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Mutations of barley β-amylase that improve substrate-binding affinity and thermostability

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Abstract Three allelic forms of barley β-amylase (Sd1, Sd2H and Sd2L) exhibit different thermostability and kinetic properties. These differences critically influence the malting quality of barley varieties. To understand the molecular basis for the different properties of these three allelic forms, Sd1 and Sd2L β-amylase cDNAs were cloned, and the effects of the amino acid substitutions between them were evaluated by site-directed mutagenesis. The results showed that an R115C mutation is responsible for the difference in kinetic properties. This substitution resulted in an additional hydrogen bond which may create a more favourable environment for substrate-binding. The different thermostabilities of the β-amylase forms are due to two amino acid substitutions (V233A and L347S), which increased the enzyme's thermostability index T_{50} by 1.9°C and 2.1°C, respectively. The increased thermostability associated with these two mutations may be due to relief of steric strain and the interaction of the protein surface with solvent water. Although both V233A and L347S mutations increased thermostability, they affected the thermostability in different ways. The replacement of L347 by serine seems to increase the thermostability by slowing thermal unfolding of the protein during heating, while the replacement of V233 by alanine appears to cause an acceleration of the refolding after heating. Because the different β-amylase properties determined by the three mutations (R115C, V233A and L347S) are associated with malting quality of barley variety, a mutant with high thermostability and substrate-binding affinity was generated by combining the three preferred amino acid residues C115, A233 and S347 together. A possible approach to producing barley varieties with better malting quality by genetic engineering is discussed.

Keywords Mutation · β-Amylase · Barley · Thermostability · Substrate-binding affinity

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Introduction

β-Amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) catalyses the release of maltose from starch. A sufficiently high level of β-amylase activity is essential for the complete hydrolysis of starch into fermentable sugars in brewing. However, in most commercial malts, β-amylase activity is insufficient, and the enzyme appears to be rate-limiting for starch hydrolysis during mashing, although the efficiency of β-amylase is influenced by other starch-degrading enzymes, particularly limit dextrinase (MacGregor et al. 1999; Stenholm and Home 1999). This lack of β-amylase activity is predominantly due to the quality, rather than the quantity, of the enzyme, since barley β-amylase, representing approximately 1–2% of total barley protein (MacGregor et al. 1971; Hejgaard and Boisen 1980), is relatively thermolabile compared to other starch-degrading enzymes. A significant proportion of β-amylase activity is lost during the mashing process. During brewing, mashing is performed either isothermally at approximately 65°C or using a ramped temperature profile from approximately 45–70°C. These elevated temperatures are required for starch gelatinisation, which in turn is necessary for rapid and complete starch degradation. β-Amylase retains maximum activity up to 55°C, but its activity diminishes rapidly at temperatures over 55°C (Yoshigi et al. 1995). Therefore, it would be beneficial to increase the thermostability and/or improve kinetic properties to enhance the hydrolytic capacity of β-amylase.

Three β-amylase alleles, termed *Bmy*1-Sd1, *Bmy*1-Sd2L and *Bmy*1-Sd2H, have been identified at the *Bmy*1 locus on chromosome 4H among cultivated barleys. The corresponding enzymes are referred to as Sd2L, Sd1 and Sd2H, and possess low, intermediate, and high thermostability, respectively (Eglinton et al. 1998). Sd1 also exhibits higher affinity for starch than Sd2L (Ma et al. 2000a). The thermostability and substrate-binding affinity of the allelic β-amylase forms remain distinct after germination during which an approximately 4-kDa peptide is cleaved from the C-terminus (Ma et al. 2000a, 2000b). The different properties of β-amylases have a significant effect on fermentability (Eglinton et al. 1998; Kihara et al. 1998, 1999). These results indicate that genetic variation in barley β-amylase directly influences barley malting quality, and an understanding of the molecular basis for the genetic variation may identify strategies for improving the enzyme's properties and, in turn, enhancing the production of fermentable sugars for brewing. The different thermostability of these three allelic β-amylase forms has been suggested to be determined by amino acid substitutions encoded by the β-amylase alleles (Kaneko et al. 2000). To identify which amino acid substitutions are responsible for the different properties of allelic β-amylase forms, Sd1 and Sd2L β-amylase cDNAs have been cloned, and the contributions of amino acid substitutions between different allelic β-amylase forms have been evaluated by site-directed mutagenesis. Here, we report the role of amino acid substitutions at positions 115, 233 and 347 on the observed variations in thermostability and kinetic properties among the three allelic barley β-amylases.

