Removal of the Four C-Terminal Glycine-Rich Repeats Enhances the Thermostability and Substrate Binding Affinity of Barley β-Amylase†

Yue F. Ma,* Jason K. Eglinton, D. Evan Evans, Sue J. Logue, and Peter Langridge

Department of Plant Science, Waite Campus, The University of Adelaide, Glen Osmond, SA 5064, Australia

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ABSTRACT: Barley β-amylase undergoes proteolytic cleavage in the C-terminal region after germination. The implication of the cleavage in the enzyme's characteristics is unclear. With purified native β-amylases from both mature barley grain and germinated barley, we found that the β-amylase from germinated barley had significantly higher thermostability and substrate binding affinity for starch than that from mature barley grain. To better understand the effect of the proteolytic cleavage on the enzyme's thermostability and substrate binding affinity for starch, recombinant barley β-amylases with specific deletions at the C-terminal tail were generated. The complete deletion of the four C-terminal glycine-rich repeats significantly increased the enzyme's thermostability, but an incomplete deletion with one repeat remaining did not change the thermostability. Although different C-terminal deletions affect the thermostability differently, they all increased the enzyme's affinity for starch. The possible reasons for the increased thermostability and substrate binding affinity, due to the removal of the four C-terminal glycine-rich repeats, are discussed in terms of the three-dimensional structure of β-amylase.

β-Amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) catalyzes the release of maltose from the nonreducing ends of starch. It is important for the production of fermentable sugars in the brewing industry where a large quantity of starch needs to be converted into fermentable sugars. The relatively thermolabile nature of barley β-amylase is a key determinant of fermentable sugar production in the brewing process (*1*). Barley β-amylase has been cloned and sequenced by Kreis et al. (*2*) and Yoshigi et al. (*3*). The three-dimensional structure of a mutant of barley β-amylase (BBA-7s)[1] revealed that the enzyme is composed of a core $(\alpha/\beta)_8$ barrel domain and a C-terminal tail on the protein surface (*4*). This C-terminal tail has four glycine-rich repeats (Figure 1).

Barley β-amylase undergoes proteolytic cleavage in the C-terminal region, and an approximately 4 kDa segment is cleaved from the C-terminus after germination (*5*, *6*). This 4 kDa cleavage (unspecified site) was reported not to affect the thermostability of barley β-amylase, but a 54-amino acid deletion (including the four C-terminal glycine-rich repeats and eight additional amino acid residues upstream of the four repeats) of a recombinant barley β-amylase resulted in a significant decrease in the thermostability (*7*). However, the C-terminal cleavage significantly increased the substrate binding affinity in both recombinant barley β-amylase (*7*) and native barley β-amylases (*8*). Moreover, soybean β-amylase is relatively thermostable compared with barley

FIGURE 1: Amino acid sequence of the C-terminal tail of barley β-amylase deduced from cDNA. Arrows show cleavage sites in the native protein (J. K. Eglinton, unpublished data), and the four glycine-rich repeats are boxed.

β-amylase (*9*). These two sequences are 66.7% identical (*3*). Their three-dimensional structures are very similar except that soybean β-amylase lacks the four glycine-rich repeats (*4*). It seems likely that the proteolytic cleavage of barley β-amylase at the C-terminal tail has both positive and negative effects on the properties of the enzyme. These various influences may be due to the different cleavage sites in the C-terminal tail. Lundgard and Svensson reported that the proteolytic cleavage of barley β-amylase is stepwise from the C-terminus, rather than specific (*5*, *10*). They isolated four major forms which terminated in A533, Q519−G528, Q508, and G493−Q497 and found that the amount of each component is dependent on the concentrations of the proteases (*10*). These results suggest that the degree of peptide cleavage in the C-terminal tail is dependent on the cleavage conditions. Therefore, it is important for malting barley improvement to clarify how the different cleavage sites affect the characteristics of barley β-amylase.

Three β-amylase alleles, *Bmy*1-Sd1, *Bmy*1-Sd2L, and *Bmy*1-Sd2H, have been identified in cultivated barley at the *Bmy*1 locus on chromosome 4H (*1*). The corresponding enzymes exhibit differences in thermostability and are termed Sd1 (intermediate thermostability), Sd2L (low thermosta-

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* To whom correspondence should be addressed. Phone: 61 8 83037286. Fax: 61 8 83037109. E-mail: yma@waite.adelaide.edu.au.

