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Partial Purification and Characterization of Trehalase from Soybean Nodules

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Summary

Soybean (*Glycine max* cv. Maple Arrow) was infected with *Bradyrhizobium japonicum* (strain 61-A-101) and grown under semi-sterile conditions. Trehalase was extracted from nodules under acidic conditions (pH 3.7) and purified 530-fold by chromatography on concanavalin A-agarose, anion exchange chromatography and gel filtration. Its native molecular weight was close to 54 kDa. Activity staining after electrophoresis yielded only one band. Isoelectric focussing under non-denaturing conditions indicated an acidic isoelectric point (ca. pH 5.2). The enzyme had a broad pH-optimum (pH 3.5–7) and a temperature optimum of 59 °C. The K_m value for trehalose was 0.28 mM, close to the data given previously for crude nodule extracts. Trehalase was found to be relatively insensitive to chelators and divalent cations, indicating that it does not require a metal cofactor. Validamycin A, a fungitoxic antibioticum isolated from *Streptomyces*, competitively inhibited trehalase with a K_i value of 2 nM.

Key words: Bradyrhizobium, carbon metabolism, nodules, soybean, trehalase, symbiosis.

Abbreviations: Bis-Tris = bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; Con A = concanavalin A; fw = fresh weight; IEF = isoelectric focussing; MES = 2-(N-morpholino)ethanesulfonic acid; PMSF = phenylmethylsulfonylfluoride; TLC = thin layer chromatography; Tris = tris(hydroxymethyl)aminomethane.

Introduction

The non-reducing disaccharide trehalose (α-D-glucopyranosyl (1-1) α-D-glucopyranoside) is a common sugar of prokaryotes as well as of many heterotrophic eucaryotes, especially fungi and invertebrates (Elbein, 1974). In the vascular plants, it occurs in certain pteridophytes (clubmosses), and several eusporangiate ferns, where it often exceeds sucrose in concentration and indeed appears to replace sucrose as a translocated and stored disaccharide (Lewis, 1984). Trehalose has not been reproducibly identified in sterile spermatophytes (Gussin, 1972; Avigad, 1990). Almost all reports on trehalose in spermatophytes deal with diseased (Keen and Williams, 1969) or symbiotic organs. The latter include ectomycorrhiza (Harley and Smith, 1983), nitrogen-fixing soybean nodules (Streeter, 1985) and actinorhizal nodules (Lopez and Torrey, 1985). In soybean nodules, the trehalose found is synthesized by the microbial partner, but diffuses, at least partly, into the plant cell (Streeter, 1985; Streeter and Salminen, 1988).

Trehalose has been found to be toxic for certain plants and plant cell cultures (Veluthambi et al., 1981). Therefore, the capacity to detoxify trehalose by hydrolysis might be essential for plants living in symbiosis with organisms that accumulate trehalose. Indeed, the presence of trehalase in plant-bacterium symbioses has been described for actinorhizal nodules (Lopez and Torrey, 1985) and for soybean root nodules (Streeter, 1982; Salminen and Streeter, 1986). Later, it was found that trehalase was absent from bacteroids but present in the plant organelles containing the bacteroids

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(called «peribacteroid units (PBUs)» or «symbiosomes») as well as in the plant cytoplasm (Mellor, 1988). A recently published report, however, suggests trehalase to be present only in the symbiosomes (Kinnback and Werner, 1991). Trehalase has so far been characterized mainly in animals (Elbein, 1974) and fungi, especially in yeast (Thevelein, 1984). Here, we present a biochemical analysis of the trehalase in soybean root nodules and provide evidence that it is a glycoprotein of plant origin.

