The Hyperdigestion of Raw Starch by a Carbohydrate-Rich Glucoamylase from a Protease- and Glycosidase-Negative Mutant of *Aspergillus awamori* var. *kawachi* F-2035

Kohsai FUKUDA,† Yuji TERAMOTO,† and Shinsaku HAYASHIDA*

Department of Agricultural Chemistry, Kyushu University, Fukuoka 812, Japan

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A protease- and glycosidase-negative mutant F-2035, of *Aspergillus awamori* var. *kawachi* has been isolated that produces a glucoamylase that contains a large carbohydrate moiety and has enhanced ability to digest raw starch (GA MU-H; MW, 110,000). This enzyme digested raw corn starch 2.5 times faster than did the parental glucoamylase I (GA I; MW, 90,000). When grown from an enriched seed culture, this mutant also produced a glucoamylase with less ability to digest raw starch (GA MU-L; MW, 110,000). Its activity was 25% of that of GA MU-H with raw starch. Both GA MU-H and GA MU-L proved to be identical to GA I in terms of adsorption to raw starch, molar activity against gelatinized starch, amino acid composition, and terminal amino acid sequence. The carbohydrate contents of GA I, GA MU-H, and GA MU-L were 17%, 33%, and 33% by weight, respectively. The carbohydrates of GA I and GA MU-H were mostly mannose, but that of GA MU-L was composed of mannose (71%) and glucose (26%). Partial removal of the carbohydrate from GA I and GA MU-H by *Turbo* glycosidase caused a parallel decrease in the ability to digest raw starch. Thus, the carbohydrate moiety of the glucoamylase molecule, in particular the mannose residues, appears to be important in the digestion of raw starch, and promote the hydration of micelles of raw starch but not the actual adsorption of the enzyme to raw starch.

*Aspergillus awamori* var. *kawachi* produces three types of glucoamylase:1) raw starch-adsorbing and raw starch-digesting GA I (MW, 90,000; type A), raw starch-nonadsorbing, and raw starch-nondigesting GA I (MW, 73,000; type B), and GA II (MW, 57,000; type C). The multiplicity of forms of the enzyme and the specific changes in ability to digest raw starch have been ascribed to the stepwise degradation of the original GA I by protease and glycosidases.2) The ability to digest raw starch requires a specific site of affinity for raw starch in addition to the active site on the GA I molecule.3) Cleavage of the site of affinity for raw starch by the bacterial protease subtilisin leads to the formation of glycopeptide I (GP-I; MW, 13,200) plus raw starch-nonadsorbing, and raw starch-nondigesting GA I.3–6)

This report describes the production of an intact glucoamylase that hyperdigests raw starch. The importance of the amount and composition of the carbohydrate bound to glucoamylase for the digestion of raw starch is demonstrated, as shown with use of a novel protease-negative, glycosidase-negative mutant F-2035. The properties of the glucoamylases produced by the mutant are also described.

**Materials and Methods**

*Measurement of enzymatic activities.* Glucoamylase activity was measured with gelatinized potato starch as the substrate.1) Reducing sugars released were assayed by the DNS (3,5-dinitrosalicylic acid) method.7) Molar activities were calculated using the molecular weight of the enzymes. The ability to digest raw starch was measured as described in a previous paper.1) The reaction mixture contained 12.5 mg of raw corn starch, 1.5 ml of deionized water, 0.25 ml of 0.1 M citrate buffer (pH 3.6), and 0.25 ml of a solution of glucoamylase (11.3 units) plus 0.1 ml of toluene, and reactions were incubated at 30°C for 10 min. Protease activity was assayed using casein as substrate.5) p-Nitrophenyl-α-mannoside and p-nitrophenyl-N-acetyl-β-D-glucosaminide were used as substrates for assays of α-mannosidase8) and N-acetyl-β-D-glucosaminidase,9) respectively.

