Commentary Article

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Computational Approach to Study Role of Active Site Amino Acids of Alpha-Amylases in Thermostability of Pyrococcus Furiosus and Bacillus sp.

Tayyaba Huma[1*], Ghulam Mustafa[2], Ayesha sethi[3], Arooma Maryam[4], Muhammad Ali[5]

[1]Department of Bioinformatics and Biotechnology, Government College University, Pakistan

[*]**Corresponding author:** Tatyana Huma, Department of Bioinformatics and Biotechnology, Government College University, Pakistan, Tel: 03006606933; Email: tayyabashahbaz@gmail.com

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Abstract

Alpha amylase has a wide range of applications in starch processing, textile industry and its role as a detergent is also well defined. In this study rational approach of protein engineering to analyse the nature of the active site of thermostable alpha amylases via in silico computational tools was excercised. Sequential and structural analysis of alpha amylases from Pyrococcus furiosus (100ºC-105ºC), Bacillus licheniformis (90-95ºC) and Bacillus amyloliquefaciens (72ºC) was carried out. Sequential analysis of hyperthermophilic and thermophilic alpha amylases of studied organisms revealed high AT content in nucleotide sequences. Ratio of charged residues such as Asp, Glu, Arg, His and Lys were found higher as compared to neutral amino residues i.e. Asn, Ser and Thr in hyperthermostable Pyrococcus furiosus and Bacillus licheniformis alpha amylases which stay active at optimum temperature of >90ºC. Role of Asp, Glu, Asp, and Tyr was also observed in activity of alpha amylases, retrieved from different organisms. Tyrosine residue was found as a conserved residue in the active sites of alpha amylases especially of Bacillus alpha amylases. Phenylalanine at position 109 and isoleucine at position 428 were observed only in hyperthermostable Pyrococcus furiosus alpha amylase. While all others were found to have Tyr and Val at position 109 and 428, respectively which could be a reason behind the thermostability of Pyrococcus furiosus alpha amylase. In addition substantial amount of aromatic residues were observed in the active site of hyperthermophilic Pyrococcus furiosus alpha amylase. A large number of aromatic residues in the active sites of these alpha amylases and their interactions are expected to be involved in the overall stability of the enzymes.

Keywords: Alpha-amylase; Thermostability, Pyrococcus furiosus; Bacillus sp; Molecular Docking

Introduction

Alpha amylase is an endo-acting enzyme that can hydrolyse and/or transglycosylase α-(1,4) and/or form α-(1,6) glycosidic bonds of starch and glycogen retaining its anomeric configuration [1-2]. In the current enzyme market alpha amylase has proved itself as a potent candidate in regard to industrial significance. This enzyme has been the prop of starch and its derivative industries influencing fields of textile, paper making, bakery, food, ethanol production, fermentation, detergents and brewing/distilling industry [3]. Alpha-amylase has also been targeted for the design of therapeutic agents against type II diabetes, obesity, hyperlipidemia and caries [4]. Amylases are also involved in anti-inflammatory reactions, including hyperamylasemia and hyperamylasuria of varying degrees that are frequently observed in patients with lung and ovarian cancers [5]. Therefore, understanding the knowledge of structure-function relationship of a protein changes with respect to changing environmental conditions is fundamentally important for both theoretical and applicative aspects.

Alpha amylases from kingdom Archea and prokaryotes with remarkable thermo stability are of special concern in starch processing and textile industry. Hyperthermophile Pyrococcus furiosus α-amylase is a member of kingdom Archea that remains active at a temperature ranging from 60-145ºC [6]. Commercially important thermostable α-amylases belonging to kingdom prokaryotes include Bacillus licheniforms α-amylase (BLA) and Bacillus amyloliquefaciens α-amylase (BAA) [7-8]. Specific applications of α-amylases including saccharification of starch, baking, detergents for dish washing and bioethanol production have attracted the researchers for its manufacturing on industrial scale [9]. Therefore, it is highly desirable to design α-amylases that remain sufficiently stable at high operating temperature (above 90°C) to provide considerable benefits in concerned industry (e.g. starch processing and textile industry) [10].

For the designing of a proposed thermostable α-amylase, it is of utmost importance to understand 3D structures of α-amylases which consist of three domains namely Domain A, B and C. Domain A is composed of eight parallel β-strands that are surrounded by eight helices. Unique arrangement of these eight parallel β-strands and α-helices form a (α/β)8 TIM barrel fold in 3D structure [11]. Domain B is important for enzyme specificity and substrate binding. Domain C is also composed of β-sheets and act as an independent domain. It stabilizes the catalytic domain by protecting the hydrophobic residues of catalytic domain from solvent and also plays role in substrate binding. Alpha amylases are also supplemented with additional domains i.e. domain D, E and N found in some of α-amylases [12].

Domain E is reported to be important for binding granular starch molecule and also plays a role towards activity of enzymes. While domain N also acts as a part of active site and confers substrate specificity to α-amylases [10]. Active site of α-amylase is present at the interface of Domain A and Domain B. It is composed of several sub-sites that interact with the substrate via side chains of amino acids. Four short conserved sequence regions cluster at Domain A and play significant role in structure conformation of particular enzyme. These regions were found to have seven fully conserved residues in their amino acid sequence while their positions in the sequence may vary among different α-amylases [10,13]. Focusing on the thermostability, it was hypothesized that in silico analysis of structural similarity and catalytic mechanism of thermostable α-amylase family may pave the way to find out the reasons of their thermostablility. Therefore, a rational approach via in silico computational tools was employed in the present study to analyse the active site and nature of thermostable α-amylases.

Current study using α-amylase provides insights into the structural and physical stabilities of this industrially vital enzyme with evidence for intermediate states in its multiple unfolding pathways that exist in a divergence from classical two-state mechanism. The 3D structure analysis of α-amylases from diverse organisms can be studied by the generation of phylogenetic trees [14]. Greater similarities are observed between reference NCBI tree and structure based phylogenetic tree compared to sequence based phylogenetic tree indicating that structures truly represent the functional aspects of proteins than from the sequence information alone.

Materials and Methods

To find out the structural determinants of activity and thermo stability in α-amylases Pyrococcus furiosus and two Bacillus species were selected and their amino acid sequences were retrieved from GenPept database.

