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α-Amylase: An Ideal Representative of Thermostable Enzymes

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Abstract The conditions prevailing in the industrial applications in which enzymes are used are rather extreme, especially with respect to temperature and pH. Therefore, there is a continuing demand to improve the stability of enzymes and to meet the requirements set by specific applications. In this respect, thermostable enzymes have been proposed to be industrially relevant. In this review, α-amylase, a well-established representative of thermostable enzymes, providing an attractive model for the investigation of the structural basis of thermostability of proteins, has been discussed.

Keywords α-Amylase · Thermostability · Structural flexibility · Rigidity · Unfolding state

Introduction

In general, the enzymes usually get denatured and lose their activities at temperatures over 50–60 °C. However, thermostable enzymes allow a higher operation temperature which is advantageous because of higher reactivity (higher reaction rate, lower diffusional restrictions), higher stability, higher process yield, lower viscosity, and fewer contamination problems [1]. Thermostable enzymes are therefore highly attractive and have increasing attention because of their potential use in biotechnological processes. Besides, these enzymes also help in ascertaining the major attributes and mechanisms of how proteins achieve extreme thermostability. The starch-degrading enzyme α-amylase (EC 3.2.1.1) plays an important role in both fields of interest [2–4]. The most widespread application of thermostable α-amylases are in the starch industry. Bacterial and fungal α-amylases, and in particular the enzymes from the *Bacillus* species, are of special interest for large-scale biotechnological processes due to their remarkable thermostability and because efficient expression systems are available for these enzymes.

The present review is an attempt to document the structural and dynamical features of α-amylase (the best studied of the amylolytic enzymes) with respect to their impact on

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thermostability. Besides discussing the reason for the thermostable character of the enzymes, the possible improvements that can be made in this regard has also been elucidated in the article.

Source of Thermostable α-Amylase

Thermostable α-amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of α-amylase, microbial sources, namely fungal and bacterial, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production, and ease of process modification and optimization [5].

Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications. Archaeal α-amylases, in particular, have to compete with the *Bacillus* α-amylases that already have excellent thermophilic properties. Table 1 shows a list of some thermostable microbial α-amylase.

Table 1 Source microorganisms and properties of thermostable α-amylase.

Organism	Optimal temp. (°C)	References
Alicyclobacillus acidocaldarius	75	[6]
Bacillus amyloliquefaciens	70	[7]
Bacillus flavothermus	60	[8]
Bacillus lentus	70	[9]
Bacillus licheniformis	100	[10]
Bacillus stearothermophilus	70–80	[11]
Bacillus subtilis	70	[12]
Chloroflexus aurantiacus	71	[13]
Desulfurococcus mucosus	100	[12]
Dictyoglomus thermophilum	90	[14]
Halothermothrix orenii	60	[15]
Lactobacillus amylovorus	60–65	[16]
Lactobacillus plantarum	65	[17]
Lipomyces kononenkoae	70	[18]
Myceliophthora thermophila	100	[19]
Pyrococcus furiosus	100	[20]
Pyrococcus woesei	100	[21]
Pyrodyctium abyssi	100	[22]
Rhizopus sp.	60–65	[23]
Rhodothermus marinus	61	[24]
Scytalidium thermophilum	60	[25]
Staphylothermus marinus	65	[12]
Thermoactinomyces vulgaris	62.5	[26]

Table 1 (continued)

Organism	Optimal temp. (°C)	References
Thermococcus aggregans	100	[12]
Thermococcus celer	90	[12]
Thermococcus fumicolans	95	[27]
Thermococcus guaymagensis	100	[12]
Thermococcus hydrothermalis	85	[27]
Thermococcus litoralis	88	[28]
Thermococcus profundus	80	[29]
Thermomyces lanuginosus	60	[30]
Thermotoga maritima	85–90	[31]
Thermus filiformis	95	[32]

