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Amylolytic enzymes: their specificities, origins and properties

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Amylases are enzymes capable of hydrolysing starch and related saccharides. The best known are α-amylase, β-amylase and glucoamylase. Basically, they belong to glycoside hydrolases in the system of which they form their own families 13, 14 and 15, respectively, although some α-amylases were classified in the family 57. While α-amylase is an α-retaining enzyme, both β-amylase and glucoamylase use the α-inverting reaction mechanism. From the structural point of view, both α-amylase and β-amylase rank among the $(\beta/\alpha)_8$-barrel enzymes (TIM-barrels), while glucoamylase adopts the structure of parallel $(\alpha/\alpha)_6$-barrel fold. β-Amylase and glucoamylase families contain only one actual specificity, whereas the α-amylase family cover more than 20 different ECs. With regard to sources of the amylolytic enzymes, they are in general produced by various microorganisms, plants and animals, the α-amylases being found in the widest spectrum of living systems. The properties of amylolytic enzymes usually reflect the characteristics of the environment occupied by the living organism, which is the source of the enzyme.

Key words: alpha-amylase, beta-amylase, glucoamylase, glycoside hydrolases, thermostability.

Introduction

Starch and cellulose belong to the most abundant carbohydrate polymers on Earth. They both consist of glucose monomer units which are, however, differently bound to form polymer chains: starch contains the glucose linked up by the α-glucosidic bonds while the glucose in cellulose is bound by the β-glucosidic linkages. Therefore these two important sources of energy for animals, plants and microorganisms are biochemically hydrolysed by two different groups of enzymes: starch by α-glycoside hydrolases and cellulose by β-glycoside hydrolases. Since starch consists of two distinct fractions: amylose – linear α-1,4-linked glucans, and amylopectin – linear α-1,4-

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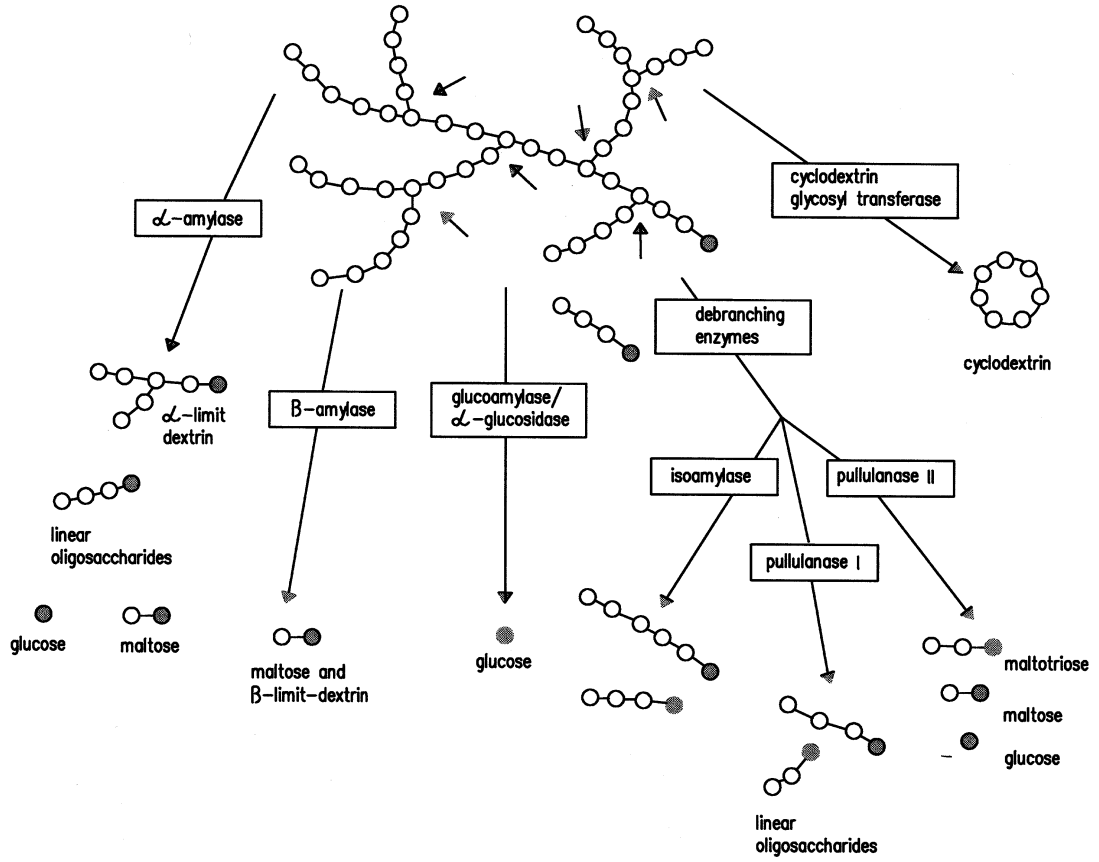


