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Insights into the catalytic properties of bamboo vacuolar invertase through mutational analysis of active site residues

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ABSTRACT

Plant acid invertases, which are either associated with the cell wall or present in vacuoles, belong to family 32 of glycoside hydrolases (GH32). Homology modeling of bamboo vacuolar invertase Boβfruct3 using Arabidopsis cell-wall invertase AtcwINV1 as a template showed that its overall structure is similar to GH32 enzymes, and that the three highly conserved motifs, NDPNG, RDP and EC, are located in the active site. This study also used site-directed mutagenesis to examine the roles of the conserved amino acid residues in these three motifs, which include Asp135, Arg259, Asp260, Glu316 and Cys317, and a conserved Trp residue (Trp159) that resides between the NDPNG and RDP motifs. The mutants W159F, W159L, E316Q and C317A retained acid invertase activity, but no invertase activity was observed for the mutant E316A or mutants with changes at Asp135, Arg259, or Asp260. The apparent K_m values of the four mutants with invertase activity were all higher than that of the wild-type enzyme. The mutants W159L and E316Q exhibited lower k_{cat} values than the wild-type enzyme, but an increase in the k_{cat} value was observed for the mutants W159F and C317A. The results of this study demonstrate that these residues have individual functions in catalyzing sucrose hydrolysis.

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1. Introduction

Sucrose is a principal product of photosynthesis, a major transport form of carbohydrates, and an important source of carbon and energy in higher plants. Utilization of sucrose for either growth or maintenance of cell activities depends on the breakdown of its glycosidic bond. Invertase (EC 3.2.1.26), which catalyzes the hydrolysis of sucrose to glucose and fructose, and sucrose synthase (EC 2.4.1.13), which converts sucrose and UDP into fructose and UDP–glucose, are the enzymes responsible for sucrose cleavage in plants. Both enzymes are crucial for plant development, growth and carbon partitioning (Sturm and Tang, 1999; Koch, 2004).

Plants possess a group of invertase isozymes that have been categorized as acid and neutral/alkaline invertases based on their pH optima (Tymowska-Lalanne and Kreis, 1998; Sturm, 1999; Roitsch and Gonzalez, 2004). The acid invertases are either vacuolar or cell-wall-associated enzymes, while the neutral/alkaline invertases are located in the cytoplasm (Tymowska-Lalanne and Kreis, 1998; Sturm, 1999), mitochondria or plastids (Murayama and Handa, 2007). The biochemical properties of the two types of acid invertases are similar, but differ from those of neutral/alkaline invertases. For example, both vacuolar and cell-wall-associated invertases are glycoproteins, but the neutral/alkaline invertases are not glycosylated. Heavy metal ions such as Hg^{2+} and Ag^{+} inhibit the activities of acid invertases but not neutral/alkaline invertases. Additionally, both types of acid invertases are β-fructofuranosidases, but neutral/alkaline invertases are not (Sturm, 1999).

In addition to their differing biochemical properties, the sequences of acid invertases show a low level of homology with neutral/alkaline invertases. Instead, acid invertases are highly homologous to fructan-synthesizing fructosyltransferases and fructan-degrading exohydrolases/fructosidases. They share several conserved regions such as the NDPNG motif, RDP motif and EC motif (Vijn and Smeekens, 1999; Ritsema and Smeekens, 2003). The importance of the conserved amino acid residues in these motifs have been proposed by a number of mutational and crystallization studies including yeast and bacterial invertases (Reddy and Maley, 1990, 1996; Alberto et al., 2004), bacterial and plant fructan-degrading hydrolases (Nagem et al., 2004; Verhaest et al., 2005), plant fructan-synthesizing fructosyltransferases (Ritsema et al., 2005; Altenbach et al., 2005), and plant cell-wall-associated invertases (Goetz and Roitsch, 2000; Verhaest et al., 2006; Le Roy et al., 2007; Matrai et al., 2008; Lammens et al., 2008). However, such studies have not been performed with plant vacuolar invertases. In this paper, we report the construction of a theoretical structure of bamboo vacuolar invertase by homology modeling and changes of conserved amino acid residues by site-directed mutagenesis. The effect of each mutation on the enzymatic activity was

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characterized by expression and purification of the recombinant vacuolar invertases in yeast *Pichia pastoris*.

