

Review

## Amylolytic Enzymes: Molecular Aspects of Their Properties

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**Abstract.** The present review describes the structural features of  $\alpha$ -amylase,  $\beta$ -amylase and glucoamylase that are the best known amylolytic enzymes. Although they show similar function, i.e. catalysis of hydrolysis of  $\alpha$ -glucosidic bonds in starch and related saccharides, they are quite different.  $\alpha$ -Amylase is the  $\alpha \rightarrow \alpha$  retaining glycosidase (it uses the retaining mechanism), and  $\beta$ -amylase together with glucoamylase are the  $\alpha \rightarrow \beta$  inverting glycosidases (they use the inverting mechanism). While  $\beta$ -amylase and glucoamylase form their own families 14 and 15, respectively, in the sequence-based classification of glycoside hydrolases,  $\alpha$ -amylase belongs to a large clan of three families 13, 70 and 77 consisting of almost 30 different specificities. Structurally both  $\alpha$ -amylase and  $\beta$ -amylase rank among the parallel  $(\beta/\alpha)_8$ -barrel enzymes, glucoamylase adopts the helical  $(\alpha/\alpha)_6$ -barrel fold. The catalytic  $(\beta/\alpha)_8$ -barrels of  $\alpha$ -amylase and  $\beta$ -amylase differ from each other. The only common sequence-structural feature is the presence of the starch-binding domain responsible for the binding and ability to digest raw starch. It is, however, present in about 10 % of amylases and behaves as an independent evolutionary module. A brief discussion on structure-function and structure-stability relationships of  $\alpha$ -amylases and related enzymes is also provided.

**Key words:** Amylase — Catalytic barrel domain — Starch-binding domain — Structure-function relationships

**Abbreviations:** CGTase, cyclodextrin glucanotransferase; SBD, starch-binding domain.

## Introduction

Amylolytic enzymes form a large group of enzymes operating on starch and related oligo- and polysaccharides. The three best known amylases are  $\alpha$ -amylase,  $\beta$ -amylase and glucoamylase (rarely  $\gamma$ -amylase). Since the starch or pullulan and glycogen belong to the important sources of energy for microorganisms, plants and animals, amylolytic enzymes are produced by a great variety of living systems (Vihinen and Mäntsälä 1989). Despite the fact that they have related function (they all catalyse the cleavage of the  $\alpha$ -glucosidic bonds in the same substrate), structurally and mechanistically they are quite different (Janeček 2000a). Both  $\alpha$ -amylase and  $\beta$ -amylase adopt the structure of a TIM-barrel fold (for a review, see Pujadas and Palau 1999), i.e. their catalytic domain consists of a  $(\beta/\alpha)_8$ -barrel formed by 8 parallel  $\beta$ -strands surrounded by 8  $\alpha$ -helices (Matsuura et al. 1984; Mikami et al. 1993), however, the barrels are not similar in details (Jespersen et al. 1991). Glucoamylase possesses the structure of an  $(\alpha/\alpha)_6$ -barrel, i.e. the helical one, consisting of the inner barrel composed of 6  $\alpha$ -helices and surrounded by further 6 ones (Aleshin et al. 1992). Strands and helices of the  $(\beta/\alpha)_8$ -barrel domain as well as the helices of the  $(\alpha/\alpha)_6$ -barrel are connected by loop regions of various length. Based on similarities and differences in their primary structure, amylolytic enzymes have been classified into families in the classification system of glycoside hydrolases (Henrissat 1991):  $\alpha$ -amylases – family 13,  $\beta$ -amylases – family 14, and glucoamylases – family 15. This classification reflects also the differences in the reaction mechanism and catalytic machinery used by the three amylases (Davies and Henrissat 1995).

Due to the enormous accumulation of new sequence data in the recent years, the  $\alpha$ -amylase family 13 has expanded so that it contains almost 30 different enzymes and proteins (e.g. pullulanase, isoamylase, neopullulanase) having sequence relatedness to  $\alpha$ -amylases (Janeček 2000a). At present all these are classified in families 13, 70 and 77 forming a glycoside hydrolase clan GH-H (Coutinho and Henrissat 2000). Moreover, there is also family 57 (Henrissat and Bairoch 1996) which contains a few  $\alpha$ -amylolytic specificities, however, with no clear sequence similarity to the main family 13 (Janeček 1998) and thus remains outside the scope of this article.

This review focuses on structural characteristics of the main representatives of amylases. Emphasis is given on their catalytic domains and machineries. The non-catalytic modules, especially the domain B of  $\alpha$ -amylases and the starch-binding domain (SBD) responsible for degradation of raw starch, are also presented. The structural features are described with respect to the function and, if possible, stability of amylolytic enzymes.

## Structure of retaining $\alpha$ -amylase and related enzymes

$\alpha$ -Amylase is the representative of the large enzyme family (glycoside hydrolase clan GH-H) well-known as the  $\alpha$ -amylase family (Janeček 2000a). It consists of

27 different enzyme specificities (Horváthová et al. 2000). Based on sequence similarities two mammalian proteins, the amino acid transport-related proteins and the 4F2 heavy-chain cell surface antigens (Wells and Hediger 1992; Bertran et al. 1992), they were proposed to belong to the  $\alpha$ -amylase family (Janeček et al. 1997). These two proteins are, however, without any catalytic potential so that they are not enzymes and may be added to the  $\alpha$ -amylase family only as structurally and evolutionarily related protein members (Janeček 2000b). The  $\alpha$ -amylase family can now be seen in detail at its own web-site ALAMY (Janeček et al. 1999) which is a database of proteins and enzymes belonging to the family.

In general, all enzymatic members of a homologous family should have in common the catalytic reaction mechanism, the catalytic machinery (identical residues on identical secondary structure elements) and the fold. The  $\alpha$ -amylase family was defined (Takata et al. 1992) as a family of enzymes that: (i) catalyse hydrolysis and/or transglycosylation at the  $\alpha$ -1,4- and  $\alpha$ -1,6-glucosidic linkages; (ii) act with the retaining mechanism with retention of the  $\alpha$ -anomeric configuration; (iii) have four highly conserved sequence regions containing all the catalytic residues and most of the substrate binding sites; and (iv) possess Asp, Glu and Asp residues as catalytic sites corresponding to Asp206, Glu230 and Asp297 of Taka-amylase A (Matsuura et al. 1984; Nakajima et al. 1986; Kuriki and Imanaka 1999). At present this definition could be slightly modified because, e.g., the enzymes acting on trehalose (trehalose synthase) and sucrose (sucrose phosphorylase), i.e. linkages other than  $\alpha$ -1,4- and  $\alpha$ -1,6-ones, have been recognised as belonging to the family (MacGregor et al. 2001). Moreover, for the  $\alpha$ -amylase family enzymes a few conserved sequence regions additional to the four well-accepted ones (Fig. 1) were shown to be of importance (Janeček 1992, 1994a, 1994b, 1995).