Materials and methods

Materials

The QuikChange site-directed mutagensis kit was obtained from Stratagene. The QIAprep spin plasmid kit was obtained from Qiagen. The mutagenic deoxy-oligonucleotide primers used to introduce the desired sequence changes were synthesized using an Applied Biosystems DNA Synthesizer (Model 381A) and subsequently purified by reverse-phase High-Performance Liquid Chromatography. The Betamyl β-amylase assay kit was obtained from Megazyme.

DNA sequencing and sequence alignment

DNAs encoding full-length Sd1 and Sd2L β-amylases were cloned and sequenced previously (Ma et al. 2000b). Two Sd2L β-amylase sequences from the barley varieties Adorra (Erkkilä et al. 1998) and Hiproly (Kreis et al. 1987), a Sd2H β-amylase sequence from the barley variety Haruna Nijo (Yoshigi et al. 1994), and a β-amylase sequence from wild barley *Hordeum vulgare* subsp. *spontaneum* NPGS PI29689 (abbreviated to *H. spontaneum*) (Erkkilä et al. 1998) were obtained from GenBank databases (the Accession Nos. are AF061203, X52321, D21349 and AF061204, respectively). A sequence alignment of the six barley β-amylases was performed using the multiple sequence alignment program Pileup available at The Australian National Genomic Information Service (ANGIS).

Site-directed mutagenesis

cDNAs encoding Sd1 and Sd2L β-amylase with a 46-amino acid deletion in the C-terminal tail were also previously cloned in the expression vector pQE-30 (Ma et al. 2000b). The plasmids were isolated using the QIAprep spin plasmid kit as recommended by the supplier (Qiagen), and used as templates for site-directed mutagenesis.

Site-directed mutagenesis was performed using a QuikChange site-directed mutagensis kit according to the instructions of the manufacturer. A total of five mutagenic primers (Table 1) were used to obtain six single mutants (five from Sd2L, and one from Sd1 β-amylase) and a double mutant (from Sd2L β-amylase). Each mutant was obtained using a 5′ sense primer and a 3′ complementary primer containing the desired mutation. For example, the Sd2L mutant at position 115 was obtained using a 5′ sense primer (5′-GGGTGCGGGACGTCGGCACGTGTGATCCCGACATTTTCTACACCG-3′) and a 3′ complementary primer corresponding to nucleotides 378–422 of barley β-amylase with a substitution that alters a codon (CGT) for arginine to a codon (TGT) for cysteine at nucleotide 398. Similarly, the Sd2L mutant at position 347 was obtained using a 5′ sense primer (5′-CGGAGATGAGGGATTCGGAGCAAAGCTCGC-3′) and a 3′ complementary primer corresponding to nucleotides1080–1109 of barley β-amylase with a substitution that changes a codon (TTG) for leucine to a codon (TCG) for serine at nucleotide 1095, and so on. Sd2L-L347S was further mutated to produce a double mutation at positions 233 and 347. To confirm that only the desired mutation had been introduced during the manipulations, each mutated cDNA was sequenced with a DNA sequencer (Model 373A, Applied Biosystems). The mutants were denoted using the one-letter amino acid code, with the wild-type residue given first, followed by the position number, and the new residue. For example, the Sd2L mutant in which R115 in the wild type is replaced with cysteine is referred to as Sd2L-R115C.

All of the mutant plasmids were expressed, recombinant proteins were purified, and the 6×His-tag was removed as described previously (Ma et al. 2000b).

Thermostability

Irreversible thermal inactivation of purified mutant and wild-type β-amylase 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 method for irreversible thermal inactivation was as follows: purified mutant or wild-type β-amylases were diluted to 0.1 mg/ml with 0.1 M maleic acid buffer

Table 1 Oligonucleotides prepared for site-directed mutagenesis

Mutant	Sequence (5′→3′)[a]	Position (bp)[b]	Mutation
R115C	GGGTGCGGGACGTCGGCACGtGTGATCCCGACATTTTCTACACCG	378–422	CGT→TGT
D165E	GCTTCAGGGAGAACATGAAAGAgTTCTTGGATGCTGG	528–564	GAC→GAG
V233A	GGGAATTTCCTAACGATGcCGGACAGTACAATGACACTCCCG	735–776	GTC→GCC
L347S	CGGAGATGAGGGATTcGGAGCAAAGCTCGC	1080–1109	TTG→TCG
V430A	GGTGGAGGGACAAAACTATGcCAATTTCAAGACCTTTGTCG	1324–1364	GTC→GCC

