Purification and Properties of Squalene-2,3-epoxide Cyclases from Pea Seedlings

Ikuro ABE, Ushio SANKAWA, and Yutaka EBIZUKA*

Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan *Received December 26, 1991*

Dramatic changes in the activities of squalene-2,3-epoxide: cycloartenol cyclase and β-amyrin cyclase were observed in germinating pea seeds. By taking advantage of this phenomenon, the two cyclases were purified from pea seedlings. The cyclases were purified to homogeneity by solubilization with Triton X-100, chromatography on hydroxylapatite and diethylaminoethyl (DEAE)-cellulose, isoelectric focusing and gel filtration. Cycloartenol cyclase was purified 471-fold to a specific activity of 167 pkat/mg protein, while β-amyrin cyclase was purified 4290-fold to a specific activity of 28 pkat/mg protein. They each showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis with a molecular mass of 55 and 35 kilodaltons (kDa), respectively. The apparent K_m values for (3*S*)-squalene-2,3-epoxide were estimated to be 25 and 50 μM, respectively. The cyclases required Triton X-100 or deoxycholate for their highest activity and each showed a broad pH optimum within the range of pH 6.5—7.5. Inhibition by *p*-chloromercuribenzene sulfonic acid and *N*-ethylmaleimide suggested involvement of an SH group at the active site of each enzyme.

Keywords *Pisum sativum*; squalene-2,3-epoxide; cyclase; cycloartenol; α-amyrin; β-amyrin; phytosterol; triterpene; pea seedling

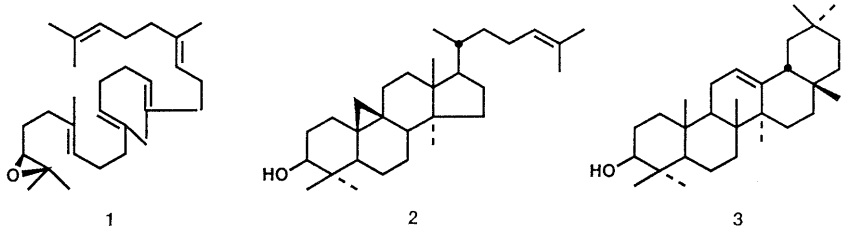
In higher plants, squalene-2,3-epoxide (**1**) undergoes enzyme-catalyzed cyclization into cycloartenol (**2**), which is the initial cyclic phytosterol precursor corresponding to lanosterol in animals. Various tetracyclic and pentacyclic triterpenenoids are widely distributed in higher plants and among them, α- and β-amyrin (**3**) are also cyclization products of squalene-2,3-epoxide. Since the "biogenetic isoprene rule" was proposed by Ruzicka and co-workers,[1] many investigations have been performed on the mechanistic and evolutionary aspects of squalene-2,3-epoxide cyclization reactions.[2,3] Membrane-bound sterol and triterpenoid cyclases have been found in many organisms, but attempts to purify the cyclases have met with difficulties in obtaining a solubilized enzyme in an active form.[4-6] The cyclases has remained incompletely characterized until our reports on the first full purification of squalene-2,3-epoxide: cycloartenol cyclase and β-amyrin cyclase, both from cell suspension cultures of *Rabdosia japonica*,[7] and lanosterol cyclase from rat liver.[8a] Recently partial[8b] and complete[8c] purifications of lanosterol cyclase from yeast have been achieved. In preliminary communications, we reported the complete purification of squalene-2,3-epoxide: cycloartenol cyclase and β-amyrin cyclase from pea (*Pisum sativum*) seedlings[9,10] and in this paper we also describe some properties of the cyclases.

Pea seedlings have been used as a source of squalene-2,3-epoxide: β-amyrin cyclase.[11] It has been reported that β-amyrin production is very active in pea seeds during development and just after the germination, whereas phytosterol synthesis become active several days after germination.[12] Concerning the dramatic switch in triterpenoid and steroid biosynthesis during germination, it has been suggested that the change in the relative activities of cycloartenol and β-amyrin cyclase may be caused by the change of electrolyte concentration,[12] while it has also been pointed out that a single protein might be responsible for the two cyclase activities, which would be switched by a modification of the same protein.[13] In order to shed light on this problem, purification and characterization of both cyclases are necessary.

Results

Time Course of Squalene-2,3-epoxide Cyclase Activities in Germinating Pea Seeds Squalene-2,3-epoxide cyclase activities of germinating pea seeds were determined under our standard assay conditions, *i.e.* in the presence of 0.1% (w/v) Triton X-100 in the assay mixture, which was favorable for the detection of cycloartenol cyclase activity. As described below, β-amyrin cyclase was more active in the presence of 0.1% deoxycholate instead of Triton X-100. As expected, the activity of β-amyrin cyclase was dominant just after the germination, with its maximum on the 2nd day after germination, while that of cycloartenol cyclase became maximum on the 4th day, and after the 5th day the major cyclization product was found to be cycloartenol (Fig. 1). The activity of α-amyrin cyclase was very low compared to those of the other cyclases at all periods. Further, it is interesting to note that β-amyrin cyclase activity was also found in mature and dried pea seeds.