*Induction and isolation of mutants.* *Aspergillus awamori* var. *kawachi* was used as the parental strain, and cells were maintained on a slant culture of potato dextrose agar medium (potato, 300 g; glucose, 20 g; agar, 20 g; and tap water, 1,000 ml) at 30°C for 10 days. Spores were collected and suspended in sterile water (not less than 106 spores/ml). Ten ml of the suspension of spores were treated with 10 ml of a freshly prepared solution of N-methyl-N-nitro-N-nitrosoguanidine (MNNG) at a concentration of 4 mg/ml in 0.1 M acetic acid buffer (pH 4.5) for 20 min, and then the mixture was centrifuged for 5 min at 12,000 g to remove the supernatant. The sedimented spores were washed once with sterile water and then inoculated into 100 ml of minimal medium (glucose, 50 g; NaNO3, 3 g; KH2PO4, 1 g; KCl, 0.5 g; MgSO4·7H2O, 0.02 g; FeSO4·7H2O, 0.01 g; and deionized water, 1,000 ml). The culture was then shaken at 30°C for 36 hr. The growing mycelia were separated by filtration through sterile glass wool. The filtrate was plated onto complete medium (glucose, 10 g; peptone, 5 g; yeast extract, 5 g; malt extract, 3 g; KH2PO4, 1 g; agar, 20 g; and deionized water, 1,000 ml), and then incubated at 30°C for 7 to 10 days. Colonies were transferred to slants of the complete medium and were further incubated at 30°C for 7 to 10 days. Several transfers were done to test the stability of the mutants. The selected mutant strains were plated individually onto casein medium (casein, 5 g; casamino acid, 0.05 g; Na2HPO4, 1.07 g; KH2PO4, 0.36 g; MgSO4·7H2O, 0.5 g; NaCl, 0.1 g; ZnCl2, 0.14 g; CaCl2, 2 mg; FeSO4·7H2O, 2 mg; agar, 20 g; and deionized water, 1,000 ml) for the primary screening of production of protease. Mutants with significantly smaller halos (clear zones) than those of the parental strain were selected and cultured on potato dextrose agar medium that contained 0.1% yeast extract and 0.1% casamino acid. Spores of the selected mutants were inoculated into liquid synthetic medium A (potato starch, 40 g; glucose, 1 g; ammonium citrate, 10 g; KH2PO4, 3 g; MgSO4·7H2O, 1 g; NaCl, 0.1 g; FeSO4·7H2O, 0.1 g; ZnCl2, 10 μg; MnSO4·7H2O, 500 μg; CoSO4·7H2O, 10 μg; CuSO4·7H2O, 10 μg; and deionized water, 1,000 ml), and shaken at 30°C for 4 days. Protease and
glycosidase activities of the culture filtrate were measured by the above mentioned procedures. The selected mutants were maintained on potato dextrose agar medium that contained 0.1% yeast extract and 0.1% casamino acids. When the mutant was used for production of enzyme, the spores were incubated in the minimal medium at 30°C for 36 hr and the filtrate, after passage through glass wool, was used as the starting inoculum.

Production and purification of parental and mutant glucoamylases. For the seed culture, the parental and mutant strains were cultivated in synthetic medium A and incubated at 30°C for 4 days on a reciprocal shaker. The main culture was done in medium A, with incubation at 30°C for 30 hr in a 200-liters stainless-steel fermentor (L.E. Marubishi Co., Ltd.). The pH of the culture was maintained above 5.5 by the addition of calcium carbonate, Gefäll (ART 2006; Merck Co., Ltd., Germany). The culture filtrates were used as crude enzyme for purification. After salting out by ammonium sulfate (60%, w/v), which was followed by treatment with acid (pH 2.0), alkali (pH 9.0), and rivanol, the crude enzyme was fractionated by chromatography on DEAE-Sephadex A-50 and Sephadex G-50 as described in a previous paper.15 The glucoamylases were finally purified by HPLC (Tri-Rotar-V model; JASCO Co., Ltd.) with a column of TSK Gel 3000SW (Tosoh Co., Ltd.).

Production and purification of GA MU-L from mutant F-2035. For the production of GA MU-L, an enriched medium, which was composed of potato starch, 40 g; glucose, 1 g; ammonium citrate, 10 g; KH₂PO₄, 3 g; MgSO₄·7H₂O, 1 g; NaCl, 0.1 g; FeSO₄·7H₂O, 0.1 g; ZnCl₂, 10 µg; MnSO₄·7H₂O, 500 µg; CoSO₄·7H₂O, 10 µg; CuSO₄·7H₂O, 10 µg; yeast extract, 5 g; casamino acid (Difco Co., Ltd.) 5 g; and deionized water, 1,000 ml, was used for the seed culture. The main culture was incubated in synthetic medium A at 30°C for 30 hr in a 200-liters stainless-steel fermentor. The pH of the culture was maintained above 5.5 by the addition of calcium carbonate. Gefäll. Purification was done as described above.