#### *Catalytic ( $\beta/\alpha$ )<sub>8</sub>-barrel domain*

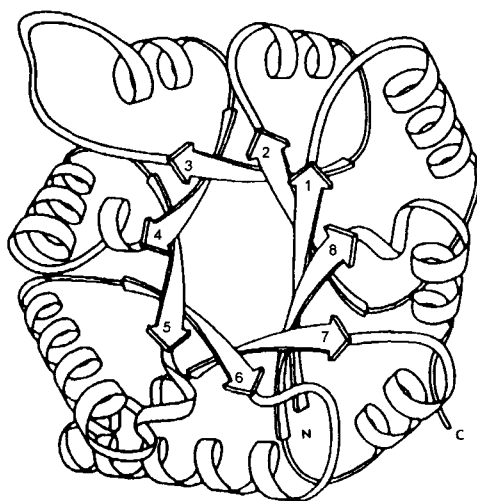
Structurally, the catalytic domain of all members of the  $\alpha$ -amylase family should adopt the structure of a parallel ( $\beta/\alpha$ )<sub>8</sub>-barrel fold (Fig. 2), first recognised in the enzyme chicken Triosephosphate IsoMerase (thus the TIM-barrel fold) twenty-five years ago (Banner et al. 1975). The ( $\beta/\alpha$ )<sub>8</sub>-barrel of the first  $\alpha$ -amylase was identified in the X-ray structure of Taka-amylase A which is the  $\alpha$ -amylase from *Aspergillus oryzae* (Matsuura et al. 1984). Currently, in addition to the structure of Taka-amylase A, the three-dimensional structures of  $\alpha$ -amylases are known for the enzymes from bacteria *Alteromonas haloplantidis* (Aghajari et al. 1998), *Bacillus licheniformis* (Machius et al. 1995), *Bacillus subtilis* (Fujimoto et al. 1998) and *Bacillus stearothermophilus* (Suvd et al. 2001), fungus *Aspergillus niger* (Brady et al. 1991), plant barley (Kadziola et al. 1994), insect yellow meal worm (Strobl et al. 1998a), and mammals, such as pig (two isozymes from pancreas; Qian et al. 1993; Gilles et al. 1996) and human (from both pancreas and saliva; Brayer et al. 1995; Ramasubbu et al. 1996). The catalytic ( $\beta/\alpha$ )<sub>8</sub>-barrel is known as the domain A.

The  $\alpha$ -amylase-type of ( $\beta/\alpha$ )<sub>8</sub>-barrel was found in several related enzymes from the  $\alpha$ -amylase family (Janeček 2000a), such as in cyclodextrin glucanotransferase (CGTase) (Klein and Schulz 1991), oligo-1,6-glucosidase (Kizaki et al. 1993),

| Enzyme/Protein                         | VI<br>β <sup>2</sup> | I<br>β <sup>3</sup> | V<br>domain B | II<br>β <sup>4</sup> | III<br>β <sup>5</sup> | IV<br>β <sup>6</sup> | VII<br>β <sup>8</sup> |
|--|----------------------|---------------------|---------------|----------------------|-----------------------|----------------------|-----------------------|
| <b><u>Hydrolases</u></b>               |                      |                     |               |                      |                       |                      |                       |
| α Amylase                              | 56_GFTAIWIT-P        | 117_DVVANH          | 173_LPDLD     | 202_GLRIIDVVKH       | 230_GVLD              | 292_FVNEHD           | 323_GIPFIYAGQ         |
| Olgo-1,6 glucosidase                   | 44_GIDVIWLS-P        | 98_DLVVNH           | 167_QPDIN     | 195_GFRMDVINP        | 255_EMPG              | 324_YVNNHD           | 360_GTFPIYQGE         |
| α Glucosidase                          | 52_GVDIIVWC-P        | 106-DLVINH          | 181_QVDIN     | 210_GFRIDTAGL        | 276_DVAR              | 344_YIENHD           | 381_GTLFVYQGS         |
| Pullulanase                            | 458_GVTHVELL-P       | 590_DVVYNH          | 632_CSDSA     | 661_GFRFDLMGY        | 694_GQWD              | 817_YVSKHD           | 859_GIAFDQQGS         |
| Cyclomaltodextrinase                   | 187_GVNAIYFN-P       | 240_DAVFNH          | 294_MPKLN     | 323_GWRIDVANE        | 356_EIMH              | 418_LLGSFD           | 450_GTPCIYYGD         |
| Isoamylase                             | 218_GVTAEFL-P        | 292_DVVYNH          | 342_GANFN     | 371_GFRFDLASV        | 435_EPWA              | 505_FIDVHD           | 574_GTFLMQGGD         |
| Dextran glucosidase                    | 44_GVMAIWLS-P        | 98_DLVVNH           | 162_QPDIN     | 190_GFRMDVIDM        | 236_EWNG              | 308_FVNNHD           | 344_GTFPIYQGE         |
| Trehalose 6-phosphate hydrolase        | 46_GVDALINLT-P       | 100_DMVFNH          | 168_QADLN     | 196_GLRIDVVMN        | 251_EHSS              | 320_FWCNHD           | 356_GTFPIYQGE         |
| Neopullulanase                         | 189_GITGIYLT-P       | 242_DAVFNH          | 295_MPKLN     | 324_GWRIDVANE        | 357_EIWH              | 419_LLGSFD           | 451_GSPCIYYGD         |
| <b><u>Transferases</u></b>             |                      |                     |               |                      |                       |                      |                       |
| Amylosucrase                           | 134_GLTYLHIM-P       | 190_DFIFNH          | 262_QWDIN     | 290_ILRMDAVAF        | 336_EAIV              | 396_YVRSHD           | 488_GLFLIYLGD         |
| Glucosyltransferase                    | 849_GITQFEDA-P       | 915_DLVFNQ          | 395_AKWDV     | 433_GVRMDAVDN        | 475_EAWS              | 542_FIRAHQ           | 614_TVTRVYYGD         |
| Cyclodextrin glucanotransferase        | 70_GVTALWISQ         | 135_DFAFNH          | 197_LADFN     | 225_GIRMDAVKH        | 257_EHFL              | 323_FIDNHD           | 354_GVPAIYYGT         |
| <b><u>Isomerases</u></b>               |                      |                     |               |                      |                       |                      |                       |
| Trehalose synthase                     | 54_GVDCLVVP-P        | 108_DFVFNH          | 178_QPDIN     | 206_GFRIDAVPY        | 252_EANQ              | 322_FLRNHD           | 385_GSPVLYYGD         |
| <b><u>Without catalytic action</u></b> |                      |                     |               |                      |                       |                      |                       |
| Amino acid transport protein           | 156_NIKTVWIT-S       | 210_DFIFNH          | 282_QPDIN     | 310_GFSLDAVKE        | ---?                  | ---?                 | 474_GTPITYYGE         |
| 4F2 Heavy chain antigen                | 154_KVKGVLVG-P       | 206_DLTFN-          | del           | 243_GFQVRDIEN        | ---?                  | ---?                 | 361_GTFVFSYGD         |

