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Full Length Research Paper

Characterization of some enzymatic properties of recombinant α-glucosidase III from the Thai honeybee, *Apis cerana indica* Fabricus

Jirattikarn Kaewmuangmoon[1], Mikio Yoshiyama[2], Kiyoshi Kimura[2], Masayuki Okuyama[3], Haruhide Mori[3], Atsuo Kimura[3] and Chanpen Chanchao[4]*

[1]Program of Biotechnology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand.
[2]Honeybee Research Group, National Institute of Livestock and Grassland Science, Ibaraki 305-0901, Japan.
[3]Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.
[4]Department of Biology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand.

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Recombinant α-glucosidase III (rHBGase III) from *Apis cerana indica* Fabricus (r*Aci*HBGase III) was expressed in the yeast *Pichia pastoris* GS115, enriched and characterized. The full length cDNA of *AciHbgase III* (~1.8 kb) was amplified by RT-PCR, cloned into the pPICZαA expression vector and used to transform *P. pastoris* GS115. The maximum secreted expression level of r*Aci*HBGase III [as an N terminal $(His)_6$ tagged chimera] was found 144 h after induction by 1% (v/v) methanol. Enrichment of the enzyme using histrap affinity purification revealed a single active glucosidase band with a molecular mass of ~68 kDa. The optimal pH and temperature for glucosidase activity of the enriched rAciHBGase III were pH 5.0 and 37°C, respectively, whilst the enzyme showed a good pH stability between pH 5.0 to 7.5, but not below pH 5.0, and a poor thermotolerance with < 10% and 0% residual activity at 40 and >50°C, respectively. The r*Aci*HBGase showed a relatively high substrate specificity for maltose (K_m of 4.5 mM) and *p*-nitrophenyl α-D-glucoside (K_m of 4.4 mM) compared to other reported HBGase enzymes.

Key words: α-Glucosidase, *Apis cerana indica*, expression, kinetics, recombinant enzyme.

INTRODUCTION

The exocarbohydrase α-glucosidase (HBGase or α-glucoside glucohydrolase; EC 3.2.1.20), a member of the glycoside hydrolase family (GH) 31 (Kimura, 2000), can cleave (hydrolyze) the α-glucosidic linkage of carbohydrate polymers to liberate α-glucose (Chiba, 1997). Various types of HBGases have been widely found in many organisms, including microorganisms (Schmidt et al., 2011), plants (Stanley et al., 2011), mammals (Moreland et al., 2012), and insects (Chiba, 1997). This enzyme is one of THE four enzymes (α-amylase, β-amylase, the debranching enzyme limit dextrinase and α-glucosidase) that are important for starch degradation and are used in biotechnology, especially in the context of brewing (Bamforth, 2009).

In addition, glycosidase can be used in the glycolsylation reactions used for carbohydrate synthesis, including various α-glucosylated compounds (Perugino et al., 2004). The method uses mild reaction conditions avoiding harsh conditions or toxic catalysts, such as heavy metals (Pal et al., 2010), which is advantageous for the food and cosmetics industry. Compared to glycosyltransferases, glycosidases are more popularly used in industry because they are less expensive, in terms of their glycosyl donors, availability and widespread occurrence in nature (Kato et al., 2002).

Nowadays, among insects, the honeybees (Genus *Apis*) have become the most popular model as a potential

*Corresponding author. E-mail: chanpen@sc.chula.ac.th. Tel: +66 2 218 5380. Fax: +66 2 218 5386.

source of new HBGase enzymes, largely since honeybees are directly involved in producing honey (principally the monosaccharides glucose and fructose) from disaccharides, especially sucrose as this is the main carbohydrate in nectar. In the European honeybee, *Apis mellifera*, α-glucosidase was reported to be a developmentally regulatory gene with the expression level depending directly on the different organs and the developmental age of the bee (Ohashi et al., 1996). The enzyme was principally synthesized in the hypopharyngeal glands, located in the head of honeybees, and was highly expressed in forager bees (older than 10 days), which are the bees that actively leave the hive to collect nectar and make honey. Therefore, the expression of the gene appears to be directly related to the behavior-mediated (honey producing or not) requirement of honeybees for the α-glucosidase enzyme.

A. mellifera originated in Europe and Africa and has been widely used as a model for research since it is commercially important for both honey and other related bee products as well as for pollination of agricultural food crop; it is easily and well managed in many areas and it has the least aggressive behavior of all honeybee species, being almost docile (Oldroyd and Wongsiri, 2006). Although it has been reported that there are three kinds of HBGases (I, II and III), which show different substrate specificities, molecular weights, nucleotide sequences and tissue locations, only the HBGase III isoform has been found in *A. mellifera* honey (*Am*HBGase III) (Kubota et al., 2004).

Thus, *Am*HBGase III is likely to be involved in honey production via the cleavage of α-glucosidic linkages in carbohydrates, generating interest in the characteristics of the native and recombinant forms for potential application in the food and biotechnology industries. The potential diversity of enzyme characteristics in HBGase isoforms is alluded to, for example, the fact that isoforms of homologous HBGases are also found in plants, such as *Arabidopsis* (Gillmor et al., 2002) and barley (Stark and Yin, 1987), suggesting a long evolutionary history with potentially different required enzyme optima.

Originally, the native form of *Am*HBGase III was enriched by ammonium sulphate salting-out followed by DEAE-cellulose, DEAE-Sepharose CL-6B, Bio-Gel P-150 and CM-Toyopearl 650M chromatographic fractionation. The enriched enzyme obtained was found to be a monomeric glycoprotein containing about 7.4% by weight of carbohydrate. Surprisingly, it was not an allosteric enzyme and showed normal Michaelis-Menten type reaction kinetics with no cooperativity (Nishimoto et al., 2001).

Currently, recombinant technology is widely used to produce enzymes that are normally expressed in low levels or are hard to otherwise enrich and so limits their commercial preparation (Zhao et al., 1993), as well as to allow genetic engineering for improved characteristics.

Accordingly, the full length cDNA of *AmHBGase* III was cloned into the pPIC3.5 or pPIC9 expression vector, transformed into *Pichia pastoris* GS115 (yeast) and expression of the recombinant (r)*Am*HBGase III enzyme was induced by 0.5% (v/v) methanol (Nishimoto et al., 2007). Under these conditions, the highest enzyme activity was found in the culture media, from where the r*Am*HBGase III was purified by ammonium sulfate salting - out followed by fractionation over successive CM Sepharose CL-6B, Bio-Gel P-100, DEAE Sepharose CL6B and Butyl-Toyopeal 650 M column chromatography (Nishimoto et al., 2007).

Other than *A. mellifera*, native HBGase III has been purified from *Apis cerana indica* (*Aci*HBGase III), which is a native honeybee species to Thailand (Chanchao et al., 2008). *A. cerana indica* is now widely managed as an economic bee in many Asian countries due to its higher disease resistance against bee mites, predators and pathogens than the introduced *A. mellifera* (Peng et al., 1987; Chen et al., 2000). In addition, many of the local Thai populace prefer the taste of the more acidic honey from *A. cerana indica*. In this paper, we aimed to obtain r*Aci*HBGase III starting from obtaining the full length cDNA of *AciHBGase III*, expressing the recombinant protein in the yeast *P. pastoris* to allow post-translational modifications, such as glycosylation, and then enriching and characterizing the r*Aci*HBGase III to evaluate the potential suitability of this isoform as a new source of HBGase III for the food and biotechnology industries.

MATERIALS AND METHODS

Sample collection

Foragers of *A. cerana indica* were collected on their return flight in front of the hive entrance in an apiary in Samut Songkram Province. Samples were kept at -80°C until used.

RNA extraction

Forager bees (50 bees) were ground with liquid nitrogen in a mortar. Total RNA was then extracted by a standard acid-guanidine thiocyanate-phenol-chloroform method, as previously reported (Nishimoto et al., 2007). The quality of the total RNA was visually assayed after resolution by 1.2% (w/v) formaldehyde/ agarose gel electrophoresis, and ultraviolet (uv) transillumination after ethidium bromide (EtBr) staining. The purity of extracted total RNA was calculated by the ratio of absorbance at 260 and 280 nm, whilst the concentration was calculated from the ratio of absorbance at 260 nm. Then, poly A^+ mRNA was isolated using the oligotex dT-30 super mRNA purification kit (Takara, Japan), as per the manufacturer's instructions. The extracted total poly A+ RNA samples were then stored at -80°C until used.

