

The effect of proline insertions on the thermostability of a barley α -glucosidase

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The thermal stability of α -glucosidase is important because the conversion of starch to fermentable sugars during industrial production of ethanol (e.g. brewing, fuel ethanol production) typically takes place at temperatures of 65–73°C. In this study we investigate the thermostability of α -glucosidases from four plant species, compare their deduced amino acid sequences, and test the effect of substituting a proline for the residue present in the wild-type enzyme on the thermostability of α -glucosidase. The α -glucosidase from barley (*Hordeum vulgare*) was significantly less thermostable than the other three α -glucosidases. A comparison of the published deduced amino acid sequences of these four α -glucosidases revealed conserved proline residues in the three most thermostable α -glucosidases that were not found in the barley enzyme. Site-directed mutagenesis was done on recombinant barley α -glucosidase to create proteins with prolines at these conserved positions. The thermostability (T_{50}) of one of these mutant enzymes, T340P, was 10°C higher than the non-mutated enzyme.

Keywords: *Hordeum vulgare*/maltase/starch degradation

Introduction

Starch degradation during seed germination is critical to the viability of cereal seeds because starch is the primary source of carbon and energy for seedlings until they become autotrophic. Degradation of cereal starch results from the concerted action of α -amylase, β -amylase, debranching enzyme and α -glucosidase. Sun and Henson concluded that during the early stages of raw starch hydrolysis in germinating seeds, α -amylase is the most important enzyme and α -glucosidase is the second most important enzyme (Sun and Henson, 1991). Cereal starch degradation by these same endogenous seed enzymes is also critical in some types of fermentation systems, e.g. those used in the production of beer.

The thermal stability of α -glucosidase is important because the conversion of starch to fermentable sugars during the industrial production of ethanol (e.g. brewing, fuel ethanol production) typically takes place at temperatures of 65–73°C. The thermostability of α -glucosidase results in either reduced

efficiency of starch breakdown at the high temperatures used for starch gelatinization or requires that the starch be cooled to a more favorable temperature for enzymatic hydrolysis after the starch is gelatinized.

In order to increase the thermostability of barley α -glucosidase, site-directed mutagenesis of the recombinant enzyme was considered. There are no data in the literature on the thermostability of other plant α -glucosidases nor is there a report of the crystal structure of any α -glucosidase. However, there have been studies of other members of the glycosyl hydrolase family 31 in which thermostability was increased by site-directed mutagenesis. The thermostability of fungal glucoamylase (GA) has been well studied. The GA of *Aspergillus awamori* is maximally active at 50°C and begins to undergo thermal denaturation at 70°C (Chen *et al.*, 1995). Chen *et al.* have succeeded in increasing the thermostability of GA by substituting alanines for asparagines (Chen *et al.*, 1994). Suzuki, working with oligo-1,6-glucosidases from several *Bacillus* species, demonstrated that an increase in the frequency of proline in β -turns and in the total number of hydrophobic residues can enhance protein thermostability (Suzuki, 1989).

In this study we investigate the thermostability of α -glucosidases from four plant species, compare their deduced amino acid sequences, and test the effect of substituting a proline for the residue present in the wild-type enzyme on the thermostability of α -glucosidase.

Materials and methods

Chemicals and reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Plant sources

Seeds of a sugarbeet (*Beta vulgaris*) breeding line (ACS9400461, Wang and Goldman, 1999) were kindly provided by Professor I. Goldman (University of Wisconsin). *Arabidopsis* (*A.thaliana*, cv. Columbia) seedlings were grown under a 10 h photoperiod at a temperature of 25°C for 3 weeks before harvesting. Spinach seeds (*Spinacia oleracea*, cv. Bloomsdale Longstanding, Northrup King) were purchased locally. Barley seeds (*Hordeum vulgare*, cv. Morex) were imbibed, germinated and kilned as described by Henson and Stone (Henson and Stone, 1989).