Structural Characteristics and Catalytic Mechanism of α-Amylase

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are endozymes which act on internal α-glycosidic bonds of polysaccharides such as starch, glycogen, etc. and hydrolyze this bond to produce α-anomeric mono- or oligosaccharides. The structure consists of a single polypeptide chain folded into three domains called A, B, and C (Fig. 1). The catalytic domain A consists of N-terminal $(\beta/\alpha)_8$ barrel which is made up of a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight α-helices. This $(\beta/\alpha)_8$ barrel has first been observed in chicken muscle triose phosphate isomerase (TIM) [33] and is therefore called the TIM barrel. It is not only present in the members of α-amylase family but has also been shown to be widespread in functionally diverse enzymes [33]. Domain B is formed by a protrusion between the third β-strand and the third α-helix of the TIM barrel. It has a rather irregular β-rich structure and varies substantially in size and structure among the α-amylases [34]. Domain B forms a large part of the substrate binding cleft and is presumed to be important for the substrate specificity differences observed between α-amylases [35]. Domain C constitutes the C-terminal part of the sequence and is a β-sandwich domain containing a Greek key motif [36]. Both domains B and C are located

Fig. 1 The domain organization of α-amylase. Domain A is shown in *green*, domain B is shown in *magenta*, and domain C is shown in *cyan*

roughly at opposite sides of the TIM barrel. In the maltogenic amylases, the C-domain is followed by a D-domain, the function of which is presently unknown [37]. The TIM barrel contains four highly conserved regions closely related to the active site present in all α-amylases [38]. They are: (1) first region: C-terminal end of β-strand 3 and histidine residue which interacts with the glucose residue of the substrate, (2) second region: β-strand 4 with Asp residue, which acts as the nucleophile during catalysis, (3) third region: β-strand 5 with glutamic acid residue acting as proton donor/acceptor, and (4) fourth region: β-strand 7 with a histidine residue and an Asp residue that may form hydrogen bonds with glucose residue of the substrate. Recent findings made by [15] on the crystal structure of a thermostable α-amylase Amy B from *Halothermothrix orenii* show that, in addition to the typical domain organization of family 13 glycoside hydrolases (i.e., A, B, and C), Amy B carries an additional N-terminal domain (N domain) that forms a large groove, the N–C groove, some 30 Å away from the active site. The structures and results from the biochemical characterization of Amy B and Amy B lacking the N domain show that the N domain increases binding of the enzyme to the raw starch. Furthermore, theoretical modeling suggests that the N–C groove can accommodate, spatially and chemically, large substrates such as starch. Compared with many different amylases that are able to hydrolyze only α-D-(1,4)-glycosidic bonds, maltogenic amylases exhibit catalytic versatility: hydrolysis of α-D-(1,4)- and α-D-(1,6)-glycosodic bonds and transglycosylation of oligosaccharides to C3-, C4-, or C6- hydroxyl groups of various acceptor mono- or disaccharides. It has been speculated that the catalytic property of the enzyme is linked to the additional ~130 residues at the N terminus that are absent in other typical α-amylases [39].

The most intensively studied α-amylase for which a three-dimensional (3D) structure has been solved (using the program MOLMOL) [40] are: *Alteromonas haloplanctis* (AHA), pig pancreatic (PPA), *Aspergillus oryzae* (TAKA), *B. subtilis* (BSUA), *B. amyloliquefaciens* (BAA), *B. licheniformis* (BLA), *Pyrococcus woesei/furiosus* (PWA/pfa). TAKA α-amylase was the first α-amylase to be structurally characterized [41]. The above mentioned α-amylases vary dramatically with respect to their individual thermostabilities. Although the corresponding 3D structures appear rather homologous at first, various proposals on the stabilizing role of structural features have been reported for the individual α-amylases [42–48]. Among these, *Bacillus* α-amylases are more thermostable and require higher temperature and longer time for inactivation.