Materials and Methods

Plant material

Seeds of soybean (*Glycine max* L. Merr.) cv. Maple Arrow were surface-sterilized by immersion in 30% H_2O_2 for 20 min followed by washing with sterile tap-water. They were grown on agar plates and infected 4 days after germination with *Bradyrhizobium japonicum* 61-A-101 (fix^+), a strain yielding effective, nitrogen-fixing nodules (Stripf and Werner, 1978). The bacteria had been grown to stationary phase in 20E-medium (Werner et al., 1975) at 27 °C on a rotary shaker (140 rpm). Infected seedlings were grown in sterilized Leonard jars filled with perlite and nutrient solution (Werner et al., 1975) in a phytotron (14 h day at 300 $\mu E\,m^{-2}s^{-1}$ and 26 °C, 10 h night at 20 °C). Nodules were harvested 5–6 weeks after planting and either used fresh or frozen and stored at −20 °C. Callus cultures were prepared from excised root tips of sterile seedlings laid on a modified solid B5 medium containing 3% (w/v) instead of 2% (w/v) sucrose and 1 mg mL^{-1} instead of 0.1 mg mL^{-1} myo-inositol (Ebel et al., 1976). After 2 weeks, growing calli were sliced into small pieces (diameter ≈ 0.5 cm) and put on solid B5 medium without carbon source or 2% (w/v) sucrose or 2% (w/v) trehalose.

Enzyme extraction and partial purification of trehalase

Enzymes were extracted from frozen soybean nodules by grinding them in ice-cold 0.1 M citrate (Na^+), pH 3.7, (2 mL g fresh $weight^{-1}$) containing 1 mM PMSF, 2 mM EDTA and insoluble polyvinylpyrrolidone (10 mg g fresh $weight^{-1}$). Where indicated, 0.1 M MES (K^+), pH 6.3, or 0.1 M Tricine (K^+), pH 8.5, were used instead. The homogenate was filtered through nylon gauze (50 µm mesh) and centrifuged for 20 min at 48,000 rpm. The resulting crude extract was either dialysed overnight against 2 mM citrate (Na^+), pH 3.7, at 4 °C and lyophilized or precipitated with 80% (v/v) ice-cold acetone at 0 °C, followed by centrifugation (13,000 g for 10 min) and drying of the pellet under an air stream.

For affinity chromatography on Con A agarose (Sigma, St. Louis, U.S.A.), the lyophilizate was resuspended in 5 mM MES (K^+), pH 6.0, containing 50 mM NaCl and 0.1 mM each $MgCl_2$, $MnCl_2$ and $CaCl_2$ and dialyzed against the same buffer. The column was equilibrated with the same buffer ten-fold concentrated (buffer A). The dialyzed sample was applied to the column after raising the salt concentration to that of buffer A. The column was washed with buffer A. Glycoproteins were eluted from the column with buffer A containing 0.2 M methyl-α-D-mannopyranoside. The flow rate was 0.5 mL min^{-1}.

For ion-exchange chromatography, samples were dialyzed against 50 mM Bis-Tris (Cl^-), pH 6, and then applied to a MonoQ column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The column was first washed with the same buffer and then eluted step-wise with 10 mL each of 0.06, 0.12, 0.25 and 0.5 M NaCl in the same buffer. The flow rate was 1 mL min^{-1}. The fractions with the highest activity were pooled, frozen and lyophilized.

The lyophilized samples were redissolved in 50 mM Tricine (K^+), pH 7.6, containing 0.15 M NaCl and portions of 200 µL were applied to column of Superose 12 (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 0.2 mL min^{-1}. The native molecular weight of soybean trehalase was estimated by comparison to marker proteins.

Enzyme assays and protein determination

In the standard assay, trehalase activity was measured by estimating the glucose produced by hydrolysis of trehalose with the glucose oxidase-peroxidase method using a test kit (Boehringer, Mannheim, Germany). Unless indicated otherwise, the reaction mixture contained 10 mM trehalose and 50 mM of an appropriate buffer, in general MES (K^+), pH 6.3, and was incubated at 37 °C for 30 min. The reaction was stopped by boiling for 3 min. Precipitates were removed by centrifugation (2000 g, 10 min). For continuous monitoring of trehalase activity, e.g. for the determination of kinetic parameters, a coupled assay was used instead. The reaction mixture contained 50 mM of MES (K^+), pH 6.3, trehalose in different concentrations, 10 U of glucose oxidase and peroxidase (Boehringer, Mannheim) and 0.4 mM guaiacol. The reaction was started by addition of the enzyme and followed by continuously measuring the absorbance at 470 nm. Enzyme and substrate blanks were subtracted. Activities are expressed in nmol trehalose hydrolyzed per second (= nkat). Possible influences of effectors on the glucose oxidase reaction were taken into account by internal standards.

