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Purification of squalene-2,3-epoxide cyclases from cell suspension cultures of *Rabdosia japonica* Hara

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Microsomes prepared from cell suspension cultures of *Rabdosia japonica* Hara showed activities for cyclizing squalene-2,3-epoxide into cycloartenol, β-amyrin and α-amyrin in the presence of Triton X-100. These activities were efficiently solubilized by treatment with Triton X-100 and separated by chromatography on hydroxy apatite, DEAE-cellulose, isoelectric focusing and gel filtration. The purified cycloartenol cyclase showed a single band on SDS-polyacrylamide gel electrophoresis with M_r = 54000, while β-amyrin cyclase gave a single band with M_r = 28000.

Squalene-2,3-epoxide; Cycloartenol cyclase; Amyrin cyclase, β-; Amyrin cyclase, α-; (*Rabdosia japonica* Hara)

1. INTRODUCTION

Squalene-2,3-epoxide (**1**) is a common biosynthetic intermediate of steroid and triterpenoid biosynthesis in higher plants. It undergoes a cyclization reaction to yield cycloartenol (**2**), the initial cyclic phytosterol precursor which corresponds to lanosterol in animals. Various skeletal types of triterpenes such as β-amyrin (**3a**) and α-amyrin (**4a**) are also cyclized products of squalene-2,3-epoxide. Since Ruzicka and co-workers [1] proposed the so-called 'biogenetic isoprene rule', many investigations have been performed on the mechanistic and evolutionary aspects of these cyclization reactions [2,3]. However, squalene-2,3-epoxide cyclase itself had remained incompletely characterized until our report of the first complete purification of squalene-2,3-epoxide: cycloartenol cyclase and β-amyrin cyclase, both being obtained form pea (*Pisum sativum*) seedlings [4,5]. Here, we describe some properties and the purification of squalene-2,3-epoxide cyclases obtained from cell suspension cultures of *Rabdosia japonica* Hara. Since these cells produce triterpenes of the olean-12-ene (**3b**) and urs-12-ene (**4b**) types, as well as phytosterols [6], we expected to observe the presence of three different enzymes which respectively catalyze the cyclization of squalene-2,3-epoxide (**1**) into cycloartenol (**2**), β-amyrin (olean-12-ene-3β-ol) (**3a**) and α-amyrin (urs-12-ene-3β-ol) (**4a**) (see scheme 1).

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2. MATERIALS AND METHODS

2.1. *Preparation of microsomal fraction*

Cells derived from stems of *R. japonica* were grown on Linsmaier-Skoog medium containing 10^{-6} M 2,4-dichlorophenoxyacetic acid in the dark at 25°C [6]. Cells were harvested during the exponential growth phase (after 3 weeks incubation). Cells (1 kg) were frozen using liquid N_2 and homogenized with a Waring blender in 1 vol. of 0.1 M potassium phosphate buffer (pH 7.4) containing sucrose (450 mM), glutathione (10 mM), $MgCl_2$ (10 mM) and Polyclar AT (3%, w/v). 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, the microsomal pellets obtained being suspended in the same buffer. 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.

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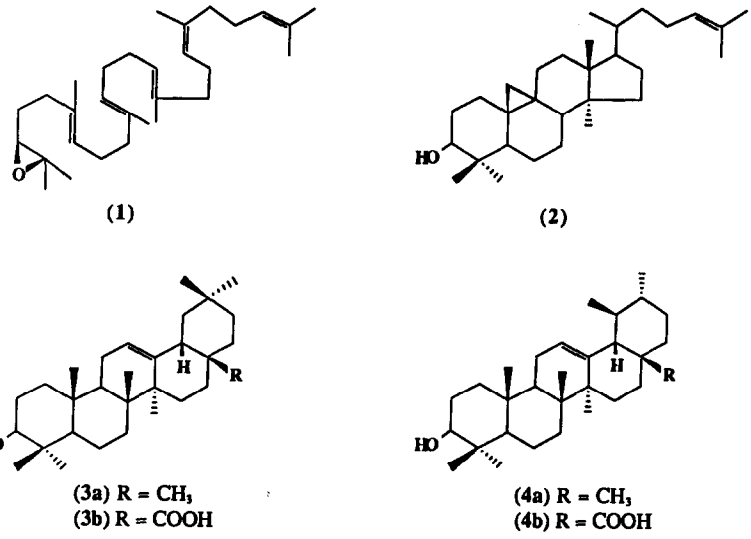

(1) (2)

(3a) R = CH_3
(3b) R = COOH

(4a) R = CH_3
(4b) R = COOH

Scheme 1. Structures of compounds 1–4.

