

***In Vitro* Stabilization and Minimum Active Component of Polygalacturonic Acid Synthase Involved in Pectin Biosynthesis**

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Polygalacturonic acid (PGA) synthase successively transfers galacturonic acid to oligogalacturonic acid by an α 1,4-linkage to synthesize PGA, the backbone of plant pectic homogalacturonan. PGA synthase has not been purified to date due to its instability *in vitro*. In this study, we found stable conditions *in vitro* and separated a minimum active component of the enzymes from pea and azuki bean epicotyls. The PGA synthase lost its activity in 500 mM of sodium chloride or potassium chloride, while it was relatively stable at low salt concentrations. Under low salt concentrations, three peaks bearing PGA synthase activity were separated, by gel filtration and sucrose density gradient centrifugation. The molecular masses of these enzymes solubilized with 3-[(3-cholamidopropyl)dimethyl-ammonio]propane-sulfonic acid were estimated to be 21,000, 5,000, and 590 kDa. The two higher molecular mass PGA synthases converted to smaller PGA synthase proteins when treated with high salt concentrations, while retaining their activity, indicating that PGA synthase has a minimum active component for its activity.

Key words: galacturonic acid; galacturonosyltransferase; homogalacturonan; pectin; polygalacturonic acid

Pectin is one of the major components in the primary cell wall of a plant cell and its chemical structure has been described.^{1,2)} It consists of three structurally distinct types of polysaccharides: homogalacturonan (HG), rhamnogalacturonan I, and rhamnogalacturonan II. All of these polysaccharides are present in pectins, but in different proportions depending on the plant species and tissue.²⁾ HG is a major pectic polysaccharide that accounts for up to 60% of the pectin of dicotyledons and non-graminaceous monocotyledons. It is a homopolymer of α 1,4-linked galacturonic acid (GalUA), partially methyl-esterified at the carboxyl groups.³⁾ HGs from some plant sources are partially *O*-acetylated at *O*-2, *O*-3, or *O*-4.⁴⁻⁶⁾

The molecular mechanism of pectin biosynthesis remains to be elucidated. Biosynthesis of HG requires at least three enzymes, polygalacturonate 4- α -galacturonosyltransferase, homogalacturonan methyltransferase, and homogalacturonan acetyltransferase.^{7,8)} These enzymes have not been purified because they are membrane proteins and are difficult to handle as such.

Genetic approaches to the identification of the genes encoding pectin synthetic enzymes have been only partially successful; mutants affected in pectin composition have been isolated, but almost all have been pleiotropic and not directly affected in pectin biosynthesis. Recently, a mutant that identifies candidate genes involved in HG biosynthesis has been found.⁹⁾ Arabidopsis plants mutated in *QUAI*, encoding a putative membrane-bound glycosyltransferase, were dwarf and showed reduced cell adhesion, lower GalUA contents, and reduction of galacturonosyltransferase and xylan synthase activities.⁹⁻¹¹⁾ A galacturonosyltransferase, GAUT1, involved in polygalacturonic acid synthesis has been functionally identified.¹²⁾ Both *QUAI* and GAUT1 belong to glycosyltransferase family 8, suggesting that quite a few glycosyltransferases in this family are related to pectin biosynthesis,¹²⁾ but structural information at the protein level on glycosyltransferases involved in PGA synthesis is still lacking. Although purification of the enzymes involved in pectin biosynthesis is a difficult task, biochemical analysis of the enzymes is crucial for understanding of the molecular mechanism of pectin biosynthesis.

Among the enzymes involved in HG synthesis, polygalacturonate 4- α -galacturonosyltransferases have been biochemically characterized in membrane suspensions of mung bean,¹³⁾ tomato, turnip,¹⁴⁾ sycamore cambial, and xylem cells,¹⁵⁾ and suspension-cultured tobacco cells.¹⁶⁾ The enzyme has been characterized in a detergent-solubilized form of suspension cultures of tobacco cells¹⁷⁾ and petunia pollen tubes.¹⁸⁾ The activity was also detected in azuki bean¹⁹⁾ and pumpkin²⁰⁾ in a detergent-permeabilized form. The solubilized enzyme

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Abbreviations: BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; DP, degree of polymerization; GalUA, galacturonic acid; HG, homogalacturonan; MES, 2-morpholinoethanesulfonic acid; MOPS, 3-morpholinopropanesulfonic acid; OGA, oligogalacturonic acid; PGA, polygalacturonic acid; PIPES, piperazine-1,4'-bis(2-ethanesulfonic acid)

has been shown to add GalUA from UDP-GalUA onto the nonreducing end of oligogalacturonic acid (OGA).^{18,20,21} It was found to be located in the lumen of the Golgi using sucrose density gradient centrifugation coupled with proteinase treatment of the Golgi-rich membrane fraction.²²

