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Purification and Biochemical Characterization of Cell Wall-bound Trehalase from Pericarp Tissues of *Actinidia deliciosa*

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A trehalase (EC 3.2.1.28) was purified from the cell walls of *Actinidia deliciosa* fruit. The purified trehalase had optimal pH of around 5, *Km* of 0.25 mM and Vmax of 5667 pkat/mg protein, and was relatively heat stable. The enzyme showed highly specific activity to trehalose and weak activity to maltose and maltotriose, but did not hydrolyze any other disaccharides. Trehalase activity was unaffected by Ca^{2+}, Na^{+}, K^{+}, Li^{+}, Mn^{2+}, Co^{2+}, and Mg^{2+} ions and EDTA, but markedly inhibited by Hg^{2+} and Fe^{3+} ions, iodoacetic acid, tris(hydroxymethyl)aminomethane (Tris), *p*-chloromercuribenzoate (PCMB), glucose and glucosamine. This cell wall-bound enzyme seems to degrade apoplastic trehalose. Another possibility is that this trehalase has additional functions such as defence against insects.

Key Words: *Actinidia deliciosa*, apoplast, cell wall, trehalase.

Introduction

The sugar content of fruits is one of the most important factors in determining their quality and in satisfying consumer preferences. Sucrose and its metabolism have been well studied, due to its large quantity and sweetness. Recently, the nonreducing disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) was noticed because of its increasing usage as a stabilizer in a number of foods. The low cariogenicity of trehalose has also been reported (Neta et al., 2000). Thus, it is possible that an increased amount of trehalose in fruit improves its shelf life and adds functionality. Trehalose is present in a variety of microorganisms and insects (Crowe et al., 1984; Strom and Kaasen, 1993); however, most plant species accumulate very small amounts of trehalose, possibly because active trehalases rapidly hydrolyze synthesized trehalose (Goddiji and Smeekens, 1998). Therefore, the study of trehalase is necessary to explore the mechanism that determines the amount of trehalose in fruits.

Trehalase (α-glucoside-1-glucohydrolase, EC 3.2.1.28) is a hydrolytic enzyme that cleaves trehalose into two glucose moieties. Trehalase activity has been detected in a variety of plants (Müller et al., 1995). In recent years, trehalase has been partially purified from the root nodules of *Glycine max* (Aeschbacher et al., 1999) and *Phaseolus vulgaris* (García et al., 2005); however, there is no recent report about the purification of trehalase from other plant organs such as the fruit of horticultural crops.

When we analyzed the cell wall of kiwifruit berries (Li et al., 2006), we observed high trehalase activity in the cell wall fraction; therefore, we chose this material to purify wall-bound trehalase. The possible function of cell wall-bound trehalase during fruit development is discussed.

Materials and Methods

Plant material

Kiwifruits (*Actinidia deliciosa* 'Hayward') were harvested from three 17-year-old trees in the National Institute of Fruit Tree Science, NARO Akitsu, Hiroshima, Japan. Four fruits were picked from each tree and 12 fruits with a similar external appearance, respectively sampled on August 15, September 8, October 14, October 20, and November 4, 2005. Fruits were peeled and the outer pericarp tissues cut into ca. 0.3 cm^3 pieces, frozen in liquid nitrogen, and stored at −80°C. At anthesis (June 1, 2006), kiwifruit flower organs and young leaves were collected from the same trees.

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Analysis of activity of trehalase and content of trehalose in kiwifruit tissues

Kiwifruit tissues (ca. 1–2 g fresh weight) were homogenized in 40 mM Hepes buffer (pH 7.0) containing 20 mM cysteine, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 10 mM sodium tetrathionate dehydrate (NaTT), 100 μM Pefabloc SC (Roche Diagnostics, Switzerland), and 2% insoluble polyvinylpolypyrrolidone (PVPP). Pefabloc SC is an irreversible proteinase inhibitor with broad specificity for serine proteinases. The homogenate was filtered through a sheet of nylon mesh with 40 μm pores, centrifuged at 12500 × *g* for 10 min, and the supernatant was designated as the symplastic fraction. For preparation of cell wall-bound enzyme, cell wall residues on the nylon mesh were washed three times with deionized water containing 5 mM NaTT and 10 μM Pefabloc SC, and then suspended overnight at 4°C in 20 mM sodium acetate buffer pH 5.4 containing 5 mM NaTT, 2 mM DTT, 1 M NaCl, and 10 μM Pefabloc SC to release wall-bound proteins. The NaCl extract was centrifuged at 12500 × g for 20 min, and the supernatant fraction used as the crude wall-bound enzyme. The trehalase assay was performed in 100 mM sodium acetate buffer (pH 5.0), 10 mM α,α-trehalose in a total volume of 150 μL. After incubation for 1 h at 37°C the reaction was terminated by boiling for 3 min. Glucose was determined using a glucose determination kit (GAGO-20, SIGMA, MO, USA). One unit of trehalase is defined as the activity of enzyme that catalyses the hydrolysis of trehalose under assay conditions giving rise to 1 nmol of glucose per second. Protein content was determined by Bio-Rad protein assay kit II (500-0002JA, Bio-Rad, CA, USA) using bovine serum albumin as the standard.