Mammalian α-amylases contain several disulfide bridges, but the bacterial enzymes are generally devoid of these. With few exceptions, all known α-amylases contain a conserved calcium ion, which is located at the interface between domains A and B [48–50] and which is known to be essential for having a stable, active enzyme [51]. Since calcium preferentially binds carboxylate and other oxygen ligands (which are the metal ligands most likely to be located on the protein surface), this metal is more likely than others to play a significant stabilizing role in proteins. Whenever removal of calcium could be achieved, the proteins lost their catalytic properties. On the other hand, restoration of calcium under appropriate condition always led to a quantitative recovery of activity. Therefore, it is proposed that α-amylases belong to a new class of metallo-enzymes characterized by a prosthetic group, i.e., an alkaline-earth metal rather than a transition element, and which plays primarily a structural role, reminiscent of that of disulfide bridges [52]. It has been suggested that the role of calcium ion is mainly structural [50, 53, 54] since it is too far away from the active site to participate directly in catalysis. The stabilizing effect of calcium ion on the thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by calcium ion in the protein, thus causing the adoption of a compact structure [55]. *Bacillus* α-amylase contains three or four calcium ions and one

sodium ion and a calcium–sodium–calcium metal triad bridge between domains A and B [44, 48]. This metal triad has been considered to be important for maintaining the protein structure in the correct conformation and for resisting thermal inactivation of the enzyme [36, 56, 57]. Three amino acid residues (Asp 262, Glu 292, and Asp 359) are known to be essential for the catalytic activity of *B. amyloliquefaciens* α-amylase. Asp 262 is the catalytic nucleophile, while Glu 292 is the catalytic hydrogen donor. A third residue (Asp 359) is believed to assist in the catalysis by hydrogen bonding to the substrate and by increasing the pKa value of Glu 292 [36]. TAKA α-amylase A (TAKA) contains two calcium ions, and PPA contains only one calcium ion. It was assumed that the Ca^{2+} binding sites should be conserved in the variant BAA enzymes as well as wild types, and tightly bound Ca^{2+} ions such as those in the metal triad should remain in the protein molecule even under conditions without $CaCl_2$ in the reaction mixture [58].

Recently, a new type of *Bacillus* α-amylase (Amy K38), a calcium-free amylase, was reported, and two sodium ions, instead of calcium ions, are used to retain the structure and function of this enzyme [59]. This report suggests that the interaction modes of BAA enzymes with ions are diverse. Besides this, *P. furiosus* and *Thermus* sp. extracellular α-amylases also do not require Ca^{2+} [60, 61]. Malhotra and coworkers [62] reported the presence of a thermostable α-amylase from *Bacillus thermooleovorans* NP54, which did not require Ca^{2+} ions for its activity or production. Most recently, a variant BLA enzyme with improved stability and activity was found as compared with the parent BLA enzyme [58].

Human salivary amylase required at least one g-atom of calcium per mole for full activity, whereas the bacterial enzyme needed four or more. This requirement for calcium in amylolysis is interpreted in the following manner: calcium confers to the amylase molecule the structural rigidity required for effective biological activity by forming a tight, intramolecular, metal-chelate structure. If one assumes that the participation of calcium in the catalytic activity of α-amylases rests on a structural basis, then an attractive explanation becomes available for the fact that *B. subtilis* α-amylase requires roughly four times more calcium for full activity than other α-amylases of nonbacterial nature. Further work is in progress in the laboratory to substantiate that calcium linkages are needed in preserving the integrity of the active center of α-amylases.

Several α-amylases contain a chloride ion in the active site, which has been shown to enhance the catalytic efficiency of the enzyme, presumably by elevating the pKa of the hydrogen-donating residue in the active site [63, 64]. Chloride ions have been found mainly in mammalian α-amylases [53, 65, 66], although a chloride ion has also been reported in a psychrophilic α-amylase (AHA) from the bacterium *A. haloplanctis* [67]. It has been observed that the affinity for the conserved calcium ion increases dramatically upon chloride binding [63], and it is therefore conceivable that chloride ion binding also induces conformational changes around the active site. A puzzling feature of chloride containing α-amylases is a serine protease-like Glu–His–Ser triad in the interface between domains A and C.

