

Functional Analysis of the Threonine- and Serine-Rich Gp-I Domain of Glucoamylase I from *Aspergillus awamori* var. *kawachi*

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Glucoamylase I (GAI) from *Aspergillus awamori* var. *kawachi* hydrolyzes raw starch efficiently and is composed of three functional domains: the amino-terminal catalytic GAI' domain (A-1 to V-469), the threonine- and serine-rich O-glycosylated Gp-I domain (A-470 to V-514), and the carboxy-terminal raw starch-binding Cp domain (A-515 to R-615). In order to investigate the role of the Gp-I domain, an additional repeat of Gp-I and internal deletions of the entire Gp-I sequence or parts of the Gp-I sequence were introduced within Gp-I. All mutant genes as well as the wild-type gene were inserted into a yeast-secretion vector, YEUp3H α , and expressed in *Saccharomyces cerevisiae*. Wild-type GAI expressed in yeast cells (GAY), GAGpI, having an extra Gp-I, and GAD470–493, lacking the A-470-to-T-493 sequences of Gp-I, were successfully secreted into the culture medium. On the other hand, GAD470–507, lacking A-470 to S-507, and GADGpI, lacking the entire Gp-I (A-470-to-V-514) sequence, failed to be secreted and remained in the yeast cells. The carbohydrate content of GAGpI was 1.2 times higher than that of GAY and 2.4 times higher than that of the original GAI. The raw starch digestibility of GAGpI was almost the same as that of GAY but was 1.5 times faster than that of GAI. Furthermore, GAGpI gained enhanced thermal stability as well as pH stability compared with GAY and GAI. On the other hand, GAD470–493 had decreased carbohydrate content (29% of that of GAY), and the raw starch digestibility of this enzyme was one-third that of GAY. These results indicate that the C-terminal portion of the Gp-I domain is critically required for secretion of GAI and, together with the Cp domain, plays an important role in raw starch digestion.

Glucoamylase (1,4- α -D-glucan glucohydrolase [EC 3.2.1.3]), which hydrolyzes both α -(1–4)- and α -(1–6)-D-glucosidic bonds to produce β -D-glucose, is one of the most important enzymes for industrial use.

Glucoamylase I (GAI) from *Aspergillus awamori* var. *kawachi* hydrolyzes both soluble and raw starches and is composed of three domains (4, 11): the catalytic GAI' domain, near the amino-terminal (A-1 to V-469) threonine- and serine-rich Gp-I domain (A-470 to V-514), and the carboxy-terminal raw starch-binding Cp domain (A-515 to R-615).

We revealed previously that the Cp domain has the ability to bind not only to raw starch but also to α -, β -, and γ -cyclodextrins. Thereby, such cyclodextrins are competitive inhibitors for binding to and digestion of raw starch of GAI (4, 8). From analyses of glucoamylases having various mutations within the Cp domain, it was demonstrated that the sequence P-560–L-W-562–Y–V–T–V–T–L–P–A-570 within the Cp domain was essentially required for digestion of raw starch, and the Trp-562 residue was critical for the formation of an inclusion complex with substrates. The region near the Trp-589 residue, which is abundant in hydrogen bond-forming amino acids as well as charged amino acids, was suggested to assist stable formation of an inclusion complex (7).

The Gp-I domain is mainly composed of Thr and Ser residues glycosylated with oligomannosaccharides (9, 14). It is known that oligosaccharides in glycoproteins are involved in maintenance of protein structure against stresses caused by heat, pH, and pressure and are also involved in secretion of proteins out of cells (1–3).

We reported previously that the Gp-I fragment obtained by proteolysis of GAI with subtilisin was enzymatically inactive but promoted the digestion of raw starch by GAI (13). Mannoside chains O linked to the Gp-I domain seem to be important for efficient digestion of raw starch, since a GAI mutant enzyme in which mannose was replaced partially with glucose showed significant decreased activity for raw starch (5). Moreover, experiments with D₂O and glycerol instead of H₂O resulted in decreased digestion of raw starch by GAI. Because D₂O is more stable than H₂O clusters and glycerol stabilizes the water clusters, the mannoside chains O linked to the Gp-I domain seem to contribute to raw starch digestion through the interaction with water (6).

In this report, we describe the characteristics of various glucoamylases having a tandemly repeated Gp-I domain or having a series of internal deletions of the Gp-I domain, hoping to make clear the role of the Gp-I domain in the digestion of raw starch.

