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Purification and Characterization of α-Glucosidase I from Japanese Honeybee (*Apis cerana japonica*) and Molecular Cloning of Its cDNA

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α-Glucosidase (JHGase I) was purified from a Japanese subspecies of eastern honeybee (*Apis cerana japonica*) as an electrophoretically homogeneous protein. Enzyme activity of the crude extract was mainly separated into two fractions (component I and II) by salting-out chromatography. JHGase I was isolated from component I by further purification procedure using CM-Toyopearl 650M and Sephacryl S-100. JHGase I was a monomeric glycoprotein (containing 15% carbohydrate), of which the molecular weight was 82,000. Enzyme displayed the highest activity at pH 5.0, and was stable up to 40 °C and in a pH-range of 4.5–10.5. JHGase I showed unusual kinetic features: the negative cooperative behavior on the intrinsic reaction on cleavage of sucrose, maltose, and *p*-nitrophenyl α-glucoside, and the positive cooperative behavior on turanose. We isolated cDNA (1,930 bp) of JHGase I, of which the deduced amino-acid sequence (577 residues) confirmed that JHGase I was a member of α-amylase family enzymes. Western honeybees (*Apis mellifera*) had three α-glucosidase isoenzymes (WHGase I, II, and III), in which JHGase I was considered to correspond to WHGase I.

Key words: Japanese honeybee α-glucosidase; honeybee isoenzymes; western honeybee α-glucosidase; allosteric enzyme

α-Glucosidase (EC 3.2.1.20, α-glucoside glucohydrolase) is an exo-carbohydrase that hydrolyzes the non-reducing terminal α-glucosidic bond and releases α-glucose from the substrate.[1,2] In the high concentrations of substrate, the enzyme also catalyzes the transferring reaction to form oligosaccharides. α-Glucosidase is widely distributed in microorganisms, plants, mammals, and insects. On the basis of substrate specificity, the enzymes are classified into three groups (type I, II, and III).[1] Type I α-glucosidase hydrolyzes heteroside substrate (sucrose and aryl α-glucoside) more rapidly than holoside substrate (maltooligosaccharides). Type II and type III enzymes prefer holoside substrate to heteroside substrate. Type III is capable of attacking polysaccharide substrate (soluble starch). According to the classification of glycoside hydrolase family (GH),[3] α-glucosidase is classified mainly into two GH groups, GH 13 and 31. Type I α-glucosidase is a member of GH 13, and type II and type III enzymes are members of GH 31.[2,4]

We purified three α-glucosidases from western honeybee (*Apis mellifera* L.), WHGase I, II, and III.[5,6] Three α-glucosidases were different in molecular size, pH-stability, temperature-stability, substrate specificity, and localization in organ.[5–10] They were glycoproteins containing sugar content of 25% (WHGase I), 15% (WHGase II), and 7.4% (WHGase III), which might

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Abbreviations: $\overline{DP}$, average degree of polymerization of glucose; GH, glycoside hydrolase family; JHGase I, Japanese honeybee α-glucosidase isoenzyme I; PAGE, polyacrylamide gel electrophoresis; *p*NPG, *p*-nitrophenyl α-glucoside; RACE, rapid amplification of cDNA end; WHGase I, WHGase II, and WHGase III, western honeybee α-glucosidase isoenzyme I, II, and III, respectively

affect solubility in ammonium sulfate separation of the purification procedure. WHGase I and II are interesting enzymes to demonstrate allosteric properties in the catalytic reaction, which are dependent on the kind of substrate. WHGase I displayed the negative cooperativity to maltose, sucrose, and aryl α-glucoside (pheny α-glucoside and *p*-nitropheny α-glucoside, *p*NPG) and the positive cooperativity to turanose and maltodextrin ($\overline{DP} = 13$),[8] while WHGase II displayed only the positive cooperativity to sucrose, turanose, kojibiose, and soluble starch.[9] WHGase III was not an allosteric enzyme showing a normal Michaelis-type reaction.[6] Recently, it was found that WHGase I, II, and III were isoenzymes having different amino-acid sequences (M. Nishimoto and A. Kimura, unpublished results). It is of interest to elucidate the molecular mechanism of allosteric properties demonstrated by WHGase I and II. However, no other α-glucosidases exhibit such unusual reactions. It is necessary to learn the structure of the same allosteric α-glucosidase from different origin.

Immunological investigation using specific antisera against α-glucosidases revealed that WHGase I, II and III were located different organs, such as in the midgut (WHGase I and II), in the haemolymph (WHGase II), and in the hypopharyngeal gland (WHGase III).[10] Kubo *et al.* reported an α-glucosidase expressed in the hypopharyngeal gland of western honeybee[11] and succeeded in the isolation of its cDNA.[12] Therefore, the enzyme found by Kubo *et al.* is considered to correspond to WHGase III. We purified an α-glucosidase from honey.[10] The properties of honey enzyme coincided completely with WHGase III, indicating a significant physiological role of WHGase III in the honey-formation process. WHGase III, secreted from hypopharyngeal gland to the nectar gathered by honeybees, was responsible for converting sucrose (main carbohydrate in nectar) to glucose and fructose (main components in honey). It is expected that the similar isoenzyme-system is also present in other honeybee species.

Western honeybee (*A. mellifera*, utilized mainly in apiculture) originates in Europe and Africa, while other species originate in the Asian area.[13,14] The eastern honeybee (*Apis cerana*) ranges widely in Asia, including Japan. In the present paper, we describe the enzymatic properties and the substrate specificity of α-glucosidase I (JHGase I) isolated from a Japanese subspecies of the eastern honeybee (*A. cerana japonica*; we describe "Japanese honeybee" in this paper) to display allosteric kinetics. In addition, cDNA encoding JHGase I was cloned. The deduced amino-acid sequence was compared with that of western honeybee α-glucosidase isoenzyme I.

Materials and Methods

Honeybees and bacterial strains. Japanese honeybees were collected in Kaneyama-cho (Fukushima Prefecture, Japan) in November 2001. The bees were immediately frozen with liquid nitrogen and stored at $-80\,^{\circ}C$ until use. *Escherichia coli* DH5α (Stratagene, La Jolla, CA) was used for plasmid construction.