The generally accepted catalytic mechanism of the α-amylase is that of the α-retaining double displacement as proposed by Koshland [68]. During the first displacement, an acid group on the enzyme protonates the glycosidic oxygen, bringing about scission of the C1–O bond and transient formation of an oxocarbenium ion-like transition state [69–71]. A nucleophilic acid group of the protein attacks at the sugar anomeric center to give a β-glycosyl enzyme intermediate, while the aglycone of the substrate leaves the active site. During the second displacement, the process just described is essentially reversed by attack, at the anomeric center, by a water molecule activated by the carboxylate form of the proton donor (Fig. 2). The second stage of the reaction proceeds via an ion-like transition state, as before, to yield a product with α-anomeric configuration and reprotonation of the original

acid group. Transglycosylation can occur if the attacking group in the second displacement of the reaction is a free hydroxyl of a sugar residue rather than water [71].

Determinants of Thermostability in α-Amylase

The term thermostability refers to the preservation of the unique chemical and spatial structure of a polypeptide chain under extremes of temperature conditions. The thermostable enzymes can be used as models for understanding thermostability. The identification of structural features involved in stability of thermostable enzymes will not only help to understand the physico-chemical principles contributing to protein stability and folding but also for designing more stable enzymes for industrial processes. Over the last two decades, α-amylase, a monomeric multi-domain protein (50–70 kDa) has become an important model system for investigating thermal adaptation of medium-sized enzymes [36, 72–75].

Site-directed mutagenesis (SDM) experiments and comparisons of structure and stability of mesophlic and thermostable protein isoforms especially in the case of homologous pairs have revealed some important factors that contribute to the remarkable stability of thermozymes [76, 77], but there is no single universal mechanism that promotes stability. The molecular mechanisms of thermostability vary and depend on the specific enzyme [78]; nevertheless, some common features, such as increased number of hydrogen bonds, greater number of ionic interactions, better hydrophobic interactions, shorter surface loops, number of disulfide bonds, metal binding, more rigid and compact packing, conformational strain release, stability of α-helix, reduced entropy of unfolding, CG-rich codons, the ratio of charged amino acids compared to uncharged amino acids, amino acid preferences and their distribution, post-translational modifications and solute accumulation, etc. can be identified as contributors to stability. However, the more general stabilization features, such as oligomeric state, number of disulfide bridges, and number and volume of cavities, i.e., compactness, do not show a correlation with thermostability in some sets of homologous α-amylases. The major factors that contribute to thermostability in α-amylase include presence of calcium, substrate, and other stabilizers [2]. The stabilizing effect of starch was observed in α-amylases from *B. licheniformis* CUMC 305 [79], *Lipomyces kononenkoae* [80], and *Bacillus* sp. WN 11 [81].

A promising approach to improve the knowledge of the physical basis of protein stability is to compare the dynamic features of mesophilic proteins and their thermophilic homologs, which share an increased sequence identity and structural similarity but are greatly different in terms of thermostability [82]. Some of the structural variations that form the basis of the increased thermostability in thermophilic proteins as compared to their mesophilic counterparts are: (a) a higher CG content in coding sequence [83, 84], (b) a

