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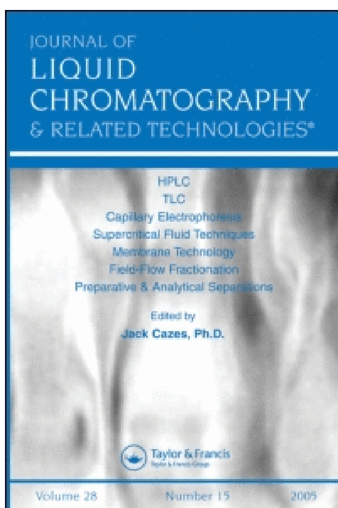

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HPLC ASSAYS FOR THE CHORISMATE UTILIZING ENZYMES CHORISMATE PYRUVATE LYASE AND *PARA*-AMINOBENZOATE SYNTHASE

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HPLC ASSAYS FOR THE CHORISMATE UTILIZING ENZYMES CHORISMATE PYRUVATE LYASE AND *PARA*-AMINOBENZOATE SYNTHASE

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ABSTRACT

High performance liquid chromatography (HPLC) assays are described for measuring the catalytic activities of chorismate-pyruvate lyase and *p*-aminobenzoate synthase (EC 4.1.3.-), enzymes involved in the ubiquinone and folate biosynthetic pathways, respectively. These assays allow rapid and accurate detection of substrate consumption and product formation. Also depletion of chorismate and the presence of other interfering chorismate utilizing enzyme activities can be observed. The detection limit for the products *p*-hydroxybenzoate and *p*-aminobenzoate is 2.5 ng for both. The suitability of the assays is demonstrated with extracts from *Escherichia coli*.

INTRODUCTION

The branching in the shikimate pathway at chorismate plays an important role in the production of a wide variety of aromatic compounds. Among these are vitamin K, ubiquinone, folate, the essential aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan, flavonoids, and pharmaceutically interesting compounds like terpenoid indole alkaloids.[1] Chorismate is the substrate for a quintet of enzymes: chorismate mutase (CM; EC 5.4.99.5), isochorismate

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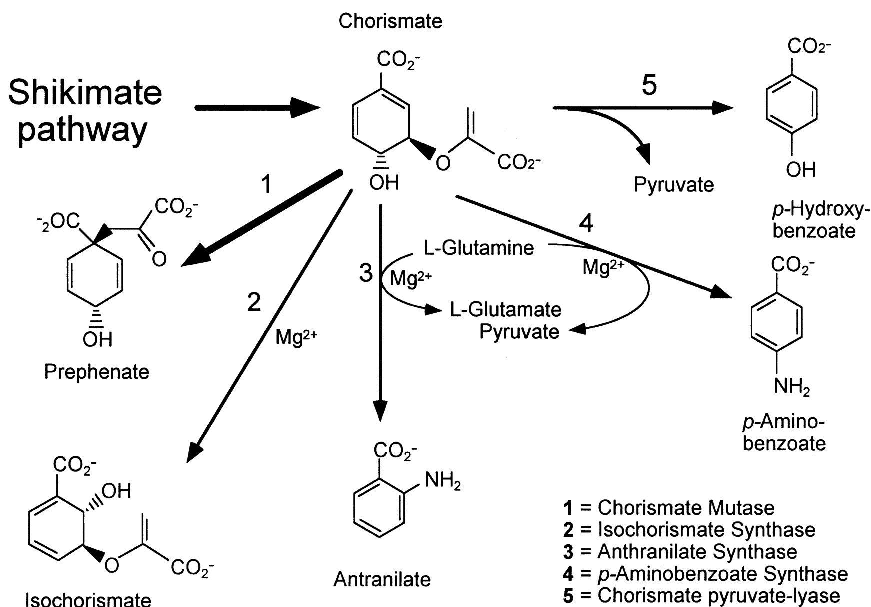

Figure 1. Chorismate as branching point for various biosynthetic pathways.

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synthase (ICS; EC 5.4.99.6), anthranilate synthase (AS; EC 4.1.3.27), *p*-aminobenzoate synthase (PABS; EC 4.1.3.-), and chorismate pyruvate-lyase (CPL) (Figure 1).