**Figure 1.** Conserved sequence regions of the selected members from the α-amylase family. The best conserved segments of the α-amylase-type (β/α)<sub>8</sub>-barrel comprise strands β<sup>2</sup>, β<sup>3</sup>, β<sup>4</sup>, β<sup>5</sup>, β<sup>7</sup> and β<sup>8</sup>. There is also a short conserved sequence stretch located near the C-terminus of the longest loop connecting strand β<sup>3</sup> and helix α<sup>3</sup> (in domain B). The three proposed catalytic residues (Asp, Glu and Asp in strands β<sup>4</sup>, β<sup>5</sup> and β<sup>7</sup>, respectively) are highlighted by inversion. Regions I, II, III and IV are the four well-known conserved sequence regions (Nakajima et al. 1986), region V is the fifth conserved sequence region identified first in the sequences of α-amylases (Janeček 1992) and then also in those of the other specificities (Janeček 1995), and regions VI and VII are the additional conserved sequence regions characteristic also for the entire α-amylase family (Janeček 1994a, 1994b). The conserved sequence regions were extracted from the following representatives of the family: α-amylase from *Aspergillus oryzae* (GenBank Accession Number D00434), olgo-1,6-glucosidase from *Bacillus cereus* (X53507), α-glucosidase from *Saccharomyces carlsbergensis* (M12601), pullulanase from *Klebsiella pneumoniae* (X52181), cyclomaltodextrinase from *Bacillus sphaericus* (X62576), isoamylase from *Pseudomonas* sp. strain SMP1 (M25247), dextran glucosidase from *Streptococcus mutans* (M77351), trehalose-6-phosphate hydrolase from *Escherichia coli* (U06195), neopullulanase from *Bacillus stearothermophilus* (M28138), amylosucrase from *Neisseria polysaccharea* (AJ011781), sucrose-utilizing glucosyltransferase from *Streptococcus downei* (M30943), cyclodextrin glucanotransferase from *Bacillus circulans* strain 8 (X68326), trehalose synthase from *Pinelobacter* sp. strain R48 (D78198), amino acid transport-related protein from *Homo sapiens* (M95548), and 4F2 heavy-chain cell surface antigen from *Homo sapiens* (M21898). Regions III and IV with the question mark for the non-enzymatic members are either not present or not easily identifiable without additional structural information, thus indicating their different function. The 4F2 heavy-chain antigens seem to have deleted the segment corresponding to domain B (indicated by “del”) of the α-amylase-type (β/α)<sub>8</sub>-barrel. Adapted from Janeček (2000a).

maltotetraohydrolase (Morishita et al. 1997), isoamylase (Katsuya et al. 1998), maltogenic α-amylase related to CGTase (Dauter et al. 1999), maltogenic amylase related to neopullulanase (Kim et al. 1999), neopullulanase (Kamitori et al. 1999), amyломaltase (Przytyl et al. 2000) and glycosyltrehalose trehalohydrolase (Feese et al. 2000). For further enzyme specificities from the α-amylase family the catalytic

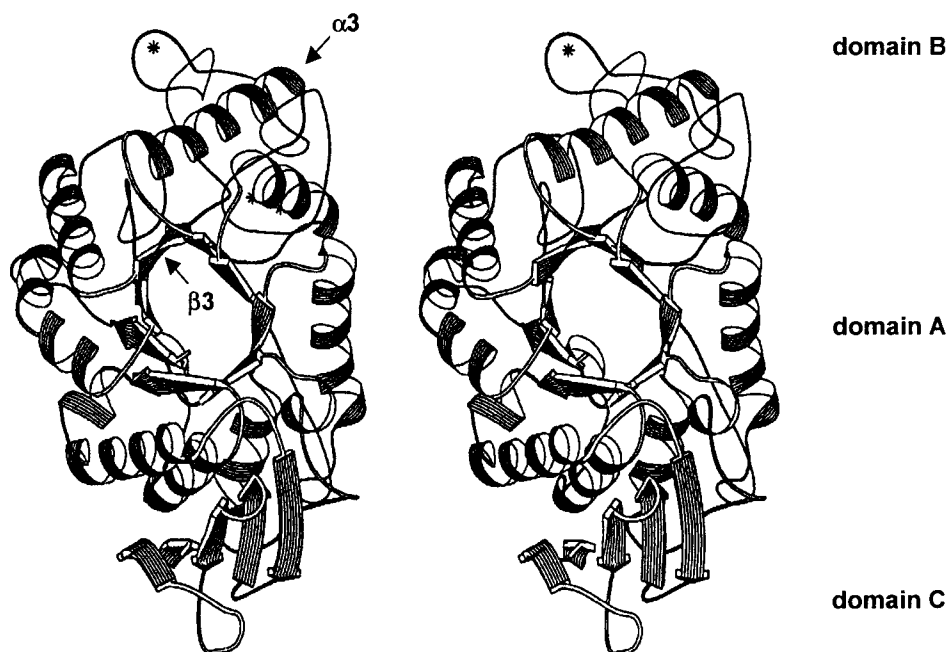


**Figure 2.** Parallel  $(\beta/\alpha)_8$ -barrel of triosephosphate isomerase This structure illustrates the classical TIM-barrel fold and is used as an illustrative example. The motif is composed of eight parallel  $\beta$ -strands (numbers 1–8) forming the inner  $\beta$ -barrel sheet that is surrounded by eight  $\alpha$ -helices in such a way that there are eight repeated  $(\beta\alpha)$ -units in a regular  $(\alpha/\beta)_8$ -barrel. The motif is seen in the end view, i.e. the C-terminal end of the  $\beta$ -barrel is toward the reader. Adapted from Richardson (1981)

$(\beta/\alpha)_8$ -barrel domain was predicted (Jespersen et al. 1991, 1993; MacGregor et al. 1996).

The main characteristic feature of the  $(\beta/\alpha)_8$ -barrel of  $\alpha$ -amylase and related enzymes is a small distinct domain, domain B, protruding of the barrel between strand  $\beta 3$  and helix  $\alpha 3$  (Fig. 3). There are or may be some other domains at both N- and C-terminal sides of the catalytic barrel (see below).

The place where catalysis by an enzyme from the  $\alpha$ -amylase family is performed is located at the C-terminal end of the parallel  $\beta$ -barrel of domain A in a cleft (Matsuura et al. 1984). However, comparison of known three-dimensional models of different enzymes from the family along with a multiple sequence alignment have suggested that the diversity in specificity arises by variation in substrate binding at the  $\beta \rightarrow \alpha$  loops (Svensson 1994). Also the active-site cleft is not always of the same shape, e.g., in neopullulanase it was found wider and shallower than the cleft of other  $\alpha$ -amylases (Kamitori et al. 1999). The core of the catalytic cleft is built up of three catalytic residues, Asp206, Glu230 and Asp297 at strands  $\beta 4$ ,  $\beta 5$  and  $\beta 7$  (Fig. 1), plus of a few additional residues depending on a concrete enzyme specificity (Janeček 1994b; Przylas et al. 2000). The  $\alpha$ -amylase-type of the active site, accommodating many related specificities (cf. e.g. Fig. 1), offers good opportunity to tailor the specificity by combination of suitable mutations of the active-site residues (e.g. Kuriki et al. 1996; Matsui and Svensson 1997).



