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Purification, Characterization, and Subsite Affinities of *Thermoactinomyces vulgaris* R-47 Maltooligosaccharide-metabolizing Enzyme Homologous to Glucoamylases

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A maltooligosaccharide-metabolizing enzyme from *Thermoactinomyces vulgaris* R-47 (TGA) homologous to glucoamylases does not degrade starch efficiently unlike most glucoamylases such as fungal glucoamylases (Uotsu-Tomita *et al.*, *Appl. Microbiol. Biotechnol.*, 56, 465–473 (2001)). In this study, we purified and characterized TGA, and determined the subsite affinities of the enzyme. The optimal pH and temperature of the enzyme are 6.8 and 60°C, respectively. Activity assays with 0.4% substrate showed that TGA was most active against maltotriose, but did not prefer soluble starch. Kinetic analysis using maltooligosaccharides ranging from maltose to maltoheptaose revealed that TGA has high catalytic efficiency for maltotriose and maltose. Based on the kinetics, subsite affinities were determined. The $A_1 + A_2$ value of this enzyme was highly positive whereas A_4–A_6 values were negative and little affinity was detected at subsites 3 and 7. Thus, the subsite structure of TGA is different from that of any other GA. The results indicate that TGA is a metabolizing enzyme specific for small maltooligosaccharides.

Key words: *Thermoactinomyces vulgaris* R-47; glucoamylase; subsite affinity; metabolism

Glucoamylase (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3, abbreviated as GA) is an exo-hydrolase that releases β-D-glucose from the non-reducing ends of starch and related oligo- and poly-saccharides. GA has been extensively used in starch-processing industries, and because of the commercial importance,[1] numerous fungal GAs have been studied. Most fungal GAs have a starch-binding domain in addition to a catalytic domain, and prefer starch over maltooligosaccharides, indicating that the primary substrate of these enzymes is starch.[2,3]

Among the bacterial and archaeal enzymes, GAs from thermophilic clostridia have been studied the most,[4–8] and the crystal structure of a clostridial species, *Thermoanaerobacterium thermosaccharolyticum*, GA has been recently determined.[9] However, except for the GAs from thermophilic clostridia, relatively little is known about the bacterial and archaeal GAs. *Thermoanaerobacterium thermosaccharolyticum* GA is reported to show almost equal levels of activity for maltotetraose, maltoheptaose, and starch.[7] In contrast, our previous report indicated that GAs from a thermophilic actinomycete, *Thermoactinomyces vulgaris* R-47 (abbreviated as TGA), and a methanogenic archaeon, *Methanococcus jannaschii*, degraded maltooligosaccharides more efficiently than starch.[10] Therefore, the enzymatic properties of the bacterial and archaeal GAs are quite diverse. Here we purified and characterized TGA, and determined the subsite affinities of the enzyme. The subsite structure of TGA was not similar to that of any other GA, and explains why TGA is a hydrolyzing enzyme specific for small maltooligosaccharides.

Materials and Methods

Substrates. Maltooligosaccharides (maltose to maltoheptaose, maltopentadecaose) and pullulan (number average molecular weight 73,000) were gifts from Hayashibara Biochemical Laboratories Inc. Maltooctaose and maltononaose were kindly provided from Nihon Shokuhin Kako Co., Ltd. Maltoheptaitol was obtained from Nikken Chemicals Co., Ltd. Soluble starch (Art. 1252), dextran T-2000 (average molecular weight 2,000,000), isomaltose and *p*-nitrophenyl α-D-maltopentaoside (*p*NP-G5) were purchased from Merck, Amersham Biosciences, Seikagaku Corporation, and SIGMA, respectively.