[a]The target nucleotides are indicated in *lower case*
[b]The positions of the oligonucleotides are numbered according to the β-amylase sequence published by Yoshigi et al. (1994)

containing 2.8 mg/ml BSA and 1 mM EDTA, pH 6.2. The concentration of BSA chosen (2.8 mg/ml) represents the mean protein content of malt extracts (Eglinton et al. 1998), since enzyme analyses are usually carried out on malted barley (germinated barley that is kiln dried) for brewing. The diluted enzymes were incubated at a range of temperatures from 50°C to 62.5°C for 30 min. After incubation, the enzyme solutions were immediately cooled on ice, and the residual enzyme activity was measured using the Betamyl assay (see below). The T_{50} value was determined by linear interpolation between the data points closest to 50% residual activity.

The temperature optima of purified mutant and wild type β-amylases were determined at temperatures ranging from 47.5°C to 65°C. Reaction mixtures contained 20 ng of β-amylase, 100 μM reduced starch and 50 mM acetate buffer pH 5.0, in a final volume of 500 μl. After incubation of the reaction mixtures at different temperatures for 10 min, the amount of reducing sugars released was determined by the Somogyi-Nelson method (Nelson 1944). One unit of activity was defined as the amount of enzyme that catalyses the release of 1 μmol of reducing sugars/min.

Betamyl assay

β-Amylase activity was determined according to the substrate manufacturer's (Megazyme) 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 substrate mixture containing α-glucosidase (200 U) and the specific "betamyl" substrate *p*-nitrophenylmaltopentaoside (PNPG5) (4.75 mg/ml). The enzyme concentrations were tested by determining the initial reaction rates that give a reaction absorbance value of 0.8. After incubation for 10 min at 40°C, the reaction was terminated by the 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 catalyses the release of 1 μmol of maltose/min.

Kinetic assay

Potato starch was reduced by the method of Takeda et al. (1993) and used as a substrate for kinetic assay. Both enzyme and substrate concentrations were tested to ensure that initial reaction rates were measured throughout. The assay was performed by mixing the purified enzymes (50 ng) with substrate solution (1–100 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. After incubation at 40°C for 3, 6, 9, and 12 min, respectively, the amount of reducing sugars was determined using the Somogyi-Nelson method (Nelson 1944). One unit of activity is defined as the amount of enzyme that catalyses the release of 1 μmol reducing sugar/min. Starch molecular weight was determined using a glucose standard curve since each starch molecule has only one reducing end.

Kinetic data were processed by a non-linear-regression analysis based on the Michaelis-Menten equation using the EZ-FIT software (Perella 1988). Variations of approximately 10% were usually observed in the kinetic analysis with starch as substrate.

Other assays

The methods for protein concentration assay and SDS-PAGE have been described previously (Ma et al. 2000a).

Structural analysis

Modelling of mutant and wild-type β-amylases was achieved using the automated SWISS-MODEL program (Peitsch 1995; 1996). The known crystal structure of the sevenfold mutant of barley β-amylase (PDB Identifier 1B1Y) (Mikami et al. 1999) was used to construct homology-based models of barley β-amylases. Structure analysis was performed using program SPDBV 3.5 (Guex and Peitsch 1997).

Results

Sequence comparison of three allelic β-amylase forms

The deduced protein sequences of Sd1 (from Franklin) and Sd2L (from Schooner) β-amylases were compared with four published barley β-amylase sequences. Amino acid differences between the three allelic β-amylase forms are listed in Table 2. The sequence of Sd2L β-amylase from Schooner is completely identical to that of the Sd2L β-amylase from the variety Hiproly (Kreis et al. 1987), which showed a single amino acid substitution at position 527 relative to another Sd2L β-amylase from variety Adorra (Erkkilä et al. 1998). The major sequence differences between the products of the three three *Bmy*1 alleles can be summarised as follows. Sd1 differs at five positions (115, 165, 347, 430 and 527) from Sd2L β-amylase and at four positions (115, 165, 347, 430 and 527) from Sd2H β-amylase. Sd2L and Sd2H sequences exhibited three amino acid substitutions at positions 233, 347 and 527. The *Bmy*1 allele of a wild barley *H. spontaneum* (Erkkilä et al. 1998) was identical in sequence to the *Bmy*1-Sd2H allele from Haruna nijo (Yoshigi et al. 1994).