Fig. 1. Action of starch-degrading enzymes. (●) Reducing α-D-glucose residue; (○) non-reducing α-D-glucose residue. Arrows indicate the α-1,6-branching points in the starch molecule. Adapted from ANTRANIKIAN (1991).

linked glucans branched with α-1,6 linkages (BALL et al., 1996; MOUILLE et al., 1996), the enzymes responsible for its hydrolysis are called amylolytic enzymes or simply amylases.

This review deals with the enzymes operating on starch. Its main goal is to provide a brief overview of the three most known amylolytic enzymes, i.e. α-amylases, β-amylases and glucoamylases. The emphasis is given on the description of their (i) specificities with regard to EC numbers (and also to families of glycoside hydrolases); (ii) producers coming from all the three domains of life (Bacteria, Archaea and Eucarya); and (iii) basic characteristic properties such as pH-stability and thermostability (with special focus on hyperstability of amylases from archaebacteria).

Amylolytic enzymes

At present there are more than about 30 different amylolytic and related enzymes (JANEČEK, 1997). Degradation of starch is essentially performed by the four groups of enzymes (GUZMÁN-MALDONADO & PAREDES-LÓPEZ, 1995): endo- and exo-amylases acting primarily on α-1,4-linkages, debranching enzymes attacking mainly the α-1,6-linkages, and cyclodextrin glycosyltransferases that degrade starch by catalysing mainly cyclisation and disproportionate reactions (Fig. 1).

Endoamylases cleave only the α-1,4-bonds in starch in the inner regions of the starch molecule by passing the α-1,6-branching points of amylopectin (VIHINEN & MÄNTSÄLÄ, 1989). The α-amylase (EC 3.2.1.1) is the best known endoamylase. It causes a rapid loss of viscosity of the starch solution. These enzymes are often divided according to degree of hydrolysis of substrate into two categories: liquifying (30–40%) and saccharifying (50–60%). This division is widely used to describe the properties of α-amylases (VIHINEN &

MÄNTSÄLÄ, 1989). Thus the products of endoamylases are oligosaccharides of varying lengths.

Exoamylases also cleave the α-1,4-bonds, e.g. β-amylase (EC 3.2.1.2), but some of them are able to attack the α-1,6-bonds, e.g. glucoamylase (EC 3.2.1.3). These enzymes act externally on substrate bonds from the non-reducing end of starch and hence produce only low molecular-weight products from starch, e.g. maltose and glucose, respectively (WIND, 1997).

Pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) may be the examples of debranching enzymes. Both are specific for α-1,6-bonds in starch (amylopectin) and related polysaccharides and branched limit dextrins. According to the inability or ability to degrade also the α-1,4-glucosidic bonds, pullulanases are classified into two categories (WIND, 1997): pullulanase I and pullulanase II, respectively. Pullulanase type II is usually referred to as α-amylase-pullulanase or amylopullulanase. However, to make it clear the specificity should be proved which enzyme it refers to.

The fourth group of starch-degrading enzymes are the cyclodextrin glycosyltransferases (CGTases, EC 2.4.1.19). They produce cyclodextrins from starch, the rings which are composed of 6, 7 or 8 glucose units bound by α-1,4-bonds (PÓCSI, 1999). The CGTases catalyse intra- and intermolecular reaction of glycosyl transfer (SVENSSON & SØGAARD, 1993).

Specificities of amylolytic enzymes

The best known amylolytic enzymes are α-amylase, β-amylase and glucoamylase. As has been indicated above, however, these three amylases are quite distinct from both functional and structural points of view. This furthermore implies that there exists a rather long evolutionary distance between them (JANEČEK, 1994a). They constitute their own independent families with no sequence similarities (PUJADAS et al., 1996; COUTINHO & REILLY, 1997; JANEČEK, 1997). Thus in the sequence-based classification of glycoside hydrolases developed almost 10 years ago (HENRISSAT, 1991), α-amylases, β-amylases and glucoamylases were found in the families 13, 14 and 15, respectively. Both β-amylases and glucoamylases are found in the only one sequence-based family, family 14 and family 15, respectively, (HENRISSAT & BAIROCH, 1993). With regard to α-amylases, however, they should be considered as the α-amylase family with many various specificities covering their main family 13 together with two related families 70 and 77 established recently (COUTINHO & HENRISSAT, 2000). Moreover, there are some α-amylases and related enzymes that have been found to exhibit no sequence similarities with the main family 13 and thus placed into the different family 57 (HENRISSAT & BAIROCH, 1996).