Primer design and RT-PCR to obtain the full length *AciHBGase* III cDNA

Primer design was based on the cDNA sequences of *AmHBGase III* (accession# NM_001011608) and *AciHBGase III* (accession# EF441271) using the Primer 3 program (http://frodo.wi.mit.edu/primer3/) and checked by eye, so as to encompass the 5' and 3'

outermost regions of *HBGase III*. The forward (F-HBG III) and reverse (R-HBG III) primers had a 5' flanking sequence (italics) containing the *Eco*RI restriction site (underlined) and six repeated codons encoding His (boldface) for the $(His)_6$ N-terminal tag in the forward primer (F-HBG III: 5'-*GGTACATGGAATTC**CATCATCATCATCATCAT***AAGGCGATAATC GTATTTTG-3') and the *Kpn*I restriction site (underlined) in the reverse primer (R-HBG III; 5'-*TTTGGTACCTT*AAAATTTCCAAATTTAGCATC-3').

RT-PCR was performed using an access RT-PCR system kit (catalog# A1250, Promega), as per the manufacturer's protocol. A reaction without the total RNA template and a reaction without reverse transcriptase were used as negative controls. The reaction mixture (25 μL final volume) was comprised of 1x AMV/ T*fl* reaction buffer, 0.2 μM of each dNTP, 0.4 μM of each primer, 1 mM of $MgSO_4$, 0.1 U of AMV reverse transcriptase, 0.1 U of T*fl* DNA polymerase, and 200 ng of RNA template. The RT-PCR reaction was performed under the previously optimized conditions (data not shown) of: 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 2 min; and finally 1 cycle of 68°C for 7 min.

The amplified RT-PCR product was resolved through 1.2% (w/v) agarose-TBE gel electrophoresis and visualized by UV transillumination following EtBr staining. RT-PCR products were then purified using a PCR purification kit (catalog# 28104, Qiagen) as per the manufacturer's protocol, and commercially direct sequenced by Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. The obtained sequence was searched for DNA sequence similarity to those sequences in the NCBI GenBank data base using the BLAST search program, and the DNA sequence identity was used to confirm the likely correct amplification of a *HBGase III* sequence.

Construction of the r(His)$_6$-*Aci*HBGase III chimeric protein encoding plasmid

The amplified RT-PCR product and the pPICZαA expression vector (Invitrogen) were digested by *Eco*RI and *Kpn*I at 37°C overnight, cleaned up by standard phenol/ chloroform extraction and 0.3 M NaOAC/ ethanol precipitation. The concentration of the digested RT-PCR product and the pPICZαA vector was estimated by their respective absorbance at 260 nm. Directional ligation of the RT-PCR product and pPICZαA with *Eco*RI and *Kpn*I compatible ends was performed at a 10:1 molar ratio of PCR product: vector using T4 DNA ligase in 1x T4 DNA ligase buffer at 16°C overnight, and then used to transform *Escherichia coli* DH5α cells, as detailed earlier. Restriction digestion with *Xho*I of the miniprep isolations of selected transformants and direct commercial sequencing was performed as stated.

Transformation of the yeast *P. pastoris*

Before transformation, 5 to 10 μg of recombinant plasmid was digested by *Sac*I at 37°C for 1 h. The *P. pastoris* GS115 (His) strain (Invitrogen) was prepared following the protocol of the Easy Select *Pichia* expression kit (Invitrogen), and then the *Sac*I-linearized plasmid was transformed into *P. pastoris* by electroporation using a Gene Pulser (Bio-Rad) according to the Invitrogen manual (methods for the expression of recombinant protein in *P. pastoris*). The electroporated yeast was spread onto YPDS-agar plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol with 2% (w/v) agar) containing 100 μg/mL Zeocin, and incubated at 30°C for 3 to 10 days until colonies formed. The His autotrophic transformants (His^+) were selected and retained on YPD agar plates (as per YPDS agar but without the sorbitol) for further study.

Expression of r(His)$_6$-*Aci*HBGase III chimera

A single colony of the selected *P. pastoris* transformant was inoculated into 25 mL of BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base, 4 μg/mL D-biotin and 1% (v/v) glycerol in 100 mM potassium phosphate buffer, pH 6.0], and grown at 30°C with shaking at 200 rpm until the O.D. at 600 nm reached 2 to 6. Cells were then harvested by centrifugation (3,000 rpm, RT for 5 min) and transferred into 100 mL of fresh buffered minimal methanol medium (BMMY) medium.

The optimum induction for protein expression was evaluated by the addition of various percentages of methanol [0 to 10% (v/v)] every 24 h and culturing for various periods. At the indicated time point, 1 mL of the induced culture was harvested by centrifugation (200 rpm, 30°C for 15 min) and the cell pellet and culture supernatant were harvested and stored separately at 4°C until assayed. Protein expression was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) resolution with an 8% (w/v) acrylamide resolving gel and Coomassie Brilliant blue staining. Glucosidase activity staining of the renatured proteins in the resolved SDS-PAGE was performed as mentioned later. The protein concentration was measured by Bradford's assay (Bradford, 1976), with detail as follows.

Purification of r(His)$_6$-*Aci*HBGase III

From the obtained optimum protein induction conditions, the transformant culture (100 mL BMMY medium) was induced by 1% (v/v) methanol for 144 h whilst incubated at 30°C with shaking at 200 rpm. The protein content in the supernatant was concentrated by ultracentrifugal filtration (8,000 rpm, 20°C for 15 min) through a 10 kDa molecular weight cut off membrane (Vivaspin 20; catalog# 28-9331-02 AB, GE Healthcare). Then, it was applied at 10 mL (22.35 mg/mL) sequentially to a HisTrap affinity column (catalog# 11-0008-88 AF, GE Healthcare), pre-equilibrated and then washed with 15 mL of 15 x binding buffer (20 mM sodium phosphate and 0.5 M NaCl at pH 7.4), which collecting 1 mL fractions.

The protein was then eluted with 10 mL of 5x elution buffer (binding buffer containing 150 mM imidazole) collecting 1 mL fractions. Fractions were screened for glycosidase activity and protein content using 50 and 15 μL aliquots, respectively, and 1 x protease inhibitor mix (Amresgo) was added to each fraction and stored at 4°C until used. The apparent homogeneity of the purified r(His)$_6$-*Aci*HBGase III was evaluated visually after SDS-PAGE (8% (w/v) acrylamide resolving gel) resolution with Coomassie Brilliant blue staining for proteins or after renaturation and glucosidase enzyme activity staining.

Enzyme assay

Glucosidase activity was monitored using *p*-nitrophenyl α-D-glucoside (PNPG, Sigma) as the substrate. The premix [100 μL of 0.1 M sodium phosphate buffer (pH 5.5), 500 μL of distilled water and 50 μL of 5 mM PNPG] was incubated at 37°C for 10 min. Then, 50 μL of the test fraction or enriched r(His)$_6$-*Aci*HBGase III (7.9 mg/mL) sample was added and the reaction was incubated at 37°C for 10 min before being stopped by the addition of 500 μL of 1 M Na_2CO_3. The control mixture was comprised of 50 μL of 5 mM PNPG, 200 μL of distilled water and 500 μL of 1 M Na_2CO_3. The absorbance at 400 nm of the reaction mixture was used to measure the release of the yellow product of *p*-nitrophenol. One unit (U) of HBGase III was defined as that which liberates 1 μM of D-glucose from PNPG per min at pH 5.5 at 37°C.

Protein determination

Protein concentration in samples was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) at various concentrations (1 to 100 µg/mL) as the calibration standard. In addition, the absorbance at 280 nm was used for monitoring the protein level in chromatography fractions.

Activity stain

After SDS-PAGE, the gel was renatured by incubating in 1.0% (v/v) Triton X - 100 with gentle shaking at RT for 2 h. The gel was then incubated in 10 mM sodium acetate buffer containing 0.5 M sucrose (pH 5.0) at 45°C for 30 min, rinsed by ddH_2O and boiled in 0.5 N NaOH containing 0.1% (w/v) triphenyltetrazolium chloride for 3 min. A red band indicated the region where HBGase activity was detected.

