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Purification and properties of trehalase in *Frankia* ArI3

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Abstract. Trehalase was purified from cultures of *Frankia* strain ArI3 grown on media with or without NH_4Cl. The purified enzyme was specific for trehalose, exhibited a broad pH optimum of pH 4.5 to 5.3 and had a K_m for trehalose of 4.2 mM. The trehalase was inhibited in vitro completely by sucrose, glucose and mannose and partially by mannitol and sorbitol. In addition to the specific trehalase, a mixture of non-specific α- and β-glucosidases which exhibited some activity with α,α-trehalose as a substrate were also partially purified in *Frankia* extracts made from nitrogen-fixing cells. These enzymes were not detected in the purifications of crude extracts made from non-nitrogen-fixing cells (grown on media supplemented with NH_4Cl). Trehalase activity in crude extracts increased over time when cells were induced to fix nitrogen, and the maximum specific activity of trehalase from nitrogen-fixing cultures was 4 times the maximum activity from non-fixing cultures. Trehalase activity was also examined in crude extracts made from *Frankia* vesicle clusters isolated from *Alnus rubra* nitrogen-fixing nodules infected with ArI3. The maximum activity of trehalase in these clusters was 6–7 times greater than in the nitrogen-fixing pure cultures of ArI3 and 26–33 times greater than the non-fixing pure cultures.

Key words: *Frankia* – Trehalose – Trehalase – Nitrogen-fixation – Carbon metabolism

Trehalose, (α-D-glucopyranosyl α-D-glucopyranoside), is found in a wide variety of organisms including yeast, fungi, insects, bacteria and plants (Elbein 1974). Among the bacteria, trehalose has been detected in the mycobacteria (Elbein and Mitchell 1973), streptomycetes (Hey and Elbein 1968; Hey-Ferguson et al. 1973), pseudomonads (Guilloux et al. 1971), *Bacillus* (Crabbe et al. 1969), and more recently in the nitrogen-fixing symbionts *Rhizobium* (Streeter 1982) and *Frankia* (Lopez et al. 1983, 1984). Trehalose serves a variety of functions in these organisms but its primary role appears to be as a stored source of easily available carbon (Panek 1963; Elbein and Mitchell 1973; Hey-Ferguson et al. 1973; Hirokazu and Shimoda 1981; Thevelein 1984). The hydrolysis of trehalose to D-glucose, except in one instance (Marechal and Belocopitow 1972), is effected by the enzyme trehalase. Properties of trehalases from different organisms are highly variable, with pH optima ranging from 3.7 to 7.5 and apparent K_m values for trehalose ranging from 400 μM to 60 mM (Elbein 1974). In this report, trehalase from the *Frankia* isolate ArI3 was purified using DEAE cellulose chromatography and its properties were investigated. In addition, crude extracts from *Frankia* vesicle clusters isolated from *Alnus rubra* nodules infected with ArI3 were assayed for trehalase activity, and a comparison of the specific activity of trehalase in nitrogen fixing and non-nitrogen fixing pure cultures of ArI3 was made.

Abbreviations. pcv, packed cell volume; DTE, dithioerythritol; PMSF, phenylmethylsulphonylfluoride; EDTA, sodium ethylenediaminetetraacetate

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Methods

Chemicals. All enzymes and substrates used in the enzyme assays and purifications were the highest purity available from Sigma Chemical Co., St. Louis, MO, USA.

Bacterial strains. Frankia strain HFP013103 hereafter referred to as ArI3 (Berry and Torrey 1979) was the strain used. Nodule material was obtained from seedlings of *Alnus rubra* grown aeroponically (Zobel et al. 1976) in the greenhouse and inoculated with the strain ArI3.

Media and cultural conditions. The BAP medium and B medium of Murry et al. (1984) supplemented with 5 mM Na propionate as a carbon source were used for growing all bacterial cells. B medium was modified by adding vitamins in the same concentrations as in BAP medium. Sodium propionate at 1 M was filter sterilized and added separately to autoclaved medium. Cultures on medium supplemented with ammonia were grown in 1 or 6 l airsparged, magnetically stirred bottles, maintained at 28°C. Logarithmic-phase cells (approximately 5–7 days old), were harvested by centrifugaiton and washed in cold 10 mM potassium phosphate buffer, pH 6.7. For obtaining nitrogen-fixing cultures a modification of the method of Murry et al. (1984) was used. Inocula were washed twice in B medium and inoculated into 6 l of B medium plus 5 mM Na propionate. Cultures were sparged with air and stirred. Approximately 3 ml packed cell volume (pcv) was used as inoculum per 1 l of medium. Nitrogenase activity was monitored by the acetylene reduction technique (Murry et al. 1984) and cells with maximum activity (unless otherwise specified) were harvested for enzyme assays and purification as described above.

