Expression of a secretory α-glucosidase II from Apis cerana indica in Pichia pastoris and its characterization

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Abstract

Background: α–glucosidase (HBGase) plays a key role in hydrolyzing α-glucosidic linkages. In Apis mellifera, three isoforms of HBGase (I, II and III) have been reported, which differ in their nucleotide composition, encoding amino acid sequences and enzyme kinetics. Recombinant (r)HBGase II from A. cerana indica (rAciHBGase II) was focused upon here due to the fact it is a native and economic honeybee species in Thailand. The data is compared to the two other isoforms, AciHBGase I and III from the same bee species and to the three isoforms (HBGase I, II and III) in different bee species where available.

Results: The highest transcript expression level of AciHBGase II was found in larvae and pupae, with lower levels in the eggs of A. cerana indica but it was not found in foragers. The full-length AciHBGase II cDNA, and the predicted amino acid sequence it encodes were 1,740 bp and 579 residues, respectively. The cDNA sequence was 90% identical to that from the HBGase II from the closely related A. cerana japonica (GenBank accession # NM_FJ752630.1). The full length cDNA was directionally cloned into the pPICZα A expression vector in frame with a (His)6 encoding C terminal tag using EcoRI and KpnI compatible ends, and transformed into Pichia pastoris. Maximal expression of the rAciHBGase II–(His)6 protein was induced by 0.5% (v/v) methanol for 96 h and secreted into the culture media. The partially purified enzyme was found to have optimal α-glucosidase activity at pH 3.5 and 45°C, with > 80% activity between pH 3.5–5.0 and 40–55°C, and was stable (> 80% activity) at pH 4–8 and at < 25–65°C. The optimal substrate was sucrose.

Conclusions: Like in A. mellifera, there are three isoforms of AciHBGase (I, II and III) that differ in their transcript expression pattern, nucleotide sequences and optimal enzyme conditions and kinetics.

Keywords: Apis cerana indica, α–glucosidase, Expression, Homology, Recombinant enzyme

Background

α–Glucosidases belong to the glycosyl hydrolase family (EC 3.2.1.20, HBGase) and catalyze the hydrolysis of non reducing terminals of substrates, such as sugars like sucrose and maltose and other glucosides including phenyl α–glucoside, to liberate α-glucose [1]. Based on their primary structure (amino acid sequences), members of Family 13 (GH13) have four regions that are important in their catalytic action [2,3]. In contrast, HBGase II enzymes do not have these four regions and belong to members of Family 31 (GH31) [4]. According to X-ray crystallographic analysis, the tertiary structures of both HBGase I and II enzymes from the bacteria Bacillus cereus and Sulfolobus sulphataricus, respectively, have a (β/α)8 barrel structure as the catalytic domain but they differ in the features of the active sites involved in the catalytic reactions [5,6].

Three isoforms of HBGase have been reported in A. mellifera (AmHBGase I, II and III), which differ in their substrate specificity, mass, nucleotide and predicted amino acid sequences, and expression patterns in different tissues and developmental stages.
Homologs of these three \textit{AmpHBGase} isoforms (I, II and type II-like HBGases) have also been reported in the yeast \textit{Saccharomyces cerevisiae}, where they were found to be located in the endoplasmic reticulum and to be involved in processing of the N-glycan core for glycoprotein biosynthesis [12]. However, these three yeast homologs differ in their molecular mass and biochemical characters.

In the biotechnology industry, HBGases are key enzymes for the preparation of alcoholic beverages and brewing, such as in the wine and sake industry. Indeed, the HBGase in rice (\textit{Oryza sativa}) is directly involved in the alcohol fermentation process in sake processing. Thus, Iwata et al. [13] purified the HBGase from \textit{O. sativa} Yamadashikij and reported that the enzyme had a different substrate specificity (nigeroside) from the HBGase I (maltotriose and maltotetraose) purified from \textit{O. sativa} cv. Shinsetsu, and also hydrolyzed nigeroside and kojibiose better than the HBGase II from \textit{O. sativa} cv. Shinsetsu.

Furthermore, Michlmayr et al. [14] reported that the aroma of wine was in part due to the action of glycosidases from \textit{Oenococcus oeni}, a wine-related lactic acid bacterium, since the precursors of the volatile monoterpenes based constituents (the primary grape aroma) were monoglucosides and diglucosides that were cleaved by the HBGase releasing the volatile monoterpenes.

Other than catalyzing the cleavage of \(\alpha\)-glucosyl residue substrates, HBGase can also catalyze transglycosylation reactions to synthesize various \(\alpha\)-glucosylated compounds. Accordingly, this enzyme-catalyzed transglycosylation is used in the biosynthesis of carbohydrates that are important for humans [15]. Zhou et al. [16] purified a novel extracellular \(\alpha\)-glucosidase II (210 kDa) with a high transglycosylation activity from \textit{Arthrobacter} sp. DL001. This enzyme could transfer glucosyl groups from donors containing an \(\alpha\)-(1,4)-glucosidic bond specific to glucosides, xylosides and alkyl alcohols in \(\alpha\)-(1,4)- or \(\alpha\)-(1,6)-linkages.

