Thermostable mutant variants of *Bacillus* sp. 406 α-amylase generated by site-directed mutagenesis

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**Abstract:** Several mutations are known to increase the thermostability of α-amylase of *B. licheniformis* and other α-amylases. Site-directed mutagenesis was used to introduce similar mutations into the sequence of the α-amylase gene from mesophilic *Bacillus* sp. 406. The influence of the mutations on thermostability of the enzyme was studied. It was shown that the Gly211Val and Asn192Phe substitutions increased the half-inactivation temperature ($T_m$) of the enzyme from $51.94\pm0.45$ to $55.51\pm0.59$ and $58.84\pm0.68^\circ C$ respectively, in comparison to the wild-type enzyme. The deletion of Arg178-Gly179 (dRG) resulted in an increase of $T_m$ of the α-amylase to $71.7\pm1.73^\circ C$. The stabilising effect of mutations was additive. When combined they increase the $T_m$ of the wild-type amylase by more than 26°C. Thermostability rates of the triple mutant are close to the values which are typical for industrial heat-stable α-amylases, and its ability to degrade starch at 75°C was considerably increased. The present research confirmed that the Gly211Val, Asn192Phe and dRG mutations could play a significant role in thermostabilization of both mesophilic and thermophilic α-amylases.

**Keywords:** Thermal stability • α-amylase • Bacillus • Site-directed mutagenesis • Protein engineering

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**Abbreviations:**
AB406 - *Bacillus* sp. 406 α-amylase;
BAA - *Bacillus amyloliquefaciens* α-amylase;
BLA - *Bacillus licheniformis* α-amylase;
BSIA - *Bacillus steaerothermophilus* α-amylase;
DNS - 3,5-dinitrosalicylic acid;
dRG - the Arg178-Gly179 residues deletion;
SD - standard deviation.

α-amylases (EC 3.2.1.1) are used in industrial processing of starch-containing materials. The majority of these reactions are conducted at high temperatures, resulting in high demand for thermostable α-amylases.

Using rational engineering methods, several scientific groups have identified amino acid substitutions that lead to enhanced thermostability of α-amylases. Considerable success in this area has been reached in thermostabilisation of α-amylase from *Bacillus licheniformis* (BLA) [2]. Declerck and co-workers combined seven stabilising mutations in BLA which resulted in a 23°C increase in the half-inactivation temperature. Another example of thermostability engineering is the deletion of a short destabilising loop structure in the B domain of *B. amyloliquefaciens* α-amylase (BAA) [3]. Arg176-Gly177 residues, which form a projecting structure on the enzyme molecule surface, were deleted and led to an increase in BAA stability. Analogous deletion also stabilised the structures of α-amylases from *Bacillus* sp. KSM-K38

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2.2 DNA isolation, manipulation and transformation

Preparation of plasmid DNA, subcloning of DNA fragments, digestion with restriction endonucleases, competent cell preparation and transformation were performed following Sambrook et al. [7].

2.3 Sequence alignment and computer modelling of the enzyme tertiary structure

Multiple sequence alignment was performed with ClustalX 1.83 [8] and was formatted using the ESPript program [9]. GenBank accession numbers for BLA, BAA, BSTA and AB406 sequences are AAA22240.1, AAA22191.1, AAA22241.1 and JX429073 respectively. The protein structure homology-modelling server, SWISS-MODEL [10] (http://www.expasy.org/swissmod/), was used to generate the three-dimensional model of AB406. The Deep View Swiss-PDB Viewer software (http://www.expasy.org/spdbv) was used to visualize and analyse the structure of the model, comparing it to the X-ray crystallographic structures of BLA and BAA (PDB accession codes: 1BLI and 3BH4).

2.4 Site-directed mutagenesis

Preparation of pALTER-1 plasmid DNA was performed by GeneJET™ Plasmid Mini-prep Kit (Fermentas). Site-directed mutagenesis was conducted using the Altered Sites® II in vitro Mutagenesis System (Promega) according to the manufacturer’s manual.