Characterization of the r(His)$_6$-*Aci*HBGase III

Substrate specificity

Evaluation of the substrate specificity followed the same method as the enzyme assay aforestated except that maltose (α1-4 glucose dimer), isomaltose (α1-6 glucose dimer), sucrose (non reducing glucose-fructose dimer), maltotriose (α1-4 glucose trimer), malto-tetraose (α1-4 glucose tetramer) and soluble starch [complex α1-4 (~75-80%) and α1-6 (20-25%) glucose polymer] were used as substrates, in addition to PNPG as a reference substrate. Substrates were evaluated over a concentration range of 1 to10 mM for maltose, 10 to 100 mM for isomaltose, 10 to 100 mM for sucrose, 10 to 90 mM for maltotriose, 10 to 60 mM for soluble starch and 5 to 20 mM for PNPG. The amount of glucose liberated from the substrates (except for PNPG where p-nitrophenol release was measured) was then determined by the glucose oxidase-peroxidase method using a glucose assay kit (catalog# GAGO20-1KT, Sigma) as per the manufacturer's instructions. In brief, 100 µL of the aforestated reaction mixture was mixed with 200 µL of the glucose assay reagent and incubated at 37°C for 30 min before the reaction was stopped by the addition of 200 µL of 12 N H_2SO_4 and the absorbance at 540 nm measured.

Optimum pH for r(His)$_6$-Aci*HBGase III activity and its pH stability (at 4°C)

A reaction mixture containing 50 µL of the enriched r(His)$_6$-*Aci*HBGase III (0.0053 U/mL; 7.9 mg/mL), 50 µL of 5 mM PNPG and 100 µL of Briton-Robinson buffer (40 mM acetic acid, 40 mM phosphoric acid and 40 mM boric acid) in the pH range of 3.0 to 7.5 was prepared (Nishimoto et al., 2001). The reaction mixture was incubated at 37°C for 10 min and then the enzyme assay was performed, monitoring the amount of glucose (or *p*-nitrophenol for PNPG) released as aforementioned. To evaluate the pH stability of the enriched r(His)$_6$-*Aci*HBGase III, the same reaction mixture as for the pH optimum aforestated was prepared but the reaction mixture was first stored at 4°C for 24 h before the enzyme was assayed.

Optimum temperature for r(His)$_6$-Aci*HBGase III activity

The same reaction mixture as for the pH optimum earlier stated was prepared except that 0.1 M sodium phosphate buffer (pH 5.5) containing 0.05% (v/v) Triton X-100 was used instead of the Briton-Robinson buffer. The reaction was then performed at various temperatures (4 to 75°C) and assayed for glucose release (or *p*-nitrophenol for PNPG), as stated earlier.

RESULTS

Construction of the r(His)$_6$-*Aci*HBGase III encoding expression vector

The full length cDNA of the *AciHBGase III* was obtained by RT-PCR, yielding an apparent single amplicon of ~1.8 kbp. The DNA sequence in the predicted coding region was 100% identical to that for the reported *AciHBGase III* cDNA (EF441271), and 99% (1689/1704 bp), 96% (1641/1708 bp) and 96% (1629/1704 bp) identical to the *HBGase III* cDNA from *A. cerana japonica* (FJ889442), *A. mellifera* (NM001011608) and *Apis dorsata* (GU224269), respectively, and with 100% identity for all these species at the predicted amino acid sequence level. An example of multiple alignment between *AciHBGase III* and *AmHBGase III* cDNA is shown in Figure 1. Thus, the obtained sequence is almost certainly the *AciHBGase III* gene.

After directional cloning (*Eco*RI/*Kpn*I directed) of the RT-PCR amplicon into the pPICZαA expression vector and selection of suitable transformants, the subsequent restriction digestion with *Xho*I of the plasmid revealed the correct sized insert, whilst sequencing revealed the complete *AciHBGase III* gene sequence (1,704 bp) in frame with the (His)$_6$ encoding N terminal tag .

Optimum conditions for r(His)$_6$-*Aci*HBGase III expression

P. pastoris GS115 transformants with the in-frame (His)$_6$-tagged *Aci*HBGase III encoding sequence were cultured in BMMY medium and the expression of the recombinant chimeric protein was induced by 1% (v/v) methanol and monitored over time in the yeast cells and the culture medium. 24 h after induction, a high specific glucosidase activity was found in cell lysate (0.72 U/mg) but not in the culture medium. However, thereafter, the specific glucosidase activity in the supernatant increased to a plateau from 72 to 120 h (~0.58 U/mg) before increasing to a maximum at 144 h (0.62 U/mg), whereas that in the cell lysate decreased to a minimum (~0.2 U/mg) from 96 h after induction onwards (Figure 2A). Thus, from 60 h after induction onwards, a higher specific glucosidase activity was found in the culture media than in the yeast cell lysate. In addition, four to six protein bands (45 to 65 kDa) were seen to increase in intensity in the culture media up to ca. 120 h after induction (Figure 2B). These bands with lower molecular mass than the targeted recombinant r(His)$_6$-*Aci*HBGase III of ~ 68 kDa may represent degraded products of the intact enzyme although protease inhibitor was used. The intensity of these bands increased after longer induction. Alternatively, these bands may be other secretory proteins from yeast itself since crude proteins from culture media were used.

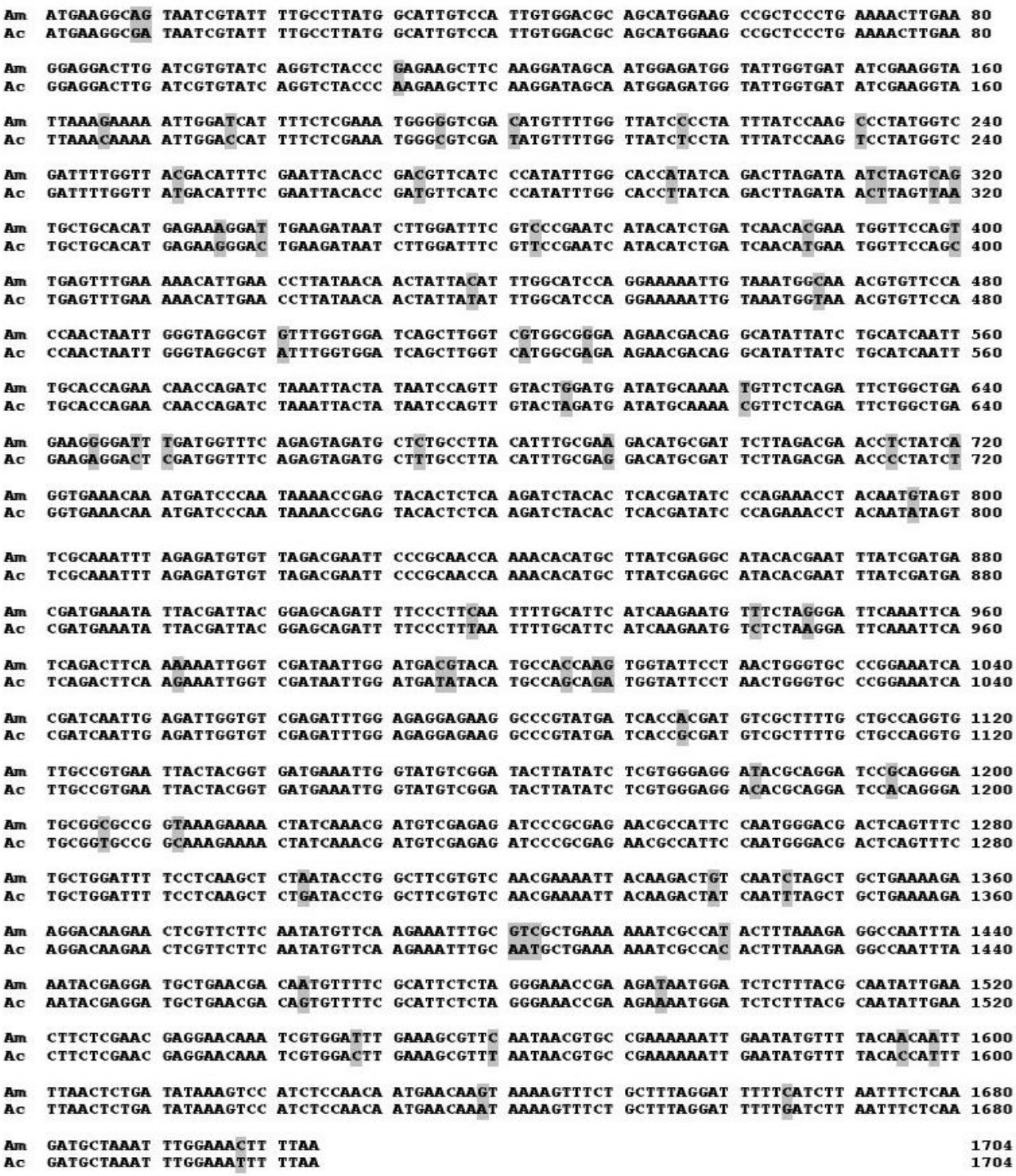

Figure 1. The multiple alignment of *AciHBGase III* and *AmHBGase III* cDNA. Ac = *AcHBGase III*; Am *AmHbgase III.* The grey shadow indicates different base residues within the aligned sequences.

