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MICROBIAL α-AMYLASES: STRUCTURE, FUNCTION AND APPLICATION – A REVIEW

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ABSTRACT

α-amylases are most important industrial enzyme and hold a major market share of enzyme sales. They hydrolyze starch molecules to small diverse products as dextrin and progressively smaller molecules of glucose units. α-amylases belong to the family 13(GH-13) of the glycoside hydrolase group of enzymes. Microbial α-amylases have a wide range of applications ranging from starch conversion to pharmaceutical applications. This article highlights on the characteristic features of α-amylase structure, function, family, primary microbial sources and uses of α-amylases in industrial purposes.

KEY WORDS: **α-amylase, glycoside hydrolases, GH-13, metalloenzymes, TIM barrel, catalytic domain.**

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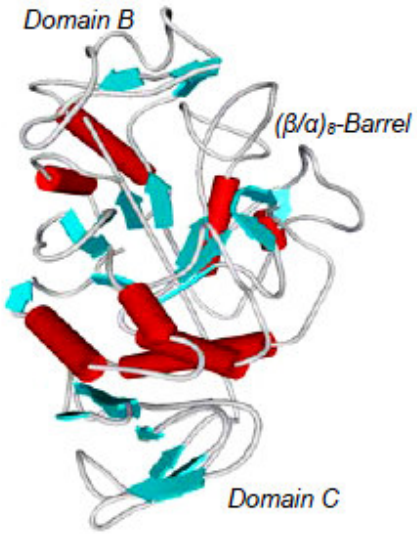
INTRODUCTION

α-amylases (1,4-α-D-glucan-glucanohydrolase, E.C.3.2.1.1.) are starch degrading enzymes that catalyze the hydrolysis of internal α-1,4-o-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products such as glucose, maltose and maltotriose units[10,16,53]. α-amylases belong to the family 13(GH-13) of glycoside hydrolase group of enzymes which is consisted of almost 30 different enzyme specificities[22]. α-amylases are one of the most important industrial enzymes with a wide variety of applications ranging from conversion of starch to sugar syrups to the production of cyclodextrins for pharmaceutical industry. These enzymes account for about 30% of the world's enzyme production[57,60]. Microorganisms specially the molds (*Aspergillus* and *Penicillium*) and bacteria (*Bacillus*) are the commercial sources of α-amylases[21,56]. Due to growing demand for these enzymes in various industries, there is increasing interest in developing enzymes with better properties such as raw starch degrading enzymes suitable for industrial applications and their cost effective production techniques. The α-amylase family represents the eight clan i.e., GH-H among the fourteen clans (A-N) defined for glycosidases and transglycosidases[36]. Structurally α-amylase enzymes are $(\beta/\alpha)_8$-barrel proteins[37,48]. Usually all these enzymes act on one type of substrate being glucose residues linked through α-1,4 and α-1,6 glycosidic bonds. However, enzymes acting on linkages other than α-1-4 and α-1-6 have also been recognized as belonging to the family[36]. The α-amylase family is roughly divided into two groups: the starch hydrolyzing enzymes and starch modifying enzymes or transglycosylating enzymes[53]. In this present review an attempt is made to document the structural and functional aspects of α-amylases, their commercial sources and industrial applications.

α-AMYLASE

α-amylase (EC 3.2.1.1) is one of the three amylases i.e., alpha, beta and gamma amylase which represents the best known endo-acting amylolytic enzyme. Amylases particularly α-amylases are one of the most important industrial enzymes with the extensive applications in various fields ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry[41]. α-amylases are starch degrading enzymes and involved in the hydrolysis of internal α-1,4-glycosidic bonds by acting at random locations in amylose and amylopectin of starch and similar compounds yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin with the retention of α-anomeric configuration in the products[10,42,57]. They also act on glycogen and related polysaccharides and oligosaccharides. The terminal glucose residues and α-1,6-linkages cannot be cleaved by α-amylase[10,13]. Since it can act anywhere on the substrate, hydrolysis by α-amylase is faster than β-amylase. On the basis of degree of hydrolysis of the substrate, α-amylases are often divided in two classes: saccharifying α-amylases hydrolyze 50 to 60% and liquefying α-amylases cleave about 30 to 40% of the glycosidic linkages of starch[1]. α-amylases are usually metalloenzymes which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability[53]. α-amylases belong to the family 13(GH-13) of the glycoside hydrolase group of enzymes and is the main representative of the family GH-13. All α-amylases possess a common $(\beta/\alpha)_8$ or TIM barrel structure (Fig.1) with the catalytic site residues and four highly conserved regions in their primary structure[32].