Solubilization of the Cyclases The activities of cycloartenol and β-amyrin cyclase obtained in the microsomal fraction were efficiently solubilized by treatment with 1.0% (w/v) Triton X-100, a non-ionic detergent, as in the case



1 2 3

Chart 1. Structures of Compounds **1**—**3**

of squalene-2,3-epoxide cyclases obtained from cell suspension cultures of *R. japonica*.[7] On the other hand, the solubilization of pea seedling β-amyrin cyclase by using deoxycholate, an anionic detergent, has been described by Corey and Oritz de Montellano.[11] The solubilized cyclases were stable in phosphate buffer containing 0.2% (w/v) Triton X-100, 20% (v/v) glycerol, 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA), and could be stored at −80 °C without significant loss of activity for at least 1 week.

Purification of Squalene-2,3-epoxide: Cycloartenol Cyclase For the purification of pea seedling cycloartenol cyclase, the cotyledons and hypocotiles of etiolated pea seedlings were used as the enzyme source, which made it possible to purify the enzyme rather efficiently. Compared with using the whole seedlings, the obtained cell-free extract contained much less contaminant proteins. The specific activity of the post-mitochondrial fraction was about 50 times higher than that of β-amyrin cyclase obtained as described above. Further, in this case about 90% of the total radioactivity of the assay reaction products was found in cycloartenol. The enzyme was purified as a soluble and homogeneous protein using methods similar to those which were successful in the purification of cycloartenol cyclase and β-amyrin cyclase, both from cell suspension cultures of *R. japonica*,[7] and lanosterol cyclase from rat liver.[8a] The solubilized enzyme from the membrane fraction was subjected to chromatography on hydroxylapatite, isoelectric focusing, diethylaminoethyl (DEAE)-cellulose chromatography and finally high performance liquid chromatography (HPLC) gel filtration. The results of the purification are summarized in Table I. As in the case of *R. japonica* cyclases, glycerol density gradient isoelectrofocusing was the most effective method for the complete separation of cycloartenol cyclase (pI = 5.1) from β-amyrin cyclase (pI = 5.6). Pea seedling cycloartenol cyclase remained active at high ionic strength, which enabled separation on a DEAE-cellulose column using increasing concentrations of phosphate buffer (10—300 mM). The cyclase was purified 471-fold to a specific activity of 167 pkat/mg protein, with 10% recovery. It gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The molecular mass estimated from SDS-PAGE was 55 kDa, while that from the elution volume on gel filtration was *ca.* 100 kDa, indicating that the native enzyme behaved as a homo-dimer in a phosphate buffer containing 0.1% (w/v) Triton X-100. *R. japonica* cycloartenol cyclase has also been reported to be a homo-dimer of 54 kDa subunits.[7]

Purification of Squalene-2,3-epoxide: β-Amyrin Cyclase For the purification of β-amyrin cyclase, pea seeds were half immersed in water and allowed to germinate for 2 d, and at this time, the whole seeds were used. Although the cyclase could be purified in a similar manner to that described above, the purification of β-amyrin cyclase was more troublesome because of the large amounts of contaminant proteins, lipids and hydrocarbons existing in the cell-free extract and more purification steps were needed to obtain a homogeneous enzyme. After solubili-

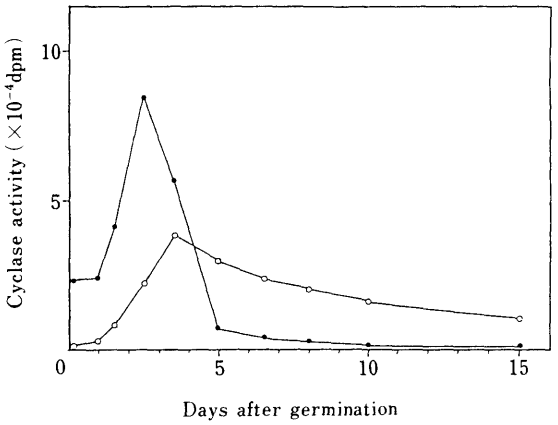


Fig. 1. Time Courses of Squalene-2,3-epoxide: Cycloartenol (○) and β-Amyrin (●) Cyclase Activities in Germinating Pea Seeds

At each developmental stage, 10 seeds were collected and microsomal fraction was prepared from the whole seeds. Total cyclase activity/seed was determined under the standard assay conditions.