[1] Abbreviations: PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BBA-7s, 7-fold mutant of barley β-amylase.

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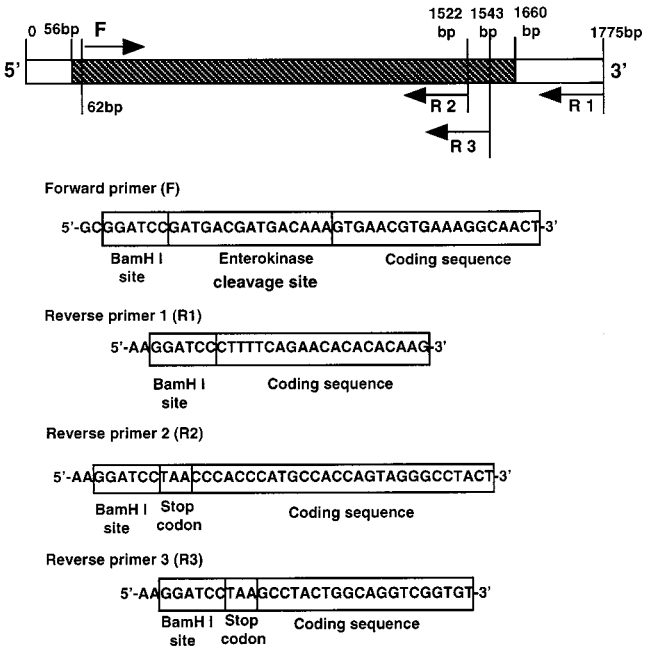

FIGURE 2: PCR primers designed for amplification of barley β-amylase cDNAs with specific deletions at the 5′- and 3′-ends: (white bar) untranslated region and (hatched bar) translated region.

bility), and Sd2H (high thermostability). To gain insight into the effects of the proteolytic cleavage on the thermostability and kinetic properties, we have purified native β-amylase forms from both mature barley grain and germinated barley of Sd1 and Sd2L barley varieties, and generated recombinant barley β-amylases with different C-terminal deletions. The effects of the proteolytic cleavage on the thermostability and kinetics properties were evaluated. In this paper, we report that the C-terminal cleavage of barley β-amylase can increase the enzyme's thermostability and substrate binding affinity for starch.

MATERIALS AND METHODS

Materials. The barley varieties Franklin (Sd1) and Schooner (Sd2L) were used to clone β-amylase cDNAs. Developing grains were harvested 23 days after anthesis, immediately frozen in liquid nitrogen, and stored at −80 °C.

Ni−NTA spin columns, anti-His antibody for detection of six-His-tagged proteins, and a pQE-30 expression vector kit containing *Escherichia coli* strain M15 were obtained from Qiagen. The pGEM-T Easy vector and *Taq* polymerase were purchased from Promega. Superscript II RT was purchased from Life Technologies. Enterokinase was a product of Novagen. The oligonucleotides used as PCR primers were synthesized on an Applied Biosystems model 381A DNA synthesizer. The β-amylase assay kit (Betamyl) was obtained from Megazyme. Native β-amylases were previously purified from mature barley grain and germinated barley of Franklin and Schooner to homogeneity (*8*).

Cloning and Sequencing of Barley β-Amylase cDNAs. Total RNA was isolated from developing barley grain by using the method of Cox and Goldberg (*11*) with minor modifications. The total RNA was used to synthesize the first-strand cDNA using SuperScript II RT. Specific PCR primers were designed on the basis of the published β-amylase nucleotide sequence (*3*) (Figure 2). These primers were used to synthesize three cDNAs encoding both Sd1 and Sd2L β-amylases with deletions at the N- and C-termini. The forward primer and reverse primer 1 were used to generate the full-length β-amylase, and the forward primer and reverse primer 3 were used to generate a β-amylase terminating at G489. A combination of the forward primer and reverse primer 2 was also used to generate a β-amylase terminating at G496. As the forward primer begins at nucleotide 62 (based on the N-terminal sequence of β-amylase from germinated barley), all recombinant β-amylases lack the first two amino acid residues (M1 and E2). PCR products were cloned into the pGEM-T Easy vector. DNA sequences were confirmed by sequencing with an ABI automated DNA Sequencer (Applied Biosystems Inc.).