Contents of trehalose in kiwifruit tissues of the pistil, stamen, petal, leaf, and fruit were identified with high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) systems (Dionex, Sunnyvale, CA, USA), as described by Timotiwu and Sakurai (2002). Plant tissues were ground in liquid N_2, and then suspended in 80% methanol containing 1% insoluble PVPP. Soluble carbohydrates were extracted at 70°C for 30 min followed by centrifugation at 13000 rpm for 10 min. The extraction was repeated once. The supernatants were collected and dried to remove methanol. The concentrated carbohydrates were dissolved in deionized water, passed through a syringe filter fitted with a 0.45 μm HT Tuffryn membrane (Pall Gelman Laboratory, NY, USA), and then injected into Dionex (DX-500, Dionex) HPLC systems to quantify trehalose content. CarboPac I column was eluted with 0.16 M NaOH at a flow rate of 0.8 ml/min.

Purification of wall-bound trehalase

Cell wall-bound trehalase was purified according to the procedures of Kotake et al. (1997) and Takeda et al. (2003) with some modifications. Pericarp tissues (ca. 200 g fresh weight) of kiwifruit berries sampled on October 20 were used to prepare cell wall-bound proteins. The NaCl-extracted fraction was precipitated by 20 to 80% ammonium sulfate, and then dialyzed against 10 mM sodium acetate buffer (pH 5.4) with 2 mM NaTT. For cation-exchange chromatography on CM sepharose CL-6B, the column (5.5 × 1.8 cm, Amersham Bioscience, NJ, USA) was eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM sodium acetate buffer (pH 5.4) containing 2 mM NaTT. The combined active fractions (no. 28–41) were concentrated at 1000 × *g* with Centricut U10 (Kurabo Co., Osaka, Japan) and applied to a Butyl-toyopearl column (5.5 × 1.8 cm, 650-M, Tosoh, Tokyo, Japan). The column was eluted with a linear gradient of 30% to 0% $(NH_4)_2SO_4$ in 20 mM sodium acetate buffer (pH 5.4). The active fractions (no. 21–27) were dialyzed against 10 mM sodium acetate buffer (pH 5.4) with 2 mM NaTT, and then applied to a Mono S HR 5/5 column (Pharmacia, Sweden) of an HPLC system (LC-10AS, Shimadzu, Japan), eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM sodium acetate buffer (pH 5.4). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The concentration of resolving gel was 12.5%. The prestained protein marker (P7708S, New England Biolabs, USA) was used as the molecular weight standard. To confirm trehalase activity during purification, protein separated on a native-PAGE gel was electroblotted to a nitrocellulose membrane in Tris-Glycine buffer containing 5% methanol, and stained with amido black (Katayama Chemical, Japan). The band was cut and placed in 10 mM trehalose solution containing 0.5% NaN_3. After incubation at 37°C for 15 h, trehalase activity was detected using a glucose determination kit (GAGO-20).

