Review

Bacterial and archaeal enzymes homologous to glucoamylase: characterization and subsite affinities of a glucoamylase from *Thermoactinomyces vulgaris* R-47

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Abstract: We have studied several bacterial and archaeal glycoside hydrolase (GH) family 15 enzymes, and in this review, mainly focus on a glucoamylase from thermophilic bacterium, *Thermoactinomyces vulgaris* R-47 (TGA). The primary structure of TGA resembled archaeal GH family 15 enzymes, but homologies with fungal glucoamylases were low. Although TGA is an exo-hydrolase that releases β-D-glucose from the non-reducing ends of substrates, as do fungal glucoamylases, this enzyme hydrolyzed maltooligosaccharides more efficiently than starch, unlike fungal glucoamylases. Subsite affinities of TGA showed that the A_1+A_2 value was highly positive whereas A_4—A_6 values were negative and little affinity was detected at subsites 3 and 7, which is different from those of not only fungal glucoamylases, but also a bacterial, *Clostridium* sp. G0005, glucoamylase. Thus, TGA is a novel metabolizing enzyme specific for small oligosaccharides, and it is likely that the difference in substrate specificities of GH family 15 enzymes is associated with the difference in their physiological roles.

Key words: glucoamylase, glucodextranase, *Thermoactinomyces vulgaris*, GH family 15.

Abbreviations: GDase, *Arthrobacter globiformis* I-42 glucodextranase; GH, glycoside hydrolase; MGA, *Methanococcus jannaschii* glucomylase; TGA, *Thermoactinomyces vulgaris* R-47 glucoamylase; TVA II, pullulan-hydrolyzing α-amylase from *Thermoactinomyces vulgaris* R-47.

Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3), classified into glycoside hydrolase (GH) family 15 (Henrissat, 1991), is an exo-hydrolase that releases β-D-glucose from the non-reducing ends of starch and related oligo- and polysaccharides (Coutinho & Reilly, 1997; Sauer et al., 2000). Glucoamylase has been extensively used in starch-processing industries, and because of its commercial importance, numerous fungal glucoamylases have been studied, whereas relatively little is known about bacterial and archaeal glucoamylases and related GH family 15 enzymes.

We have studied several bacterial and archaeal GH family 15 enzymes, namely, two glucoamylases – one from a thermophilic bacterium, *Thermoactinomyces vulgaris* R-47, and another one from hyperthermophilic archaeon, *Methanococcus jannaschii*, (abbreviated as TGA and MGA, respectively) – and a glucodextranase from *Arthrobacter globiformis* I-42 (GDase). We have demonstrated that their enzymatic properties are markedly different from those of fungal glucoamylases despite their structural similarities, and these enzymes hydrolyze starch less efficiently than typical glucoamylases. In particular, we mainly describe in this review the molecular cloning and characterization of TGA.

Molecular cloning of the TGA gene and a comparison of the primary structures with other GH family 15 enzymes

At the beginning of this study, we studied enzymes which hydrolyze polysaccharide, pullulan, and cyclic maltooligosaccharides, cyclodextrins. Although most α-amylases barely hydrolyze pullulan and cyclodextrins, *Thermoactinomyces vulgaris* R-47 produces two pullulan-hydrolyzing α-amylases, and one of these enzymes, TVA II, also efficiently hydrolyzes cyclodextrins and maltooligosaccharides. The characterization and three-dimensional structure of TVA II have been reported (Tonozuka et al., 1995; 2002; Kamitori et al.,

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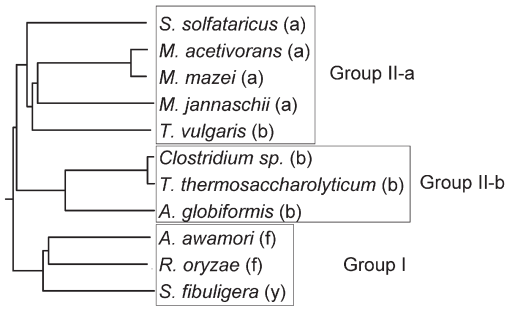

Fig. 1. Phylogenetic analysis of GH family 15 enzymes from various origins. Abbreviations designations are (a) archaeal, (b) bacterial, (f) fungal, (y) yeast GH family 15 enzymes. Groups I, II-a, and II-b are boxed. Branch lengths correspond to evolutionary distances. The phylogenetic tree was built from catalytic domain sequences. Various glucoamylases from *Sulfolobus solfataricus* (GeneBank GI number 15897867), *Methanococcus jannaschii* (1592211), *Thermoactinomyces vulgaris* R-47 (8777462), *Clostridium* sp. G0005 (231542), *Themoanaerobacterium thermosaccharolyticum* (3243238), *Aspergillus awamori* (166505), *Rhizopus oryzae* (1168453), and *Saccharomycopsis fibuligera* (171611), a glucodextranase from *Arthrobacter globiformis* I-42 (12249093), and two putative glucoamylases MA4050 protein from *Methanosarcina acetivorans* C2A (19918146) and MM0864 protein from *Methanosarcina mazei* Goe1 (20905279) were used in this analysis.