2. Results

2.1. Sequence alignment and homology modeling

Plant vacuolar invertases belong to the glycoside hydrolase family 32 (GH32) in the carbohydrate-active enzymes database (http://www.cazy.org). Fig. 1 shows the multiple sequence alignment of bamboo vacuolar invertase Boβfruct3 with other GH32 enzymes whose crystal structures have been resolved (Alberto et al., 2004; Nagem et al., 2004; Verhaest et al., 2005, 2006). As expected, several conserved regions can be clearly noted. In order to obtain a reasonable theoretical structure of Boβfruct3, protein homology modeling was performed by using the cell-wall invertase AtcwINV1 from *Arabidopsis thaliana* (PDB id: 2AC1, Verhaest et al., 2006) as a template. The modeling procedure was only applied to residues Q118-N631 of Boβfruct3. The leader sequence and both the N-terminus and C-terminus of the mature protein were omitted for better accuracy. The primary model was further structurally aligned with Arabidopsis AtcwINV1 D239A mutant in complex with sucrose (PDB id: 2QQU, Lammens et al., 2008) to predict the theoretical position of sucrose when binding Boβfruct3. As shown in Figs. 2A and B, the overall structure of Boβfruct3 (residues Q118-N631) with substrate is similar to that observed for GH32 enzymes, with the N-terminus composed of a five-bladed β-propeller module while a β-sandwich structure can be observed in the C-terminus. The three major conserved motifs, namely NDPNG, RDP and EC, are located in the active site of the enzyme (Fig. 2C).

2.2. Mutagenic replacement and expression of recombinant mutant proteins

To assess the role of the conserved amino acid residues in or around the active site of Boβfruct3, site-directed mutagenesis

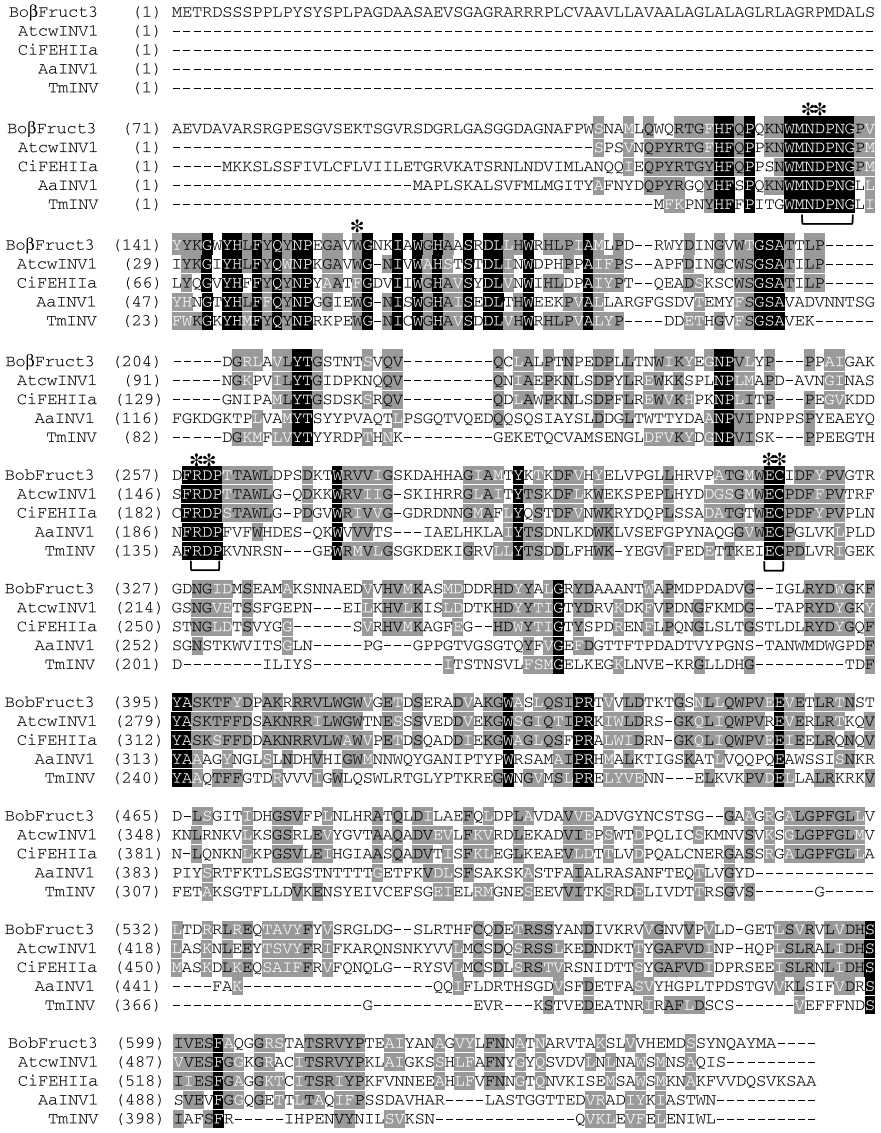

Fig. 1. Alignment of the deduced amino acid sequences of bamboo vacuolar invertase Boβfruct3 and four other GH32 enzymes with known structures. The sequences compared are *Arabidopsis thaliana* cell-wall invertase 1 (AtcwINV1, accession number NP566464), *Cichorium intybus* fructan 1-exohydrolase IIa (CiFEHIIa, CAC37922), *Aspergillus awamori* exo-inulinase (AaInul, CAC44220) and *Thermotoga maritime* invertase (TmINV, NP229215). Identical residues are shown on a black background. The characteristic conserved motifs of acid invertases and fructan-related fructosyltransferases are indicated by brackets below the sequences. The amino acid residues selected for site-directed mutagenesis are denoted by asterisks.