Measurement of molecular weight. The molecular weight was measured by SDS-PAGE on 5% acrylamide gels by the method of Weber and Osborn.16

Carbohydrate analysis. The total carbohydrate content of glucoamylases was measured by the phenol-sulfuric acid method.17 The carbohydrate composition of each enzyme was measured by gas-liquid chromatography (Hitachi model 163) as previously described.46

Protein measurement. Protein was measured by the method of Lowry et al.12 using the Bio-Rad Protein assay kit (Bio-Rad Laboratories), with bovine serum albumin as the standard.

Partial removal of the carbohydrate moiety of glucoamylase. Purified glucoamylase (60 mg) was digested with 6 mg of a protease-free glucoamylase from *Turbo cornutus* (Seikagaku Kogyo Co., Ltd.) in 0.2 M citrate buffer (pH 4.0) at 32°C for 48 hr. The digested enzyme was put on a column of DEAE-Sephadex A-50, and then eluted with a linear gradient of 0–0.6 M NaCl. The second digestion of the glucoamylase by *Turbo* glucoamylase was done by repeating this procedure.

Amino acid analysis. Glucoamylase was hydrolyzed with 6 N HCl (Ishizu Pharmaceutical Co., Ltd.) at 110°C for 24 hr, and the amino acid composition of the hydrolysate was analyzed with a Hitachi Model 638-30 amino acid analyzing system by the procedure of Spackman.13

Analysis of terminal amino acids. The N-terminal amino acid was identified on a protein sequencer (Beckman model 890 M/E) and analyzed by System Gold (Beckman Co., Ltd.). The C-terminal amino acid was identified after digestion with carboxypeptidase Y (Seikagaku Kogyo Co., Ltd.). Experimental details can be found in an earlier paper.41

Immunoprecipitation test. Antiserum against the purified GA I was prepared by immunizing a rabbit with three intradermal injections of a mixture of 1 mg of GA I and 1 ml of Freund’s complete adjuvant (Difco Co., Ltd.) at weekly intervals. After 4 weeks, 1 ml of phosphate-buffered saline containing 1 mg of GA I was injected into the animal by the intravenous route. The rabbit was bled one week later, and the serum was collected by centrifugation and stored at -20°C. The double-immunodiffusion test was done by the method of Langone.44

Results

Selection of mutants

*Aspergillus awamori* var. *kawachi* was used as the parental strain. Twenty-two colonies with smaller halos on the casein medium plates were initially selected from about 2,000 colonies that had grown less in minimal medium for 36 hr. The selected mutants were cultivated on synthetic medium A at 30°C for 4 days.

The mutant F-2035 was finally selected. As shown in Table I, no protease or mannosidase activities were detected in the culture filtrate of mutant F-2035 after a 4-day culture. Most of the N-acetyl-β-D-glucosaminidase activity disappeared as the result of mutation. The mutant F-2035 produced twice as much glucoamylase activity as the
parental strain. The spores of mutant F-2035 were denser, and blacker, and the sporulating activity was slightly lower than that of the parental strain.

Production and purification of two glucoamylases that differ in their abilities to digest raw starch from mutant F-2035

The parental strain and mutant F-2035 were cultivated in synthetic medium A to produce both a seed culture and a main culture in a 200-liters fermentor, as shown in Fig. 1. The glucoamylase from mutant F-2035 was purified, as GA MU-H (Table II), to homogeneity as assessed by SDS-PAGE and HPLC. Mutant F-2035 was also cultivated in enriched medium for a seed culture with a subsequent main culture in synthetic medium A. In this case, the total activity and specific activity of the culture filtrate and the purified enzyme were 2 × 10⁶ units and 30 units/mg protein, and 1.7 × 10⁵ units and 140 units/mg protein, respectively. The glucoamylase (GA MU-L) was purified in the same manner to homogeneity as assessed by SDS-PAGE and HPLC. These glucoamylases showed a difference in their abilities to digest raw starch, the former having higher and the latter having lower ability than the parental GA I. These isozymes were, therefore, designated as GA MU-H and GA MU-L, respectively.

