Microbial acid-stable α-amylases: Characteristics, genetic engineering and applications

Archana Sharma, T. Satyanarayana*

Department of Microbiology, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110021, India

ABSTRACT

Among the wide variety of amylolytic enzymes synthesized by microorganisms, α-amylases are the most widely used biocatalysts in starch saccharification, baking industries and textile desizing. These enzymes randomly cleave the α-1,4-glycosidic linkages in starch, generating maltose and malto-oligosaccharides. The commercially available α-amylases have certain limitations, such as limited activity at low pH and Ca²⁺-dependence, and therefore, the search for novel acid-stable and thermostable amylases from extremophilic microorganisms and the engineering of the already available enzymes have been the major areas of research in this field over the years. Several attempts have been made to find suitable microbial sources of acid-stable and thermostable α-amylases. Acid-stable α-amylases have been reported in fungi, bacteria and archaea. α-Amylases that are active at elevated temperatures have been reported in bacteria as well as in archaea. α-Amylases that possess both characteristics, to the extent required for their various applications are very scarce. The developments that have been made in molecular biology, directed evolution and structural conformation studies of α-amylases for improving their properties to suit various industrial applications are discussed in this review.

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1. Introduction

Starch is the second major polysaccharide food reserve in nature after cellulose. Plants are unique in synthesizing this α-glucan, which serves as an important source of nutrition for other living organisms. Starch is the most easily available source of carbon and energy on the Earth and is synthesized by plants in the presence of sunlight and water through photosynthesis. Starch is synthesized in plastids, which are present in leaves, and accumulates as insoluble granules in higher and lower plants. It is synthesized as semi-crystalline granules with different polymorphic types and degrees of crystallinity. The granules vary in size from 2 to 100 μm and exhibit round, oval and irregular shapes. Starch is a major component of most staple foods and is used in many food and non-food industries. It is mainly utilized in the textile, paper, pharmaceutical, beverage and alcohol industries.

In terms of its structural organization, starch is mainly composed of two high molecular weight compounds, amylose and amylopectin, and both of these contain α-D-glucose as the sole monomer. Amylose is a linear, water-insoluble polymer of glucose subunits that are joined by α-1,4-bonds (99%), and it possesses a molecular weight of ∼1×10^6 to 1×10^8. In contrast, amylopectin is a branched, water-soluble polysaccharide with short α-1,4-linked (∼95%) linear chains of 10–60 glucose units and α-1,6-linked (∼5%) side chains with 15–45 glucose units that form the volume of the starch molecule [1,2]. The levels of amylose and amylopectin vary among starches, but representative percentages of amylose and amylopectin are 25–28% and 72–75%, respectively.

Starch is mainly consumed after processing for domestic or industrial purposes. Hydrolyzed starch has applications in the food, beverage, pharmaceutical, textile and detergent industries. Until the 19th century, starch saccharification was achieved by acid hydrolysis using dilute HCl because the understanding of the potential advantages of biological catalysts was limited. Enzymatic starch processing has advantages over chemical starch hydrolysis, as the latter has drawbacks including a requirement for high temperature, low pH and corrosion-resistant vessels, low glucose yields, and the formation of unwanted color and bitter tasting compounds [3]. Today, starch saccharification is totally enzyme-based.

The world market for industrial enzymes is estimated to be around 3 billion US dollars [4], and it is estimated to be even greater from the products obtained from these enzymes. The total enzyme market was 5.1 billion US dollars in 2009 [5]. Acid-stable extracellular enzymes are required because they are utilized in the degradation of polymeric or oligomeric carbon sources, which occurs at a pH ranges from 3.2 to 4.5 [6]. The promising property of enzymes from thermoacidophiles is activity at low pH and high temperature, and therefore, these enzymes can be used in starch, textile and fruit juice industries. The demand for enzymes from extremophiles may increase in the future because of their activity under harsh industrial conditions.

A large number of bacteria, fungi and yeasts produce extracellular enzymes that degrade starch in different environmental niches. Variety of polysaccharide hydrolyzing enzymes that are suitable for various industrial applications have emerged in the last few decades, leading to the screening of these enzymes for novel properties. In this review, we review the developments in the genetic engineering of acidic microbial α-amylases to improve their characteristics to suit varied applications.

2. Starch hydrolyzing enzymes

Amyloytic enzymes (α-glucanases) hydrolyze the glycosidic linkages in various α-glucans. They belong to 3 families of glycoside hydrolases (GHS) [7]: GH 13 (α-amylases) [8,9], GH 14 (β-amylases) [10] and GH 15 (glucoamylases) [11]. The enzymes differ in their amino acid sequences, reaction mechanisms, catalytic activities and structural characteristics.

Based on their mode of action, the enzymes are divided into two categories: endoamylases (α-amylases, pullulanases, isoamylose) and exoamylases (β-amylase, glucoamylase).