Protein was assayed according to Bradford (1976).

Electrophoresis, isoelectric focussing and activity staining

Trehalase was analyzed on non-denaturing gels using 6% (w/v) polyacrylamide in 0.375 M Tris (Cl^-), pH 9, for the gels and 0.192 M glycine adjusted to pH 9 with Tris base as running buffer. The samples contained 10% (v/v) glycerol and 10 pm bromophenol blue. Electrophoresis was performed at 10 mA 4 °C in a minigel-apparatus (Biorad, Richmond, Cal., USA).

Trehalase activity was detected by staining the gels with an agar overlay (10 mL) of 1% (w/v) low melting agar containing trehalose, buffer, glucose oxidase and peroxidase as described for the coupled assay, with the only exception that 20 mg 4-chloro-1-naphthol dissolved in 2 mL methanol were used instead of guaiacol. For controls, trehalose was omitted.

Isoelectrical focussing (IEF) in liquid medium was performed in a Biorad Rotaphor apparatus using 2% (w/v) Biolyte (pH range 3–10, 2200 Vh), as specified by the supplier. After the run, pH and trehalase activity were measured in the collected fractions.

Chemicals

The chemicals were purchased (when not otherwise indicated) from Fluka or Merck in the highest available purity. Validamycin A was provided by CIBA Geigy AG, Basel.

Results

Trehalase in Soybean Callus Cultures

As previously reported (Veluthambi et al., 1981), callus cultures of soybean were able to grow on trehalose as sole carbon source, but the growth rate was lower than on sucrose. Indeed, at the moment of harvesting, the calli grown on trehalose had a biomass of abbout 35% of those grown

Table 1: Trehalase activity in supernatants from soybean nodules after extraction at different pH values and centrifugation at 48,000 g for 20 min.

Buffer	Enzyme activity nkat per g fresh weight	nkat per mg protein
Tricine (K^+) pH 8.5	10.55	0.57
MES (K^+) pH 6.3	9.91	1.06
Citrate (Na^+) pH 3.7	10.12	2.14

Table 2: Partial purification of trehalase from soybean nodules.

Step	Total protein (mg)	Trehalase activity total (nkat)	specific (nkat mg prot.$^{-1}$)	activity ratio pH 6.3/ pH 3.7	Purification Factor
1. Crude extract (pH 3.7)	373	1303	3	0.8	1
2. Dialysis (pH 3.7)	243	1335	5	1	1.6
3. Lyophilization	114	1285	11	1.1	3
4. Chromatography on Con A	10	776	77	1.1	23
5. Chromatography on MonoQ	1.4	364	263	1.3	75
6. Gel filtration on Superose 12	0.13	201	1596	1.3	530

on sucrose. On the plates without additional carbon source, no growth was observed. Crude extracts from 8-week-old soybean callus cultures grown on sucrose exhibited an activity similar to uninfected roots (0.32 nkat · g fw^{-1}). In callus cultures grown on trehalose, this activity was approximately doubled (0.7 nkat · g fw^{-1}). This activity was far below that measured in nitrogen fixing nodules.

Trehalase in Soybean Root Nodules

Trehalase activity was measured in crude extracts (pH 6.3) from 5-week-old nodules and from uninfected roots of the same age. The activity was about 30 times higher in nodules than in roots (10.1 nkat · g fw^{-1} vs. 0.3 nkat · g fw^{-1}). Trehalase thus may be considered as a nodule-stimulated protein. In callus culture, and root and nodule extracts, trehalase activity was completely inhibited by 20 µM validamycin A, a potent inhibitor of fungal trehalases (Asano et al., 1987). To study nodule trehalase in more detail, the enzyme was partially purified from 5–6-week-old nodules.

