ELSEVIER

Biochimica et Biophysica Acta 1546 (2001) 1–20

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Review

Relationship of sequence and structure to specificity in the α-amylase family of enzymes

E. Ann MacGregor [a], Štefan Janeček [b], Birte Svensson [c,*]

[a] *Department of Chemistry, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada*
[b] *Institute of Microbiology, Slovak Academy of Sciences, Stefanikova 3, SK-81434 Bratislava, Slovakia*
[c] *Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark*

Received 19 October 2000; accepted 20 December 2000

Abstract

The hydrolases and transferases that constitute the α-amylase family are multidomain proteins, but each has a catalytic domain in the form of a $(\beta/\alpha)_8$-barrel, with the active site being at the C-terminal end of the barrel β-strands. Although the enzymes are believed to share the same catalytic acids and a common mechanism of action, they have been assigned to three separate families – 13, 70 and 77 – in the classification scheme for glycoside hydrolases and transferases that is based on amino acid sequence similarities. Each enzyme has one glutamic acid and two aspartic acid residues necessary for activity, while most enzymes of the family also contain two histidine residues critical for transition state stabilisation. These five residues occur in four short sequences conserved throughout the family, and within such sequences some key amino acid residues are related to enzyme specificity. A table is given showing motifs distinctive for each specificity as extracted from 316 sequences, which should aid in identifying the enzyme from primary structure information. Where appropriate, existing problems with identification of some enzymes of the family are pointed out. For enzymes of known three-dimensional structure, action is discussed in terms of molecular architecture. The sequence–specificity and structure–specificity relationships described may provide useful pointers for rational protein engineering.

Keywords: α-Amylase family; Glycoside hydrolase family 13; Glycoside hydrolase family 70; Glycoside hydrolase family 77; Sequence motif; Specificity; α-1,4 Glucoside bond; α-1,6 Glucoside bond

1. Introduction

Comparisons of amino acid sequences of glycoside hydrolases and transglycosylases have allowed a classification scheme to be established for these enzymes, based on structure rather than specificity [1–3], i.e., the enzymes have been grouped into more than 80 families, where the members of one family share a common three-dimensional structure and mechanism, and have from a few to many sequence similarities. Families 13, 70 and 77 in this classification contain structurally and functionally related enzymes catalysing hydrolysis or transglycosylation of α-linked glucans, with retention of anomeric configuration. Many of these enzymes act on starch, and one of the most important starch-degrading enzymes, α-amylase, is also the most widely-studied member of family 13. Hence this group of enzymes, here con-

* Corresponding author. Fax: +45-3327-4708;
E-mail: bis@crc.dk

0167-4838/01/$ – see front matter
PII: S0167-4838(00)00302-2

sidered to consist of families 13, 70 and 77, is often known as the α-amylase family of enzymes, but is, in fact, comprised of enzymes with almost 30 different specificities. In some cases, specificities of different enzymes in the family overlap and this, coupled with amino acid sequence similarities, has led to confusion in identification, particularly of family 13 enzymes. In this review, we attempt to clarify the relationship between sequence and specificity, and discuss, where information is available, key structural features that contribute to specificity.

2. Domain architecture

The enzymes are multidomain proteins, but share a common catalytic domain in the form of a $(\beta/\alpha)_8$-barrel, i.e., a barrel of eight parallel β-strands surrounded by eight helices, the so-called domain A (Fig. 1). This structure has been demonstrated by X-ray crystallography in several enzymes of the α-amylase family (Table 1), although in one instance only seven of the eight helices in the barrel fold are present [16]. In addition, studies of amino acid sequence similarities have led to the prediction that many other enzymes belong to this family and have a similar catalytic domain (Table 1). Usually, the loops that link β-strands to the adjacent helices carry amino acid residues of the active site; some of these loops may be long enough to be considered as domains in their own right. Thus, in most cases where the structure has been determined by crystallography, a large loop between the third β-strand and third helix is discussed as a separate domain, domain B. This loop has an irregular structure that varies from enzyme to enzyme, and, it has been argued, should not always be considered a separate domain but, in some cases, should be thought of as part of a structural unit containing other loops [16]. Similarities in domain B amongst members of sub-groups of the α-amylase family have, however, been found, e.g., various glucosidases resemble each other, while a relationship can be demonstrated between enzymes such as neopullulanase and cyclomaltodextrinase [31,32].

In one set of enzymes, the glucan sucrases (often called glucosyltransferases) and dextran sucrases, similarities to α-amylase family proteins can be demonstrated in the amino acid sequences of the catalytic domains, yet well-recognised sequence segments appear in a different order. It has been proposed that a circular permutation of the barrel elements occurred during evolution to give enzymes where the first element of the barrel is a helix equivalent to helix 3 of an α-amylase barrel and the last element is a strand equivalent to strand 3 of α-amylases, i.e., instead of B1-H1-B2-H2.....B8-H8 as in α-amylases (where B and H represent strand and helix, respectively), we have in glucan sucrases H3-B4-H4.....B8-H8-B1-H1-B2-H2-B3 [36]. In the enzyme classification scheme based on structure, such enzymes belong to family 70 and are said to form a 'clan' GH-H with family 13 and family 77 proteins [35].

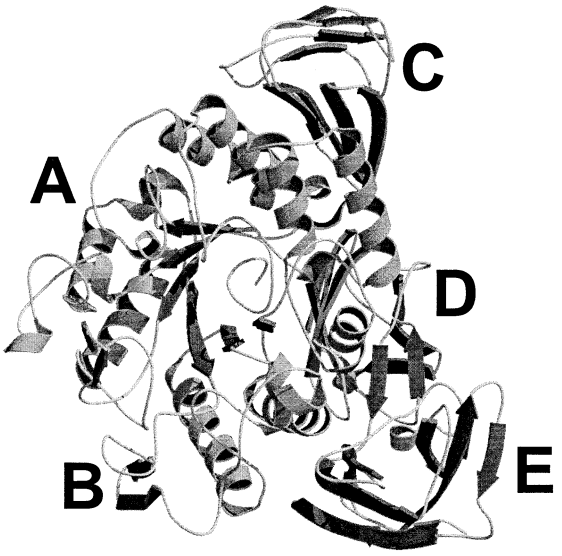

Fig. 1. Ribbon model illustrating the individual domains in CGTase, A is the catalytic $(\beta/\alpha)_8$ domain, B protruding from that domain. C, D, and E are antiparallel β-strand domains of which E can bind starch [23].

In most, but not all, α-amylases, domain A occurs at the N-terminal end of the protein. However, several other members of the family have a distinct domain, domain N, preceding the catalytic domain. The role of this domain is as yet uncertain, and is most probably not identical in all enzymes. In isoamylase and malto-oligosyltrehalose trehalohydrolase, domain N is close to the loop linking β-strand 3 of the barrel to helix 3, and in the former enzyme, amino acid residues of domain N may form part of the active site [16,20]. (Unfortunately, this domain has been labelled domain E in malto-oligosyltrehalose

trehalohydrolase [20], in disagreement with earlier nomenclature discussed below.) In contrast, it has been suggested in an 'α-amylase II' from *Thermoactinomyces vulgaris* that domain N is too far from the active site to participate in enzyme activity, and the domain does indeed appear to be located in a different position, relative to the rest of the molecule, from that found in isoamylase [16,19]. In the crystal structure of the closely related maltogenic 'amylase' (also referred to as cyclodextrinase or CDase) from *Thermus* sp., the domain N of one monomer of a dimer has been suggested to be involved in the enzyme specificity through its interaction with the active site region of the other monomer [18]. Such a situation may also hold for the 'α-amylase II', since it is found by X-ray crystallography to exist as a similar dimer, and its domain N occupies a position analogous to that of the equivalent domain in the maltogenic 'amylase' [19]. The malto-oligosyltrehalose trehalohydrolase appears to function as a covalent dimer, although dimerisation is not necessary for activity or specificity [20].

The vast majority of members of the enzyme family have another domain, domain C, following the catalytic β/α-barrel. This domain is made up of β-strands and is thought to stabilise the catalytic domain by shielding hydrophobic residues of domain A from the solvent. It has also been suggested that domain C may aid in substrate binding [17,23]. Some amylomaltases, however, appear to lack this

Table 1
Structural information on the α-amylase family of enzymes

Enzyme	EC Number	Ref.
(a) Enzymes of known three-dimensional structure		
α-Amylase	3.2.1.1	[4–13]
Oligo-1,6-glucosidase	3.2.1.10	[14]
Maltotetraohydrolase	3.2.1.60	[15]
Isoamylase	3.2.1.68	[16]
Maltogenic amylase[a]	3.2.1.133	[17,18]
Neopullulanase[b]	3.2.1.135	[19]
Malto-oligosyltrehalose trehalohydrolase	3.2.1.141	[20]
Amylosucrase	2.4.1.4	[21]
Cyclodextrin glucanotransferase	2.4.1.19	[22–26]
Amylomaltase (4-Glucanotransferase)	2.4.1.25	[27]
(b) Enzymes predicted to belong to the α-amylase family		
α-Glucosidases[c]	3.2.1.10	[28]
Pullulanase (Limit dextrinase)	3.2.1.41	[29,30]
Amylopullulanase	3.2.1.1/41	[29,30]
Cyclomaltodextrinase[a,b]	3.2.1.54	[31,32]
Dextran glucosidase	3.2.1.70	[29,30]
Trehalose-6-phosphate hydrolase	3.2.1.93	[31]
Maltohexaohydrolase	3.2.1.98	[1]
Maltotriohydrolase	3.2.1.116	[33,34]
Maltopentaohydrolase	3.2.1.-	[29]
Sucrose phosphorylase	2.4.1.7	[35]
Branching enzyme	2.4.1.18	[29,30]
Glucan debranching enzyme	2.4.1.25/3.2.1.33	[30]
Maltosyl transferase	2.4.1.-	[35]
Dextran sucrase/Alternansucrase	2.4.1.5/2.4.1.140	[36,138]
Maltooligosyl trehalose synthase[d]	5.4.99.15	[33]
Trehalose synthase[d]	5.4.99.16	[35]

[a]α-Amylase-type (AM-type), see Section 5.1 [17]. Neopullulanase-type (N-type), see Section 5.3 [18].
[b]Neopullulanase, resembles closely the maltogenic amylase (N-type), see Section 5.3: referred to also as cyclodextrinase [18].
[c]Only α-glucosidases belonging to family 13 are considered here; other α-glucosidases belong to family 31.
[d]These are considered to be isomerases.

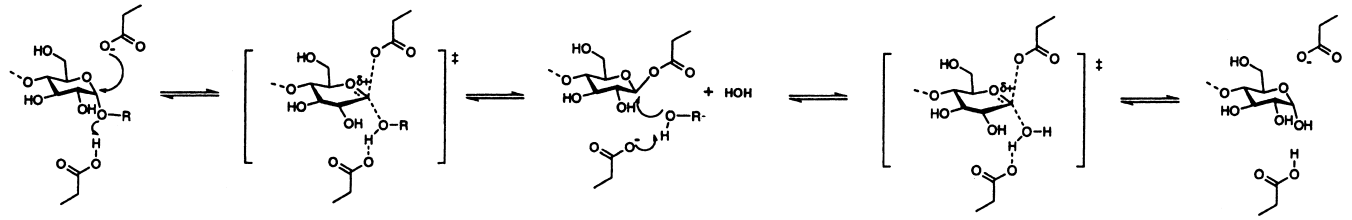

Fig. 2. Catalytic steps in glycoside bond cleavage in retaining enzymes. The proton donor protonates the glycosidic oxygen and the catalytic nucleophile attacks at C1 leading to formation of the first transition state. The catalytic base promotes the attack of the incoming molecule ROH (water in hydrolysis or another sugar molecule in transglycosylation) on the formation of the covalent intermediate resulting in a second transition state, leading to hydrolysis or transglycosylation product.

domain; it cannot therefore be necessary for activity or stability in all cases [27].

