Stabilization of Aspergillus awamori glucoamylase by proline substitution and combining stabilizing mutations

Martin J. Allen, Pedro M. Coutinho and Clark F. Ford

Introduction
Aspergillus awamori glucoamylase [α-1,4 d-glucan gluco-hydrolase, EC 3.2.1.3] (GA) is an enzyme that catalyses the release of β-glucose from the nonreducing ends of starch and related oligosaccharides. Glucoamylase is used in, and defines the rate-limiting step of, the commercial conversion of starch to high glucose syrups that may be converted to fructose syrups by glucose isomerase or used in fermentations to produce ethanol. Its application to these processes make GA one of the most heavily used industrial enzymes.

Glucoamylase is used industrially at 55–60°C; at higher temperatures the enzyme is rapidly and irreversibly inactivated. A more stable GA, which could be used at elevated temperatures, is industrially desirable. Advantages of using elevated temperatures include increased reaction rate (or decreased amounts of enzyme required to give the same reaction rate as at lower temperatures), decreased microbial contamination of reaction vessels, and decreased viscosity of reaction syrups.

GA is composed of a catalytic domain (amino acids 1–470), a highly glycosylated linker that overlaps the catalytic domain (amino acids 441–512) and a starch-binding domain (amino acids 513–616) (Svenssson et al., 1983). The structure of the A. awamori var. X100 GA catalytic domain has been solved by X-ray crystallography (Aleshin et al., 1992) and the active site has been described (Harris et al., 1993; Aleshin et al., 1994; Stoffer et al., 1995). The highly α-helical catalytic domain folds into a 12-helix α/α barrel (Aleshin et al., 1992) with the core of the barrel defining a pocket containing the catalytic acid and base (Harris et al., 1993).

Previous work has shown that the natural stability of oligo-1,6-glucosidase (Suzuki et al., 1987) and pullulanase (Suzuki et al., 1991) can be positively correlated to the mole percent proline present in the protein, and a general rule for protein stability has been proposed (Suzuki, 1989). This work has been extended to show that bacteriophage T4 lysozyme (Matthews et al., 1987) and Bacillus cereus ATCC 7064 oligo-1,6-glucosidase (Watanabe et al., 1994) can be stabilized by engineering proline into selected sites thereby decreasing the protein’s conformational entropy of unfolding.

We have applied a similar strategy to our studies of GA stability. We have previously reported the results of proline substitution at the following positions in GA: Ala27, Ala393, Ala435, Ser436 and Ser460 (Li et al., 1997). Only Ser436→Pro demonstrated increased GA thermostability. The current report presents the results of three additional proline substitution mutations. Based on structural and evolutionary considerations, Ser30, Asp345 and Glu408 were mutated to proline (S30P, D345P and E408P respectively). Mutations at these sites were constructed using the cloned A. awamori gene (Innis et al., 1985) and the proteins were expressed in Saccharomyces cerevisiae (Cole et al., 1988). As measured by resistance to irreversible thermostabilization at various temperatures we show that S30P strongly stabilizes GA without decreasing the enzyme’s activity.

In addition to S30P we have also previously shown that the following mutations significantly stabilize GA without decreasing enzyme activity: Asn20→Cys/Ala27→Cys (Li, 1996) (referred to as S–S in this work), which creates a disulfide bond between positions 20 and 27, and Gly137→Ala (Chen et al., 1996) (G137A). To investigate whether individual stabilizing mutations could cumulatively stabilize GA, we prepared various combined mutant enzymes and here we report the significant and cumulative stabilization of GA without activity loss.

Materials and methods
The yeast expression plasmid YepPM18 (Cole et al., 1988) and S. cerevisiae C468 (α leu2-3 leu 2-112 his 3-11 his 3-15 mat′) (Innis et al., 1985) were gifts from Cetus. All restriction enzymes were from Promega (Madison, WI). Acrbose was a gift from Miles Laboratories and Maltrin M100 DE 10 maltodextran was a gift from the Grain Processing Corporation.