MATERIALS AND METHODS

Microorganism and plasmids. *Escherichia coli* JM109 [*recA1 endA2 glyA96 thi hsdR17 supE44 relA1 Δ (lac-proAB) F'* [*traD36 proAB⁺ lacI^q lacZ Δ M15*]] was used as a cloning host. *Saccharomyces cerevisiae* CG379 (*MAT α ura3 leu2 trp1 his3*) was used as a host for expression of mutated GAI genes. The plasmid YEUp3H α was used as an expression vector for secretion of various mutant glucoamylases as described previously (7).

Construction of GAI mutants having an additional repeat of Gp-I or series of internal deletions in the Gp-I domain. cDNAs suitable for expression in yeast cells were amplified by the PCR, as shown in Fig. 1. The reaction conditions and template DNA were described previously (7). For wild-type and mutant glucoamylases, the following 10 primers were synthesized with a DNA synthesizer (Applied Biosystems, Inc., model 392): primer 1, 5'-ACAGAATTCAGCTTC TGCGACCTTGGATTCTGGTGG-3'; primer 2, 5'-AAGAATTCAGCTTCTAC CGCCAGGTGTCGGT-3'; primer 3, 5'-ATAGGATCCGCTACTGGTGGCAC C-3'; primer 4, 5'-CTCGGATCCTACGGCAGTGGGGGT-3'; primer 5, 5'-TG

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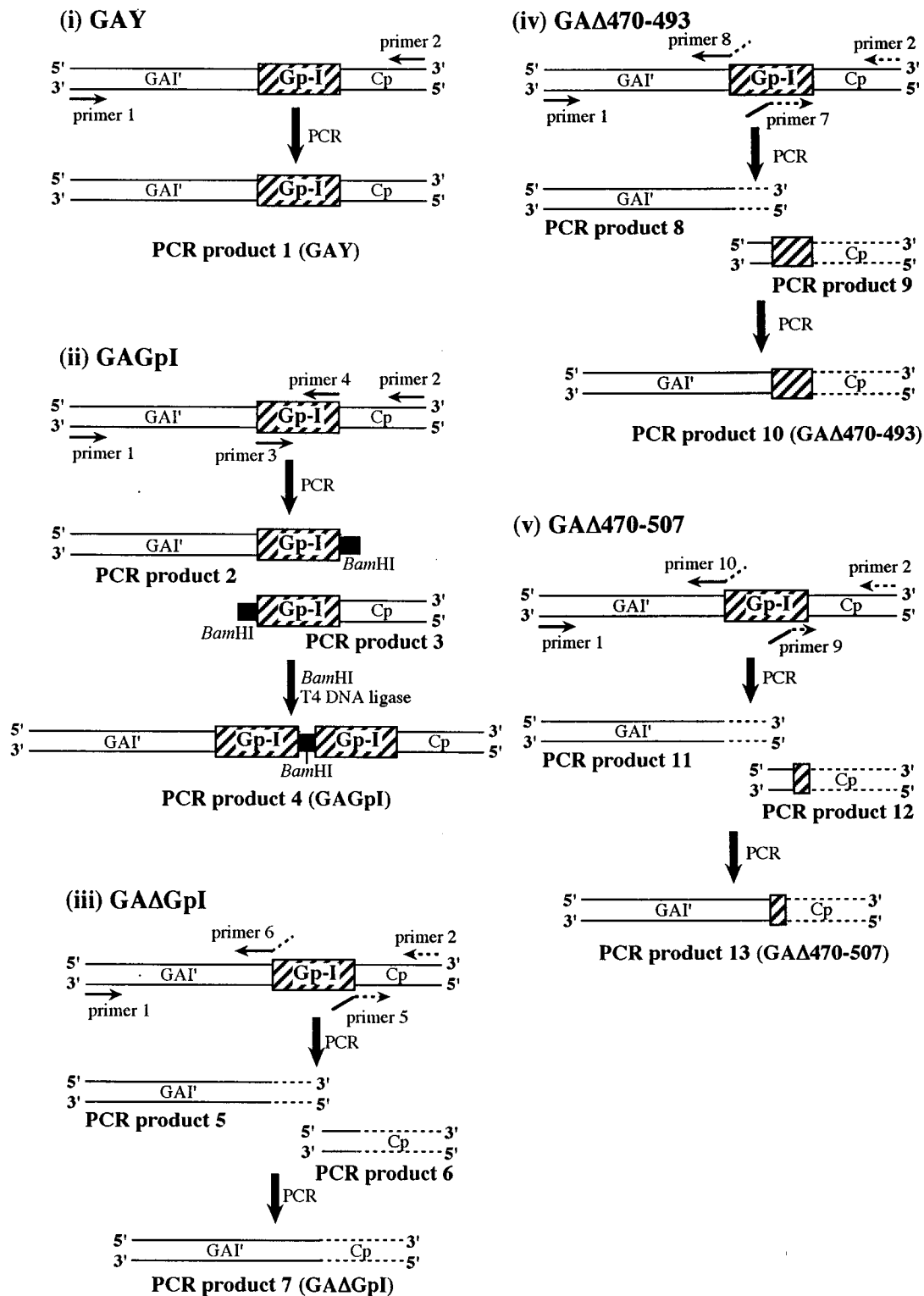


