Mutant barley (1→3,1→4)-β-glucan endohydrolases with enhanced thermostability

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The similar three-dimensional structures of barley (1→3)-β-glucan endohydrolases and (1→3,1→4)-β-glucan endohydrolases indicate that the enzymes are closely related in evolutionary terms. However, the (1→3)-β-glucanases hydrolyze polysaccharides of the type found in fungal cell walls and are members of the pathogenesis-related PR2 group of proteins, while the (1→3,1→4)-β-glucanases function in plant cell wall metabolism. The (1→3)-β-glucanases have evolved to be significantly more stable than the (1→3,1→4)-β-glucanases, probably as a consequence of the hostile environments imposed upon the plant by invading microorganisms. In attempts to define the molecular basis for the differences in stability, eight amino acid substitutions were introduced into a barley (1→3,1→4)-β-glucanase using site-directed mutagenesis of a cDNA that encodes the enzyme. The amino acid substitutions chosen were based on structural comparisons of the barley (1→3)- and (1→3,1→4)-β-glucanases and of other higher plant (1→3)-β-glucanases. Three of the resulting mutant enzymes showed increased thermostability compared with the wild-type (1→3,1→4)-β-glucanase. The largest increase in stability was observed when the histidine at position 300 was changed to a proline (mutant H300P), a mutation that was likely to decrease the entropy of the unfolded state of the enzyme. Furthermore, the three amino acid substitutions which increased the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII were all located in the COOH-terminal loop of the enzyme. Thus, this loop represents a particularly unstable region of the enzyme and could be involved in the initiation of unfolding of the (1→3,1→4)-β-glucanase at elevated temperatures.

Keywords: barley/β-glucan endohydrolases/site-directed mutagenesis/thermal stability/three-dimensional structure

Introduction

The three-dimensional structures of barley (1→3)-β-glucanase isoenzyme GII (EC 3.2.1.39) and (1→3,1→4)-β-glucanase isoenzyme EII (EC 3.2.1.73) have been solved to high resolution by X-ray crystallography (Varghese et al., 1994). The Ca atoms of the two enzymes are almost 100% superimposable, with a mean r.m.s. deviation of 0.65 Å over 280 residues. Furthermore, the enzymes share 52% positional identity and there is compelling evidence to suggest that they result from the divergent evolution of a common ancestral enzyme (Varghese et al., 1994; Høj and Fincher, 1995). Both are members of the family 17 group of glycoside hydrolases and are classified in the 4/7 superfamily and GH-A clan (Henrissat, 1998).

Despite the similarities in their primary sequences and three-dimensional structures, the two enzymes have vastly different functions. The (1→3)-β-glucanases are believed to play a major role in protecting the plant against pathogen attack, through their ability to hydrolyze linear, substituted and branched (1→3)-β-glucans of the type that are widely distributed in fungal cell walls (Hrmova and Fincher, 1993; Høj and Fincher, 1995). In contrast, the (1→3,1→4)-β-glucanases are primarily involved in endosperm cell wall degradation in germinated grain or in cell wall turnover in elongating vegetative tissues (Høj and Fincher, 1995).

Plant (1→3)-β-glucanases are classified among the pathogenesis-related (PR) proteins because they are synthesized and usually secreted from the cell as part of a general plant defense strategy against invading pathogens (Boller, 1987; Mauch et al., 1988; Leah et al., 1991; Stintzi et al., 1993). In the extracellular environment, the (1→3)-β-glucanases encounter relatively harsh conditions of pH and temperature and are subject to attack by degradative enzymes of microbial origin. In many cases the PR proteins have evolved to be inherently stable at extremes of pH and temperature (Stintzi et al., 1993). Indeed, barley (1→3)-β-glucanase isoenzyme GII is significantly more thermostable than barley (1→3,1→4)-β-glucanase isoenzyme EII (Hrmova and Fincher, 1993; Chen et al., 1995). Thus, the (1→3)-β-glucanase loses little activity over 1 h at 50°C, in contrast to the (1→3,1→4)-β-glucanase, which is almost completely inactivated after 15 min at the same temperature (Chen et al., 1995).

The structural similarities between the two enzymes (Varghese et al., 1994) provide an opportunity to examine the evolution of key properties, such as substrate specificity and enzyme stability, which are linked to enzymic function. In describing the high degree of structural similarity between barley (1→3)-β-glucanase isoenzyme GII and (1→3,1→4)-β-glucanase isoenzyme EII, Varghese et al. noted that the differences in substrate specificity and function were achieved without any major changes in the conformation of the polypeptide backbone (Varghese et al., 1994). Instead, a relatively small number of amino acid differences in the substrate-binding grooves of the two barley enzymes are probably responsible for the distinct substrate specificities of the enzymes (Varghese et al., 1994).