**Figure 3.** Overall structure of barley  $\alpha$ -amylase in stereo. The structure consists of three domains: A, a parallel  $(\beta/\alpha)_8$ -barrel, B, an irregular fold stabilised by three calcium ions (stars); and C, a 5-stranded anti-parallel  $\beta$ -sheet. The third  $\beta$ -strand ( $\beta 3$ ) and third  $\alpha$ -helix ( $\alpha 3$ ) are also marked. Adapted from Kadziola et al. (1994)

With regard to the reaction mechanism by which the  $\alpha$ -glycosidic bond is cleaved, all the enzymes from the  $\alpha$ -amylase family should use the anomer retaining mechanism (McCarter and Withers 1994; Henrissat and Davies 1997; Kuriki and Imanaka 1999), so that they are classified as the  $\alpha \rightarrow \alpha$  retaining glycosidases (Sinnot 1990; Kuriki 2000). Very recently Uitdehaag et al. (1999) have shown how catalysis in the  $\alpha$ -amylase family proceeds by solving the crystal structure of the complexes of CGTase from *Bacillus circulans* strain 251 with an intact substrate and a covalently bound reaction intermediate using the inactive CGTase mutant. The mechanism by which  $\alpha$ -amylase perfectly produces only  $\alpha$ -anomers in hydrolysis has been explained by Kaneko et al. (1998). The details of the active-site models of different members of the  $\alpha$ -amylase family can be deduced from published three-dimensional structures solved as complexes of their native and/or mutant forms with various substrates, substrate analogues or inhibitors, e.g. for the  $\alpha$ -amylases from *A. haloplanctis* (Aghajari et al. 1998), *B. subtilis* (Fujimoto et al. 1998), *A. oryzae* (Matsuura et al. 1984; Brzozowski and Davies 1997), barley (Kadziola et al. 1998; Vallee et al. 1998), yellow meal-worm (Strobl et al. 1998b), and pig pancreas (Qian et al. 1993, 1997; Wiegand et al. 1995; Bompard-Gilles et al. 1996; Gilles

et al. 1996; Machius et al. 1996), maltotetraohydrolase from *Pseudomonas stutzeri* (Yoshioka et al. 1997), and CGTases from *B. circulans* strain 8 (Klein et al. 1992; Parsiegla et al. 1998; Schmidt et al. 1998), *B. circulans* strain 251 (Lawson et al. 1994; Knegtel et al. 1995; Strokopytov et al. 1995; 1996) and *Thermoanaerobacterium thermosulfurigenes* (Wind et al. 1998b).

#### *Domain B protruding of the barrel*

The loops connecting the  $\beta$ -strands to the adjacent  $\alpha$ -helices in the catalytic ( $\beta/\alpha$ )<sub>8</sub>-barrel domain of the enzymes from the  $\alpha$ -amylase family are usually short, consisting of several amino acid residues only. The loop between the third  $\beta$ -strand and third  $\alpha$ -helix is, however, long enough to be considered as a distinct domain, known as domain B (Klein and Schulz 1991; Qian et al. 1993). This domain has an irregular structure varying from well-defined (certain number of  $\alpha$ -helices and  $\beta$ -strands) present in, e.g., *B. circulans* strain 8 CGTase (Klein and Schulz 1991) and pig pancreatic  $\alpha$ -amylase (Qian et al. 1993), to a random-coil-type (without defined secondary structure elements) found in the barley  $\alpha$ -amylase (Kadziola et al. 1994). For instance, in the *Pseudomonas amyloclavata* isoamylase structure, the long  $\beta 3 \rightarrow \alpha 3$  region cannot be considered to be an independent domain because this loop forms a globular cluster together with the loop between the fourth  $\beta$ -strand and fourth  $\alpha$ -helix (Katsuya et al. 1998). Furthermore, amylomaltase from *Thermus aquaticus* has been shown to contain longer regions in the loops of the barrel to form subdomains B1 and B2 (Przytyl et al. 2000).

Although domain B of the  $\alpha$ -amylase family members varies substantially in both length and sequence (Jespersen et al. 1993), it has also been found that the variation may be related to the enzyme specificity (Janeček et al. 1997). Domain B in different forms, moreover, may still have evolved from a common ancestor. There is a short conserved sequence stretch (Fig. 1), positioned near the C-terminus of domain B, e.g. 173<sub>LPDLN</sub> in Taka-amylase A, containing the almost invariant aspartate residue (Asp175), involved in most cases in the binding of a calcium ion (Boel et al. 1990).

With regard to this conserved sequence region (Janeček 1992, 1995), there is a characteristic change from QPDLN to MPKLN via the MPDLN for the members of the  $\alpha$ -amylase family (Table 1). Structurally, the domain B of these members should resemble very closely the domain B structure of oligo-1,6-glucosidase from *B. cereus* (Janeček et al. 1997). The sequence QPDLN (or QxDLN) is typical for a large group of  $\alpha$ -amylase family enzymes, such as  $\alpha$ -glucosidase, dextran glucosidase, trehalose-6-phosphate hydrolase, amylosucrase and trehalose synthase in addition to oligo-1,6-glucosidase for which also the three-dimensional structure is known (Kizaki et al. 1993). Remarkably, the mammalian proteins that induce the transport of dibasic and neutral amino acids across cell membranes also unambiguously contain this conserved sequence region (Fig. 1, Table 1). The members of the family with characteristic motif in the fifth conserved sequence region MPKLN (or MPKIN and MPKLR), such as cyclomaltodextrinases, maltogenic amylases and neopullulanases, have shorter domain B and presumably do not bind a calcium

