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Communication

Galactinol Synthase is an Extravacuolar Enzyme in Tubers of Japanese Artichoke (*Stachys sieboldii*)[1]

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ABSTRACT

Galactinol synthase (GS, UDP-α-D-galactose:1L-*myo*-inositol-1-*O*-α-D-galactopyranosyltransferase) is a key enzyme in the biosynthetic pathway of the raffinose family of oligosaccharides. The subcellular location of GS was studied in the parenchyma of stachyose-storing tubers of Japanese artichoke (*Stachys sieboldii*) by isolation of protoplasts and vacuoles. A comparison of the activities of GS, malate dehydrogenase, and alcohol dehydrogenase (extravacuolar markers) and α-mannosidase and β-*N*-acetylglucosaminidase (vacuolar markers) in parenchyma protoplasts with those of vacuoles isolated from them showed that GS was an extravacuolar enzyme.

GS[2] (UDP-α-D-galactose:1L-*myo*-inositol-1-*O*-α-D-galactopyranosyltransferase) catalyzes the first committed step in the biosynthetic pathway of the RFO [(α-D-galactosyl-1–6)$_n$-sucrose] by the reaction of

UDP-α-D-galactose + *myo*-inositol → UDP
+ galactinol (1L-1-*O*-α-D-galactopyranosyl-*myo*-inositol).

It thus provides activated galactosyl substrate for the synthesis of RFO. Since its first discovery in maturing pea seeds (2), GS has been found in a number of plant species and tissues. GS activities showed a positive correlation with RFO levels and a negative correlation with sucrose levels in leaves of 20 plant species (4). During plant development, GS activities were also positively correlated with RFO levels in cucumber leaves (8) and with RFO accumulation in soybean seeds (4, 7, 10). These correlations strongly suggest an important regulatory role of GS in the channeling of carbon into RFO.

Even though GS has been purified and biochemically characterized (3, 9, 11, 12), its subcellular location has not been determined. GS has been suggested to be a cytosolic enzyme in cucumber leaves, mainly on the grounds of its good solubility in cell-free extracts and neutral pH optimum (3). Knowledge of a more exact location, however, is needed and is a prerequisite for a better understanding of its role, regulation, and function *in vivo*.

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[2] Abbreviations: GS, galactinol synthase; RFO, raffinose family of oligosaccharides.

In an earlier study (6), we showed that galactinol, the main product of GS, is present in the extravacuolar space of the typical RFO-storing parenchyma cells of Japanese artichoke tubers (*Stachys sieboldii*). Furthermore, we showed that stachyose, the RFO tetrasaccharide, is stored in the vacuoles of these tubers (6) and transported across the tonoplast via an active sugar carrier (5). A possible interpretation of these three findings is that the whole synthetic pathway of the RFO in Japanese artichoke tubers occurs outside the vacuole. The aim of this study was to find direct evidence for an extravacuolar location of GS, the first enzyme of the RFO synthesis, using the established method for the isolation of protoplasts and vacuoles of Japanese artichoke tubers (6).

MATERIALS AND METHODS

Plant Material

Tubers of Japanese artichoke (*Stachys sieboldii* Miq.) were grown outdoors in a nursery garden of the Institute of Plant Biology, University of Zürich, and were harvested in November. They were stored in moist sand in the dark at 5°C for up to 4 months.

Isolation of Protoplasts and Vacuoles

Protoplasts and vacuoles from the storage parenchyma of resting tubers were prepared exactly as described previously (5). Briefly, protoplasts were prepared from chopped tuber parenchyma by enzymic digestion of the cell walls with Cellulase Y-C and Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan). Vacuoles were released from purified protoplasts by gentle, selective lysis of the plasmalemma. Yields and purity of the vacuoles were estimated by comparison of extravacuolar markers (malate dehydrogenase, alcohol dehydrogenase) with vacuolar markers (α-mannosidase, β-*N*-acetylglucosaminidase) as described by Keller and Matile (6).

Enzyme Extraction

Tuber parenchyma (1 g) was homogenized in 2 mL of ice-cold extraction buffer (50 mM MOPS [KOH] [pH 7.0], 10 mM DTT, and 0.1% [w/v] BSA) in a glass tissue grinder. The homogenate was centrifuged at 14,000*g* for 5 min. The supernatant was either directly desalted as described below or ultracentrifuged at 100,000*g* for 30 min. After ultracentrifugation, the supernatant was also desalted, whereas the pellet

was resuspended in extraction buffer by sonication at 4°C for 1 min.