2002). In order to find enzymes metabolically related to TVA II, the flanking region of TVA II was sequenced, and a gene was found whose primary structure was homologous to glucoamylase (Uotsu-Tomita et al., 2001). Furthermore, the entire genome of a hyperthermophilic archaeon *Methanococcus jannaschii* has been sequenced and a putative glucoamylase gene, MJ1610, was reported (Bult et al., 1996). The primary structure of this enzyme, MGA, resembled that of TGA and sequence identity between both proteins was 27%. Also, sequence identity between TGA and GDase (N-terminal portion of mature form, residues 29-712) was 15%.

All GH family 15 enzymes have five conserved regions proposed for glucoamylase, the (DL/ER)-WEE motif, which consists of the catalytic center, being found in region III. Bacterial and archaeal GH family 15 enzymes have an extra domain ($\sim$ 250 amino acid residues; designated as domain N) at their N-terminus, and GDase also has an additional region composed of two domains (designated as domains B and C) at the C-terminus (Mizuno et al., 2004). Based on the analysis of phylogenetic tree of glucoamylases and glucodextranases, but not taking into account their enzymatic properties, these enzymes seem to cluster into two groups (Fig. 1); a group of fungal and yeast glucoamylases (Group I), and a group of archaeal and bacterial glucoamylases and glucodextranases (Group II). In Group II, the archaeal and bacterial enzymes are categorized into two subgroups (designated as Groups II-a and II-b). Group II-a mainly consists of archaeal glucoamylases, while Group II-b consists of bacterial glucoamylases and glucodextranases. Interestingly, TGA appears to belong to Group II-a, although TGA is derived from a bacterium, and the primary structure of TGA is closer to that of archaeal glucoamylases, such as MGA, rather than to those of other bacterial glucoamylases and glucodextranases. FASTA search analysis also showed that TGA most resembles two archaeal proteins, MM0864 from *Methanosarcina mazei* Goe1 (29.8% identity) and MA4050 from *Methanosarcina acetivorans* C2A (29.7% identity), although the (DL/ER)-WEE motif is not found in those proteins, and the corresponding residues are identified as DL-WET.

Comparison of substrate specificities of TGA, MGA, and GDase with those of other GH family 15 enzymes

To clarify the substrate specificities of TGA, MGA and GDase, MGA gene was cloned by using two plasmids AMJBX50 and AMJKW69 (which were purchased from ATCC), a recombinant MGA expression system was constructed in *E. coli*, and the substrate specificities of these three enzymes were compared to those of glucoamylases from two fungi, *Aspergillus niger* and *Rhizopus niveus* (Table 1) (Uotsu-Tomita et al., 2001). TGA and MGA most preferred maltooligosaccharides, such as maltotetraose and maltose, and their substrate specificities were similar. Recently, a hyperthermophilic archaeon, *Sulfolobus solfataricus* glucoamylase was also reported to prefer maltotriose to starch (Kim et al., 2004). On the other hand, fungal glucoamylases hydrolyzed starch more efficiently than maltooligosaccharides. The substrate specificity of GDase was quite different from that of all glucoamylases. GDase preferred substrates whose cleavage points are α-(1$\rightarrow$6)-glucosidic linkages of dextran. *Thermoanaerobacterium thermosaccharolyticum* glucoamylase was reported to show almost the same levels of activity for maltotetraose, maltoheptaose, and starch (Ganghofner et al., 1998). Thus, although TGA is a bacterial enzyme, the substrate specificity of TGA is also similar to those of archaeal enzymes, rather than that of fungal and bacterial glucoamylases and glucodextranases.

Is TGA a glucoamylase?

TGA preferred maltooligosaccharides such as maltotetraose and maltose rather than starch. The substrate specificity of TGA more closely resembled that of α-glucosidases, which are also known to hydrolyze maltooligosaccharides efficiently and produce glucose. The term "amylase" originally means an enzyme which hydrolyzes starch; thus, TGA may not be a glucoamylase if this definition is applied. To determine whether TGA is a glucoamylase or an α-glucosidase, we would like to define "glucoamylase" as follows: (i) glucoamylase liberates β-D-glucose from substrate (while α-glucosidase liberates α-D-glucose); (ii) glucoamylase is

Table 1. Comparison of the activities of TGA and related GH15 enzymes for various saccharides.[a]

	G2	G4	Starch	Dextran
TGA	33	100	2.6	N.D.[b]
MGA	64	100	6.2	N.D.
GDase	1.9	1.9	1.0	100
*Rni*GA	12	85	100	N.D.
*Ani*GA	15	85	100	N.D.