Here, we investigated the molecular basis for the difference in heat stability between the structurally similar barley (1→3)- and (1→3,1→4)-β-glucanases. This was aimed not only at gaining some insight into the evolution of thermostable enzymes, but also to generate a more thermostable (1→3,1→4)-β-glucanase that could have commercial potential. A barley (1→3,1→4)-β-glucanase with enhanced heat stability might prove useful in overcoming filtration problems and other difficulties encountered in the malting and brewing industries as
To increase the thermostability of barley (1→3,1→4)-β-glucanase isozyme EII, amino acid substitutions were introduced into the wild-type form of the enzyme and the relative thermostabilities of the resulting mutant enzymes were measured. Predicting which amino acid substitutions were necessary to increase the thermostability of barley (1→3,1→4)-β-glucanase isozyme EII required a comprehensive understanding of the factors which affect protein folding and stability in general. Protein engineering studies, together with structural comparisons of proteins from thermophilic and mesophilic organisms, have revealed several key principles that govern protein stability (Fersht and Serrano, 1993; Vriend and Eijssink, 1993; Fágán, 1995; Matthews, 1996; Querol et al., 1996; Vieille and Zeikus, 1996). The amino acid substitutions designed for enhancing the thermostability of barley (1→3,1→4)-β-glucanase isozyme EII were based on these principles of protein stability in the first instance, but were further rationalized by comparing the amino acid sequence of the enzyme with those of several plant (1→3)-β-glucanases, including barley (1→3)-β-glucanase isozyme GI. Thus, if there were several possible substitutions in a particular region of the enzyme and one of these was conserved in the relatively stable (1→3)-β-glucanases, that substitution was chosen in preference to other possibilities. Molecular modeling was used to confirm that the substitutions would not destabilize the enzyme’s three-dimensional conformation. Finally, the proposed amino acid substitutions were introduced into barley (1→3,1→4)-β-glucanase isozyme EII at the cDNA level using site-directed mutagenesis. The resulting mutant enzymes were expressed in *Escherichia coli*, purified and analyzed for thermostability.

**Materials and methods**

**Materials**

The pET3a-HT plasmid was generously provided by Dr Helen Healy, Department of Plant Science, University of Adelaide, South Australia. *E.coli* strain BL21 (DE3) pLysS was obtained from Novagen (Madison, WI) and *E.coli* XL1-Blue cells from Stratagene (La Jolla, CA). Ni-NTA spin columns and imidazole were purchased from Qiagen (Chatsworth, CA). DNA oligonucleotide primers were synthesized using an Applied Biosystems (Foster City, CA) DNA Synthesizer. Restriction enzymes, DNA ligase and Vent DNA polymerase were purchased from Qiagen (Chatsworth, CA). DNA oligonucleotides were purchased from Novagen (Madison, WI). DNA ligase was provided by Pharmacia (Uppsala, Sweden). The remaining enzymes were purchased from Novagen (Madison, WI) and were used in the pET3a-HT plasmid and transformed into competent *E.coli* XL1-Blue cells. The recombinant plasmid was designated pET3a-HT/EII. The nucleotide sequences of all constructs were checked by the dideoxynucleotide chain termination procedure (Sanger et al., 1977).

For expression, pET3a-HT/EII was independently transformed into *E.coli* BL21 (DE3) pLysS using standard transformation protocols (Sambrook et al., 1989). Expression medium was prepared as follows: 50 ml of a sterile solution of 0.17 M KH2PO4 and 0.72 M K2HPO4 was added to 450 ml of autoclaved Terrific Broth (containing 6 g of bacto-tryptone, 12 g of bacto-yeast extract and 2 ml of glycerol in 450 ml of sterile water) in a 2 L Erlenmeyer conical flask. A 1 ml volume of the cell suspension was added to the medium and cells were grown at 37°C in the presence of 100 µg/ml ampicillin and 25 µg/ml chloramphenicol, with shaking at 250 r.p.m. When the OD600 reached ~0.6, a 1 ml aliquot of the cells was centrifuged and the pellet was resuspended in 50 µl of SDS loading buffer [50 mM Tris–HCl buffer, pH 7.8, containing 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% (v/v) glycerol]. The resuspended cells, representing the ‘uninduced’ sample, were frozen at −20°C. Expression was induced with the addition of IPTG to a final concentration of 1.0 or 0.25 mM and incubation was continued at either 23 or 37°C for up to 4 h. At the end of the incubation period, a 1 ml aliquot was centrifuged and the pellet was resuspended in 100 µl of SDS loading buffer and stored at −20°C as the ‘induced’ sample. The remaining cells were transferred to 50 ml centrifuge tubes and harvested by centrifugation at 3000 r.p.m. for 10 min at 4°C. Cell pellets were drained and stored at −20°C.

