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Enzymes of sucrose, maltose, and α,α-trehalose catabolism in soybean root nodules *

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Abstract. Crude, Sephadex-filtered extracts of soybean (*Glycine max* (L.) Merr.) root nodules contained invertase (E.C. 3.2.1.26) activity with pH optima at 5.4 and 7.8, α,α-trehalase (E.C. 3.2.1.28) activity with pH optima at 3.8 and 6.6, and maltase (E.C. 3.2.1.20) activity with a broad pH optimum between 4.5 and 5.0. Bacteroids and cytosol were separated using Percoll density gradients. Cellulase and pectinase were employed to separate protoplasts from the infected region from the nodule cortex, which remained intract. Assays of disaccharidases from these nodule fractions indicated the following localization of enzymes: (1) Bacteroids lack invertase activity (pH 5.4 and 7.8). (2) Much, if not most, of the invertase activity may be localized in the nodule cortex; this is especially likely for acid invertase. However, there was substantial invertase activity in cytosol from the infected region. (3) Most of the maltase activity (pH 5.0) and trehalase activity (pH 3.8 and 6.6) were localized in the cytosol. It is likely that most of these disaccharidase activities are in the cytosol of the infected region, in contrast to invertase. (4) Bacteroids contain maltase (pH 5.0) and trehalase (pH 3.8 and 6.6), but the amount of these enzyme activities was less than 15% of total activity in nodules. Bacteroids and nodule cortex were capable of in-vivo hydrolysis of [^{14}C]trehalose and [^{14}C]maltose. These disaccharides were also hydrolyzed by soybean roots and hypocotyls. Therefore, while α,α-trehalose in soybean nodules is probably synthesized by the bacteroids, the capability for utilization of trehalose was not restricted to the bacteroids.

Key words: Bacteroid – *Glycine* – *Rhizobium* – Root nodule enzymes.

Introduction

Soybean nodules contain high concentrations of sucrose, small amounts of maltose and α,α-trehalose (Streeter 1980). Sucrose is the principal sugar translocated in soybeans (Burley 1961), maltose is thought to be formed during the hydrolysis of nodule starch (Streeter 1981), and α,α-trehalose is of special interest because it is essentially restricted to the nodules (Streeter 1980), is formed by *Rhizobium japonicum* (this paper), and accumulates in nodules during nodule senescence (Streeter 1981). Free trehalose has not been conclusively demonstrated in higher plants (Gussin 1972) and the above findings indicate that nodule trehalose may be synthesized by the bacteroids.

The catabolism of the above three disaccharides in soybean nodules was studied by determining the activity and location of invertase (E.C. 3.2.1.26), maltase (E.C. 3.2.1.20) and trehalase (E.C. 3.2.1.28), and the ability of various nodule compartments to hydrolyze ^{14}C-labeled maltose and trehalose. Invertase activity in nodules has been previously studied in lupins (Robertson and Taylor 1975; Kidby 1966).

Material and methods

Plant growth; protoplast preparation. Soybean seeds (*Glycine max* (L.) Merr) cv. Beeson were a gift from D.L. Jeffers, this Department. Plants were grown in silica sand using a nitrogen-free nutrient solution (Streeter 1981), in a greenhouse with natural light supplemented with 200 quanta m^{-2} s^{-1} (400–700 nm) from fluorescent and incandescent lamps throughout the photoperiod of 15 h. Day-night temperature was 25°–19° ± 2° C, and root nodules were established using commercial soybean inoculant (Agricultural Laboratories, Columbus, O., USA).

Protoplasts were isolated by incubating thin (< 1 mm) slices of nodules with commercial cellulase and pectinase as described in Streeter (1980). The medium of Meyer (1974) was used with Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), KH_2PO_4, $CaCl_2$, and KNO_3 (Broughton et al. 1976) and a pH of 5.8. The incubations with hydrolytic enzymes were 3.5–4 h; there was very little apparent hydrolysis of cell walls in the cortex

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resulting in the formation of rings of cortex tissue which were free of infected cells.

The protoplasts were rinsed two or three times with 10-fold volumes of medium lacking enzymes. They were allowed to settle and the supernatant was drawn off with a Pasteur pipette (no centrifugation). The cortex rings were also washed two or three times with medium lacking enzymes. These rinses are important because the cellulase and pectinase from two sources (Yakult Biochemicals, Nishinomiya, Japan; Calbiochem-Behring, San Diego, Cal., USA) were found to contain abundant maltase and small amounts of invertase and trehalase activity.