α-Amylases are extracellular enzymes that catalyze the hydrolysis of α-1,4-glycosidic linkages in starch liberating linear and branched oligosaccharides of varying chain lengths as well as glucose; the end products have an α-conformation at C1 [12]. These are categorized based on their end product as saccharifying and liquefying α-amylases. Saccharifying α-amylases produce free sugars but reduce the viscosity of starch pastes slowly. On the contrary, liquefying α-amylases rapidly reduce the viscosity of starch pastes without producing free sugars as compared to saccharifying α-amylases. The saccharifying α-amylases are further classified as malto-amyloses (Bacillus coeureus) [13], maltolutaose-forming (Pseudomonas sp. IMD 353) [14], maltotetraose-forming (Bacillus cereus NY-14) [15] and maltotetraose-forming (Bacillus stearothermophilus US100) [16] α-amylases based on the end products formed. Liquefying α-amylases are reported from Sclerotinia sclerotiorum [17,18] and B. stearothermophilus [19] and saccharifying α-amylases are known from Streptococcus bovis JB 1 [20] and Scytalidium thermophilum [21].

Based on their optimal pH for activity, α-amylases are also classified as acid, neutral or alkaline. The optimal pH of α-amylases ranges from 2 to 12. Most α-amylases are active in the neutral range [22]. The α-amylases from Bacillus subtilis AX20, Bacillus licheniformis, Micromonospora melanospora and Geobacillus thermoleovorans display their highest activities at pH of 6.0, 6.5, 7 and 8, respectively [23–25]. Acidic, neutral and alkaline α-amylases are suited to different industrial applications. The search for α-amylases with the desired kinetic properties for diverse applications is encouraged to improve the industrial process in terms of economics and feasibility [26].

3. Acid-stable α-amylases

The demand for α-amylases that produce high levels of maltose is increasing, as they have diverse commercial applications [27]. The α-amylases currently used in starch processing are active at 95°C and pH 6.8 and are stabilized by Ca²⁺; therefore, the industrial processes with these enzymes cannot be performed at low pH (3.2–4.5), the pH of native starch [28]. In order to be compatible with the optimal pH of the enzyme used for liquefaction, the pH of the starch slurry is raised from its native pH 3.2–4.5 to 5.8–6.2, and furthermore, Ca²⁺ is added to enhance the activity and/or stability of enzyme. The next saccharification step requires another pH adjustment to pH 4.2–4.5. Both of these steps (pH adjustment and salts removal) should be omitted, as they are time consuming and increase the cost of the products [12]. The need is, therefore, to identify extrezoymes from extremophiles that are naturally endowed with the properties required for specialized industrial applications [29] (Fig. 1). α-Amylases are widely distributed in plants, animals and microorganisms. Among amylases derived from various sources, microbial enzymes are known to fulfill industrial demands.

4. Production of α-amylases

The production of α-amylase in submerged and solid state fermentations has been studied extensively. The growth and enzyme production of various microorganisms are affected by a number of physical and chemical parameters, such as carbon, nitrogen and phosphate sources, metal ions, temperature, pH, agitation, aeration, inoculum age and size. Most α-amylases are inducible enzymes.
Fig. 1. Conventional and ideal starch saccharification processes.

Their expression is induced in the presence of starch, maltose and other carbon sources, including lactose, trehalose and α-methyl-D-glycoside. Maltose induces α-amylase in Aspergillus spp., but in Aspergillus oryzae (NRC 401013) and A. oryzae (DSM 63303), both maltose and starch act as inducers [30,31]. Catabolite repression has been reported to involve glucose and other sugars. Carbon sources, such as glucose and maltose have been used in the production of α-amylase, but the use of starch is ubiquitous [32–34]. Industrially important enzymes have traditionally been produced in submerged fermentation, but recently, these enzymes have been produced by solid state fermentation. The combination of low molecular weight dextran with Tween-80 increases α-amylase production by 2.7-fold [35]. Soybean meal, casamino acids [36], corn steep liquor [37] and meat extract [32] have all been employed for the economical production of α-amylase. The level of phosphate in the medium significantly affects growth and enzyme production [34,38]. Various metal ions, such as Ca$^{2+}$, Fe$^{2+}$, Mg$^{2+}$ and K$^+$ are added to the medium for α-amylase production [32,33]. The presence of Ca$^{2+}$ in the production medium supports 13-fold higher biomass, but reduces the enzyme yield [39]. Traditionally, the ‘one-variable-at-a-time’ approach has been used to optimize the production of α-amylase [40], but it is time consuming and does not permit an understanding of the interactions among the process parameters [41]. Statistical approaches have proven to be very useful in optimizing medium components and cultural variables for maximizing enzyme titer in B. acidolica [34], Bacillus sp. KR 8104 [38] and Aspergillus awamori [42].