Site-directed mutagenesis
Site-directed mutagenesis was performed according to the Muta-Gen phagemid in vitro mutagenesis kit from Bio-Rad, which is based on the method of Kunkel et al. (1987). For the proline substitution mutants, a 1.7-kb XhoI–BamHI DNA fragment coding for the GA catalytic domain was cloned into a pBluescript II KS(+) vector from Stratagene. The following oligonucleotides were used as mutagenic
primers: CAGAGTCCCGGCCCAGCAACACACACGCTC (S30P), AACCTCCGACACACACACACGCTC (D345P) and CGACGCCAAAAGCTCGGCCCAGCAGTGCACG-AC (E408P). The underlined nucleotides in the primers indicate mismatches creating the proline substitution mutations. For the S/S-S30P combined mutant, a 1.7-kb Xhol–BamHI DNA fragment coding for the wild-type GA catalytic domain was cloned into a pBluescript II KS (+) vector from Stratagene. The following oligonucleotide was used as a mutagenic primer: CCGGCGGCCAGACACACACGCCCAGGATACG- TCCGCCCAGGTATCGACACATGGC, the underlined nucleotides represent DNA mismatches that create the S30P, A27C an N20C mutations, respectively. For the S30P/G137A mutant GA, the 1.7-kb Xhol–BamHI DNA fragment coding for the previously constructed S30P GA catalytic domain was cloned into a pGEM-7zf(+) vector from Promega. The following oligonucleotide was used as a mutagenic primer: ACTGCTATGCTACGCTTCTCGAGCTGAAAAG. The underlined nucleotides indicate DNA mismatches that create the G137A amino acid substitution. The S/S–S30P/G137A combined mutant was constructed using the S/S–S30P oligonucleotide listed above and a DNA template derived from a pBluescript II KS (+) vector with a 1.7-kb Xhol–BamHI DNA fragment coding for the GA catalytic domain, which already contained mutations conferring the S30P and G137A amino acid substitutions. The presence of the individual mutations was confirmed by sequencing, and each mutated GA gene fragment was cloned into YEpPM18 (Cole et al., 1988) and transformed into S.cerevisiae.

**Enzyme production and purification**

Wild-type, proline substitution mutants and S–S/S30P GAs were produced by growing yeast at 30°C in 5.3 l SD media [1.7 mg/ml yeast nitrogen base without amino acids or ammonium sulfate (Difco), 5 mg/ml ammonium sulfate, 2% glucose, 0.1 mg/ml L-histidine] for 72 h at pH 4.5 in a 5 l fermentor. After 48 h, 100 g glucose and 22 g (NH₄)₂SO₄ in 300 ml H₂O was added as a supplement (Chen et al., 1994a). S30P/G137A and S–S/S30P/G137A mutant GAs were produced by growing yeast in SD + His media at 30°C for 5 days in a shaking incubator without pH control or the addition of supplement to the media. Following growth, the cultures were centrifuged to remove yeast cells, the supernatants were concentrated by ultrafiltration, dialyzed against H₂O, further concentrated by ultrafiltration and dialyzed against 0.5 M NaCl/0.1 M NaOAc, pH 4.5, and GA purified by acarbose–Sepharose affinity chromatography (Chen et al., 1994b). GA was eluted with 1.7 M Tris–HCl, pH 7.6, dialyzed against H₂O, further concentrated by ultrafiltration and dialyzed against 0.05 M NaOAc buffer, pH 4.5. The protein concentration was determined according to the Pierce bicinchoninic acid protein assay (Smith et al., 1985) using bovine serum albumin as a standard.

**Enzyme assays**

Enzyme kinetic assays were done as described by Li et al. (1997) using maltose as a substrate at pH 4.5 and 35°C. Maltose concentrations used ranged from 0.2 to 4 Kₘ, in 0.005 M NaOAc buffer. Kinetic parameters were analyzed by the program ENZFITTER (Elsevier-Biosoft).