All this means that the amylases (and related enzymes) belonging to families 13 (plus 70 and 77), 14 and 15 differ from each other by their amino acid sequences, i.e. also by their three-dimensional structures. α-Amylases, β-amylases and glucoamylases use furthermore different reaction mechanisms (Fig. 2) and catalytic machineries for cleaving the glucosidic bonds (HENRISSAT & DAVIES, 1997; KANEKO et al., 1998).

α-Amylase glycoside hydrolase family 13

The recent list of the members of the α-amylase family is shown in Table 1. There are not only hydrolases (EC 3) but also transferases and isomerases from the enzyme classes 2 and 5, respectively. It is worth mentioning that not all of these enzymes are attacking the glucosidic bonds in starch. They all have in common (MACGREGOR, 1993; SVENSSON, 1994; JANEČEK, 1997; KURIKI & IMANAKA, 1999): (i) sequence similarities (so-called conserved sequence regions) covering the equivalent elements of their secondary structure (especially the β-strands); (ii) catalytic machinery (Asp, Glu and Asp residues at β-strands β4, β5 and β7, respectively); (iii) retaining reaction mechanism (the resulting hydroxyl group retains the α-configuration); and (iv) three-dimensional fold (TIM-barrel).

The first three-dimensional structure of an α-amylase solved was that of the Taka-amylase A, i.e. the α-amylase from *Aspergillus oryzae* (MATSUURA et al., 1984). The enzyme adopts the so-called TIM-barrel fold, which is the motif, found firstly in the structure of triosephosphate isomerase (BANNER et al., 1975) and now found in about 50 different enzymes and proteins (REARDON & FARBER, 1995, JANEČEK & BATEMAN, 1996; PUJADAS & PALAU, 1999). The motif, called also $(\beta/\alpha)_8$-barrel, consists of eight parallel β-strands forming the inner β-barrel which is surrounded by the outer cylinder composed of eight α-helices so that the individual β-strands and α-helices alternate and are connected by loops (Fig. 3a).

Although all the members of the α-amylase family listed in Table 1 should share the characteristics given above, some of them have been classified in the new families of glycosidases in the sequence-based classification system (COUTINHO

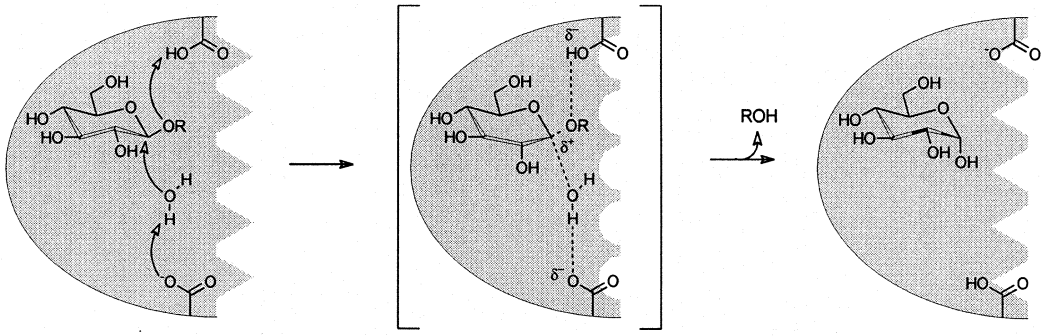
& Henrissat, 2000). Thus the sucrose-utilising glucosyltransferases (EC 2.4.1.5) have been placed into the family 70 because their catalytic domain was predicted to contain a circularly per-

Table 1. Classification of the α-amylase family (glycoside hydrolase families 13, 70 and 77) members according to EC numbers.[a]