Characterization of purified kiwifruit cell wall trehalase

The molecular mass of native enzyme was estimated by gel filtration on a 3.2 × 300 mm Superdex 200 PC 3.2/30 column (Amersham Bioscience), with a flow rate of 40 μl min^{-1} in 50 mM sodium phosphate buffer (pH 7) containing 0.15 M NaCl. A calibration curve was made using a calibration kit for molecular mass determination (18–300 KDa, Boehringer Manheim, Germany). To study substrate specificity, enzyme optimum pH, pH stability, thermostability, and inhibition by reagents, the reaction mixture remained the same as above except for the buffers and temperature of the enzymatic reaction. Substrate specificity of kiwifruit trehalase was examined by measuring the reaction rate of 15 U purified trehalase with 1.0% of various glucosides (trehalose, sucrose, isomaltose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, cellobiose, laminaribiose, laminarin, gentiobiose, pullulan) for 1 h at 37°C. Activity toward each substrate was replicated three times and expressed as a relative percentage of the

activity to trehalose reaction. Trehalose and cellobiose were obtained from Katayama Chemical (Japan). Laminarin (from *Laminaria digitata*), maltotetraose, maltohexaose, maltoheptaose, and methyl D-α-glucose were from SIGMA, pullulan from Nakalai Tesque Inc. (Kyoto, Japan), and isomaltose and laminaribiose from Seikagaku Corporation, Japan.

The effect of pH on trehalase activity was assayed using 100 mM citrate/Na_2HPO_4 buffer pH from 2.5 to 8. Trehalase pH stability was assayed after preincubating the enzyme in various pH of citrate/Na_2HPO_4 buffer for 20 h at 4°C, then the substrate was added and enzyme activity measured. To test thermostability, the enzyme was preincubated without trehalose in 100 mM sodium acetate buffer (pH 5.0) at various temperatures (28, 37, 45, 60, and 77°C) for 30 min, and then the substrate was added and the enzyme activity measured. The inhibition of trehalase by various reagents was assayed after incubating the enzyme for 30 min with the following substances: EDTA, NaCl, KCl, LiCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $ZnSO_4$, NH_4Cl, $HgCl_2$, $AlCl_3$, $FeCl_3$, iodoacetic acid, tris(hydroxymethyl)aminomethane (Tris), *p*-chloromercuribenzoate (PCMB), N-ethyl maleimide (NEM), glucose, methyl-α-glucose, methyl-α-mannose, *p*-nitrophenyl-α-glucose, glucosamine, *N*-acetyl-glucosamine, sucrose, and fructose. The results are expressed as the relative percentage of activity to the control reaction without effectors.

Results and Discussion

The activity of trehalase and content of trehalose in kiwifruit flower and fruit

The activity of trehalase was identified in the petals and stamen of male and female flowers, female pistil and young leaves at anthesis (Fig. 1A). In the petals of male and female flowers, the specific activity of trehalase in the symplastic fraction was higher than in the wall-bound fraction. The total activity of trehalase in female petals was higher than in male petals. No activity was observed in the wall-bound fraction of female stamen. Total trehalase activity in female stamen was much lower than in male stamen which exhibited no difference in activity of the symplastic and wall-bound fractions. In contrast to the activity in female stamen, higher activity of trehalase was observed in pistil, especially in the wall-bound fraction. By contrast, trehalase activity in young leaves was too low to be detected.

Developing kiwifruit berries showed high trehalase activity from fruit enlargement until maturity (Fig. 1B). Wall-bound trehalase from cell wall residues of fruits showed 1.3–7 times higher activity than that from the symplastic fraction. Trehalase activity decreased at harvest time; thus, we used fruits sampled on October 20 to purify wall-bound trehalase. The content of trehalose in kiwifruit tissues was very low ($< 50\ \mu g \cdot g^{-1}$ FW), especially in pistil tissue (Table 1). Female stamen had a higher content of trehalose than male stamen; however, there was no significant difference in trehalose content between male and female leaves or between male and female petals.

Purification of cell wall-bound trehalase

The purification of the cell wall-bound form of trehalase from pericarp tissues of kiwifruit is shown in Table 2. The enzyme was released with 1 M NaCl from cell wall residues and concentrated by 20–80% ammonium-sulfate precipitation, achieving a recovery of 31% and a purification factor of 2.3. Its activity persisted for five months in the ammonium sulfate-precipitated fraction at 4°C. To desalt, fractions containing protein were dialyzed against 20 mM sodium acetate buffer (pH 5.4) overnight or concentrated by ultrafiltration. These methods recovered more activity than those passing through a PD-10 (Amersham

