Preparative Nondenaturing Gel Electrophoresis of 4S-Limonene Synthase, a Monoterpene Cyclase from Spearmint (*Mentha spicata*)

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Introduction

Several hundred naturally occurring monoterpenes are known, and all are biosynthesized from geranyl pyrophosphate, the ubiquitous C10 intermediate of the isoprenoid pathway.[1] Monoterpene cyclases, or synthases, catalyze the reactions by which geranyl pyrophosphate is cyclized to the various monoterpene carbon skeletons. Monoterpene cyclases, like most enzymes involved in the biosynthesis of natural products, occur in very low abundance in specialized tissues. Consequently, the purification of these enzymes by multiple liquid chromatographic steps generally does not provide high enough levels of protein to permit additional analysis such as N-terminal sequencing, production of antibodies, etc.

Nondenaturing PAGE is a very selective purification technique because fractionation is based on net charge as well as size and shape of the proteins. A preparative nondenaturing gel electrophoresis system which affords superior resolution could reduce the number of steps that are typically required to isolate monoterpene cyclases, and thus improve the overall yield of purified protein. The multiphasic zone electrophoresis system MZE.3328.IV of Jovin has considerable potential for the purification and analysis of many different classes of enzymes because N-terminal modification of proteins is prevented, and it has afforded excellent recovery of pinene cyclase activity.[2,3,4] Other discontinuous nondenaturing PAGE systems that employ higher pH are not useful for this purpose because the recoveries of enzyme activity are often unacceptably low.

Here we report purification of the monoterpene cyclase 4S-limonene synthase by approximately 80-fold using Bio-Rad's Model 491 Prep Cell with the MZE.3328.IV electrophoresis system of Jovin.2 Slab gel electrophoresis was employed to test the utility of the MZE.3328.IV electrophoresis system for purifying 4S-limonene synthase from a crude enzyme preparation. Subsequently, the Model 491 Prep Cell was employed to scale up the purification so that higher levels of protein could be obtained, thus facilitating antibody production and amino acid sequencing.

Methods

LIMONENE SYNTHASE ISOLATION AND ASSAY

4S-Limonene synthase was extracted from spearmint (Mentha spicata) plants as described previously.[5] Initially, young spearmint leaves (40 g) were subjected to an epidermal abrasion technique to remove the glandular secretory cell clusters from the epidermis. Polyvinylpyrrolidone (mw = 40,000) and polystyrene resin (Amberlite® XAD-4) were included in the isolation buffer to adsorb phenolic and lipophilic materials present in the crude enzyme extract. The extraction mixture was filtered through a series of nylon meshes to separate the secretory cell clusters from the glass beads, polymeric adsorbents, and residual plant material. After sonication of the secretory cell clusters to disrupt the cells, the crude enzyme preparation was centrifuged at 27,000g (pellet discarded) and again at 195,000g to provide a supernatant used as the enzyme source.

The soluble supernatant was dialyzed against a buffer containing 10 mM Tris-Cl (pH 6.8), 1 mM KH_2PO_4, 1 mM sodium ascorbate, 0.5 mM dithiothreitol, and 1% (w/v) sorbitol, and concentrated by lyophilization. The lyophilized enzyme was suspended in a minimal volume of sample loading buffer (125 mM Tris-Cl (pH 6.8), 0.5 mM dithiothreitol, and 10% (v/v) glycerol) and dialyzed against sample loading buffer for 2 hours. The enzyme solution was clarified by centrifugation (16,000g for 15 minutes) and bromophenol blue was added to a final concentration of 0.005% (w/v) prior to electrophoresis.

NONDENATURING SLAB GEL ELECTROPHORESIS

A 10% polyacrylamide slab gel (16 cm x 18 cm x 1 mm) employing the multiphasic zone electrophoresis system MZE.3328.IV of Jovin was used as described by Lewinsohn.[4] The slab gels were pre-electrophoresed for 30 minutes at 20 mA in gel buffer containing 0.1 mM thioglycolate to remove residual persulfate. This buffer was discarded and replaced with upper (44 mM TES (2-{[tris-(hydroxymethyl) methyl]amino} ethane sulfonic acid), 113 mM Bis-Tris (pH 7.25)) and lower (63 mM Bis-Tris (pH 5.9)) electrophoresis buffers. Several 50 µl samples were loaded in adjacent

1 cm-wide wells, and electrophoresed overnight at 4 °C at 25 V, followed by an increase to 250 V to complete the run.