Sequence Alignment

Protein sequences of α-amylases from P. furiosus (100-105ºC), B. licheniforms (90-95ºC) and B. amyloliquefaciens (72ºC) were retrieved from GenPept. While carrying out sequence alignment, P. furiosus α-amylase with highest optimum temperature of 105ºC was selected as first member and taken as reference sequence for performing alignment. Multiple sequence alignment was performed using Geneious v7.0.6 [15]. Along with the multiple sequence alignment, consensus sequence and information graph were also shown to define the conserved regions among the sequences of above mentioned α-amylases. From these regions, highly conserved residues and invariant residues were elucidated.

Docking Studies

In order to find out residues involved in the activity and thermostability, structure files of P. furiosus, B. licheniforms and B. amyloliquefaciens α-amylases were retrived from Protein Data Bank in PDB format. Literature related to α-amylases was reviewed and one putative inhibitor and six substrates were choosen for the study. Acarbose was the only inhibitor while amylose, amylopectin, maltose, maltotriose, maltotetrose and maltopentose were selected as substrates for docking analysis. 3D structures and selected ligands were then subjected to docking studies. Molecular Operating Environment (MOE v2009.10) software was used to study binding sites and affinities of different ligands with selected α-amylases.

3D protonation of enzymes was carried out using the MOE software. Protein structure was minimized using AMBER99 Forcefield. 3D structures of selected substrates were optimized by adding hydrogen atoms and removing water molecules. AMBER99 Forcefield was also used for energy minimization of substrates. After protein docking, the resultant complexes were subjected to hydrogen bonding analysis. LigX feature of MOE was used to find interactions among ligand and receptor proteins.

Results

Primary Structure Analysis

Nucleotide and protein sequences of α-amylases from Bacillus species and Archea were saved in different text files and multiple sequence alignments were carried out. Analysis of multiple sequence alignment was completed by finding conserved regions using Geneious software. Sequence alignment snapshots and findings of conserved regions of Bacillus sp. and archea are given below.

Analyses of primary structures of α-amylases from Archea and Prokaryotes

For multiple sequence alignment, text files containing nucleotide and protein sequences of α-amylases from P. furiosus, B. licheniformis, and B. amyloliquefaciens were prepared. Thus in the multiple sequence alignment analyses of α-amylases from archea and prokaryotes, P. furiosus α-amylase was choosen as a reference sequence for comparative study.

According to the results (Table 1)

Table 1: Nucleotide based conserved regions in MSA of Arhea: Prokaryotes α-amylases. Archea i.e. P. furiosus; Prokaryotes i.e. B. licheniformis and B. amyloliquefaciens.

Regions	Position	Sequence	Segment Length
Region1	1514 to 1516	TGA	3
Region2	1790 to 1792	TGG	3
Region3	1973 to 1975	TGG	3

only three nucleotide conserved regions are present among α-amylases from P. furiosus, B. licheniformis and B. amyloliquefaciens. Various identical amino acids among P. furiosus, B. licheniformis and B. amyloliquefaciens at different positions were also observed. This is due to the presence of five cysyteine residues in P. furiosus α-amylase unlike B. licheniformis α-amylase that contains no cysteine residue.

Primary structure analysis of alpha amylases from bacillus species

Two files having nucleotide and protein sequences of α-amylase gene of two Bacillus species i.e. B. licheniformis and B. amyloliquefaciens were saved in FASTA format. During multiple sequence alignment of Bacillus species files, primary structure of B. licheniformis α-amylase was taken as a reference sequence for sequence comparison as it has the highest optimum temperature (90-95ºC) than other α-amylases of Bacillus species.

Multiple sequence alignment, information graph along with conserved regions analysis of these files were performed and results are shown in figure 1 and figure 2.

Figure 1: (a) Multiple sequence alignment of nucleotide sequences of α-amylases from Archea and Prokaryotes. Archea i.e. P. furiosus (Accession no. U96622.1); Prokaryotes i.e. B. licheniformis (Accession no. M38570.1), Geobacillus stearothermophilus (Accession no. X02769.1), B. amyloliquefaciens (Accession no. J01542.1), B. subtilis (Accession no. K00563.1) and B. velezensis (Accession no. CP020805.1).

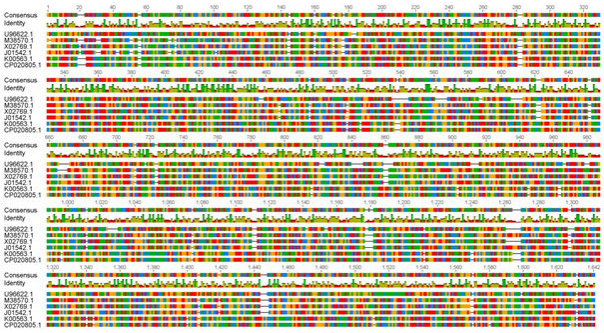


Figure 1: (b) Multiple sequence alignment of protein sequences of α-amylases from

Archea and Prokaryotes. Archea i.e. P. furiosus (Accession no. O08452); Prokaryotes i.e. B. licheniformis (Accession no. P06278), G. stearothermophilus (Accession no. P06279), B. amyloliquefaciens (Accession no. AAA22191.1), B. subtilis (Accession no. AAA22234.1) and B. velezensis (Accession no. ARJ73763.1).

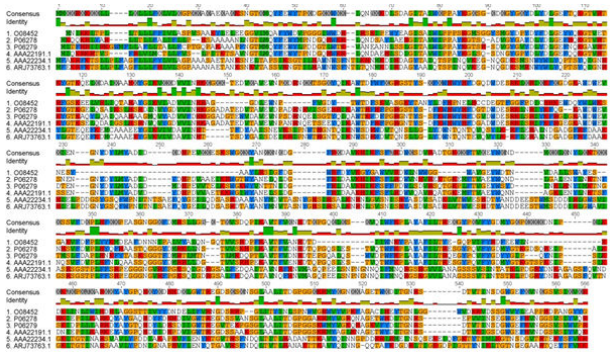
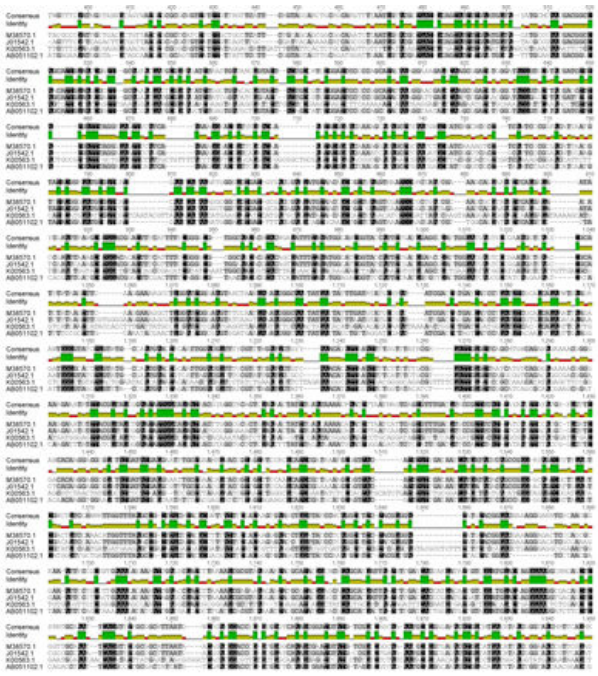


Figure 2: Multiple sequence alignment and information scan of nucleotide sequences of Bacillus specie α-amylases.gb file. (Bacillus species i.e. B. licheniformis, B. amyloliquefaciens and B. subtilis).