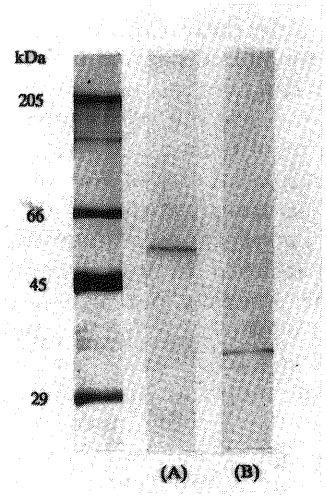


Fig. 2. SDS-PAGE (7.5% T) of Purified Squalene-2,3-epoxide: Cycloartenol (A) and β-Amyrin (B) Cyclase from Pea Seedlings

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

Fraction	Total volume (ml)	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification (fold)	Yield (%)
Post-mitochondria	290	928	330	0.36	1	100
Microsomes	51	237	159	0.67	2	48
Solubilized enzyme	50	195	140	0.72	2	42
Hydroxylapatite	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

TABLE II. Purification of 2,3-Oxidosqualene: β-Amyrin Cyclase from Pea Seedlings

Fraction	Total volume (ml)	Total protein (mg)	Total activity (pkat)	Specific activity (μkat/kg)	Purification (fold)	Yield (%)
Post-mitochondria	555	15984	105.4	0.007	1	100
Microsomes	58	2088	62.3	0.030	4	59
Solubilized enzyme	51	775	61.2	0.079	12	58
Hydroxylapatite (1st)	53	93.6	57.7	0.616	93	55
DEAE-cellulose	5.8	2.26	33.1	14.7	2222	31
Isoelectric focusing (1st)	3.6	1.16	32.4	27.8	4220	31
Hydroxyl apatite (2nd)	6.4	0.32	7.2	22.6	3429	7
Isoelectric focusing (2nd)	4.1	0.10	2.5	24.8	3754	2
HPLC G3000SW+G4000SW	3.0	0.08	2.2	28.3	4290	2

zation with Triton X-100, the cyclase was subjected to chromatography on hydroxylapatite (1st), DEAE-cellulose and isoelectric focusing (1st). These three steps were particularly effective, yeilding 4220-fold enrichment in specific activities. The active fractions were further subjected to hydroxylapatite chromatography (2nd), isoelectric focusing (2nd) and finally HPLC gel filtration. The results are summarized in Table II. The enzyme was purified 4290-fold to a specific activity of 28 pkat/mg protein, with 2% recovery, and showed a single band on SDS-PAGE (Fig. 2). The molecular mass estimated from SDS-PAGE was 35 kDa, and the cyclase was indicated to form a tetramer in the presence of Triton X-100. It has been reported that *R. japonica* β-amyrin cyclase is a tetramer of 28 kDa subunits.[7]

Properties of Squalene-2,3-epoxide Cyclases from Pea Seedlings Squalene-2,3-epoxide cyclase activities have previously been shown to depend not only on detergents but also on the salt concentration in the assay medium.[4,5] The three cyclases (cycloartenol, β-amyrin and α-amyrin) obtained from *R. japonica* required 0.1% (w/v) Triton X-100 and low salt concentration for their optimal activities.[7] In agreement with these reports, both of the pea seedling cyclase activities were significantly enhanced in the presence of 0.1% (w/v) Triton X-100. At this concentration, cycloartenol cyclase was 13 times more active than in the absence of the detergent (Fig. 3A). Triton X-100 could be substituted by deoxycholate. In particular, β-amyrin cyclase activity was further enhanced in the presence of 0.1% (w/v) deoxycholate (Fig. 3B). The effect of ionic strength on the cyclase activities was tested by addition of KCl up to 1.0 M to the assay mixture in the presence of the optimal concentration of the two detergents. Cycloartenol cyclase was active at 1.0 M KCl in the presence of either Triton X-100 or deoxycholate (Fig. 4A, B), in contrast with *R. japonica* cyclases which showed markedly reduced activities under this condition.[7] On the other hand, high ionic strength was inhibitory to β-amyrin cyclase, especially in the presence of deoxycholate (Fig. 4B). The cyclases showed a broad pH optimum within the range of pH 6.5—7.5 under the standard assay conditions. The apparent K_m values for (3*S*)-squalene-2,3-epoxide were determined, assuming that only the (3*S*)-enantiomer was accepted as the substrate,[14] to be 25 μM for cycloartenol cyclase and 50 μM for β-amyrin cyclase. The reported values for *R. japonica* cyclases were 50 and 40 μM respectively,[7] and that for lanosterol cyclase from rat liver was 55 μM.[8a]

