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Review article

# Properties and applications of starch-converting enzymes of the $\alpha$ -amylase family

Marc J.E.C. van der Maarel <sup>a,b,d,\*</sup>, Bart van der Veen <sup>a,d</sup>,  
Joost C.M. Uitdehaag <sup>c,d</sup>, Hans Leemhuis <sup>a</sup>, L. Dijkhuizen <sup>a,d</sup>

<sup>a</sup> *Microbial Physiology Research Group, Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kercklaan 30, 9751 NN Haren, The Netherlands*

<sup>b</sup> *Department of Carbohydrate Technology, TNO Nutrition and Food Research, Groningen, The Netherlands*

<sup>c</sup> *Department of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Haren, The Netherlands*

<sup>d</sup> *Centre for Carbohydrate Bioengineering TNO-RUG, P.O. Box 14, NL-9750 AA Haren, The Netherlands*

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## Abstract

Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. A large-scale starch processing industry has emerged in the last century. In the past decades, we have seen a shift from the acid hydrolysis of starch to the use of starch-converting enzymes in the production of maltodextrin, modified starches, or glucose and fructose syrups. Currently, these enzymes comprise about 30% of the world's enzyme production. Besides the use in starch hydrolysis, starch-converting enzymes are also used in a number of other industrial applications, such as laundry and porcelain detergents or as anti-staling agents in baking. A number of these starch-converting enzymes belong to a single family: the  $\alpha$ -amylase family or family13 glycosyl hydrolases. This group of enzymes share a number of common characteristics such as a  $(\beta/\alpha)_8$  barrel structure, the hydrolysis or formation of glycosidic bonds in the  $\alpha$  conformation, and a number of conserved amino acid residues in the active site. As many as 21 different reaction and product specificities are found in this family. Currently, 25 three-dimensional (3D) structures of a few members of the  $\alpha$ -amylase family have been determined using protein crystallization and X-ray crystallography. These data in combination with site-directed mutagenesis studies have helped to better understand the interactions between the substrate or product molecule and the different amino acids found in and around the active site. This review illustrates the reaction and product diversity found within the  $\alpha$ -amylase family, the mechanistic principles deduced from structure–function relationship structures, and the use of the enzymes of this family in industrial applications. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:**  $\alpha$ -Amylase; Starch; Starch-converting enzymes; Anti-staling of bread; Starch industry; Glycosylhydrolases

\* Corresponding author. Tel.: +31-50-363-2113; fax: +31-50-363-2154.

E-mail address: [maarelmj@biol.rug.nl](mailto:maarelmj@biol.rug.nl) (M.J.E.C. van der Maarel).

## 1. Introduction

Starch-containing crops form an important constituent of the human diet and a large proportion of the food consumed by the world's population originates from them. Besides the use of the starch-containing plant parts directly as a food source, starch is harvested and used as such or chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch or maltodextrin derivatives, or cyclodextrins. In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The major industrial sources are maize, tapioca, potato, and wheat. In the European Union, 3.6 million tons of maize starch, 2 million tons of wheat starch, and 1.8 millions tons of potato starch were produced in 1998 (DeBaere, 1999).

## 2. Starch

Plants synthesize starch as a result of photosynthesis, the process during which energy from the sunlight is converted into chemical energy. Starch is synthesized in plastids found in leaves as a storage compound for respiration during dark periods. It is also synthesized in amyloplasts found in tubers, seeds, and roots as a long-term storage compound. In these latter organelles, large amounts of starch accumulate as water-insoluble granules. The shape and diameter of these granules depend on the botanical origin. For commercially interesting starch sources, the granule sizes range from 2–30 (maize starch) to 5–100  $\mu\text{m}$  (potato starch) (Robyt, 1998).

Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. This glycosidic bond is stable at high pH but hydrolyzes at low pH. At the end of the polymeric chain, a latent aldehyde group is present. This group is known as the reducing end. Two types of glucose polymers are present in starch: (i) amylose and (ii) amylopectin. Amylose is a linear polymer consisting of up to 6000 glucose units with  $\alpha$ ,1-4 glycosidic bonds. The num-

ber of glucose residues, also indicated with the term DP (degree of polymerization), varies with the origin. Amylose from, e.g. potato or tapioca starch has a DP of 1000–6000 while amylose from maize or wheat amylose has a DP varying between 200 and 1200. The average amylose content in starches can vary between almost 0 and 75%, but a typical value is 20–25%. Amylopectin consists of short  $\alpha$ ,1-4 linked linear chains of 10–60 glucose units and  $\alpha$ ,1-6 linked side chains with 15–45 glucose units. The average number of branching points in amylopectin is 5%, but varies with the botanical origin. The complete amylopectin molecule contains on average about 2 000 000 glucose units, thereby being one of the largest molecules in nature. The most commonly accepted model of the structure of amylopectin is the cluster model, in which the side chains are ordered in clusters on the longer backbone chains (see Buléon et al., 1998; Myers et al., 2000).

Starch granules are organized into amorphous and crystalline regions (Fig. 1). In tuber and root starches, the crystalline regions are solely composed of amylopectin, while the amylose is present in the amorphous regions. In cereal starches, the amylopectin is also the most important component of the crystalline regions. The amylose in cereal starches is complexed with lipids that form a weak crystalline structure and reinforce the granule.

While amylopectin is soluble in water, amylose and the starch granule itself are insoluble in cold water. This makes it relatively easy to extract starch granules from their plant source. When water–starch slurry is heated, the granules first swell until a point is reached at which the swelling is irreversible. This swelling process is termed gelatinization. During this process, amylose leaches out of the granule and causes an increase in the viscosity of the slurry. Further increase in temperature then leads to maximum swelling of the granules and increased viscosity. Finally, the granules break apart resulting in a complete viscous colloidal dispersion. Subsequent cooling of concentrated colloidal starch dispersion results in the formation of an elastic gel. During retrogradation, the starch substance undergoes a change from a dissolved and dissociated state to an asso-

ciated state. Retrogradation is primarily caused by the amylose; amylopectin, due to its highly branched organization, is less prone to retrogradation.

### 3. Starch-converting enzymes

A variety of different enzymes are involved in the synthesis of starch. Sucrose is the starting point of starch synthesis. It is converted into the nucleotide sugar ADP-glucose that forms the actual starter molecule for starch formation. Subsequently, enzymes such as soluble starch synthase and branching enzyme synthesize the amylopectin and amylose molecules (Smith, 1999). These enzymes will not be discussed in this review. In bacteria, an equivalent of amylopectin is found in the form of glycogen. This has the same structure as amylopectin. The major difference lies within the side chains: in glycogen, they are shorter and about twice higher in number. A large variety of bacteria employ extracellular or intracellular enzymes able to convert starch or glycogen that can thus serve as energy and carbon sources (Fig. 2).

There are basically four groups of starch-converting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases.

#### 3.1. Endo and exoamylases

Endoamylases are able to cleave  $\alpha$ ,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain.  $\alpha$ -Amylase (EC 3.2.1.1) is a well-known endoamylase. It is found in a wide variety of microorganisms, belonging to the Archaea as well as the Bacteria (Pandey et al., 2000). The end products of  $\alpha$ -amylase action are oligosaccharides with varying length with an  $\alpha$ -configuration and  $\alpha$ -limit dextrins, which constitute branched oligosaccharides.