5. Purification and kinetics of acid-stable α-amylases

A number of conventional and rapid, modern strategies used for the purification of α-amylases are listed in Table 1. The methods often used for the concentration and purification of acid-stable α-amylases are ammonium sulfate precipitation and ion exchange chromatography. The commercial use of α-amylases does not require the enzyme to be in a purified form, but their application in the pharmaceutical and clinical sectors demand high purity. The purified enzyme is also required in studies of structure–function relationships and biochemical properties. Some purified microbial acid-stable α-amylases and their characteristics are listed in Table 2.

Table 1

<table>
<thead>
<tr>
<th>Source</th>
<th>Purification strategy</th>
<th>Fold purification/% recovery</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alicycloabacillus sp. A4</td>
<td>Ultrafiltration with 6kDa (Motianmo, Tianjin, China)</td>
<td>21.8</td>
<td>[129]</td>
</tr>
<tr>
<td>Bacillus circulans GRS 313</td>
<td>HiTrap SP XL column (ion exchange) (Amersham Pharmacia, Uppsala, Sweden)</td>
<td>2.54</td>
<td>[52]</td>
</tr>
<tr>
<td>Bacillus licheniformis NCIB 6346</td>
<td>DEAE-Cellulose DE52 (pH 5.3)</td>
<td>33/66</td>
<td>[130]</td>
</tr>
<tr>
<td>Bacillus sp. B3</td>
<td>Affinity chromatography with alginic acid-CELBEDS (pH 5.0)</td>
<td>–</td>
<td>[131]</td>
</tr>
<tr>
<td>Bacillus sp. KR-8104</td>
<td>Ammonium sulfate precipitation DEAE-Sephrose, phenyl sepharose</td>
<td>–</td>
<td>[32]</td>
</tr>
<tr>
<td>Bacillus sp. WN11</td>
<td>60% (NH$_4$SO$_4$), DEAE Sepharose (pH 5.3)</td>
<td>Amy I = 65/13</td>
<td>[132]</td>
</tr>
<tr>
<td>Bacillus sp. YK-1</td>
<td>Ammonium sulfate precipitation DEAE Sepharose fast flow Sephadex G-75</td>
<td>34</td>
<td>[31]</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>Affinity chromatography, Sepharose 6B (pH 5.5)</td>
<td>–</td>
<td>[133]</td>
</tr>
</tbody>
</table>

*: data not available.
Several characteristics of acid-stable α-amylases differ from those of neutral α-amylases (Table 3).

6. Characterization of α-amylases

6.1. Substrate specificity

The substrate specificity of α-amylases vary among microorganisms. α-Amylases exhibit their highest specificity toward starch compared to other substrates, including amylase, amylopectin, cyclodextrin, glycinin and maltotriose [12].

6.2. Optimal temperature and pH

α-Amylases display activity over a broad pH range from 2.0 to 12.0 [43]. The optimal pH of most of the α-amylases falls in the acidic and neutral range [22]. The acid-stable α-amylases from microorganisms are listed in Table 2.

The thermostability of these enzymes is an important characteristic and determines the primary structure of the protein. Optimal temperatures ranging between 45 and 115 °C have been observed for α-amylases (Table 2). A number of acid-stable α-amylases have been purified and characterized from different microorganisms. It has been observed that acid-stable α-amylases contain 30% less acidic and basic amino acids compared to neutral α-amylases, and

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Characteristics of acidstable microbial α-amylases.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td><strong>Molecular mass (kDa)</strong></td>
</tr>
<tr>
<td>Alicyclobacillus sp. A4</td>
<td>64</td>
</tr>
<tr>
<td>A. acidocaldarius</td>
<td>160</td>
</tr>
<tr>
<td>Bacillus acidocaldarius</td>
<td>66</td>
</tr>
<tr>
<td>B. caldotylicus</td>
<td>70</td>
</tr>
<tr>
<td>Bacillus circulans GR631</td>
<td>48</td>
</tr>
<tr>
<td>Bacillus licheniformis NH1</td>
<td>58</td>
</tr>
<tr>
<td>Bacillus steatorrhoeicus</td>
<td>–</td>
</tr>
<tr>
<td>B. steatorrhoeicus US 100</td>
<td>–</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>53</td>
</tr>
<tr>
<td>Bacillus sp. KR8104</td>
<td>59</td>
</tr>
<tr>
<td>Bacillus sp. WN 11</td>
<td>Amy 1 – 76</td>
</tr>
</tbody>
</table>