Recently, a new assay method for polygalacturonate 4- α -galacturonosyltransferase was developed using fluorescence-labeled OGAs as acceptor substrates.^{18–20} When the enzyme was incubated with fluorescence-labeled OGA and UDP-GalUA, it transferred more than 10 GalUA units onto the fluorogenic OGA.¹⁸ The glycosyltransferases involved in the biosynthesis of cell wall polysaccharides have been classified into two general categories: glycan synthases and glycosyltransferases.²³ The former includes those enzymes that make up the polysaccharide backbone by successive glycosyl transfer reactions, and the latter includes those enzymes that transfer a specific sugar from a sugar nucleotide to a specific position on an acceptor molecule. Polygalacturonate 4- α -galacturonosyltransferase from petunia pollen tube successively transferred GalUA to synthesize PGA,¹⁸ leading to the classification of this enzyme as a glycan synthase. Therefore, it is appropriate to call this enzyme PGA synthase, a name that has been used in a previous report.¹⁵

Although PGA synthase has been enzymatically identified, no information on its molecular structure is available. In this paper, we offer further enzymatical characterization and structural analysis of PGA synthase. On the basis of the results of this enzymatical characterization and on the hydrodynamic properties of PGA synthase, we propose conditions for stabilizing the enzyme and present evidence suggesting that PGA synthase exists as a high molecular mass protein.

Materials and Methods

Materials. UDP-GalUA was enzymatically prepared as reported elsewhere.²⁴ Ribosomes from *Escherichia coli* MRE600 strain were prepared as described previously.²⁵ Seeds of pea (*Pisum sativum*) and azuki bean (*Vigna angularis*) were purchased from Snow Brand Seed (Okayama, Japan) and Watanabe Seed (Miyagi, Japan) respectively. Epicotyls were grown in moist vermiculite at 25 °C in the dark. Q Sepharose and Sephacryl S-500 were obtained from GE healthcare (Piscataway, NJ), and TSKgel DEAE-5PW (0.75 \times 7.5 cm) from Tosoh (Tokyo).

Preparation of pyridylaminated (PA-)OGA. PA-OGA was prepared by the method of Akita *et al.*¹⁸ with slight modifications. PGA (5 g) suspended in 500 ml distilled water was adjusted to pH 4.2 with NaOH. The PGA in this solution was partially hydrolyzed in an autoclave at 121 °C for 30 min, and then the solution was adjusted to pH 2.0 with hydrochloric acid. The supernatant obtained after centrifugation at 4,000 \times g for 20 min was lyophilized.

The lyophilized OGA (1 g) was dissolved in 50 ml of 20 mM ammonium acetate buffer, pH 4.5. To this solution, 50 ml of a coupling reagent (prepared by mixing 55.2 g of 2-aminopyridine with 20 ml of acetic acid) was added, and the reaction mixture was heated at 90 °C for 60 min. The resulting Schiff base was reduced with 175 ml of a reducing agent (freshly prepared by mixing 150 g of dimethylamine-borane complex, 60 ml of acetic acid, and 37.5 ml of water) at 80 °C for 30 min. Excess reagents were extracted with 200 ml of water-saturated chloroform: phenol (1:1, v/v), and further with 200 ml of chloroform. Each PA-OGA was separated on a Q Sepharose column (2.5 \times 45 cm) using a linear gradient of 60 mM to 1 M ammonium acetate buffer, pH 6.5. The effluent was monitored by fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm). The peaks corresponding to each authentic PA-OGA were pooled. Each PA-OGA was lyophilized and further purified on a TSKgel DEAE-5PW column at a flow rate 1.0 ml/min. Two gradients of ammonium acetate buffer, pH 4.8, were used. For separating PA-OGA by degree of polymerization (DP) 4 to 10, the concentration of ammonium acetate was 60 mM for 3 min increased linearly to 134 mM over 2 min, to 282 mM over 20 min, to 467 mM over 40 min, and then to 541 mM over 10 min. For separating PA-OGA of DP more than 11, the concentration of the ammonium acetate was 60 mM for 3 min, increased linearly to 134 mM over 2 min, to 282 mM over 5 min, to 467 mM over 40 min, and then to 541 mM over 10 min.

Preparation of solubilized enzyme. The solubilized enzyme was prepared from pea or azuki bean epicotyls, as described by Akita *et al.*¹⁸ The epicotyls (10 g), ground with a mortar and a pestle under liquid nitrogen, were homogenized with a grinding buffer (50 mM HEPES–NaOH, pH 7.0, 50% glycerol, 25 mM KCl, 2 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 0.7 μ g/ml pepstatin) at 4 °C for 15 min. The homogenate was centrifuged at 8,630 \times g at 4 °C for 20 min. The supernatant was centrifuged at 103,000 \times g at 4 °C for 1 h, and the pellet was washed with the grinding buffer and recentrifuged at 103,000 \times g at 4 °C for 1 h. The pellet was solubilized with a buffer consisting of 20 mM HEPES–NaOH, pH 7.0, 25% glycerol, 25 mM KCl, 2 mM EDTA, and 0.5% Triton X-100 or 20 mM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS). The supernatant obtained after centrifugation at 103,000 \times g at 4 °C for 1 h was used as the solubilized enzyme.