Thus, it is of relevance to find new sources of HBGases with different enzyme properties, like the conformational stability to heat, pH and denaturants, a wide range of or different specific substrate specificities, catalysis of transglycosylation reactions, and enzymatic synthesis of novel oligosaccharides, so as to better fit the specific requirements needed by each respective application. Accordingly, HBGase enzymes have been purified from many organisms, but especially from microorganisms [17]. Cihan et al. [17] successfully purified HBGases from \textit{Geobacillus} sp. HTA-35, originally isolated from a deep sea sediment. The mimic glycolipids produced could be used as biosurfactants and materials in a diverse array of biological applications by changes to their physicochemical properties.

In this research, the transcript expression pattern and sequence determination of the \textit{HBGase II} from \textit{Apis cerana indica} (\textit{AciHBGase II}) together with the expression, purification and characterization of the \textit{rAciHBGase II} enzyme (as a C-terminal (His)\textsubscript{6} tagged chimera) activity was evaluated. To our knowledge, this is the first report of the \textit{AciHBGase} gene and recombinant enzyme from this Thai native and economic honeybee species, although the native form has not yet been purified and characterized. The outcome from this research is a potential new source of HBGase II that may be applied in the glucose related industries.

**Methods**

**Sample collection**

\textit{A. cerana indica} were collected from an apiary in Samut Songkram province. Eggs, larvae and pupae were collected directly from hives while foragers were collected from the returning flight at the entrance area of the hive. Samples were kept at -80°C until used.

**RNA extraction**

Samples (eggs, larvae, pupae and forager bees) were taken from -80°C storage and were separately ground with a pestle in a mortar under liquid nitrogen and then total RNA was extracted from the ground samples using a standard acid-guanidine thiocyanate-phenol-chloroform method [19]. The quality of RNA was visually assayed under UV-transillumination after resolution by 1.2% (w/v) formaldehyde/ agarose gel electrophoresis and ethidium bromide (EtBr) staining. After that, poly A\(^+\) mRNA was isolated using the oligotex (dT)\textsubscript{30} super kit (catalog # 9086, Takara) as per the manufacturer’s instructions. To monitor the RNA concentration and its relative purity, the absorbance at 260 and 280 nm was measured.
Transcript expression pattern and sequence analysis of AciHBGase II

Primer design was based on the cDNA sequence of AmiHBGase II (GenBank accession # NM_001040259). All primers (Table 1) were designed using the Primer 3 program (http://frodo.wi.mit.edu/primer3/) and manually checked. In addition, as a template control, a 350 bp fragment of the 28S rDNA gene was amplified using the primers shown in Table 1. RT-PCR was performed using an Access RT-PCR system kit (catalog # A1250, Promega) as per the supplier’s instructions, and reactions without the RNA template or without reverse transcriptase were used as negative controls. For analysis of the AciHBGase II transcript expression pattern, the extracted total RNA from eggs, larvae, pupae and foragers was used, while for sequence analysis the total RNA extracted from the pupae was used as the RT-PCR template. The reaction mixture (25 μl final volume) was comprised of 1 x AMV / Tfl reaction buffer, 0.2 μM of each dNTP, 0.4 μM of each forward (F) and reverse (R) primer, 1 mM MgSO4, 0.1 units (U) of AMV reverse transcriptase, 0.1 U Tfl DNA polymerase and 200 ng of RNA template. All RT-PCR reactions were performed under previously optimized conditions as follows: 1 cycle of 48°C for 45 min and 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 expected RT-PCR product was observed by UV transillumination after resolution through a 1% (w/v) agarose-TBE gel and EtBr staining. The desired RT-PCR product was isolated and ligated into pPICZαA (Invitrogen), which had been separately digested by EcoRI and KpnI at 37°C overnight, using a 3:1 (w/w) ratio of PCR product: vector and 1 U T4 ligase in 1 x T4 ligase buffer (6 μl total volume) at 16°C overnight, and then transformed to P. pastoris. Note that this construct places an in-frame C-terminal (His)6 tag onto the encoded protein, so in this case a rAcIHBGase II-(His)6 chimeric enzyme is encoded for.

