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Purification and kinetic properties of elisabethatriene synthase from the coral *Pseudopterogorgia elisabethae*

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Abstract

The Bahamian octocoral *Pseudopterogorgia elisabethae* is the source of pseudopterosins, diterpene glycosides with potent anti-inflammatory activity. The first committed step in pseudopterosin biosynthesis comprises the cyclisation of the universal diterpene precursor geranylgeranyl diphosphate to elisabethatriene. This reaction is catalysed by elisabethatriene synthase, which was purified to homogeneity from a crude coral extract. This represents the first purification to apparent homogeneity of a terpene cyclase from any marine source. The reaction kinetics of elisabethatriene synthase was examined using a steady state approach with ^{3}H-labelled isoprenyldiphosphates varying in carbon chain length (C_{10}, C_{15}, C_{20}). For the reaction of elisabethatriene synthase with its natural substrate geranylgeranyl diphosphate, values of K_m (2.3×10^{-6} M), V_{max} (3.4×10^4 nM elisabethatriene$*s^{-1}$) and the specificity constant ($k_{cat}/K_m=1.8\times10^{-10}$ $M^{-1}*s^{-1}$) were comparable with diterpene cyclases from terrestrial plants. Elisabethatriene synthase also catalysed the conversion of C_{15} and C_{10} isoprenyldiphosphate analogues to monoterpene and sesquiterpene olefins, respectively. Kinetic parameters indicated that substrate specificity and K_m of elisabethatriene synthase decreased with decreasing isoprenoid carbon chain length. Furthermore, GC–MS analysis showed increased product diversity with decreasing isoprenoid carbon chain length.

Keywords: Octocoral; Elisabethatriene synthase; Protein purification; Kinetic properties; Terpene

1. Introduction

The pseudopterosins are a family of diterpene glycosides that was isolated from the marine octocoral *Pseudopterogorgia elisabethae* from several locations in the West Indian region (Look et al., 1986; Fenical, 1987). Pseudopterosins continue to be of commercial interest due to their excellent anti-inflammatory and analgesic activities (Mayer et al., 1998). Although the exact mechanism of action of pseudopterosins is still uncertain (Look et al., 1986; Mayer et al., 1998) in vitro assays suggest that the pharmacological target of pseudopterosins may be phospholipase A_2 (PLA_2), since pseudopterosin A was able to inhibit pancreatic PLA_2 ($IC_{50}=3$ μM) at a concentration comparable to other PLA_2 inhibitors (Petrosaspongiolide M: $IC_{50}=0.6$ μM, manoalide: $IC_{50}=7.5$ μM) (Dal Piaz et al., 2002). Animal model studies have shown that pseudopterosin A was a more potent anti-inflammatory ($ED_{50}=8$ μg/ear) than classical cyclooxygenase inhibiting NSAIDs, such as indomethacin ($ED_{50}=80$ μg/ear) (Mayer et al., 1998).

Pathway elucidation studies in our lab have largely uncovered the biosynthetic origin of the pseudopterosins. We have reported the identity of the cyclisation product elisabethatriene (1) and described its subsequent aromatization to erogorgiaene (2) (see Scheme 1) (Coleman and Kerr, 2000; Kohl and Kerr, 2003). We have also identified the oxidation products of erogorgiaene and have characterised the key intermediates involved in the ring closure of the serrulatane ring system to the amphilectane skeleton of the pseudopterosins (Ferns and Kerr, 2005).

Abbreviations: CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate; Dpm, Disintegrations/minute; DTT, Dithiothreitol; ELS, Elisabethatriene synthase; FPLC, Fast protein liquid chromatography; K_m, Michaelis constant; K-P, Phosphate buffer; k_{cat}, Turnover number; k_{cat}/K_m, Specificity constant; NSAID, Non-steroidal anti-inflammatory drug; SDS–PAGE, Sodium-dodecylsulphate–polyacrylamide gel electrophoresis; V_{max}, Maximal velocity.

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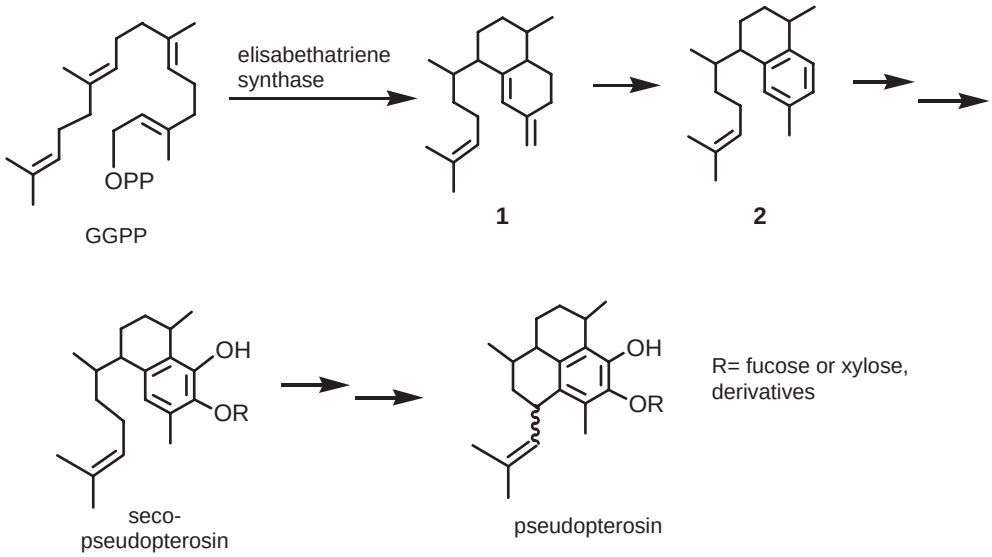