Frozen cell pellets containing recombinant (1→3,1→4)-β-glucanase were thawed on ice for 15 min and resuspended in 1 ml of 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl, 25 mM imidazole and 1 mM PMSF. The viscous cell suspension was incubated on ice for a further 30 min and complete cell lysis was facilitated by sonication for 30 s. The resulting cell lysate was centrifuged at 10 000 r.p.m. for 20 min at 4°C to remove insoluble cell debris and inclusion bodies. To purify the expressed protein under non-denaturing conditions, 600 µl of the soluble cell lysate were loaded on to an Ni-NTA spin column which had been pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl and 25 mM imidazole (Buffer I). The column was centrifuged for 2 min at 2000 r.p.m. and washed twice with 600 µl of Buffer I at room temperature. The bound protein was eluted from the column using 200 µl of Buffer I adjusted to 200 mM imidazole. The eluted protein was collected in a 2 ml plastic tube and stored on ice. Protein yield was determined spectrophotometrically using the Coomassie Protein Assay Reagent and bovine serum albumin as a standard.

**SDS–PAGE**

SDS–PAGE was performed on 12.5% polyacrylamide gels as described by Laemmli (Laemmli, 1970). Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol–acetic acid–water (40:7:53) and destained in a solution containing 20% (v/v) ethanol and 7% (v/v) acetic acid.

**Sequence alignment and selection of mutation sites**

To identify those amino acids which might be responsible for the apparent stability of plant (1→3)-β-glucanases, an amino acid sequence alignment of 41 related plant β-glucanases was performed using PILEUP in the University of Wisconsin GCG suite of programs (Devereux et al., 1984). The aligned sequences encoded (1→3)-β-glucanases from barley (seven isoforms), potato (three isoforms), tomato (four isoforms), tobacco (10 isoforms), *Nicotiana* species (three isoforms),...
Fig. 1. Schematic view of barley (1→3,1→4)-β-glucanase isoenzyme EII showing secondary structure and the positions of various amino acid substitutions. The three-dimensional structure of barley (1→3,1→4)-β-glucanase isoenzyme EII is shown with α-helices as yellow tapes, β-strands as blue arrows and loop regions as green coils. The NH₂- and COOH-termini are labeled N and C, respectively (red) and the COOH-terminal loop region is shown in white. The various amino acid substitutions described are indicated in black. (A) ‘Bottom’ view of the enzyme looking from the underside of the substrate binding cleft; (B) ‘side’ view of the enzyme showing the substrate binding cleft and representing a 90° rotation of the enzyme compared with the orientation in frame A. The figure was constructed using MOLSCRIPT.

Arabidopsis species (four isoforms) and Brassica species (two isoforms) and one enzyme each from soybean, pea and bean. Also included in the alignment were sequences encoding barley (1→3,1→4)-β-glucanase isoenzymes EI and EII and one related enzyme each from wheat, maize and oats which have not been unequivocally assigned a substrate specificity. The aligned sequences were obtained from GenBank/EMBL DNA databases using the following codes in the ‘BrowseCode’ search program in the University of Wisconsin GCG suite of programs (Devereux et al., 1984): blyglnhi, blygeh, hv13geiiii, blyglu2x, blyglnhiv, blyglnhvi, mze13bgcln, blyglnhvi, hvglb1, hvglb2, tabegluu, asbgluan, tobo2a, tobglucb, nstps41b, nstps41a, tobo135a, tobo13b, tobo13gla, peabaglu, pv13bdgl, tobo2bcreg, nplgub, npb13gg, tobo13b, tobo13b, tobo13b, uo1900, uo1901, uo1902, tobo13gub, nteh32139, leqa, leqb, soybl3endg, ahthbg1a, ahthbg2a, ahthbg3a, bcgbl, atagblu, bnaa6blu and nribgluc.

Files of crystal structure coordinates describing the three-dimensional structures of barley (1→3,1→4)-β-glucanase isoenzyme EII were obtained from the Brookhaven Protein Database and protein structures were analyzed using the ‘O’ software (Jones et al., 1991) on a Silicon Graphics Iris Indigo Elan 4000 workstation. Potential substitutions were introduced ‘hypothetically’ into the enzyme and rationalized using the ‘O’ program (Jones et al., 1991). Substitutions that caused steric hindrance with atoms of neighboring residues or disrupted hydrogen bonding were avoided. Substitutions involving residues in close proximity to substrate binding or catalytic regions were also avoided.

Site-directed mutagenesis

The USE method for site-directed mutagenesis was employed to introduce the proposed amino acid substitutions into the barley (1→3,1→4)-β-glucanase isoenzyme EII cDNA in pET3a-HT/EII, as described by the manufacturer. Mutant plasmids were transformed into E.coli strain BL21 (DE3) plpLysS and expression was induced as described above. Expressed fusion proteins were purified using Ni-NTA resin and analyzed by SDS–PAGE.