Preparation of crude extract. Crude recombinant TGA was prepared from *E. coli* MV1184 cells carrying

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Abbreviations: GA, glucoamylase; TGA, glucoamylase from *Thermoactinomyces vulgaris* R-47; *p*NP-G5, *p*-nitrophenyl α-D-maltopentaoside; DTT, dithiothreitol

the plasmid pTGA6060.[10] MV1184 cells carrying pTGA6060 were precultured in 40 ml of LB medium with ampicillin (50 μg/ml) at 37°C overnight, and then 10 ml of the cells was added to 1 liter of LB medium with ampicillin (50 μg/ml). After 1.5 hr of shaking at 37°C, 1 ml of 0.5 M isopropyl β-D-thiogalactoside (IPTG) was added, and the culture continued at 37°C overnight. The cultured cells were collected by centrifugation at 9,000 × *g* for 15 min and suspended in 50 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT. The suspended cells were disrupted by sonication and the supernatant obtained by centrifugation at 9,000 × *g* for 15 min was pooled as the crude TGA solution.

Purification. The crude TGA solution was incubated at 50°C for 30 min to denature *E. coli* proteins, and the supernatant was collected following centrifugation at 9,000 × *g* for 15 min (heat-treated TGA). The heat-treated TGA was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT at 4°C overnight. The dialyzed TGA was applied to a Q-Sepharose FF 16/10 anion-exchange column (1.6 × 10 cm, Amersham Biosciences), and eluted with a linear gradient of 10 mM Tris-HCl buffer, 1 mM DTT, and 0–0.4 M NaCl (pH 7.5). The active fraction was dialyzed, and separated using a Q-Sepharose HP 16/10 anion-exchange column (1.6 × 10 cm, Amersham Biosciences) under the same conditions as with Q-Sepharose FF. The active fraction was concentrated using Centriprep YM-50 (Millipore) and applied to a Sephacryl S-200 HR 26/60 gel filtration column (2.6 × 60 cm, Amersham Biosciences) equilibrated in 10 mM Tris-HCl buffer, 1 mM DTT, and 0.15 M NaCl (pH 7.5), and eluted at a flow rate of 1.3 ml/min. The active fraction was pooled as purified enzyme.

Protein assay. Protein concentrations were determined by the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

Enzyme assay. TGA activity was assayed as follows. Twenty microliters of TGA in 10 mM Tris-HCl buffer (pH 7.5) and 80 μl of 0.5% substrate in 0.1 M sodium phosphate buffer (pH 6.5) were mixed and incubated at 40°C for 30 min. The reaction was quenched by mixing with an equal volume of 2 M Tris-HCl buffer (pH 7.5). The amount of glucose released was assayed using the mutarotase-glucose oxidase-peroxidase method[12] with a Glucose C-II Test Wako (Wako Pure Chemical Ind., Ltd.). One unit was defined as the amount of enzyme that produces 1 μmol of glucose from maltotetraose per minute.

Effect of pH and temperature. The pH stability was examined as below. Ten microliters of TGA in 10 mM Tris-HCl buffer (pH 7.5) was mixed with 10 μl of 0.1 M glycine-HCl buffer (pH 2.5–3.5), sodium citrate buffer (pH 3.5–6.0), sodium phosphate buffer (pH 6.0–8.0), bicine-NaOH buffer (pH 8.0–9.0), glycine-NaOH buffer (pH 9.0–10.5), and glycine-NaOH (pH 11.0), respectively, and kept at 4°C for 30 min. Eighty microliters of 0.2 M sodium phosphate buffer (pH 6.5) was added to the treated TGA, and the residual activity for 0.4% maltotetraose was measured.

Thermal stability was assayed as follows. TGA in 10 mM Tris-HCl buffer (pH 7.5) was treated at 40–70°C for 30 min. After cooling in an ice bath, 20 μl of the treated TGA was added to 80 μl of 0.5% maltotetraose in 0.1 M sodium phosphate buffer (pH 6.5) and the residual activity was measured.

Optimal pH and temperature was assayed for 30 min at various pH values (3.5–10.5) and temperatures (30–70°C), respectively.