CPL catalyzes the conversion of chorismate to *p*-hydroxybenzoate and pyruvate, which is the first step in ubiquinone biosynthesis in microorganisms.[2] The gene encoding CPL has been cloned from *Escherichia coli* and the enzyme has been purified.[3,4] CPL of *E. coli* is a soluble enzyme with a M_r of 18,800.[3] Although the shikimate pathway is unique for microorganisms and plants, it seems that they do not share the same route of *p*-hydroxybenzoate biosynthesis. No CPL-activity has been found in plants and several reports describe that here *p*-hydroxybenzoate is formed via L-phenylalanine.[5,6,7]

PABS is involved in the biosynthesis of folic acid, a building block for tetrahydrofolate, which is a highly versatile carrier of activated one-carbon units and is necessary for the production of the nucleotide precursors involved in purine and pyrimidine synthesis.[8] In *E. coli,* PABS is composed of three sub-units, encoded by the genes *pabA*, *pabB,* and *pabC*.[9] The gene *pabA* encodes a glutamine amidotransferase with a relative molecular weight of 20,000. The

subunit encoded by *pabB* (50 kDa) catalyzes the conversion of the substrate chorismate to 4-amino-4-deoxychorismate (ADC). The *pabC* product, aminodeoxychorismate lyase (= 50 kDa),[10,11] converts ADC to *p*-aminobenzoate and pyruvate.

We are studying the biosynthesis of terpenoid indole alkaloids in the higher plant *Catharanthus roseus*, and are interested in the regulation of the chorismate utilizing enzymes. To complement the available assays for the other chorismate utilizing enzymes CM, AS, and ICS, we have developed HPLC assays to measure the catalytic activity of PABS and CPL.

EXPERIMENTAL

Chemicals

Chemicals used were of analytical grade and were obtained from Merck (Darmstadt, Germany), except for leupeptin and phenylmethylsulfonyl fluoride (PMSF), which were from Boehringer (Mannheim, Germany). Yeast extract and tryptone were from Difco (Detroit, U.S.A); barium-chorismate, polyvinylpolypyrrolidone (PVPP), reactive yellow 3-agarose, and bovine serum albumin (BSA) were from Sigma (St. Louis, U.S.A.). PD-10 columns, Sephacryl 200, Q-Sepharose FF and molecular weight markers were obtained from Pharmacia (Uppsala, Sweden). Blue A dye gel was from Amicon (Beverly, U.S.A.).

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High Performance Liquid Chromatography System

The HPLC system consisted of a 2248 HPLC pump from LKB (Bromma, Sweden), a Rheodyne 7125 injection valve with a 20 µL loop, a 2158 Uvicord SD detector from LKB, equipped with an 8 µL flow cell operating at 280 nm or 254 nm, and a Model RF-530 fluorescence detector from Shimadzu Co. (Kyoto, Japan) with a 12 µL flow cell. The excitation/emission wavelengths were 273 nm/320 nm and 279 nm/335 nm for *p*-hydroxy- and *p*-amino-benzoate, respectively. All analyses were carried out at room temperature on a 4.0 mm (i.d.) x 125 mm LiChrospher RP-select B column (Merck), with a particle size of 5 µm, and at a flow rate of 1 mL min^{-1}. A guard column (Merck) was used in combination with the analytical column.

The mobile phase consisted of 50 mM H_3PO_4 in water/methanol (65/35, v/v), pH 2.5, and was filtered through a 0.45 µm nylon (RC55) filter (Schleicher & Schüell, Dassel, Germany) and degassed under vacuum before use.

A photodiode array detector (type 990, (Waters, Milford, MA, USA)) was used to confirm compound identity.

Preparation of Bacterial Extracts

Escherichia coli strain BN117 was transformed with plasmids pBN168Δ3,[9] pSZD52, pSZD51[3] or pJMG30.[11]

Preparation of the extracts was performed as described by Nichols and Green[3] with some modifications. Plasmid-bearing extracts *E. coli* strains were grown over night in a 500 mL medium containing 1% tryptone, 0.5% yeast extract, and 0.8% NaCl, at 37°C and 250 rpm. Cells were harvested by 10 min centrifugation at 2,500 g at 4°C and washed with 100 mL ice cold 0.85% NaCl. The cell pellet was re-suspended in 20 mL 50 mM Tris-HCl pH 7.5, 5 mM DTT and sonicated on ice (5x30 sec). The sonicated extract was centrifuged for 30 min at 30,000 g at 4°C and the supernatant was used for further experiments. Crude extract was desalted using Sephadex G-25 (PD-10 columns) before enzyme assays.