(1→3,1→4)-β-Glucanase activity

(1→3,1→4)-β-Glucanase activity was assayed reductometrically (Nelson, 1944; Somogyi, 1952) at 37°C using 0.2% (w/v) barley (1→3,1→4)-β-glucan (Deltagen) in 50 mM sodium acetate buffer, pH 5.0, containing 200 µg/ml bovine serum albumin. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol reducing equivalents per minute. The thermostabilities of mutant and wild-type (1→3,1→4)-β-glucanases were determined by incubating the enzymes in 50 mM sodium acetate buffer, pH 5.0, containing 0.2 mg/ml BSA, for 15 min over a range of temperatures and measuring the residual activity. Thermostability was described by T50, which represents the temperature at which 50% of the initial enzyme activity remains after incubation for 15 min. Kinetic analyses were performed over a substrate concentration range 0.2–2.0 mg/ml. The kinetic parameters K_m, V_max and k_cat were derived from Lineweaver–Burk plots.

Table I. Summary of amino acid alignment of 41 plant β-glucanases

<table>
<thead>
<tr>
<th>Residue number in barley (1→3,1→4)-β-glucanase</th>
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<tbody>
<tr>
<td>17</td>
</tr>
<tr>
<td>Residue type</td>
</tr>
<tr>
<td>K</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>T17D</td>
</tr>
</tbody>
</table>

The deduced amino acid sequences of 41 plant β-glucanases were aligned and the number of sequences containing a particular residue at each position is shown (see text for details). Residue positions are numbered according to the barley (1→3,1→4)-β-glucanase isoenzyme EII primary structure and only those positions at which amino acid substitutions were made in this study are shown. Standard one-letter amino acid codes are used.
Site-directed mutagenesis of barley \((1\rightarrow3,1\rightarrow4)\)-\(\beta\)-glucanase isoenzyme EII

Eight amino acid substitutions were designed for increasing the thermostability of barley \((1\rightarrow3,1\rightarrow4)\)-\(\beta\)-glucanase isoenzyme EII. These substitutions were based on the amino acid sequence alignments of higher plant \((1\rightarrow3)\)-\(\beta\)-glucanases shown in Table I and comparisons with the barley \((1\rightarrow3)\)-\(\beta\)-glucanase isoenzyme GII. A brief description of how each substitution was rationalized is presented below.

A surface lysine to arginine substitution: \(K23R\)

It has been reported that surface lysine residues are susceptible to glycation and that this can adversely affect thermostability (Mrabet et al., 1992). Mrabet et al. have also shown that substitution of surface arginines increases heat stability in the absence of sugar derivatives and suggested that lysine to arginine substitutions are important elements in protein stability through the stabilization of electrostatic interactions. Amino acid residue K23 is located on the surface of \((1\rightarrow3,1\rightarrow4)\)-\(\beta\)-glucanase isoenzyme EII where its side chain group is exposed to the solvent (Figures 1 and 2a). Barley \((1\rightarrow3)\)-\(\beta\)-glucanase isoenzymes GI–GIV all have an arginine residue at amino acid position 23, which indicates that this residue may be important for protein stability.

Changes in conformational entropy: \(G44R, A79P\) and \(H300P\)

The amino acid substitutions G44R, A79P and H300P were chosen in attempts to decrease the entropy of the unfolded state of \((1\rightarrow3,1\rightarrow4)\)-\(\beta\)-glucanase isoenzyme EII and hence to increase its overall stability (Matthews et al., 1987). Barley \((1\rightarrow3)\)-\(\beta\)-glucanase isoenzymes GI, GII, GV and GVI all have an arginine residue at amino acid position 44 and arginine
is also highly conserved at this position in other plant β-glucanases (Table I). Introduction of the bulky side chain of an arginine residue was therefore expected to reduce the freedom of rotation around bonds in the Cα chain at this position (Figure 2b). The amino acid substitution A79P was chosen because a proline is well conserved in this position in all of the barley (1→3)-β-glucanases, including the intrinsically stable isoenzyme GII (Table I). In addition, the A79P substitution is located at the end of a major α-helix that is a common position for stabilizing substitutions of this type (Serrano et al., 1992; Figure 2c). The H300P substitution was chosen on the basis that proline is conserved at position 300 in five of the seven barley (1→3)-β-glucanases, including barley (1→3)-β-glucanase isoenzyme GII and in 60% of the aligned plant (1→3)-β-glucanases that were examined (Xu et al., 1992; Table I; Figure 2d).
Increasing packing efficiency and hydrophobicity: F85Y, N290H

In barley (1→3,1→4)-β-glucanase isoenzyme EII, the amino acid substitution F85Y potentially creates a hydrogen bond between the hydroxyl group of the introduced tyrosine and the carbonyl oxygen of amino acid 76 or 80 (Figure 2e) and may thereby help in the packing efficiency of the hydrophobic core (Rose and Wolfenden, 1993). Furthermore, phenylalanine represents the most common residue at this position in the plant β-glucanases generally (Table I), which suggests that residues with relatively large side chains may be important here for packing efficiency. Another substitution located in the protein core, N290H, was selected on the basis that there is a strong bias for histidines (67%) at the corresponding position in other plant (1→3)-β-glucanases (Table I) and because the imidazole side chain might increase packing density in the region (Figure 2f).