Thirteen conserved regions of nucleotides were predicted among the α-amylases of Bacillus species (Table 2)

Table 2: Nucleotide based conserved regions in MSA of Bacillus species α-amylases (Bacillus species i.e. B. licheniformis and B. amyloliquefaciens).

Conserved Regions	Position	Sequence	Segment Length
Region1	479 to 482	TGAT	4
Region2	633 to 635	ACG	3
Region3	660 to 662	ATT	3
Region4	865 to 867	TCC	3
Region5	924 to 926	TGG	3
Region6	1174 to 1176	GGG	3
Region7	1242 to 1244	ATT	3
Region8	1268 to 1271	GATT	4
Region9	1327 to 1330	TTGG	4
Region10	1519 to 1521	TGT	3
Region11	1707 to 1709	AAA	3
Region12	1806 to 1809	GGAA	4
Region13	1834 to 1836	CAG	3

while no conserved region of amino acids was seen among other members under study i.e. B. licheniformis and B. amyloliquefaciens.

Structures and activity relationship of alpha amylases

For docking studies 3D structures of α-amylases from three species were selected and docked with seven different ligands. Three dimensional structures of α-amylases from P. furiosus, B. licheniformis (BLA) and B. amyloliquefaciens (BAA) were

retrieved from PDB. Seven different ligands used in this study were amylose, amylopectin, maltose, maltotriose, maltotetrose, maltopentose and acarbose (inhibitor molecule). Among all these substrates amylose is a linear polymer of glucose units linked via α-(1, 4) glycosidic bonds and amylopectin is composed of short α-(1, 4) linked linear chains of 10–60 glucose units and α-(1, 6) linked side chains with 15-45 glucose units while maltose is a disaccharide formed from two units of glucose joined through α-(1, 4) glycosidic bonds.

Pyrococcus furiosus α-amylase

Active site of P. furiosus α-amylase is composed of twenty amino acid residues (Table 3).

Table 3: Active site residues of P. furiosus, B. licheniformis and B. amyloliquefaciens.

	Pyrococcus furiosus	Bacillus licheniformis	Bacillus amyloliquefaciens
Active site residues	Asp35, Pro60, Glu61, Gly62, Val63, Trp64, His65, Arg155, Glu156, Phe158, Tyr160, Asp441, Arg443, Arg457, Arg464, Ile471, Glu475, Leu482, Leu486	Trp13, Asp53, Trp56, His105, Leu196, Met197, Tyr198, Asp231, Asp232, His235, Glu261, Trp263, His327, Asp328, Ser334, Leu335	Tyr12, Tyr194, Leu197, Met198, Arg230, Asp232, Ala233, Lys235, His236, Glu262, His328, Asp329, Ser335, Leu336

Ratio of charged residues (Asp, Glu, Arg, His and Lys) is higher in the active site of P. furiosus α-amylase as compared to hydrophobic amino acid residues (Phe, Ile, Leu). Along with the involvement of residues from helices and beta sheets of catalytic domain, role of third domain was also observed. It is proposed that the active site of highly thermostable P. furiosus α-amylase is composed of residues from Domain A and Domain N. It is reported that Domain N is present in some members of α-amylase family. According to the results, maltotetrose and acarbose have shown interactions with all the essential catalytic residues. Maltotetrose, an oligosaccharide binds with Glu156 which acts as an acid catalyst during reaction. Asp35 is an acidic amino acid and acts as nucleophile during the enzymatic breakdown of sugar residues. Asp155 is involved in stabilization of enzyme-substrate complex and supports the hydrolysis of maltotetrose by making hydrogen bond with the sugar substrate. Beside these three invariant catalytic residues Arg443, Arg88, Val63, Tyr486 and Glu61 were also bound to the substrate.

Acarbose is a potent α-amylase inhibitor and bound by Glu475, Asp35, Asp155, Tyr486, Arg464, Arg443 and Val63. All these residues are the key residues required for the degradation of substrate. Amino residues that are closer to the ligand molecule are Trp12, Leu197, Met198, Ser335, Leu336 and Trp264. Well docked structure of acarbose with P. furiosus α-amylase showed that it is a potential inhibitor of P. furiosus α-amylase as it occupies enzyme active site by interacting with its maximum catalytic residues.

Furthermore, Arg443 and Tyr486 residues were observed to have stacking interactions of variable bond strengths with maltotetrose and acarbose. Networks of these stacking interactions were only observed for docking structures of P. furiosus α-amylase. It was found that interaction of hydrophobic Val63 and presence of Phe158 and Pro60 in close vicinity with the catalytic residue contributes to the preference of P. furiosus α-amylase for transglycosylation.

In the docking results of P. furiosus α-amylase as shown in figure 3 and figure 4 with amylose, maltose, maltotriose and maltopentose; maximum residues from active site of P.

Figure3: Docking complex of P. furiosus alpha amylase with (A) Acarbose (B) Amylopectin (C) Amylose (D) Maltose (E) Maltopentose (F) Maltotetrose (G) Maltotriose. The figure is showing binding modes of ligand molecules within the binding pocket of alpha amylase receptor protein.