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Partial Purification of Bacterial Extracts Containing CPL

Partial purification of the extracts was based on the method described by Nichols and Green,[3] and performed at 4°C. The bacterial extract was desalted on Sephadex G-25 (PD-10 columns) equilibrated with 100 mM Tris-HCl, pH 7.5. Five mL of the extract was applied on a Blue A (Amicon) column (1.5 x 10.0 cm) equilibrated in buffer (100 mM Tris-HCl, 5 mM DTT, 5% glycerol) with a flow rate of 0.33 mL min^{-1}. Using a flow rate of 0.4 mL min^{-1}, the column was washed with 15 mL buffer and the protein was eluted with a 30 mL linear gradient from 0 to 1 M KCl, and fractions of 1 mL were collected. Fractions were assayed for CPL activity and purity was analyzed by SDS-PAGE. The purified fractions were used for further analysis.

Partial Purification of Bacterial Extracts Containing PABS

The bacterial extracts were desalted on Sephadex G-25 (PD-10 columns) equilibrated with 100 mM Tris-HCl, pH 7.5. The pabA containing extract was purified by gel filtration on a Sephacryl S-300 column (2.6 x 90.0 cm) equilibrated and eluted with A-buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol) by collecting 10 mL fractions. An extract containing pabB was purified by dye ligand chromatography using a reactive yellow No. 3-agarose column (1.0 X 7.0 cm) equilibrated and eluted with A-

buffer by collecting 1.3 mL fractions. PabB did not bind to the column material and was recovered in the unbound fractions. An extract containing aminodeoxychorismate lyase (pabC) was purified by anion exchange chromatography on a Q-Sepharose Fast Flow column (1.5 x 8.5 cm) equilibrated and washed with A-buffer and eluted with a 150 mL linear gradient from 0 to 1 M NaCl in A-buffer. Five mL fractions were collected. Fractions were tested for enzyme activity and analyzed by SDS-PAGE for purity.

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Chorismate Pyruvate-Lyase Assay

The chorismate pyruvate-lyase assay was based on the assay described by Nichols and Green.[3] The incubation mixture, with a total volume of 250 µL, contained 100 mM Tris-HCl pH 7.5, 1 mM chorismate, 5 mM EDTA, 5 mM DTT and crude desalted extract. The incubation was started by adding chorismate to the incubation mixture. After incubation for 30 min at 37°C the assay was stopped by adding 62.5 µL of 1 M H_3PO_4. Blanks were made by adding H_3PO_4 before incubation. As a substrate blank, 100 mM Tris-HCl pH 7.5 was used instead of desalted crude extract. After stopping the assay, the samples were centrifuged for 5 min at 14,000 g and analysed by HPLC. The detector attenuation of the fluorescence detector was adapted to the amount of enzyme activity.

p-Aminobenzoate Synthase Assay

The *p*-aminobenzoate assay was based on the procedure described by Nichols *et al.,*[9] with several modifications. The incubation mixture, with a total volume of 250 µL, contained 100 mM Tris-HCl pH 7.5, 1 mM chorismate, 10 mM $MgCl_2$, 10 mM L-glutamine, 5 mM DTT, and crude desalted extracts. The incubation was started by adding chorismate to the incubation mixture. After incubation for 30 min at 37°C, the assay was stopped by adding 62.5 µL of 1 M H_3PO_4. Blanks were made by adding H_3PO_4 before incubation. As a substrate blank, 100 mM Tris-HCl pH 7.5 was used instead of desalted crude extracts. After stopping the assay, samples were centrifuged for 5 min at 14,000 g and analysed by HPLC. The detector attenuation of the fluorescence detector was set according to the amount of enzyme activity.

Protein Methods

Protein concentration was determined as described by Peterson[13] using BSA as standard. SDS-PAGE was performed according to Laemmli[14]. Proteins were stained with Coomassie brilliant blue.