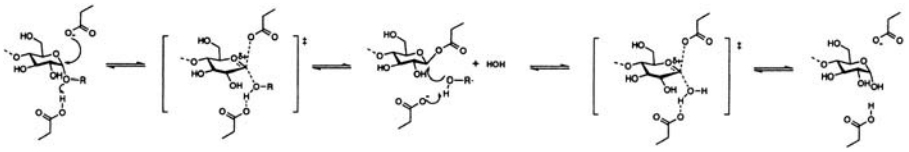

Fig. 2 Catalytic steps in glycoside bond cleavage in retaining enzymes. The proton donor protonates the glycosidic oxygen, and the catalytic nucleophile attacks at C1 leading to formation of the first transition state. The catalytic base promotes the attack of the incoming molecule (water in hydrolysis or another sugar molecule in transglycosylation) on the formation of the covalent intermediate resulting in a second transition state, leading to hydrolysis or transglycosylation product

higher ratio of charged and polar amino acid such as Glu, Arg, Tyr, Asp, and Lys in thermophilic proteins as compared to Ala, Asn, Glu, Thr, Ser, and Val [85, 86], (c) greater number of ionic interactions [87], (d) higher number of disulfide bridges [88], (e) additional networks of hydrogen bonds [89], (f) increased packing density [90] leading to a lower level of thermal motion and less flexibility than in the mesophilic counterparts at a given temperature, (g) higher core hydrophobicity [91], (h) decreased lengths of the surface loops [92], (i) stabilization by heat-stable chaperones [93], (j) a general shortening of length [94, 95], (k) reduction in the difference in entropy between folded and unfolded proteins which in practice means reducing the number of possible conformations in the unfolded state [96], (l) reduction in the area of water-accessible hydrophobic surface [97], (m) amino acid substitutions within and outside the secondary structures [98–100], (n) increased occurrence of proline residues [99, 101, 102], and (o) decreased occurrence of thermolabile residues [90], etc. Protein characteristic is believed to be related with amino acid composition, and some of these structural factors seem to be obtained with the exchange of some amino acids [103, 104]. Thus, the modifications in primary structure, i.e., amino acid composition, are believed to alter the intrinsic stability of proteins, and comparison of sequence and structure of thermophilic and mesophilic proteins has formed the basis of theoretical efforts in elucidating the thermostability mechanisms [105]. Thermozymes are not only characterized by a higher thermal stability but, in general, also by a higher temperature of maximum enzyme activity as compared to their mesophilic analogs (thermal adaptation) [82, 106]. Facchiano and coworkers [107] compared the X-ray structures of 13 thermophilic proteins with their mesophilic homologues and found that the only factor occurring significantly in the thermostable proteins was the lack of β-branched residues (Val, Ile, Thr) in α-helix. Thermostable proteins are found to have a larger fraction of their residues in the α-helical conformation, and they avoid Pro in their α-helices to a greater extent than the mesophiles. It has been noticed that thermophilic enzymes are more resistant to proteolysis than their mesophilic homologues [108], probably owing to their greater rigidity. ΔG values determined for thermoenzymes are larger as compared to mesophilic species with differences in stabilization strategies (between mesophilic and thermophilic sp.; $\Delta\Delta G_{\text{stab}}$ of ~30–50 kJ/mol) [109, 110]. Yokota and coworkers [111] performed a comparative analysis of 47 homologous pairs of thermophilic and mesophilic proteins in order to investigate the factors responsible for protein thermostability and found higher frequencies of Arg, Glu, and Tyr on the surface of thermophilic proteins. Computational methods have also been used with some success to design proteins with enhanced thermostability [112].

Although the organism *B. licheniformis* itself is not a thermophile, it contains a rather thermostable α-amylase (BLA) [2]. The 3D structure of BLA has been determined to atomic resolution by X-ray crystallography [48, 50]. BLA is a monomer of ~58 kDa exhibiting an α/β-barrel as a central part. As a mesophilic homolog, α-amylase from *B. amyloliquefaciens*, with nearly the same molecular mass (58 kDa) as BLA, was chosen. Sequence alignment of BLA and BAA performed by Machius and coworkers [48] revealed a very high homology. Although the crystal structure of BAA has not yet been determined, the sequence alignment and the comparison of Fourier transform infrared (FTIR) spectra suggest that the 3D structures of BAA can be said to be rather similar to those of BLA [113]. The composition of secondary structure elements of the two α-amylases are very similar. As compared by faster H/D exchange and by picosecond dynamics, a fairly higher structural flexibility of the thermophilic BLA was found as compared to the mesophilic BAA. Because for different mesophilic–thermophilic pairs different mechanisms of increased thermostability have been found, it is assumed that nature uses different strategies to adapt enzymes to their specific environment. This might explain the