Production of mutant β-amylases

Using Sd2L β-amylase cDNA (encoding the proteolytically cleaved form of the enzyme) as a template, the codons for arginine (CGT) at position 115, aspartic acid (GAC) at position 165, leucine (TTG) at position 347 and valine (GTC) at position 430 were replaced by the corresponding codons TGT for cysteine, GAG for glutamic acid, TCG for serine and GCC for alanine found in Sd1 β-amylase, respectively. In addition, codon GTC for valine at position 233 was replaced by codon GCC for alanine found in Sd2H β-amylase, since Sd2L and Sd1 β-amylase had the same sequence at this position (Table 2). Thus, five single mutants (Sd2L-R115C, Sd2L-D165E, Sd2L-V233A, Sd2L-L347S and Sd2L-V430A) were constructed (Fig. 1). A double mutant

Table 2 Amino acid substitutions in the allelic forms of barley β-amylase

Variety	Allele	Positions					
		115	165	233	347	430	527
Franklin	*Bmy*1-Sd1	C	E	V	S	A	M
Schooner	*Bmy*1-Sd2L	R	D	V	L	V	I
Hiproly	*Bmy*1-Sd2L	R	D	V	L	V	I
Adorra	*Bmy*1-Sd2L	R	D	V	L	V	M
Haruna nijo	*Bmy*1-Sd2H	R	D	A	S	V	M
H. spontaneum	*Bmy*1-Sd2H	R	D	A	S	V	M

(Sd2L-V233A/L347S) was also generated by further replacing V233 of mutant Sd2L-L347S with alanine. Thus, the resultant mutant had exactly the same sequence as Sd2H β-amylase (Table 2). Moreover, an Sd1 mutant (Sd1-V233A) was also produced (Fig. 1).

After confirming the amino acid substitutions in each mutant by DNA sequencing, the plasmids were expressed in *E. coli* as active and soluble enzymes, and purified to homogeneity and the His-tag used to facilitate purification was removed (Fig. 2).

Thermostability of mutant and wild type β-amylases

The thermostability of mutant and wild type enzymes was measured by plotting irreversible thermal inactivation curves (Fig. 3) which were used to determine the T_{50} values (Table 3). The T_{50} values for Sd2H (Sd2L-V233A/L347S), Sd1 and Sd2L β-amylases were 59.2°C, 57.3°C and 55.2°C, respectively. Among the five Sd2L single mutants, two mutations (V233A and L347S) resulted in an increase in the T_{50} values by 1.9°C and 2.1°C, respectively, compared with the wild type. All the remaining mutants had little effect on the T_{50} values.

The importance of V233A and L347S mutations for β-amylase thermostability was further demonstrated in the double mutant Sd2L-V233A/L347S, in which L347 and V233 in Sd2L β-amylase were replaced with alanine and serine, respectively. As shown in Fig. 3a and Table 3, this combination of mutations enhanced the T_{50} by 4°C, which is the sum of individual contributions of the two individual mutations Sd2L-V233A and Sd2L-L347S. This result indicates that the two residues had a simple additive effect on the thermostability of β-amylase. This additive effect was further confirmed by the Sd1 mutant (Sd1-V233A). Wild-type Sd1 β-amylase, with a serine residue at position 347, had a T_{50} value 2.1°C higher than wild type Sd2L β-amylase, which has a leucine residue at this position (Table 2). The single

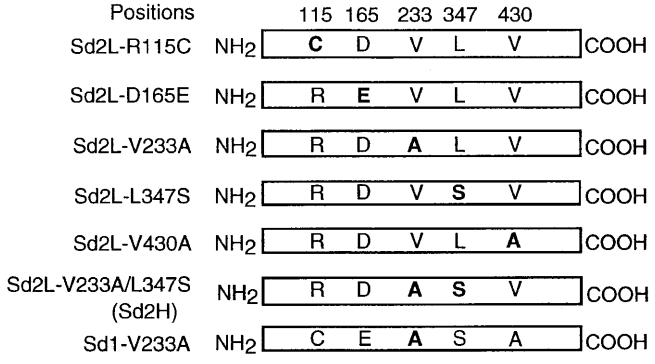

Fig. 1 Schematic representations of barley β-amylase mutants. Mutated residues are shown in *bold*