Similar amounts of trehalase were extracted from the nodules when using buffers ranging from pH 8.8 to 3.7. Extraction at pH 3.7 yielded about four times less protein than extraction at pH 8.8 after centrifugation for 20 min at 48,000 g (Table 1). Overnight dialysis against citrate buffer at pH 3.7 increased this factor to about 6. Trehalase activity in crude extracts was completely stable for at least 3 months at −20 °C and also upon dialysis and lyophilization. Precipitation by 80 % acetone at 0 °C was a rapid and useful method to concentrate and desalt the enzyme. After resuspension of the pellet in appropriate buffer, the entire trehalase activity was recovered. After ultracentrifugation at 105,000 g for 1 h all activity remained in the supernatant; thus, soybean trehalase is thought to be a soluble enzyme.

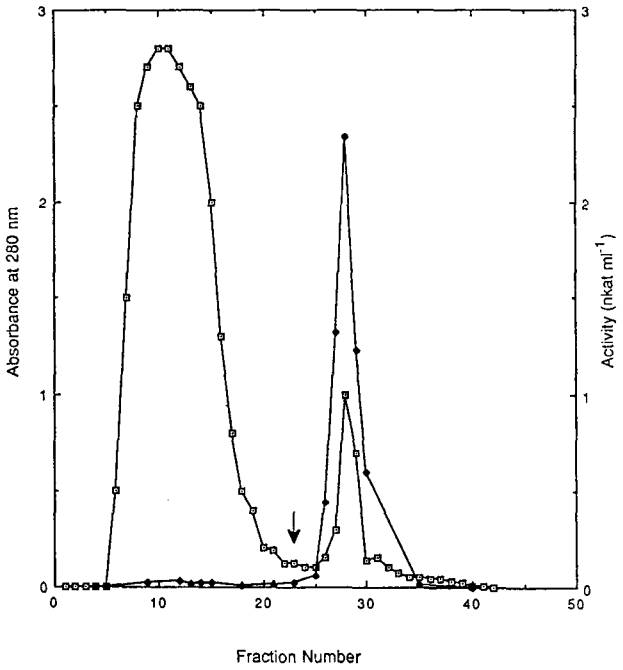

Fig. 1: Concanavalin A agarose column chromatography of a trehalase preparation from soybean nodules. Elution with 200 mM α-methylmannopyranoside started at the time indicated by an arrow. Activity applied, 6.5 nkat; flow rate, 0.5 mL min^{-1}; fraction volume, 1 mL. Absorbance at 280 nm (□) and trehalase activity (●) are indicated. Trehalase activity was measured at 37 °C and pH 6.3.

Partial purification of soybean nodule trehalase

Trehalase was partially purified by lectin affinity chromatography, ion exchange chromatography and gel filtration (Table 2). Trehalase activity was completely retained on a Con A agarose column and could be eluted by α-methylmannopyranoside (Fig. 1) providing seven-fold purification with about 60 % yield in the main fractions. Ion exchange chromatography on MonoQ yielded a single peak of trehalase activity and resulted in a four-fold increase of specific activity. After dialysis and lyophilization, pooled peak fractions were applied to a Superose 12 column, which resulted in a six-fold increase in specific activity (Table 2). A molecular mass of 54 kDa was estimated (Fig. 2).

Overall, these procedures resulted in a 530-fold purification with ca. 15 % yield (Table 2). Interestingly, the ratio of trehalase activity at pHs 3.7 and 6.3 changed only little during purification (Table 2).

Characterization of soybean root nodule trehalase

On non-denaturing polyacrylamide gel electrophoresis, both dialyzed crude extracts and partially purified enzyme exhibited one activity band (Fig. 3 A). IEF in liquid medium gave an activity optimum at about pH 5.2 (Fig. 3 B).

