# ลักษณะสมบัติและการแสดงออกของรีคอมบิแนนต์แอลฟากลูโคซิเดสชนิดที่ I, II และ III ในผึ้งไทย *Apis cerana indica*

นางสาวจิรัฏติกาล แก้วเมืองมูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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# CHARACTERIZATION AND EXPRESSION OF RECOMBINANT α-GLUCOSIDASE I, II and III IN THE THAI HONEYBEE *Apis cerana indica*

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**จิรัฏติกาล แก้วเมืองมูล: ลักษณะสมบัติและการแสดงออกของรีคอมบิแนนต์แอลฟากลูโคซิเดส ชนิดที่ I, II และ III ในผึ้งไทย *Apis cerana indica* (CHARACTERIZATION AND EXPRESSION OF RECOMBINANT α-GLUCOSIDASE I, II and III IN THE THAI HONEYBEE *Apis cerana indica*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, 157 หน้า**

แอลฟากลูโคซิเดส (HBGase) สามารถ catalyse สารตั้งต้นที่มี non-reducing end เพื่อให้ได้แอลฟาดีกลูโคสและเอนไซม์ชนิดนี้มี 3 ชนิดคือ HBGase I, II และ III ตามความจำเพาะต่อสารตั้งต้น ในงานวิจัยนี้สนใจที่จะศึกษา recombinant HBGase I, II และ III จาก *Apis cerana indica* (r*Aci*HBGase I, II และ III) เพื่อค้นหาแหล่งใหม่ของเอนไซม์ ผึ้งชนิดนี้ยังเป็นผึ้งพื้นเมืองและผึ้งเศรษฐกิจของประเทศไทยอีกด้วย ก่อนหน้านี้มีรายงานถึง cDNA ที่ยาวเต็มสายของ ORF และ deduced amino acid ของ *Aci*HBGase III ว่ามีความยาว 1,704 คู่เบสและ 567 residues ในงานวิจัยนี้ได้ cDNA ที่ยาวเต็มสายแบบ ORFและ deduced amino acid ของ *Aci*HBGase I (1,734 คู่เบสและ 577 residues) และ *Aci*HBGase II (1,740 คู่เบสและ 579 residues) โดยเทคนิค RT-PCR หลังจากนั้น cDNA ที่ยาวเต็มสายของ *Aci*HBGase II และ III ถูกโคลนเข้าสู่ pPICZαA ซึ่งเป็น expression vector และประสบผลสำเร็จในการแสดงออกใน *Pichia pastoris* GS115 การแสดงออกที่สูงสุดของ r*Aci*HBGase II และ III ถูกชักนำโดยเมทานอลที่ 0.5% (ปริมาตรต่อปริมาตร) เป็นเวลา 96 ชั่วโมงและเมทานอลที่ 1% (ปริมาตรต่อปริมาตร) เป็นเวลา 144 ชั่วโมง ตามลำดับ อย่างไรก็ตาม cDNA ที่ยาวเต็มสายของ *Aci*HBGase I ถูกโคลนเข้าสู่ pEcoli ซึ่งเป็น expression vector และประสบผลสำเร็จในการแสดงออกใน *E. coli* Rosetta (DE3) หลังจากถูกชักนำด้วย IPTG ที่ 1 มิลลิโมลาร์เป็นเวลา 3 ชั่วโมง r*Aci*HBGase I, II และ III ที่บริสุทธิ์แสดงค่า pH ที่เหมาะสมที่สุดที่ 3.5, 3.5, 5.0 และสามารถเสถียรในช่วง pH ที่ 3.5-5.0, 5.0-7.0, 5.0-7.5 ตามลำดับ นอกจากนั้น r*Aci*HBGase I และ II แสดงอุณหภูมิที่เหมาะสมที่สุดที่ 40 และ 45 องศาเซลเซียสตามลำดับ แอกทิวิตีของ r*Aci*HBGase I และ II ยังคงอยู่ในสภาพแวดล้อมที่เป็นกรดและทนต่อกรดได้ เอนไซม์เหล่านี้สามารถทำงานได้ดีที่อุณหภูมิค่อนข้างสูง (35-50 และ 45-55 องศาเซลเซียสตามลำดับ) ในทางตรงข้าม r*Aci*HBGase III แสดงแอกทิวิตีที่เหมาะสมที่สุดที่ 37 องศาเซลเซียสพร้อมทั้งไม่สามารถทนอุณหภูมิสูงๆ ได้ ยิ่งไปกว่านั้นความจำเพาะต่อสารตั้งต้นของ r*Aci*HBGase I และ II คือซูโครสแต่ของ r*Aci*HBGase III คือมอลโทสและ PNPG จึงสรุปได้ว่า r*Aci*HBGase มีประโยชน์และสามารถเป็นแหล่งใหม่เพื่อการประยุกต์ใช้ต่อไป

**สาขาวิชา .....เทคโนโลยีชีวภาพ…….** **ลายมือชื่อนิสิต ……..**

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**KEY WORD: ALPHA-GLUCOSIDASE / *Apis cerana indic*a / EXPRESSION / KINETICS / PURIFICATION / RECOMBINANT ENZYME**

**JIRATTIKARN KAEWMUANGMOON: CHARACTERIZATION AND EXPRESSION OF RECOMBINANT α-GLUCOSIDASE I, II and III IN THE THAI HONEYBEE *Apis cerana indica.* ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO. 157 pp.**

α-Glucosidase (HBGase) catalyzes the non-reducing end of a substrate to liberate α-D-glucose and can be classified into three types of HBGase I, II and III according to the substrate specificity. Recombinant HBGase I, II and III from *A. cerana indica* (r*Aci*HBGase I, II and III) were focused in this research in order to find a new source of enzyme. Also, this bee species is native and economic to Thailand. Previously, it was reported that the full length ORF cDNA and predicted deduced amino acid of *Aci*HBGase III was 1,704 bp and 567 residues. In this research, the full length ORF cDNA and predicted deduced amino acid of *Aci*HBGase I (1,734 bp and 577 residues) and *Aci*HBGase II (1,740 bp and 579 residues) were obtained by RT-PCR. Later, the full length cDNA of *Aci*HBGase II and III were cloned into pPICZαA expression vector and were successfully expressed in *Pichia pastoris* GS115. The highest expression of r*Aci*HBGase II and III was induced by 0.5% (v/v) methanol for 96 h and 1.0% (v/v) methanol for 144 h, respectively. However, the full length cDNA of *Aci*HBGase I was cloned into pEcoli expression vector and was successfully expressed in *E. coli* Rosetta (DE3) after induced by 1 mM IPTG for 3 h. The purified r*Aci*HBGase I, II and III showed an optimum pH of 3.5, 3.5, 5.0 and was stable in the pH range of 3.5-5.0, 5.0-7.0, 5.0-7.5, respectively. In addition, r*Aci*HBGase I and II showed a thermal optimum at 40 and 45°C, respectively. Theirs activity still remained active in an acidic environment and was acid tolerant. Also, they could work well at the relatively high temperatures (35-50 and 45-55°C, respectively). In contrast, r*Aci*HBGase III showed an optimum activity at 37°C with no thermo tolerance. Moreover, substrate specificity of r*Aci*HBGase I and II were sucrose but of r*Aci*HBGase III was maltose and PNPG. In conclusion, r*Aci*HGAases are useful and can be a new source for further application.

**Field of Study: ..Biotechnology.....** **Student's Signature: ……**

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# CHAPTER I

## INTRODUCTION

α-Glucosidase (HBGase; EC 3.2.1.20) can hydrolyze the α-glucosidic linkage of non reducing end of substrates such as carbohydrate polymers and other glucosides including phenyl α-glucoside to liberate α-glucose (Takewaki *et al*., 1980). The enzymes are classified into 3 isoforms of HBGase I, II and III which are based on their substrate specificity (Giannesi *et al*., 2006; Nishimoto *et al*., 2007). They are divided into two groups of the GH13 (HBGase I) and GH31 (HBGase II and III) families based on their sequence homology within the carbohydrate-active enzyme database (Kimura, 2000; Nimpiboon *et al*., 2001). HBGases are useful in biotechnological, clinical, and microbiological approaches. These enzymes come from various sources like microorganisms, plants, mammals and insects (Nakai *et al*., 2007; Nimpiboon *et al*., 2001; Nishimoto *et al*., 2007; Okuyama *et al*., 2001).

Nowadays, *Apis mellifera* (European honeybee) has been widely used as a model for a new source of HBGases in insects. It was also reported that there were three kinds of HBGases (I, II and III) which were different in substrate specificity, molecular weights, nucleotide sequences and tissue locations (Kubota *et al*., 2004). The native HBGases from *A. mellifera* (*Am*HBGase) were successfully purified by DEAE-cellulose, DEAE-Sepharose CL-6B, Bio-Gel P-150 and CM-Toyopeal 650 M chromatographies. All enriched *Am*HBGase I, II and III were found to be a monomeric glycoprotein containing about 25%, 15% and 7.4%, respectively (Kimura *et al*., 1992; Takewake *et al*., 1993; Nishimoto *et al*., 2001). Besides *A. mellifera*, three isoforms were also reported in *A. cerana japonica* (Wongchawalit *et al*., 2006).

Recently, recombinant technology or genetic engineering becomes popular in order to provide enough HBGase production for industrial application. Expression of recombinant enzyme in *Pichia pastoris* (yeast) expression system has been widely used since this eukaryotic system achieves a low production cost and highly efficient enzyme production. Unlike a bacterial expression system, the recombinant enzyme from this system is free of bacterial endotoxins and lipopolysaccharides. By using *P. pastoris* expression system, it can establish the correct folding and glycosylation of new born protein. For example, in honeybee research, recombinant HBGases I, II and III from *A. mellifera* were produced by using pPICZαA as an expression vector which was, later, transformed into *P. pastoris* GS115. The enriched recombinant enzymes were induced by 0.5% (v/v) methanol due to the designed vector containing AOX1 regions (Nishimoto *et al*., 2007).

Currently, *A. cerana indica* is wildly managed as economic bees in many Asian countries because it can be more resistant to mites, predators and pathogens than the introduced *A. mellifera*. Besides, it can adapt to the local environment and can survive without supplementary feeding and medication (Chen *et al*., 2000; Peng *et al*., 1987; Pothichot and Wongsiri, 1993). In Thailand, there were a few researches on enzymes from *A. cerana indica*. Only native HBGase III was purified and characterized (Chanchao *et al*., 2008). Native HBGase III from *A. cerana indica* (*Aci*HBGase III) was purified and characterized. Briefly about this work, by RT-PCR, the full length cDNA sequence of *AciHBGase III* was 1,704 bp encoding for the predicted polypeptide of 567 amino acid (GenBank, accession # EF441271). Furthermore, *Aci*HBGase III was purified by DEAE-cellulose and Sephadex 200 chromatographies (2.2 U/mg and 1.8 U/mg, respectively). Moreover, such native

enzyme was also purified from *A. florea* (Chanchao *et al*., 2007) and *A. dorsata* (Kilaso *et al*., 2011).

In this research, two more forms of HBGases were reported. The transcriptional expression pattern and sequence analysis of *Aci*HBGase I and II were evaluated. Moreover, the properties of recombinant *Aci*HBGase I, II and III (r*Aci*HBGase I, II and III) were obtained after cloning the full length cDNA sequence into an expression vector, induction by methanol and purification by a single step affinity column. Based on our knowledge, this is the first report on *HBGases* and their recombinant enzymes from this native and economic honeybee species in Thailand. The outcome from this research will reveal a new source of HBGase which may be applied in food industry and biotechnology hopefully.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Biology of *Apis cerana*

*A. cerana* (Asiatic honeybee; Eastern honeybee), is a species of honeybee found in southern and southeastern Asia. This species is the sister species of *A. koschevnikovi* and both are in the same subgenus as the European honeybee *A. mellifera*. *A. cerana* can be classified into eight subspecies which are 1) *A. cerana cerana* Fabricius found in Afganistan, Pakistan, north India, China and north Vietnam; 2) *A. cerana heimifeng* Engel; 3) *A. cerana indica* Fabricius found in South India, Sri Lanka, Bangladesh, Burma, Malaysia, Indonesia and Thailand; 4) *A. cerana japonica* Fabricius found in Japan; 5) *A. cerana javana* Enderlein; 6) *A. cerana johni* Skorikv; 7) *A. cerana nuluensis*, Tingek; and 8) *A. cerana skorikovi* (or *himalaya*) Engel found in central and eastern Himalayan mountains (Engel, 1999).

In Thailand, there are five species of honeybees. Four species are native which are *A. andreniformis* Smith, 1858; *A. florea* Fabricius, 1787; *A. cerana indica* Fabricus, 1787 and *A. dorsata* Fabricius, 1793. Only one species is imported which is *A. mellifera* Linnaeus, 1758. *A. cerana indica* looks similar to *A. mellifera* except the color and body size. *A. cerana indica* has black stripes on its abdomen but not *A. mellifera*. For the body size, *A. mellifera* is larger. Both of them like nesting close to hilly areas and are sometimes seen in plain regions. They can potentially colonize in temperate areas with prolonged winter or cold temperature. Both are less aggressive and also perform less swarming behavior than other wild bees like *A. dorsata* and *A. florea*. Thus, both can be easily managed for beekeeping (Oldroyd and Wongsiri,

2006). Both bees can be well adapted to live in any cavities like a tree trunk, a house, a temple and in purpose-made hives (Fig. 2.1) (Dyer and Seeley, 1991).

Considering bee products, honey of both bee species is popular among consumers. In contrast, while propolis of *A. mellifera* has been widely used in folk medicine, *A. cerana* does not produce propolis. However, its beeswax was able to treat and heal wounds (Oldroyd and Wongsiri, 2006).

In term of communication, like *A. mellifera*, *A. cerana* communicates to their nest members about the distance and location of food and water sources by means of waggle dance and round dance languages (Engel, 1999: Oldroyd and Wongsiri, 2006). The waggle and round dances of *A. cerana* are performed in the dark and on a vertical comb surface of a multiple-comb composing nest. Thus, they usually convey their wagging motions in the dark via vibrations. In general, they have a foraging range of about 1 km in diameter.

Furthermore, *A. cerana* can maintain the internal hive temperature precisely. It uses the similar mechanism to *A. mellifera*. The blood temperature of *A. cerana* is also stable in the range of 33-35 $^{0}$C even though the ambient temperature can be varied in the range of 12-36 $^{0}$C. This mechanism clearly shows that they possess effective thermoregulation systems in the hive. In summer, *A. cerana* employs evaporation cooling, where the worker bees cluster outside the nest in the hot weather and fan their wings. By doing this, it can remove the excess heat out of the hive and bring the moisture into the nest instead. Finally, it can decrease the hive temperature (Oldroyd and Wongsiri, 2006).

Even though *A. cerana* and *A. mellifera* share a lot in common in many behaviors, there are still some different behaviors such as reproductive swarming. *A. cerana* has more migrating and absconding behavior than *A. mellifera*. Absconding will start when there is not enough pollen and nectar. After the last brood emerges, the adult bees will fill their honey crop from the stored honey. Then, they will swarm eventually to establish a new nest at a new location (Oldroyd and Wongsiri, 2006). Moreover, *A. cerana* nest size is similar or somewhat smaller than the nest of *A. mellifera*. *A. cerana* usually does not store much honey in the hive so honey yield is less. Also, that may be because the bees form a smaller colony.

In term of defensive behavior, *A. cerana* could act against a predatory wasp like *Vespa velutina* and *Vespa magnifica* (Abrol, 2006). It can kill many predatory wasps by clumping into a well organized ball. The bee mortality is higher when a few wasps invade an apiary due to an unorganized defense. However, when the intensity of attack was severe, fewer bees can kill a lot of wasps due to an organized defense.

**Figure 2.1** Multiple-comb nest of *A. cerana indica* in a coconut tree trunk found in an apiary in Samut songkram province, Thailand. Photo by J. Kaewmaungmoon.

*A. cerana indica* is classified as below (Wongsiri, 1989; Oldroyd and Wongsiri, 2006).

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hymenoptera

Suborder: Apocrita

Superfamily: Apoidea

Family: Apidea

Subfamily: Apinae

Tribe: Apini

Genus: *Apis*

Species: *Apis cerana*

Subspecies: *Apis cerana indica*

## 2.2 α-Glucosidase (HBGase)

α-Glucosidase (HBGase; EC3.2.1.20) as an exocarbohydrase can cleave or hydrolyze the α-glucosidic linkage of carbohydrate polymers to liberate glucose (Chiba, 1997). Moreover, HBGase can perform transglucosylation reaction *in vitro* (Frandsen and Svensson, 1998; Yamamoto *et al*., 2004). This enzyme has been classified into two families (I and II). Family I (GH13) enzyme has four conserved regions for catalytic reaction (1-4) and these regions are shared by glycoside hydrolase family 13. While family II (GH31) enzyme has only two conserved sequence regions (1 and 2) which the two essential residues (D481 and D647) are found within the amino acid sequence and belong to the glycoside hydrolase family 31. Furthermore, HBGase can be divided into three types of HBGase I, II and III based on their substrate specificity (Giannesi *et al*., 2006; Nishimoto *et al*., 2007). HBGase I (a member of the GH13 family) prefers to hydrolyze aryl glucosides and sucrose as substrates whereas HBGase II and III (members of the GH31 family) show the preference for substrate like maltose and isomaltose. Only the HBGase III group can hydrolyze polysaccharide like starch and amylase (Nimpiboon *et al*., 2011).

### 2.2.1 Homologous HBGase

Homologous HBGase could occur in several reasons. For example, the different isoform could be derived from different genes like HBGase I, II and III in *Apis cerana japonica* (*Acj*HBGase) (Wongchawalit *et al*., 2006), or from differential post transcriptional or post translational modifications of the same gene. For example, the alternative mRNA splicing of the HBGase II mRNA could yield HBGase III and the post-translational proteolysis of the HBGase I could yield HBGase I in the

ripening seeds of *Oryza sativa* L. (Nakai *et al*., 2007). Furthermore, the putative *N*-glycosylation motif (N-Xa.a.-S/T) for the addition of various oligosaccharides onto asparagine residues was found in the predicted primary amino acid sequence of HBGase I, II and III in *A. mellifera* (*Am*HBGase) (Nishimoto *et al*., 2007).

### 2.2.2 Source of HBGase

It was reported that microorganisms, plants, mammals and insects were sources for various isoforms of HBGases (Chiba, 1997; Nakai *et al*., 2007; Nimpiboon *et al*., 2001; Nishimoto *et al*., 2007; Okuyama *et al*., 2001). The potential diversity of enzyme characteristics in HBGase isoforms was frequently found. Isoforms of homologous HBGase were 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.

Moreover, they found homologs of these three HBGase isoforms (I, II and type II-like HBGase) in yeast *Sporothrix schenckii*, 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. However, they were different in their molecular mass and biochemical characters (Torres-Rodriguez *et al*., 2012).

### 2.2.3 HBGase in honeybee

In honeybee, there were three kinds of HBGases (I, II and III) which were different in substrate specificity, mass weight, nucleotide sequences and tissue location. Moreover, these three enzymes were different in optimum pH, pH stability,

thermal stability and sugar content (Kimura *et al*., 1990; Nishimoto *et al*., 2001; Takewaki *et al*., 1993).

In worker honeybee, three enzymes (HBGase I, II and III) were different in tissue location. HBGase I was found in ventriculus. HBGase II was found in both ventriculus and haemolymph while HBGase III was found in hypopharyngeal glands (Fig. 2.2). Interestingly, due to substrate specificity, HBGase III was mostly the same as HBGase found in honey (Kubota *et al*., 2004). It could be implied that HBGase III was synthesized in hypopharyngeal glands and, later, was secreted into a honey crop in order to hydrolyse sucrose in nectar to be fructose and glucose. Alternatively, it could be said that *Am*HBGase III involved directly in making up honey which was full of monosaccharides.

More than the above, these *Am*HBGases were different in nucleotide sequences. The cDNA encoding *Am*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 sequences were 588, 580 and 567 in length, respectively (Nishimoto *et al*., 2007; Ohashi *et al*., 1996).

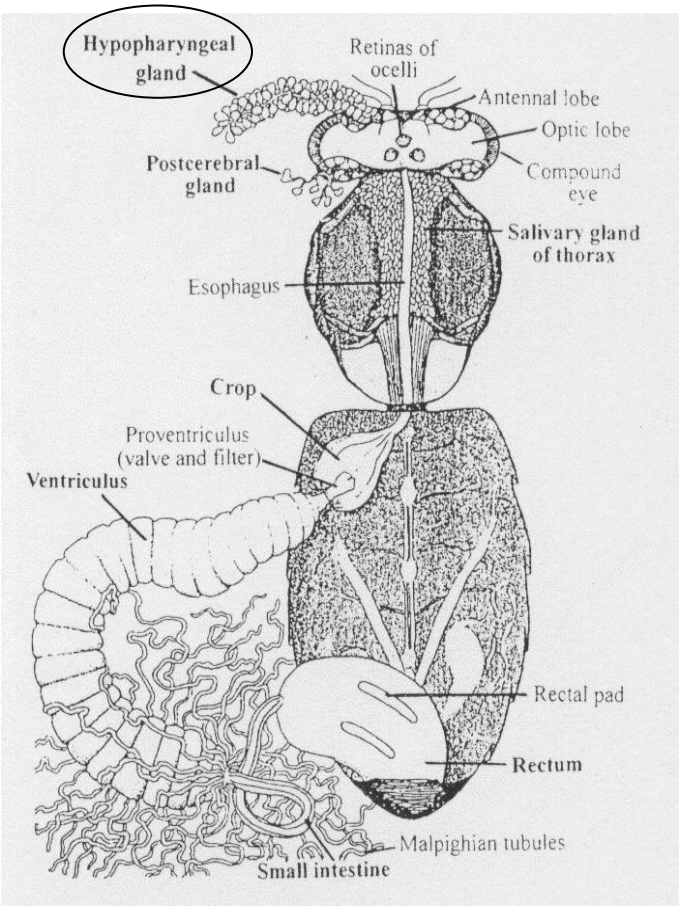


**Figure 2.2** Dissected anatomy of *A. mellifera* worker bee. Hypopharyngeal glands, ventriculus and honey crop as locations for *Am*HBGase I, II and III were indicated (modified from Kubota *et al*., 2004).

### 2.2.4 Native HBGases from honeybee

Native forms of three *Am*HBGases were already purified and characterized. The native *Am*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-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 diethylpyrocarbonate (DEPC). Furthermore, it was proved that *Am*HBGase I had a single catalytic site and there was one histidyl residue at or near the active site (Kimura *et al*., 1992).

Native *Am*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 *Am*HBGase I but both were different in substrate specificity. The *Am*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 *Am*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 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).

In addition, besides *A. mellifera*, native HBGases were already purified from other honeybee species like *A. cerana japonica* which is native to Japan. *Acj*HBGase I was purified by using CM-Toyopeal 650 M and Sephacryl S-100. It was a monomeric glycoprotein containing 15% carbohydrate with the molecular weight of 82 kDa. The full length cDNA of *Acj*HBGase I was 1,930 bp and deduced amino acid was 577 residues (Wongchawalit *et al*., 2006).

