Thermostable mutant variants of \textit{Bacillus} sp. 406 \( \alpha \)-amylase generated by site-directed mutagenesis

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\textbf{Abstract:} Several mutations are known to increase the thermostability of \( \alpha \)-amylase of \textit{B. licheniformis} and other \( \alpha \)-amylases. Site-directed mutagenesis was used to introduce similar mutations into the sequence of the \( \alpha \)-amylase gene from mesophilic \textit{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\( \pm \)0.45 to 55.51\( \pm \)0.59 and 58.84\( \pm \)0.68\( ^\circ \)C respectively, in comparison to the wild-type enzyme. The deletion of Arg178-Gly179 (dRG) resulted in an increase of \( T_m \) of the \( \alpha \)-amylase to 71.7\( \pm \)1.73\( ^\circ \)C. The stabilising effect of mutations was additive. When combined they increase the \( T_m \) of the wild-type amylase by more than 26\( ^\circ \)C. Thermostability rates of the triple mutant are close to the values which are typical for industrial heat-stable \( \alpha \)-amylases, and its ability to degrade starch at 75\( ^\circ \)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 \( \alpha \)-amylases.

\textbf{Keywords:} Thermal stability • \( \alpha \)-amylase • Bacillus • Site-directed mutagenesis • Protein engineering

\( \alpha \)-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 \( \alpha \)-amylases. Using rational engineering methods, several scientific groups have identified amino acid substitutions that lead to enhanced thermostability of \( \alpha \)-amylases. Considerable success in this area has been reached in thermostabilisation of \( \alpha \)-amylase from \textit{Bacillus licheniformis} (BLA) \cite{2}. Declerck and co-workers combined seven stabilising mutations in BLA which resulted in a 23\( ^\circ \)C increase in the half-inactivation temperature. Another example of thermostability engineering is the deletion of a short destabilising loop structure in the \( \beta \)-domain of \textit{B. amyloliquefaciens} (BAA) \cite{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 \( \alpha \)-amylases from \textit{Bacillus} sp. KSM-K38.

\textbf{1. Introduction}

Nowadays, thermostable enzymes are used in various biotechnological processes \cite{1}. Increased stability of enzymes to extreme temperatures allows the amount of enzyme in the reaction to be reduced. Increasing the reaction temperature improves the reaction yield and reduces the time taken for product accumulation.
α-amylase from Bacillus sp. 406 (AB406) is a mesophilic amyloytic enzyme, which is synthesized by soil Bacillus isolate. AB406 is significantly less thermostable than closely related therophilic α-amylases from B. licheniformis, B. amylyoliquefaciens, and B. stearothermophilus. Despite the relatively low level of sequence identity between the given thermostable amylases and AB406 (66-73%), the structure of AB406 contains three sites similar to sites that affected the thermal stability of BLA and BAA. Two of these are the amino acid residues Asn192 and Gly211. When analogous amino acids in BLA were replaced (substitutions Asn190Phe and Ala209Val, respectively), an increase in thermostability was observed [2]. Another potential site of interest is amino acids Arg178-Gly179. Presence of the analogous mutations in mutant variants that combine the above-mentioned was studied. We also obtained and characterised AB406 mutant variants of 178Arg-179Gly (dRG) on thermostability of the enzyme substitutions Asn192Phe and Gly211Val and the deletion of residues 178-179 were selected as target sites for AB406 mutagenesis to study the influence of these mutations (identified in BLA and BAA) on thermostability of the mesophilic α-amylase. In the present work we report construction of five mutant variants of AB406. Effect of amino acid substitutions Asn192Phe and Gly211Val and the deletion of residues 178-179 were selected as target sites for AB406 mutagenesis to study the influence of these mutations (identified in BLA and BAA) on thermostability of the mesophilic α-amylase.

2. Experimental Procedures

2.1 Bacterial strains, plasmids and culture conditions

*E. coli* XL1-Blue (F::Tn10(Tet)), *proA+B*, *lacI*, Δ(lacZ) M15/recA1, endA1, gyrA96, *thi*-1, *hsdR*17 (κ- κκ-), *glvV*44, *relA*1, *lac* cells were used for cloning and expression of amylase genes. Recombinant plasmid pUC18-amy6-28 contained AB406 gene in 2.8 kb fragment of *Bacillus* sp. 406 chromosome, which was inserted into HindIII restriction site of pUC18. Bacterial strains *E. coli* ES1301mutS (lacZ53, mutS201::Tn5, thyA36, rha-5, metB1, deoC, IN(mD-rM))E. coli JM109 (endA1, *glvV*44, thi-1, *relA*1, *gyrA*96(Nal'), recA1, *mcrB*+, Δ(lac-pro*AB*), e14. [F', traD36, *proAB*+, *lacI*, lacZΔM15], *hsdR*17 (κ- κκ-)), and plasmid pALTER-1 were received as a part of the Altered Sites® II in *vitro* Mutagenesis System (Promega) and used for site-directed mutagenesis. Bacterial cells were grown in LB broth.

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 [9] 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 plALTER-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’-CCGCATTTTTAATTCCAGGAAAAGCGTGGGACTG-3’ (for the dRG deletion), 5’- CGCGCGGCTTGTGTTGTTGAGCACTAAGGGAGCCGATTCCCC-3’ (for the Gly211Val substitution) and 5’-GTATCAAGTGAATTCGGCAACTATGA-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* ES1301mutS. Segregation of native and mutant variants of the gene was conducted in *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’-CTTTCAGGCGGACTAC-3’ and Cycle Reader™ Auto DNA Sequencing Kit (Fermentas). Separation and detection of PCR products was