Purification of r(His)$_6$-*Aci*HBGase III

After enrichment of the r(His)$_6$-*Aci*HBGase III protein from the culture media by histrap affinity column chromatography, the enzyme activity in each fraction was assayed using PNPG as the substrate. Among the five unbound fractions, fraction 3 provided the highest glucosidase activity at 0.02 U/mL, while among the 10 bound frac-

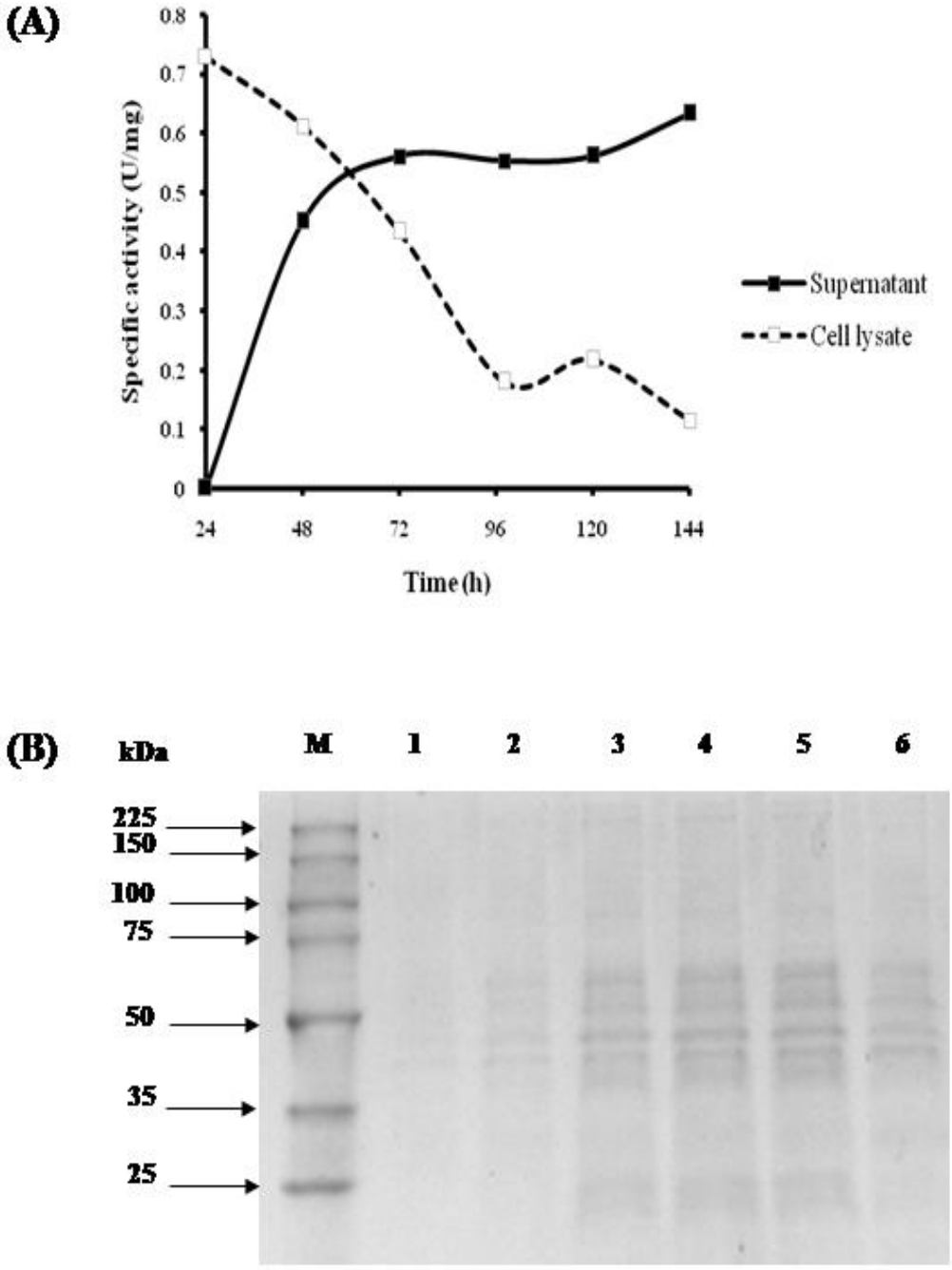

Figure 2. Expression of the methanol-induced r(His)$_6$-*Aci*HBGase III expression. (**A**) Glucosidase activity in the culture media and yeast cell lysate with time since methanol (1% (v/v)) induction. (**B**) Coomassie Brilliant blue stained SDS-PAGE resolution of the culture media proteins with increasing time since methanol induction. Lane M, protein marker; lanes 1 to 6, 2 μg crude protein from the culture media at 24, 48, 72, 98, 120 and 144 h after induction, respectively.

tions, fraction 10 (fifth eluted of the bound fractions) provided a higher glucosidase activity at 0.068 U/mL with a lower likely protein content (A_{280} value) and so likely higher specific activity (Figure 3A). Thus, fraction 10 was selected for characterization of the r(His)$_6$-*Aci*HBGase III enzyme. However, although a single main band of ~68 kDa was present on both the Coomassie Brilliant blue stained SDS-PAGE resolved gel (Figure 3B), and on the glucosidase activity stained gel (Figure 3C), several other protein bands were visible (Figure 3B) and so the enriched r(His)$_6$-*Aci*HBGase III preparation of fraction 10 was not purified to homogeneity.