Cyclodextrin glucanotransferases and a second type of maltogenic 'amylase', from *Bacillus stearothermophilus* and quite distinct from that of *Thermus* sp., have additional β-sheet domains, domains D and E, after domain C [17,22–26]. While no role has been assigned to domain D, domain E occurs in a variety of other starch-degrading enzymes, not all from family 13, i.e., glucoamylases (family 15) and a few β-amylases (family 14) and α-amylases [29]. This domain is believed to be important for binding granular starch and has also been proposed to provide an extension to the active site in cyclodextrin glucanotransferases [22,29].

Much of the specificity of each enzyme is, however, thought to be defined by amino acid residues of domains A and B, and characteristic differences in these domains will be discussed here.

3. Catalytic mechanism and substrate binding at the active site

Throughout the family, the enzymes are believed to have a similar mechanism of action, and so the catalytic amino acid residues are thought to be common to all the enzymes [37]. Anomeric configuration is retained when the substrate is converted to product, i.e., the enzymes act on α-linkages in glucans or glucosides and yield α-linked products. The reaction is believed to proceed by a double displacement mechanism (Fig. 2). During the first displacement an acid group on the enzyme protonates the glycosidic oxygen, bringing about scission of the C1-O bond and transient formation of an oxocarbenium ion-like transition state [38–40]. A nucleophilic acid group of the protein attacks at the sugar anomeric centre to give a β-glycosyl enzyme intermediate, while the aglycone of the substrate leaves the active site. In hydrolysis, during the second displacement, the process just described is essentially reversed by attack, at the anomeric centre, by a water molecule activated by the carboxylate form of the former proton donor (Fig. 2). This second stage of the reaction proceeds via an ion-like transition state, as before, to yield a product with α-anomeric configuration and reprotonation of the original acid group. Transglycosylation can occur if the attacking group in the second displacement of the reaction is a free hydroxyl of a sugar residue rather than water [40,41].

In Taka-amylase A, the first protein of this family to be examined by X-ray crystallography, three acidic residues, i.e., one glutamic and two aspartic acids were found at the centre of the active site [8], and subsequent mutational studies have shown that these residues are essential for catalysis [33,37]. The glutamic acid residue is now believed to be the proton donor, while the first of the two conserved aspartic acids appearing in the amino acid sequence of an α-amylase family member is thought to act as the nucleophile. The role of the second aspartic acid is less certain, but it has been suggested to be involved in stabilising the oxocarbenium ion-like transition state and also in maintaining the glutamic acid in the correct state of protonation for activity [40].

The active site of an enzyme belonging to the α-amylase family is considered to be made up of a number of subsites, each subsite being capable of interaction with one glucose residue of the substrate (Fig. 3). The subsites themselves are composed of side chains of amino acid residues situated on loops in the enzyme structure that connect the C-terminal ends of β-strands to the N-terminal ends of the ad-

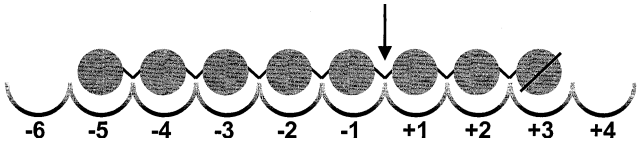

Fig. 3. Schematic of subsite arrangement with oligosaccharide occupying subsites −5 through +3. Cleavage occurs between subsites −1 and +1 as indicated by the arrow. The reducing end group is bound at subsite +3.

jacent helices of the $(\beta/\alpha)_8$-barrel of the catalytic domain. In the case of malto-oligosyltrehalose trehalohydrolase, however, an α-β loop, i.e., joining a helix to the next β-strand of the barrel, is unusually long and may contribute to the specificity of the active site [20]. Because the architecture of the β-α loops varies from enzyme to enzyme, the number and nature of the subsites at the active site also varies, and is characteristic of a particular enzyme. Different enzymes of the α-amylase family act on different substrates and the only shared feature of the substrates of the different enzymes is an α-linked glucose residue that should bind at subsite −1. Hence strong similarities should be found in the residues making up this subsite.

In Taka-amylase A the proton donor (Glu230), the catalytic nucleophile (Asp206), the second conserved aspartic acid (Asp297), and two histidine residues (His122 and His296) were postulated as being important at subsite −1 and this has been confirmed by investigations on mutated proteins, although the His residues are not invariant throughout the family [33,36,37]. These five residues occur near the ends of strands 3, 4, 5 and 7 of the β/α-barrel and are found in four short sequences, long-recognised as being conserved in α-amylase family enzymes. Typical examples of these sequences are shown in Fig. 4.

Further, less obvious conservations of short sequence segments can be found at strands 2 and 8 and in loop 3 between strand 3 and helix 3 (i.e., domain B of many enzymes) [33]. The conservations may be related to maintenance of structure while variations within these segments may be characteristic of certain enzyme specificities [33]. In many enzymes of the α-amylase family, the conserved sequence at strand 2 is characterised by a glycine residue followed eight or nine amino acid residues later by proline, although this is not invariant. It has been suggested that this region can be used to distinguish cyclodextrin glucanotransferases from α-amylases, since the former enzymes usually have eight residues between the Gly and Pro, while the latter enzymes have seven [42]. This is, however, not always the case, and several exo-α-amylases have eight residues between Gly and Pro [43–45], and one CGTase appears to have seven [46].

4. Conserved sequences and specificity

Different enzymes of the family have different specificities; some are active only on α-1,4 glycosidic bonds between glucose residues, others on α-1,6 bonds exclusively, some on both bond types, yet others can cleave sucrose, while there are related enzymes that can hydrolyse or form the inter-glucose link in trehalose (Fig. 5). For all of the enzymes, activity involves binding a glucose residue of the substrate at subsite −1, while the nature of the portion of the substrate binding at subsites +1 and +2 varies with the specificity of the enzyme, particularly with respect to the type of bond to be formed or destroyed, e.g., whether α-1,4 or α-1,6. Thus the amino acid residues making up subsites +1 and +2 of an enzyme may be expected to vary with the specificity of the enzyme.

From structural studies with mutated enzymes and substrates or inhibitors, it appears that amino acids of conserved sequence segments II and III (Fig. 4) generally constitute subsites +1 and +2 [17,40,47–54]. In particular these acids are 209, 210, 231, 232 and possibly 233 and 234 (Taka-amylase A numbering will be used throughout), and occur on loops 4 and 5 of the $(\beta/\alpha)_8$ barrel [8]. Thus residues at these positions may be characteristic for specificity with respect to bond-type. Of 175 sequences of enzymes

a.

I	116 VDVVANH 122	II	202 GLRIDTVKH 210
III	226 YCIGEVLD 233	IV	292 FVENHD 297

b. More generally

I	XDXXXNH	II	GXRXDXXZZ
III	XXX(G/A)EZZZ	IV	XXBBHD

Fig. 4. Short conserved amino acid sequences of α-amylase family enzymes: a, for Taka-amylase A; b, a generalised set of sequences. X, usually a hydrophobic residue; B, usually a hydrophilic residue; Z, a residue important for specificity.

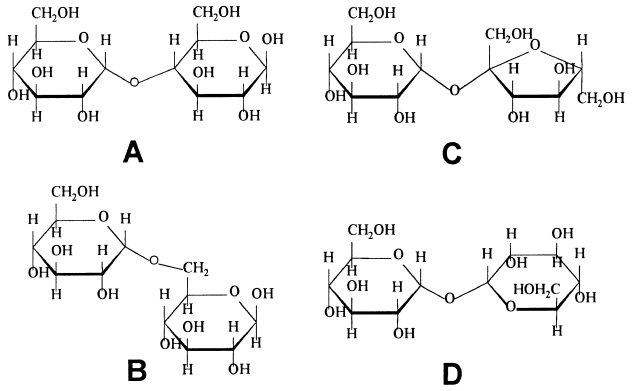

Fig. 5. Four possible substrates for members of clan GH-H. A: maltose, B: isomaltose, C: sucrose and D: trehalose.

from families 13 and 77 that are specific for α-1,4 glucosidic bonds, 160 or 91% have Lys or Arg at the position equivalent to 209 of Taka-amylase A, while these two residues are conspicuously absent from this position in enzymes with specificity for other α-glucosidic bonds. Further, 170 or 97% of the α-1,4-bond-specific enzymes have His or Gly at the position equivalent to 210. Where Gly is present at 210, an aromatic residue is found at 207 and seems to replace, in the three-dimensional structure, the His ring otherwise found at 210 in α-1,4-bond-specific enzymes [9,15]. Amongst the α-amylases, all the archaeal and plant enzymes have Gly at position 210 [55], but the significance of this is not understood. A view of a substrate analogue bound at the active site of an α-amylase is shown in Fig. 6.

In the case of enzymes acting on α-1,6-linkages only, the situation is less clear-cut. In 28 sequences, the most common amino acid residues at 209 are Gly or Ser found in 20 or 71% of these proteins. Gly is also found at this position, however, in some α-glucosidases that appear not to be active on α-1,6-linkages [47,56] and in sucrose phosphorylases that catalyse the phosphorolysis of sucrose [57–60]. In addition, 26 or 93% of the enzymes that are specific for α-1,6-bonds have a hydrophobic residue, Val, Leu or Met, at position 207.

At position 210, in α-1,6-link-specific enzymes, 24 or 86% of the proteins have a hydrophobic or aromatic amino acid. Enzymes that can act on both α-1,4 and α-1,6 bonds at a single active site, such as neopullulanases and some amylopullulanases, have mainly Asn at 209 and Glu at 210 (in 16 out of 22, or 68% of the enzymes) but other possibilities are known.

Enzymes acting on sucrose can be considered in two groups - the glucan sucrases with a permuted β-barrel, and the sucrose phosphorylases and amylosucrase with a 'normal' barrel. In the first group, all of the sequences examined have Asp–Asn at positions equivalent to 209–210 of Taka-amylase A, while in the second group, all have Gly or Ala at 209, followed by an aromatic Phe or Tyr at 210. Thus there is more than one architecture possible, in the sucrose-binding enzymes, for subsite +1, where the fructose might be expected to bind.

Most of the glucosidases (24 out of 31, or 77%), whether oligo-1,6-glucosidase, α-glucosidase, dextran glucosidase or trehalose 6-phosphate hydrolase, have a large hydrophobic or aromatic residue at the position equivalent to 210 of Taka-amylase A. This might allow for interaction with a variety of sugar or even non-sugar residues at subsite +1.

At positions 231 and 232, the most obviously distinctive sequence is the Trp-Tyr/Phe that occurs in cyclodextrin glucanotransferases (CGTases) and one maltogenic 'amylase', but none of the other family 13 enzymes (Table 2). The latter enzyme lacks other key features of the CGTases (see below) and has no cyclising activity [17,61].