[a] TGA, *Thermoactinomyces vulgaris* R-47 glucoamylase; MGA, *Methanococcus jannaschii* glucomylase; GDase, *Arthrobacter globiformis* I-42 glucodextranase; *Rni*GA, *Rhizopus niveus* glucoamylase; *Ani*GA, *Aspergillus niger* glucoamylase. G2, maltose; G4, maltotetraose. Numbers were calculated by designating the values for each saccharide as 100%. Table reproduced from Uotsu-Tomita et al. (2001) with permission from Springer-Verlag.
[b] Not detected.

an exo-hydrolase, and releases the product from the non-reducing end; and (iii) glucoamylase efficiently hydrolyzes α-(1→4)-glucosidic linkages of starch and related oligo- and polysaccharides.

The anomeric forms of TGA hydrolyzates were compared with those of fungal glucoamylase and α-glucosidase (Fig. 2). After the addition of an alkaline solution during the reaction, the anomeric equilibrium between the α- and β-forms of the products was reached immediately and the anomeric form of the liberated glucose could be determined. The optical rotation was increased in the reaction of TGA and fungal glucoamylase (Fig. 2B), indicating TGA liberated β-D-glucose from the substrate.

To determine whether TGA releases the product from the non-reducing end or the reducing end of the substrate, the patterns of hydrolysis for maltoheptaitol and *p*-nitrophenyl α-D-maltopentaoside were tested (Fig. 3) (Ichikawa et al., 2004). If TGA released the product from the reducing end of the substrate, the hydrolysis for these two substrates was expected to be inefficient. For maltoheptaitol, only glucose was produced as the reducing sugar in the reaction (Fig. 3B, lanes 5 and 7). Also, the patterns of hydrolysis for maltoheptaitol and maltoheptaose were almost identical (Fig. 3A). For *p*-nitrophenyl α-D-maltopentaoside, glucose and *p*-nitrophenyl α-D-maltotetraoside were observed as the major products in the early reaction (Fig. 3C, lane 3), and glucose and *p*-nitrophenyl α-D-glucopyranoside remained in the reaction mixture (Fig. 3C, lane 6). These findings indicated that TGA hydrolyzes β-D-glucose from the non-reducing end of the substrates, showing that TGA is a glucoamylase.

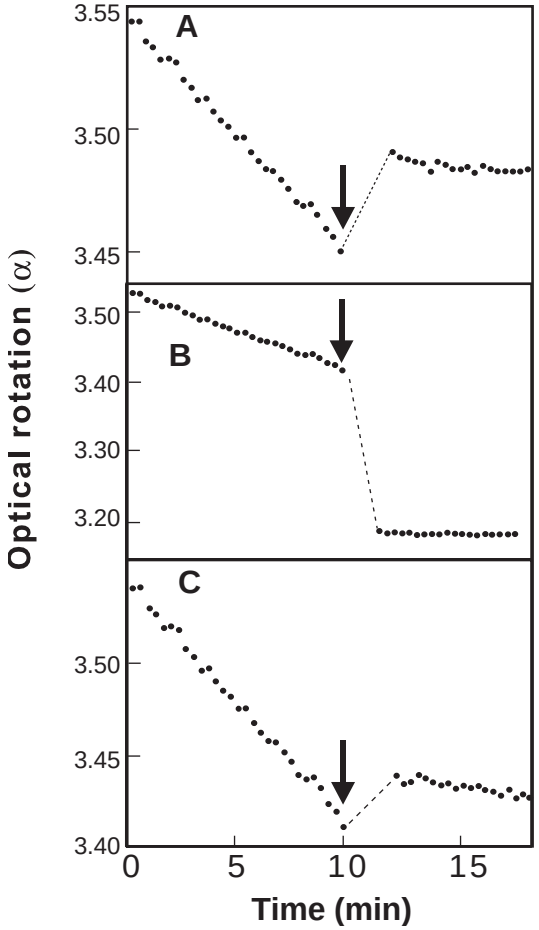

Fig. 2. Alterations in optical rotations during the hydrolysis of maltose catalyzed by glucoamylase from *Rhizopus niveus* (A), α-glucosidase (B), and TGA (C). After the reaction mixture containing 200 μL of enzyme and 800 μL of 3% maltose was incubated for 10 min, 20 μL of 15 M ammonium hydroxide (arrows) were added and the change in the optical rotation was observed. Figure reproduced from Uotsu-Tomita et al. (2001) with permission from Springer-Verlag.