**Table 1.** Short conserved stretch near the C-terminal end of domain B in the  $\alpha$ -amylase family enzymes

| Enzyme                                      | EC        | Sequence  | GenBank  |
|---|-----------|-----------|----------|
| Taka-amylase A                              | 3 2 1 1   | 173 LPDLD | D00434   |
| Oligo-1,6-glucosidase                       | 3 2 1 10  | 167 QPDLN | X53507   |
| Alpha-glucosidase                           | 3 2 1 20  | 169 QPDLN | M30442   |
| Dextran glucosidase                         | 3 2 1 70  | 162 QPDLN | M77351   |
| Trehalose-6-phosphate hydrolase             | 3 2 1 93  | 170 QADLN | Z54245   |
| Amylosucrase                                | 2 4 1 4   | 262 QWDLN | AJ011781 |
| Sucrose phosphorylase                       | 2 4 1 7   | 164 QIDID | D90314   |
| Trehalose synthase                          | 5 4 99 16 | 178 QPDLN | D78198   |
| Cyclomaltodextrinase                        | 3 2 1 54  | 294 MPKLN | X62576   |
| Maltogenic amylase                          | 3 2 1 133 | 295 MPKLN | AF115340 |
| Neopullulanase                              | 3 2 1 135 | 293 MPKLR | D13178   |
| Alpha-amylases                              | 3 2 1 1   |           |          |
| <i>Bacillus megaterium</i>                  |           | 174 MPDLN | X07261   |
| <i>Thermotoga maritima</i>                  |           | 186 MPDLN | Y11359   |
| <i>Xanthomonas campestris</i> (periplasmic) |           | 165 MPDLN | D38228   |
| <i>Dictyoglomus thermophilum</i> AmyC       |           | 181 MPDLN | X15948   |
| <i>Dictyoglomus thermophilum</i> AmyB       |           | 276 MPKIN | X13199   |
| Amino acid transport protein                | —         | 282 QPDLN | M95548   |

ion due to Asp  $\rightarrow$  Lys substitution (Janeček et al 1997). Indeed, it has recently been shown that the longer side-chain of lysine, that in neopullulanase corresponds to the calcium-binding aspartate of TAA, may play the stabilizing role of calcium by occupying its position and fixing the relative orientation and location of the catalytic  $(\beta/\alpha)_8$ -barrel and domain B (Kamitori et al 1999). Finally, the members of the  $\alpha$ -amylase family containing the intermediary sequence MPDLN, especially some particular  $\alpha$ -amylases from *B. megaterium* (Metz et al. 1988), *Xanthomonas campestris* (Abe et al. 1996) and *Thermotoga maritima* (Liebl et al. 1997), may exhibit the mixed substrate specificity of  $\alpha$ -amylase, cyclodextrinase and neopullulanase (Brumm et al. 1991; Abe et al. 1996; Janeček 1997). In this respect, the two  $\alpha$ -amylases from *Dictyoglomus thermophilum*, designated as AmyB and AmyC (Horinouchi et al. 1988), containing MPKIN and MPDLN, respectively (Table 1), should be of great interest (Janeček 2000a).

The possible function of domain B was most deeply studied using the barley  $\alpha$ -amylase isozymes 1 and 2 (Rodenburg et al. 1994; Juge et al. 1995). The amino acid sequences of these two plant  $\alpha$ -amylase pH isozymes (Rogers and Milliman 1983; Rogers 1985) exhibit 80 % identity (Kadziola et al. 1994) and differ in some physico-chemical properties. It has been revealed that domain B determines several functional and stability properties, such as stability at low pH, characteristic of



AMY1, and sensitivity to barley amylase/subtilisin inhibitor, specific to AMY2 (Rodenburg et al. 1994; Juge et al. 1995). In a most recent study, sensitivity to inhibitor was gained in the insensitive isozyme hybrid by site-directed mutagenesis mimicking the sequence of the sensitive isozyme AMY2 (Rodenburg et al. 2000). In general, in  $\alpha$ -amylases domain B contributes several residues functioning as substrate binding-sites (e.g., Matsuura et al. 1984) and it is involved in binding of the structural calcium ion (e.g., Boel et al. 1990). However, not all members of the  $\alpha$ -amylase family are  $\text{Ca}^{2+}$ -binding proteins (Jespersen et al. 1991; Kizaki et al. 1993; Katsuya et al. 1998; Kamitori et al. 1999).

#### *Other non-catalytic domains*

As it was indicated above, the  $\alpha$ -amylase family members may contain some additional N- as well as C-terminal domains. Most members of the  $\alpha$ -amylase family contain, in addition to domains A (catalytic TIM-barrel) and B (small distinct domain in the loop 3), the third domain, domain C, just after the  $(\beta/\alpha)_8$ -barrel (Fig. 3). This antiparallel  $\beta$ -sheet domain could protect the hydrophobic residues of the  $(\beta/\alpha)_8$ -barrel from solvent thus stabilising the catalytic domain (Katsuya et al. 1998). Amylomaltase, however, was recently found to miss domain C (Przylas et al. 2000).

The CGTases have usually two more domains (D and E) in comparison with most  $\alpha$ -amylases (e.g., Klein and Schulz 1991). This seems to be also the case with maltogenic  $\alpha$ -amylase from *Bacillus stearothermophilus* (Dauter et al. 1999). While no role has been assigned to domain D with the immunoglobulin-like fold (Jespersen et al. 1991), much more is revealed with respect to domain E that is well-known as SBD (Svensson et al. 1989). Both structural and evolutionary features of this interesting domain connecting the retaining and inverting amylases (Janeček and Ševčík 1999) will be described in the next section.

On the other hand, isoamylase, neopullulanase and maltogenic amylase were found to have a special domain N, originally called domain F, which precedes the  $(\beta/\alpha)_8$ -barrel (Jespersen et al. 1991). This N-terminal domain may play different roles in different enzymes. In isoamylase (Katsuya et al. 1998) a part of the domain N makes a wall of the active-site cleft together with the long loop between strand  $\beta 3$  and helix  $\alpha 3$ . In neopullulanase (Kamitori et al. 1999) domain N is one of the driving forces in the formation of the dimer structure of this enzyme. The role of this N-domain in dimerisation and consequently in the active site has been described in the structure of maltogenic amylase (Kim et al. 1999) in which the N-terminal domain of one subunit covers a part of the top of the  $(\beta/\alpha)_8$ -barrel of the other subunit corresponding to the active site cleft. According to the recent sequence and structural comparison (Park et al. 2000) this unique N-terminal domain apparently contributes to the active site structure of neopullulanase and maltogenic amylase resulting in the distinct substrate specificity through a dimer formation.

## Structure of inverting $\beta$ -amylase and glucoamylase

Both  $\beta$ -amylase and glucoamylase belong to  $\alpha \rightarrow \beta$  inverting glycosidases (Sinnot 1990; Kuriki 2000). Sequentially, these two amylases do not contain any of the conserved regions characteristic of the  $\alpha$ -amylase family (Fig. 1). Although they both are exo-amylases, their amino acid sequences and three-dimensional structures are different (Aleshin et al. 1992; Mikami et al. 1993) so that they form their own independent families in the sequence-based classification of glycoside hydrolases (Henrissat 1991; Coutinho and Henrissat 2000):  $\beta$ -amylase – family 14, glucoamylase – family 15. Structurally,  $\beta$ -amylase ranks along with  $\alpha$ -amylase among the large family of parallel  $(\beta/\alpha)_8$ -barrel proteins (Pujadas and Palau 1999), while glucoamylase belongs to a smaller family of proteins adopting the structure of  $(\alpha/\alpha)_6$ -barrel fold (Ševčík et al. 1998).

### *( $\beta/\alpha$ )<sub>8</sub>-Barrel domain of $\beta$ -amylase*

The first determined three-dimensional structure of  $\beta$ -amylase was that of soybean  $\beta$ -amylase (Mikami et al. 1993). At present the structures of  $\beta$ -amylases from sweet potato (Cheong et al. 1995), barley (Mikami et al. 1999b) and *B. cereus* (Mikami et al. 1999a; Oyama et al. 1999) are known in addition to the structure of soybean enzyme.