Isolation of vesicle clusters. A modification of the methods of Akkermans et al. (1981) and Benson (1982) was used to

isolate endophytic vesicle clusters from *Alnus rubra* nodules. Nodules were harvested, washed in distilled water and placed in an appropriate volume of ice cold 0.2 M glycylglycine buffer, pH 7.5+4% soluble polyvinylpyrrolidone in a Waring blender. The entire blender apparatus was placed inside a glove box flushed with nitrogen. The nodules were homogenized, filtered through a 102 μm Nitex filter and the filtrate was then passed through a 32 μm Nitex filter. The residue, which contained the intact vesicle clusters, was washed 3 times with ice cold buffer and then resuspended in an appropriate buffer for the enzyme assay. Homogenization and filtration were done anaerobically to minimize damage to enzymes from oxidizable phenolic compounds which are present in abundance in alder nodules. Once the vesicle clusters were isolated, the phenolic substances were left in the filtrate which was then discarded.

Preparation of crude cell-free extracts for assays. Cells and vesicle clusters were harvested and washed as described above. The pellet was resuspended in the buffer to be used in a particular assay or in 10 mM potassium phosphate pH 6.7 and kept on ice. Approximately 1 ml pcv to 2 ml of buffer was used. The probe from a Braun sonicator was precooled in ice and the cell suspension was sonicated at 400 W on ice for a total of 1 min in 15 s bursts in between which the probe was cooled in ice. The sonicate was then centrifuged at 13,000 rpm ($10{,}000 \times g$) in a Beckman refrigerated centrifuge for 15 min. The pellet was discarded and the supernatant was considered the cell-free extract. Cell-free extracts were always kept on ice.

Determination of protein concentration in cell-free extracts and cultures. Protein concentrations were estimated by using the method of Bradford (1976) with reagents commericially prepared by Bio-Rad Laboratories (CA, USA).

Enzyme assays. Reaction mixtures contained, in a final volume of 1 ml; 200 – 1,000 μmol glycylglycine or potassium phosphate buffer, of specified pH, 10 – 150 μmol trehalose and cell-free extract or purified enzyme. Controls were run with boiled extract or enzyme. The reaction mixtures were incubated for 2 h at 33° C, unless otherwise stated, and then boiled for 10 min to stop the reaction. Glucose was determined by using the glucose oxidase assay commercially prepared by Sigma Chemical Co., St. Louis, MO, USA. All assays were run in duplicate, with duplicate controls. Apparent K_m values were determined by linear regression analysis of double reciprocal plots obtained from three separate experiments performed on enzyme from two separate purifications. Each plot consisted of 10 data points representing the mean value between two replicate treatments.

Extraction and assay of free trehalose. Free trehalose was extracted and assayed according to previously published methods (Lopez et al. 1984).

Purification of trehalase. Trehalase was purified using a modification of the method of Hill and Sussman (1963). Cells were harvested as described above and 150 – 200 ml pcv were sonicated at 400 W for 1 min in 30 s bursts in an equal amount of 0.1 M Tris/HCl buffer pH 7.5 or in 1 M potassium phosphate buffer pH 7.5 plus 0.001 M dithioerythritol (DTE), 0.001 M phenylmethylsulphonylfluoride (PMSF), 0.003 M sodium ethylenediaminetetraacetate (EDTA) over ice. The sonicator probe was cooled in ice in between each burst of sonication. The crude extract was then centrifuged at $10{,}000 \times g$ for 15 min in a refrigerated centrifuge at 3° C. The pH of the supernatant was adjusted if necessary to 7.5 with dilute KOH or HCl. Ammonium sulfate was then added slowly to 80% saturation and the mixture was centrifuged at $13{,}000 \times g$ for 15 min to pellet precipitated protein. The pellet was redissolved in 50 ml of 0.01 M Tris/HCl or 0.01 M potassium phosphate buffer, pH 7.5 plus 0.001 M DTE. The 50 ml sample was dialyzed for 2 h and then overnight at 5° C each time against 5 l of 0.01 M Tris/HCl or potassium phosphate buffer, pH 7.5 plus 0.001 M DTE. The dialyzed sample of protein was layered onto a column of 3×15 cm DEAE cellulose that had been previously equilibrated with 400 ml of 0.01 M Tris/HCl or potassium phosphate buffer pH 7.5 plus 20% glycerol. The proteins were eluted with 400 ml of a linear gradient of 0.01 M Tris/HCl or potassium phosphate buffer pH 7.5 plus 0.001 M DTE and 20% glycerol; and 0.1 M Tris/HCl or potassium phosphate buffer pH 7.5 plus 0.3 – 0.5 M NaCl, 20% glycerol and 0.001 M DTE. The flow rate was set at approximately 1 ml/min and eighty 5 ml fractions were collected. The elution was done at room temperature. Immediately after collecting the fractions, 1 ml of glycerol was added to each and the fractions were stored overnight at −15° C.

