Volume 304, number 1, 1–3 FEBS 11085 June 1992

Review Letter

α-Amylases and approaches leading to their enhanced stability

Štefan Janeček and Štefan Baláž

Department of Biochemical Technology, Slovak Technical University, Radlinského 9, CS-81237 Bratislava, Czech and Slovak Federal Republic

Received 16 March 1992; revised version received 10 April 1992

The recent state of the knowledge of properties and structure of α-amylases is reviewed with the aim of elucidation the basis for their stabilization. Three principal ways for obtaining stable α-amylases (isolation of enzymes from extremophiles, production of extremophilic enzymes in mesophiles, and modification of mesophilic enzymes) are discussed separately. Detailed experimental examples are given for modification approaches.

α-Amylase; Structure-function relationship; Enzyme stabilization

1. INTRODUCTION

Amylases are enzymes capable of hydrolyzing starch. They are produced by animals, plants, and microorganisms. Starch being a higher plants' polymer composed of α-1,4-linked α-D-glucose units is formed by amylose and amylopectin. These two starch components differ from each other in the degree of branching. Amylose is a mainly linear polysaccharide whereas the structure of amylopectin is highly α-1,6-branched.

In spite of a long history of these enzymes [1] and their wide distribution in nature as well as utilization in several industries, the structure–function and structure–stability relationships of amylases have been elucidated only for a few of them [2–7].

Since α-amylase (EC 3.2.11) is the most frequently utilized and studied amylolytic enzyme, its basic physico-chemical and structural characterization is given in the first part of this review. Although there are some microbial α-amylases which exhibit exceptional stability, those from thermophiles, acidophiles, alkalophiles, and halophiles, most α-amylases derived from animals, plants as well as microorganisms keep their unique properties only in the normal physiological conditions. Scientific investigations looking for possibilities to enhance the stability of α-amylases are reviewed in the second part of this contribution.

Correspondence address: Š. Janeček, Department of Biochemical Technology, Faculty of Chemical Technology, Slovak Technical University, Radlinského 9, CS-81237 Bratislava, Czech and Slovak Federal Republic.

2. PHYSICOCHEMICAL PROPERTIES AND STRUCTURE OF α-AMYLASES

α-Amylases catalyze the hydrolysis of α-1,4-glucosidic bonds of starch predominantly in a random manner. These endoenzymes are able to by-pass the branching points (α-1,6-bonds) in amylopectin. Formed reducing glycosidic groups are liberated in α-configuration.

All α-amylases are metalloenzymes containing at least one Ca^{2+} ion per enzyme molecule, which is essential for activity and stability. The amount of bound calcium varies from one to about ten [8]. Ca^{2+} ion in Taka-amylase A from *Aspergillus oryzae* is located near the cleft between its two structural domains, possibly playing a role in stabilizing the cleft architecture [8]. A similar situation has been identified in the porcine pancreatic α-amylase where Ca^{2+} ion appears to stabilize the cleft by inducing an ionic bridge between the domains [10]. The affinity of Ca^{2+} ions to α-amylase is much stronger than that of other cations. It is still unclear whether calcium can be replaced by other cations [8]. In addition to calcium zinc has been reported to induce formation of multiple forms of *Bacillus subtilis* α-amylase [11].

α-Amylases are generally stable in the pH range 5.5–8.0 but exceptions exist on both sides of the pH scale, mainly in the enzymes of microbial origin. As examples of this behaviour are an acidophilic α-amylase from *Bacillus* sp. 11-1S with the pH optimum of 2.0 [12] and an alkalophilic α-amylase from *Bacillus* No. A-40-2 with the optimum pH of 10.5 [13]. This broad range of optimum pH values for activity of individual α-amylases indicates their evolutionary adaptability to environmental circumstances [8].

The lowest temperature optimum for activity has been reported to be 25°C for *Fusarium oxysporum* α-amylase [14] and the highest for the enzyme from *Bacillus licheniformis* CUMC 305 to be 90°C [15]. Most of the temperature optima of α-amylases, however, are encountered between 50 and 60°C.

Molecular weights of these enzymes vary from 15,600 [16] to 139,300 [17] but the values are usually 45,000 to 60,000. Moreover, *Bacillus macerans* α-amylase with the molecular weight of 139,300 has been classified as a transferase [17].