Isolation of crude extracts from plants

Crude extracts from malted barley, seeds of sugarbeet and spinach, and leaves from *Arabidopsis* were isolated using published protocols (Chiba *et al.*, 1978; Im and Henson, 1995; Sugimoto *et al.*, 1995; Monroe *et al.*, 1999). The extracts were dialyzed (16 h, 4°C) against 50 mM sodium-succinate (pH 4.5).

Enzyme assay

α -Glucosidase activities were measured by the release of glucose from maltose. Unless otherwise stated, the enzyme

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was incubated for 1 h at 30°C with 25 mM maltose in 50 mM sodium-succinate (pH 4.5) during which time substrate hydrolysis rates were linear. The glucose released was quantified by determining the reduction of NAD with the coupled reactions of hexokinase and glucose-6-dehydrogenase (Im and Henson, 1995).

Thermostability testing of plant extracts

Enzyme extracts were incubated for 10 min at temperatures ranging from 5 to 75°C. The residual rate of maltose hydrolysis was assayed for 1 h at 30°C.

Alignment of α-glucosidase sequences from four plant species

Alignment of the published α-glucosidase deduced amino acid sequences from barley (GenBank accession number U22450), spinach (D86624), sugarbeet (D89615) and *Arabidopsis* (AF014806) was done using the program Align Plus-Version 2.0 (Scientific and Educational Software).

Mutagenesis

Mutagenesis was done using the Muta-Gene kit (BIO-RAD, Hercules, CA). Barley α-glucosidase cDNA (Tibbot and Skadsen, 1996) was sub-cloned into the *Eco*RI site of the phagemid pTZ18U (BIO-RAD). *Escherichia coli* strain CJ236 was used to generate dU-substituted DNA, and single-stranded DNA was isolated using the helper phage M13K07 (BIO-RAD). Generation of the mutant R336P used the oligonucleotide CCGTGAAGTTGACAGGATCCAAGGTGAAG (5', reverse complement) to replace the codon for arginine (CGT) with a codon for proline (CCT) and to remove a Tth111I site. The mutant T340P was generated using the oligonucleotide GAGCTCGGCGGCGGGGAAGTTTACACGGTC to replace the codon for threonine (ACC) with a codon for proline (CCC) and to remove a Tth111I site. To generate the mutant A742P we used the oligonucleotide CCAGGAGGTGGAACGGGTCCGGCGC to replace the codon for alanine (GCG) with a codon for proline (CCG) and to remove a *Rsr*II site.

Sequencing

The mutated cDNA was sequenced using the Sanger method (Sanger *et al.*, 1977) with an automatic sequencer by the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL.

Expression

The mutated cDNA was subcloned into the *Eco*RI site of the *Pichia pastoris* vector pPIC9K (Invitrogen, CA) and transformed into *P.pastoris* GS115 using the *Pichia* EasyComp kit (Invitrogen). Ten histidine autotrophs (His+) were induced with methanol following the instructions in the *Pichia* Expression kit (Invitrogen). *Pichia* colonies that secreted measurable α-glucosidase activity were used for thermostability studies.

Thermostability testing of wild-type and mutated α-glucosidases

Enzyme extracts from non-mutated, recombinant α-glucosidase (rAGL), T340P and A742P were incubated for 10 min at temperatures ranging from 0 to 60°C at a pH of either 6.0 or 4.0. The residual rate of maltose hydrolysis was assayed for 18 h at 30°C at pH 4.5.

Arrhenius plot

Enzyme extracts from rAGL and T340P (pH 6.0) and substrate (pH 4.5) were incubated separately (10 min) at temperatures

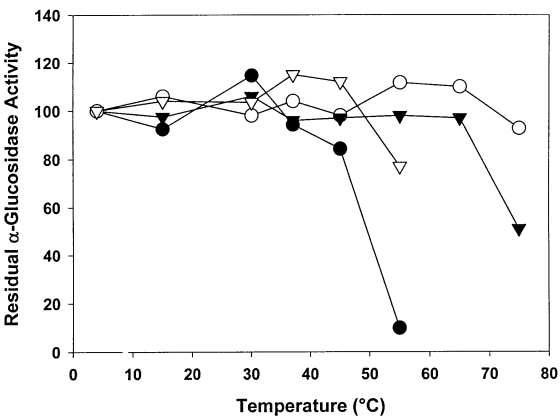


Fig. 1. Effect of temperature on the activity of α-glucosidases extracted from barley (closed circle), sugarbeet (open circle), spinach (closed triangle) and *Arabidopsis* (open triangle). Enzyme extracts were incubated for 10 min (pH 4.5) at temperatures ranging from 5 to 75°C. The residual rate of maltose hydrolysis was assayed for 1 h at 30°C (pH 4.5). Residual activity is based on percent of non-heated control.