Assay for PGA synthase. PGA synthase activity was measured as described by Akita *et al.*¹⁸ Briefly, the reaction mixture (total volume 30 μ l) containing the enzyme solution, 33 mM HEPES–NaOH, pH 7.0, 17 mM KCl, 5 mM MnCl₂, 0.13 M sucrose, 0.033% bovine serum albumin (BSA), 0.17% Triton X-100, 1 mM UDP-GalUA, and 5 μ M PA-OGA, was incubated at 30 °C for

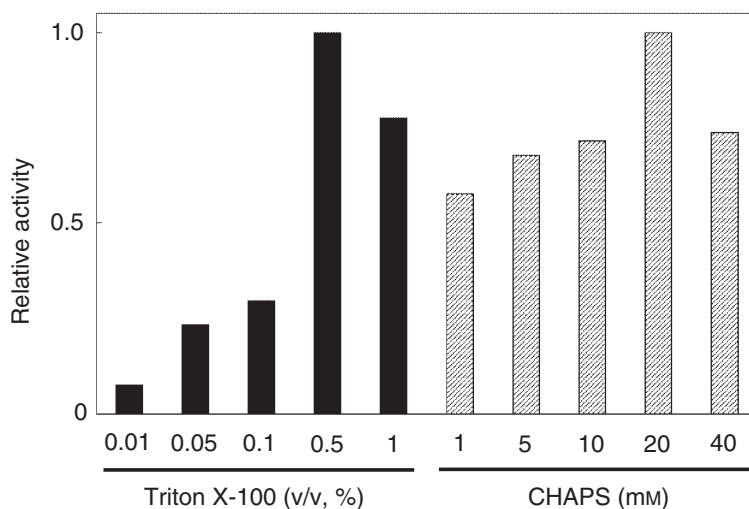


Fig. 1. Effects of Triton X-100 and CHAPS Concentration on Solubilization of PGA Synthase.

The enzyme was solubilized with buffer containing Triton X-100 (black bars) or CHAPS (shaded bars) at various concentrations and assayed. The enzyme activity is shown as relative activity to that of the enzyme solubilized with the buffer containing 0.5% Triton X-100.

30 min. It was analyzed by DEAE-HPLC with a gradient of ammonium acetate buffer, as described above. One unit of enzyme activity was defined as the amount of the enzyme that transferred 1 μmol of GalUA from UDP-GalUA to PA-OGA with a DP of 14 ($5\ \mu\text{M}$) per min under the conditions described above. The apparent K_m and relative V_{max} values of the PGA synthase for PA-OGAs of DP 7, DP 11, and DP 14 were determined by assay with various concentrations of PA-OGA of DP 7 (5.5 to $83\ \mu\text{M}$), DP 11 (1.3 to $19\ \mu\text{M}$), and DP 14 (0.26 to $26\ \mu\text{M}$) respectively, in the presence of 1 mM UDP-GalUA and 1.1 μunits of the solubilized enzyme. The apparent K_m value for UDP-GalUA was determined with various concentrations of UDP-GalUA (0.38 to 1.9 mM) in the presence of $19\ \mu\text{M}$ PA-OGA of DP 11 and 1.1 μunits of the solubilized enzyme.

Gel filtration. The solubilized enzyme was loaded onto a Sephacryl S-500 column (5.0×85 cm) equilibrated with buffer (20 mM HEPES-NaOH, pH 7.0, 25 mM KCl, 15% glycerol, 10 mM CHAPS), and eluted with the same buffer at a flow rate of 1.6 ml/min. Fractions (17.5 ml) were collected and assayed for PGA synthase activity. The void volume and the internal volume (the volume accessible to the buffer) were determined from the elution positions of polybeads (polystyrene microspheres, $0.50\ \mu\text{m}$, Poly Science, Warrington, PA) and acetone respectively. The Stokes radii of the PGA synthase-detergent complexes were estimated using the relationship between the Stokes radius and the inverse error function complement of the partition coefficient, K_D .²⁶⁾ It is known that this relationship holds well for molecules eluted near the void volume of the column.²⁶⁾