| **Fungi** | **Molecular mass (kDa)** | **pH/stability** | **Temperature/stability** | **pH** | **Km, Vmax, kcat** | **Reference** |
| Aspergillus oryzae M13 | 52 | 4.0 | 50 | 4.0 | 0.13% (Km) | [142,143,144] |
| A. awamori ATCC 22342 | 54 | 4.8–5.0 | 50/40 (60 min) | 1.0 mg ml⁻¹ (Km) | [145,146] |
| A. chalaraus NSPRI | 68 | 5.5 | 40/60 (15 min) | 0.19 mg ml⁻¹ (Km) | [147] |
| A. chalaraus NSPRI | 68 | 5.5 | 40/60 (15 min) | – | 0.19 mg ml⁻¹ (Km) | [148] |
| A. flavus | – | 5.25/5.0–8.0 | 50/55 (10 min) | – | – | [149] |
| A. foetidus ATCC 10254 | 41.5 | 5.0 | 45/35 (60 min) | – | 1.14 mg ml⁻¹ (Km) | [149] |

| **Yeast** | **Molecular mass (kDa)** | **pH/stability** | **Temperature/stability** | **pH** | **Km, Vmax, kcat** | **Reference** |
| Cryptococcus flavus | 84.5 | 5.5 | 50 | – | 0.056 mg ml⁻¹ (Km) | [151] |
| Cryptococcus sp. S-2 | 66 | 6.0 | 37 | 4.2 | – | [153] |

– not available.

The thermodynamic studies of these enzymes is an important characteristic and determines the primary structure of the protein. Optimal temperatures ranging between 45 and 115 °C have been observed for α-amylases (Table 2). A number of acid-stable α-amylases have been purified and characterized from different microorganisms. It has been observed that acid-stable α-amylases contain 30% less acidic and basic amino acids compared to neutral α-amylases, and

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Distinguishable properties between acid-stable and neutral α-amylases.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Properties</strong></td>
<td><strong>Acid-stable α-amylases</strong></td>
</tr>
<tr>
<td>pH range</td>
<td>3.0–6.0</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>40–115</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>41–160</td>
</tr>
<tr>
<td>pH</td>
<td>5.6–8.0</td>
</tr>
<tr>
<td>Acid-stability</td>
<td>3.5–5.5</td>
</tr>
<tr>
<td>Thermostability (°C)</td>
<td>60–80</td>
</tr>
<tr>
<td>Release of CNP from CNP-α-G3</td>
<td>Suppressed by KSCN</td>
</tr>
<tr>
<td>Cleavage of G5</td>
<td>GGG(α) + G</td>
</tr>
<tr>
<td>Number of subsite</td>
<td>5</td>
</tr>
</tbody>
</table>
this feature prevents the electrostatic repulsion of charged groups at acidic pHs and contributes to the acid stability of the proteins [44]. As the nature of acid-stable α-amylases depends on their ionizable groups, the pH lower than the isoelectric point signifies that their basic amino acids carry a large number of positive charges, resulting in the expansion of the protein structure, which consequently affects the activity of the catalytic center [45]. Charged amino acids make up only 18% of the A. niger α-amylase, suggesting the acid resistance of this enzyme [46]. Most of the known acid-stable α-amylases lack thermostability at elevated temperatures, which is a major constraint for their application in the starch industry. Research is in progress to isolate extremophilic microorganisms that produce enzymes with this desired property.

6.3. Molecular weight

The molecular weights of microbial α-amylases range from 12.5 to 160 kDa (Table 2). The acidic α-amylase from A. niger has a lower molecular weight than that of acidic α-amylase from other molds. Most acid-stable α-amylases are high molecular weight enzymes as reported in B. stearothermophilus US 100[16], Lactobacillus manihotivorans [47], G. thermoleovorans [48], Bacillus sp. KR8104 [38] and B. acidificola [49].

6.4. Inhibitors

Heavy metal ions, sulfhydryl group reagents, N-bromosuccinimide (NBS), p-hydroxy mercuribenzoic acid, iodoacetate, EDTA and EGTA are known to inhibit α-amylases [50]. Many α-amylases are also inhibited by the Hg²⁺ ion [51] indicating the presence of a carboxyl group in the enzyme molecules [52]. Furthermore, Hg²⁺ is known to oxidize indole rings and to interact with the aromatic rings present in tryptophan residues [53]. The inhibition of enzyme activity by NBS demonstrates the catalytic role of tryptophan [49]. Dithiothreitol and β-mercaptoethanol are reducing agents, which suggest the role of —SH groups in the catalytic activity of these enzymes. There are reports where DTT has stimulated and inhibited the activities of α-amylases [54]. DTT has no effect on maltogenic α-amylase from Bacillus sp. WP0616, indicating that —SH groups are not involved in its catalytic activity or that this enzyme has no free, accessible —SH groups [55]. The inhibition of α-amylase by Woodward’s reagent K (WRK) signifies the involvement of acidic amino acids in the active site of the enzyme [56,57].