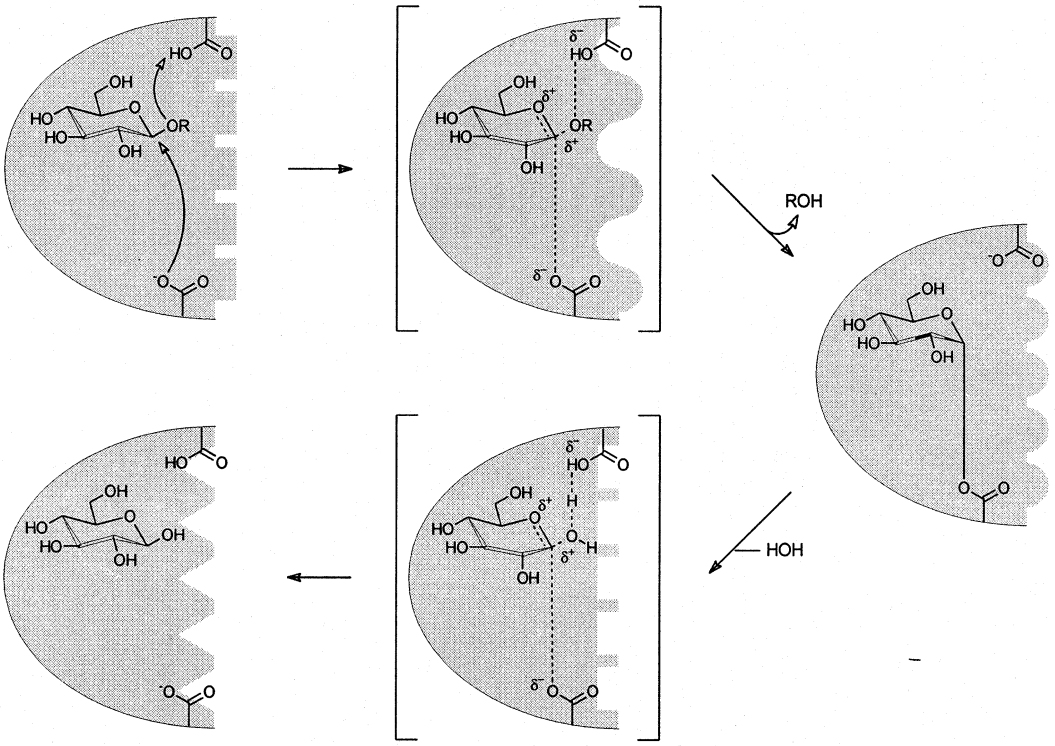
Enzyme class	Enzyme name	EC number
Hydrolases	α-Amylase	3.2.1.1
	Oligo-1,6-glucosidase	3.2.1.10
	α-Glucosidase	3.2.1.20
	Pullulanase	3.2.1.41
	Amylopullulanase	3.2.1.1/41
	Cyclomaltodextrinase	3.2.1.54
	Maltotetraohydrolase	3.2.1.60
	Isoamylase	3.2.1.68
	Dextran glucosidase	3.2.1.70
	Trehalose-6-phosphate hydrolase	3.2.1.93
	Maltohexaohydrolase	3.2.1.98
	Maltotriohydrolase	3.2.1. 116
	Maltogenic amylase	3.2.1.133
	Neopullulanase	3.2.1.135
	Maltooligosyltrehalose hydrolase	3.2.1.141
	Maltopentaohydrolase	3.2.1.-
Transferases	Amylosucrase	2.4.1.4
	Glucosyltransferase	2.4.1.5
	Sucrose phosphorylase	2.4.1.7
	Glucan branching enzyme	2.4.1.18
	Cyclodextrin glucanotransferase	2.4.1.19
	4-α-Glucanotransferase	2.4.1.25
	Glucan debranching enzyme	2.4.1.25/3.2.1.33
	Alternansucrase	2.4.1.140
	Maltosyltransferase	2.4.1.-
Isomerases	Maltooligosyltrehalose synthase	5.4.99.15
	Trehalose synthase	5.4.99.16

[a]Based on the close sequence similarities with oligo-1,6-glucosidases, the mammalian proteins that induce transport of dibasic and neutral amino acids across cell membranes, and the mammalian 4F2 heavy-chain cell surface antigens (both without any enzymatic activity) have been shown to be in close evolutionary relationships with the α-amylase family enzymes (Janeček et al., 1997) forming thus together one protein structural family.

Fig. 2. Proposed mechanism of inverting and retaining glycoside hydrolases. α-Amylase uses a retaining mechanism, while both β-amylase and glucoamylase belong to inverting enzymes. The inverting glycosidases use a direct displacement mechanism, while the catalysis by retaining glycosidases proceeds via a two-step double-displacement mechanism. In the inverting mechanism the two active-site carboxylic acid residues are suitably oriented so that one assists as a general base to the attack of water, while the other serves as a general acid to cleavage of the glycosidic bond. In the retaining mechanism, involving the formation and hydrolysis of a covalent glycosyl-enzyme intermediate, the roles of the two active-site carboxylic acid residues are somewhat different in comparison with the inverting mechanism. One, playing the role of the nucleophile, attacks at the sugar anomeric centre to form the glycosyl-enzyme species, while the other acts as an acid/base catalyst, protonating the glycosidic oxygen in the first step (general-acid catalysis) and deprotonating the water in the second step (general-base catalysis). Reaction in both mechanisms proceeds through an oxocarbenium ion-like transition states (shown in square brackets). The distance between the two catalytic residues $\sim$0.95 nm in the inverting mechanism is presumably just right to allow the water and the substrate to bind simultaneously. In the retaining mechanism the shorter distance between the two residues ($\sim$0.55 nm) is consistent with the need for direct attack of the nucleophile. Adapted from Ly & Withers (1999).