Sucrose density gradient centrifugation. A linear density gradient of sucrose was prepared as previously described.²⁷⁾ The enzyme solution ($50\ \mu\text{g/ml}$, 1 ml)

was loaded onto 35 ml of a linear gradient of 10 to 50% (w/w) sucrose in buffer (20 mM HEPES-NaOH, pH 7.0, 25 mM KCl, and 10 mM CHAPS). The sucrose gradient was centrifuged at 28,000 rpm for 3 h or 12 h in a Beckman SW-28 rotor. After centrifugation, the gradient was separated into 2-ml fractions (18 fractions in total) at 4°C , and each fraction was assayed for PGA synthase activity. The sedimentation coefficient is given by the following equation, provided by McEwen:²⁸⁾

$$S_{20,w} \int_0^t \omega^2 dt = \int_{z_1}^{z_2} \frac{\eta(z, T)}{\eta_{20,w}} \cdot \frac{\rho_C - \rho_{20,w}}{\rho_C - \rho(z, T)} \cdot \frac{1}{z - z_0} \cdot dz$$

where S is the sedimentation coefficient, t the total running time, ω the angular velocity, z the sucrose concentration, z_0 the sucrose concentration obtained by extrapolation of the linear sucrose gradient to a radius of zero, z_1 the sucrose concentration at the top of the sucrose gradient, z_2 the sucrose concentration at the sedimented position of the enzyme activity, η the viscosity, ρ the particle density, ρ_C the particle density of the PGA synthase-CHAPS complex, and T the temperature. The sedimentation coefficient of the enzyme was estimated using the calculated integral values at the right-hand of the equation. It was assumed that the particle density of the PGA synthase-CHAPS complex is $1.3\ \text{g/cm}^3$, the average value for membrane proteins solubilized with CHAPS.²⁹⁻³²⁾

Calculation of the molecular mass of the PGA synthase-CHAPS complex. The molecular mass, M_C , of the PGA synthase-CHAPS complex was calculated by the following equation, described by Clarke:³³⁾

$$M_C = \frac{S_{20,w} \cdot \eta_{20,w} \cdot 6\pi \cdot N \cdot R_S}{1 - \rho_{20,w}/\rho_C}$$

where R_S is the Stokes radius of the PGA synthase-CHAPS complex, and N is Avogadro's number.

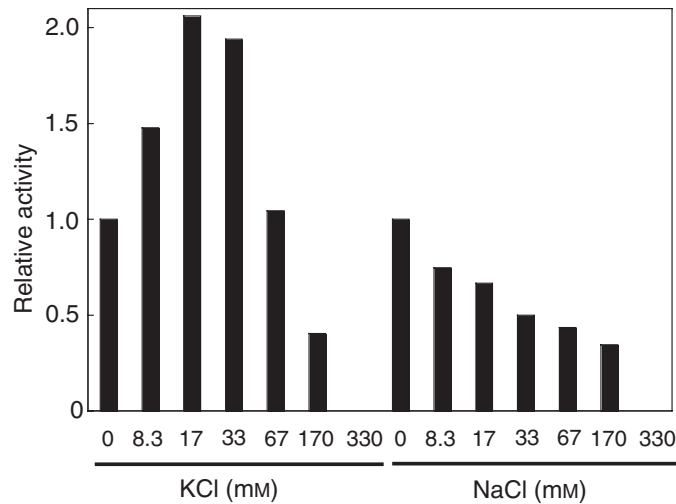


Fig. 2. Effects of Potassium Chloride and Sodium Chloride on PGA Synthase Activity.

Enzyme activity was assayed in a reaction mixture supplemented with various concentrations of potassium chloride or sodium chloride. Enzyme activity is shown as the relative activity to that of the enzyme assayed without these extra salts.

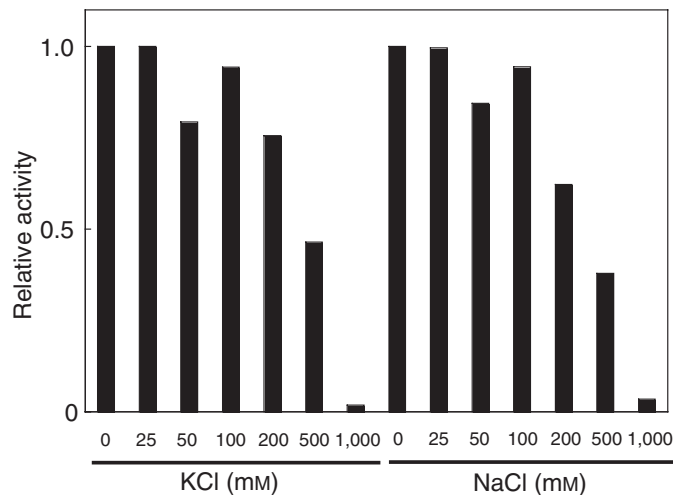


Fig. 3. Effects of Preincubation with Potassium Chloride or Sodium Chloride on PGA Synthase Activity.

The solubilized enzyme was preincubated in buffer containing potassium chloride or sodium chloride at various concentrations at 30 °C for 30 min. The preincubated enzyme was then assayed for PGA synthase activity. The enzyme activity is shown as activity relative to that of the enzyme preincubated without these salts.