The ability of digest raw starch of GA I, GA MU-H, and GA MU-L are shown in Fig. 2. With adjustments to a common unit of activity against gelatinized potato starch (11.3 units), GA MU-H digested raw corn starch to an extent of hydrolysis of 46%, while GA I and GA MU-L digested it only to 18% and 12%, respectively, in 18 hr at 30°C. However, these three glucoamylases had the same abilities to adsorb to raw starch (0.9 units/mg raw corn starch). Furthermore, GA I and GA MU-L also digested raw corn starch to nearly 100% under other conditions with higher levels of glucoamylase activity (data not shown).

Comparison of properties between parental and mutant glucoamylases

i) Molecular weight. The molecular weights of the parent GA I, mutant GA MU-H, and GA MU-L were estimated to be 90,000, 110,000, and 110,000, respectively, by SDS-PAGE.

ii) Carbohydrate content and composition. The total carbohydrate contents of GA I, GA MU-H, and GA MU-L were estimated to be 17%, 33%, and 33%. As shown in Table III, more than 90% of the total carbohydrate of GA I and GA MU-H was composed of mannose residues (79 mol and 196 mol per mol, respectively). Thus, the increase in carbohydrate contents was responsible for the increase in molecular weight. The carbohydrate of GA MU-L was found to be different from that of GA I and GA MU-H, and it was composed of both mannose (71%) and glucose (26%).

iii) Amino acid composition. Amino acid compositions of GA I, GA MU-H, and GA MU-L were completely identical, as shown in Table IV.

iv) Analyses of terminal amino acids. N- and C-terminal amino acids of GA I, GA MU-H, and GA MU-L were proved to be identical and to be Ala and Arg, respectively.

v) Thermal stabilities. GA MU-H was more stable to

Table I. Enzymatic Activities of the Parent Strain and the Mutant F-2035

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucoamylase (units/ml)</th>
<th>Protease (units/ml)</th>
<th>Glicosidase (units/ml)</th>
<th>Glucosease</th>
<th>α-Mannosidase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pH 3</td>
<td>pH 7</td>
<td>pH 9</td>
<td>Glc-</td>
<td>NAcase</td>
</tr>
<tr>
<td>Parent</td>
<td>35</td>
<td>10</td>
<td>20</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Mutant</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>F-2035</td>
<td></td>
<td></td>
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</table>

A. awamori var. kawachi and mutant F-2035 were cultivated in 100 ml of synthetic medium A13 in Sakaguchi flasks at 30°C for 4 days on a reciprocal shaker and the enzymatic activities of the culture filtrates were measured.

* N-Acetyl-β-D-glucosaminidase.

![Fig. 2. Courses of Digestion of Raw Starch by GA I, GA MU-H, and GA MU-L.](image)

Symbols: GA I (○), GA MU-H (●), GA MU-L (◆). The experimental details are described in the text.
Role of Carbohydrate in Digestion of Raw Starch by Glucoamylase

Table IV. Amino Acid Compositions of Parental and Mutant Glucoamylases

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>GA I</th>
<th>GA MU-H</th>
<th>GA MU-L</th>
<th>GA I</th>
<th>GA MU-H</th>
<th>GA MU-L</th>
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<tbody>
<tr>
<td></td>
<td>(10^{-4} nmol)</td>
<td></td>
<td></td>
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<tr>
<td>Asx</td>
<td>49.27</td>
<td>41.08</td>
<td>43.08</td>
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<td>Thr</td>
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<td>46.10</td>
<td>49.13</td>
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<td>28.42</td>
<td>29.44</td>
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<td>45</td>
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<tr>
<td>Pro</td>
<td>16.65</td>
<td>14.02</td>
<td>14.25</td>
<td>22</td>
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<tr>
<td>Gly</td>
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<td>31.40</td>
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<tr>
<td>Ala</td>
<td>42.69</td>
<td>36.60</td>
<td>37.99</td>
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<tr>
<td>Leu</td>
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<td>26.07</td>
<td>27.98</td>
<td>42</td>
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<tr>
<td>Tyr</td>
<td>20.34</td>
<td>16.68</td>
<td>18.07</td>
<td>27</td>
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<td>27</td>
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<tr>
<td>Phe</td>
<td>15.16</td>
<td>12.81</td>
<td>13.44</td>
<td>20</td>
<td>20</td>
<td>20</td>
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<tr>
<td>Lys</td>
<td>9.23</td>
<td>7.81</td>
<td>7.70</td>
<td>12</td>
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</tr>
<tr>
<td>His</td>
<td>3.37</td>
<td>2.90</td>
<td>3.53</td>
<td>5</td>
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<td>5</td>
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<tr>
<td>Arg</td>
<td>14.43</td>
<td>11.69</td>
<td>12.76</td>
<td>19</td>
<td>19</td>
<td>19</td>
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<tr>
<td>Total</td>
<td>435.39</td>
<td>367.89</td>
<td>387.42</td>
<td>587</td>
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<td>587</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of Partial Removal of Carbohydrate from GA I and GA MU-H on Their Ability to Digest Raw Starch.
Symbols: GA I (○), GA MU-H (●), glycosidase-digested GA I (△), glycosidase-digested GA MU-H (■). Details of experimental conditions are in the text.

