รายงานวิจัยฉบับสมบูรณ์

โครงการ การทำโคลนนิ่ง การแสดงออกและการสกัดบริสุทธิ์ของแอลฟากลูโคซิเดสชนิดที่ 3
ของผึ้งโพรง (*Apis cerana*)

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เดือน พฤษภาคม ปี 2554 ที่เสร็จโครงการ

สัญญาเลขที่ RMU5180042

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและ
สำนักงานกองทุนสนับสนุนการวิจัย

(ความคิดเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abtract

In this research, it aimed to focus on recombinant HBGase I, II, and III from *A. cerana indica* due to economic honeybees. In order to determine a pattern of expression, honeybees at different stages of development (eggs, larvae, pupae, and forager bees) were chosen. It showed that the highest expression of both *HBGase I* and *III* were in forager bees. The highest expression of *HBGase II* was in larvae and pupae but lower expression was found in forager bee together with no expression in eggs. At present, 3' end of *HBGase III* together with partial cDNA sequences of *HBGase I* and *II* at 942 and 834 bp were obtained. Considering the sequence homology, *HBGase I* was similar to the sequence of *A. cerana japonica* isozyme I (NM_AB260890.1) at 98% while *HBGase II* was similar to the sequence of *A. cerana japonica* isozyme II (NM_FJ752630.1) at 100%. The full length of *HBGase III* (1,704 bp) was cloned into pPICZαA (an expression vector) and was transformed into *Pichia pastoris* GS115 (yeast). The expression of recombinant HBGase III was induced by 1% methanol after 144 hours (7 days). The obtained activity of secreted HBGase III was up to 6 U from the large scale culture (1.2 Liters). Also, pH activity, pH stability, and thermal stability of the enzyme were found out to be at 5.0, 4.5-7.5, and $<50^{0}$C, respectively. Considering substrate specificity, they were maltose and p-nitrophenyl α-D-glucoside (PNPG). In the future, kinetics of HBGase III will be obtained and recombinant clones of *HBGase I* and *II* will be expressed and characterized.

Key words: *Apis cerana indica*, α-glucosidase, *Pichia pastoris*, expression, recombinant enzyme

บทคัดย่อ

ในงานวิจัยนี้ มุ่งเน้นที่ recombinant HBGase I, II และ III จากผึ้งโพรงไทย (*Apis cerana indica*) ซึ่งเป็นผึ้งเศรษฐกิจอย่างหนึ่ง ในการตรวจสอบรูปแบบการแสดงออกของยีน ทำการเก็บตัวอย่างผึ้งที่อยู่ในระยะการเจริญต่างๆ ได้แก่ ระยะไข่ ตัวอ่อน ดักแด้และตัวเต็มวัย พบว่ายีน *HBGase I* และ *III* มีการแสดงออกสูงสุดในผึ้งตัวเต็มวัยที่เป็นผึ้งออกหาอาหาร ส่วน *HBGase II* มีการแสดงออกสูงสุดในผึ้งระยะตัวอ่อนและดักแด้ แต่มีระดับการแสดงออกในระยะตัวเต็มวัยที่น้อยกว่าและไม่มีการแสดงออกเลยในระยะไข่ จากการค้นหาลำดับนิวคลีโอไทด์ที่ยาวเต็มสายของยีน ได้ส่วนปลายสายของ *HBGase III* ในส่วนของ *HBGase I* ได้ลำดับนิวคลีโอไทด์บางส่วนที่มีความยาว 942 bp และในส่วนของ *HBGase II* ได้ลำดับนิวคลีโอไทด์บางส่วนที่มีความยาว 834 bp เมื่อมาพิจารณาที่ความเหมือนของลำดับนิวคลีโอไทด์ พบว่าลำดับนิวคลีโอไทด์ของ *HBGase I* มีความเหมือนกับยีนดังกล่าวของ *A. cerana japonica* isozyme I (NM_AB260890.1) ถึง 98% ในขณะที่ลำดับนิว คลีโอไทด์ของ *HBGase II* มีความเหมือนกับยีนดังกล่าวของ *A. cerana japonica* isozyme II (NM_FJ752630.1) ถึง 100% ต่อมาทำการโคลน *HBGase III* (ความยาวของยีนเต็มสาย 1,704 bp) เข้าสู่ pPICZαA (ซึ่งเป็น expression vector) แล้วทำการ transform เข้าสู่ยีสต์ *Pichia pastoris* GS115 พบว่า recombinant HBGase III มีการแสดงออกสูงสุดเมื่อถูก induce ด้วย 1% methanol เป็นเวลา 144 ชั่วโมง (7 วัน) ได้ activity ของ HBGase III ที่หลั่งออกนอกเซลล์ 6 U จากการเลี้ยงยีสต์ที่ 1.2 ลิตร นอกจากนี้ยังพบว่าเอนไซม์ดังกล่าวมี pH activity, pH stability, และ thermal stability ที่ 5.0, 4.5-7.5, และ < 50^0C ตามลำดับ เมื่อมาพิจารณาที่ substrate specificity พบว่า maltose และ p-nitrophenyl α-D-glucoside (PNPG) เป็นสารตั้งต้นที่เหมาะสมที่สุด

คำสำคัญ: ผึ้งโพรงไทย แอลฟากลูโคซิเดส ยีสต์ การแสดงของยีน เอนไซม์แบบรีคอมบิแนนท์

Executive Summary

1. Rationale

Honey is one of economic honeybee products which can bring income to bee farmers. It can be applied in cosmetics, apitherapy, and traditional medicine since it can provide many bioactivities such as antimicrobial, antiproliferative, anti-oxidant activities. Consuming as food, it can supply a lot of energies, vitamins, monosaccharides, disaccharides, minerals, etc. As known, monosaccharides can be absorbed into our body directly. Honeybees can invert disaccharides in nectar to be monosaccharides in honey by the function of alpha-glucosidase III (HBGase III) which it can hydrolyse alpha-glucosidic linkages, especially alpha-1, 4 – linkage. Thus, this enzyme may be useful in food industry or may be replaced the same enzyme obtained from bacteria. In this research, it was focused on recombinant HBGase III since *in vitro* expression can produce large amount of enzyme and provide no need to kill a lot of honeybees like native enzyme purification. In addition, we can keep and multiply the clones for the long term use.

2. Objectives

2.1 Purify native HBGase III from forager bees of *Apis cerana indica*

2.2 Characterize native HBGase III

2.3 Perform *in vitro* expression of recombinant HBGase III

2.4 Characterize recombinant HBGase III

2.5 Compare the characterizations and properties between native and recombinant HBGase III

3. Methodology

Native HBGase III: Native HBGase III was purified. Then, it was characterized in term of pH activity, pH stability, temperature stability, and substrate specificity. Also, deglycosylation of the enzyme was determined.