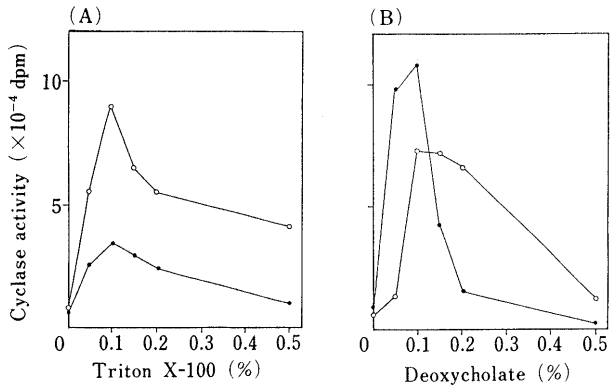


Fig. 3. Effect of Detergents on Squalene-2,3-epoxide: Cycloartenol (○) and β-Amyrin (●) Cyclase Activities in the Assay Medium

Triton X-100 (A) and deoxycholate (B) were added to the assay mixture. Microsomal fraction of pea seedlings 5 d after germination was used (0.8 mg protein/assay).

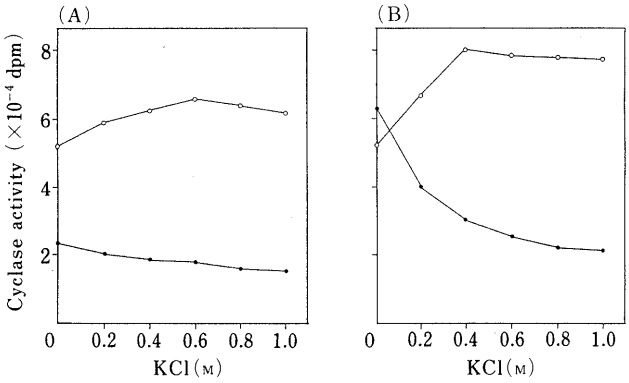


Fig. 4. Effect of Ionic Strength on Squalene-2,3-epoxide: Cycloartenol (○) and β-Amyrin (●) Cyclase Activities in the Assay Medium

KCl up to 1.0 M was added to the assay mixture in the presence of 0.1% (w/v) Triton X-100 (A), or 0.1% (w/v) deoxycholate (B). Microsomal fraction of pea seedlings 5 d after germination was used (0.8 mg protein/assay).

The presence of an essential SH group in the active site of the enzyme has been suggested for several squalene-2,3-epoxide cyclases,[6,15] and indeed the pea seedling cyclases suffered 100% inhibition by SH reagent, either 1 mM *p*-chloromercuribenzenesulfonic acid or 1 mM *N*-ethylmaleimide, in the assay medium, which the addition of 5 mM DTT caused 150% enhancement of both cyclase activities, also suggesting the involvement of an essential SH group. AMO 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methylchloride), a plant growth

retardant which is known to be an inhibitor of several squalene-2,3-epoxide cyclases,[16,17] caused 94% inhibition of cycloartenol cyclase at 1 mM concentration, whereas it caused only 35% inhibition of β-amyrin cyclase in our assay system. The same results have been obtained with *R. japonica* cyclases.[18]

Discussion

It was demonstrated for the first time that cycloartenol cyclase and β-amyrin cyclase from pea seedlings are distinct proteins; they were purified as two different proteins having molecular masses of 55 kDa (dimer) and 35 kDa (tetramer), respectively, which are responsible for the dramatic switch in β-amyrin and cycloartenol production in germinating pea seeds. It has also been reported that the purified cycloartenol cyclase obtained from cell suspension cultures of *R. japonica* had a different molecular mass (54 kDa, dimer) from that of β-amyrin cyclase (28 kDa, tetramer).[7] Under all assay conditions tested, cycloartenol was the sole product of the purified cycloartenol cyclase, and β-amyrin that of the purified β-amyrin cyclase. However, since the structural relationship between the two cyclases is not clear yet, we can not absolutely exclude the possibility that a single protein coded by the same gene may be responsible for the two cyclase activities, which are switched by a modification of the protein by proteolytic action, oxidation of SH groups, change of salt concentration, *etc.*[13] Concerning the effect of salt concentration, cycloartenol cyclase was active at high ionic strength, in the presence of 1.0 M KCl in the assay medium, whereas β-amyrin cyclase showed decreased activity under such a condition, which seems inconsistent with the previous suggestion that β-amyrin production may be a consequence of the increasing electrolyte concentration in the developing pea seed as dehydration occurs during maturation.[12] The activity of β-amyrin cyclase was also detected in the dormant seed, suggesting that the enzyme is one of the stored proteins which are synthesized during development of the seed, and probably, during the initial stages of germination. Although similar changes in triterpenoid and steroid biosynthesis in developing seed have been reported with *Sorghum bicolor*,[19] it is not known whether it is a common phenomenon in nature. It should be noted that, in higher plants, triterpenoids are regarded as species-specifically produced "secondary metabolites", and that their physiological role and regulation of their biosynthesis are not well understood at present.