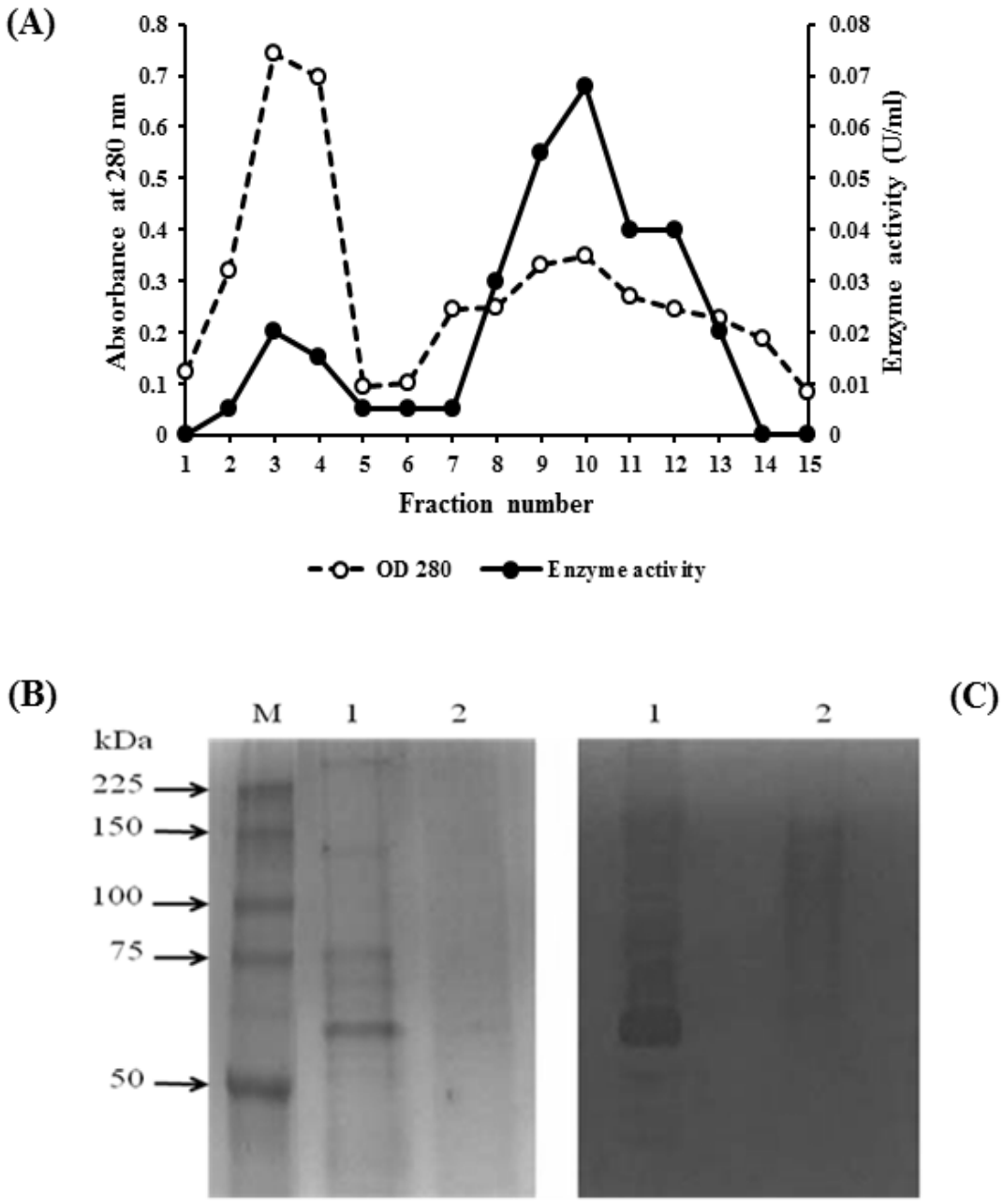

Figure 3. Enrichment of the r(His)$_6$-*Aci*HBGase III. (A) Elution profile from the histrap affinity column, showing the A_{280} (protein level) and glucosidase activity level for the five unbound (fractions# 1-5) and 10 bound (fractions# 6-15) fractions. (**B** and **C**) SDS-PAGE resolved gel of fraction 3 and fraction 10 after staining (**B**) with Coomassie Brilliant blue or (**C**) for glucosidase activity after renaturation. Lane M, protein marker; lane 1, 2 μg of bound fraction# 10; lane 2, 2 μg of unbound fraction 3.

Characterization of the r(His)$_6$-*Aci*HBGase III

The pH and temperature optima and tolerance of the r(His)$_6$-AciHBGase III

The effect of the substrate pH and temperature on the glucosidase activity in the enriched r(His)$_6$-*Aci*HBGase III preparation was measured using PNPG as the substrate. The optimal pH for enzyme activity was found to be 5.0, with > 70% and > 40% residual activity in the pH range of 4.5 to 7.5 and pH 3.0 to 7.5, respectively (Figure 4A). With respect to the enzyme stability at 4°C for 24 h, the enzyme was stable over the pH range of 5.0 to 6.0, with >70% activity at pH 5 to 7.5 but it showed little or no

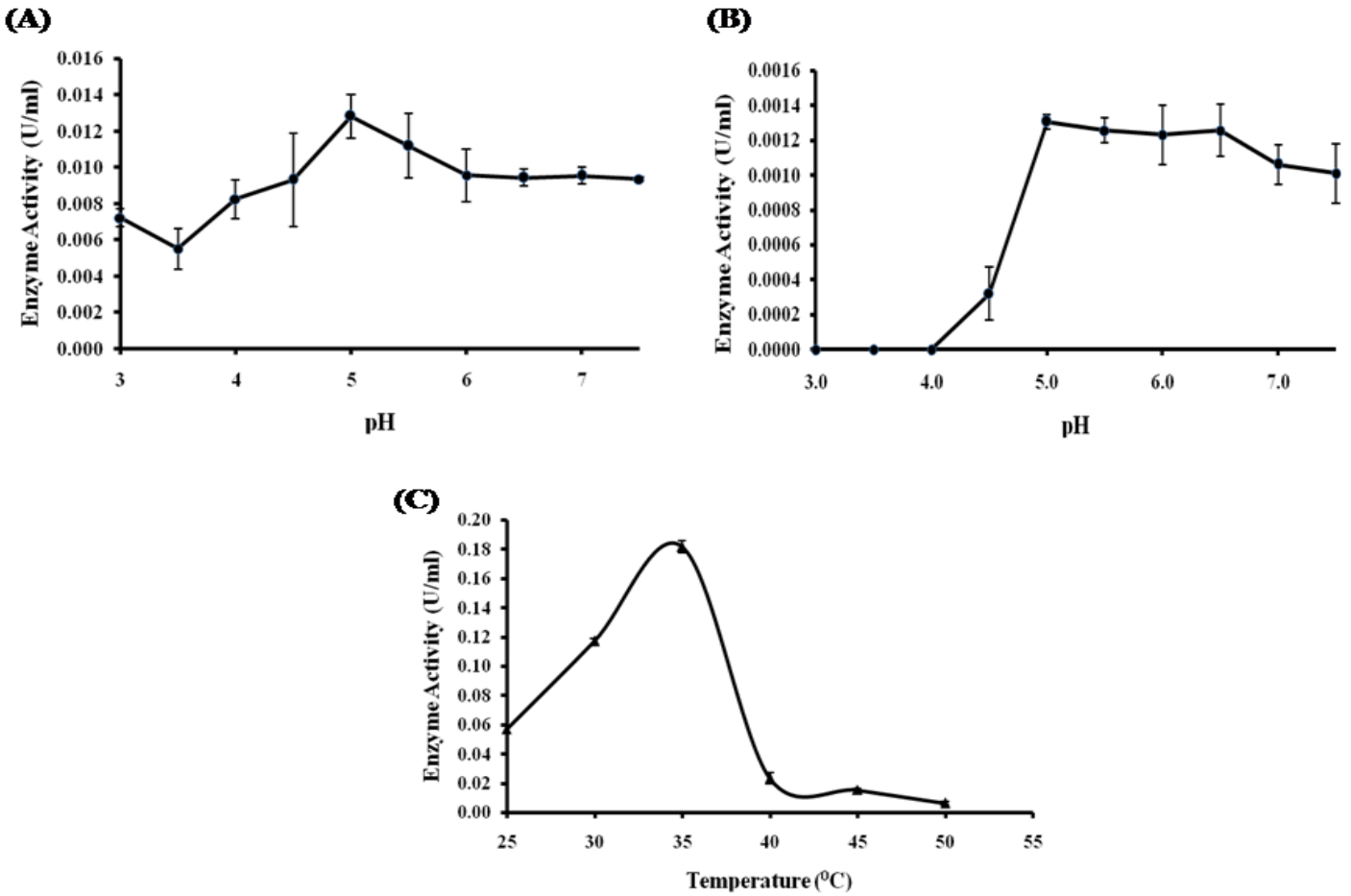

Figure 4. The optimum (A) pH and (B) pH stability of the enzyme to a 24 h pretreatment at 4°C and the (C) temperature for glucosidase activity in the enriched r(His)$_6$-AciHBGase III preparation. All assays used PNPG as the substrate and the data represent the mean ± 1 SD derived from three independent repeats.

stability to prior treatment at pH 4.5 or below, respectively (Figure 4B). For the optimal temperature, the r(His)6-*Aci*HBGase III showed an optimal activity at 37°C, but no thermotolerance with ~33% residual activity at 25°C, and less than 15% and no activity at 40 to 50°C and 55°C, respectively (Figure 4C).