Recombinant HBGase III: Full length of cDNA of *HBGase III* was obtained and analysed. Then, recombinant HBGase III expression vector was constructed. Then, it was induced to express and purify. Finally, the recombinant enzyme was characterized in term of pH activity, pH stability, temperature stability, and substrate specificity.

4. Results

Native HBGase III was purified and characterized. The full length of nucleotide of *HBGase III* was obtained, including 3' end of the gene. The *HBGase III* was cloned into pPICZα A and transformed into *Pichia pastoris*. The highest expression was induced by 1% MeOH for 144 h. The enzyme activity was 0.028 U/ml. After purified by His-trap affinity column, higher activity at0.052 U/ml was obtained. The pH activity was 5.0. The pH stability was 4.5-7.5. The thermal stability was less than 50 ^{0}C. The preferable substrates were maltose and PNPG. The Km for maltose was 1 mM while the Km for PNPG was 0.5 mM.

5. Future work: The full length of nucleotides of *HBGase I* and *II*, including 5' and 3' ends, will be obtained. The recombinant HBGase I and II will be expressed and purified. Then, they will be characterized. Finally, the characterizations and properties of native HBGase III, recombinant HBGase III, recombinant HBGase I, and recombinant HBGase II will be compared.

เนื้อหางานวิจัย

Introduction

Nowadays, honeybee is the most popular model to be a new source of α-glucosidase (HBGase) among insects. European honeybee (*Apis mellifera*) has been widely used as a model for native and recombinant HBGase. It was reported that there were three kinds of HBGase (I, II, and III) which were different in substrate specificity, mass weight, nucleotide sequences, and tissue location (Kubota *et al*., 2004). Moreover, these three enzymes were different in optimum pH, pH stability, thermal stability, and sugar content (Kimura *et al*., 1990; Takewaki *et al*., 1993; Nishimoto *et al*., 2001). More than the above, these enzymes were different in nucleotide sequences. The full length of cDNA encoding *HBGase I, II,* and *III* were 1,986, 1,910, and 1,915 bp, respectively. Those included the open reading frames (ORFs) of 1,767, 1,743, and 1,704 bp, respectively. According to the mentioned ORFs, the deduced amino acid sequences were 588, 580, and 567 in length, respectively (Ohashi*et al*., 1996; Nishimoto*et al*., 2007).

Native forms of these three enzymes in *Apis mellifera* (European honeybee) were already purified and characterized. The native HBGase I was purified by salting-out chromatography with 60-80 % saturation of ammonium sulfate and was characterized as an allosteric enzyme. It was a monomeric protein and a glycoprotein containing sugar content of 25 %. This enzyme demonstrated the negative cooperativity to maltose, sucrose, and aryl α-glucoside (phenyl α-glucoside and *p*-nitrophenyl α-D-glucoside, PNPG) and the positive cooperativity to turanose and maltodextrin (Kimura *et al.,* 1990). In addition, this enzyme was inactivated by using chemical modification with

diethyl-pyrocarbonate (DEPC). Furthermore, it was proved that HBGase I had a single catalytic site and there was one histidyl residue at or near the active site (Kimura *et al*., 1992).

Native HBGase II was purified by salting-out chromatography with 60-80 % and 30-50 % ammonium sulfate, and further by non-adsorbed active fraction on DEAE-cellulose. It was an allosteric protein as well. It had the same properties for the cleavages of several kinds of substrates as HBGase I but both were different in substrate specificity. The HBGase II displayed only the positive cooperativity to sucrose, turanose, kojibiose, and soluble starch. It was also a monomeric protein and a glycoprotein containing about 15 % carbohydrate (Takewaki *et al*., 1993).

Native HBGase III was purified by using salting-out chromatography, DEAE-cellulose, DEAE-Sepharose CL-6B, Bio-Gel P-150, and CM-Toyopearl 650M chromatographies. The enzyme was also a monomeric protein and glycoprotein containing about 7.4 % of carbohydrate. Surprisingly, it was not an allosteric enzyme. Although it showed a normal Michaelis-Menten type reaction, it showed no cooperativity (Nishimoto *et al*., 2001).

Moreover, HBGase was also performed in Japanese honeybee *A. cerana japonica*. Native HBGase I was purified by using CM-Toyopeal 650M and Sephacryl S-100. It was also a monomeric protein and glycoprotein containing 15 % carbohydrate. Furthermore, it showed unusual kinetics, the negative cooperative behavior on the intrinsic reaction on the cleavage of sucrose, maltose, and *p*-nitrophenolα-glucoside and the positive cooperative behavior on turanose. It was considered to correspond to HBGase I from *A. mellifera*. Moreover, the isolated cDNA encoding *HBGase I* of *A.*

cerana japonica was 1,930 bp in length and was deduced to be 577 amino acid residues (Wongchawalit*et al*., 2006).

Currently, the recombinant technique was popularly used to synthesize interesting enzymes. For example, in *A. mellifera*, the full length cDNAs of three HBGases were cloned into expression vectors of pPIC3.5 or pPIC9. Later, it was transformed into *P. pastoris* GS115 (yeast). The recombinant clones were induced by 0.5% methanol. The highest activities of culture media were assayed. The obtained result presented that recombinant HBGase II and III could be produced but not HBGase I. After that, recombinant HBGase II and III were purified by salting-out chromatography with ammonium sulfate and by CM Sepharose CL-6B, Bio-Gel P-100, DEAE Sepharose CL6B, and Butyl-Toyopeal 650 M columns (Nishimoto *et al*., 2007).

Most of researches on HBGase were from *A. mellifera* and some were from *A. cerana japonica*. In contrast, no researches on HBGase were from our Thai native species (*A. dorsata*, *A. andreniformis*, and a lot of stingless bee species), except a few researches on native HBGase III from *A. cerana indica* (Chanchao *et al*., 2008) and *A. florea* (Chanchao *et al*., 2007). Recently, *A. cerana indica* is wildly managed as economic bees due to its disease resistance against bee mites, predators, and pathogens (Pothichot and Wongsiri, 1993).

It is interesting to determine the properties of recombinant HBGase I, II, and III from *A. cerana indica* although native HBGase I and II have not been obtained. In this research, it will be focused on obtaining the full length cDNA of all *HBGase I, II,* and *III*. Then, they will be cloned into an expression vector (pPICZαA) and will be transformed into *P. pastoris*. The recombinant HBGase will be expressed after methanol induction.