BARLEY	GFKDFTLDRVNF TAAELRPFVDR LHRNAQKYVLILDPIRVDPIDATYGT (377)
S. BEET	aFKDFTLDpVhFpIdkmgqFvTkLHRNgQrYvPILDPGI---ntnksYGT (409)
SPINACH	ayKDFTLDpVNFpIdkmgqFvnnLHKNgQKYVvILDPGI---stnkTYeT (405)
ARABID.	GyKDFTLDpVNFpedkmqsFVDtLHKNgQKYVLIILDPGI---gvDssYGT (399)
BARLEY	SGRYTAYWTGDNAATWGLDRYSINTLSFGLFGMPMIGADICGFNGNTTE (573)
S. BEET	SGkYTAhWTGDNAArWdDLqYSIPtMLnFGLFGMPMIGADICGFaesTTE (607)
SPINACH	SGkYTAhWTGDNAATWnDLvSIPsMLdFGLFGiPMvGADICGFIGNTTE (603)
ARABID.	SGkYTAhWTGDNAAKWeDLaySIpgiLnFGLFGiPMvGADICGFshdTTE (597)
BARLEY	TILPLQQSALTTSRRRTAFHLLVALAEDGTASGYLFLDDGDSPEYGR-R (772)
S. BEET	nIvamQgeAmTTqaARsTpFHLlLvmsdhvastGeLFLDnGiemdiGg-p (806)
SPINACH	nILamQgkAmTTqaARkTpFHLlLvmsdcGasfGeLFLDDGvevtmGvnr (803)
ARABID.	sIvamQgeALTTrdARkTpyqLLVvasrlenISGeLFLDDGenlrmaG-g (796)

Fig. 2. Portions of the sequence alignment of α-glucosidases from barley (GenBank accession No. U22450), spinach (D86624), sugarbeet (D89615) and *Arabidopsis* (AF014806). Alignment was done using the program Align Plus-Version 2.0 (Scientific and Educational Software). Gaps to optimize alignment are designated by (-). Altered residues are highlighted and underlined in the barley α-glucosidase sequence.

ranging from 0 to 55°C and assayed for 3.5 h at the same temperatures. The results were plotted as log V_i versus $10^{-3}/T$ (K).

Results and discussion

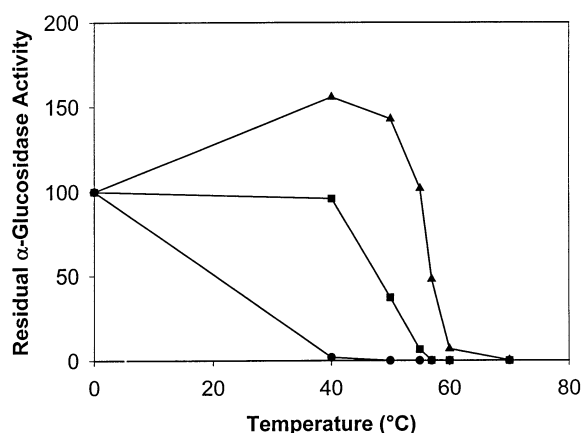
In order to increase the thermostability of α-glucosidase, site-directed mutagenesis of the recombinant protein was considered. However, since no crystal structure has been determined for any α-glucosidase of the glycosyl hydrolase family 31, selection of targets for mutagenesis was based on comparisons of the thermostabilities and deduced amino acid sequences of α-glucosidases from four plant species.