FIG. 1. Construction of wild-type (GAY) and mutant genes of GAI having various mutations in the Gp-I domain. Primers 5, 6, 7, 8, 9, and 10 were designed such that their 3' ends hybridize to template sequence on one side of deletion and the 5' ends were complementary to template sequence on the other side of the deletion (15).

GCCGAGCATCGTGGCTGTGACCTTTGAT-3'; primer 6, 5'-ATCAAAGGT CACAGCCACGATGCTCGGCCA-3'; primer 7, 5'-TGGCCGAGCATCGTG ACAACTGCTAGTAAG-3'; primer 8, 5'-CTTACTAGCAGTTGTTCACGATG CTCGGCCA-3'; primer 9, 5'-TGGCCGAGCATCGTGACCACCCCACTG

CC-3'; and primer 10, 5'-GGCAGTGGGGGTGTCACGATGCTCGGCCA-3'. The *EcoRI* site is underlined, the *HindIII* site is double-underlined, the newly introduced *BamHI* site is in boldface type, and the extra CT nucleotides and the stop codon are in italics. Primers 1 and 3 were designed to corresponding to the

5' sequences of GAI' and the Gp-I region, respectively (7, 12). Primers 2 and 4 were complementary to the 3' sequences of Cp and the Gp-I region, respectively. Primers 5, 6, 7, 8, 9, and 10 were designed such that their 3' ends hybridized to template sequence on one side of deletion and their 5' ends were complementary to template sequence on the other side of the deletion (15).

Full-length glucoamylase (GAY). PCR product 1, encoding the full-length, wild-type GAI (GAY) sequence, was amplified with primers 1 and 2.

Glucoamylase in which an additional Gp-I region is tandemly introduced (GAGpI). PCR product 2, coding for GAI' and Gp-I, and PCR product 3, coding for Gp-I and Cp, were amplified with primers 1 and 4 and primers 2 and 3, respectively. The PCR products thus obtained (products 2 and 3) were digested with *Bam*HI and ligated with T4 DNA ligase (Toyobo Co., Ltd., Osaka, Japan) to generate PCR product 4, in which an additional Gp-I could be introduced.

Glucoamylase which lacks the entire Gp-I region (GAΔGpI). The deletion of amino acid sequences within Gp-I was performed according to the method of Ho et al. (15). PCR product 5, coding for GAI' with primers 1 and 6, and PCR product 6, coding for the Cp region with primers 2 and 5, were individually amplified. The PCR products thus obtained (products 5 and 6) share overlapping sequences at their respective 3' and 5' ends. The second PCR was performed with PCR products 5 and 6 and primers 1 and 2 to generate PCR product 7, in which the entire Gp-I sequence was deleted.

Glucoamylase which lacks 24 amino acids within the Gp-I region (GAΔ470-493). PCR product 8, coding for GAI' with primers 1 and 8, and PCR product 9, coding for the C-terminal half of Gp-I and Cp with primers 2 and 7, were individually constructed. PCR product 10 was constructed with PCR products 8 and 9 and primers 1 and 2, in which the nucleotide sequences coding for the 24 N-terminal amino acids within Gp-I (45 amino acids total) were deleted.