Enzymes belonging to the second group, the exoamylases, either exclusively cleave  $\alpha$ ,1-4 glycosidic bonds such as  $\beta$ -amylase (EC 3.2.1.2) or cleave both  $\alpha$ ,1-4 and  $\alpha$ ,1-6 glycosidic bonds like amyloglucosidase or glucoamylase (EC 3.2.1.3) and  $\alpha$ -glucosidase (EC 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and  $\alpha$ -glucosidase), or maltose and  $\beta$ -limit dextrin ( $\beta$ -amylase).  $\beta$ -Amylase and glucoamylase also convert the anomeric configuration of the liberated maltose from  $\alpha$  to  $\beta$ . Glucoamylase and  $\alpha$ -glucosidase differ in their substrate preference:  $\alpha$ -glucosidase acts best on short maltooligosaccharides and liberates glucose with an  $\alpha$ -configuration while glucoamylase hydrolyzes long-chain polysaccharides best.  $\beta$ -Amylases and glucoamylases have also been found in a large variety of microorganisms (Pandey et al., 2000).

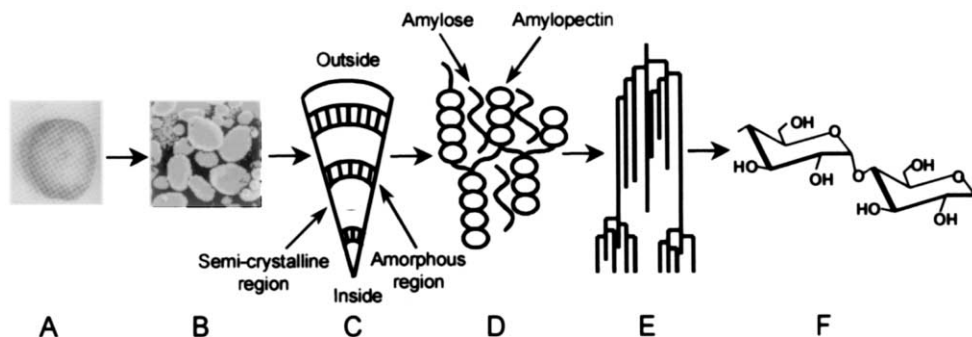


Fig. 1. Zoom in of how a potato starch tuber is built-up. A, tuber; B, electron microscopic image of starch granules; C, slice of a starch granule showing the growth rings consisting of semi-crystalline and amorphous regions; D, detail of the semi-crystalline region; E, organization of the amylopectin molecule into the tree-like structure; F, two glucose molecules with an  $\alpha$ ,1-4 glycosidic bond.

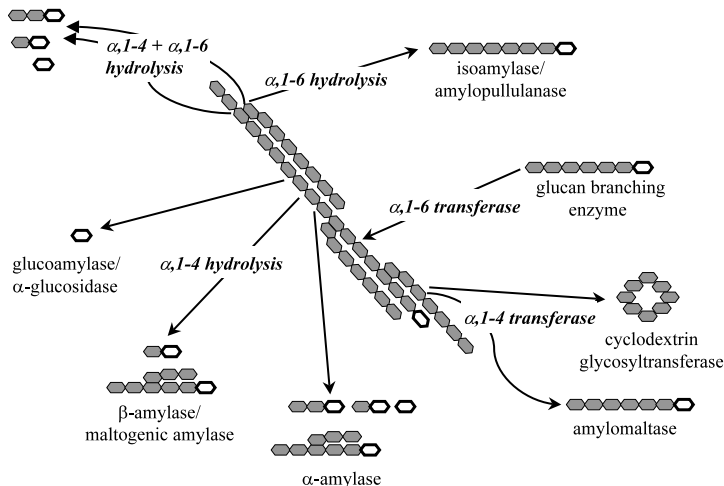


Fig. 2. Different enzymes involved in the degradation of starch. The open ring structure symbolizes the reducing end of a polyglucose molecule.

Other exo-acting amylolytic enzymes are cyclodextrin glycosyltransferase (EC 2.4.1.19), an enzyme that additionally has a transglycosylation activity, maltogenic  $\alpha$ -amylase (glucan 1,4- $\alpha$ -glucanhydrolase, EC 3.2.1.133), an amylase from *Bacillus stearothermophilus* releasing maltose (Diderichsen and Christiansen, 1988), and maltotriose forming amylases such as the maltotetraose forming enzyme from *Pseudomonas stutzeri* (EC 3.2.1.60; Robyt and Ackerman, 1971) or the maltohexaose forming amylase (EC 3.2.1.98) from *Klebsiella pneumoniae* (Momma, 2000).

### 3.2. Debranching enzymes

The third group of starch-converting enzymes are the debranching enzymes that exclusively hydrolyze  $\alpha$ ,1-6 glycosidic bonds: isoamylase (EC 3.2.1.68) and pullanase type I (EC 3.2.1.41). The major difference between pullulanases and isoamylase is the ability to hydrolyze pullulan, a polysaccharide with a repeating unit of maltotriose that is  $\alpha$ ,1-6 linked (Bender et al., 1959; Israilides et al., 1999). Pullulanases hydrolyze the  $\alpha$ ,1-6 glycosidic bond in pullulan and amylopectin, while isoamylase can only hydrolyze the  $\alpha$ ,1-6 bond in amylopectin. These enzymes exclusively degrade amylopectin, thus leaving long lin-

ear polysaccharides. From *Sclerotium rolfsii*, a glucoamylase has been identified that also has a significant action on pullulan (Kelkar and Deshpande, 1993).

There are also a number of pullulanase type enzymes that hydrolyze both  $\alpha$ ,1-4 and  $\alpha$ ,1-6 glycosidic bonds. These belong to the group II pullulanase and are referred to as  $\alpha$ -amylase–pullulanase or amylopullulanase. The main degradation products are maltose and maltotriose. A special enzyme belonging to this group of pullulanases is neopullulanase, which can also perform transglycosylation with the formation of a new  $\alpha$ ,1-4 or  $\alpha$ ,1-6 glycosidic bond (Takata et al., 1992).

### 3.3. Transferases

The fourth group of starch-converting enzymes are transferases that cleave an  $\alpha$ ,1-4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amyломaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC 2.4.1.19) form a new  $\alpha$ ,1-4 glycosidic bond while branching enzyme (EC 2.4.1.18) forms a new  $\alpha$ ,1-6 glycosidic bond.

Cyclodextrin glycosyltransferases have a very low hydrolytic activity and make cyclic oligosac-

charides with 6, 7, or 8 glucose residues and highly branched high molecular weight dextrans, the cyclodextrin glycosyltransferase limit dextrans. Cyclodextrins are produced via an intramolecular transglycosylation reaction in which the enzyme cleaves the  $\alpha$ ,1-4 glycosidic bond and concomitantly links the reducing to the non-reducing end (Takaha and Smith, 1999; Van der Veen et al., 2000a).

Amylomaltases are very similar to cyclodextrin glycosyltransferases with respect to the type of enzymatic reaction. The major difference is that amylomaltase performs a transglycosylation reaction resulting in a linear product while cyclodextrin glycosyltransferase gives a cyclic product. Amylomaltases have been found in different microorganisms in which they are involved in the utilization of maltose or the degradation of glycogen (Takaha and Smith, 1999).