Figure 1
Taka amylase (α-amylase) from Aspergillus oryzae (PDB code: 2TAA; Matsuura et al., 1984)



THE α-AMYLASE FAMILY

Glycoside hydrolases have been divided into families based on their well defined amino acid sequence similarities and families into larger groups, termed as 'clan'[20]. A 'clan' is a group of families that possess significant similarity in their tertiary structure, catalytic residues and mechanism. Among the fourteen clans (A-N) defined for glycosidases and transglycosidases, α-amylase family belong to the eight clan, the clan GH-H[35]. The clan GH-H is now consisted of three families viz., GH-13, GH-70 and GH-77[35,36]. At present all the GH families are incorporated in the CAZy web-server[6] which also covers other carbohydrate-active enzymes[22]. Most of the starch converting enzymes belongs to GH-13 family. α-amylases belong to the family 13 i.e., GH-13 which is commonly known as α-amylase family. The main representative of the clan (GH-H) is the GH-13 family which is the largest and most complex family containing hydrolases, transferases and isomerases[35]. The family, at present is consisted of 30 different enzyme specificities and has more than 6,000 sequences[6]. Initially, the concept of the family was given by Takata *et al.* (1992) as family GH-13 and the α-amylase family was documented as a group of starch hydrolases and associated enzymes (such as α-amylase, cyclodextrin glucanotransferase, neopullulanase, etc.) having sequence similarities and generally predicted TIM-barrel fold[22,58]. The family GH-13 was the first constituent of clan GH-H[17], but later the families GH-70 and GH-77 were included to form the currently well established GH-H clan[22,35]. Most of the α-amylase family enzymes attack the glycosidic bond in starch with some exceptions which are active towards the analogous bonds in glycogen, pullulan and other related poly- and oligosaccharides like trehalose, sucrose, etc.[36]. The family GH-77 contains only one enzyme specificity i.e., amylomaltase (EC 2.4.1.25) and the family GH-70 consists of two specificities – glucosyltransferase (EC 2.4.1.5) and alternansucrase (EC 2.4.1.140). The family GH-13 includes all the remaining enzyme specificities constituting the main α-amylase family[22]. The enzymes of this family have to fulfill the following requirements (i) they catalyze hydrolysis and/or transglycosylation at the α-1,4- and α-1,6- glucosidic linkages (ii) retention of the α-anomeric configuration in the products (iii) usually have four highly conserved amino acid sequence regions that contain all the catalytic residues and most of the substrate binding sites (iv) possess Asp, Glu and Asp residues as catalytic sites forming catalytic triad, corresponding to Asp206, Glu230 and Asp297 of Taka amylase A (the α-amylase from *Aspergillus oryzae*) and (v) possess a $(\beta/\alpha)_8$ or TIM-barrel catalytic domain[22,23,32,37,60]. A rapid increase of the number of GH-13 members to several thousands offered a great variety in both substrate and product specificities and sequence diversity leading to update the above criteria[6,22]. For example – enzymes of α- amylase family are also active on α-1,1- , α-1,2- , α-1,3- and α-1,5-glucosidic linkages. Moreover, in addition to the four well-accepted conserved sequence regions, another three conserved sequence regions are also discovered in some α-amylases[24,25] which can often help to assign the correct enzyme specificity of α-amylase family members[23].

