Expression and Functional Analysis of a Hyperglycosylated Glucoamylase in a Parental Host, Aspergillus awamori var. kawachi

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A modified glucoamylase gene (glaA) with an extra Thr- and Ser-rich Gp-I domain (T. Semimaru, M. Goto, K. Furukawa, and S. Hayashida, Appl. Environ. Microbiol. 61:2885–2890, 1995) was introduced into a mutant parental host, Aspergillus awamori var. kawachi, in which the original glaA gene had been completely deleted and replaced with the hygromycin phosphotransferase gene. The modified glaA was successfully expressed and secreted. The modified glucoamylase possessed higher digestibility of raw corn starch and higher stabilities in response to heat and extreme pH.

Filamentous fungi, Aspergillus strains are used in industrial enzyme production because of their superior ability to secrete proteins. Some of these strains are traditionally used for saccharification during sake and shochu brewing. Specifically, Aspergillus awamori var. kawachi is widely used for shochu making in Kyushu, Japan.

Glucoamylase I (GAI) of A. awamori var. kawachi is an extracellular enzyme and shows efficient saccharification of raw starch. GAI consists of three domains (1, 4, 6, 8): an amino-terminal catalytic domain covering amino acids 1 to 469, an O-glycosylated Thr- and Ser-rich Gp-I domain, including amino acids 470 to 514, and a carboxy-terminal raw starch-binding domain of amino acids 515 to 615. The functional analysis of the Gp-I domain expressed in Saccharomyces cerevisiae allowed us to conclude that it is involved in the secretion, enzyme stability, and digestion of raw starch (16). The tandem introduction of an extra Gp-I domain and the expression of the modified GAI (designated GAGpI) in S. cerevisiae resulted in further enhancement of the digestibility for raw starch. Moreover, GAGpI showed increased thermal and pH stabilities (16).

However, expression of the heterologous glaA gene in S. cerevisiae was much lower (less than 4 mg/liter of culture) than that in original Aspergillus host (130 mg/liter of culture), and the excreted GA was hypermannosylated (4, 16). Tanner and Lehle (17) reported that O-glycosylated proteins in S. cerevisiae possessed a linear carbohydrate chain of up to five mannose residues. On the other hand, Hayashida et al. (8) reported that in A. awamori var. kawachi, Thr and Ser residues were O glycosylated with an average of two mannoses. Thus, some structural and functional discrepancies can be seen between the GA expressed in the original Aspergillus host and that expressed in the Saccharomyces host.

In this report, we expressed GAGpI in the parental Aspergillus host and investigated the function of an extra Gp-I domain in comparison with the GAI and GAGpI expressed in the Saccharomyces host. For this purpose, a glaA disruptant of A. awamori var. kawachi was first constructed, and GAGpI was expressed in glaA mutant background.

A plasmid, pAN7.1, carrying the hygromycin phosphotransferase gene (hph) as a selection marker for fungi was kindly provided by Punt et al. (14). pBS-hph was obtained by cloning of a 1.0-kb EcoRI fragment and a 2.0-kb EcoRI-HindIII fragment containing an expression cassette of the hph gene from pAN7.1 into the EcoRI and HindIII sites of pBluescript-SKII (Stratagene, La Jolla, Calif.). pUC-PglaA was constructed by introduction of a 1.8-kb SalI fragment containing a promoter sequence of the glaA gene (9) into SalI- and XhoI-digested pUC119. pUC-TglaA was constructed by introduction of a 1.0-kb EcoRI-SalI fragment containing a terminator sequence of the glaA into pUC119. pHSG-PtglaA was obtained by introduction of 1.8- and 1.0-kb EcoRI-HindIII fragments containing the promoter and the terminator sequences from pUC-PglaA and pUC-TglaA, respectively, into the EcoRI site of pHSG396 (Takara Shuzo Co., Ltd., Kyoto, Japan). A vector for disruption of the endogenous glaA gene, pMG12, was constructed by introduction of a 3.5-kb BsiHI-HindIII fragment containing the hph gene into pHSG-PtglaA.

A plasmid, p3SR2, carrying the amdS gene involved in acetamide utilization as a selection marker for fungi was obtained from the Fungal Genetic Stock Center (University of Kansas Medical Center) (10). pHSG-amdS was constructed by insertion of a 5.2-kb EcoRI-SalI fragment containing the amdS gene from p3SR2 into the EcoRI and Xhol sites of pHSG396. pMG21 was obtained by insertion of 1.8- and 1.0-kb EcoRI-SalI fragments containing the promoter and the terminator sequences, respectively, from pUC-PglaA and pUC-TglaA into the EcoRI site of pHSG-amdS. A vector for expression of the modified glaA gene, pMG23, was constructed by introduction of a 1.7-kb SalI fragment containing the modified glaA cDNA from YEUp-GAGpI (16) into the SalI site of pMG21. A positive control vector for expression of the wild-type glaA cDNA, pMG22, was also constructed by the method described for pMG23.