2.2. *Solubilization and purification of enzyme*

2.2.1. Cycloartenol cyclase

A microsomal preparation obtained as described above was treated with 1% (w/v) Triton X-100 for 60 min to solubilize the enzyme. In the following purification procedures, all phosphate buffers contained Triton X-100 (0.2%, w/v), glycerol (20%, v/v), DTT (1 mM) and EDTA (1 mM). Solubilized enzyme was first subjected to chromatography on a hydroxy apatite column (Bio-Rad, Bio-gel HT, 40 × 100 mm), equilibrated with 5 mM phosphate buffer and eluted with 50 mM phosphate buffer. The sample was then subjected to isoelectric focusing (glycerol density gradient, 110 ml column) in a 1% LKB Ampholine carrier ampholyte solution which was prepared by mixing pH 4–6 and pH 3.5–10 ampholytes in the ratio 4:1. Cycloartenol cyclase appeared in the fractions of pH 6.1. It was further subjected to a second stage of chromatography on a hydroxyapatite column (40 × 30 mm) and eluted with a linear gradient of 5–150 mM phosphate buffer. Active fractions were finally purified by rapid gel filtration using columns on TSK-gel G3000SW (7.5 × 600 mm) and G4000SW (7.5 × 300 mm) (TOSOH) connected in series.

2.2.2. β-Amyrin cyclase

This enzyme was purified similarly to cycloartenol cyclase. After solubilization with Triton X-100, the solubilized enzyme was chromatographed on a hydroxyapatite column (Bio-Rad, Bio-gel HT, 52 × 65 mm), equilibrated with 10 mM phosphate buffer. The non-adsorbed fraction was subjected to DEAE-cellulose column (Whatman DE52, 27 × 130 mm) chromatography and eluted with a linear gradient of 10–300 mM phosphate buffer. The active fractions were then subjected to isoelectric focusing (glycerol density gradient, 110 ml column) in a 1% LKB Ampholine carrier ampholyte solution prepared by mixing pH 4–6 and pH 3.5–10 ampholytes in the ratio 4:1. β-Amyrin cyclase appeared in the fractions of pH 5.4. Further purification was achieved on a second hydroxyapatite column (15 × 20 mm), which was eluted with a linear gradient of 5–150 mM phosphate buffer. Active fractions were pooled and then subjected to a second isoelectric focusing stage in a 1% LKB Ampholine carrier ampholyte solution (pH 4–6). Finally, the enzyme was purified by rapid gel filtration using columns of TSK-gel G3000SW (7.5 × 600 mm) and G4000SW (7.5 × 300 mm) (TOSOH) connected in series.

2.3. *Enzyme assay*

As described in [4], the standard assay mixture contained (total volume, 1.0 ml): 0.1 M, potassium phosphate buffer (pH 7.4), Triton X-100 (0.1%, w/v), (*RS*)-[3-^{3}H]squalene-2,3-epoxide (50 nmol, 3 × 10^6 dpm) and enzyme. 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) and separated from the substrate by preparative TLC (Merck, no. 11798, CH_2Cl_2) and then subjected to HPLC (column, TSK-gel ODS120T, 4.6 × 250 mm; eluent, 95% aqueous CH_3CN; flow rate, 1.5 ml/min; column temperature, 40°C; detection, UV 205 nm) after addition of non-labeled carriers. The peaks corresponding to β-amyrin (R_t = 21.6 min), α-amyrin (R_t = 24.2 min) and cycloartenol (R_t = 26.7 min) were collected separately and then radioactivities 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 estimated from double-reciprocal plots.

2.4. *Analytical methods*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [7]. The molecular mass for the purified enzyme was determined 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).

Protein concentration was determined by a modified Lowry method [8], with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

3.1. *Solubilization and purification of cyclases*

Microsomal preparations obtained from cell suspension cultures of *R. japonica* converted squalene-2,3-epoxide into cycloartenol, β-amyrin and α-amyrin in the presence of 0.1% (w/v) Triton X-100 (table 1). The activity of α-amyrin cyclase was very low compared to those of the other cyclases.