Subsite affinities of TGA

Kinetic parameters (k_0 and K_m) of TGA for maltooligosaccharides (maltose to maltoheptaose) were evaluated to determine subsite affinities. Based on kinetic data, the subsite affinities A_i of TGA were evaluated according to the method of Hiromi et al. (1973). As shown in Figure 4, the A_1+A_2 value of TGA was strongly positive, while the A_4-A_6 values were negative. Also, little affinity was detected at subsites 3 and 7. The pattern of subsite affinities was markedly different from that of other glucoamylases, whose subsite structures have been reported, for example, glucoamylases from *Aspergillus awamori* (Sierks et al., 1989), *Rhizopus niveus* (Tanaka et al., 1983), and *Clostridium* sp. G0005 (Ohnishi et al., 1992). In the fungal glucoamylases, namely those from *A. awamori* and *R. niveus*, the A_1 values are slightly negative, while the A_2 values are most positive, and the A_3-A_7 values are also positive. In the bacterial glucoamylases, the subsite structure of *Clostridium* sp. G0005 glucoamylase is available, and the primary structure of the enzyme has 95% identity with that of *Thermoanaerobacterium thermosaccharolyticum* glucoamylase whose three-dimensional structure has been re-

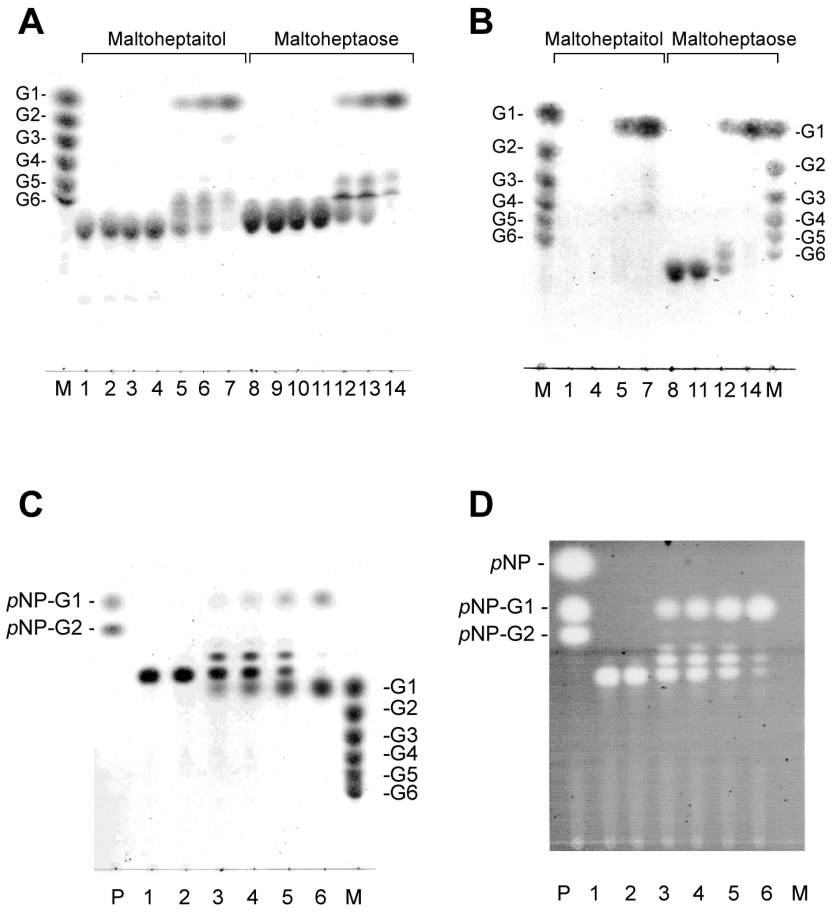

Fig. 3. Thin-layer chromatograms showing the action of TGA on maltoheptaitol, maltoheptaose and *p*-nitrophenyl α-D-Maltopentaoside. Spots of carbohydrate (A, C), reducing sugar (B) and *p*-nitrophenol (D) were detected. Lane M, maltooligosaccharide markers: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose. (A)(B) Action of TGA on maltoheptaitol (lanes 1-7) and maltoheptaose (lanes 8-14). Lanes 1 and 8, substrate only. Lanes 2-4 and 9-11, substrate incubated (no TGA added). Lanes 5-7, and 12-14, substrate incubated with TGA. Reaction mixtures were incubated for 0.5 h (lanes 2, 5, 9, and 12), 1 h (lanes 3, 6, 10, and 13), and 3 h (lanes 4, 7, 11, and 14). (C)(D) Lane P, *p*-nitrophenyl maltooligosaccharide markers: *p*NP, *p*-nitrophenol; *p*NP-G1, *p*-nitrophenyl α-D-glucopyranoside; *p*NP-G2, *p*-nitrophenyl α-D-maltoside. Lane 1, *p*NP-G5 only. Lane 2, *p*NP-G5 incubated for 17 h. Lanes 3, 4, 5, and 6, reaction mixture containing TGA and *p*NP-G5 incubated for 0.5, 1, 3, and 17 h, respectively. Figure reproduced from Ichikawa et al. (2004) with permission from Japan Society for Bioscience, Biotechnology, and Agrochemistry.

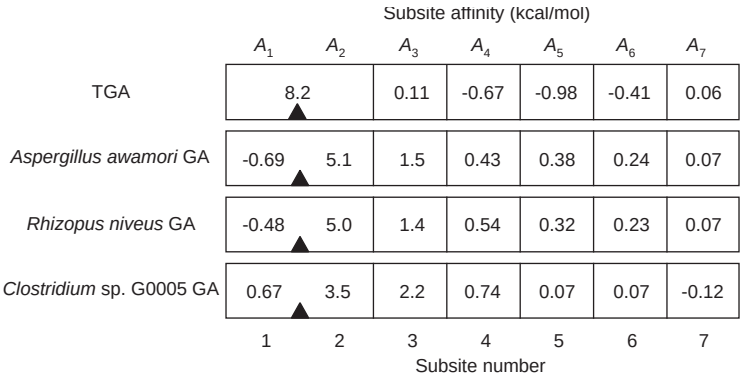