Production of Purified Recombinant β-Amylases. *Bam*HI fragments were cut from the pGEM-T Easy vector, subcloned into the expression vector pQE-30, and then transformed into *E. coli* host strain M15. Cell lines containing the correct orientation of the cDNA inserts were selected for production of the recombinant β-amylases. Expression of the β-amylase cDNAs and affinity purification of the recombinant proteins were performed according to the manufacturer's instructions.

Potential interference by the six-His tag in thermostability and kinetic assays was prevented by digesting 0.2 mg of purified recombinant proteins with 4 units of enterokinase at 23 °C for 16 h. The undigested recombinant proteins were removed by loading the digestion mixture on a Ni−NTA column. The complete cleavage of the six-His tag from the recombinant β-amylases was monitored with anti-His antibodies. Enterokinase was removed by Bio-gel P60 (Bio-Rad) size exclusion chromatography.

Irreversible Thermal Inactivation. Irreversible thermal inactivation of purified recombinant β-amylases was assayed by the determination of T_{50} values. The T_{50} value is the temperature at which 50% of initial enzymatic activity is lost after heat treatment. The assay was carried out as follows. Purified β-amylases were diluted to a final concentration of 0.1 mg/mL with 0.1 M maleic acid buffer containing 2.8 mg/mL BSA (pH 6.2). The diluted enzymes were incubated at a range of temperatures from 50 to 62.5 °C for 30 min. After incubation, the enzyme solutions were immediately cooled in ice, and the residual β-amylase activity was measured using the Betamyl assay. The T_{50} value was determined by linear interpolation between the data points closest to 50% remaining activity.

Betamyl Assay. β-Amylase activity was determined according to the manufacturer's instructions. The assay was performed by mixing the diluted enzyme (50 μL) in 0.1 M maleic acid buffer (pH 6.2) with 50 μL of the substrate mixture containing α-glucosidase (200 units) and the specific "Betamyl" substrate *p*-nitrophenylmaltopentaoside (PNPG5) (4.75 mg/mL). The enzyme concentrations were adjusted by the initial reaction rates which are linear only with enzyme concentrations that give a reaction absorbance value of 0.8. After incubation for 10 min at 40 °C, the reaction was terminated by addition of 750 μL of Trizma base (1%, w/v). The absorbance was read at 410 nm. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of maltose/min.

Kinetic Assay. Potato starch was reduced by the method of Takeda et al. (*12*) and used as a substrate for kinetic assay. Both enzyme and substrate concentrations were tested to

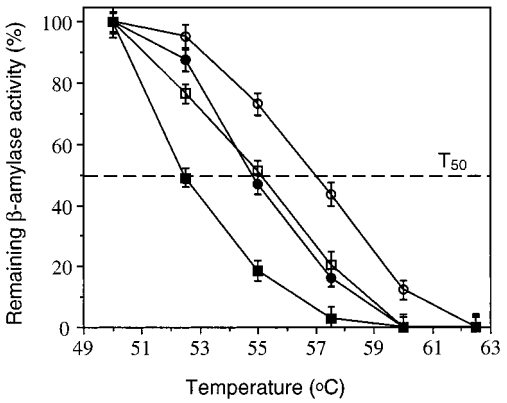

FIGURE 3: Irreversible thermal inactivation of purified native barley β-amylases. Activity is expressed as a percentage of initial activity. Values are the means of three independent determinations with the standard deviation shown as bars: (○) β-amylase from Sd1 germinated barley, (●) β-amylase from Sd1 barley grain, (□) β-amylase from Sd2L germinated barley, and (■) β-amylase from Sd2L barley grain.

force the rate of hydrolysis to measurable levels, and special care was taken to ensure that initial reaction rates were measured throughout. The assay was performed by mixing the purified enzymes (50 ng) with a substrate solution (10−200 μM) containing 50 mM acetate buffer (pH 5.0) in a final volume of 500 μL. Under these conditions, reaction rates were linear for at least 15 min. The amount of reducing sugars was determined using the Somogyi−Nelson method (*13*). One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 μmol of reducing sugar/min. Calculation of starch concentration in units of molar is based on the determination of starch molecular weight by a series of starch solutions of different concentrations (milligrams per milliliter). The reducing ends were assessed by the Somogyi−Nelson method (*13*) using a glucose standard curve. This value should be treated as an approximation as the starch substrate is expected to be heterogeneous.