Chemicals. Maltose (SP-grade), *p*NPG, and soluble starch were purchased from Nacalai Tesque (Kyoto, Japan); sucrose, from Wako Pure Chemical (Osaka, Japan); isomaltose, from Hayashibara Biochemicals (Okayama, Japan); turanose, from Sigma (St. Louis, MO). Malto-triose, -tetraose, and -pentaose were kindly supplied by Nihon Shokuhin Kako (Tokyo, Japan). To remove possible impurities, maltose and sucrose were further purified though repeated recrystallizations, and soluble starch was washed with ice-cold water. Nigerose and kojibiose were synthesized by buckwheat α-glucosidase-catalyzed transglucosylation.[15]

Assay of enzyme activity. The reaction mixture containing 0.2 ml of 0.5% maltose (or 0.5% isomaltose), 0.2 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.1 ml of enzyme solution was incubated at 37 °C. The reaction was stopped by adding 1 ml of 2 M Tris–HCl (pH 7.0). Glucose liberated from substrate was determined by the glucose oxidase-peroxidase method modified by us.[8] One unit of maltase activity (or isomaltase activity) was defined as the amount of enzyme hydrolyzing 1 μmole of substrate per min under the conditions described above.

Analytical methods. The concentration of JHGase I was determined spectrophotometrically using $E^{1\%}_{1\,cm,\,280\,nm}$ of 5.5, which was obtained from the relationship between dry weight and absorbance at 280 nm of the purified enzyme preparation.[16] The carbohydrate content of JHGase I (protein used, 93 μg) was estimated as mannose or glucose by the phenol-sulfuric acid method.[17] Polyacrylamide gel electrophoresis (native-PAGE) and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 8.0% separating gel were performed according to our previous paper,[5] basically followed by the method of Reisfied *et al.*[18] JHGase I of 5 μg and 2.5 μg was applied for native-PAGE and SDS–PAGE, respectively, using Mini-protean III (Bio-Rad, Richmond, CA). Protein was stained by Rapid Kanto CBB (Kanto Kagaku, Tokyo, Japan). After native-PAGE, activity staining was done by immersing the gel in 0.2% 4-methylumbelliferyl α-glucoside (Nacalai Tesque) containing 0.1 M sodium acetate buffer (pH 5.0), followed by the detection of 4-methylumbelliferone with a UV transilluminator. For deglycosylation of JHGase I, the purified enzyme (2.5 μg) was treated with endoglycosidase H (5 mU; Roche Diagnostics, Indianapolis, IN) at pH 5.5 and 37 °C for 24 h, and then subjected to SDS–PAGE for analysis of molecular weight.

Effects of pH and temperature.

pH-Activity. The reaction mixture containing 0.05 ml of purified JHGase I (0.56 μg), 0.05 ml of 2% maltose, and 0.4 ml of Briton-Robinson buffer (pH 2.5–13; pH of the acid mixture consisting of 40 mM acetic acid, 40 mM phosphoric acid, and 40 mM glycine was adjusted with sodium hydroxide) was incubated at 37 °C for 10 min.

pH-Stability. The purified enzyme was diluted with Briton-Robinson buffer (pH 2.5 to 13), followed by the treatment at 4 °C for 24 h. The mixture (0.1 ml containing 0.56 μg of JHGase I) was taken and incubated with 0.05 ml of 2% maltose and 0.35 ml of 1 M sodium acetate buffer (pH 5.0) at 37 °C for 10 min. The residual α-glucosidase activity was determined by measuring glucose.

Thermal stability. After 0.1 ml of JHGase I (1.1 μg) in 10 mM sodium acetate buffer (pH 5.5) was treated at 4–70 °C for 15 min, 0.05 ml of the enzyme solution was incubated with 0.1 ml of 0.1 M sodium acetate buffer (pH 5.5) and 0.1 ml of 0.5% maltose at 37 °C for 10 min to estimate the residual activity.

Kinetic studies. The reaction mixture composed of 0.02 ml of substrate solution, 0.02 ml of 0.05 M sodium acetate buffer (pH 5.0), and 0.01 ml of purified JHGase I (0.04 to 0.5 μg) was incubated at 37 °C. The ranges of substrate concentration (s) were 0.5–10.0 mM for maltose, 0.25–6.0 mM for maltotriose, 0.25–4.0 mM for maltotetraose, 1.0–10.0 mM for maltopentaose, 0.1–8.0 mM for kojibiose, 0.1–3.0 mM for *p*NPG, 0.25–15.0 mM for sucrose, and 2.0–20 mM for turanose. The initial velocity (v) was expressed as mg glucose liberated from substrate per min per mg of protein (for kojibiose, glucose from the non-reducing end of substrate). Measurement of *p*-nitrophenol from *p*NPG was performed by the method described in our previous paper.[5] Glucose and fructose from sucrose were assayed with F-Kit D-Glucose/D-Fructose (R-Biopharm AG, Darmstadt, Germany). The molecular activity (k_0) and Michaelis–Menten constant (K_m) were estimated from the relationship between s and v using Lineweaver–Burk plots ($1/s$ *versus* $1/v$). Experimental errors were less than 2%.

Analysis of internal amino-acid sequence. Purified JHGase I (2 nmol) was reduced and *S*-pyridylethylated with 150 mM 2-mercaptoethanol and 140 mM 4-vinylpyridine in 0.01 M Tris–HCl (pH 8.0) containing 8 M urea and 10 mM EDTA. To remove excess 2-mercaptoethanol and 4-vinylpyridine, the reaction mixture was dialyzed to 10 mM sodium acetate buffer (pH 4.5), and then 0.01 M Tris–HCl (pH 8.0). *S*-Pyridylethylated JHGase I was digested with 50 pmol Lysyl Endopeptidase (Lys-C protease; Wako Pure Chemical) in 0.01 M Tris–HCl (pH 8.0) containing 4 M urea at 30 °C for 24 h. Peptides were isolated by reversed-phase HPLC on a Capcell Pac C_{18} column (UG120; 4.6 × 150 mm; Shiseido, Tokyo, Japan) using a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid. The sequence analysis was performed with a protein sequencer (490 Procise; Applied Biosystems, Foster City, CA).