The core of each  $\beta$ -amylase structure is formed by the catalytic  $(\beta/\alpha)_8$ -barrel domain, as seen first in the structure of triosephosphate isomerase (Fig. 2), followed by the C-terminal loop region. Although this loop surrounds the N-terminal side of the  $(\beta/\alpha)_8$ -barrel and may stabilise the whole  $\beta$ -amylase molecule, it is not concerned with the catalysis (Mikami 2000). As it has been pointed out above, the  $(\beta/\alpha)_8$ -barrel of  $\beta$ -amylase itself differs in detail from that of  $\alpha$ -amylase and its related enzymes from the glycoside hydrolase family 13 (and clan GH-H), and basically resembles rather the single domain structure of triosephosphate isomerase (Mikami 2000). The  $\beta$ -amylase from *B. cereus* (Mikami et al. 1999a; Oyama et al. 1999) contains SBD at the C-terminus (see the next section).

Although there is only about 30 % of identical residues between *B. cereus* and soybean  $\beta$ -amylases in the  $(\beta/\alpha)_8$ -barrel domain (Nanmori et al. 1993), the amino acid residues important for catalysis and substrate binding are well conserved in bacterial and plant enzymes (Cheong et al. 1995). The two amino acid residues responsible for catalysis are the two glutamates, Glu186 and Glu380 (soybean  $\beta$ -amylase numbering), positioned near the C-terminus of strand  $\beta_4$  and in strand  $\beta_7$  of the  $(\beta/\alpha)_8$ -barrel domain, respectively (Mikami et al. 1994). Totsuka and Fukazawa (1996) described further the indispensable roles for Asp101 and Leu383 in addition to the two catalytic glutamic acid residues, and also the SH-group should be concerned with substrate binding in  $\beta$ -amylases (Shinke 2000). Analyses of the  $(\beta/\alpha)_8$ -barrel fold of  $\beta$ -amylases from both the evolutionary (Pujadas et al. 1996) and structural (Pujadas and Palau 1997) points of view are also available.

### $(\alpha/\alpha)_6$ -Barrel domain of glucoamylase

Glucoamylase structure was solved for the two eukaryotic enzymes from the fungus *A. awamori* (Aleshin et al. 1992) and the yeast *Saccharomycopsis fibuligera* (Ševčík et al. 1998). This amylase adopts a helical catalytic domain consisting of 12  $\alpha$ -helices that form the so-called  $(\alpha/\alpha)_6$ -barrel fold. It consists of an inner core of six mutually parallel  $\alpha$ -helices that are connected to each other through a peripheral set of six  $\alpha$ -helices which are parallel to each other but approximately antiparallel to the inner core of the  $\alpha$ -helices (Aleshin et al. 1992). This fold is not so frequent as the TIM-barrel fold (Farber and Petsko 1990; Janeček and Bateman 1996; Pujadas and Palau 1999), however, the  $(\alpha/\alpha)_6$ -barrel has also been found in different proteins and enzymes, such as enzymes from the glycoside hydrolase families 8 and 9 (Juy et al. 1992; Alzari et al. 1996), and farnesyltransferase (Park et al. 1997). Some glucoamylases, similar to some  $\alpha$ -amylases (and related enzymes) and  $\beta$ -amylases, contain SBD (Svensson et al. 1989). The structure of this domain isolated from the catalytic  $(\alpha/\alpha)_6$ -barrel was solved by NMR (Sorimachi et al. 1996) and will be discussed below.

Based on an analysis of the glucoamylase amino acid sequences, Coutinho and Reilly (1997) found seven subfamilies of glucoamylases taxonomically corresponding to bacterial (1), archaeal (1), yeast (3) and fungal (2) origin.

As evidenced by the crystal structures of the complexes of *A. awamori* glucoamylases (Harris et al. 1993; Aleshin et al. 1994, 1996; Stoffer et al. 1995) as well as the structure of *S. fibuligera* glucomylase with bound Tris (Ševčík et al. 1998), the two glutamates, Glu179 and Glu400 (fungal enzyme numbering), act as the key catalytic residues. The next most deeply studied is glucoamylase from *A. niger* (e.g., Christensen et al. 1996; Frandsen et al. 1996) which is practically identical to the *A. awamori* counterpart.

### Starch-binding domain

As indicated above, in amylolytic enzymes there is a domain, so-called raw-starch-binding domain (SBD), that is common for all the amylase families although not each member of a family contains the motif. SBD is present in about 10 % of  $\alpha$ -amylase,  $\beta$ -amylase and glucoamylase families (Janeček and Ševčík 1999). It is present in all CGTases (domain E), maltogenic  $\alpha$ -amylase (Dauter et al. 1999) and in a few  $\alpha$ -amylases mainly from actinomycetes (Janeček 1997). This module is responsible for the ability to bind and digest native raw, granular starch (Svensson et al. 1982; Penninga et al. 1996). In  $\alpha$ -amylases SBD may govern the enzyme thermostability (Iefuji et al. 1996), however, this motif seems to have nothing to do with thermostability in glucoamylases (Chen et al. 1995). SBD has been demonstrated (Dalmia et al. 1995) to independently retain its function even if fused to a protein other than amylase. It may also disrupt the starch surface, thereby enhancing the amylolytic rate (Southal et al. 1999). Almost exclusively, it is positioned at the C-terminal part of an amylase. The only known exception is glucoamylase from *R.*