Kinetic data were processed by a nonlinear regression analysis based on the Michaelis−Menten equation using EZ-FIT software (*14*). Variations of approximately 10% were usually observed in the kinetic analysis with the starch substrate.

Modeling. The determined crystal structure of BBA-7s (PDB entry 1B1Y) (*4*) was used to construct a homology-based model of the three-dimensional structure of Sd2L β-amylase by using Swiss-Model (*15, 16*). Molecular visualization and analysis were performed using the program SPDBV 3.5 (*17*).

Other Methods. The methods for protein concentration and SDS−PAGE have been described previously (*8*).

RESULTS

Thermostability of Native Barley β-Amylases. The thermostability of native barley β-amylases was measured by irreversible thermal inactivation curves (Figure 3) which were used to determine the T_{50} values. The T_{50} values for β-amylases from Sd1 barley grain, Sd1 germinated barley, Sd2L barley grain, and Sd2L germinated barley were 54.7, 57.0, 52.6, and 55.0 °C, respectively. Therefore, β-amylases from Sd1 and Sd2L germinated barley had T_{50} values 2.3 and 2.4 °C higher than those of their respective β-amylases from barley grain. The difference in T_{50} values between the Sd1 and Sd2L enzymes is maintained after germination.

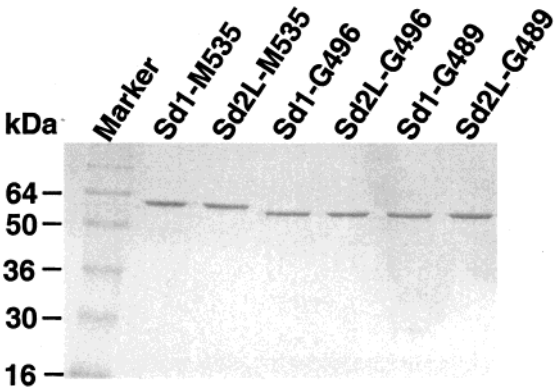

FIGURE 4: SDS−PAGE of purified barley recombinant β-amylases stained with Coomassie blue R250.

Production of Purified Recombinant β-Amylases. Protein sequencing of barley β-amylases revealed that both the N- and C-termini were cleaved after germination (J. K. Eglinton, unpublished data). The first two amino acid residues were removed at the N-terminus, and there were three cleavage sites (a major site at G489 and two additional sites at H483 and G496) and many ragged ends at the C-terminal tail. On the basis of the cleavage sites, cDNA fragments with these specific deletions at the 5′- and 3′-ends were amplified from both Sd1 and Sd2L barley varieties to elucidate whether these deletions affect thermostability and kinetic properties, except for that with deletion at H483 because the effect of a deletion at Q481 (only two residue differences from H483) on thermostability and affinity for starch has been reported (*7*). A fragment amplified by the forward primer and reverse primer 1 began at nucleotide 62 and ended at nucleotide 1775, encoding the full-length β-amylase without M and E at positions 1 and 2, respectively (533 amino acid residues from V3 to M535). The second fragment amplified with the forward primer and reverse primer 3 was 1492 bp long, from nucleotide 62 to nucleotide 1522. This cDNA encodes the β-amylase containing 487 amino acid residues from V3 to G489, the dominant form after proteolytic cleavage. The third fragment amplified with the forward primer and reverse primer 2 was 1503 bp long, from nucleotide 62 to 1543. This cDNA encodes the β-amylase containing 494 amino acid residues from V3 to G496, one of the minor species from the proteolytic cleavage.

These cDNAs were expressed in *E. coli* as soluble and catalytically active proteins. The recombinant enzymes were designated as Sd1-M535, Sd1-G496, Sd1-G489, Sd2L-M535, Sd2L-G496, and Sd2L-G489 on the basis of the terminal amino acid number and the β-amylase allele. Complete removal of the six-His tag and enterokinase cleavage site was confirmed with an immunoblot using the anti-His antibody, where no bands were detected (data not shown). The purified recombinant β-amylases are shown in Figure 4.