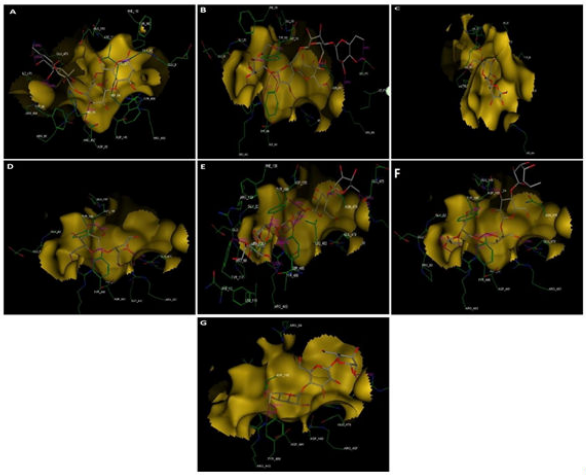
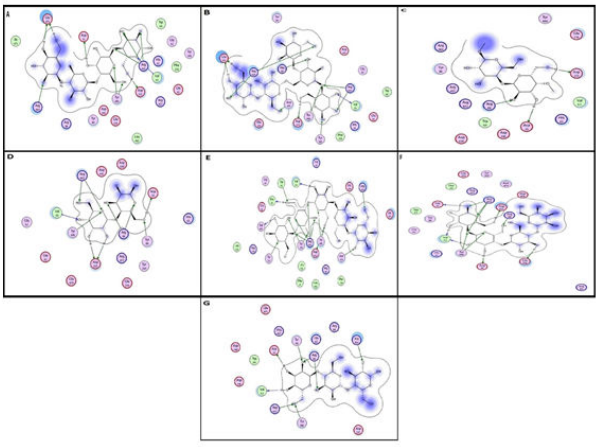


Figure 4: LigX interaction diagram of active site residues of P. furiosus α-amylase with (A) Acarbose (B) Amylopectin (C) Amylose (D) Maltose (E) Maltopentose (F) Maltotetrose (G) Maltotriose. Pink color is showing polar interactions while green, red and blue colors are showing hydrophobic, exposed and mild polar bondings respectively of ligands with binding pocket of alpha amylase.



furiosus α-amylase are interacting with these substrates but bonding with potential and invariant glutamic residue is missing although Glu479, Glu61 and Glu156 are lying very close to the exposed surface of ligands. So, amylose, maltose, maltotriose

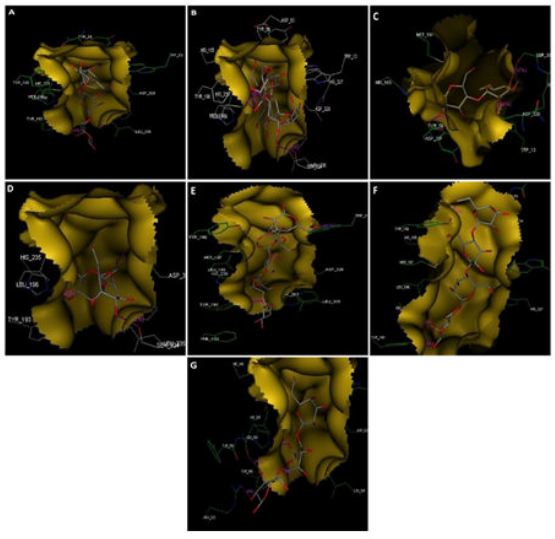
and maltopentose are rendered as weak substrates for P. furiosus α-amylase.

Bacillus licheniformis α-amylase

Sequence analysis of BLA showed that nucleotide sequence of BLA is composed of 1948 bp while protein sequence is composed of 512 amino acids. Like BAA, 3D structure of BLA is also composed of 3 domains i.e. Domain A, B and C. Docking study Of BLA with six different carbohydrate related substrates showed that active site of BLA is composed of sixteen amino acid residues (Table 3). Except Trp13 and Trp56 all the active site residues are same in BAA and BLA only their positions are varying. Majority of these active site residues are located on β-turns except Glu261 which is present at fifth β-strand of domain A and acting as catalytic residue. BLA involves His105 residue from domain B as well.

Asp231 acts as acidic chain acceptor of proton and shares its electron pair and interacts with amylopectin through covalent bonding. Besides Asp231, Asp328 also assists in catalytic mechanism of hydrolysis by acting as acidic side chain acceptor residue and makes hydrogen bond with the substrate. Tyr198 in BLA structure again interacts with the substrate as in the BAA-substrate complex, only its position varies here (Figure 5)

Figure 5: Docking complex of B. licheniformis α-amylase with (A) Acarbose (B) Amylopectin (C) Amylose (D) Maltose (E) Maltopentose (F) Maltotetrose (G) Maltotriose. The figure is showing binding modes of ligand molecules within the binding pocket of alpha amylase receptor protein.



which indicates its major role in catalysis of substrate by BLA. Maltotriose interacts with all the critical residues required for binding and distortion of substrate as shown in fig 5 but lacks interaction with catalytic Glu261 so it is unable to degrade maltotriose. Likewise in maltose and BLA complex, bonding with major catalytic residue Asp231 (nucleophile) and Asp328 were found missing.

Other maltooligosaccharides i.e. maltotetrose and maltopentose are also poor substrates of BLA. Both the substrates do not have bonds between catalytic residues (Asp328 and Glu261) and glucose subunits.

Docking results of amylose indicated least interactions with the active site of BLA which showed that amylopectin is the only potential substrate of BLA as compared to the amylose. No significant interactions were observed between the active site residues of BLA and acarbose inhibitor. So in terms of potential substrate, amylopectin is the only substrate which is being catalysed by B. licheniformis α-amylase as shown in figure 5 and figure 6.

Figure 6: LigX interaction diagram of active site residues of B. licheniformis α- amylase with (A) Acarbose (B) Amylopectin (C) Amylose (D) Maltose (E) Maltopentose (F) Maltotetrose (G) Maltotriose. Pink color is showing polar interactions while green, red and blue colors are showing hydrophobic, exposed and mild polar bondings respectively of ligands with binding pocket of alpha amylase.