Phosphorylated oligonucleotides 5’-CCGCATTTTTTAATTCCACAGGAAAAGCGTGGGACTG-3’ (for the Gly211Val substitution), 5’ - C C G A T G T T G T G A C A C G A A A T G - 3 ‘ (for the Gly211Val substitution) and 5’-GATCAAGTGAACTCGCAACTATAGA-3’ (for the Asn192Phe substitution) were used to generate corresponding mutations. A 2.8 kb DNA fragment containing the AB406 gene was inserted into plasmid pALTER-1 and used for introduction of the above-mentioned mutations. Plasmid pALTER-1-amy406 was subjected to denaturation and hybridization with mutagenesis oligonucleotides, and then treated with DNA-polymerase and T4 DNA-ligase. The ligation mixture was used to transform E. coli JM109 cells.

DNA sequencing of the mutated gene region was used to select plasmids containing changed variants of the AB406 gene. Sequencing was performed using fluorescent Cy5-primer 5’-CTTTCCAGGTCGCGGGACTG-3’ and Cycle Reader™ Auto DNA Sequencing Kit (Fermentas). Separation and detection of PCR products was...
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continued using an automated DNA sequencer ALFexpress II (Amersham Pharmacia Biotech) with proper manufacturer’s software. Finally, mutant variants of AB406, which encoded the appropriate deletion or substitutions were subcloned into the pUC18 vector for expression in E. coli XL1-blue cells and further studies.

2.5 Partial purification of enzymes
The plasmids containing mutated genes were transformed to E. coli XL1-blue. Expression and partial purification of the mutated enzymes was performed using the same procedure as the wild type.

For α-amylase purification E. coli XL1-Blue cells with appropriate plasmid were cultivated in LB medium with 1% soluble starch and 100 μg/ml ampicillin. Freshly transformed E. coli cells were incubated at 37°C for 16 hours. Partially purified preparations of amylase variants were extracted from the cell periplasm by osmotic shock procedure [11]. Periplasmic fractions were diluted with equal volumes of 0.1 M Na-phosphate buffer (pH 7.0).

2.6 Enzyme assay
Amylase activity assays were performed using the 3,5-dinitrosalicylic (DNS) acid method [12] with minor modifications. The reaction mixture contained 1% (w/v) soluble starch and 0.2 mM CaCl₂ in 50 mM Na-phosphate buffer (pH 7.0) and 0.025-0.055 units of enzyme in a final volume of 0.5 ml. The temperature of activity measurement was 50°C, as optimal temperature for activity performance of the wild-type enzyme in the presence of stabilizing starch. After incubation for 15 min 1.5 ml of DNS reagent was added. After boiling for 12 min and cooling the absorbance at 540 nm was measured. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute under the assay conditions. D-Glucose was used to construct a standard curve. Amylase activities represent the mean value of at least three independent determinations.

2.7 Determination of temperature profile
The temperature optimum of AB406 was determined by measuring the activity of the amylase at temperatures from 27 to 72°C. The values of relative activity are shown as percentages of the maximal activity, which is taken as 100%.

2.8 Determination of pH-profiles
The effect of pH on α-amylase activity was studied by enzyme activity assay at 50°C in reaction mixtures with different pH. The following buffer systems were used: 50 mM Na-acetate buffer, pH 4.0-5.0; 50 mM Na-phosphate buffer, pH 6.0-7.0; 50 mM Tris-HCl buffer, pH 7.0-8.0; 50 mM glycine–NaOH buffer, pH 9.0–10.0. The values of activity are shown as percentages of the maximal activity, which is taken as 100%.

2.9 Thermostability tests
For thermostability studies the thermoinactivation of the wild-type and mutant AB406 variants was performed by incubating 0.5-1.1 units ml⁻¹ of amylase in 0.2 mM CaCl₂ and 50 mM Na-phosphate buffer (pH 7.0) at high temperatures in a water-bath, cooling on ice for 15 min and measuring residual amylase activities.