Construction of the rAciHBGase II-(His)6 encoding expression vector for P. pastoris

In order to amplify the full-length AciHBGase II cDNA the F and R PCR primers were designed to encompass the 5’ and 3’ outermost regions of the AciHBGase II, based upon the sequences for the related AmiHBGase II (HBGase II (5’ CAAAATGAATTCTTCTCCAGACGGACGAATTTAGTTTGCTTGTG) and AmiHBGase IIR (5’ CGAAGATACCTCAGCAGCGCAAAATGGAATTCTTCTCCAGACGGACGAATTTAGTTTGCTTGTG). Note that to facilitate the directional cloning the F and R primers contained 5’ flanking extension sequences to yield EcoRI and KpnI restriction sites (underlined), respectively. The RT-PCR reaction was performed under previously optimized conditions as follows: 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 expected RT-PCR product was observed by UV transillumination after resolution through a 1% (w/v) agarose-TBE gel and EtBr staining. The desired RT-PCR product was isolated and ligated into pPICZαA (Invitrogen), which had been separately digested by EcoRI and KpnI at 37°C overnight, using a 3:1 (w/w) ratio of PCR product: vector and 1 U T4 ligase in 1 x T4 ligase buffer (6 μl total volume) at 16°C overnight, and then transformed to P. pastoris. Note that this construct places an in-frame C-terminal (His)6 tag onto the encoded protein, so in this case a rAciHBGase II-(His)6 chimeric enzyme is encoded for.

Transformation of P. pastoris

Before transformation, 5–10 μg of the recombinant plasmid was digested by SacI at 37°C for 1 h. P. pastoris GS115 (His) strain (Invitrogen) was prepared following the protocol of the EasySelect Pichia expression kit (catalog # K1740-01, Invitrogen). The SacI-linearized plasmid was transformed into P. pastoris by electroporation using Gene Pulser (Bio-Rad), as per the recommendation of the Invitrogen manual (Methods for the expression of recombinant protein in P. pastoris). The electroporated yeast were spread onto YPDS plates (YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 2% (w/v) agar,) with 1 M sorbitol) containing 100 μg/ml Zeocin and incubated at 30°C for 3 - 10 days until colonies formed. The His autotrophic transformants (His+) were selected and were retained on a YPD agar plate for further study. As a negative control, transformation was also performed using only the sacI linearized empty pPICZαA vector.

Table 1 Primers used for the RT-PCR to obtain the full-length cDNA of AciHBGase II

| Primer name | Forward primer (5’ → 3’) | Reverse primer (5’ → 3’) | Ta (°C) | Size (bp) |
|-------------|--------------------------|--------------------------|---------|----------|
| Pair 1_AciHBGase II | AATGCCGGAATTTAGTTTGCTTG | TGGAGTTTACGCTTGTGAG | 53 | 783 |
| Pair 2_AciHBGase II | AGTACATGTGGCCGGGAG | GAAGCCTTGAACGGCGAG | 52 | 503 |
| Pair 3_AciHBGase II | CCGGGAATTTAGTTTGCTTG | GGTGAACGCTTGTGAG | 42 | 581 |
| Pair 4_AciHBGase II | CGAGGAGTTTCCAAGACAGC | CCAAAATGGAATTCTTCTCCAGACGGACGAATTTAGTTTGCTTGTG | 48 | 634 |

Notes: Ta = annealing temperature used in the RT-PCR reaction. 
Size = expected amplicon size.
Expression of the rAciHBGase II-(His)_6 enzyme
After a few transformants were selected to see the level of expression, a single transformed P. pastoris colony presenting the highest expressed enzyme level was inoculated into 25 ml of BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% (w/v) yeast nitrogen base, 4 μg/ml D-biotin and 1% (v/v) glycerol). To form the inoculum, the culture was grown at 30°C, 200 rpm until the O.D. at 600 nm reached 2-6. Cells, collected by centrifugation at 704 x g, room temperature (RT) for 5 min, were then transferred into 50 ml of BMMY medium (BMGY except with methanol in place of glycerol) containing various percentages of methanol (0–10% (v/v)), and this amount of methanol was added every 24 h for various times of incubation (0–144 h). At the indicated time point, 1 ml of the induced culture was collected by centrifugation (13,226 x g, RT, 3 min) and the pellet and supernatant were stored separately at -20°C until assayed. Protein expression was determined by resolution through a reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8% (w/v) acrylamide resolving gel) and visualized by Coomassie blue staining. The protein concentration was calculated by the absorbance at 280 nm and Bradford’s assay [20].

Partial purification of the rAciHBGase II-(His)_6 enzyme
The transformants were first cultured in 25 ml of BMGY medium at 30°C for 24 h with agitation at 200 x rpm to form the inoculum, and then the induction of rAciHBGase II-(His)_6 was performed by the addition of the optimal level of methanol (% (v/v) every 24 h determined as above) in 1.2 liter of BMMY medium for 96 h. The sample was collected by centrifugation (5,009 x g, 4°C, 10 min), and then 100 ml of the supernatant was concentrated (~100-fold) through a 10 kDa molecular weight cut off membrane (Vivaspin 20; catalog# 28-9331-02 AB, GE Healthcare) to ~1 ml. The sample composition was adjusted to that of the binding buffer (20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 20 mM imidazole) and applied (200 μl at a time) sequentially to a HisTrap affinity column (1 ml in size, GE Healthcare). After that, 15 ml of binding buffer was used to wash the column and then the rAciHBGase II-(His)_6 protein was eluted with 5 ml of elution buffer (binding buffer except with 150 mM imidazole). Fractions (1 ml) were collected, mixed with 1 x protease inhibitor cocktails (Amresco) and stored at 4°C. The apparent homogeneity of the rAciHBGase II-(His)_6 protein was evaluated by resolution of 1 μg of sample per track of a reducing SDS-PAGE (8% (w/v) acrylamide resolving gel). Renaturation and α-glucosidase activity staining (zymogen) within resolved SDS-PAGE gels was performed as reported previously [21].