Nowadays nearly 50 complete amino acid sequences of α-amylases are available [3] but only those of *Aspergillus oryzae* [18] and porcine pancreas enzymes [19,20] have been determined by direct protein sequencing. The others have been deduced from the nucleotide sequences. Sequence homology varies from 10% (microorganisms, plants, and animals) to 80–90% (animals) [21]. The recent study on classification of glycosyl hydrolases and related enzymes [22] has revealed 35 structural families formed by 291 amino acid sequences. It has been pointed out, however, that arrangement of the catalytic site of amylases might be homologous in spite of the lack of homology in their amino acid sequences [23]. This seems to be true because all studied α-amylases, cyclodextrin glucanotransferases and related enzymes [2,24,25] have probably the same basic $(\alpha/\beta)_8$ structure, i.e. a barrel domain of eight parallel β-strands surrounded by eight α-helices. This motif was first observed in triose phosphate isomerase [26]. This structure was recently determined also for the soybean β-amylase [27]. The $(\alpha/\beta)_8$ barrel domains in α-amylases and β-amylases are different [25]. The barrels of α-amylases containing the active sites are built up from approximately 400 amino acid residues of the N-terminal regions [2].

Three-dimensional structures have been reported for Taka-amylase A from *Aspergillus oryzae* [9], porcine pancreatic α-amylase [10], and for an acid α-amylase from *Aspergillus niger* [28]. The structures of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* [29] as well as human salivary α-amylase [30] are under way. The α-amylases with known three-dimensional structure are multidomain single polypeptide-chain proteins. Besides the $(\alpha/\beta)_8$ barrel they all comprise an antiparallel β-stranded domain [9,10,28], and the porcine pancreatic enzyme has even a small distinct domain which is inserted between the third β-strand and the third α-helix of the $(\alpha/\beta)_8$ barrel [10]. The existence of this small domain interrupting the barrel is consistent with the prediction of a long third loop stretch for all α-amylases [2].

Active sites have been localized in the cleft of the $(\alpha/\beta)_8$ barrel domain with Asp-206, Glu-230, and Asp-297 (Taka-amylase A numbering) as the residues playing the catalytic role whereas His-122 and His-296 might bind to glucosyl residues of substrate [9,10,28].

3. STABILIZATION OF MESOPHILIC α-AMYLASES

In principle, there are three ways how to obtain stable α-amylases: (i) isolation from extremophilic microorganisms; (ii) production in genetically manipulated non-extremophilic microbial producents; and (iii) stabilization of unstable α-amylases by their modification. The last approach can be realized in three ways: (i) by immobilization: (ii) by chemical modification; and (iii) by protein engineering.

α-Amylases produced by extremophilic microorganisms can be classified according to their origin as thermophilic, acidophilic, alkalophilic, and halophilic enzymes. Only the first category has found a large scale industrial use [8].

Production of α-amylases with given properties, e.g. thermostability, in microorganisms is highly attractive also from point of view of a decrease in possible health risks. Facilities in this direction are offered especially using the recombinant DNA techniques with yeasts as the expression hosts [31].

Many stabilization studies of α-amylase have reported protective effect of different additives, such as bovine serum albumine [32], Na^+, K^+, NH_4^+ cations [33], 2-mercaptoethanol and glycerol [34], polyols, dimethyl formamide, and dimethyl sulfoxide [35]. Stabilizing effect of calcium is generally known [8].

The extra thermostability of thermophilic *Bacillus licheniformis* α-amylase has been found to be mainly due to additional salt bridges involving a few specific lysine residues [36]. Similarly the *Bacillus stearothermophilus* α-amylase [35] as well as a mutant α-amylase from *Bacillus amyloliquefaciens* [4] have been suggested to be stabilized against thermal denaturation through ionic interaction.

It has been pointed out [37] that immobilization need not always lead to enzyme stabilization. Using the results from covalent immobilization of *Aspergillus oryzae* α-amylase to the γ-aminopropyl silica and aminomethyl polystyrene [38], a novel view on protein stabilization has emerged recently. Each enzyme molecule should possess a specific site where the unfolding process caused by denaturation starts. This 'unfolding nucleus' can be stabilized by covalent or noncovalent bonds after immobilization thus blocking the native unfolding pathway [38].