Pea seedling squalene-2,3-epoxide cyclases were solubilized and purified by using almost the same method which was successful in the purification of cycloartenol cyclase and β-amyrin cyclase from cell suspension cultures of *R. japonica*[7] and lanosterol cyclase from rat liver (75 kDa, dimer).[8a] Prokaryotic squalene: hopene cyclase from *Bacillus acidocaldarius* has also been solubilized and purified to homogeneity using the non-ionic detergent Triton-X-100 (75 kDa, dimer).[20,21] It is expected that other types of cyclase might be purified in a similar manner. All of these cyclases required 0.1% (w/v) Triton X-100 in the assay medium for their highest activities; the detergent is considered to function as a stabilizer of the membrane-bound cyclases and to present the water-insoluble substrate to the active site of the enzyme. Such interaction with the detergent is influenced by the ionic strength of the assay medium. Usually the cyclases are susceptible to high salt concentration, as in the case of

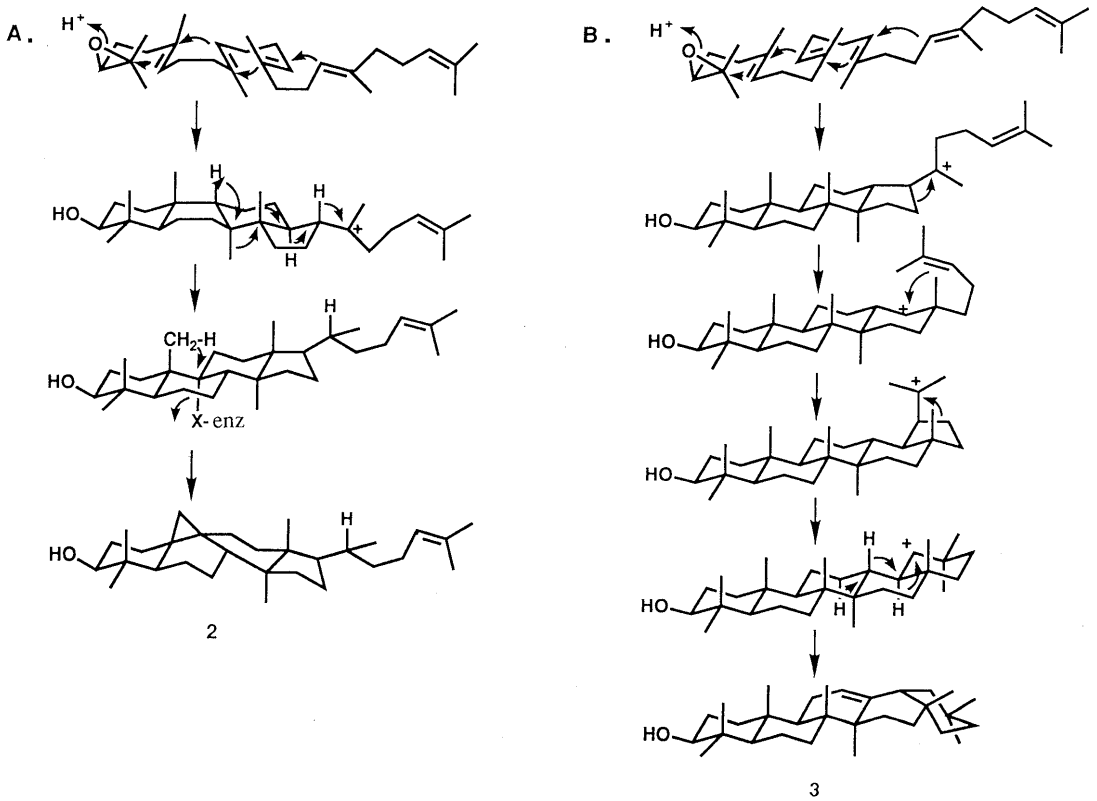


Chart 2. Proposed Mechanism for the Cyclization of Squalene-2,3-epoxide into Cycloartenol (A) and β-Amyrin (B)