Assay for rAciHBGase II-(His)_6 enzyme activity
The α-glucosidase activity was determined using p-nitrophenyl α-D-glucoside (PNPG) as the substrate. The premix (0.1 ml of 0.1 M sodium phosphate buffer (pH 5.5), 0.05 ml of d-H2O and 0.05 ml of 5 mM PNPG) was incubated at 37°C for 10 min and then 0.05 ml of the test sample (e.g. column chromatography fraction or the rAciHBGase II-(His)_6 preparation) was added and incubated at 37°C for 10 min. The reaction was then stopped by adding 0.5 ml of 1 M Na2CO3. The control reaction was performed as above but with the addition of the sample buffer only and not the test sample (enzyme). The absorbance at 400 nm was monitored so as to measure the release of the yellow p-nitrophenol. One U of HBGase II activity was defined as that which liberates 1 μM of D-glucose from PNPG per min at pH 5.5 at 37°C.

Characterization of the rAciHBGase II-(His)_6 enzyme preparation
To evaluate the optimum reaction pH for α-glucosidase activity, the reaction mixture contained 0.05 ml of the rAciHBGase II-(His)_6 preparation, 0.05 ml of 5 mM PNPG and 0.1 ml of Briton-Robinson buffer (10 mM acetic acid, 10 mM phosphoric acid and 10 mM boric acid) at the desired pH (range of 3.0–7.5). The reaction mixture was incubated at 37°C for 10 min, and then stopped and assayed as above. For evaluation of the pH stability, the rAciHBGase II-(His)_6 preparation was prepared in Briton-Robinson buffer at the desired pH (range 3.0–12.0) and then stored at 4°C for 24 h before being adjusted to the previously determined optimal pH and assayed for α-glucosidase activity as above. The α-glucosidase activity was expressed as the% of the residual activity compared to the control sample that was not pretreated at 4°C for 24 h.

To evaluate the optimum reaction temperature for α-glucosidase activity, the reaction mixture was prepared as above except that the reaction was performed at pH 5.5 and at 25, 30, 35, 40, 45, 50, 55, 60 or 70°C. Moreover, to determine the thermal stability of the enzyme, the rAciHBGase II-(His)_6 preparation was prepared in 0.1 M sodium phosphate buffer containing 0.05% (v/v) Triton X-100 at pH 5.5 and kept at the indicated temperature (range 4–85°C) for 15 min before then being assayed for α-glucosidase activity as above. In each experiment, three independent repeats were done.

rAciHBGase II-(His)_6 enzyme kinetics
To determine the enzyme’s substrate specificity, the α-glucosidase activity was determined as above except that the substrate used was varied from maltose, maltooltriose, maltotetraose, isomaltose, sucrose and soluble starch at the indicated concentrations (s) of 0–60 mM for maltose and maltooltriose, 0–30 mM for maltotetraose, 0–100 mM for isomaltose, 0–25 mM for PNPG, 0–20 mM

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for sucrose and 0–40 mM for soluble starch. In addition, for the non-PNPG substrates, the α-glucosidase activity was determined in terms of the amount of glucose liberated. To this end 0.1 ml of the reaction mixture was mixed with 0.2 ml of glucose assay reagent (Sigma) and incubated at 37°C for 30 min. Then, the reaction was stopped by the addition of 0.2 ml of 12 N H₂SO₄ and the absorbance at 540 nm was measured. In each experiment, three independent repeats were done. The amount of glucose liberated from the substrate was determined by the glucose oxidase-peroxidase method (glucose assay kit, Sigma) as in Eq. (1):

\[
\text{Glucose (mg)} = \frac{[A_{540\text{nm}} \text{ of test}] (\text{standard glucose in mg})}{(A_{540\text{nm}} \text{ of standard})}
\]

(1)

For the non-PNPG substrates, the α-glucosidase activity was determined in terms of the amount of glucose liberated. To this end 0.1 ml of the reaction mixture was mixed with 0.2 ml of glucose assay reagent (Sigma) and incubated at 37°C for 30 min. Then, the reaction was stopped by the addition of 0.2 ml of 12 N H₂SO₄ and the absorbance at 540 nm was measured. In each experiment, three independent repeats were done. The amount of glucose liberated from the substrate was determined by the glucose oxidase-peroxidase method (glucose assay kit, Sigma) as in Eq. (1):

\[
\text{Glucose (mg)} = \frac{[A_{540\text{nm}} \text{ of test}] (\text{standard glucose in mg})}{(A_{540\text{nm}} \text{ of standard})}
\]