Inverting mechanism



Retaining mechanism

muted version of the α-amylase-type $(\beta/\alpha)_8$-barrel (MacGregor et al., 1996). This is also the case for the very recent member of the α-amylase family, alternansucrase (Argüello-Morales et al., 2000). Furthermore, some amylomaltases (EC 2.4.1.25) with sequences exhibiting low sequence similarities with the main α-amylase family members have become the basis for the new family 77 (Coutinho & Henrissat, 2000). However, the three-dimensional structure of amylomaltase from *Thermus aquaticus* (Przylas et al., 2000) confirmed that also this enzyme possesses the $(\beta/\alpha)_8$-barrel structure with the arrangement of the catalytic side-chains (two Asp residues and one Glu residue) similar to that of the α-amylase family. At present the α-amylase family therefore covers in the sequence-based classification system a clan of three related families of glycoside hydrolases designated as the clan GH-H (Coutinho & Henrissat, 2000).

With regard to the quarternary structure, a remarkable behaviour was described for the α-amylase from *Bacillus subtilis*, which is able to form dimers in the presence of zinc (Fogarty, 1983). The conditions necessary for obtaining the subunit forms of this α-amylase were given by Robyt & Ackerman (1973).

α-Amylase glycoside hydrolase family 57

More than ten years ago a sequence of a heat-stable amylase from a thermophilic bacterium *Dictyoglomus thermophilum* was published (Fukusumi et al., 1988). Despite this sequence has coded for an α-amylase, it did not exhibit any detectable similarities with the known sequences belonging to the α-amylase family. Later a similar sequence of the α-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* was determined (Laderman et al., 1993). These two sequences became the basis for a new family of α-amylases, glycoside hydrolase family 57 (Henrissat & Bairoch, 1996). The main reason for establishing the new family was the fact that the peculiar α-amylases lack the conserved sequence regions (Janeček, 1994a) characteristic for the main α-amylase family 13.

At present the family 57 contains about ten members mostly from extremophiles (Coutinho & Henrissat, 2000; Janeček et al., 2000) having the α-amylase, 4-α-glucanotransferase and amylopullulanase specificities (Tab. 2). Although the prediction attempts aimed at revealing the eventual relatedness between the α-amylase families 13 and 57 have neither been confirmed nor disproved yet (Dong et al., 1997; Janeček, 1998), the common retaining mechanism used by both families (Coutinho & Henrissat, 2000) still preserves the possibility to classify them into a larger superfamily or a common clan. The final answer concerning the relatedness of these two seemingly unrelated α-amylase families will be possible to obtain only by the results of site-directed mutagenesis and crystallographic studies.

β-Amylase and glucoamylase: families 14 and 15, respectively

The structural differences between α-amylase, β-amylase and glucoamylase predicted by Jespersen et al. (1991) were confirmed by the determination of the first crystal structures of *Aspergillus awamori* glucoamylase (Aleshin et al., 1992) and soybean β-amylase (Mikami et al., 1993) in addition to the several known structures of α-amylases and cyclodextrin glucanotransferases from the α-amylase family (Matsuura et al., 1984; Qian et al., 1993; Hofmann et al., 1989; Brady et al., 1991; Kubota et al., 1991). Remarkably, β-amylase adopts also the structure of TIM-barrel like the α-amylase (Fig. 3a), however, these two $(\beta/\alpha)_8$-barrels have been found to be quite different in agreement with the predictions (Jespersen et al., 1991). These differences reflect the differences in the amino acid sequences of both α-amylases and β-amylases (Janeček, 1994a). Then it is not surprising that the catalytic machinery of β-amylase consisting of two Glu residues (Mikami et al., 1994; Totsuka & Fukazawa, 1996) is different from that of α-amylase.

Glucoamylase has completely different structure from both α-amylase and β-amylase. It adopts a helical $(\alpha/\alpha)_6$-barrel fold (Fig. 3b), which consists of six mutually parallel α-helices forming an inner core (helical barrel mimicking the inner β-barrel of α-amylase and β-amylase), which is covered by a peripheral set of six further α-helices (Aleshin et al., 1992; Ševčík et al., 1998). The peripheral α-helices are parallel to each other, but antiparallel to the inner core of α-helices. Two Glu residues are responsible for catalysis (Harris et al., 1993).