RESULTS AND DISCUSSION

Chorismate is the substrate for a quintet of enzymes (Figure 1), which complicates measurement of the individual enzyme activities. Nevertheless, suitable assay systems are available for anthranilate synthase (AS),[15,16] chorismate mutase (CM),[17,18] and isochorismate synthase (ICS).[19,20] Because we are interested in the regulation of the chorismate utilizing enzymes, we needed a sensitive and practical assay for measuring chorismate pyruvate-lyase (CPL) and *p*-aminobenzoate synthase (PABS) activity. Chorismate is mainly converted by chorismate mutase into prephenate, thereby hampering the detection of CPL and PABS.

Due to the extremely low levels of the enzyme in microbial wild type cells[21,22] and problems with stability of the enzyme,[21] we isolated these enzymes from an *Escherichia coli* mutant strain transformed with plasmids over-expressing the genes encoding CPL or PABS.[3,9,11] The purification of the enzymes from crude bacterial extracts was monitored by enzyme assays, SDS-PAGE, and protein quantitation. Blue A dye-ligand chromatography yielded highly purified CPL.

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A similar HPLC system as was used for measuring AS activity,[23] was modified to separate and detect *p*-hydroxybenzoate (4-HBA) and *p*-aminobenzoate (PABA), the products of CPL and PABS activity, respectively. 4-HBA could be detected by UV_{280nm}, although UV_{254nm} was about threefold more sensitive. With a fluorescence detector ($\lambda_{ex.}$273nm, $\lambda_{\varepsilon\mu}$ 320 nm) 4-HBA could specifically be detected with a similar sensitivity as with UV_{280nm} detection.

The t_R of 4-HBA was 3.6 min, and the t_R of chorismate and anthranilate were 2.8 min and 4.4 min, respectively. A standard curve of 4-HBA showed linearity from 0.90 µM (2.5 ng) to 0.4 mM. After addition of H_3PO_4 to stop the enzyme reaction, the product 4-HBA was stable for at least 10 h at room temperature.

The described assay system was tested with crude desalted, and highly purified bacterial extracts. A typical chromatogram is shown in Figure 2. Decomposition of chorismate readily occurs,[1] and as the commercially available chorismate is contaminated with 4-HBA, blanks are necessary for the correction of chemical decomposition. Other possibilities are the purification of chorismate as described by Conneley and Siehl[24] or extraction as mentioned by Siebert *et al.*[25] However, these methods are more cumbersome and the stability of pure chorismate is poor.[1]

We did not observe an inhibitory effect of 4-HBA on CPL activity as described by Siebert *et al.*[25] This could be caused by the higher concentration