pea seedling β-amyrin cyclase, *R. japonica* cyclases,[7] lanosterol cyclase[5,8a] and hopene cyclase.[20] In this respect, pea seedling cycloartenol cyclase seems rather exceptional, since it showed higher activity in the presence of 1.0 M KCl. In our assay system, the apparent K_m values for (3*S*)-squalene-2,3-epoxide estimated for the above-described squalene-2,3-epoxide cyclases were within the range of 25—55 μM,[7,8a] which is higher than that for the purified squalene cyclase, 3 μM.[21] Inhibition by SH reagents *p*-chrolomercuribenzenesulfonic acid and *N*-ethylenemaleimide, and enhancement by the addition of DTT, suggested the presence of an essential SH group in the active site of the enzyme, as has been postulated for several cyclases.[6,15,20] AMO 1618, a plant growth retardant which is known to be an inhibitor of squalene-2,3-epoxide cyclases from rat liver and tobacco seedlings[16,17] and of squalene: hopene cyclase from *B. acidocaldarius*,[20] also strongly inhibited pea seedling cycloartenol cyclase, whereas it showed only partial inhibition toward β-amyrin cyclase. Although it has been reported that AMO 1618 was not inhibitory toward pea seedling β-amyrin cyclase,[22] the same result was obtained with *R. japonica* cyclases in our assay system.[18]

According to the "biogenetic isoprene rule",[1] enzymatic cyclization of squalene-2,3-epoxide into cycloartenol proceeds in its pre–chair–boat–chair conformation first to produce the C-20 protosteryl carbocation. After a 120° rotation of the alkyl side chain around the C-17–C-20 bond in order to achieve the (20*R*) stereochemistry of cycloartenol,[23] a series of hydride and methyl migrations follows. Concerning the final cyclopropane ring formation, involvement of a transient C-9 lanostenyl cation stabilized by a nucleophilic group of the enzyme is postulated before the elimination of the C-19 hydrogen (Chart 2).[2] On the other hand, cyclization into β-amyrin proceeds in all–pre–chair conformation of squalene-2,3-epoxide. The proton initiated cyclization first produces the tetracyclic dammarenyl C-20 cation, and the subsequent rearrangement leads to the pentacyclic oleanyl cation *via* the baccharenyl, and lupenyl cationic intermediates. Finally a series of 1,2-hydride shifts with elimination of the H-12α proton gives the β-amyrin framework. It is a matter of great interest to investigate the relationship between the cyclization mechanism and enzyme structure. Determination of the amino acid sequence of the active site of several cyclases is prerequisite for such study. Further it is also of interest from the view-point of molecular evolution of triterpenoid cyclases, as proposed by Ourisson and co-workers.[3] To date we have purified five squalene-2,3-epoxide cyclases and we are currently examining in more detail and structures of these cyclases and their catalytic properties. Recently, gene cloning and sequencing of squalene: hopene cyclase from *B. acidocaldarius* has been reported.[24] Comparison of the sequences of the cyclases is expected to yield exciting results.

Experimental

Chemicals (*RS*)-[3-^{3}H]Squalene-2,3-epoxide (specific activity, 1.3 GBq/mmol) was chemically synthesized by the known method.[25] Triton X-100 was purchased from Nakarai; deoxycholate, DTT, *p*-chloromercuribenzenesulfonic acid, *N*-ethylmaleimide, AMO 1618 from Wako; Polyclarl AT (insoluble polyvinylpyrrolidone) from Gokyo Sangyo; Sephadex G-25 from Pharmacia; hydroxylapatite (Bio gel HT) from Bio-Rad; Ampholine (pH 3.5—10, pH 4—6) from LKB, and DEAE-cellulose (DE52) from Whatman.

Plant Material Pea (*Pisum sativum*) seeds (Snap pea "Sugar Snap", U.S.A.) were purchased from Sakatanotane Co., Japan. They were kept moist with water and allowed to germinate at 25°C in the dark.

Enzyme Assay The standard assay mixture contained (total volume, 1.0 ml): 0.1 M potassium phosphate (pH 7.4), 0.1% (w/v) Triton X-100, (*RS*)-[3-^{3}H]squalene-2,3-epoxide (50 nmol, 50 GBq) and an appropriate amount of enzyme preparation. Reaction mixtures were incubated anaerobically for 1 h at 30 °C followed by termination of the reaction by addition of 6% (w/v) KOH in EtOH (1 ml). Reaction products were extracted with cyclohexane (2 ml), separated from the substrate by preparative thin layer chromatography (TLC) (Merck, No. 11798, CH_2Cl_2) and then subjected to HPLC (column, TSK-gel ODS 120T, 4.6 × 250 mm; eluent, 95% aqueous CH_3CN; flow rate, 1.5 ml/min; column temperature, 40°C; detection, ultraviolet (UV) 205 nm) after addition of non-labeled carriers. The peaks corresponding to β-amyrin (t_R = 21.6 min), α-amyrin (t_R = 24.2 min) and cycloartenol (t_R = 26.7 min) were collected separately, then the radioactivities were determined in a liquid scintillation counter (Aloka, LSC-670). For determination of K_m values, substrate concentrations of 2—200 μM were employed under the standard assay conditions and the K_m values were estimated from double-reciprocal plots. For inhibition experiments, microsomal fraction of pea seedlings at the 5th day after germination was used (0.8 mg protein/assay).