Salt bridge: M298K/T17D

In the relatively stable barley (1→3)-β-glucanase isoenzyme GI, residues K298 and D17 potentially constitute an ion pair which would link the COOH-terminal loop to the main body of the enzyme (Figure 2g). These residues are not observed at the corresponding positions of barley (1→3,1→4)-β-glucanase isoenzyme EII. In addition, 52% of the plant β-glucanases examined have an aspartic acid or glutamic acid residue at position 17 and a lysine or arginine at position 298. Furthermore, five of the seven barley (1→3)-β-glucanases contain a potential ion pair at positions 298 and 17 (Xu et al., 1992). The amino acid substitutions M298K and T17D were therefore introduced into (1→3,1→4)-β-glucanase isoenzyme EII in an attempt to form a salt bridge and hence to stabilize the COOH-terminal loop region (Figure 2g).

Triple mutant: H300P, N290H and M298K/T17D

The effects of single amino acid substitutions which give increases in protein stability are often additive (Matsumura and Alb, 1985; Matsumura et al., 1988; Eijsink et al., 1991; Watanabe et al., 1994). The three substitutions which increased the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII (N290H, M298K/T17D and H300P) were therefore combined in an attempt to take advantage of any additive effects on thermostability.

Expression and purification of mutant (1→3,1→4)-β-glucanases

Mutations causing amino acid substitutions K23R, G44R, A79P, F85Y, N290H, M298K/T17D and H300P were introduced at the cDNA level using site-directed mutagenesis and confirmed by DNA sequencing. The eight mutant (1→3,1→4)-β-glucanases, including the ‘triple’ mutant (containing H300P, N290H and M298K/T17D), were expressed in E.coli and purified using affinity chromatography on Ni-NTA resin.

The expressed wild-type protein had a molecular weight of ~33.5 kDa (Figure 3), which corresponds with the calculated molecular weight for barley (1→3,1→4)-β-glucanase isoenzyme EII (~32.5 kDa) fused to the polyhistidine tag (~1.0 kDa). Four hours after the induction of expression at 37°C, more than 85% of the wild-type (1→3,1→4)-β-glucanase fusion protein was found in the insoluble fraction as inclusion bodies (data not shown). To increase the solubility of the recombinant (1→3,1→4)-β-glucanase, the expression temperature was decreased from 37 to 23°C. This significantly increased the solubility of the recombinant wild-type (1→3,1→4)-β-glucanase, with at least five times the amount of recombinant protein residing in the soluble fraction following expression at 23°C, compared with 37°C (data not shown). For subsequent experiments involving recombinant mutant (1→3,1→4)-β-glucanases, expression was routinely induced with 0.25 mM IPTG at 23°C for 4 h. It was estimated from SDS–PAGE analysis of the four mutant enzymes N290H, H300P, M298K/T17D and the triple mutant, together with the recombinant wild-type (1→3,1→4)-β-glucanase, that the five enzymes were all >95% pure (Figure 3).

Yields of the recombinant enzymes varied for each of the mutants and between independent experiments, but were generally between 2 and 5 mg of protein per liter of bacterial culture. Mutants with relatively low thermostabilities were generally less soluble and were therefore purified in lower yields from the soluble bacterial cell lysate. This was probably a direct reflection of their compromised stability. All purified recombinant enzymes were stored at 4°C at a concentration of 0.25–1.0 mg/ml in 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM sodium chloride. Under these conditions, the enzymes showed no appreciable decrease in specific activity over several weeks (data not shown).

