Sucrose Synthase from *Camellia japonica* Pollen : Properties and Possible Roles in Sucrose Metabolism

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Some properties of partially purified sucrose synthase from *Camellia japonica* pollen were studied to verify whether the enzyme is involved in UDP-glucose (UDPG) synthesis, sucrolysis, during pollen growth. The enzyme showed a molecular mass of 380 kDa and an isoelectric point of 4.6-4.8. The enzyme had a sharp optimum for sucrolysis at pH 5.5. The sucrolysis activity was enhanced about three times by Mg^{2+} and Ca^{2+}, and inhibited by fructose and glucose. Km values for UDP and sucrose were 46 μM and 256 mM, respectively, being lower than the levels of these substrates in the pollen cell. Enzyme activity increased in pollen grown on sucrose or maltose medium, but not in pollen on glucose medium. These results suggest that sucrose synthase may participate in UDPG synthesis in the growth of camellia pollen. The role of this enzyme in sucrose metabolism is discussed in special relation to UDPG pyrophosphorylase reaction.

Key word: *Camellia japonica*, Pollen tube growth, Sucrose, Sucrose synthase, UDP-glucose.

Mature pollen grains of *Camellia japonica* reserve sucrose but not starch and utilize it as an energy source and a substrate of polysaccharide synthesis for pollen growth[1]. During pollen growth, sucrose must be converted to UDP-glucose (UDPG), which is a precursor of tube wall polysaccharides, directly by sucrose synthase or indirectly through the sequential reactions of invertase, hexose kinases, phosphoglucoisomerase, phosphoglucomutase and UDPG pyrophosphorylase. In either case, the cytoplasmic UDPG supply may affect pollen tube growth, primarily depending on synthesis of such major tube wall components as β-1,3-glucan, callose[2]. It remains unsettled, however, what pathway of sucrose to UDPG is predominant in growing pollen. UDPG synthesis by a coupled reaction of UDPG pyrophosphorylase with inorganic pyrophosphatase was thought important in *Typha latifolia* pollen[3]. In contrast, our study of camellia pollen UDPG pyrophosphorylase suggests that the enzyme is involved in UDPG degradation[4]. Thus, in camellia pollen, sucrose synthase rather than UDPG pyrophosphorylase may serve for UDPG synthesis. Since the discovery of sucrose synthase in wheat germ by Cardini et al.[5], there are many studies on the enzyme from various plant materials[6]. As to pollen sucrose synthase, however,there is only a paper describing some properties of the crude camellia enzyme studied by assay of sucrose synthesis activity[7]. Here we report partial purification

of sucrose synthase from camellia pollen and some of its properties studied in both directions of sucrose synthesis and sucrolysis, i.e., UDPG synthesis, and also discuss the physiological role of this enzyme in pollen growth.

Materials and Methods

Plant materials – Mature pollen grains of *Camellia japonica* were collected, dried at 30－35℃ overnight, and stored at －20℃ until use as described previously[1].

Crude enzyme preparation – Extraction and purification of enzyme were carried out at 0－4℃. Mature pollen grains (0.2－2.0 g), previously washed with acetone, were suspended in 40 ml of 50 mM HEPES-KOH(pH 7.0) containing 5 mM 2-mercaptoethanol and 1mM EDTA, and disrupted in a French press (130－150 MPa). The homogenate was centrifuged (10000 *g*, 20 min), and the residue was extracted twice by the above procedure. The combined extract was used as a crude enzyme preparation immediately after desalting by Sephadex G-25 gel filtration.

Purification of enzyme – The crude enzyme prepared from 1.0－2.0 g of pollen grains was fractionated by addition of $(NH_4)_2SO_4$, and the pellet at 35－45% saturation was collected by centrifugation (10000 *g*, 20 min), and dissolved in 2－3 ml of the same buffer as the one used in extraction. This solution was applied to a Sepharose CL-6B column (2.6×91cm) equilibrated with the same buffer and 5 ml fractions were collected. The active fractions (25 ml) were pooled and applied to a Q Sepharose fast flow column (1.6×8.0cm) equilibrated with the same buffer. After the column was washed with 80 ml of the same buffer, proteins were eluted with 200 ml of a linear 0－0.25 M KCl gradient, and 5 ml fractions were collected. The active fractions (25 ml) was used immediately as the partially purified enzyme.

Enzyme assays – The enzyme activity of sucrose cleavage was usually measured, but the reverse reaction was measured in the process of enzyme purification.