differences in dynamic properties between the BAA–BLA pair and the comparison of other mesophilic–thermophilic pairs. BLA is far more thermostable than the corresponding enzymes from *B. stearothermophilus* and *B. amyloliquefaciens* despite the strong sequence similarity between these three proteins [114, 115].

Besides the well-approved features which are known to play an important role for the thermostabilization of α-amylases, structural and dynamical features of the unfolded state as well as of the unfolding transition also show a major impact on thermostability. Temperature exerts a profound influence on the balanced interplay of structural flexibility and rigidity. While structural or conformational flexibility is responsible for the proper functioning of proteins, rigidity is required for preserving and maintaining the specific and unique 3D structure of a protein.

Usually, the native and active protein structures are held together by a subtle balance of non-covalent factors or interactions such as hydrogen bonds, ion pairs, and hydrophobic and van der Waals interactions. However, at elevated temperatures, these non-covalent interactions get too weak or become counterbalanced by other interactions, and proteins start to unfold. This protein unfolding can be observed by many different techniques like circular dichroism, differential scanning calorimetry, fluorescence FTIR, nuclear magnetic resonance spectroscopies, sedimentation techniques, viscosity, and migration patterns. Generally, a native protein structure is efficiently packed and appears considerably compact, and it exhibits a rather restricted conformational freedom in the interior of the structure. However, upon unfolding, the structure becomes less compact accompanied by a larger degree of conformational freedom. Small monomeric proteins (like α-amylase) commonly unfold via a two-state transition where the unfolding intermediates are not or barely detectable. Some proteins regain their native and active conformation upon cooling, and this is called thermodynamically reversible unfolding. However, larger multi-domain proteins generally exhibit a different behavior called irreversible unfolding. The major factor behind these irreversible unfolding is the formation of aggregates. It is assumed that mainly the exposed hydrophobic and charged residues which are normally buried in the native protein are responsible for the aggregation.

At least two approaches can help to overcome this problem. First is fluorescence correlation spectroscopy, which significantly reduces the aggregation of unfolded states. This technique measures diffusion coefficients and has already been employed successfully in studies on unfolded proteins [116]. Second, the molecular chaperones can suppress aggregation of non-native or unfolded proteins [117, 118]. The mild conditions of sol–gel encapsulation provide a further technique to reduce aggregation of unfolded states [119]. These approaches have a number of limitations, e.g., limited temperature range, and would be applicable only for some of the relevant aspects. However, studies with homologous α-amylase using these techniques have great potential for progress in this field of application.

Strategies for Improving Thermostabilization

Enzyme thermostability encompasses thermodynamic and kinetic stabilities. Thermodynamic stability is defined by the enzyme's free energy of stabilization (ΔG_{stab}) and by its melting temperature (T_m, the temperature at which 50% of the protein is unfolded), while kinetic stability is often expressed as the enzyme's half-life ($t_{1/2}$) at defined temperature. Two types of protein stability (thermodynamic and long term) are of interest from an applied perspective.

Increasing the thermodynamic stability is the main issue when an enzyme is used under denaturing conditions (i.e., high temperature or organic solvents). Industrialists need active enzymes rather than enzymes that are in a reversibly inactivated state.