With regard to reaction mechanism, both β-amylase and glucoamylase use the inverting mechanism (Fig. 2), i.e. they invert the anomeric configuration of the resulting hydroxyl group to β.

Origins of amylolytic enzymes

Enzymes capable of hydrolysing starch and related saccharides are produced by both prokaryotes and eukaryotes, i.e. by organisms belonging

Table 2. Classification of the α-amylase family 57 members according to EC numbers.[a]

Enzyme class	Enzyme name	EC number
Hydrolases	α-Amylase	3.2.1.1
	Pullulanase	3.2.1.41
	Amylopullulanase	3.2.1.1/41
Transferases	4-α-Glucanotransferase	2.4.1.25

[a]The glycoside hydrolase family 57 contains also several ORFs of putative proteins obtained mainly from sequencing of the complete genomes of, e.g., *Methanococcus jannaschi* (Bult et al., 1996), *Aquifex aeolicus* (Deckert et al., 1998) and *Treponema pallidum* (Fraser et al., 1998). These putative proteins exhibit sequence similarities with the enzymes from the table (see the URL: http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf_57.html).

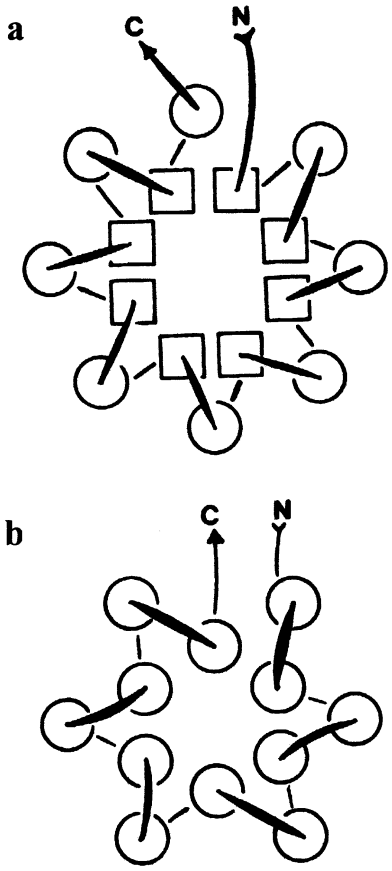


Fig. 3. Schematic representation of secondary structure of a $(\beta/\alpha)_8$-barrel fold (a) characteristic of α-amylase and β-amylase, and an $(\alpha/\alpha)_6$-barrel fold (b) characteristic of glucoamylase. The squares and circles represent the β-strands and α-helices, respectively. Adapted from Aleshin et al. (1992).

to all the three domains of life: Bacteria, Archaea and Eucarya. In other words, different amylolytic enzymes are known to be of animal, plant and microbial origins. Since degradation of starch usually requires co-operation of several amylolytic enzymes, starch-degrading organisms often have several amylolytic activities (Vihinen & Mäntsälä, 1989). On the other hand, not all the living organisms able to utilise starch and/or related saccharides always produce all the enzymes necessary for complete degradation of these substrates.

α-Amylases and related enzymes (e.g. α-glucosidases, pullulanases; see Table 1) as well as glucoamylases have been reported to occur in a wide variety of organisms, especially of microorganisms (Fogarty, 1983; Vihinen & Mäntsälä, 1989). They are produced also by animals and plants (Janeček & Baláž, 1992; Pandey, 1995). On the other hand, β-amylases have been found to be distributed in higher plants and some microorganisms only (Adachi et al. 1998; Mikami et al., 1999).

Due to the improvement of the industrial starch degradation process there has been a great interest in extremely thermostable amylolytic enzymes, especially in glucoamylase involved in the second step, i.e. in conversion of starch dextrin to glucose (Legin et al., 1998; Reilly, 1999). In the first step, degradation of starch into the limit dextrin, the highly thermostable α-amylase from *Bacillus licheniformis* is used at about 95 °C (Legin et al., 1998). The used glucoamylase, which is produced by filamentous fungi, works at 60 °C (Reilly, 1999). Having thermostable glucoamylase suitable for the industrial use would reduce the cost of the starch degradation process by reducing the process to a single step. Since Archaea were found to be a good source of hyperextremostable enzymes (Woese, 1987), many efforts have been aimed at finding, isolation and biochemical characterisation of amylolytic enzymes, especially glucoamylase, of archaeal origin. Unfortunately, although there were found a lot of

α-amylases and related enzymes (see Tab. 1) produced by various archaebacteria (Sunna et al., 1997; Bauer et al., 1998; Niehaus et al., 1999; Lévêque et al., 2000), archaeal glucoamylase was reported only as a putative protein derived from the complete genome sequencing of the methanogenic archaeon *Methanococcus jannaschii* (Bult et al., 1996). It is remarkable that from the evolutionary point of view, archaeal hyperthermostable α-amylases were found to be most closely related to their plant counterparts with thermostability around 40 °C (Janeček et al., 1999). The putative glucoamylase from *M. jannaschii* representing archaeal glucoamylases, which is at present a subject of biochemical characterisation (Reilly, 1999), seems to be related to eubacterial counterparts (Coutinho & Reilly, 1997). However, since no sequence of a plant glucoamylase has been available, the image of glucoamylases relationships cannot be completed.