Like immobilization approaches, the prediction how the thermal stability of an enzyme will be affected by its chemical modification is difficult [39]. Stabilization of *Bacillus subtilis* α-amylase has been achieved by acetylation with *p*-nitrophenyl acetate [40] and by acylation with esters based on *N*-hydroxysuccinimide and 1-hydroxybenzotriazole [41]. The increase in enzyme stability has been qualitatively correlated with the polarity of the introduced acyl group [41] whereas in the case of the acetylation the thermostability has been increased only

at temperatures higher than 70°C supporting the idea of existence of so-called isokinetic temperature, T_i, with no effect at T_i and opposite effects at both sides of T_i [40]. This temperature (63–64°C) has also been recently found in investigations of thermoinactivation kinetics of *Bacillus subtilis* α-amylase stabilized by modification with D-glucono-δ-lactone [42].

4. CONCLUDING REMARKS

α-Amylase is a member of $(\alpha/\beta)_8$ barrel enzyme family [43] which probably contain all starch hydrolases and related enzymes as indicated by three-dimensional structures of cyclodextrin glucanotransferase [44,45] and β-amylase [27].

Stability of mesophilic α-amylases has not yet been increased substantially up to the level of stability of naturally thermostable α-amylases, as it has been documented in the case of proteases [46].

Stabilization of α-amylases should be oriented mainly towards utilization of their known structural features in combination, for instance, with the methods of chemical modification or site-directed mutagenesis. Various single amino acid changes resulting in the increase of thermostability of different proteins have already been reported. The examples demonstrate the role of hydrogen bonding [47], hydrophobic effect [48], disulfide [49] and salt [50] bridges. Studies on α-amylases have been aimed at elucidation of the role of individual amino acid residues [4–7]. They should allow to achieve successful stabilization of these enzymes.