Fig. 4. Comparison of subsite affinities of TGA with those of *A. awamori*, *R. niveus* and *Clostridium* sp. G0005 glucoamylases. The subsite affinities of *R. niveus* glucoamylase are from Tanaka et al. (1983). Subsite affinities of glucoamylases from *A. awamori* and *Clostridium* sp. G0005 were calculated using the results of Sierks et al. (1989) and Ohnishi et al. (1992), respectively. Figure reproduced from Ichikawa et al. (2004) with permission from Japan Society for Bioscience, Biotechnology, and Agrochemistry.

ported (Aleshin et al., 2003). Although similarities in primary and three-dimensional structures between these bacterial and fungal glucoamylases are quite low, the pattern of subsite affinities of *Clostridium* sp. G0005 glucoamylase resembles that of the fungal glucoamylases rather than that of TGA. These results also provide evidence that TGA is an intracellular enzyme engaged in metabolizing small oligosaccharides.

Implications for the physiological role of TGA

It is reasonable to assume that TGA is a member of the system metabolizing maltooligosaccharides, cyclodextrins, and related sugars in *T. vulgaris* R-47 (Yopi et al., 2002). Genes encoding an intracellular α-amylase (TVA II), a cyclodextrin-binding protein, and TGA are contiguously located as a gene cluster. Similar systems have been reported in various bacteria and archaea, for example, in *Escherichia coli* (Boos et al., 1998), *Klebsiella oxytoca* (Fiedler et al., 1996), *Alicyclobacillus acidocaldarius* (Hülsmann et al., 2000), and *Thermococcus* sp. B1001 (Hashimoto et al., 2001).

Many enzymes, whose primary structures and enzymatic properties are homologous to TVA II, have been reported (Tapio et al., 1991; Feederle et al., 1996; Matzke et al., 2000). Although these enzymes have slight differences in their substrate specificities, their primary structures resemble that of TVA II and

they also prefer to hydrolyze oligosaccharides, as TVA II does. However, proteins homologous to TGA are uncommon and not found in these four organisms, while proteins homologous to TVA II and cyclodextrin-binding protein are commonly found in such metabolic systems. In a sugar metabolic system lacking protein homologous to TGA, multiple enzymes are generally required to utilize maltose. For example, *Escherichia coli* metabolize maltose by the combined action of amylomaltase, maltodextrin phosphorylase, and maltodextrin glucosidase. In contrast, our results demonstrate that TGA has the ability to utilize not only maltooligosaccharides longer than maltose, but also maltose. It is an intriguing question why proteins homologous to TGA are not widely distributed in all of the sugar metabolic systems of bacteria and archaea.

Acknowledgements

This study was supported in part by the Novozymes Japan Research Fund.

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Received October 8, 2004
Accepted March 18, 2005