In some, but not all, enzymes with marked transferase activity an acidic residue, either Asp or Glu is

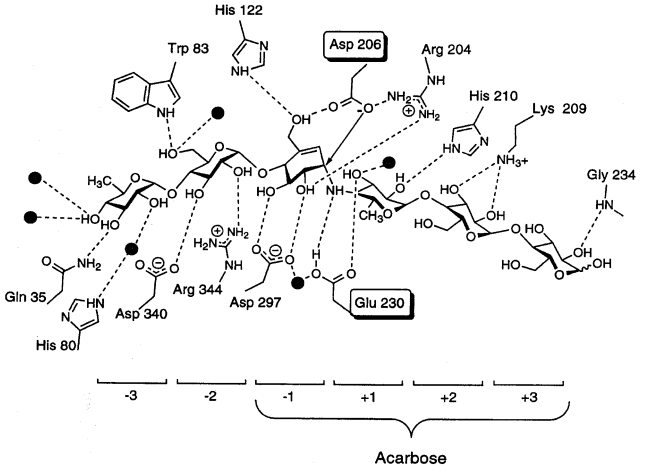

Fig. 6. Schematic diagram of the interactions of acarbose with Taka-amylase A. Hydrogen bonds are shown as dotted lines and water molecules as spheres. (Reproduced from [54] with permission.)

found at 231. Such an arrangement of two contiguous acidic residues (231 following the catalytic Glu at 230) is not found in hydrolases of the family (see Table 2).

Janecek et al. have already pointed out that residues characteristic of a particular type of enzyme can be located at other positions in the amino acid sequence, e.g., an Ile at position 203 is found in cyclodextrin glucanotransferases [42], but not in other 1,4-bond-specific members of the family, notably α-amylases and the above-mentioned maltogenic 'amylase'.

A close examination of over 300 amino acid sequences of α-amylase family members (listed in [62]) has enabled a table to be drawn up that shows sequences characteristic of known specificities of enzymes within this family (Table 2). In several cases, insufficient structural information is available for it to be possible to offer an explanation of the relationship between these sequences and enzyme specificity.

5. Structure–specificity relationships in individual enzymes

5.1. Enzymes specific for α-1,4 glycosidic bonds

5.1.1. α-Amylases

α-Amylases are generally considered to be endo-acting enzymes. There are enzymes, however, that hydrolyse starch polysaccharides to products with the α-anomeric configuration and are believed to act preferentially at one end of a polysaccharide chain to give primarily one size of small oligosaccharide, i.e., are exo- rather than endo-acting hydrolases. Some of these enzymes now have Enzyme Commission numbers distinct from that of α-amylase, e.g., maltogenic 'amylase' (3.2.1.133), maltotriohydrolase (3.2.1.116), maltotetraohydrolase (3.2.1.60), and maltohexaohydrolase (3.2.1.98) producing α-maltose, maltotriose, maltotetraose and maltohexaose, respectively. With the exception of some so-called maltogenic 'amylases', these enzymes cannot be distinguished from endo-acting α-amylases on the basis of amino acid residues in conserved sequence regions II and III (Fig. 4). This is to be expected, since the exo-activity is conferred, at least in the case of the maltotetraohydrolase where the structure is known, by residues on loop 3, while residues in conserved regions II and III form the beginnings of loops 4 and 5 of the β-barrel [63]. At this stage in our understanding of structure-function relationships in family 13 enzymes it is not possible to predict which residues of the other exo-acting members of the family ensure exo- rather than endo-activity, although they are likely to be in loops 1, 2, 3, 7 and 8, since these loops contribute to binding glucose residues of the substrate on the glycone side of the bond on which the enzyme acts [6,48,63,64].

Consideration of the maltogenic 'amylases' illustrates some of the problems of confusion and nomenclature that have arisen in this family. In fact, the enzymes classed as maltogenic 'amylases' under EC number 3.2.1.133 appear to be of two distinct types, one which has the characteristic signature of a-1,4-linkage-specific enzymes of Lys–His at positions 209–210 [65,66] and the other with the Asn–Glu characteristic of enzymes active on both α-1,4 and α-1,6 bonds [67–70] (Table 2). There is evidence that enzymes of the latter type are indeed active on both types of linkage [68,71] – hence, in this case, the name amylase, usually reserved for 1,4-bond-specific enzymes, is misleading. The differences between the two types of maltogenic 'amylase' extend much further than simple signature sequences. The *B. stearothermophilus* enzyme of the first type (hereafter called type AM, i.e., amylase-like) has been studied by X-ray crystallography, and the overall structure has been found to be remarkably similar to that of a cyclodextrin glucanotransferase, with five domains, the first being the catalytic (β/α)-barrel [17]. The authors suggest that the enzyme attacks by an endo-mechanism, but that the final product is mainly maltose. Indeed they consider that the enzyme should be classified as an α-amylase. The structure shows key differences in loop 3 of the barrel between this enzyme and a CGTase (see below). Such differences make it less likely that the maltogenic 'amylase' AM could hydrolyse a hexa-, hepta- or octa-saccharide from the non-reducing end of a substrate, as would be required for cyclodextrin formation, or that cyclisation could take place. A second maltogenic 'amylase' (from *B. subtilis*) has the Lys–His characteristic of an α-1,4-linkage-specific enzyme and is considered here as an AM-type enzyme. It has an amino acid sequence long enough and similar enough to the *B. stearothermophilus* enzyme that it too may

Table 2
Sequences at β→α 4 and 5 of domain A characteristic for enzymes in clan GH-H of different specificity

Enzyme	Residues at position number (Taka-amylase A numbering)												No. of sequences
	203	205	207	208	209	210	231	232	233	234	236	237	
Enzymes acting on sucrose													
Amylosucrase			A	V	A	F							1
Glucan sucrase			A	V	D	N	M/D/A						14
Sucrose phosphorylase	A		A/V	G	Y/F								4
	A		F	A	Y								2
Enzymes acting on trehalose													
Maltooligosyl trehalose synthase			H	P/T/L/I	D	G							8
Trehalose-6-phosphate hydrolase		L	V	I/V	N	L			S		V/L		2
Trehalose synthase			A	I/V	P	Y		N	M/Q				5
Enzymes acting on α-1,4- and α-1,6-bonds													
Branching enzyme			A/G	I/V/T	A/T/V	S/N	E/D						26
Glycogen debranching enzyme			Y/N	C	H	S							3
Neopullulanase			A	A	M/Q	H/Y							2
			T	H	P	Y							1
			V	P	D	C							1
			V	A	N	E	[a]					No K/L	4
Amylopullulanase			V	A/E	N	E						K/L	4
Maltogenic 'amylase' (N-type)			V	A	N	E	[a]					No K/L	4
Cyclomaltodextrinase[b]			V	A	N	E	[a]					No K/L	4
			C	G	H	D							2
α-Glucosidases			A	L/V	P	Y/H		Y	S/T				5
			A/V	G/I	S	H/L/M							3
			Q/V	A/L/V	H	G/L/M							4
			V	L	W	L	[c]						1
			A	I	T	H							1
			V	I	N	A/Q							2
			S/T	A/I	A/G/N	F/L/M							4
Enzymes acting on α-1,6-bonds only													
Oligo-1,6-glucosidase			V	I	G/N	F/M/S							5
			V	L	W	L	[c]						1
Dextran glucosidase			V	I	D	L/M							2
		No L	V	I	N	L			A		I		1
Pullulanase			L	M	A	L							1
			V	P	N	E							1
(Limit dextrinase)			L/M/Q	L/M	G	D/H/I/L/Y							13
Isoamylase			L	A	S	I/V							6
Enzymes acting on α-1,4-bonds only													
Maltooligosyltrehalose trehalohydrolase			A	V	H	A							6
Maltosyl transferase			M	G	H	A	E						1
CGTases	I		A	I/V	K	H	W						18

Table 2 (*continued*)

Enzyme	Residues at position number (Taka-amylase A numbering)												No. of sequences
	203	205	207	208	209	210	231	232	233	234	236	237	
Maltogenic 'amylases'	L		A	V	K	H	W						1
(AM-type)			A	A	K	H	I	[d]		E			1
4-Glucanotransferase			H	F	R	G	D						5
			H	V	M	S/G	D						2
			A	A	K	H	I			E			2
α-Amylase			S	L/V	E/L/Q	E/Q							3
			H/F/Y	A/V	K/R/T	G							31
			A	A/S	T	H							5
	No I		A/S/T	A/C/I/S/V	A/K/L/R/V	H	No W			No E			106

[a]These three cannot be distinguished on basis of sequence of segments II and III (Fig. 4).
[b]Generally considered to act on α-1,4 bonds only, but see text.
[c]These two differ in only 9 amino acid residues of 529.
[d]Like a *Bacillus subtilis* α-amylase (see text).

be a 5-domain protein [65]. Curiously, the sequence is 97% identical, over the first three domains, to that of a *B. subtilis* α-amylase of known three-dimensional structure [6]. The α-amylase is, however, truncated with respect to the maltogenic 'amylase' and lacks the last 192 residues of the latter enzyme, i.e., possible domains D and E. Such truncations in *B. subtilis* α-amylase (resulting from base deletions in the gene) have been observed previously [72] and are not believed to affect specificity on soluble substrates, but decrease activity on starch granules. The enzyme produces maltose as a major, but not sole, product of hydrolysis of soluble starch. We suggest that the *B. subtilis* maltogenic 'amylase' also may be considered as an α-amylase.

X-ray crystallographic studies of one of the second type of maltogenic 'amylase' (called N-type, i.e., neopullulanase-like) [18] shows a different domain organisation, with the enzyme having a domain N before the catalytic β-barrel, and lacking domains D and E. Again the enzyme gives maltose as the major, but not only, product of starch hydrolysis. In addition, the enzyme is shown to be active on both α-1,4 and α-1,6-linkages between glucose residues, and its amino acid sequence is closely related to those of another maltogenic 'amylase' [68] and a neopullulanase [73] known to have dual bond-type specificity (see later).

Thus, at present, the name maltogenic amylases and EC number 3.2.1.133 are being used for two distinct enzymes, neither of which produce exclusively maltose from starch.

Further confusion arises from the description of two enzymes from *Thermoactinomyces vulgaris* as 'pullulan-hydrolysing' α-amylases, TVAI and TVAII. Specificity studies on both enzymes show that they can hydrolyse α-1,4 glucosidic linkages, giving, for example, panose from pullulan. Both enzymes, however, can hydrolyse cyclodextrins and α-1,6 linkages in small oligosaccharides, i.e., they show activities characteristic of neopullulanases. In addition, the amino acid sequences of the enzymes show a marked similarity to a neopullulanase and a cyclodextrinase [74]. The structure of TVAII has been determined by X-ray crystallography and shows the domain N that is rare in α-amylases, but common in dual bond-specificity enzymes [19]. For this review, we have considered both TVAI and TVAII as neopullulanases.

The true α-amylases act only on α-1,4-glucosidic linkages, bringing about hydrolysis of starch polysaccharides or transglycosylation with small oligosaccharides that are poor substrates for the enzymes [75–77]. While there are exo-α-amylases producing mainly one size of oligosaccharide from the non-reducing end of a long α-1,4-linked glucan chain, the endo-α-amylases also give greater yields of some oligosaccharides than others by preferential action near chain ends. This action can be the combined result of the number of subsites at the active site and the

tendency to multiple attack, i.e., the scission of more than one glucosidic bond during a single enzyme-substrate encounter. Enzymes giving high yields of maltose and maltotriose during amylose hydrolysis, e.g., α-amylases from mammals, some insects and bacteria, are generally considered to have 5 important subsites at the active site (−3 to +2 of Fig. 3) and in several cases this has been confirmed by X-ray crystallography of enzyme-oligosaccharide or enzyme-carbohydrate inhibitor complexes [4,6,10,13,49,50,78]. In some enzymes a sixth subsite at +3 is believed to be present [17]. The ability to bind long substrates with five or six contiguous internal glucose residues interacting directly with the enzyme gives these enzymes endo- rather than exo-character. In addition, some mammalian α-amylases are believed capable of high levels of multiple attack, accentuating the production of small oligosaccharides such as maltose or maltotriose early in amylose hydrolysis [79]. This multiple attack may be facilitated by the presence, in mammalian α-amylases, of a movable loop, within loop 7 of the β-barrel, that can interact with the substrate at subsites −2 and +2 [13,49,50,80] and has been observed in two distinct conformations by X-ray crystallography. Differences both in the level of multiple attack and in the detailed action on oligosaccharides among the highly conserved mammalian α-amylases are believed to be due to a small number of amino acid substitutions at the active site [13].