Physicochemical properties of amylolytic enzymes

Properties of enzymes hydrolysing starch and related saccharides vary and are more or less linked to the environmental niche occupied by the producing organisms (Vihinen & Mäntsälä, 1989). This is especially true for microbial amylolytic enzymes which can be classified as those produced by mesophiles or extremophiles. Extremophilic enzymes can be further specified as those originated from thermophiles, psychrophiles, alkaliphiles, acidophiles and halophiles. The higher hydrostatic pressure as a further potential environmental factor, i.e. the microorganisms called barophiles, should also be taken into account. The resistance of enzymes to the effects of organic solvents is of special interest with regard to their physicochemical properties. General aspects of molecular adaptation to extreme conditions were reviewed by Jaenicke (1991), whereas Klibanov (1989, 1997) reviewed the possibilities of using the enzymes for work in anhydrous conditions. Some of the extremophilic enzymes may exhibit combined properties, e.g. thermostability and acidophily (Rolfsmeier & Blum, 1995), mainly the amylolytic enzymes produced by Archaea (Lévêque et al., 2000).

In general, fungal enzymes can be characterised by higher acidostability while bacterial enzymes are more thermostable. Amylolytic enzymes produced by plants and animals are usually stable at pH close to physiological conditions (around 7.0) and at temperatures not dramatically elevated above 40–50 °C. Microorganisms have been found to be the best sources of amylases and related enzymes exhibiting their optimal properties at extreme conditions (Vihinen & Mäntsälä, 1989): (i) thermophilic (psychrophilic) microorganisms produce more (less) thermostable enzymes in comparison with mesophiles; (ii) enzymes from halophilic microorganisms require salts to be active and are most active at such a salt concentration that inhibits other amylases; (iii) alkaliphiles and acidophiles produce enzymes which are most active at extreme pH values. Of these extremostable enzymes, thermostable enzymes have been the most deeply studied and have become the remarkable tools for studying protein stability and for developing commercial biotechnologies (Zeikus et al., 1998). These enzymes come from thermophilic (growth at temperatures above 60 °C) and hyperthermophilic (growth at temperatures above 80 °C) organisms (Stetter, 1999). They maintain their optimal activity at very high temperatures: from >60 °C to 120 °C (Zeikus et al., 1998).

Thermostable and thermolabile amylolytic enzymes

The properties of amylolytic enzymes produced by extremophilic microorganisms (of both bacterial and archaeal origins) can be found in several recent reviews (e.g. Sunna et al., 1997; Bauer et al., 1998; Niehaus et al., 1999; Lévêque et al., 2000). Hyperthermostable amylolytic enzymes may expand the horizons of new frontiers in microbiology, biochemistry and biotechnology (Zeikus et al., 1998). It is worth mentioning that some of the hyperthermophiles contain the amylases and related enzymes that are at the molecular level different from their ordinary counterparts. This was the case of the thermostable α-amylases from thermophilic bacterium *Dictioglomus thermophilum* (Fukusumi et al., 1988) and the hyperthermophilic archaeon *Pyrococcus furiosus* (Laderman et al., 1993), the sequences of which exhibited no obvious homology to usual α-amylases from the family 13, and thus a new family 57 of glycoside hydrolases was established (Tab. 2). Similarly, Galichet & Belarbi (1999) reported a new α-glucosidase gene from a hyperthermophilic archaeon *Thermococcus hydrothermalis* that is also different from usual α-glucosidases. In addition to amylases from hyperthermophilic archaea, a special interest has been evoked by studies on the amylolytic enzymes from hyperthermophilic bacterium *Thermotoga maritima* (Liebl et al., 1997; Bibel et al., 1998), since this bacterium is capable of cell division at temperatures up to 90 °C. Very recently a CGTase (which is a

member of the α-amylase family 13; see Table 1) from the hyperthermophilic archaeon *Thermococcus* sp. B1001 was isolated, purified and characterised (Tachibana et al., 1999; Yamamoto et al., 1999) and also sequenced (Fujiwara et al., 1999).