was performed on Asp135, Trp159, Arg259, Asp260, Glu316 and Cys317. The plasmids containing either the mutated or wild-type Boβfruct3 cDNA were transformed into *Pichia pastoris* for expression of the recombinant proteins in a secreted extracellular form. After induction by methanol for 24 h, His-tagged recombinant proteins were detected in the growth medium of each transformed *P. pastoris* strain by western analysis using an anti-$(His)_6$ antibody (data not shown). Expression levels for different mutants varied, but each was obtained in soluble form. The wild-type enzyme and the mutants W159F, W159L, E316Q and C317A possessed different levels of acid invertase activity (data not shown). However, no invertase activity was observed either for the mutant E316A or for mutants with changes at Asp135, Arg259, or Asp260.

2.3. Purification and characterization of the recombinant invertases

To avoid the effect of differential protein expression by various strains on the observed activities of crude enzyme preparations, the recombinant wild-type and mutant invertases were all purified from the growth media of methanol-induced cultures using

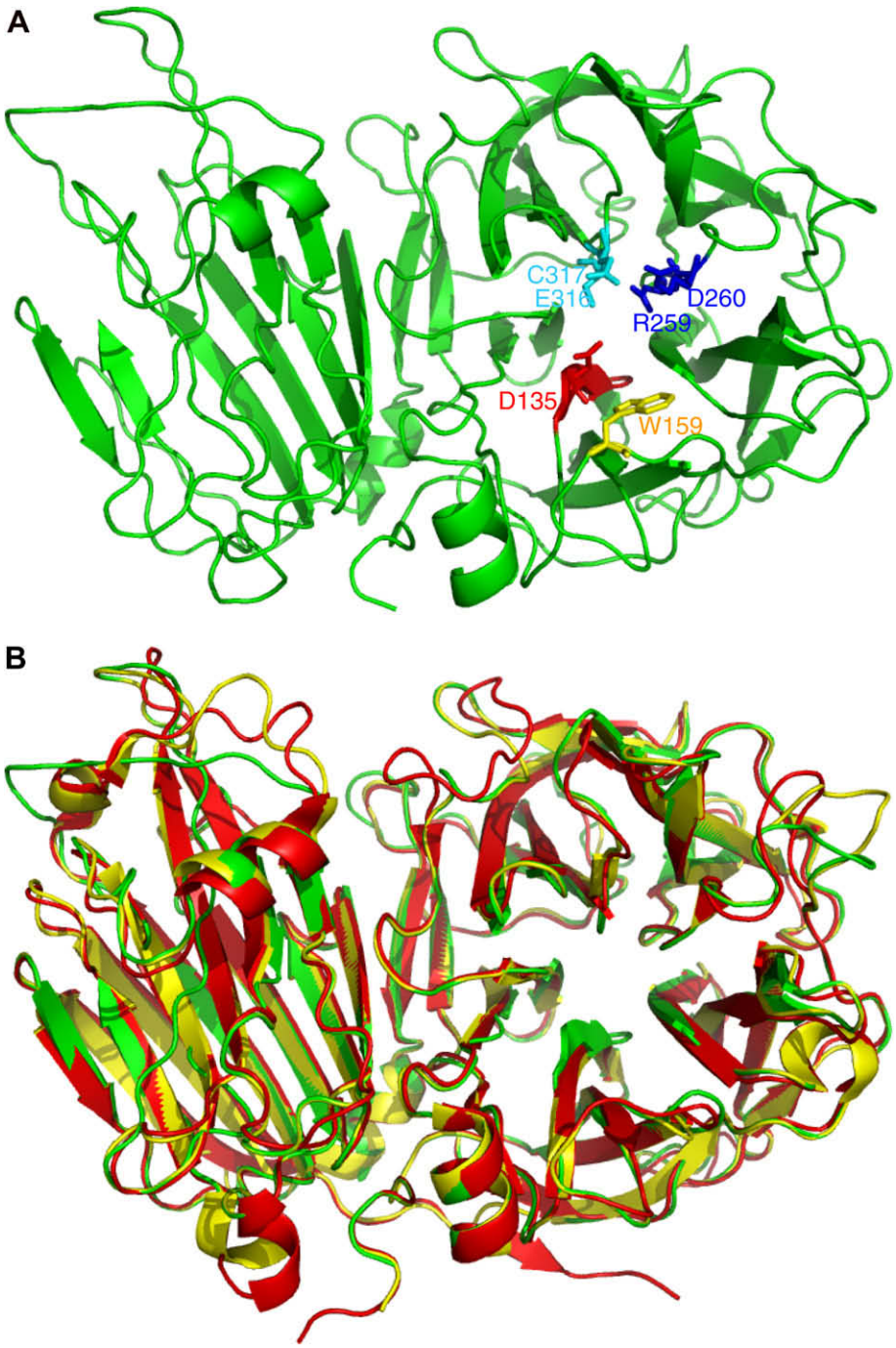

Fig. 2. Theoretical structure of bamboo vacuolar invertase Boβfruct3 constructed by homology modeling: (A) Theoretical model of Boβfruct3 (Q118-N631) based on the known structure of cell-wall invertase AtcwINV1 of Arabidopsis. The conserved motifs NDPNG, RDP and EC are highlighted with red, blue and cyan, respectively. (B) Superposition of Boβfruct3 (green), AtcwINV1 (yellow) and *Cichorium intybus* fructan 1-exohydrolase IIa (red). (C) Close-up view of the conserved residues at and around the active site. The primary model of Boβfruct3 in panel A was further structurally aligned with Arabidopsis AtcwINV1 D239A mutant in complex with sucrose. The residues in Boβfruct3, wild-type AtcwINV1 and AtcwINV1 D239A mutant are shown in green, yellow and red, respectively.

