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Note

High-performance liquid chromatography of shikimate pathway intermediates

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Aromatic amino acids, folic acid, ubiquinone, the benzoic acids and a large number of secondary metabolites are synthesized in plants and micro-organisms by the shikimate pathway[1] (Fig. 1). Microbial utilization of quinic acid occurs by transformation to shikimate pathway intermediates[2] and quinic acid biosynthesis may utilize the pathway, although there is evidence for a separate but as yet undefined route[3].

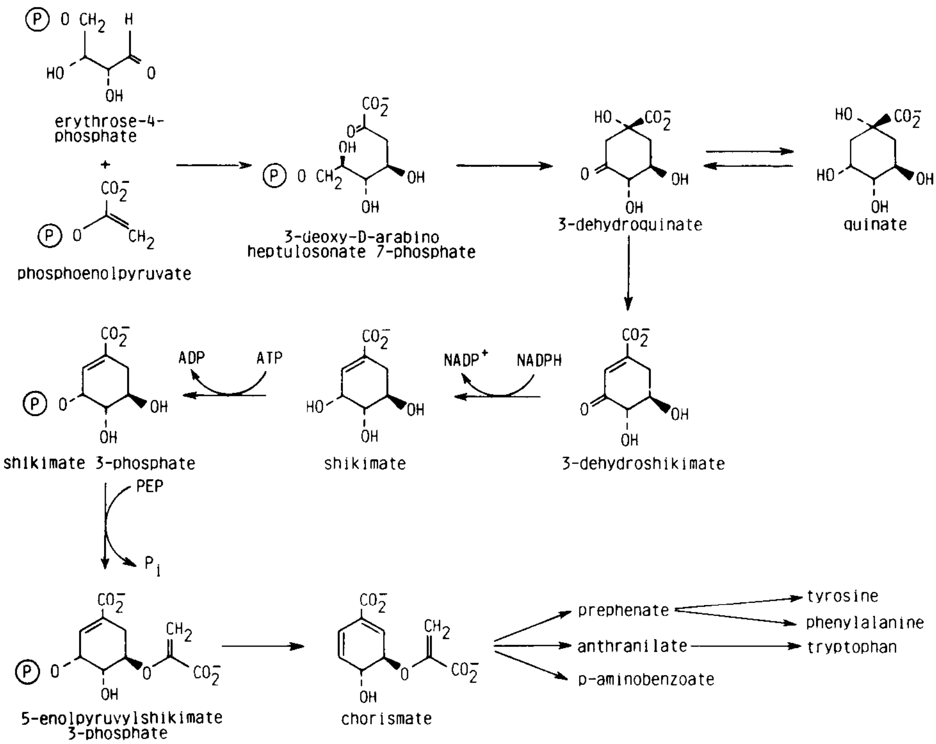

Fig. 1. The shikimate (pre-aromatic) pathway in plants and micro-organisms.

Shikimic and quinic acids accumulate in a variety of higher plant species and have been investigated using paper[4], thin-layer[5] and ion-exchange column[3] chromatography. A thin-layer chromatographic separation of shikimic, 3-dehydroquinic and chorismic acids, shikimate 3-phosphate and 5-enolpyruvylshikimate 3-phosphate has been reported[6]. Shikimic acid has also been quantitatively determined in plant tissues by high-performance liquid chromatography (HPLC)[7]. Here we describe isocratic HPLC methods for separating the alicyclic intermediates of the common shikimate pathway intermediates and quinic and anthranilic acids.

EXPERIMENTAL

Chromatographic techniques

Aminex HPX-87H Organic Acids Analysis columns (300 × 7.8 mm I.D.; 9 μm particle diameter) were purchased from Bio-Rad Labs. (Watford, U.K.), Spherisorb Amino bonded-phase columns (250 × 4.6 mm I.D.; 5 μm particle diameter) from Michrom Technology (Stanmore, U.K.) and μBondapak C_{18} columns (300 × 3.9 mm I.D.; 10 μm particle diameter) from Waters (Northwich, U.K.). The mobile phases were: for Aminex HPX-87H, 1.25–5.0 m*M* sulphuric acid; for Spherisorb Amino, 5–400 m*M* potassium dihydrogen orthophosphate (pH 4.0 with orthophosphoric acid); for μBondapak C_{18}, 10 m*M* ammonium dihydrogen orthophosphate (pH 3.0). A flow-rate of 1 ml/min was provided by a Model 303 pump (Gilson, Villiers-le-Bel, France) and the column eluate was monitored at 215 or 275 nm using an M300 UV detector (Michrom). Chromatography was performed at room temperature.