Preparation of Microsomal Fraction Pea seedlings were frozen with liquid N_2 and homogenized with a Waring blender (30 s × 3 times, with 2 min intervals) in 1 volume of 0.1 M potassium phosphate buffer (pH 7.4) containing 450 mM sucrose, 10 mM glutathione, 10 mM $MgCl_2$, and 3% (w/v) Polyclarl AT. The homogenate was passed through 3 layers of cotton gauze, then centrifuged at 6000 *g* for 15 min. The supernatant was further centrifuged at 105000 *g* for 60 min. The soluble supernatant was removed, and the microsomal pellets were suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM DTT and 1 mM EDTA. All procedures were carried out at 4 °C unless otherwise stated. These preparations could be stored at −80 °C without significant loss of activities for at least 1 month.

Solubilization and Purification of Cycloartenol Cyclase The cotyledons and hypocotiles of etiolated pea seedlings at the 8th day after germination (250 g) were used as the enzyme source. The microsomal fraction prepared as described above was supplemented with 1.0% (w/v) Triton X-100 and gently stirred for 60 min. Then the homogenate was centrifuged at 105000 *g* for 60 min to obtain the solubilized enzyme, which could be stored at −80 °C without significant loss of activity for at least 1 week. The solubilized cyclase was first passed through a Sephadex G-25 column (void volume (v.v.) 90 ml) equilibrated with 5 mM buffer A (potassium phosphate buffer (pH 7.4), containing 0.2% (w/v) Triton X-100, 10% (v/v) glycerol and 1 mM DTT), and then loaded on a hydroxylapatite column (40 × 50 mm) equilibrated with the same buffer. The column was washed with the buffer and eluted with 50 mM buffer A. The activity recovered in the fraction of electroconductivity 0.15—0.50 mmho, was further subjected to isoelectric focusing (glycerol density gradient, column volume 110 ml) at a constant voltage of 800 V for 36 h. The column was filled with a 1% carrier ampholyte solution which was prepared by mixing pH 4—6 and pH 3.5—10 ampholytes in the ratio of 4 : 1, and 0.2% (w/v) Triton X-100, 1 mM DTT and 1 mM DETA were also added. Cycloartenol cyclase appeared in the fraction of pH 5.1, while β-amyrin cyclase appeared in the fraction of pH 5.6. The active fraction was passed through a Sephadex G-25 column (v.v. 27 ml) equilibrated with 5 mM buffer B (potassium phosphate buffer (pH 7.4) containing 0.2% (w/v) Triton X-100, 20% (v/v) glycerol, 1 mM DTT and 1 mM EDTA), then applied to a DEAE-cellulose column equilibrated with the same buffer and eluted with a linear gradient of 5—150 mM phosphate buffer. The pooled active fraction (electroconductivity 0.31—0.36 mmho) was concentrated using a Centricon 10 membrane filter (Amicon) and finally purified by HPLC gel filtration using columns of TSK-gel G3000SW (7.5 × 600 mm) and G4000SW (7.5 × 300 mm) (Tosoh) connected in series. It was eluted with 50 mM buffer C (potassium phosphate buffer (pH 6.8) containing 0.1% (w/v) Triton X-100, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA and 0.2 M KCl) at a flow rate of 0.7 ml/min. The cyclase was eluted in the fraction of t_R = 35—38 min.

Solubilization and Purification of β-Amyrin Cyclase Pea seeds (1 kg) were half immersed in water and allowed to germinate for 2 d, then used as an enzyme source. The solubilized enzyme prepared as described above was passed through a Sephadex G-25 column (v.v. 250 ml) equilibrated with 5 mM buffer A and applied to a hydroxylapatite column (40 × 50 mm)

equilibrated with the same buffer. The column was washed and eluted with 50 mM buffer A. The active fractions (electroconductivity 0.15—0.55 mmho) were then passed through a Sephadex G-25 column (v.v. 90 ml) equilibrated with 10 mM buffer B and loaded on a DEAE-cellulose column (27 × 75 mm) equilibrated with the same buffer. Elution with a linear gradient of 10—300 mM phosphate buffer provided the activity in the fractions of electroconductivity 0.39—0.49 mmho. Then the active fractions were subjected to isoelectric focusing as described above. β-Amyrin cyclase appeared in the fraction of pH 5.6. It was further passed through a Sephadex G-25 column (v.v. 27 ml) equilibrated with 5 mM buffer A, and applied to a second hydroxylapatite column (15 × 30 mm) equilibrated with the same buffer. This time the enzyme was eluted with a linear gradient of 5—150 mM phosphate buffer. The pooled active fractions (electroconductivity 0.20—0.31 mmho) were subjected to a second isoelectric focusing in a 1% ampholyte solution (pH 4—6). Finally, the enzyme was purified by HPLC gel filtration as described above and recovered in the fraction of $t_R = 33$—36 min.