Thermostability profiles were obtained by incubating the samples for 20 min at various temperatures and plotting the residual amylase activity versus incubation temperature. The transition midpoint temperature at which half of the enzyme activity is lost (Tₘ) was estimated from the thermostability profiles.

For thermostinactivation kinetics studies, the samples were incubated for various times at the temperature range 50-80°C, and natural logarithmic values of residual activity plotted against incubation time (T). The apparent first-order rate constant of thermal inactivation (kᵣ) was evaluated from the slope of the inactivation curves. The half-life time (t₁/₂) of the enzymes was calculated from kᵣ. The activation energy (Eₐ) for thermal inactivation was calculated from an Arrhenius plot (dependence natural logarithm of kᵣ versus 1/T) [13]. The Gibbs free energy change (ΔG°), the enthalpy of activation (ΔH°) and the entropy of activation (ΔS°) were determined from an Eyring plot (dependence natural logarithm of kᵣ/T versus kᵣ/T) [14].

2.10 Productivity test
To determine the productivity (amount of reducing groups formed as products of starch degradation), soluble starch was hydrolyzed by AB406 variants at 75°C for 5.5 h [15]. The reaction was initiated by adding enzyme (0.62 ml of periplasmic extract) to 15 ml of substrate solution, containing 6% (w/v) soluble starch and 0.2 mM CaCl₂ in 50 mM Na-acetate buffer, pH 5.5. Non-enzymatic degradation of starch was determined by adding 50 mM Na-phosphate buffer (pH 7.0) instead of enzyme. During the incubation, samples were withdrawn every 30 min and the concentration of reducing sugars in reaction solutions were assayed by DNS acid method.

2.11 Statistical evaluation
Statistical data were analyzed by Microsoft Office Excel 2003 software, using Student’s test and linear least squares regression fitting [16]. All experimental
data were results of at least three independent determinations.

3. Results and Discussion

The optimum temperature for activity of wild-type α-amylase from *Bacillus* sp. 406 was determined by assaying enzyme activity at different temperatures. AB406 has optimum activity at 60°C (Figure 1A). A study of the effect of pH on AB406 activity showed that the enzyme had maximum activity in the pH range 6.0 to 7.0 and retains more than 70% activity in the pH range 5.0 to 9.0.

Examining AB406 inactivation at 50°C in the presence of 0, 0.5, 0.75 and 1% soluble starch indicates that starch has a stabilizing effect on AB406 thermal stability (Figure 1B). 84.9±6.4% of enzyme activity was retained after 60 min incubation in the presence of 1% starch.

To improve the thermostable properties of α-amylase from *Bacillus* sp. 406 a search was performed to identify target sites for site-specific mutagenesis. The level of sequence homology with previously studied α-amylases from *Bacillus* is relatively low. Based on BLAST search results, the amino acid sequence of AB406 is most closely related to *B. cereus*, *B. thuringiensis* and *B. megaterium* α-amylases (78-80% identity, 85-86% similarity), while homology with BLA (73% identity, 84% similarity) is slightly lower.

Based on amino acid sequence alignment (Figure 2) and modelling of the three-dimensional structure of AB406, we identified residues Arg178-Gly179 that are analogous to residues Arg176-Gly177 in the BAA structure. These residues are known to destabilise the structure of BAA and other amylases [3-5], and are missing in the more thermostable BLA [3]. In addition, AB406 also contains residues Asn192 and Gly211. Altering of analogous amino acids in BLA (Asn190Phe and Ala209Val respectively) resulted in stabilization of the enzyme [2]. 3D structure modelling of AB406 revealed similar structural conditions around Asn192 and Gly211. Point substitutions Asn192Phe and Gly211Val and the deletion of residues in positions 178-179 were chosen for thermostabilisation of AB406 (Figure 2).