|             |     |                         |                          |                               |      |      |
|-------------|-----|-------------------------|--------------------------|-------------------------------|------|------|
| Rhior gmy   | 9   | vqldsnyndgstfsg-klyv    | 27 45                    | dnwnnnngntiaasysapisgsnyeywtf |      |      |
| Strlm amy   | 442 | QTSASFHVNATTAWGENIYVTC  | QDAALGNWDPARALKL-----    | DPAAYFPVWKL                   |      |      |
| Cloth bmy   | 420 | IPVTFTINNATTYYGQNVYI    | VGSTDLGNWNTTYARGP-----   | ASCPNYPTWTI                   |      |      |
| Aspng gmy   | 514 | AVAVTFDLTATTYYGENIYLV   | GSISQLGDWETSDGIALSAD---  | KYTSSDPLWYV                   |      |      |
| BacstA mga  | 581 | SVVFTVKSAPPTNLGDKIYLT   | GNIPELGNWSTDTSGAVNNAQ    | GPLLAPNYPDWFY                 |      |      |
| Psesz m4h   | 430 | VSVSFRCDNGATQMGSVYAV    | GNVSQLGNWSPAALRL-----    | TDTSGYPTWKG                   |      |      |
| Bac1011 cgt | 587 | VTVRFVINNATTALGQNVFLT   | GNVSELGNWDPNNAIGPMYNQ--- | VVYQYPTWYY                    |      |      |
| Klepn cgt   | 530 | QSINFTCNNGYTISSGQSVYI   | IGNIPQLGQWDLTKAVKI-----  | SPTQYEPWSA                    |      |      |
|             |     | -β1-                    | -β2-                     | -β3-                          | -β   |      |
| Rhior gmy   |     | sasingikefylykyev-----  | sgktyydnnpnsanvqvst      | 104                           |      |      |
| Strlm amy   |     | DVPLAAGTPFQYKYLKRDAA--- | GKAVWESGANRTATVGTT--     | GALTINDTWRG                   | 538  |      |
| Cloth bmy   |     | TLNLLPGEQIQFAVKIDSS---  | GNVTWEGGSNHTYTVPTS--     | GTGSVTITWQN                   | 519  |      |
| Aspng gmy   |     | TVTLPAGESFEYKFIRIESD--- | DSVEWESDNREYTVPQACGT     | STATVDTWR                     | 616  |      |
| BacstA mga  |     | VFSVPAGKTIQFKFIKRA---   | GTIQWENGSNHVATPTG--      | ATGNITVTWQN                   | 684  |      |
| Psesz m4h   |     | SIALPAGQNEEWKCLIRNEAN   | ATQVRQWGGANNSLTPSE----   | GATTVGRL                      | 526  |      |
| Bac1011 cgt |     | DVSVPAGQTIEFKFLKKQG---  | STVTWEGGANRTFTTPTS--     | GTATVNVNWQP                   | 686  |      |
| Klepn cgt   |     | SLELPSDLNVEWKCVKRNETNP  | TANVEWQSGANNQFNSND----   | TQTNGSF                       | 625  |      |
|             |     | 4-                      | -β5--                    | -β6                           | -β7- | -β8- |

**Figure 4.** Starch-binding domain of amylolytic enzymes Sources of the enzymes Rhior gmy, glucoamylase from *Rhizopus oryzae* (GenBank Accession Number D00049), Strlm amy,  $\alpha$ -amylase from *Streptomyces limosus* (M18244), Cloth bmy,  $\beta$ -amylase from *Clostridium thermosulfurogenes* (M22471), Aspng gmy, glucoamylase from *Aspergillus niger* (X00712), BacstA mga, maltogenic  $\alpha$ -amylase from *Bacillus stearothermophilus* (M36539), Psesz m4h, maltotetraohydrolase from *Pseudomonas stutzeri* (M24516), Bac1011 cgt, cyclodextrin glucanotransferase from *Bacillus* sp. strain 1011 (M17366), Klepn cgt, cyclodextrin glucanotransferase from *Klebsiella pneumoniae* (M15264) Gaps are indicated by dashes The consensus residues are highlighted by inversion The sequence of SBD from *R. oryzae* positioned at the N-terminus is shown in lower-case letters Secondary structure elements are indicated under the alignment blocks Adapted from Svensson et al (1989)

*oryzae* (Ashikari et al. 1986) which contains this domain at its N-terminus. While the sequence conservation as well as the basic arrangement of secondary structure elements of SBD in different amylases (Fig. 4) have been well recognised (Tanaka et al. 1986; Svensson et al. 1989; Coutinho and Reilly 1994a, 1994b, 1997), the evolutionary relationships of the SBD motifs from individual amylases were described only recently (Janeček and Ševčík 1999). Three-dimensional structure is also known for SBD from all the three amylase families 13, 14 and 15 (Klein and Schulz 1991; Sorimachi et al. 1996; Mikami et al. 1999a).

#### *Structural features and evolutionary implications of SBD*

The SBD motif consists of several  $\beta$ -strand segments forming an open-sided, distorted  $\beta$ -barrel structure (Klein and Schulz 1991; Sorimachi et al. 1996, 1997; Mikami et al. 1999a). The consensus invariant residues (Fig. 4), identified by Svensson et al. (1989), seem to be still well-conserved in all the three amylase families (Janeček and Ševčík 1999). Recent analysis of SBD sequences has indicated that

there could be two types of SBD: fungal type and bacterial type, regardless of the fact whether SBD comes from an  $\alpha$ -amylase,  $\beta$ -amylase or glucoamylase (Janeček and Ševčík 1999). Despite the high degree of sequence identity (similarity) between *Aspergillus* (fungal) and *Bacillus* (bacterial) SBDs, which is about 37 % (64 %), strand  $\beta 3$  of fungal SBD is not present in the bacterial SBD, whereas strand  $\beta 6$  of bacterial SBD is not present in the fungal SBD. On the other hand, the sequence of *R. oryzae* SBD exhibits a very low degree of similarity with both representative SBDs and contains a major insertion between strands  $\beta 2$  and  $\beta 3$  (Fig. 4). Nevertheless, this sequence was demonstrated to be responsible for adsorbing to raw starch and degrading this substrate (Takahashi et al. 1985; Ashikari et al. 1986).

Despite the substantial sequence conservation among all known SBD sequences (Tanaka et al. 1986; Svensson et al. 1989; Nanmori et al. 1993; Coutinho and Reilly 1997), from the evolutionary point of view, SBD behaves independently with regard to the catalytic domains of  $\alpha$ -amylases,  $\beta$ -amylases and glucoamylases (Janeček and Ševčík 1999). This means that the taxonomy is respected. In the evolutionary tree based on sequence alignment of SBDs fungi and actinomycetes formed their own separate parts surrounded by bacteria that were also clustered according to taxonomy (Janeček and Ševčík 1999). The N-terminal SBD of *R. oryzae* is most distantly related to all the other present-day C-terminal SBDs. This "evolutionary solitude" can be, however, a consequence of lack of data, since *R. oryzae* SBD may represent a newer-type SBD at the N-terminus (Coutinho and Reilly 1997). The differences in sequences between the N- and C-terminal SBDs may reflect the possibility that during their molecular evolution the *Rhizopus* and *Aspergillus* glucoamylases obtained their abilities to adsorb to raw starch independently (Tanaka et al. 1986).

It might be concluded that the evolution of SBD in the three amylolytic families (glycoside hydrolase families 13, 14 and 15) reflects evolution of species rather than evolution of the individual amylases. The fact that the *Aspergillus kawachii* SBD originated from  $\alpha$ -amylase was clustered together with the rest of the other *Aspergillus* SBDs originated, however, from glucoamylases, can serve as the most striking example of the remarkable behaviour of this motif (Janeček and Ševčík 1999). The present forms of SBD may constitute the modern "descendants" of a domain that might either have joined to or removed from these proteins during the evolution (Svensson et al. 1989). According to the most recent observations by Rodriguez Sanoja et al. (2000) and Sumitani et al. (2000), there may exist a new type of SBD located at the C-terminus of some  $\alpha$ -amylases from bacilli and lactobacilli. This new SBD formed by two or more repeat unit regions seems to be structurally different from SBD shown in Fig. 4. Tibbot et al. (2000) have furthermore isolated a functional SBD, different from that shown in Fig. 4, too, in the low pI isozyme of barley  $\alpha$ -amylase. The SBD in this case corresponds with the C-terminal part of the  $\alpha$ -amylase covering the end of the catalytic  $(\beta/\alpha)_8$ -barrel (from the strand  $\beta 7$ ) and the entire domain C (Tibbot et al. 2000).