(a) Sucrose cleavage reaction: The reaction mixture (200 μl) containing 0.2 μmol UDP, 20 μmol sucrose, 10 μmol MES-KOH (pH 5.5) and the enzyme solution was incubated for 60 min at 30℃. The reaction was stopped by adding 10 μl of 24% perchloric acid and by cooling the mixture to 0－5℃ in an ice-bath. The cooled mixture was centrifuged (1500 *g*, 7 min), and 100 μl of the supernatant was neutralized with 50 μl of 1 M Tris-HCl (pH 8.4) containing saturated KCl. The neutralized solution was centrifuged (1500 *g*, 7 min), and the supernatant obtained was diluted to 1/10 with water. The solution was analyzed for UDPG by a HPLC system on a reverse-phase column (4.6×150 mm, TOSO ODS-80T) using 100 mM sodium phosphate buffer (pH 6.9－7.0) as the mobile phase (flow rate, 1.0 ml; column temperature, 30℃).

One enzyme unit is defined as the activity necessary to produce 1 μmol of UDPG min^{-1}.

(b) Sucrose synthesis reaction – The reaction mixture (200 μl) contained 0.5 μmol UDPG, 2 μmol fructose, 1 μmol $MnCl_2$, 10 μmol Tris-HCl (pH 7.5) and the enzyme solution. The reaction was stopped by adding 200 μl of 5N NaOH and then the mixture was heated in a boiling water bath for 10 min. Sucrose produced was determined by the thiobarbituric acid method[8].

Molecular mass determination – The molecular mass of the enzyme was estimated by gel filtration on a Sepharose CL-6B column (2.6×91cm)[9]. The molecular mass markers were Ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), hen egg albumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome c (12 kDa).

Isoelectric point determination – The pI of the enzyme was determined by electrofocusing on a Rotofor apparatus (Bio-Rad Laboratories) using 2% carrier ampholite in the pH range of 4.0–6.0. The active fractions were collected and subjected again to the electrofocusing to make sure the pI .

Protein determination – Proteins was determined by the Bradford's method[10] with bovine serum albumin as standard.

Chemicals – Sephadex G-25, Sepharose CL-6B, and Q Sepharose fast flow were purchased from Pharmacia LKB Biotechnology. UDP and UDPG were of Sigma chemicals Co. Protein assay kit and carrier ampholite were obtained from Bio-Rad Laboratories. Other chemicals were from Wako Pure Chemical Ind.

Results

Purification of enzyme – Camillia pollen sucrose synthase was purified about 370-fold by ammonium sulfate fractionation, Sepharose CL-6B gel filtration and Q Sepharose fast flow ion-exchange chromatography (Table 1) . This partially purified enzyme was very unstable; the storage in 20% glycerol at −25℃ resulted in the activity loss of about 80% after 3 day and of about 100 % after 5 day. Attempts to stabilize the enzyme were unsuccessful and therefore, it was used immediately after preparation.

Molecular mass and isoelectric point of the enzyme – The molecular mass of the enzyme was estimated to be 380 kDa (Fig. 1); it was similar to the values of 360–405 kDa for the enzymes from wheat germ[11], *Phaseolus aureus* seedlings[12], *Vicia faba* cotyledons[13], soybean nodules[14], and peach fruit[15],but different from 280–290 kDa for bamboo shoot[16] and potato tubers[17]. The pI of the enzyme was determined to be 4.6–4.8; the value was rather acidic than 5.2–5.5 for the enzymes from bamboo shoot[16] and *Vicia faba* cotyledons[13].

Effects of pH, cations and metabolites on the enzyme activity – The optimum pH of sucrose synthase activity in sucrose cleavage was 5.5 (Fig. 2), while the optimum for the activity in sucrose synthesis has been reported to be pH 8.0[7]. Thus, the effects of pH on the activity of camellia pollen sucrose synthase

Table 1. Purification of sucrose synthase from pollen grains of *Camellia japonica*

Step	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
Crude extract	610	311	1.96	100	1
Ammonium sulfate fraction (35–45% saturation)	281	22.6	12.4	46	6.3
Sepharose CL-6B gelfiltration	286	3.12	91.7	47	47
Q Sepharose fast flow ion-exchange chromatography	219	1.31	719	36	367

were considerably different in the direction of reactions. Similarly, the optima in the range of pH 6.0−7.3 for sucrolysis and pH 8.0−9.5 for sucrose synthesis have been reported for the enzyme from potato tubers[17], maize kernels[18], morning-glory callus cells[19], soybean nodules[14], and wheat leaves[11].