The three target sites were changed using site-directed mutagenesis. Sequencing of the AB406 gene in the mutant variants confirmed the sequence is identical to the parent gene with the exception of the mutations introduced.

Initially the influence of the dRG, Asn192Phe and Gly211Val mutations was studied on AB406 activity in the pH range 4.0–10.0. No statistically significant differences were found in the pH-profiles of the mutant variants dRG, Asn192Phe and Gly211Val compared to the original enzyme.

To calculate the transition midpoint temperature (Tₘ) or temperature of half-inactivation the stability of the enzymes was determined following 20 min treatment at 45-80°C (Figure 3). Tₘ of the Gly211Val mutant increased to 55.51±0.59°C, while Tₘ of wild type amylase was equal to 51.94±0.45°C. Tₘ of the Asn192Phe variant was raised to 58.84±0.68°C. The deletion of Arg178-Gly179 in AB406 increased the Tₘ to 71.7±1.73°C.

**Figure 1.** A - Profile of *Bacillus* sp. 406 α-amylase activity at various temperatures (mean of relative activity ± SD); B - Time course of *Bacillus* sp. 406 amylase inactivation (mean of residual activity ± SD) at 50°C in the presence of 0 (▲), 0.5 (▲), 0.75 (■) and 1 (○) % soluble starch.
Temperature inactivation kinetic studies were carried out in the temperature range 50–60°C for Asn192Phe and Gly211Val enzyme variants, and in the range 60–75°C for the dRG variant, due to the considerable stabilizing effect of the dRG deletion. The time-course obtained of residual activity decay versus the activity of untreated enzyme is presented as natural logarithms (Figure 4A–D), which allows an estimate of the kinetic constants of inactivation ($k_D$). High determination coefficient values ($R^2$) were obtained for all inactivation curves (Table 1). Thermodynamic parameters of inactivation at various temperatures were estimated from Arrhenius and Eyring plots. The calculated constants are presented in Table 1.

Compared to the wild-type enzyme, the variant with the Gly211Val substitution was found to have slightly increased thermostability. The activation energy of denaturation ($E_a$) of the mutant variant was raised from 136.7±4.7 kJ mol$^{-1}$ for the wild-type to 146.0±4.6 kJ mol$^{-1}$ under the conditions used for incubation. Half-life ($t_{1/2}$) at 50°C increased from 25.88±2.32 to 48.2±7.6 min.

Stabilization of BLA by the introduction of substitution Ala209Val was explained by improved hydrophobic core packing through filling of a small groove on the protein.
Figure 3. Temperature stability profiles of Bacillus sp. 406 amylase variants (mean of residual activity ± SD): (●) – Wild type, (○) – G211V, (▲) – N192F, (◊) – dRG, (■) – dRG-N192F, (Δ) – dRG-N192F-G211V.

Figure 4. Comparison of time course of Bacillus sp. 406 amylase variants thermoinactivation (mean of log(residual activity) ± SD) at 50 (●), 55 (◊), 60 (▲), 65 (○), 70 (■), 75 (Δ) and 80 (♦) °C: A - Wild type, B - G211V, C - N192F, D - dRG, E - dRG-N192F, F - dRG-N192F-G211V.

In the AB406 three-dimensional model, the analogous Gly211 residue in AB406 is also located on the side of a similar small cavity, formed by Asp206, Lys239, Pro241, Asp245, Lys215, Ala213 and Pro208 (Figure 6).

The Asn192Phe substitution also improved the thermal stability of AB406. The activation energy of denaturation \( (E_a) \) of the mutant variant increased to 161.2±5.1 kJ mol\(^{-1}\). Half-life \( (t_{1/2}) \) at 50°C increased from 25.88±2.32 to 99.3±11.7 min, compared with the wild-type enzyme.
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<th>R²</th>
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Table 1. Kinetic and thermodynamic parameters of Bacillus sp. 406 amylase variants (± SD).