Glucan branching enzymes are involved in the synthesis of glycogen in many microorganisms. They are responsible for the formation of  $\alpha$ ,1-6 glycosidic bonds in the side chains of glycogen. Although glycogen has been found in a large number of microorganisms (Preiss, 1984), only a limited number of microbial glucan branching enzymes have been characterized (Kiel et al., 1991, 1992; Takata et al., 1994; Binderup and Preiss, 1998).

#### 4. The $\alpha$ -amylase family: characteristics and reaction mechanism

Most of the enzymes that convert starch belong to one family based on the amino acid sequence homology: the  $\alpha$ -amylase family or family 13 glycosyl hydrolases according to the classification of Henrissat (1991). This group comprises those enzymes that have the following features: (i) they act on  $\alpha$ -glycosidic bonds and hydrolyze this bond to produce  $\alpha$ -anomeric mono- or oligosaccharides (hydrolysis), form  $\alpha$ ,1-4 or 1-6 glycosidic linkages (transglycosylation), or a combination of both activities; (ii) they possess a  $(\beta/\alpha)_8$  or TIM barrel (Fig. 3) structure containing the catalytic site residues; (iii) they have four highly conserved regions in their primary sequence (Table 1) which contain the amino acids that form the catalytic site, as well as some amino acids that are essential for the stability of the conserved TIM barrel topology (Kuriki and Imanaka, 1999). The enzymes that match the above-mentioned criteria and belong to the  $\alpha$ -amylase family are listed in Table 2.

##### 4.1. The catalytic mechanism

The  $\alpha$ -glycosidic bond is very stable having a spontaneous rate of hydrolysis of approximately

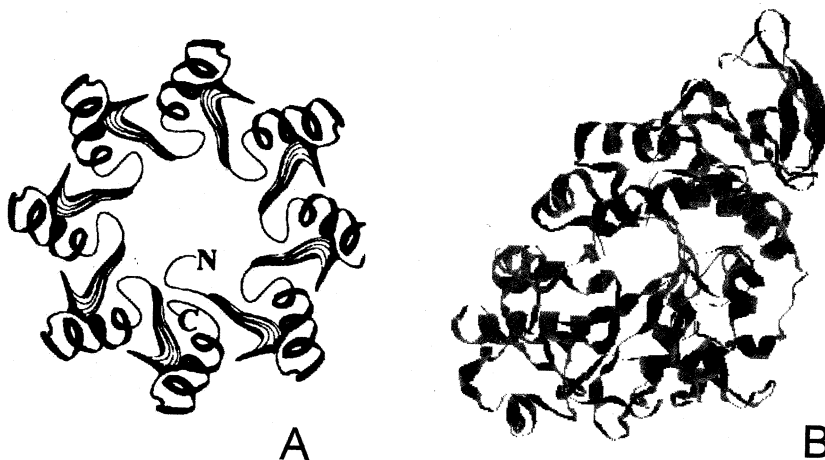


Fig. 3. Schematic representation of the  $(\beta/\alpha)_8$  barrel (A) and 3D structure of the  $\alpha$ -amylase of *Aspergillus oryzae* or Taka amylase (B), obtained from the Protein Database.

Table 1

The four conserved regions and the corresponding  $\beta$ -sheets found in the amino acid sequence of  $\alpha$ -amylase family enzymes

	I $\beta$ 2	II $\beta$ 4	III $\beta$ 5	IV $\beta$ 7
Amylomaltase	EALGIRIIGMPIFVAED	LFHLVRIDHFRG	VPVLAEDLGVI	VVYTGTHDNDT
Amylosucrase	HEAGISAVVDFIFNHTSN	GVDILRMDAVAF	VFFKSEAIVHP	VNYVRSDDIG
CGTase	HAKNIKVIIDFAPNHTSP	GIDGIRMDAVKH	VFTFGFWFLGV	VTFIDNHDMER
CMDase	HDNGIKVIFDAVFNHCGY	DIDGWRLDVANE	AIIVGEVWHDA	FNLIGSHDTER
BE	HQAGIGVILDWVPGHFCK	HVDGFRVDAVAN	ILMIAEDSTDW	FILPFSHDEVV
Isoamylase	HNAGIKVYMDVVYNHTAE	GVDGFRFDLASV	LDLFAEPWAIG	INFIDVHDGMT
M. amylase	HQKAIKRVMLDAVFNHSGY	DIDGWRLDVANE	AYILGEIWHDA	FNLLGSHDTPR
Pullulanase	HAHGVRVILDGVFNHTGR	GVDGWRLDVPNE	AYIVGEIWEWA	MNLLTSHDTPR
Sucrose Pase	LGECSHLMFDFVCNHMSA	GAEYVRLDAVGF	TVIITITNVPH	FNFLASHDGIG
BLamylase	HERGMYLMVDVVANHMGY	SIDGLRIDTVKH	VYCIGEVLDGD	GTFVENVHNDPR

Highlighted are the conserved catalytic amino acid residues. The following enzymes were used for the alignment: amylomaltase of *Thermus aquaticus* (Terada et al., 1999); amylosucrase of *Neisseria polysaccharea* (Büttcher et al., 1997); CGTase: cyclodextrin glycosyltransferase of *Bacillus circulans* 251 (Lawson et al., 1994); CMDase: cyclomaltodextrinase of *Clostridium thermohydrosulfuricum* 39E (Podkovyrov and Zeikus, 1992); BE: branching enzyme of *Bacillus stearothermophilus* (Kiel et al., 1991); isoamylase of *Pseudomonas amyloclavata* (Amemura et al., 1988); M. amylase: maltogenic  $\alpha$ -amylase of *Bacillus stearothermophilus* (Cha et al., 1998); pullulanase of *Bacillus flavocaldarius* KP 1228 (Kashiwabara et al., 1999); Sucrose Pase: sucrose phosphorylase of *Escherichia coli* K12 (Aiba et al., 1996); BLamylase:  $\alpha$ -amylase of *Bacillus licheniformis* (Kim et al., 1992).  $\beta$ 2,  $\beta$ 4,  $\beta$ 5, and  $\beta$ 7 indicate the  $\beta$ -sheet in which this region is present.

$2 \times 10^{-15} \text{ s}^{-1}$  at room temperature (Wolfenden et al., 1998). Members of the  $\alpha$ -amylase family enhance this rate so enormously that they can be considered to belong to the most efficient enzymes known. Cyclodextrin glycosyltransferase, e.g. has a rate of hydrolysis of  $3 \text{ s}^{-1}$  (Van der Veen et al., 2000b) and thereby increases the rate by  $10^{15}$  fold.

The generally accepted catalytic mechanism of the  $\alpha$ -amylase family is that of the  $\alpha$ -retaining double displacement. The mechanism involves two catalytic residues in the active site; a glutamic acid as acid/base catalyst and an aspartate as the nucleophile (Fig. 4). It involves five steps: (i) after the substrate has bound in the active site, the glutamic acid in the acid form donates a proton to the glycosidic bond oxygen, i.e. the oxygen between two glucose molecules at the subsites  $-1$  and  $+1$  and the nucleophilic aspartate attacks the C1 of glucose at subsite  $-1$ ; (ii) an oxocarbenium ion-like transition state is formed followed by the formation of a covalent intermediate; (iii) the protonated glucose molecule at subsite  $+1$  leaves the active site while a water molecule or a new glucose molecule moves into the active site and attacks the covalent bond between the glu-

cose molecule at subsite  $-1$  and the aspartate; (iv) an oxocarbenium ion-like transition state is formed again; (v) the base catalyst glutamate accepts a hydrogen from an incoming water or the newly entered glucose molecule at subsite  $+1$ , the oxygen of the incoming water or the newly entered glucose molecule at subsite  $+1$  replaces the oxocarbenium bond between the glucose molecule at subsite  $-1$  and the aspartate forming a new hydroxyl group at the C1 position of the glucose at subsite  $-1$  (hydrolysis) or a new glycosidic bond between the glucose at subsite  $-1$  and  $+1$  (transglycosylation). Recently, studies with cyclodextrin glycosyltransferase from *Bacillus circulans* 251 have shown that the intermediate indeed has a covalently linked bond with the enzyme (Uitdehaag et al., 1999).