Results

pH optima of crude extract and elution pattern of purified trehalase. Figure 1a, b show the pH optima of trehalase activity in crude extracts of ArI3 cells. A biphasic response to pH was observed with optima at pH 5.0 and pH 7.5. The relative proportion of specific activity at each optimum varied with the cultural conditions of the organism. When combined nitrogen was omitted from the medium (i.e. B medium), ArI3 was induced to differentiate specialized structures termed vesicles and to fix dinitrogen (see Murry et al. 1984; Lopez et al. 1984 and Fig. 4 for typical kinetics of induction). ArI3 cells grown in BAP medium containing NH_4Cl (not fixing nitrogen) consistently showed a much greater proportion of trehalase activity at the acid optimum (Fig. 1a). Cells which had been induced to fix nitrogen by the omission of NH_4Cl from the growth medium showed a different pattern, with a more equal distribution of specific activity between the acidic and the alkaline pH optima (Fig. 1b).

When crude extracts from ArI3 were purified on DEAE cellulose, two different patterns were obtained, depending on whether the cells had been supplemented with NH_4Cl or not. Figure 2 shows a typical elution pattern obtained with a crude extract made from non-nitrogen-fixing cells grown in medium containing NH_4Cl. The fractions were assayed at pH 5.0 and 7.5, the optima for trehalase activity observed with the crude extract (Fig. 1). Trehalase activity was obtained only at pH 5.0 and in fractions 64 – 70. No activity was obtained with any fraction at pH 7.5.

Figure 3 shows a typical elution pattern of a crude extract made from nitrogen-fixing cells grown on media lacking NH_4Cl. At pH 5.0, two widely separated peaks of trehalase activity were obtained (Fig. 3a) and the first one was confined essentially to one 5 ml fraction. The second peak occurred in fractions 61 through approximately 68 and appeared to have a very similar elution pattern to that seen

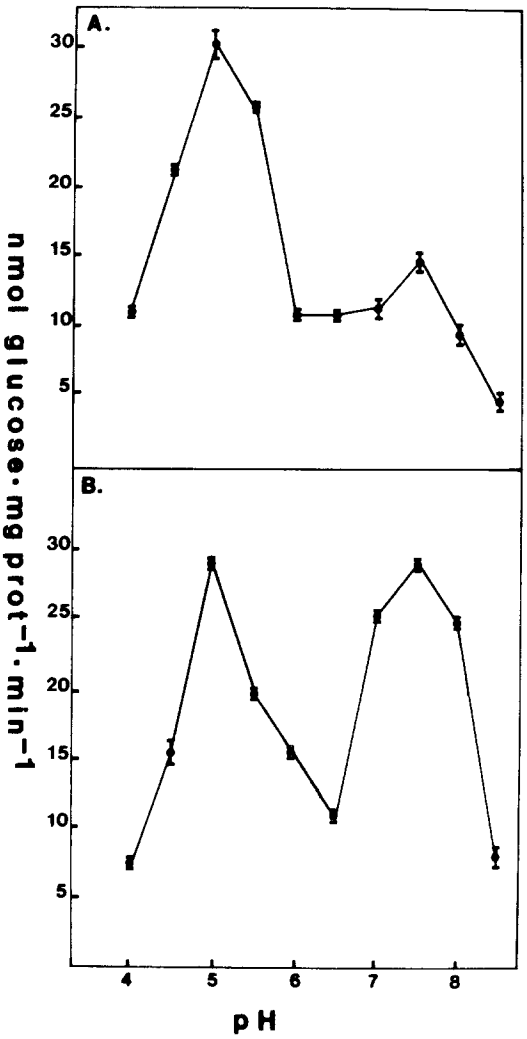

Fig. 1A, B. Effect of pH on trehalase activity in crude extracts of ArI3. **A** Non-N_2-fixing culture grown on BAP (i.e., $+NH_4Cl$) medium; **B** N_2-fixing culture grown on B (i.e., $-NH_4Cl$) medium. Nitrogen-fixing cultures had nitrogenase activity (acetylene reduction) of a minimum of 100 nmol ethylene evolved · mg prot^{-1} · h^{-1}