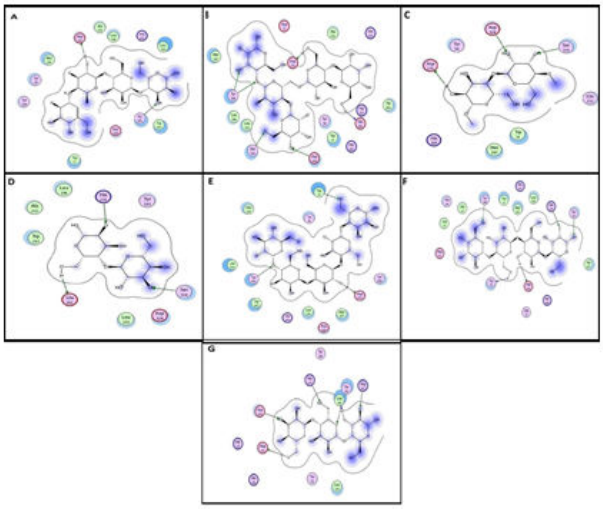
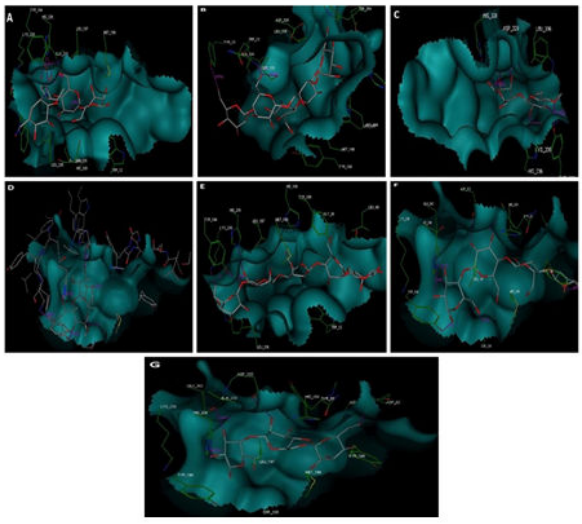


Figure7: Docking complex of B. amyloliquefaciens α-amylase with (A) Acarbose (B) Amylopectin (C) Amylose (D) Maltose (E) Maltopentose (F) Maltotetrose (G) Maltotriose. The figure is showing binding modes of ligand molecules within the binding pocket of alpha amylase receptor protein.

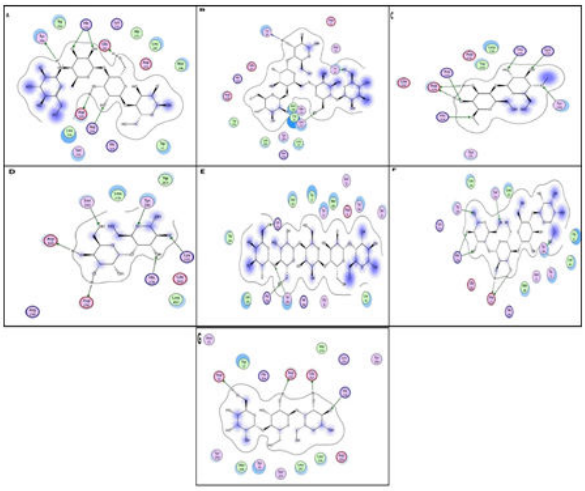


Bacillus amyloliquefaciens α-amylase

Nucleotide and protein sequences of BAA are composed of 2084 bp and 659 aa respectively. BAA has three distinct domains (Doman A, B and C) in its 3D structure. Active site of BAA is composed of fourteen amino acid residues (Table 3). Two dimensional and three dimensional structural analyses showed that Glu262 is located at 3rd α-helix while Lys235 and His236 are located at 5th α-helix of domain A. Aspartic acid at position 232 in protein sequence is found to be present at 6th β-strand of BAA structure. Rest of the active site residues were found to be located on β- and γ- turns of loops that connect the helices and β-strands of TIM barrel structure. Most of the charged residues are involved in the composition of active site of BAA structure while less number of hydrophobic residues were predicted.

Dotted lines around the substrate show proximity contour (Figure 8).

Figure8: LigX interaction diagram of active site residues of B. amyloliquefaciens α-amylase with (A) Acarbose (B) Amylopectin (C) Amylose (D) Maltose (E) Maltopentose (F) Maltotetrose (G) Maltotriose. Pink color is showing polar interactions while green, red and blue colors are showing hydrophobic, exposed and mild polar bondings respectively of ligands with binding pocket of alpha amylase.



Tyr194, Arg230, His236, Glu262 and Asp329 form hydrogen bonds of different strengths with acarbose molecule. Catalytic residues of BAA Glu262 and Asp329 are involved in the hydrolysis of α-(1, 4) glycosidic bonds present in the acarbose molecule at sub site-1. Glu262 is present at helix 3 of TIM barrel structure of BAA and acts as an acid catalyst during reaction and is one of the conserved catalytic residues of alpha amylases. Asp329 is an acidic amino acid and acts as a nucleophile during the enzymatic breakdown of sugar residues. Tyrosine at position 194 is a polar aromatic residue whose side chain carboxylic group is behaving as donor of hydroxyl ion to acarbose molecule and makes a hydrogen bond with the substrate at the expense of water molecule. Arg230 is a basic, positively charged amino acid which shares its hydrogen ion with hydroxyl group of acarbose to develop polar bond with the substrate. Asp232 is in close proximity with the acarbose but do not interacts directly with the acarbose. Amino residues that are close to the ligand molecule are Trp12, Leu197, Met198, Ser335, Leu336 and Trp264.

Well docked structure of acarbose with BAA shows that it has ability to inhibit BAA as it occupies enzyme active site by interacting with its maximum catalytic residues (Fig 7). Maltose is linked via α-(1, 4) glycosidic bonds and it shows interaction with Tyr194, Asp232, His236, Lys235, Asp329 and Ser335 residues. All of the residues are located on doman A. Three hydrophobic residues i.e. Leu336, Leu197 and Trp264 are located away from catalytic residues in the active site which indicates that B. amyloliquefaciens alpha amylase may carry out hydrolysis of maltose. Glu262 does not interacts directly with maltose but it lies very close to the substrate. Glu262 can be made to interact with maltose by introducing some kind of interaction and BAA can be exploited to carry out the hydrolysis of maltose. But here poorly docked complex of maltose and BAA shows that it is not a potent substrate of BAA due to the lack of interaction between catalytic Glu262 residue of BAA and maltose.

Amylose is reported to be the main substrate of the BAA. Amylose has shown strong interaction with maximum residues of active site i.e. Tyr194, Arg230, Asp232, His236, Lys234, and His328. Among them Lys235 and His236 are located in helix 3 of catalytic domain and Tyr194, Arg230 and His328 belong to different β-turns. These residues capture amylose and act as side chain acceptors of electron pairs and donate their hydrogen ions for building interactions of different bond strengths. Only Asp329 acts as a side chain donor by donating electron pairs and sharing its hydroxyl ions with the substrate. Arg230, His328 and Asp232 are important for amylose binding and distortion at sub site-1. While His236 is a calcium binding residue and it has important role in structure stabilization. Amylose has shown to be properly docked within the given active site but amylose lacks interaction with Glu262 so it is supposed that Glu262 which lies close to the nucleophile might interact with the substrate by employing other protein-protein interactions.