Stabilization of the enzymes can be achieved by: screening intrinsically stable enzymes, adding stabilizing agents, chemical modification, immobilization, protein engineering, etc. Protein engineering is currently a very active area of research, both for the commercial production of enzymes of improved thermostability and for furthering our understanding of protein structure–function relationship. Several stabilization strategies are at hand to increase the thermostability of enzymes, e.g., better core packing, surface ion pairing, surface loop stabilization, and reduction of the entropy of unfolding, but these do not have comparable potential for protein stability engineering today. The high conservation of the protein core (mostly defined by α-helices and β-strands) between mesophilic and thermophilic protein homologues suggests that the protein core is already quite optimized for stability, even in mesophilic enzymes. For this reason, mutations in the protein core are often destabilizing, with stabilizing effects often being masked by destabilizing conformational constraints or repulsive van der Waals interactions. The stability gain from α-helix stabilization by introducing residues with high helix propensity is also usually small [120]. Thus, the most promising strategy for thermostabilization in this case is site-directed mutagenesis having a comparable potential for protein engineering, which can be of any type. One method is introduction of mutations Gly → Xaa and Xaa → Pro. Another method is to anchor the loops to the protein surface, either by non-covalent interactions or with a disulfide bridge. The creation of additional nonlocal ion pairs linking non-adjacent sequences in a protein has a great stabilizing potential, and since they can be designed on the protein surface, they do not tend to create as many destabilizing conformational constraints or repulsive van der Waals interactions as substitutions of buried residues. Metal-mediated protein cross linking can also be a stabilizing strategy which stabilizes the protein by reducing the entropy of the denatured state.

Cloning and expression of thermostable genes in suitable hosts are being used routinely by major enzyme production companies to produce enzymes of improved stability to temperature. Genetic engineering has been used extensively for cloning α-amylase genes from amylase-producing strains. A great deal of work has been done on the cloning of α-amylase genes in different microbes, mostly in *E. coli* or *Saccharomyces cerevisiae*. α-Amylase was one of the first proteins adopted for molecular biological studies for several reasons like existence of easy screening assays, availability of amylase negative strains, and the profound knowledge of the genetics, protein production, and fermentation techniques of α-amylase genes in organisms like *B. subtilis*. Hybrids of two homologous strains of the *B. licheniformis* and *B. amyloliquefaciens* was generated [121], and two regions that are important for thermostability has been identified. Introduction of disulphide bonds in the enzyme and alteration of the amino acids prone to oxidation by an amino acid that is unaffected by oxidative agents [122] leads to improved stability of the enzymes [36].

With the advent of directed evolution techniques, protein engineering has received a fresh impetus. The term “directed evolution” encompasses a series of experimental techniques that reproduce, on an accelerated timescale in the test tube, the evolution of natural diversity and environmental adaptation. This is achieved through mutation and recombination and by giving the process a “direction” towards the optimization of one or more properties of interest. Either a selective pressure is applied, or in each round of mutagenesis and/or recombination, the library of variants obtained is screened for the desired trait [123]. To date, directed evolution has proven to be a much more powerful engineering method than SDM as no knowledge about the enzyme structure is necessary.

This method has also been used for a variety of other needs, such as developing enzymes active in solvents, enzymes with altered substrate specificity [124, 125], or thermostable enzymes with increased activity at 20–37 °C. Two directed evolution methods have been developed: (a) the first one is the random mutagenesis method called DNA shuffling which involves random fractionation of a gene with DNase followed by polymerase chain reaction (PCR)-mediated reassembly of the full gene, (b) the second method involves error-prone PCR together with DNA shuffling, i.e., each cycle of error-prone PCR is followed by screening for the desirable trait, and the variants with the best characteristics are then recombined by the DNA shuffling technology.

A third approach for engineering thermostability is the consensus concept which is based on the hypothesis that at a given position in an amino acid sequence alignment of homologous proteins, the respective consensus amino acid contributes more than average to the stability of the protein than the nonconsensus amino acids. Thus, the substitution of nonconsensus by consensus amino acids may be a feasible approach for improving the thermostability of a protein. Pantoliano and coworkers [126] were the first to apply this consensus concept.