Escherichia coli JM109 was used as a cloning host for propagation of plasmids. A. awamori var. kawachi was maintained on a slant of potato dextrose agar medium. Medium A was used for production of GA (7). For selection of transformants, Aspergillus minimal medium supplemented with 200 μg of hygromycin B per ml and acetamide medium, consisting of the Aspergillus minimal medium containing 20 mM acetamide and 15 mM CsCl, were used (18).

Preparation of protoplasts of A. awamori var. kawachi was

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basically performed according to the methods described by Gomi et al. (3) with a slight modification.

Transformation of \textit{A. awamori} var. \textit{kawachi} was performed according to the method described by Yelton et al. (22). Southern analysis of genomic DNA from the selected transformants was done with the DIG (digoxigenin) DNA labeling and detection kit according to the manufacturer’s recommendation (Boehringer Mannheim GmbH, Mannheim, Germany). Fungal DNA was prepared by the method described by Raeder and Broda (15).

Purification and assay of purified GAs were performed as previously described (5, 16). GA activity in the liquid culture was assayed by incubation of 0.5 ml of culture filtrate with 0.5 ml of 0.25 M McIlvaine buffer (pH 3.8) and 1 ml of 1% soluble starch at 50°C for 10 min. The amount of glucose in the reaction mixture was quantified with a glucose oxidase kit (Katayama Co., Ltd., Osaka, Japan) according to the protocol of the supplier.

Thermal and pH stabilities of enzymes were determined according to a method described previously (16).

The molecular mass of GA was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (12), with high-molecular-mass standard mixtures (SDS-6H; Sigma, St. Louis, Mo.) as molecular mass markers. The total carbohydrate content of GA was measured by the phenol-sulfuric acid method (2).

A \textit{glaA} disruptant of \textit{A. awamori} var. \textit{kawachi} was first constructed in order to express the modified \textit{glaA} gene coding for GAGpI, which contained tandemly repeated Gp-I domains. In order to replace the \textit{glaA} structural gene, the \textit{hph} gene was inserted between the 1.8-kb upstream (5′) and 1.0-kb downstream (3′) regions of the \textit{glaA} gene to construct pMG12. The long 5′ and 3′ flanking sequences of the \textit{hph} gene in pMG12 facilitate efficient targeting of homologous recombination with the original \textit{glaA} locus in the parental genome. A linearized form of pMG12 digested with \textit{KpnI} and \textit{DraI} was used for transformation of \textit{A. awamori} var. \textit{kawachi} with an efficiency of 4.4 transformants/µg of DNA. No transformant was obtained when the circular form was used. Southern blot analysis revealed that a DNA probe containing the terminator sequence of the \textit{glaA} gene was hybridized to a 5.4-kb EcoRI DNA fragment from the parental strain and a 2.5-kb EcoRI DNA fragment from the hygromycin B-resistant (HygB r) transformants.
2G1, 15A1, and 15B1 (Fig. 1). These results indicated that the glaA gene in strains 2G1, 15A1, and 15B1 was completely replaced with the hph gene expression cassette. Since the strains 2G1, 15A1, and 15B1 showed the same morphological and physiological properties, 2G1 was used for further study.

We could then successfully introduce the modified glaA gene into the glaA disruptant strain 2G1. In general, integration of foreign DNA in the genome of filamentous fungi occurs randomly by nonhomologous recombination (11). To achieve nonhomologous recombination, pMG22 carrying an expression cassette of the wild-type glaA gene and pMG23 carrying the modified glaA gene were constructed. These glaA genes were sandwiched by 5′ upstream and 3′ downstream regions of the original glaA structural gene. The transformants were selected by growth on acetamide as the sole source of nitrogen in the presence of CsCl due to the vector-borne amdS gene. Transformation efficiencies of strain 2G1 with pMG22 and pMG23 were as low as 1.2 and 1.5 transformant/μg of DNA, respectively. Integration of pMG22 and pMG23 was confirmed by Southern blot analysis with the glaA structural gene as the probe (Fig. 2). The 1.6-kb band corresponding to the cDNA for GAI was detected for the SacII-digested genomic DNA of 2G1(pMG22), and the 1.7-kb band of GAGpl cDNA was detected for those of 2G1(pMG23). The integration of the glaA gene in 2G1(pMG22) and 2G1(pMG23) seemed to be multiple, since a number of bands that hybridized with the glaA probe were always observed (Fig. 2), while only one band was observed for the parental strain.