As shown in Fig. 3, the enzyme was activated about three times by 5 mM Ca^{2+} and Mg^{2+}, but inhibited by a low concentration of Zn^{2+}. A slight activation of sucrose synthase by these ions was found in bamboo shoots[16], soybean nodules[14], and peach fruit[15], although these ions inhibited the enzyme of morning-glory callus cells[19].

The enzyme activity was inhibited by 10 mM glucose, fructose and 1 mM ATP, and enhanced by 1 mM pyrophosphate; other metabolites in glycolysis had a little or no effect (Table 2).

The growth of camellia pollen is influenced by exogenous sugars[20]. Fig. 4 shows difference in the sucrose synthase activity in pollen grown on different sugar media. The enzyme activity increased when the pollen were incubated on sucrose and/or maltose media. The increment under each of these disaccharides was similar and the sum was nearly equal to that under copresence of both the sugars. However, glucose had no positive effect on the enzyme activity.

Enzyme kinetics − The saturation curve of camellia pollen sucrose synthase for sucrose was sigmoidal rather than hyperbolic (Fig. 5), but the Hill coefficient was 1.1. The apparent Km value for sucrose of this enzyme was determined to be 256±94 mM from Lineweaver-Burk's plot at the range of 50−250 mM sucrose. Similar sigmoidal curves have been reported for the enzymes from rice grains[21], potato tubers[21] maize kernels[18], and *Vicia faba* cotyledons[13], but the hyperbolic curves for those from many other plant tissues[12, 14, 17, 19, 22]; the Km values reported are 17−290 mM. On the other hand, the saturation curve of the camellia pollen enzyme for UDP was hyperbolic and the Km value

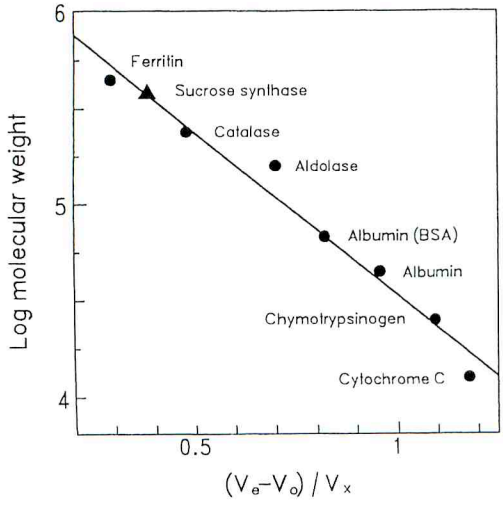

Fig. 1. **Estimation of the molecular mass of camellia pollen sucrose synthase by Sepharose CL-6B gel filtration.**

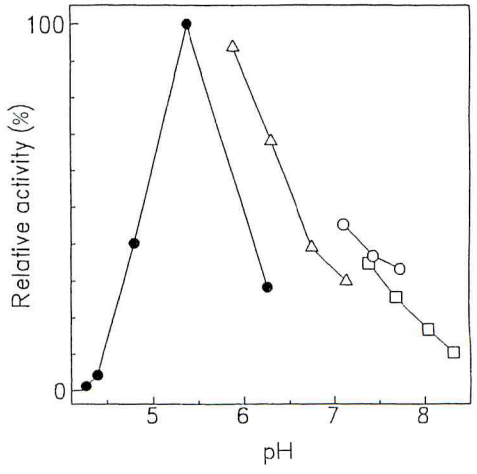

Fig. 2. **Effect of pH on the sucrose synthase activity for sucrolysis.** Enzyme activities were assayed under standard conditions but with sodium acetate-acetic acid (●), MES-KOH (△), HEPES-KOH (○) and Tris-HCl (□) buffers.

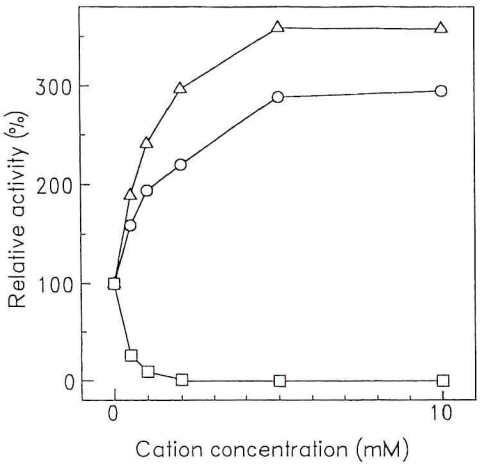

Fig. 3. Effect of cations on the sucrose synthase activity for sucrolysis. Enzyme activities were assayed with changing concentrations of $CaCl_2$ (△), $MgCl_2$ (○) or $ZnCl_2$ (□) under standard conditions.