Other endo-acting α-amylases give larger oligosaccharides as products of starch degradation, mainly because scission of α-1,4-linkages close to the non-reducing end of a substrate molecule is difficult for these enzymes. Such α-amylases, notably from *Aspergillus oryzae*, barley and *Bacillus licheniformis*, have more extended active sites where the architecture of loops 1, 2, 3, 7 and 8 of the β-barrel is different for each enzyme and unlike that of the mammalian amylases [5,8,9,11–13,54,80,81]. These are the loops critical for interaction with substrate on the glycone side of the bond to be broken. Modelling of a complex between barley α-amylase and an extended amylose fragment suggests, for example, that a tyrosine on loop 3 of the barrel may be important for interaction with a glucose residue at subsite −6 [51], hence ensuring that barley α-amylases produce maltodextrins smaller than maltohexaose from amylopectin (i.e., from non-reducing substrate chain ends) only with great difficulty [82].

Other α-amylases are known that appear to lack several of the short conserved sequences of family 13 enzymes. Such amylases are found in thermophilic bacteria and archaea, and form family 57 of the classification scheme for glycoside hydrolases. A possible distant sequence relationship between the two families has been discussed [83].

5.1.2. Cyclodextrin glucanotransferases

Another subgroup of enzymes of the α-amylase family that have been widely studied are the cyclodextrin glucanotransferases, and several three-dimensional structures have been determined, for free enzymes, mutated enzymes and complexes with oligosaccharides [22–26,40,41,48,52,53,84]. As stated earlier, these enzymes have five domains, the first domain at the N-terminal end of the protein being the catalytic $(\beta/\alpha)_8$-barrel, while the last domain, E, may carry amino acid residues important for guiding a long substrate to the active site [85]. These enzymes are capable of bringing about some hydrolysis of glucan chains, but differ from α-amylases primarily in their ability to produce cyclodextrins by cyclising an oligosaccharide of 6–8 glucose residues cut from the non-reducing end of a chain. Mutation studies indicate that a Phe or Tyr residue (amino acid 195 in the CGTase of *B. circulans* [22,23]) in loop 3 of the β-barrel, that is lacking in loop 3 of α-amylases, is important for cyclisation activity in CGTases [86]. This critical residue is absent from the maltogenic 'amylase' AM of *B. stearothermophilus* that resembles a CGTase in structure [17,66]. Detailed structural studies of CGTase with bound oligosaccharides or cyclodextrin have led to the conclusion that when a long substrate is bound, the enzyme active site adopts a conformation similar to that of an α-amylase where a Lys residue (209 of Fig. 4) is important for substrate binding at subsite +2 (Fig. 3). After bond scission, the glycone part of the substrate – 6, 7 or 8 glucose residues long, with the size preference depending on the source of the CGTase – remains covalently bound to an Asp residue (206 of Fig. 4) [87], and a conformational shift takes place in the enzyme that forces the non-reducing-end glucose of the substrate into subsite +1, while the penultimate glucose occupies subsite +2. The substrate, however,

is now further away from the Lys at subsite +2 and residues specific for CGTase become more important for substrate binding at this subsite, e.g., a phenylalanine of loop 3 (residue 183 of *B. circulans* CGTase [22,23]) and another on loop 5, equivalent to residue 232 of Taka-amylase A [40,41,48,53]. Cyclisation can then take place by glucosidic bond formation between C1 and C4 of the glucose residues at subsites −1 and +1, respectively – essentially a reversal of bond scission. The first of these phenylalanine residues is missing from the maltogenic 'amylase' AM, and both are absent from α-amylases, presumably rendering these enzymes unable to form cyclodextrins [66]. Mutational studies indicate that critical residues in loop 3 may influence the size of the cyclodextrins produced by the CGTase, i.e., whether predominantly α-, β- or γ-cyclodextrin [48], although other residues can have an effect [88].

5.1.3. 4-α-Glucanotransferases

Other enzymes, in addition to cyclodextrin glucanotransferases, are primarily transferases, but without cyclising activity, e.g., maltosyl transferase and 4-glucanotransferases. They act on linear α-1,4-linked glucans, removing a fragment from one and transferring it to another. Although the structure of one of these 4-glucanotransferases has been determined, the relationship between structure and activity is not yet understood [27]. The enzyme clearly, however, belongs to the α-amylase family in terms of its structure and putative catalytic amino acid residues, and it seems an unnecessary complication that the enzyme is not included in family 13 of the glycoside hydrolase classification scheme, but has been assigned to a new family, family 77, even if that family is considered as part of the α-amylase 'clan' GH-H [35]. Several, but not all of these transferases, share an unusual feature at sequence I of Fig. 4, i.e., His 122 forming an important part of subsite −1 is not conserved, but may be replaced by Thr in maltosyl transferase or Pro, Val and Ser in 4-glucanotransferases. It is not yet possible to say whether this is relevant to transfer activity.

5.2. Enzymes specific for α-1,6 glycosidic bonds

At present, structures of oligosaccharide complexes with α-1,6-linkage-specific enzymes have not been obtained. In general, however, these enzymes have relatively large residues, Leu, Val or Gln following the first catalytic Asp residue (206 of Fig. 4) in the sequence. In both known structures of α-1,6-bond-specific enzymes, isoamylase and oligo-1,6-glucosidase, this residue could interfere with binding of a 4-linked glucose on the aglycone side of the bond to be broken and may help to determine bond specificity [14,16]. In α-amylases, when a large residue follows the Asp, e.g., phenylalanine in barley α-amylase, the side chain of this residue is closer to the position of the common His210 of several 1,4-bond-specific enzymes and helps rather than hinders binding at subsite +1 [64]. Part of the active site of the oligo-1,6-glucosidase appears to be in the form of a pocket which may be able to bind the single 1,6-linked glucose residue to be split from the substrate [14], while that of the isoamylase is a shallow groove more characteristic of the active site of a polysaccharide endo-hydrolase [16]. Isoamylase easily removes long branches from amylopectin, but the common plant debranching enzymes, known as limit dextrinases, are better adapted to the hydrolysis of α-1,6-linkages in small oligosaccharides resulting from the hydrolysis of starch by α- and β-amylases during seed germination. These plant debranching enzymes resemble most closely, in sequence, the bacterial pullulanases (30–40% identity) and are now often called plant pullulanases. The structural basis for the specificity differences between isoamylases, limit dextrinases and bacterial pullulanases is not yet understood.

5.3. Enzymes acting on both α-1,4 and α-1,6 glycosidic bonds.

Enzymes acting on both α-1,4- and α-1,6-linkages may do so in a very specific way, e.g., branching enzymes, that usually rupture an α-1,4-bond and make a l,6-linkage, create branches in glycogen or amylopectin by removing an oligosaccharide from the non-reducing end of a straight 1,4-linked glucan chain and reattaching the oligosaccharide as a branch.

Two major groups of starch branching enzymes are found in plants (see for example [89]), with the A group (also known as SBE-II) having a much longer domain N than the B group (SBE-I). In general

the A-type enzymes are more active on amylopectin than amylose, while the opposite is true for the B-type branching enzymes. In addition, the former enzymes seem to transfer shorter chains to form branches than the latter [90]. It is not yet known whether domain N can form an extension of the active site and hence be important for specificity, but in the maize enzymes both the N-terminal and C-terminal regions of the proteins seem to exert an influence [90,91].

As pointed out earlier, the catalytic glutamic acid of branching enzymes is usually followed, in the amino acid sequence, by a second acidic residue, either glutamic or aspartic acid. Mutation of this second acidic amino acid in *Escherichia coli* branching enzyme showed that it is important, but not essential, for activity and has an effect on specificity [92].

Glycogen debranching enzymes are believed to have two active sites, one for breaking and making 1,4-linkages and the other for removal of a 1,6-linked glucose residue, to release β-glucose. The first active site is thought to resemble that of an α-amylase-family enzyme. Indeed the aspartic acid residue acting as the catalytic nucleophile has been identified for the rabbit enzyme [93]. The second site, in contrast, forms a product with inversion of anomeric configuration and so must differ substantially from a family 13 active site. Its amino acid residues have not yet been identified.

Neopullulanases, certain maltogenic 'amylases' (N-type) and most amylopullulanases, however, can hydrolyse and synthesise both α-1,4- and α-1,6-linkages at a single active site. Unfortunately a large protein with two active sites, one specific for α-1,4-bonds and the other for α-1,6-bonds, has also been called an amylopullulanase, leading to confusion with the enzymes possessing a single active site [94–96]. Further confusion has arisen from the practice of calling pullulanases, that are α-1,6-linkage-specific, 'pullulanase type I' enzymes and amylopullulanases, with a single active site that processes α-1,4- and α-1,6-bonds, 'pullulanase type II' enzymes [97].

Where enzymes can act on more than one type of linkage, there must be some flexibility at the active site, particularly at subsite +1, since a 4-linked glucose and a 6-linked glucose cannot fit in an identical way into an active site. In many 'dual-specificity' enzymes, Asp, Asn or Gln is found at position 209 (Fig. 4) and an Asp or Glu at 210. Mutation of these residues has been shown to alter bond specificity [98,99] and relative amounts of hydrolysis versus transglycosylation [100], but the effect on enzyme structure has not yet been determined.

Cyclomaltodextrinases are usually considered to act on α-1,4-linkages only, but there is a report that they can hydrolyse other α-bonds [101]. They have been included here with dual bond-type specific enzymes because of close sequence similarities with enzymes such as neopullulanases. Cyclodextrinases are defined as enzymes that hydrolyse cyclodextrins and some linear maltodextrins quickly, but have little activity on starch. Some maltogenic 'amylases' (N-type) also can hydrolyse cyclodextrins, as well as starch, [68,71, 102] and so the specificities of the two types of enzymes are not distinctly different. Several maltogenic 'amylases' N, notably those with sequence VANE at positions 207–210 (Taka-amylase A numbering, Fig. 4) can also act on α-1,6 bonds in addition to α-1,4-linkages [68,71, 102,103]. Neopullulanases are defined as enzymes hydrolysing primarily α-1,4-bonds of pullulan to give panose, but are also known to be active on α-1,6-linkages. Since maltogenic 'amylases' N have been shown to give panose from pullulan [68,71,102,103], again there is an overlap in specificity between neopullulanases and the maltogenic 'amylases' N that act on more than one type of linkage. In addition, the amino-acid sequences of some maltogenic 'amylases' N and neopullulanases are very similar: 86% identity for example between a *Bacillus stearothermophilus* neopullulanase and a maltogenic 'amylase' from *Thermus* species [70,73] and 99% identity between a putative neopullulanase from *Bacillus subtilis* and a maltogenic 'amylase' from the same organism [69,104]. In this latter case it seems very likely that the two sequences correspond to isozymes of the same enzyme. As has been pointed out earlier in this review, structures of a maltogenic 'amylase' N from *Thermus* sp. and an 'α-amylase II', here considered to be a neopullulanase, from *Thermoactinomyces vulgaris* appear very similar [18,19]. Thus it may not always be possible to distinguish between maltogenic 'amylases' (N-type) and neopullulanases, and the names may have been assigned simply on the basis of the first substrates studied.

