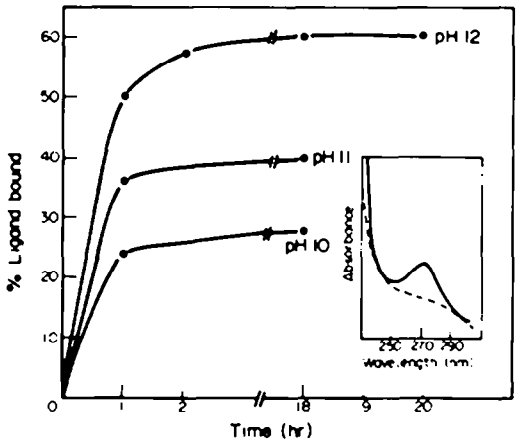


Fig. 1. Effect of pH on the coupling of ethyl-2-(*tert*-butoxycarbonylaminooxy)-3-(4'-hydroxyphenyl) propionate to epoxy-activated Sepharose 6B. 60% binding of ligand was equivalent to reaction of 84% of available epoxy groups. Insert shows the UV spectra of unreacted, ethanolamine-blocked epoxy-Sepharose (- - - -) and ligand-substituted, ethanolamine-blocked Sepharose ( ) observed after suspending the gel beads in 60% sucrose.

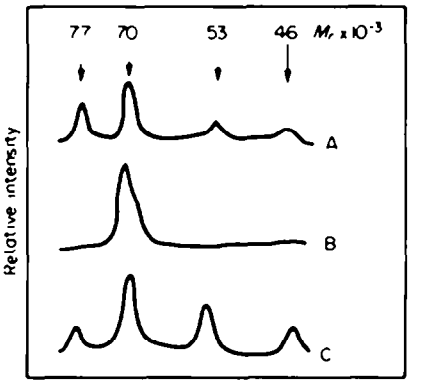


Fig. 2. M_r distribution of PAL subunits released from the affinity matrix by prolonged incubation with 10 mM AOPP (a), boiling in SDS-polyacrylamide gel sample buffer (b) or eluting with 6 M urea (c). Subunits were analysed on 8% polyacrylamide gels in the presence of SDS, and gels scanned by densitometry after staining with Coomassie brilliant blue.

by the formation of cinnamic acid in the eluant, presumably due to the exposure of one of the active sites of the tetrameric protein. However, material was eluted following prolonged incubation (16 hr) with 10 mM AOPP in the presence of 0.4 M NaCl from which activity could be recovered after dialysis against saturated phenylalanine (Table 1). Recovery of activity was low, mainly reflecting the avidity of the binding. Alternatively, the PAL could be removed under denaturing conditions, for example by boiling the gel in SDS-polyacrylamide gel electrophoresis sample buffer or by elution of the column with 6 M urea. This led to high recoveries of enzyme subunits suitable for molecular studies.

Using these rather prolonged or harsh treatments the enzyme subunit released exhibited heterogeneity of M_r following analysis on SDS-polyacrylamide gels (Fig. 2).

Table 1. Affinity purification of phenylalanine ammonia-lyase from cell suspension cultures of *Phaseolus vulgaris**

Stage	Fraction	Total activity (nkat)	Total protein (mg)	Specific activity (μkat kg^{-1})	Purification (-fold)	Recovery (%)
1	Crude extract	28.4	461	61	1	100
2	40–55% $(NH_4)_2SO_4$/Sephacryl S-300	26.5	15.5	1710	28	95
3	AOPP-Sepharose 6B†	3.9	0.6	6500	106	14

*Elicitor-treated cells [1] (200 g) were used as enzyme source.
†72.5% of the activity from stage 2 bound to the affinity column.

The M_r of the newly synthesized PAL subunit is 77000 [1] but partial degradation products of M_r 70000, 53000 and 46000 are readily formed subsequently *in vivo* and can be generated *in vitro* in partially purified preparations by freezing and thawing [2]. The identity of these polypeptides as PAL has been confirmed by peptide mapping and immunological techniques, and enzyme composed of M_r 70000 partially degraded subunits appears to retain significant catalytic activity [2]. All these partially degraded subunits are represented in differing proportions in the enzyme eluted from the column. No polypeptides other than PAL subunit forms could be recovered from the column.

The affinity matrix here described could be of great value in the purification to homogeneity of PAL to be used in the production of antibody probes for molecular studies. The matrix has also been used for the measurement of active-site-intact subunits in studies on the regulation of PAL by its product cinnamic acid [4]. It is likely that the highly specific affinity of this immobilized ligand for PAL results from coupling via the aromatic ring (phenolic group) rather than through the side-chain as with phenylalanyl Sepharoses [5, 6].