Glucoamylase which lacks 38 amino acids within the Gp-I region (GAΔ470-507). PCR product 11, coding for GAI', was constructed with primers 1 and 10, and PCR product 12, coding for the C-508-to-R-615 sequence of GAI, was constructed with primers 2 and 9. PCR product 13 was then constructed with PCR products 11 and 12 and primers 1 and 2, in which the nucleotide sequences coding for the N-terminal 38 amino acids within the Gp-I region were deleted.

The amplified PCR products 1, 4, 7, 10, and 13 were digested with *Eco*RI and were introduced into the *Eco*RI site of pUC19 to generate pUC-GAY, pUC-GAGpI, pUC-GAΔGpI, pUC-GAΔ470-493, and pUC-GAΔ470-507, respectively, and mutated sequences were confirmed by a DNA sequencer (Applied Biosystems Inc., model 373A). The *Hind*III fragments including the respective glucoamylase genes were inserted into the *Hind*III-digested YEUp3Hα to generate YEUp-GAY, YEUp-GAGpI, YEUp-GAΔGpI, YEUp-GAΔ470-493, and YEUp-GAΔ470-507, respectively. These plasmids were transformed into *S. cerevisiae* CG379 by electroporation (model 600; BTX Co., Ltd., San Diego, Calif.).

Purification of enzymes. Culture filtrates from the recombinant yeast cells were brought to 60% (wt/vol) saturation with ammonium sulfate. GAY, GAGpI, and GAΔ470-493 were purified by column chromatographies on Sephacryl S-200 (Pharmacia, Tokyo, Japan), α-cyclodextrin-Sepharose CL-4B, and TSK-G3000 SW (Tohso, Tokyo, Japan) as described previously (7). Since GAΔGpI and GAΔ470-507 were not secreted in the culture broth from yeast cells, we could not purify these enzymes in active forms.

Assay of glucoamylase activity. Treatment of glucoamylases with α-mannosidase and the purification of modified enzymes were performed as described previously (6).

Glucoamylase activity was assayed by the method described previously (19). Briefly, a mixture of 0.5 ml of enzyme solution, 2.5 ml of 1% soluble starch, and 1 ml of 10 mM Mcllvaine buffer (pH 3.8) was incubated at 50°C for 10 min. The reducing sugar formed was quantified by the 3,5-dinitrosalicylic acid method (14). One unit of glucoamylase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per min under these conditions.

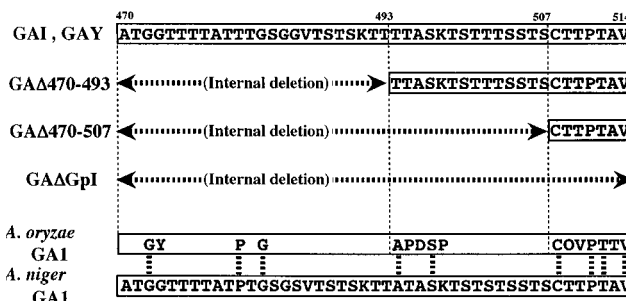
Adsorption of glucoamylases to raw starch. Raw cornstarch (200 mg) was added to 1 ml of enzyme solution in 0.1 M citrate buffer (pH 4.0). The mixture was incubated at 4°C for 30 min with occasional shaking. After centrifugation, the glucoamylase activity of the supernatant was measured, and the relative adsorption of the enzyme to raw starch was calculated (6, 7).

Digestion of raw starch. The digestibility of raw starch was measured as described previously (7). The reaction mixture, containing 12.5 mg of raw cornstarch and 2 ml of glucoamylase in 0.1 M citrate buffer (pH 3.6), was covered with 0.1 ml of toluene. Reactions were carried out at predetermined temperatures without shaking. At suitable intervals, the reducing sugar formed was quantified by the 3,5-dinitrosalicylic acid method, and the extent of hydrolysis was calculated.

Measurement of thermostability and pH stability of glucoamylases. Purified GAI, GAY, and GAGpI in 50 mM phosphate buffer at pH 5.5 (each at 5.0 U/ml) were incubated at temperatures ranging from 30 to 70°C. After 30 min of incubation at the respective temperature, samples were chilled in ice and glucoamylase activities were assayed. The same enzymes were also incubated at various pHs. KCl-HCl buffer was used at pH 1.5, 2.0, and 2.5; citrate-NaOH buffer was used at pH 3.0 and 3.5; Mcllvaine buffer was used at pH 4.0, 5.0, 6.0, and 7.0; Tris-HCl buffer was used at pH 8.0, 9.0, and 10.0; and NaOH-Na₂HPO₄ buffer was used at pH 11.0. Enzyme solution at the respective pH was kept at 4.0°C for 24 h, and glucoamylase activity was assayed in 0.2 M Mcllvaine buffer (pH 3.8).