Thin-layer chromatography. Twenty microliters of TGA (60 ng/μl) in 10 mM Tris-HCl buffer (pH 7.5) was added to 20 μl of 1% *p*NP-G5, or 80 μl of maltoheptaitol or maltoheptaose in 0.1 M sodium phosphate buffer (pH 6.5), and the solutions were incubated at 40°C. The reaction was stopped by boiling. The hydrolyzates were separated by thin-layer chromatography (TLC) on Silica gel 60 F_{254} (Merck) using developing solvent with *1*-butanol/ethanol/water (5/5/3, v/v/v). The carbohydrates were detected by charring with 5% sulfuric acid in methanol. The spots of *p*-nitrophenol were detected by exposure of UV. Detection of reducing sugar was performed by the method of Kim and Sakano.[13]

Kinetic analysis. Kinetic parameters of TGA for maltooligosaccharides (maltose to maltoheptaose) were determined as follows. Thirty microliters of TGA in 10 mM Tris-HCl buffer (pH 7.5) and 120 μl of substrate at various concentrations in 0.1 M sodium phosphate buffer (pH 6.5) were mixed. The samples reacted at 40°C were taken at 2-minute intervals and the reaction was quenched by addition to an equal volume of 2 M Tris-HCl buffer (pH 7.5). The amount of glucose produced was measured as described above.

Results

Purification of TGA

Recombinant TGA was purified in four steps (Heat treatment, Q-Sepharose FF, Q-Sepharose HP, and Sephacryl S-200 HR chromatography). After the second step with the Q-Sepharose FF column, the majority of impurities were removed (Fig. 1, lane 3). Finally, the enzyme was purified 3.5-fold with 24.1% yield, and had a specific activity of 20.2 U/mg (Table 1). SDS-PAGE with CBB staining gave a single band (Fig. 1, lane 5). The purified enzyme was used for the subsequent experiments.

Effect of pH and temperature

The effect of pH and temperature on TGA activity was examined using maltotetraose as a substrate. The

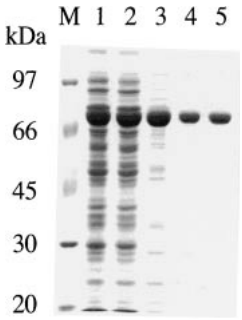

Fig. 1. SDS-PAGE of TGA in Each Purification Step.

Lane M, Low molecular mass markers (phosphorylase b, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa); Lane 1, crude extract; Lane 2, after heat treatment; Lane 3, after Q-Sepharose FF; Lane 4, after Q-Sepharose HP; Lane 5, after Sephacryl S-200 HR.

Table 1. Purification of *Thermoactinomyces vulgaris* R-47 Glucoamylase

Purification step	Total unit (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	738	128	5.77	100
Heat treatment	724	116	6.24	98.1
Q-Sepharose FF	399	32.7	12.2	54.1
Q-Sepharose HP	281	15.5	18.1	38.1
Sephacryl S-200 HR	178	8.83	20.2	24.1

TGA activity in each purification step was measured using maltotetraose as a substrate.

optimal pH and temperature of TGA were 6.8 and 60°C, respectively (Fig. 2B, 2D). The enzyme was stable in the range from pH 5.5 to 9.5 (Residual activity > 90%, Fig. 2A). TGA was stable at 60°C, but retained only 4.5% of its activity at 68°C, and completely lost its activity at 70°C (Fig. 2C). The activation energy of TGA from 30 to 55°C was 43.1 ± 2.0 kJ/mol (Fig. 2F).

TGA activity for various substrates

TGA activity for various substrates was measured with the same quantity of enzyme. TGA showed maximal activity towards maltotriose (Table 2). When maltooligosaccharides longer than maltose were tested, the relative activity decreased as the maltooligosaccharide chains increased in length. No hydrolyzing activity for soluble starch, pullulan, dextran and isomaltose was detected under the conditions. Activity for four substrates was investigated further by increasing the amount of TGA by 3-fold. The enzyme hydrolyzed soluble starch slightly, but not the others at all. These results indicated that TGA hydrolyzes α-(1→4)-linkages of saccharides efficiently not α-(1→6)-linkages. Also, TGA prefers maltooligosaccharides to polysaccharides like soluble starch.