EXPERIMENTAL

Ethyl-2-(tert-butoxycarbonylaminooxy)-3-(4'-hydroxyphenyl)-propionate (3). *tert*-Butyl *N*-hydroxycarbamate (0.87 g) was added to NaOEt (from 0.166 NaH) in dry EtOH (30 ml). After 0.5 hr stirring **2** (2.42 g) was added and the mixture stirred at 25° overnight then at 40° for 3 hr, diluted with H_2O, acidified with citric acid and extracted with Et_2O. The extracts were washed with H_2O, dried and concentrated to a yellow-orange oil (2.57 g). This was dissolved in dry THF (25 ml), tetrabutylammonium fluoride (3.06 g) was added, the soln stirred overnight and poured into aq. $NaHCO_3$. The mixture was acidified with citric acid and extracted with Et_2O. The extracts were washed with H_2O and concentrated to an orange oil from which **3** was isolated as a viscous oil (0.54 g) by prep. TLC [silica gel, EtOAC petrol (60–80°), 2:3]. ^{1}H NMR (100 MHz, $CDCl_3$): δ1.20 (3H, *t*), 1.47 (9H, *s*) 3.06 (2H, *m*), 4.16 (2H, *m*), 4.63 (1H, *m*), 6.76 (2H, *m*), 7.10 (2H, *m*), 7.08 (1H, *br s*), 7.88 (1H, *s*); EIMS (probe) 70 eV, *m/z* (rel. int.): 193 (30), 107 (70), 57 (100), 29 (55); CIMS (NH_3, probe) 50 eV, *m/z* (rel. int.): 343 $[M + NH_4]^+$ (5), 326 $[M + H]^+$ (1), 287 $[M + NH_4 - C_4H_8]^+$ (100), 270 $[M + H - C_4H_8]^+$ (11), 226 (50).

Ethyl 2-bromo-3-(4'-tert-butyldimethylsilyloxyphenyl)propionate (2). To a soln of ethyl 2-bromo-3-(4'-hydroxyphenyl)propionate (**1**) (5.26 g) in dry DMF (40 ml) at 0° was added *tert*-butyldimethylsilyl chloride (3.48 g) followed by imidazole (3.28 g). After overnight stirring at 25° H_2O was added followed by citric acid to pH 4.0 and the mixture extracted with Et_2O. The extracts were washed with H_2O, dried and concentrated to a yellow oil (6.0 g) from which **2** (2.43 g) was isolated as a pale yellow oil by vacuum liquid chromatography [10] (silica gel, toluene). IR ν_{max}^{film} cm^{-1}: 1742 (C=O), 1262 (Si–O).

Coupling of ligand to epoxy-Sepharose 6B. 15 mg of protected ligand was coupled to 2.0 ml epoxy-Sepharose 6B in 1.5 ml 0.1 M Na_2CO_3, pH 12.0, containing 10% (v/v) dimethylformamide at 40° for 18 hr. The *tert*-butoxycarbonyl groups, and any ethyl groups remaining after the basic coupling step, were removed by hydrolysis for 3 days at 60° in trifluoroacetic acid, pH 3.0, to yield 2-aminooxy-3-(4'-hydroxyphenyl)propionic acid linked to the spacer via the phenolic group. Unreacted epoxy groups were blocked by treatment with 1 M ethanolamine, pH 10, overnight and the column washed extensively with 0.1 M sodium acetate, pH 4.0, followed by 0.1 M sodium borate, pH 8.0 (both buffers containing 0.5 M NaCl), before use.

Purification of PAL. PAL was partially purified from suspension cultured cells of bean (*Phaseolus vulgaris* cv Canadian Wonder), that had been exposed to a fungal elicitor from *Colletotrichum lindemuthianum*, by $(NH_4)_2SO_4$ precipitation and gel filtration on Sephacryl S300 [1, 2]. This enzyme preparation (10 ml) was cycled through a column (1 × 5 cm) of the immobilized ligand equilibrated with 50 mM Tris HCl, pH 8.0, at 4° for at least 3 hr. The column was washed with 20 ml 50 mM Tris HCl, pH 8.0, followed by 20 ml 50 mM Tris–HCl, pH 8.0, containing 1 M NaCl. Enzyme was released by either (a) incubation of the column with 50 mM Tris HCl, pH 8.0, containing 10 mM AOPP and 0.4 M NaCl for 16 hr before elution or (b) elution with 50 mM Tris–HCl, pH 8.0, containing 6 M urea. Enzyme activity was recovered from fractions eluted with AOPP by dialysis against saturated phenylalanine at 37° [8]. Enzyme activity was assayed spectrophotometrically [1, 2] and protein determined by the method of [11]. Fractions were extensively dialysed against 20 mM Tris HCl buffer, pH 7.0, before analysis by SDS-PAGE [1, 2].

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