(1)

Results

AciHBGase II transcript expression pattern

Comparison of the AciHBGase I and II transcript levels among egg, larvae, pupae and adult forager bees by semi-quantitative RT-PCR using primer pair 3_HBGase I and primer pair 2_HBGase II, respectively, was performed. Under the previously determined optimum condition for RT-PCR (data not shown), the highest expression of AciHBGase I transcripts was seen in adult forager bees since no detectable expression was found in eggs, larvae or pupae (Figure 1A), in keeping with the proposed role for this isoform in honey formation via hydrolysis of nectar derived sucrose in forager bees. In contrast, AciHBGase II transcripts were expressed in eggs and, especially, in larvae and pupae, but with no detectable expression in foragers (Figure 1B). As a template control, the expression of a fragment of the 28S rDNA was observed in all samples at essentially the same level (Figure 1C). Of course this is the average transcript expression level across all larval, pupal

![Figure 1 Expression patterns of AciHBGase I and II in A. cerana indica. The RT-PCR products amplified by the specific primer pairs for (A) AciHBGase I and (B) AciHBGase II with (C) the control profile of 28S rDNA amplification. In each figure, lane M contained 100 bp DNA ladder marker, lane C was the negative control (no reverse transcriptase), and lanes 1–4 contained RT-PCR products from eggs, larvae, pupae and adult foragers, respectively. Gels shown are representative of those seen from three independent trials.](image-url)
and adult foraging bee tissues and so excludes any more subtle developmentally dependent tissue specific expression patterns.

**Full length cDNA sequence and its homology**

To obtain the full length cDNA nucleotide sequence of the *AciHBGase II* gene, primers were designed from various locations based upon the available *AmHBGase II* sequence (GenBank accession # NM_001040259). The expected RT-PCR products were successfully amplified by the designed primers (Table 1) (data not shown), and then direct sequenced and assembled using the Clustal X program to obtain the full length consensus cDNA sequence of *AciHBGase II* at 1,740 bp (Figure 2). The sequence has been deposited at GenBank with accession code # JX468895. From the predicted ORF of the cDNA sequence the deduced amino acid sequence of 579 amino acids was obtained (Figure 2). This predicted amino acid sequence has been deposited at GenBank with accession # NM_001040259. The sequence was 90% identical to that of the *HBGase II* from the closely related *A. cerana japonica* bee (GenBank accession # NM_FJ752630.1). When the deduced amino acid sequence of *AciHBGase II* was aligned to the amino acid sequences of homologs with similar annotated functions, a high amino acid sequence similarity was revealed (Figure 2).

**Expression of *AciHBGase II* (as a C-terminal (His)₆ tagged chimera)**

The expression plasmid pPICZαA harboring the cDNA encoding the full length *AciHBGase II* was transformed into *P. pastoris* GS115. The selected transformant was cultured in BMMY medium (50 ml) and was induced with methanol (0–10% (v/v)) in order to express the r*AciHBGase II*-His₆ enzyme. The optimal methanol concentration, in terms of inducing the highest secretory enzyme activity, was found searching the NCBI GenBank database using the megabLASTn algorithm (http://www.ncbi.nlm.nih.gov) revealed that the *AciHBGase II* cDNA nucleotide sequence was 90% identical to that of the *HBGase II* from the closely related *A. cerana japonica* bee (GenBank accession # NM_FJ752630.1). When the deduced amino acid sequence of *AciHBGase II* was aligned to the amino acid sequences of homologs with similar annotated functions, a high amino acid sequence similarity was revealed (Figure 2).

The predicted start (ATG) and stop (TAA) codons are shown in bold, giving a predicted open reading frame (ORF) of 1,740 bp for a deduced 579 residue amino acid sequence. The conserved amino acid regions associated with the α-amylase family of enzymes [3].

Searching the NCBI GenBank database using the megabLASTn algorithm (http://www.ncbi.nlm.nih.gov) revealed that the *AciHBGase II* cDNA nucleotide sequence was 90% identical to that of the *HBGase II* from the closely related *A. cerana japonica* bee (GenBank accession # NM_FJ752630.1). When the deduced amino acid sequence of *AciHBGase II* was aligned to the amino acid sequences of homologs with similar annotated functions, a high amino acid sequence similarity was revealed (Figure 2).