Cloning and sequencing of JHGase I cDNA. Total RNA was extracted from 50 Japanese honeybees by the acid guanidinium thiocyanate–phenol–chloroform method.[19] Poly(A)$^+$ RNA was purified by Oligotex dT-30 super (Takara, Otsu, Japan). First strand cDNA for rapid amplification of cDNA 3′ and 5′ ends (3′-RACE and 5′-RACE) was prepared using SuperScript II RNase H$^-$ (Invitrogen, Carlsbad, CA) and 3′-AP primer (5′-GGCCACGCGTCGACTAGTACT$_{17}$-3′), according to the procedure indicated by the manufacturer. The forward primer (5′-GGTATTAAGGATAAGCTTTCA-CACTTC-3′, synthesized according to Gly51-Phe59 of WHGase I; corresponding to G164-C190 in Fig. 4) and AUAP-primer (5′-GGCCACGCGTCGACTAGTAC-3′) were used for 3′-RACE. Amplified product was sequenced by the dideoxy chain termination method using a 310 Genetic Analyzer (Applied Biosystems) in both directions. For 5′-RACE, a homopolymeric tail of dA$_{17}$ was added to the 3′ end of the first strand cDNA by terminal deoxynucleotide transferase, and then it was used as a template. PCR amplification was done using 5′-AP primer (5′-GACTCGAGTCGACATCGAT$_{17}$-3′), and followed by nest PCR with 5′-AUAP primer (5′-GACTCGAGTCGACATCG-3′) and specific anti-sense primer (5′-GACTTCGATAAATTGGTGATAACCATA-3′, synthesized based on the product of 3′-RACE; covering T213-C239 in Fig. 4). The 5′-RACE product was sequenced. Sense primer (5′-GATCATTCAAATC-3′; covering the 5′ end sequence in Fig. 4) and AUAP-primer were used for amplification of the full length of JHGase I cDNA (accession no. AB260890).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis of tryptic peptides obtained by in-gel digestion. JHGase I (244 pmol) was treated with endoglycosidase H (10 mU) under the conditions above-mentioned to remove *N*-linked sugar chains. The deglycosylated enzyme was applied to 8% SDS–PAGE, from which we excised the stained gel pieces (1 mm square). The gel was destained by washing three times with 100 μl of 50 mM NH_4HCO_3 in 50% acetonitrile for 20 min at 37 °C. To gel dehydrated by vacuum centrifugation, 25 μl of trypsin solution (60 pmol, TPCK-treated type; Sigma) containing 50 mM NH_4HCO_3 (pH 7.8) was added, followed by the incubation at 30 °C overnight. Peptides were recovered from the gel by extraction three times with 100 μl of 60% acetonitrile at 37 °C for 20 min, and then dried. Further analysis of tryptic peptides (dissolved with 3 μl of 0.1% trifluoroacetic acid) using MALDI-TOF MS was performed as described in our previous paper.[20]

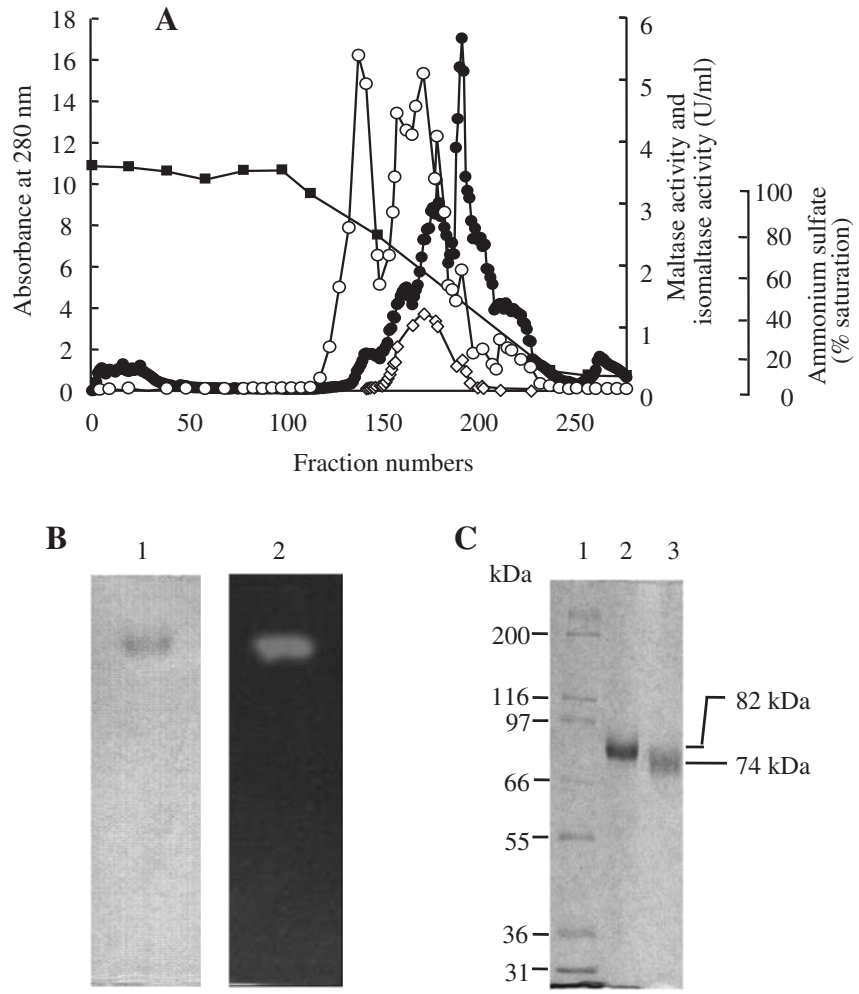

Fig. 1. Salting-Out Chromatography (A) and Electrophoretical Analysis of Japanese Honeybees α-Glucosidase I (B and C).

Panel-A: Flow rate, 20 ml/h; fraction, 15 g; closed circle, absorbance at 280 nm; open circle, maltase activity; open rhombus, isomaltase activity; closed squire, saturation degree of ammonium sulfate. Panel-B: Lanes 1 and 2, native-PAGE (5 µg protein for each lane) visualized by protein-staining and activity-staining, respectively. Panel-C: Lane 1, SDS–PAGE of molecular size makers: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), glutamate dehydrogenase (55 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrolase (31 kDa); lanes 2 and 3, SDS–PAGE of intact and deglycosylated JHGase I (2.5 µg for each lane), respectively.