Moreover, native HBGase III from *A. cerana indica* (*Aci*HBGase III), *A. florea* (*Af*HBGase III) and *A. dorsata* (*Ad*HBGase III) which were native to Thailand were already purified. *Aci*HBGase III and *Af*HBGase III were purified by diethyaminoethyl-celluose and Superdex 200 gel filtration chromatography (Chanchao *et al*., 2007; Chanchao *et al*., 2008). The full length cDNA of *Aci*HBGase III and *Af*HBGase III were 1,704 and 1,701 bp predicting for 567 and 566 amino acids, respectively. Furthermore, *Ad*HBGase III was purified and characterized by diethylaminoethyl-cellulose ion exchange and Superdex 75 ion exchange chromatography. The full length cDNA of *Ad*HBGase III was 1,704 bp predicting for 567 amino acids (Kilaso *et al*., 2011).

## 2.3 Application of HBGases

HBGase is one of four enzymes (α-amylase, β-amylase, the de-branching dextrinase and α-glucosidase) those are important for starch degradation and are used in biotechnology (Bamforth, 2009), especially in the context of brewing.

In addition, glycosidase can be used in the glycosylation reactions used for carbohydrate synthesis, including various α-glucosylated compounds (Perugino *et al*., 2004). It is advantageous for food and cosmetics industry because, in glycosylation reaction, it uses mild reaction conditions avoiding harsh conditions or toxic catalysis like heavy metals (Pat *et al*., 2010). Moreover, a method using glycosidase is less expensive and more popularly used in industry more than glycosyltransferases in term of their glycosyl donors, availability and widespread occurrence in nature (Kato *et al*., 2002).

For biotechnology industry, HBGase from rice (*Oryza sativa*) is directly involved directly in the alcohol fermentation in sake processing. Thus, it is still challenging to find a new source of HBGase which is the key enzyme for preparation of alcoholic beverages and brewing. In 2003, Iwata *et al*. found that HBGase from *O. sativa* Yamadanishiki had different substrate specificity (nigerose) from the HBGase I (maltotriose and maltotetraose) purified from *O. sativa* cv. Shinsetsu, and also hydrolyzed nigerose and kojibiose better than the HBGase II from *O. sativa* cr. Shinsetsu**.**

For clinical purpose, since HBGase I could remove the outermost α-1,2 glucose unit from substrates, it was required in many organisms to survive *in vivo*. For example, human pathogenic yeast *C. albicans* used this enzyme to synthesize the

mannoproteins in the outer layer of its cell wall. This step was important in cell adhesion to host tissue, dimorphism, recognition by the host immune system and virulence (Mora-Montes *et al*., 2009).

**2.4 Recombinant HBGase**

Recombinant technique is wildly used to produce an enzyme that is normally expressed in a low level, is hard to enrich or has a limit in the commercial preparation (Zhao *et al*., 1993). Recombinant HBGases were reported. Recently, recombinant HBGase (rHBGase) produced by *Geobacillus sp*. HTA-462 which was isolated from deep sea sediment could be successful in synthesizing novel artificial glycolipids (Konishi *et al*., 2011). The mimic glycolipids produced could be used as biosurfactants and materials in a diverse array of biological applications by changing their physiochemical properties.

In honeybee, this technique was already used to characterize recombinant HBGase I, II and III from *A. mellifera* (r*Am*HBGase I, II and III). Each of the full length cDNA of *Am*HBGases was cloned into pPIC3.5 or pPIC9 expression vector. Then, it was transformed into *P. pastoris* GS115. The recombinant construct containing *Am*HBGase cDNA was induced by 0.5% (v/v) methanol (Nishimoto *et al*., 2007). After that, these recombinant enzymes were purified by using ammonium sulfate salting-out followed by fractionation over successive CM Sepharose CL-6B, Bio-Gel P-100, DEAE Sepharose CL6B and Butyl-toyopeal 650 M column chromatography.

### 2.4.1 Yeast expression system, *P. pastoris*

*P. pastoris* has become a highly successful system for the expression of heterologous genes. This system has a promoter derived from the alcohol oxidase I gene (*AOXI*) of *P. pastoris* that is uniquely suited for the controlled expression of foreign genes. Besides, with the similar strategy for the molecular genetic manipulation, *Saccharomyces cerevisiae* could also be used as one of the best-characterized expression systems in modern biology.

*P. pastoris* is a single-celled microorganism that is easy to manipulate and culture. However, it is also a eukaryote and capable of many posttranslational modifications performed in higher eukaryotic cells such as proteolytic processing, folding, disulfide bond formation and glycosylation. Thus, many proteins that end up as inactive inclusion bodies in bacterial systems are produced as biologically active molecules in *P. pastoris* (Cregg *et al.*, 2000).

Moreover, the *P. pastoris* yeast expression system has gained widespread use for production of proteins for structural or functional studies. Proteins that are normally secreted or required mammalian post-translational modifications cannot be produced in an active form in *E. coli*. Since *P. pastoris* can also grow rapidly in inexpensive media similar to *E. coli*, it is feasible to quickly and cheaply synthesize and examine the large numbers of both native and mutant proteins. Moreover, *P. pastoris* can be a suitable host in order to prepare high yield of glycosylated proteins. However, these sugars can be removed by cleavage with endoglycosidase H (Kim *et al.*, 1997; Nilsen *et al*, 1997).

In addition, *P. pastoris* has the methanol metabolic pathway, which appeared to be similarly found in all yeasts. It involves in a unique set of enzyme pathways (Veenhuis *et al*., 1983). The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde, generating hydrogen peroxide in the process by the alcohol oxidase (AOX). AOX is a homo-octomer which each subunit contains one noncovalently bound FAD (flavin adenine dinucleotide) cofactor. Although AOX has a poor affinity to $O_2$, methylotrophic yeasts appear to compensate this deficiency by synthesizing large amounts of the enzyme.

Furthermore, there are 2 genes (*AOX1* and *AOX2*) in *P. pastorsi* coding for AOX, but AOX1 gene is responsible for the vast majority of alcohol oxidase activity in the cell (Cregg *et al*., 1989). Approximately 5% of AOX from total soluble protein would be reached in methanol-grown shake-flask culture, but over than 30% of the AOX could be obtained in cells fed with methanol at the growth limiting rates in a fermenter culture (Couderc and Baratti, 1980).

GS115 (*his4*) is the most commonly used strains of *P. pastoris* as an expression host. It is wild type regarding to the *AOX1* and *AOX2* genes and grows on methanol at the wild type rate (methanol utilization plus $Mut^+$ phenotype) (Cregg and Madden, 1987).

Moreover, there are several plasmid vectors designed for heterologous protein expression in *P. pastoris*. For secretion of foreign proteins, a vector has been constructed to contain the *S. cerevisiae* α-factor, a signal DNA sequence for secretion, just immediately after the *AOX1* promoter (Cereghino and Cregg, 2000; Higgins and Cregg, 1998; Laroche *et al*., 1994).

Other than α-factor, a vector is designed to have a drug-resistant gene as a primary screening marker. At the beginning, it is used to separate a clone containing an insert and no insert. Later, multiple copies of foreign gene can be obtained after transformation and culture. For example, pPIC3K and pPIC9K vectors contain the bacterial kanamycin-resistant gene. Thus, any bacteria containing either vector can confer the resistance to high levels of G418. The more resistant strains contain more multiple copies of either vector (Cereghino and Cregg, 2000; Clare *et al*., 1991). For pPICZα series, each vector contains the *Sh ble* gene from *Streptoalloteichus hindustanus*. This gene is short (375 bp in length) and confers resistance to Zeocin drug. A host for this vector can be *E. coli*, yeasts (including *P. pastoris*) and other eukaryotic cells (Cereghino and Cregg, 2000; Higgins and Cregg, 1998).

The series of pPICZα vector is supplied with the multiple cloning site in three reading frames which are pPICZαA (3,593 bp), B (3,597 bp) and C (3,598 bp). They are different in multiple cloning sites. Also, *Pst*I and *Cla*I restriction sites were appeared only in pPICZα B and C vectors, respectively (Fig. 2.3). These vectors are used to express and secrete recombinant protein in *P. pastoris*. Recombinant proteins are expressed as a fusion protein because the sequence was attached to an N-terminal peptide encoding the *S. cerevisiae* secretion signal α-factor. This vector allows high-level of methanol induced protein of interest in *Pichia* strain.

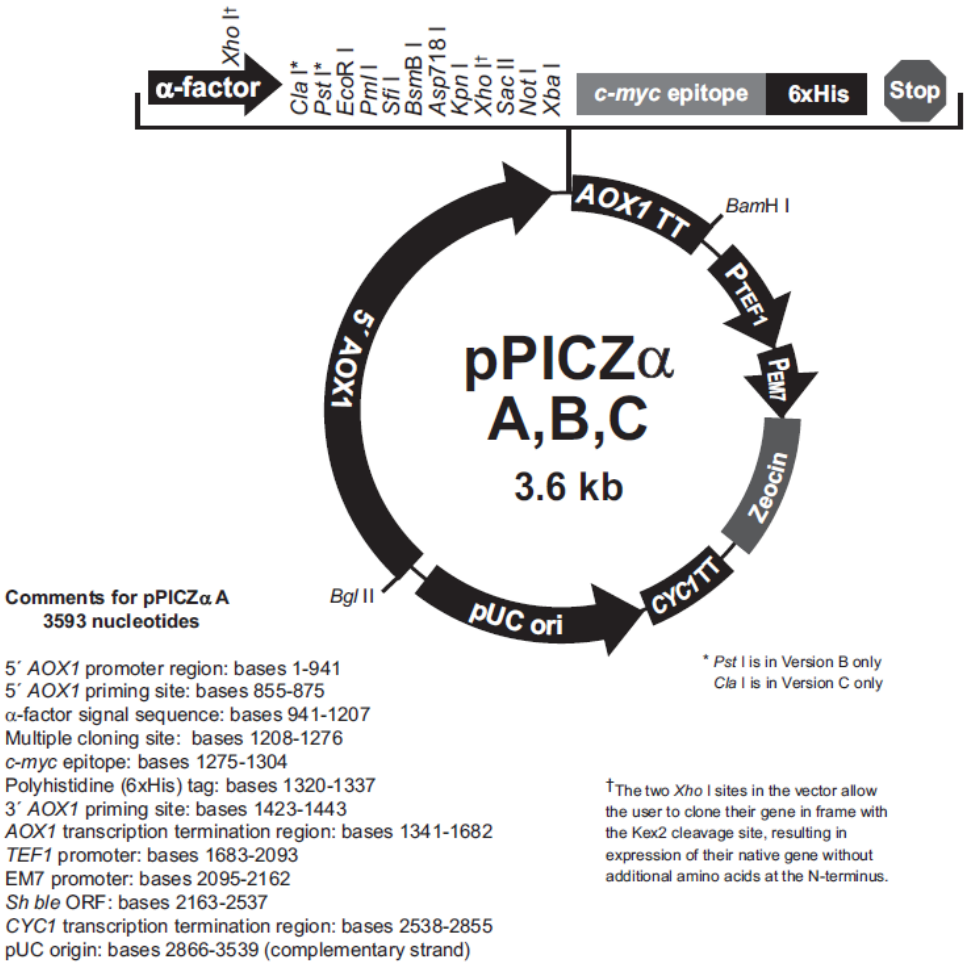


**Figure 2.3** A map of multiple cloning sites for C-terminal pPICZαA, B and C vectors. The map is from Invitrogen User Manual EasySelect™ Pichia Expression Kit.

### 2.4.2 *Escherichia coli* as a bacterial expression system

During the proteomic era, the production of recombinant proteins has been increased. A bacterial expression system for heterologous protein production is attractive due to low cost, high productivity, simplicity and rapid process. Nevertheless many bacterial systems are not able to modify proteins or posttranslational modification such as glycosylation, folding, cleavage. In case the posttranslational modification is essential for an enzyme activity, a bacterial expression system cannot be used for heterologous protein production. Alternative hosts such as yeast, filamentous fungi, insect cells and mammalian cells need to be considered (Terpe, 2006).

*E. coli,* the gram-negative bacterium, is the most commonly used for heterologous protein production in pharmaceutical industry because its genetics are well studied and the easiness for the safety handling in a laboratory. Thus, a large scale production system is established. However, a disadvantage still remains. Often, endotoxins and lipopolysaccharides (LPS) from *E. coli* which are pyrogenic to humans and other mammals can be contaminated to the produced products. Thus, it is necessary that synthesized protein from this process must be purified in the second step until it becomes endotoxin-free (Petsh and Anspach, 2000).

*E. coli* BL21 and K12 are most frequently used as hosts for recombinant protein expression. Unlike *E. coli* K12, *E. coli* BL are *lon* and *ompT* protease deficient (Phillips *et al.*, 1984). Besides posttranslational modification, more troubles may appear during the gene expression in a foreign host. Since there is the difference between the codon usage of *E. coli* and the over expressed protein, rare codons may

make a trouble. If the mRNA of heterologous target gene is trended to be over-expressed in *E. coli*, the difference in the codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the expression host (Goldman *et al*., 1995; Kane, 1995).

In order to enhance the expression of eukaryotic proteins or foreign proteins those contain any amino acids rarely used in *E. coli*, many *E. coli* were engineered to overcome this problem. Also, additional tRNAs under control of their native promoters will be supplied. For example, *E. coli* BL21 (DE3) derivatives were especially designed for the over expression of membrane proteins (Miroux and walker, 1996). Also *E. coli* Rosetta DE3, which is derived from *E. coli* BL21 can enhance the expression of eukaryotic protein that contains codons rarely used in *E. coli* such as AUA (isoleucine), AGG (arginine), AGA (arginine), CUA (leucine), CCC (proline) and GGA (glycine). Also, it is deficient in *lon* and *ompT* proteases.

Furthermore, in order to increase the heterologous expression in *E. coli*, T7 RNA polymerase is most widely used (Rosenberg *et al*., 1987; Studier and Moffatt, 1986; Studier *et al*., 1990). It elongates a chain about five times faster than *E. coli* RNA polymerase. In this research, pEcoli expression system was used for cloning and expression of *Aci*HBGase I (Fig. 2.4). This system which is based on the inducible T7 expression system (pET) contains the hybrid of T7 *lac* promoter and *lacI* gene which encodes Lac repressor. This combination reduces the expression in the absence of an inducer while it allows for rapid induction upon addition of IPTG to the bacterial culture. It also can reduce the background of expression level since Lac repressor

binds to the *lac* operator locating adjacent to the T7 promoter only (Dubendorff and Studier, 1991).

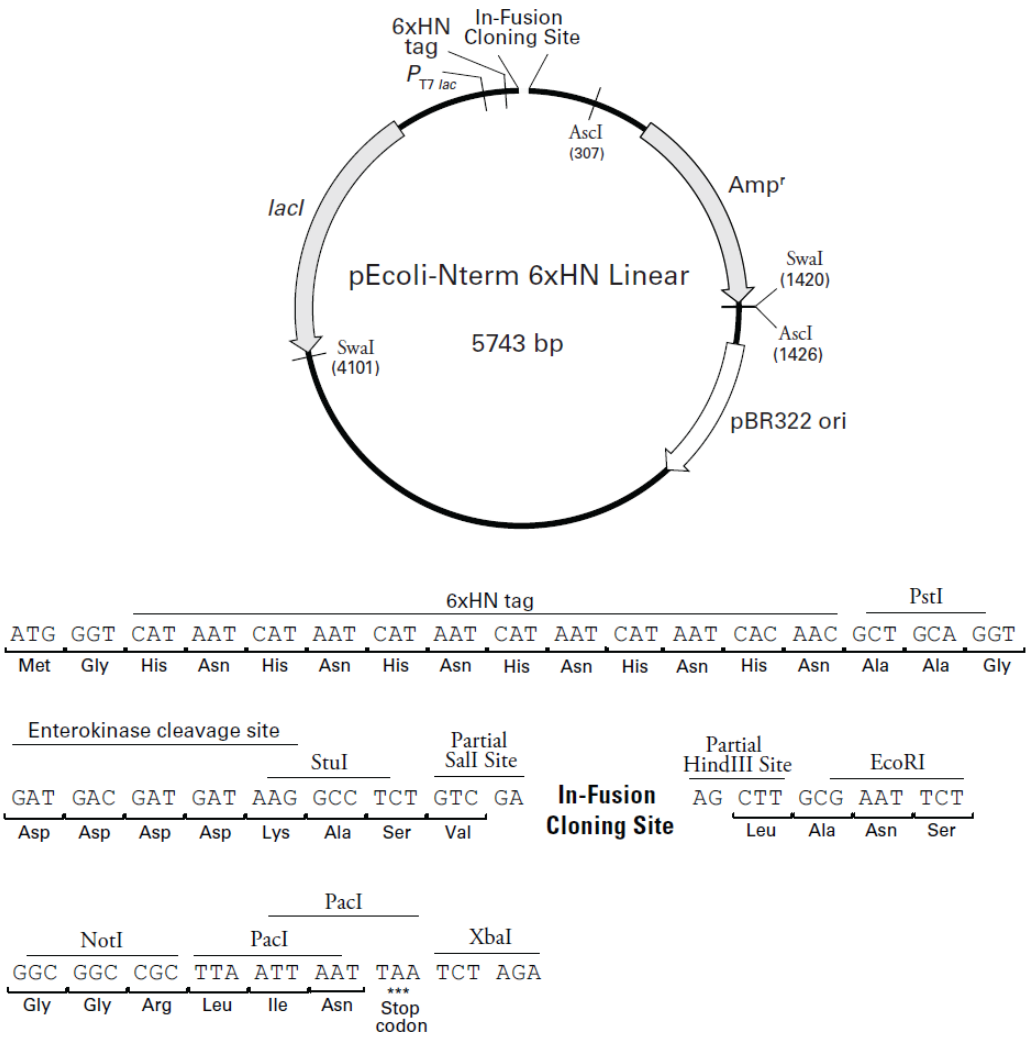


**Figure 2.4** A map of N-terminal pEcoli expression system presenting multiple cloning sites. This map is from pEcoli-Nterm 6xHN Vector Information (Clontec).

## 2.5 Recombinant protein purification

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. The staring material is usually biological tissue or microbial culture. Recombinant DNA technology is used to develop cells in low abundance to produce large quantities of desired protein. Recombinant expression allows the protein to be tagged like histidine-tag in order to facilitate the purification (Ehle and Horn, 1990; Porath *et al.*, 1975; Terpe, 2003).

Normally, there are three analytical purification properties to separate protein. First, protein may be purified according to its isoelectric point by running it through a pH graded gel. Second, protein can be separated according to its size or molecular weight via size exclusion chromatography or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) analysis. In addition, two dimensional PAGE (2D – PAGE) gel is often used to purify proteins with close mass weight. And third, protein may be separated by polarity/hydrophobicity via ion exchange chromatography or hydrophobic interaction chromatography. Eventually, the amino acid sequence of the target protein can be analysed by tryptic digestion assay and peptide mass fingerprinting to establish the protein identity (Regnier, 1983).

Other than the columns mentioned above, more columns are widely used. For example, ion exchange chromatography can separate protein based on its type and strength of charge. Affinity chromatography can separate protein based upon molecular conformation by specific resins and immune-affinity chromatography can selectively separate protein with the specific binding between antibody and a target protein.

### 2.5.1 Polyhistidine-tag (His-tag)

Immobilized metal affinity chromatography (IMAC) is another technique used to purify recombinant protein. It is based on the interaction between a transition metal ion immobilized on a matrix and specific amino acid side chain (Porath *et al*., 1975). This method contains a sequence of 6-8 histidines at either the N- or C-terminal of protein. The polyhistidine binds strongly to divalent metal ion such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions which binds to the histidine-tag protein. All untagged proteins pass through the column. The histidine-tag protein can be eluted by imidazole which competes the bound histidine-tag protein. Imidazole at low concentration is commonly mixed in the binding and the wash buffer to minimize the binding of host cell proteins. For the same reason, it is also important to add imidazole to a sample (generally, at the same concentration as in the wash buffer). At somewhat higher concentrations, imidazole may decrease the binding of histidine-tagged protein. The imidazole concentration must therefore be optimized to ensure the best balance of high purity (low binding of host cell proteins) and high yield (strong binding of histidine-tagged target protein). This optimal concentration is different for different histidine-tagged protein (Terpe, 2003).

## 2.6 Enzyme kinetic study

Enzyme kinetic is the study of a chemical reaction that is catalysed by enzyme**.** The study of enzyme kinetics can help us understand how metabolic pathways are controlled and which conditions under an enzyme is active. Moreover, kinetic study can yield information about a mechanism of an enzymatic reaction. In 1913, Michaelis

and Menten derived a rate law that governed enzyme kinetics. As enzyme-catalysed reactions are saturated, their rate of catalysis does not show a linear response to an increasing concentration of substrate. If the initial rate of the reaction is measured over a range of substrate concentrations [*S*], the reaction rate [*v*] will coincide to [*S*] as in Figure 2.5.

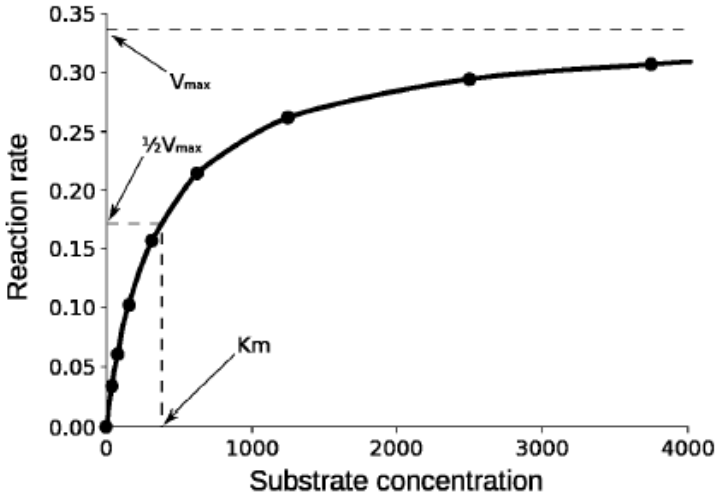


**Figure 2.5** Michaelis-Menten graph. It shows the saturation curve for an enzyme with the relation between the concentration of substrate [S] and reaction rate (*v*).

Michaelis-Menten enzyme kinetic can be modeled by the following equation.

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Where *v* represents the reaction velocity. *Vmax* represents the maximum reaction velocity. *Km* presents the Michalis-Menten constant. And [*S*] represents the substrate concentration. When looking at the equation, one can readily see that the velocity of the reaction (*v*) is dependent on the substrate concentration or [*S*]. In fact, the Michaelis-Menten equation is rationally functional which can be difficult to work with graph. The Michaelis-Menten equation can be transformed into a linear equation by taking the reciprocal plot of both sides as follow.

$$\frac{1}{v} = \frac{K_m + [S]}{V_{max}\,[S]} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

This new equation is called the Lineweaver-Burk equation after Michaelis and Menten in 1934. The Lineweaver-Burk equation is a linear equation (Fig. 2.6) where 1/*v* is a linear function of 1/[*S*] instead of *v* is a rational function of [*S*]. This equation can be readily represented graphically to determine the values of $K_m$ and $V_{max}$.