Extraction and assay of disaccharidases. Chilled nodules were ground in a mortar in 0.1 M sodium phosphate buffer, pH 7.5 (2 ml/g nodules) and moist, insoluble polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, Mo., USA) was mixed with the homogenate (lg polyvinylpyrrolidone/lg nodules). The slurry was filtered with four layers of cheesecloth and centrifuged at 40,000 *g*. Portions of the supernatant were filtered with Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J., USA), using 0.01 M sodium phosphate buffer, pH 7.0 for equilibration and elution of the column. Blue dextran (Sigma) was used to mark the protein when leghemoglobin was not present.

Reaction mixtures contained 0.5 ml of buffer (50 mM glycine, 50 mM sodium phosphate, 50 mM sodium citrate) of variable pH, 10 µmol of maltose or trehalose or 20 µmol sucrose, and Sephadex-filtered protein, in a volume of 0.75 ml. The control was boiled protein. The substrates must be free of glucose. The sucrose and trehalose (Sigma) were found to be essentially free of glucose, and the maltose (Calbiochem) was purified as described in Messer and Dahlqvist (1966).

The reaction mixtures were incubated for 1 h at 30° C and then boiled. Glucose formation was determined as described in Lloyd and Whelan (1969) using glucose oxidase (Sigma; Type V). Standards were run with boiled protein with every set of reaction mixtures. The method permits quantitation of glucose down to 2 µg glucose mixture. Glucose formation was linear over these protein concentrations: trehalase, 5–50 µg, invertase or maltase, 15–100 µg. Glucose formation in all three assays was linear for 60 min. Protein concentration was determined as described in Lowry et al. (1951).

Washed protoplasts from 8 g of nodules were chilled (2° C) and suspended in cold 0.1 M sodium-phosphate buffer, pH 7.5, containing 10 mM mercaptoethanol. The protoplasts were ruptured and transferred to a centrifuge tube by using a Pasteur pipette with a constricted tip, and centrifuged (0° C) at 40,000 *g*. The supernatant was used as the cytosol fraction. The cortex rings were chilled (2° C) and ground in a mortar with 0.1 M sodium phosphate, pH 7.5. The supernatant after centrifugation at 40,000 *g* was used as cortex extract. The extracts were filtered through Sephadex G-25.

The purification of bacteroids on Percoll (Sigma) gradients was as described in Reibach et al. (1981). The bacteroids were washed twice with 3- and 12-fold volumes of 0.2 M sodium phosphate buffer, pH 7.5. The bacteroids were then suspended in the same buffer, two drops of Triton X-100 (Sigma) added, and the mixture was sonicated four times for 3 min at 0° C. After centrifugation at 40,000 *g*, the supernatant (bacteroid extract) was filtered (Sephadex G-25) as was the cytosol collected from the Percoll gradient.

Analysis of ^{14}C-labeled carbohydrates. Uniformly labeled [^{14}C]trehalose ($36 \cdot 10^5$ Bq/µmol) and [^{14}C]maltose ($79 \cdot 10^3$ Bq/µmol) were from ICN Chemical and Radiosotope Division, Irvine, Cal., USA, and Amersham Corp., Arlington Heights, Ill., USA, respectively. Tissues or cells were repeatedly extracted with ethanol, and the extracts were purified on ion exchange resins and analyzed by thin-layer chromatography. The previously described thin-layer chromatography system (Streeter and Bosler 1976) was modified to permit separation of pinitol and glucose (Kindl and Hoffmann-Ostenhof 1966) and much more sensitive detection of cyclitols (Nemec et al. 1969). The radioactivity was determined by liquid scintillation.

Culture of Rhizobium japonicum. Rhizobium japonicum strain 110 (Cell Culture and Nitrogen Fixation Laboratory, Plant Physiology Institute, Beltsville, Md., USA) was grown in (g/l): K_2HPO_4 (0.35), KH_2PO_4 (0.35), $MgSO_4 \cdot 7H_2O$ (0.2), NaCl (0.1), Na gluconate (2.0), yeast extract (Difco Laboratories, Detroit, Mich., USA) (0.1), mannitol (2 or 0), and glucose (8 or 10); pH was adjusted with KOH to 6.9. The cultures were incubated (21° C) on a rotary shaker (100 cycles/minute) for 12 d and the yield of cells was 1.1 ± 0.1 cm^3. The cells were washed twice, repeatedly extracted with ethanol, and the extracts were analyzed by gas-liquid chromatography as described in Streeter (1980).