To locate enzyme activity, one lane was cut into 7 mm segments and each was soaked for 1 hour at 0 °C in 1 ml of chilled assay buffer that contained 20 mM MOPSO (pH 7.0), 15 mM $MgCl_2$, 0.5 mM dithiothreitol, and 10% (v/v) glycerol. This wash buffer was discarded and the gel slices were crushed with a glass rod. Subsequently, 1 ml fresh assay buffer and 10 µM [1-3H]geranyl pyrophosphate was added to initiate the reaction. After incubation for 2 hours at 30 °C with gentle agitation, the reaction was stopped and the products analyzed as described (Alonso *et al.*, 1992). Limonene synthase activity eluted as a single peak centered in gel slices 3 and 4, which corresponds to an Rf = 0.82.

PREPARATIVE NONDENATURING GEL ELECTROPHORESIS

For preparative nondenaturing gel electrophoresis, an 11 cm high 10% acrylamide (30 acrylamide: 0.8 bis-acrylamide) resolving gel, and a 2 cm high 3.75% acrylamide stacking gel, were cast in the 28 mm ID gel tube of the Model 491 Prep Cell. The resolving and stacking gels were polymerized in 123 mM Bis-Tris-HCl, pH 6.61, using 450 µg/ml ammonium persulfate and 0.50 µl/ml TEMED, and cooled during polymerization as described in the Model 491 Prep Cell Instruction Manual.

The gel was pre-electrophoresed for 30 minutes at 40 mA in gel buffer containing 0.1 mM thioglycolate to remove residual persulfate. This buffer was discarded and replaced with upper (44 mM TES, 113 mM Bis-Tris (pH 7.25)) and lower (63 mM Bis-Tris (pH 5.9)) electrophoresis buffers. The elution buffer reservoir was filled with a buffer containing 113 mM Bis-Tris (pH 7.0), 0.5 mM dithiothreitol, and 10% (v/v) glycerol. The lower electrophoresis buffer was cooled to 15 °C by circulation through 22 m of 1/8" ID Tygon tubing submerged in a salt/ice/H_2O bath (-5 °C) prior to re-entering the gel chamber. The sample, 10 mg total protein in 2.5 ml buffer, was loaded on top of the stacking gel, and then electrophoresed at a constant power of 10 W for 5 hours (initial voltage 250 V, final voltage 390 V).

FRACTION COLLECTION AND ANALYSIS

The elution chamber outlet was pumped at 1 ml/min to a fraction collector and 6 ml fractions were collected. Fraction number one, the first fraction containing bromophenol blue marker dye, was collected after about 3 hours of electrophoresis. Samples were desalted to assay conditions and analyzed for limonene synthase activity.[5] Maximum limonene synthase activity eluted as a single peak in fractions 10–12. See Figure 2. Protein was determined by the method of Bradford using the Bio-Rad Protein Assay Kit with lysozyme as standard.[6]

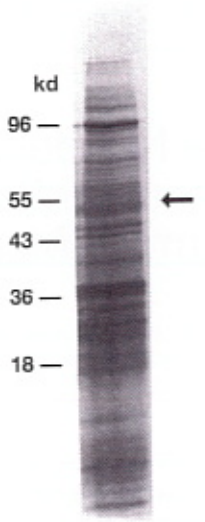

Fig. 1. SDS-PAGE analysis of crude extract prior to preparative native PAGE. The arrow shows the location of enzyme to be purified.

ANALYSIS OF PROTEINS SEPARATED BY PREPARATIVE NONDENATURING GEL ELECTROPHORESIS USING THE MODEL 491 PREP CELL

Aliquots of fractions collected after preparative electrophoresis were dialyzed against water, lyophilized, resuspended in SDS-PAGE sample buffer, and heated on a steam bath for 10 minutes before electrophoresis.[7] SDS-PAGE was carried out according to Laemmli in 10% polyacrylamide vertical slab gels (16 cm x 18 cm x 1 mm).[7] Gels were stained with 0.25% (w/v) Coomassie® Brilliant Blue R-250 in methanol:acetic acid: H_2O (30:10:60) and destained in methanol:acetic acid: H_2O (35:10:55), or silver stained according to Wray.[8] See Figure 3.