In the above-mentioned double displacement mechanism as proposed by Koshland (1953), only two of the three conserved catalytic residues directly play a role. The third conserved residue, a second aspartate, binds to the OH-2 and OH-3 groups of the substrate through hydrogen bonds and plays an important role in the distortion of the substrate (Uitdehaag et al., 1999). Other con-

served amino acid residues can be histidine, arginine, and tyrosine. They play a role in positioning the substrate into the correct orientation into the active site, proper orientation of the nucleophile, transition state stabilization, and polarization of the electronic structure of the substrate (Nakamura et al., 1993; Lawson et al., 1994; Strokopytov et al., 1996; Uitdehaag et al., 1999).

Besides the four conserved amino acid sequence regions, an additional fifth conserved region can be identified in members of the  $\alpha$ -amylase family (Janecek, 1992, 1995). This region also contains an aspartate that acts as calcium ligand.

#### 4.2. Domain organization

A characteristic feature of the enzymes from the  $\alpha$ -amylase family is that they all employ the  $\alpha$ -retaining mechanism but that they vary widely in their substrate and product specificities. These differences can be attributed to the attachment of different domains to the catalytic core (Table 2)

or to extra sugar-binding subsites around the catalytic site. The most conserved domain found in all  $\alpha$ -amylase family enzymes, the A-domain, consists of a highly symmetrical fold of eight parallel  $\beta$ -strands arranged in a barrel encircled by eight  $\alpha$ -helices. The highly conserved amino acid residues of the  $\alpha$ -amylase family that are involved in catalysis and substrate binding are located in loops at the C-termini of  $\beta$ -strands in this domain. The  $(\beta/\alpha)_8$  barrel has first been observed in chicken muscle triose phosphate isomerase (Banner et al., 1975) and is therefore also called the TIM barrel. It is not only present in members of the  $\alpha$ -amylase family but it has also been shown to be widespread in functionally diverse enzymes (Svensson and Sogaard, 1991). All enzymes of the  $\alpha$ -amylase family have a B-domain that protrudes between  $\beta$  sheet no 3 and  $\alpha$  helix no 3. It ranges in length from 44 to 133 amino acid residues and plays a role in substrate or  $\text{Ca}^{2+}$  binding.

Besides the A- and B-domains, nine other domains have been identified in members of the

Table 2

Enzymes of the  $\alpha$ -amylase family that act on glucose-containing substrates, their corresponding EC number, the domain organization as far as it has been described, and main substrates

Enzyme	EC number	Domains	Main substrate
Amylosucrase	2.4.1.4		Sucrose
Sucrose phosphorylase	2.4.1.7		Sucrose
Glucan branching enzyme	2.4.1.18	A, B, F	Starch, glycogen
Cyclodextrin glycosyltransferase	2.4.1.19	A, B, C, D, E	Starch
Amylomaltase	2.4.1.25	A, B1, B2	Starch, glycogen
Maltopentaose-forming amylase	3.2.1.–	A, B, I	Starch
$\alpha$ -Amylase	3.2.1.1	A, B, C	Starch
Oligo-1,6-glucosidase	3.2.1.10	A, B	Amylopectin
$\alpha$ -Glucosidase	3.2.1.20		Starch
Amylopullulanase	3.2.1.41 or 3.2.1.1	A, B, H, G, 1	Pullulan
Cyclomaltodextrinase	3.2.1.54	A, B	Cyclodextrins
Isopullulanase	3.2.1.57		Pullulan
Isoamylase	3.2.1.68	A, B, F, 7	Amylopectin
Maltotetraose-forming amylase	3.2.1.60	A, B, C, E	Starch
Glucodextranase	3.2.1.70		Starch
Trehalose-6-phosphate hydrolase	3.2.1.93		Trehalose
Maltohexaose-forming amylase	3.2.1.98		Starch
Maltogenic amylase	3.2.1.133	A, B, C, D, E	Starch
Neopullulanase	3.2.1.135	A, B, G	Pullulan
Malto-oligosyl trehalase hydrolase	3.2.1.141		Trehalose
Malto-oligosyl trehalase synthase	5.4.99.15		Maltose



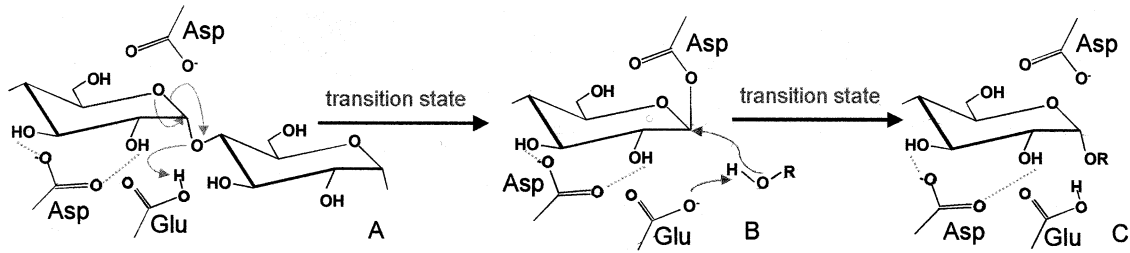


Fig. 4. The double displacement mechanism and the formation of a covalent intermediate by which retaining glycosylhydrolases act.

$\alpha$ -amylase family. A second protrusion of the A-domain (domain 2 or 7) is present in a number of enzymes that hydrolyze interior  $\alpha$ ,1-6 glycosidic bonds. Other domains that can be present in front or behind the A domain are the domains C to I. The function of the C-domain is not known, but mutations in the C-domain of the  $\alpha$ -amylase of *Bacillus stearothermophilus* suggest that it is involved in enzyme activity (Holm et al., 1990). In cyclodextrin glycosyltransferase, the C-domain contains a maltose-binding site that is involved in the binding of raw starch (Lawson et al., 1994; Penninga et al., 1996). In the maltogenic  $\alpha$ -amylase and cyclodextrin glycosyltransferase, the C-domain is followed by a D-domain. The function of this D-domain is also presently unknown. A number of  $\alpha$ -amylase family enzymes have a raw starch binding or E-domain that interacts with the substrate (Dalmia et al., 1995; Knegt et al., 1995; Penninga et al., 1996). Other characteristic domains of the  $\alpha$ -amylase family are N-terminal F-, H-, or G-domains found in the enzymes that have an endo action or those that hydrolyze  $\alpha$ ,1-6 glycosidic linkages of branched substrates.