In contrast, the cyclodextrinases seem to be less

closely related to maltogenic 'amylases' and neopullulanases, with sequence identities usually less than 50% [67,105–107]. A similarity in domain B between a cyclomaltodextrinase and a neopullulanase has, however, been found [31]. Certainly, it is not always possible to distinguish cyclodextrinases from maltogenic 'amylases' (N-type) and neopullulanases on the basis of the short sequences shown in Fig. 4, and a strong resemblance between maltogenic 'amylases' (N-type) and cyclomaltodextrinases has, indeed, been suggested [68]. Further, it has been postulated that some enzymes of a group of cyclodextrinases, maltogenic 'amylases' and neopullulanases have been misnamed [106]. More recently it has been suggested that, since neopullulanases, maltogenic 'amylases' (N-type) and cyclomaltodextrinases all hydrolyse cyclodextrins and share marked sequence similarities, they may be considered a cyclodextrin-metabolising subfamily of the α-amylase enzyme family [32].

Interestingly, a pullulanase from *Bacillus flavocaldarius* has many sequence features resembling those of a neopullulanase [32,108], but appears to lack the domain N usually found in pullulanases and neopullulanases.

The amylopullulanases are much larger proteins, in general, than neopullulanases, cyclodextrinases and N-type maltogenic 'amylases', e.g., molecular masses of amylopullulanase are well over 100 000 (ca. 140 000–180 000) while the other enzymes have molecular masses in the range 60 000–80 000. Amylopullulanases can bring about hydrolysis of α-1,4-linkages in amylose and α-1,6-linkages in pullulan [109], but it is not yet possible to relate this activity to structural features.

5.4. α-Glucosidases

α-Glucosidases are enzymes that remove an α-linked glucose, usually from the non-reducing end of a chain, although at least one enzyme appears to remove glucose from the reducing end of a chain [110]. The α-glucosidases that act at non-reducing chain ends may not be specific for the aglycone part of the substrate – for example, an α-glucosidase from *Candida albicans* can hydrolyse maltose, sucrose and α-methyl maltoside, but not trehalose, nigerose or isomaltose [47]. Again, there must be some flexibility at the active site for binding substrate at subsite +1.

Some confusion seems to have arisen, however, when classifying an enzyme as an α-glucosidase or as an oligo-1,6-glucosidase, specific for hydrolysing an α-1,6-linked glucose residue from a substrate. An 'oligo-1,6-glucosidase' from *Bacillus* sp. F5 has been shown to be active on both α-1,4- and α-1,6-linkages [111], as has an 'α-glucosidase' from *Bacillus* sp. SAM 1606 [112]. It is interesting that both of these enzymes have the sequence Gly-Gly at positions equivalent to 232–233 of Taka-amylase A, i.e., in conserved sequence III of Fig. 4 [111,113], while glucosidases that hydrolyse either α-1,4- or α-1,6-bonds (but not both) do not. Studies of mutations of the first of these glycine residues in the 'α-glucosidase' have shown that this residue has a marked influence on specificity [114]. Further, a *Bacillus flavocaldarius* oligo-1,6-glucosidase, for which the specificity has been studied [115,116], is 98% identical in sequence to a *Thermus caldophilus* 'α-glucosidase' [117]. It seems unlikely, given such a high sequence similarity, that the specificities of the two enzymes are substantially different, and so it is probable that the 'α-glucosidase' in this case will prove to be an oligo-1,6-glucosidase.

The glucosidases of family 13, i.e., oligo-1,6-glucosidase, α-glucosidase, dextran glucosidase and trehalose 6-phosphate hydrolase, show some strong sequence similarities [118], and may be considered a sub-group of the α-amylase family. Thus any prediction of specificity based on sequence comparisons should be carried out with great care.

5.5. Enzymes acting on sucrose

Enzymes, other than cyclodextrin glucanotransferases, function as transferases, but some transfer glucose from a sucrose molecule to a growing polysaccharide chain. These are amylosucrase that synthesises an α-1,4-bond, and glucan sucrase (also known as glucosyl transferase). In the case of the second enzyme, however, the new bond formed may be an α-1,2-, α-1,3-, or α-1,6-linkage. The basis of this specificity is not yet understood.

While it has been suggested that secondary structure elements of the $(\beta/\alpha)_8$-barrel of glucan sucrases are permuted [36], the contrary argument has been

made that the β-barrel elements of these enzymes appear in the same order as in α-amylases [119]. The sequences on which the latter suggestions were based, however, are very variable in the glucan sucrases and, while occurring in one such enzyme, are not found in others. In contrast, the sequences on which the postulate of circular permutation was based are common to all the glucan sucrases [36]. At present, no explanation can be offered as to why amylosucrase should function with a 'normal' β-barrel while glucan sucrases require a permuted barrel.

As in some other transferases, one of the conserved histidine residues making up subsite −1, i.e., His122 of Taka-amylase A (Fig. 4), is replaced, by a glutamine residue in glucan sucrases. Its relevance to activity is not known, although mutational studies indicate an involvement in specificity with acceptor molecules [120].

Even less is known of sucrose phosphorylase, which might have been expected, from the reaction catalysed, to resemble a phosphorylase more than an α-amylase, yet the sequence bears the hallmarks of an α-amylase family enzyme.

6. Relevance to protein engineering

The large diversity of specificity and the different types of reaction catalysed by enzymes in glycoside hydrolase families 13, 70, and 77 – or clan GH-H – invite rational engineering of the enzyme specificity. Early mutational analyses investigated structure/function relationships [37] and protein engineering moreover addressed important industrial goals such as improvement of thermostability or changing the pH activity dependence [121–123]. Similarly, modification of the product specificity has major industrial relevance. One issue in the case of cyclodextrin glucanotransferases has been to engineer the product composition to have higher contents of a given cyclodextrin and indeed CGTases have been successfully developed by rational design to produce specific cyclodextrins [41,48,52,84,124]. Neopullulanases represent family 13 members in which substrate specificity changes have been attempted. These enzymes possess dual bond-type specificity, acting on both α-1,4- and α-1,6-bonds; moreover they catalyse both hydrolytic and transglycosylation reactions [74,98,125,126]. At an early point an α-amylase was altered to enhance transglycosylation, probably by slow product release which allowed attack of a new substrate donor molecule [127,128]. Random mutation at a localised substrate binding region of the polypeptide chain, e.g., in β→α loops 4 [129] and 7 [130,131] in barley α-amylase shifted the position of the preferred productive binding mode of the oligosaccharide substrates. In addition mutant enzymes were obtained with altered substrate specificity, including increased specific activity towards insoluble starch [130,131]. Several examples are reported on the usefulness of chimaeric enzymes from this family, including chimaeras of related *Bacillus* α-amylases [132,133], barley α-amylase isozymes [134,135] and starch branching enzyme isozymes from maize endosperm [90,91]. These chimaeric enzymes all have modulated specificity – typically intermediate between the two or similar to one of the parent enzymes – and their properties enable tentative correlation of structural features with specificity variation. Cyclodextrin glucanotransferase specificity was rationally engineered in an α-amylase of *B. stearothermophilus* which was closely related to a CGTase in sequence from the same organism [136]. Recently the mutation of a glutamine – normally found as a transition state stabilising histidine at subsite −1 in family 13 – to histidine in glucansucrase from *Streptococcus downei* (a circularly permuted family 70 enzyme) altered the acceptor reaction to produce new oligosaccharides [120]. Interestingly the reverse mutation of histidine to glutamine in the related amylosucrase from *Neisseria polysaccharea* – which is a non-circularly permuted family 13 member – was the superior of a series of replacements and gave an enzyme with enhanced sensitivity to glycogen activation [137].

7. Conclusions and recommendations

Enzymes of the α-amylase family can bring about scission and synthesis of α-1,4-, α-1,6- and less commonly α-1,2- and α-1,3-glucosidic linkages, as well as act on sucrose and trehalose. The enzyme active sites may be considered to be composed of subsites, each capable of interacting with one monosaccharide residue. All enzymes of the family require an α-linked

glucose residue in the substrate to interact with the glycone-binding subsite −1 adjacent to the catalytic acids. Enzymes of different specificities, however, act on substrates containing different aglycones; hence the aglycone-binding subsites and the amino acid residues that constitute them are characteristic of particular specificities. The table relating specificity to short sequences that are likely to contain such amino acids has been compiled using some 300 representative enzyme sequences. New primary structures may be obtained that do not conform to any of the signatures given here. In such a case, we would recommend detailed examination of the specificity of the protein involved. Indeed it is possible that enzymes may be found, in future, with a specificity that combines the specificities of more than one known member of family 13, or that is intermediate between the specificities of known enzymes.

Although short sequences that are characteristic of certain specificities have been defined here, in many cases it is not possible to offer a rational explanation for these sequence–specificity relationships. More work is required on three-dimensional structures, particularly of enzyme–carbohydrate complexes, before the relationships will become clear.

It is unfortunate that some enzymes, clearly demonstrated to be active on both α-1,4- and α-1,6-glucosidic linkages are described in the literature as α-amylases, which by Enzyme Commission definition, catalyse hydrolysis of α-1,4-bonds only. We recommend that the name α-amylase be restricted to those enzymes that fit the definition.

Where a single name, e.g., maltogenic amylase is used for what are demonstrably two different types of enzymes, we recommend that the name be used only for the type of enzyme that can be considered as a specialised α-amylase and is inactive on α-1,6-glucosidic linkages. Where enzymes are shown to act on both α-1,4- and α-1,6-glucosidic linkages and produce maltose from starch, it is likely they can also hydrolyse cyclodextrins and pullulans [32]. In this case, the preferred substrate should be identified – this is more likely to be cyclodextrin or pullulan rather than starch [32] – and the enzyme named accordingly, i.e., either cyclodextrinase or neopullulanase.

For the enzymes that catalyse hydrolysis of α-1,4-linkages in amylose and α-1,6-linkages in pullulan, we recommend the proteins with a single active site be called amylopullulanases, while the enzymes with two active sites, each active site being specific for one bond type, could be called α-amylase-pullulanases.

Acknowledgements

M.T. Jensen and J.G. Olsen are thanked for help with Figs. 1,3 and 6. E.A.MacG. wishes to thank the University of Manitoba for the provision of office space and computer facilities. S.J. thanks the Slovak Grant Agency for Science (VEGA Grant No. 2/6045/99), FEBS for a short-term fellowship, and the Slovak literary Fund for financial support. B.S. was supported by the EU 4th Framework Programme (BIO4-CT98-0022).

References

[1] B. Henrissat, A classification of glycosyl hydrolases based on amino acid sequence similarities, Biochem. J. 280 (1991) 309–316.
[2] B. Henrissat, A. Bairoch, New families in the classification of glyosyl hydrolases based on amino acid sequence similarities, Biochem. J. 293 (1993) 781–788.
[3] B. Henrissat, A. Bairoch, Updating the sequence-based classification of glycosyl hydrolases, Biochem. J. 316 (1996) 695–696.
[4] N. Aghajari, G. Feller, C. Gerday, R. Haser, Crystal structures of the psychrophilic α-amylase from *Alteromonas haloplanctis* in its native form and complexed with an inhibitor, Protein Sci. 7 (1998) 564–572.
[5] M. Machius, G. Wiegand, R. Huber, Crystal structure of calcium-depleted *Bacillus licheniformis* α-amylase at 2.2 Å resolution, J. Mol. Biol. 246 (1995) 545–559.
[6] Z. Fujimoto, K. Takase, N. Doui, M. Momma, T. Matsumoto, H. Mizuno, Crystal structure of a catalytic-site mutant α-amylase from *Bacillus subtilis* complexed with maltopentaose, J. Mol. Biol. 277 (1998) 393–407.
[7] R.L. Brady, A.M. Brzozowski, Z.S. Derewenda, E.J. Dodson, G.G. Dodson, Solution of the structure of *Aspergillus niger* acid α-amylase by combined molecular replacement and multiple isomorphous replacement methods, Acta Cryst. B47 (1991) 527–535.
[8] Y. Matsuura, M. Kusunoki, W. Harada, M. Kakudo, Structure and possible catalytic residues of Taka amylase A, J. Biochem. 95 (1984) 697–702.
[9] A. Kadziola, J. Abe, B. Svensson, R. Haser, Crystal and molecular structure of barley α-amylase, J. Mol. Biol. 239 (1994) 104–121.