The specific activity of the recombinant (1→3,1→4)-β-glucanase purified from E.coli was 14.5 units/mg, which is similar to the specific activity (16.0 units/mg) of the native barley (1→3,1→4)-β-glucanase isoenzyme EII (Table II; Woodward and Fincher, 1982). The kinetic parameters for the recombinant (1→3,1→4)-β-glucanase were determined at 37°C using 0.2–2.0 mg/ml barley (1→3,1→4)-β-glucan as a substrate. The recombinant (1→3,1→4)-β-glucanase had a $K_m$ value of 1.3 mg/ml, which is slightly lower than that of native barley (1→3,1→4)-β-glucanase isoenzyme EII of 3.4 mg/ml (Table II; Woodward and Fincher, 1982). The $k_{cat}$ values for the recombinant (1→3,1→4)-β-glucanase and the native barley (1→3,1→4)-β-glucanase isoenzyme EII were 156.0 and 193.0 s$^{-1}$, respectively (Table II). The differences in kinetic properties might result from the absence of N-glycosylation on the recombinant enzyme (Doan and Fincher, 1992).
Thermostabilities of the mutant enzymes were defined using $T_{50}$, which is defined as the temperature at which 50% of the initial activity remains after incubation for 15 min in 50 mM sodium acetate buffer, pH 5.0, containing 200 μg/ml BSA. As shown in Figure 4, the wild-type recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII had a $T_{50}$ of 47.5°C.

Three of the eight mutant barley (1→3,1→4)-β-glucanases displayed enhanced thermostability. The substitutions N290H, A79P and F85Y had negative effects on the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII and showed $T_{50}$ values 1.3–3.0°C lower than the wild-type enzyme (Figure 4).

Kinetic analysis of mutant H300P

Because mutant enzyme H300P displayed the largest increase in thermostability compared with the wild-type enzyme, it was further analyzed in terms of progressive activity decay at 48°C and its kinetic properties. The thermostable mutant enzyme H300P retained measurable activity at least three times longer than the corresponding wild-type enzyme at 48°C (Figure 5). The $K_m$ and $k_{cat}$ values for mutant H300P were 1.0 mg/ml and 166.0 s⁻¹, respectively, which were similar to the values of 1.2 mg/ml and 156.0 s⁻¹ obtained for the recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII (Table II). The optimum pH for both the wild-type and mutant H300P enzymes was ~4.5 (data not shown).

Discussion

The wild-type recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII was expressed in E.coli and purified using affinity chromatography. The thermostabilities of the two enzymes were determined by either measuring the residual activity after 15 min incubation at a range of different temperatures (A) or by measuring the progressive loss of activity over time at 48°C (B). Error bars indicate standard errors calculated from at least five independent experiments.

Table II. Kinetic parameters and specific activities for recombinant and native barley (1→3,1→4)-β-glucanase isoenzyme EII

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (units/mg)</th>
<th>$K_m$ (mg/ml)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Native barley isoenzyme EII</td>
<td>16.0</td>
<td>3.4</td>
<td>193.0</td>
</tr>
<tr>
<td>Rec. EII + histidine tag</td>
<td>14.5</td>
<td>1.2</td>
<td>156.0</td>
</tr>
<tr>
<td>Rec. EII + histidine tag</td>
<td>13.8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Rec. EII/H300P + histidine tag</td>
<td>12.5</td>
<td>1.0</td>
<td>166.0</td>
</tr>
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</table>

*Not determined.

Thermostability of mutant (1→3,1→4)-β-glucanases

Thermostabilities of the mutant enzymes were defined using $T_{50}$, which is defined as the temperature at which 50% of the initial activity remains after incubation for 15 min in 50 mM sodium acetate buffer, pH 5.0, containing 200 μg/ml BSA. As shown in Figure 4, the wild-type recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII had a $T_{50}$ of 47.5°C.

Three of the eight mutant barley (1→3,1→4)-β-glucanases displayed enhanced thermostability. The substitutions N290H, M298K/T17D and H300P increased the displayed enhanced thermostability. The substitutions N290H, A79P, F85Y and K23R had negative effects on the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII and showed $T_{50}$ values 1.3–3.0°C lower than the wild-type enzyme (Figure 4).

Kinetic analysis of mutant H300P

Because mutant enzyme H300P displayed the largest increase in thermostability compared with the wild-type enzyme, it was further analyzed in terms of progressive activity decay at 48°C and its kinetic properties. The thermostable mutant enzyme H300P retained measurable activity at least three times longer than the corresponding wild-type enzyme at 48°C (Figure 5). The $K_m$ and $k_{cat}$ values for mutant H300P were 1.0 mg/ml and 166.0 s⁻¹, respectively, which were similar to the values of 1.2 mg/ml and 156.0 s⁻¹ obtained for the recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII (Table II). The optimum pH for both the wild-type and mutant H300P enzymes was ~4.5 (data not shown).

Discussion

The wild-type recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII was expressed in E.coli with a maltose-binding protein (MBP) fused to its NH₂-terminus (Chen et al., 1995); this MBP fusion protein had a $T_{50}$ value of 47.5°C. In the present work the poly(histidine) tag was removed from the expressed enzyme with thrombin, but no increase in thermostability could be detected after removal of the tag (data not shown). Similarly, removal of the MBP component of the fusion protein generated by Chen et al. (Chen et al., 1995) had no effect on thermostability.