Substrate specificity

The substrate specificity of the enriched r(His)$_6$-*Aci*HBGase III enzyme preparation was examined using six different glucose based substrates at either 5 or 10 mM, in comparison to PNPG for reference (Figure 5). The different substrates varied in their linkage types and polymer sizes, as detailed in the methods section. The results reveal that the best hydrolyzed substrate was sucrose

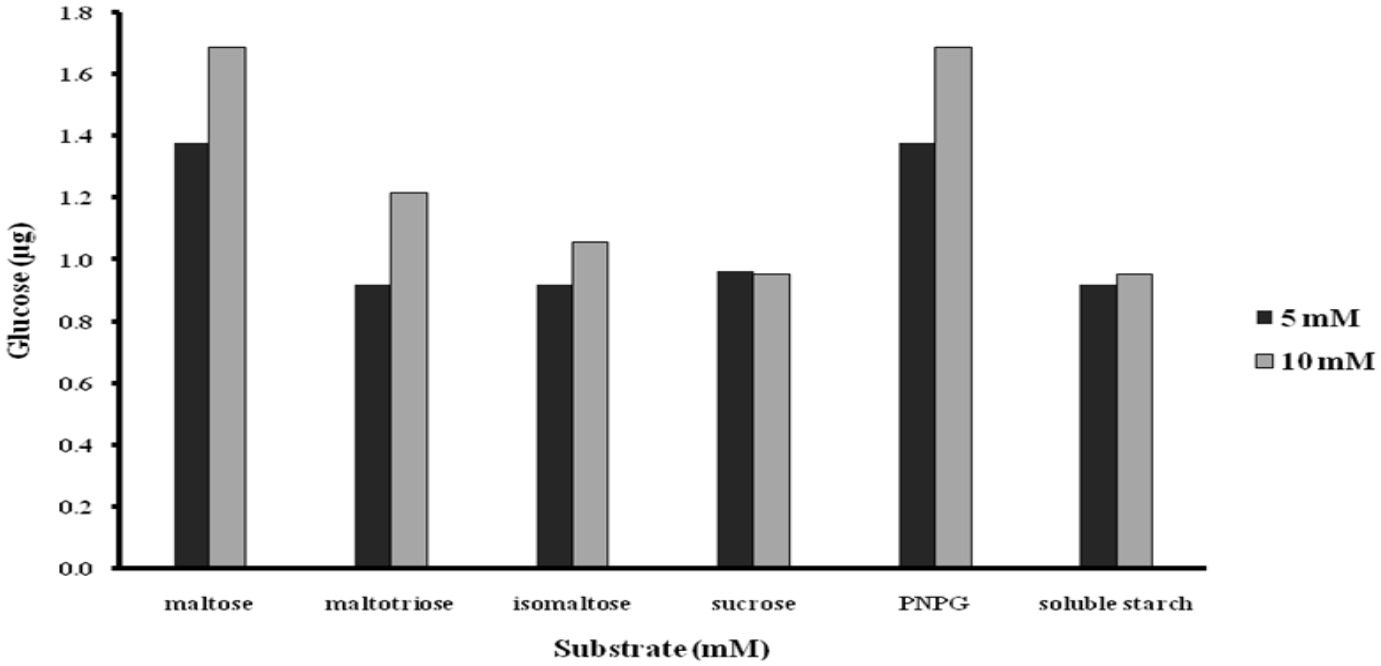

Figure 5. Substrate specificity of the enriched r(His)$_6$-*Aci*HBGase III enzyme preparation with six different glucose containing polymers at 5 mM (black bar) or 10 mM (grey bar) in comparison to the reference PNPG substrate.

followed by maltose and PNPG. At both 5 and 10 mM substrate concentration, the highest glucose yield was obtained from maltose, which was essentially comparable to that of the reference PNPG (in terms of glucose equivalents). Changing the linkage of the two glucose molecules from α(1-4) to α(1-6) in isomaltose resulted in a significant decrease in the amount of glucose released, whilst increasing the α(1-4) linked glucose polymer size to three (maltotriose) or four (maltotetraose) also reduced the amount of released glucose. However, the complex mixture of many units of both α(1-4) and α(1-6) linkages (starch) revealed a similar level of released glucose as that for isomaltose.

Thus, the slight trend of a reduced hydrolysis efficiency with increasing polymer length and with α(1-4) to α(1-6) linkage changes is not universal or strong. Whilst the glucose level released from the non-reducing sugar sucrose was similar to that from starch and slightly lower than isomaltose, it should be bore in mind that an equimolar proportion of fructose as glucose is produced upon hydrolysis and so the number of bonds hydrolyzed is actually higher than that for maltose, at around 1.9 mg of glucose equivalents. Given that the source of this enzyme is from foraging honeybees and so, it is likely to be mainly involved in sucrose cleavage (from nectar) to form glucose and fructose, for honey; this highest bond cleaving activity on sucrose is to be expected. Thus, it is of interest that the r(His)$_6$-*Aci*HBGase III can cleave not only small α(1-4) and α(1-6) linkage glucose units, but also large polymers like starch. With respect to the substrate concentration, except for sucrose, a higher amount of glucose was always obtained with 10 mM substrate than with 5 mM hydrolyzed substrate, as expected. The enzyme kinetics for the enriched r(His)$_6$-*Aci*HB-Gase III enzyme preparation was evaluated using the same six substrates, in comparison to PNPG, over a range of concentrations as mentioned in the materials and methods, and measuring the amount of released glucose (or p-nitrophenol for PNPG) over time. The data were then plotted as standard Michaelis-Menten plots [substrate concentration (s) versus the velocity (v)], and in all cases revealed a hyperbolic curve that reached a plateau, except for when isomaltose was used as the substrate where a plateau was still not reached at the highest tested concentration of 100 mM (Figure 6). The data was then replotted as double reciprocal plots, or Lineweaver-Burk plots (1/*s* versus 1/*v* plots) (Lineweaver and Burk, 1934), where a linear relationship was found on all the substrates (Figure 7). From the intercepts and slope of the best fit linear lines, the estimated kinetic parameters K_m, k_0 and V_{max} for the hydrolysis of each substrate were then derived and are reported in Table 1. The best K_m values were from maltose (4.5 mM) and PNPG (4.4 mM).

DISCUSSION

After the complete sequence and partial annotation of the genome, *A. mellifera* was released (The Honeybee Genome Sequencing Consortium, 2006); it allowed, by standard molecular approaches including degenerate PCR, the rapid derivation of the nucleotide and assumed amino acid sequences of genes designated to be of