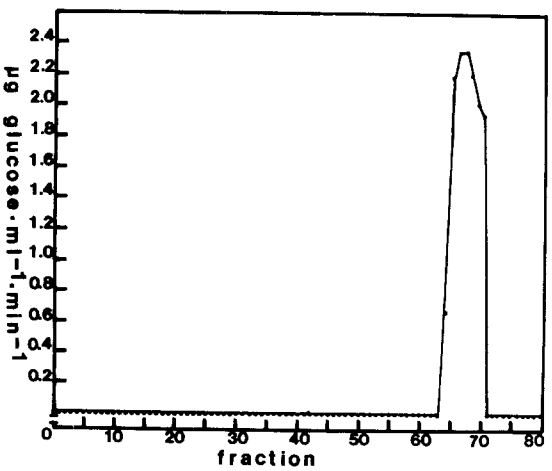

Fig. 2. Elution pattern of trehalase activity from ArI3 cells grown in media supplemented with NH_4Cl (not fixing nitrogen). Fractions were assayed at pH 5.0 and 7.5. Pattern from assays at pH 5.0 is shown. No activity was detected in any fraction assayed at pH 7.5

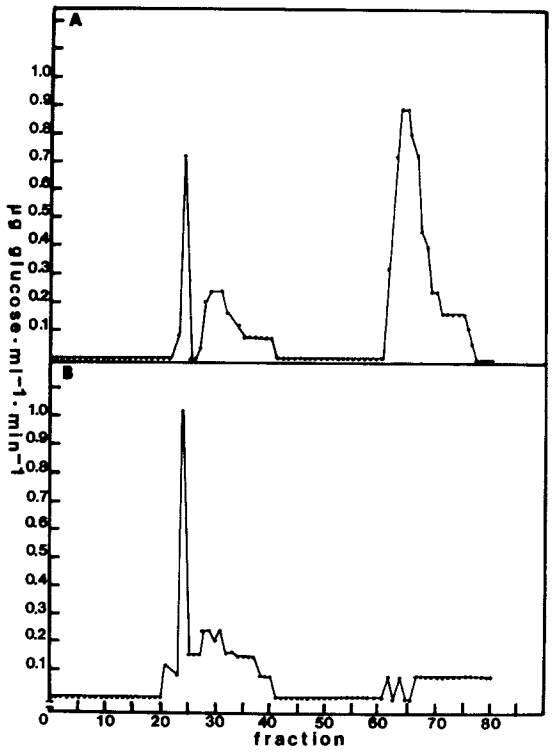

Fig. 3A, B. Elution pattern of trehalase activity from ArI3 cells fixing nitrogen. **A** Fractions assayed at pH 5.0; **B** fractions assayed at pH 7.5. Nitrogen-fixing cells had nitrogenase activity (acetylene reduction) of a minimum of 100 nmol ethylene evolved · mg prot^{-1} · h^{-1}

Table 1. Purification of trehalose-cleaving enzymes from ArI3

Fraction	Specific activity[a]	Purification factor
Crude extract	6.3	–
After 80% ammonium sulfate precipitation and dialysis	132.2	20.9
After DEAE cellulose chromatography		
fraction 24	348.4	55.3
fraction 63	247.2	39.2

[a] nmol glucose·mg prot^{-1}·min^{-1}; all fractions were assayed at pH 5.0

Data shown were obtained with fractions from the purification depicted in Fig. 2

in the extract made from ammonia-grown cells (Fig. 2). When the fractions obtained from the nitrogen-fixing cells were assayed at pH 7.5, only the first peak was observed and the activity was again confined to one 5 ml fraction, number 24 (Fig. 3b). The purifications of crude extracts from fixing and non-fixing cells were each performed twice and the elution patterns for similar extracts were nearly identical (data not shown). Table 1 summarizes the purification achieved in the elution from Fig. 3. After DEAE cellulose chromatography, the trehalose cleaving enzymes were purified by a factor of 55 and 39 for fractions 24 and 63 respectively.

Table 2. Substrate specificity of trehalose-cleaving enzymes from ArI3

Substrate	Specific activity[a]	
	Fraction 24	Fractions 62–66 combined
Trehalose	87.6	95.2
Sucrose	<1	<1
Lactose	464.9	<1
Maltose	154.8	<1
Cellobiose	154.8	<1