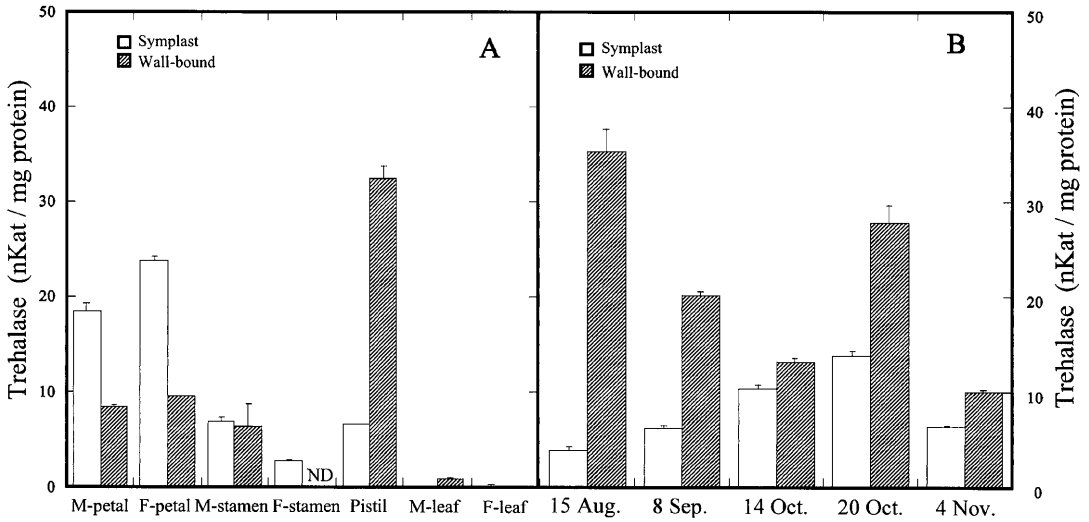

Fig. 1. Trehalase activity in the symplastic and wall-bound fraction of flower and fruit tissues of *A. deliciosa*. A, flower and young leaf tissues. B, berry pericarp tissues during development. M, male; F, female; ND, not detected. Vertical bars indicate SD (n = 3).

Bioscience) column. The second step in cation-exchange chromatography on CM Sepharose (Fig. 2A) of the ammonium-sulfate precipitated extract resulted in four-fold purification and a yield of 20%. Positively charged protein with trehalase activity was collected in the elution of 0.25 to 0.35 M NaCl fractions. In the third step of Butyl-Toyopearl column chromatography (Fig. 2B), the collected protein having trehalase activity was hydrophobic. In the final step of Mono S column chromatography (Fig. 2C), more than half of the activity retained on the column. We extracted trehalase from the cell wall fraction using 1 M NaCl as a solubilizer, suggesting that this enzyme ionically binds to acidic cell wall polysaccharides. This is consistent with binding of the enzyme to the Mono S cation exchange column. It appeared that the first peak of activity (flowthrough fraction) was due to incomplete binding of the enzyme; thus, this activity was not further analyzed. After purification, 57 μg of trehalase was obtained from the cell walls of 200 g fw of kiwifruit pericarp tissues with activity enriched about 24-fold.

Molecular mass determination

Samples from each purification step (lane 2 to 5: 6 μg, lane 6: 3.5 μg) were applied to SDS-PAGE (Fig. 3). With progressive purification, the band at ca. 26 kDa became prominent. In the step of final purification, the density of the single band at 26 kDa was relatively weak. This may have been due to the weak stainability of the protein with silver. Some modification of the protein, such as

Table 1. Content of trehalose in tissues of *A. deliciosa* plant.

Tissues	Trehalose (μg·g^{-1} FW)
Flowers[z]	
Male stamen	11.1 ± 3.6[y]
Female stamen	35.4 ± 13.8
Pistil	ND[x]
Male petal	9.3 ± 6.6
Female petal	20.3 ± 6.3
Young leaves[z]	
Male leaves	16.0 ± 5.1
Female leaves	17.5 ± 2.8
Fruit[z]	10.9 ± 6.4

[z] Flowers and young leaves were sampled on May 31, and fruits were sampled on September 28, 2006.
[y] Values shown are the means ± SD (n = 3).
[x] ND, not detected.