Enzyme specific activities were determined at 50°C using 4% maltose in 0.05 M NaOAc buffer, pH 4.5, as substrate. One international unit (IU) of enzyme activity was defined as the amount of enzyme required to produce 1 µmol/min glucose at assay conditions. Following mixing of enzyme with substrate, six 100-µl samples were removed at 7 min intervals over 42 min, the reaction was stopped by adding 40 µl of 4.0 M Tris–HCl, pH 7.0, and the glucose concentration was determined by a glucose oxidase/o-dianisidine assay (Banks et al., 1971).

**Irreversible thermostability**

Duplicate aliquots of 40 µg/ml purified wild-type and mutant enzymes were subjected to inactivation at six or more temperatures between 65 and 80°C (except E408P which was inactivated at five temperatures between 58.5 and 68.5°C) at intervals of 2.5°C. Samples were removed at six different times, immediately placed on ice and stored at 4°C for 24 h to eliminate the possibility that the inactivation observed was reversible. The residual activity of the inactivated samples, along with a corresponding sample which had not been subjected to thermostabilization, was determined as described for enzyme specific activities, but at 35°C.

**Saccharification analysis**

Saccharifications were performed in duplicate using stirring heating blocks and tightly sealed vials to prevent evaporation. Wild-type and mutant GAs (8 µg/ml) were assayed using 28% (w/v) DE 10 maltodextrin in 0.05 M NaOAc, pH 4.5, as substrate. At various times, a sample was removed, diluted appropriately in 0.05 M NaOAc, pH 4.5, and the reaction was stopped by adding 100 µl diluted sample to 40 µl 4.0 M Tris–HCl, pH 7.0. The glucose concentration was determined by a glucose oxidase/o-dianisidine assay (Banks et al., 1971).

**Results**

**Irreversible thermostability**

Wild-type and mutant GAs were subjected to thermostabilization at pH 4.5 between 65 and 80°C. Semilogarithmic plotting of residual activity versus inactivation time was used to determine inactivation rate coefficients (kᵢ) for wild-type, S30P, D345P and E408P GAs. Data points represent the average of duplicate analysis.

![Fig. 1. Effect of temperature on first-order thermostabilization rate coefficients (kᵢ) of wild-type, S30P, D345P and E408P GAs. Data points represent the average of duplicate analysis.](Image 321x587 to 542x760)
Stabilization of Aspergillus awamori

Fig. 2. Effect of temperature on first-order thermoinactivation rate coefficients ($k_d$) of wild-type, S30P, D345P and E408P GAs. Data for the G137A and S–S mutant GAs are shown for comparison and were taken from Chen et al. (1996) and Li et al. (1996) respectively. Data points represent the average of duplicate analysis.

extrapolation from the thermoinactivation plots, and transition-state theory was used to calculate activation energies for thermal inactivation ($\Delta G^+$) at 65°C and temperatures at which the enzyme is 50% inactivated after 10 min ($\Delta T_{50}$) relative to wild-type GA.

Table I. Changes in activation energies for thermodenaturation ($\Delta G^+$) at 65°C and temperatures at which the enzyme is 50% inactivated after 10 min ($\Delta T_{50}$) relative to wild-type GA.

<table>
<thead>
<tr>
<th>GA form</th>
<th>$\Delta G^+$ (kJ/mol)</th>
<th>$\Delta T_{50}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S30P</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>D345P</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>E408P</td>
<td>-7.2</td>
<td>-6.7</td>
</tr>
<tr>
<td>S30P/G137A</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>S–S/S30P</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td>S–S/S30P/G137A</td>
<td>4.4</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Table II. Kinetic parameters for wild-type and mutant GAs at pH 4.5 and 35°C using maltose as a substrate.