Results

Optimal buffer conditions for solubilization and assay of PGA synthase

The buffer conditions used by Akita *et al.*¹⁸⁾ to extract and assay PGA synthase activity were optimized in this study. In solubilizing PGA synthase from the microsomal pellet of pea epicotyls, 0.5% Triton X-100 in the solubilization buffer yielded maximum activity (Fig. 1). As a substitute for Triton X-100, CHAPS, a reagent frequently used in solubilizing membrane proteins to be purified by chromatography, also worked well in solubilizing the enzyme. The optimal concentration of CHAPS for solubilizing it was 20 mM (Fig. 1), and the enzyme activity at this concentration was similar to that at 0.5% Triton X-100. Solubilizing the enzyme with a

Table 1. Kinetics of PGA Synthase Activity from Pea Epicotyles

The apparent K_m and relative V_{max} values of PGA synthase for PA-OGAs of DP 7, 11, and 14 were determined as described in "Materials and Methods."

Substrate	K_m (μ M)	Relative V_{max}
PA-OGA of DP 7	16	1.0
PA-OGA of DP 11	6.4	1.9
PA-OGA of DP 14	5.4	3.2
UDP-GalUA	170	0.7

buffer containing 2 mM EDTA, which works as an inhibitor of metalloprotease in crude enzyme solution, increased the activity 1.7-fold. A mixture of aprotinin, leupeptin, and pepstatin was less effective (1.3-fold) for solubilization.

The buffer conditions for assay of the enzyme were also optimized. The enzyme showed similar activity in HEPES, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris), and 2-morpholinoethanesulfonic acid (MES) buffers, while the enzyme activities in piperazine-1,4'-bis(2-ethanesulfonic acid) (PIPES), 3-morpholinopropanesulfonic acid (MOPS), and *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffers were 74%, 64%, and 41% respectively of that in HEPES buffer. The enzyme activity was increased 2.0-fold by the addition of 17 or 33 mM potassium chloride to the reaction mixture, but was reduced to about 60% by addition of sodium chloride at a similar concentration (Fig. 2). These results indicate that potassium ion at an appropriate concentration activates PGA synthase. The enzyme exhibited lower or no activity in the presence of higher concentrations (more than 170 mM) of potassium chloride and sodium chloride (Fig. 2). The optimal concentration of manganese ion for enzyme activity was 5 mM, similar to that for the petunia pollen tube enzyme.¹⁸⁾ For the remainder of the study, we used the following buffer compositions: solubilization buffer, 20 mM HEPES-NaOH, pH 7.0, 25% glycerol, 25 mM KCl, 2 mM EDTA, and 20 mM CHAPS; enzyme reaction buffer, 33 mM HEPES-NaOH, pH 7.0, 17 mM KCl, 5 mM MnCl₂, 0.13 M sucrose, 0.033% BSA, and 0.17% Triton X-100.

Characterization of PGA synthase

The optimal temperature for the pea epicotyl enzyme under the conditions used in this study was 30 °C. The optimal pH for enzyme activity was 7. The apparent K_m values for PA-OGAs of DP 7, 11, and 14 were 16, 6.4, and 5.4 μM respectively, and the value for UDP-GalUA was 170 μM (Table 1). The relative V_{max} values for PA-OGAs of DP 7, 11, and 14 were 1.0, 1.9, and 3.2 respectively (Table 1). These values are similar to those for the petunia pollen tube enzyme.¹⁸⁾ The pea epicotyl enzyme also successively transferred multiple GalUA to PA-OGAs, as was found for the petunia pollen tube enzyme (data not shown).

Influence of salt concentration on PGA synthase stability

When PGA synthase was incubated with low concentrations (25 to 100 mM) of salts (potassium chloride or sodium chloride) at 30 °C for 30 min, enzyme activity was almost entirely retained (Fig. 3). Meanwhile, the enzyme incubated with 500 mM salts lost about 60% of its activity, and the enzyme incubated with 1 M of salts lost more than 95% of its activity (Fig. 3). No difference between the effect of potassium chloride and that of sodium chloride on enzyme stability was observed. These results indicated that PGA synthase is relatively stable under low salt concentration conditions. They also suggest that intermolecular ionic interactions within PGA synthase are important for enzyme stability.