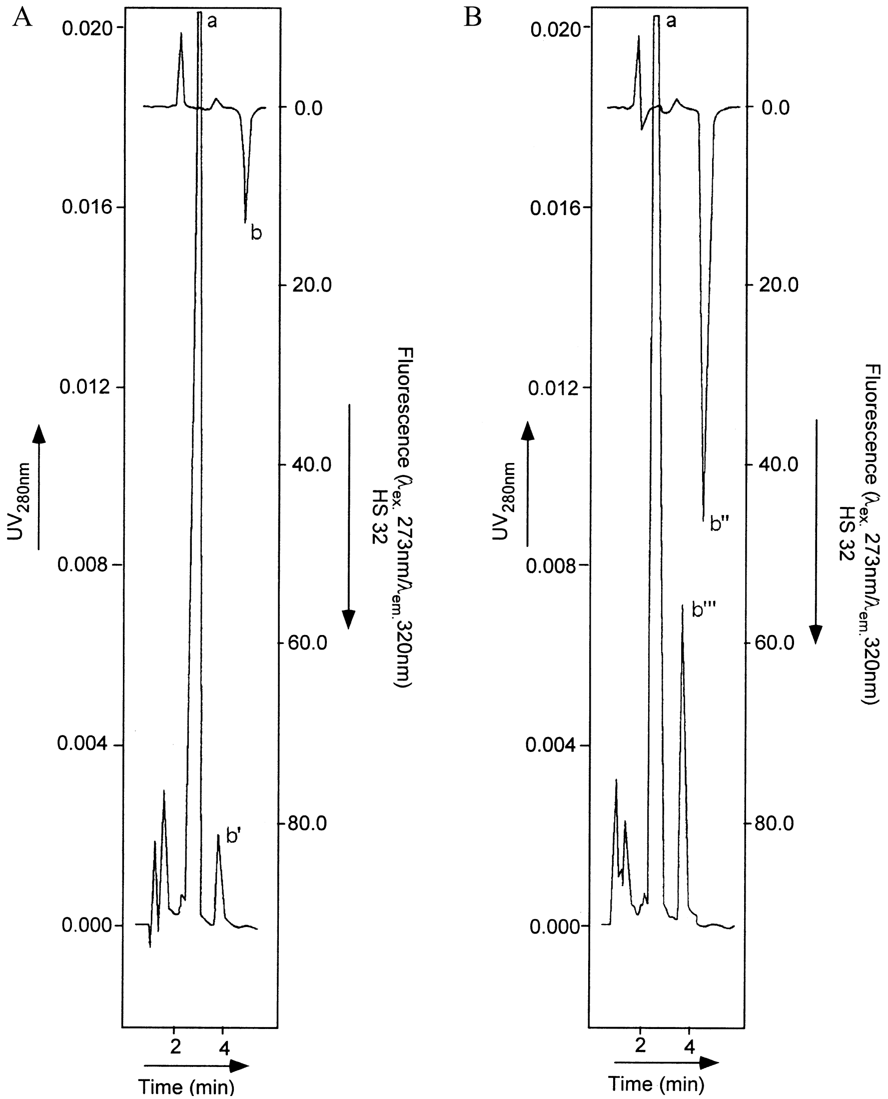

Figure 2. HPLC chromatograms of a chorismate pyruvate-lyase assay using *Escherichia coli* extract (B) and its blank (A). The trace a corresponds to chorismate detected by means of UV 280 nm, b'/b", and b/b" correspond to 4-hydroxybenzoic acid detected by means of UV 280 nm and fluorescence, respectively.

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of chorismate in the assay, as they reported an reverse inhibition upon the addition of excess of chorismate.[25] The assay reaction was not influenced by extra added protein (BSA) concentrations from 0 to 100 μg.

The HPLC assay is accurate and fast as the incubation mixture does not need to be extracted and evaporated before analysis, but can be analyzed immediately. Moreover, the presence of other chorismate utilizing enzyme activities can be detected.

In plants 4-HBA is an important compound, not only for the biosynthesis of naphthoquinones[5,6] and ubiquinones, but 4-HBA is also synthesized upon fungal elicitor treatment in carrot suspension cells.[7] Its biosynthesis is reported to occur (mainly) via phenylpropanoid precursors,[26] in contrast to *E. coli,* where 4-HBA is derived directly via chorismate.[3,27] Nevertheless, we used our assay to study possible CPL activity in plants. Until now, no CPL activity could be detected in *Catharanthus roseus*, *Arabidopsis thaliana,* and *Zea mays,* corresponding to the reports that in *Lithospermum* cell suspension cultures no direct conversion of chorismic acid into 4-HBA occurs.[6,28]

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PABA could not be detected using UV_{280nm} because it had almost the same t_R as chorismate. Nevertheless, by using double detection with UV_{280nm} and a fluorimeter ($\lambda_{ex.}$279nm, $\lambda_{em.}$335nm), it was possible to detect chorismate as well as PABA. A standard curve of PABA was linear from 0.90 μM to at least 0.4 mM, and PABA was stable in a stopped assay mixture at room temperature for over 8 h. The detection limit of PABA in this system was 2.5 ng.

As detailed characterisation of PABS is missing due to the extremely low levels of the enzyme and problems with stability of the enzyme;[21,22] we used a mixture of partially purified bacterial extracts, containing *pabA*, *pabB*, and *pabC* gene products, to test the described system (Figure 3). With a detection limit of 2.5 ng PABA the sensitivity the HPLC system was similar to the PABA assay described by Gil *et al.*[29] but had the additional advantages of requiring less enzyme and no product derivatization. HPLC separation of the incubation mixture enabled monitoring of PABA formation and chorismate disappearance, and, therefore, also detection of substrate depletion. Compared with the HPLC method of Kastel *et al.*,[22] this assay was 100-fold more sensitive and enables detection of other chorismate utilizing enzymes.

