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Exploring and exploiting starch-modifying amylomaltases from thermophiles

T. Kaper*†, M.J.E.C. van der Maarel*‡[1], G.J.W. Euverink*† and L. Dijkhuizen*†

*Centre for Carbohydrate Bioengineering TNO-RUG, P.O. Box 14, 9750 AA Haren, The Netherlands, †Microbial Physiology Research Group, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands, and ‡Innovative Ingredients and Products Department, TNO Nutrition and Food Research, Rouaanstraat 27, 9723 CC Groningen, The Netherlands

Abstract

Starch is a staple food present in water-insoluble granules in many economically important crops. It is composed of two glucose polymers: the linear α-1,4-linked amylose and amylopectin with a backbone of α-1,4-glycosidic bonds and α-1,6-linked side chains. To dissolve starch completely in water it needs to be heated; when it cools down too much the starch solution forms a thermo-irreversible gel. Amylomaltases (EC 2.4.1.25) are enzymes that transfer a segment of an α-1,4-D-glucan to a new 4-position in an acceptor, which may be glucose or another α-1,4-D-glucan. Acting upon starch, amylomaltases can produce cycloamylose or a thermoreversible starch gel, both of which are of commercial interest.

Introduction

Starch is an important energy reserve in plants and is composed of the α-glucans amylose, which is α-1,4-linked and linear, and amylopectin, with a backbone of α-1,4-glycosidic bonds and α-1,6-linked side chains. It is abundantly present in seeds, tubers and roots of plants like rice, maize, wheat, potato and cassava and has always accounted for a large proportion of the dietary energy of humans and animals [1]. The world's annual starch production has been estimated to be 1.4×10^9 tons (according to the Food And Agriculture Organization of the United Nations; [2]). Purified starch is nowadays used in the food, pharmaceutical, textile and paper industries.

In the plant, starch is stored in crystalline form in compact spherical granules and is completely insoluble in water at ambient temperatures. The shape and size of these granules depend on the botanical origin. Upon heating in water, the crystalline order is lost and the granules swell as the amylose and amylopectin chains are hydrated [3]. Depending on the properties, complete solubilization of starch is reached at 70–105°C. Subsequent cooling leads to retrogradation, a process in which the amylose chains interact by hydrogen bonding, resulting in the formation of a gel. The process of retrogradation is irreversible, i.e. heating of the gel does not result in its dissolution.

The industrial enzymic processing of starch is based on (partial) hydrolysis to maltodextrins, maltose and glucose syrups [4]. Since solubilization of starch is desired for such enzymic treatment, the applied enzymes need to be stable and active at temperatures above 65–70°C. A natural source for extreme thermostable and thermoactive enzymes are (hyper)thermophiles that have their optimal temperature for growth above 60°C [5]. Well-known starch-acting enzymes are α-amylases and pullulanases, which degrade it to malto-oligosaccharides and glucose. Recently, a thermostable cyclodextrin glycosyl transferase (EC 2.4.1.19) that produces circular cyclodextrins was introduced on the market [6]. This enzyme performs a transglycosylation or transferase reaction instead of a hydrolysis. Two other starch-modifying transferases, i.e. glucan-branching enzymes (E.C. 2.4.1.18) and amylomaltases (E.C. 2.4.1.25), are explored for their potential applications, as can be judged from the number of patent applications in recent years. In the rest of this communication, we describe the basic characteristics of amylomaltases and some recently developed applications of this enzyme.

Amylomaltases

Amylomaltases are intracellular 4-α-glucanotransferases: they catalyse the transfer of a segment of an α-1,4-D-glucan to a new 4-position in an acceptor, which may be glucose or another α-1,4-D-glucan. This reaction is a variation of the α-retaining mechanism [7]. Several amylomaltases of various sources have been studied in detail (Table 1). In plants the enzyme is known as a disproportionating enzyme or D-enzyme. It is presumed that in plants the enzyme is involved in starch metabolism, although its precise role in this process is less clear [8,9]. In a number of micro-organisms, e.g. *Aquifex aeolicus*, the presence of an amylomaltase-encoding gene is highly correlated with that of α-1,4-glucan-branching enzyme and glycogen phosphorylase, which suggests a role for amylomaltase in glycogen synthesis [10]. In other micro-organisms, e.g. *Escherichia coli*, amylomaltase is essential for the metabolism of maltose [11].