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*NP-G5 were tested. If TGA releases the product from the reducing end of the substrate, the hydrolysis for these two substrates was expected to be inefficient. For

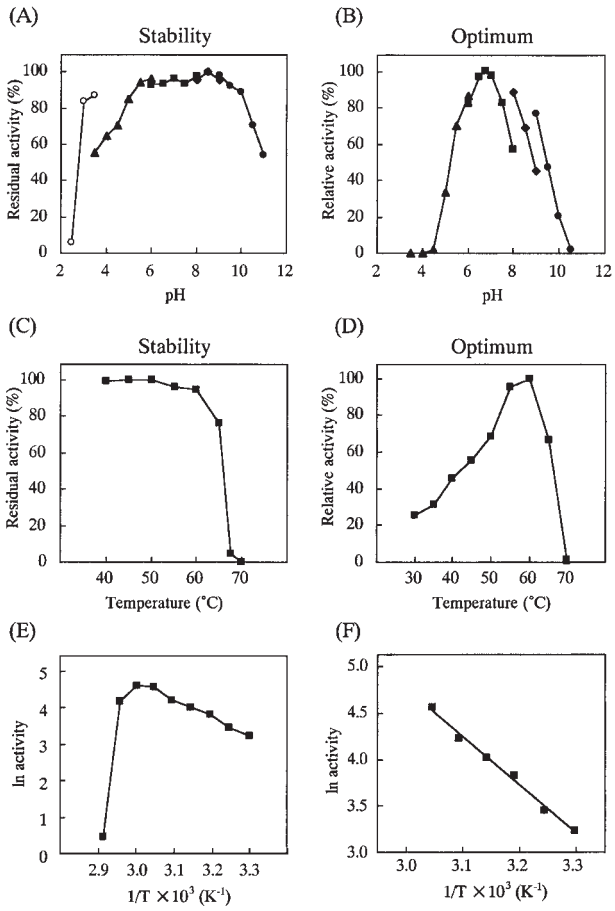

Fig. 2. Effect of pH (A, B) and Temperature (C, D, E, F) on TGA Activity.

Symbols: Glycine-HCl buffer (○), sodium citrate buffer (▲), sodium phosphate buffer (■), bicine-NaOH buffer (◆), glycine-NaOH (●).

Table 2. TGA Activities for Various Substrates

Substrate	Relative activity (%)
Maltose (G_2)	23.5
Maltotriose (G_3)	100
Maltotetraose (G_4)	98.7
Maltopentaose (G_5)	58.6
Maltohexaose (G_6)	37.5
Maltoheptaose (G_7)	28.4
Maltooctaose (G_8)	26.9
Maltononaose (G_9)	22.5
Maltopentadecaose (G_{15})	11.0
Soluble starch	1.9
Pullulan	N.D.[a]
Dextran	N.D.
Isomaltose	N.D.

Relative activity was defined as follows:

$$\frac{\text{Amount of } G_1 \text{ produced from substrate per amount of TGA}}{\text{Amount of } G_1 \text{ produced from } G_3 \text{ per amount of TGA}} \times 100\ (\%)$$

[a] Not detected.