## Structure-activity relationships in amylolytic enzymes

The structures of all the three amylases have been solved at high resolution and, moreover, for most of them also the structures of the complexes were determined. It is not surprising, therefore, that the structure-activity relationships of  $\alpha$ -amylase,  $\beta$ -amylase and glucoamylase have been well-recognised (Svensson et al. 1995; MacGregor 1996; Reilly 1999; Pandey et al. 2000). Many studies focused on the determination of the roles the individual amino acid residues may play in the stability and/or stabilisation of these enzymes. And especially in the frame of the  $\alpha$ -amylase family, which consists of almost 30 related specificities, many efforts have been spent to investigate the possibilities to change the functionally important residues in order to modify the final specificity of the enzyme.

### Function

Since details of catalysis brought about by the active-sites of the individual amylases can be found in the reports on their three-dimensional structures solved as complexes of their native and/or mutant forms with various substrates, substrate analogues and inhibitors (see above), here the emphasis is given on presenting the studies trying to improve, modify and alter the specificity of an amylolytic enzyme.

Using  $\alpha$ -amylase from *B. stearothersmophilus*, Takase (1993) found that mutagenesis of residues near the catalytic groups may lead to improving the enzyme activity. The replacement of the lysine residue positioned near the catalytic Asp from strand  $\beta 4$  (Fig. 1) by arginine and asparagine resulted in the alteration of bond-cleavage pattern in *S. fibuligera*  $\alpha$ -amylase (Matsui et al. 1992a). This mutant  $\alpha$ -amylase exhibited also enhanced activity specific for short substrates (Matsui et al. 1992b). Modulated action pattern was obtained by random mutagenesis in the equivalent place (i.e. region of strand  $\beta 4$ ) of the structure of barley  $\alpha$ -amylase (Matsui and Svensson 1997). Conrad et al. (1995) prepared the series of single and mosaic hybrid  $\alpha$ -amylases from *B. amyloliquefaciens* and *B. licheniformis* in order to find their new properties (thermostability, temperature and pH optima and substrate specificity) and to localise the regions responsible for the changes. Saab-Rincón et al. (1999) mutated  $\alpha$ -amylase from *B. stearothersmophilus* at residue Ala289 to phenylalanine and tyrosine, and found that this position may be implicated in the control of the transferase activity which was not present in the wild-type  $\alpha$ -amylase.

Works on other amylolytic enzymes have also been performed. For instance, the mutant CGTase of *B. ohbensis* having tryptophane instead of Tyr188 produced 15 % of  $\gamma$ -cyclodextrin which was about twice as much as the amount produced by the wild-type enzyme (Sin et al. 1994). Cyclodextrin product specificity was changed also in mutated CGTases from *T. thermosulfurigenes* (Wind et al. 1998b) and *B. circulans* (van der Veen et al. 2000; Uitdehaag et al. 2000). A shift from CGTase to  $\alpha$ -amylase specificity was achieved using two related bacterial CGTases by site-directed mutagenesis (Wind et al. 1998a) as well as by chemical modification (Alcalde et al. 1999). The site-directed mutagenesis approach was used to converse neopullulanase from *Thermoactinomyces vulgaris* into an amylopullulanase-

type enzyme (Ibuka et al 1998) Kuriki et al (1996) were able to control the transglycosylation activity of *B. stearothermophilus* neopullulanase by manipulating hydrophobicity in its active centre. Important results concerning the functional properties were achieved on glucansucrases (Monchois et al 1999, 2000a,b) and amylosucrase (Sarcabal et al 2000).

It seems, however, that the most important roles are played by the residues located in the conserved sequence regions (Fig. 1). It was indicated that these residues may discriminate also between very close specificities, such as, e.g.,  $\alpha$ -amylases and CGTases (Janeček et al 1995), or  $\alpha$ -glucosidase and oligo-1,6-glucosidase (Inohara-Ochiai et al 1997).

### Stability

There is a great interest in stable and/or stabilised amylolytic enzymes evoked especially by their great industrial potential (Levêque et al 2000). Different approaches with various methods and techniques have been applied to obtain stable amylases and related enzymes (Janeček and Balaž 1992), such as immobilisation, chemical modification and protein engineering, as well as use of additives (for a review, see Mozhaev et al 1988). In the recent years the enzymes from hyperthermophilic microorganisms have attracted special attention due to the fact that these protein molecules are stable at temperatures near and above 100°C (for reviews, see Adams 1993, Niehaus et al 1999).

An interesting approach to thermal stabilisation of oligo-1,6-glucosidases (members of the  $\alpha$ -amylase family) has been developed by Suzuki (1989) who proposed so-called "proline rule" that should be applicable to other proteins, too, as a general strategy for their stabilisation. The rule states that thermostability of a globular protein can be additively increased by increasing the frequency of the occurrence of proline residues at special positions on the surface of the protein and by clustering prolines around the flexible protein regions (Suzuki 1999). Based on a comparison of the amino acid sequences of three *Bacillus*  $\alpha$ -amylases, differing in their thermostability, Janeček (1993) calculated that one way leading to stabilisation of a protein may be to increase the hydrophobicity of the protein interior and/or to decrease the hydrophobicity of the protein exterior. Bacterial  $\alpha$ -amylases from *Bacillus* were used also in studying the mechanism of their irreversible thermal inactivation (Tomazic and Klibanov 1988a, Brosnan et al 1992) as well as the reasons responsible for their resistance to this process (Tomazic and Klibanov 1988b). Site-directed mutagenesis approach was used to study the thermostability of the naturally thermostable  $\alpha$ -amylase from *B. licheniformis* with focus on Gln178 (Suzuki et al 1989) and six histidine residues (Declerck et al 1990, Joyet et al 1992). Declerck et al (1995, 1997, 2000) prepared hyperthermostable mutants of this  $\alpha$ -amylase and discussed also the structural consequences of their mutations.

### Conclusions

The best known amylolytic enzymes,  $\alpha$ -amylase,  $\beta$ -amylase and glucoamylase, form a large group of enzymes that have great biotechnological importance. Despite

their quite closely related function (catalysis of hydrolysis of  $\alpha$ -glucosidic bonds) these amylases are quite different from both sequence-structural and evolutionary points of view. They are well-studied, i.e. at least one representative structure of  $\alpha$ -amylase,  $\beta$ -amylase and glucoamylase has been determined at high resolution. Among them,  $\alpha$ -amylase deserves a special attention because this amylase is the leading member of a large family (or clan), well-known as the  $\alpha$ -amylase family. Many related specificities belonging to this family having the same catalytic machinery, reaction mechanism and fold offer opportunities for studies focused on tailoring new properties according to industrial requests.

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