REFERENCES

[1] Payen, F.W. and Persoz, J.F. (1833) Ann. Chim. Phys. 53, 73–92.
[2] MacGregor, E.A. (1988) J. Protein Chem. 7, 399–415.
[3] Svensson, B. (1991) Denpun Kagaku 38, 125–135.
[4] Suzuki, Y., Ito, N., Yuuki, T., Yamagata, H. and Udaka, S. (1989) J. Biol. Chem. 264, 18933–18938.
[5] Holm, L., Koivula, A.K., Lehtovaara, P.M., Hemmink, A. and Knowles, J.K.C. (1990) Protein Eng. 3, 181–191.
[6] Vihinen, M., Ollikka, P., Niskanen, J., Meyer, P., Suominen, I., Karp, M., Holm, L., Knowles, J. and Mäntsälä, P. (1990) J. Biochem. 107, 267–272.
[7] Declerck, N., Joyet, P., Gaillardin, C. and Masson, J.-M. (1990) J. Biol. Chem. 265, 15481–15488.
[8] Vihinen, M. and Mäntsälä, P. (1989) Crit. Rev. Biochem. Mol. Biol. 24, 329–418.
[9] Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) J. Biochem. 95, 697–702.
[10] Buisson, G., Dueé, E., Haser, R. and Payan, F. (1987) EMBO J. 6, 3909–3916.
[11] Robyt, J.F. and Ackerman, R.J. (1973) Arch. Biochem. Biophys. 155, 445–451.
[12] Uchino, F. (1982) Argric. Biol. Chem. 46, 7–13.
[13] Horikoshi, K. (1971) Agric. Biol. Chem. 35, 1783–1791.
[14] Chary, S.J. and Reddy, S.M. (1985) Folia Microbiol. 30, 452–457.
[15] Krishnan, T. and Chandra, A.K. (1983) Appl. Environ. Microbiol. 46, 430–437.
[16] Manning, G.B., Campbell, L.L. and Foster, R.J. (1961) J. Biol. Chem. 236, 2958–2961.
[17] De Pinto, J.A. and Campbell, L.L. (1968) Biochemistry 7, 114–120.
[18] Toda, H., Kondo, K. and Narita, K. (1982) Proc. Japan Acad. 58B, 208–212.
[19] Kluh, I. (1981) FEBS Lett. 136, 231–234.
[20] Passero, L., Mazzei-Pierron, Y., Abadie, B., Chicheportiche, Y. and Marchis-Mouren, G. (1986) Biochim. Biophys. Acta 869, 147–157.
[21] Nakajima, R., Imanaka, T. and Aiba, S. (1986) Appl. Microbiol. Biotechnol. 23, 355–360.
[22] Henrissat, B. (1991) Biochem. J. 280, 309–316.
[23] Friedberg, F. (1983) FEBS Lett. 152, 139–140.
[24] MacGregor, E.A. and Svensson, B. (1989) Biochem. J. 259, 145–152.
[25] Jespersen, H.M., MacGregor, E.A., Sierks, M.R. and Svensson, B. (1991) Biochem. J. 280, 51–55.
[26] Banner, D.W., Bloomer, A.C., Petsko, G.A., Phillips, D.C., Pogson, C.I., Wilson, I.A., Corran, P.H., Furth, A.J., Milman, J.D., Offord, R.E., Priddle, J.D. and Waley, S.G. (1975) Nature 255, 609–614.
[27] Mikami, B., Shibata, T., Hirose, M., Aibara, S., Sato, M., Katsube, Y. and Morita, Y. (1991) Denpun Kagaku 38, 147–151.
[28] Boel, E., Brady, L., Brzozowski, A.M., Derewenda, Z., Dodson, G.G., Jensen, V.J., Petersen, S.B., Swift, H., Thim, L. and Woldike, H.F. (1990) Biochemistry 29, 6244–6249.
[29] Suzuki, A., Yamane, T., Ito, Y., Nishio, T., Fujiwara, H. and Ashida, T. (1990) J. Biochem. 108, 379–381.
[30] Ramasubbu, N., Bhandary, K.K., Scannapieco, F.A. and Levine, M.J. (1991) Proteins: Struct. Funct. Genet. 11, 230–232.
[31] Tubb, R.S. (1986) Trends Biotechnol. 4, 98–104.
[32] Ogasahara, K., Yutani, K., Imanishi, A. and Isemura, T. (1970) J. Biochem. 67, 83–89.
[33] Galabova, D. and Velcheva, P. (1985) Acta Microbiol. Bulg. 16, 67–72.
[34] Augustin, J. and Šmogrovičová, D. (1988) Biotekhnol. Biotekh. 3, 45–46.
[35] Brumm, P.J. and Teague, W.M. (1989) Biotechnol. Lett. 11, 541–544.
[36] Tomazic, S.J. and Klibanov, A.M. (1988) J. Biol. Chem. 263, 3092–3096.
[37] Klibanov, A.M. (1979) Anal. Biochem. 93, 1–25.
[38] Schellenberger, A. and Ulbrich, R. (1989) Biomed. Biochim. Acta 48, 63–67.
[39] Torchillin, V.P. and Martinek, K. (1979) Enzyme Microb. Technol. 1, 74–82.
[40] Urabe, I., Nanjo, H. and Okada, H. (1973) Biochim. Biophys. Acta 302, 73–79.
[41] Hora, J. (1973) Biochim. Biophys. Acta 310, 264–267.
[42] Janeček, Š., Baláž, Š., Rosenberg, M. and Stredanský, M. (1992) Biotechnol. Tech. 6, 173–176.
[43] Farber, G.K. and Petsko, G.A. (1990) Trends Biochem. Sci. 15, 228–234.
[44] Hofmann, B.E., Bender, H. and Schultz, G.E. (1989) J. Mol. Biol. 209, 793–800.
[45] Klein, C. and Schultz, G.E. (1991) J. Mol. Biol. 217, 737–750.
[46] Mozhaev, V.V., Šikšnis, V.A., Melik-Nubarov, N.S., Galkantaite, N.Z., Denis, G.J., Butkus, E.P., Zaslavsky, B.Yu., Mestechkina, N.M. and Martinek, K. (1988) Eur. J. Biochem. 173, 147–154.
[47] Alber, T., Dao-pin, S., Wilson, K., Wozniak, J.A., Cook, S.P. and Matthews, B.W. (1987) Nature 330, 41–46.
[48] Kellis Jr., J.T., Nyberg, K., Šali, D. and Fersht, A.R. (1988) Nature 333, 784–786.
[49] Matsumura, M., Becktel, W.J., Levitt, M. and Matthews, B.W. (1989) Proc. Natl. Acad. Sci. USA 86, 6562–6566.
[50] Erwin, C.R., Barnett, B.L., Oliver, J.D. and Sullivan, J.F. (1990) Protein Eng. 4, 87–97.