Purification and characterization of recombinant HBGase will be performed. The outcome from this research will be a new source of HBGase which may be applied in food industry.

Materials and Methods

Sample collection

Honeybees were from an apiary in Samut Songkram province. Eggs, larvae, and pupae were collected directly from *A. cerana indica* hives while foragers were collected from the returning flight in front of a hive. Then, samples were kept at $-80^{0}C$ until used.

RNA extraction

Fresh samples (eggs, larvae, pupae, and forager bees) were grinded with liquid nitrogen in a mortar. Total RNA was extracted by an acid-guanidine thiocyanate-phenol-chloroform method (Nishimoto *et al*., 2007). The quality of total RNA was assayed by 1.2 % (w/v) formaldehyde/ agarose gel electrophoresis and Ethidium Bromide (EtBr) staining. After that, poly A^{+} mRNA was isolated by using the oligotex mRNA mini kit (Qiagen).

Primer design and RT-PCR to obtain full length cDNA

Primer design was based on the cDNA sequence of *HBGase I, II,* and *III* in *A. mellifera* (accession# NM_001040236, NM_001040259, and NM_001011608, respectively). All primers were designed by using Primer 3 program

(http://frodo.wi.mit.edu/primer3/) and by eyes (Table 1). RT-PCR was performed by using an access RT-PCR system kit (Promega). Reaction without RNA template was used as a negative control. 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 forward primer (F) and reverse primer (R), 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. All RT-PCR reactions were performed under optimal conditions as follows: 1 cycle of 48°C for 45 min and 94°C for 2 min; 30 cycles of 94°C for 30 sec, 42°C for 30 sec, and 68°C for 2 min; and finally 1 cycle of 68°C for 7 min. RT-PCR products were assayed on 1.2 % (w/v) agarose gel electrophoresis and EtBr staining. After that, they were purified by using QIAquick PCR purification kit (Qiagen) and direct sequencing by Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. The obtained sequences were blasted to the recorded HBGase sequences in GenBank in order to confirm the results.

Synthesis of the first strand cDNA from mRNA for RACE-PCR

In one tube, the mRNA (6 μl) is mixed with 1 μl of 10 mM dNTPs while the other tube contains 4 μl of 20 μM 3'-AP primer (5'-GGCCACGCGTCGACTAGTAC T_{17}-3'). Both tubes are incubated at 65°C for 10 min, quick chilled on ice, and quick spun. One μl of RNaseOut and 2 μl of 20 μM heated 3'-AP (from the second tube) is transferred to the first tube. Then, it is mixed with 8 μl of buffer mixture (2 μl of 10 x RT buffer, 4 μl of 25 mM $MgCl_2$, and 2 μl of 0.1 M DTT). The mixture is incubated at 42 °C for 2 min. After that, 1 μl of Superscript II reverse transcriptase is added. It is

incubated at 42°C for 50 min and, then, is incubated at 70°C for 15 min. One μl of RNase H is added. After it is incubated at 37°C for 20 min, it is stored at -20°C.

Poly A addition at 5'-end of the first strand cDNA for RACE-PCR

The method is followed by a protocol of TdT recombinant (Invitrogen). In each reaction, the first strand cDNA is mixed with 1 x TdT buffer (tailing buffer), 0.2 mM of dATP, and 30 U of TdT. The reaction is incubated at 37 ^{o}C for 15 min and later at 80 ^{o}C for 3 min. At last, the first strand cDNA containing poly A at the 5'-end is stored at -20 ^{o}C.

The 5'-rapid amplification of cDNA end (5'-RACE)

The first strand cDNA with poly A at the 5'- end is used as template. For the first PCR, 25 μl of mixture consists of 1 x PCR master mix (5 Prime), 0.1 mM of dNTP, 0.3 μM of 5'-AP primer (5'-GACTCGAGTCGACATCGAT$_{17}$-3'), 0.35 μl of the poly (A)$^{+}$ tailed cDNA. PCR is carried out under the condition of 94 ºC for 1 min, 42 ºC for 1 min, and 68 ºC for 1 min. It is followed by the addition of 0.6 μM of 5'-AUAP (5'-GACTCGAGTCGACATCG-3') and 0.6 μM of reverse primer. The PCR amplification is continuously performed by 30 cycles of the thermal procedure at 94 ºC for 30 sec, 42 ºC for 30 sec, and at 68 ºC for 2 min for extension and then hold at 4ºC. The PCR product is visible under the U.V. light on 1 % agarose gel after being stained with 10 μg/ml EtBr. Expected PCR products are purified and directly sequenced.

The 3'-rapid amplification of cDNA end (3'-RACE)

The first strand cDNA is amplified by forward primer and a universal primer (3'- followed by 30 cycles of denaturation at 96 ºC for 30 sec, annealing at 55 ºC for 20 sec, and extension at 74 ºC for 2 min before holding at 4 ºC. The PCR product is visible under the U.V. light on 1 % agarose gel after being stained with 10 μg/ml EtBr. Expected PCR products are purified and directly sequenced. AUAP, 5'-GGCCACGCGTCGACTAGTAC-3') which shares the common 5' end sequence to 3'-AP primer. A PCR reaction is prepared as mentioned above by using Prime star master mix. The PCR is carried out under the condition of 96 ºC for 2 min,

Construction of expression vector

In order to amplify the full length cDNA and facilitate the cloning, forward and reverse primers were designed to encompass the 5' and 3' outermost regions of each *HBGase*, respectively. The nucleotide sequence of forward primer of *HBGase I, II* and *III* were as followed: F_Hbg I as 5' AATCA TGGAA TTCTC CGATC GGCCT TGGCG CCG 3'; F_Hbg II as 5' CAAAA TGGAA TTCTT TCGAG CGACG ATAGT TA 3'; and F_Hbg III as 5' GGTAC ATGGA ATTCC ATCAT CATCA TCATC ATCAT AAGGC GATAA TCGTA TTTTG 3'. At the 5' end, forward primer contained *Eco*RI restriction site (underline) and six repeated codons encoding His (boldface). Also, at the 3' end of reverse primer, it contained *Kpn*I restriction site (underline). The nucleotide sequence of reverse primers was as followed: R_Hbg I as 5' AAGCG GTACC TCGAT ACCGT CGACC TCGAG 3'; R_Hbg II as 5' CGAGG TACCC AACCA GTCTA CACCT TGCC 3'; and R_Hbg III as 5' TTTGG TACCT TAAAA TTTCC AAATT TAGCA TC 3'. The RT-

PCR reaction was performed under optimal conditions as follows: 1 cycle of 94°C for 2 min; 30 cycles of 94°C for 30 sec, 52°C for 30 sec, and 68°C for 2 min; and finally 1 cycle of 68°C for 7 min. The expected RT-PCR product of about 1.9 kb was observed on 1 % agarose gel after EtBr staining. The amplified RT-PCR product and pPICZαA as an expression vector (Invitrogen) were separately digested by *Eco*RI and *Kpn*I at 37°C for overnight. Ligation reaction between RT-PCR product and pPICZαA with compatible ends of *Eco*RI and *Kpn*I was performed by T4 ligase and 1x T4 ligase buffer at 16°C for overnight.

