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PURIFICATION OF 2,3-OXIDOSQUALENE:CYCLOARTENOL CYCLASE FROM PEA SEEDLINGS

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2,3-Oxidosqualene:cycloartenol cyclase (E.C.5.4.99.8) is a membrane bound enzyme prevalent in the plant kingdom.It acts as a key enzyme in phytosterol biosynthesis. Cycloartenol cyclase was purified from pea seedlings in six steps as a soluble and homogeneous enzyme. The purified enzyme showed a single band in sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a molecular weight of 55 K Da and had a Km value of 25 μM. Cycloartenol cyclase requires Triton X-100 or deoxycholate for its highest activity.

KEYWORDS —— cycloartenol; cycloartenol cyclase; 2,3-oxidosqualene; Pisum sativum; phytosterol

2,3-Oxidosqualene is a versatile intermediate in steroid and triterpenoid biosynthesis in plants. In phytosterol biosynthesis 2,3-oxidosqualene undergoes cyclization reaction to form cycloartenol which corresponds to lanosterol in higher animals and yeast.[1] Enzymes responsible for the cyclization of 2,3-oxidosqualene have been extensively studied by many workers. However, their studies were carried out at the microsomal level and reports dealing with soluble enzyme preparations are scanty. Yamamoto et al. reported the properties of solubilized pig liver 2,3-oxidosqualene:lanosterol cyclase[2] and some properties of solubilized yeast cyclase was reported by Shechter et al.[3] 2,3-Oxidosqualene: cycloartenol cyclase was partially purified from a phytoflagellate, Ochromonas malhamensis, and its properties were discussed in comparison with those of lanosterol cyclase in pig liver and yeast.[4] Difficulties in obtaining solubilized enzyme in an active state prevented purification studies of the membrane-bound sterol and triterpenoid cyclases. We wish to report here the first complete purification of 2,3-oxidosqualene:cycloartenol cyclase from pea (Pisum sativum) seedlings.

Pea seedlings have been used as a source of 2,3-oxidosqualene:β-amyrin cyclase.[5] β-Amyrin production was very active in pea seeds during development and just after the germination, whereas phytosterol synthesis became active several days after germination.[6] Baisted has suggested that the change in the relative activities of cycloartenol cyclase and β-amyrin cyclase may be caused by the change of electrolyte concentration,[6] whereas Goad has pointed out that a single

protein may be responsible for the two cyclase activities, which are switched by the modification of the same protein.[7] The dramatic change in the activities of two cyclases makes it possible to obtain a desired enzyme source for the purification of the two cyclases. In order to determine the cyclase activities in pea seedlings, assay conditions for enzyme reactions were investigated with a labelled substrate, (RS)-[3-^{3}H]-2,3-oxidosqualene, which was synthesized by a known method.[8] The assay mixture in 0.1 M potassium phosphate buffer (pH 7.4, 1 ml) contained (RS)-[3-^{3}H]-2,3-oxidosqualene (50 n mol, 3 x 10^6 dpm), Triton X-100 (0.1%) and enzyme. The assay mixture was incubated for 1 h at 30°C. The reaction products were separated by preparative TLC and then applied on HPLC after the addition of non-labeled carriers (Fig. 1).

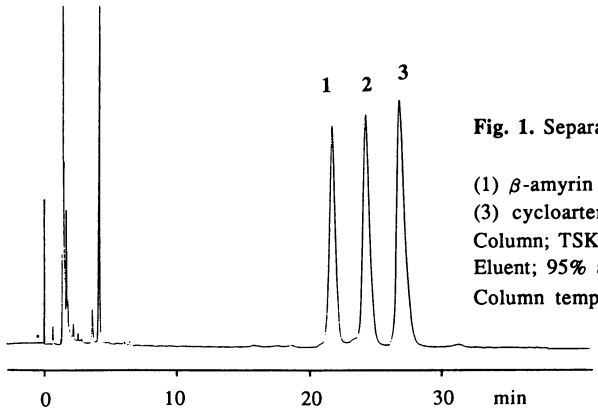

Fig. 1. Separation of Cyclization Products by HPLC

(1) β-amyrin (Rt=21.6 min), (2) α-amyrin (Rt=24.2 min), (3) cycloartenol (Rt=26.7 min).
Column; TSKgel ODS-120T 4.6 mm x 25 cm
Eluent; 95% aq. CH_3CN: Flow rate; 1.5 ml/min
Column temperature; 40 C: Detection; UV 205 nm

The corresponding peaks of β-amyrin, α-amyrin and cycloartenol were collected separately and submitted to radioactivity counting. The activity of α-amyrin cyclase was very low compared to the other cyclases. Triton X-100 could be substituted by deoxycholate (0.1-0.2%), but the enzyme activity was very low in the absence of the detergents. The time course changes of the activities of β-amyrin and the cycloartenol cyclases appear in Fig. 2. It is surprising that the activity of β-amyrin cyclase was found in mature and dried pea seeds, i.e. at the 0 time in Fig. 2. It became a maximum on the 3rd day after germination. The activity of cycloartenol cyclase reached its maximum on the 4th day after the germination, then it fell to 1/3 of the maximum and maintained the same level. The pea seedlings of the 8th day after germination were used as the enzyme source of cycloartenol cyclase.