* $\Delta G^\ddagger$ determined at 60°C for wild-type, G211V and N192F variants, and at 70°C for dRG, dRG-N192F and dRG-N192F-G211V variants.

The analogous amino acid substitution (Asn190Phe) in BLA resulted in the establishment of a triple stacking interaction between aromatic residues Phe190, Tyr193 and His235 and the removal of the amide group of asparagine [18]. In the model of the mutant AB406, the equivalent residues Phe192, Tyr195 and His237 are also located at the appropriate distances of 3.54 and 3.42 Å (Figure 7).

The dRG deletion led to a noticeable increase in stability of AB406. The activation energy of denaturation ($E_a$) of the dRG variant increased from 136.7±4.7 kJ mol⁻¹ for the wild-type to 177.1±4.3 kJ mol⁻¹ for the mutant. The half-life ($t_{1/2}$) at 60°C was raised from 5.62±0.39 to 172.9±14.8 min. Of all the structural changes introduced into AB406, the dRG deletion resulted in the highest improvement in thermostability.

The deletion of two amino acid residues in similar positions led to stabilization of other α-amylases. Stabilization of BStA and BAA was explained by decreased flexibility within the region located near one of the bound Ca²⁺ ions, which is involved in strengthening of the enzyme tertiary structure [5,19]. This shortened loop in BLA seems to be the important determinant of its high thermostability [3].

To explore the possible cumulative effect of the characterized mutations, the double mutant dRG-N192F...
Figure 5. Arrhenius plots of Bacillus sp. 406 amylase variants kD (mean of log(kD) ± SD): (●) – Wild type, (○) – G211V, (▲) – N192F, (∗) – dRG, (■) – dRG-N192F, (Δ) – dRG-N192F-G211V.

Figure 6. Amino acid residues surrounding the Gly211Val mutation site (Gly211Val in AB406 and Ala209Val in BLA). 3D structures of the amylases were superimposed by The Deep View Swiss-PDB Viewer software. Residues are named and numbered as in AB406 sequence. Residues of BLA, that differ from those in AB406, are given in brackets.

Figure 7. Amino acid residues surrounding the Asn192Phe mutation site (Asn192Phe in AB406 and Asn190Phe in BLA). 3D structures of the amylases were superimposed by The Deep View Swiss-PDB Viewer software. Residues are named and numbered as in AB406 sequence. Residues of BLA, that differ from those in AB406, are given in brackets.
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was obtained on basis of the mutant variant dRG after introducing the Asn192Phe substitution. The third stabilizing mutation, Gly211Val, was introduced into the dRG-N192F variant to generate the triple mutant. Kinetic and thermodynamic parameters of inactivation were determined for the double and triple mutant variants (Table 1) by analysis of inactivation curves (Figure 4E-F).

The effect of mutations Gly211Val, Asn192Phe and dRG is additive. The AB406 double mutant, in which mutations dRG and Asn192Phe were combined, had an activation energy of denaturation (E<sub>a</sub>) of 195.5±4.8 kJ mol<sup>-1</sup>, while the half-life (t<sub>1/2</sub>) of the double mutant was 65.9±5.8 min at 70°C. The activation energy of denaturation (E<sub>a</sub>) of the dRG-N192F-G211V variant, which includes all three stabilizing mutations, was 205.3±8.0 kJ mol<sup>-1</sup>. The half-life (t<sub>1/2</sub>) was 113.0±12.5 at 70°C and 989 min at 60°C as calculated from the Arrhenius plot. The T<sub>m</sub> was calculated as 78.5±2.4°C, compared with a T<sub>m</sub> for the wild-type of 51.9±0.4°C.

Introduction of the three mutations increased the thermostability of AB406 to the values appropriate for industrial thermostable α-amylases from *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* (Table 2) [20].