[a] nmol glucose·mg prot^{-1}·min^{-1}; all assays were performed at pH 5.0

Data shown were obtained with fractions from the purification depicted in Fig. 2

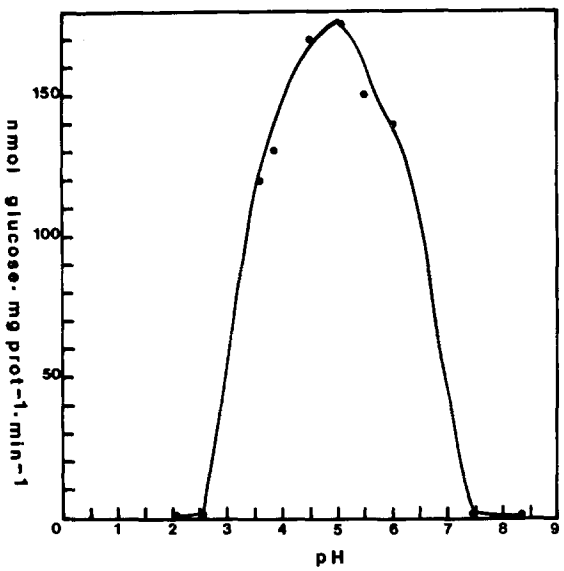

Fig. 4. Effect of pH on purified trehalase from ArI3. The pH curve shown was obtained with the combined fractions 62–66 from the purification shown in Fig. 2

Specificity of the purified trehalase(s). Fractions 24 and 62–66 combined from the purification shown in Fig. 3 were assayed for substrate specificity at pH 5.0 and these results are presented in Table 2. The enzyme(s) collected in fraction 24 showed activity with 4 out of 5 of the substrates tested, including trehalose, lactose, maltose and cellobiose. No activity was obtained with sucrose as a substrate. The activity with lactose was 3–4 times greater than with the other substrates and the activity with trehalose was the lowest. This suggests that there were at least two enzymes present in fraction 24 since the substrates with which activity was observed included disaccharides with α- and β-glucose or galactose linkages. The combined fractions 62–66 however were highly specific for trehalose (Table 2) and showed no detectable activity with any of the other substrates tested.

pH optimum and kinetic properties of the specific trehalase. Figure 4 shows the effect of pH on the purified trehalase from fractions 62–66. A broad pH optimum was obtained, with highest activity occurring between pH 4.5 and 5.3. The responses to trehalose concentration of the specific trehalases obtained from three separate purifications were assayed and a K_m of 4.7 mM was obtained by linear regression analysis with a standard error of less than 10% and r values between 0.79 and 0.98.

Table 3. Inhibition of specific trehalase by other metabolites and effect of c AMP

Carbon source[a]	% inhibition
Sucrose	100
Mannose	100
Glucose	100
Maltose	46
Fructose	29
Mannitol	28
Sorbitol	22
Gluconate	0
Lactose	0
Cellobiose	0
Pyruvate	0
Acetate	0
Malate	0
Succinate	0
Propionate	0
Citrate	0
c AMP[b]	0

[a] All carbon sources were tested at 12 mM final concentration in assay mixture

[b] % inhibition or stimulation

Final concentrations of reactants in assay mixture: 10 mM $MgSO_4$, 4 mM ATP, 0.06 mM cAMP (VanLaere and Hendrix 1983)

Inhibition of the specific trehalase by other metabolites and effect of cyclic AMP. The susceptibility of the specific trehalase from fractions 62–66 to inhibition by other metabolites was tested since inhibition of trehalases from other organisms by sugars and sugar alcohols has been observed (Elbein 1974). Table 3 shows the effect of various sugars, sugar alcohols and organic acids on the specific activity of the purified trehalase from ArI3. Sucrose, mannose and glucose at a concentration of 12 mM completely inhibited trehalase activity. Maltose inhibited the activity by roughly 50% and fructose, mannitol and sorbitol had roughly a 20–30% inhibition on trehalase activity. None of the other substances tested had a significant effect on the activity of the purified trehalase. Other workers have reported an activation of trehalase by cylic AMP (Van Laere and Hendrix 1983; Thevelein 1984). We investigated the effect of cAMP on the purified trehalase from ArI3 and did not find a stimulation or an inhibition under the conditions we tested (Table 3).

Trehalase activity over a time course of nitrogenase induction in culture and activity of trehalase and vesicle clusters. A preliminary investigation of trehalase activity during nitrogenase induction was made. Free trehalose concentration, acetylene reduction (nitrogenase activity), and trehalase specific activity at pH 5.0 and 7.5 were followed over a time course of nitrogenase induction in ArI3 cells grown on B (−NH_4Cl) medium. Figure 5 shows data from a typical experiment. The trehalose levels in the cells at the beginning of the experiment were low.