(A)

A. awamori var. *kawachi*



(B)

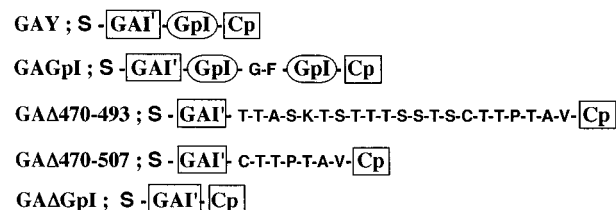


FIG. 2. Comparison of the Gp-I (GpI) domains in three *Aspergillus* glucoamylases (A) and schematic representation of various mutant glucoamylases (B).

General analytical procedures. The molecular masses of glucoamylases were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (19), with a high-molecular-mass standard mixture (SDS-6H; Sigma, St. Louis, Mo.) as molecular mass markers. Protein concentration was quantified from A_{280} . The total carbohydrate content of glucoamylases was measured by the phenol-sulfuric acid method (6).

RESULTS

Effect of glycosylation on secretion of various glucoamylase mutants. We constructed GAGpI, which has two tandemly repeated Gp-I domains, and mutant glucoamylases, which have a series of internal deletions within the Gp-I domain of the GAI molecule (Fig. 2). All glucoamylases used in this study possessed an extra serine residue at the amino terminus to adjust the sequence frame. In addition, GAGpI had extra glycine and phenylalanine residues between the two Gp-I regions, which were generated by ligation of two PCR products (products 2 and 3) at the *Bam*HI site.

GAY, GAGpI, and GAΔ470-493 gave broad bands for SDS-PAGE because of microheterogeneity due to glycosylation. It is known that heterologous expression of proteins in yeast cells results, in many cases, in hyperglycosylation of the proteins (16). GAY, for instance, contained double amounts of carbohydrates compared with GAI.

The molecular masses of GAY, GAGpI, and GAΔ470-493 were estimated to be 120, 135, and 85 kDa, respectively (Table 1). Since GAGpI has an extra 47 amino acids which correspond to a molecular mass of 5 kDa, the 15-kDa difference in molecular mass between GAGpI and GAY implies that the extra Gp-I region should contribute to the hyperglycosylation (up to 40.2%) in GAGpI.

These glucoamylases were expressed in yeast cells as precursor proteins, and mature glucoamylases were successfully secreted except for GAΔGpI and GAΔ470-507. Although GAΔGpI and GAΔ470-507 were not secreted in active forms

TABLE 1. Properties of glucoamylases from *A. awamori* var. *kawachi* and *S. cerevisiae*-expressed transformants^a

Glucoamylase or transformant	Mol wt	Carbohydrate content (%)	Activity for soluble starch (U/nmol)	Raw starch	
				Adsorbability (%)	Digestibility (mM/h) ^b
GAI	90,000	17.0	6.11 ± 0.05	77.0 ± 0.5	0.295 ± 0.010
GAY	120,000	34.2	6.24 ± 0.06	77.3 ± 0.4	0.450 ± 0.006
α-Mannosidase-treated GAY	110,000	20.4	6.19 ± 0.07	76.5 ± 0.5	0.268 ± 0.003
GAGpI	135,000	40.2	6.52 ± 0.09	74.9 ± 0.6	0.434 ± 0.011
α-Mannosidase-treated GAGpI	120,000	28.3	5.98 ± 0.06	74.3 ± 0.5	0.344 ± 0.011
GAΔ470–493	85,000	9.85	5.92 ± 0.06	72.2 ± 0.7	0.156 ± 0.011

^a All experiments were done in triplicate as described in text.

^b Raw starch digestibility represents an initial velocity of raw starch digestion by an enzyme, which was the slope of a line from 0 to 1 h taken from Fig. 3 and 4.

from yeast cells, they were identified in yeast cells by Western blot (immunoblot) analysis with anti-GAI antibody (data not shown).