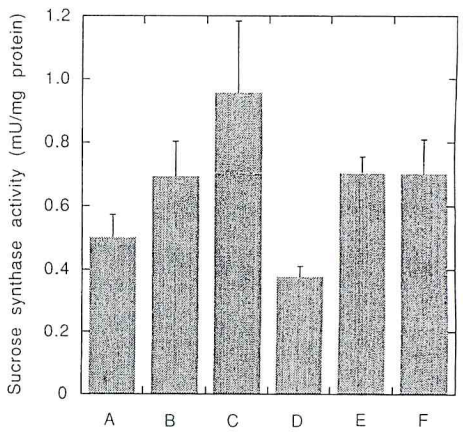

Fig. 4. Activities of sucrose synthase in pollen grown on various sugar media. The crude enzyme samples were from the quiescent pollen grains (A), or from the pollen incubated for 6 h at 25℃ on the medium containing 0.1 M sucrose (B), 0.1 M sucrose plus 0.1 M maltose (C), 0.1 M glucose (D), 0.1 M glucose plus 0.1 M maltose (E) or 0.1 M maltose (F) ; the mean pollen tube lengths were 4.3 (B), 4.2 (C), 3.3 (D), 1.8 (E) or 0.5 mm (F), respectively. The samples were prepared as described in the method, except that the growing pollen were disrupted in a glass homogenizer. Results are means of 3 experiments ; bars indicate the standard error.

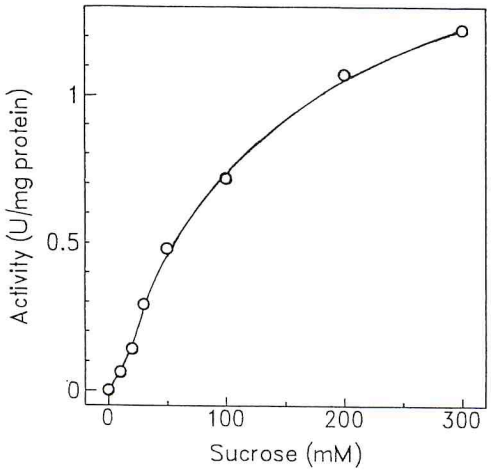

Fig.5. Effect of sucrose concentration on sucrose synthase activity.

was determined to be 46±13 μM from Lineweaver-Burk's plot at the range of 0.02−0.1 mM UDP. This value was much lower than those for the enzymes from other plant tissues [11, 17, 23], and nearly equal to that for the maize kernel enzyme [18].

Discussion

Although most properties of the camellia pollen sucrose synthase were not very different from those reported for the enzymes from other materials, some seemed to be remarkable.

Ca^{2+} or Mg^{2+} enhanced 3.5- or 3.0-fold the sucrolytic activity of camellia pollen enzyme (Fig. 3). Such a marked activation of sucrose synthase by these cations has never been reported and furthermore, is contrasting with the fact that the activation degree in the sucrose synthesis by this enzyme was ca. 30−60% [7]. Ca^{2+} is generally stimulative for pollen growth and Ca has been reported to occur mostly in the apical part of growing pollen tubes [24]. These facts may suggest the relation between Ca^{2+}-stimulated UDPG synthesis from sucrose and pollen growth.

Glucose and fructose inhibited the sucrose synthase-catalyzed sucrolysis (Table 2). Fructose inhibition has also been reported for the enzymes from other sources [13, 18, 23]. Since fructose, a product of sucrolysis enters in metabolism after being phosphorylated, the reaction of sucrose synthase can be regulated indirectly by that of fructokinase or hexokinase. It is noteworthy, in this connection, that these occurs a high activity of fructokinase in camellia pollen [25].