The cotyledons and hypocotils of etiolated pea seedlings (250 g) were collected on the 8th day after the germination and stored at -80°C. They were further frozen with liquid nitrogen and homogenized with a Waring blender in 0.1 M phosphate buffer containing sucrose (0.45 M), glutathione (10 mM), $MgCl_2$ (10 mM) and Polyclar AT (3% W/V). Ca. 90% of the total radioactivity of the reaction products was found in cycloartenol. A microsomal preparation obtained from the homogenate was treated with 1% Triton X-100 for 60 min to give a solubilized enzyme. In order to prevent aggregation and to stabilize the enzyme activity, phosphate buffer containing Triton X-100 (0.2%), glycerol (10%), DTT (1 mM) and EDTA (1 mM) was

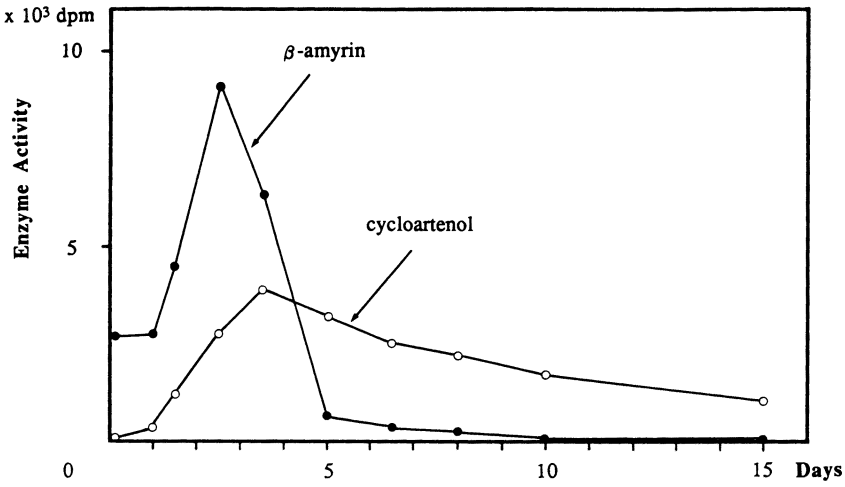

Fig. 2. Time Course Changes of 2,3-Oxidosqualene Cyclases in Pea Seedlings after Germination

mainly used in the following purification steps. In some steps EDTA was omitted from the buffer solution. The solubilized enzyme obtained by the treatment was further purified with a hydroxyl apatite column. The enzyme adsorbed on the column was eluted with 50 mM phosphate buffer. It was subjected to isoelectric focusing in a 1% LKB Ampholine carrier ampholyte solution which was prepared by mixing pH 4-6 and pH 3.5-10 ampholytes in a ratio of 4:1. The enzyme appeared in the fractions of pH 5.1. These two steps were effective for purification and each step gained 10-fold enrichment in specific activities. The active fractions obtained from isoelectric focusing were subjected to a DEAE-cellulose column and eluted gradiently with increasing concentrations of phosphate buffer (10 mM - 300 mM). Active fractions were further purified with HPLC by using a tandemly connected column of G3000SW and G4000SW (TOSOH). The results of the purification are summarized in Table I.

Table I. Purification of 2,3-Oxidosqualene:Cycloartenol Cyclase from Pea Seedlings

Fraction	Total volume (ml)	Total protein (mg)	Total activity (p kat)	Specific activity (p kat/mg)	Purification (folds)	Yield (%)
Post-mitochondria	290	928	330	0.36	1	100
Micorsome	51	237	159	0.67	2	48
Solubilized enzyme	50	195	140	0.72	2	42
Hydroxyl apatite	15	12.5	129	10.2	29	39
Isoelectric focusing	8.3	1.56	135	86.7	244	41
DEAE-cellulose	16	0.47	56	118	331	17
HPLC G3000SW+G4000SW	8.4	0.20	33	167	471	10

The enzyme was finally purified up to 471-fold with 10% yield and gave a single band in sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) (Fig. 3). The molecular weight estimated from SDS-PAGE was 55 K Da, while that from the elution time of HPLC was ca. 100 K, indicating that the cyclase forms a dimer in a

phosphate buffer containing 0.1% Triton X-100. The Km value estimated from double reciplocal plots was 25 μM.

Pea seedlings are an excellent source of the 2,3-oxidosqualene:cycloartenol cyclase, which is useful for investigating the structure-activity relationships of 2,3-oxidosqualene analogues and selective inhibitors of steroid biosynthesis. To shed light on the dramatic switch in triterpenoid and steroid biosynthesis during germination, we have also been engaged in the purification of the β-amyrin cyclase using the same assay system. The results will be published in a forthcoming paper.

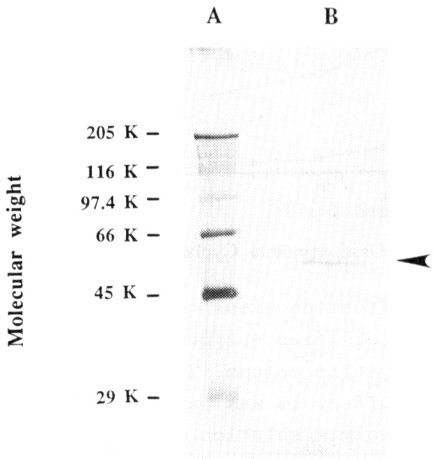

Fig. 3. SDS-PAGE (7.5% T) of Purified 2,3-Oxidosqualene: Cycloartenol Cyclase
(A) Molecular weight marker. (B) Purified enzyme.

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