In BAA-maltotriose complex as shown in fig 7 Asp52, Glu262, Asp232 are catalytic residues and act as binding and distortion residues. His236, a metal binding residue also interacts with the substrate in active site pocket of BAA and plays its role in catalytic mechanism. On the basis of docking results as shown in fig 7 and fig 8 it is proposed that BAA is able to breakdown maltotriose. Presence of hydrophobic residues near catalytic residues in the active site may change the reaction specificity of BAA from hydrolysis to transglycosylation.

Other substrates which were used for docking studies of BAA include amylopectin, maltotetrose and maltopentose. Least interactions were observed in all these above mentioned substrates. In case of amylopectin only three residues i.e. Tyr13, Tyr55 and Ser355 were predicted to have 55%, 14% and 62% bond strengths with substrate, respectively. No other catalytic residue (Asp, Glu, Asp) was present at ligand exposed areas.

Results of maltotetrose have shown interaction of a single catalytic residue (Asp232) along with His236 (calcium binding residue) but lacks exposure to Glu262 (acid/base catalyst) and Asp329. While in the complex of maltopentose and BAA, only three residues showed interactions including Tyr194, Lys235 and His236 (calcium binding residue). Conserved acid/base catalyst i.e. Glu232 and Asp329 does not interact with maltotetrose,

amylopectin and maltopentose. That is why these substrates showed improper binding with the active site of BAA. Hence amylopectin, maltotetrose and maltopentose are rendered as poor substrates of α-amylase.

Discussion

The present study was based on sequential and structural analysis of α-amylases from hyperthermophilic and thermophilic species which show activities at different physical conditions. Thermostability of α-amylases are found to be influenced by various cations, stabilizers and various substrates [16]. Calcium ion alters the thermostability of α-amylase by altering its activity [17]. Alpha amylases are generally defined as a group of enzymes that can hydrolyse and/or transglycosylase α-(1, 4) and/or α-(1, 6) glycosidic bonds of starch related polysaccharides. It is predicted from various studies that α-amylase having desired kinetic properties can play a diverse role in different industries in terms of economy and feasibility [18]. Alpha amylase family accounts for 30% of the world's enzyme production. Initially α- amylases were categorized into different types on the basis of their physical characteristics i.e. temperature, pH and activities. Keeping in view the pH required for optimal activity α-amylases can be categorized as acidic, neutral or alkaline which ranges from 2 to 12. Most α-amylases are found more active in neutral range [19]. After categorization of α-amylase family members, α-amylase was selected due to its broad temperature range, enzyme stability and having broad range of industrial applications. Owing to its high demand in industrial processes, it is highly desirable to design α-amylases that remain sufficiently stable at high operating temperature of above 90ºC to provide direct benefits in starch processing and textile industry. In this study, rational approach of protein engineering was used to analyse the nature of the active site of thermostable α-amylases via in silico computational softwares. This study involves the primary structure (nucleotide and amino acid) and 3D structural analysis of α-amylase from P. furiosus [20], B. licheniformis [21] and B. amyloliquefaciens [22]. For understanding the activity of structural determinants and thermo stability, DNA and protein sequence analysis and docking studies were performed. Purpose of designing this study was to find out the potential substrates and residues involved in activity of α-amylase at their optimum temperature and pH.

Multiple sequence alignment of P. furiosus, B. licheniformis, and B. amyloliquefaciens including nucleotide and protein sequences were carried out where P. furiosus α-amylase was choosen as a reference sequence for comparative study. Alpha amylase of P. furiosus is the only enzyme having optimum activity at 100-105ºC while all other α-amylases under study have optimum activity below 100ºC [23-24]. Sequence alignment revealed that three nucleotide conserved regions are present among α-amylases from P. furiosus, B. licheniformis and B. amyloliquefaciens. Various identical amino acids among P. furiosus, B. licheniformis and B. amyloliquefaciens at different positions were also observed. Pyrococcus furiosus α-amylase has 43% nucleotide identity and 29% amino acid sequence identity with B. licheniformis α-amylase [25]. Less amino acid sequence resemblance between P. furiosus α-amylase and B. licheniformis α-amylase might be responsible for their difference in optimum temperature. According to a study by Savchenko et al. [20] calcium independent P. furiosus α-amylase is more thermotolerant enzyme as compared to bacterial homologue B. licheniformis α-amylase which is also indicated by low level of primary structure similarity. This is due to the presence of five cysytein residues in P. furiosus α-amylase unlike B. licheniformis α-amylase that contain no cysteine residue. Cys165 is involved in Zn+2 binidng and is essential for stability of P. furiosus α-amylase.

Pairwise sequence alignments of Bacillus species files revealed that B. licheniformis α- amylase has 64.5% nucleotide sequence identity with B. amyloliquefaciens α-amylase. While protein sequence of B. licheniformis α-amylase has shown 60.4% similarity with amino acid sequences of α-amylases belonging to B. amyloliquefaciens. The information available on protein helps in predicting properties of protein using computational approaches [26]. Current findings were supported by the finding of Chen et al. [27] indicating that B. licheniformis α-amylase and B. amyloliquefaciens α-amylase are more closely related to each other. Alpha-amylases isolated from thermococcus are optimally active at temperatures close to 80°C. Alpha amylase isolated from archaebacterium P. furiosus is a hyperthermophilic enzyme because pyrococcus enzymes are optimally active around 100°C [28]. Haki and Rakshit, [29] reported that the maximum activity of α-amylase was observed at an optimum temperature range of 95-100°C from P. furiosus. Calcium dependent P. furiosus has wide range of substrate specificity [25]. Sequence analysis showed that length of nucleotide and protein sequence of P. furiosus α-amylase is 1740 bp and 460 aa respectively while its 3D structure is composed of three domains i.e. domain A, B and N [30]. Unique organisation of active site in highly thermo stable P. furiosus α-amylase can be regarded as one of its distinguishing property as compared to other prokaryotic and eukaryotic α-amylases [30].