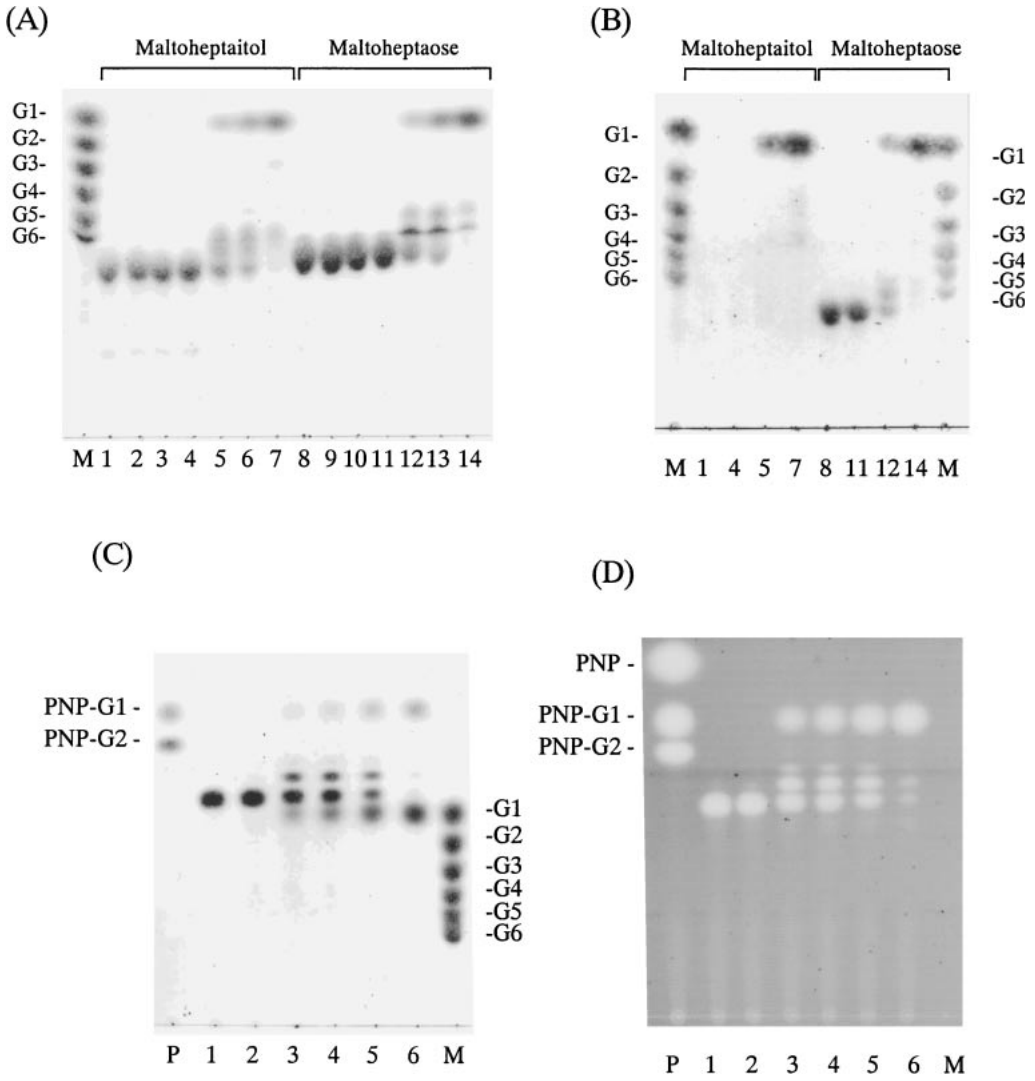

Fig. 3. Thin-layer Chromatograms Showing That the Action of TGA on Maltoheptaitol, Maltoheptaose and *p*-Nitrophenyl α-D-Maltopentaoside. The spots of carbohydrate (A, C), reducing sugar (B) and *p*-nitrophenol (D) were detected. Lane M, maltooligosaccharide markers: G1, glucose; G2, maltose; G3, maltotriose; 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 hr (lanes 2, 5, 9, and 12), 1 hr (lanes 3, 6, 10, and 13), and 3 hr (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 hr. Lanes 3, 4, 5, and 6, reaction mixture containing TGA and *p*NP-G5 incubated for 0.5, 1, 3, and 17 hr, respectively.