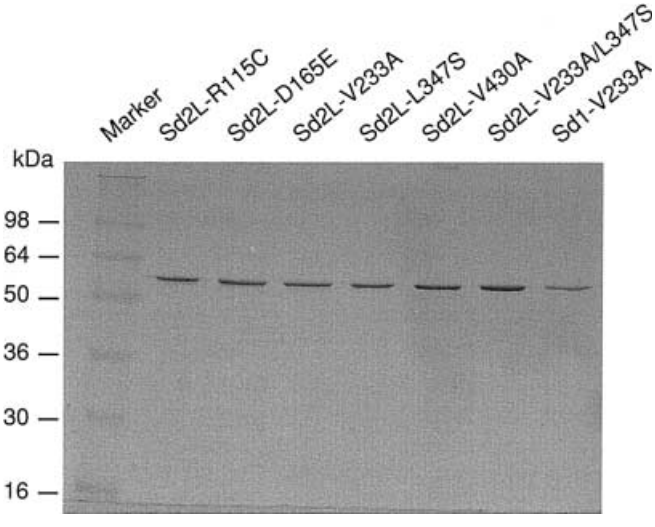

Fig. 2 SDS-PAGE of purified mutant β-amylases stained with Coomassie blue R250

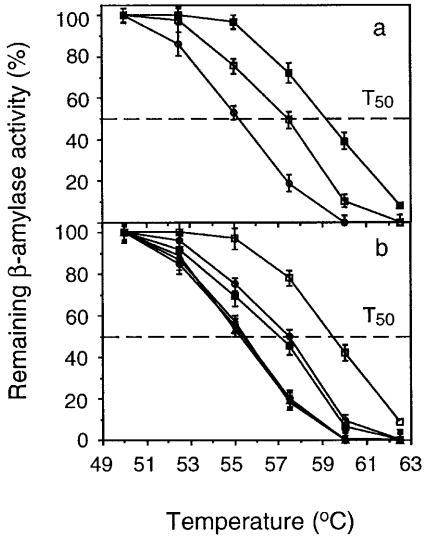

Fig. 3a, b Irreversible thermal inactivation. **a** Wild type β-amylases. *Open squares*, Sd1; *open circles*, Sd2L; *filled squares*, Sd2H. **b** Mutant β-amylases. *Open squares*, Sd1-V233A; *open circles*, Sd2L-L347S; *filled squares*, Sd2L-V233A; *filled triangles*, Sd2L-R115C; *filled circles*, Sd2L-D165E; *filled pentagons*, Sd2L-V430A. Activity is expressed as a percentage of initial activity. Values are the means of three independent determinations, with the standard deviations indicated by the *error bars*

Table 3 T_{50} values of wild-type and mutant β-amylases

Purified β-amylase	T_{50} (°C)	ΔT_{50} (°C)[a]
Sd2L (wild-type)	55.2 ± 0.4	–
Sd2L-R115C	55.3 ± 0.4	+0.1
Sd2L-D165E	55.2 ± 0.2	0
Sd2L-V233A	57.1 ± 0.3	+1.9
Sd2L-L347S	57.3 ± 0.4	+2.1
Sd2L-V430A	55.3 ± 0.3	+0.1
Sd2L-V233A/L347S (Sd2H)	59.2 ± 0.5	+4.0
Sd1 (Wild-type)	57.3 ± 0.3	–
Sd1-V233A	59.4 ± 0.4	+2.1

[a] ΔT_{50} is the change in T_{50} value of the mutant proteins relative to the wild-type protein

Sd1 mutant (Sd1-V233A) resulted in an another 2.0°C increase in the T_{50} value (Fig. 3 and Table 3).

Although both V233A and L347S mutations increased T_{50} values, only the substitution of L347 by serine enabled maintenance of significantly higher activity than the wild type at higher assay temperatures (Fig. 4). The Sd2L-L347S mutant lost activity more slowly than the wild type at temperatures over 55°C. Only approximately 10% and 40% activity was lost from Sd2L-L347S when the temperature was raisd from 55°C to 57.5°C and 60°C, respectively, compared with approximately 30% and 60% for the wild type (Fig. 4). The Sd2L-V233A mutant, however, did not have a significant effect on the ability to retain activity when the temperature was increased (Fig. 4).