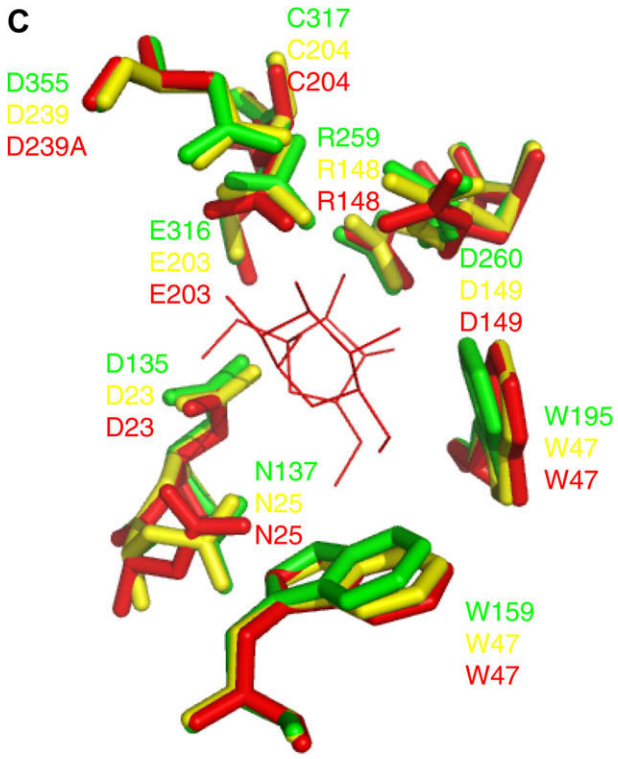

Fig. 2 (*continued*)

ammonium sulfate fractionation and cobalt-based IMAC (Fig. 3). The chromatograms of cobalt-based IMAC for the wild-type and mutant enzymes were similar (data not shown). The specific activities of the purified enzymes are shown in Table 1. As observed in the crude preparations, mutagenesis at Asp135 (in the NDPNG motif), Arg259 and Asp260 (both in the RDP motif) or replacement of Glu316 (in the EC motif) with alanine generated inactive enzyme, and only mutants W159F, W159L, E316Q and C317A possessed invertase activity. The mutants W159L and E316Q retained 11.7% and 67.3% of wild-type activity, respectively, while the specific activities of W159F and C317A were 4.7-fold and 2.5-fold, respectively, of that of the wild-type enzymes.

The four mutants possessing invertase activity exhibited the same pH optimum (pH 5.0) and substrate specificity as the wild-type enzyme did. They all hydrolyzed sucrose and raffinose but could not use cellobiose, maltose, lactose and inulin as substrates. However, the relative rates of hydrolysis of raffinose by the mutants were all lower than that of the wild-type enzyme. The activities toward raffinose were 41.0%, 33.2%, 24.3%, 15.5% and 20.7% of sucrose for wild-type enzyme, mutant W159F, W159L, E316Q and C317A, respectively. The effects of mutation on the K_m value for sucrose and the turnover number (k_{cat}) of the enzyme were also determined (Table 2). The apparent K_m values of the four mutants possessing invertase activity were all higher than that of the wild-type enzyme. The mutants W159L and E316Q exhibited lower k_{cat} values than the wild-type enzyme, but an increase in k_{cat} value was observed for mutants W159F and C317A. The k_{cat}/K_m ratios of W159F, W159L, E316Q and C317A were 148%, 2.9%, 25.3% and 94.4% of the wild-type enzyme, respectively (Table 2).