## 5. Utilization of $\alpha$ -amylase family enzymes

### 5.1. Industrial production of glucose and fructose from starch

A large-scale starch processing industry has emerged since the mid-1900s. Before further processing can take place, the starch-containing part of the plants have to be processed and the starch harvested (see Bergthaller et al., 1999). Besides

starch, sugars, pentosans, fibres, proteins, amino acids, and lipids are also present in the starch-containing part of the plant. A typical composition of a potato is as follows: 78% water; 3% protein and amino acids; 0.1% lipids; 1% fibers; and 17% starch. In the beginning, starch was hydrolyzed into glucose syrups using acid treatment. In 1811, the German scientist Kirchhoff found that sweet-tasting syrup was obtained when starch–water suspension was treated with diluted acid. It took several decades before a large-scale starch-hydrolyzing industry developed.

Only in 1921, Newkirk described a commercial process for the production of glucose from starch. In this batch process, starch is mixed with water, boiled to dissolve the starch granules and release the amylose and amylopectin into the water, and treated with acid for a certain period depending on the degree of hydrolysis that is desired. Instead of boiling, a jet-cooker can be used in which starch is pasted by mixing steam under pressure at 100–175 °C with the starch slurry. Under such conditions, the starch slurry is rapidly heated within a few seconds. The heated starch slurry can then pass directly into a hydrolysis reactor for further (enzymatic) treatment. The enzyme, if not thermally inactivated, can be added to the starch slurry before it enters the jet-cooker. The starch granules are more extensively fragmented and dispersed in the jet-cooker process than in the batch operation. Industrial scale jet-cookers were introduced in the 1950s.

The sweetness of a starch syrup depends on the degree of hydrolysis. Complete hydrolysis results in the formation of only glucose or dextrose, a term commonly used in UK and USA. The

amount of dextrose in syrup is given by the DE or dextrose equivalent. The DE value gives the amount of reducing equivalents expressed as glucose per unit dry weight and can be calculated using the formula:  $DE = 180 / (162 \times n + 18) \times 100$ , where  $n$  is the average DP. Glucose has a DE of 100, maltose of 53, maltotriose of 36, and starch of almost 0. So the higher the DP, the lower the DE value.

The acid hydrolysis method for the production of glucose has been replaced recently by enzymatic treatment with three or four different enzymes (Fig. 5; Crabb and Mitchinson, 1997; Crabb and Shetty, 1999). For the complete conversion into high glucose syrup, the first step is the liquefaction into soluble, short-chain dextrans. A 30–35% dry solids starch slurry of pH 6 is mixed with  $\alpha$ -amylase and passed through a jet-cooker after which the temperature is kept at 95–105 °C for 90 min. A temperature above 100 °C is preferred to assure the removal of lipid–starch complexes. Initially, the  $\alpha$ -amylase of *Bacillus amyloliquefaciens* was used but this has been replaced by the  $\alpha$ -amylase of *Bacillus stearothermophilus* or *Bacillus licheniformis*. The DE value of a starch–hydrolysate syrup depends on the time of incubation and the amount of enzyme added. If the hydrolysate is used for the production of glucose, usually the final DE value is between 8 and 10.

The drawback of the  $\alpha$ -amylases used currently is that they are not active at a pH below 5.9 at the high temperatures used. Therefore, the pH has to be adjusted from the natural pH 4.5 of the starch slurry to pH 6 by adding NaOH. Also  $Ca^{2+}$  needs to be added because of the  $Ca^{2+}$ -dependency of these enzymes. *Pyrococcus furiosus* has an extracellular  $\alpha$ -amylase enzyme that shows promising characteristics for applications in the starch industry. The enzyme is highly thermostable in the absence of metal ions, active even at a temperature of 130 °C, and shows a unique product pattern and substrate specificity (Jorgensen et al., 1997).

The next step is the saccharification of the starch–hydrolysate syrup to a high concentration glucose syrup, with more than 95% glucose. This is done by using an exo-acting glucoamylase, that

hydrolyzes  $\alpha$ ,1-4 glycosidic bonds from the non-reducing end of the chain. Most commonly used are glucoamylases of *Aspergillus niger* or a closely related species. This glucoamylase has a pH optimum of 4.2 and is stable at 60 °C. To run an efficient saccharification process, the pH of the starch–hydrolysate syrup is adjusted to 4.5 using hydrochloric acid. Depending on the specifications of the final product, this step is performed for 12–96 h at 60–62 °C. A practical problem in this process is that the glucoamylase is specialized in cleaving  $\alpha$ ,1-4 glycosidic bonds and slowly hydrolyzes  $\alpha$ ,1-6 glycosidic bonds present in maltodextrins. This will result in the accumulation of isomaltose. A solution to this problem is to use a pullulanase that efficiently hydrolyzes  $\alpha$ ,1-6 glycosidic bonds. A prerequisite is that the pullulanase has the same pH and temperature optimum as the glucoamylase. A second problem is caused by the high dry solid contents that need to be used during the process in order to make the production of high glucose syrups (>95% glucose) economically feasible. The glucoamylase can easily form reversion products such as maltose and isomaltose at the expense of the amount of glucose. The current solution is to balance the amount of enzyme, the temperature, and the time of incubation (Crabb and Mitchinson, 1997).

A third step in industrial starch processing is the conversion of a high glucose syrup into a high fructose syrup. Fructose is an isomer of glucose and is almost twice as sweet as glucose. This conversion is done using the enzyme D-xylose-ketol isomerase (EC 5.3.1.5), better known as glucose isomerase. The high glucose syrup is first refined, carbon filtered, concentrated to over 40% dry solids and adjusted to pH 7–8. In a continuous process, this adjusted high glucose syrup is passed over an immobilized column containing glucose isomerase on a solid support. Maximum levels of fructose are about 55%. An excellent review on this enzyme and its industrial application has been published by Bhosale et al. (1996).

## 5.2. Bakery and anti-staling

The baking industry is a large consumer of starch and starch-modifying enzymes. Bread bak-