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*NP-G5, 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 were finally remained in the reaction mixture (Fig. 3C, lane 6). These findings suggested that TGA liberates glucose from the non-reducing end of the substrate.

Kinetic analysis

Kinetic parameters (k_0 and K_m) of TGA for maltooligosaccharides (maltose to maltoheptaose) were determined (Table 3). Dependence of $\log k_0$, $\log(k_0/K_m)$, and pK_m on degree of polymerization is shown in Fig. 4. Molecular activity k_0 was almost equal except for maltose (Table 3, Fig. 4). Michaelis constant, K_m, values for maltooligosaccharides from maltose to maltohexaose increased as the degree of polymerization increased, but no significant change was detected between maltohexaose and maltoheptaose. Based on the kinetic data, the subsite affinities A_i of TGA were evaluated according to the method of Hiromi *et al.*[14] The A_3–A_7 values were calculated using the following formula.

$$A_{n+1} = RT\{\ln(k_0/K_m)_{n+1} - \ln(k_0/K_m)_n\}$$

According to this equation, the subsite affinities A_3, A_4, A_5, A_6, and A_7 were determined to be 0.11, −0.67,

Table 3. Kinetic Parameters for Hydrolysis of Maltooligosaccharides by TGA at pH 6.5 and 40°C

Substrate	k_0 (s^{-1})	K_m (mM)	k_0/K_m ($s^{-1}\cdot mM^{-1}$)
Maltose	16.9 ± 0.4[a]	0.101 ± 0.008	167 ± 14
Maltotriose	76.4 ± 1.1	0.380 ± 0.016	201 ± 9
Maltotetraose	70.7 ± 1.8	1.03 ± 0.064	68.7 ± 4.6
Maltopentaose	56.3 ± 0.8	3.94 ± 0.25	14.3 ± 0.9
Maltohexaose	55.0 ± 1.0	7.43 ± 0.48	7.41 ± 0.50
Maltoheptaose	61.6 ± 0.8	7.59 ± 0.38	8.12 ± 0.42

[a] standard deviation.

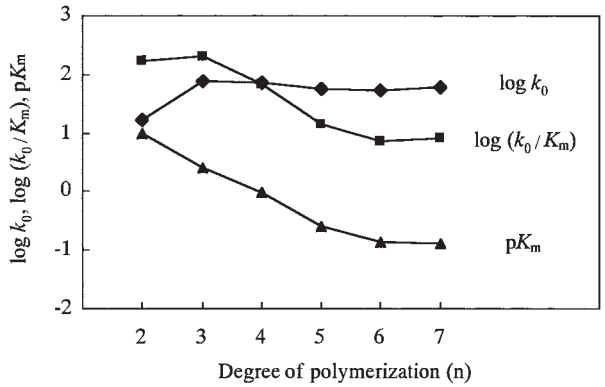

Fig. 4. Dependence of Kinetic Parameters on Degree of Polymerization.

The reaction was done at pH 6.5 and 40°C. k_0, molecular activity; K_m, Michaelis constant.

−0.98, −0.41, and 0.06 kcal/mol, respectively (Fig. 5).

Generally in GA, A_1 and k_{int} (intrinsic rate constant of substrate hydrolysis in a productive complex) values can be calculated using the linear line obtained from plots of $\exp(A_{n+1}/RT)$ vs. $1/k_0$. However, in this case a linear line could not be drawn. In TGA, maltose must occupy subsites 1 and 2 because little affinity from subsite 3 to 7 was detected. Therefore, the $A_1 + A_2$ value was calculated on the assumption that $k_{int} = (k_0)_2$ according to the following equation.

$$(k_0/K_m)_2 = 0.018 k_{int} \exp\{(A_1 + A_2)/RT\}$$

From this equation, the $A_1 + A_2$ value was estimated to be 8.2 kcal/mol.