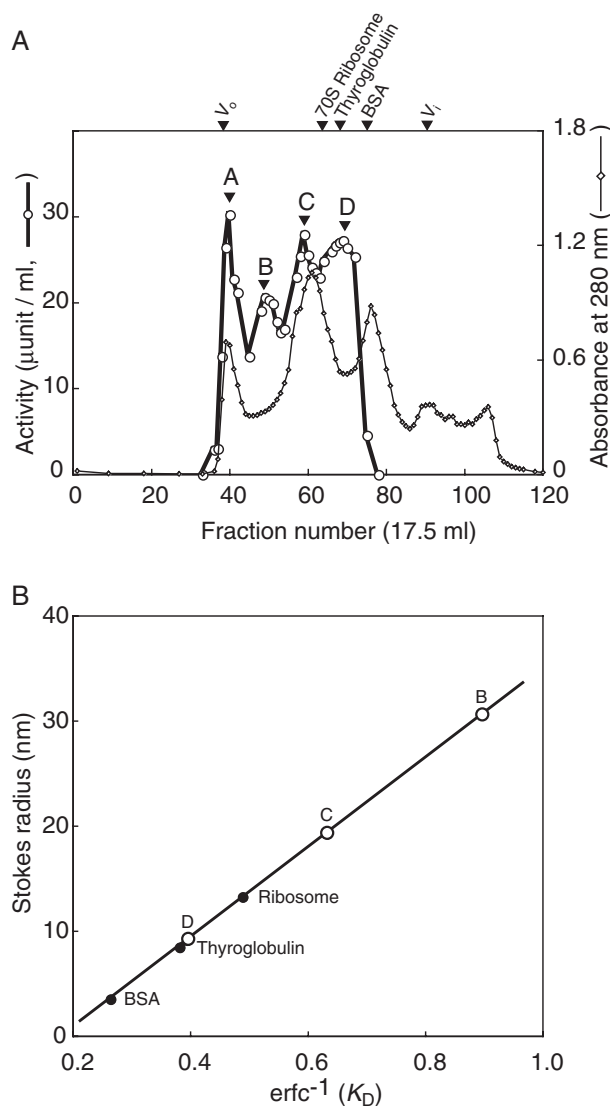


Fig. 4. Gel Filtration of PGA Synthase-CHAPS Complex on Sephacryl S-500.

A, The solubilized enzyme was loaded onto Sephacryl S-500 (5.0 × 85 cm) equilibrated with a buffer (20 mM HEPES-NaOH, pH 7.0, 25 mM KCl, 15% glycerol, 10 mM CHAPS), and was eluted with the same buffer at a flow rate of 1.6 ml/min. Fractions (17.5 ml each) were collected and assayed for PGA synthase activity (open circles) and protein content (open diamonds). Arrowheads indicate the void volume (V_0), the internal column volume (V_i), and standard proteins (*E. coli* 70S ribosome, 13.3 nm; Thyroglobulin, 8.5 nm; BSA, 3.7 nm). V_0 and V_i were determined from the elution positions of polybeads (polystyrene microspheres, 0.50 μm , Poly Science, Warrington, PA) and acetone respectively. Each fraction bearing PGA synthase activity (Fractions B, C, and D) was collected and further analyzed in sucrose density gradient centrifugation experiments. B, The closed circles show the plots of the Stokes radii against $\text{erfc}^{-1}(K_D)$ of the standard proteins, where erfc^{-1} is the inverse error function complement and K_D is the partition coefficient. The open circles indicate $\text{erfc}^{-1}(K_D)$ of the PGA synthase enzyme activity.

Structural characterization of the PGA synthase-CHAPS complex

To determine the structural characteristics of PGA synthase, we used azuki bean epicotyls instead of pea

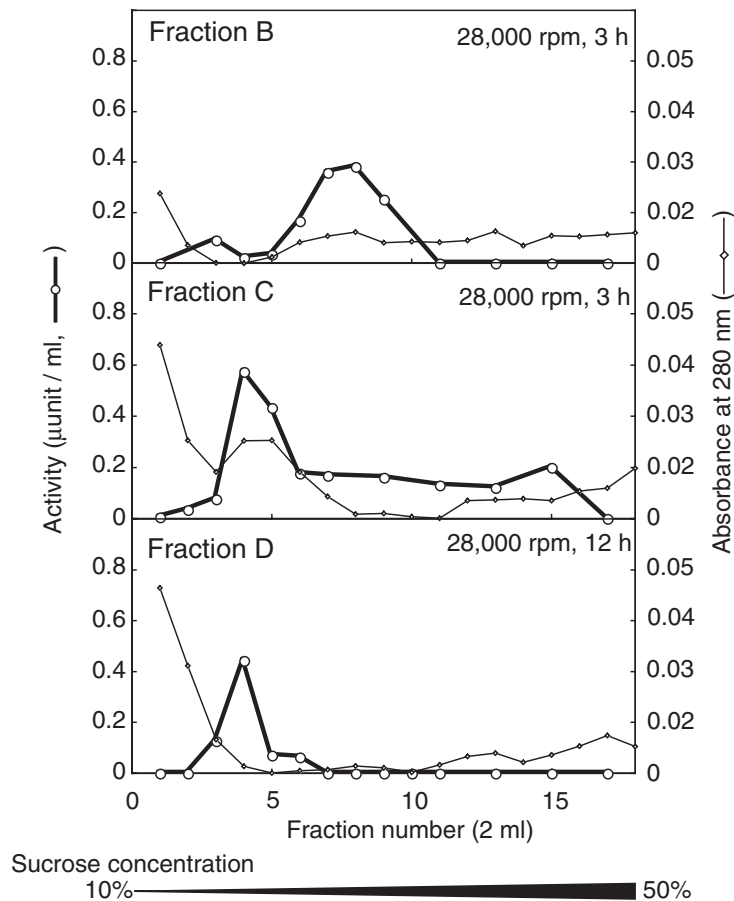


Fig. 5. Sucrose Density Gradient Centrifugation of PGA Synthase-CHAPS Complexes Separated by Sephacryl S-500 Gel Filtration.