Table 1
Specific activities of the purified wild-type and mutant invertases.

Enzyme	Specific activity[a] (nkatal/mg)
Wild type	109.7
D135N	0
D135E	0
W159F	512.3
W159L	12.8
R259K	0
R259L	0
D260N	0
D260E	0
E316Q	73.9
E316A	0
C317A	271.7

[a] Sucrose was used as the substrate to determine the activity.

Table 2
Kinetic parameters for the wild-type and mutant invertases.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild type	53.68	501.7	9.35
W159F	169.77	2342.2	13.80
W159L	213.58	57.86	0.27
E316Q	142.56	338.41	2.37
C317A	140.66	1242.6	8.83

3. Discussion

Based on the stereochemical outcomes of the hydrolysis reaction, glycoside hydrolases can be classified as either inverting or retaining enzymes. The inverting enzymes act via a general acid/base-catalyzed direct displacement mechanism which results in overall inversion of the anomeric configuration at the site of cleavage. The retaining enzymes operate through a double-displace-

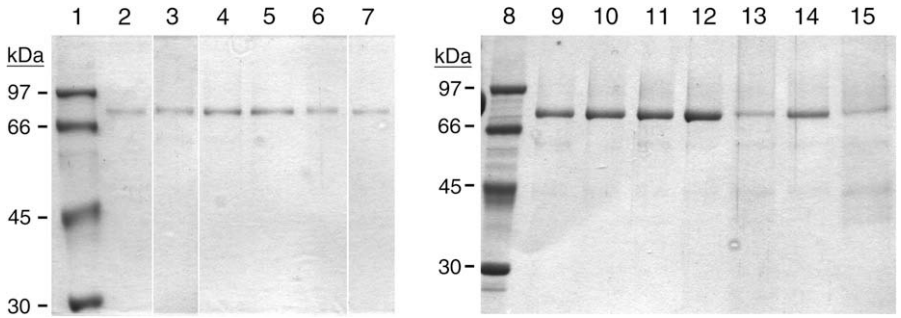

Fig. 3. SDS–PAGE analysis of the purified wild-type and mutated invertases. The recombinant wild-type and mutant invertases, purified by ammonium sulfate fractionation and cobalt-based IMAC, were separated on 10% SDS–polyacrylamide gels, which were then stained with Coomassie blue R-250. Lanes 1 and 8, molecular mass markers; lanes 2 and 9, wild-type Boβfruct3; lanes 3–7, mutants W159F, W159L, E316Q, E316A and C317A, respectively; lanes 10–15, mutants D135N, D135E, D260N, D260E, R259K and R259L, respectively.

ment mechanism with a net retention of the anomeric configuration. The retaining mechanism involves two active site residues acting as a nucleophile and an acid/base catalyst. Attack of the anomeric center by the nucleophile results in the formation of a covalent glycosyl-enzyme intermediate, which is subsequently hydrolyzed in a general acid/base-catalyzed manner (Sinnott, 1990; Davies and Henrissat, 1995; White and Rose, 1997). Acid invertases are retaining enzymes. By affinity labeling and site-directed mutagenesis, Reddy and Maley (1990, 1996) first proposed that the nucleophile and the acid/base catalyst in the active site of yeast invertase were the Asp residue in the NDPNG motif and the Glu residue in the EC motif, respectively. The proposed catalytic mechanism is as follows: the Glu residue protonates the glycosidic oxygen of sucrose and the nucleophile Asp attacks C-2 of the fructose moiety, forming a fructosyl-enzyme intermediate and releasing glucose. Subsequently, the Glu abstracts a proton from water, which enables the hydroxyl group to displace fructose and restore the original active center (Reddy and Maley, 1996). The NDPNG and EC motifs are highly conserved across invertases and fructan-related fructosyltransferases. Replacing the homologs Asp with Asn or Glu with Ala, which resulted in inactive enzymes, has also been reported for other invertase and fructosyltransferases (Goetz and Roitsch, 2000; Ozimek et al., 2004; Ritsema et al., 2005). Analyses of crystal structures confirmed that these two amino acid residues, together with the Asp residue of RDP motif, are located in the active site of invertases and fructan-metabolizing enzymes (Alberto et al., 2004; Nagem et al., 2004; Verhaest et al., 2005; Verhaest et al., 2006; Lammens et al., 2008). By homology modeling, we have shown that the theoretical structure of bamboo vacuolar invertase Boβfruct3 (residues Q118-N631) is similar to the resolved crystal structures of Arabidopsis cell-wall invertase and the other three GH32 enzymes. The homologs of the nucleophile Asp and the acid/base catalyst Glu in Boβfruct3 are Asp135 and Glu316, respectively. The critical requirement of Asp135 was supported by the results of site-directed mutagenesis. No catalytic activity was detected in mutant D135N (Table 1). Even when Asp135 was replaced with Glu, a residue with only one more $-CH_2$ in the side-chain, the enzymatic activity was not retained (Table 1), indicating the importance of the structural distance and orientation for nucleophilic attack.