STRUCTURAL CHARACTERISTICS OF α-AMYLASES

The α-amylases are monomeric, calcium-containing enzymes, with a single polypeptide chain folded into three domains (A-C). The molecular weight of microbial α-amylases usually ranges between 40-70 kDa[16]. The X-ray crystallographic structures of α-amylases characterized from different sources show that most α-amylases are multidomain proteins with three major domains, generally designated as A, B and C[17,18,32,36]. The domain A is the largest and placed centrally which forms the core of the molecule and it has a $(\beta/\alpha)_8$ barrel (TIM-barrel) structure. The other two domains, B and C are positioned nearly at opposite ends of this barrel. The domain A possesses the catalytic function and is the most conserved domain in the α-amylase family. The active centre of the catalytic domain is found to be located at the C-terminal end of the β-barrel in the $(\beta/\alpha)_8$ structure[11]. This $(\beta/\alpha)_8$-barrel or TIM-barrel (named for the first enzyme i.e., Triosephosphate Isomerase where the structure was observed, Banner *et al*., 1975) consists of eight stretches of parallel β-strand arranged in a barrel encircled by eight α-helices[55]. The β-strands and the α-helices are connected through irregular loops and it was observed that the first set of these loops forms the active site of the enzyme concerned. In most of the α-amylases, the catalytic domain (domain A) characteristically occurs towards the N-terminnus of the protein. The three catalytic and substrate binding residues i.e., Asp206, Glu230 and Asp297 are present on the C-terminal end loops of the 4^{th}, 5^{th} and 7^{th} β-strand of the barrel respectively[38] A typical triad is formed by these three catalytic residues with a distance between carboxylate groups ranging from 5 to 7 Å, but no direct hydrogen bond formation occur with each other[38]. The linking loops of β-strands to the nearby α-helices are usually short and may hold the active site amino acids. However, often loop 3 that connects β-strand 3 to helix 3 of the TIM barrel, is sufficiently long to fold as an independent unit which is considered as the domain B (loop 3)[60]. But, in some enzymes the loop 3 is short without any characteristic folding to be considered as a domain. In general, the domain B of α-amylases possesses several residues as substrate binding sites[39] and it is also reported to involve in binding of the structural calcium ion[5]. Studies have also revealed that several functional and stability properties, like stability at low pH, characteristic of AMY1, and sensitivity to barley amylase inhibitor, specific to AMY2 are determined by domain B[27,50]. Antiparallel β sheets present at the C-terminal part of the protein forms the domain C. It is considered that the hydrophobic residues of the $(\beta/\alpha)_8$-barrel are perhaps protected by the domain C from solvent, consequently stabilizing the catalytic domain[28].

THE CATALYTIC MECHANISM OF α-AMYLASE

The catalytic mechanism of α-amylases is well characterized with respect to Taka amylase and the α-amylase family enzymes always carry strictly conserved

three essential catalytic residues, also described as catalytic triad[39]. Of these three residues, Glu230 functions as an acid catalyst donating a proton to the glucosidic bond of the substrate, leading to cleavage and Asp206 acts as a base catalyst (nucleophile)[26,32,38,55]. The third residue, Asp297 is involved centrally to establish binding of the substrate, generating a deformation of the substrate ring at the cleavage point, which is obligatory for catalysis[38]. However, a fourth invariantly conserved GH-13 residue, i.e., β-4 arginine equivalent to the Arg204 in Taka-amylase A is also found in α-amylases which is positioned two residues preceding the catalytic nucleophile[23]. The catalytic mechanism of the α-amylase family is that of the α-retaining (the stereochemistry of the donor's anomeric bond is retained, α→α) double displacement (which would cause two inversions about the anomeric carbon for a net retention of stereochemistry) which was proposed by Koshland in 1953[19,60]. However, in the double displacement mechanism only two (Asp206 and Glu230) of the three conserved catalytic residues directly play a role. The third conserved residue, Asp297, forms hydrogen bonds with the substrate through hydrogen bonds inducing distortion of the substrate[59]. Although all α-amylases show the same catalytic mechanism, but vary widely in their reaction specificities. The attachment of different domains to the catalytic site or to extra sugar binding subsites around the catalytic site is the prime reason for these differences[60].