The thermostability profiles of barley, sugarbeet, spinach and *Arabidopsis* α-glucosidases are shown in Figure 1. Sugarbeet α-glucosidase was the most thermostable and still retained greater than 90% of its maximal activity following exposure to 75°C for 10 min. The spinach α-glucosidase was the second most thermostable, followed by those from *Arabidopsis* and barley. The barley enzyme had only 10% of its maximal activity after exposure to 55°C for 10 min.

A portion of the alignment of the α-glucosidase deduced amino acid sequences from barley, sugarbeet, spinach and *Arabidopsis* is shown in Figure 2. An analysis of sequence identity between the enzymes showed that, even though barley had a relatively high sequence identity with sugarbeet and spinach (50.8 and 53.6%, respectively), key differences between the sequences of the enzymes existed. The sugarbeet

Table I. Secondary structure predictions for α -glucosidase from barley, sugarbeet, spinach and *Arabidopsis* made using the Peptide Structure program based on the theories of Garnier *et al.* (Garnier *et al.*, 1978)

	Barley	Sugarbeet	Spinach	<i>Arabidopsis</i>
336	β -Turn	Undefined	β -Sheet	Undefined
340	α -Helix (1st turn)	α -Helix (1st turn)	α -Helix (1st turn)	α -Helix (1st turn)
547	β -Sheet	β -Sheet	β -Sheet	Undefined
742	α -Helix (2nd turn)	Undefined	Undefined	Undefined

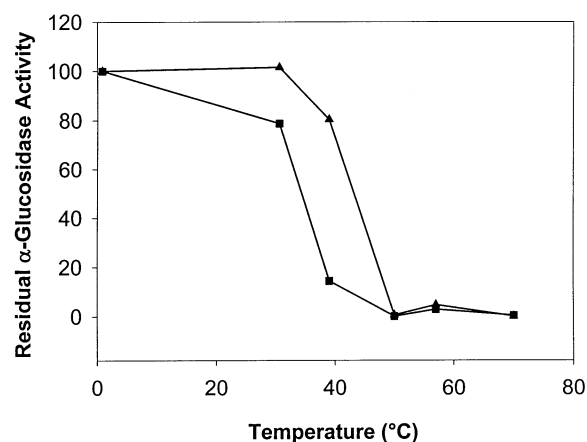
**Fig. 3.** Effect of temperature on the activity of recombinant barley α -glucosidase (rAGL, square), the T340P mutant enzyme (triangle) and the A742P mutant enzyme (circle) at pH 6.0. rAGL, T340P and A742P were incubated for 10 min at temperatures ranging from 0 to 60°C at pH 6. The residual rate of maltose hydrolysis was assayed for 18 h at 30°C at pH 4.5. Residual activity is based on percent of non-heated control (rAGL = 0.7 nmol/min/ml, T340P enzyme = 0.9 nmol/min/ml, A742P enzyme = 1.65 nmol/min/ml).

(with the highest thermostability), spinach and *Arabidopsis* sequences had four conserved proline residues that were not found in the barley enzyme (which had the lowest thermostability) including prolines at position 336, 340, 547 and 742 (based on the barley sequence).

Because proline residues are important for thermostability (Matthews *et al.*, 1987; Suzuki *et al.*, 1989), mutations were made in recombinant barley α -glucosidase to insert proline residues in positions where they were present in the more thermostable α -glucosidases. Mutations were made to add prolines to positions 336, 340 and 742. While T340P and A742P had α -glucosidase activity, a mutation at position 336 (R336P) resulted in an inactive protein (data not shown).

No mutation was made at position 547 because secondary structure predictions made using the program Peptide Structure (Wisconsin GCG Package, Madison, WI), which is based on the theories of Garnier *et al.* (Garnier *et al.*, 1978), placed this residue in the middle of a β -sheet (Table I). According to Watanabe *et al.* the insertion of a proline residue in the middle of a β -sheet would not enhance the thermostability of the enzyme (Watanabe *et al.*, 1991).