Kinetic properties of mutant and wild-type β-amylases

The kinetic constants K_m and k_{cat} for each mutant and wild-type β-amylase were determined from initial rate measurements for hydrolysis of soluble starch. Table 4 summarises the kinetic parameters for starch hydrolysis catalysed by the mutant and wild-type β-amylases. Sd1 β-amylase exhibited a 2.5-fold lower K_m value than Sd2L and Sd2H β-amylases. Results for the mutant forms showed that replacement of R115 with cysteine resulted in a decrease in the K_m value of the enzyme from 8.31 to 3.6 μM. Thus, this mutant has almost the same K_m value for starch as Sd1 β-amylase, which has a cysteine residue at this position. For all of the other mutants, however, the K_m values are almost unchanged. No significant differences in the k_{cat} value between the mutant and wild type enzymes were observed.

Structure of mutant and wild type β-amylases

The structural basis of the observed thermostability and kinetic properties of barley β-amylase was investigated using a model based on the known crystal structure for the sevenfold mutant of barley β-amylase (PDB Identifier 1B1Y) (Mikami et al. 1999). The structural quality was ensured by the use of a template that shares 97.3% sequence identity with the mutant and wild-type Sd2L barley β-amylases. Superposition of all atoms of the three mutant structures with the wild-type protein structure resulted in root-mean-square differences for the backbone of 0.112, 0.106 and 0.108 Å for Sd2L-R115C, Sd2L-V233A and Sd2L-L347S, respectively. This indicates that a single amino acid substitution is not likely significantly to affect the backbone structure of the protein. Some small differences in root-mean-square appear to be the logical consequence of the smaller side-chains in the mutants.

Figure 5a shows the location of residues R115, V233 and L347 in the wild-type enzyme (Sd2L β-amylase). R115 is located at the C-terminal turn of the α-helix of Loop 3 (shown in red), which is a loop 1-α-helix-loop 2 motif. The long side-chain of arginine extends into the solvent. Inspection of the model showed that the main-chain nitrogen atom of R115 makes a hydrogen bond (distance 3.2 Å) with the main-chain oxygen atom of the adjacent V112 in the α-helix. In the mutant Sd2L-R115C, the replacement of R115 by cysteine resulted in the formation of an additional hydrogen bond (distance, 2.4 Å) because the SH group of the introduced cysteine residue has the capacity to act as a hydrogen bond donor.

Both V233 and L347 residues are located in a loop region (Fig. 5). V233 lies on Loop 4 (shown in pink) pointing to the interior. The side-chain of this residue is close to surrounding residue F245. L347, however, lies on Loop 6 (shown in blue) pointing into the solvent. The side-chain of this residue is completely exposed to the solvent. Structural analysis shows that neither residue is involved in hydrogen bond interactions with neighbouring residues in either wild-type Sd2L β-amylase, or in the Sd2L-V233A or Sd2L-L347S mutant.

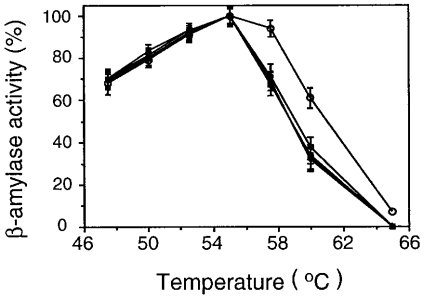

Fig. 4 Effect of assay temperature on the activities of mutant and wild-type barley β-amylases. *Open circles*, Sd2L-L347S; *filled triangles*, Sd2L-R115C; *filled circles*, Sd2L-D165E; *filled squares*, Sd2L-V233A; *filled diamonds*, Sd2L-V430A. Activity is expressed as a percentage of initial activity. Values are the means of three independent determinations, with the standard deviations indicated by the *error bars*

Discussion

The sequence comparison of Sd1, Sd2L and Sd2H β-amylases revealed six amino acid exchanges at

Table 4 Kinetic parameters for the hydrolysis of starch by wild-type and mutant barley β-amylases

Purified β-amylase	Kinetic parameter[a]	
	k_{cat} (s^{-1})	K_m (μM)
Sd2L (wild-type)	299 ± 12	8.31 ± 0.58
Sd2L-R115C	309 ± 8	3.60 ± 0.28
Sd2L-D165E	319 ± 9	8.56 ± 0.61
Sd2L-V233A	315 ± 11	8.25 ± 0.71
Sd2L-L347S	309 ± 9	8.34 ± 0.68
Sd2L-V430A	319 ± 10	8.38 ± 0.75
Sd2L-V233A/L347S (Sd2H)	308 ± 7	8.30 ± 0.62
Sd1 (wild-type)	305 ± 9	3.30 ± 0.28
Sd1-V233A	314 ± 7	3.17 ± 0.56