Discussion

TGA was optimally active at pH 6.8, which is almost neutral. On the other hand, optimal pH values of most of the fungal GAs were 4.0 to 6.0. Recently, several GAs have been found in thermoacidophilic archaea, *Thermoplasma acidophilum, Picrophilus torridus* and *Picrophilus oshimae*, and their optimal pH values were reported to be 2.0.[15] Only a few microorganisms, like *Methanococcus jannaschii*[10] and *Thermomucor indicae-seudaticae*,[16] are known to produce neutral GAs. Generally, the pH optimum is related to the physiological role of the enzyme. We have already reported that an extracellular α-amylase from *T. vulgaris* R-47, TVA I, which has strong hydrolyzing activity for starch, is optimally active under acid conditions (pH 4.5–5.0).[17,18] The findings support that, unlike TVA I, TGA is an intracellular enzyme, the physiological role of which is different from that of acidic GAs. The p*K*a values of two catalytic glutamate residues of GAs, which are a catalytic base and a catalytic acid, may well be responsible for the optimal pH values. The p*K*a values of these two residues depend on their surrounding environment. Fang *et al.* reported that the mutation of Ser411 in *Aspergillus awamori* GA elevated its optimal pH value.[19] Ser411 was found to interact with catalytic base Glu400 according to its three-dimensional structure.[20–24] As shown in Fig. 6, position 411 of *A. awamori* GA is either a Ser or Gly in known GA sequences[2] except Trp in TGA.[10] It is likely that the difference of the amino acid

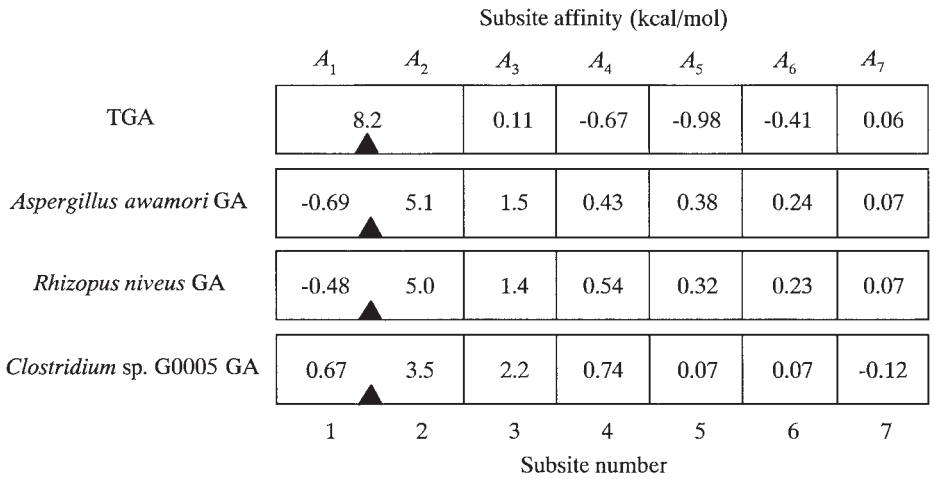

Fig. 5. Comparison of Subsite Affinities of TGA with Those of *A. awamori, R. niveus* and *Clostridium* sp. G0005 GAs.

The subsite affinities of *R. niveus* GA are from [26]. Subsite affinities of *A. awamori* GA and *Clostridium* sp. G0005 GA were recalculated from [25] and [6], respectively.

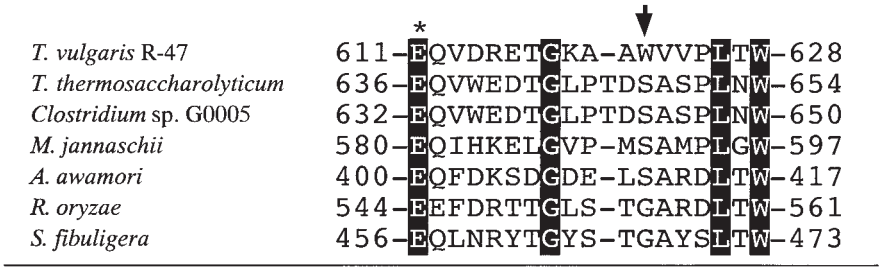

Fig. 6. Sequence Alignment among Various Glucoamylases Centered around Ser411 in *A. awamori* Glucoamylase.
∗, catalytic base;[23] ↓, amino acid residues corresponding to Ser411 in *A. awamori* glucoamylase. Black boxes represent identical amino acid residues.

residue is one of the reasons why TGA is optimally active in the neutral pH range.