Results and discussion

For invertase, pH optima of 7.8 and 5.2 were observed, with much greater activity for the "alkaline" invertase (Fig. 1). Robertson and Taylor (1973) reported a similar pH optimum for alkaline invertase from nodules of *Lupinus angustifolius* using the same buffer as used in this study. Neither Kidby (1966) nor Robertson and Taylor (1973) reported the presence of an "acid" invertase in lupin nodules.

Maltase activity had a broad pH optimum between pH 4 and 5 (Fig. 1). For trehalase, pH optima

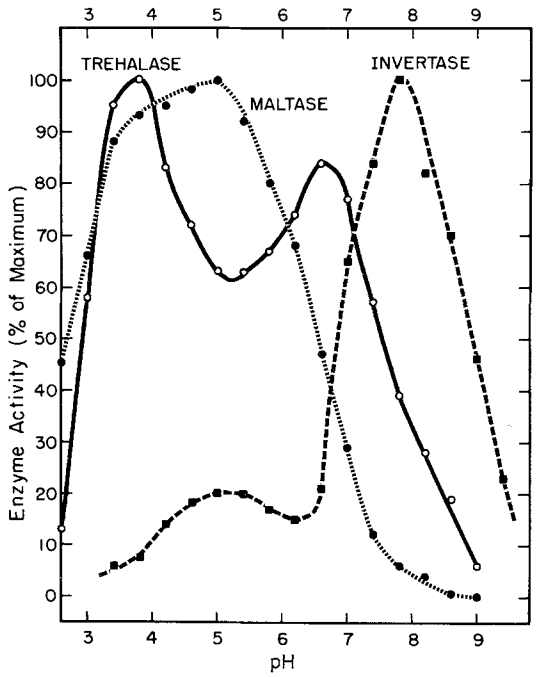

Fig. 1. Activity of trehalase, maltase, and invertase in crude, Sephadex-filtered extracts of soybean nodules as a function of pH. Non-enzymatic hydrolysis of sucrose below pH 5.0 and maltose below pH 3.5 is significant and was subtracted from total hydrolysis to obtain the curves shown. The same buffer (citrate + phosphate + glycine, each 50 mM) was used at all pHs

of 3.8 and 6.6 were observed, with slightly greater activity at the lower pH. Trehalase from other sources has a wide variety of pH optima, generally falling between 5.5 and 6.5 (see review by Elbein 1974). The only trehalases previously reported with pH optima of 4.0 or below are from the fungi (Elbein 1974). The pH optima shown in Fig. 1 were checked using extracts of bacteroids purified on Percoll gradients. For trehalase, the curves for the bacteroids and the cytosol were almost exactly coincident, both curves showing pH optima at 3.8 and 6.6. For maltase, the highest activities in the bacteroid extract were observed between pH 4.4 and 6.2, inclusive. The response of these enzyme activities to substrate over a 35- to 75-fold range of concentrations was determined. Invertase (pH 5.4 and 7.8), maltase (pH 5.0), and trehalase (pH 3.8 and 6.6) had apparent Km values of, in the order given, 13.0, 9.1, 1.0, 0.94, and 0.38 mM.

The bacteroids prepared on Percoll density gradients contained no detectable invertase activity at either pH 5.4 or pH 7.8 (Table 1). The bacteroids purified on Percoll gradients have high viability, contain no phosphoenol-pyruvate carboxylase, and contain virtually all of the asparaginase, alanine dehydrogenase and β-hydroxybutyrate dehydrogenase found in the crude homogenates of soybean nodules (Reibach et al. 1981). The results in Table 1 indicate that invertase in soybean nodules is completely host-derived. The trace of invertase reported by Robertson and Taylor (1973) in the bacteroids from lupin nodules may have been a consequence of contamination by the cytosol.

In contrast to invertase, the specific activity of maltase and trehalase was greater in the bacteroids than in the cytosol (Table 1). However, with much greater quantities of protein in the cytosol than the bacteroids, the total activity of maltase and trehalase was much greater in the cytosol than the bacteroids. Recovery of the protein from the bacteroids after sonication was variable, and was also probably incomplete. Thus the specific activity of maltase and trehalase in the bacteroids was variable. Results for total activity are considered more meaningful, and in an experiment where the yield of bacteroid protein was high, the maltase and trehalase activities in the cytosol were still about 7-fold greater than these enzyme activities in the bacteroids.