Fig. 4. Double-immunodiffusion Patterns of GA I and GA MU-H with Antiserum against GA I.
Symbols: antiserum against GA I (A), GA I (B), GA MU-H (C).

heating at a high temperature than GA I. About 90% of the activity of GA MU-H was destroyed by heating at 75°C for 15 min, while GA I completely lost its activity after heating at 75°C for 15 min.

vi) Molar activity. The solutions of the same molarity (1 × 10^{-5} M) of GA I, GA MU-H, and GA MU-L had almost the same glucoamylase activities (140 units/ml) against gelatinized potato starch.

Effects of the partial removal of carbohydrate on the ability to digest raw starch

The digestion by glycosidase of GA I and GA MU-H decreased their carbohydrate contents from 17% to 12% and from 33% to 22%, respectively. These glycosidase-digested enzymes had 40% lower abilities to digest raw starch than native GA I and GA MU-H. The twice glycosidase-digested GA MU-H gave the same carbohydrate content (17%) and the same ability to digest raw starch as those of GA I (Fig. 3). These three glycosidase-digested glucoamylases had the same N- and C-terminal amino acids. The ability to digest raw starch, therefore, decreased in parallel with the removal of carbohydrate from the glucoamylase molecules.

Immunological comparison

As shown in Fig. 4, GA MU-H formed a precipitate with the antiserum against GA I. No spur of precipitate was observed. These results indicate that the surface structures of the GA I and GA MU-H molecules are similar.

Discussion

To elucidate the mechanism responsible for the occurrence of multiple types of glucoamylase, a protease-negative, glycosidase-negative mutant F-2035 from Aspergillus awamori var. kawachi was obtained as a producer of intact glucoamylase. Differing from the parental strain, this mutant did not produce multiple glucoamylases, but it produced a glucoamylase GA MU-H, which had an elevated ability to digest raw starch. GA MU-H digested raw starch 2.5 times more effectively and had a carbohydrate content that was 2 times greater than the parental GA I,
although this GA MU-H was identical to GA I as far as molar activity against gelatinized potato starch was concerned and as far as the adsorption to raw starch granules was concerned.

When an enriched medium for the seed culture was used, this mutant produced a glucoamylase with lower ability to digest raw starch. In this case, the composition of the carbohydrate moiety of the glucoamylase was found to have been modified by the culture conditions. This enzyme contained 26% glucose residues in its carbohydrate moiety, and it had only 25% of the ability to digest raw starch of GA MU-H. However, it was identical to GA MU-H in terms of total carbohydrate content, molecular weight, amino acid composition, terminal amino acids, molar activity, and ability to adsorb to raw starch. The partial conversion of mannose residues to glucose in the carbohydrate moiety led to a significant decrease in the ability to digest raw starch.

Removal of 30% of the carbohydrate from GA I and GA MU-H by glycosidase decreased the ability to digest raw starch by 40%. In addition, the twice glycosidase-digested GA MU-H and the intact GA I, which contained the same amount of carbohydrate, both had the same ability to digest raw starch.

All the data clearly show that the content and composition of the carbohydrate moiety, in particular the mannose residues, are important in the digestion of raw starch by glucoamylase. As we have reported previously, the Ga-I region of the site of affinity for raw starch was rich in mannose residues, and they promote the digestion of raw starch by GA I. The hydration of starch micelles could be promoted by dissociation of the water cluster, and a hydrated starch micelle could become suitable for hydrolysis at the active site of glucoamylase. This paper supports the previously described model of a "water-cluster-dissociating model of the Ga-I region" for the digestion of raw starch by glucoamylase.

References