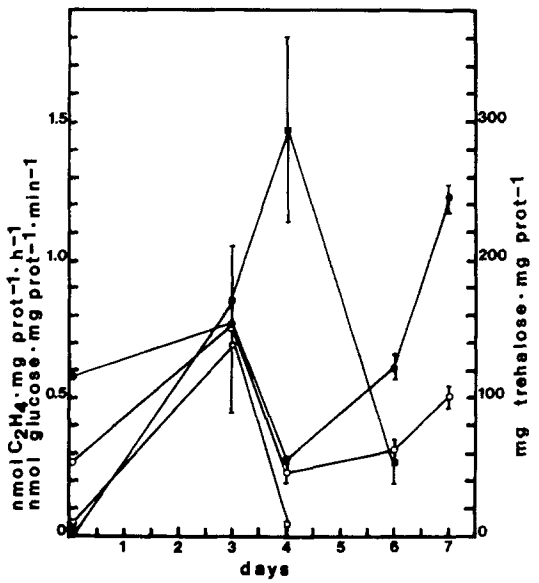

Fig. 5. Changing concentrations of free trehalose and trehalase and nitrogenase activity (acetylene reduction) with time along a nitrogenase induction curve in ArI3. ●, Trehalase activity at pH 5.0; ○, trehalase activity at pH 7.5; ■, nitrogenase activity; □, free trehalose concentration

Table 4. Maximum specific activities of trehalase observed in ArI3 nitrogen-fixing (−NH_4Cl medium) and non-nitrogen-fixing (+NH_4Cl medium) cells and in vesicle clusters isolated from *A. rubra* nodules inoculated with ArI3

Treatment	Specific activity[a]	
	pH 5.0	pH7.5
A. ArI3 grown in BAP medium (+NH_4Cl)	58.0	59.4
ArI3 grown in B medium (−NH_4Cl)	245.0	259.3
B. ArI3 vesicle clusters	1,925.1	1,578.0

[a] nmol glucose·mg prot^{-1}·min^{-1}
Results show the highest specific activity observed in 1 – 12 separate experiments with replicate assays for each treatment
Nitrogen-fixing cultures had nitrogenase activity (acetylene reduction) of a minimum of 100 nmol ethylene evolved·mg^{-1} prot·h^{-1}

As was previously reported (Lopez et al. 1984), the free trehalose concentrations peaked (day 3), when nitrogenase activity was increasing slowly. The *specific activity* (trehalase activity · mg prot^{-1} · ml^{-1} of cell free extract) of trehalase at both pH's also increased during this time, paralleling the rise in free trehalose, suggesting that the rate of trehalose synthesis was also accelerating. From day 3 to day 4, as nitrogenase activity reached its peak, both free trehalose concentrations and trehalase specific activity declined. From day 4 to day 6, a steep drop in nitrogenase activity was observed while trehalase activity increased, and the activity at pH 5.0 increased to its highest levels in the time course by day 7. Overall, it appeared that trehalase specific activity at pH 5.0 increased significantly over the total time course of nitrogenase induction. However, the specific activity of trehalase dropped during the period from day 3 to day 4 when nitrogenase activity was rising sharply. Trehalase activity at pH 7.5, which probably more accurately represents broad spectrum glucosidase activity, roughly paralleled the trehalase activity at pH 5.0 but did not reach such high levels at the end of the time course.

Table 4 shows the maximum specific activities of trehalase at pH 5.0 and 7.5 in nitrogen-fixing cells compared to the maximum specific activities in non-fixing cells grown on BAP (+NH_4Cl) medium. These activities were dramatically different. Cells fixing N_2 had roughly 4 times the activity at both pH's of the non-fixing cells at their maximum. The activity of trehalase in fixing cells was not always higher than that in non-fixing cells however, since trehalase activity varied greatly depending on the age of the culture (Fig. 5).

As described in the methods, a variation of the techniques of Akkermans et al. (1981) and Benson (1982) was used to isolate vesicle clusters of *Frankia* from *Alnus rubra* nodules inoculated with ArI3. The activity of trehalase at pH 5.0 and 7.5 in these vesicle clusters was investigated and the results are shown in Table 4. The maximum activity of trehalase at both pH's in vesicle clusters was very high in comparison to the pure cultures of ArI3. The activity in the isolated clusters was 6 – 7 times that in the nitrogen-fixing pure cultures and 26 – 33 times that observed in the non-fixing cultures at each pH respectively.

Discussion

The purification of trehalose-cleaving enzymes from crude extracts of *Frankia* ArI3 resulted in the elution of two groups of enzymes, a specific trehalase and an unresolved mixture of non specific α- and β-glucosidases that could also hydrolyze α,α-trehalose. We found that the presence of the non-specific glucosidases was correlated with the physiological state of the cultures. Cultures that had been induced to fix nitrogen resulted in crude extracts from which we could partially purify a mixture of α- and β-glucosidases and cultures that were not fixing nitrogen (grown in media supplemented with NH_4Cl) resulted in crude extracts that yielded an elution pattern lacking these enzymes in quantities that we could detect. We were, on the other hand, able to purify a specific trehalase with consistent kinetic and physical properties from both types of cultures. The presence of the non-specific trehalose-cleaving enzymes in nitrogen-fixing cultures was reflected in the biphasic pH optimum observed in the crude extracts from these cultures. The partially purified non-specific glucosidases had consistently high trehalose-hydrolyzing activity at pH 7.5, whereas the purified specific trehalase had a pH optimum of 5.0 and essentially no activity at pH 7.5.