The minimal α-saccharide that D-enzyme from potato can use as a donor is maltotriose and maltose is the minimal transferred glucan unit [12,13]. Glucose and maltose can only serve as an acceptor. The bonds at the non-reducing

Key words: amylomaltase, enzyme, gel, 4-α-glucanotransferase, glycosidic linkage, starch.

[1]To whom correspondence should be addressed, at the Innovative Ingredients and Products Department, TNO Nutrition and Food Research (e-mail maarel@voeding.tno.nl).

Table 1 | Properties and applications of amylomaltases and 4-α-glucanotransferases
GH, glycoside hydrolase (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html).

Organism	GH family	T_{opt} (°C)	GenBank acc. no.	Applications	Reference
Arabidopsis	77	30*	AB019236		[9]
Chlamydomonas reinhardtii	77	55*	AF307843		[8]
Potato	77	37*	X68664	Cycloamylose	[13,16,17]
Escherichia coli ML	77	30*	–		[14]
Thermus aquaticus	77	75	AB016244	Cycloamylose	[15]
Thermus thermophilus	77	80	–	Starch gels	[29]
Aquifex aeolicus	77	90	AE000704	Cycloamylose starch gels	[18]
Thermotoga maritima	13	80	Z50813	Isomalto-oligosaccharides	[25,31]
Thermococcus litoralis	57	90	D88253		[32]
Thermococcus kodakaraensis	57	90*	–	Cycloamylose	[33]

*Reported assay temperature.

end and the penultimate bond at the reducing end of the donor substrate are never acted upon by the potato enzyme. Consequently, maltose is never produced [12,13]. Upon lengthy incubation with maltotriose, amylaceous products are formed which are able to complex with iodine [14]. On the contrary, the amylomaltases from *E. coli* and *Thermus aquaticus* are able to use maltose as a donor, although at a low rate [14,15]. *T. aquaticus* amylomaltase is able to transfer oligosaccharides from starch to glucose [15]. In addition, D-enzyme and amylomaltase catalyse an intramolecular transglycosylation reaction with amylose and amylopectin as substrates, which results in circular α-glucans (see also applications, below; Figure 1) [15–17]. The amylomaltase from *Aquifex aeolicus* is the most thermoactive described in literature to date (Table 1) [18].

Based on sequence similarities, amylomaltases have been placed in family 77 of the glycoside hydrolases (GH77), according to Henrissat's classification, and form the α-amylase superfamily together with α-amylase family GH13 and glucansucrase family GH70 [19,20]. As for GH13, the sequence homology within GH77 is relatively low (approx. 15%). In contrast to GH13, only one enzymic activity, i.e. the amylomaltase activity, has been assigned to the GH77 family. The 4-α-glucanotransferase activity, however, is not exclusive for GH77, but is also found among members of GH13 and unrelated GH57 (Table 1; see also the Carbohydrate-Active Enzymes server at [21]). Compared with GH13, the enzymes of GH77 have a simpler modular organization, consisting of a catalytic domain A with a $(\beta\alpha)_8$-barrel fold with inserted B1, B2 and B3 domains only. Analogous to GH13 enzymes, GH77 enzymes are characterized by four conserved regions with fully conserved carboxylic residues, which have proposed roles in substrate binding and catalysis [22].

The three-dimensional structures of the amylomaltases from *T. aquaticus* ATCC 33923 (PDB code 1CWY) [23] and *Thermus thermophilus* HB8 (PDB code 1FP8 and 1FP9; J.C.M. Uitdehaag, unpublished work) have been determined by X-ray crystallography. The core structure of the enzyme consists of a $(\beta\alpha)_8$-barrel, which is interrupted by several insertions between the barrel strands, of which a 100-residue α-helical B2 subdomain is unique to amylomaltases. A C-terminal domain, which is present in all GH13 enzymes, is absent. The putative catalytic nucleophile and acid/base residues are located at the end of β-strands 5 and 6,

Figure 1 | Top (A) and side (B) views of the molecular structure of cycloamylose consisting of 26 glucose molecules
PDB code 1C58 [26]. Picture was generated using Rasmol.

