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

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Recombinant α-glucosidase III (rHBGase III) from Apis cerana indica Fabricus (rAciHBGase 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 rAciHBGase 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 thermostolerance with < 10% and 0% residual activity at 40 and >50°C, respectively. The rAciHBGase showed a relatively high substrate specificity for maltose (Kₘ of 4.5 mM) and p-nitrophenyl α-D-glucoside (Kₘ 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-glucosi-

dase) 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 glycol-sylation 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
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 (AmHBGase III) (Kubota et al., 2004).

Thus, AmHBGase 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 AmHBGase 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)AmHBGase 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 rAmHBGase 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-Toyopearl 650 M column chromatography (Nishimoto et al., 2007).

Other than A. mellifera, native HBGase III has been purified from Apis cerana indica (AciHBGase 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 rAciHBGase 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 rAciHBGase 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-guanidinium 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 EcoRI 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' GGTACATGGAATTCATCATCATCATCATCATGTTTGGATATG-3') and the KpnI restriction site (underlined) in the reverse primer (R-HBG III: 5'- GGTACCTTAAATTCTCAATTAGCTAC-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/Taq reaction buffer, 0.2 μM of each dNTP, 0.4 μM of each primer, 1 mM of MgSO4, 0.1 U of AMV reverse transcriptase, 0.1 U of Taq 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-AcHGBase III chimeric protein encoding plasmid

The amplified RT-PCR product and the pPICZαA expression vector (Invitrogen) were digested by EcoRI and KpnI 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 EcoRI and KpnI 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 Xhol 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 SacI 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 SacI-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 YPDSagar 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-AcHGBase 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-AcHGBase 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 (Amresco was added to each fraction and stored at 4°C until used. The apparent homogeneity of the purified r(His)6-AcHGBase 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 ρ-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-AcHGBase 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 Na2CO3. The control mixture was comprised of 50 μL of 5 mM PNPG, 200 μL of distilled water and 500 μL of 1 M Na2CO3. The absorbance at 400 nm of the reaction mixture was used to measure the release of the yellow product of ρ-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 ddH2O 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-AciHBGase III

Substrate specificity

Evaluation of the substrate specificity followed the same method as the enzyme assay aforementioned except that maltose (α1-4 glucose dimer), isomaltose (α1-6 glucose dimer), sucrose (non reducing glucose-fructose dimer), maltotriose (α1-4 glucose trimer), maltotetraose (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 to 100 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# GAGO201KT, Sigma) as per the manufacturer's instructions. In brief, 100 μL of the aforementioned 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 H2SO4 and the absorbance at 540 nm measured.

Optimum pH for r(His)6-AciHBGase III activity and its pH stability (at 4°C)

A reaction mixture containing 50 μL of the enriched r(His)6-AciHBGase 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-AciHBGase III, the same reaction mixture as for the pH optimum aforementioned 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-AciHBGase 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-AciHBGase 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 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 (FJ899442), A. mellifera (NM001011608) andApis 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 AmyHBGase III cDNA is shown in Figure 1. Thus, the obtained sequence is almost certainly the AciHBGase III gene.

After directional cloning (EcoRI/KpnI directed) of the RT-PCR amplicon into the pPICZαA expression vector and selection of suitable transformants, the subsequent restriction digestion with XhoI 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-AciHBGase III expression

P. pastoris GS115 transformants with the in-frame (His)6-tagged AciHBGase 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 in the cell lysate increased 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-AciHBGase 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.
Purification of r(His)_6-AciHBGase III

After enrichment of the r(His)_6-AciHBGase 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)$_6$-AciHBGase 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)$_6$-AciHBGase 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)$_6$-AciHBGase III preparation of fraction 10 was not purified to homogeneity.
Characterization of the r(His)$_6$-AciHBGase 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)$_6$-AciHBGase 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 3. Enrichment of the r(His)$_6$-AciHBGase 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.
stability to prior treatment at pH 4.5 or below, respectively (Figure 4B). For the optimal temperature, the r(His)6-AciHBGase III showed an optimal activity at 37°C, but no thermostolerance 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-AciHBGase 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 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.
Figure 5. Substrate specificity of the enriched r(His)$_6$-AciHBGase 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 borne 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)$_6$-AciHBGase 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)$_6$-AciHBGase 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 were 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)$_6$-AcHBGase 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)$_6$-AcHBGase 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 pPICZ$\alpha$A expression vector provided an N terminal (His)$_6$ sequence on the rAcHBGase III protein allowing histrap affinity column enrichment. Any role of this N-terminal (His)$_6$ sequence on the stability and kinetics of the AcHBGase III enzyme has not yet been evaluated. For large (commercial) scale produc-
Figure 7. Lineweaver-Burk plots for the enriched r(His)$_6$-AciHBGase 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)$_6$-AciHBGase III.

<table>
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<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_0$ (s$^{-1}$)</th>
<th>$V_{max}$ (µmol/min/mg protein)</th>
<th>$k_0/K_m$ (mM$^{-1}$·s$^{-1}$)</th>
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<tr>
<td>PNPG</td>
<td>4.4</td>
<td>3.3</td>
<td>3.0</td>
<td>0.76</td>
</tr>
<tr>
<td>Maltose</td>
<td>4.5</td>
<td>4.5</td>
<td>4.0</td>
<td>1.01</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>46.5</td>
<td>1.2</td>
<td>1.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>23</td>
<td>3.9</td>
<td>3.4</td>
<td>0.17</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>26.6</td>
<td>2.1</td>
<td>1.8</td>
<td>0.08</td>
</tr>
</tbody>
</table>
tion of rAcHMGase 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-Toyopearl 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 AcHGBase III enzyme (Chanchao et al., 2008) and the r(His)$_6$-AcHGBase 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 AcHGBase III compared to 35°C and with no thermal stability for the r(His)$_6$-AcHGBase III form.

The enriched r(His)$_6$-AcHGBase 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$_m$ 1.7-2.4 mM), which is used during starch formation (Naested et al., 2006). However, comparing the K$_m$ of r(His)$_6$-AcHGBase III, in this research, and of the rHGBase III from A. mellifera (r-AmHGBase 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)$_6$-AcHGBase 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 AcHGBase I and II will be reported.

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