The native (1→3,1→4)-β-glucanase isoenzyme EII purified from germinated barley grain (Woodward and Fincher, 1982) has a $T_{50}$ of 52°C, which is 4.5°C higher than that of the recombinant wild-type enzyme purified from E.coli. This
difference in thermostability is probably due to the different glycosylation states of the respective enzymes. barley (1→3,1→4)-β-glucanase isoenzyme EII is naturally glycosylated (Woodward and Fincher, 1982; Doan and Fincher, 1992; Harthill and Thomsen, 1995), whereas the recombinant enzyme expressed in E.coli remains unglycosylated. Doan and Fincher have demonstrated that glycosylation contributes significantly to the stability of barley (1→3,1→4)-β-glucanase isoenzyme EII (Doan and Fincher, 1992). Results from related studies have also shown that glycosylation can increase protein stability (Olsen and Thomsen, 1991; Meldgaard and Svendsen, 1994).

Site-directed mutagenesis was used to introduce amino acid substitutions into barley (1→3,1→4)-β-glucanase isoenzyme EII in order to increase the enzyme’s thermostability. It should be noted that comparisons of thermostability were performed against the ‘wild-type’ enzyme, which was expressed from the unmodified cDNA in E.coli and purified on an Ni-NTA column in the same way as the mutant enzymes. The amino acid substitutions G44R, A79P, F85Y and K23R caused a decrease in thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 4). For substitutions A79P and G44R, the new residues are significantly different from the replaced residues in terms of size and/or polarity. Alanine and glycine have relatively small uncharged side chains, whereas arginine and proline are relatively large amino acids. Thus, the G44R and A79P substitutions might have destabilized the enzyme by causing steric hindrance with the atoms of neighboring residues or by disrupting other non-covalent interactions in folding intermediates of the folding pathway. The molecular modeling was based on the folded molecule.

The F85Y substitution was designed to form an additional hydrogen bond with the carbonyl oxygen of residues 76 or 80 (Figure 4). The decreased thermostability resulting from the F85Y substitution may be due to the hydroxyl group of the tyrosine disrupting the spatial arrangement of hydrophobic residues in the core of the enzyme (Karpusas et al., 1989; Sandberg and Terwilliger, 1989; Shortle et al., 1990). The amino acid substitution K23R also had a negative effect on the stability of barley (1→3,1→4)-β-glucanase isoenzyme EII. This substitution was based on the general conservation of arginine at this position in barley (1→3)-β-glucanases, including the relatively thermostable (1→3)-β-glucanase isoenzyme GII. In addition, a computer-based structural analysis of barley (1→3,1→4)-β-glucanase isoenzyme EII suggested that an arginine at amino acid position 23 should be tolerated within the enzyme structure. Although it is unlikely that the relatively conservative lysine to arginine substitution caused conformational strain in the polypeptide backbone, it is possible that hydrogen bonds in the vicinity of the substitution were disrupted.

Three of the mutant barley (1→3,1→4)-β-glucanases exhibited enhanced thermostability (Figure 4). The small increase in stability (0.7°C) that was associated with the N290H substitution may result from an increase in the packing efficiency of the protein due to the larger size of the histidine residue compared with the asparagine residue of the wild-type enzyme. There are several examples where increased packing efficiency of the protein core has led to increased protein stability (Davies et al., 1993; Rose and Wolfenden, 1993). Alternatively, the nitrogen atoms of the histidine may form extra hydrogen bonds with neighboring atoms.

The M298K/T17D substitution also resulted in a small increase in thermostability. This mutant was designed to stabilize the COOH-terminal loop by forming a salt bridge between the top of the loop and the body of the enzyme (Figure 2g). Although salt bridges can make a major contribution to the stability of proteins (Anderson et al., 1990; Dao-Pin et al., 1991; Davies et al., 1993; Ishikawa et al., 1993; Kelly et al., 1993), the increase in thermostability attributable to the M298K/T17D substitution was only 0.2°C (Figure 4).

The amino acid substitution H300P showed the largest increase in thermostability (Figures 4 and 5). This effect can be attributed to a reduction in the entropy of the unfolded state of the enzyme (Matthews et al., 1987; Eijsink et al., 1991; Hardy et al., 1993; Watanabe et al., 1994; Watanabe, 1996; Watanabe and Suzuki, 1998). The H300P mutant exhibited a Tm value that was 3.7°C higher than that of the wild-type enzyme and remained active at 48°C about three times longer than the wild-type enzyme (Figures 4 and 5). The kinetic properties of the H300P mutant were similar to those of the wild-type enzyme (Table II).