Fractions B, C, and D separated by Sephacryl S-500 gel filtration were loaded onto the top of a 10–50% sucrose gradient. Fractions B and C were centrifuged at 28,000 rpm for 3 h. Fraction D was centrifuged at 28,000 rpm for 12 h. After centrifugation, each gradient was divided into 18 fractions (2 ml each). Each fraction was assayed for PGA synthase activity (open circles) and protein content (open diamonds).

epicotyls as an enzyme source because the specific activity of the crude solubilized azuki bean enzyme was much higher (2.1-fold) than that of pea. The enzymatic characteristics of the azuki bean enzyme were similar to those of the petunia pollen tube enzyme¹⁸⁾ and the pea epicotyl enzyme (data not shown).

The azuki bean microsomal proteins solubilized with CHAPS were applied to a Sephacryl S-500 gel filtration column under low salt concentration conditions. Four distinct peaks of enzyme activity were separated (Fig. 4A). The relative amount of peak A, which eluted in the void volume of the column, was not reproducible, while the relative amounts of the other three peaks were reproducible. This suggests that the PGA synthase in peak A was not cleaned from surrounding membrane components. From this elution profile, the Stokes radii of the PGA synthase-CHAPS complexes in fractions B, C, and D were estimated to be 31, 19, 9.2 nm respectively (Fig. 4B).

Next, the PGA synthase-CHAPS complexes fractionated by gel filtration were analyzed by 10 to 50% (w/w) sucrose density gradient centrifugation (Fig. 5). PGA synthase activity sedimented at the appropriate positions for various enzyme-detergent complexes. The sedimentation

coefficients of the PGA synthase-CHAPS complexes were estimated to be 140, 52, and 13 S for fractions B, C, and D respectively.

The molecular masses of the PGA synthase-CHAPS complexes were calculated from their hydrodynamic parameters, as described in "Materials and Methods." The molecular masses of the PGA synthase-CHAPS complexes in fractions B, C, and D were calculated to be 21,000 kDa, 5,000 kDa, and 590 kDa respectively.

The PGA synthase-CHAPS complexes in fractions B, C, and D were incubated with 500 mM sodium chloride on ice for 30 min, and then each fraction was analyzed by gel filtration. Elution of PGA synthase-CHAPS complexes in fraction B was shifted toward the same position as fraction D (Fig. 6B), while the enzyme in fraction B incubated without salt was not shifted (data not shown). Elution of fraction C was also shifted toward the same position as fraction D (Fig. 6C). The enzyme in fraction D incubated with 500 mM of salts was not shifted (Fig. 6D). The recovery of PGA synthase activity was 43%, 63%, and 95% for fractions B, C, and D respectively. This suggests that the PGA synthase-CHAPS complex in fraction D was a component of the complexes in fractions B and C.