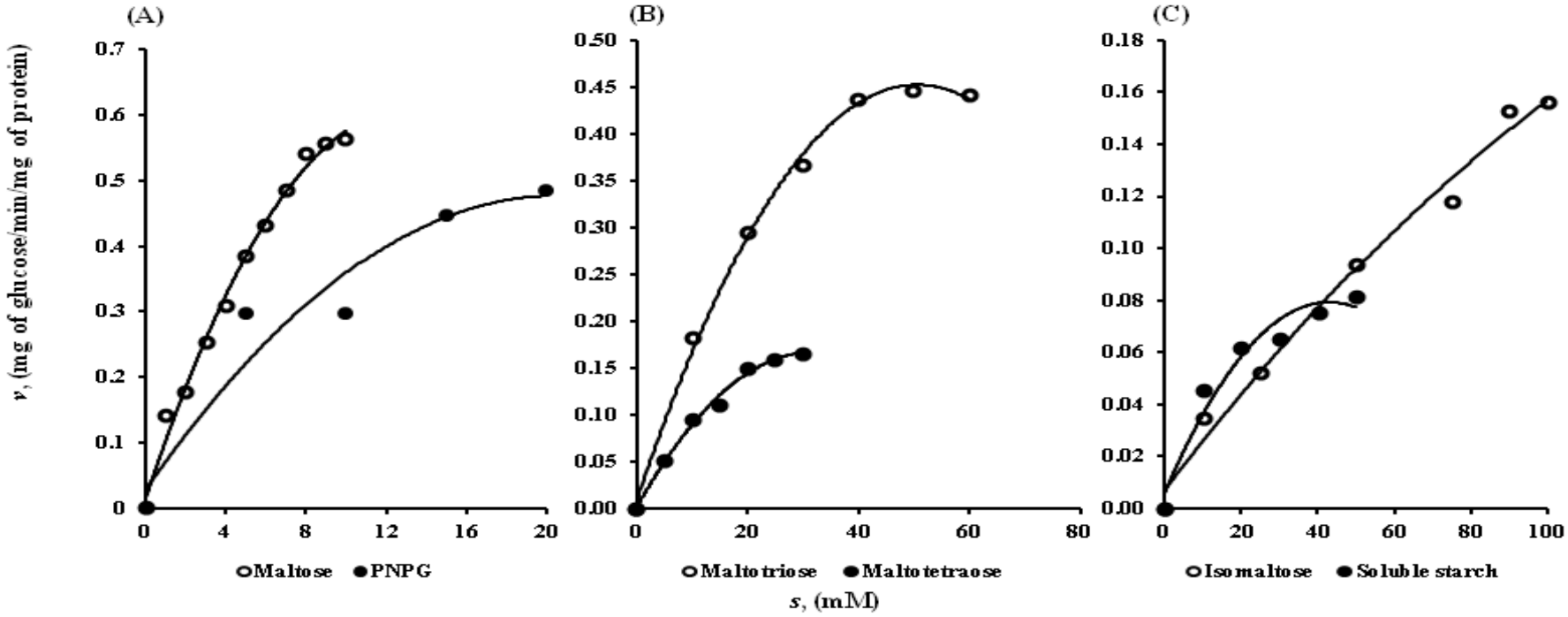

Figure 6. Michaelis-Menten plots (*s* versus *v*) for the enriched r(His)$_6$-*Aci*HBGase III enzyme with (A) PNPG and maltose, (B) maltotriose and maltotetraose and (C) soluble starch and isomaltose, as substrates.

interest in other *Apis* sp. For example, with respect to HBGase III, the nucleotide and predicted amino acid sequences of the *AmHBGase III* (GenBank, accession# D79208.1), and those from *A. cerana japonica* (GenBank, accession# FJ889442), *A. dorsata* (GenBank, accession# GU224269) and *Apis florea* (GenBank, accession# EF586680) are now available. This brings the benefit of allowing homologous and functional analysis across different species for each gene of interest, as well as in improving the design of degenerative PCR primers to amplify the homolog's from other more distant species or across less conserved regions. However, in eukaryotes, the targeted protein is not always readily deduced from the DNA sequence due to variations in intron-exon junction recognition and alternative splicing, as examples, and so the open reading frame (ORF) is usually analyzed from the corresponding cDNA sequences.

In this research, forager bees were selected for use due to the fact that this developmental stage has the highest expression level of *HBGase III*. Here, to allow post-translational glycosylation, we used the yeast *P. pastoris* rather than *E. coli* as the recombinant host, as this has been reported to be a suitable system before (Chen et al., 2010). The estimated molecular mass of the r(His)$_6$-*Aci*HBGase III, at a little bit higher than 68 kDa, is a little over 6.5% higher than the predicted mass from the deduced amino acid sequence (allowing for the (His)$_6$ tag), and this may then be due to the glycosylation of the protein. In accord, the *AmHBGase III* homolog is a glycoprotein with a sugar content of 7.4% by weight.

The utilization of the pPICZαA expression vector provided an N terminal (His)$_6$ sequence on the r*Aci*HBGase III protein allowing histrap affinity column enrichment. Any role of this N-terminal (His)$_6$ sequence on the stability and kinetics of the *Aci*HBGase III enzyme has not yet been evaluated. For large (commercial) scale produc-

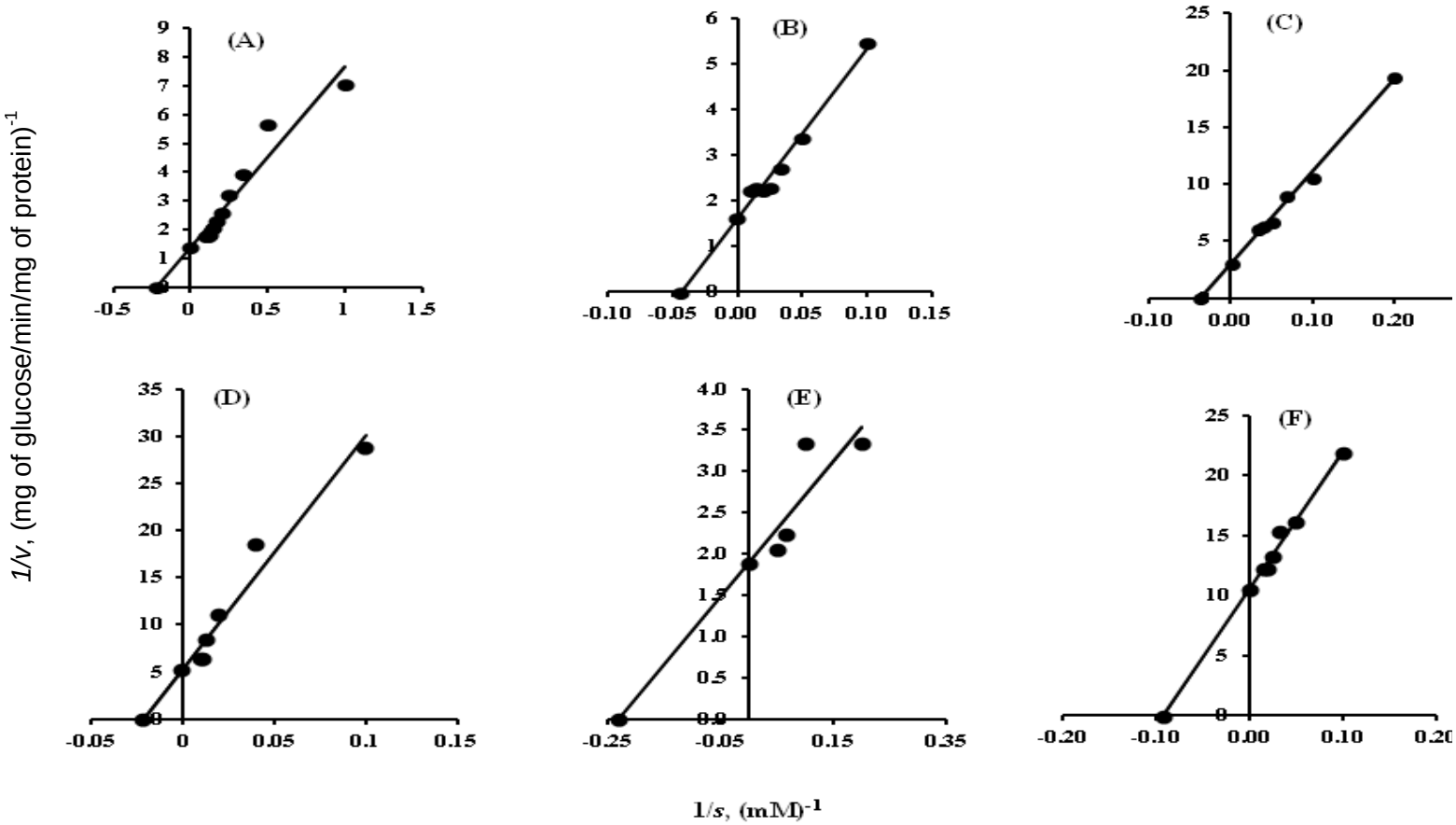

Figure 7. Lineweaver-Burk plots for the enriched r(His)$_6$-*Aci*HBGase III enzyme with (A) maltose, (B) maltotriose, (C) maltotetraose, (D) isomaltose, (E) PNPG and (F) soluble starch, as substrates.

Table 1. Kinetic parameters for the hydrolysis of different substrates by the r(His)$_6$-*Aci*HBGase III.

Substrate	K_m (mM)	k_0 (s^{-1})	V_{max} (µmol/min/mg protein)	k_0/K_m ($mM^{-1}\cdot s^{-1}$)
PNPG	4.4	3.3	3.0	0.76
Maltose	4.5	4.5	4.0	1.01
Isomaltose	46.5	1.2	1.0	0.03
Maltotriose	23	3.9	3.4	0.17
Maltotetraose	26.6	2.1	1.8	0.08

tion of r*Aci*HMGase III, where the carbohydrate and $(His)_6$ tag might also affect the purification procedure (Nishimoto et al., 2001), fractionation over DEAE-Sepharose CL-6B, Bio-Gel P-150, CM-Toyopeal 650 M and Sephacryl S-100 may be more suitable (Takewaki et al., 1993; Nishimoto et al., 2001; Wongchawalit et al., 2006; Nishimoto et al., 2007). The previously reported activity of the native *Aci*HBGase III enzyme (Chanchao et al., 2008) and the r$(His)_6$-*Aci*HBGase III form (this study) were assayed using different methods, making direct comparisons equivocal. The native form was assayed by Momose's method (Momose and Inaba, 1961) and the recombinant form with PNPG as the substrate, respectively. However, the same pH optimum for enzyme activity (pH 5.0) was revealed, whilst the optimal temperature was different, being 50°C for the native form of *Aci*HBGase III compared to 35°C and with no thermal stability for the r$(His)_6$-*Aci*HBGase III form.