6.5. Metal ion and stability of α-amylase

Various cations, substrates and other stabilizers influence the thermostability of these enzymes [43]. α-Amylases are metal activated enzymes that have a high affinity for the Ca²⁺ ion. The Ca²⁺ ion alters the activity and thermal stability of most α-amylases, and it is known that their thermal stability is usually enhanced in the presence of the Ca²⁺ ion [58,59]. The number of bound Ca²⁺ ions varies from 1 to 10. Usually one Ca²⁺ ion is sufficient to stabilize the enzyme. However, the crystalline TAKA amylase A contains ten Ca²⁺ ions but only one is tightly bound [60]. Dialysis against EDTA can remove Ca²⁺ and the Ca²⁺ ion free enzyme can be reactivated with the addition of calcium ions. Although α-amylase is known to be Ca²⁺ dependent, there are reports of Ca²⁺ independent acid-stable α-amylases that do not require Ca²⁺ for stability and activity [32,48,51].

7. α-Amylase gene cloning and expression

α-Amylase was one of the first proteins adopted for molecular biological studies because of the existence of an easy screening assay, the availability of amylase negative strains, the extensive knowledge of its genetics, protein production and fermentation technologies for the α-amylase of B. subtilis. Sajedi et al. [61] reported the cloning of the α-amylase gene (1328 bp) from Bacillus sp. KR8104, which encodes 440 amino acids without 20 amino acids at its N- and C- termini. The α-amylase gene of B. acidificola with an N- and C- terminal truncation has been recently cloned into the pET28a(+) vector and expressed in E. coli [49]. The 62 kDa recombinant α-amylase is optimally active at pH 4.0 and 60 °C. The gene encoding the 460 aa extracellular α-amylase from Pyrococcus furiosus was also cloned in E. coli. The P. furiosus α-amylase is a liquefying enzyme with a specific activity of 39000 U mg⁻¹ at 98 °C. It is optimally active at pH 5.5–6.0 and 100 °C with a T½ of 13 h at 98 °C and does not require Ca²⁺ for its activity [58]. Another α-amylase gene, Amy N, from B. licheniformis NH1 was also cloned, sequenced and expressed in E. coli using the pDEST17 expression system. This recombinant α-amylase shows high thermostability at 85 °C (60 min) compared to the native B. licheniformis NH1 α-amylase (8 min) [62]. The gene encoding the acid-stable α-amylase from Aspergillus niger was cloned in the pPIC9K vector and expressed in Pichia pastoris with a very high production of 2838 U ml⁻¹. The 58 kDa recombinant α-amylase is optimally active at pH 4.0 and 70 °C [46]. A 1920 bp gene encoding 640 amino acids of an acid-stable α-amylase was cloned from Aspergillus kawachii IP04308.

8. Structural conformation studies

Circular dichroism (CD) spectroscopy and X-ray crystallography are extensively used techniques for acquiring information about protein structure and conformation. α-Amylases have 3 domains. A central (α/β)₃ TIM-barrel (named after triosephosphate isomerase, a protein fold consisting of eight α-helices and eight parallel β-strands in an alternating pattern of α-helices and β-strands in a single domain), known as domain A forms the core of the molecule and contains three active site residues D₂₃₁, E₂₆₁ and D₁₂₈ [B. licheniformis α-amylase (BLA) numbering], while domains B and C are situated at opposite sides of this TIM-barrel. The amino acid residues in the active site are strictly conserved, but a few positional changes particularly in the catalytic residues were observed when the B. stearothermophilus α-amylase (BSTA) was superimposed with BLA. This finding indicates the flexible nature of the catalytic residues, which plays an important role in catalytic reactions. Domain B is a projection between the third strand and the third helix of the TIM barrel and forms an irregular β-like structure that is possibly responsible for the differences in substrate specificity and stability between α-amylases. The C-terminal portion of the sequence is present in domain C and contains a Greek key motif (a frequent beta sheet motif found in protein structures and is formed from four adjacent antiparallel beta strands), C-terminal truncation has been observed in some glycosyl hydrolases such as the α-amylases from B. subtilis, Pseudomonas stutzeri and α-amylase 1 in malt [63,64] while artificial truncation has been performed on various amyloolytic enzymes from B. subtilis, Bacillus sp. and B. stearothermophilus [65–67] to study the function of the C-terminal region of α-amylase. The C-terminus has been shown to be involved in the translocation of the enzyme across the outer membrane of E. coli, as reported in Alteromonas haloplanktis [68] and Bacillus sp. KR8104 [69], the binding of the enzyme to raw starch [70] and its thermal stability [66,67,70]. In contrast, Salimi et al. [71] suggested that C-terminal truncation does not affect the thermal stability, optimal pH or end products of starch hydrolysis. Some amylases contain a carbohydrate-binding module (CBM) for the hydrolysis of insoluble starch. A CBM is a noncatalytic ancillary domain of 40–200 amino acids with a discrete fold that aids in the binding
of enzyme to insoluble polysaccharide surfaces and thus improves hydrolysis [72,73]. CBM with affinity for starch are known as starch binding domains (SBDs). Approximately 10% of amylolytic enzymes possess a separate domain for binding to raw starch. Florencio et al. [74], Morlon-Guyot et al. [75] and Nakamura et al. [76] reported the presence of SBDs in three α-amylases from Lactobacillus. The genes encoding the α-amylases have been sequenced [77], and amino acid sequence analysis of these enzymes showed more than 96% identity.