Results and Discussion

Purification of Japanese honeybee α-glucosidase

The purification procedure was performed at 4 °C. Frozen Japanese honeybees (150 g) were homogenized with 30 mM sodium phosphate buffer, pH 6.3 (buffer-A). After stirring it overnight at 4 °C, the homogenate was centrifuged at 15,500 × *g* for 20 min. The precipitate was extracted twice with buffer-A. All supernatant obtained was combined (1,000 ml; crude extract). Ammonium sulfate (70 g per 100 ml of crude extract; 100% saturation) was slowly added to the crude extract to precipitate protein with gentle stirring overnight at 4 °C. Precipitant, recovered by centrifugation at 15,500 × *g* for 20 min, was dissolved with 600 ml of buffer-A, followed by centrifugation to discard insoluble materials. To the resultant supernatant, ammonium sulfate was slowly added to 95% saturation, and the suspension was left overnight in a cold room. The turbid solution was centrifuged at 15,500 × *g* for 30 min to collect precipitate. The precipitate was mixed with a suspension of Celite 545 in buffer-A containing 95% saturation of ammonium sulfate solution (buffer-B), and was subjected to subsequent salting-out column chromatography.[21] The suspension was gently packed into a column (4.7 × 25 cm), and then washed with buffer-B. The enzyme was eluted by reverse gradient of 95–0% saturation of ammonium formed by buffer-B and buffer-A. As shown in Fig. 1A, α-glucosidase activities were separated into two components: component I (nos. 120 to 150, about 420 ml) and component II (nos. 155 to 200, about 700 ml) which were eluted in ranges of about 80–95% and 40–80% saturation of ammonium sulfate, respectively. Component I was then dialyzed against 10 mM sodium acetate buffer (pH 5.8) and again dialyzed overnight against 50 mM sodium acetate buffer

Table 1. Purification Procedure for Japanese Honeybee α-Glucosidase I

Procedure	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Crude extract[a]	52,700	6,700[a]	0.13	
100% $(NH_4)_2SO_4$[a]	24,100	4,800[a]	0.20	
Salting-out chromatography	560	760	1.4	100
CM-Toyopearl 650M	93	610	6.6	80
Sephacryl S-100HR	29[b]	370	13	49

[a]These steps included α-glucosidase component II.
[b]The value was calculated on the basis of the fact that $E^{1\%}_{1\,cm}$ at 280 nm was 5.5. Other values were estimated from the assumption that $E^{1\%}_{1\,cm}$ at 280 nm was 10.

(pH 4.7). The dialyzed solution (1,250 ml) was subjected to a CM-Toyopearl 650M column (3.0 × 30 cm) equilibrated with 50 mM sodium acetate buffer (pH 4.7). A majority of α-glucosidase activity (85%) did not adsorb this column, but this step was useful for the removal of the resin-adsorbed α-glucosidase that might have originated from component II. The non-adsorbed fraction was concentrated to 8 ml with Ultrafiltration Cell using PM-10 (Millipore, Bedford, MA). Gel filtration was conducted on a Sephacryl S-100HR column (2.9 × 65 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.8) containing 0.15 M sodium chloride. The active fractions were pooled and used as a purified enzyme, which was designated Japanese honeybee α-glucosidase I (JHGase I). The purification procedures of JHGase I are summarized in Table 1. JHGase I was isolated with a specific activity of 13 U/mg and the overall yield of 49%.

The enzyme preparation was subjected to electrophoresis to confirm the purity of enzyme. The enzyme migrated as a single protein band on native-PAGE, which displayed α-glucosidase activity (Fig. 1B). JHGase I was a monomeric protein having a molecular weight (M_r) of 82,000 (Fig. 1C). This M_r was the intermediate value of WHGase I, WHGase II, and WHGase III (M_r of WHGase I, 98,000; WHGase II, 76,000; WHGase III, 68,000; the values included sugar moieties, since the three enzymes were glycoproteins).[5,6] Treatment with endoglycosidase H gave a deglycosylated product (Fig. 1C), of which the M_r (74,000) was smaller than that of the intact enzyme, indicating that JHGase I was a glycoprotein. The carbohydrate content was measured to be 15% as mannose or 21% as glucose. The $E^{1\%}_{1\,cm}$ of the enzyme at 280 nm was 5.5.

Effects of pH and temperature

The effects of pH and temperature on the activity were determined using maltose as substrate. The optimum pH was 5.0. JHGase I was stable in a pH-range from 4.5 to 10.5 (treatment at 4 °C for 24 h) and a temperature-range up to 40 °C (15 min of incubation). The activity decreased significantly at 60 °C. These properties are almost identical to those of WHGase I, except for a minor difference in pH-stability (pH 4.9–12.0 for WHGase I).[5]

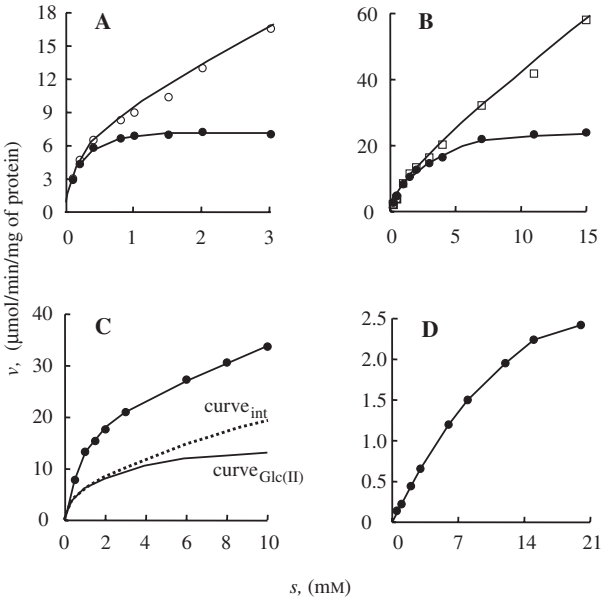

Fig. 2. *s versus v* Plots for Reactions on *p*NPG (A), Sucrose (B), Maltose (C), and Turanose (D) by Japanese Honeybee α-Glucosidase I.

Detail reaction conditions are described in the text. Enzyme of 0.047 μg, 0.043 μg, 0.047 μg, or 0.5 μg was used for *p*NPG, sucrose, maltose, or turanose, respectively. The initial velocity was expressed as μmol glucose (closed circle, A–D), *p*-nitrophenol (open circle, A) or fructose (open square, B) liberated from substrate per min per mg of protein. As described in the text, $curve_{Glc(II)}$ and $curve_{int}$ in panel-C display the theoretical liberation velocities of Glc(II) (produced by hydrolysis) and Glc(I) (produced by intrinsic reaction for cleavage of maltose), respectively.