GA secretion from strains 2G1(pMG22) and 2G1(pMG23) was detected in the culture medium (Fig. 3). In medium A containing 4% soluble potato starch, the secretion profile of GA by strain 2G1(pMG22) was almost the same as that of the parental strain. On the other hand, secretion of GAGpl by strain 2G1(pMG23) was lower than that of the parental strain. In the case of strain 2G1(pMG22), the wild-type glaA cDNA was also integrated randomly with more than 10 copies (Fig. 2). In spite of high numbers of copies of the genes, the secretion levels of GAI from 2G1(pMG22) and GAGpl from 2G1(pMG23) were a little lower than that of the parental strain. The reason for the low secretion levels in these transformants remains to be elucidated, but the cDNA introduced into the genome may be somewhat rearranged. Integration of the genomic glaA gene with multiple copies was also reported in Aspergillus niger (20), in which no rearrangement was observed. We cannot exclude the possibility that hyperglycosylation affects the secretion of GAGpl. The yield of GAGpl from strain 2G1(pMG23) was 85 mg/liter of culture. This value was 22 times higher than that of GAGpl secreted from the S. cerevisiae host (Table 1).

GAI and GAGpl expressed in the Aspergillus host contained 17 and 36.9% carbohydrates, respectively (Table 1). On the

### Table 1. Properties of GAs expressed in the original Aspergillus host and the Saccharomyces host

<table>
<thead>
<tr>
<th>GA</th>
<th>Mol mass (Da)</th>
<th>% Carbohydrate content</th>
<th>Activity for soluble starch (U/nmol)</th>
<th>% Raw starch adsorbability</th>
<th>Yield of GA (mg/liter of culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. awamori var. kawachi</strong></td>
<td></td>
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<td></td>
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<tr>
<td>GAI</td>
<td>90,000</td>
<td>17.0</td>
<td>6.1</td>
<td>77.0</td>
<td>130</td>
</tr>
<tr>
<td>GAGpl</td>
<td>130,000</td>
<td>36.9</td>
<td>6.2</td>
<td>73.7</td>
<td>85</td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAI</td>
<td>120,000</td>
<td>34.2</td>
<td>6.2</td>
<td>77.3</td>
<td>3.6</td>
</tr>
<tr>
<td>GAGpl</td>
<td>135,000</td>
<td>40.2</td>
<td>6.5</td>
<td>74.9</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Yield of GA was calculated from the activity in the supernatants of *A. awamori* var. *kawachi* and *S. cerevisiae* cultures.

* Properties of GA expressed in *S. cerevisiae* were cited in a previous paper (16).

* Raw starch adsorbability was determined in a reaction mixture containing 100 μg of GA per ml and 20% (wt/vol) raw corn starch.
other hand, the same enzymes expressed in the \textit{S. cerevisiae} host were hypermannosylated and contained much higher carbohydrate levels of 34.2 and 40.2\%, respectively (16). The molecular mass of GAGpI expressed in 2G1(pMG23) was 130 kDa, which was much higher than that of the original GAI (90 kDa). This can be explained by the introduction of an extra Gp-I domain containing 30 residues of O-glycosylated Ser and Thr out of a total of 45 amino acids. Catalytic activity of GAGpI toward soluble starch was comparable with that of the original GAI. The extent of adsorption of GAGpI toward raw corn starch was also comparable to that of the original GAI (Table 1). Thus, the extra Gp-I domain of GAGpI did not significantly affect both the catalytic activity for soluble starch and the capability of adsorption to raw starch. It should be noted that GAGpI expressed in the \textit{Aspergillus} host could digest raw corn starch 1.2 times faster than the original GAI (Fig. 4). However, the digestibility of GAGpI expressed in \textit{Aspergillus} was 80\% compared to that of GAGpI expressed in \textit{S. cerevisiae} (16). These results indicate that the Gp-I domain is involved in efficient digestion of raw starch and the content of mannose moieties O linked to the Gp-I region is correlated in response to heat and extreme pH. However, \textit{Aspergillus} GAGpI showed less enzyme stability than \textit{S. cerevisiae} GAGpI, probably because of the difference of hyperglycosylation.

Generally, glycosylation acts to aid the folding of the nascent polypeptide chain and to stabilize the conformation of the mature glycoprotein (13). Wang et al. (21) reported that the destabilization effect of deglycosylation seemed to depend on the carbohydrate content, irrespective of the types (N linked or O linked) or patterns (mono- or multibranched) of the covalently attached carbohydrate chains. Our study has provided additional evidence concerning the effect of glycosylation on enzymatic stability as well as the enzymatic functions of the \textit{Aspergillus} GA. This information will be useful for improving GA for industrial uses.

REFERENCES