SOURCES OF α-AMYLASES

α-amylases are occurring all through the animal, plant and microbial kingdoms where they play a central role in carbohydrate metabolism. Besides the universal distribution of α-amylases, microbial sources, namely, fungal and bacterial, are extensively used for industrial production due to manifold advantages such as cost effectiveness, consistency, less time and space and easy manipulation of both genetic and cultural factors required for optimum production[10,16,53,57]. Among bacteria, *Bacillus* sp. and its mutant stains are extensively employed for thermostable α-amylase production to meet industrial needs. *Bacillus subtilis, B. licheniformis, B. stearothermophilus and B. amyloliquefaciens* are widely used for commercial production of α-amylase[61]. Recently *Bacillus polymyxa, B. mesentericus, B. megaterium, B. vulgaris* are also being exploited through solid state fermentation for α-amylase production[42,53]. Some Halobacteria are also being experimented to get better α-amylase activity at high saline conditions e.g., *Chromohalobacter* sp*., Halobacillus* sp*., Halomonas meridiana* etc.[10,54]. Among the fungi, mold species have been widely used for commercial production of α-amylase and a variety of other enzymes as they are known to be prolific producer of extracellular enzymes. Most reports about fungi producing α-amylases have been limited to a species of mesophilic fungi and are confined to terrestrial isolates of *Aspergillus* and *Penicillium*[57]. Among *Aspergillus, A. niger* and *A. oryzae* have wide use in large scale production of α-amylase. *A. oryzae* has received greater importance as a favourable host for the production of heterologous proteins because of its ability to secret a huge quantity of high value proteins and industrial enzymes[10,16,53]. *A. niger* has high acid tolerance (pH<3) during fermentation which allows the avoidance of bacterial contamination. The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of thermostable amylase[31]. The fungal α-amylases are considered ideal than other microbial sources due to their more accepted GRAS (Generally Recognized As Safe) status[56].

FERMENTATION METHOD

Low cost medium is always desirable for industrial production of microbial metabolites. Traditionally, amylases have been obtained from submerged fermentation (SmF). Many renowned biotechnology companies like Novozymes, MAPS etc. are employing SmF for the production of microbial enzymes. However, Solid State Fermentation (SSF) method is gaining its attention because of easy handling and greater control of environmental factors such as temperature, pH etc. for which SSF is now becoming a highly efficient method of enzyme production[57]. In Taiwan and other Asian countries, the koji process has been used to produce various enzymes by growing molds on cereals or their brans. Usually for the production of amylases by Submerged Fermentation (SmF), synthetic media have been used[16,29,53,57]. Since the contents of synthetic media such as nutrient broth, soluble starch, etc. are expensive, these could be replaced by various cheaper agricultural by-products through SSF to reduce the cost of the medium. SSF is similar to natural microbiological process such as composting and ensiling and can be utilized in a controlled way to get the desired product. The microorganisms grow on moist solid substrates with little free water, provide support and nutrition. SSF represents a promising alternative of SmF, since the metabolites so produced are concentrated leading to less costly purification procedures[44,45,46,53]. SSF is preferred to SmF because of simple technique, low capital investment, lower levels of catabolite repression and end product inhibition, less waste water output, better product recovery and high quality production[34]. Substrates that have been tried for SSF of α-amylase are wheat bran, sunflower meal, rice husk, cottonseed meal, soybean meal, pearl millet, and rice bran[47,49], among these wheat bran has been reported to be most promising[53]. SSF technique is generally confined to the process involving fungi. However, some *Bacillus* sp. have also shown good growth and production of α-amylase in SSF[52] which may require less fermentation time, 24-48 hrs.[43] leading to considerable reduction in the capital and recurring expenditure. Researches on Agro-industrial residues as suitable substrate for SSF have been receiving outmost importance due to the potential advantages for filamentous fungi to penetrate and colonize the solid substrates easily. In addition, Agro-industrial wastes not only provide an attractive alternative substrate but also help in solid waste management[57].