**Figure 2** The full-length *AciHBGase II* cDNA and deduced amino acid sequence of the encoded *AciHBGase II* enzyme. The predicted start (ATG) and stop (TAA) codons are shown in bold, giving a predicted open reading frame (ORF) of 1,740 bp for a deduced 579 residue amino acid sequence. The conserved amino acid regions associated with the α-amylase family are underlined.
to be 0.5% (v/v) (data not shown). At this optimal methanol concentration (0.5% (v/v) added every 24 h), α-glucosidase activity (assumed to be due to the rAcIHBGase II-(His)₆ enzyme) was found in both the cell lysate and supernatant and was maximal 96 h after induction, declining thereafter especially in the cell lysate (Figure 3). However, at all time points, including at the maximal expression time of 96 h after induction, a significantly greater α-glucosidase activity (2.5-fold) was obtained in the culture medium (supernatant) than in the cell lysate (~0.026 U/ml) (Figure 3). When the culture was scaled up to 1.2 l a 1.92-fold lower maximal α-glucosidase activity was obtained (0.026 U/ml) under otherwise the same conditions (data not shown).

Characterization of the rAcIHBGase II-(His)₆ enzyme

Optimum pH and temperature

The effects of pH and temperature on the α-glucosidase activity of the partially purified rAcIHBGase II-(His)₆ preparation were measured using PNPG as the substrate. The optimal activity was found at pH 3.5, although >80% was observed at pH 3.5 – 5.0 (Figure 5A) and the enzyme was stable (>90% residual activity) to a 24 h exposure at 4°C to pH in the range of 5.0 – 7.0 (Figure 5B). In addition, the optimal enzyme activity at pH 3.5 was found at 45°C, although 90% activity was found in the 40–55°C range (Figure 5C). The enzyme preparation was stable (>90% residual activity) to a 15 minute exposure of <45°C, although >80% residual activity was maintained after exposure to up to 65°C for 15 min, but decreased rapidly at higher temperatures (Figure 5D).

Substrate specificity

The substrate specificity of the partial purified rAcIHBGase II-(His)₆ preparation was examined using seven different kinds of substrates, each at two different concentrations (5 and 10 mM), from which the best hydrolyzed substrate was found to be sucrose at both concentrations (Figure 6). Considering Figures 7 and 6 together, since the enzyme could hydrolyze sucrose very well, the deduced amino acid sequence of this enzyme was searched against the amino sequences in the NCBI GenBank database using the megaBLASTp algorithm, to check in particular for related sucrases or other enzymes that hydrolyze sucrose efficiently. The result showed that the enzyme was similar to β-fructosidase (sucrase) of Thermotoga maritima MS8 (GenBank accession # O33833.1), sucrose-6-phosphate hydrolase of Lactobacillus animalis KCTC (GenBank accession # ZP_08548622.1), sucrose-6-phosphate hydrolase of Lactobacillus ruminis ATCC 25644 (GenBank accession # ZP_08079921.1) at 38%, 37% and 71% amino acid identity, respectively. This may also support the broad specificity of the enzyme.

Enzyme kinetics

When the substrate concentration (s) was plotted against the experimentally determined velocity (v) (Additional file 1: Figure S1), three possibilities could be observed. The first one, standard single-substrate Michaelis-Menten shapes were observed for maltotriose, maltotetraose and sucrose. In contrast, for isomaltose, PNPG and soluble starch, since the curve decreased after attaining a maximum, polynomial shapes as the second one was revealed. For maltose, the plateau phase was not reached within the concentration ranges tested (0–60 mM). Thus, higher substrate concentrations must be used in order to establish the saturation [22].

Lineweaver-Burk plots (1/v versus 1/s plots) [23] revealed a linear regression for all the evaluated substrates.
The derived kinetic parameters ($K_m$, $k_0$ and $V_{\text{max}}$) for the hydrolysis of those substrates are reported in Table 3, except for maltose since the reaction was clearly far off reaching a saturation in the concentration range tested (Additional file 1: Figure S1).

Although the $K_m$ values for maltotriose and maltotetraose were very similar, the $k_0$ and $V_{\text{max}}$ values were 9.4-fold larger for maltotriose than maltotetraose. The enzyme showed high activities towards PNPG and sucrose as substrates. The lowest $K_m$ value was from PNPG (4.2 mM) although the $V_{\text{max}}$ value was also the lowest (0.28 mg glucose/min/mg protein). This may indicate the enzyme could bind to PNPG very well leading to catalysis but that the reaction was not efficient or had a slow off rate. Whilst PNPG is not a biological substrate, such traits, if found for real substrates, would potentially be useful where some cellular compartments had only a low concentration of that sugar substrate. In the future, more experiments on determining the importance of a substrate presenting (i) low $K_m$ and low $V_{\text{max}}$ values, (ii) high $K_m$ and low $V_{\text{max}}$ values, (iii) low $K_m$ and high $V_{\text{max}}$ and (iv) high $K_m$ and high $V_{\text{max}}$ to cells should be performed.