Analytical Methods SDS-PAGE was carried out by the method of Laemmli.[26] Proteins were visualized by silver staining.[27] The molecular mass of the purified enzyme was determined by using standard markers [myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa)]. For the estimation of the molecular mass from the elution volume on HPLC gel filtration as described above, the following standard markers were used: glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140 kDa), enolase (67 kDa), adenyl kinase (32 kDa) and cytochrome c (12.4 kDa). Protein concentration was determined by a modified Lowry method,[28] with bovine serum albumin a the standard.

References

1) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chem. Acta*, **38**, 1890 (1955).
2) T. W. Goodwin, "Biosynthesis of Isoprenoid Compounds," Vol. 1, ed. by J. W. Porter and S. L. Spurgeon, John Wiley and Sons, New York, 1980, pp. 443—480 and references cited therein.
3) G. Ourisson, M. Rohmer, and K. Poralla, *Ann. Rev. Microbiol.*, **41**, 301 (1987).
4) S. Yamamoto, K. Lin, and K. Bloch, *Proc. Natl. Acad. Sci. U.S.A.*, **63**, 110 (1969).
5) I. Schechter, F. W. Sweat, and K. Bloch, *Biochim. Biophys. Acta*, **220**, 463 (1970).
6) G. H. Beastal, H. H. Rees, and T. W. Goodwin, *FEBS Letters*, **18**, 175 (1971).
7) I. Abe, Y. Ebizuka, S. Seo, and U. Sankawa, *FEBS Letters*, **249**, 100 (1988).
8) *a*) M. Kusano, I. Abe, U. Sankawa, and E. Ebizuka, *Chem. Pharm. Bull.*, **39**, 239 (1991); *b*) T. Hoshino, H. J. Williams, Y. Chung, and A. I. Scott, *Tetrahedron*, **47**, 5925 (1991); *c*) E. J. Corey and S. P. T. Matsuda, *J. Am. Chem. Soc.*, **113**, 8172 (1991).
9) I. Abe, Y. Ebizuka, and U. Sankawa, *Chem. Pharm. Bull.*, **36**, 5031 (1988).
10) I. Abe, U. Sankawa, and Y. Ebizuka, *Chem. Pharm. Bull.*, **37**, 536 (1989).
11) E. J. Corey and P. R. Oritz de Montellano, *J. Am. Chem. Soc.*, **89**, 3362 (1967).
12) D. J. Baisted, *Biochem. J.*, **124**, 375 (1971).
13) L. J. Goad, *Biochem. Soc. Trans.*, **1983**, 548.
14) D. H. R. Barton, T. R. Jarman, K. C. Watson, D. A. Widowson, R. B. Boar, and K. Damps, *J. Chem. Soc., Perkin Trans. 1*, **1975**, 1134.
15) H. S. Penefsky and A. Tzagoloff, *Methods Enzymol.*, **22**, 204 (1971).
16) T. J. Douglas and L. G. Paleg, *Phytochemistry*, **17**, 705 (1978).
17) W. D. Nes, T. J. Douglas, J.-T. Lin, E. Heftmann, and L. G. Paleg, *Phytochemistry*, **21**, 575 (1982).
18) I. Abe, Dissertation, The University of Tokyo, Tokyo, Japan (1989).
19) M. A. Palmer and B. N. Bowden, *Phytochemistry*, **16**, 459 (1977).
20) B. Seckler and K. Poralla, *Biochim. Biophys. Acta*, **881**, 356 (1986).
21) D. Ochs, C. H. Tappe, P. Gärtner, R. Kellner, and K. Poralla, *Eur. J. Biochem.*, **194**, 75 (1990).
22) A. Duriatti, P. Bouvier-Nave, P. Benveniste, F. Schuber, L. Delprino, G. Balliano, and L. Cattel, *Biochem. Pharmacol.*, **34**, 2765 (1985).
23) J. W. Cornforth, *Angew. Chem.*, **23**, 977 (1968).
24) D. Ochs, Dissertation, Universität Tübingen, Tübingen, Federal Republic of Germany (1990).
25) M. Rohmer, C. Anding, and G. Ourisson, *Eur. J. Biochem.*, **112**, 541 (1980).
26) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
27) H. Blum, *Electrophoresis*, **8**, 93 (1987).
28) A. Bensadoun and D. Weinstein, *Anal. Biochem.*, **70**, 241 (1976).