The thermostability profiles (pH 6) of wild-type recombinant barley α -glucosidase (rAGL), and the mutant enzymes T340P and A742P, are shown in Figure 3. The thermostability profile of rAGL shown in Figure 3, which is from a crude extract, was almost identical to the thermostability profile of the purified rAGL previously published by Muslin *et al.* (Muslin *et al.*, 2000). In fact, the T_{50} was 47.8°C for the crude enzyme and 47.6°C for the purified enzyme. Therefore, we concluded that it was not necessary to purify any of the enzyme extracts used in this study.

**Fig. 4.** Effect of temperature on the activity of rAGL (square) and the T340P mutant enzyme (triangle) at pH 4.0. rAGL and the T340P enzyme were incubated for 10 min at temperatures ranging from 0 to 60°C at pH 4.0. The residual rate of maltose hydrolysis was assayed for 18 h at 30°C at pH 4.5. Residual activity is based on percent of non-heated control (rAGL = 0.8 nmol/min/ml, T340P enzyme = 1.5 nmol/min/ml).

The A742P enzyme had a T_{50} of 20°C and was completely inactive after exposure to 40°C. In contrast, the T340P enzyme showed the highest thermostability and had no decrease in activity until it was heated to temperatures above 50°C. The T_{50} for the T340P enzyme was 58°C, which represents an increase in the T_{50} of 10°C from the wild-type enzyme. The activities of rAGL, T340P and A742P α -glucosidases prior to heating were 0.7, 0.9 and 1.65 nmol/min/ml, respectively.

In order to determine whether the thermostability is affected by pH, a thermostability profile was generated for the wild-type enzyme and the T340P enzyme at pH 4.0, the pH at which rAGL is the most thermolabile (Muslin *et al.*, 2000). At this pH the wild-type enzyme had a T_{50} of 36°C and the T340P enzyme had a T_{50} of 43°C (Figure 4). It is apparent that while the thermostability of both enzymes was reduced by low pH, the T340P enzyme was still more thermostable than the wild-type enzyme. The original (non-heated) activity of rAGL and the T340P enzyme at pH 4 was 0.8 and 1.5 nmol/min/ml, respectively. At pH 4, the rAGL enzyme was 14% more active than at pH 6 and the T340P enzyme was 36% more active at pH 4 than at pH 6. This increase in activity at the lower pH was expected based on the pH profile of rAGL (Muslin *et al.*, 2000). In this profile the pH optimum of the enzyme was ~4.

An Arrhenius plot was generated for the wild-type enzyme and the T340P enzyme and the results are shown in Figure 5. The Arrhenius energy of activation (E_a) for rAGL was 17.5 while for the T340P enzyme it was 16.5.

Taken together the results presented in this study indicate that the substitution of a proline for a threonine in position 340 enhances the thermostability of α -glucosidase from barley.

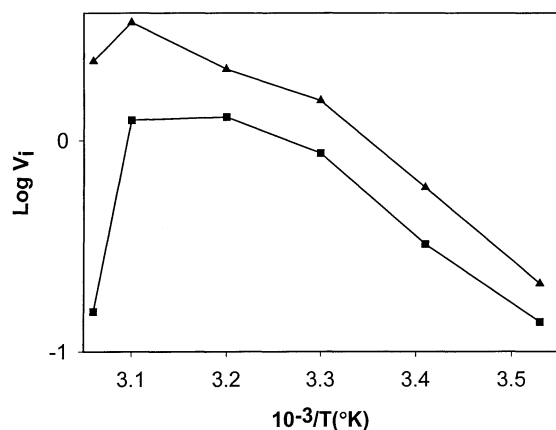


Fig. 5. Arrhenius plot of rAGL (square) and the T340P mutant enzyme (triangle). rAGL and the T340P mutant enzyme, at pH 6.0 and substrate, at pH 4.5, were incubated separately (10 min) at temperatures ranging from 0 to 55°C and assayed for 3.5 h at the same temperatures at pH 4.5.