Thermostability of Recombinant β-Amylases. As shown in Figure 5, T_{50} values for recombinant β-amylases Sd1-G489, Sd1-G496, Sd1-M535, Sd2L-G489, Sd2L-G496, and Sd2L-M535 were 57.6, 55.0, 55.0, 55.2, 52.7, and 52.6 °C, respectively. Thus, both Sd1-G489 and Sd2L-G489 had T_{50} values 2.6 °C higher than those of Sd1-M535 and Sd2L-M535. However, the T_{50} values of Sd1-G496 and Sd2L-G496

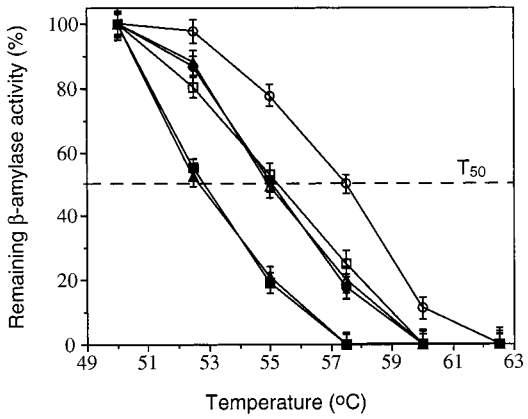

FIGURE 5: Irreversible thermal inactivation of purified recombinant β-amylases. Activity is expressed as a percentage of initial activity. Values are the means of three independent determinations with the standard deviation shown as bars: (○) Sd1-G489, (△) Sd1-G496, (●) Sd1-M535, (□) Sd2L-G489, (▲) Sd2L-G496, and (■) Sd2L-M535.

Table 1: Kinetic Parameters for the Hydrolysis of Starch by Recombinant β-Amylases

β-amylase	K_m (μM)	k_{cat} (units/mg)
Sd1-M535	16.23 ± 0.61	300 ± 8
Sd2L-M535	16.03 ± 0.84	300 ± 8
Sd1-G496	3.35 ± 0.18	310 ± 10
Sd2L-G496	8.42 ± 0.71	320 ± 7
Sd1-G489	3.20 ± 0.28	310 ± 9
Sd2L-G489	8.31 ± 0.58	320 ± 6

were almost the same as those of Sd1-M535 and Sd2L-M535, respectively. Comparison of Figures 3 and 5 shows that the T_{50} values of recombinant β-amylases Sd1-M535 and Sd2L-M535 were very similar to those of Sd1 and Sd2L grain β-amylases (54.7 vs 55.0 °C and 52.6 vs 52.6 °C), although the first two amino acids at the N-terminus were missing in Sd1-M535 and Sd2L-M535 compared with Sd1 and Sd2L grain β-amylases. The thermostability of recombinant β-amylases with or without the six-His tag and the enterokinase recognition site (17 amino acid residues) at the N-terminus of the protein was similar (data not shown). The higher T_{50} value of Sd1 compared to that of Sd2L β-amylase was maintained after the deletions.

Kinetic Parameters of Recombinant β-Amylases. As shown in Table 1, Sd1-G489 and Sd2L-G489 had lower K_m values than Sd1-M535 and Sd2L-M535. The K_m values of Sd1-G489 and Sd2L-G489 were 3.20 and 8.31 μM, respectively, compared to 16.23 (Sd1-M535) and 16.03 μM (Sd2L-M535). Sd1-G496 and Sd2L-G496 had K_m values very similar to those of Sd1-G489 and Sd2L-G489. No differences in k_{cat} values were observed between recombinant β-amylases.

Structure of the C-Terminal Region of Barley β-Amylase. Only Sd2L β-amylase was modeled since the sequence of Sd1 β-amylase is 99.1% identical with that of Sd2L β-amylase, and has the same cleavage sites at both the N- and C-termini as Sd2L β-amylase (J. K. Eglinton, unpublished data). The modeling of Sd2L β-amylase was based on the determined crystal structure of BBA-7s (PDB entry 1B1Y) (*4*). Sd2L β-amylase only exhibited 13 amino acid differences from BBA-7s, which includes two amino acid substitutions (V233A and L347S), four amino acid residues missing (positions 2−5), and seven mutated amino acids in