[10] S. Strobl, K. Maskos, M. Betz, G. Wiegand, R. Huber, F.X. Gomis-Ruth, R. Glockshuber, Crystal structure of yellow meal worm α-amylase at 1.64 Å resolution, J. Mol. Biol. 278 (1998) 617–628.
[11] M. Qian, R. Haser, F. Payan, Structure and molecular model refinement of pig pancreatic α-amylase at 2.1 Å resolution, J. Mol. Biol. 231 (1993) 785–799.
[12] G.D. Brayer, Y. Luo, S.G. Withers, The structure of human pancreatic α-amylase at 1.8 Å resolution and comparisons with related enzymes, Protein Sci. 4 (1995) 1730–1742.
[13] N. Ramasubbu, V. Paloth, Y. Luo, G.D. Brayer, M.J. Levine, Structure of human salivary α-amylase at 1.6 Å resolution: implications for its role in the oral cavity, Acta Cryst. D52 (1996) 435–446.
[14] H. Kizaki, Y. Hata, K. Watanabe, Y. Katsube, Y. Suzuki, Polypeptide folding of *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase revealed by 3 Å resolution X-ray analysis, J. Biochem. 113 (1993) 646–649.
[15] Y. Morishita, K. Hasegawa, Y. Matsuura, Y. Katsube, M. Kubota, S. Sakai, Crystal structure of a maltotetraose-forming exo-amylase from *Pseudomonas stutzeri*, J. Mol. Biol. 267 (1997) 661–672.
[16] Y. Katsuya, Y. Mezaki, M. Kubota, Y. Matsuura, Three-dimensional structure of *Pseudomonas isoamylase* at 2.2 Å resolution, J. Mol. Biol. 281 (1998) 885–897.
[17] Z. Dauter, M. Dauter, A.M. Brzozowski, S. Christensen, T.V. Borchert, L. Beier, K.S. Wilson, G.J. Davies, X-ray structure of Novamyl, the five-domain 'maltogenic' α-amylase from *Bacillus stearothermophilus*: maltose and acarbose complexes at 1.7 Å resolution, Biochemistry 38 (1999) 8385–8392.
[18] J.S. Kim, S.S. Cha, H.J. Kim, T.J. Kim, N.C. Ha, S.T. Oh, H.S. Cho, M.J. Cho, M.J. Kim, H.S. Lee, J.W. Kim, K.Y. Choi, K.H. Park, B.H. Oh, Crystal structure of a maltogenic amylase provides insights into a catalytic versatility, J. Biol. Chem. 274 (1999) 26279–26286.
[19] S. Kamitori, S. Kondo, K. Okuyama, T. Yokota, Y. Shimura, T. Tonozuka, Y. Sakano, Crystal structure of *Thermoactinonyces vulgaris* R-47 α-amylase II (TVAII) hydrolyzing cyclodextrins and pullulan at 2.6 Å resolution, J. Mol. Biol. 287 (1999) 907–921.
[20] M.D. Feese, Y. Kato, T. Tamada, M. Kato, T. Komeda, Y. Miura, M. Hirose, K. Hondo, K. Kobayashi, R. Kuroki, Crystal structure of glycosyltrehalose trehalohydrolase from the hyperthermophilic archaeum *Sulfolobus solfataricus*, J. Mol. Biol. 301 (2000) 451–464.
[21] L. Skov, O. Mirza, A. Henriksen, G. Potocki de Montalk, M. Remaud-Simeon, P. Sarbacal, R.-M. Willemot, P. Monsan, M. Gajhede, Structure of the glucan synthesising enzyme amylosucrase, in: International Carbohydrate Symposium 2000, Hamburg, abstract C-012.
[22] B.E. Hofmann, H. Bender, G.E. Schulz, Three-dimensional structure of cyclodextrin glucosyltransferase from *Bacillus circulans* at 3.4 Å resolution, J. Mol. Biol. 209 (1989) 793–800.
[23] C.L. Lawson, R. van Montfort, B. Strokopytov, H.J. Rozeboom, K.H. Kalk, G.E. de Vries, D. Penninga, L. Dijkhuizen, B.W. Dijkstra, Nucleotide sequence and X-ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form, J. Mol. Biol. 236 (1994) 590–600.
[24] Y. Matsuura, M. Kubota, Crystal structure of cyclodextrin glucanotransferase from *Bacillus stearothermophilus* and its carbohydrate binding sites, in: Enzyme Chemistry and Molecular Biology of Amylases and Related Enzymes, CRC Press, London, 1995, pp. 153–162.
[25] R.M.A. Knegtel, R.D. Wind, H.J. Rozeboom, K.H. Kalk, R.M. Buitelaar, L. Dijkhuizen, B.W. Dijkstra, Crystal structure at 2.3 Å resolution and revised nucleotide sequence of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EMl, J. Mol. Biol. 256 (1996) 611–622.
[26] K. Harata, K. Haga, A. Nakamura, M. Aoyagi, K. Yamane, X-ray structure of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011. Comparison of two independent molecules at 1.8 Å resolution, Acta Cryst. D52 (1996) 1136–1145.
[27] I. Przylas, K. Tomoo, Y. Terada, T. Takaha, K. Fujii, W. Saenger, N. Sträter, Crystal structure of amylomaltase from *Thermus aquaticus*, a glycosyltransferase catalysing the production of large cyclic glucans, J. Mol. Biol. 296 (2000) 873–886.
[28] E.A. MacGregor, B. Svensson, A super-secondary structure predicted to be common to several α-1,4-D-glucan-cleaving enzymes, Biochem. J. 259 (1989) 145–152.
[29] H.M. Jespersen, E.A. MacGregor, M.R. Sierks, B. Svensson, Comparison of the domain-level organization of starch hydrolases and related enzymes, Biochem. J. 280 (1991) 51–55.
[30] H.M. Jespersen, E.A. MacGregor, B. Henrissat, M.R. Sierks, B. Svensson, Starch- and glycogen-debranching and branching enzymes: prediction of structural feature of the catalytic $(\beta/\alpha)_8$-barrel domain and evolutionary relationship to other amylolytic enzymes, J. Protein Chem. 12 (1993) 791–805.
[31] S. Janecek, B. Svensson, B. Henrissat, Domain evolution in the α-amylase family, J. Mol. Evol. 45 (1997) 322–331.
[32] K.H. Park, T.J. Kim, T.K. Cheong, J.W. Kim, B.H. Oh, B. Svensson, Structure, specificity and function of cyclomaltodextrinase, a multispecific enzyme of the α-amylase family, Biochim. Biophys. Acta 1478 (2000) 165–185.
[33] S. Janecek, α-Amylase family: molecular biology and evolution, Prog. Biophys. Mol. Biol. 67 (1997) 67–97.
[34] S. Janecek, Structural features and evolutionary relationships in the α-amylase family, in: M. Ohnishi, T. Hayashi, S. Ishijima, T. Kuriki (Eds.), Glycoenzymes, Japan Scientific Societies Press, Tokyo, 2000, pp. 19–54.
[35] P.M. Coutinho, B. Henrissat, Carbohydrate-active Enzymes server, at URL: http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html (1999)
[36] E.A. MacGregor, H.M. Jespersen, B. Svensson, A circularly permuted α-amylase-type α/β-barrel structure in glucan-syn-

thesizing glucosyltransferases, FEBS Lett. 378 (1996) 263–266.
[37] B. Svensson, Protein engineering in the α-amylase family: catalytic mechanism, substrate specificity, and stability, Plant Mol. Biol. 25 (1994) 141–157.
[38] G. Davies, M.L. Sinnott, S.G. Withers, Glycosyl transfer, in: M. Sinnott (Ed.), Comprehensive Biological Catalysis, Academic Press, New York, 1998, pp. 119–208.
[39] H.D. Ly, S.G. Withers, Mutagenesis of glycosidases, Annu. Rev. Biochem. 68 (1999) 487–522.
[40] J.C.M. Uitdehaag, R. Mosi, K.H. Kalk, B.A. van der Veen, L. Dijkhuizen, S.G. Withers, B.W. Dijkstra, X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase elucidate catalysis in the α-amylase family, Nat. Struct. Biol. 6 (1999) 432–436.
[41] J.C.M. Uitdehaag, K.H. Kalk, B.A. van der Veen, L. Dijkhuizen, B.W. Dijkstra, The cyclization mechanism of cyclodextrin glycosyltransferase (CGTase) as revealed by a γ-cyclodextrin-CGTase complex at 1.8 Å resolution, J. Biol. Chem. 274 (1999) 34868–34876.
[42] S. Janecek, E.A. MacGregor, B. Svensson, Characteristic differences in the primary structure allow discrimination of cyclodextrin glucanotransferases from α-amylases, Biochem. J. 305 (1995) 685–686.
[43] J. Zhou, T. Baba, T. Takano, S. Kobayashi, Y. Arai, Nucleotide sequence of the maltotetrahydrolase gene from *Pseudomonas saccharophila*, FEBS Lett. 255 (1989) 37–41.
[44] A. Candussio, G. Schmid, A. Bock, Biochemical and genetic analysis of a maltopentaose-producing amylase from an alkalophilic Gram-positive bacterium, Eur. J. Biochem. 191 (1990) 177–185.
[45] O. Shirokizawa, T. Akiba, K. Horikoshi, Nucleotide sequence of the G6-amylase gene from alkalophilic *Bacillus* sp. H-167, FEMS Microbiol. Lett. 70 (1990) 131–136.
[46] T. Yamamoto, S. Fujiwara, Y. Tachibaha, M. Takagi, K. Fukui, T. Imanaka, Alteration of product specificity of cyclodextrin glucanotransferase from *Thermococcus* sp. B1001 by site-directed mutagenesis, J. Biosci. Bioeng. 89 (2000) 206–209.
[47] A. Geber, P.R. Williamson, J.H. Rex, E.C. Sweeney, J.E. Bennett, Cloning and characterization of a *Candida albicans* maltase gene involved in sucrose utilization, J. Bacteriol. 174 (1992) 6992–6996.
[48] B. Strokopytov, R.M.A. Knegtel, D. Penninga, H.J. Rozeboom, K.H. Kalk, L. Dijkhuizen, B.W. Dijkstra, Structure of cyclodextrin glycosyltransferase complexed with a maltononaose inhibitor at 2.6 Å resolution. Implications for product specificity, Biochemistry 35 (1996) 4241–4249.
[49] M. Qian, R. Haser, G. Buisson, E. Duée, F. Payan, The active center of a mammalian α-amylase. Structure of the complex of a pancreatic α-amylase with a carbohydrate inhibitor refined to 2.2 Å resolution, Biochemistry 33 (1994) 6284–6294.
[50] V. Nahoum, G. Roux, V. Anton, P. Rougé, A. Puigserver, H. Bischoff, B. Henrissat, F. Payan, Crystal structures of human pancreatic α-amylase in complex with carbohydrate and proteinaceous inhibitors, Biochem. J. 346 (2000) 201–208.
[51] G. André, A. Buleon, R. Haser, V. Tran, Amylose chain behavior in an interacting context III. Complete occupancy of the AMY2 barley α-amylase cleft and comparison with biochemical data, Biopolymers 50 (1999) 751–762.
[52] G. Parsiegla, A.K. Schmidt, G.E. Schulz, Substrate binding to a cyclodextrin glycosyltransferase and mutations increasing the γ-cyclodextrin product, Eur. J. Biochem. 255 (1998) 710–717.
[53] A.K. Schmidt, S. Cottaz, H. Driguez, G.E. Schulz, Structure of cyclodextrin glycosyltransferase complexed with a derivative of its main product β-cyclodextrin, Biochemistry 37 (1998) 5909–5915.
[54] A.M. Brzozowski, G.J. Davies, Structure of the *Aspergillus oryzae* α-amylase complexed with the inhibitor acarbose at 2.0 Å resolution, Biochemistry 36 (1997) 10837–10845.
[55] S. Janecek, E. Leveque, A. Belarbi, B. Haye, Close evolutionary relatedness of α-amylases from archaea and plants, J. Mol. Evol. 48 (1999) 421–426.
[56] S.H. Hong, J. Marmur, Primary structure of the maltase gene of the MAL6 locus of *Saccharomyces carlsbergensis*, Gene 41 (1986) 75–84.
[57] K.R. Piper, B. von Bodman, D.M. Cook, I. Hwang, H. Kim, S.K. Farrand, GenBank data base, accession no. AF010180 (1998).
[58] P. Fournier, P. de Ruffray, L. Otten, Natural instability of *Agrobacterium vitis* Ti plasmid due to unusual duplication of a 2.3-kb DNA fragment, Mol. Plant Microbe Interact. 7 (1994) 164–172.
[59] H. Aiba, T. Baba, K. Fujita, K. Hayashi, T. Inada, K. Isono, T. Itoh, H. Kasai, K. Kashimoto, S. Kimura, M. Kitakawa, M. Kitagawa, K. Makino, T. Miki, K. Mizobuchi, H. Mori, T. Mori, K. Motomura, S. Nakade, Y. Nakamura, H. Nashimoto, Y. Nishio, T. Oshima, N. Saito, G. Sampei, Y. Seki, S. Sivasundaram, H. Tagami, J. Takeda, K. Takemoto, Y. Takeuchi, C. Wada, Y. Yamamoto, T. Horiuchi, GenBank data base, accession no. D90768 (1996).
[60] R.N. Trethewey, A.R. Fernie, A. Bachmann, H. Fleischer-Notter, P. Geigenberger, L. Willmitzer, GenBank data base, accession no. AF158367 (1999).
[61] C. Christophersen, D.E. Otzen, B.E. Norman, S. Christensen, T. Schafer, Enzymic characterization of Novamyl, a thermostable α-amylase, Starch 50 (1998) 39–45.
[62] Tables of enzymes studied are given at URL: http://nic.savba.sk/~umikstef/bba
[63] Y. Yoshioka, K. Hasegawa, Y. Matsuura, Y. Katsube, M. Kubota, Crystal structures of a mutant maltotetraose-forming exo-amylase cocrystallized with maltopentaose, J. Mol. Biol. 271 (1997) 619–628.
[64] A. Kadziola, M. Søgaard, B. Svensson, R. Haser, Molecular structure of a barley α-amylase-inhibitor complex: implications for starch binding and catalysis, J. Mol. Biol. 278 (1998) 205–217.
[65] Y.W. Kim, T.J. Kim, J.W. Kim, K.H. Park, GenBank data base, accession no. AF116581 (1998).