Furthermore, AB406 variants with increased thermal stability were able to efficiently form the products of starch cleavage at temperatures that caused inactivation of the original amylase. The most thermostable variants, dRG-N192F and dRG-N192F-G211V, produced increased amounts of reducing sugars compared with the wild-type enzyme (260 and 288% respectively), without significant loss in activity, after 4.5 hours reaction at 75°C (Figure 8). The single AB406 mutants generated reduced amounts of the products at 75°C, obviously due to rapid irreversible denaturation.

Interestingly, the stabilization effects of amino acid substitutions Asn192Phe and Gly211Val in AB406, which did not originally exhibit large thermostability values, were quantitatively comparable to the effects of analogous mutations introduced into the highly thermostable BLA (Asn190Phe and Ala209Val) [2]. For both enzymes, the first and second substitutions increase T<sub>m</sub> by 6°C and 3°C respectively (Table 2). We suspect that similar mutations could be used to stabilize other enzymes that contain a microenvironment structure analogous to Asn190 and Gly209 in BLA.

According to the thermodynamic parameters calculated (Table 2), higher stability of AB406 mutant variants corresponds to higher Gibbs free energy of activation (ΔG<sup>f</sup>), compared to the wild-type enzyme. The increase in ΔG<sup>f</sup> observed could be attributed either by increased activation enthalpy (ΔH<sup>f</sup>) or by reduced activation entropy (ΔS<sup>f</sup>) [21]. The present results suggest that the stabilization effects of the Asn192Phe, Gly211Val and dRG mutations are caused by an increase in the activation enthalpy of the mutant variants denaturation. Activation enthalpy of a protein reflects the total energy of interactions in the folded protein structure, which combines ionic, electrostatic interactions, hydrogen bonds and van der Waals forces [22]. Stabilization of AB406 with the Asn192Phe, Gly211Val substitutions and the dRG deletion most likely results from the establishment of additional intramolecular interactions. Comparison of inactivation thermodynamics of the original BLA and the variant with the Ala209Val mutation showed that this substitution was also accompanied by an increase in both ΔH<sup>f</sup> and ΔS<sup>f</sup> [17]. Unfortunately, there was no data regarding changes of BLA and BAA thermodynamic parameters following introduction of the Asn190Phe and dRG mutations respectively. However, the tertiary structure changes identified in the A209V and N192F mutant variants of BLA [18], as well as the stabilization effect

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<td><em>B. sp.</em> 406</td>
<td>136.7</td>
<td>52</td>
</tr>
<tr>
<td><em>B. sp.</em> 406 (dRG-N192F-G211V variant)</td>
<td>205.3</td>
<td>78.5</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>262.0</td>
<td>86</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em> (BAA)</td>
<td>255.4</td>
<td>86</td>
</tr>
<tr>
<td><em>B. licheniformis</em> (BLA)</td>
<td>363.7</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 2. E<sub>a</sub> and T<sub>m</sub> comparison of thermostable α-amylases.

![Figure 8](image-url) Time course of reducing sugar formation during starch hydrolysis by *Bacillus* sp. 406 amylase variants (mean of concentration ± SD): (●) – Wild type, (□) – G211V, (▲) – N192F, (○) – dRG, (■) – dRG-N192F, (Δ) – dRG-N192F-G211V.
observed following shortening of the loop between domains A and B in BSTA [5] and BAA [19], suggest the formation of additional intramolecular interactions. As mentioned above, the Asn192Phe and dRG mutations lead to a significant increase in thermal stability of AB406. If combined they increase Tm of the enzyme by more than 26°C. This example demonstrates that great stability change could be reached through slight structural changes. The present research confirmed the notable influence of mutations analogous to Asn192Phe and dRG on thermostability of both mesophilic and thermophilic amylases.

Application of site-directed mutagenesis allowed us to significantly improve the thermal stability of the α-amylase of Bacillus sp. strain 406. Following the introduction of three mutations in the amylase gene, thermostability rates of the enzyme are close to the values which are typical for industrial heat-stable α-amylases.

References

Thermostable mutant variants of *Bacillus* sp. 406 α-amylase generated by site-directed mutagenesis