TGA had maximal activity for maltotriose, and its activity decreased with an increase of the maltooligosaccharide chain length. The enzyme scarcely degraded soluble starch (1.9% of the maltotriose hydrolysis). In contrast, *Aspergillus niger* GA hydrolyzes starch most efficiently, and *T. thermosaccharolyticum* GA was reported to show almost equal levels of activity for maltotetraose, maltoheptaose, and starch.[7] A comparison of their subsite structures explains the difference of substrate specificity more clearly. We determined the subsite affinities of TGA based on the kinetics towards maltooligosaccharides ranging from maltose to maltoheptaose. The $A_1 + A_2$ value was strongly positive, while the A_4–A_6 values were negative, and also little affinity was detected at subsites 3 and 7. The pattern of subsite affinities is markedly different from that of other GA whose subsite structure has been reported, for example GAs from *Aspergillus awamori*,[25] *Rhizopus niveus*,[26] and *Clostridium* sp. G0005.[6] In the fungal GAs, namely *A. awamori* and *R. niveus* GAs, 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 GAs, the subsite structure of *Clostridium* sp. G0005 GA is available (Fig. 5), and the primary structure of the enzyme has 95% identity with that of *T. thermosaccharolyticum* GA whose three-dimensional structure has been reported.[9] Although similarities of primary and three-dimensional structure between these bacterial and fungal GAs are quite low, the pattern of subsite affinities of *Clostridium* sp. G0005 GA resembles that of the fungal GAs rather than that of TGA. These results also provide evidence that TGA is an intracellular enzyme and engages in metabolizing small oligosaccharides.

Here the values of k_{int}, A_1 and A_2 of TGA could not be determined. Similar observation has been reported in the study of almond β-glucosidase.[27] Tanaka *et al.* concluded that the hydrolysis of cellooligosaccharides by almond β-glucosidase may not be applicable to the subsite theory proposed by Hiromi *et al.* Recently, the A_1 and A_2 values of GA from *Aspergillus awamori* were reevaluated based on the stopped-flow fluorescence kinetics,[28] and the A_1 value is higher than A_2, which conflicts with the values determined using Hiromi's model. The hydrolysis of maltooligosaccharides by TGA may not be also suited to the theory.

We have already reported that TGA is a member of the system metabolizing maltooligosaccharides, cyclodextrins, and related sugars in *T. vulgaris* R-47.[29] Genes encoding an intracellular α-amylase (TVA II), a cyclodextrin-binding protein (CBP), and TGA are contiguously located as a gene cluster. Similar systems have been reported in various bacteria and archaea, for example, *Escherichia coli*,[30] *Klebsiella oxytoca*,[31] *Alicyclobacillus acidocaldarius*,[32] and *Thermococcus* sp. B1001.[33] However, proteins homologous to TGA are uncommon and not found in these four organisms, while proteins homologous to TVA II and CBP are commonly found in such metabolic systems. In the sugar metabolic system lacking protein homologous to TGA, multiple enzymes are generally required to utilize maltose. For example, *Escherichia coli* metabolizes maltose by the combined action of amylomaltase, maltodextrin phosphorylase, and maltodextrin glucosidase. In contrast, our results demonstrate that TGA has an ability to utillize not only maltooligosaccharides longer than maltose but also maltose. It is an intriguing question why the proteins homologous to TGA are not widely distributed in all of the sugar metabolic systems of bacteria and archaea.

Acknowledgments

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