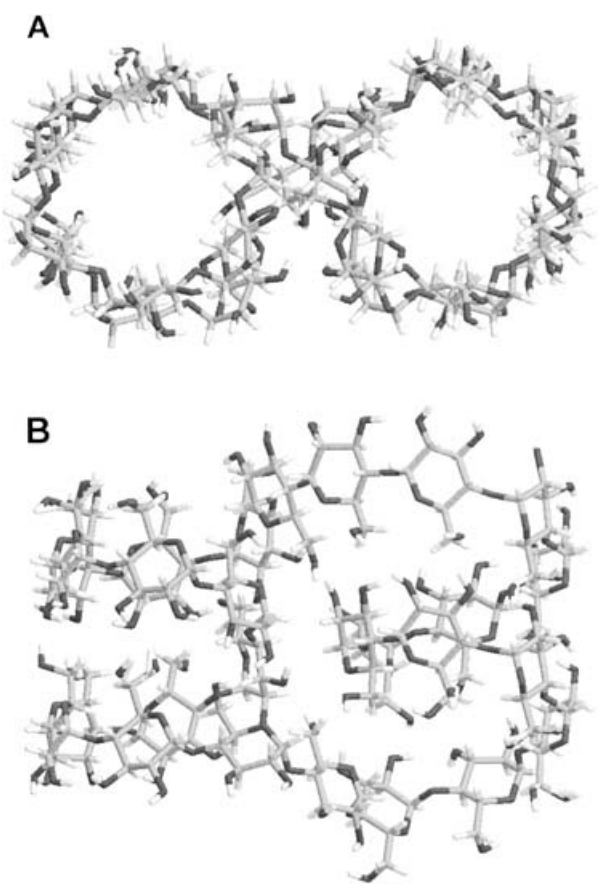
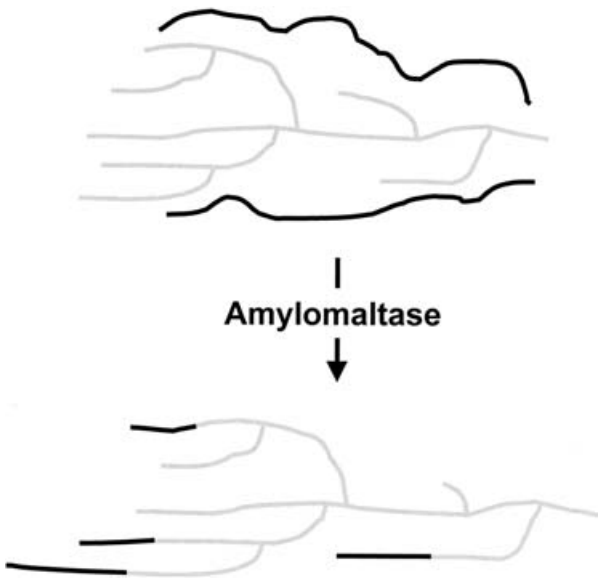


Figure 2 | Action of amylomaltase on starch
The black lines represent the amylose, the grey lines the amylopectin. As a result of the enzyme's action, the final product has some side chains which have been shortened and others which have been elongated.