The described HPLC system allowed the rapid and accurate analysis of *p*-aminobenzoate synthase (PABS) and chorismate pyruvate-lyase (CPL) activity. The combined UV and fluorimetric detection enabled registration of substrate disappearance and product formation, and the detection of other chorismate uti-

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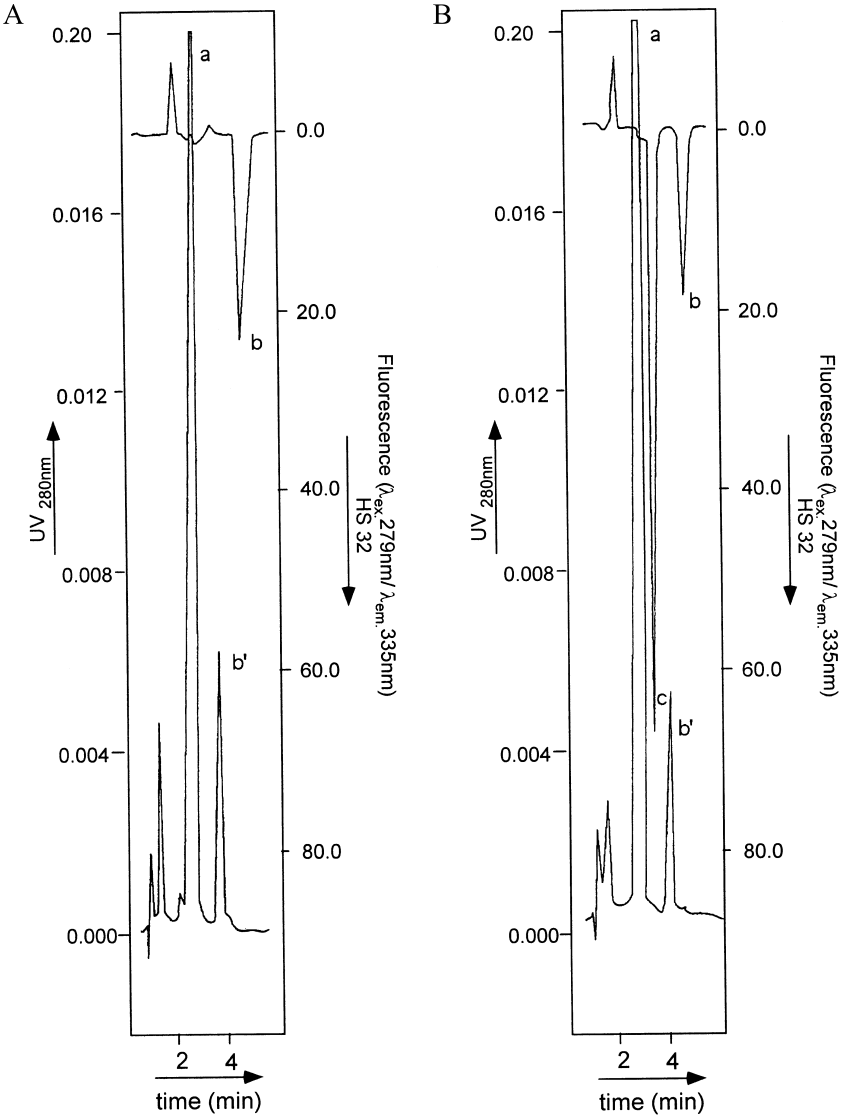

Figure 3. Typical HPLC chromatograms of a *p*-aminobenzoic acid synthase assay using purified *Escherichia coli* extract (B) and its blank (A). The trace a and b' correspond to chorismate and 4-hydroxybenzoic acid detected by means of UV 280 nm respectively; b and c correspond to 4-hydroxybenzoic acid and *p*-aminobenzoic acid detected by means of fluorescence.

lizing enzyme activities. The detection limits for the products *p*-hydroxybenzoate and *p*-aminobenzoate were both 2.5 ng.

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