### 2.6.1 Key parameters from the Michalis-Menten equation

$K_m$ is one of parameters that provide an indication of the binding strength of an enzyme to its substrate. The less *Km* value indicates the better binding between an enzyme and a substrate.

$V_{max}$ is the maximum velocity ($v$) that an enzyme could achieve.

$K_{cat}$ or $k_0$ is the turnover number of an enzyme which is the measurement of the maximum catalytic production of the product under saturating substrate conditions per unit of time and per unit of enzyme.

The ratio of $K_{cat}/K_m$ is defined as the catalytic efficiency and can be taken as the measurement of the substrate specificity (Rogers and Gibon, 2009).

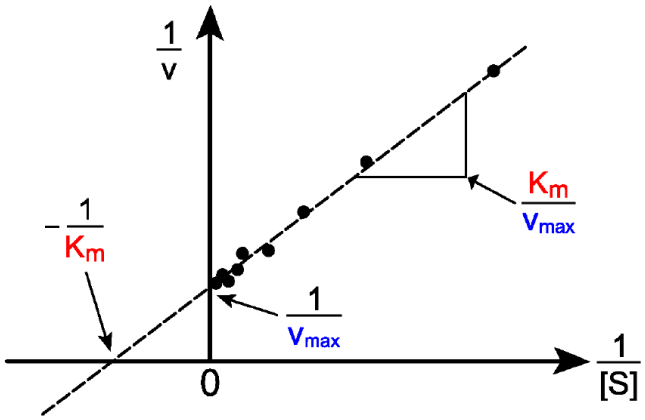


**Figure 2.6** Lineweaver-Burk or double-reciprocal plots of kinetic data, showing the significance of the axis intercepts and gradient.

### 2.6.2 Enzyme assay

An enzyme assay is a laboratory procedure that measures the rate of enzyme reaction. Since an enzyme is not consumed by a reaction it catalyses, its assay usually follows the change in the concentration of either substrates or products to measure the rate of reaction. There are many methods of measurement. Spectrophotometric assay which the change in the absorbance of light between products and reactants is observed are the most convenient. They allow the rate of the reaction to be measured continuously (Danson and Eisenthal, 2002).

There are several factors to be controlled in enzyme assays such as salt concentration, temperature, pH and substrate saturation. Normally, most enzymes cannot tolerate extremely high salt concentrations. An ion can interfere the weak ionic bonds of protein. Typical enzymes are active in 1-500 mM of salt concentration, except enzymes in the halophilic algae and bacteria. All enzymes work within a range of temperature specific to an organism. In general, the increasing temperature leads to the increasing reaction rates. However, there is a limit in increasing temperature because too high temperature leads to a sharp decrease in reaction rates due to the denaturation (alteration) of protein structure. Eventually, it will result in breaking the weak ionic and hydrogen bond that stabilizes the three dimensional structure of the enzyme, especially its active site (Daniel *et al*., 2010). All enzymes have an optimum pH, most of them are sensitive to pH and have specific ranges of activity. The pH can stop enzyme activity by denaturating (altering) the three dimensional shape of the enzyme by breaking ionic and hydrogen bond. In addition, an enzyme has the

saturation point of reaction rate where increasing substrate concentration cannot increase the rate of enzymes activity.

### 2.6.3 Glucose oxidase/peroxidase assay

Principle

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{D-Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Reduced } o\text{-dianisidine (colorless)} \xrightarrow{\text{Peroxidase}} \text{Oxidized } o\text{-dianisidine (brown)}$$

$$\text{Oxidized } o\text{-dianisidine (brown)} \xrightarrow{H_2SO_4} \text{Oxidized } o\text{-dianisidine (pink)}$$

For this method, glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase (from *Aspergillus niger*). Hydrogen peroxide reacts to *o*-dianisidine in the presence of peroxidase (from horseradish) to form a brown product. After that, oxidized *o*-dianisidine reacts to sulfuric acid to form a more stable colored product. Then, the intensity of the pink color is measured at 540 nm which is proportional to the original glucose concentration (Bergmeyer and Bernt, 1974).

# CHAPTER III

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

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**ABSTRACT**

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

**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 four enzymes (α-amylase, β-amylase, the debranching enzyme limit dextrinase and α-glucosidase) that are important for starch degradation and are used in biotechnology, especially in the context of brewing (Bamforth, 2009). In addition, glycosidase can be used in the glycosylation 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 crops, it is easily and well managed in many areas and it has the least aggressive behavior of all honeybee species, being almost docile (Oldroyd and Wongsiri, 2006). Although it has been reported that there are three kinds of HBGases (I, II and III), which show different substrate specificities, molecular weights, nucleotide sequences and tissue locations, only the HBGase III isoform has been found in *A. mellifera* honey (*Am*HBGase III) (Kubota *et al*., 2004).

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

Originally, the native form of *Am*HBGase III was enriched by ammonium sulphate salting-out followed by DEAE-cellulose, DEAE-Sepharose CL-6B, Bio-Gel P-150 and CM-Toyopearl 650M chromatographic fractionation. The enriched enzyme obtained was found to be a monomeric glycoprotein containing about 7.4% by weight of carbohydrate. Surprisingly, it was not an allosteric enzyme and showed normal

Michaelis-Menten type reaction kinetics with no cooperativity (Nishimoto *et al*., 2001).

Currently, recombinant technology is widely used to produce enzymes that are normally expressed in low levels or are hard to otherwise enrich and so limits their commercial preparation (Zhao *et al*., 1993), as well as to allow genetic engineering for improved characteristics. Accordingly, the full length cDNA of *AmHBGase* III was cloned into the pPIC3.5 or pPIC9 expression vector, transformed into *Pichia pastoris* GS115 (yeast) and expression of the recombinant (r)*Am*HBGase III enzyme was induced by 0.5% (v/v) methanol (Nishimoto *et al*., 2007). Under these conditions, the highest enzyme activity was found in the culture media, from where the r*Am*HBGase III was purified by ammonium sulfate salting-out followed by fractionation over successive CM Sepharose CL-6B, Bio-Gel P-100, DEAE Sepharose CL6B and Butyl-Toyopeal 650 M column chromatography (Nishimoto *et al*., 2007).

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

suitability of this isoform as a new source of HBGase III for the food and biotechnology industries.

## MATERIALS AND METHODS

### Sample collection

Foragers of *A. cerana indica* were collected on their return flight in front of the hive entrance in an apiary in Samut Songkram province. Samples were kept at -80 $^0$C until used.

### RNA extraction

Forager bees (50 bees) were ground with liquid nitrogen in a mortar. Total RNA was then extracted by a standard acid-guanidine thiocyanate-phenol-chloroform method, as previously reported (Nishimoto *et al*., 2007). The quality of the total RNA was visually assayed after resolution by 1.2% (w/v) formaldehyde/ agarose gel electrophoresis, and ultraviolet (uv) transillumination after ethidium bromide (EtBr) staining. The purity of extracted total RNA was calculated by the ratio of absorbance at 260 and 280 nm, whilst the concentration was calculated from the ratio of absorbance at 260 nm. Then, poly $A^+$ mRNA was isolated using the oligotex dT-30 super mRNA purification kit (Takara, Japan), as per the manufacturer's instructions. The extracted total poly A+ RNA samples were then stored at -80 $^0$C until used.

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

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

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

RT-PCR was performed using an access RT-PCR system kit (catalog# A1250, Promega), as per the manufacturer's protocol. A reaction without the total RNA template and a reaction without reverse transcriptase were used as negative controls. The reaction mixture (25 μL final volume) was comprised of 1x AMV/ T*fl* reaction buffer, 0.2 μM of each dNTP, 0.4 μM of each primer, 1 mM of $MgSO_4$, 0.1 U of AMV reverse transcriptase, 0.1 U of T*fl* DNA polymerase, and 200 ng of RNA template. The RT-PCR reaction was performed under the previously optimized conditions (data not shown) of: 1 cycle of 94°C for 2 min; 30 cycles of 94°C for 30 sec, 52°C for 30 sec, 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)$_6$-AciHBGase III chimeric protein encoding plasmid**

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

**Transformation of the yeast *Pichia pastoris***

Before transformation, 5-10 μg of recombinant plasmid was digested by *Sac*I at 37 $^{o}$C for 1 h. The *P. pastoris* GS115 (His) strain (Invitrogen) was prepared following the protocol of the Easy Select *Pichia* expression kit (Invitrogen), and then the *Sac*I-linearized plasmid was transformed into *P. pastoris* by electroporation using a Gene Pulser (Bio-Rad) according to the Invitrogen manual (methods for the expression of recombinant protein in *P. pastoris*). The electroporated yeast was spread onto YPDS-agar plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol with 2% (w/v) agar) containing 100 μg/mL Zeocin, and incubated at 30

$^{o}$C for 3-10 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$(His)_6$-AciHBGase 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 $^{o}$C with shaking at 200 rpm until the O.D. at 600 nm reached 2-6. 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 - 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 $^{o}$C for 15 min) and the cell pellet and culture supernatant were harvested and stored separately at 4 $^{o}$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), as detailed below.

**Purification of r(His)$_6$-AciHBGase 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 $^{o}$C with shaking at 200 rpm. The protein content in the supernatant was concentrated by ultracentrifugal filtration (8,000 rpm, 20 $^{0}$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), 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 (see below) and protein content using 50 and 15 μL aliquots, respectively, and 1x protease inhibitor mix (Amresgo) was added to each fraction and stored at 4 $^{o}$C until used. The apparent homogeneity of the purified r(His)$_6$-*Aci*HBGase III was evaluated visually after SDS-PAGE (8% (w/v) acrylamide resolving gel) resolution with Coomassie Brilliant blue staining for proteins or after renaturation and glucosidase enzyme activity staining.

**Enzyme assay**

Glucosidase activity was monitored using *p*-nitrophenyl α-D-glucoside (PNPG, Sigma) as the substrate. The premix (100 μL of 0.1 M sodium phosphate buffer (pH 5.5), 500 μL of distilled water and 50 μL of 5 mM PNPG) was incubated at 37 $^{o}$C for 10 min. Then, 50 μL of the test fraction or enriched r(His)$_6$-*Aci*HBGase III (7.9 mg/mL) sample was added and the reaction was incubated at 37 $^{o}$C for 10 min

before being stopped by the addition of 500 μL of 1 M $Na_2CO_3$. The control mixture was comprised of 50 μL of 5 mM PNPG, 200 μL of distilled water and 500 μL of 1 M $Na_2CO_3$. The absorbance at 400 nm of the reaction mixture was used to measure the release of the yellow product of *p*-nitrophenol. One unit (U) of HBGase III was defined as that which liberates 1 μM of D-glucose from PNPG per min at pH 5.5 at 37$^oC$.

**Protein determination**

Protein concentration in samples was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) at various concentrations (1-100 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 gently 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 $^oC$ for 30 min, rinsed by dd$H_2O$ and boiled in 0.5 N NaOH containing 0.1% (w/v) triphenyltetrazolium chloride for 3 min. A red band indicated the region where HBGase activity was detected.

## Characterization of the r(His)$_6$-AciHBGase III

### *Substrate specificity*

Evaluation of the substrate specificity followed the same method as the enzyme assay above 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 1-10 mM for maltose, 10-100 mM for isomaltose, 10-100 mM for sucrose, 10-90 mM for maltotriose, 10-60 mM for soluble starch and 5-20 mM for PNPG. The amount of glucose liberated from the substrates (except for PNPG where p-nitrophenol release was measured) was then determined by the glucose oxidase-peroxidase method using a glucose assay kit (catalog# GAGO20-1KT, Sigma) as per the manufacturer's instructions. In brief, 100 μL of the above reaction mixture was mixed with 200 μL of the glucose assay reagent and incubated at 37$^o$C for 30 min before the reaction was stopped by the addition of 200 μL of 12 N $H_2SO_4$ and the absorbance at 540 nm measured.

### *Optimum pH for r(His)$_6$-AciHBGase III activity and its pH stability (at 4$^o$C)*

A reaction mixture containing 50 μL of the enriched r(His)$_6$-*Aci*HBGase III (0.0053 U/mL; 7.9 mg/mL), 50 μL of 5 mM PNPG and 100 μL of Briton-Robinson buffer (40 mM acetic acid, 40 mM phosphoric acid and 40 mM boric acid) in the pH range of 3.0-7.5 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 above. To evaluate the pH stability of the enriched r(His)$_6$-*Aci*HBGase III, the same reaction mixture as for the pH optimum above was prepared but the reaction mixture was first stored at 4°C for 24 h before the enzyme was assayed.

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

The same reaction mixture as for the pH optimum above was prepared except 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 (4-75°C) and assayed for glucose release (or *p*-nitrophenol for PNPG), as above.

## 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 for the reported *AciHBGase III* cDNA (EF441271), and 99% (1689/1704 bp), 96% (1641/1708 bp) and 96% (1629/1704 bp) identical to the *HBGase III* cDNA from *A. cerana japonica* (FJ889442), *A. mellifera* (NM001011608) and *A. dorsata* (GU224269), respectively, and with 100% identity for all these species at the predicted amino acid sequence level. An example of multiple alignment between *AciHBGase III* and *AmHBGase III* cDNA is shown in Figure 3.1. Thus, the obtained sequence is almost certainly the *AciHBGase III* gene.

After directional cloning (*Eco*RI/*Kpn*I directed) of the RT-PCR amplicon into the pPICZαA expression vector and selection of suitable transformants, the subsequent restriction digestion with *Xho*I of the plasmid revealed the correct sized insert, whilst sequencing revealed the complete *AciHBGase III* gene sequence (1,704 bp) in frame with the (His)$_6$ encoding N terminal tag (data not shown).

```
Am  ATGAAGGCAG TAATCGTATT TTGCCTTATG GCATTGTCCA TTGTGGACGC AGCATGGAAG CCGCTCCCTG AAAACTTGAA 80
Ac  ATGAAGGCGA TAATCGTATT TTGCCTTATG GCATTGTCCA TTGTGGACGC AGCATGGAAG CCGCTCCCTG AAAACTTGAA 80

Am  GGAGGACTTG ATCGTGTATC AGGTCTACCC GAGAAGCTTC AAGGATAGCA ATGGAGATGG TATTGGTGAT ATCGAAGGTA 160
Ac  GGAGGACTTG ATCGTGTATC AGGTCTACCC AAGAAGCTTC AAGGATAGCA ATGGAGATGG TATTGGTGAT ATCGAAGGTA 160

Am  TTAAAGAAAA ATTGGATCAT TTTCTCGAAA TGGGGGTCGA CATGTTTTGG TTATCCCCTA TTTATCCAAG CCCTATGGTC 240
Ac  TTAAACAAAA ATTGGACCAT TTTCTCGAAA TGGGCGTCGA TATGTTTTGG TTATCTCCTA TTTATCCAAG TCCTATGGTC 240

Am  GATTTTGGTT ACGACATTTC GAATTACACC GACGTTCATC CCATATTTGG CACCATATCA GACTTAGATA ATCTAGTCAG 320
Ac  GATTTTGGTT ATGACATTTC GAATTACACC GATGTTCATC CCATATTTGG CACCTTATCA GACTTAGATA ACTTAGTTAA 320

Am  TGCTGCACAT GAGAAAGGAT TGAAGATAAT CTTGGATTTC GTCCCGAATC ATACATCTGA TCAACACGAA TGGTTCCAGT 400
Ac  TGCTGCACAT GAGAAGGGAC TGAAGATAAT CTTGGATTTC GTTCCGAATC ATACATCTGA TCAACATGAA TGGTTCCAGC 400

Am  TGAGTTTGAA AAACATTGAA CCTTATAACA ACTATTACAT TTGGCATCCA GGAAAAATTG TAAATGGCAA ACGTGTTCCA 480
Ac  TGAGTTTGAA AAACATTGAA CCTTATAACA ACTATTATAT TTGGCATCCA GGAAAAATTG TAAATGGTAA ACGTGTTCCA 480

Am  CCAACTAATT GGGTAGGCGT GTTTGGTGGA TCAGCTTGGT CGTGGCGGGA AGAACGACAG GCATATTATC TGCATCAATT 560
Ac  CCAACTAATT GGGTAGGCGT ATTTGGTGGA TCAGCTTGGT CATGGCGAGA AGAACGACAG GCATATTATC TGCATCAATT 560

Am  TGCACCAGAA CAACCAGATC TAAATTACTA TAATCCAGTT GTACTGGATG ATATGCAAAA TGTTCTCAGA TTCTGGCTGA 640
Ac  TGCACCAGAA CAACCAGATC TAAATTACTA TAATCCAGTT GTACTAGATG ATATGCAAAA CGTTCTCAGA TTCTGGCTGA 640

Am  GAAGGGGATT TGATGGTTTC AGAGTAGATG CTCTGCCTTA CATTTGCGAA GACATGCGAT TCTTAGACGA ACCTCTATCA 720
Ac  GAAGAGGACT CGATGGTTTC AGAGTAGATG CTTTGCCTTA CATTTGCGAG GACATGCGAT TCTTAGACGA ACCCCTATCT 720

Am  GGTGAAACAA ATGATCCCAA TAAAACCGAG TACACTCTCA AGATCTACAC TCACGATATC CCAGAAACCT ACAATGTAGT 800
Ac  GGTGAAACAA ATGATCCCAA TAAAACCGAG TACACTCTCA AGATCTACAC TCACGATATC CCAGAAACCT ACAATATAGT 800

Am  TCGCAAATTT AGAGATGTGT TAGACGAATT CCCGCAACCA AAACACATGC TTATCGAGGC ATACACGAAT TTATCGATGA 880
Ac  TCGCAAATTT AGAGATGTGT TAGACGAATT CCCGCAACCA AAACACATGC TTATCGAGGC ATACACGAAT TTATCGATGA 880

Am  CGATGAAATA TTACGATTAC GGAGCAGATT TTCCCTTCAA TTTTGCATTC ATCAAGAATG TTTCTAGGGA TTCAAATTCA 960
Ac  CGATGAAATA TTACGATTAC GGAGCAGATT TTCCCTTTAA TTTTGCATTC ATCAAGAATG TCTCTAAGGA TTCAAATTCA 960

Am  TCAGACTTCA AAAAATTGGT CGATAATTGG ATGACGTACA TGCCACCAAG TGGTATTCCT AACTGGGTGC CCGGAAATCA 1040
Ac  TCAGACTTCA AGAAATTGGT CGATAATTGG ATGATATACA TGCCAGCAGA TGGTATTCCT AACTGGGTGC CCGGAAATCA 1040

Am  CGATCAATTG AGATTGGTGT CGAGATTTGG AGAGGAGAAG GCCCGTATGA TCACCACGAT GTCGCTTTTG CTGCCAGGTG 1120
Ac  CGATCAATTG AGATTGGTGT CGAGATTTGG AGAGGAGAAG GCCCGTATGA TCACCGCGAT GTCGCTTTTG CTGCCAGGTG 1120

Am  TTGCCGTGAA TTACTACGGT GATGAAATTG GTATGTCGGA TACTTATATC TCGTGGGAGG ATACGCAGGA TCCGCAGGGA 1200
Ac  TTGCCGTGAA TTACTACGGT GATGAAATTG GTATGTCGGA TACTTATATC TCGTGGGAGG ACACGCAGGA TCCACAGGGA 1200

Am  TGCGGCGCCG GTAAAGAAAA CTATCAAACG ATGTCGAGAG ATCCCGCGAG AACGCCATTC CAATGGGACG ACTCAGTTTC 1280
Ac  TGCGGTGCCG GCAAAGAAAA CTATCAAACG ATGTCGAGAG ATCCCGCGAG AACGCCATTC CAATGGGACG ACTCAGTTTC 1280

Am  TGCTGGATTT TCCTCAAGCT CTAATACCTG GCTTCGTGTC AACGAAAATT ACAAGACTGT CAATCTAGCT GCTGAAAAGA 1360
Ac  TGCTGGATTT TCCTCAAGCT CTGATACCTG GCTTCGTGTC AACGAAAATT ACAAGACTAT CAATTTAGCT GCTGAAAAGA 1360

Am  AGGACAAGAA CTCGTTCTTC AATATGTTCA AGAAATTTGC GTCGCTGAAA AAATCGCCAT ACTTTAAAGA GGCCAATTTA 1440
Ac  AGGACAAGAA CTCGTTCTTC AATATGTTCA AGAAATTTGC AATGCTGAAA AAATCGCCAC ACTTTAAAGA GGCCAATTTA 1440

Am  AATACGAGGA TGCTGAACGA CAATGTTTTC GCATTCTCTA GGGAAACCGA AGATAATGGA TCTCTTTACG CAATATTGAA 1520
Ac  AATACGAGGA TGCTGAACGA CAGTGTTTTC GCATTCTCTA GGGAAACCGA AGAAAATGGA TCTCTTTACG CAATATTGAA 1520

Am  CTTCTCGAAC GAGGAACAAA TCGTGGATTT GAAAGCGTTC AATAACGTGC CGAAAAAATT GAATATGTTT TACAACAATT 1600
Ac  CTTCTCGAAC GAGGAACAAA TCGTGGACTT GAAAGCGTTT AATAACGTGC CGAAAAAATT GAATATGTTT TACACCATTT 1600

Am  TTAACTCTGA TATAAAGTCC ATCTCCAACA ATGAACAAGT AAAAGTTTCT GCTTTAGGAT TTTTCATCTT AATTTCTCAA 1680
Ac  TTAACTCTGA TATAAAGTCC ATCTCCAACA ATGAACAAAT AAAAGTTTCT GCTTTAGGAT TTTTGATCTT AATTTCTCAA 1680

Am  GATGCTAAAT TTGGAAACTT TTAA                                                            1704
Ac  GATGCTAAAT TTGGAAATTT TTAA                                                            1704
```

**Figure 3.1** The multiple alignment of *AciHBGase III* and *AmHBGase III* cDNA. Ac = *AcHBGase III*; Am *AmHbgase III*. The grey shadow indicates different base residues within the aligned sequences.

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

*P. pastoris* GS115 transformants with the in-frame (His)$_6$-tagged *Aci*HBGase III encoding sequence were cultured in BMMY medium and the expression of the recombinant chimeric protein was induced by 1% (v/v) methanol and monitored over time in the yeast cells and the culture medium. Twenty-four hours after induction a high specific glucosidase activity was found in cell lysate (0.72 U/mg) but not in the culture medium. However, thereafter the specific glucosidase activity in the supernatant increased to a plateau from 72 to 120 h (~0.58 U/mg) before increasing to a maximum at 144 h (0.62 U/mg), whereas that in the cell lysate decreased to a minimum (~0.2 U/mg) from 96 h after induction onwards (Fig. 3.2A). 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 (45-65 kDa) were seen to increase in intensity in the culture media up to ca. 120 h after induction (Fig. 3.2B). These bands with lower molecular mass than the targeted recombinant r(His)$_6$-*Aci*HBGase III of ~ 68 kDa may represent degraded products of the intact enzyme although protease inhibitor was used. The intensity of these bands increased after longer induction. Alternatively, these bands may be other secretory proteins from yeast itself since crude proteins from culture media were used.