Shikimate pathway intermediates

Shikimic acid, caffeic acid and chlorogenic acid were purchased from Sigma (Poole, U.K.) and quinic and anthranilic acids from BDH (Poole, U.K.). 3-Dehydroquinic acid, prepared by oxidation of quinic acid[8], and 3-dehydroshikimic acid, prepared by acid-catalyzed dehydration of dehydroquinic acid[8], were the gifts of Dr. S. Chaudhuri (Department of Biochemistry, University of Glasgow, Glasgow, U.K.). Chorismic acid was prepared from *Aerobacter aerogenes* strain ATCC 25306[9] and shikimate 3-phosphate from *A. aerogenes* strain ATCC 25597[10]. 5-Enolpyruvylshikimate 3-phosphate, prepared enzymatically from shikimate 3-phosphate[11,12], was a gift of Dr. A. Lewendon (Department of Biochemistry, University of Glasgow). A mixture of the 3- and 4-phosphates of shikimic acid was prepared by acid-catalyzed isomerization of shikimate 3-phosphate[13]. Injections of the aqueous solutions were made in 25 μl containing 5–150 nmol.

RESULTS AND DISCUSSION

The phosphorylated intermediates shikimate 3-phosphate and 5-enolpyruvylshikimate 3-phosphate were excluded from the strong cation-exchange Aminex HPX-87H resin (Table I). Shikimic, 3-dehydroshikimic, quinic and 3-dehydroquinic acids were retained with capacity factors (k') in the range 0.3–1.8; retention times increased with increasing molarity of sulphuric acid in the mobile phase. Chorismic acid eluted considerably later (k' = 4.8–5.9) than its biosynthetic precursors (Table

TABLE I

EFFECT OF MOBILE PHASE COMPOSITION ON RETENTION OF SHIKIMATE PATHWAY INTERMEDIATES ON AMINEX HPX-87H

Intermediate	*Retention time (min)*		
	1.25 mM H_2SO_4	*2.5 mM H_2SO_4*	*5.0 mM H_2SO_4*
Shikimate 3-phosphate	4.0	4.0	4.0
5-Enolpyruvylshikimate 3-phosphate	4.0	4.0	4.0
3-Dehydroquinic acid	5.3	5.3	5.6
Quinic acid	6.4	6.4	6.5
Shikimic acid	8.1	8.2	8.3
3-Dehydroshikimic acid	9.4	10.2	11.0
Chorismic acid	23.2	26.5	27.6

TABLE II

EFFECT OF MOBILE PHASE COMPOSITION ON RETENTION OF SHIKIMATE PATHWAY INTERMEDIATES ON SPHERISORB AMINO

Intermediate	*Retention time (min)*		
	5 mM KH_2PO_4	*200 mM KH_2PO_4*	*400 mM KH_2PO_4*
Shikimic acid	16.0	4.0	3.4
3-Dehydroquinic acid	20.9	4.1	3.5
3-Dehydroshikimic acid	21.8	4.1	3.6
Quinic acid	23.2	4.3	3.8
Shikimate 3-phosphate	>60	7.9	4.9
Anthranilic acid	22.4	5.8	5.5
Chorismic acid	>60	10.3	6.3
5-Enolpyruvylshikimate 3-phosphate	>60	33.4	11.1

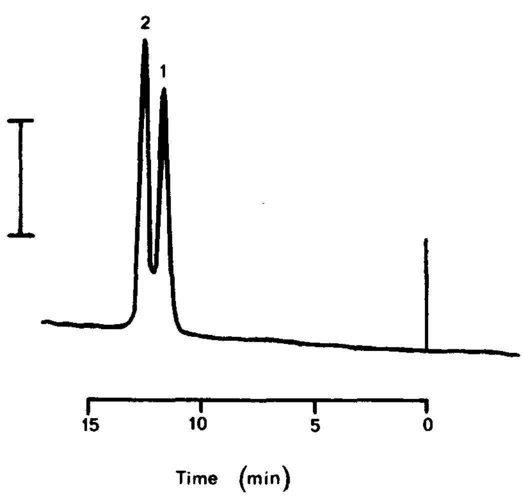

Fig. 2. Separation of the 3- and 4-isomers of shikimate phosphate. Column: Spherisorb Amino; mobile phase: 100 m*M* phosphate (pH 4.0). Vertical bar: 0.01 absorbance unit (215 nm). 1 = Shikimate 4-phosphate; 2 = shikimate 3-phosphate.

I). Chlorogenic acid (5-O-caffeoylquinic acid), the major metabolite of quinic acid[1], could be recovered from the Aminex resin if acetonitrile was added to the mobile phase; in acetonitrile–5 m*M* sulphuric acid (20:80) chlorogenic acid (k' = 3.4) was separated from quinic acid (k' = 0.8) and from caffeic acid (k' = 6.0).