Crude soybean trehalase manifested a broad pH optimum between 3.7 and 7 in all buffer systems tested (Fig. 4). As indicated in Table 2, the acidic optimum of activity did not disappear during purification. The maximal enzyme activity oc-

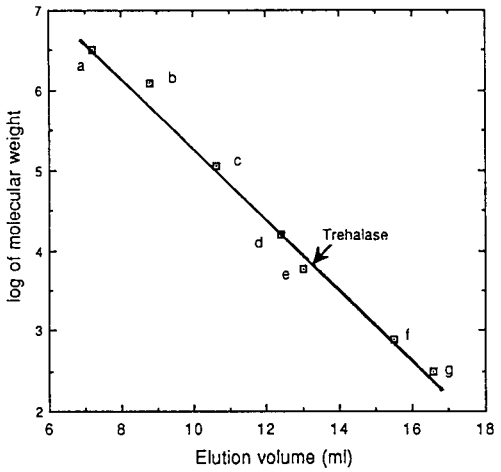

Fig. 2: Estimation of the molecular mass of partially purified trehalase from soybean nodules by comparison with marker proteins in high performance gel filtration. 400 µg of partially purified enzyme (Table 2, step 5) were passed through a Superose 12 column. Fractions of 0.5 mL each were collected. The column was calibrated with (a) thyroglobulin (669 kDa), (b) ferritin (440 kDa), (c) aldolase (158 kDa), (d) bovine serum albumin (67 kDa), (e) ovalbumin (43 kDa), myoglobulin (18 kDa) and cytochrome c (12 kDa). The position of the marker proteins was determined by measuring the absorption of 280 nm, and the position of trehalase was determined by measuring enzymatic activity at 37 °C and pH 6.3.

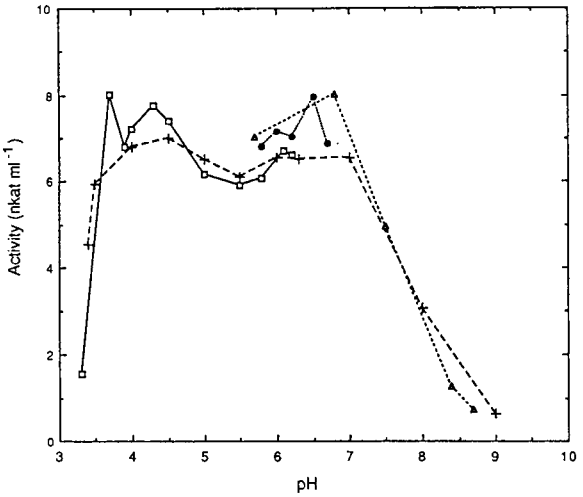

Fig. 4: pH dependence of trehalase activity from soybean nodules. As enzyme source, a dialyzed and lyophilized crude extract was used. The tested buffer systems were citrate (Na^+) (□), MES (K^+) (●) and Tricine (K^+) (△), 100 mM each or a ternary buffer system (methylpiperazine, MES and Tricine, 125 mM each) (+). The reaction mixtures were incubated at 37 °C and stopped after 30 min.

curred at approximately 59–65 °C. The energy of activation, calculated from the activity data between 0° and 50 °C according to the Arrhenius equation, was 10.8 kcal · mole^{-1}. The K_m value for trehalase was 0.28 mM (Fig. 5 A). Interestingly, soybean trehalase is strongly inhibited by the antibioticum validamycin A, a trehalose analogue known to inhibit fungal trehalases (Asano et al., 1987). The inhibition was competitive with a K_i of 2 nM (Fig. 5 B). Metal cations, chelators (EDTA, phenanthroline) and sulfhydryl reagents (Iodoacetate, E-64) had no effect on activity (Table 3). Phosphate did not stimulate trehalase activity. The enzyme was not inactivated by treatment with 5 mM EDTA followed by desalting.