Reaction on maltose, sucrose, pNPG, and turanose

As shown in Fig. 2, *s versus v* plots show unusual curves in the reaction on *p*NPG, sucrose, and maltose, where the liberation rates of *p*-nitrophenol, fructose, and glucose, respectively, were accelerated by increasing of substrate concentration. The curves were not of the typical Michaelis–Menten shape without reaching a plateau. Their Lineweaver–Burk plots (Fig. 3A, B, and C), therefore, displayed convex curves under the high substrate concentration. Liberation of glucose from *p*NPG and sucrose depicted ordinary hyperbolic Michaelis–Menten curves (Fig. 2A and B), which resulted in linearity in $1/s$ *versus* $1/v$ plots (Fig. 3A and B). Under the low substrate concentration, the liberation velocities of glycon (glucose) and aglycon (*p*-nitrophenol or fructose) agreed well (Fig. 2A and B; see also Fig. 3A and B), implying that JHGase I catalyzed only the hydrolytic reaction below a certain range of *s*. To estimate the K_m and k_0 values for hydrolysis of those three substrates, we extrapolated the linear portion of Lineweaver–Burk plots to the $1/s$-axis and $1/v$-axis, respectively.

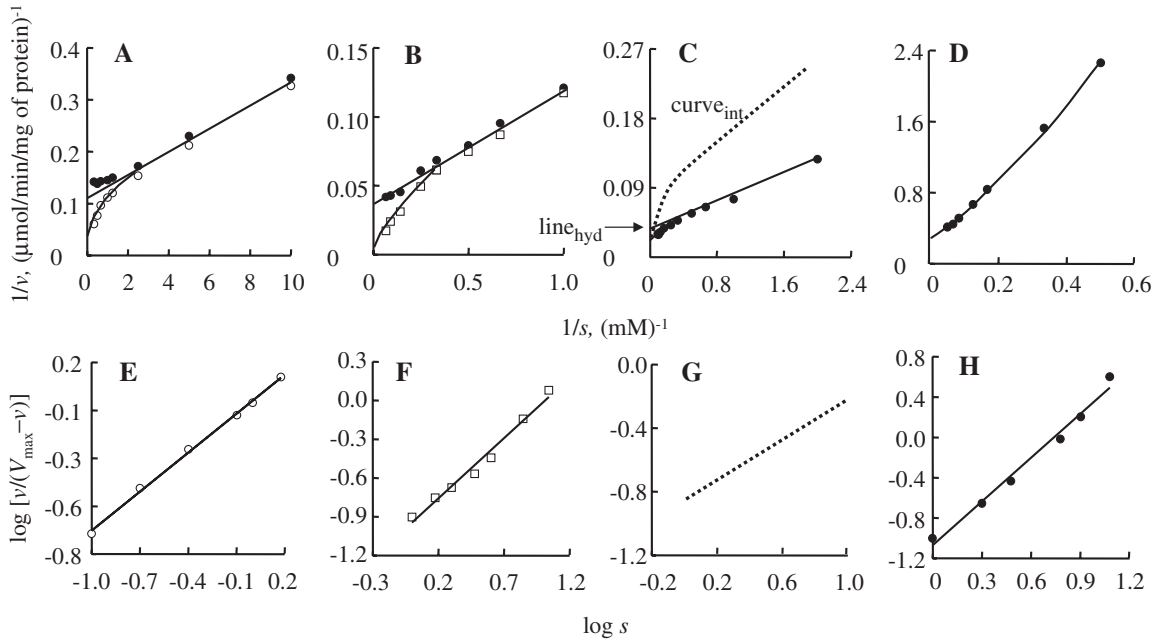

Fig. 3. Lineweaver–Burk Plots (A to D) and Hill Plots (E to H) for Reactions on *p*NPG (A and E), Sucrose (B and F), Maltose (C and G), and Turanose (D and H) by Japanese Honeybee α-Glucosidase I.

The reaction conditions and symbols are the same as in Fig. 2. In panel-C, line$_{hyd}$ and curve$_{int}$ indicate the reciprocal rates of glucose produced by hydrolysis [Glc(I) and Glc(II) formed by hydrolytic reaction] and by intrinsic cleavage reaction [Glc(I) formed by hydrolysis and transglycosylation], respectively. The dot line in panel-G was estimated from curve$_{int}$ in panel-C.

In the cases of sucrose and *p*NPG, the hydrolysis and transglucosylation are shown by the following two schemes:

$$\text{Glc-Agl} + \text{H}_2\text{O} \rightarrow \text{Glc} + \text{Agl (hydrolysis)},$$

$$\text{Glc-Agl} + \text{Glc-Agl} \rightarrow \text{Glc-Glc-Agl} + \text{Agl (transglucosylation)},$$

where Glc-Agl, Glc, and Agl indicate substrate, glucose, and aglycon (fructose or *p*-nitrophenol), respectively. The rate of hydrolysis is the liberation velocity of glucose. The difference in the liberation velocities of glucose and aglycon corresponds to the rate of transglucosylation. Aglycon is produced by both reactions, so that the intrinsic v value for the cleavage of glucosidic linkage of substrate is the liberation rate of aglycon. The maximum velocity for intrinsic cleavage of the substrate (intrinsic V_{max}) was estimated by extrapolating the convex curve of $1/s$ *versus* $1/v$ plots to the $1/v$-axis (Fig. 3A and B). The reaction on maltose is also demonstrated by the following two schemes:

$$\text{Glc(II)-Glc(I)} + \text{H}_2\text{O} \rightarrow \text{Glc(II)} + \text{Glc(I) (hydrolysis)},$$

$$\text{Glc(II)-Glc(I)} + \text{Glc-Glc} \rightarrow \text{Glc(II)-Glc-Glc} + \text{Glc(I) (transglucosylation)},$$

where Glc(II)-Glc(I), Glc-Glc, Glc(II), and Glc(I) represent maltose of donor, maltose of acceptor, glucose of non-reducing side, and glucose of reducing side, respectively. The experimentally measured glucose includes Glc(I) and Glc(II) produced by hydrolysis and Glc(I) produced by transglucosylation. The rate of intrinsic reaction (v_{int}), which is the liberation velocity of Glc(I) formed by both hydrolysis and transglucosylation, is calculated by the following equation:

$$v_{int} = v_{obs} - v_{Glc(II)} = v_{obs} - v_{hyd}/2 \text{ (eq. 1)}^*,$$

where v_{obs}, $v_{Glc(II)}$, and v_{hyd} indicate the experimentally observed v value, the v value for liberated Glc(II), and the v value for Glc(I) and Glc(II) produced by hydrolysis, respectively. Since the hydrolytic reaction forms the equimolar amount of Glc(I) and Glc(II), half of v_{hyd} becomes $v_{Glc(II)}$. The v_{hyd} value under a high concentration of maltose (the nonlinear portion in Fig. 3C) is predicable from the linear line$_{hyd}$ in Fig. 3C (the arithmetically obtained $v_{Glc(II)}$ is also displayed as a hyperbolic curve$_{Glc(II)}$ in Fig. 2C), from which v_{int} is calculated from eq. 1 (curve$_{int}$ in Fig. 2C and 3C). The intrinsic V_{max} for the total cleavage of maltose was estimated by extrapolating curve$_{int}$ in Fig. 3C to its $1/v$-axis. Consequently, JHGase I exhibited the negative kinetic cooperativity in the cleavage of these three substrates.