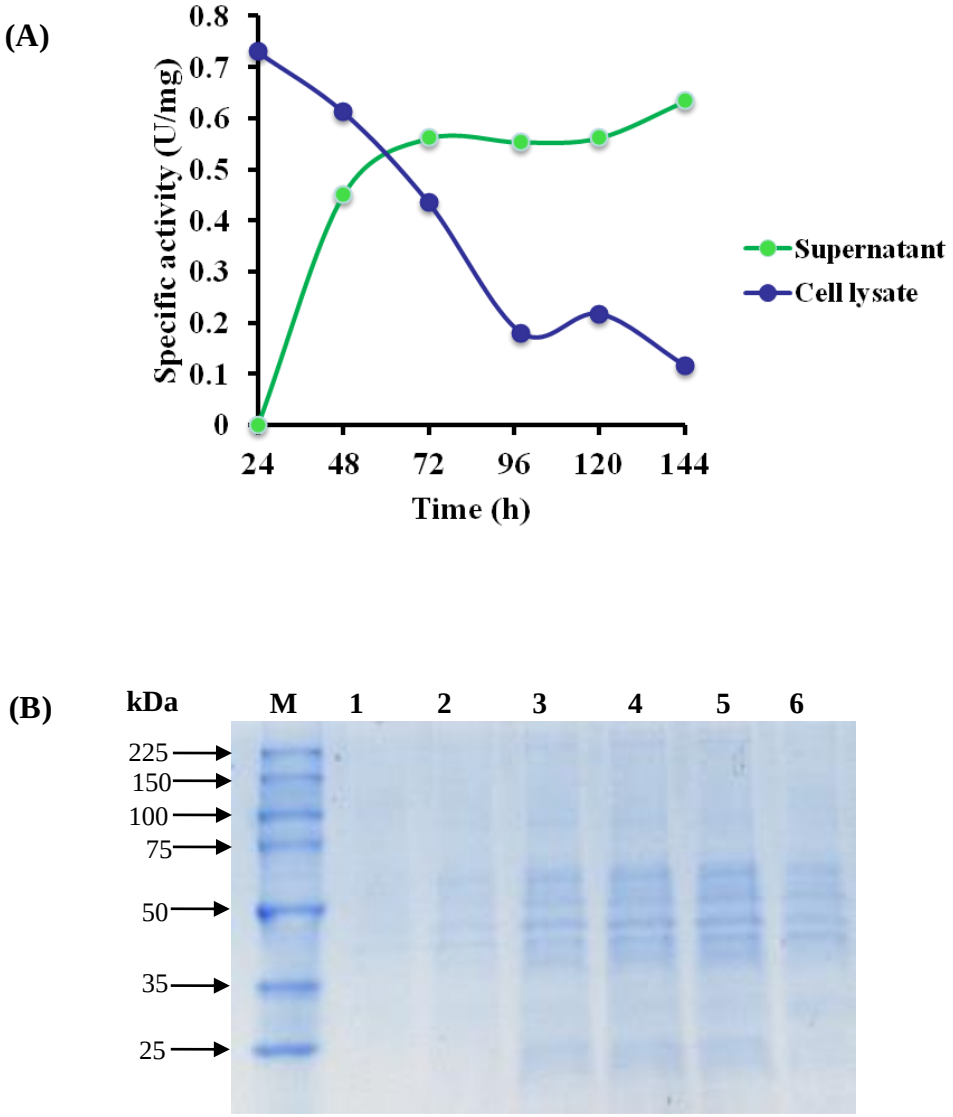


**Figure 3.2** Expression of the methanol-induced r(His)$_6$-*Aci*HBGase III expression. (A) Glucosidase activity in the culture media and yeast cell lysate with time since methanol (1% (v/v)) induction. (B) Coomassie Brilliant blue stained SDS-PAGE resolution of the culture media proteins with increasing time since methanol induction. Lane M: protein marker; lanes 1-6: 2 μg crude protein from the culture media at 24, 48, 72, 98, 120 and 144 h after induction, respectively.

**Purification of r(His)$_6$-AciHBGase III**

After enrichment of the r(His)$_6$-*Aci*HBGase III protein from the culture media by histrap affinity column chromatography the enzyme activity in each fraction was assayed using PNPG as the substrate. Among the five unbound fractions, fraction 3 provided the highest glucosidase activity at 0.02 U/mL, while among the 10 bound fractions, 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 (Fig. 3.3A). Thus, fraction 10 was selected for characterization of the r(His)$_6$-*Aci*HBGase III enzyme. However, although a single main band of ~68 kDa was present on both the Coomassie Brilliant blue stained SDS-PAGE resolved gel (Fig. 3.3B), and on the glucosidase activity stained gel (Fig. 3.3C), several other protein bands were visible (Fig. 3.3B) and so the enriched r(His)$_6$-*Aci*HBGase III preparation of fraction 10 is not purified to homogeneity.

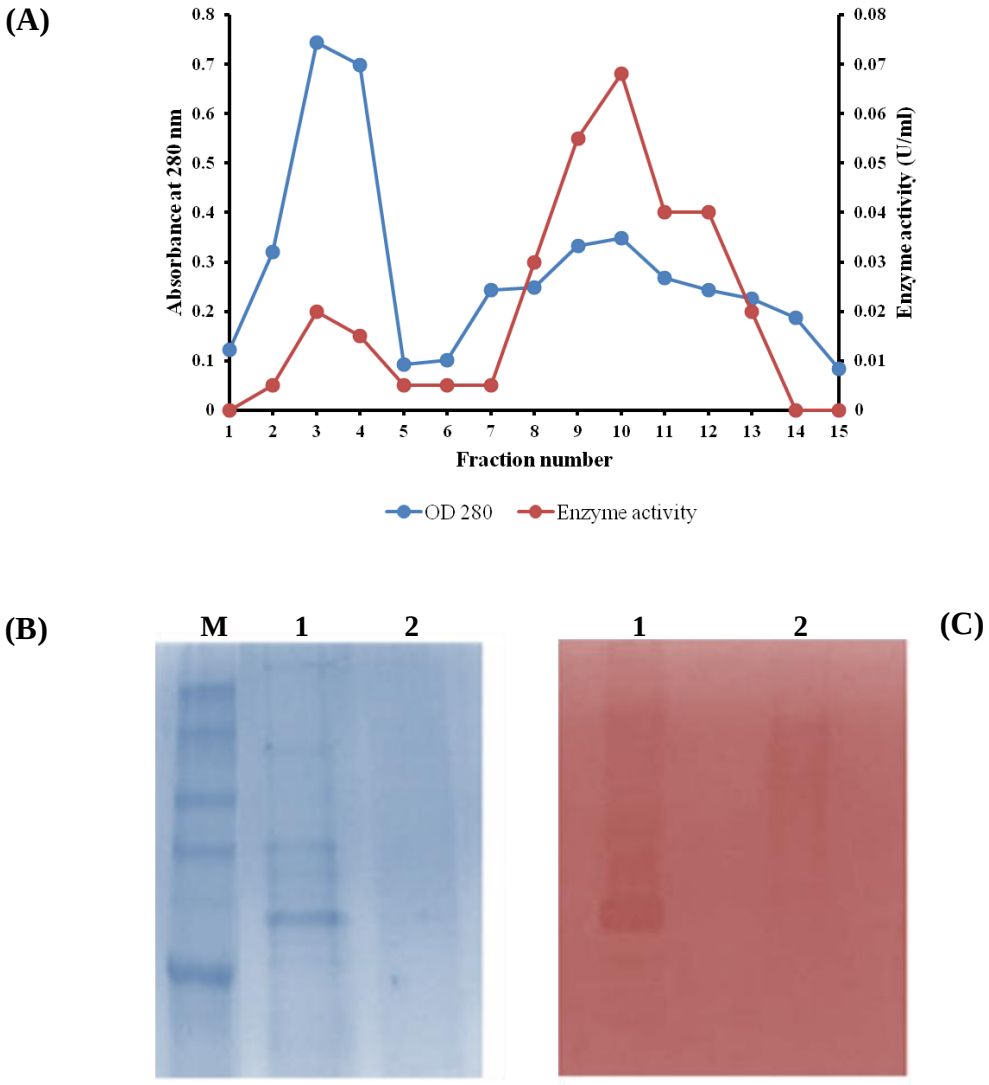


**Figure 3.3** Enrichment of the r(His)$_6$-*Aci*HBGase III. (**A**) Elution profile from the histrap affinity column, showing the $A_{280}$ (protein level) and glucosidase activity level for the five unbound (fractions# 1-5) and 10 bound (fractions# 6-15) fractions. (**B**, **C**) SDS-PAGE resolved gel of fraction# 3 and fraction# 10 after staining (**B**) with Coomassie Brilliant blue or (**C**) for glucosidase activity after renaturation. Lane M, protein marker; lane 1, 2 µg of bound fraction# 10; lane 2, 2 µg of unbound fraction# 3.

## Characterization of the r(His)$_6$-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)$_6$-*Aci*HBGase III preparation was measured using PNPG as the substrate. The optimal pH for enzyme activity was found to be 5.0, with > 70% and > 40% residual activity in the pH range of 4.5 - 7.5 and pH 3.0 - 7.5, respectively (Fig. 3.4A). With respect to the enzyme stability at 4$^o$C for 24 h, the enzyme was stable over the pH range of 5.0 - 6.0, with >70% activity at pH 5 – 7.5 but it showed little or no stability to prior treatment at pH 4.5 or below, respectively (Fig. 3.4B). For the optimal temperature, the r(His)$_6$-*Aci*HBGase III showed an optimal activity at 37$^o$C, but no thermotolerance with ~33% residual activity at 25$^o$C, and less than 15% and no activity at 40-50$^o$C and 55$^o$C, respectively (Fig. 3.4C).

### *Substrate specificity*

The substrate specificity of the enriched r(His)$_6$-*Aci*HBGase III enzyme preparation was examined using six different glucose based substrates at either 5 or 10 mM, in comparison to PNPG for reference (Fig. 3.5). The different substrates varied in their linkage types and polymer sizes, as detailed in the methods section. The results revealed that the best hydrolyzed substrate was sucrose 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 born in mind that an equimolar proportion of fructose as glucose is produced upon hydrolysis and so the number of bonds hydrolyzed is actually higher than that for maltose, at around 1.9 mg of glucose equivalents. Given that the source of this enzyme is from foraging honeybees and so it is likely to be mainly involved in sucrose cleavage (from nectar) to form glucose and fructose for honey, this highest bond cleaving activity on sucrose is to be expected. Thus, it is of interest that the r$(His)_6$-*Aci*HBGase III can cleave not only small α(1-4) and α(1-6) linkage glucose units, but also large polymers like starch. With respect to the substrate concentration, except for sucrose, a higher amount of glucose was always obtained with 10 mM substrate than with 5 mM hydrolyzed substrate, as expected.

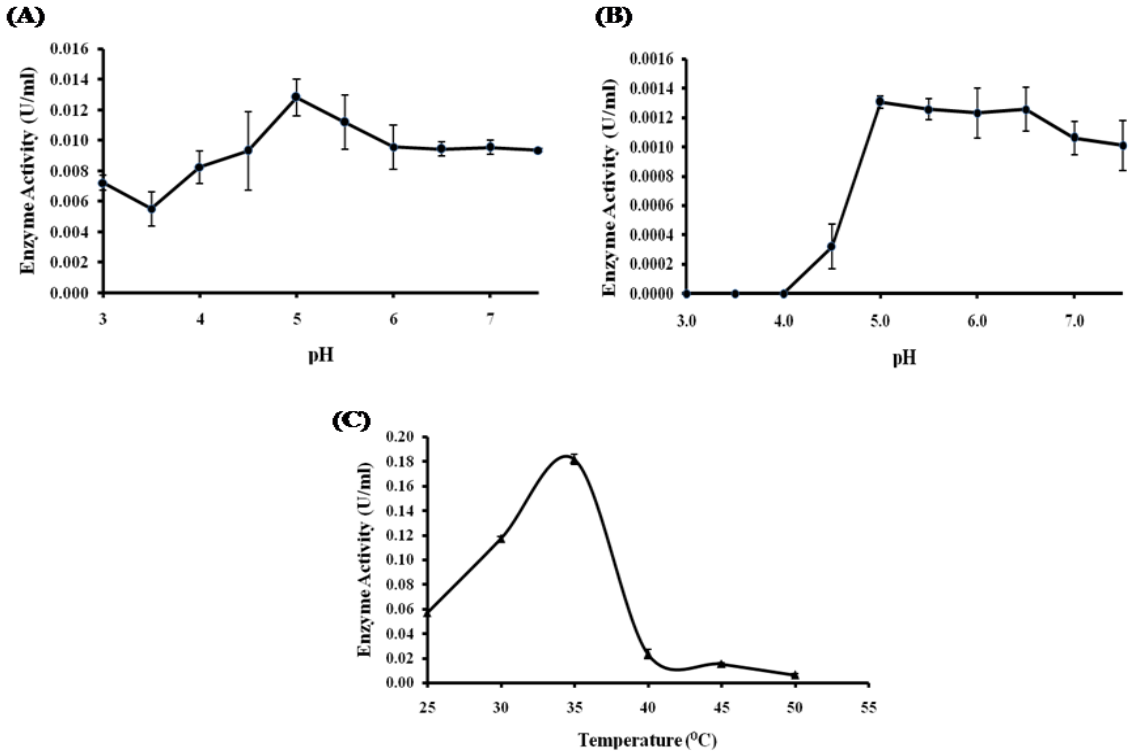


**Figure 3.4** The optimum (**A**) pH and (**C**) temperature for glucosidase activity in the enriched r(His)$_6$-AciHBGase III preparation, and the (**B**) pH stability of the enzyme to a 24 h pretreatment at 4°C. All assays use PNPG as the substrate and the data represent the mean $\pm$ 1 SD and are derived from three independent repeats.

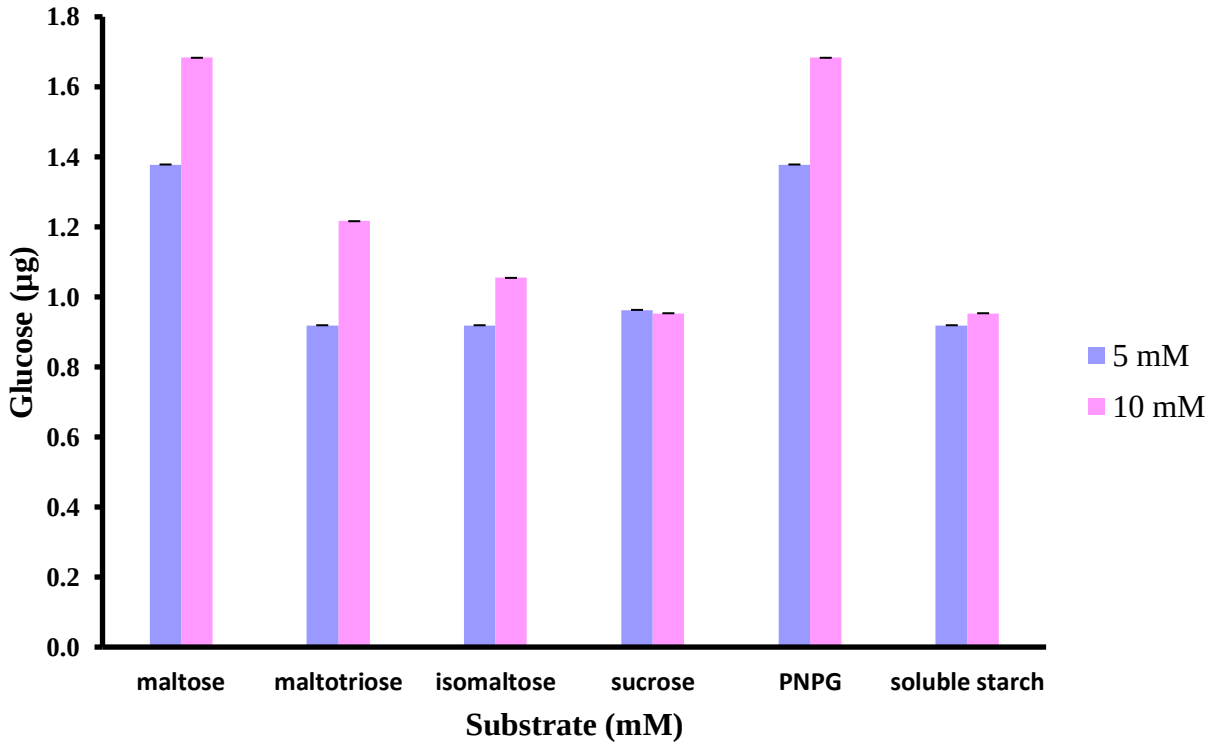


**Figure 3.5** Substrate specificity of the enriched r(His)$_6$-*Aci*HBGase III enzyme preparation with six different glucose containing polymers at 5 mM (black bar) or 10 mM (grey bar) in comparison to the reference PNPG substrate.

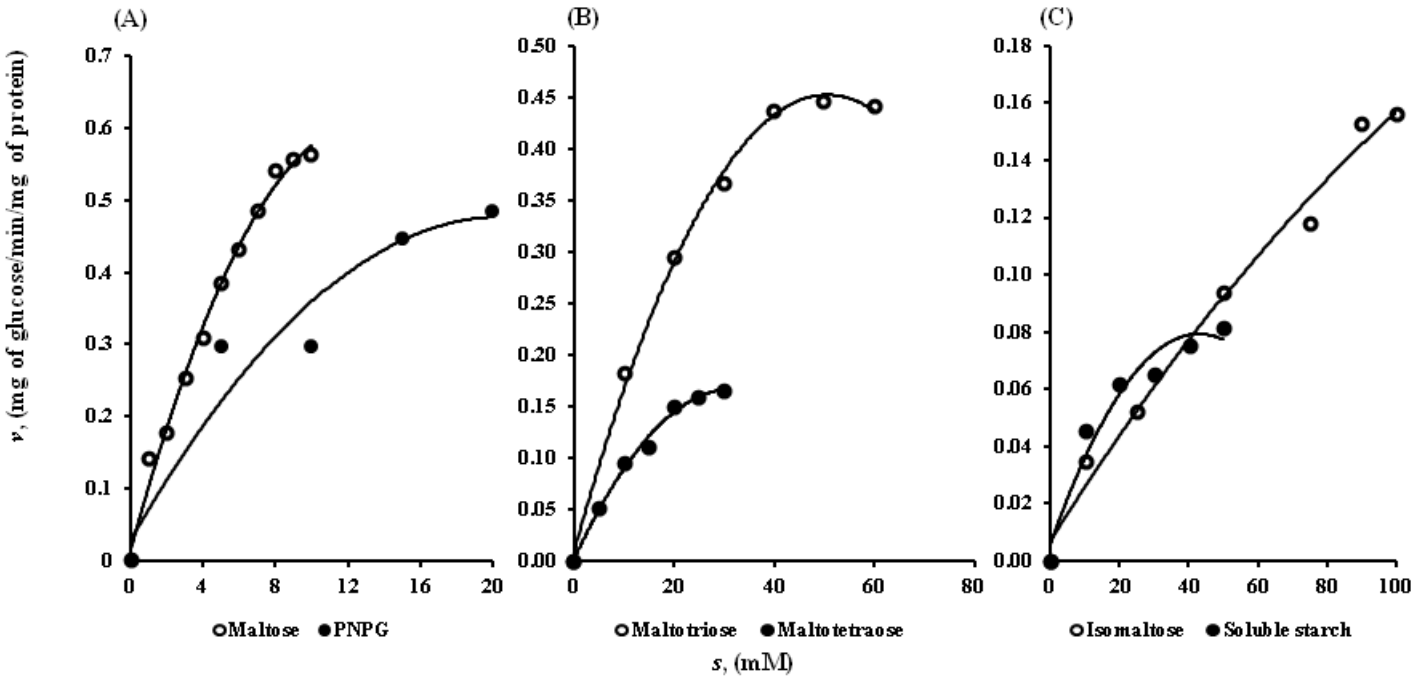


**Figure 3.6** Michaelis-Menten plots (*s* versus *v*) for the enriched r(His)$_6$-*Aci*HBGase III enzyme with (**A**) PNPG and maltose, (**B**) maltotriose and maltotetraose and (**C**) soluble starch and isomaltose, as substrates.

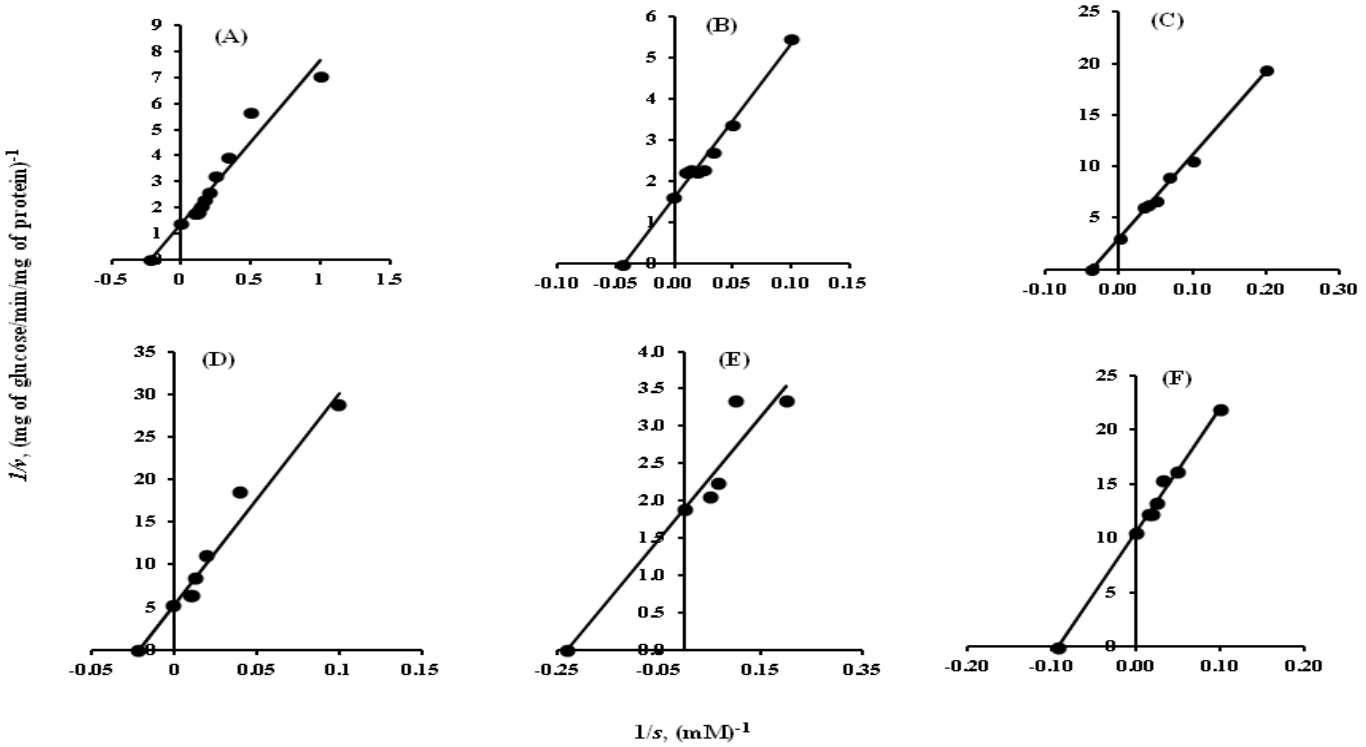


**Figure 3.7** Lineweaver-Burk plots for the enriched r(His)$_6$-*Aci*HBGase III enzyme with (**A**) maltose, (**B**) maltotriose, (**C**) maltotetraose, (**D**) isomaltose, (**E**) PNPG and (**F**) soluble starch, as substrates.