The results of mutagenesis at Glu316 also suggested its importance in the active site but its specific role remains uncertain. Replacement of Glu316 with Ala generated an inactive enzyme; however, the mutant E316Q retained 67% activity of the wild-type enzyme (Table 1). In Arabidopsis AtcwINV1, Glu203 was identified as the acid/base catalyst in the active site. The mutant E203A exhibited a drastic decrease in k_{cat} value (Le Roy et al., 2007) and mutant E203Q was inactive (Matrai et al., 2008; Lammens et al., 2008). The X-ray crystallographic structure of the E203Q in complex with sucrose showed that the sugar binding and the catalytic pocket arrangement is significantly altered in this mutant (Matrai et al., 2008; Lammens et al., 2008). Although the theoretical structure of Boβfruct3 (residues Q118-N631) is similar to the revolved structures of AtcwINV1, the difference in enzymatic activity between Boβfruct3 E316Q mutant and AtcwINV1 E203Q mutant suggests that the two homologous residues may not have exactly same role in the two enzymes. The reaction catalyzed by the retaining glycoside hydrolases usually involves two carboxyl groups acting as a nucleophile and an acid/base catalyst (Sinnott, 1990; Davies and Henrissat, 1995; White and Rose, 1997) but variants of the acid/base catalyst exist in some enzymes (Zechel and Withers, 2001; Burmeister et al., 1997). For example, in plant myrosinase (thioglucoside glucohydrolase), which belongs to family 1 of glycoside hydrolases and also is a retaining enzyme, the Glu residue at the usual position of the acid–base catalyst is naturally replaced by Gln. The role of this Gln is thought to ensure a precise positioning of a water molecule, rather than to provide general base assistance. The positioning of the water molecule is sufficient to hydrolyze the glycosyl-enzyme intermediate. Ascorbate, a cofactor for myrosinase, can substitute for the function of the catalytic base (Burmeister et al., 1997, 2000). We propose that the Glu316 in wild-type Boβfruct3 functions as an acid/base catalyst that enhances the rate of sucrose hydrolysis. When Glu316 is replaced with another amino acid residue that can provide the hydrogen-bond network for positioning the water, the hydrolysis reaction still can occur, but at lower rate, in acidic condition in which protonation of the glycosidic oxygen of sucrose by an acid/base catalyst could be unnecessary. However, it requires further investigation.

In addition to the two amino acid residues that may be directly involved in catalysis in the active site, other conserved residues in or around the active site were also examined, including Trp159, Arg259, Asp260 and Cys317.

Trp159 is located between the NDPNG and RDP motifs. The corresponding residue in *T. maritime* invertase was reported to form a hydrogen-bond with the hydroxyl group of the C6 of fructose (Alberto et al., 2004). Replacing Trp159 with either Leu or Phe resulted in increases in K_m values for sucrose, confirming its role in fructose binding. Interestingly, the two mutations had opposite effects on the turnover number and specificity constant of the enzyme (Table 2). There were 8.7-fold and 34.6-fold reductions in the k_{cat} and k_{cat}/K_m values of W159L, but 4.7-fold and 1.5-fold increases in the k_{cat} and k_{cat}/K_m values of W159F as compared to the wild-type enzyme. The results indicated that the aromatic side-chain of the residue in this position was important for efficient catalysis. The increase in the k_{cat} value of W159F can be explained by assuming that the interaction between Phe and fructose is weaker than that between Trp and fructose. Therefore, once the enzyme is saturated with sucrose and the catalytic reaction has occurred, the second product, fructose, can be released more readily from the active site of W159F than from that of the wild-type enzyme.

Both Arg259 and Asp260 are part of the RDP motif. The Asp residue is conserved and occurs in the active site of invertases and fructan-metabolizing enzymes. However, it was suggested that this residue does not have a direct function during catalysis but may affect the nucleophilicity of the nucleophile Asp in the NDPNG motif (Nagem et al., 2004; Verhaest et al., 2006). Alberto et al (2004) proposed that the Arg and Asp in the RDP motif (Arg137 and Asp138 in *T. maritime* invertase) play crucial roles in substrate binding and recognition since the former is hydrogen-bonded to the glucose O4 and the latter forms hydrogen-bonds to O3 and O4 of the fructose unit. In AtcwINV1, the Asp149 of the RDP motif was reported to have a role in stabilization of the fructose ring; Arg148, together with W82, D239 and K242, have an influence on the orientation of glucose (Lammens et al., 2008). In this study, the Arg259 was changed to Lys or Leu and Asp260 was replaced with Asn or Glu. The mutations all resulted in the complete loss of enzymatic activity, in spite of the retention of positive and negative charges by Lys and Glu, respectively. Since the RDP motif is connected by two flexible coils and turn structures, mutations at Arg259 or Asp260 may cause conformational disturbances and a less stable local structure, which consequently lead to changes in substrate binding orientation and prevention of catalysis.