<table>
<thead>
<tr>
<th>GA form</th>
<th>$K_m$ (mM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.09 ± 0.1</td>
<td>8.91 ± 0.2</td>
<td>8.21 ± 0.5</td>
</tr>
<tr>
<td>S30P</td>
<td>1.49 ± 0.1</td>
<td>10.24 ± 0.3</td>
<td>6.89 ± 0.4</td>
</tr>
<tr>
<td>D345P</td>
<td>1.27 ± 0.1</td>
<td>11.19 ± 0.4</td>
<td>8.78 ± 0.6</td>
</tr>
<tr>
<td>E408P</td>
<td>1.29 ± 0.1</td>
<td>12.00 ± 0.4</td>
<td>9.28 ± 0.6</td>
</tr>
<tr>
<td>S30P/G137A</td>
<td>1.55 ± 0.1</td>
<td>11.18 ± 0.3</td>
<td>7.23 ± 0.4</td>
</tr>
<tr>
<td>S–S/S30P</td>
<td>1.31 ± 0.1</td>
<td>9.95 ± 0.3</td>
<td>7.58 ± 0.4</td>
</tr>
<tr>
<td>S–S/S30P/G137A</td>
<td>1.29 ± 0.1</td>
<td>10.57 ± 0.2</td>
<td>8.19 ± 0.3</td>
</tr>
</tbody>
</table>

Table III. Specific activities of wild-type and mutant GAs at pH 4.5 and 50°C using maltose as substrate.

<table>
<thead>
<tr>
<th>GA form</th>
<th>Specific activity (IU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>21.1 ± 0.1</td>
</tr>
<tr>
<td>S30P</td>
<td>20.3 ± 0.9</td>
</tr>
<tr>
<td>D345P</td>
<td>21.2 ± 0.5</td>
</tr>
<tr>
<td>E408P</td>
<td>21.7 ± 0.5</td>
</tr>
<tr>
<td>S30P/G137A</td>
<td>24.0 ± 1.2</td>
</tr>
<tr>
<td>S–S/S30P</td>
<td>21.2 ± 0.5</td>
</tr>
<tr>
<td>S–S/S30P/G137A</td>
<td>24.5 ± 0.2</td>
</tr>
</tbody>
</table>

*Standard deviation resulting from three or more assays.

strate that the S30P mutation stabilizes the enzyme, and that combining S30P with S–S and G137A mutations can cumulatively stabilize GA.

Enzyme activities

Table II shows kinetic analysis of the mutants and wild-type at 35°C. Although $K_{cat}/K_m$ values were similar for the mutants and wild-type, $K_{cat}$ values for the mutants were almost 10% higher than that of the wild-type. Table III shows specific activities of the wild-type and mutant GAs at 50°C and pH 4.5 using maltose as substrate. None of the mutant GAs demonstrated reduced enzyme activity; however, the S30P/G137A and S–S/S30P/G137A mutants were somewhat more active than wild-type at 50°C. To determine if the apparent increased activity was due to differential survival of active enzyme at the assay temperature, the activities of these mutant enzymes were assayed at various temperatures between 35 and 68°C. The S30P/G137A and S–S/S30P/G137A mutant GAs were 10–20% more active than wild-type at all temperatures examined (not shown). A possible explanation for this observation will be discussed below.

Saccharification analysis

Figure 3 shows the results of saccharification analysis at 55 and 65°C for wild-type, S30P/G137A and S–S/S30P/G137A GAs using the industrial DE 10 maltodextrin substrate Maltrin...
M100 (28% w/v) from the Grain Processing Corporation. Complete conversion of 28% w/v DE 10 maltodextrin to glucose would result in a 1.71 M glucose syrup. Industrial saccharifications typically result in 96% maximum conversion to glucose (O’Rourke et al., 1996). However, previous saccharification analyses in our laboratory have demonstrated that recombinant wild-type GA produced by yeast typically results in approximately 90% theoretical maximum glucose yield at 55°C (Fang et al., 1988a,b; Liu et al., 1998). The difference is apparently due to the lack of secondary enzymes (α-amylase and pullulanase) present in the industrial reactions (O’Rourke et al., 1996). At 55°C no significant difference in glucose production was observed between the wild-type and mutant enzymes (all of which resulted in approximately 90% conversion of DE 10 maltodextrin to glucose), indicating that thermostabilization of wild-type was not rate-determining and that specific activity differences between wild-type and the mutants did not affect the final glucose yield over the time course of the reaction. At 65°C, however, the mutant GAs produced 8–10% more glucose than wild-type GA, although none of the enzymes tested produced as much glucose as at 55°C probably due to thermostabilization of the enzymes at the elevated reaction temperature.