The microsomal preparation could be stored at −80°C without significant loss of activities for at least 1 month. Cyclases obtained in the microsomal fraction were efficiently solubilized by treatment with 1% (w/v) Triton X-100 (table 1) and were purified to homogeneity using methods similar to those described in [4,5]. In order to prevent aggregation and to stabilize the solubilized enzymes, phosphate buffer containing Triton X-100 (0.2%, w/v), glycerol (20%, v/v), DTT (1 mM) and EDTA (1 mM) was used throughout all purification procedures. Cycloartenol cyclase was purified as a soluble and homogeneous protein in 4 steps: chromatography on hydroxyapatite (1 st), isoelectric focusing, hydroxyapatite (2nd) and final purification by rapid gel filtration. Isoelectric focusing (glycerol density gradient) was the most effective procedure for purifying the cyclases, resulting in the complete separation of cycloartenol cyclase (pI = 6.1) from β-amyrin cyclase (pI = 5.4). The results obtained on purification are summarized in table 2. Cycloartenol cyclase was purified up to 139-fold with yield of 8% and gave a single band on SDS-PAGE (fig.1). The molecular mass estimated from SDS-PAGE was 54 kDa, whereas the value determined from the elution volume on gel filtration (in the presence of Triton X-100) was approx. 110 kDa, indicating that the cyclase forms a dimer in the presence of Triton X-100. It should be noted that the purified pea cycloartenol cyclase had almost the same molecular mass (55 kDa) as reported in [4].

β-Amyrin cyclase was also purified to homogeneity in a similar manner to the above. After Triton X-100 treatment solubilized enzyme was subjected to chromatography on hydroxyapatite (1st), DEAE-cellulose, isoelectric focusing (1st), hydroxyapatite (2nd), isoelectric focusing (2nd), and finally purified by rapid gel filtration. The results obtained for purification are summarized in table 3. β-Amyrin cyclase was purified to a final value of up to 541-fold with 7% yield and resulted in a single band on SDS-PAGE (fig.1). The molecular mass estimated from SDS-PAGE was 28 kDa. As determined from the elution volume on gel filtration, this enzyme was indicated as forming a tetramer in the presence of Triton X-100.

3.2. *Properties of cyclases*

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 mixture [9,10]. In agreement with these reports, the three cyclases required 0.1% (w/v) Triton X-100, a non-ionic detergent, and low salt concentration for optimal activities.

When Triton X-100 was added to the assay medium, all three cyclase activities were significantly enhanced within a narrow concentration range (0.05–0.2%, w/v) with the optimal effect being at 0.1% (w/v). At this concentration, cyclo-

Table 1

Squalene-2,3-epoxide cyclase activities obtained from cell suspension cultures of *Rabdosia japonica*

Fraction	Total volume (ml)	Total protein (mg)[a]	Total activity (pkat)		
			β-Amyrin	α-Amyrin	Cycloartenol
Post-mitochondrial	11.7	45.4	7.25	0.98	9.95
Microsomal	7.3	13.8	2.67	0.50	8.55
Solubilized	7.1	5.5	3.50	0.45	8.25

[a] Starting from 7.5 g cultured cells

Table 2

Purification of squalene-2,3-epoxido:cycloartenol cyclase

Purification step	Total volume (ml)	Total protein[a] (mg)	Total activity (pkat)	Specific activity (μkat/kg)	Purification (-fold)	Yield %
(1) Microsome	54	405	47.4	0.117	1	100
(2) Solubilized enzyme	53	246	54.9	0.223	2	116
(3) Hydroxyapatite (1st)	20	15.8	12.3	0.778	7	26
(4) Isoelectric focusing	7.7	1.02	16.0	15.7	134	34
(5) Hydroxyapatite (2nd)	12	0.31	5.2	16.7	142	11
(6) HPLC G3000SW + G4000SW	9.2	0.24	3.9	16.3	139	8