On the other hand, in the reaction on turanose, JHGase I showed the positive kinetic cooperativity. As shown in Fig. 2D, the sigmoidal curve appeared in s *versus* v plots for liberated glucose, resulting in the depiction of a concave curve in the $1/s$ *versus* $1/v$ plots (Fig. 3D). The intrinsic V_{max} was also obtained by extrapolation of the concave curve to the $1/v$-axis. JHGase I is an allosteric enzyme exhibiting both positive and negative kinetic cooperativities.

* Equation 1 is recommended for the paper,[8] where the intrinsic k_0 of maltose for WHGase I is estimated to be 90.5 s^{-1}.

Table 2. Kinetic Parameters (K_m, k_0, and k_0/K_m) for Hydrolysis of Substrates by Japanese Honeybee α-Glucosidase I

Substrate	JHGase I			WHGase I[a]		
	K_m (mM)	k_0 (s^{-1})	k_0/K_m ($mM^{-1}\cdot s^{-1}$)	K_m (mM)	k_0 (s^{-1})	k_0/K_m ($mM^{-1}\cdot s^{-1}$)
Maltose	1.5	22	15	0.85	45	53
Maltotriose	0.40	15	36	0.65	51	82
Maltotetraose	1.3	13	10	2.0	53	26
Maltopentaose	5.5	15	2.7	8.0	50	6.3
Kojibiose	8.7	8.7	1.0	25	37	1.5
*p*NPG	0.21	13	61	0.31	57	184
Sucrose	2.4	40	17	4.2	43	10

[a]Kinetic parameters of western honeybee α-glucosidase I.[8)]

Table 3. Kinetic Parameters (K_m, V_{max}, and k_0) and Hill Coefficient (n_H) for Intrinsic Reaction on Cleavage of Substrates by Japanese Honeybee α-Glucosidase I

Substrate	K_m[a] (mM)	V_{max} (μmol/min/mg protein)	k_0 (s^{-1})	n_H
Maltose	15	50[b]	70[b]	0.63[b]
*p*NPG	1.0	18[c]	25[c]	0.67[c]
Sucrose	14	110[d]	150[d]	0.75[d]
Turanose	6.2	2.4[e]	3.4[e]	1.5[e]

[a]The K_m value is the substrate concentration giving one half of intrinsic V_{max}.
[b]Obtained from the calculated value as the glucose liberated from the reducing end residue of maltose.
[c,d,e]Obtained from the liberation of *p*-nitrophenol[c], fructose[d], and glucose[e].

Table 2 summarizes the kinetic parameters for the hydrolysis of sucrose, *p*NPG, maltose, and turanose, which were estimated by the method above-mentioned (extrapolation of the linear portion in $1/s$ *versus* $1/v$ plots to abscissa and ordinate). The kinetic parameters for intrinsic cleavage of substrates, which were estimated by aglycons, are listed in Table 2, while those for maltose and turanose were done by the calculated Glc(I) and glucose, respectively. The K_m value for the intrinsic reaction was defined as the substrate concentration to give half of intrinsic V_{max}. To evaluate the degree of allosteric cooperativity, the Hill coefficient (n_H)[22–24)] was calculated from Hill plots (lower panels in Fig. 3) using the following equation:

$$\log[v/(V_{max} - v)] = n_H \log s - \log K,$$

where V_{max} is the intrinsic V_{max} and K, the dissociation constant for a hypothetical binding of a certain number of molecules of the ligand to the enzyme. The slope of the plots in coordinate $\log s$ *versus* $\log[v/(V_{max} - v)]$ gives the Hill coefficient. The n_H values obtained are shown in Table 3. Since the evaluated n_H values were smaller than unity in the intrinsic reactions of sucrose, *p*NPG, and maltose, JHGase I exhibited the negative cooperative behavior for these substrates. The apparent n_H for turanose is larger than unity, indicating the positive kinetic cooperativity.

WHGase I showed two types of allosteric properties, the positive kinetic cooperativity for the reaction of turanose and maltodextrin ($\overline{DP} = 13$) and the negative kinetic cooperativity for the cleavage of phenyl α-glucoside, *p*NPG, sucrose, and maltose. Unusual kinetic behavior of α-glucosidase from western honeybee toward sucrose and *p*NPG was also reported by Huber and Thompson.[25)] They explained the reactions by transglucosylation: under the high concentration of sucrose and *p*NPG, in addition to hydrolysis, the transglucosylation occurred independently. This reaction mechanism, by which Huber and Thompson interpret the acceleration of aglycon liberation, however, cannot account for the positive-cooperative kinetic. Interestingly, JHGase I and WHGase I are monomeric proteins, while two enzymes display both positive and negative kinetic cooperativities. It was a rare case that monomeric glycosylase exhibited the allosteric reaction.[26)] Relatively slow conformational change of an enzyme molecule during a reaction is thought to be related to the allosteric kinetic.[27)] Studies of the cooperative behavior of these monomeric enzymes are in progress through analysis of the structural changes induced by substrate molecules.