Treatment of GAY and GAGpI with α-mannosidase resulted in decreases in their molecular masses to 110 and 120 kDa, respectively, which corresponded to the decreases in their carbohydrate contents to 20.4 and 28.3%, respectively.

Hydrolysis of soluble starch. The catalytic activities of GAY, GAGpI, and GAΔ470–493 for soluble starch were almost the same as that of GAI (Table 1). This result indicates that an extra Ser residue at the amino terminus in GAY, GAGpI, and GAΔ470–493 and extra Gly and Phe residues between the two Gp-I regions in GAGpI did not affect catalytic activity and that, more importantly, the Gp-I domain having carbohydrates was not essentially responsible for catalytic function.

Adsorption of glucoamylases to raw starch. The extent of adsorption of GAY, GAGpI, and GAΔ470–493 to raw starch was also comparable to that of GAI. Moreover, partial removal of carbohydrates, mainly mannoside chains O linked to the Gp-I domain by α-mannosidase, did not significantly affect either the catalytic activity for soluble starch or adsorption to raw starch. These results also indicate that the hypermannosylated Gp-I region is not involved in adsorption to raw starch.

Digestion of raw starch. When the same number of units of enzyme (10 U to soluble starch) was used, GAY digested raw cornstarch 1.5 times faster than did GAI (Table 1 and Fig. 3). GAGpI digested the same substrate as fast as GAY. GAΔ470–

493 digested raw cornstarch at a rate one-third that of GAY. On the other hand, the digestibilities of raw cornstarch by α-mannosidase-treated GAY and GAGpI were decreased to 59.2 and 77.6%, respectively, compared with those by the untreated enzymes (Table 1 and Fig. 4).

Effect of carbohydrate contents in the Gp-I region on thermal and pH stability. The carbohydrate contents of GAI, GAY, and GAGpI were 17.0, 34.2, and 40.2%, respectively. As shown in Fig. 5, thermal stability and pH stability of these enzymes increased as the carbohydrate contents increased. After inactivation at 50°C for 30 min, GAGpI retained full activity, but the activities of GAY and GAI decreased to 95 and 88%, respectively. Likewise, at 55°C, GAGpI retained 92% activity, but the activities of GAY and GAI decreased to 70 and 60%, respectively. The pH stability of GAGpI was also superior to those of GAI and GAY. For instance, GAGpI retained more than 90% activity, even at pH 1.5 or 10. The optimal temperature to hydrolyze soluble starch by GAI was 60°C, but the optimal temperature to hydrolyze soluble starch by GAY and GAGpI was 65°C (data not shown).

Raw starch digestion at higher temperature. Digestion of raw starch was performed at various temperatures at 30, 40, 50, and 60°C. The initial velocities of digestion of raw starch by GAY and GAGpI were 1.5 to 2.0 times faster than that by GAI at each temperature (Fig. 6). This result coincided well with thermal stability (Fig. 5), although all enzymes were completely denatured after 20 h of incubation at 60°C.

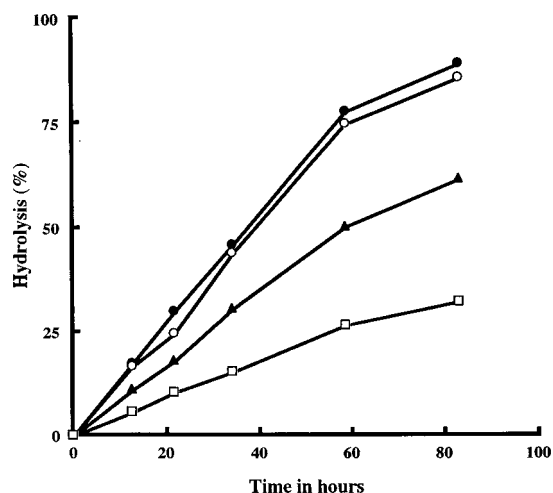


FIG. 3. Digestion of raw cornstarch by wild-type and mutant glucoamylases. The incubations were carried out at 30°C. ●, GAY; ○, GAGpI; ▲, GAI; □, GAΔ470–493.