Several studies with other enzymes show an increase in thermostability due to the addition of proline residues at key positions. Allen *et al.* made three proline substitution mutations in *A. awamori* GA (Allen *et al.*, 1998). One of these mutations, S30P, stabilized the enzyme. The S30P mutant increased the T_{50} of the enzyme by 1.7°C. Similarly, Watanabe *et al.* introduced nine proline substitutions into the oligo-1,6-glucosidase gene from *B. cereus* ATCC 7064 (Watanabe *et al.*, 1994). These mutations resulted in a 5.1°C increase in the protein's T_{50} .

Although no three-dimensional structure has been reported for an α -glucosidase, the secondary structure of the barley protein was predicted using the program Peptide Structure. Table I shows the secondary structure predictions for the residues at positions 336, 340, 547 and 742 (numbers based on barley sequence) in α -glucosidases from barley, sugarbeet, spinach and *Arabidopsis*. According to Table I, the threonine at position 340 of barley α -glucosidase is in the first turn of an α -helix. Similarly, the prolines in the same position in the sugarbeet, spinach and *Arabidopsis* enzymes are also found in the first turn of an α -helix. According to Watanabe, the presence of prolines in the first turn of α -helices and at the second sites of β -turns often results in protein stabilization (Watanabe *et al.*, 1991). Therefore, it makes sense that the addition of a proline in the first turn of an α -helix in the barley α -glucosidase made the enzyme more thermostable.

Residue 742 in barley α -glucosidase was predicted to be in the second turn of an α -helix (Table I). According to Watanabe *et al.*, the influence of proline on nearby residues is so strong that the imprudent substitution of proline for another amino acid might result in the destruction of both secondary and tertiary structures as well as the loss of protein function and/or stability (Watanabe *et al.*, 1996). The substitution of a proline in the second turn of an α -helix (as was done with the mutation A742P) apparently had a destructive effect on the thermostability of the enzyme but not on the enzyme's activity (Figure 3).

According to the secondary structure program Peptide Structure, residue 336 in barley α -glucosidase is in the second site of a β -turn (Table I). Thus, we predicted the insertion of a proline at this position would positively affect the enzyme's thermostability. However, the mutation R336P resulted in an inactive enzyme. It should be noted that the secondary structure predictions made using the method of Chou and Fassman

(Chou and Fassman, 1978) indicate that residue 336 is in the last turn of an α -helix (data not shown). Therefore, according to these structural predictions, the addition of a proline at residue 336 could destabilize the enzyme. Such a destabilization occurred as evidenced by the R336P mutation resulting in an inactive α -glucosidase. The secondary structure predictions (Peptide Structure) for sugarbeet and *Arabidopsis* at position 336 indicate that these prolines are in undefined regions; therefore, the effect these prolines have on enzyme stability cannot be anticipated. According to the program Peptide Structure, the proline at position 336 in spinach is in a β -sheet. According to Watanabe, this proline should not enhance the protein's thermostability, possibly indicating that it is not this residue that is the cause of this enzyme's thermostability.

The difference in T_{50} values between the mutant and wild-type enzymes was decreased from 10°C at pH 6 to 7°C at pH 4. These data could indicate that different mechanisms are responsible for thermostability at these two pHs. Such has been documented to be the case for lysozyme. Ahern and Klivanov found that the thermoinactivation of lysozyme can be attributed to one or more of the following mechanisms: deamidation of asparagine residues, hydrolysis of peptide bonds at aspartic acid residues, destruction of disulfide bonds and the formation of non-functional aggregates or other incorrect structures (Ahern and Klivanov, 1985). The relative contributions of these mechanisms to thermoinactivation are dependent upon the individual protein and its environment, e.g. pH. They determined that at pH 4.0 the dominant mechanism of thermoinactivation of lysozyme is deamidation of asparagine residues. At pH 8 destruction of cysteine residues and formation of incorrect structures become the dominant mechanisms of thermoinactivation.

According to an analysis of the deduced amino acid sequences of barley, sugarbeet, spinach and *Arabidopsis* α -glucosidases, the sugarbeet and spinach enzymes are the most related (Monroe *et al.*, 1999). Perhaps the presence of the conserved prolines in these dicot species, specifically the proline at position 340, represents an evolutionary trend towards a more thermostable enzyme.

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