[a]Kinetic data were fitted to the Michaelis-Menten relationship to evaluate K_m and k_{cat} values. Values are the means of three independent determinations ± standard deviations

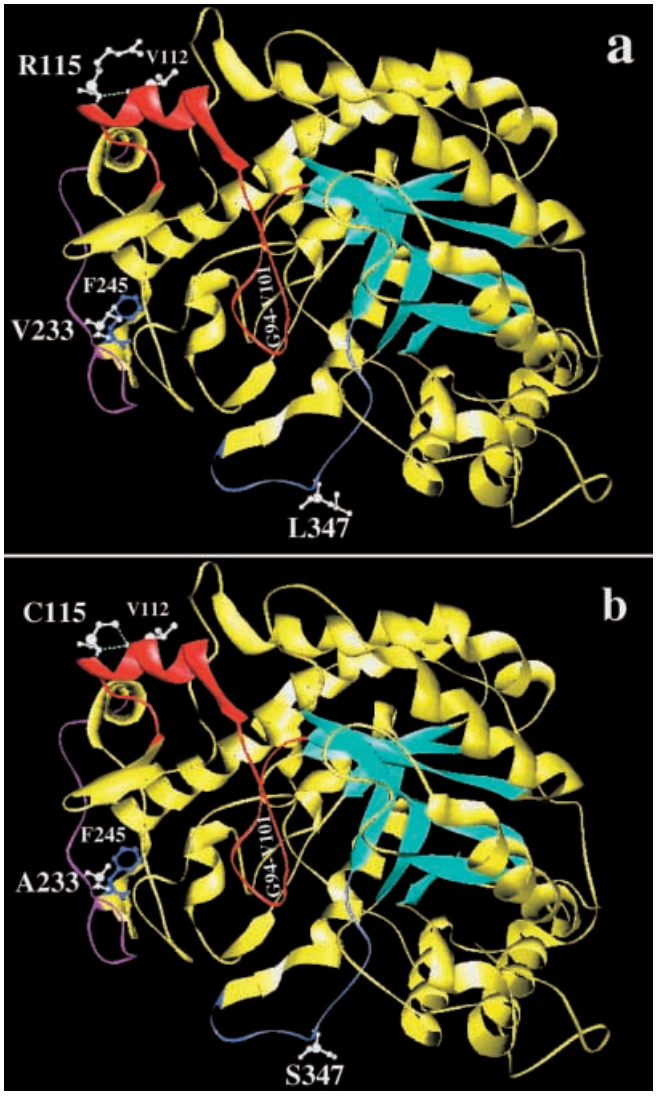

Fig. 5a, b Three-dimensional structures of wild type Sd2L β-amylase (**a**) and Sd1-V233A mutant (**b**). Loop 3 is coloured in red, Loop 4 is coloured in pink, and Loop 6 is coloured in blue. The pocket is coloured in aqua. The segment encompassing residues G94–V101 is labelled, as are the residues affected in the various allelic forms of the protein. Hydrogen bonds are shown as *green dotted lines*. The Figure was generated using the program SPDBV 3.5 (Guex and Peitsch 1997)

positions 115, 165, 233, 347, 430 and 527 (Table 2). The substitution at position 527 is not responsible for the observed differences in the thermostability and kinetic properties, because this residue, lying within the C-terminal region, is proteolytically cleaved after germination, and the differences in the thermostability and kinetic properties of β-amylase allelic forms are retained after germination (Ma et al. 2000a, 2000b). The contribution of the remaining five amino acid substitutions to the thermostability and kinetic properties of barley β-amylase was evaluated by individually replacing the residues in Sd2L β-amylase with the corresponding ones in Sd1 or Sd2H β-amylase.

Recombinant Sd1 β-amylase has a lower K_m value than Sd2H and Sd2L β-amylases (Table 4), indicating that this allelic β-amylase form has a higher affinity for substrate than Sd2H and Sd2L β-amylases. This is consistent with the results obtained with native purified β-amylases (Ma et al. 2000a). Among the mutants, only the replacement of R115 by cysteine caused a reduction in the K_m value by approximately 2.5-fold. Thus, the mutant exhibited a very similar K_m value to that of Sd1 β-amylase, suggesting that this substitution is responsible for the kinetic differences between the three allelic barley β-amylase forms. A structural analysis shows that the R115C mutation occurs in the α-helix of Loop 3 (Fig. 5). Loop 3 is an essential part of the active centre (Mikami et al. 1993, 1994; Laederach et al. 1999). In particular, the open-and-close movement of the loop segment (residues 94–101, corresponding to residues 96–103 in soybean β-amylase) is involved in the binding of the substrate to the active site of the enzyme (Laederach et al. 1999). The R115C mutation results in the formation of an additional hydrogen bond with the main-chain oxygen atom of V112 (Fig. 5b). Although there is no experimental evidence that the removal of the positively charged R115 affects the dipoles across the α-helix electric field, it seems likely that the α-helix in the mutant is stabilised by the additional hydrogen bond. This may create a more favourable environment for substrate-binding.