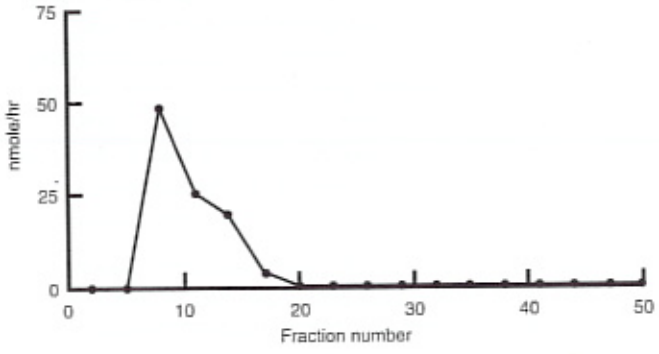

Fig. 2. 4S-limonene synthase activity after preparative nondenaturing electrophoresis using the Model 491 Prep Cell with the MZE.3328.IV system of Jovin. Fractions were assayed for activity as described in the Methods section. Maximum limonene synthase activity eluted as a single peak centered about fractions 10–12.

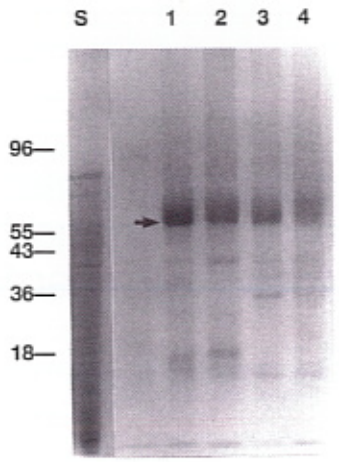

Fig. 3. Analytical SDS-PAGE of 4S-limonene synthase purified by preparative nondenaturing gel electrophoresis using the Model 491 Prep Cell. Lanes 1–4 contain aliquots from pooled prep cell fractions 7–9, 10-12, 13–15, and 16–19, respectively. Lane S is the crude enzyme sample prior to preparative electrophoresis, and the arrow indicates the location of the purified 4S-limonene synthase.

Results

4S-limonene synthase was purified by approximately 80-fold using the Model 491 Prep Cell with the multiphasic zone electrophoresis system, MZE.3328.IV.2 Initially, nondenaturing analytical slab gels employing the MZE.3328.IV electrophoresis system were used to test the suitability of this system for the purification of 4S-limonene synthase from a crude enzyme preparation. The 4S-limonene synthase activity was readily identified by assay because electrophoresis is carried out at neutral pH values which are not detrimental to enzyme activity. Analytical SDS-PAGE of the nondenaturing gel slices containing cyclase activity indicated the outstanding separation of 4S-limonene synthase from most contaminants in the crude enzyme preparation.

Nondenaturing preparative electrophoresis of 4S-limonene synthase samples on the Model 491 Prep Cell was carried out under the conditions deemed optimal for analytical slab gel electrophoresis. A single peak of cyclase activity eluted from the gel in fractions 7–19 (Figure 2) and excellent recovery (>90%) was achieved. These fractions (7–19), containing approximately 400 µg of total protein, were analyzed by Coomassie-stained analytical SDS-PAGE (Figure 3). 4S-Limonene synthase is the major protein band in lanes 2, 3, and 4, as well as the lower band of the doublet (56 and 59 kDa) in lane 1. The resolution of 4S-limonene synthase from its nearest contaminants was satisfactory for N-terminal sequencing and the production of polyclonal antibodies from the isolated protein.

These experiments suggest that the combination of the Model 491 Prep Cell and MZE.3328.IV electrophoresis system is suitable for the purification of monoterpene cyclases from crude preparations with an excellent yield of activity. Although this high resolution technique cannot provide single step purification of all crude enzyme samples, it will obviate several liquid chromatography steps in most fractionation schemes, and it should have great utility in the analysis of proteins that are difficult to purify.

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