Santiago et al. [78] reported the involvement of five-tandem-module SBD, which act not only as distinct modules but also as a part of the whole amylase. Three steps are involved in the catalytic mechanism for retaining glycosyl hydrolyses [79,80]. First, the glycosidic oxygen is protonated by the proton donor (E261) followed by a nucleophilic attack on the C1 of the sugar residue in subsite-1 by D231. Once the aglycon group of the substrate leaves, a water molecule is activated presumably by the deprotonated E261. This water molecule hydrolizes the covalent bond between the nucleophilic oxygen and the Cl of the sugar residue in subsite-1, thereby completing the catalytic cycle [81].

The 3D structure and amino acid sequence of acidic α-amylase from A. niger was described by a group at the company Novo [82]; this enzyme displayed a 3D structure that is similar to that of TAKA amylase A (TAA). Several applications involving α-amylases are performed at pH that differs from the optimal pH of α-amylases and therefore, there is a compelling need to change the pH performance profile of α-amylases and their related enzymes. Binding and light scattering experiments using β-o-naphthylamine and β-amyloliquafaciens α-amylase (BAA) revealed that at an acidic pH, B. amyloliquefaciens α-amylase (BAA) unfolds in such a way that its hydrophobic surface is exposed to a greater extent than the native form at a neutral pH. In addition, quenching of the intrinsic tryptophan residues in the protein molecules with acrylamide indicates that at pH 3.0, the protein is in a partially unfolded conformation with more tryptophan residues exposed to the solvent compared to the native conformation at a neutral pH. Ca2+ is required for the refolding of the molten globule state to the native form.

All known α-amylases contain a calcium ion, which is situated at the interface between domains A and B [82], and is required for the activity and/or stability of the enzyme. The function of the conserved Ca2+ has been suggested to be structural [83,84], as it is too distant from the active site to participate in catalysis. One or more Ca2+ ions are present in many structures, the first calcium ion (Ca I) is strictly conserved in all distantly related α-amylases and it connects domain A to domain B and helps to stabilize the active site structure and controls the formation of the extended substrate binding site. The second calcium ion (Ca II) is situated close to Ca I and in the presence of sodium ions, a Ca–Na–Ca arrangement was observed in BLA [83], BAA [85] and BSTA [86]. By comparing the metal-containing and metal-free crystal structures of BLA, it was observed that the loss of metal ions causes numerous conformational changes around the metal triad and the active site, which contains 21 residues. In addition to Ca2+, chloride ions have been shown to enhance the catalytic efficiency of the enzyme. The deduced amino acid sequence of the Ca2+-independent α-amylase from Bacillus sp. KR-8104 (KRA) revealed maximum sequence homology to the BAA [85% identity and 90% similarity] and BLA [81% identity and 88% similarity] α-amylases. The 3D structure of KRA shows one amino acid substitution in comparison with BLA and BAA in the region engaged in calcium binding, while at the interface of the A and B domains and around the metal triad and active site, many amino acid differences between BLA and KRA exist. The presence of chloride ions in the active sites is predominant in mammalian α-amylases [84,87].

9. Protein engineering of α-amylase by site directed mutagenesis

Information from 3D structures, chemical modifications and other studies aids in understanding the functions of various enzyme domains and offers ways to tailor the key amino acids for catalytic activity, and to improve stability under stressed conditions. Site directed mutagenesis is a technique that alters the properties of an enzyme based on its structural information. Rational protein engineering and site-directed mutagenesis studies on α-amylases have been carried out to tailor glycosyl hydrolyses in terms of increasing their thermostability [88–90], altering their pH activity profile [81,91–94], and altering their product specificity [9,95].