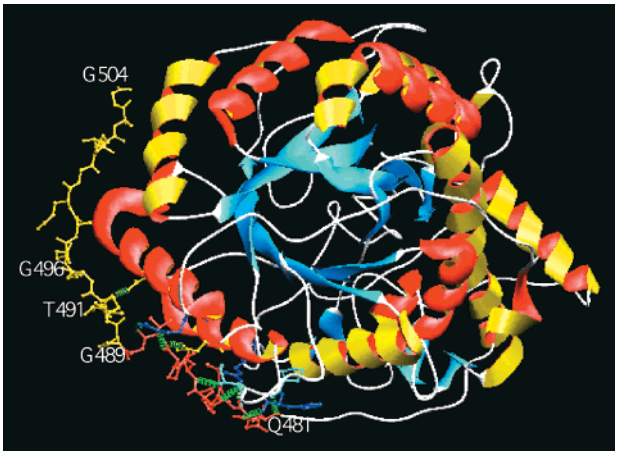

FIGURE 6: Ribbon diagram of Sd2L β-amylase showing hydrogen bond interactions of the C-terminal residues between Q481 and G504 with other parts of the protein. Hydrogen bonds are shown as green dotted lines. The residues from Q481 to V488 (upstream of the four C-terminal glycine-rich repeats) are colored in red, and the residues from G489 to G504 of the four C-terminal glycine-rich repeats are colored in yellow. The figure was generated using the program SPDBV 3.5 (*17*).

BBA-7s (M185L, S295A, I297V, S350P, S351P, Q352D, and A376S). Such a high degree of sequence homology provides a high degree of confidence in the model structure. The quality of the model was confirmed by its root-mean-square (rms) backbone deviation from that of BBA-7s with a value of 0.11 Å for Cα atoms (*18*).

The residues beyond G504 are absent in the model as they were not included in the structure of BBA-7s. Thus, only the first one and one-half repeats (residues between G489 and G504) of the four C-terminal glycine-rich repeats are visible in the model of Sd2L β-amylase. Figure 6 is a ribbon model of Sd2L β-amylase showing the hydrogen bond interactions of residues Q481−G504 with other parts of the protein. There was only one interchain hydrogen bond detected within the first one and one-half repeats (G489−G504). Upstream of the four repeats (from V488), however, more hydrogen bonds are formed. Eight hydrogen bonds were predicted between residues Q481 and V488. Of the eight, seven are interchain hydrogen bonds, V488 O−R438 N (3.1 Å), V488 N−N390 OD1 (2.8 Å), P487 O−R438 NE (3.0 Å), D485 OD1−I401 N (3.0 Å), E482 O−R345 NH2 (2.6 Å), Q481 NE2−H399 O (3.1 Å), and Q481 N−P398 O (3.0 Å), and one is an intrachain hydrogen bond, D485 OD1−L486 N (3.2 Å) (Figure 7).

DISCUSSION

A significant increase in thermostability was observed from both purified Sd1 and Sd2L native β-amylases after the proteolytic cleavage after germination (Figure 3). This cleavage also increased the native enzyme's affinity for starch (*8*). Both N- and C-termini of barley β-amylases were proteolytically cleaved after germination (J. K. Eglinton, unpublished data). The removal of the first two amino acid residues (M1 and E2) at the N-terminus appeared not to be a determinant of the changes in β-amylase properties because no differences in the T_{50} values was observed between the native grain β-amylases and the recombinant β-amylases Sd1-M535 and Sd2L-M535 in which the first two amino acids at the N-terminus were removed. Moreover, the

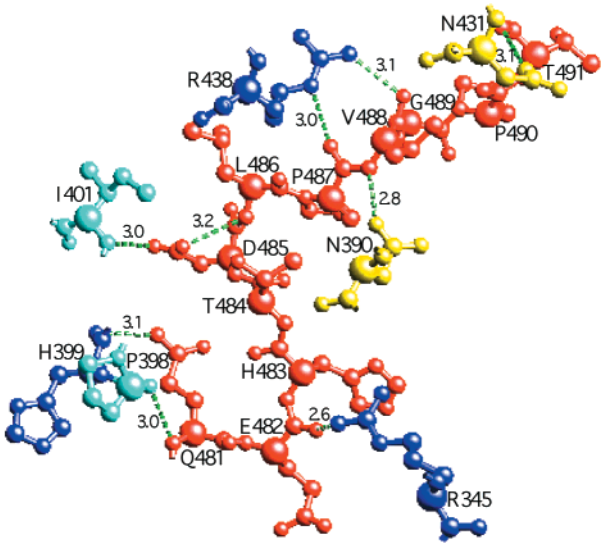

FIGURE 7: Closeup view of the hydrogen bonds from residues between Q481 and T491. Hydrogen bonds are shown as green dotted lines, and bond lengths are shown in angstroms. The C-terminal residues (Q481−T491) are colored in red. Other residues are colored in yellow (polar residues), blue (basic residues), and light blue (nonpolar residues). The figure was generated using the program SPDBV 3.5 (*17*).