To localize cytosolic disaccharidase activities more precisely, nodule cortex was separated from the protoplasts of the infected region. The specific activity of invertase at pH 7.8 was greater in the nodule cortex than in the cytosol from the infected region (Table 2). Conversely, the specific activity of maltase and trehalase was greater in the cytosol from the infected region than in the cortex (Table 2). The calculation of total enzyme activity is risky in this experiment because large portions of the protein were lost during isolation and washing of the protoplasts. Microscope examination of the cortex tissue indicated little disruption of the cells during protoplast isolation and it is probable that most of the protein lost was from the cytosol. If this is true, the localization of invertase in the cortex is less pronounced than indicated in Table 2, while the localization of maltase and trehalase in the cytosol from the infected region is more pronounced than indicated in Table 2.

Table 1. Activity of disaccharidases in the bacteroids and cytosol of soybean root nodules prepared by Percoll density gradient centrifugation. Data are averages of two experiments which gave very similar results. In the second experiment, where crude extract was analyzed, 94% of the soluble protein and an average (five enzyme activities) of 93% of enzyme activity applied to the gradients was recovered in the two fractions. (Crude extract was also sonicated prior to Sephadex filtration and enzyme assays.)

	Invertase		Maltase	Trehalase	
	pH 5.4	pH 7.8	pH 5.0	pH 3.8	pH 6.6
Specific activity (μmol disaccharide mg^{-1} protein h^{-1})					
Bacteroid	0	0	1.68	2.45	3.86
Cytosol	0.41	6.30	0.48	1.18	1.18
Total activity (μmol disaccharide fraction^{-1} h^{-1})					
Bacteroid	0	0	2.7	3.6	6.1
Cytosol	28.1	416.2	33.2	80.2	80.2

Table 2. Activity of disaccharidases in soybean nodules and nodule fractions prepared after isolation of protoplasts. Values shown are μmol disaccharide mg^{-1} protein h^{-1}

	Invertase		Maltase	Trehalase	
	pH 5.4	pH 7.8	pH 5.0	pH 3.8	pH 6.6
Whole nodules[a]	1.0	5.3	0.89	1.6	1.5
Fractions (protein, mg/ml)[b]					
Cytosol[c] (0.14)	0.88	1.1	4.7	10.6	8.5
Cortex (0.71)	16.4	3.4	0.96	1.5	1.3

[a] Average of two or three samples

[b] Volume of each fraction was about 15 ml. However, total enzyme activity was not calculated because cytosol protein was lost during protoplast isolation when some protoplasts rupture

[c] From protoplasts of the infected region. May include a small amount of cytoplasm from the inner cortex

Bacteroids, infected protoplasts, rings of nodule cortex, roots (without nodules), and segments of hypocotyl were incubated with [^{14}C]maltose or [^{14}C]trehalose for four periods from 0 to 100 min. Although the rate of disaccharide hydrolysis and accumulation of ^{14}C in glucose varied widely among the tissue and cell types, all of the tissues and cells were capable of some hydrolysis of both disaccharides. Zero-time samples showed that the analytical methods or contamination of the [^{14}C] disaccharide substrates could not account for the accumulation of the ^{14}C in glucose.

The catabolism of trehalose is of interest because, in some mutualistic and parasitic associations involving higher plants, the invading fungus synthesizes trehalose, utilizing carbohydrate from the host (Lewis and Harley 1965; Long and Cooke 1974). Furthermore, the ability to metabolize trehalose is almost entirely restricted to the fungus, thus sequestering carbohydrate for the nearly exclusive use of the fungal symbiont (Lewis and Harley 1965). The sequestering of carbohydrate in several symbiotic systems has been reported (see review by Smith et al. 1969), but the concept has not been examined in legume nodules.

The average concentration of carbohydrates in the cells of *Rhizobium japonicum* grown in liquid culture was (mg carbohydrate/cm^3 of cells): α,α-trehalose, 5.85; glucose, 0.12; maltose and fructose, <0.1. The yeast extract in the medium contained about 3 mg of trehalose. With a yield of about 1.1 cm^3 of cells/l, this added trehalose could not account for the quantity of trehalose in the cells. Thus, *R. japonicum* synthesized large amounts of trehalose in liquid culture, using mannitol or glucose as a carbon source.

The synthesis of trehalose by *Rhizobium japonicum* in culture, the high concentration of trehalose in soybean nodules relative to other plant organs (Streeter 1980), and the accumulation of trehalose in senescing nodules (Streeter 1981) indicate that trehalose in nodules might represent carbohydrate sequestered by the microsymbiont. However, the hydrolysis of [^{14}C]trehalose by the nodule cortex and the concentration of trehalase in the cytosol (Table 2) demonstrates that the utilization of trehalose is not restricted to the bacteroids. The hydrolysis of [^{14}C]trehalose by the root and hypocotyl tissues also supports the proposition that trehalose can be metabolized outside of the bacteroids.

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