Because amino acid substitutions which increase thermostability are often additive (Eijsink et al., 1991; Watanabe et al., 1994), a mutant of isoenzyme EII containing the substitutions H300P, M298K/T17D and N290H was engineered. However, the thermostability of the resulting ‘triple mutant’ enzyme was the same as that of the enzyme containing the single substitution H300P (Figure 4). This non-cumulative effect is also evident in the neutral proteases from Bacillus stearothermophilus, where the mutational effects of stabilizing substitutions are not always additive (Vriend and Eijsink, 1993). The relatively high ΔTm value for mutant H300P (3.7°C), compared with those of M298K/T17D (0.4°C) and N290H (0.7°C), suggests that the H300P substitution had an overriding effect on the stability of the COOH-terminal loop, where each of these mutations were affected.

The three amino acid substitutions which increased the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII (H300P, N290H and M298K/T17D) were all located in the COOH-terminal loop of the enzyme (Figure 1). This solvent-exposed loop region, which includes residues 286–306 (Varghese et al., 1994), lacks any well-defined secondary structure (Varghese et al., 1994) and may represent a ‘weak link’ in the enzyme that could ‘unfold’ early in the thermal inactivation process. Because one of the potential catalytic amino acid residues, E288, is close to the COOH-terminus of the enzyme (Varghese et al., 1994), unfolding of this region would have an immediate effect on enzyme activity. Thus, stabilizing this region would necessarily produce a more thermostable enzyme if the COOH-terminus were the first part of the enzyme to unfold at elevated temperatures. Eijsink et al. found that the crucial interactions responsible for the relatively high thermostability of a bacterial theromolsin were all localized to a solvent-exposed loop region in the NH2-terminal domain of the protein, suggesting that peripheral loops may be involved in the early steps of the unfolding process (Eijsink et al., 1991). Similarly, Welfle et al. found (Welfle et al., 1996) that several amino acid substitutions which contributed to the thermostability of a hybrid bacterial (1→3,1→4)-β-glucanase (Olsen et al., 1991) were all located in the NH2-terminal loop region. Finally, proline residues are responsible for the extremely high thermostability of a β-glucosidase from Bacillus thermoglucosidasius also occur with high frequency in loop regions (Watanabe et al., 1994).

Thus, three of the eight amino acid substitutions that were introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII
Mutant barley (1→3,1→4)β-glucan endohydrolases with enhanced thermostability

resulted in mutant enzymes that displayed a higher thermostability than the corresponding wild-type enzyme. These mutant enzymes contained substitutions corresponding to residues found in a high proportion of the naturally thermostable (1→3)-β-glucanases. The three amino acid substitutions which caused an increase in heat stability were clustered at the COOH-terminal loop of barley (1→3,1→4)-β-glucanase isoenzyme EI, suggesting that this loop may represent an unstable region of the enzyme. However, the cumulative effect of the three substitutions did not account for the difference in thermostability between the barley (1→3,1→4)-β-glucanase isoenzyme EI and the barley (1→3)-β-glucanase isoenzyme GH. The latter enzyme has a $T_{50}$ value which is $-15^\circ C$ higher than that of the (1→3,1→4)-β-glucanase (Chen et al., 1995). Thus, although the stability of the COOH-terminal loop of the enzymes clearly contributes to the overall enzyme stability, there must be other regions in the three-dimensional structure of the barley (1→3)-β-glucanase that have become more stable during the process of evolution. Whether the regions identified here for the barley (1→3,1→4)-β-glucanase are also important for the thermostability of other family 17 enzymes or more generally in the GH-A clan of glycoside hydrolases remains to be demonstrated.

Finally, the production of a thermostable barley (1→3,1→4)-β-glucanase has implications for the brewing industry. At the elevated temperatures used for the malting and brewing processes, endogenous barley (1→3,1→4)-β-glucanases are rapidly inactivated and thus do not completely break down residual (1→3,1→4)-β-glucans that might be present in malt extracts. If barley expressing a thermostable (1→3,1→4)-β-glucanase could be engineered, many of the difficulties associated with residual (1→3,1→4)-β-glucans (Bamforth, 1994) might be overcome. The data here show that at temperatures of say 50°C the H300P substitution has a dramatic effect on the number of catalytic events one would expect from a given amount of enzyme. Furthermore, preliminary data from simulated mashing experiments at 65°C show that the H300P mutant enzyme retains activity at least five times longer than the wild-type enzyme (data not shown). Transgenic barley plants carrying the H300P DNA construct have been generated and the thermostability of expressed (1→3,1→4)-β-glucanases can now be evaluated.

Acknowledgements

This work was supported by grants from the Australian Research Council and the Grains Research and Development Corporation of Australia. R.J.S. acknowledges the receipt of a Grains Research and Development Corporation postgraduate scholarship. We thank Professor Peter Colman for his interest and support.

References


Received October 24, 2000; revised January 16, 2001; accepted January 26, 2001