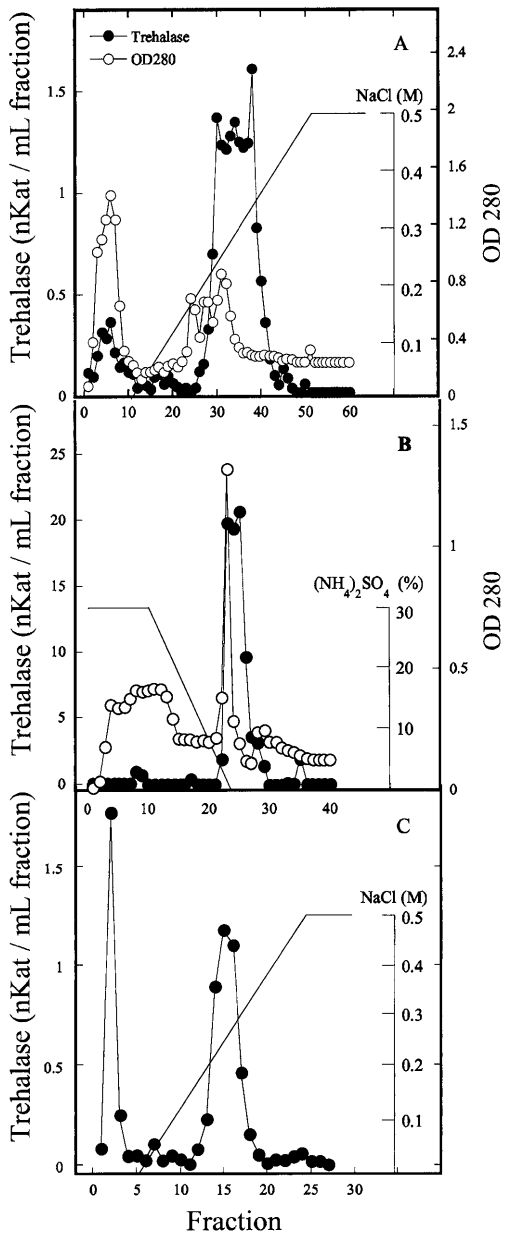

Fig. 2. Purification of *A. deliciosa* cell wall-bound trehalase. A, CM Sepharose CL-6B column, eluted with a gradient of 0–0.5 M NaCl solution in 20 mM sodium acetate buffer (pH 5.4), collecting 3 mL per fraction. Fractions 28–41 were used for further purification. B, Butyl toyopearl column, eluted with a gradient of 30–0% ammonium sulfate in 20 mM sodium acetate buffer (pH 5.4), collecting 3 mL per fraction. Fractions 21–27 were used for further purification. C, Mono S HR column, eluted with a gradient of 0–0.5 M NaCl solution in 20 mM sodium acetate buffer (pH 5.4), collecting 1 mL per fraction.

Table 2. Purification of wall-bound trehalase from *A. deliciosa* fruit.

Purification step	Total Protein (mg)	Total activity (nkat)	Specific activity (nkat/mg protein)	Yield (%)	Fold purified
1. NaCl-extract	147.22	320.90	2.18	100	1.0
2. Ammonium sulfate	19.88	99.18	4.99	30.91	2.3
3. CM Sepharose	7.03	64.90	9.23	20.22	4.2
4. Butyl-Toyopearl	1.30	30.31	23.33	9.40	10.7
5. Mono S HR 5/5	0.06	2.98	51.88	0.93	23.8

glycosylation, may explain this result. The molecular mass of native enzyme was estimated to be 36 kDa by gel filtration on Superdex 200. The molecular mass of our enzyme was slightly lower than those of *P. vulgaris* (45 kDa) and *G. max* (54 kDa).

Effect of pH and thermostability

Trehalase from kiwifruit cell walls showed a broad pH optimum, ranging from ca. 5 to 6 in citrate/Na_2HPO_4 buffer (Fig. 4). The enzyme was stable from pH 4 to 8.

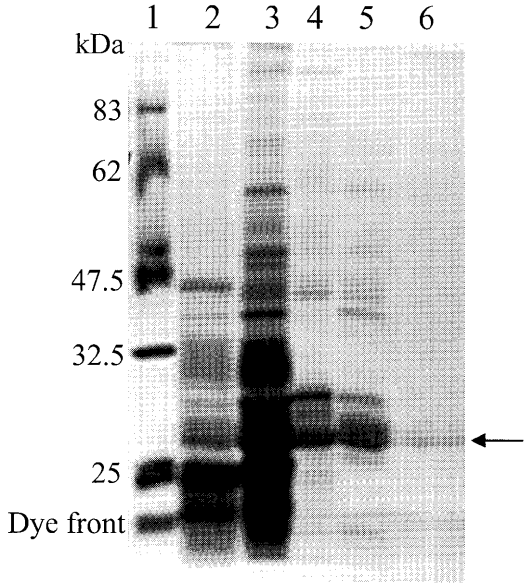

Fig. 3. SDS-PAGE of *A. deliciosa* cell wall-bound trehalase preparation. Lane 1, molecular weight standards; Lane 2, protein from 1 M NaCl-extract fraction; Lane 3, protein from 80% ammonium sulfate precipitated fraction; Lane 4, protein from CM Sepharose chromatography; Lane 5, protein from Butyl-Toyopearl chromatography; Lane 6, protein from Mono S chromatography. Arrow shows purified trehalase.