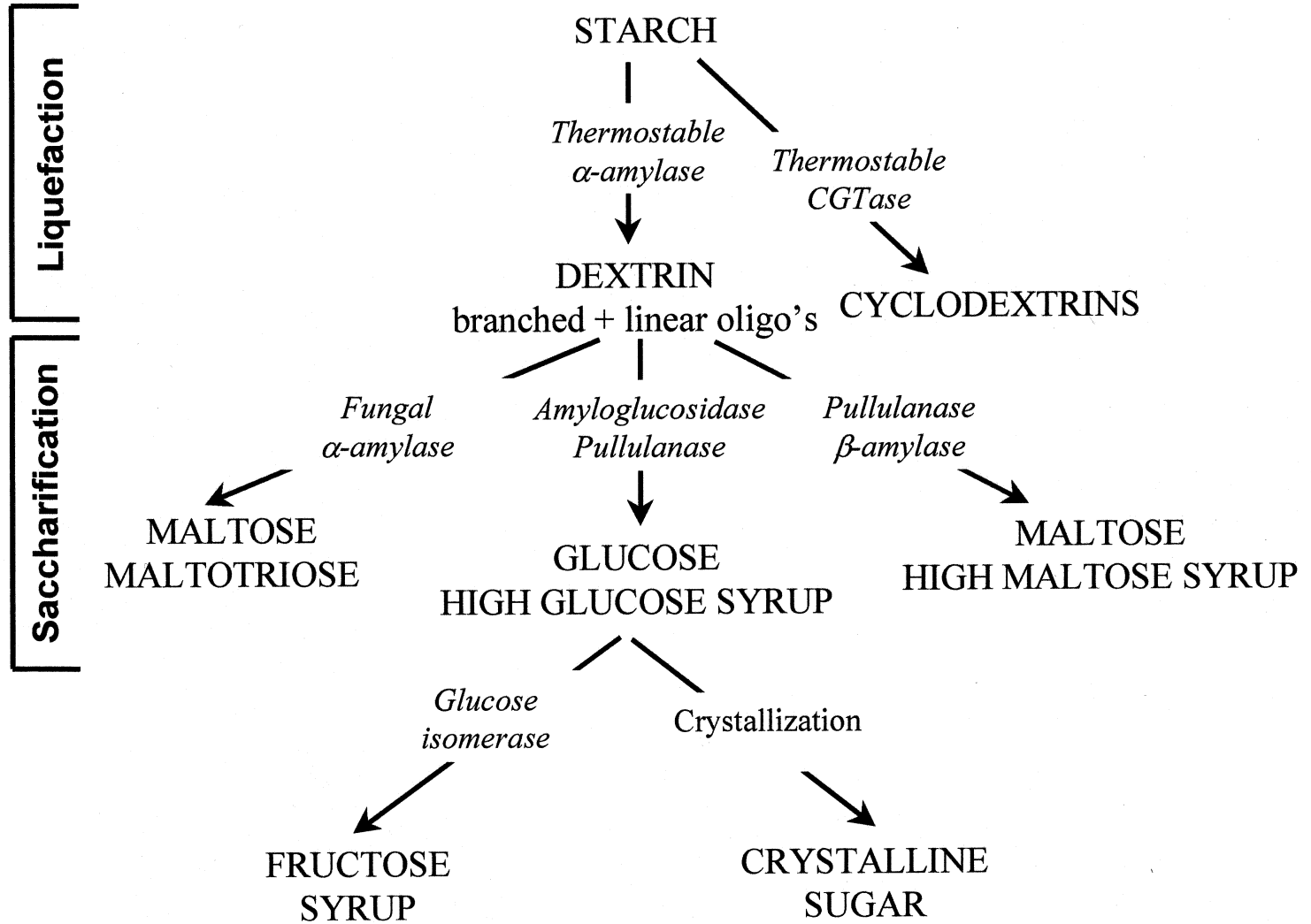


Fig. 5. Overview of the industrial processing of starch into cyclodextrins, maltodextrins, glucose or fructose syrups and crystalline sugar.

ing starts with dough preparation by mixing flour, water, yeast and salt and possibly additives. Flour consists mainly of gluten, starch, non-starch polysaccharides and lipids. Immediately after dough preparation, the yeast starts to ferment the available sugars into alcohols and carbon dioxide, which causes rising of the dough. Amylases can be added to the dough to degrade the damaged starch in the flour into smaller dextrans, which are subsequently fermented by the yeast. The addition of malt or fungal  $\alpha$ -amylase to the dough results in increased loaf volume and improved texture of the baked product (homepage Novo Nordisk).

After rising, the dough is baked. When the bread is removed from the oven, a series of changes start which eventually leads to the deterioration of quality. These changes include increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb and loss of bread flavor. All undesirable changes that do occur upon storage together are called staling. Retrogradation of the starch fraction in bread is considered very important in staling (Kulp and Ponte, 1981). Especially the extent of amylopectin retrogradation correlates strongly with the firming rate of bread (Champenois et al., 1999). Staling is of considerable economic importance for the baking industry since it limits the shelf life of baked products. In USA, for instance, bread worth more than US\$1 billion is discarded annually (homepage Novo Nordisk).

To delay staling, to improve texture, volume and flavor of bakery products, several additives may be used in bread baking. These include chemicals, small sugars, enzymes or combinations of these. Well-known additives are: milk powder, gluten, emulsifiers (mono- or diglycerides, sugar esters, lecithin, etc.), granulated fat, oxidant (ascorbic acid or potassium bromate), cysteine, sugars or salts (Spendler and Jørgensen, 1997). Rapid advances in biotechnology have made 'new' enzymes available for the industry. Since enzymes are produced from natural ingredients, they will find greater acceptance by the consumers because of their demand for products without chemicals. Several enzymes have been suggested to act as dough and/or bread improvers, by modifying one of the major dough components. Ex-

amples are glucose oxidase, hemicellulase, lipase, protease and xylanase. These enzymes, however, do not act on the starch fraction itself. Enzymes active on starch have been suggested to act as anti-staling agents. Examples are:  $\alpha$ -amylases (De Stefanis and Turner, 1981; Cole, 1982), branching (Okada et al., 1984) and debranching (Carroll et al., 1987) enzymes, maltogenic amylases (Olesen, 1991),  $\beta$ -amylases (Würsch and Gumy, 1994), and amyloglucosidases (Vidal and Gerrity, 1979).

Originally,  $\alpha$ -amylases were added during dough preparation to generate fermentable compounds. Besides generating fermentable compounds,  $\alpha$ -amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods (De Stefanis and Turner, 1981; Cole, 1982; Sahlström and Bråthen, 1997). Despite a possible anti-staling effect, the use of  $\alpha$ -amylases as anti-staling agent is not widespread because even a slight overdose of  $\alpha$ -amylase results in sticky bread. Positive effects of delayed staling, on the contrary, are measured only after 3–4 days (Olesen, 1991). The increased gummyness of  $\alpha$ -amylase treated bread is associated with the production of branched maltodextrins of DP20-100 (De Stefanis and Turner, 1981). Debranching enzymes are claimed to decrease strongly the problems associated with the use of  $\alpha$ -amylases as anti-staling agents in baking. In this method a thermostable pullulanase, and an  $\alpha$ -amylase are used together. The pullulanase rapidly hydrolyzes the branched maltodextrins of DP20-100 produced by the  $\alpha$ -amylase, while they have little effect on the amylopectin itself (Carroll et al., 1987). Pullulanase thus specifically removes the compound responsible for the gummyness associated with  $\alpha$ -amylase treated bakery products.

Branching enzyme is claimed to increase shelf life and loaf volume of baked goods (Okada et al., 1984; Spendler and Jørgensen, 1997). These effects are achieved by modifying the starch material in the dough during baking. Improved quality of baked products is also obtained when the branching enzyme is used in combination with other enzymes, such as  $\alpha$ -amylase, maltogenic amylase, cyclodextrin glycosyltransferase,  $\beta$ -amylase, cellulase, oxidase and/or lipase (Spendler and Jørgensen, 1997).

The use of cyclodextrin glycosyltransferase as dough additive is claimed to increase the loaf volume of the baked product (Van Eijk and Mutstaers, 1995). The effect is suggested to result from the gradual formation of cyclodextrins in the dough after mixing.

Exoamylases, such as  $\beta$ -amylase and amyloglucosidase, shorten the external side chains of amylopectin by cleaving maltose or glucose molecules, respectively. Both enzymes are suggested to delay bread staling by reducing the tendency of the amylopectin compound in bakery products to retrograde (Würsch and Gumy, 1994). Anti-staling effects of amylo-glucosidase in baking are claimed in a few patents (Van Eijk, 1991; Vidal and Gerrity, 1979). The synergetic use of  $\alpha$ - and  $\beta$ -amylase is also claimed to increase the shelf life of baked goods (Van Eijk, 1991).