Cys317 and the preceding Glu316 (the acid/base catalyst) reside in the EC motif. The cysteine in the EC motif is thought to play a role in transition state stabilization and/or the catalytic residue microenvironment in *T. maritime* invertase, since it forms hydrogen-bonds to the two Asp residues in the active site (Alberto et al., 2004). However, replacement of this conserved cysteine with other residues has different effects on the activity of invertases from different species. In yeast, the mutant C205A exhibited

decreased affinity for sucrose and k_{cat}, and a 6-fold reduction in the k_{cat}/K_m value (Reddy and Maley, 1996), while in *C. rubrum*, the mutant C237S was inactive (Goetz and Roitsch, 2000). In this study, the mutant C317A of Boβfruct3 was found to exhibit higher K_m and k_{cat} values than the wild-type enzyme, but the specificity constant was not significantly changed. The results indicate that the function of this residue in transition state stabilization and/or the catalytic residue microenvironment was not significant, and it may instead have a role in substrate binding.

Recent studies on the AtcwINV1 mutants showed that next to the highly conserved residues of the NDPNG, EC and RDP motif, Trp82, Asp239 and Lys242 are additional key residues in stabilization of the substrate molecule (Le Roy et al., 2007; Matrai et al., 2008; Lammens et al., 2008). The Asp239 can interact directly with the glucose moiety of sucrose. Substitution of Asp239 with alanine alters substrate specificity of AtcwINV1 into a 1-kestose (Le Roy et al., 2007). In Boβfruct3, the Trp82, Asp239 and Lys242 homologs are Trp159, Asp355 and Arg358, respectively. Whether the functions of these residues are conserved in different invertases or not will be investigated in the future.

4. Conclusions

The results of this study demonstrate that the conserved residues in or around the active site of bamboo vacuolar invertase Boβfruct3 have individual functions in catalyzing sucrose hydrolysis. Our results also suggest that, as long as the conformation of the active site is properly maintained, decreasing the affinity of the enzyme for substrate by mutagenesis at Trp159 and Cys317 can increase the turnover number of the enzyme, probably by facilitating the release of reaction products from the enzyme. Although this finding requires further investigation, it suggests a possible way to engineer this enzyme for application.

5. Experimental

5.1. General experimental procedures

The QuickChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The cobalt-based immobilized metal affinity chromatography (IMAC) resin was from BD Biosciences Clontech (Palo Alto, CA). Yeast extract, peptone and yeast nitrogen base were obtained from Becton, Dickinson and Company (Sparks, MD). Other chemicals were from Sigma-Aldrich (St. Louis, MO).

5.2. Homology modeling and structure prediction

Residues of plant vacuolar invertases Boβfruct3 from Q118-N631 were submitted to the Swiss-Model server (http://swissmodel.expasy.org) to perform sequence analysis, and a protein from *A. thaliana* (PDB id: 2AC1) showing 47% sequence identity was thus determined as the best template for model construction. The model returned from the server was further structurally aligned with Arabidopsis AtcwINV1 D239A mutant in complex with sucrose (PDB id: 2QQU) with Pymol 0.99rc6 (DeLano Scientific, CA, USA) in order to predict the theoretical position of sucrose when binding with Boβfruct3. A final model was proposed after performing energy minimization with DeepView 3.7 (Guex and Peitsch, 1997).

5.3. Plasmid and site-directed mutagenesis

Plasmid pBoIT3, which was constructed by insertion of the coding region of Boβfruct3 cDNA into the *Eco*RI/*Xba*I site of the *Pichia* expression vector pPICZαB (Hsieh et al., 2006), was used for expression of the wild-type recombinant invertase in yeast, and as a template DNA for the generation of mutated plasmids. Site-directed mutagenesis was carried out using the QuickChange site-directed mutagenesis kit and mutagenic primer pairs (Table 3).

Table 3
Primers used for the mutagenesis of *Boβfruct3* cDNA.