**Discussion**

The *AciHBGase III* transcript expression pattern [24] and nucleotide sequence have been reported previously [25]. Here we report that for the *AciHBGase II* isoform. Since some *AciHBGase II* transcript expression was
observed in eggs and a higher expression level was found in larvae and pupae, but not in adult foraging bees (Figure 1), then AciHBGase II is not likely to play a role in honey synthesis through the hydrolysis of sucrose from nectar in the honey crop [7], although we did not formerly test for expression levels in specific tissues including the hypopharangeal and salivary glands and honey crop, but rather screened whole adults. Nevertheless, it is more likely that AciHBGase II is involved in other functions and possibly to do with the development of this honeybee. Hercovics [26] reported that endoplasmic reticulum (ER) - resident HBGase I and II played a sequential action on removing the terminal α-(1,2) – linked and the two more internal α-(1,3) – linked glucose residues from post-modified glycoproteins in the ER. If HBGase is defective, it can lead to a negative effect on cellular functions [27].

The full length deduced open reading frame (ORFs) of the obtained AciHBGase II cDNA was 1,740 bp while the ORF of this gene homolog in A. mellifera was 1,743 bp [19], with the four highly conserved regions found in α-amylase family members being present. With respect to the nucleotide and predicted amino acid sequences, AciHBGases can be classified into three isoforms (AciHBGase I, II and III) as per the HBGases in the closely related A. mellifera and A. cerana japonica honey bees [19,28]. Different isoforms of enzymes can display marked differences in their kinetics and especially in their substrate specificities, as well as in the tissue or developmental stage dependent control of their expression levels or response to stimuli [29].

The secreted form of the rAciHBGase II (as a chimeric protein with a C-terminal (His)₆ tag) could be enriched by only a few purification steps (Figure 4 and Table 3). The partially purified rAciHBGase II–(His)₆ protein appeared to be active under relatively acidic conditions with a pH optima of 3.5, and with > 80% activity at pH 3.5–5.0, as well as being more acid-stabile than neutral to alkali. This is somewhat like that reported for the mannose 6-phosphate containing acid HBGase purified

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**Table 2 Purification of the rAciHBGase II–(His)₆ enzyme**

| Procedure                                  | TotalPrProtein (mg) | Total activity (U) | Specific activity (mU/mg)* | Yield (%) | Purification fold |
|---------------------------------------------|---------------------|--------------------|----------------------------|-----------|-------------------|
| Negative control (Empty vector used)        | 3720                | 1.0                | 0.26                       | -         | -                 |
| Supernatant (100 ml)                        | 2326                | 2.6                | 0.11                       | 100       | 1.0               |
| Concentrated with Viva spin 20              | 151.5               | 0.20               | 1.3                        | 7.69      | 1.20              |
| Histrap column chromatography               | 11.25               | 0.018              | 1.6                        | 0.69      | 1.45              |

* mU = 10⁻³ U.

The rAciHBGase II–(His)₆ enzyme was partially purified from 100 ml of a 1.2 liter culture of transformed P. pastoris after 96 h induction with 0.5% (v/v) methanol.
from bovine testes [30] and the human placental lysosomal acid HBGase that hydrolyzes glycogen to glucose [31]. As such it is potentially consistent with the proposed role of AciHBGase II in the ER rather than in sucrose metabolism for honey production. Pompe disease, an autosomal recessive metabolic myopathy in the glycogen storage disease type II category, is caused by a deficiency of acid HBGase activity in humans. Thus, other than industrial enzyme production, heterogeneous expression of this gene in \textit{P. pastoris} may be useful for this disease treatment. Furthermore, the optimum temperature for \(\alpha\)-glucosidase activity of the rAciHBGase II-(His)_6 enzyme was at 45°C (with 80–90% residual activity at 40–55°C), which is much higher than that previously reported by Wu et al. [32], and higher than the physiological temperature of the bees. However, within extreme thermophiles, Nashiru et al. [33] reported a novel HBGase from \textit{Thermus caldophilus} GK24 with an optimal temperature of 90°C, whilst the intracellular and extracellular HBGases from \textit{G. toebii} strain E134 showed an optimum activity of 65°C and 70°C, respectively [34].

The enriched rAciHBGase II-(His)_6 enzyme showed a reasonably wide substrate specificity encompassing \(\alpha-(1,1)\), \(\alpha-(1,2)\), \(\alpha-(1,4)\) and \(\alpha-(1,6)\)-glucosidic linkages, but rapidly hydrolyzed PNPG \(\alpha-(1,6)\) and sucrose \(\alpha-(1,2)\). Similar to the native AmHBGase II that displayed positive cooperativity to sucrose, turanose \(\alpha-(1,3)\), kojibiose \(\alpha-(1,2)\) and soluble starch \(\alpha-(1,4)\) [10], the rAciHBGase II-(His)_6 enzyme hydrolyzed isomaltose and soluble starch but with different \(K_m\) values. This is expected since the substrate specificity of HBGases is reported to differ greatly with the enzyme source [35], one of the reasons for searching for new sources and thus isozymes with novel properties. Also, it may imply a difference in the recognition mechanism for allosteric ligands between enzymes or in the structures of the substrate-binding and catalytic sites of enzymes. Nevertheless, the difference in enzyme products could also be explained by different mechanisms employed by recombinant and native enzymes [36], and so it awaits the purification and characterization of the native AciHBGase II for comparison.