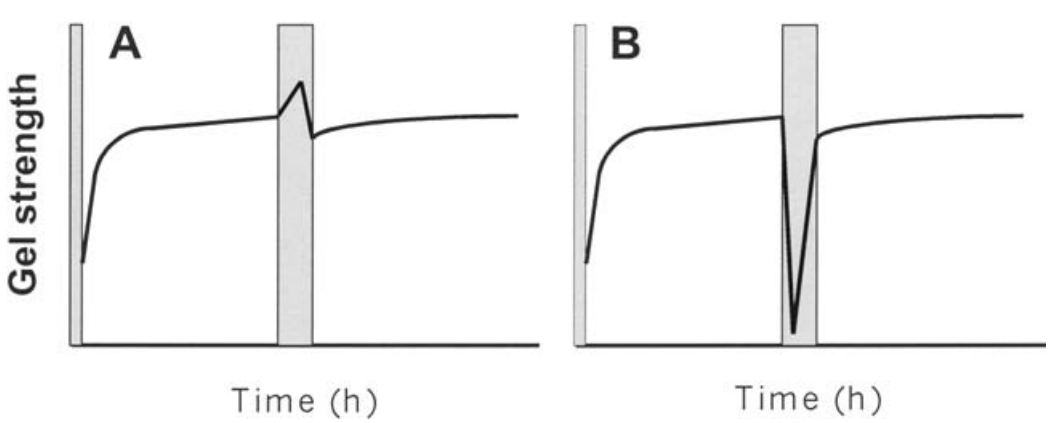
respectively. The active site is located on the surface of the protein and partially shielded from the environment by two loops. Formation of circular amylose products is proposed to occur by curling of the substrate around these loops, defining the minimal size of the cycloamylose products (degree of polymerization >22). At least four substrate-binding subsites have been identified in the *T. aquaticus* enzyme from an acarbose inhibitor bound in the active site [24].

Applications using amylomaltases

A number of interesting and promising applications using thermostable amylomaltases have been reported over recent years. Lee et al. [25] reported on the combined use of a maltogenic amylase from *Bacillus stearothermophilus* and an α-glucanotransferase of *Thermotoga maritima* in the production of isomalto-oligosaccharides from starch. Syrups of isomalto-oligosaccharides have a low viscosity, are resistant to crystallization and have a reduced sweetness. They can be applied as a substitute sugar for diabetics, to improve the intestinal microflora, or to prevent dental caries. The role of the α-glucanotransferase in the proposed process was 2-fold: it produced longer (iso)malto-oligosaccharides that served as a substrate for the amylase and elongated the isomalto-oligosaccharides produced by the amylase. The advantage of using an amylase and an α-glucanotransferase was a reduction of the number of processing steps involved and of the reaction time in combination with a higher yield of isomalto-oligosaccharides [25].

A second application of amylomaltase is its use in the production of cyclic α-1,4-glucans with a degree of polymerization ranging from 17 to a few hundred (cycloamylose). Cyclic glucans are produced by an intermolecular transglycosylation reaction performed by the enzyme. Terada et al. [15] reported on the production of cycloamylose using an amylomaltase such as that from *T. aquaticus* ATCC 33923. The structure of cycloamylose with a degree of polymerization of 26 was elucidated, showing that it consists of two short, left-handed amylose helices in an anti-parallel arrangement (Figure 1) [26]. Along the axis of the helices runs a hydrophobic channel of 5–5.5 Å. In this way a hydrophobic channel is created that can form complexes with hydrophobic guest molecules [27]. Cycloamyloses resemble cyclodextrins, which are short cyclic α-1,4-linked glucans. Cyclodextrins are used to change the solubility, stability or volatility of certain compounds such as flavours. Another possible application of cycloamylose is as an artificial chaperone for protein refolding [28]. This concept is based on the incorporation of a detergent into the cycloamylose molecules. This detergent prevents aggregation of chemically denatured enzymes and also promotes proper protein folding.

Figure 3 | Rheological behaviour of untreated potato starch (A) and amylomaltase-treated potato starch (B) illustrating the thermoreversible gelling properties of the latter
Grey bars indicate heating steps.



A third application of amylomaltases is in the production of a thermoreversible starch gel that can be used as a substitute for gelatin [29]. Thermostable amylomaltases are required, since they have to perform at gelatinization temperatures (Table 1). When gelatinized potato starch was treated with the amylomaltase from *T. thermophilus*, a product free of amylose and containing amylopectin with shortened and elongated side chains was obtained [29,30] (Figure 2). This product could be dissolved in water and formed after heating and cooling a firm gel. The gel could be dissolved again by a new heating step (Figure 3). The thermoreversible behaviour of the amylomaltase-modified potato starch product is very similar to gelatin, a product derived from the bone marrow of cows. Due to its animal origin, gelatin suffers from a disputable reputation and is not accepted by vegetarians and certain religious groups as a food ingredient.

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