Amylopectin is being degraded by Asp 231, Glu261, Asp328, Tyr198 and Ser334. Glu261 in the docking complex which is acting as backbone acceptor that donates a proton for the cleavage of substrate [21]. Side chain of the tyrosine residue is also helpful in improving of stacking interactions of BLA and strengthen the network of aromatic–aromatic interactions that is probably important for maintaining the proper folding of domain A [31-32]. Catalytic residues i.e. Asp231, Glu261 and Asp238 from the active site of BLA were also reported by the Vihinen and Mantsala, [33]. It is reported that arginine in protein structure is responsible for the charge stabilization of protein molecule during substrate binding. His236 is part of domain A and due to its calcium binding residue property it acts as a stabilization agent of BAA structure [34]. Johansson et al. [35] reported that position 105 in human glutathione transferase P1-1 affect the thermal stability of the enzyme at 50˚C. Similary, Wang et al. [36] mutated some of the residues from the active site of Streptomyces xylanase with proline and glutamic acid which resulted in imporved thermostability of the enzyme. When functionally important residues in the active site of T4 lysozyme were mutated, the thermal stability of enzyme was foud to be increased from 0.7-1.7 kcal mol-1 [37]. Therefore, the

nature of amino acids of active sites (i.e. substrate binding sites) strongly affect the thermal stability of enzymes.

The meanings of "protein thermo stability" can be of two types according to the mechanistic point of view. First one is thermodynamic thermo stability (folded and unfolded equilibrium of protein) and the second is kinetic thermo stability (Activation time of a protein at elevated temperature before going to irreversible denaturation) [38].The mostly known factors responsible for protein thermo stability includes hydrogen bonding, ion pairing, loops shortening, salt bridge formation, high secondary structure contents and better hydrophobic packing [39]. All of these factors are thought to be responsible in increasing the structural rigidity of the folded state. In contradiction to all existing factors, the thermophiles organism's protein is considering more flexible as compared to the thermophiles microorganisms [40]. Moreover, the study of the protein stability and active site point, from all over the protein facilitates to comprehend the significance of certain residues in determined positions.

Conclusion

Presence of more charged residues in the active site of thermo tolerant α-amylases could be a reason for carrying out activity of the these enzymes at high temperatures. Charged residues are evident in the active sites of α-amylases from B. licheniformis and B. amyloliquefaciens as compared to less thermos table α-amylases. Among α-amylases of prokaryotes, it was observed that charged residues of BLA are specifically rich in histamine residues which are helpful in stability of the enzyme. Alpha amylases from hyperthermostable P. furiosus and B. licheniformis the involvement of residues from domains other than the catalytic domain was also seen. Substantial amount of aromatic residues were observed in the active site of BLA. These aromatic residues were expected to be involved in the overall stability by aromatic-aromatic residue interaction. In glycosyl hydrolases, side chains of aromatic serve as a platform for stacking interactions in sugar moieties of the substrate. From sequence alignment analysis it was deduced that Leu18, Ala77, Val78, Gln120, Asp242, Tyr250 were present only in BLA. Most of these residues are located with hydrophobic side chains. So hydrophobicity of these residues might have some role in making a protein thermos table.

References

1. M. S. Paula and O. M. Pérola, "Application of microbial α-amylase in industry", Brazilian Journal of Microbiology, vol. 41, pp. 850-861, 2010.
2. D. Raul, T. Biswas, S. Mukhopadhyay, S. Kumar Das and S. Gupta, "Production and partial purification of alpha amylase from Bacillus subtilis (MTCC 121) using solid state fermentation". Biochemistry Research International, 2014.
3. K. Singh and A. M. Kayastha, "Optimal immobilization of α-amylase from wheat (Triticum aestivum) onto DEAE-cellulose using response surface methodology and its characterization". Journal of Molecular Catalysis B: Enzymatic, vol. 104, pp. 75–81, 2014.
4. K. Singh, M. Shandilya, S. Kundu and A. M. Kayastha, "Heat, acid and chemically induced unfolding pathways, conformational stability and structure-function relationship in wheat α-amylase". PloS One, vol. 10, e0129203, 2015a.
5. S. Singh and L. Guruprasad, "Structure and sequence based analysis of alpha-amylase evolution". Protein and Peptide Letters, vol.. 21, pp. 948–956, 2014.
6. N. Reddy, A. Nimmagadda and K. S. Rao, "An overview of the microbial α-amylase family". Afr J Biotechnol, vol. 2, pp. 645-648, 2004.
7. P. V. Aiyer, "Amylases and their applications". Afr J Biotechnol, vol. 4, 2005.
8. M. Asgher, M. J. Asad, S. Rahman and R. A. Legge, "A thermostable α-amylase from a moderately thermophilic Bacillus subtilis strain for starch processing". Journal of Food Engineering, vol. 79, pp. 950-955, 2007.
9. I. Sani, A. Abdulhamid, F. Bello, M. Yahaya and A. I. Bagudo, "Isolation, partial purification and characterization of α-amylase from Bacillus subtilis", J Microbiol Biotechnol, vol. 4, pp. 49-54, 2017.
10. O. Prakash and N. Jaiswal, "α-Amylase: an ideal representative of thermostable enzymes". Applied Biochemistry and Biotechnology, vol. 160, pp. 2401-2414, 2010.
11. P. M. de-Souza, "Application of microbial α-amylase in industry-A review". Brazilian Journal of Microbiology, vol. 41, pp. 850-861, 2010.
12. K. Ohdan, T. Kuriki, H. Takata, H. Kaneko and S. Okada, "Introduction of Raw Starch-Binding Domains into Bacillus subtilis α-Amylase by Fusion with the Starch-Binding Domain of Bacillus Cyclomaltodextrin Glucanotransferase". Applied and Environmental Microbiology, vol. 66, pp. 3058-3064, 2000.
13. M. J. Van Der Maarel, B. Van Der Veen, J. Uitdehaag, H. Leemhuis and L. Dijkhuizen, "Properties and applications of starch-converting enzymes of the α-amylase family". J Biotechnol, vol. 94, 137-155, 2002.
14. T. Huma, A. Maryam and S. ur Rehman, "Phylogenetic and Comparative Sequence Analysis of Thermostable Alpha Amylases of kingdom Archea, Prokaryotes and Eukaryotes". Bioinformation, vol. 10, pp. 443-448, 2014.
15. M. Kearse, R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock et al. "Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data". Bioinformatics, vol. 28, pp. 1647-1649, 2012.
16. R. R. Zohra, S. A. Qader, S. Pervez and A. Aman, "Influence of different metals on the activation and inhibition of α-amylase from thermophilic Bacillus firmus KIBGE-IB28". Pak J Pharm Sci. vol. 29, pp. 1275-1278, 2016.
17. L. Li, J. Yang, J. Li, L. Long, Y. Xiao, X. Tian, F. Wang and S. Zhang, "Role of two amino acid residues' insertion on thermal stability of thermophilic α-amylase AMY121 from a deep sea bacterium Bacillus sp. SCSIO 15121". Bioprocess and Biosystems Engineering, vol. 38, pp. 871-879, 2015.
18. K. Singh, G. Srivastava, M. Talat, O. N. Srivastava and A. M. Kayastha, "α-Amylase immobilization onto functionalized graphene nanosheets as scaffolds: its characterization, kinetics and potential applications in starch based industries". Biochem Biophys Rep, vol. 3, pp. 18-25, 2015b.