thermostability and kinetic properties of the recombinant β-amylases with the six-His tag were similar to those without them, and similar to those of the native enzyme (data not shown).

The C-terminal deletions, however, resulted in a significant increase in the enzyme's affinity for starch. Both deletions at G489 and G496 decreased the K_m values for starch by 2−5-fold (Table 1). A deletion at Q481 (two amino acid differences from H483) was also reported to decrease the K_m values for starch by 2.6-fold (*7*). Therefore, different deletions at the C-terminal region all act at a kinetic level by increasing the enzyme's affinity for starch. This result suggests that that the removal of the C-terminal tail may create a more favorable environment for substrate binding, presumably by reducing the level of steric hindrance in starch binding.

The C-terminal deletion also affected the thermostability of barley β-amylase, although different deletions had different effects. The deletion at G489 significantly increased β-amylase's thermostability, while the deletion at G496 did not change the thermostability of the enzyme. Yoshigi et al. reported that the deletion at Q481 (upstream of the four repeats) resulted in a significant decrease in the thermostability of the enzyme (*7*). It appears that G489 is an important proteolytic cleavage site for optimizing the thermostability of the enzyme. Significantly, G489 is precisely located at the beginning of the four glycine-rich repeats (Figure 1). Thus, the cleavage completely removes the four C-terminal glycine-rich repeats. Soybean β-amylase, which has a relatively higher thermostability than barley β-amylase (*9*), also lacks the four glycine-rich repeats. Therefore, it would be interesting to know if the four C-terminal glycine-rich repeats destabilize the protein.

To determine a possible explanation for why the four C-terminal glycine-rich repeats destabilize the β-amylase structure, Sd2L β-amylase was modeled using the crystal structure of BBA-7s (PDB entry 1B1Y) (*4*). The four glycine-rich repeats include residues G489−P532. The three-dimensional structure revealed that there is only one hydrogen bond interaction with the other part of the protein between G489 and G504 in comparison with many more hydrogen bonds upstream of the four-repeat region (from V488) (Figure 6). This observation indicates that the first one and one-half repeats (G489−G504) do not form a rigid structure. For remaining repeats, Mikami et al. (*4*) has reported an incomplete electron density map, indicating a highly disordered main chain structure beyond G504. Thus, the four C-terminal glycine-rich repeat region is an unstable and unstructured peptide chain.

The structural instability of the four C-terminal glycine-rich repeats most probably results from the high degree of chain flexibility imparted by the abundant glycine. Glycine lacks a β-carbon, and thus has more backbone conformational flexibility and greater configurational entropy than other amino acid residues. Highly flexible chains cannot form defined structures (*19*). It has been demonstrated that an increase in the rigidity of a flexible region of a protein by replacing glycines with other residues (*20*, *21*) can enhance the protein thermostability. Therefore, it is necessary to completely remove the four glycine-rich repeats of barley β-amylase to reduce the level of chain movement of this region and stabilize the protein. Even a short peptide (P490−G496) containing four glycines is sufficient to destabilize the protein.

More hydrogen bonds are present upstream of the four glycine-rich repeats that reinforce the hydrogen bond network of the region. Therefore, any deletion upstream of the four repeats, such as at Q481, would destroy the hydrogen bond network. Hydrogen bonding plays an important role in protein stability, and an increase in the number of hydrogen bonds is a principal determinant of increased thermal stability of 16 families of proteins, including $(\alpha/\beta)_8$ barrel proteins (*22*). It seems likely that the decreased thermostability of barley β-amylase by the deletion at Q481 (*7*) is due to the disruption of the hydrogen bonds between Q481 and V488. Therefore, an appropriate cleavage site, rather than the length of the C-terminal tail, appears to determine the thermostability of β-amylase.