Scheme 1. Key early steps in pseudopterosin biosynthesis.

We have recently described the partial purification and preliminary characterisation of the enzyme elisabethatriene synthase (ELS), responsible for the cyclisation of geranylgeranyl diphosphate (GGPP) to elisabethatriene, from a crude coral extract (Kohl and Kerr, 2004). ELS is a soluble, monomeric protein with a molecular weight of 47 kDa and a pH optimum of 7.1 (*p*I ~5.1), which requires Mg^{2+} as a metal-cofactor for catalysis and as such shares common features with plant diterpene synthases (=cyclases) (Dueber et al., 1973; Frost and West, 1977; Hezari et al., 1995; Saito et al., 1995; Huang et al., 1998; Williams et al., 2000). As observed with other diterpene synthases (Hill et al., 1996; Vogel et al., 1996; Hamano et al., 2002), ELS catalysis seems to be highly specific, with elisabethatriene as the sole reaction product (Coleman and Kerr, 2000; Kohl and Kerr, 2003, 2004). At present, mechanistic and structural studies necessary to understand and control ELS catalysis are limited by the relatively small yields gained from extracts of *P. elisabethae* and by the presence of protein contaminants (Kohl and Kerr, 2004). Although a broad range of mechanistic and structural studies on monoterpene (C_{10}) and sesquiterpene (C_{15}) synthases are available (Bohlmann et al., 1998; Cane et al., 1995; Cane et al., 1996a, 1996b; Davis and Croteau, 2000; Starks 1997; Steele et al., 1998; Rynkiewicz et al., 2001, 2002; Little and Croteau, 2002; Seemann et al., 2002; Whittington et al., 2002; Deligeorgopoulou and Allemann, 2003; Phillips et al., 2003; El-Tamer et al., 2003), there is more limited information on native diterpene cyclases (LaFever et al., 1994; Martin et al., 2004; Starks et al., 1997; Peters et al., 2002; Peters and Croteau, 2002a,b).

Generally these proteins are expressed as soluble monomeric or homodimeric forms ranging from 40 to 60 (Hezari et al., 1995; Saito et al., 1995; Frost and West, 1977; Cane et al., 1996a) and 80 to 120 kDa (Huang et al., 1998; Williams et al., 2000; Hamano et al., 2002; Phillips et al., 2003), respectively. Terpene cyclases bind a flexible polyisoprene substrate (C_{10}, C_{15} or C_{20}) through a multistep catalytic cascade that is initiated by the generation and propagation of a highly reactive carbocation, followed by the nucleophilic attack by a carbon–carbon double bond and often accompanied by rearrangements of subsequent carbocations (Martin et al., 2004; Peters and Croteau, 2002b).

To investigate the kinetic properties of the novel diterpene synthase ELS, a revised purification procedure to a homogeneous preparation was developed. Further, we report the effects of substrate carbon chain length on the reaction dynamics of ELS, when the C_{20} isoprenoid GGPP (natural substrate) is substituted with its C_{15} and C_{10} analogues, farnesyl diphosphate (FPP) and geranyl diphosphate (GPP), respectively. The kinetic data with the natural substrate GGPP, is somewhat different from our previous report (Kohl and Kerr, 2004), which we attribute to the higher purity of the enzyme preparation using our improved isolation strategy. Our new kinetic data is in close agreement with the kinetic properties of taxadiene synthase and other model terpene synthases of plant origin (Frost and West, 1977, Little and Croteau, 2002, Huang et al., 1998). This study represents the first kinetic study of any marine-derived terpene cyclase.

2. Experimental

2.1. Materials

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), except [C_1-^{3}H] labelled GGPP, FPP and GPP which were purchased from American Radiolabelled Chemicals (ARC Inc., St. Louis, MO, USA). For protein purification and analysis, the silver staining kit (Cat. no.: 161-0443), precision plus protein standards (Cat. no: 161-0363) and the CHT 5-I hydroxyapatite FPLC column (Cat. no.: 751-0023) were from Bio-Rad (Hercules, CA, USA). Q-Sepharose, Phenyl-Sepharose Cl-B (fast flow) chromatography matrices and the Mono-Q 5/5 HR (Cat. no.: 17-0546-01) FPLC column were purchased from Amersham (Piscataway, NJ, USA).