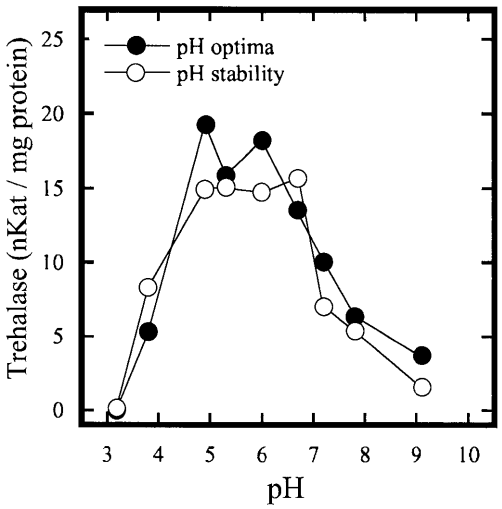

Fig. 4. pH optima and stability of purified *A. deliciosa* cell wall-bound trehalase. The enzyme was reacted with trehalose in citrate/Na_2HPO_4 buffer of various pH. Values are the average of two different experiments.

The optimum temperature was determined by comparing hydrolysis rates of trehalose at 28–77°C in 100 mM sodium acetate buffer pH 5.0 (Fig. 5). When heated at various temperatures for 30 min, the enzyme was stable up to 45°C; however, it lost 10% of its activity at 60°C, and lost almost 90% of its activity at 77°C. These results suggest that the enzyme was relatively thermostable.

Substrate specificity and kinetic parameters

Purified trehalase is highly substrate specific (Table 3). It effectively hydrolyzed trehalose, but weakly

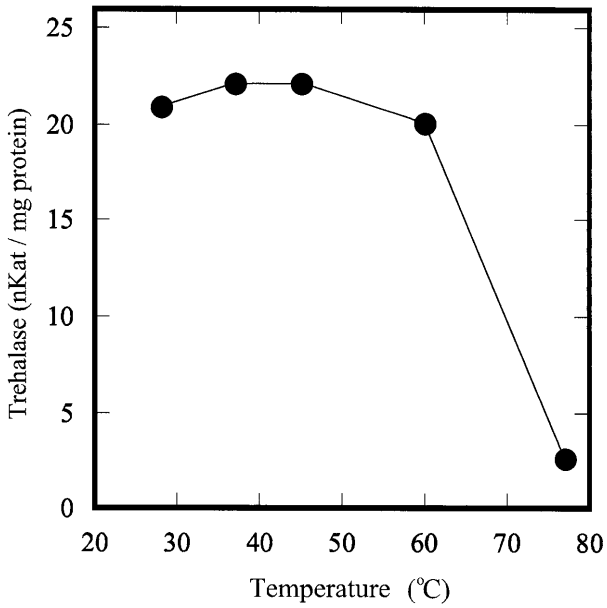

Fig. 5. Thermostability of *A. deliciosa* cell wall-bound trehalase. The enzyme was kept in 100 mM sodium acetate buffer (pH 5) at various temperatures for 30 min, and then the substrate was added and activity measured. Values are the average of two different experiments.

Table 3. Substrate specificity of purified trehalase.

Carbohydrates	Linkage	Relative rate of hydrolysis (%)
Trehalose	α-1,1	100
Maltose	α-1,4	13.7 ± 4.5
Isomaltose	α-1,6	0
Maltotriose	α-1,4	8.0 ± 1.8
Maltotetraose	α-1,4	0
Maltopentaose	α-1,4	0
Maltohexaose	α-1,4	0
Maltoheptaose	α-1,4	0
Pullulan	α-1,4; α-1,6	0
Sucrose	α-1,2	0
Curdlan	β-1,3	0
Gentiobiose	β-1,6	0
Cellobiose	β-1,4	0
Laminarin (*Laminaria digitata*)	β-1,3	0
Laminaribiose	β-1,3	0

Values shown are the means ± SD (n = 4).

hydrolyzed maltose and maltotriose. The enzyme did not split other malto-oligosaccharides, laminarin, pullulan, and disaccharides such as sucrose, cellubiose, laminaribiose, and gentiobiose. These results indicate that the trehalase showed a strong preference for trehalose. The substrate specificity of this trehalase is different from that of *P. vulgaris* nodule trehalase that shows maximal activity with sucrose and maltose besides trehalose (García, et al., 2005). The *G. max* nodule trehalase has specificity for trehalose and maltose (Müller, et al., 1992).