Since  $\alpha$ -amylases cause stickiness of baked goods, especially when overdosed, it was suggested that these problems could be solved using an exoamylase, since they do not produce the branched maltooligosaccharides of DP20-100. Such enzymes, called maltogenic amylases, produce linear oligosaccharides of 2–6 glucose residues. Maltogenic amylases producing maltose (Olesen, 1991), maltotriose (Tanaka et al., 1997) and maltotetraose (Shigeji et al., 1999a,b) are claimed to increase the shelf life of bakery products by delaying retrogradation of the starch compound. Currently, a thermostable maltogenic amylase of *Bacillus stearothermophilus* (Diderichsen and Christiansen, 1988) is used commercially in the bakery industry. Although this enzyme has some endo-activity (Christophersen et al., 1998), it does act as an exo-acting enzyme during baking, modifying starch at a temperature when most of the starch starts to gelatinize (Olesen, 1991).

### 5.3. Cyclodextrin/cycloamylose formation

Cyclodextrins are cyclic  $\alpha$ ,1-4 linked oligosaccharides mainly consisting of 6, 7, or 8 glucose residues ( $\alpha$ -,  $\beta$ -, or  $\gamma$ -cyclodextrin, respectively). The glucose residues in the rings are arranged in such a manner that the inside is hydrophobic thus resulting in an apolar cavity while the outside is hydrophilic. This enables cyclodextrins to form

inclusion complexes with a variety of hydrophobic guest molecules. Specific ( $\alpha$ -,  $\beta$ -, or  $\gamma$ -) cyclodextrins are required for complexation of guest molecules of specific sizes. The formation of inclusion complexes leads to changes in the chemical and physical properties of the guest molecules, such as stabilization of light- or oxygen-sensitive compounds, stabilization of volatile compounds, improvement of solubility, improvement of smell or taste, or modification of liquid compounds to powders. These altered characteristics of the encapsulated compounds have led to various applications of cyclodextrins in analytical chemistry (Armstrong, 1988; Loung et al., 1995), agriculture (Saenger, 1980; Oakes et al., 1991), biotechnology (Allegre and Deratani, 1994; Szejtli, 1994), pharmacy (Albers and Muller, 1995; Thompson, 1997), food (Allegre and Deratani, 1994; Bicchi et al., 1999) and cosmetics (Allegre and Deratani, 1994).

A major drawback for the application of cyclodextrins on a large scale is that all enzymes used today produce a mixture of cyclodextrins. Two different industrial approaches are used to purify the cyclodextrin mixtures: selective crystallization of  $\beta$ -cyclodextrin, which is relatively poorly water-soluble, and selective complexation with organic solvents. Major disadvantages of the latter method are the toxicity, flammability, and need for solvent recovery (Pedersen et al., 1995). This makes the production of cyclodextrins too costly for many applications. Additionally, the use of organic solvents limits applications involving human consumption.

For the industrial production of cyclodextrins, starch is first liquefied by a heat-stable  $\alpha$ -amylase and then the cyclization occurs with a cyclodextrin glycosyltransferase from *Bacillus macerans* (Riisgaard, 1990) sp. A major drawback of this process is that the cyclization reaction has to be performed at lower temperatures than the initial liquefaction because of the low thermostability of the bacillus cyclodextrin glycosyltransferase. The use of cyclodextrin glycosyltransferase from thermophilic microorganisms can solve this problem. Thermostable cyclodextrin glycosyltransferases have been found in a *Thermoanaerobacter* species

(Starnes, 1990; Norman and Jorgensen, 1992; Pedersen et al., 1995), *Thermoanaerobacterium thermosulfurigenes* (Wind et al., 1995), and *Anaerobranca bogoriae* (Prowe et al., 1996).

Cyclodextrin glycosyltransferases can also be used for the production of novel glycosylated compounds, making use of the transglycosylation activity. A commercial application is the glycosylation of the intense sweetener stevioside, isolated from the leaves of the plant *Stevia rebaudania*, thereby increasing solubility and decreasing bitterness (Pedersen et al., 1995).

Other cyclic products that can be generated from starch are cycloamyloses. These large cyclic glucans (DP > 20) contain antiparallel helices, providing long cavities with a diameter similar to that of  $\alpha$ -cyclodextrin. Unlike cyclodextrins, cycloamylose is formed by all the transglycosylating enzymes of the  $\alpha$ -amylase family (Takaha et al., 1996; Takata et al., 1996; Terada et al., 1997, 1999). Formation of cyclodextrins occurs by an intramolecular transglycosylation reaction whereas the formation of large cycloamylose molecules is the result of an intramolecular transglycosylation. To form cycloamylose, low concentrations of high molecular weight amylose in the micromolar range are incubated with a relatively high amount of enzyme. This reaction is therefore not based on a novel catalytic mechanism but is a direct effect of the limited availability of acceptor molecules. Production of cycloamylose is currently not done on an industrial scale.

#### 5.4. Miscellaneous applications

$\alpha$ -Amylase, pullulanase, cyclodextrin glycosyltransferase, and maltogenic amylase are nowadays widely used by industry in various applications (Table 3).  $\alpha$ -Amylase probably has the most widespread use. Besides their use in the saccharification or liquefaction of starch, these enzymes are also used for the preparation of viscous, stable starch solutions used for the warp sizing of textile fibers, the clarification of haze formed in beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility. A growing new area of application of  $\alpha$ -amylases is in the fields of laundry and dish-washing detergents. A modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At these lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergents with  $\alpha$ -amylases optimally working at moderate temperatures and alkaline pH can help solve this problem.

Two starch-modifying enzymes of the  $\alpha$ -amylase family that do not find large-scale application yet are amyloamylase and branching enzyme. Several patents exist describing the potential use of branching enzyme in bread as an anti-staling agent (Spendler and Jørgensen, 1997), or for the production of low-viscosity, high molecular weight starch for, e.g. the coating of paper (Bruinenberg et al., 1996) or warp sizing of textile fibers, thus making the fibers stronger (Hendriksen et al., 1999). Application of branching en-

Table 3  
Different fields of application of enzymes belonging to the  $\alpha$ -amylase family

Application	Enzyme
Starch liquefaction	$\alpha$ -Amylase
Starch saccharification	Amyloglucosidase, pullulanase, maltogenic $\alpha$ -amylase, $\alpha$ -amylase, isoamylase
Laundry detergent and cleaners; reduction of haze formation in juices, baking, brewing, digestibility of animal feed, fiber and cotton desizing, sanitary waste treatment	$\alpha$ -Amylase
Cyclodextrin production	Cyclodextrin glycosyltransferase
Thermoreversible starch gels	Amylomaltase
Cycloamylose	Amylomaltase, branching enzyme, cyclodextrin glycosyltransferase

zymes is limited by the lack of commercially available enzymes that are sufficiently thermostable.

A potentially interesting industrial application of amyloamylase is the production of thermoreversible starch gels. As already indicated above, a normal untreated starch gel cannot be dissolved in water after it has retrograded. However, starch that has been treated with amyloamylase has obtained thermoreversible gelling characteristics: it can be dissolved numerous times upon heating. This behavior is very similar to gelatine. Van der Maarel et al. (2000) described this process using the amyloamylase from the hyperthermophilic bacterium *Thermus thermophilus*. Currently, no amyloamylases are commercially available and the thermoreversible starch gel is not produced on an industrial scale.