19. F. Khodayari, Z. Cebeci and B. D. Ozcan, Optimization of xylanase and α-amylase production by alkaline and thermophilic Bacillus isolate KH-13". J Entomol Zool Stud, vol. 2, pp. 295-303, 2014.

20. A. Savchenko, C. Vieille, S. Kang and J. G. Zeikus, "Pyrococcus furiosus α-amylase is stabilized by calcium and zinc". Biochemistry, vol. 41, pp. 6193-6201, 2002.

21. M. H. Rivera, A. Lopez-Munguia, X. Soberon and G. Saab-Rincon. "α-Amylase from Bacillus licheniformis mutants near to the catalytic site: effects on hydrolytic and transglycosylation activity". Protein Engineering, vol. 16, pp. 505-514, 2003.

22. J. Alikhajeh, K. Khajeh, B. Ranjbar, H. Naderi-Manesh, Y. H. Lin, E. Liu et al., "Structure of Bacillus amyloliquefaciens-amylase at high resolution: implications for thermal stability". Acta Crystallographica Section F: Structural Biology and Crystallization Communications, vol. 66, pp. 121-129, 2010.

23. B. T. Abd-Elhalem, M. El-Sawy, R. F. Gamal and K. A. Abou-Taleb, "Production of amylases from Bacillus amyloliquefaciens under submerged fermentation using some agro-industrial by-products". Annals of Agricultural Sciences, vol. 60, pp. 193-202, 2015.

24. S. Afrisham, A. Badoei-Dalfard, A. Namaki-Shoushtari and Z. Karami, "Characterization of a thermostable, CaCl 2-activated and raw-starch hydrolyzing alpha-amylase from Bacillus licheniformis AT70: Production under solid state fermentation by utilizing agricultural wastes". Journal of Molecular Catalysis B: Enzymatic, vol. 132, pp. 98-106, 2016.

25. S. Jorgensen, C. E. Vorgias and G. Antranikian, "Cloning, Sequencing, Characterization, and Expression of an Extracellular α-Amylase from the Hyperthermophilic Archae on Pyrococcus furiosus in Escherichia coli and Bacillus subtilis". J Biol Chem. Vol. 272, pp. 16335-16342, 1997.

26. V. S. Rao, K. Srinivas, G. N. Sujini and G. N. Kumar, "Protein-protein interaction detection: methods and analysis". International Journal of Proteomics, 2014.

27. J. Chen, X. Chen, J. Dai, G. Xie, L. Yan, L. Lu and J. Chen, "Cloning, enhanced expression and characterization of an α-amylase gene from a wild strain in B. subtilis WB800". Int J Biol Macromol, vol. 80, pp. 200-207, 2015.

28. A. Upgade, A. Nandeshwar and L. Samant, "Assessment of fungal protease enzyme from French bean using A. niger by Solid State Fermentation", J Microbiol Biotechnol, vol. 1, pp. 45-51, 2011.

29. G. D. Haki and S. K. Rakshit, "Developments in industrially important thermostable enzymes: a review". Bioresource Technology, vol. 89, pp. 17-34, 2003.

30. J. T. Park, H. N. Song, T. Y. Jung, M. H. Lee, S. G. Park, E. J. Woo et al., "A novel domain arrangement in a monomeric cyclodextrin-hydrolyzing enzyme from the hyperthermophile Pyrococcus furiosus". Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, vol. 1834, pp. 380-386, 2013.

31. N. Declerck, M. Machius, P. Joyet, G. Wiegand, R. Huber and C. Gaillardin, "Hyperthermostabilization of Bacillus licheniformis α-amylase and modulation of its stability over a 50 C temperature range". Protein Engineering, vol. 16, pp. 287-293, 2003.

32. S. Janecek, B. Svensson and B. Henrissat, "Domain evolution in the α-amylase family". Journal of Molecular Evolution, vol. 45, pp. 322-331, 1997.

33. M. Vihinen and P. Mantsala, "Characterization of a thermostable Bacillus stearothermophilus alpha-amylase". Biotechnol Appl Biochem, vol. 12, pp. 427-435, 1990.

34. H. Hagihara, Y. Hayashi, K. Endo, K. Igarashi, T. Ozawa, S. Kawai et al., "Deduced amino-acid sequence of a calcium-free α-amylase from a strain of Bacillus". Eur J Biochem, vol. 268, pp. 3974-3982, 2001.

35. A..S. Johansson, G. Stenberg, M. Widersten and B. Mannervik. "Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105". J Mol Biol, vol. 278, pp. 687-698, 1998.

36. K. Wang, H. Luo, J. Tian, O. Turunen, H. Huang, P. Shi, et al. "Thermostability improvement of a Streptomyces xylanase by introducing proline and glutamic acid residues". Appl Environ Microbiol, vol. 80, pp. 2158-2165, 2014.

37. B.K. Shoichet, W.A. Baase, R. Kuroki and B.W. Matthews. "A relationship between protein stability and protein function". Proceedings of the National Academy of Sciences, vol. 92, pp. 452-456, 1995.

38. C. Ó'Fágáin. "Engineering protein stability. In: Walls D, Loughran ST, editors. Protein Chromatography: Springer; pp. 103–36, 2011.

39. M.M. Gromiha, M.C. Pathak, K. Saraboji, E.A. Ortlund and E.A. Gaucher. "Hydrophobic environment is a key factor for the stability of thermophilic proteins". Proteins, vol. 81, pp. 715–721, 2013.

40. C. Danciulescu, R. Ladenstein and L. Nilsson. "Dynamic arrangement of ion pairs and individual contributions to the thermal stability of the cofactor-binding domain of glutamate dehydrogenase from Thermotoga maritima. Biochemistry, vol. 46, pp. 8537–8549, 2007.