To test substrate specificity, partially purified trehalase was incubated with different glucosides. As Table 4 shows, apart from trehalose only maltose was significantly cleaved by the Con A-purified enzyme preparations. Maltase activity, however, was separated from trehalase in subsequent purification steps. As reported in Table 5, maltase activity decreased to about 3 % of the trehalase activity in the Superose 12 fraction. Whereas the K_m value for maltose hydrolysis was in the same order of magnitude as the K_m for trehalose hydrolysis, maltase activity was only weakly sensitive to va-

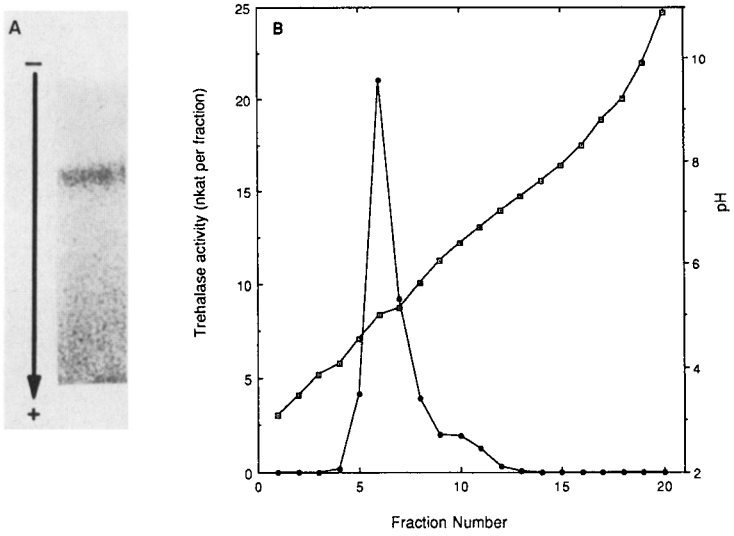

Fig. 3: A. Activity staining of trehalase from soybean nodules after gel electrophoresis under non-denaturing conditions (PAGE at pH 9, 1 nkat per lane). B. Isoelectric focussing under non-denaturing conditions in liquid medium (Biorad rotaphor, pH 3–10, 2200 Vh); 40 nkat were applied. The activity was measured at 37 °C and pH 6.3. Enzyme activity (●) and pH of the fractions (□) are indicated.

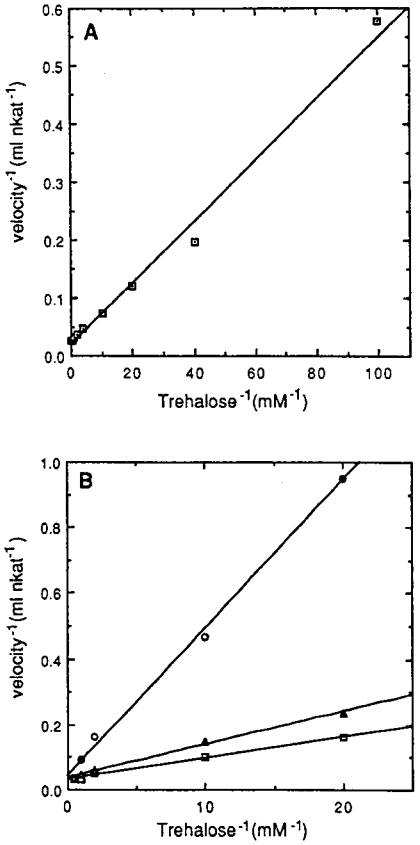

Fig. 5: Enzyme kinetics of trehalase purified by MonoQ chromatography (step 5, Table 1). The assays were performed using a coupled assay as described in Material and Methods. A. Determination of the K_m value for trehalose in a Lineweaver-Burk-plot. B. Determination of the K_i for validamycin A in a Lineweaver-Burk-plot. The following concentrations of validamycin A were used: 0 nM (□); 2 nM (△); 20 nM (○).

Table 3: Influence of various additions on trehalase activity in dialyzed and lyophilized extracts from soybean nodules. The preparation was incubated at pH 6.3 and 37 °C (activity without additions: 100 %). The concentration of the tested effectors was 10 mM if not otherwise indicated. Cations were used as Cl^--salts, anions as Na^+-salts.

Treatment	Activity (%)
Ca^{2+}	100
Mg^{2+}	100
Zn^{2+}	106
Phosphate	85
Borate	93
Succinate	87
Phenanthroline (0.1 mM)	118
EDTA (2 mM)	105
Iodoacetic acid	99
E-64 (0.1 mM)	110
Validamycin A (20 µM)	< 0.1

lidamycin A, the K_m/K_i ratios being about 4 orders of magnitude lower than for trehalase (Table 5).