Recombinant Sd2H, Sd1 and Sd2L β-amylases exhibited high, intermediate and low T_{50} values, respectively (Fig. 3a and Table 3), reflecting the differences in their thermostability as native β-amylases. The replacement of V233 and L347 with alanine and serine increased the T_{50} values by 1.9°C and 2.1°C, respectively. The contributions of the two residues to the thermostability of barley β-amylase were additive. All other mutations had little effect on the T_{50} values. These results suggest that the two amino acid substitutions at positions 233 and 347 are responsible for the different thermostability of the three allelic β-amylase forms, and that alanine and serine at the two positions are the preferred residues for enhancing thermostability. Soybean β-amylase, which has a relatively high thermostability compared to barley β-amylase (Yoshigi et al. 1995), also contains alanine and serine at these two positions (Totsuka and Fukazawa 1993).

Both V233A and L347S substitutions are "large-to-small" exchanges. Residue 233 points into the interior, and its side-chain is very close to F245, which may cause some unfavourable strain (Fig. 5a). As shown in Figure 5b, the mutant side-chain of alanine is much smaller than that of valine. This reduction in the side-chain volume in the mutant may relieve some steric strain. Thus, the increased thermostability caused by V233A mutation may be due to the reduction of the side-chain volume. In the case of the L347S substitution, the size of the side-chain may not be important for structural stability because this residue is completely exposed to the solvent, and does not have any interactions with

neighbouring residues. In addition to the difference in volume, the L347S substitution is an exchange from hydrophobic to hydrophilic. The polar residue serine has a hydroxyl group with the ability to hydrogen bond to solvent water. A protein that makes more hydrogen bonds with the solvent should gain energetic stability. It is therefore likely that the increased thermostability of the Sd2L-L347S mutant is correlated with the hydrophilic property, especially the interactions of the protein surfaces with the surrounding water.

Although both V233A and L347S mutations led to increased thermostability, they affected the thermostability in different ways. The replacement of L347 with serine significantly increased the capability to retain activity as the assay temperature was increased (Fig. 4). This suggests that this serine residue is crucial for slowing the thermal unfolding of the protein and maintaining a heat-stable conformation of β-amylase. In contrast, the Sd2L-V233A mutant did not improve the capability to retain activity at higher temperatures (Fig. 4), suggesting that this replacement does not slow thermal unfolding of the protein during heating. Thus, the increased thermostability associated with the V233A mutation is most probably due to an acceleration of refolding after heating.

Our results have demonstrated that the different thermostability and kinetic properties of the three allelic barley β-amylase forms are due to different combinations of three preferred residues – C115, A233 and S347. Sd2L β-amylase, with all three unfavourable residues R115, V233 and L347, has low thermostability and affinity for substrate. Sd1, containing preferred residue C115, showed high affinity for substrate, but its thermostability was lower than that of Sd2H β-amylase. Sd2H β-amylase, carrying two preferred residues A233 and S347, exhibited high thermostability, but its affinity for substrate was lower than that of Sd1 β-amylase. These β-amylase properties determined by the three residues appear to be associated with the malting quality of barley varieties. Sd2L varieties have typically low fermentability, and the fermentability of Sd1 and Sd2H varieties are generally very similar (Eglinton et al. 1998). Thus, a β-amylase mutant (Sd1-V233A) containing all the three preferred residues was generated. Results showed that this mutant has almost the same T_{50} value as Sd2H β-amylase and a very similar K_m value to Sd1 β-amylase, indicating that the mutant combined both the high thermostability and high substrate-binding affinity. At present, no barley cultivar carries all three preferred residues. Techniques for barley transformation are available (Wan and Lemaux 1994). Introduction of an β-amylase gene containing all three preferred residues is expected to produce barley varieties with better malting quality.

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