For β-amylases and glucoamylases there have been substantially less reports on their thermostability. The most thermostable β-amylase can be the one from thermophilic bacterium *Clostridium thermosulfurogenes* (Kitamoto et al., 1988) with 75 °C as the optimal temperature for its activity (Hyun & Zeikus, 1985). With regard to glucoamylases, the one from thermophilic fungus *Thermomyces lanuginosus* (Mishra & Maheshwari, 1996), reported to be fully active at 60 °C for 7 hours can be considered as the most thermostable glucoamylase.

As far as the amylases from psychrophilic microorganisms are concerned (for a review, see Gerday et al., 1997), the thermolabile α-amylase from Antarctic psychrotroph *Alteromonas haloplanctis*, with the optimal temperature for its activity between 20–30 °C (Feller et al., 1992), is the best studied amylolytic enzyme (Feller et al., 1994; 1996; 1999). Despite the fact that its three-dimensional structure is known to adopt the classical α-amylase-type TIM-barrel fold (Aghajari et al., 1998) it was revealed by two independent studies (Feller et al., 1994; Janeček, 1994b) to exhibit close sequence similarity to animal α-amylases, i.e. from the evolutionary point of view, this thermolabile bacterial α-amylase is closely related also to human salivary and pancreatic α-amylases.

Further examples of extremophilic amylolytic enzymes

Acidophilic microorganisms that have adapted to the acid conditions (growth at pH values between 1–4) by maintaining their cytoplasmic pH at a value close to neutrality (Matzke et al., 1997) are the sources of acidostable amylolytic enzymes. Although the most acidophilic α-amylase is probably the amylase isolated from a thermophilic acidophilic *Bacillus* sp. 11-1S with the pH optimum 2.0 (Uchino, 1982), the most deeply studied is the α-amylase from *Alicyclobacillus acidocaldarius* with pH optimum 3.0 (Koivola et al., 1993; Schwermann et al., 1994).

Alkaliphiles, on the other hand, are microorganisms that like to grow at high values of pH (for a review, see Horikoshi, 1996). For instance an interesting alkaline amylopullulanase from alkaliphilic *Bacillus* sp. KSM-1378 was reported (Ara et al., 1995; 1996) which consists of two functional domains: one for the amylase activity (for the α-1,4 hydrolysis) and the other one for the pullulanase activity (for the α-1,6 hydrolysis). Remarkably, the amylase activity of this amylopullulanase was completely inhibited by incubation of the enzyme at 40 °C and pH 9.0 within 4 days, whereas the pullulanase activity remained at the original level under the same conditions (Ara et al., 1995). As a further example, the archaeal maltotrioseforming amylase from *Natronococcus* sp. Ah-36 (Kobayashi et al., 1992) can be used with pH optimum 8.7. This amylase, which is the member of the α-amylase family (Kobayashi et al., 1994; cf. Table 1), has also a halophilic character because it exhibited its maximal activity in the presence of 2.5 M NaCl (Kobayashi et al., 1992). Coronado et al. (2000a) have isolated and biochemically characterised an α-amylase from a halophilic bacterium *Halomonas meridiana* for which a considerable amylase activity was detected even at 30% NaCl. Interestingly, this halophilic bacterial α-amylase exhibits sequence similarity to the animal group of α-amylases (Coronado et al., 2000b), alike the psychrophilic α-amylase from *A. haloplanctis* (Feller et al., 1994; Janeček, 1994b).

With regard to barophilic amylolytic enzymes, according to our knowledge no report on an amylase with barophilic character has been published yet. Some studies were done with fungal and animal α-amylases on the effect of pressure on their activity and stability (Makimoto et al., 1989; Matsumoto et al., 1997). The potential sources of barophilic proteins are living organisms (especially microorganisms) occupying the deep sea conditions, which include pressures up to 120 MPa (for reviews, see Gross & Jaenicke, 1994; Robb & Clark, 1999).

Conclusion

The present review deals with the amylolytic enzymes, which can be considered to be one of the most widely, distributed enzymes in nature. It is focused on their specificities, origins and properties with a special emphasis given to the three best known amylases: α-amylase, β-amylase and glucoamylase. Despite the fact that these enzymes are quite close by their functions (all operate on starch and related saccharides), not all of them share common structure, reaction mechanism and evolution. They are produced by a great variety of living organisms covering all the three domains of life, two of them being prokaryotic (microbes from Bacteria and Archaea) and the third one be-

ing eukaryotic (Eucarya with fungi, plants, insects, birds, mammals). This biodiversity yields almost all possible properties including those characteristic of mesophiles, thermophiles, psychrophiles, acidophiles, alkaliphiles, halophiles, and potentially also barophiles.

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