9.1. Active site residues

The three catalytic residues (D231, E261 and D294) in BLA are fully conserved residues among all α-amylase family enzymes in both their amino acid sequences and 3D structures [96]. Hasegawa et al. [97] studied the possible role and mechanism of the catalytic residues D193, E219 and D294 of the P. stutzeri maltotetraosforming α-amylase. D193 acts as base catalyst (nucleophile) whose side chain oxygen atom lies close to the C-1 atom of glucose-4 (Glc4), which is involved in the formation of the intermediate in the hydrolysis reaction. Residue D294 aids in tightly binding the substrate to form a twisted and distorted glucose ring at the −1 position (Glc4). The hydrogen bond between the side chain atom of E219 and the O-1 atom of Glc4 suggests the possible role of E219 as an acid catalyst (proton donor). This residue is protonated at the optimal pH for catalysis [98] (Fig. 2). The active site residues of the α-amylase of B. subtilis N7 (D176, E208 and D269) were mutated to their amide forms to evaluate the role of these active site residues in catalysis. Mutations resulted in a 15,000-fold decrease in the specific activity of the enzyme, with no effect on ability of the mutants to bind the substrate [99]. Vihinen et al. [100] showed that a specific mutation in the active site (D131E) leads to an almost complete inactivation of the α-amylase from B. stearothermophilus.

9.2. Thermostability

Residues involved in salt-bridges, calcium binding or potential deamination processes were reported to affect the thermostability of the protein and were subjected to site-directed mutagenesis, based on informational suppression. The mutations responsible for altering the thermostability are mainly present in domain B and its interface with domain A, where the triad metal site (Ca–Na–Ca) and substrate binding site are located [90]. The triad metal site is highly sensitive to any modification that can change the network of electrostatic interactions that entraps the metal ions. Priyadarshini et al. [101] also reported that the region around the calcium binding site of BLA is sensitive to any modification. The amino acid residues encoding the calcium-binding site (N104, D161, D163, D200 and D293), which extends from residues 104 to 200 in the loop regions of domain B and D293 in domain C, were changed to D104 and N161, N163, N200 and N293 by site-directed mutagenesis and the resultant mutant amylase showed improved specific activity at pH 5.0 and 70 °C and decreased activity at 30 °C. However, the stability of the amylase was unaffected by the mutations N104D and D293N. Another mutation, D161N, greatly decreased the thermostability of amylase.

In another study, site-directed mutagenesis was used to enhance the thermal stability and calcium independence of a mesophilic α-amylase from Bacillus megaterium WHO. Mutations (A53S and H58L) in the calcium-binding site affected the α-amylase. The A35S mutant showed that the enhanced calcium binding is most likely responsible for the increased stability of the enzyme.
In contrast, a calcium-independent mutant (H58I) possessed high thermostability. Mutation of H234D in the \textit{B. stearothermophilus} \(\alpha\)-amylase, which is involved in Ca\(^{2+}\) and substrate binding, resulted in decreased specific enzyme activity and thermal stability. However, Ca\(^{2+}\) had a greater effect on the catalytic efficiency, \(k_{cat}/K_m\), and half-life (at 60 °C) of the A235S mutant [102]. Structure-based mutagenesis of BLA revealed the significance of seven residues (D121, N126, D164, N192, D200, D204, and A205), which were sensitive to any amino acid substitutions. Replacement of three asparagine residues (N172, N188 and N190) significantly enhanced the thermostability of the mutant. The substitution of phenylalanine to asparagine at position 190, led to a 6 fold increase in the half-life of enzyme at 80 °C [90]. Similarly, the mutations of H133A and A200V were the most favorable substitutions for enhancing the thermostability of \(\alpha\)-amylases [88]. Salt bridge residues D121–R127 also contributed to increasing the thermostability of BLA compared to other homologous bacterial \(\alpha\)-amylases.

In a protein, hydrophobic residues are unfavorable at solvent-exposed sites, and amino acid substitutions on the surface have less of an impact on protein thermostability. Interestingly, hyper-thermostable variants of BLA were obtained by incorporating hydrophobic residues at the surface [103]. There are several reasons why the protein is stabilized at high temperatures including increased hydrophobic packing (generally in cavities and surface indentations), aromatic–aromatic interactions on the surface of the protein, stabilization of the intrinsic metal binding site and \(\beta\)-sheets, an extended network of salt bridges and hydrogen bonds, and the replacement of the residues responsible for irreversible chemical alterations of the protein structure.