INDUSTRIAL APPLICATIONS OF α-AMYLASES

α-amylases are the most important hydrolytic enzymes for all starch based industries and the commercialization of amylases is the oldest among the enzymes with first production in 1894 by Dr. J. Takamine form *Aspergillus oryzae*[53]. In the present day scenario, α-amylases find potential applications in a number of industrial processes such as in food, baking, brewing, detergent, textile and paper industries[57]. α-amylases have promising applications in medical, clinical and molecular biology fields as well[16,41]. It is noteworthy that microbial amylases have completely replaced chemical hydrolysis in the starch processing industry[16]. The global market for enzymes was USD 4,411.6 million in 2013 and is estimated to increase to USD 7,652.0 million by 2020, growing at a compound annual growth rate (CAGR) of 8.3% from 2014 to 2020. North America is the dominated global market for enzymes accounting about 37.4% of total market revenue in 2013[14]. Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) are the main industries, which use about 75% of industrially produced enzymes[9].

1. Starch conversion (liquefaction and saccharification)

The most widespread application of α-amylases has in the starch processing industry, which converts starch into fructose and glucose syrups[16]. Owing to their high sweetening property, these are used in large quantities in the beverage industry as sweeteners for soft drinks. The process requires highly thermo stable α-amylase for starch liquefaction. Initially, the α-amylase of *Bacillus amyloliquefaciens* was used but it has been substituted by thermostable α-amylase of *B. stearothermophilus* or *B. licheniformis*[60].

2. Detergent industry

Enzyme based detergents are also known as green chemicals. Detergent industries are the one of the primary consumers of enzymes, in respect of both volume and value. The presence of enzymes in detergents formulations improves the detergents ability to remove tough satins converting the detergent environmentally safe[10]. Nowadays, 90% of the liquid detergents contain these enzymes with a rising demand in automatic dishwashing detergents. Generally α-amylases are sensitive to oxidants which are components of detergents and for their activity lower temperatures and alkaline pH and maintaining the necessary stability under oxidative detergent conditions are most important criteria for using in detergents[10]. These limitations can be overcome by using α-amylase from genetically modified organisms[54]. The major detergent enzyme suppliers Novozymes and Genecore International have used protein engineering to improve the bleach stability of the amylases from *B. licheniformis*[16].

3. Food and feed and starch processing industries.

α-amylases are utilized extensively in food processing industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups[10]. Thermostable α-amylases from *Bacillus* spp. have extensive use in these fields. In baking industry, the addition of α-amylase to the dough of bread degrades flour starch into smaller dextrins resulting enhanced rate of fermentation and reduce viscosity of dough which improves the volume and texture of the product. Furthermore, it produces additional sugar in the dough that improves the taste, crust, colour and toasting qualities of the bread[16,60]. Besides the generation of fermentable compounds, α-amylases also have an anti-staling effect in bread baking thereby improves the softness of baked goods increasing the shelf life of these products[1]. α-amylases are also used for the clarification of beer or fruit juices. These are used for the preparation of animal feed to improve the digestibility of fiber as feed additives too[21]. The production of starch hydrolysates such as glucose and fructose utilizes a large quantity of α-amylase. High glucose and fructose syrup are produced by starch liquefaction process applying thermostable α-amylase. Because of high sweetening property, glucose syrup is used in the beverage industry as sweeteners for soft drinks[1,54].

4. Fuel alcohol production

Ethanol, the most utilized liquid biofuel, is produced from the low cost substrates of starch. The process of bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar by using either an amylolytic microorganism or α-amylase enzyme. This is followed by ethanol fermentation that converts sugar into ethanol by Yeast, *Saccharomyces cerevisiae*[10,41]. In the economy of Brazil, the production of ethanol by yeast fermentation plays a very important role. α-amylase obtained from *B. licheniformis* or *B. subtilis* is used during the first step of hydrolysis of starch suspension[51].