Assay of GS Activity

Enzyme samples (500 μL) were desalted by passage through 3-mL Bio-Gel P-6 (medium) columns, preequilibrated with extraction buffer. For compartmentation studies, the desalted samples were concentrated three- to ninefold by ultrafiltration through Ultrafree-MC filter units (10,000 nominal mol wt limit; Millipore AG, Kloten, Switzerland). The faster flowing 10-kD units were chosen because preliminary experiments had shown that similar GS activities were obtained when ultrafilters with cutoff points of 10 and 30 kD were used, respectively. GS activity was measured radiometrically using a modification of the assay described by Pharr *et al.* (9). The reaction mixture (110 μL) contained 50 μL desalted sample and, expressed as final concentrations, 25 mM MOPS (KOH) (pH 7.0), 5 mM DTT, 0.05% BSA (w/v), 1 mM UDP-[U-^{14}C]galactose (1 μCi/μmol), 1 mM $MnCl_2$, and 0 or 20 mM *myo*-inositol. After incubation at 25°C for 40 min, reactions were terminated by addition of 400 μL of absolute ethanol. Unreacted UDP-[^{14}C]galactose was removed by addition of 200 μL of a 1:1 (v/v) slurry of water:Dowex-1 × 8 (100–200 mesh, formate form) and occasional shaking for 20 min. After centrifugation at 13,000*g* for 2 min, 250-μL aliquots of the supernatant were added to 3 mL of the scintillation cocktail Ready Safe (Beckman, Zürich, Switzerland). The radioactivity was determined in a Beckman LS 7800 liquid scintillation counter. GS activity was calculated from the inositol-dependent increase of radioactivity. GS activity of all the cell fractions tested (total homogenate, protoplast lysate, vacuolar fraction) was linear with time for at least 40 min.

Enzyme Units

One unit of enzyme activity catalyzes the formation of 1 μmol of the measured product per min.

RESULTS AND DISCUSSION

Vacuoles were isolated from protoplasts of the storage parenchyma of tubers of *S. sieboldii* by our well-established method (5, 6). An average yield of vacuoles of 35.9% of the original vacuoles present in the protoplasts was obtained as assessed by the recovery of the vacuolar markers α-mannosidase and β-*N*-acetylglucosaminidase in the vacuolar fraction (Table I). Only 6.7 and 4.4% of the activities of the extravacuolar markers malate dehydrogenase and alcohol dehydrogenase, respectively, cosedimented with the vacuoles, indicating that the purification procedure was highly effective. Yield and purity of the isolated vacuoles were, therefore, similar to that of earlier reports (5, 6) and were sufficiently high to allow compartmentation analysis. Table I shows that 9.2% of the GS activity was associated with the vacuoles, which is similar to the extravacuolar contamination (4.4–6.7%). It might, therefore, be concluded that GS is an extravacuolar enzyme. A loose association with the cytosolic side of the tonoplast *in vivo* and an artifactual dissociation during isolation cannot be completely ruled out.

No attempt was made to exactly localize GS in the extravacuolar space, *i.e.* to distinguish between cytosolic and organellar location in the cytoplasm. When a crude cell homogenate was ultracentrifuged at 100,000*g* for 30 min, 93.8% of the total GS activity (4.8 milliunits/g fresh weight) was found in the supernatant and 6.2% in the pellet. This indicates that GS is a soluble or readily solubilized enzyme. However, a definite location in the cytoplasm cannot be assigned to GS with this approach and has to await more detailed cell fractionation studies.

The physiological role of GS in *Stachys* tubers is not very clear at the moment. The most obvious one is that of a key enzyme to commit imported sucrose to stachyose synthesis during tuber formation. Even though GS activity seems to be positively correlated with tuber development (9.9, 21.7, and 6.1 milliunits/g fresh weight for small, medium [both growing] and large [dormant] tubers, respectively), it is relatively low compared with published activities for other RFO plants (about 100 milliunits/g fresh weight for developing soybean seeds [1, 10] and up to 2500 milliunits/g fresh weight for leaves [4]). Furthermore, preliminary measurements did not show any detectable enzyme activity of the subsequent steps of RFO synthesis, *i.e.* raffinose synthase and stachyose synthase, in *Stachys* tubers. The bulk of the large amounts of stachyose present in *Stachys* tubers, therefore, seems not to be the result of *de novo* synthesis but of direct phloem import of stachyose. Phloem unloading is probably of the symplastic

Table I. *Subcellular Distribution of Galactinol Synthase and Marker Enzymes in Protoplasts Isolated from Tubers of Japanese S. sieboldii*

The enzyme activities in the vacuolar fraction are expressed as the percentages of the respective total activities in the protoplast lysate used. The association of enzymes with the vacuoles was calculated assuming α-mannosidase and β-*N*-acetylglucosaminidase to be 100% vacuolar in location (average). Values are means ± SE of four to six independent experiments.