[66] B. Diderichsen, L. Christiansen, Cloning of a maltogenic α-amylase from *Bacillus stearothermophilus*, FEMS Microbiol. Lett. 56 (1988) 53–60.
[67] A.P. Kelly, GenBank data base, accession no. Z22520 (1993).
[68] H.J. Cha, H.G. Yoon, Y.W. Kim, H.S. Lee, J.W. Kim, K.S. Kweon, B.H. Oh, K.H. Park, Molecular and enzymic characterization of a maltogenic amylase that hydrolyzes and transglycosylates acarbose, Eur. J. Biochem. 253 (1998) 251–262.
[69] H.Y. Cho, T.J. Kim, J.W. Kim, K.H. Park, GenBank data base, accession no. AF115340 (1998).
[70] T.J. Kim, J.W. Kim, K.H. Park, GenBank data base, accession no. AF060204 (1998).
[71] I.C. Kim, J.H. Cha, J.R. Kim, S.Y. Jang, B.C. Seo, T.K. Cheong, D.S. Lee, Y.D. Choi, K.H. Park, Catalytic properties of the cloned amylase from *Bacillus licheniformis*, J. Biol. Chem. 267 (1992) 22108–22114.
[72] K. Yamane, Y. Hirata, T. Furusato, H. Yamakazi, A. Nakayama, Changes in the properties and molecular weights of *Bacillus subtilis* M-type and N-type α-amylases resulting from a spontaneous deletion, J. Biochem. 96 (1984) 1849–1858.
[73] T. Kuriki, T. Imanaka, Nucleotide sequence of the neopullulanase gene from *Bacillus stearothermophilus*, J. Gen. Microbiol. 135 (1989) 1521–1528.
[74] T. Tonozuka, S. Mogi, Y. Shimura, A. Ibuka, H. Sakai, H. Matsuzawa, Y. Sakano, T. Ohta, Comparison of primary structures and subsite specificities of two pullulan-hydrolyzing α-amylases, TVA I and TVA II, from *Thermoactinomyces vulgaris* R-47, Biochim. Biophys. Acta 1252 (1995) 35–42.
[75] J.F. Robyt, D. French, The action pattern of porcine pancreatic α-amylase in relationship to the substrate binding site of the enzyme, J. Biol. Chem. 245 (1970) 3917–3927.
[76] T. Suganuma, R. Matsuno, M. Ohnishi, K. Hiromi, A study of the mechanism of action of Taka amylase A on linear oligosaccharides by product analysis and computer simulation, J. Biochem. 84 (1978) 293–316.
[77] A.W. MacGregor, J.E. Morgan, E.A. MacGregor, The action of germinated barley α-amylases on linear maltodextrins, Carbohydr. Res. 227 (1992) 301–313.
[78] N. Aghajari, R. Haser, Reactivity of psychrophilic α-amylase in the crystalline state, in: H.J. Gilbert, G.J. Davies, B. Henrissat, B. Svensson (Eds.), Proceedings of Recent Advances in Carbohydrate Bioengineering, Newcastle, Royal Society of Chemistry, 1999, pp. 175–185.
[79] J.F. Robyt, D. French, Multiple attack hypothesis of α-amylase action: action of porcine pancreatic, human salivary, and *Aspergillus oryzae* α-amylases, Arch. Biochem. Biophys. 122 (1967) 8–16.
[80] M. Machius, L. Vértesy, R. Huber, G. Wiegand, Carbohydrate and protein-based inhibitors of porcine pancreatic α-amylase: structure analysis and comparison of their binding characteristics, J. Mol. Biol. 260 (1998) 409–421.
[81] M. Machius, N. Declerck, R. Huber, G. Wiegand, Activation of *Bacillus licheniformis* α-amylase through a disorder → order transition of the substrate-binding site mediated by a calcium-sodium-calcium metal triad, Structure 6 (1998) 281–292.
[82] R. Bird, R.H. Hopkins, The action of some α-amylases on amylose, Biochem. J. 56 (1954) 86–99.
[83] S. Janecek, Sequence of archaeal *Methanococcus jannaschii* α-amylase contains features of families 13 and 57 of glycosyl hydrolases: a trace of their common ancestor?, Folia Microbiol. 43 (1998) 123–128.
[84] B.A. van der Veen, J.C.M. Uitdehaag, D. Penninga, G.J.W.M. van Alebeek, L.M. Smith, B.W. Dijkstra, L. Dijkuizen, Rational design of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 to increase α-cyclodextrin production, J. Mol. Biol. 296 (2000) 1027–1038.
[85] D. Penninga, B.A. van der Veen, R.M.A. Knegtel, S.A.F.T. van Hijum, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra, L. Dijkuizen, The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251, J. Biol. Chem. 271 (1996) 32777–32784.
[86] R.D. Wind, R.M. Buitelaar, L. Dijkhuizen, Engineering of factors determining α-amylase and cyclodextrin glycosyltransferase specificity in the cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1, Eur. J. Biochem. 253 (1998) 598–605.
[87] R. Mosi, S. He, J. Uitdehaag, B.W. Dijkstra, S.G. Withers, Trapping and characterization of the reaction intermediate in cyclodextrin glycosyltransferase by use of activated substrates and a mutant enzyme, Biochemistry 36 (1997) 9927–9934.
[88] T. Yamamoto, S. Fujiwara, Y. Tachibana, M. Takagi, K. Fukui, T. Imanaka, Alteration of product specificity of cyclodextrin glucanotransferase from *Thermococcus* sp. B1001 by site-directed mutagenesis, J. Biosci. Bioeng. 89 (2000) 206–209.
[89] R.A. Burton, J.D. Bewley, A.M. Smith, M.K. Bhattacharyya, H. Tatge, S. Ring, V. Bull, W.D.O. Hamilton, C. Martin, Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development, Plant J. 7 (1995) 3–15.
[90] T. Kuriki, D.C. Stewart, J. Preiss, Construction of chimeric enzymes out of maize endosperm branching enzymes I and II, J. Biol. Chem. 272 (1997) 28999–29004.
[91] S. Hong, J. Preiss, Localization of C-terminal domains required for the maximal activity or for determination of substrate preference of maize branching enzymes, Arch. Biochem. Biophys. 378 (2000) 349–355.
[92] K. Binderup, J. Preiss, Glutamate-459 is important for *Escherichia coli* branching enzyme activity, Biochemistry 37 (1998) 9033–9037.
[93] C. Braun, T. Lindhorst, N.B. Madsen, S.G. Withers, Identification of A549 as the catalytic nucleophile of glycogen-debranching enzyme via trapping of the glycosyl-enzyme intermediate, Biochemistry 35 (1996) 5458–5463.
[94] Y. Hatada, K. Igarashi, K. Ozaki, K. Ara, J. Hitomi, T. Kobayashi, S. Kawai, T. Watabe, S. Ito, Amino acid se-

quence and molecular structure of an alkaline amylopullulanase from *Bacillus* that hydrolyses α-1,4 and α-1,6 linkages in polysaccharides at different active sites, J. Biol. Chem. 271 (1996) 24075–24083.

[95] K. Ara, K. Igarashi, K. Saeki, S. Ito, An alkaline amylopullanase from alkalophilic *Bacillus* sp. KSM 1378; kinetic evidence for two independent active sites for the α-1,4 and α-1,6 hydrolytic reactions, Biosci. Biotechnol. Biochem. 59 (1995) 662–666.

[96] K. Ara, K. Igarashi, H. Hagihara, K. Sawada, T. Kobayashi, S. Ito, Separation of functional domains for the α-1,4 and α-1,6 hydrolytic activities of a *Bacillus* amylopullulanase by limited proteolysis with papain, Biosci. Biotechnol. Biochem. 60 (1996) 634–639.

[97] C.H. Kim, Specific detection of pullulanase type I in polyacrylamide gels, FEMS Microbiol. Lett. 116 (1994) 327–332.

[98] T. Kuriki, H. Takata, S. Okada, T. Imanaka, Analysis of the active center of *Bacillus stearothermophilus* neopullulanase, J. Bacteriol. 173 (1991) 6147–6152.

[99] A. Ibuka, T. Tonozuka, H. Matsuzawa, H. Sakai, Conversion of neopullulanase-α-amylase from *Thermoactinomyces vulgaris* R-47 into an amylopullulanase-type enzyme, J. Biochem. 123 (1998) 275–282.