Barley β-amylase is heat-labile compared with other starch-degrading enzymes such as α-amylase. The activity diminished rapidly at >55 °C (*9*). During brewing, however, mashing is performed either isothermally at approximately 65 °C or using a ramped temperature profile from approximately 45 to 70 °C, which is required for starch gelatinization, which in turn is necessary for rapid and complete starch degradation. The thermal instability of barley β-amylase reduces the efficiency of the saccharifying process in commercial beer production. Improvement of β-amylase thermostability has become an important target for protein engineering (*9*, *23*). Our results demonstrate that the complete removal of all four glycine-rich repeats at the C-terminal tail significantly increases the thermostability of barley β-amylase. Therefore, control of barley germination conditions is worthy of further investigation to ensure the complete removal of the four glycine-rich repeats at the C-terminal tail. In addition, our results show that the Sd1 β-amylase has higher thermostability than Sd2L β-amylase regardless of the C-terminal deletions. The characterization of the structural significance of amino acid substitutions between

the β-amylase alleles may also lead to further increases in barley β-amylase thermostability. This work is currently in progress.

REFERENCES

1. Eglinton, J. K., Langridge, P., and Evans, D. E. (1998) *J. Cereal Sci. 28*, 301−309.
2. Kreis, M., Williamson, M., Buxton, B., Pywell, J., Hejgaard, J., and Svendsen, I. (1987) *Eur. J. Biochem. 169*, 517−525.
3. Yoshigi, N., Okada, Y., Sahara, H., and Koshino, S. (1994) *J. Biochem. 115*, 47−51.
4. Mikami, B., Yoon, H.-J., and Yoshigi, N. (1999) *J. Mol. Biol. 285*, 1235−1243.
5. Lundgard, R., and Svensson, B. (1986) *Carlsberg Res. Commun. 51*, 487−491.
6. Evans, D. E., MacLeod, L. C., Eglinton, J. K., Gibson, C. E., Zhang, X., Wallace, W., Skerritt, J. H., and Lance, R. C. M. (1997) *J. Cereal Sci. 25*, 229−239.
7. Yoshigi, N., Sahara, H., and Koshino, S. (1995) *J. Biochem. 117*, 63−67.
8. Ma, Y. F., Stewart, D. C., Eglinton, J. K., Logue, S. J., Langridge, P., and Evans, D. E. (2000) *J. Cereal Sci. 31*, 335−344.
9. Yoshigi, N., Okada, Y., Maeba, H., Sahara, H., and Tamaki, T. (1995) *J. Biochem. 118*, 562−567.
10. Lundgard, R., and Svensson, B. (1987) *Carlsberg Res. Commun. 52*, 313−326.
11. Cox, K. H., and Goldberg, R. B. (1988) in *Plant Molecular Biology, A Practical Approach* (Shaw, C. H., Ed.) pp 1−8, IRL Press, Washington, DC.
12. Takeda, Y., Guan, H. P., and Preiss, J. (1993) *Carbohydr. Res. 240*, 253−263.
13. Nelson, N. (1944) *J. Biol. Chem. 153*, 375−380.
14. Perella, F. W. (1988) *Anal. Biochem. 174*, 437−447.
15. Peitsch, M. C. (1995) *Bio/Technology 13*, 658−660.
16. Peitsch, M. C. (1996) *Biochem. Soc. Trans. 24*, 274−279.
17. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis 18*, 2714−2723.
18. Chothia, C., and Lesk, A. M. (1986) *EMBO J. 5*, 823−826.
19. Schulz, G. E., and Schirmer, R. H. (1984) in *Principles of protein structure* (Cantor, C. R., Ed.) pp 149−165, Springer-Verlag, New York.
20. Hardy, F., Vriend, G., Veltman, O. R., van der Vinne, B., Venema, G., and Eijsink, V. G. H. (1993) *FEBS Lett. 317*, 89−92.
21. Matthews, B. W., Nicholson, H., and Becktel, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6663−6667.
22. Vogt, G., Woell, S., and Argos, P. (1997) *J. Mol. Biol. 269*, 631−643.
23. Okada, Y., Yoshigi, N., Sahara, H., and Koshino, S. (1995) *Biosci., Biotechnol., Biochem. 59*, 1152−1153.

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