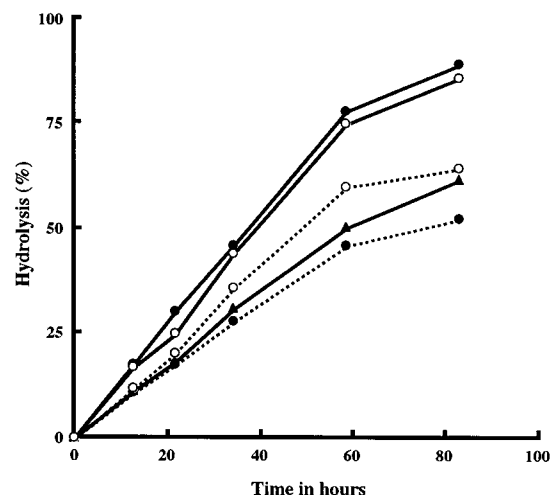


FIG. 4. Digestion of raw starch by α-mannosidase-treated glucoamylases. ● (solid line), GAY; ○ (solid line), GAGpI; ▲, GAI; ● (dashed line), α-mannosidase-treated GAY; ○ (dashed line), α-mannosidase-treated GAGpI.

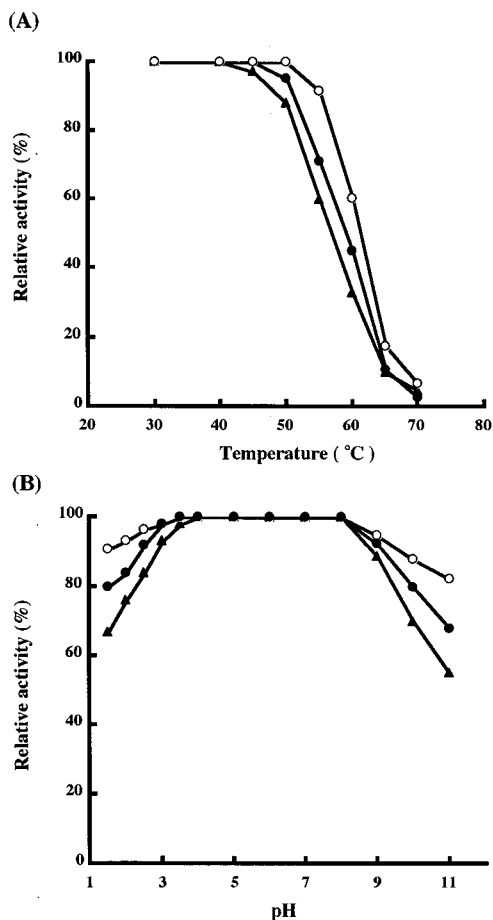


FIG. 5. Relative thermal (A) and pH (B) stabilities of glucoamylases. Thermal inactivation data were determined after 30 min of incubation. Stability in response to pH was determined at 4°C after 24 h of incubation. ●, GAY; ○, GAGpI; ▲, GAI.

DISCUSSION

As previously reported (14), the Gp-I region of *A. awamori* var. *kawachi* GAI has an unusual structure; 30 of 45 amino acids are composed of Thr (22 residues) and Ser (8 residues) residues O glycosylated with oligomannosaccharides. In this communication, we report the results of an investigation of the role of Gp-I within the GAI molecule in terms of raw starch digestibility, secretion, and enzyme stability.

GAY, GAGpI, having an extra Gp-I region, and GAΔ470-493, lacking 24 amino acids within the Gp-I domain, showed almost the same digestibility of soluble starch compared with fungal GAI. The raw starch-binding activities were also similar among them. However, GAY and GAGpI digested raw cornstarch 1.5 times faster than GAI. On the other hand, GAΔ470-493 digested the same substrate at a rate one-third that of GAY. The digestibilities of raw starch by GAY and GAGpI, when these enzymes were treated with α -mannosidase, decreased to 59.2 and 77.6%, respectively. These results indicate that the mannoside chains O linked to the Gp-I domain play an important role in efficient digestion of raw starch by GAI and thus support the previously proposed water-cluster-dissociating model: that is, the numerous mannoside chains, which are arranged in parallel and are extraordinarily close together in the Gp-I domain, would dissociate water clusters around starch micelles and promote the partial hydration of raw starch (6, 14).

Although the carbohydrate content of GAGpI reached as high as 40.2% when the Gp-I domain was tandemly introduced, the digestibility of raw cornstarch by GAGpI was not further enhanced compared with that of GAY. The possible explanations of this are as follows. (i) Additional hydration of raw starch by the extra Gp-I region of GAGpI is no more effective in raw starch digestion, since GAY is already hypermannosylated. (ii) As Williamson et al. reported, the Thr- and Ser-rich Gp-I domain has an extended conformation (21), making the distance between the catalytic domain and raw starch-binding domain of GAGpI along one compared with that in GAY. (iii) Alternatively, introduction of an extra Gp-I region may change the configuration of catalytic and raw starch-binding domains.