The enzyme kinetics for the enriched r(His)$_6$-*Aci*HBGase 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 (Fig. 3.6).

The data was then replotted as double reciprocal plots, or Lineweaver-Burk plots (1/*s* versus 1/*v* plots) (Lineweaver and Burk, 1934), where a linear relationship was found on all the substrates (Fig. 3.7). 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).

**Table 3.1** Kinetic parameters for the hydrolysis of different substrates by the r(His)$_6$-*Aci*HBGase III.

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

## DISCUSSION

After the complete sequence and partial annotation of the genome of *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 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 *A. florea* (GenBank, accession# EF586680) are now available. This brings the benefit of allowing homologous and functional analysis across different species for each gene of interest, as well as in improving the design of degenerative PCR primers to amplify the homolog's from other more distant species or across less conserved regions. However, in eukaryotes the targeted protein is not always readily deduced from the DNA sequence due to variations in intron-exon junction recognition and alternative splicing, as examples, and so the open reading frame (ORF) is usually analyzed from the corresponding cDNA sequences.

In this research, forager bees were selected for use due to the fact that this developmental stage has the highest expression level of *HBGase III*. Here, to allow post-translational glycosylation, we used the yeast *P. pastoris* rather than *E. coli* as the recombinant host, as this has been reported to be a suitable system before (Chen *et al*., 2010). The estimated molecular mass of the r(His)$_6$-*Aci*HBGase III, at a little bit higher than 68 kDa, is a little over 6.5% higher than the predicted mass from the

deduced amino acid sequence (allowing for the $(His)_6$ tag), and this may then be due to the glycosylation of the protein. In accord, the *AmHBGase III* homolog is a glycoprotein with a sugar content of 7.4% by weight.

The utilization of the pPICZαA expression vector provided an N terminal $(His)_6$ sequence on the r*Aci*HBGase III protein allowing histrap affinity column enrichment. Any role of this N-terminal $(His)_6$ sequence on the stability and kinetics of the *Aci*HBGase III enzyme has not yet been evaluated. For large (commercial) scale production of r*Aci*HMGase III, where the carbohydrate and $(His)_6$ tag might also affect the purification procedure (Nishimoto *et al*., 2001), fractionation over DEAE-Sepharose CL-6B, Bio-Gel P-150, CM-Toyopeal 650 M and Sephacryl S-100 may be more suitable (Takewaki *et al*., 1993; Nishimoto *et al*., 2001; Wongchawalit *et al*., 2006; Nishimoto *et al*., 2007).

The previously reported activity of the native *Aci*HBGase III enzyme (Chanchao *et al*., 2008) and the r$(His)_6$-*Aci*HBGase III form (this study) were assayed using different methods, making direct comparisons equivocal. The native form was assayed by Momose's method (Momose and Inaba, 1961) and the recombinant form with PNPG as the substrate, respectively. However, the same pH optimum for enzyme activity (pH 5.0) was revealed, whilst the optimal temperature was different, being 50$^{o}$C for the native form of *Aci*HBGase III compared to 35$^{o}$C and with no thermal stability for the r$(His)_6$-*Aci*HBGase III form.

The enriched r$(His)_6$-*Aci*HBGase III showed normal Michaelis-Menten type reaction kinetics on all tested substrates including the complex polysaccharide soluble starch, in agreement with previous reports (Chiba, 1997, 1998). The substrate

specificity was relatively high in maltose ($K_m$ of 4.5) and PNPG ($K_m$ of 4.4 mM) but was still lower than the α-glucosidase from barley endosperm ($K_m$ of 1.7-2.4 mM), which is used during starch formation (Naested *et al*., 2006). However, comparing the $K_m$ of r(His)$_6$-*Aci*HBGase III, in this research, and of the rHBGase III from *A. mellifera* (r-*Am*HBGase III) (Nishimoto *et al*., 2007), the recombinant enzyme in this research showed a higher substrate specific activity in maltose, maltotriose and PNPG.

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

# CHAPTER IV

## 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* (r*Aci*HBGase 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, *Aci*HBGase 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 pPICZαA expression vector in frame with a $(His)_6$ encoding C terminal tag using *Eco*RI and *Kpn*I compatible ends, and transformed into *Pichia pastoris*. Maximal expression of the r*Aci*HBGase 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$^{o}$C, with > 80% activity between pH 3.5-5.0 and 40-55$^{o}$C, and was stabile (> 80% activity) at pH 4-8 and at < 25-65$^{o}$C. The optimal substrate was sucrose.

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

## INTRODUCTION

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 (Takewake *et al*., 1980). Based on their primary structure (amino acid sequences), members of Family 13 (GH13) have four regions that are important in their catalytic action (Chiba, 1997; Svensson, 1994). In contrast, HBGase II enzymes do not have these four regions and belong to members of Family 31 (GH31) (Henrissat, 1991). 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 $(\beta / \alpha)_8$ barrel structure as the catalytic domain but they differ in the features of the active sites involved in the catalytic reactions (Ernst *et al*., 2006; Watanabe *et a*l., 1997).

Three isoforms of HBGase have been reported in *A. mellifera* (*Am*HBGase 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 in the insect (Kubota *et al*., 2004; The honeybee Genome Sequencing Consortium, 2006). Moreover, these three enzymes showed different pH and temperature optima for enzyme activity, as well as pH and thermal stabilities, and

sugar substrate preferences (Kimura *et al*., 1990; Nishimoto *et al*., 2001; Takewaki *et al*., 1993).

Homologs of these three *Am*HBGase isoforms (I, II and type II-like HBGases) have also been reported in the yeast *Sporothrix schenckii*, 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 (Torres-Rodriguez *et al*., 2012). 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 (*Oryza sativa*) is directly involved in the alcohol fermentation process in sake processing. Thus, Iwata et al. (2003) purified the HBGase from *O. sativa* Yamadanishiki and reported that the enzyme had a different substrate specificity (nigerose) from the HBGase I (maltotriose and maltotetraose) purified from *O. sativa* cv. Shinsetsu, and also hydrolyzed nigerose and kojibiose better than the HBGase II from *O. sativa* cv. Shinsetsu.

Furthermore, Michlmayr et al. (2012) reported that the aroma of wine was in part due to the action of glycosidases from *Oenococcus oeni*, a wine-related lactic acid bacterium, since the precursors of the volatile monoterpene 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 α–glucosyl residue substrates, HBGase can also catalyze transglycosylation reactions to synthesize various α-glucosylated compounds. Accordingly, this enzyme-catalyzed transglycosylation is used in the

biosynthesis of carbohydrates that are important for humans (Perugino *et al*., 2004). Zhou et al. (2012) purified a novel extracellular α–glucosidase II (210 kDa) with a high transglycosylation activity from *Arthrobacter* sp. DL001. This enzyme could transfer glucosyl groups from donors containing an α-(1,4)-glucosidic bond specific to glucosides, xylosides and alkyl alcohols in α-(1,4)- or α(-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 transglucosylation 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 (Cihan *et al*., 2011). Cihan et al. (2011) successfully purified HBGases from *Geobacillus toebii* strain E134, isolated from a hot spring. The intracellular HBGase showed an optimal enzyme activity at 65$^{o}$C and pH 7.0, while the extracellular one showed an optimal activity at 70$^{o}$C and pH 6.8. Interestingly, both enzymes were active over the temperature and pH ranges of 35-70$^{o}$C and 4.5-11.0, respectively.

Recently, in order to supply sufficient HBGase for industrial production, not only native HBGase from new sources has been explored, but recombinant (r)HBGase has gained in interest. Heterogeneous expression of rHBGase in the *Pichia pastoris* expression system has been widely used since it achieves a low production cost and highly efficient production, whilst maintaining glycosylation and correct folding, and is free of potential bacterial endotoxins and lipopolysaccharides unlike bacterial expression systems. Konishi et al. (2011) successfully synthesized novel artificial

glycolipids using the rHBGase from *Geobacillus* sp. HTA-462, 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 *HBGase II* from *Apis cerana indica* (*AciHBGase II*) together with the expression, purification and characterization of the r*Aci*HBGase II enzyme (as a C-terminal $(His)_6$ tagged chimera) activity was evaluated. To our knowledge, this is the first report of the *Aci*HBGase 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.

## MATERIALS AND METHODS

### Sample collection

*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 $^0$C until used.

### RNA extraction

Samples (eggs, larvae, pupae and forager bees) were taken from -80$^o$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 (Nishimoto *et al*., 2007). 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 $(dT)_{30}$ 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 *AmHBGase II* (GenBank accession # NM_001040259). All primers (Table 4.1) 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 $MgSO_4$, 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, Ta° C (see Table 4.1) for 30 s, and 68°C for 2 min; and finally

1 cycle of 68$^{o}$C for 7 min. RT-PCR products were resolved by 1.2% (w/v) agarose-TBE gel electrophoresis and visualized by UV-transillumination after EtBr staining. After that, they were purified using a QIAquick PCR purification kit (catalog # 28104, Qiagen) as per the manufacturer's protocol and then direct sequenced commercially at the Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand, using the same F and R primers (separate reactions). The obtained consensus sequences were searched against the NCBI GenBank data base for homologous sequences using the MegaBLASTn algorithm.

**Table 4.1** 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)[a] | Size (bp)[b] |
|---|---|---|---|---|
| Pair 3_HBGase I | AATGGCGAGAATTTTGTGGAC | TGGAGTTTACGCTGCTTGTG | 53 | 783 |
| Pair 1_HBGase II | ATGTTTCGAGCGACGATAGTTAC | AAGACGCTGAGCCAATTGTT | 52 | 503 |
| Pair 2_HBGase II | CGAGGAGTTTCCAAGACAGC | CTCGAACATGTGGTTGATGG | 42 | 581 |
| Pair 3_HBGase II | AGTACTACGTGTGGCGGGAC | GGACTTGAACGCCACGTAAT | 52 | 983 |
| Pair 4_HBGase II | CGTGATGCTGACGTTGACTT | TTACAACCAGTCTACACCTTGCC | 48 | 634 |
| 28S rDNA | AAAGATCGAATGGGGATATTC | CACCGGGTCCGTACCTCC | 44 | 350 |

[a]Ta = annealing temperature used in the RT-PCR reaction

[b]Size = expected amplicon size

**Construction of the r*Aci*HBGase 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 *Am*HBGase II (*HBGase II*F (5' CAAAATGGAATTCTTTCGAGCGACGATAGTTA 3') and *AmHBGase II*R (5' CGAGGTACCCAACCAGTCTACACCTTGCC 3'). Note that to facilitate the directional cloning the F and R primers contained 5' flanking extension sequences to yield *Eco*RI and *Kpn*I 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 *Eco*RI and *Kpn*I 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 r*Aci*HBGase II-$(His)_6$ chimeric enzyme is encoded for.

**Transformation of *P. pastoris***

Before transformation, 5-10 μg of the recombinant plasmid was digested by *Sac*I 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 *Sac*I-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 *sac*I linearized empty pPICZαA vector.

**Expression of the r*Aci*HBGase 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$^{o}$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 (Bradford, 1976).

**Partial purification of the r*Aci*HBGase II-(His)$_6$ enzyme**

The transformants were first cultured in 25 ml of BMGY medium at 30$^{o}$C for 24 h with agitation at 200 x rpm to form the inoculum, and then the induction of r*Aci*HBgase 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 $^{0}$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 r*Aci*HBGase 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 (Amresgo) and stored at 4ºC. The apparent homogeneity of the r*Aci*HBGase 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 (Kilaso *et al*., 2011).

**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-$H_2O$ and 0.05 ml of 5 mM PNPG) was incubated at 37ºC for 10 min and then 0.05 ml of the test sample (e.g. column chromatography fraction or the r*Aci*HBGase II-$(His)_6$ preparation) was added and incubated at 37ºC for 10 min. The reaction was then stopped by adding 0.5 ml of 1 M $Na_2CO_3$. 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 37ºC.

**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 r*Aci*HBGase 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 37ºC for 10 min, and then stopped and assayed as above. For

evaluation of the pH stability, the r*Aci*HBGase II-$(His)_6$ preparation was prepared in Briton-Robinson buffer at the desired pH (range 3.0-12.0) and then stored at 4$^{o}$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$^{o}$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 $^{0}$C. Moreover, to determine the thermal stability of the enzyme, the r*Aci*HBGase 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$^{o}$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, maltotriose, maltotetraose, isomaltose, sucrose and soluble starch at the indicated concentrations (*s*) of 0-60 mM for maltose and maltotriose, 0-30 mM for maltotetraose, 0-100 mM for isomaltose, 0-25 mM for PNPG, 0-20 mM 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 $H_2SO_4$ 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):

Glucose (mg) = [($A_{540}$ of test)(standard glucose in mg)] / ($A_{540}$ 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 (Fig. 4.1A), 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 (Fig. 4.1B). As a template control, the expression of a fragment of the *28S rDNA* was observed in all samples at essentially the same level (Fig. 4.1C). Of course this is the average transcript expression level across all larval, pupal and adult foraging bee tissues and so excludes any more subtle developmentally dependent tissue specific expression patterns.

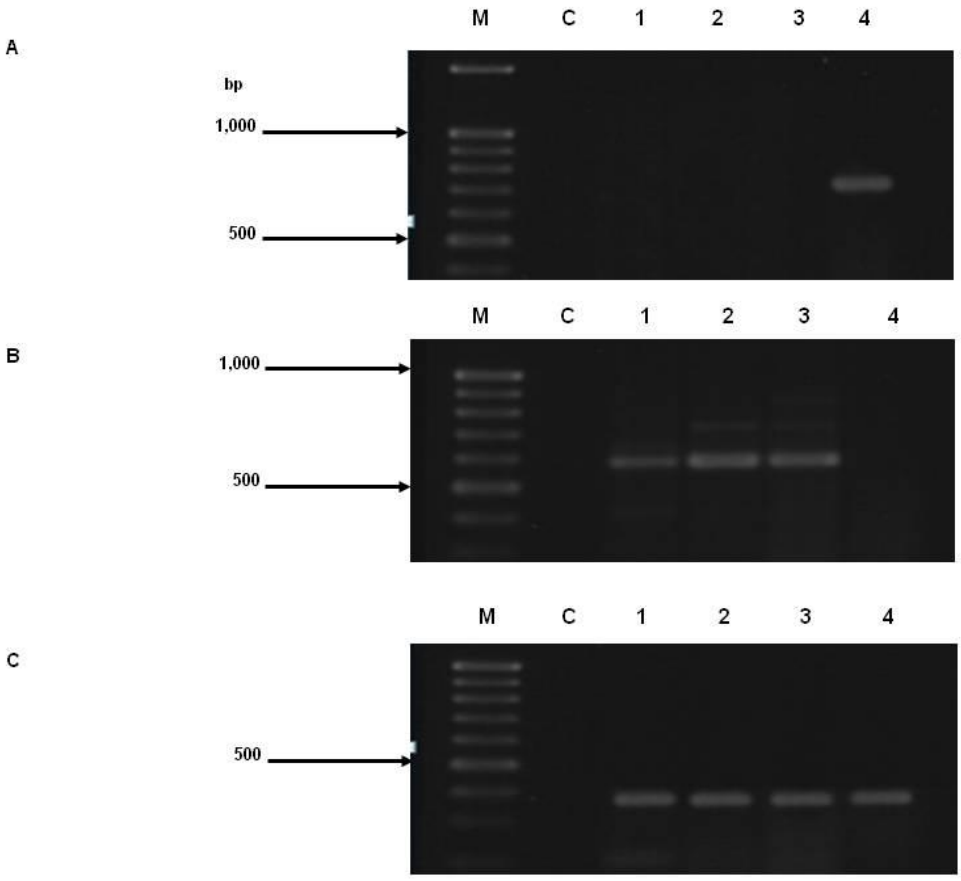


**Figure 4.1** Expression patterns of *AciHBGase I* and *II* in *A. cerana indica*. The RT-PCR products amplified by the specific primer pairs for (A) *AciHBGase I* and (B) *AciHBGase II* with (C) the control profile of *28S rDNA* amplification. In each figure, lane M contained 100 bp DNA ladder marker, 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.

**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 successful 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 (Fig. 4.2). The sequence has been deposited at GenBank with accession code # JX468895. From the predicted ORF of the cDNA sequence the deduced encoded amino acid sequence of 579 amino acids was obtained (Fig. 4.2). This predicted primary structure of *Aci*HBGase II showed the conserved regions associated with α–amylase family of enzymes (Svensson, 1994). 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 *Aci*HBGase II was aligned to the amino acid sequences of homologs with similar annotated functions, a high amino acid sequence similarity was revealed (Fig. 4.3).

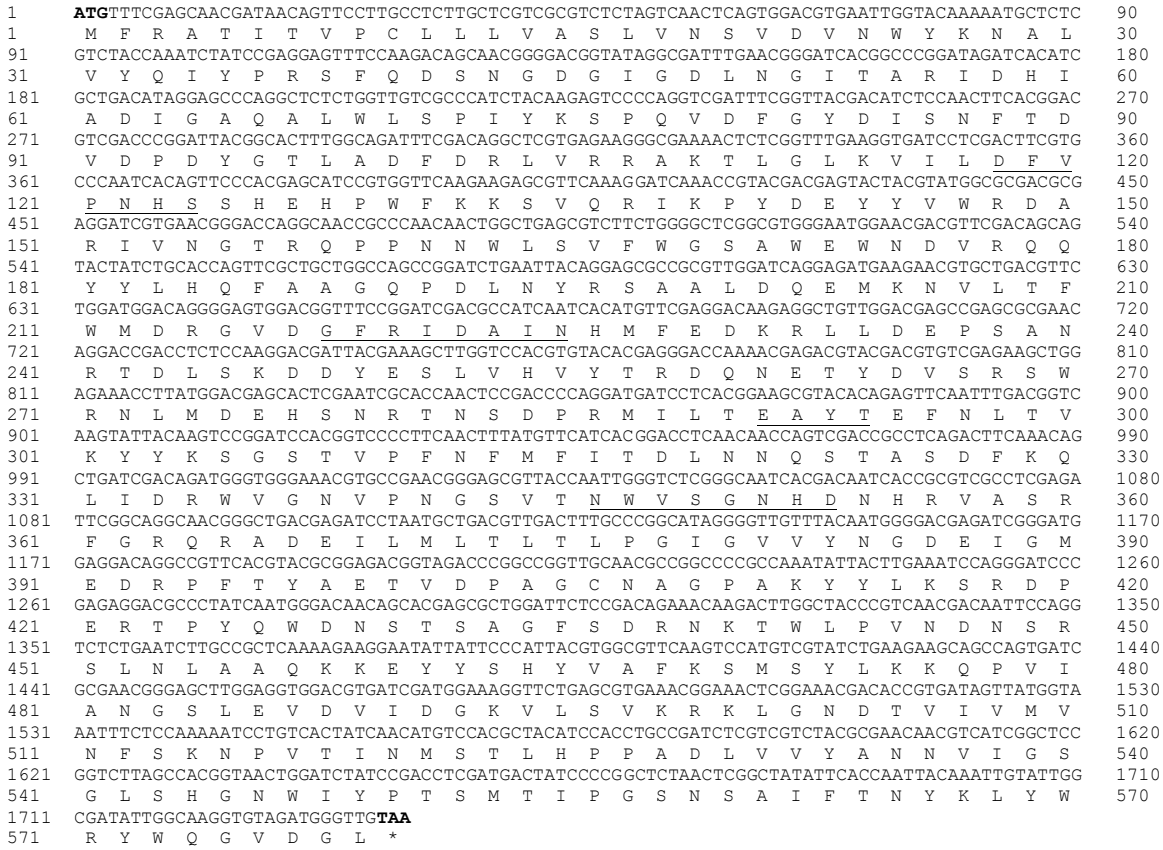


**Figure 4.2** The full-length *AciHBGase II* cDNA and deduced amino acid sequence of the encoded *Aci*HBGase II enzyme. The predicted start (ATG) and stop (TAA) codons are shown in bold, giving a predicted open reading frame (ORF) of 1,740 bp encoding for a deduced 579 residue amino acid sequence. The conserved amino acid regions associated with the α-amylase family are underlined.

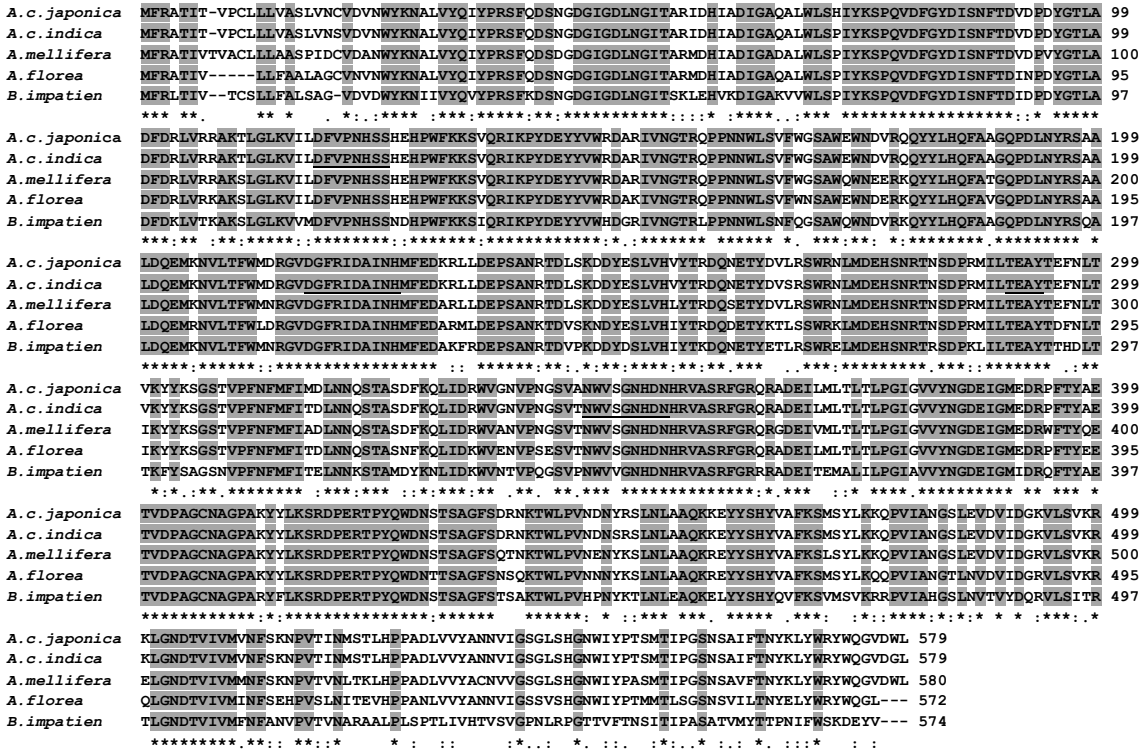


**Figure 4.3** Multiple amino acid sequence alignment of *Aci*HBGase II with other HBGase II homologs. Shown are HBGase II sequences from *Apis cerana indica* (*Ac. indica*) in comparison with those from the Japanese honeybee *A. cerana japonica* (*Ac. japonica*, GenBank accession # ACN 63343.1), European honeybee *A. mellifera* (*A. mellifera*, GenBank accession # NP_001035349.1), predicted maltase 2-like of *Apis florea* (*A. florea*, GenBank accession # XP_003691502.1) and predicted maltase 2-like of *Bombus impatiens* (*B. impatien*, 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.