Mutation	Nucleotide sequence
D135N	S: 5′-CAGAAGAACTGGATGAACAACCCTAACGGGCCTGTG-3′
	As: 5′-CCCGTTAGGGTTGTTCATCCAGTTCTTCTGAGGCTG-3
D135E	S: 5′-CAGAAGAACTGGATGAACGAGCCTAACGGGCCTGTG-3′
	As: 5′-CCCGTTAGGCTCGTTCATCCAGTTCTTCTGAGGCTG-3′
W159F	S: 5′-GAGGGCGCGGTGTTCGGCAACAAGATCGCCTGGG-3′
	As: 5′-CTTGTTGCCGAACACCGCGCCCTCCGGGTTGTAC-3′
W159L	S: 5′-GGCGCGGTGCTGGGCAACAAGATCGCCTGGG-3′
	As: 5′-GATCTTGTTGCCCAGCACCGCGCCCTCCGG-3′
R259K	S: 5′-GCCAAGGACTTCAAGGACCCCACCACCGCCTGG-3′
	As: 5′-GGTGGGGTCCTTGAAGTCCTTGGCGCCGATGGC-3′
R259L	S: 5′-GCCAAGGACTTCCTGGACCCCACCACCGCCTGG-3′
	As: 5′-GGTGGGGTCCAGGAAGTCCTTGGCGCCGATGGC-3′
D260N	S: 5′-AAGGACTTCCGCAACCCCACCACCGCCTGGC-3′
	As: 5′-GGTGGTGGGGTTGCGGAAGTCCTTGGCGCCG-3′
D260E	S: 5′-AAGGACTTCCGCGAGCCCACCACCGCCTGG-3′
	As: 5′-GGTGGTGGGCTCGCGGAAGTCCTTGGCGCC-3′
E316Q	S: 5′-GCCACCGGCATGTGGCAGTGCATCGACTTCTACCCC-3′
	As: 5′-GAAGTCGATGCACTGCCACATGCCGGTGGCCGGGAC-3′
E316A	S: 5′-GCCACCGGCATGTGGGCCTGCATCGACTTCTACCCC-3′
	As: 5′-GAAGTCGATGCAGGCCCACATGCCGGTGGCCGGGAC-3′
C317A	S: 5′-GGCATGTGGGAGGCCATCGACTTCTACCCC-3′
	As: 5′-GAAGTCGATGGCCTCCCACATGCCGGTGGC-3′

5.4. Expression and purification of the wild-type and mutated invertases in yeast

Pichia pastoris strain X-33 was transformed with plasmid pBoIT3 or mutated plasmids by electroporation. The transformed cells were grown in buffered glycerol-complex medium [0.1 M potassium phosphate, pH 6.0, 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base (YNB), 4×10^{-5}% (w/v) biotin and 1% (v/v) glycerol] at 30 °C until the A_{600} value reached 1.0. The cells were collected by centrifugation and then resuspended in the buffered MeOH-complex medium [0.1 M potassium phosphate, pH 6.0, 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin and 0.5% (v/v) MeOH] containing 1% casamino acid, and were grown at 30 °C for 24 h.

For purification of the recombinant invertases secreted in the medium, the methanol-induced cultures were centrifuged at 6000*g* for 30 min to remove cells. The proteins in the supernatants were precipitated by 80% saturation of ammonium sulfate followed by centrifugation. The precipitates were then dissolved in PB-7.0 (50 mM sodium phosphate, pH 7.0) and dialyzed against PB-7.0 containing 0.3 M NaCl. The resultant enzyme solution was mixed with cobalt-based IMAC resin and incubated for 1 h at 4 °C. The enzyme–resin mixture was then packed into a column, washed with PB-7.0 containing 0.3 M NaCl and 10 mM imidazole, and eluted with PB-7.0 containing 0.3 M NaCl and 150 mM imidazole. Proteins in each fraction were analyzed by 12.5% SDS–PAGE. After electrophoresis, the proteins on the gels were stained with Coomassie Blue R-250 or were transferred onto PVDF membranes for immunodetection using an anti-$(His)_6$ antibody. Fractions containing the recombinant invertase proteins were collected.

5.5. Acidic invertase assay and enzyme kinetics

The reaction mixture contained 0.1 M sodium acetate, pH 5.0, 0.1 M sucrose and an appropriate amount of enzyme. After incubation at 37 °C, the amount of reducing sugars (glucose and fructose) produced was measured by the method of Somogyi-Nelson (Nelson, 1944). For routine assay of invertase activity during puri-

fication of the enzyme, the incubation time was 20 m. For determination of kinetic parameters, the reactions were performed for various time periods to obtain initial velocities. The sucrose concentrations used in kinetic analysis were 0, 20, 40, 60, 80, 100, 150, 200 and 300 mM. One unit of activity was defined as the amount of enzyme that catalyzed the formation of l μmol of reducing sugar from sucrose in 1 h at 37 °C. The kinetic constants were determined from the Eadie-Hofstee plot using the program Enzyme Kinetics!Pro (ChemSW Software, CA). The k_{cat} values were calculated on the basis of a molecular mass of 76,200 Da, as determined by gel filtration chromatography.

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