[100] T.J. Kim, C.S. Park, H.Y. Cho, S.S. Cha, J.S. Kim, S.B. Lee, T.W. Moon, J.W. Kim, B.H. Oh, K.H. Park, Role of the glutamate 332 residue in the transglycosylation activity of *Thermus* maltogenic amylase, Biochemistry 39 (2000) 6773–6780.

[101] S. Kitahata, S. Okoda, Hydrolytic action on various maltosides by an enzyme from *Bacillus coagulans*, Carbohydr. Res. 137 (1985) 217–225.

[102] T.J. Kim, M.J. Kim, B.C. Kim, J.C. Kim, T.K. Cheong, J.W. Kim, K.H. Park, Modes of action of acarbose hydrolysis and transglycosylation catalysed by a thermo-stable maltogenic amylase, the gene for which was cloned from a *Thermus* strain, Appl. Environ. Microbiol. 65 (1999) 1644–1651.

[103] H.Y. Cho, Y.W. Kim, T.J. Kim, H.S. Lee, D.Y. Kim, J.W. Kim, Y.W. Lee, S.B. Lee, K.H. Park, Molecular characterization of a dimeric intracellular maltogenic amylase of *Bacillus subtilis* SUH4-2, Biochim. Biophys. Acta 1478 (2000) 333–340.

[104] F.C. Denizot, GenBank data base, accession no. Z94043 (1997).

[105] K. Igarashi, K. Ara, K. Saeki, K. Ozaki, S. Kawai, S. Ito, Nucleotide sequence of the gene that encodes a neopullulanase from an alkalophilic *Bacillus*, Biosci. Biotechnol. Biochem. 56 (1992) 514–516.

[106] J. Matzke, A. Herrmann, E. Schneider, E.P. Bakker, Gene cloning, nucleotide sequence and biochemical properties of a cytoplasmic cyclomaltodextrinase (neopullulanase) from *Alicyclobacillus acidocaldarius*, reclassification of a group of enzymes, FEMS Microbiol. Lett. 183 (2000) 55–61.

[107] T. Oguma, A. Matsuyama, M. Kikuchi, E. Nakano, Cloning and sequence analysis of the cyclomaltodextrinase gene from *Bacillus sphaericus* and expression in *Escherichia coli* cells, Appl. Microbiol. Biotechnol. 39 (1993) 197–203.

[108] S. Kashiwabara, S. Ogawa, N. Miyoshi, M. Oda, Y. Suzuki, Three domains comprised in thermostable molecular weight 54,000 pullulanase of type I from *Bacillus flavocaldarius* KP1228, Biosci. Biotechnol. Biochem. 63 (1999) 1736–1748.

[109] S. Mathupala, B.C. Saha, J.G. Zeikus, Substrate competition and specificity at the active site of amylopullulanase from *Clostridium thermohydrosulfuricum*, Biochem. Biophys. Res. Commun. 166 (1990) 126–132.

[110] R. Peist, C. Schneider-Fresenius, W. Boos, The Mal-T dependent and malZ-encoded maltodextrin glucosidase of *Escherichia coli* can be converted into a dextrinyl transferase by a single mutation, J. Biol. Chem. 271 (1996) 10681–10689.

[111] M. Yamamoto, K. Horikoshi, Nucleotide sequence of alkalophilic *Bacillus* oligo-1,6-glucosidase gene and the properties of the gene product by *Escherichia coli* HB101, Denpun Kagaku 37 (1990) 137–144.

[112] M. Nakao, T. Nakayama, M. Harada, A. Kakudo, H. Ikemoto, S. Kobayashi, S. Shibano, Purification and characterization of a *Bacillus* sp. SAM 1606 thermo-stable α-glucosidase with transglucosylation activity, Appl. Microbiol. Biotechnol. 41 (1994) 337–343.

[113] M. Nakao, T. Nakayama, A. Kakudo, M. Inohara, M. Harada, F. Omura, Y. Shibano, Structure and expression of a gene coding for a thermostable α-glucosidase with a broad substrate specificity from *Bacillus* sp. SAM 1606, Eur. J. Biochem. 220 (1994) 293–300.

[114] M. Inohara-Ochiai, T. Nakayama, R. Goto, M. Nakao, T. Ueda, Y. Shibano, Altering substrate specificity of *Bacillus* sp. SAM 1606 α-glucosidase by comparative site-specific mutagenesis, J. Biol. Chem. 272 (1997) 1601–1607.

[115] Y. Suzuki, H. Fujii, H. Uemura, M. Suzuki, Purification and characterization of extremely thermostable exo-oligo-1,6-glucosidase from a caldoactive *Bacillus* sp. KP1228, Starch 39 (1987) 17–23.

[116] S. Kashiwabara, Y. Matsuki, T. Kishimoto, Y. Suzuki, Clustered proline residues around the active-site cleft in thermostable oligo-1,6-glucosidase of *Bacillus flavocaldarius* KP1228, Biosci. Biotechnol. Biochem. 62 (1998) 1093–1102.

[117] O. Nashiru, S.Y. Lee, D.S. Lee, GenBank data base, accession no. AF096282 (1998).

[118] Y. Takii, K. Takahashi, K. Yamamoto, Y. Sogabe, Y. Suzuki, *Bacillus stearothermophilus* ATCC12016 α-glucosidase specific for α-1,4 bonds of maltosaccharides and α-glucans shows high amino acid sequence similarities to seven α-D-glucohydrolases with different substrate specificity, Appl. Microbiol. Biotechnol. 44 (1996) 629–634.

[119] K.S. Devulapalle, S.D. Goodman, Q. Gao, A. Hemsley, G. Mooser, Knowledge-based model of a glucosyl-transferase from the oral bacterial group of mutans streptococci, Protein Sci. 6 (1997) 2489–2493.

[120] V. Monchois, M. Vignon, P.-C. Escalier, B. Svensson, R.R.B. Russell, Involvement of Gln937 of *Streptococcus*

downei GTF-I glucansucrase in transition-state stabilization, Eur. J. Biochem. 267 (2000) 4127–4136.

[121] N. Declerck, M. Machius, R. Chanbert, G. Wiegand, R. Huber, C. Gaillardin, Hyperthermostable mutants of *Bacillus licheniformis* α-amylase: thermodynamic studies and structural interpretation, Prot. Eng. 10 (1997) 541–549.

[122] J.E. Nielsen, L. Beier, D. Outzen, T.V. Borchert, H.B. Frantzen, K.V. Andersen, A. Svendsen, Electrostatics in the active site of an α-amylase, Eur. J. Biochem. 264 (1999) 816–824.

[123] N. Declerck, M. Machius, G. Wiegand, R. Huber, C. Gaillardin, Probing structural determinants specifying high thermostability in *Bacillus licheniformis* α-amylase, J. Mol. Biol. 301 (2000) 1041–1057.

[124] N. Ishii, K. Haga, K. Yamane, K. Harata, Crystal structure of alkalophilic asparagine 233-replaced cyclodextrin glucanotransferase complexed with an inhibitor, acarbose, at 2.0 A resolution, J. Biochem. 127 (2000) 383–391.

[125] T. Kuriki, H. Kaneko, M. Yanase, H. Takata, J. Shimada, S. Handa, T. Takada, H. Umeyama, S. Okada, Controlling substrate preference and transglycosylation activity of neopullulanase by manipulating steric constraint and hydrophobicity in active center, J. Biol. Chem. 271 (1996) 17321–17329.

[126] T. Tonuzuka, Y. Sakano, Crystal structure of the pullulan-hydrolyzing α-amylase from *Thermoactinomyces vulgaris* R-47, and manipulation of substrate specificities, in: H.J. Gilbert, G.J. Davies, B. Henrissat, B. Svensson (Eds.), Recent Advances in Carbohydrate Bioengineering, Newcastle, Royal Society of Chemistry, 1999, pp. 150–158.

[127] I. Matsui, K. Ishikawa, S. Miyari, S. Fukui, K. Honda, An increase in transglycosylation activity of *Saccharomycopsis* α-amylase altered by site-directed mutagenesis, Biochim. Biophys. Acta 1077 (1991) 416–419.

[128] I. Matsui, K. Ishikawa, S. Miyari, S. Fukui, K. Honda, Alteration in bond-cleavage pattern in the hydrolysis catalyzed by *Saccharomycopsis* α-amylase altered by site-directed mutagenesis, Biochemistry 31 (1992) 5232–5236.

[129] I. Matsui, B. Svensson, Improved activity and modulated action pattern obtained by random mutagenesis at the fourth β-α loop involved in substrate binding to the catalytic $(\beta/\alpha)_8$-barrel domain of barley α-amylase 1, J. Biol. Chem. 272 (1997) 22456–22463.

[130] B. Svensson, K.S. Bak-Jensen, M.T. Jensen, J. Sauer, T.E. Gottschalk, K.W. Rodenburg, Studies on structure, function, and protein engineering of starch-degrading enzymes, J. Appl. Glycosci. 46 (1999) 49–63.

[131] B. Svensson, K.S. Bak-Jensen, H. Mori, J. Sauer, M.T. Jensen, B. Kramhøft, T.E. Gottschalk, T. Christensen, B.W. Sigurskjold, N. Aghajai, R. Haser, N. Payre, S. Cottaz, H. Driguez, The engineering of specificity and stability in selected starch degrading enzymes, in: H.J. Gilbert, G.J. Davies, B. Henrissat, B. Svensson (Eds.), Recent Advances in Carbohydrate Bioengineering, Newcastle, Royal Society of Chemistry, 1999, pp. 274–281.

[132] Y. Suzuki, N. Ito, T. Yuuki, H. Yamagata, S. Udaka, Amino acid residues stabilizing a *Bacillus* α-amylase against irreversible thermodenaturation, J. Biol. Chem. 264 (1989) 18933–18938.

[133] B. Conrad, V. Hoang, A. Polley, J. Hofemeister, Hybrid *Bacillus amyloquefaciens* X *Bacillus licheniformis* α-amylases; construction, properties and sequence determinants, Eur. J. Biochem. 230 (1995) 481–490.

[134] K.W. Rodenburg, N. Juge, X.-J. Guo, M. Søgaard, J.-C. Chaix, B. Svensson, Domain B protruding at the third β-strand of the α/β-barrel in barley α-amylase confers distinct isozyme-specific properties, Eur. J. Biochem. 221 (1994) 277–294.

[135] N. Juge, K.W. Rodenburg, X.-J. Guo, J.-C. Chaix, B. Svensson, Isozyme hybrids within the protruding third loop domain of the barley α-amylase $(\beta/\alpha)_8$-barrel. Implication for BASI sensitivity and substrate affinity, FEBS Lett. 363 (1995) 299–303.

[136] L. Beier, A. Svendsen, C. Andersen, T.P. Frandsen, T.V. Borchert, J.R. Cherry, Conversion of the maltogenic α-amylase Novamyl into a CGTase, Protein Eng. 13 (2000) 509–513.

[137] P. Sarcabal, M. Remaud-Simeon, R.-M. Willemot, G. Potocki de Montalk, B. Svensson, P. Monsan, Identification of key amino acid residues in *Neisseria polysaccharea* amylosucrase, FEBS Lett. 474 (2000) 33–37.

[138] M.A. Arguello-Morales, M. Renaud-Simeon, S. Pizzut, P. Sarcabal, R.-M. Willemot, P. Monsan, Sequence analysis of the gene encoding alternansucrase from *Leuconostoc mesenteroides* NRRLB-1355, FEMS Microbiol. Lett. 182 (2000) 81–85.