Transformation to P. pastoris

Before transformation, 5-10 μg of recombinant plasmid was digested by *Sac*I at 37°C for 1 h. *P. pastoris* GS115 (His) strain (Invitrogen) was prepared by following the protocol of EasySelect*Pichia* expression kit (Invitrogen). *Sac*I-linearized plasmid was transformed into *P. pastoris* by electroporation using Gene Pulser (Bio-Rad) according to the recommendation of Invitrogen, a manual of methods for the expression of recombinant protein in *P. pastoris*. The electroporated yeast was spreaded onto YPDS plates (YPD with 1 M sorbitol) containing 100 μg/ml Zeocin. It was incubated at 30°C for 3-10 days until colonies formed. The His autotrophic transformants (His^{+}) were selected and were retained on an YPD agar plate (1 % yeast extract, 2 % peptone, 2 % dextrose, and 2 % agar) for further study.

Expression of recombinant HBGases

A single colony of transformant was inoculated into 25 ml of BMGY medium (1 % yeast extract, 2 % peptone, and 100 mM potassium phosphate buffer (pH 6.0), 1.34 % Yeast Nitrogen Base, 4 μg/ml D-biotin, and 1 % glycerol). The culture was grown at 30°C, 200 rpm until the O.D. at 600 nm reached 2-6. Cells, collected by centrifugation at 3,000 rpm, RT for 5 min, were transferred into 100 ml of BMMY medium containing 1.0 % methanol. Induction of protein expression was achieved by adding 1.0 % methanol at every 24 h for 144 h (7 days) in order to maintain an induction. It was cultivated at 200 rpm, 30°C for 15 min. At the indicated time point, 1 ml of the induced culture was collected. Pellet and supernatant were stored at -20°C until assay. Protein expression was determined by 8% SDS PAGE and Coomassie blue staining. The protein concentration was measured by Bradford's assay.

HBGase purification

The transformants were cultured in 50 ml of BMGY medium at 200 rpm, 30°C for 24 h. The induction was performed in 1.2 l of BMMY medium for 144 h. The sample was collected by centrifugation at 8,000 rpm, RT for 10 min. Protein in supernatant was concentrated by Vivaspin 20 (GE Healthcare) and was applied sequentially to HisTrap affinity column (GE Healthcare). The purified protein was stored at 4°C by adding protease inhibitor cocktails (Amresgo). The homogeneity of the purified recombinant HBGase was confirmed by 8 % SDS-PAGE. Also, the activity of the enzyme was assayed.

Enzyme activity assay

The *p*-nitrophenyl α-D-glucoside (PNPG, Sigma) was used as substrate. The premix (0.1 ml of 0.1 M Sodium phosphate buffer at pH 5.5, 0.5 ml of distilled water, and 0.05 ml of 5 mM PNPG) was incubated at 37°C for 10 min. Then, 0.05 ml of HBGase III was added. The reaction was further incubated at 37°C for 10 min. After that, the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. Control mixture was comprised of 0.05 ml PNPG, 0.2 ml water, and 0.5 ml Na_2CO_3. The absorbance at 400 nm of the reaction mixture was used to measure the release of the yellow *p*-nitrophenol. One unit of HBGase will liberate 1 μM of D-glucose from PNPG per min at pH 5.5 at 37°C. In order to obtain a substrate activity, a method was followed from the above but maltose, maltotriose, isomaltose, sucrose, and soluble starch was used instead of PNPG. After that, it was stopped by Na_2CO_3. Reaction mixture (0.1 ml) was transferred into a new tube and was mixed with 0.2 ml of Glucose assay reagent (Sigma). It was incubated at 37°C for 30 min. Then, the reaction was stopped by 0.2 ml of 12 N H_2SO_4. The absorbance at 540 nm was measured. Glucose liberated from substrate was determined by the glucose oxidase-peroxidase method (Glucose Assay Kit, Sigma).

Protein determination

Bradford assay was used (Bradford, 1976). Bovine serum albumin (BSA) at various concentrations was prepared. Then, it was mixed with Bradford solution (Biorad) at the volume ratio of 1:10. The mixture was incubated at RT for 5 min. The absorbance at 595 nm was measured. The standard curve of BSA was drawn. The preparation and

measurement of diluted HBGase was the same as those of BSA. Moreover, protein concentration was also estimated by determining the absorbance at 280 nm.

Characterization of recombinant HBGases

For determination of pH activity, a reaction mixture containing 0.05 ml of HBGase III, 0.05 ml of 5 mM PNPG and 0.1 ml of Briton-Robinson buffer (40 mM acetic acid, 40 mM phosphoric acid, and 40 mM boric acid) providing varied pH in the range of 3.0-7.5 (Nishimoto *et al*., 2001). The reaction mixture was incubated at 37°C for 10 min. Furthermore, for determination of pH stability, HBGase III was prepared in Briton-Robinson buffer (pH 3.0-7.5) and was stored at 4°C for 24 h before assayed.

For determination of thermal stability, HBGase was prepared in 0.1 M sodium phosphate buffer at pH 5.5, containing 0.05 % Triton X-100. It was kept at 4-70°C. After 15 min, the mixture was chilled at 0°C. Then, the mixture was pre-incubated at 37°C for 3 min. The activity of HBGase was later assayed.

Results

Expression pattern of HBGase I and II

After RNA extraction, the quality of total RNA from eggs, larvae, pupae, and forager bees were determined by 1.2% formaldehyde/ agarose gel electrophoresis. After that, 200 ng RNA of each sample was used to detect the expression pattern by semi-quantitative RT-PCR. In term of *HBGase I* and *II*, primer pair 3_HBG I and primer pair 2_HBG II were used, respectively (Table 1). Under the optimum condition for RT-PCR, the highest expression of *HBGase I* was in forager bees (Fig. 1A) which provided the

same result as in *HBGase III* (Srimawong, 2003). The highest expression of *HBGase II* was in larvae and pupae but lower expression was found in forager bee together with no expression in eggs (Fig. 1B). The amplification of *28S rRNA* was used as control (Fig. 1C).