## 6. Engineering of commercial enzymes for improved stability

The conditions prevailing in the industrial applications in which enzymes are used are rather extreme, especially with respect to temperature and pH. Therefore, there is a continuing demand to improve the stability of the enzymes and thus meet the requirements set by specific applications. One approach would be to screen for novel microbial strains from extreme environments such as hydrothermal vents, salt and soda lakes, and brine pools (Sunna et al., 1997; Niehaus et al., 1999; Veille and Zeikus, 2001). This is being used successfully by a number of academic and industrial groups and has resulted in the submission of a number of patent applications such as a thermostable pullulanase from *Fervidobacterium pen- navorans* (Bertoldo et al., 1999) or an  $\alpha$ -amylase from *Pyrococcus woesei* (Antranikian et al., 1990). Although these enzymes have better thermostability than the currently available commercial enzymes, none have been introduced onto the market yet. One of the reasons being that besides thermostability and activity other factors such as activity with high concentrations of starch, i.e. more than 30% dry solids, or the protein yields of the industrial fermentation are important criteria

for commercialization (Schäfer et al., 2000). Most, if not all  $\alpha$ -amylase family enzymes found by screening new, exotic strains do not meet these criteria.

A second approach to find new and potentially interesting enzymes is to use the nucleotide or amino acid sequence of the conserved domains in designing degenerated PCR primers. These primers can then be used to screen microbial genomes for the presence of genes putatively encoding the enzyme of interest. This approach has been used successfully by Tsutsumi et al. (1999) to find and express a novel thermostable isoamylase enzyme from two *Sulfolobus* species and *Rhodothermus marinus*.

A third approach that is used with more success is to engineer commercially available enzymes. Several different engineering approaches have been described. A short overview of some of the results obtained by engineering the protein will be given below, without the intention of being comprehensive.

To find out what specific regions are of importance for a given property, hybrids of two homologous enzymes can be generated or detailed comparisons of the amino acid sequence can be made. Suzuki et al. (1989), e.g. made a hybrid of the *B. licheniformis* and the *B. amyloliquefaciens*  $\alpha$ -amylase and the identified two regions that are of importance for thermostability. A similar approach was used by Conrad et al. (1995). They identified the amino acid regions 34–76, 112–142, 174–179, and 263–276 as important for the thermostability of the *B. licheniformis*  $\alpha$ -amylase. Another method for finding regions contributing to a specific property was used by Borchert et al. (1999). They compared the active sites and the surroundings of different  $\alpha$ -amylases active at medium and high temperatures and identified a number of regions that could be of importance for the functioning of the *B. licheniformis*  $\alpha$ -amylase (Termamyl) at medium temperatures. Besides the regions identified by Conrad et al. (1995) and Suzuki et al. (1989), they postulated that regions 181–195, 141–149, 456–463, and the individual amino acids at positions 311, 346, 385 and mutations therein or deletions thereof contribute to improved pH stability at a pH from 8 to 10.5,

improved  $\text{Ca}^{2+}$  stability at pH 8–10.5, or increased specific activity at 30–40 °C.

It has been described that the introduction of prolines in loop regions can have a stabilizing effect on proteins in general, due to the lowering of the entropy of the unfolded state more than the entropy of the folded state (Matthews et al., 1987). This has been used to replace the arginine residue at position 124 of an  $\alpha$ -amylase of an alkalophilic *Bacillus* species into a proline, resulting in a more stable enzyme (Bisgard-Frantzen et al., 1996). The introduction of disulfide bonds in the enzyme can also lead to improved stability as was described by Day (1999). Another important stability criterium is the effect of oxidative agents as, e.g. found in cleaning agents on the enzyme. Altering amino acids prone to oxidation, such as methionine, tryptophane, cysteine, histidine, of tyrosine by an amino acid that is not affected by an oxidizing agent can cause increased stability in the presence of bleach, peracids, or chloramine (Barnett et al., 1998). Engineering  $\alpha$ -amylase enzymes for changed pH–activity profiles is a continuing challenge because many applications and industrial processes in which these enzymes are used are carried out at diverse, usually extreme, pH values. Nielsen and Borchert (2000) have recently published a comprehensive overview of a number of experiments that have been done to engineer pH–activity profiles.

A currently fashionable approach for engineering protein is random mutagenesis coupled to high-throughput screening (Chen, 2001). In this approach, point mutations generated by error-prone PCR lead to such a change in the triplet codon that a new amino acid is built into the protein. Because of the random nature of this method, a large collection of mutants needs to be screened to find those that are of interest. Shaw et al. (1999) reported on the use of this method to improve the stability of the *B. licheniformis*  $\alpha$ -amylase at pH 5.0 and 83 °C 23 times when the beneficial mutations found by random mutagenesis were combined with the already known beneficial.

All the above-mentioned engineering approaches are aimed at increasing stability of the enzyme at a given condition. Using the currently

available insights into the structure–function relationships of the  $\alpha$ -amylase family enzymes as described in Section 4, protein engineering via site-directed mutagenesis has been used to change the product specificity of the cyclodextrin glycosyltransferase (Dijkhuizen et al., 1999; Schulz and Candussio, 1995) or of the maltogenic  $\alpha$ -amylase (Cherry et al., 1999) used as an anti-staling agent in bread. Van der Veen et al. (2000a) gave an excellent overview of the engineering of cyclodextrin glycosyltransferase reaction and product specificity. Therefore, this will not be discussed further in this review. Cherry et al. (1999) described in detail the 3D structure of the maltogenic  $\alpha$ -amylase and used this to claim specific amino acid modifications to obtain variants of the enzyme with improved product specificity, altered pH optimum, improved thermostability, increased specific activity, altered cleavage pattern and thus have an increased ability to reduce retrogradation of starch or staling of bread.

## 7. Conclusions

The  $\alpha$ -amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate, being glucose residues linked through an  $\alpha$ ,1-1,  $\alpha$ ,1-4, or  $\alpha$ ,1-6 glycosidic bond. Members of this family share a number of common characteristics but at least 21 different enzyme specificities are found within the family. These differences in specificities are based not only on subtle differences within the active site of the enzyme but also on the differences within the overall architecture of the enzymes. The  $\alpha$ -amylase family can roughly be divided into two subgroups: the starch-hydrolysing enzymes and the starch-modifying or -transglycosylating enzymes.

During the last three decades,  $\alpha$ -amylases have been exploited by the starch-processing industry as a replacement of acid hydrolysis in the production of starch hydrolysates. This enzyme is also used for the removal of starch in beer, fruit juices, or from clothes and porcelain. Another starch-hydrolysing enzyme that is used in a large scale is the thermostable pullulanase for the debranching



of amylopectin. A new and recent application is maltogenic amylase as an anti-staling agent to prevent the retrogradation of starch in bakery products.

Only one type of starch-modifying enzyme has found its way to the commercial market: cyclodextrin glycosyltransferase either for the production of cyclodextrins for non-food applications or for the hydrolysis of starch during the saccharification process. Other starch-modifying enzymes, i.e. amyloamylase and branching enzyme, are not yet used by the industry, although potentially interesting applications have been described in patent and scientific literature. It is probably a matter of time before these enzymes are also used in commercial applications.

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