5. Textile industry

In textile industry, α-amylases are employed for desizing process. Application of sizing agents like starch to yarn before fabric production ensures a fast and secure weaving by preventing the loss of string by friction, cutting and generation of static electricity on the string due to laid down wrap[2,54]. After weaving starch is removed by applying α-amylase which selectively removes the size i.e. starch layer, without attacking the fiber. The α-amylase converts starch into dextrins that are water soluble and can be removed by washing[16]. α-amylases from *Bacillus* strain have been employed in textile industries for a quit long time[10].

6. Paper industry

In pulp and paper industry, α-amylase is used for the modification of starch of coated paper, i.e. for the production of low viscosity, high molecular weight starch. Like textile industry, sizing of paper is carried out to protect the paper against mechanical damage during processing. This improves the quality of finished paper, enhancing the stiffness and strength in paper[1,41]. This also makes the surface of the paper adequately smooth

and improves the writing quality of the paper. The sizing process requires a temperature range of 45-60^0 C and different paper grades are made by varying the starch viscosity[16]. The high viscosity of natural starch, unsuitable for paper coating is adjusted by partially degrading the polymer with α-amylases in a batch or continuous process. Following products of α-amylases obtained from microorganisms are being used in paper industries: Amizyme® (PMP Fermentation Products, Peoria, USA), Termany(®, Fungamyl, BAN® (Novozymes, Denmark) and α-amylases G9995® (Enzyme Biosystems, USA)[10].

7. ***Elimination of environmental pollutants***

Starch occurs extensively in waste materials produced from the processing of plant raw materials in food and other industries. Starch-processing waste is generated in huge quantities and causes environmental problems. To purify starch containing effluents microbial amylolytic enzyme or microorganisms producing amylolytic enzymes can be employed[41].

8. ***Medical and Clinical Chemistry***

With the introduction of new fields in biotechnology, the applications of α-amylase have expanded into many other areas, such as clinical, medicinal and analytical chemistry[16]. Several processes in medicinal and clinical areas have been developed that involve the application of amylases. In the Ciba Corning Express clinical chemistry α-amylase based liquid stable reagent has been applied[4]. The use of commercially available α-amylase compounds to inhibit and remove *Staphylococcus aureus* biofilms was described[8]. Some other processes that have been developed using amylases are – detection of higher oligosaccharides[12], biosensors with an electrolyte isolator semiconductor capacitor transducer for process monitoring[40], disposable α-amylase biosensor based on ferrocene as an electron transfer mediator[7] etc.

10. Molecular applications

Reporter gene assay is indispensable for the study of gene regulatory elements and gene expression. In molecular biology, the presence of amylase can assist the method of selecting for successful integration of a reporter construct in addition to antibiotic resistance. Insertion of a foreign DNA into this gene will result in a loss of amylolytic activity in the host cell which can be assayed with a simple and inexpensive iodine staining procedure[9,41].

CONCLUSION

α-amylases are among the most widely used industrial enzymes. Carbohydrase, primarily amylases and cellulases, was the leading consumed enzyme accounting for over 45% of the global market in 2013, mainly used in industries such as the starch, textile, detergent and baking industries[14]. With the advent of new frontiers in biotechnology, there is a continuous rise in the enzyme market. Due to the expansion of new spectrum of applications of α-amylases in medical and clinical chemistry, molecular biology and bioremediation of pollutants etc., the demand is for α-amylase with high specificity, stability and efficiency. The focus of present research is on developing thermotolerant and pH tolerant α-amylase from microorganisms, modifying them genetically or applying site-directed mutagenesis to obtain desired properties in the enzyme. Commercially major portion of α-amylase is produced by submerged fermentation method, but solid-state fermentation is being looked at as a potential alternative method for its production, specially applying agroindustrial residues as substrate which may contribute to the solid waste management.

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