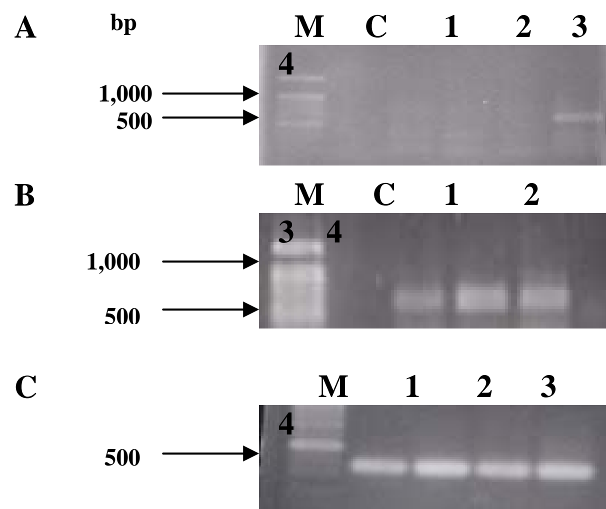

Fig. 1. Expression patterns of *HBGase I* and *II* in *A. cerana indica*. Profiles of RT-PCR products amplified by specific primer pairs of *HBGase I* were in (1A) while that of *HBGase II* were in (1B). The control profile of *28S rRNA* amplification was in (1C). In each figure, lane M contained 100 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 forager bees, respectively.

Primer design and RT-PCR

To obtain the nucleotide sequence of *HBGase I* and *II* from *A. cerana indica*, primers were designed by using primer 3 program (http://frodo.wi.mit.edu/primer3) and by eyes (Table 1). They were based on cDNA sequences of *A. mellifera*

(NM_001040236 and NM_001040259). Before this, the complete cDNA length of *HBGase III* in the length of 1,704 bp was obtained. That encoded a predicted polypeptide of 567 amino acid (Chanchao *et al*., 2008). The results showed that RT-PCR products were successful detected by primer pair 1, 2, and 3 of *HBGase I* and primer pair 2, 3, and 4 of *HBGase II*, respectively. After that, Clustal X program was used to assemble cDNA sequence. The partial cDNA sequences of *HBGase I* and *II* were 942 bp and 834 bp, respectively. Moreover, they were blasted against the recorded sequences in the GenBank by using Blast*N* (http://www.ncbi.nlm.nih.gov). The data showed that *HBGase I* was similar to the sequence of *A. cerana japonica* isozyme I (NM_AB260890.1) at 98% while *HBGase II* was similar to the sequence of *A. cerana japonica* isozyme II (NM_FJ752630.1) at 100%. In addition, it was found that the annealing temperature for RT-PCR amplification of *HBGase I* (53°C) and *II* (42°C) were different.

Table 1. Primers design for RT-PCR to obtain full length cDNA of *HBGase I* and *II*.

Primer name	Forward primer (5' → 3')	Reverse primer (5' → 3')	Size (bp)
Pair 1_HBG I	ATGAA GAGCC TCGTC GTGG	GCGCT TTCGA ATAAA TGTGG	716
Pair 2_HBG I	TGCAG AAGCG AAGAA ACAGA	CTGGC GTGGA AGATG AATTT	689
Pair 3_HBG I	AATGG CGAGA ATTTT GTGGA C	TGGAG TTTAC GCTGC TTGTG	783
Pair 4_HBG I	TCGCT GAAAT TTGCT TGAA	TTACC GTTGG AAAAA AGATA	482
Pair 1_HBG II	ATGTT TCGAG CGACG ATAGT TAC	AAGAC GCTGA GCCAA TTGTT	503
Pair 2_HBG II	CGAGG AGTTT CCAAG ACAGC	CTCGA ACATG TGGTT GATGG	581
Pair 3_HBG II	AGTAC TACGT GTGGC GGGAC	GGACT TGAAC GCCAC GTAAT	983
Pair 4_HBG II	CGTGA TGCTG ACGTT GACTT	TTACA ACCAG TCTAC ACCTT GCC	634

3' end of HBGase III

After the full length of HBGase III was obtained, it was recorded in GenBank as accession number EF441271. Later, 3' end of the end was revealed by doing RACE PCR as shown in Figure 2. At present, 5' end of the gene is in the process.

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ATGAAGGCGATAATCGTATTTTGCCTTATGGCATTGTCCATTGTGGACGCAGCATGGAAGCCGCTCCCTG
AAAACTTGAAGGAGGACTTGATCGTGTATCAGGTCTACCCAAGAAGCTTCAAGGATAGCAATGGAGATGG
TATTGGTGATATCGAAGGTATTAAACAAAAATTGGACCATTTTCTCGAAATGGGCGTCGATATGTTTTGG
TTATCTCCTATTTATCCAAGTCCTATGGTCGATTTTGGTTATGACATTTCGAATTACACCGATGTTCATC
CCATATTTGGCACCTTATCAGACTTAGATAACTTAGTTAATGCTGCACATGAGAAGGGACTGAAGATAAT
CTTGGATTTCGTTCCGAATCATACATCTGATCAACATGAATGGTTCCAGCTGAGTTTGAAAAACATTGAA
CCTTATAACAACTATTATATTTGGCATCCAGGAAAAATTGTAAATGGTAAACGTGTTCCACCAACTAATT
GGGTAGGCGTATTTGGTGGATCAGCTTGGTCATGGCGAGAAGAACGACAGGCATATTATCTGCATCAATT
TGCACCAGAACAACCAGATCTAAATTACTATAATCCAGTTGTACTAGATGATATGCAAAACGTTCTCAGA
TTCTGGCTGAGAAGAGGACTCGATGGTTTCAGAGTAGATGCTTTGCCTTACATTTGCGAGGACATGCGAT
TCTTAGACGAACCCCTATCTGGTGAAACAAATGATCCCAATAAAACCGAGTACACTCTCAAGATCTACAC
TCACGATATCCCAGAAACCTACAATATAGTTCGCAAATTTAGAGATGTGTTAGACGAATTCCCGCAACCA
AAACACATGCTTATCGAGGCATACACGAATTTATCGATGACGATGAAATATTACGATTACGGAGCAGATT
TTCCCTTTAATTTTGCATTCATCAAGAATGTCTCTAAGGATTCAAATTCATCAGACTTCAAGAAATTGGT
CGATAATTGGATGATATACATGCCAGCAGATGGTATTCCTAACTGGGTGCCCGGAAATCACGATCAATTG
AGATTGGTGTCGAGATTTGGAGAGGAGAAGGCCCGTATGATCACCGCGATGTCGCTTTTGCTGCCAGGTG
TTGCCGTGAATTACTACGGTGATGAAATTGGTATGTCGGATACTTATATCTCGTGGGAGGACACGCAGGA
TCCACAGGGATGCGGTGCCGGCAAAGAAAACTATCAAACGATGTCGAGAGATCCCGCGAGAACGCCATTC
CAATGGGACGACTCAGTTTCTGCTGGATTTTCCTCAAGCTCTGATACCTGGCTTCGTGTCAACGAAAATT
ACAAGACTATCAATTTAGCTGCTGAAAAGAAGGACAAGAACTCGTTCTTCAATATGTTCAAGAAATTTGC
AATGCTGAAAAAATCGCCACACTTTAAAGAGGCCAATTTAAATACGAGGATGCTGAACGACAGTGTTTTC
GCATTCTCTAGGGAAACCGAAGAAAATGGATCTCTTTACGCAATATTGAACTTCTCGAACGAGGAACAAA
TCGTGGACTTGAAAGCGTTTAATAACGTGCCGAAAAAATTGAATATGTTTTACACCATTTTTAACTCTGA
TATAAAGTCCATCTCCAACAATGAACAAATAAAAGTTTCTGCTTTAGGATTTTTGATCTTAATTTCTCAA
GATGCTAAATTTGGAAATTTTTAACTTTGTGCTGATCGCATGGTCATCTAGCACCGGCGACGCATCTTTC
AAATGTCTGCCTTATCAACTGTCGATGGTAGGTTCTGCGCCTACCATGGTTGTAACGGGGTAACGGGGAA
TCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACAGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAA
ATTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAAAAAAAAAAACCTATAGGGAGTCGTATTAATTCTG
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Figure 2. The 3' end of *HBGase III* in *A. cerana indica*. The underlined nucleotides were obtained from 3' RACE.