Substrate specificity

In addition to the four substrates described the previous section, JHGase I exhibited hydrolytic activities toward maltooligosaccharides (malto-triose, -tetraose, and -pentaose having the α-1,4-glucosidic bond) and kojibiose (having the α-1,2-glucosidic bond), but no activities were observed toward isomaltose (α-1,6-glucosidic bond), nigerose (α-1,3-glucosidic bond), or soluble starch. No allosteric kinetic cooperativity was found in the reaction for kojibiose or maltooligosaccharide (except for maltose). Kinetic parameters (K_m, k_0, and k_0/K_m) for the hydrolysis of these substrates are summarized in Table 2. The heteroside substrates (sucrose and *p*NPG) were hydrolyzed more quickly than the holoside substrates (maltose and maltotriose), implying that JHGase I is a member of type I α-glucosidase preferring heterogeneous substrates.[1)] WHGase II and WHGase III were capable of hydrolyzing isomaltose and nigerose[5,6,9)] (soluble starch was hydrolyzed only by WHGase II[9)]), indicative of the difference in the substrate specificity of JHGase I. Table 2 also shows the kinetic parameters of WHGase I for comparison.[8)] WHGase I showed a higher K_m value than JHGase I for the hydrolysis of all substrates, with the exception of maltose. However, WHGase I had better efficiency (k_0/K_m) than JHGase I due to the low k_0 values of JHGase I.

Isolation of α-glucosidase cDNA

PCR works isolated JHGase I cDNA, which comprised an ORF of 1,734 bp encoding 577 amino acid residues, a 5′-untranslated sequence of 13 bp, and a 3′-untranslated sequence of 183 bp (Fig. 4). Two polyadenylation signals were found in the 3′-end region.

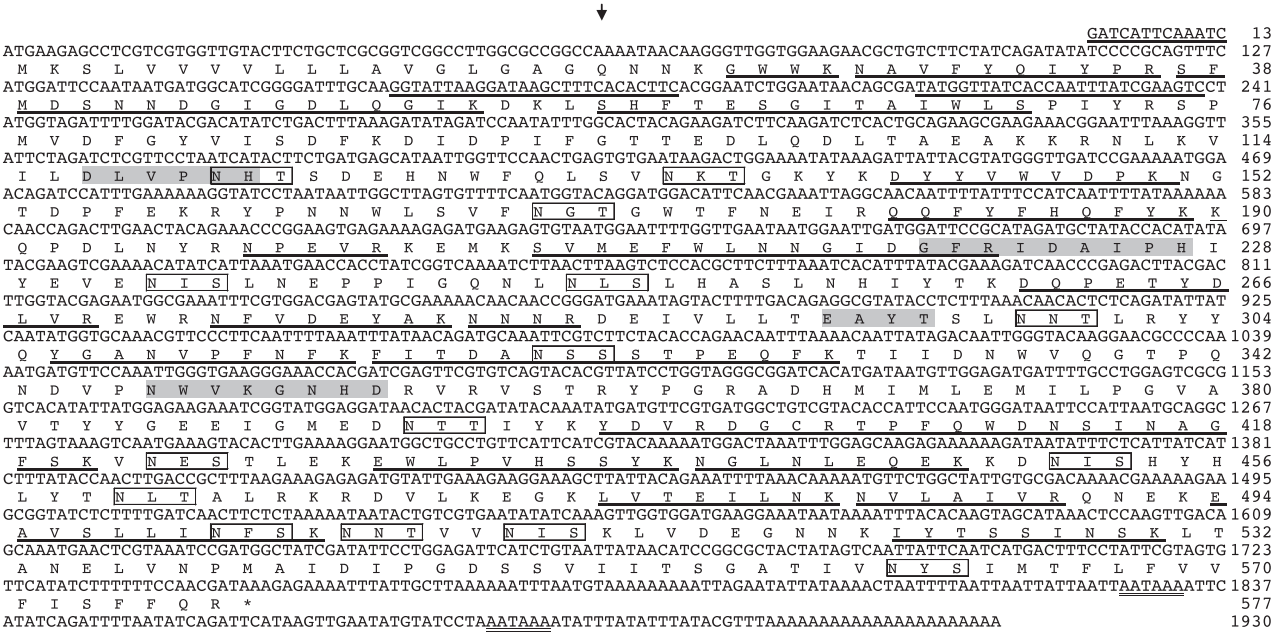

Fig. 4. Nucleotide and Amino-Acid Sequences of Japanese Honeybee α-Glucosidase I.

The oligonucleotide primers are indicated by underlining below the DNA sequence. Two putative polyadenylational signals and stop codon are double-underlined and asterisked, respectively. Amino-acid sequences determined using peptide fragments are underlined (Ser57-Ser70, Asp142-Lys150, Phe316-Thr325, and Tyr399-Gly418 by Lys-C protease digestion; others by tryptic digestion which includes Asp142-Lys150 and Phe316-Lys330). Predicted N-terminal amino acid and potential *N*-glycosylation sites are shown by arrow and box, respectively. Gray-background sequences are the catalytic regions conserved in α-amylase family enzymes.

The N-terminal amino acid sequence of purified JHGase I could not be analyzed by Edman's degradation method because of possible modification of the N-end amino group. The signal peptide prediction program (SignalP)[28] judged that Gln19 was an N-terminal amino acid residue. The molecular weight estimated from amino-acid sequence was 66,515.21, which agreed with M_r of the deglycosylated enzyme (74,000, Fig. 1C). As shown in Fig. 4, the deduced amino-acid sequence contained four internal sequences of Lys-C protease-digested peptides and 18 internal sequences of tryptic peptides, which were determined by Edman's degradation analysis and by MALDI-TOF MS analysis combined with in-gel digestion, respectively. Both approaches, finding about 39% (226 residues) of the whole sequence, indicate that the cloned cDNA is a gene of JHGase I.

JHGase I possessed four conserved regions responsible for the catalytic reaction, region 1 to 4, observed in GH 13 enzymes (so called α-amylase family enzymes).[29,30] Region 1 was located in Asp117-His122; region 2 in Gly219-His227; region 3 in Glu292-Thr295; region 4 in Asn347-Asp354 (Fig. 4), where the catalytic residues were thought to be Asp223, Glu292, and Asp354. α-Amylase family members shared a common structure composed of three domains: domain N, the catalytic domain formed by a $(\beta/\alpha)_8$-barrel; domain S, a long loop connecting between β-strand 3 and α-helix 3 of domain N; domain C, antiparallel β-sheets followed by domain N.[30] The three-dimensional structure of GH 13 α-glucosidase was available only in *Bacillus cereus* oligo-1,6-glucosidase.[31] Compared with oligo-1,6-glucosidase on the basis of secondary structure, most of the structural elements were well-conserved in JHGase I (Fig. 5; the whole amino-acid sequence identity being 26% between the two enzymes). Domain N was predicted to consist of Gln19-Thr123 and Pro199-Gly473, domain S of Ser124-Asn198, and domain C of Lys474-Arg577. The overall sequence of JHGase I was very similar to that of WHGase I (M. Nishimoto and A. Kimura, unpublished results) with 76% identity (Fig. 5). Before this study, we expected to find extremely high sequence identity between JHGase I and WHGase I, perhaps more than 95%. There is a high identity of 76%, while replacement and deletion of 34% and 29% amino acid residues appear in domains S and C, respectively. In catalytic domain N, the replaced amino acids (15% of residues) were located mainly in Pro199 to Ala308 of JHGase I. In particular, variation was concentrated on four structural elements (one α-helix and three loops): Pro199-Asn215 of α-helix 3 in JHGase I, Ile225-Gln243 of $\beta\rightarrow\alpha$ loop 4, Lys280-Asn283 of $\alpha\rightarrow\beta$ loop 4, and Gln305-Ala308 of $\alpha\rightarrow\beta$ loop 5. Alternation or conservation of amino acids leads us to predict the structural element(s) responsible for allosteric behavior. For this, we are making an attempt to achieve the heterogeneous expression of isoenzyme I gene.