[a] Starting from 1 kg cultured cells

artenol cyclase was 9-times more active vs the absence of the detergent. On the other hand, when the anionic detergent deoxycholate was employed, β- and α-amyrin cyclase activities increased to some extent (2–3-fold) at the optimum concentration (0.1%, w/v) while cycloartenol cyclase was not activated, as occurred with β- and α-amyrin cyclase, at any concentration of this detergent. 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 Triton X-100 (0.1% w/v). High ionic strength (> 0.2 M KCl) was inhibitory to all of the cyclase activities. In particular, cycloartenol cyclase activity declined sharply with increasing KCl concentration, indicating that this enzyme is more suspceptible toward high ionic strength compared with two triterpene cyclases. The requirement of Triton X-100 as detergent and low ionic strength for the optimal activity demonstrated for *R. japonica* cyclases resembles that reported for yeast lanosterol cyclase [10] but contrasts with

Table 3

Purification of squalene-2,3-epoxide:β-amyrin cyclase

Purification step	Total volume (ml)	Total protein (mg)[a]	Total activity (pkat)	Specific activity (μkat/kg)	Purification (-fold)	Yield (%)
(1) Microsome	70	644	49.4	0.076	1	100
(2) Solubilized enzyme	64	307	51.7	0.168	2	105
(3) Hydroxyapatite (1st)	180	18.0	23.6	1.31	17	48
(4) DEAE-cellulose	15	0.88	17.0	19.3	254	34
(5) Isoelectric focusing (1st)	9.4	0.43	11.1	25.8	339	22
(6) Hydroxyapatite (2nd)	8.7	0.17	4.8	28.2	372	10
(7) Isoelectric focu sing (2nd)	16	0.12	3.6	30.0	395	7
(8) HPLC G3000SW + G4000SW	15	0.08	3.2	41.0	541	7

[a] Starting from 1 kg cultured cells

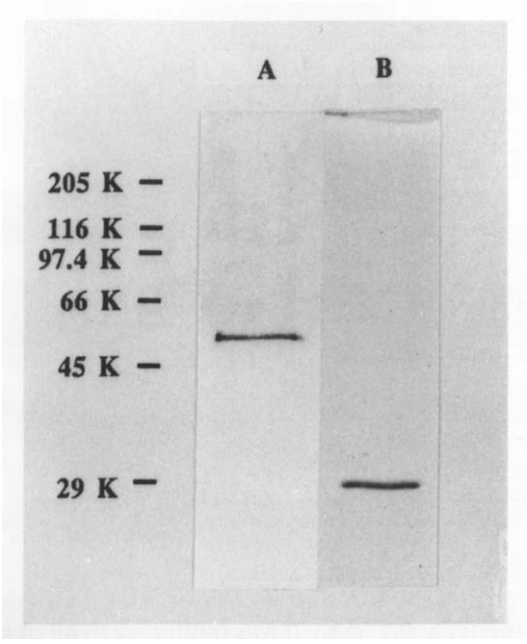

Fig.1. SDS-polyacrylamide gel electrophoresis (7.5%) of purified squalene-2,3-epoxide cyclases. (A) Cycloartenol cyclase. (B) β-Amyrin cyclase.

those for pea seedling cycloartenol and β-amyrin cyclases. The latter two enzymes were active at 1.0 M KCl in the presence of either Triton X-100 or deoxycholate [4,5]. All three types of cyclase from *R. japonica* showed a broad pH optimum within the range 6.5–7.5 under standard assay conditions. The apparent K_m values for (3*S*)-squalene-2,3-epoxide were determined to be 50 μM for cycloartenol cyclase and 40 μM for β-amyrin cyclase, the reported values for pea cyclases being 25 and 50 μM, respectively [4,5].

It has been demonstrated that the purified cycloartenol cyclase has a different molecular mass (54 kDa) from that of β-amyrin cyclase (28 kDa) as in the case of pea squalene-2,3-epoxide cyclases (55 and 35 kDa), respectively) [4,5]. According to the biogenetic isoprene rule, of Ruzicka et al., cyclization of squalene-2,3-epoxide into cycloartenol required that it adopts a chair-boat-chair conformation, while cyclization to yield β-amyrin requires an all-chair conformation. The cyclization reactions are subsequently followed by the corresponding stereospecific backbone rearrangements [1]. It is a matter of great interest to investigate the relationship between these conformational differences in the cyclization process and enzyme structure from the viewpoint of the catalytic mechanism and the molecular evolution of these enzymes. To date we have purified four plant squalene-2,3-epoxide cyclases and are now attempting to eludicate the structural relationship between these enzymes.

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