Conditions for expressed recombinant HBGases

The full length PCR product of *HBGase I, II* and *III* was successfully cloned into pPICZαA. At the beginning, a recombinant *HBGase III* clone was transformed into *P. pastoris* GS115. The transformant was cultured in BMMY medium and was induced by

methanol in order to express a recombinant enzyme. After 60 h, recombinant HBGase III was secreted into the supernatant. The highest specific activity (0.62 U/mg) was detected at 144 h (7 days) after 1 % methanol induction (Fig. 3A). Furthermore, protein expression was confirmed by Coomassie blue - stained SDS-PAGE (Fig. 3B). In addition, the obtained activity of secreted HBGase III was up to 6 U from the large scale culture (1.2 l).

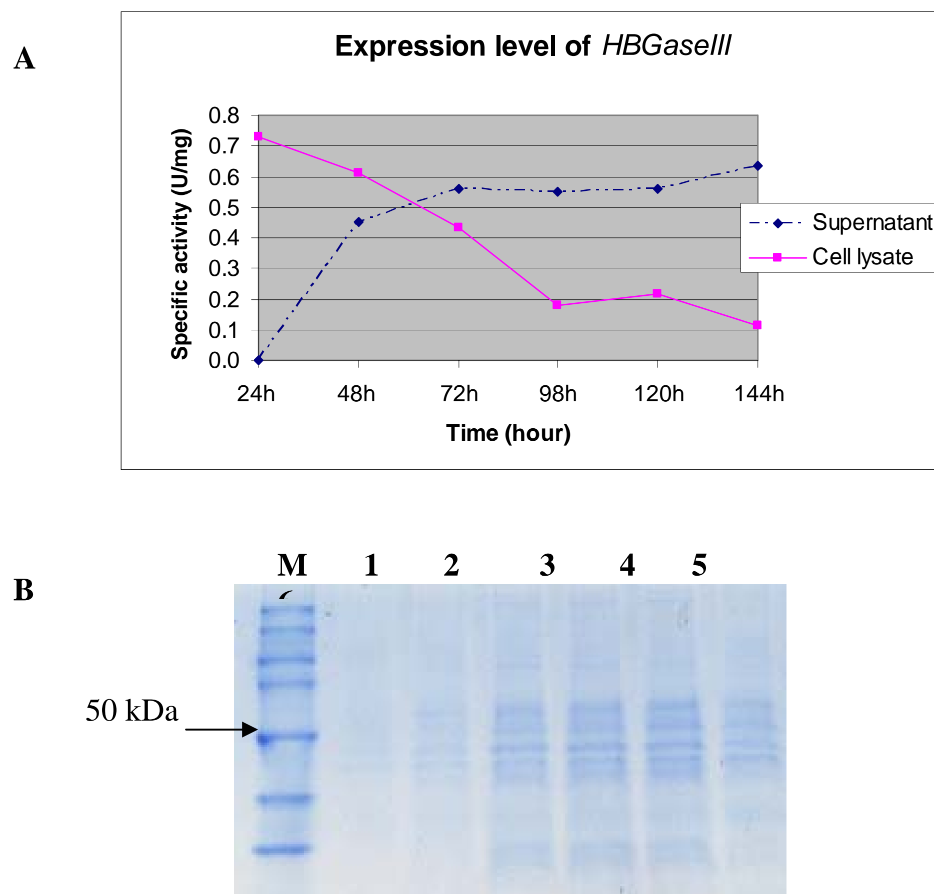

Fig. 3. Expression of recombinant HBGase III. In panel 2A, recombinant HBGase III was found in both supernatant and cell lysate. In panel 2B, the induced protein band was visible on SDS PAGE. Lane M contained protein marker.

Purification of recombinant HBGase III

Secreted protein in supernatant and that in cell lysate was separately concentrated by Vivaspin20 (GE Healthcare). Concentrated recombinant HBGase III was purified by using Histrap affinity column (GE Healthcare) in a small scale. Ten fractions were colleted and kept at -20°C. An enzyme activity was assayed by using PNPG as substrate. The unbound fraction# 3 provided the highest enzyme activity (052 U/ml) so the enzyme solution of this fraction was used for characterization. Considering fraction# 8, it contained eluted protein. Although it provided the lower activity as only 0.0053 U/ml (Fig. 4A), by SDS PAGE analysis, the single band was visible on the gel (Fig. 4B).

