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Review

Improvement of microbial α-amylase stability: Strategic approaches

Tapati Bhanja Dey[a], Arvind Kumar[a], Rintu Banerjee[b], Piyush Chandna[a], Ramesh Chander Kuhad[a,*,1]

[a] Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India
[b] Microbial Biotechnology and Downstream Processing Lab, Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur, 721302, India

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ABSTRACT

Microbial α-amylase being a vital enzyme in industrial biotechnology, has also received enormous attention in academic field. Although a huge number of α-amylases from different sources have been mentioned in the literature, only few of them are able to meet the industrial demands. Thermostability, pH tolerance, calcium independency and oxidant stability and very high starch hydrolyzing efficiency are the important criteria for the diverse applications of α-amylase in starch based industries. Owing to biotechnological importance, its stability is a major concern for its economic viability. Stable microbial α-amylase can be obtained: (i) from extremophiles and genetically manipulated non extremopliles, and (ii) through improving stability by immobilization, chemical modification, protein engineering and augmenting with different additives. The present review is an attempt to compile the different strategies employed till date to obtain stable α-amylase.

Contents

Abbreviations: TAKA/TAA, α-amylase from *Aspergillus oryzae*; BLA, α-amylase from *Bacillus licheniformis*; BSUA, α-amylase from *Bacillus subtilis*; BAA, α-amylase from *Bacillus amyloliquefaciens*; AHA, α-amylase from *Pseudoalteromonas haloplanctis*; PWA, α-amylases from *Pyrococcus woesei*; PFA, α-amylase from *Pyrococcus furiosus*; BStA, α-amylase from *Bacillus stearothermophilus*; TIM, Triosephosphate isomerase; ILs, ionic liquids.

* Corresponding author.
E-mail addresses: rb@iitkgp.ac.in (R. Banerjee), kuhad85@gmail.com (R.C. Kuhad).
[1] Present address: Central University of Haryana, Jant-Pali, Mahendergarh District, Pali, Haryana, 123029, India.

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1. Introduction

α-Amylase (EC 3.2.1.1) is one of the oldest industrial enzymes of great significance for industrial biotechnology. Despite of their wide distribution across animals, plants and microbes, α-amylases of microbial origin have proved to be of great value in all starch based industries such as food, agricultural, fermentation, textiles, pharmaceuticals, detergent, brewing, baking and paper [1]. Other potential applications include treatment of starch processing waste water, bio-ethanol production, and the manufacturing of oligosaccharides mixtures and high molecular weight branched dextrins for production of powdery foods, rice cakes, etc. [2]. The major advantages of microbial α-amylases are their cost-effective bulk production, an easy manipulation of the microorganisms for desired enzyme characteristics and high productivity, less time and space requirement, and broad spectrum of stability and specificity [3]. In most of the industrial sectors, α-amylase mediated reactions are carried out under extreme conditions at very low or high pH and temperature. The conventional starch hydrolysis bioprocessing involves two steps: liquefaction and saccharification, where thermostable and acid stable α-amylases are required [4]. On the other hand alkaline and oxidative stabilities, calcium ion independency and activity at a broader temperature range are important aspects need to be possesses by α-amylase for their use in detergent industry [5]. Therefore, there is a continuous demand of stable α-amylases to meet requirements of such specific applications in various industrial sectors [6]. In this context, the discovery of new and robust α-amylases, with respect to mainly their pH and temperature tolerance, has become a trend in biotechnology. Such enzymes can be obtained from extremophiles or can be produced by the genetic manipulated of non extremophiles. Moreover, the stability of α-amylases can be improved by different processes such as immobilization, chemical modification, protein engineering and by different additives. Modification of α-amylase properties by means of protein engineering is a relatively popular and routine practice for both academic and industrial sectors. In the present review, an emphasis has been given on compiling different strategies employed for improving the stability of α-amylase.

2. Major amylases and their common properties

α-Amylase (1,4-α-D-glucan glucanohydrolase) is one of the extensively studied amylolytic enzymes and most frequently utilized in all starch based industries. Most α-amylases belong to the family 13 of glycoside hydrolases (GH13) [7] which randomly cleave the α-1,4 linkages between adjacent glucose units in the linear amylose and amylopectin chain and ultimately generate glucose, maltose, maltotriose and small dextrins.