**Expression of rAciHBGase 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 by methanol (0-10% (v/v)) in order to express the r*Aci*HBGase II-$(His)_6$ enzyme. The optimal methanol concentration, in terms of inducing the highest secretory enzyme activity, was found 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 r*Aci*HBGase 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 (Fig. 4.4). 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 (~ 0.05 *vs*. ~ 0.02 U/ml, respectively). 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).

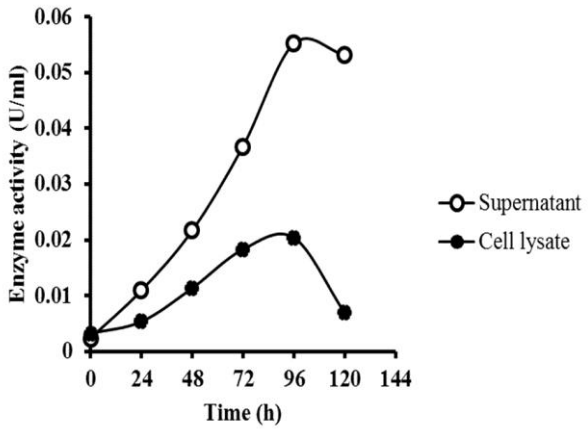


**Figure 4.4** Induction and expression of α-glucosidase activity. α-Glucosidase activity levels (assumed to be from the r*Aci*HBGase II-$(His)_6$ enzyme) in the culture medium (supernatant) and *P. pastoris* cell lysate after being induced by 0.5% (v/v) MeOH in 50 ml cultures.

**Table 4.2** Purification of the r*Aci*HBGase II-$(His)_6$ enzyme.

| **Procedure** | **TotalProtein (mg)** | **Total activity (U)** | **Specific activity (mU/mg)[a]** | **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 |

[a] $mU = 10^{-3}$ 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.

**Purification of the rAciHBGase II-$(His)_6$ enzyme**

After centrifugal filtration based concentration of the culture medium, the secreted r*Aci*HBGase II–$(His)_6$ enzyme (1 ml) was further purified by Histrap affinity column chromatography (GE Healthcare). Eleven fractions (2 ml per fraction) were collected (kept at -20$^o$C until use) and assayed for α-glucosidase activity using PNPG as the substrate. From the protein and enzyme activity elution profile (Fig. 4.5A), fraction # 10 provided the highest enzyme specific activity (1.6 mU/mg). Although only a one step purification procedure was used, a single HBGase active band could be observed (Fig. 4.5B and 4.5C). Thus, this fraction was used for further characterization. The enrichment data are summarized in Table 4.2.

**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 r*Aci*HBGase 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 (Fig. 4.6A) and the enzyme was stable (> 90% residual activity) to a 24 h exposure at 4$^o$C to pH in the range of 5.0 - 7.0 (Fig. 4.6B). In addition, the optimal enzyme activity at pH 3.5 was found at 45 $^0$C, although 90% activity was found in the 40-55$^o$C range (Fig. 4.6C). The enzyme preparation was stable (> 90% residual activity) to a 15 minute exposure of < 45 $^0$C, although > 80% residual activity was maintained after exposure to up to 65$^o$C for 15 min, but decreased rapidly at higher temperatures (Fig. 4.6D).

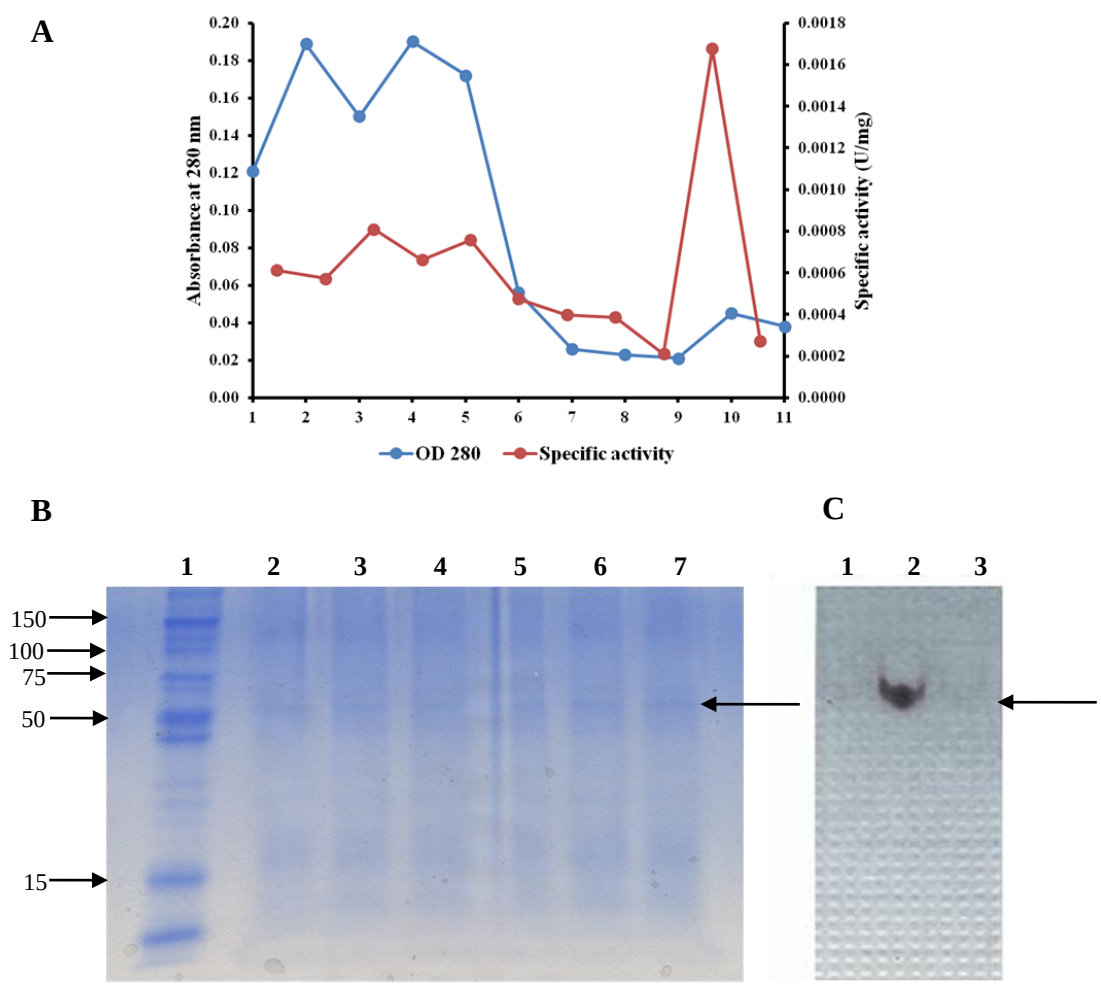


**Figure 4.5** Enrichment profile for the r*Aci*HBGase II–$(His)_6$ protein by Histrap affinity column. (A) Elution profile from the Histrap column. The washed fractions contained a high content of protein while eluted fraction # 10 presented the highest specific activity of HBGase II. (B) SDS-PAGE resolved and coomassie blue stained protein profile of selected fractions from the Histrap column elution. Lane 1: standard protein marker, lanes 2-6: contained 1 μg of protein in fractions # 2, 4, 6, 8 and 10, respectively. Lane 7 is a duplication of lane 6. (C) Glucosidase activity stain of the renatured SDS-PAGE resolved protein samples (Zymograph). Lanes 1-3, fractions # 9-11, respectively. Arrows in (B) and (C) indicate the ~ 73 kDa band.

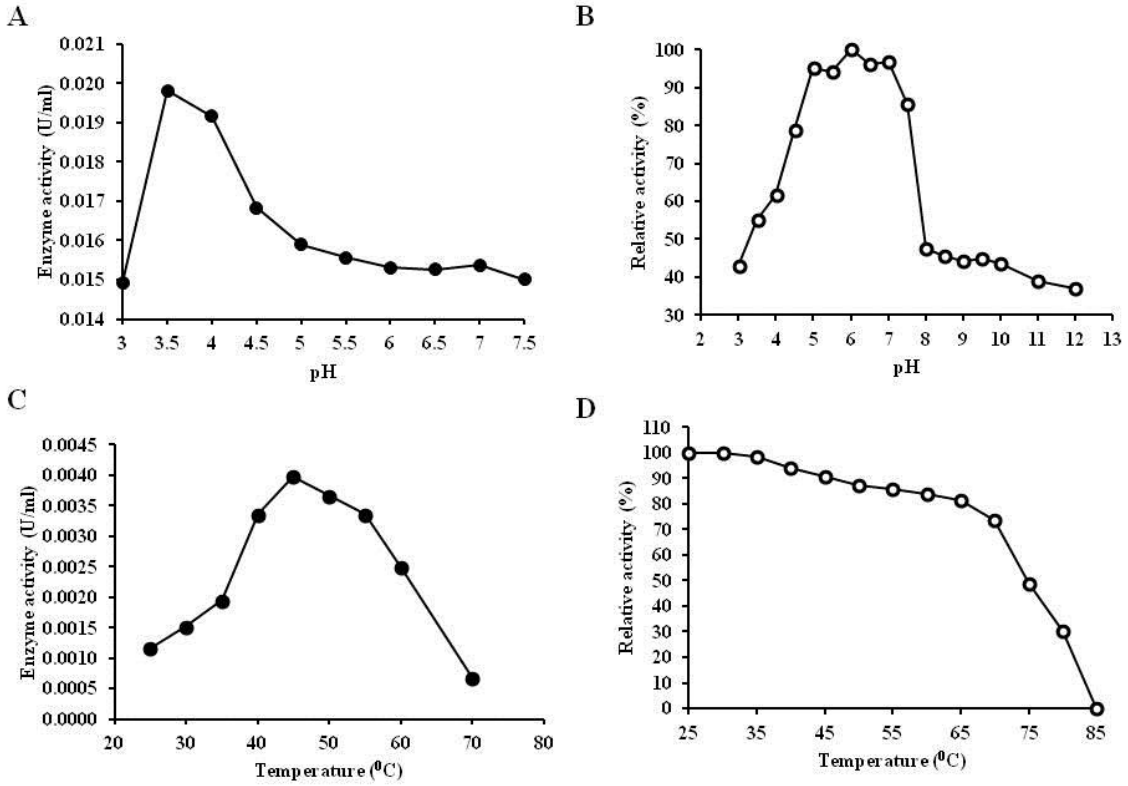


**Figure 4.6** The optimum conditions for α-glucosidase activity of the partially purified r*Aci*HBGase II–$(His)_6$ enzyme. The optimal (A) pH and (C) temperature and the (B) pH or (D) thermal stability.

***Substrate specificity***

The substrate specificity of the partial purified r*Aci*HBGase 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 (Fig. 4.7). Considering Figures 4.3 and 4.7 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* MSB8 (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*) (Fig. 4S1; Additional file 1), three possibilities could be observed. The first one, standard single-substrate Michaelis-Menten shape curves 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 (Tseng and Hsu, 1990).

Lineweaver-Burk plots (1/*s* versus 1/*v* plots) (Lineweaver and Burk, 1934) revealed a linear regression for all the evaluated substrates (Fig. 4S2; Additional file 2). The derived kinetic parameters ($K_m$, $k_0$ and $V_{max}$) 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 (Fig. 4S1).

Although the $K_m$ values for maltotriose and maltotetraose were very similar, the $k_0$ and $V_{max}$ 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 $V_{max}$ 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 $V_{max}$ values, (ii) high $K_m$ and low $V_{max}$ values, (iii) low $K_m$ and high $V_{max}$ and (iv) high $K_m$ and high $V_{max}$ to cells should be performed.

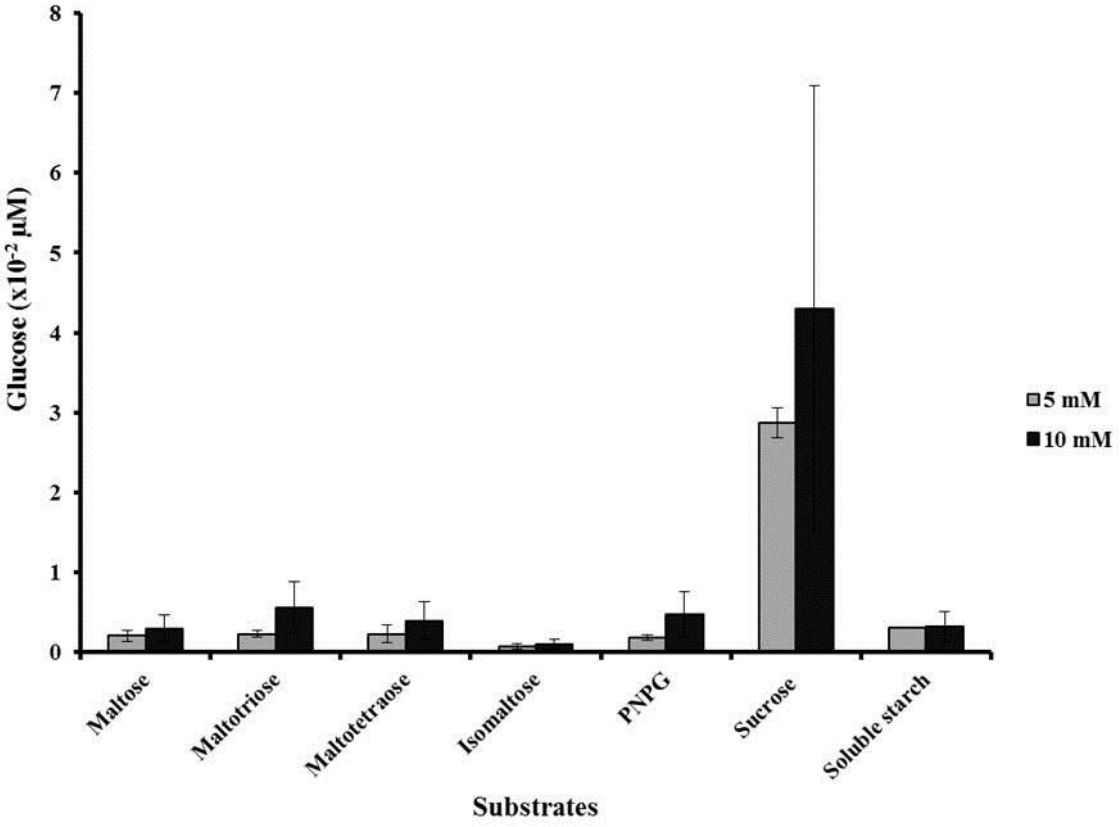


**Figure 4.7** Substrate specificity of the partially purified r*Aci*HBGase II-$(His)_6$ preparation. For PNPG each molecule of *p*-nitrophenol released is equated to one glucose molecule. Data are shown as the mean $\pm$ 1 SD, and are derived from three replications.

**Table 4.3.** Kinetic parameters[a] for the hydrolysis of different substrates by the partial purified r*Aci*HBGase II-$(His)_6$ enzyme.

| **Substrate** | $K_m$[b] **(mM)** | $k_0$ **($s^{-1}$)** | $V_{max}$[c] **(mg glucose/min/mg protein)** | $k_0/K_m$ **($mM^{-1}\cdot s^{-1}$)** |
|---|---|---|---|---|
| Maltotriose | 26.7 ± 1.96 | 26.6 ± 0.88 | 3.94 ± 0.13 | 1.00 |
| Maltotetraose | 25.0 ± 3.10 | 2.84 ± 0.31 | 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 | 22.0 ± 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 |

[a]Data are shown as the mean ± 1 SD and are derived from three independent repeats.

[b]The $K_m$ value is the substrate concentration giving one half of the intrinsic $V_{max}$.

[c]$V_{max}$ was obtained from the calculated value as the glucose liberated from the reducing end residue of substrates.

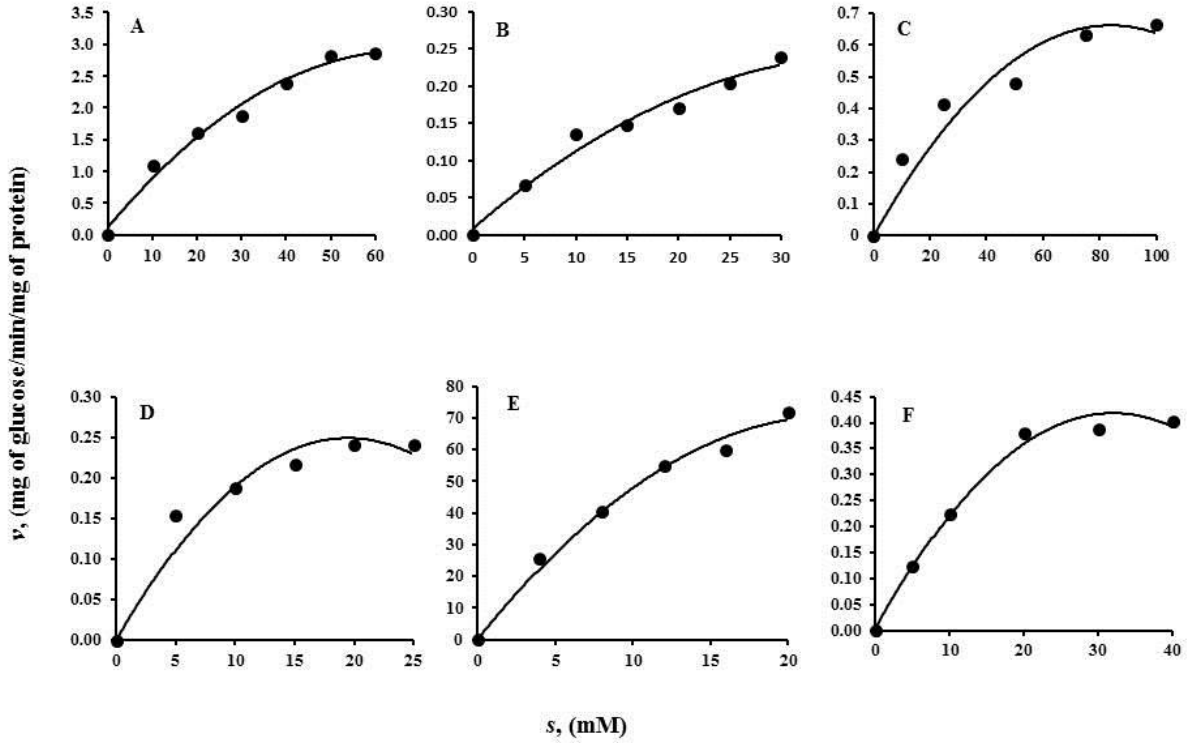


**Figure 4S1** Substrate (*s*) versus enzyme velocity (*v*) plots for the hydrolysis reaction with the partially purified r*Aci*HBGase II-$(His)_6$ preparation. Shown are with the results for, (**A**) maltotriose (0-60 mM), (**B**) maltotetraose (0-30 mM), (**C**) isomaltose (0-100 mM), (**D**) PNPG (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.

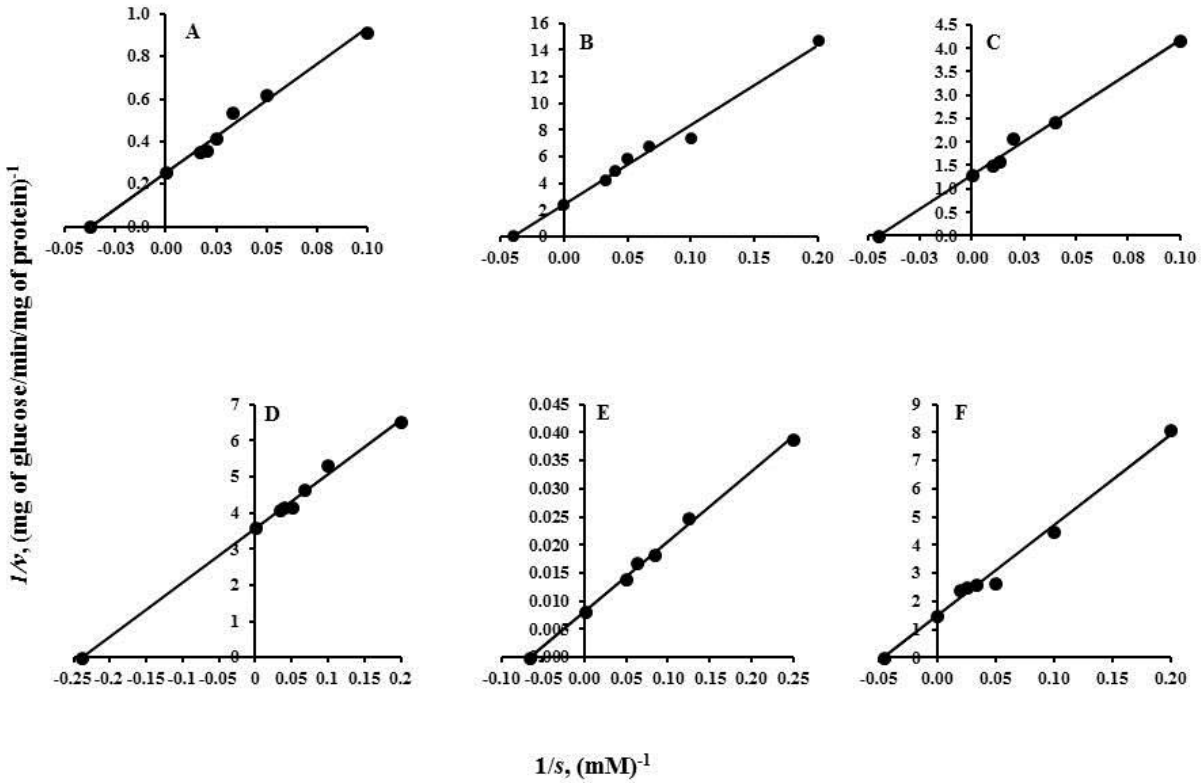


**Figure 4S2** Lineweaver-Burk plots for the hydrolysis reaction with the partial purified r*Aci*HBGase II-$(His)_6$ preparation. Substrates shown are (**A**) maltotriose (0-60 mM), (**B**) maltotetraose (0-30 mM), (**C**) isomaltose (0-100 mM), (**D**) PNPG (0-25 mM), (**E**) sucrose (0-20 mM), and (**F**) soluble starch (0-40 mM). For PNPG each molecule of *p*-nitrophenol released is equated to one glucose molecule. The linear regression coefficiemt ($R^2$) and equation of the best for line shown are given in each panel.