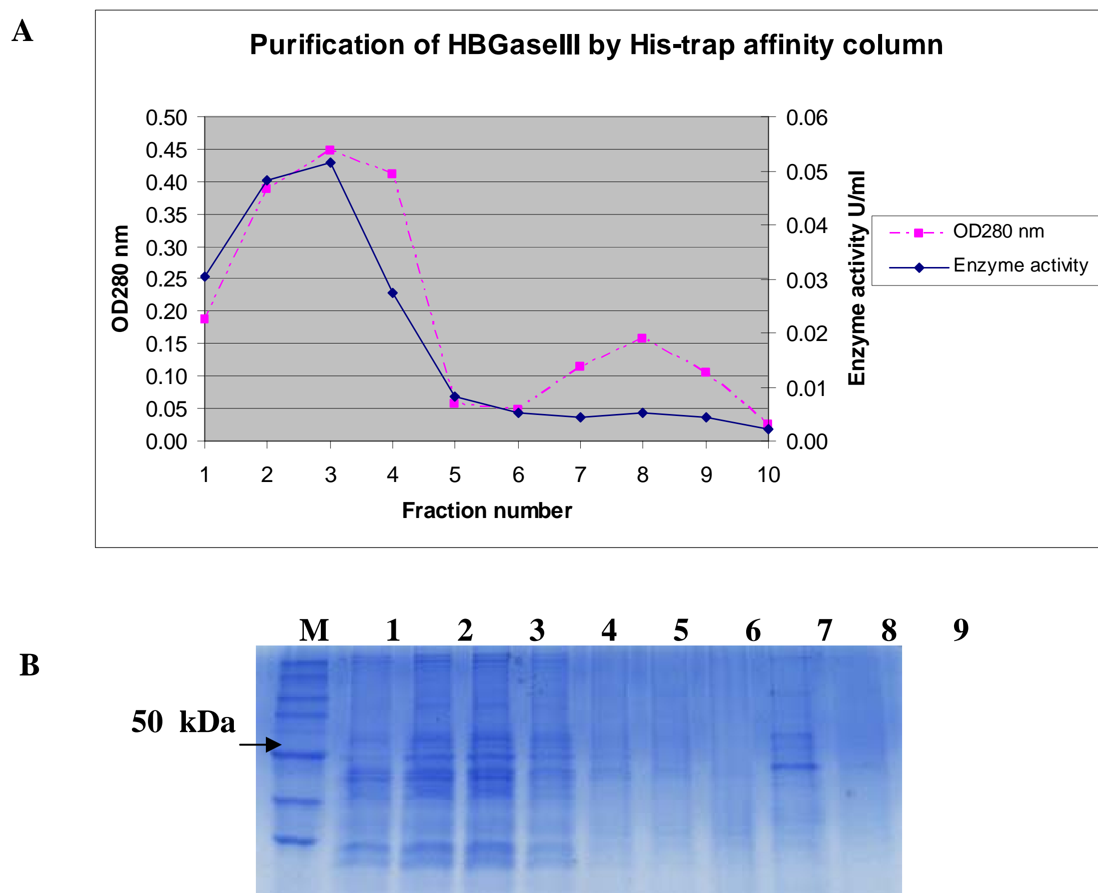

Fig. 4. Purification of HBGase III by Histrap affinity column (3A) and the HBGase pattern on SDS PAGE (3B).

Characterization of recombinant HBGase III

Effect of pH and temperature

The effects of pH and temperature on activity of HBGase III were measured by using p-nitrophenyl α-D-glucoside (PNPG, Sigma). As shown in Fig. 5, the pH-activity was 5.0 (Fig. 5A) and the pH-stability was stable in the pH range of 4.5-7.5 (Fig. 5B). For thermal stability, the enzyme was stable up to 50°C but the activity was totally lost at 55°C after 15 min incubation (Fig. 5C).

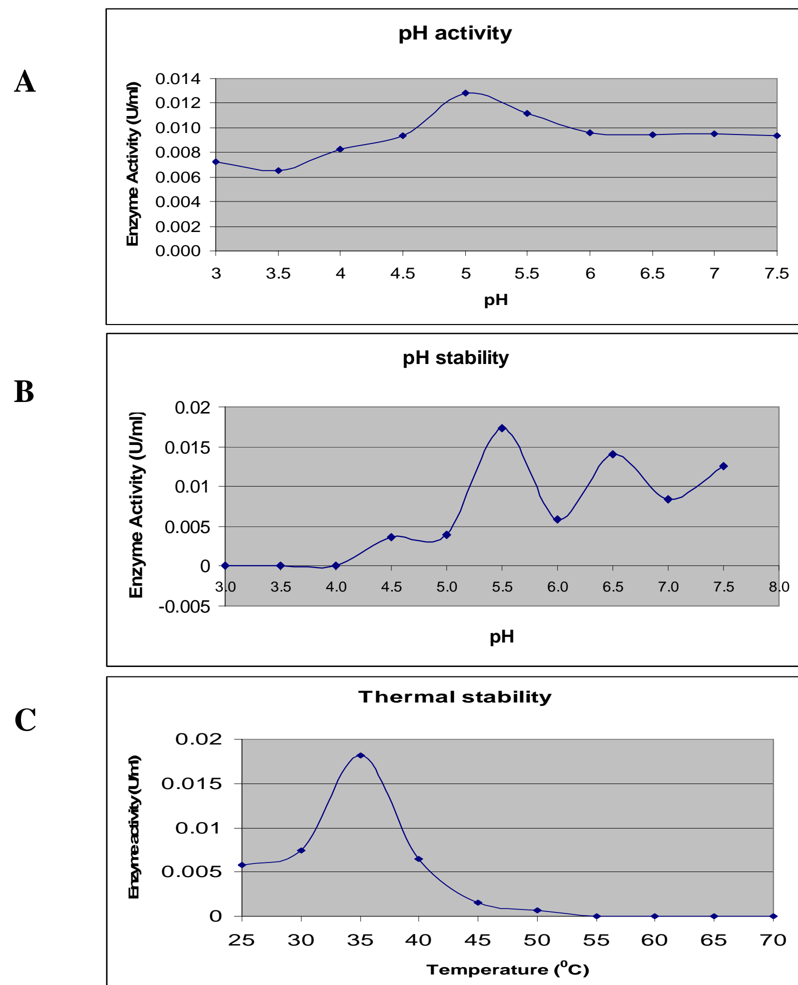

Fig. 5. The pH activity (5A), the pH stability (5B), and the thermal stability (5C) of recombinant HBGase III were indicated.

Substrate specificity

The substrate specificity of recombinant HBGase III was examined by using several kinds of substrates. Six substrates were used (Fig. 6). The results presented that the best hydrolyzed substrate were maltose and PNPG at the concentrations of 5 mM and 10 mM while low hydrolyzed substrates were maltotriose, isomaltose, sucrose, and soluble starch, respectively.