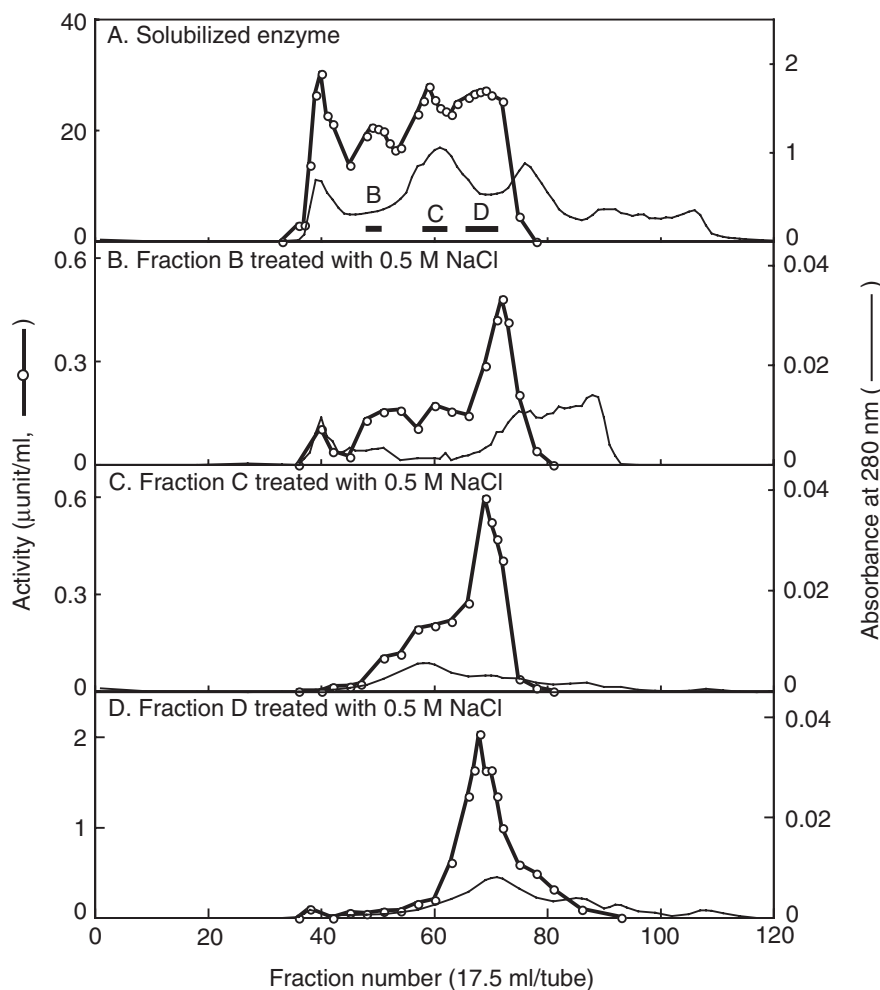


Fig. 6. Effects of High Salt Concentrations on the Structural Features of PGA Synthase.

A, The solubilized enzyme was fractionated by Sephacryl S-500 gel filtration. The fractions of each peak (B to D) indicated by horizontal bars were pooled and further analyzed. B–D, Each fraction (B–D), treated with 500 mM sodium chloride on ice for 30 min, was fractionated by Sephacryl S-500 gel filtration. The thick line with circles and the thin line indicate PGA synthase activity and protein content respectively.

Discussion

The enzymatic characteristics of pea epicotyl PGA synthase, including optimal temperature, pH, and manganese ion concentration, and the apparent K_m values for PA-OGAs and UDP-GalUA, are similar to those of PGA synthase from petunia pollen tube.¹⁸⁾ The elution patterns of the pea epicotyl enzyme and the petunia pollen tube enzyme on gel filtration chromatography were also similar to that of the azuki bean epicotyl enzyme (data not shown). Therefore, the PGA synthases from petunia pollen, pea epicotyl, and azuki bean epicotyl might have similar enzymatic and structural characteristics.

During the course of this study, the PGA synthase activity from pea and azuki bean epicotyls was found to be stimulated by potassium ion at an appropriate concentration (Fig. 2), as has been found for some other plant enzymes.³⁴⁾ The mechanism of activation of the enzyme in the presence of potassium ion remains to be elucidated. In contrast, the activity of HG-methyltrans-

ferase was decreased by the presence of 25 mM potassium ion in the homogenization buffer,³⁵⁾ indicating that the enzyme activities of PGA synthase and HG-methyltransferase might be coregulated by potassium ion.

In this study, we provided the first structural information on PGA synthase. At least three peaks bearing PGA synthase activity were separated by gel filtration and sucrose density gradient (Figs. 4, 5). The molecular masses of these PGA synthase solubilized with CHAPS were estimated to be 590 kDa to 21,000 kDa. In addition, the high molecular mass PGA synthase-CHAPS complexes, incubated with 500 mM salt, were converted to smaller enzyme-detergent complexes but retained their activity (Fig. 6). This suggests that PGA synthase is composed of several subunits, and that one of the subunits is a catalytic component. The instability of the enzyme at higher salt concentrations (Figs. 2, 3) also supports this idea, in that it might be caused by disruption of intermolecular ionic interactions between the subunits of PGA synthase, as is the case for murine

melanocyte tyrosinase and human 26S proteasome.^{36,37)}

Although PGA synthase had no activity in a buffer containing 330 mM salt (Fig. 2), the enzyme preincubated in 500 mM salt at 30 °C for 30 min had activity when it was assayed in an enzyme reaction buffer containing 17 mM potassium chloride (Fig. 3). This result indicates that inactivation of the enzyme at these salt concentrations is reversible. We speculate that during the assay, the quaternary structure of PGA synthase is reconstituted from the salt-inactivated form of the enzyme.

However, because of the impurity of this enzyme, separated by gel filtration and sucrose density gradient centrifugation, the exact nature of this complex, such as its composition, and the stoichiometry and amino acid sequences of the components, could not be determined. Further purification of PGA synthase is needed for a more detailed structural study. Since Kauss and Swanson³⁸⁾ proposed that PGA synthase and HG-methyltransferase are intimately associated, the latter may be a part of the PGA synthase protein complex. Purification of PGA synthase should allow testing of this hypothesis.

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