**DISCUSSION**

The *AciHBGase III* transcript expression pattern (Chanchao *et al*., 2008) and nucleotide sequence have been reported previously (Chanchao *et al*., 2006). 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 *Aci*HBGase II is not likely to play a role in honey synthesis through the hydrolysis of sucrose from nectar in the honey crop (Kubota *et al*., 2004), although we did not formerly test for expression levels in specific tissues including the hyopharangeal and salivary glands and honey crop, but rather screened whole adults. Nevertheless, it is more likely that *Aci*HBGase II is involved in other functions and possibly to do with the development of this honeybee. Hercovics (1999) 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 (Zhang *et al*., 2008).

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 (Nishimoto *et al*., 2007), with the four highly conserved regions found in α–amylase family members being present. With respect to the nucleotide and predicted amino acid sequences, *Aci*HBGases can be classified into three isoforms (*Aci*HBGase I, II and III) as per the HBGases in the closely related *A. mellifera* and *A. cerana japonica* honey bees (Nishimoto *et al*., 2007; Wongchawalit

*et al*., 2006). 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 (Harzer *et al*., 2012).

The secreted form of the r*Aci*HBGase II (as a chimeric protein with a C-terminal $(His)_6$ tag) could be enriched by only a few purification steps (Figure 4.5 and Table 4.3). The partially purified r*Aci*HBGase 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 from bovine testes (Van der Ploeg *et al*., 1991) and the human placental lysosomal acid HBGase that hydrolyzes glycogen to glucose (Moreland *et al*., 2012). As such it is potentially consistent with the proposed role of *Aci*HBGase 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 *P. pastoris* may be useful for this disease treatment. Furthermore, the optimum temperature for α-glucosidase activity of the r*Aci*HBGase II-$(His)_6$ enzyme was at 45$^{o}$C (with 80-90% residual activity at 40-55$^{o}$C), which is much higher than that previously reported by Wu et al. (Wu *et al*., 2010), and higher than the physiological temperature of the bees. However, within extreme thermophiles, Nashiru et al. (Nashiru *et al*., 2001) reported a novel HBGase from *Thermus caldophilus* GK24 with an optimal temperature of 90$^{o}$C, whilst the

intracellular and extracellular HBGases from *G. toebii* strain E134 showed an optimum activity of 65$^{o}$C and 70$^{o}$C, respectively (Cihan *et al*., 2012).

The enriched r*Aci*HBGase II-$(His)_6$ enzyme showed a reasonably wide substrate specificity encompassing α-(1,1)-, α-(1,2)-, α-(1,4)- and α-(1,6)-glucosidic linkages, but rapidly hydrolyzed PNPG α-(1,6) and sucrose α-(1,2). Similar to the native *Am*HBGase II that displayed positive cooperativity to sucrose, turanose α-(1,3), kojibiose α-(1,2) and soluble starch α-(1,4) (Takewaki *et al*., 1993), the r*Aci*HBGase 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 (Chiba, 1998), 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 (Minetoki *et al*., 1995), and so it awaits the purification and characterization of the native *Aci*HBGase 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 *P. pastoris*.

# CHAPTER V

## Over-expression and Characterization of recombinant alpha-glucosidase I from *Apis cerana indica* in *E. coli*

*A version of this Chapter was accepted to published in Journal of Apiculture*

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## ABSTRACT

α-Glucosidases (HBGases) catalyze the non-reducing end of a substrate to liberate α-D-glucose, and are classified into the three types of HBGase I, II and III according to their substrate specificity. The full length ORF cDNA (1,734 bp) and the deduced encoded amino acid sequence (577 residues) of the HBGase I from *Apis cerana indica* (*Aci*HBGase I) was obtained by RT-PCR. The deduced amino acid sequence showed 99% and 80% sequence identity to the HBGase I from the closely related honey bees, *A. cerana japonica* and *A. mellifera*, respectively. After cloning in the pEcoli-Nterm-6xHN expression vector and transforming into *E. coli* Rosetta(DE3) cells the maximal glucosidase activity (assumed to be due to the expression of the recombinant (r)$(His)_6$-*Aci*HBGase I enzyme) was found in the cell lysate (0.054 U/ mL; equivalent to 2.16 U/ mL culture medium) after 1 mM IPTG induction for 3 h. The partially purified r$(His)_6$-*Aci*HGase I enzyme displayed an optimal glucosidase activity, in terms of the hydrolysis of *p*-nitrophenyl α-D-glucoside, at pH 3.5 and 40$^o$C, whilst it was stable (> 90% residual activity) at pH 3.5-5.5 (24 h at 4$^o$C) and up to 45 $^0$C (15 min exposure at pH 5.5). The most preferable substrate was sucrose ($K_m$ value of 10.82 ± 0.39 mM).


## INTRODUCTION

α-Glucosidases (HBGase) or α-D-glucoside glucohydrolases belong to the group of glucosidases that hydrolyze O- and S-glycosyl compounds (EC 3.2.1.20). HBGases not only hydrolyze the terminal α1-4-glucosidic linkage to liberate α-D-glucose, but they also can perform transglucosylation reactions in vitro (Frandsen and Svensson, 1998;

Yamamoto *et al*., 2004). The enzymes are classified into the two groups of the GH13 and GH31 families (Nakai *et al*., 2005; Tsujimoto *et al*., 2007), based on their sequence homology within the carbohydrate-active enzyme database (CAZY). Members of the GH13 family have four conserved sequence regions (1-4) that are important for catalytic activity (Kimura, 2000), whilst those from GH31 have two conserved sequence regions (1 and 2) within which the two essential residues, D481 and D647, are found (Okuyama *et al*., 2001). Based on their substrate specificity, HBGases can be divided into the three types of HBGase I, II and III (Giannesi *et al*., 2006; Nishimoto *et al*., 2007). The preferred substrate for HBGase I (a member of the GH13 family) are aryl glucosides and sucrose, whereas HBGase II and III (members of the GH31 family) show a preference for substrates like maltose and isomaltose, but only the HBGase III group can hydrolyze polysaccharides like amylose and starch (Nimpiboon *et al*., 2011).

Isoforms of HBGase are found in many organisms, including bacteria, yeasts, insects and plants (Nakai *et al*., 2007; Nimpiboon *et al*., 2011; Nishimoto *et al*., 2007; Okuyama *et al*., 2001). The different isoforms can be derived from different genes, such as for HBGase I, II and III in *Apis cerana japonica* (*Acj*HBGase) (Wongchawalit *et al*., 2006), or from differential post transcriptional or post translational modifications of the same gene, such as the alternative mRNA splicing of the HBGase II mRNA to yield HBGase III and the post-translational proteolysis of the HBGase II to yield HBGase I in the ripening seeds of *Oryza sativa* L. (Nakai *et al*., 2007). Furthermore, the putative *N*-glycosylation motif (N-Xa.a.-S/T) for the addition of various oligosaccharides onto

asparagine residues was found in the predicted primary amino acid sequences of HBGase I, II and III in *A. mellifera* (*Am*HBGase) (Nishimoto *et al*., 2007).

Of the three isoforms of HBGases, HBGase I is interesting in that it can remove the outermost α1,2-glucose unit from substrates. In vitro, this enzyme is or has the potential to be important in biotechnological, clinical, and microbiological fields. In vivo, it is required in many organisms, for example in the human pathogenic yeast *C. albicans* to synthesize the mannoproteins in the outer layer of its cell wall that are important in cell adhesion to host tissue, dimorphism, recognition by the host immune system and virulence (Mora-Montes *et al*., 2009).

In this research, we focused on the HBGase I of *A. cerana indica* (*Aci*HBGase I), an economic and native honey bee species in Thailand. The full length ORF was obtained by RT-PCR, cloned and sequenced. The homology of the deduced amino acid sequence to that of other proteins in the NCBI GenBank data base was evaluated, whilst the substrate specificity of the recombinant enzyme, expressed as a N-terminal $(His)_6$ tagged chimera (r$(His)_6$*Aci*HBGase I) in *Escherichia coli*, was evaluated *in vitro*.

## MATERIALS AND METHODS

### Sample collection

Foragers of *A. cerana indica* were collected from an apiary in Samut Songkram province, as previously reported (Kaewmuangmoon *et al*., 2012). They were stored in dry ice and then at - 80$^{o}$C until used.

### RNA extraction

Total RNA from forager bees was extracted using the RNeasy® Plus Mini kit (Qiagen). The quality of the obtained total RNA was assayed visually following 1.2% (w/v) formaldehyde/agarose gel electrophoresis and ethidium bromide (EtBr) staining with UV transillumination. The concentration of the extracted RNA was calculated by the absorbance at 260 nm, and its purity from the ratio of the absorbance at 260 and 280 nm. The extracted total RNA sample was kept at -80$^{o}$C until used.

### Primer design and RT-PCR

Primer design was based on the mRNA sequence of *AcjHBGase I* as obtained from GenBank (accession # AB260890). All primers were designed using the Primer 3 program (http://frodo.wi.mit.edu/primer3/) and then checked manually (Table 1). After that, RNA was reversed transcribed to first-strand cDNA using the SuperScript™ III First-strand Synthesis SuperMix for qRT-PCR (Invitrogen), as per the manufacturer's protocol. The full length cDNA of *AciHBGase I* was then amplified by PCR using the five primer sets shown in Table 1 to yield overlapping contigs. All PCR reactions were

performed under previously optimized conditions of 94ºC for 2 min; 30 cycles of 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 2 min; and finally 1 cycle of 72ºC for 7 min. A reaction as above but without the reverse transcriptase (so no cDNA) was performed as a negative control. After being resolved by 1.5% (w/v) agarose-TBE gel electrophoresis, checked for expected amplicon size and purified, the PCR product was direct sequenced by Bigdye® terminator using a 3730 DNA Analyzer (Applied Biosystems). The obtained overlapping (contiguous) sequences were aligned in Sequencher 4.1.4 software to derive the complete sequence, which was then searched for homologous sequences, including any recorded *HBGase I* sequences, in the NCBI GenBank database using the on line megaBLASTn algorithm.

**Construction of the *AciHBGase I*-pEcoli-Nterm-6xHN expression vector**

In order to amplify the full length cDNA and facilitate the cloning, the F (5'-AATCGTCGACAAGAGCCTCGTCGTGGTTGTA-3') and R (5'-TCTCTGTC GACTCGTTGGAAAAAAGATAT-3') primers were designed to encompass the 5' and 3' outermost regions of *AciHBGase I*, respectively. Both primers contained 5' flanking regions with a *Sal*I restriction site (underlined) to allow in-frame cloning in the pEcoli-Nterm-6xNH expression vector, but as with the initial amplification note that the assumed ATG initiation codon and following 12 nucleotides (four amino acids) are fixed by the primer rather than the actual sequence. The PCR reaction was performed under the previously optimized conditions as described for the RT-PCR above, except that the annealing temperature was 53 $^{0}$C. PCR reactions were checked for amplicons of the

expected size by resolving a 5 µL aliquot by 1.5% (w/v) agarose-TBE gel electrophoresis. The remaining amplified PCR product and the pEcoli-Nterm-6xHN expression vector (Clontech) were separately digested by *Sal*I (1 µg DNA/ U) at 37ºC for 2 h. The vector was then dephosphorylated with alkaline phosphatase at 37 $^0$C for 30 min and the reaction stopped by incubating at 65 $^0$C for 30 min. The *Sal*I restricted PCR product and *Sal*I restricted plus dephosphorylated pEcoli-Nterm-6xHN vector were ligated by T4 DNA ligase and 2x rapid ligation buffer (Promega) for 1 h at room temperature (RT), as per the supplier's protocol.

**Expression of the r(His)$_6$-*Aci*HBGase I enzyme**

The *AciHBGase I*-pEcoli-Nterm-6xHN construct was initially transformed into *E. coli* (DH5α) by heat shock at 42 $^0$C for 45 sec. Single colonies on LB-agar (1% (w/v)) plates containing 100 µg/ mL ampicillin were checked by colony PCR for the presence of the *AciHBGase I* construct in the correct orientation. The selected transformant clone was grown overnight in 2 mL LB-ampicillin (100 µg/ mL) at 37ºC with shaking at 130 rpm prior to extraction of the plasmid DNA using the QIAprep® Miniprep (Qiagen) kit as per the supplier's protocol, and then transformed into *E. coli* Rosetta(DE3) expression cells. To express the r(His)$_6$-*Aci*HBGase I enzyme, an overnight 1 mL culture of the transformed DE3 cells was diluted 1:49 (v/v) with fresh LB-ampicillin and incubated as above until an $OD_{600}$ of 0.6-0.7 was attained. A 1 mL aliquot was kept on ice to serve as a non-induced control whilst the remainder was supplemented with IPTG to 1 mM final concentration (except where stated otherwise, when it set within the range of 0.5 - 10mM)

and incubated as above for 5 h (except where stated differently, when it was within the 1-5 h range). Cells were then harvested by centrifugation (3,000x g, 4 $^0$C, and 15 min) and the supernatant discarded. The total protein expression was evaluated by resuspending the cell pellet in PBS to ten-fold the original culture volume and then mixing with an equal volume of 2x SDS loading buffer. Moreover, the solubility of the recombinant protein in the host Rosetta (DE3) cells was analyzed following the protocol in the pEcoli Expression Systems User Manual (Clontech). The supernatant (soluble proteins) and the pellet (insoluble proteins) were separately harvested and assayed for the total protein concentration by measuring the absorbance at 280 nm and Bradford's assay (Bradford, 1976), and for the protein profile by resolution of 5 μg total protein by SDS-PAGE (8% (w/v) acrylamide resolving gel) with Coomassie blue staining.

**Partial purification of the chimerical r(His)$_6$-*Aci*HBGase I enzyme**

The selected Rosetta (DE3) transformant with a high expression level of glucosidase activity was cultured in 25 mL of LB medium with 100 μg/ mL ampicillin at 37 $^0$C and shaking at 150 rpm overnight. This culture was then added to 1,225 mL of fresh LB/ ampicillin (100 μg/ mL) medium and cultured as above until the $OD_{600}$ reached 0.6-0.7 when 1 mM IPTG was added and further cultured as above for 1 - 5 h. After this induction period the cells were collected by centrifugation (3,000x g, 4 $^0$C and 15 min), the cell pellet was further lysated and extracted following the protocol in the pEcoli Expression Systems User Manual (Clontech). The resultant supernatant harvested and five-fold concentrated through a Vivaspin 20, 10 kDa cut off filter (GE Healthcare), to a

sample volume of 5 mL. The concentrated sample was then applied (500 μL at a time) onto a HisTALON$^{TM}$ gravity column (Clontech) and washed with 8 mL of equilibration buffer (HisTALON buffer set catalog # 635651) containing 10 mM imidazole, followed by 7 mL of wash buffer and 8 mL of elution buffer containing 150 mM imidazole under gravity feed (elution rate of ~ 0.3 mL/ min). Fractions were evaluated for protein content by following the absorbance at 280 nm, and glucosidase enzyme activity using the hydrolysis of *p*-nitrophenyl α-D-glucoside (PNPG, Sigma), as detailed below. The partially purified protein was then mixed with a commercial protease inhibitor cocktail (Amresgo) and stored at 4$^{o}$C until used, whilst the apparent degree of homogeneity and (subunit) molecular weight of the partially purified r*Aci*(His)$_6$HBGase I was evaluated following SDS-PAGE resolution and either Coomassie blue staining or, after renaturation in the gel, glucosidase activity staining (zymograph) as detailed below.

**Glucosidase (enzyme) activity (Zymography)**

For assaying the glucosidase activity of the r(His)$_6$-*Aci*HBGase I preparations, PNPG was used as the substrate. The premix (0.1 mL of 0.1 M sodium phosphate buffer at pH 5.5, 0.05 mL of d-$H_2O$ and 0.05 mL of 5 mM PNPG) was pre-incubated at 37$^{o}$C for 3 min. Then, 50 μL of the respective r(His)$_6$*Aci*HBGase I preparation was added and further incubated at 37$^{o}$C for 10 min before the reaction was stopped by the addition of 0.5 mL of 1 M $Na_2CO_3$. The absorbance at 400 nm of the reaction mixture was measured to follow the release of *p*-nitrophenol. A blank control reaction was performed as above but with the addition of the enzyme preparation free solvent instead of the enzyme. One

unit of r(His)$_6$*Aci*HBGase I is defined as that that which liberates 1 μM of D-glucose (followed as 1 μM of *p*-nitrophenol) from PNPG per min at pH 5.5 and 37$^o$C.

**Glucosidase (enzyme) activity stained SDS-PAGE gels (Zymograph)**

After denaturing SDS-PAGE (8% (w/v) acrylamide resolving gel) resolution of the respective enzyme preparation (5 μg per lane), samples were renatured by incubating the gel in 1.0% (v/v) Triton X-100 with gentle shaking at RT for 2 h followed by 10 mM sodium acetate buffer containing 0.5 M sucrose (pH 5.0) at 45 $^0$C for 30 min to allow the enzyme activity to proceed. Then the gels were rinsed in double distilled (dd)-$H_20$ and developed by boiling in 0.5 M NaOH containing 0.1% (w/v) 2, 3, 5-triphenyltetrazolium chloride until a red band, indicative of glucosidase activity, was detected.

**Characterization of the partially purified r(His)$_6$*Aci*HBGase I enzyme**

*pH optimum and stability of the glucosidase activity*

The glucosidase assay reaction mixture contained 50 μL of the partially purified enzyme preparation, 50 μL of 5 mM PNPG and 100 μL of Briton-Robinson buffer (10 mM acetic acid, 10 mM phosphoric acid and 10 mM boric acid) at the desired pH (range of pH 3 – 12). The reaction mixture was incubated at 37 $^o$C for 10 min and then assayed for glucosidase activity as outlined above. To determine the pH stability, the partially purified r(His)$_6$*Aci*HBGase I preparation was first incubated in the appropriate pH Briton-Robinson buffer (range of pH 3 – 12) at 4 $^o$C for 24 h, and then assayed for glucosidase activity as detailed above.

*Thermal optimum and stability of the glucosidase activity*

The same reaction mixture was used as that for determination of the pH optimum except that the pH was constant at pH 5.5 and the incubation temperature was adjusted to one of 25, 30, 35, 40, 45, 50, 55, 60 or 70 $^0$C. To evaluate the thermal stability, the partially purified enzyme preparation was prepared in 0.1 M sodium phosphate buffer (pH 5.5) containing 0.05% (v/v) Triton X-100 and incubated at the desired temperature (range of 4 – 85 $^o$C) for 15 min before then being assayed for glucosidase activity as described above.

*Substrate specificity*

To evaluate the substrate specificity, six natural carbohydrate substrates (maltose, maltotriose, maltotetraose, isomaltose, sucrose and soluble starch) plus PNPG were tested, each at the two concentrations of 5 and 10 mM. The reaction mixture (50 μL of 25 or 50 mM substrate, 50 μL of dd-$H_2O$ and 100 μL of 0.1 M sodium phosphate buffer, pH 5.5) were mixed with 50 μL of the partially purified r*Aci*HBGase I preparation and incubated at 37 $^0$C for 10 min before being stopped by the addition of 500 μL of 1 M $Na_2CO_3$. The amount of glucose liberated from the six carbohydrate substrates was determined by the glucose oxidase - peroxidase method using a commercial Glucose Assay Kit (Sigma). For this, 100 μL of the reaction mixture was mixed with 200 μL of the Glucose assay reagent and incubated at 37$^o$C for 30 min before being stopped by the addition of 0.2 mL of 12 N $H_2SO_4$. The absorbance at 540 nm was then 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):

Glucose (mg) = [($A_{540}$ of test)(standard glucose in mg)] / ($A_{540}$ of standard) (1)

In order to evaluate the enzyme kinetics, the same method that was used for determining the substrate specificity above was followed using the commercial glucose assay kit, except the final substrate concentrations (*s*) were assayed over a range of 5 - 30 mM for maltose and PNPG, 5 - 50 mM for maltotriose and maltotetraose, 4 - 20 mM for sucrose and 5 - 100 mM for soluble starch. Note that isomaltose was not assayed.

## RESULTS

### Full length cDNA of *Aci*HBGase I

PCR amplification of the cDNA with the five sets of primers (Table 5.1) designed to give overlapping sequences, all gave amplicons of the expected size. After direct sequencing, the contiguous sequences were assembled to form the complete *Aci*HBGase I cDNA sequence (and subsequently aligned with other sequences obtained from GenBank) using the Clustal X and Sequencher 4.1.4 software followed by manual checking. The resultant full length cDNA had a predicted ORF of 1,734 bp encoding for a deduced amino sequence of 577 residues (Fig. 5.1), with an unmodified predicted molecular weight ($M_W$) of 66.54 kDa. Both the nucleotide and amino acid sequences are deposited in GenBank with the accession code # KC149921. Note, however, as stated before that the first five N terminal amino acids (and the corresponding sequence of 15 nucleotides)

would have been fixed by the 5' forward primer used in the PCR reaction, including the assumed ORF initiation ATG/methionine.

**Table 5.1** Primers used in the RT-PCR to obtain the full-length cDNA of *AciHBGase I*.

| **Primer pairs** | **Primer sequence (5' to 3')** | **Position (5' to 3')[a]** | **Size (bp)[b]** |
|---|---|---|---|
| *Aci*HBGase I-A | F: TCAAATCATGAAGAGCCTCGT | 7 - 27 | 458 |
| | R: TTTTCGGATCAACCCATACG | 445 - 464 | |
| *Aci*HBGase I-B | F: TCACTGCAGAAGCGAAGAAA | 321 - 340 | 632 |
| | R: ATTGAAGGGAACGTTTGCAC | 933 - 952 | |
| *Aci*HBGase I-C | F: ACAACAACCGGGATGAAATAG | 854 - 875 | 456 |
| | R: GCAGCCATTCCTTTTCAAGT | 1291 - 1310 | |
| *Aci*HBGase I-D | F: TATGATGTTCGTGATGGCTGT | 1208 - 1228 | 433 |
| | R: CGATAGCCATCGGATTTACG | 1621 - 1640 | |
| *Aci*HBGase I-E | F: AATCGTGACAAGAGCCTCGTCGTGGTTGTA | 4 - 24 | 1,500 |
| | R: TCTCTGTCGACTCGTTGGAAAAAAGATAT | 1,714 - 1,731 | |

[a] Primer positions and [b] expected amplicon size are based upon the *Acj*HBGase 1 sequence (GenBank accession code AB260890). Underlined bases in *Aci*HBGase I-E represented *Sal*I restriction site.