The sucrose synthase activity of camellia pollen increased after growing for 6 h on sucrose or maltose medium, but almost unchanged in case of glucose culture (Fig. 4). The increase in sucrose synthase activity by sucrose has been observed in potato tubers [26] and *Phaseolus vulgaris* suspension cultured cells [27], and in the former, that has been indicated to be induction of the enzyme protein by sucrose [26]. Thus the cellular level of sucrose can regulate that of sucrose synthase, and this may relate to the fact that sucrose is the most effective sugar for the growth of camellia pollen. Maltose enhanced

Table 2. Effects of metabolites on the activity of camellia pollen sucrose synthase

Metabolites	Relative activity (%)
Control	100
10 mM Glucose	66±15
10 mM Fructose	53±11
1 mM Glucose-1-phosphate	109±23
1 mM Fructose-6-phosphate	117±10
1 mM Fructose-1,6-bisphosphate	111± 8
0.1 mM Fructose-2,6-bisphosphate	117±15
1 mM 3-Phophoglycerate	114± 8
1 mM Phosphoenolpyruvate	111± 9
1 mM Pyruvate	103±13
1 mM ATP	67± 9
1 mM CTP	100±21
1 mM ADP	87± 9
1 mM pyrophosphate	139±21
1 mM inorganic phosphate	116±17

Enzyme activities for sucrolysis were assayed under standard conditions.
The values are the means±SD of three experiments.

the specific activity of sucrose synthase (Fig. 4) but not those of phosphoglucomutase and invertase (data not shown) and UDPG pyrophosphorylase[4] in growing camellia pollen. The enhancement degree by maltose was about the same as that by sucrose and the effect was nearly additive. Although the results may suggest a possibility that these sugars act independently on different causal sequences, further experiments in combination at various sugar concentrations must be done. Maltose has no such effect on sucrose synthase in potato tubers[26] ; this is probably due to the occurrence in potato tubers of maltase, which is almost undetectable in the growing camellia pollen[20].

The Km values of camellia pollen sucrose synthase for sucrose and UDP were 256 mM and 46 μM, respectively, whereas those for fructose and UDPG have been reported to be 71 mM and 2.9 mM, respectively[7] ; thus, this enzyme has a higher affinity for UDP than for UDPG. In this connection, the UDP level in the pollen grown on sugar-free medium for 6 h was 80 μM (unpublished data). The affinity for sucrose of this enzyme was lower than that for fructose. However, the mature quiescent pollen grains contain about 0.8 M or more of sucrose[1] and therefore, the sucrose level in the early stage of pollen growth is probably sufficient for sucrose cleavage by this enzyme.

It seems likely from the results described above that in camellia pollen, sucrose synthase will promote UDPG synthesis from sucrose. In addition, UDPG pyrophosphorylase has been supposed to be involved in degradation rather than synthesis of UDPG in camellia pollen[4]. Taking account of all these results, it seems probable that sucrose degradation in camellia pollen is catalyzed jointly by sucrose synthase and UDPG pyrophosphorylase, as similarly reported for cultured sycamore[28] and *Catharanthus roseus* cells[29], and that the main pathway of UDPG synthesis is the direct one by sucrose synthase rather than the sequential one by invertase and hexose kinases. On the other hand, glucose and fructose are also effective for pollen growth, and in this case, UDPG must be synthesized by UDPG pyrophosphorylase. For this reaction to be directed toward UDPG synthesis, UDPG and pyrophosphate (PPi) formed must be consecutively utilized for the the reactions by such UDPG- or PPi-utilizing enzymes as β-glucan synthase, UDPG 4-epimerase and PPi-dependent phosphofructokinase. For the present, since a high activity of PPi-dependent phosphofructokinase has been found in camellia pollen[30], coupling of UDPG pyrophosphorylase reaction with PPi-dependent phosphofructokinase reaction seems to be favorable for UDPG formation, eventually for pollen tube growth. These inferences have to be verified by further experiments under various conditions for pollen growth.

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ツバキ花粉のショ糖合成酵素の性質とショ糖代謝における役割

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ツバキ花粉が成長する際に，ショ糖合成酵素がUDP－グルコース（UDPG）の合成，つまりショ糖の分解に関与しているかどうかを確かめるために，この酵素を部分精製し，その性質を調べた。この酵素の分子量は380 kDaで，等電点はpH 4.6－4.8であった。酵素の最適pHは，ショ糖分解反応おいてはpH 5.5であった。ショ糖分解反応はCa^{2+}とMg^{2+}により約3倍に活性化され，グルコースとフルクトースにより阻害された。Km値はUDPに対して46 μM，ショ糖に対して256mMで，その値はこれら基質の花粉内濃度よりも低かった。ショ糖やマルトース培地で成長した花粉ではショ糖分解活性が増加したが，グルコース培地で成長した花粉では活性の増加はみられなかった。これらの結果はショ糖合成酵素がUDPG合成に関与していることを示しており，ショ糖代謝におけるこの酵素の役割について，とくにUDPGピロホスホリラーゼの反応との関連について考察した。