During reversed-phase chromatography at low pH on C_{18} columns shikimic, 3-dehydroshikimic and 3-dehydroquinic acids, shikimate 3-phosphate and 5-enolpyruvylshikimate 3-phosphate were all eluted rapidly ($k' < 0.25$); the less polar cho-

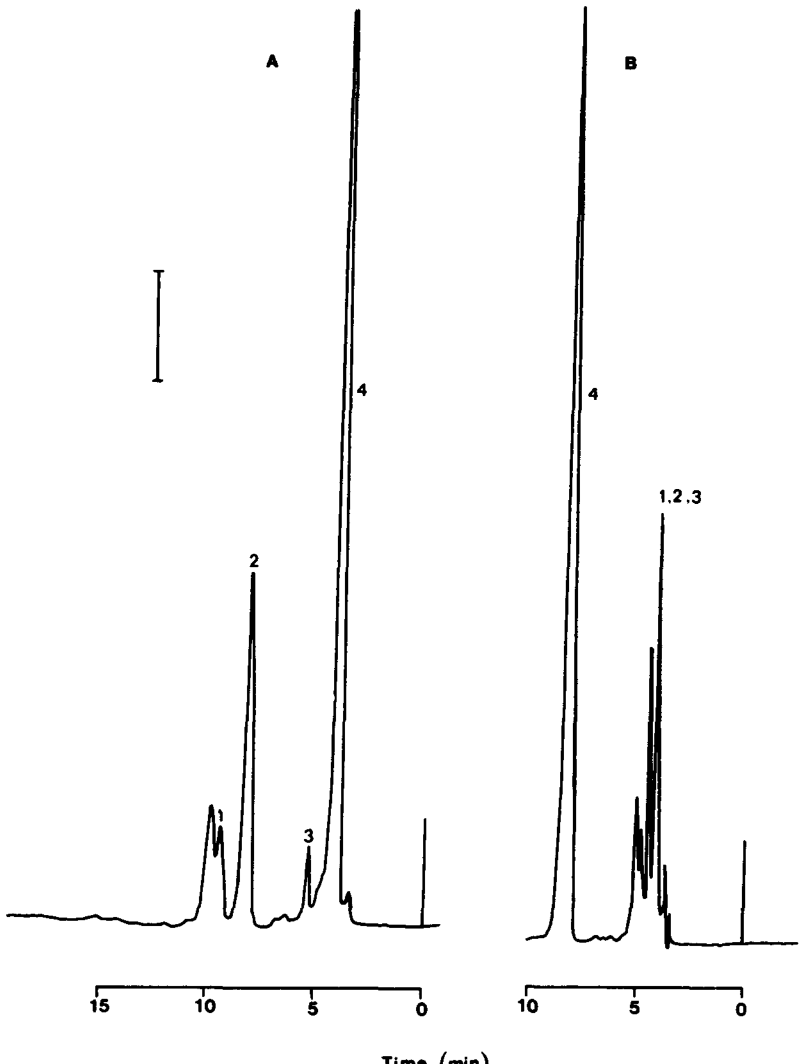

Fig. 3. Chromatograms of culture filtrate of *A. aerogenes* strain ATCC 25597. (A) Aminex HPX-87H, 1.25 m*M* sulphuric acid as mobile phase; (B) Spherisorb Amino, 200 m*M* phosphate (pH 4.0) as mobile phase. A volume of 5 μl culture filtrate was injected. Peaks on chromatograms correspond to retention times of: (1) 3-dehydroshikimic acid; (2) shikimic acid; (3) 3-dehydroquinic acid; (4) shikimate 3-phosphate. Vertical bar: 0.01 absorbance unit (215 nm).

rismic acid ($k' = 11.4$) and anthranilic acid ($k' = 8.6$) could be resolved and separated from the other shikimate pathway intermediates.

With the Spherisorb Amino bonded-phase material used as a weak anion-exchanger (at pH 4.0) shikimate 3-phosphate and 5-enolpyruvylshikimate 3-phosphate were retained and were clearly separated from one another (Table II). The non-phosphorylated intermediates were in general poorly retained at high phosphate concentrations; however, the dicarboxylic acid chorismic acid and the aromatic anthranilic acid were again more strongly retained. Reducing the phosphate concentration in the mobile phase increased the retention times of all the intermediates. At 200 m*M* phosphate chorismic acid was separated from anthranilic acid; at 5 m*M* phosphate shikimic acid was separated from its biosynthetic precursors and from quinic acid (Table II). Shikimate 3-phosphate and its not naturally occurring isomer shikimate 4-phosphate could be distinguished (Fig. 2); the separation of these isomers has not previously been reported.

The complementary use of the Aminex and the amino-modified C_{18} columns gives straightforward separations of all the cyclic intermediates of the shikimate pathway up to and including anthranilic acid. This may be of value in the preparation of isotopically labelled intermediates and in the analysis of endogenous levels and biosynthetic relationships. An example of their use is given in Fig. 3. *A. aerogenes* strain ATCC 25597 when grown on a glucose medium supplemented with the aromatic amino acids accumulates millimolar concentrations of shikimate 3-phosphate in the medium. We have used HPLC analysis for monitoring the accumulation of shikimate derivatives during bacterial growth.

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