Other interesting features of hypermannosylated GAY and GAGpI are as follows. (i) The optimal temperature of GAY and GAGpI was increased to 65°C, compared with that of original fungal GAI (60°C). The higher optimal temperatures of these enzymes may be due to the protection of the active center by the increased oligomannosaccharides. (ii) Furthermore, GAGpI gained remarkable thermal and pH stability compared with fungal GAI and GAY. In this sense, GAGpI could be more advantageous in industrial use.

Although GAΔGpI and GAΔ470-507 were expressed by using a yeast secretion vector, YEUp3H α , which possesses a yeast-MF α signal sequence (18), they were not secreted. It is interesting to compare two glucoamylases from *Aspergillus oryzae* (AO-GAI) and *A. awamori* var. *kawachi*, focusing on the structure and function of their Gp-I domains. Although the catalytic and the raw starch-binding domains of these two glu-

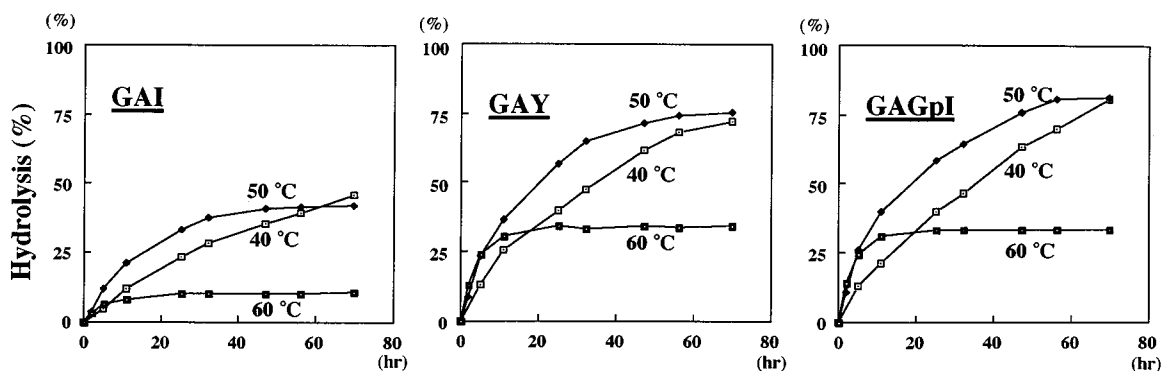


FIG. 6. Digestion of raw cornstarch by GAI, GAY, and GAGpI at higher temperatures.

coamylases share a high degree of similarity (74%) in their amino acid sequences, a remarkable discrepancy can be seen in the Gp-I regions. AO-GA1 has only 16 amino acids in the Gp-I domain, in which there are only 1 Thr and 2 Ser residues (Fig. 2A). However, AO-GA1 expressed in *S. cerevisiae* was secreted from yeast cells (10). Our study showed that GA Δ 470–493, which has 21 amino acids in the C-terminal half of the Gp-I domain, was successfully secreted, but GA Δ 470–507, which has 7 amino acids in the C-terminal region of the Gp-I domain, failed to be secreted. These data indicate that the C-terminal half of the Gp-I domain and an extended stretch between the catalytic and C-terminus binding domains are necessary for secretion of *Aspergillus* glucoamylase. Recent studies also postulated that correct folding of protein is important for transportation from endoplasmic reticulum membrane to endoplasmic reticulum lumen when the proteins are secreted properly (17). In GA Δ 470–507, there are only seven amino acids between the catalytic and binding domains, so that the distance between these two domains might be too short for them to be folded correctly, which may result in the failure of secretion of this protein. In this connection, it seems apparent that AO-GA1 has evolutionarily retained the minimum size (16 amino acids) of Gp-I domain which is functional for secretion. On the other hand, it is said that sugars are not essential for protein secretion (20), but they make protein separate from endoplasmic reticulum membrane, resulting in an increase in the secretion rate. Oligosaccharides bound to the Gp-I domain in GA1 and GA Δ 470–493 seem to play a similar role.

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