A biphasic response of trehalase to pH was previously observed in crude extracts from a fungus, *Coprinus lagopus* (Rao and Niederpruem 1969) and in *Rhizobium japonicum* bacteroids (Streeter 1982). In *Coprinus*, a variation was observed in the pH optima with extracts made from cultures at different stages in the life cycle. Rao and Niederpruem (1969) speculated that some of the trehalase activity observed in their cultures might have been due to non-specific α-glucosidase activity. It is interesting that the proportion of non-specific glucosidase enzyme activity was consistently higher in nitrogen-fixing cultures of *Frankia*. This fact suggests that disaccharides other than trehalose may be actively metabolized during nitrogen-fixation. *Frankia*

cultures store glycogen (Benson and Eveleigh 1979; Lopez et al. 1984), and some of the glucosidase activity may be an indication of maltase activity associated with the metabolism of glycogen. Fontaine et al. (1984) found that very young (presumably immature) *Frankia* vesicles contained large amounts of glycogen but that mature, presumably functional vesicles seldom contained glycogen. Vesicles are purported to be the site of nitrogenase activity (Tjepkema et al. 1980; Murry et al. 1984).

A variety of trehalases from other organisms have reported to be inhibited by metabolites such as glucose, glucose-6-phosphate, sucrose and mannitol (Avigad et al. 1965; Gussin and Wyatt 1965; Gilby et al. 1957; Paulsen 1971; Lefebvre and Huber 1970; Horikoshi and Ikeda 1966). The inhibition of *Frankia* trehalase by sucrose and glucose poses some interesting questions about the regulation of trehalose catabolism in symbiotic *Frankia* within nitrogen-fixing nodules. Alder nodules contain large amounts of sucrose (Lopez, unpublished data) and sucrose and fructose were the only carbon sources tested that stimulated nitrogen fixation in intact nodule slices from *Alnus rubra* nodules infected with ArI3 (Lopez, unpublished data). Trehalase activity in vesicle clusters isolated from similar nodules, however, was very high (this paper).

The inhibition of trehalase by mannitol and sorbitol suggests that these sugar alcohols may be involved in the metabolism of trehalose in *Frankia*. Mannitol is a carbon reserve stored by many fungi but there are no published reports of mannitol accumulation by *Frankia*. Efforts are presently underway in this laboratory to investigate this possibility.

The catabolism of trehalose is of interest since trehalose is stored as a reserve form of carbon in many organisms (Elbein 1974; Thevelein 1984), including *Frankia* (Lopez et al. 1983, 1984). In most cases trehalose is accumulated during the time when differentiation processes and slower growth rates are occurring, and is broken down during or just before the induction of rapid growth of other energy-requiring processes such as spore germination. Lopez et al. (1984) showed that free trehalose levels in ArI3 were inversely correlated with a sharp increase in nitrogenase activity. In this report, a similar pattern was observed. In addition, trehalase activity in these cells was examined and although the specific activity of trehalase increased dramatically over the total time course of nitrogenase induction, trehalase specific activity dropped during the period when nitrogenase activity was increasing sharply. This result suggests that the drop in free trehalose levels during the period of sharp increase in nitrogenase activity was probably due to a decrease in trehalose synthesis rather an increase in trehalose hydrolysis. High specific activities of trehalase in nitrogen-fixing cultures of ArI3 and especially in vesicle clusters of ArI3 isolated from *Alnus rubra* nodules suggest that trehalose catabolism may be important in maintaining an energy supply for nitrogen fixation, although perhaps not directly.