The use of a mini-fermentor could be considered for scale-up experiments instead of culture in a shaken flask in order to improve the efficiency of product production and the level of expression of foreign protein in \textit{P. pastoris}.
cVmax was obtained from the calculated value as the glucose liberated from the reducing end residue of substrates.

The a data are shown as the mean ± 1 SD and are derived from three independent repeats.

Figure 7 Multiple amino acid sequence alignment of AciHBGase II with other HBGase II homologs. Shown are HBGase II sequences from Apis cerana indica (Ac. indica) in comparison with those from the Japanese honeybee A. cerana japonica (Ac. japonica), European honeybee A. mellifera (A. mellifera, GenBank accession # ACN 63349.1), predicted maltase 2-like of Apis florea (A. florea, GenBank accession # KP_001035349.1), predicted maltase 2-like of AciHBGase II-(His)6 (Ac. indica) in comparison with those from the Japanese honeybee A. cerana japonica (Ac. japonica, GenBank accession # XP_003493603.1). Sequences were aligned by the Clustal X program and the similarity across the aligned sequences is shown as identical (*), conserved (:), and semi-conserved (•). The conserved amino acid regions associated with the α-amylase family are underlined.

Table 3 Kinetic parametersa for the hydrolysis of different substrates by the partial purified AciHBGase II-(His)6 enzyme

| Substrate      | \(k_m^0\) (mM) | \(k_0\) (s⁻¹) | \(V_{\text{max}}\) (mg glucose/min/mg protein) | \(k_0/k_m^0\) (mM⁻¹s⁻¹) |
|----------------|----------------|---------------|---------------------------------------------|-------------------------|
| Maltotriose    | 26.7 ± 1.96    | 26.6 ± 0.88   | 3.94 ± 0.13                                 | 1.00                    |
| Maltotetraose  | 25.0 ± 3.10    | 284.0 ± 0.06  | 0.42 ± 0.04                                 | 0.11                    |
| Isomaltose     | 22.3 ± 3.49    | 5.2 ± 0.46    | 0.77 ± 0.07                                 | 0.23                    |
| Sucrose        | 15.3 ± 0.87    | 845.0 ± 0.00  | 125.0 ± 0.00                                | 55.4                    |
| Soluble starch | 220 ± 1.28     | 4.60 ± 0.14   | 0.68 ± 0.04                                 | 0.21                    |
| PNPG           | 4.20 ± 0.18    | 1.89 ± 0.11   | 0.28 ± 0.02                                 | 0.45                    |

aData are shown as the mean ± 1 SD and are derived from three independent repeats.

The \(k_m^0\) value is the substrate concentration giving one half of the intrinsic \(V_{\text{max}}\).

\(V_{\text{max}}\) was obtained from the calculated value as the glucose liberated from the reducing end residue of substrates.
at 45–55°C) suggest it could be of interest for enzyme applications and in biotechnological processes when the conformational stability of the enzyme to heat and pH are required.

Additional files

Additional file 1: Figure S1. Substrate (s) versus enzyme velocity (v) plots for the hydrolysis reaction with the partially purified rAChHBGase II-(His)6 preparation. Shown are with the results for, (A) maltotriose (0–60 mM), (B) maltotetraose (0–30 mM), (C) isomaltose (0–100 mM), (D) PNPG (0–25 mM), (E) sucrose (0–20 mM), and (F) soluble starch (0–40 mM) as substrates. For PNPG each molecule of p-nitrophenol released is equated to one glucose molecule.

Additional file 2: Figure S2. Lineweaver-Burk plots for the hydrolysis reaction with the partially purified rAChHBGase II-(His)6 preparation. Substrates shown are (A) maltotriose (0–60 mM), (B) maltotetraose (0–30 mM), (C) isomaltose (0–100 mM), (D) PNPG (0–25 mM), (E) sucrose (0–20 mM), and (F) soluble starch (0–40 mM). For PNPG each molecule of p-nitrophenol released is equated to one glucose molecule. The linear regression coefficient (R2) and equation of the best for line shown are given in each panel.

Abbreviations

HBGase: α-glucosidase; rAChHBGase: α-glucosidase from A. cerana indica; rAnHBGase: α-glucosidase from A. mellifera; (r)HBGase: Recombinant α-glucosidase; rAChHBGase II-(His)6: Recombinant α-glucosidase from A. cerana indica; rAChHBGase II-(His): rAChHBGase as a C-terminal (His)6 tagged chimera.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

JK and MK performed the experiments, analyzed and interpreted the data. JK and MK contributed in drafting the manuscript. The helpful suggestions of three anonymous reviewers contributed significantly in improving the manuscript. The final version of the manuscript was approved by all authors.

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