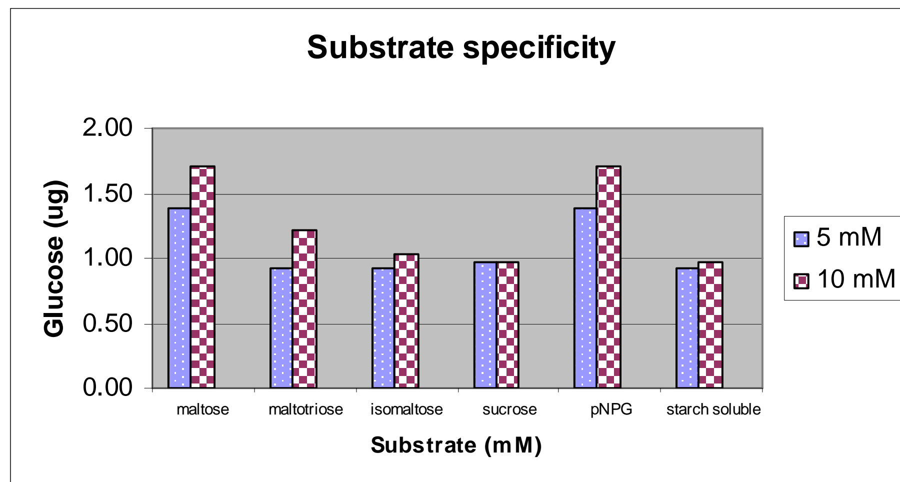

Fig. 6. Substrate specificity of HBGase III on maltse, maltotriose, isomaltose, PNPG, and soluble starch were presented. Chosen concentrations of each substrate were 5 mM and 10 mM.

Discussion

Expression pattern of α-glucosidase

Considering *α-glucosidase I* (*HBGase I*), the highest expression was found in forager bees which was coincided to the highest expression of *HBGase III.* Among *Apis* spp., the same result was obtained from *A. cerana indica* (Srimawong, 2003), *A. mellifera* (Kubo *et al*., 2006), and *A. florea* (Chanchao *et al*., 2006). As known, *HBGase III* was presented in hypopharyngeal glands and was secreted into honey crop (Kubota *et al*., 2004). *HBGase I* and *II* were the highest expressed in forager bee, but not in honey. It indicated that HBGase III was involved directly in honey production. For *α-glucosidase II* (*HBGase II*), the highest expression of *HBGase II* was in larvae and pupae but the lower expression was found in forager bees. In addition, no expression

was detected in eggs. Considering the obtained data, forager bees were the appropriate stage for the study of HBGases.

cDNA sequence of HBGase I and II

At present, the cDNA sequences of *HBGase I* and *II* were not completed yet. Only, the nucleotide at 942 and 834 bp, respectively, was obtained. The mentioned nucleotide sequences were mostly related to the sequences of the same genes from *A. cerana japonica*. *HBGase I* was mostly similar to *A. cerana japonica* isozyme I (NM_AB260890.1) at 98% while *HBGase II* was similar to the sequence of *A. cerana japonica* isozyme II (NM_FJ752630.1) at 100%. This was supported by the researches of Nishimoto *et al.* (2007) and Wongchawalit *et al*. (2006) which presented that HBGase I from European honeybees (*A. mellifera*) was mostly similar to HBGase I from Japanese honeybee. In over all, it indicated the close relatedness of honeybees in *Apis* spp.

Expression and optimum conditions of recombinant α-glucosidase III

Optimum conditions of recombinant HBGase III in *A. cerana indica* and in *A. mellifera* were different. For example, HBGase III of *A. cerana indica* was secreted to supernatant after 60 h and the highest activity was found after 144 h induction. HBGase II and III of *A. mellifera* was secreted after 50 h and the highest activity was found after 200 h induction (Nishimoto *et al*., 2007).

Since pPICZαA as an expression vector already contained histidine-tagged sequence, HisTrap affinity column (GE Healthcare) was used to purify recombinant

HBGase III. The highest activity of recombinant HBGase III was found in unbound fractions (fractions# 1-4) and a single band was visible on SDS PAGE (Fig. 2). In the future, large scale for purification will be performed and another columns such as DEAE cellulose, Superdex 200, etc. will be used according to Chanchao *et al*. (2007, 2008), DEAE-Sepharose CL-6B, Bio-Gel P-150, CM-Toyopeal 650 M, and Sephacryl S-100 (Takewaki *et al*., 1980; Nishimoto *et al*., 2001; Wongchawalit *et al*., 2006; Nishimoto *et al*., 2007).

Activity of native HBGase III (Chanchao *et al*., 2008) and recombinant HBGase III were assayed by using different methods. Native form was assayed by Momose's method (Momose & Inaba, 1961) and recombinant form was assayed by *p*-nitrophenol (PNPG) method, respectively. Although two forms of HBGases were assayed differently, the same optimum pH at 5.0 could be revealed. The similar result was also obtained in native HBGase I from *A. cerana japonica* (Wongchawalit *et al*., 2006) and both native HBGase I and II from *A. mellifera* (Takewake *et al*., 1980).

Besides, the obtained HBGase III in this research was compared to recombinant HBGase II and III from *A. mellifera* and to native HBGase I from *A. cerana japonica*. The thermal stability of HBGase III from *A. cerana indica* was below 50°C while it was below 37°C for the former one and below 40°C for the latter one. It might indicate that recombinant HBGase III from *A. cerana indica* could be tolerant to higher temperature.

From the above mentioned data, it was confirmed that recombinant HBGase III could be in *vitro* expressed in yeast, *P. pastoris* expression system. Later, the enzymes kinetics of HBGase III will be obtained and recombinant clones of HBGase I and II will be expressed and characterized.

Acknowledgments

This research was supported by Thailand Research Fund (grant # RMU5180042), Center of Excellence in Biodiversity, Faculty of Science, Chulalongkorn University, and Graduate School of Chulalongkorn University.

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Output ที่ได้จากโครงการ

Umthong, S., Phuwapraisirisan, P., Puthong, S., and **Chanchao, C.** (2011) *In vitro* antiproliferative activity of partially purified *Trigona laeviceps* propolis from Thailand on human cancer cell lines. BMC Complementary and Alternative Medicine. 11: 37. doi: 10.1186/1472-6882-11-37. **(unofficial 2009 impact factor 1.94,** indexed in Pubmed**)**

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