Discussion

Sites of mutation

Figure 4 shows the sites of mutation in the catalytic domain of A. awamori var. X100 GA whose structure is known (Aleshin et al., 1992). Based on this structure, we chose three sites for proline substitution, which met the following criteria: (i) Ramachandran (φ,ψ) angles (Ramachandran et al., 1963) were within allowed values for proline. For his work the φ and ψ angles at the substituted site were restrained to the broad range φ = −90° to −40°, ψ = 120° to 180° or φ = −90° to −40°, ψ = −50° to 10°. (ii) Residues were highly solvent-exposed, since mutation of residues in the core of the enzyme were thought to be more likely to decrease the enzyme’s catalytic efficiency. (iii) Residues did not participate in hydrogen bonding with other amino acids. Additionally, based on sequence alignments with GAs from other organisms (Coutinho and Reilly, 1994), only residues that met the above structural criteria and were not well conserved were selected for mutation. Ser30 could be aligned with proline in GAs from Humicola grisea var. thermoidea and Hormoconis resinae GamP (Coutinho and Reilly, 1994), which made it particularly attractive for proline substitution. The criteria used to select the sites of mutation for the S–S and G137A mutations have been discussed in detail by Chen et al. (1996) (G137A), and Li (1996) (S–S) and will not be repeated. Briefly, the mutations Asn20→Cys and Ala27→Cys form a disulfide bond between the C-terminus of α-helix 1 and an extended loop between α-helices 1 and 2 (Li, 1996), and G137A was designed to stabilize the enzyme by reducing its conformational entropy of unfolding and is the most stabilizing in a series of Gly→Ala mutations (Chen et al., 1996).

It is of particular importance to note the positions of the
S30P and the disulfide bond-forming mutations. The disulfide bond is formed between positions 20 and 27, relatively close to position 30. The fact that both the disulfide bond-forming mutations and the S30P stabilize GA suggests that this region of the enzyme is critical for irreversible thermoactivation and may represent a region of local unfolding important for thermoactivation. Additionally, previous investigators have suggested that a disulfide bond should not be engineered within four amino acids of a proline in the primary sequence (Balaji et al., 1989). Our work demonstrates that this rule is not absolute since thiol analysis showed that the disulfide bond was formed in the S-S/S30P and S-S/S30P/G137A combined mutants (not shown), and thermoactivation studies showed the stabilizing effects of the mutations were cumulative.

**Enzyme activity**

None of the proline substitution mutations decreased enzyme activity as shown by kinetic analysis at 35°C and by specific activity measurements at 50°C. This suggests that these mutations did not significantly alter the enzyme’s structure around the active site or alter its interaction with substrate. Additionally, neither S–S nor G137A mutant GAs had enzyme activity significantly different from wild-type GA at 50°C (Li, 1996; Chen et al., 1996). The S30P/G137A and S-S/S30P/G137A combined mutants, however, had slightly increased activity compared with wild-type GA at all temperatures tested between 35 and 68°C. A possible explanation for this may be the existence of active and inactive conformations due to local unfolding and refolding of the molecule at the assay temperature. Introduction of stabilizing mutations may result in a greater population of molecules in an active conformation, thus resulting in higher specific activity for the mutant enzymes.

**Stability of the proline substitution mutants**

The proline substitution mutants had different thermostabilities when measured by their resistance to irreversible thermoactivation. When compared with wild-type GA, E408P decreased, D345P did not significantly alter and S30P increased GA stability (Figure 1 and Table I).