Table 4: Saccharidase activities of a trehalase-containing enzyme preparation from soybean root nodules after partial purification on ConA agarose. The release of glucose was measured at pH 6.3 and 37 °C using various glucosides (100 mM) as substrates (activity on trehalose: 100 %).

Substrate	Activity (%)
Trehalose	100
Lactose	2.5
Sucrose	1.7
Turanose	< 0.1
Cellobiose	3.6
Maltose	12.2
Maltotriose	3.9
Raffinose	0.7
Soluble starch (0.5 %)	< 0.1

Table 5: Maltase activity in trehalase preparations of different degrees of purity. The release of glucose was measured at pH 6.3 and 37 °C (activity on trehalose: 100 %) by standard assays containing maltose (TLC purity) between 20 and 0.2 mM.

Step	Maltase activity (%)	K_m (maltose) (mM)	K_i (Validamycin A) (mM)
Crude (dial., lyo.)	39.4	0.43	0.083
Con A	12.2	0.48	0.110
MonoQ	7.5	0.57	0.073
Superose 12	3.2	0.65	0.090

Discussion

Our observations show that trehalase occurs in soybean roots and that its activity strongly increases in nodules, indicating that it is a nodule-stimulated protein. The following lines of evidence suggest that the nodule-stimulated trehalase is a plant enzyme: (i) The enzyme is present in callus cultures and sterile roots, (ii) The enzyme binds to Con A and can be eluted by α-methylmannoside, indicating that it is a glycoprotein, (iii) The enzyme is present not only in the infected zone, but also in the nodule cortex (data not shown) and (iv) Both bacteroids and free-living bacteria (Mellor, 1988 and unpublished data) lack trehalase activity. Soybean trehalase appears to be highly specific for trehalose, at least concerning the tested series of glucosides. The maltase activity present at low levels in partially purified trehalase preparations appeared to be due to a different contaminating enzyme. This enzyme may be related to one of the α-glucosidases purified from sugar beet seeds (Yamasaki and Suzuki, 1980). Since phosphate has only little effect on soybean trehalase activity, the trehalose-splitting activity is certainly not due to a phosphotrehalase, as partially purified from *Euglena* (Maréchal and Belocopitow, 1972).

The properties of soybean trehalase are similar to those of *Lilium* pollen trehalase (Gussin and Cormack, 1970), both enzymes having a broad pH optimum (4–6), a high temperature optimum (58 °C) and a K_m in the same order of magnitude. In the kingdom of fungi, two classes of trehalases have been characterized from several species (Londesborough and Varimo, 1984; see also Thevelein, 1984). The fungal acid trehalases have greater similarities to the soybean enzyme than to fungal neutral trehalases. Like soybean trehalase, the acid

trehalase purified from yeast (Mittenbühler and Holzer, 1988) is a glycoprotein, is insensitive to sulfhydryl reagents and zinc ions and does not require metal cofactors. The native molecular mass of yeast acid trehalase, however, is much higher than the molecular mass of the soybean enzyme. In contrast to yeast acid trehalase and the soybean enzyme, yeast neutral trehalase has completely different properties. It is highly sensitive to Zn^{2+}, sulfhydryl reagents and EDTA (1 mM), is not a glycoprotein and is activated by phosphorylation (Londesborough and Varimo, 1984). The K_m of the purified enzyme (34.5 mM) is two orders of magnitude higher than the K_m of the soybean enzyme (App and Holzer, 1989).

The question whether trehalase activity in soybean nodules is due to one enzyme or several distinct ones remains unresolved. Based on the pH optimum curve (Fig. 4), on the nearly parallel copurification of activity at pHs 3.7 and 6.3 (Table 2) and on the activity staining results, we favour the hypothesis that trehalase activity was caused by one enzyme rather than by two as suggested by previous reports (Streeter, 1982; Mellor, 1988). More investigations will be necessary to provide a definitive answer to this question and to reveal the role of trehalase and trehalose in symbiosis.

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