It was established that the value of Michaelis constant *Km* for trehalose was 0.25 mM at 37°C in sodium acetate buffer (pH 5.0), with the maximum velocity Vmax of 5667 pkat/mg protein. The catalytic properties of *A. deliciosa* wall-bound trehalase, such as the broad pH optimum, low *Km* and heat stability, resemble those of *Lilium* pollen (Gussin and Cormack, 1970) and *Phaseolus* nodule (García et al., 2005) enzymes, both of which are of plant origin.

Effects of inhibitors

Effects of various substances as activators or inhibitors on trehalase activity were assayed (Table 4). Metal ions, Na^+, K^+, Li^+, Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, NH_4^+, and EDTA, had no effect, but Al^{3+} slightly and Fe^{3+} markedly reduced the activity, whereas Tris strongly inactivated the activity. SH-blocking reagents, such as PCMB, iodoacetic acid, and $HgCl_2$, almost completely inactivated the activity, and NEM at 1 mM modestly reduced the activity. Glucose and glucosamine strongly reduced the activity of kiwifruit wall-bound trehalase, but *p*-nitrophenyl-α-glucose, methyl-α-D-glucose, methyl-α-D-mannose, fructose, and sucrose had almost no effect on enzyme activity.

Possible role of trehalose in A. deliciosa fruit

What is the role of trehalase in the fruit of *A. deliciosa*? The high activity of apoplastic trehalase in *A. deliciosa* fruit may keep the apoplastic trehalose concentration low; however, we have not examined whether trehalose is actively transported into the apoplast fraction in the fruit of *A. deliciosa*. Further studies of the overall metabolism (synthesis and degradation) of trehalose in this fruit are needed. Such studies will be helpful to modify the trehalose content of this fruit.

Alternatively, it is possible that our trehalase has another simultaneous function, such as defense against insects. Plant storage tissues often contain anti-insect proteins. For example, it is well known that the α-amylase inhibitor in plant seeds hinders the digestion of starch by insects (Moreno et al., 1990). In *P. vulgaris*, Dayler et al. (2005) have identified a novel α-amylase inhibitor that shows simultaneous chitinase activity. It is possible that our trehalase shows additional functions, such as the inhibition of digestive enzymes within the body of insects when fruits are damaged by feeding.

Table 4. Influence of various effectors and inhibitors on the enzyme activity of cell wall-bound trehalase of *A. deliciosa*.

Reagents	Concentration (mM)	Relative percent of activity (%)
None	—	100.0
EDTA	10	96.6±4.5
Na^+	10	99.5±6.8
K^+	10	97.1±5.5
Li^+	10	98.2±4.9
Mg^{2+}	10	94.6±6.6
Mn^{2+}	10	99.8±6.1
Ca^{2+}	10	102.4±6.8
Co^{2+}	10	98.6±5.9
Zn^{2+}	10	94.1±4.5
NH_4^+	10	93.0±6.4
Hg^{2+}	10	29.0±7.6
Al^{3+}	10	79.5±7.0
Fe^{3+}	10	6.4±4.3
Tris	10	30.5±4.5
Iodoacetic acid	10	10.5±4.5
PCMB	2.5	0
NEM	1.0	82.6±0.3
Glucose	5	0
Methyl-α-glucose	10	93.9±2.7
Methyl-α-mannose	10	90.2±1.5
p-Nitrophenyl-α-glucose	10	86.5±2.5
Glucosamine	10	0
N-acetyl-glucosamine	10	81.7±0.8
Sucrose	10	91.4±2.2
Fructose	10	92.7±5.5

Values shown are the means±SD (n=3).

Trehalose is known as the main hemolymph sugar in insects. Thus, it is also possible that trehalase activity influences insects directly upon feeding. Examination of the effect of our trehalase on the growth of insects is needed.

Acknowledgements

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