E408P destabilized GA. As was first suggested by Schimmel and Flory (1968), and has been expanded by others (MacArthur and Thornton, 1991; Hurley et al., 1992), proline not only restricts the \( \phi, \psi \) values for the site at which it exists, but also the \( \phi, \psi \) values of the preceding residue. These reports suggest that the \( \phi, \psi \) values for the residue preceding proline should be restricted to approximately \( \phi = -180 \) to \( -55 \)° and \( \psi = 55 \) to \( 180 \)° or \( \phi = -180 \) to \( -55 \)° and \( \psi = -30 \) to \( -70 \)° for all residues in Xaa-Pro except for Xaa=Gly, for which the preceding still applies, but is extended to include \( \phi = 45 \) to \( 180 \)°. In the published A.awamori var. X100 catalytic domain structure (Aleshin et al., 1992), Asp408 (\( \phi = -65 \), \( \psi = 146 \)), which aligns with Glu408 in A.awamori GA, has \( \phi, \psi \) values in ranges acceptable for proline. However, the preceding residue Gly407 (\( \phi = 80 \), \( \psi = -5 \)) has \( \phi, \psi \) outside acceptable ranges for positions preceding proline. Therefore, it was not surprising that the E408P mutation destabilized GA. Additionally, X-ray crystallography suggests that position 408 lies within a \( \beta \)-strand in the closely related A.awamori var. X100 GA, a site not well suited for proline substitution.

Asp345 (\( \phi = -65 \), \( \psi = -26 \)) and the preceding Thr344 (\( \phi = -116 \), \( \psi = 178 \)) have \( \phi, \psi \) angle values well within allowed values for proline substitution at position 345. However, the D345P mutant GA did not demonstrate stability significantly different from wild-type GA. This is particularly interesting since position 345 lies at the N-terminus of an \( \alpha \)-helix (Aleshin et al., 1992), a position previously shown to be particularly favorable for proline substitution (Watanabe et al., 1994). A possible explanation for this observation is that Asp345 is at the N-terminus of \( \alpha \)-helix 11 (Li, 1996; Chen et al., 1996) in the A.awamori var. X100 GA structure (Aleshin et al., 1992). Replacing Asp345 with Pro could disrupt the \( \alpha \)-helix dipole. Therefore, any entropic stabilization brought about by the proline substitution might be offset by the disruption in the \( \alpha \)-helix dipole. An alternative explanation for this observation that \( \alpha \)-helix 11 is not part of the core 12-helix \( \alpha/\alpha \) barrel structure, and therefore stabilization of this helix may not be reflected in our functional stability assay. Interestingly, Li (1996) showed that another mutation in this region, Lys352→Arg, which was predicted to increase GA stability but failed to do so in our functional assay, did increase one thermal unfolding transition by 1.5°C when measured by differential scanning calorimetry. This suggests that the region including \( \alpha \)-helix 11 may represent a subdomain that unfolds independently of the rest of the catalytic domain. In other words, it may be possible that slight stabilization or destabilization of \( \alpha \)-helix 11 does not result in an enzyme with significantly altered functional stability since this helix is not part of the core \( \alpha/\alpha \) barrel.

Ser30 (\( \phi = -49 \), \( \psi = 130 \)) is preceded by Val29 (\( \phi = -127 \), \( \psi = 46 \)) both of which have acceptable \( \phi, \psi \) angle values except Val29 \( \psi = 46 \), which is slightly smaller than ideal for proline substitution at position 30. Upon characterization, the S30P substitution increased GA thermostability. Position 30 lies on an extended loop between \( \alpha \)-helices 1 and 2 in the A.awamori var. X100 GA structure (Aleshin et al., 1992), and can be aligned with proline in other GAs as discussed above. When this loop region is examined more closely, position 30 is at the second position of a type II \( \beta \)-turn using the definition of Wilmot and Thornton (1998). This is in agreement with the observations of Watanabe et al. (1994), who have suggested that the second position of \( \beta \)-turns is particularly favorable for proline substitution. These investigators have reported increases in \( T_m \) of 0.8–1.4°C per proline residue for substitutions made at the second position of \( \beta \)-turns in Bacillus cereus ATCC 7064 oligo-1,6-glucosidase.