The enriched r$(His)_6$-*Aci*HBGase III showed normal Michaelis-Menten type reaction kinetics on all tested substrates including the complex polysaccharide soluble starch, in agreement with previous reports (Chiba, 1997, 1998). The substrate specificity was relatively high in maltose (K_m of 4.5) and PNPG (K_m of 4.4 mM) but was still lower than the α-glucosidase from barley endosperm (K_m of 1.7-2.4 mM), which is used during starch formation (Naested et al., 2006). However, comparing the K_m of r$(His)_6$-*Aci*HBGase III, in this research, and of the rHBGase III from *A. mellifera* (r-*Am*HBGase III) (Nishimoto et al., 2007), the recombinant enzyme in this research showed a higher substrate specific activity in maltose, maltotriose and PNPG.

It can be concluded that r$(His)_6$-*Aci*HBGase III can be expressed *in vitro* and enriched from the *P. pastoris* yeast expression system. Although α-glucosidases have been recognized as important enzymes in the carbohydrate industry, challenges still exist to explore more α-glucosidases with better activity, either transglycosylation or product hydrolysis. Since we are interested in HBGases in honeybees, in the future, the expression and primary structure of *Aci*HBGase I and II will be reported.

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REFERENCES

Bamforth CW (2009). Current perspectives on the role of enzymes in brewing. J. Cereal Sci. 50:353-357.

Bradford MM (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Chanchao C, Pilalam S, Sangvanich P (2008). Purification and characterization of α-glucosidase in *Apis cerana indica*. Insect Sci. 15:217-224.

Chen DL, Tong X, Chen SW, Chen S, Wu D, Fang SG, Wu J, Chen J (2010). Heterogenous expression and biochemical characterization of α-glucosidase from *Aspergillus niger* by *Pichia pastoris*. J. Agr. Food Chem. 58: 4819-4824.

Chen YW, Wang CH, An J, Ho KK (2000). Susceptibility of the Asian honeybee, *Apis cerana*, to American foulbrood, *Paenibacillus larvae larvae*. J. Apicult. Res. 39:169-175.

Chiba S (1997). Molecular mechanism in α-glucosidase and glucoamylase. Biosci. Biotechnol. Biochem. 61:1233-1239.

Chiba S (1998). α-glucosidase. In: The Amylase Research Society of Japan (eds) Handbook of Amylases and Related Enzymes. Oxford: Pergamon Press, pp. 104-116.

Gillmor CS, Poindexter P, Lorieau J, Palcic MM, Somerville C (2002). α-Glucosidase I is required for cellulose biosynthesis and morphogenesis in Arabidopsis. J. Cell Biol. 156:1003-1013.

Kato N, Suyama S, Shirokane M, Kato M, Kobayashi T, Tsukagoshi N (2002). Novel α-glucosidase from *Aspergillus nidulans* with strong transglycosylation activity. Appl. Environ. Microbiol. 68:1250-1256.

Kimura A (2000). Molecular anatomy of α-glucosidase. Trends Glycosci. Glyc. 12: 373-380.

Kubota M, Tsuji M, Nishimoto M, Wongchawalit J, Okuyama M, Mori H, Matsui H, Surarit R, Svasti J, Kimura A, Chiba S (2004). Localization of α-glucosidase I, II and III in organs of European honeybees, *Apis mellifera* L., and the origin of α-glucosidase in honey. Biosci. Biotechnol. Biochem. 68:2346-2352.

Lineweaver H, Burk D (1934). The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658-666.

Momose T, Inaba A (1961). Mechanism of the color reaction of 3,6-dinitrophthalic acid with reducing sugars. Chem. Pharm. Bull. 9:263-266.

Moreland RJ, Higgins S, Zhou A, VanStraten P, Cauthron RD, Brem M, McLarty BJ, Kudo M, Canfield WM (2012). Species-specific differences in the processing of acid α-glucosidase are due to the amino acid identity at position 201. Gene 491:25-30.

Naested H, Kramhoft B, Lok F, Bojsen K, Yu S, Svensson B (2006). Production of enzymatically active recombinant full-length barley high pI α-glucosidase of glycoside family 31 by high cell-density fermentation of *Pichia pastoris* and affinity purification. Protein Expres. Purif. 46:56-63.

Nishimoto M, Kubota M, Tsuji M, Mori H, Kimura A, Matsui H, Chiba S (2001). Purification and substrate specificity of honeybee, *Apis mellifera* L., α-glucosidase III. Biosci. Biotechnol. Biochem. 65:1610-1616.

Nishimoto M, Mori H, Moteki T, Takamura Y, Iwai G, Miyaguchi Y, Okuyama M, Wongchawalit J, Surarit R, Svasti J, Kimura A, Chiba S (2007). Molecular cloning of cDNAs and genes for three α-glucosidase from European honeybee, *Apis mellifera* L. and heterologous production of recombinant enzymes in *Pichia pastoris*. Biosci. Biotechnol. Biochem. 71:1703-1716.

Ohashi K, Sawata M, Takeuchi H, Natori S, Kubo T (1996). Molecular cloning of cDNA and analysis of expression of the gene for α-glucosidase from the hypopharyngeal gland of the honeybee *Apis mellifera* L. Biochem. Bioph. Res. Co. 221:380-385.

Oldroyd BP, Wongsiri S (2006). Asian Honey Bees: Biology, Conservation and Human Interactions. Harvard University Press.

Pal S, Banik SP, Ghorai S, Chowdhury S, Khowala S (2010). Purification and characterization of a thermostable intra-cellular β-glucosidase with transglycosylation properties from filamentous fungus *Termitomyces clypeatus*. Bioresour. Technol. 101:2412-2420.

Peng CYS, Fang Y, Xu S, Ge L (1987). The resistance mechanism of the Asian honeybee, *Apis cerana* Fabr., to an ectoparasitic mite, *Varroa jacobsoni* Oudemans. J. Invertebr. Pathol. 49:54-60.

Perugino G, Trincone A, Rossi M, Moracci M (2004). Oligosaccharide synthesis by glycosynthases. Trends Biotechnol. 22:31-37.

Schmidt S., Rainieri S., Witte S, Matern U, Martens S (2011). Identifica-

tion of a *Saccharomyces cerevisiae* glucosidase that hydrolyzes flavonoid glucosides. Appl. Environ. Microb. 77:1751-1757.
Stanley D, Rejzek M, Naested H, Smedley M, Otero S, Fahy B, Thorpe F, Nash RJ, Harwood W, Svensson B, Denyer K, Field RA, Smith AM (2011). The role of α-glucosidase in germinating. Plant Physiol. 155:932-943.
Stark JR, Yin XS (1987). Evidence for the presence of maltase and α-glucosidase isoenzymes in barley. J. I. Brewing. 93:108-112.
Takewaki S, Kimura A, Kubota M, Chiba S (1993). Substrate specificity and subsite affinities of honeybee α-glucosidase II. Biosci. Biotechnol. Biochem. 57:1508-1513.
The Honeybee Genome Sequencing Consortium (2006). Insights into social insects from the genome of the honeybee *Apis mellifera. Nature*. 443:931-949.
Wongchawalit J, Yamamoto T, Nakai H, Kim YM, Sato N, Nishimoto M, Okuyama M, Mori H, Saji O, Chanchao C, Wongsiri S, Surarit R, Svasti J, Chiba S, Kimura A (2006). Purification and characterization of α-glucosidase I from Japanese honeybee (*Apis cerana japonica*) and molecular cloning of its cDNA. Biosci. Biotechnol. Biochem. 70:2889-2898.
Zhao X, Qu Y, Gao P (1993). Acceleration of ethanol production from paper mill waste fiber by supplementation with β-glucosidase. Enzyme Microb. Tech. 15:62-65.