The X-ray crystallography elucidating the structures of α-amylases isolated from *A. oryzae* (TAKA/TAA), *B. llicheniformis* (BLA), *B. subtilis* (BSUA), *B. amyloliquefaciens* (BAA), *P. haloplanktis* (AHA), *P. woesei* (PWA), and *P. furiosus* (PFA) revealed that these enzymes share common structural features such as: (i) they possess a $(\beta/\alpha)_8$ or TIM barrel structure containing the catalytic site residues, (ii) they have four highly conserved regions in their primary sequence which contains all the catalytic and most of the important substrate-binding sites, and (iii) contain Asp, Glu and Asp residues as catalytic sites [8,9]. However, a truncated mutant of *B. stearothermophilus* US100 α-amylase (AmyTM) is the only exception which contains the βαβα instead of $(\beta/\alpha)_8$ unit as the minimal subdomain associated to an enzymatic function. Moreover, it is devoid of the above mentioned three catalytic residues but still retains catalytic activity with the regeneration of a catalytic site [10].

As shown in Fig. 1, α-amylase consists of two large domains, namely N-terminal and C-terminal domains. N-terminal domain is further divided into two domains, viz., A and B. The catalytic domain A contains $(\beta/\alpha)_8$ or TIM barrel structure and domain B consists of three-stranded antiparallel β-sheet structures and protrudes between β_3 and α_3 of domain A. Domain B is involved in functional diversity and stability. Domain C with a β-sheet structure is located in the C-terminal part of the polypeptide chain [8,11] . Similar to all TIM-barrel enzymes, the α-amylase family carries the catalytic and substrate binding residues at the C-terminal of β-strands and in loops that extend from these strands [12].

3. Stability of α-amylases

The stability and functionality of native proteins are maintained by a subtle balance among the non-covalent forces or interactions, such as H bonds, ion pairs, hydrophobic and van der Waals interactions and weakening of these will result in unfolding or denaturation of the protein. According to the commonly accepted notion (Lumry-Eyring model), enzyme inactivation is a two-stage process that involves an early reversible unfolding step followed by an irreversible step [9] (Eq. (1)):

$$\mathrm{N(folded)} \leftrightarrow \mathrm{U(unfolded)} \rightarrow \mathrm{D(finaldenaturedstate)} \quad (1)$$

Irreversibility is generally caused by aggregation, misfolding, and chemical modification or in the absence of chaperones [13]. Reports are available on the stability and irreversible inactivation of the α-amylases mostly on TAKA, BLA, BAA, BStA and PFA. In some cases, thermal unfolding transitions of α-amylases have shown a partial reversibility due to the use of high concentrations of co-solvents and chemical denaturants [14–17]. Strucksberg et al. [18] found reversible unfolding of TAKA in alkaline pH and Gnd-HCl, whereas, for BLA it was only under high concentrations of protecting osmolytes (glycerol). This phenomenon supports that aggregation of the unfolded state works as the main obstacle for a proper refolding.

3.1. Sources of stable α-amylases

Stable microbial α-amylases can be obtained from two sources: (i) extremophiles (thermophiles, hyperthermophiles, halophiles, alkaliphiles, acidophiles, piezophiles, metallophiles and psychrophiles), and (ii) genetically manipulated non extremophiles. Compared to other extremophiles, thermophiles and their thermostable enzymes are in great demand in different industrial sectors. Therefore, screening of thermozymes following conventional methods is still going on as an alternative over the tedious bioengineering procedures applied for enzymes of mesophilic origin [19]. Some important and recently isolated thermophiles with efficient thermostable (optimum temperature $\geq 60\,^{\circ}C$) α-amylase production capacity are listed in Table 1. Among them *B. amyloliquefaciens* (BAA), *B. licheniformis* (BLA), *B. stearothermophilus* (BStA) and *Pyrococcus furiosus* (PFA) are the most potent producers of thermostable α-amylase at 37 °C to 60 °C. The isolation and maintenance of pure cultures of extremophiles are difficult tasks and most of them do not possess significant starch hydrolyzing efficiency. Hence, a more convenient approach has been explored for a long time to clone extremophiles genes into suitable mesophilic hosts. This gives the advantages of high productivity and high stability [20]. Some of the examples of genetic manipulations of mesophiles for the production of thermostable α-amylases are mentioned in Table 2. In this process, α-amylases were produced at ambient temperature by genetically manipulated mesophilic hosts like *Escherichia coli* or *Pichia pastoris* retaining all the properties of the thermostable enzyme. The developments of genetically modified thermophilic hosts could help in overexpreesion of the