**Cumulative stabilization**

Previous work in our laboratory has shown that combining two stabilizing mutations does not necessarily stabilize GA (Chen et al., 1996). The present study shows that the three mutations chosen here can be combined to cumulatively stabilize the enzyme even though two of the mutations (S-S and S30P) are very close together in the protein. Thus, cumulative thermostabilization must be assessed on a case by case basis.

S30P combined with G137A showed more than additive stabilization at low temperatures (65–70°C), but less than additive stabilization at higher temperatures (77.5–80°C) (Figure 2A). At 80°C the inactivation rate for the S30P/G137A combined mutant was nearly identical to that of the S30P individual mutant protein. This indicates that both regions are very important for low temperature thermostabilization, but at high temperatures inactivation became governed by other processes.

It was somewhat surprising that combining the S30P with the disulfide bond-forming mutations resulted in cumulative stabilization. This is not only because the engineered disulfide
bond is so close to the engineered proline as discussed above, but also because both are targeting the same region of the protein (i.e. the extended loop between α-helices 1 and 2). If either the disulfide bond or S30P stabilized this region maximally, further stabilization at this site would not result in roughly additive stabilization at all temperatures examined between 65 and 80°C. This raises the possibility that further stabilization may result from the addition of more stabilizing mutations in this region.

The S–S/S30P/G137A combined mutant was no more stable than S30P/G137A GA at low temperatures (65–70°C), but it was slightly more stable at higher temperatures (75–80°C) (Figure 2C). Interestingly, the S–S/S30P GA was also more stable than S30P/G137A GA at high temperatures. Therefore, it appears that the introduced disulfide bond is particularly effective at stabilizing GA at high temperatures. The cause for this observation remains unclear. However, given the position of the bond, it may serve to anchor the extended loop to the first α-helix, thereby preventing it from unfolding even at high temperatures.

Industrial application
Carbohydrates are known to stabilize proteins (Shein, 1990; Butler and Falke, 1996), including GA (Taylor et al., 1978; Sinitisyn et al., 1978; Przybyt and Sugier, 1988). To determine whether the most stabilizing mutations, S30P/G137A and S–S/S30P/G137A, would enhance GA performance at conditions more closely resembling those which the enzyme would encounter industrially, we subjected the enzymes to thermoinactivation analysis, we estimate that the S30P/G137A and S–S/S30P/G137A mutant GAs could be used industrially at temperatures 3–4°C higher than wild-type GA. This could stabilize of an oligosaccharide substrate. Based on irreversible thermoinactivation analysis, we estimate that the S30P/G137A and S–S/S30P/G137A mutant GAs could be used industrially at temperatures 3–4°C higher than wild-type GA. This could greatly increase the rate of saccharification reactions (or decrease amounts of enzyme required to give the same reaction rate as at 60°C), decrease microbial contamination of reaction vessels and decrease the viscosity of reaction syrups. Conversely, the mutant GA could be used at the same temperature as wild-type but for much longer reaction times, which would significantly decrease the amount of enzyme required to carry out starch saccharification.

Conclusions
We have demonstrated significant and cumulative stabilization of A. awamori GA by proline substitution mutagenesis and by combining individual stabilizing mutations. It is significant that the most stabilizing mutations in GA identified to date all stabilize regions that are highly conserved, that the most stabilizing mutations in GA identified to date all stabilize regions that are highly conserved, that the cause for this observation remains unclear. However, given the position of the bond, it may serve to anchor the extended loop to the first α-helix, thereby preventing it from unfolding even at high temperatures.

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