Demands for α-amylases with different physiological and biochemical properties by different industries necessitate the search and development of enzymes with increased thermostable properties. Enzyme/protein engineering is known to be a promising technique to achieve this goal, which consists of integrating desired properties in the appropriate gene. Introduction of three mutations Asn172 → Arg, His156 → Tyr, and Ala181 → Thr was reported to increase the thermostability of α-amylase of *B. licheniformis* by 5-fold [127]. Thermostabilty at low pH was achieved by substitutions Met15 → Thr/Asn 188 → Ser [128]. Stabilizations of the proteins have also been done by the insertion of prolines in loop regions [96]. Error-prone PCR in combination with gene shuffling of α-amylase gene of *B. amyloliquefaciens* and two of its mutants resulted in another mutant, which was active over a broader pH range than the wild type, also exhibiting a 5-fold higher activity at pH 10 [129]. It has also been shown that the incorporation of hydrophobic residues at the surface of the enzyme of *B. licheniformis* has resulted in increased resistance to high temperature [130].

In particular, a number of mutations within domain B have improved the stability of BLA to the extent that it can be used for almost any high-temperature application. It is difficult to rationalize the stabilizing effect for several of the stabilizing mutations, and the processes leading to the irreversible denaturation of the α-amylases are still unknown. In this respect, there are still many questions to be answered regarding the stability of bacterial α-amylases. With the construction of the highly stable *Bacillus* α-amylases, it is likely, however, that the future will see a change in the focus of α-amylase engineering from stability engineering to pH activity profile engineering and substrate specificity engineering. Due to the continuing research efforts in the α-amylases fields, it seems likely, however, that such theories and methods will be rapidly developed and result in the construction of α-amylases with novel and improved properties.

Application of Thermostable α-Amylases in Starch Industry

Today, the annual market for thermostable enzymes represents ~US $250 million, and it is likely that the potential value of thermostable α-amylase is greater, in view of the diverse capability of this enzyme. One of the concerns of the thermostable α-amylase is in starch industry. Although α-amylases used in the commercial production of sugar syrups from starch are sufficiently stable at the high operating temperatures of reactor, the enzymes

employed for saccharification and for debranching of amylopectin are more thermolabile. The enzymatic conversion of all starch includes (1) gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension, (2) liquefaction, which involves partial hydrolysis and loss in viscosity, and (3) saccharification, involving the production of glucose and maltose via further hydrolysis. Gelatinization is achieved by heating starch granules in a jet cooker at 105–110 °C for 5 min in aqueous solution (pH 5.8–6.5). During liquefaction, the partial hydrolysis of starch at α-1,4 linkages with a thermostable α-amylase at 95 °C is done for 2–3 h. If the gelatinization temperature drops below 105 °C, incomplete starch gelatinization occurs, which causes filtration problems in the downstream process. Thus, it is desirable that α-amylases should be active at high temperatures of gelatinization (100–110 °C) and liquefaction (80–90 °C) to economize the process; therefore, there has been a need and continual search for more thermophilic and thermostable α-amylase [5] as the development of α-amylases that are active at high temperatures (i.e. ~90 °C) would directly benefit the starch processing industries.

Conclusion

Thermostable α-amylases are not only of extraordinary interest at the fundamental level to investigate the thermodynamic stability of proteins but also to understand the relationship between stability, flexibility or plasticity, and their catalytic efficiency. Despite the fact that α-amylases have been used in a wide variety of technical applications and for several years, there have not been many new developments. The available enzymes are good and have fulfilled, until recently, the needs of the customers. The interest in new and improved α-amylase is growing, and consequently, the research is intensified as well. Research is focused on developing more thermotolerant α-amylases modifying them genetically or applying site-directed mutagenesis to acquire desired property in the enzymes.

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