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References

Akkermans ADL, Huss-Danell K, Roelofsen W (1981) Enzymes of the tricarboxylic acid cycle and the malate-aspartate shuttle in the N_2-fixing endophyte of *Alnus glutinosa*. Physiol Plant 53:289–294

Avigad G, Ziv G, Newfeld EF (1965) Intracellular trehalase of a hybrid yeast. Biochem 97:715–722

Benson DR (1982) Isolations of *Frankia* strains from alder actinorhiza root nodules. Appl Env Microbiol 44:461–465

Benson DR, Eveleigh DE (1979) Ultrastructure of the nitrogen-fixing symbiont of *Myrica pensylvanica* L. (Bayberry) root nodules. Bot Gaz 140:(Suppl) S15–S21

Berry A, Torrey JG (1979) Isolation and characterization in vivo and in vitro of an actinomycetous endophyte from *Alnus rubra* Bong. In: Gordon JC, Wheeler CT, Perry DA (eds) Symbiotic nitrogen fixation in the management of temperate forests. Forest Research Lab., Oregon State University, Corvallis, OR

Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein binding. Anal Biochem 72:248–254

Crabbe LH, Gussin AES, Tyrell EA (1969) Trehalase induction in *Bacillus cereus*. Experientia 25:793

Elbein A (1974) The metabolism of α,α-trehalose. Adv Carbo Chem Biochem 30:227–256

Elbein AD, Mitchell M (1973) Levels of glycogen and trehalase in *Mycobacterium smegmatis* and the purification and properties of the glycogen synthetase. J Bacteriol 113:863–873

Fontaine MF, Lancelle SA, Torrey JG (1984) Initiation and ontogeny of vesicles in cultured *Frankia* sp. strain HFPArI3. J Bacteriol 160:921–927

Gilby AR, Wyatt SS, Wyatt GR (1967) Trehalases from the cockroach, *Blaberus discoidales*: activation, solubilization and properties of the muscle enzyme and some properties of the intestinal enzyme. Acta Biochim Pol 14:83–100

Guilloux E, Arcila MA, Courtois JE, Mourmrikoff V (1971) La trehalase de *Pseudomonas fluorescens*. Biochimie 53:853–857

Gussin AES, Wyatt GR (1965) Membrane bound trehalase from *Cecropia* silk moth muscle. Arch Biochem Biophys 112:626–634

Hey A, Elbein AD (1968) Partial purification and properties of a trehalase from *Streptomyces hygroscopicus*. J Bacteriol 96:105–110

Hey-Ferguson A, Mitchell M, Elbein AD (1973) Trehalose metabolism in germinating spores of *Streptomyces hygroscopicus*. J Bacteriol 116:1084–1085

Hill EP, Sussmann AS (1963) Purification and properties of trehalase(s) from *Neurospora*. Arch Biochem Biophys 102:389–396

Hirokazu I, Shimoda C (1981) Changes in trehalose content and trehalase activity during spore germination in fission yeast, *Schizosaccharomyces pombe*. Arch Microbiol 129:19–20

Horikoshi K, Ikeda Y (1966) Trehalase in conidia of *Aspergillus oryzae*. J Bacteriol 91:1883–1887

Lefebvre YA, Huber RE (1970) Solubilization, purification and some properties of trehalase from honeybee (*Apis mellifera*). Arch Biochem Biophys 140:514–520

Lopez MF, Whaling CS, Torrey JG (1983) The polar lipids and free sugars of *Frankia* in culture. Can J Bot 61:2834–2842

Lopez MF, Fontaine MS, Torrey JG (1984) Levels of trehalose and glycogen in *Frankia* sp. HFPArI3 (Actinomycetales). Can J Microbiol 30:746–752

Marechal LR, Belocopitow E (1972) Metabolism of trehalose in *Euglena gracilis*. I. Partial purification and some properties of trehalose phosphorylase. J Biol Chem 247:3223–3228

Murry MA, Fontaine MS, Torrey JG (1984) Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArI3 grown in batch culture. Plant Soil 78:61–78

Panek A (1963) Function of trehalose in backer's yeast (*Saccharomyces cerevisiae*). Arch Biochem Biophys 100:422–425

Paulsen R (1971) Characterization of trehalase from labial glands of ants. Arch Biochem Biophys 142:170–176

Rao PS, Niederpruem DJ (1969) Carbohydrate metabolism during morphogenesis of *Corprinus lagopus* (sensu Buller). J Bacteriol 100:1222–1228

Streeter JG (1982) Enzymes of sucrose, maltose, and α,α-trehalose catabolism in soybean root nodules. Planta 155:112–115

Thevelein JM (1984) Regulation of trehalose mobilization in fungi. Microbiol Rev 48:42–59

Tjepkema JD, Ormerod W, Torrey JG (1980) Vesicle formation and acetylene reduction activity in *Frankia* sp. CpI1 cultured in defined nutrient media. Nature (Lond) 287:633–635

VanLaere AJ, Hendrix P (1983) Cyclic AMP-dependent in vitro activation of trehalase from dormant *Phycomyces blakesleeanus* spores. J Gen Microbiol 129:3287–3290

Zobel RW, DelTredici P, Torrey JG (1976) Method for growing plants aeroponically. Plant Physiol 57:344–346

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