9.3. Determinants of the pH activity profile

A string of substitutions have been made in BLA to stabilize the protein and improve its performance at acidic pHs [88–90,93,94,103–105]. Site-directed mutagenesis and saturated mutagenesis have been employed to tailoring the optimal pH of a number of enzymes, including the \(\alpha\)-amylase from \textit{B. licheniformis} [106] and soybean \(\beta\)-amylase [107], but the catalytic rate of these enzymes was affected. Liu et al. [104] described the relationships between acid resistance and the structural features of different mutants of BLA with changes at two crucial residues, L134 and S220 (Fig. 3). The combined effect of the double mutant L134R/S220A indicated that the amino acids inserted at each site contribute independently to the overall stability of the protein, as is generally seen for stabilizing mutations in protein structures [108–111]. Changes in the electrostatic field due to charged groups play a significant role in determining the stability of BLA in strongly acidic environments. However, Neilson et al. [105] reported a significant change in the pH activity profile of \(\alpha\)-amylase of \textit{Bacillus} Ba2 between neutral–neutral mutations and neutral–charged mutations. It was concluded that the dynamic aspects of the active site other than electrostatics are most likely responsible for the pH activity profiles of \(\alpha\)-amylases. Mutations around the catalytic nucleophile in KRA that decreased its pKa value are as follows: D188R, D246K, D260N, N280K, and Q291H (increased positive or decreased negative charges) and A328S, V154N, L121T, Y205N, T297S, and A376S (decreased hydrophobic effects) which may be responsible for the shift in its pH activity profile [61].

10. Alteration of other properties of \(\alpha\)-amylases by directed evolution

Naturally occurring enzymes are wonderful biocatalysts with abundant potential applications in industry and medicine. To be compatible with the specific requirements for an application, the catalytic properties of the enzyme must be tailored. Directed evolution mimics Darwinian evolution and has emerged as a powerful tool for engineering enzymes with new or improved functions. This technique can be used to modify various enzyme properties, such as activity, selectivity, substrate specificity, stability and solubility.
11. Correlation between the molecular dynamics of active site residues and the pH activity profile

The conformational dynamics of protein molecules are programmed into their structures and are a significant element of their function. Alteration of the pKa values of catalytic residues affects the pH-profile of the enzyme. There are a number of microenvironmental determinants of the pKa of a titratable group in proteins such as electrostatic effects [104], hydrophobic effects (desolvation effects) [61], hydrogen binding, helix dipole interactions, as well as the dynamic aspects of the active site [105], which can be changed to influence the pKa.

BLA catalyzes the enzyme substrate reaction at a low pH by protonating the nucleophile (D231) and at a high pH by deprotonating hydrogen donor (E261); the correlation between the activity of the enzyme and the pH is determined by the pKa values of these two active site groups [119].

12. Computational modeling

The availability of basic information on a protein aids in improving the properties of the protein by computational modeling. A number of computer programs have been employed to model α-amylase including MODELLER 9 VB [120], Frodo, Hydra (Polygen Waltham, Mass.), Insight (Biosym Technologies, San Diego, Calif.), Gromos (Biosoft b.v., Groningen, The Netherlands). For validation of the model, PROCHECK [http://nihserver.mbi.ucla.edu/SAVES/Info.php], WHATIF [https://swift.cmbi.ru.nl/servers/html/index.html], ProSA [http://prosa.services.came.sbg.ac.at] and RAMPAGE [http://mordred.bioc.cam.ac.uk/] have been employed and Swiss pdbViewer ver.3.7 [121] and MOLMOL [122] programs have been used to analyze and compare 3D structures.

13. Applications of α-amylases

Presently, amylases have the major world market share for enzymes [123]. Many amylase preparations are available from various enzyme manufacturers for specific applications, such as starch saccharification, baking, bioethanol production, textile desizing, in laundry and dish-washing detergents [22,124–127]. Bacterial amylases are generally preferred over fungal amylases due to the characteristic advantages that the former offers [25,128]. Acid-stable α-amylases are preferred, as their application minimizes contamination risk.

The acid-stable and Ca$^{2+}$-independent α-amylases are preferred over the currently used enzymes in starch processing because the latter are active at 95°C and pH 6.8 and stabilized by Ca$^{2+}$. The process cannot be performed at low pH (3.2–4.5), the pH of the native starch [28]. The stress is, therefore, on extremozymes from extremophiles that are naturally endowed with the properties needed for specific industrial applications [29].

To prevent the staling of bread and other baked goods and to improve their texture and shelf-life, the dough is supplemented with α-amylase. The bacterial maltogenic and acidic α-amylases with intermediate thermostability have been reported to act as antistaling agents, thereby reducing the crumb firmness during storage and improving the volume, texture, flavor and shelf-life of bread by shortening the amylopectin chain length due to the formation of malto-oligosaccharides (DP 2–12) [124].

14. Conclusions

α-Amylase is one of the most important enzymes employed in the starch processing industry for the production of starch hydrolysates. The pH of native starch ranges between 3.2 and 4.5, and therefore, thermostable acidic and Ca$^{2+}$-independent α-amylases are better suited for conversion of starch to various sugar syrups. Acidic α-amylases are produced by bacteria, archaea and fungi. α-Amylases are also used in the removal of starch from beer, fruit juices, textiles and porcelain. Maltogenic amylase is used as an antistaling agent to prevent the retrogradation of starch in bakery products. Food and starch-based industries are the major markets for their application of α-amylases, but they are now gaining importance in biopharmaceutical applications, and their demand of these enzymes is expected to rise in the future.

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