Fifteen *N*-glycosylation sites (sequon, Asn-X-Ser/Thr: X, an amino acid other than Pro or Asp) were found

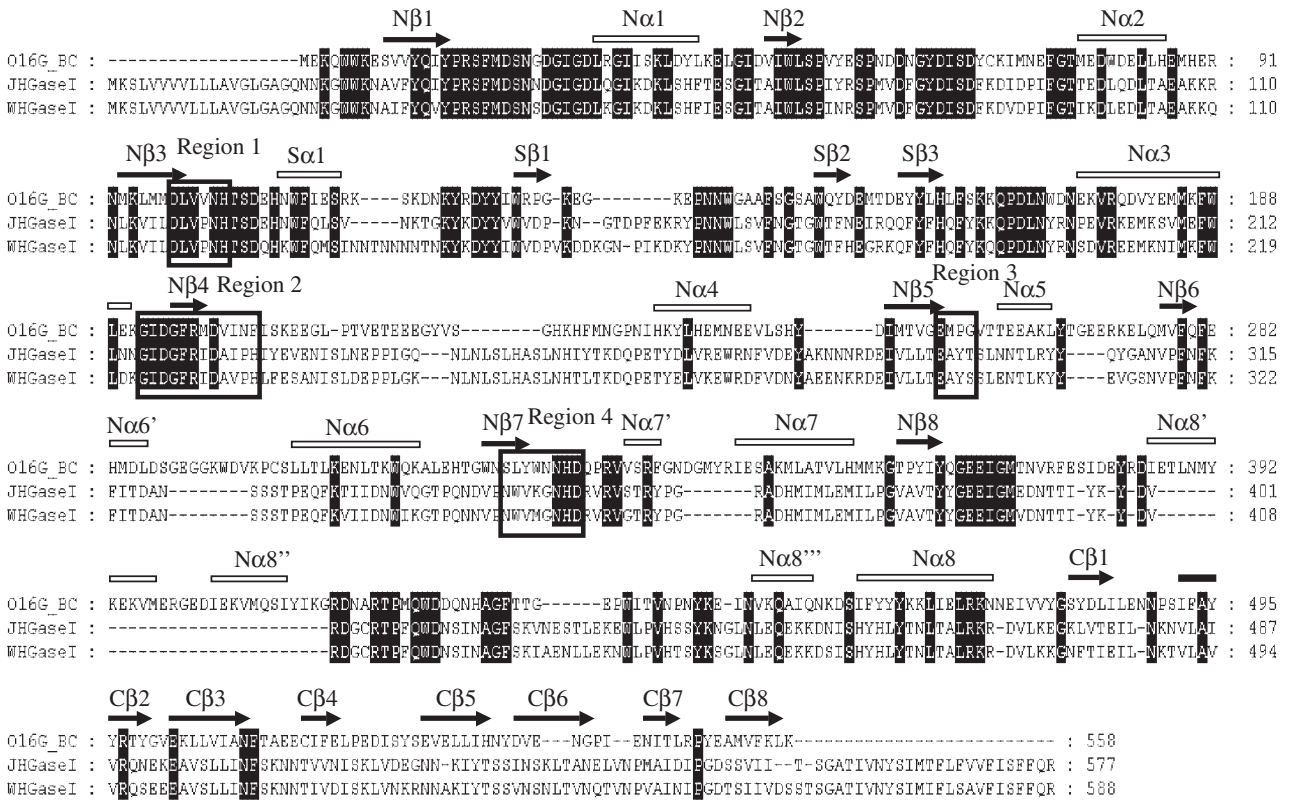

Fig. 5. Multiple Alignment of Oligo-1,6-glycosidase from *Bacillus cereus* (O16G_BC), Japanese Honeybee α-Glucosidase I (JHGaseI), and Western Honeybee α-Glucosidase I (WHGaseI).

The alignment was done based on the similarity of secondary structure.[32] The symbols above the sequence indicate the secondary structures found in oligo-1,6-glucosidase from *B. cereus*[31] (bar, α-helix; arrow, β-strand). Black background residue is conserved in the three primary sequences. Four conserved catalytic regions in the α-amylase family are boxed.

in the deduced amino-acid sequence of JHGase I (Fig. 4). Most Asn in these sequons were situated in loops. It is of interest that one sequon (Asn121-His122-Thr123) overlaps with region 1, where His122 is essential for substrate recognition in the α-amylase family enzyme. WHGase I also had the identical *N*-glycosylation motif (Asn121-His122-Thr123, Fig. 5), while no sugar chain was linked to its Asn121, which was elucidated by Edman's degradation analysis of a peptide (Val114-Lys128) obtained by Lys-C protease digestion of WHGase I (M. Nishimoto and A. Kimura, unpublished results). The biochemical mechanism for no addition of *N*-linked sugar chain is obscure. It may be essential to the catalytic reaction that the structural hindrance due to *N*-linked oligosaccharide is excluded from the active center. It is thought that Asn in region 1 of JHGase I is not modified by the sugar chain.

Salting-out chromatography separated maltase activity into two fractions (components I and II in Fig. 1A). Component II exhibited isomaltase activity, which JHGase I was devoid of, indicating the existence of different α-glucosidase(s) in Japanese honeybee. In order to learn whether the α-glucosidase isoenzyme-system is general in honeybee species, the characterization of the enzyme in component II is in progress.

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