Enzymes	Protoplast Lysate	Vacuolar Fraction	Recovery in Isolation Gradient	Association with Vacuoles
	milliunits/mL		%	
Extravacuolar markers				
NAD-malate dehydrogenase	295 ± 64	2.40 ± 0.21	129 ± 6	6.70 ± 0.57
Alcohol dehydrogenase	90.6 ± 7.7	1.58 ± 0.64	126 ± 19	4.41 ± 1.78
Vacuolar markers				
α-Mannosidase	51.6 ± 11.4	36.3 ± 3.0	93.4 ± 7.5	101 ± 8
β-*N*-Acetylglucosaminidase	70.4 ± 19.2	35.4 ± 3.3	83.2 ± 3.3	98.7 ± 9.1
GS	0.439 ± 0.102	3.28 ± 0.59	98.0 ± 29.6	9.15 ± 1.65

type as indicated by three recent observations: (a) in *Stachys* tuber parenchyma, an extremely high frequency of plasmodesmata is observed, (b) stachyose does not cross the plasmalemma of isolated parenchyma protoplasts (K. Schmit, personal communications), and (c) an active stachyose carrier is present at the tonoplast (5).

From these results and considerations, the following roles might be allotted to GS in *Stachys* tubers: (a) to assist in the channeling of carbon from surplus sucrose (from the phloem) into RFO during tuber formation and (b) to sustain a possible slow turnover of RFO during tuber dormancy. The extravacuolar location of GS found in this study would be in support of both of these roles.

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LITERATURE CITED

1. **Castillo EM, de Lumen BO, Reyes PS, de Lumen HZ** (1990) Raffinose synthase and galactinol synthase in developing seeds and leaves of legumes. J Agric Food Chem **38:** 351–355
2. **Frydman RB, Neufeld EF** (1963) Synthesis of galactosylinositol by extracts from peas. Biochem Biophys Res Commun **12:** 121–125
3. **Handley LW, Pharr DM** (1982) Ion stimulation, UDP inhibition and effects of sulfhydryl reagents on the activity of galactinol synthase from leaves of cucumber, *Cucumis sativus* L. Z Pflanzenphysiol **108:** 447–455
4. **Handley LW, Pharr DM, McFeeters RF** (1983) Relationship between galactinol synthase activity and sugar composition of leaves and seeds of several crop species. J Am Soc Hort Sci **108:** 600–605
5. **Keller F** (1992) Transport of stachyose and sucrose by vacuoles of Japanese artichoke (*Stachys sieboldii*) tubers. Plant Physiol *98:* 442–445
6. **Keller F, Matile P** (1985) The role of the vacuole in storage and mobilization of stachyose in tubers of *Stachys sieboldii.* J Plant Physiol **119:** 369–380
7. **Lowell CA, Kuo TS** (1989) Oligosaccharide metabolism and accumulation in developing soybean seeds. Crop Sci **29:** 459–465
8. **Pharr DM, Sox HN** (1984) Changes in carbohydrate and enzyme levels during the sink to source transition of leaves of *Cucumis sativus* L., a stachyose translocator. Plant Sci Lett **35:** 187–193
9. **Pharr DM, Sox HN, Locy RD, Huber SC** (1981) Partial characterization of the galactinol forming enzyme from leaves of *Cucumis sativus* L. Plant Sci Lett **23:** 25–33
10. **Saravitz DM, Pharr DM, Carter Jr TE** (1987) Galactinol synthase activity and soluble sugars in developing seeds of four soybean genotypes. Plant Physiol **83:** 185–189
11. **Smith PT, Kuo TM, Crawford CG** (1991) Purification and characterization of galactinol synthase from mature zucchini squash leaves. Plant Physiol **96:** 693–698
12. **Webb JA** (1982) Partial purification of galactinol synthase from leaves of *Cucurbita pepo.* Can J Bot **60:** 1054–1059