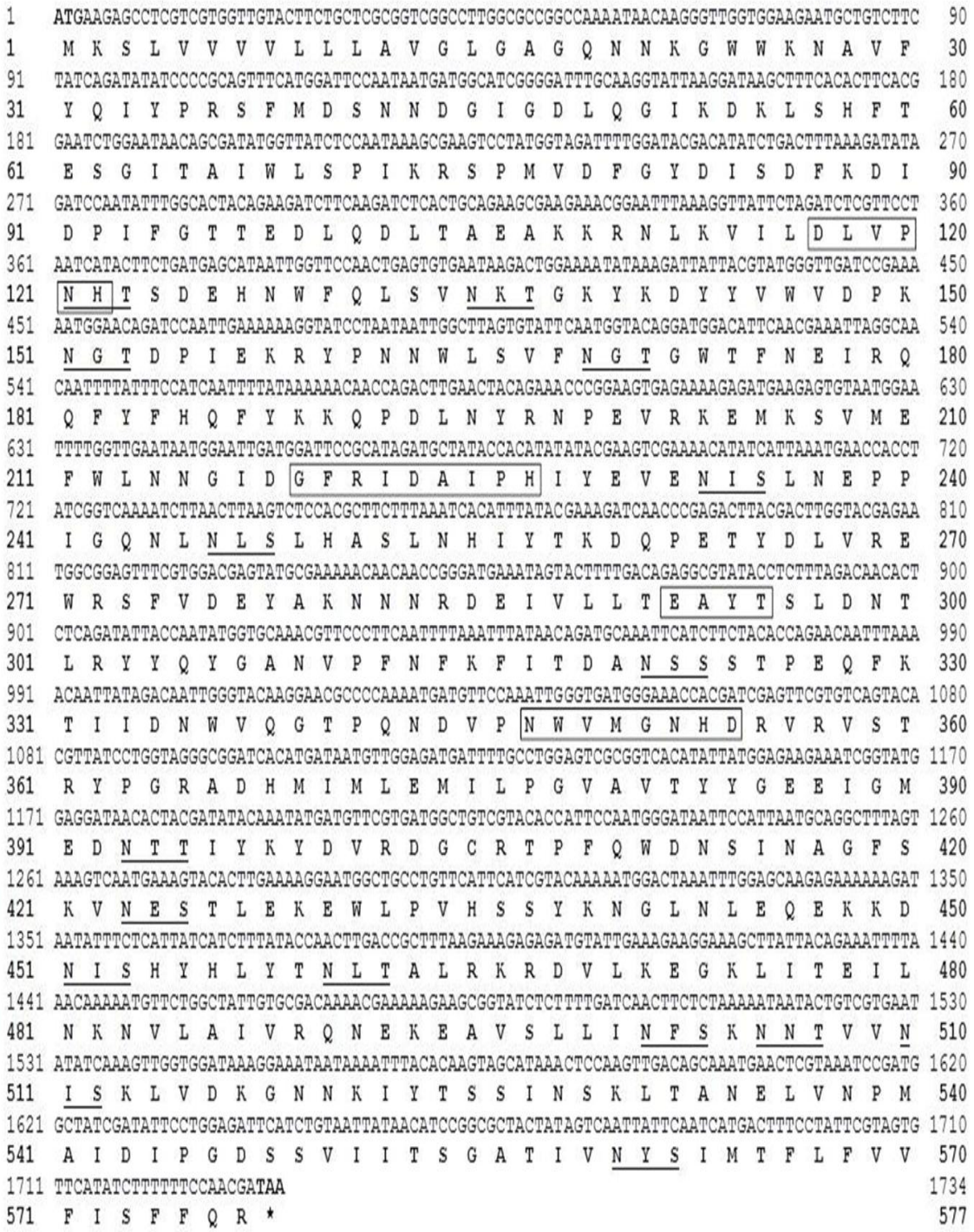

1 ATGAAGAGCCTCGTCGTGGTTGTACTTCTGCTCGCGGTCGGCCTTGGCGCCGGCCAAAATAACAAGGGTTGGTGGAAGAATGCTGTCTTC 90
1 M K S L V V V V L L L A V G L G A G Q N N K G W W K N A V F 30
91 TATCAGATATATCCCCGCAGTTTCATGGATTCCAATAATGATGGCATCGGGGATTTGCAAGGTATTAAGGATAAGCTTTCACACTTCACG 180
31 Y Q I Y P R S F M D S N N D G I G D L Q G I K D K L S H F T 60
181 GAATCTGGAATAACAGCGATATGGTTATCTCCAATAAAGCGAAGTCCTATGGTAGATTTTGGATACGACATATCTGACTTTAAAGATATA 270
61 E S G I T A I W L S P I K R S P M V D F G Y D I S D F K D I 90
271 GATCCAATATTTGGCACTACAGAAGATCTTCAAGATCTCACTGCAGAAGCGAAGAAACGGAATTTAAAGGTTATTCTAGATCTCGTTCCT 360
91 D P I F G T T E D L Q D L T A E A K K R N L K V I L D L V P 120
361 AATCATACTTCTGATGAGCATAATTGGTTCCAACTGAGTGTGAATAAGACTGGAAAATATAAAGATTATTACGTATGGGTTGATCCGAAA 450
121 N H T S D E H N W F Q L S V N K T G K Y K D Y Y V W V D P K 150
451 AATGGAACAGATCCAATTGAAAAAAGGTATCCTAATAATTGGCTTAGTGTATTCAATGGTACAGGATGGACATTCAACGAAATTAGGCAA 540
151 N G T D P I E K R Y P N N W L S V F N G T G W T F N E I R Q 180
541 CAATTTTATTTCCATCAATTTTATAAAAAACAACCAGACTTGAACTACAGAAACCCGGAAGTGAGAAAAGAGATGAAGAGTGTAATGGAA 630
181 Q F Y F H Q F Y K K Q P D L N Y R N P E V R K E M K S V M E 210
631 TTTTGGTTGAATAATGGAATTGATGGATTCCGCATAGATGCTATACCACATATATACGAAGTCGAAAACATATCATTAAATGAACCACCT 720
211 F W L N N G I D G F R I D A I P H I Y E V E N I S L N E P P 240
721 ATCGGTCAAAATCTTAACTTAAGTCTCCACGCTTCTTTAAATCACATTTATACGAAAGATCAACCCGAGACTTACGACTTGGTACGAGAA 810
241 I G Q N L N L S L H A S L N H I Y T K D Q P E T Y D L V R E 270
811 TGGCGGAGTTTCGTGGACGAGTATGCGAAAAACAACAACCGGGATGAAATAGTACTTTTGACAGAGGCGTATACCTCTTTAGACAACACT 900
271 W R S F V D E Y A K N N N R D E I V L L T E A Y T S L D N T 300
901 CTCAGATATTACCAATATGGTGCAAACGTTCCCTTCAATTTTAAATTTATAACAGATGCAAATTCATCTTCTACACCAGAACAATTTAAA 990
301 L R Y Y Q Y G A N V P F N F K F I T D A N S S S T P E Q F K 330
991 ACAATTATAGACAATTGGGTACAAGGAACGCCCCAAAATGATGTTCCAAATTGGGTGATGGGAAACCACGATCGAGTTCGTGTCAGTACA 1080
331 T I I D N W V Q G T P Q N D V P N W V M G N H D R V R V S T 360
1081 CGTTATCCTGGTAGGGCGGATCACATGATAATGTTGGAGATGATTTTGCCTGGAGTCGCGGTCACATATTATGGAGAAGAAATCGGTATG 1170
361 R Y P G R A D H M I M L E M I L P G V A V T Y Y G E E I G M 390
1171 GAGGATAACACTACGATATACAAATATGATGTTCGTGATGGCTGTCGTACACCATTCCAATGGGATAATTCCATTAATGCAGGCTTTAGT 1260
391 E D N T T I Y K Y D V R D G C R T P F Q W D N S I N A G F S 420
1261 AAAGTCAATGAAAGTACACTTGAAAAGGAATGGCTGCCTGTTCATTCATCGTACAAAAATGGACTAAATTTGGAGCAAGAGAAAAAAGAT 1350
421 K V N E S T L E K E W L P V H S S Y K N G L N L E Q E K K D 450
1351 AATATTTCTCATTATCATCTTTATACCAACTTGACCGCTTTAAGAAAGAGAGATGTATTGAAAGAAGGAAAGCTTATTACAGAAATTTTA 1440
451 N I S H Y H L Y T N L T A L R K R D V L K E G K L I T E I L 480
1441 AACAAAAATGTTCTGGCTATTGTGCGACAAAACGAAAAAGAAGCGGTATCTCTTTTGATCAACTTCTCTAAAAATAATACTGTCGTGAAT 1530
481 N K N V L A I V R Q N E K E A V S L L I N F S K N N T V V N 510
1531 ATATCAAAGTTGGTGGATAAAGGAAATAATAAAATTTACACAAGTAGCATAAACTCCAAGTTGACAGCAAATGAACTCGTAAATCCGATG 1620
511 I S K L V D K G N N K I Y T S S I N S K L T A N E L V N P M 540
1621 GCTATCGATATTCCTGGAGATTCATCTGTAATTATAACATCCGGCGCTACTATAGTCAATTATTCAATCATGACTTTCCTATTCGTAGTG 1710
541 A I D I P G D S S V I I T S G A T I V N Y S I M T F L F V V 570
1711 TTCATATCTTTTTTCCAACGATAA 1734
571 F I S F F Q R * 577

**Figure 5.1** The predicted full length cDNA of *AciHBGase I* and deduced amino acid sequence of *Aci*HBGase I. The predicted start (ATG) and stop (TAA) codons are shown in bold, giving a predicted open reading frame (ORF) of 1,734 bp encoding for a deduced 577 residue amino acid sequence. The underlined amino acid sequences indicate the putative motifs (N-Xa.a.-S/T) for *N*-glycosylation. The catalytic regions conserved in α-amylase family enzymes are boxed. Note, however, that the sequence of the first 5' 15 nucleotides and five amino acids including the assumed ATG/M initiation codon were fixed by the primer, and so (assuming the ATG initiation to be correct) may vary from the AAGAGCCTCGT and KSLV sequences shown in the actual *AciHBGase I* and *Aci*HBGase I sequences.

Fifteen putative N-glycosylation motifs (N-Xa.a-S/T) were located in the deduced amino acid sequence of *Aci*HBGase I (Fig. 5.1), indicating that this enzyme might be subject to post-translational N-glycosylation at asparagine residues, but which if any of these potential sites is actually glycosylated in the bee and the function of such are unknown. However, one sequon (Asn121-His122-Thr123) was quite interesting because His122 was believed to be essential for substrate recognition in the α-amylase family enzyme (Wongchawalit *et al*., 2006). In addition, the four highly conserved sequence regions of the α-amylase GH13 family were present as DLVPNH (Asp117-His122), GFRIDAIPH (Gly219-His227), EAYT (Glu292-Thr295) and NWV<u>M</u>GNHD (Asn347-Asp354), the fourth region varying at one amino acid (underlined) from that found in *A. cerana japonica* (NWV<u>K</u>GNHD). In the sequence, the catalytic residues were likely to be Asp223, Glu292 and Asp354.

The deduced amino acid sequence (Fig. 5.1) from the ORF was then used to search for similar homologous sequences in the NCBI GenBank data base using the megaBLASTp and tBLASTn algorithms (http://www.ncbi.nlm.nih.gov). The deduced amino acid sequence of *Aci*HBGase I had a high similarity to that in related organisms, with 99, 81 and 80% similarity to those from *A. cerana japonica, A. florea* and *A. mellifera*, respectively (Fig. 5.2), as expected. In contrast, the similarity of the amino acid sequences between *A. cerana indica* and *Bombus terrestris* was only 61%. As marked in Figure 5.2, α-amylase family members shared a common structure composed of three domains. The first is domain N, which is the catalytic domain formed by a (β/α)8-barrel. The second is domain S, which is a long loop located within domain N of β-strand 3 and α-helix 3. The last is domain C, which is antiparallel β-sheets and is located next to the last domain N (Nα8). Overall, according to the projected secondary structure, most of the structural elements were well conserved in HBGase I.

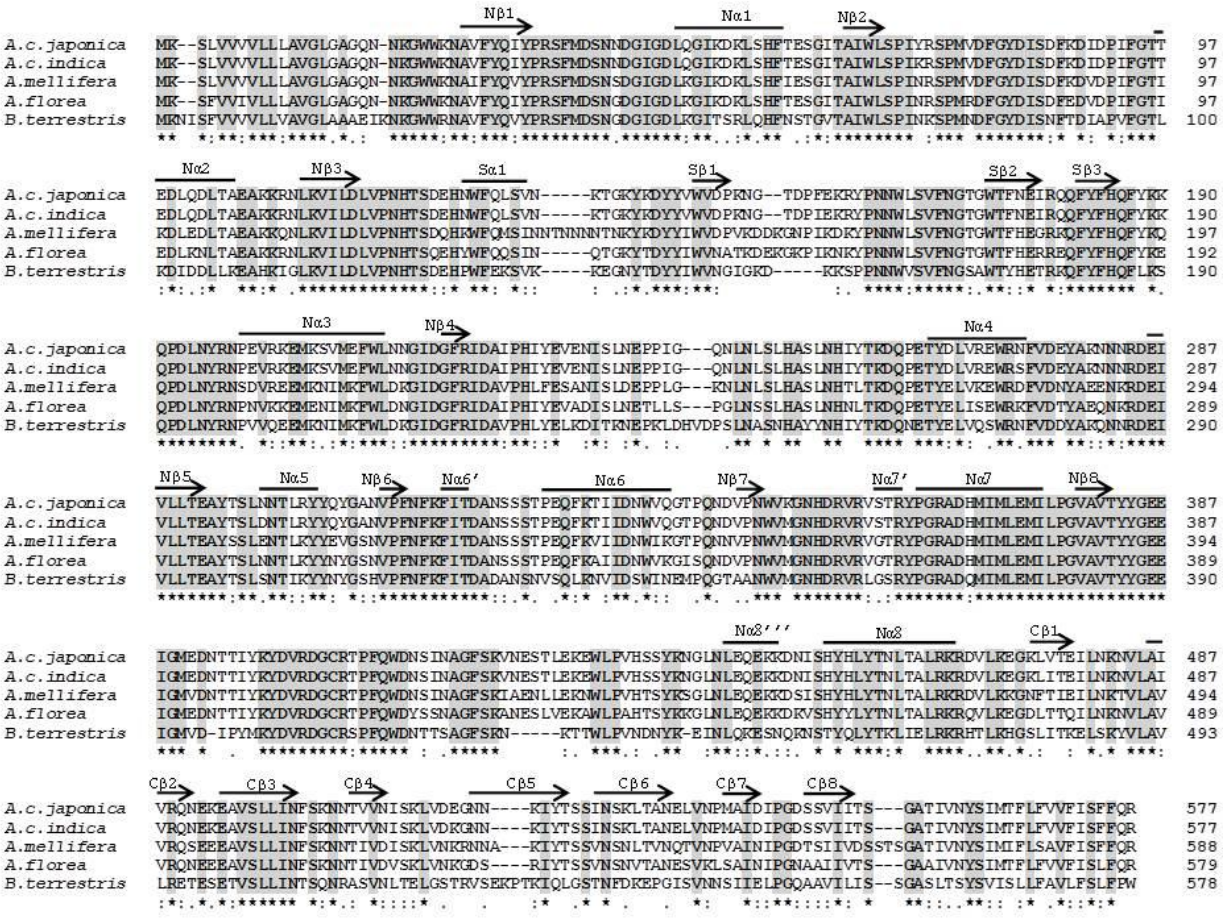


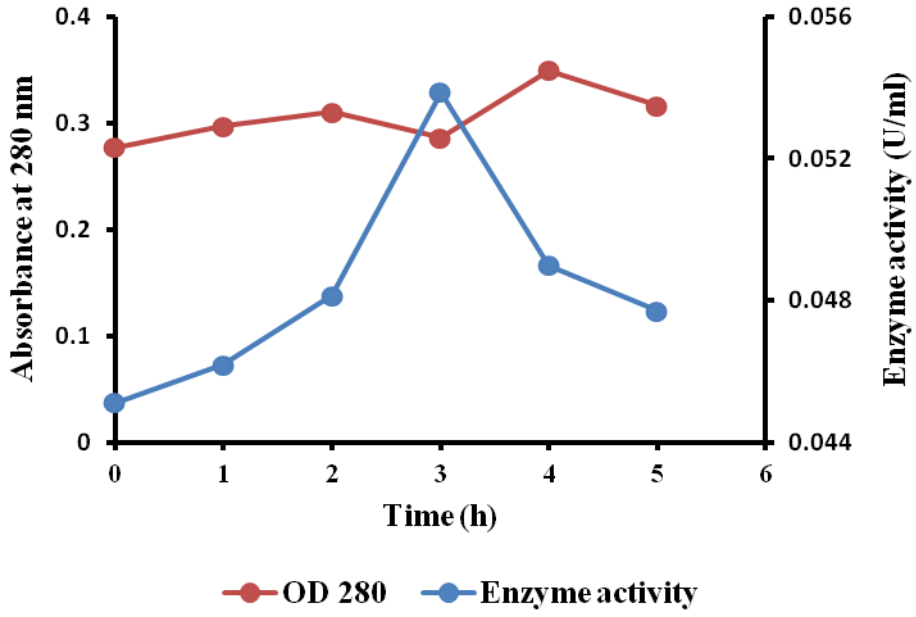

**Figure 5.2** Multiple alignment of homologous HBGase I amino acid sequences from closely related species. Sequences shown are from *Apis cerana indica* (*A.c. indica*; this study), *A. cerana japonica* (*A.c. japonica*, accession # BAF44218.1), *A. mellifera* (*A. mellifera*, accession # NP_001035326.1), predicted maltase 1-like of *A. florea* (*A. florea*, accession # XP_003692294.1) and predicted maltase 1-like of *Bombus terrestris* (*B. terrestris*, accession # XP_003395914.1). The similarity across the aligned sequences is shown as identical (*), conserved (:) and semi-conserved (.).

**Expression of r(His)$_6$*Aci*HBGase I**

The full length cDNA of *AciHBGase I* (1,734 bp) was successfully cloned into the pEcoli-Nterm-6xHN expression vector and transformed into *E. coli* Rosetta (DE3) cells. Five transformants (N1B, N2B, C1D, NN3 and NN6) were randomly selected and the glucosidase activity of their cell lysates (0.049, 0.054, 0.053, 0.037 and 0.037 U/ mL cell lysate, respectively), in terms of the ability to hydrolyze PNPG, was assayed as outlined in the methods section. In addition, a transformant containing only vector was randomly selected and the glucosidase activity of its cell lysate was found to be significantly (just over 140-fold) lower at 0.00026 U/ mL cell lysate, suggesting the glucosidase activity was essentially all derived from the recombinant plasmid and not the host cell genome.

The N2B and C1D transformants presented the highest glucosidase enzyme activity at 0.054 and 0.053 U/ mL cell lysate (2.16 and 2.12 U/L culture medium), respectively. Accordingly, the N2B transformant was used to determine the optimum concentration of IPTG and induction time, in terms of the highest production level of glucosidase activity and so assumed r(His)$_6$*Aci*HBGase I expression level. From the assayed conditions, induction with 1 mM IPTG for 3 h yielded the highest glucosidase activity (~0.062 U/ mL cell lysate; 2.48 U/ mL culture medium) and so assumed induction of r(His)$_6$*Aci*HBGase I expression (Fig. 5.3).

**A**


0.4
0.3
0.2
0.1
0
Absorbance at 280 nm
0.056
0.052
0.048
0.044
Enzyme activity (U/ml)
0
1
2
3
4
5
6
Time (h)
OD 280
Enzyme activity

**B**

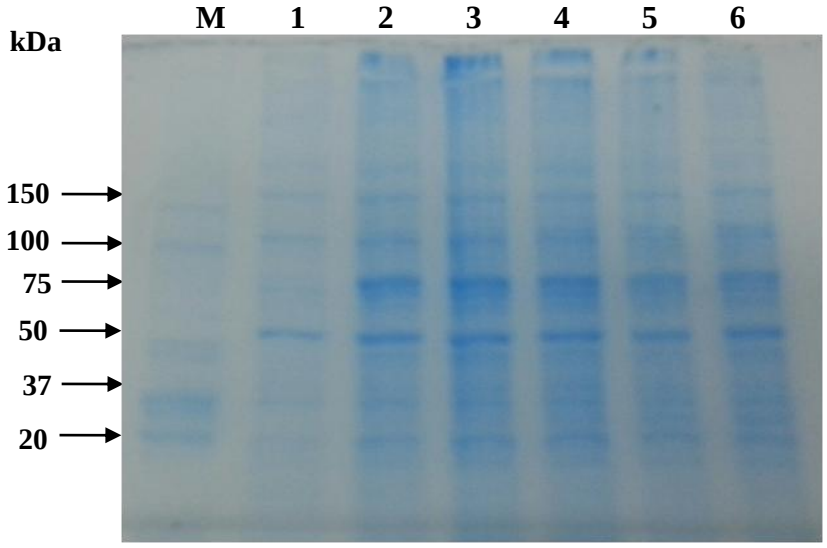

M
1
2
3
4
5
6
kDa
150
100
75
50
37
20

**Figure 5.3** (A) Expression of glucosidase activity (assumed r(His)$_6$*Aci*HBGase I levels) and total protein levels in transformed Rosetta(DE3) cells after being induced with 1 mM IPTG for various incubation times (0-5 h). Cultures were assayed for protein concentration by the absorbance of 280 nm and glucosidase activity (U/ mL cell lysate) by the hydrolysis of PNPG. (B) The protein pattern (5 μg total protein per lane) after reducing SDS-PAGE (8% (w/v) acrylamide resolving gel) and Coomassie blue staining. Each lane contained 5 μg protein. Lane M: protein standard markers, lane 1: non-induced culture, lanes 2-6 cultures after being induced for 1, 2, 3, 4 and 5 h, respectively.

**Partial purification of the r*(His)$_6$Aci*HBGase I enzyme**

The N2B transformant, selected due to its higher apparent glucosidase activity, was cultured at the larger scale of 1.25 L, yielding a significantly (1.6-fold) higher glucosidase activity of 0.097 U/ mL cell lysate (3.87 U/L culture media) (Table 5.2). The cell lysate (50 mL) was concentrated five-fold by centrifugal filtration through a 10 MWCO membrane and purified using a HisTALON$^{TM}$ gravity column (Clontech). Of the 20 collected 1-mL fractions, two separate peaks of protein were observed (Fig. 5.4A); one from unbound fractions # 2 - 4 (peak I) and the other from eluted fraction # 17 (peak II). Surprisingly, the unbound peak showed the highest glucosidase activity (Fig. 5.4A) with a specific activity of 0.0078 U/mg, which was just over a 20-fold and 35-fold higher total and specific activity, respectively, than that for the bound fraction after elution (fraction # 17) (Table 5.2). The r(His)$_6$-*Aci*HBGase I would have been expected to bind to the $Co^{2+}$-resin based HisTALON column and so to be in the bound fractions and eluted with the $Ni^{2+}$ based elution buffer, and not in the unbound fraction. It remains plausible that the enzyme structure is refolded to mask the six N-terminal histidine residues, or else that they have been truncated.

**Table 5.2** Summary of the partial purification stages of r*Aci*$(His)_6$HBGase I from a 1.2 L culture of transformed *E. coli* Rosetta(DE3) cells.

| Step | Total protein (mg) | Total activity (U) | Specific activity (mU/mg)[a] | Yield (%) | Enrichment (fold) |
|---|---|---|---|---|---|
| Cell lysate (50 mL) | 1,027.5 | 4.887 | 4.8 | 100% | 1.0 |
| Viva spin 20 concentration (5 mL) | 118.25 | 0.622 | 5.3 | 12.7% | 1.10 |
| HisTALON gravity column | | | | | |
| - Unbound fraction (4 mL) | 24.0 | 0.187 | 7.8 | 3.83% | 1.63 |
| - Bound fraction (2 mL) | 4.1 | 0.009 | 2.2 | 0.48% | 0.046 |

**[a] mU = $10^{-3}$ U**

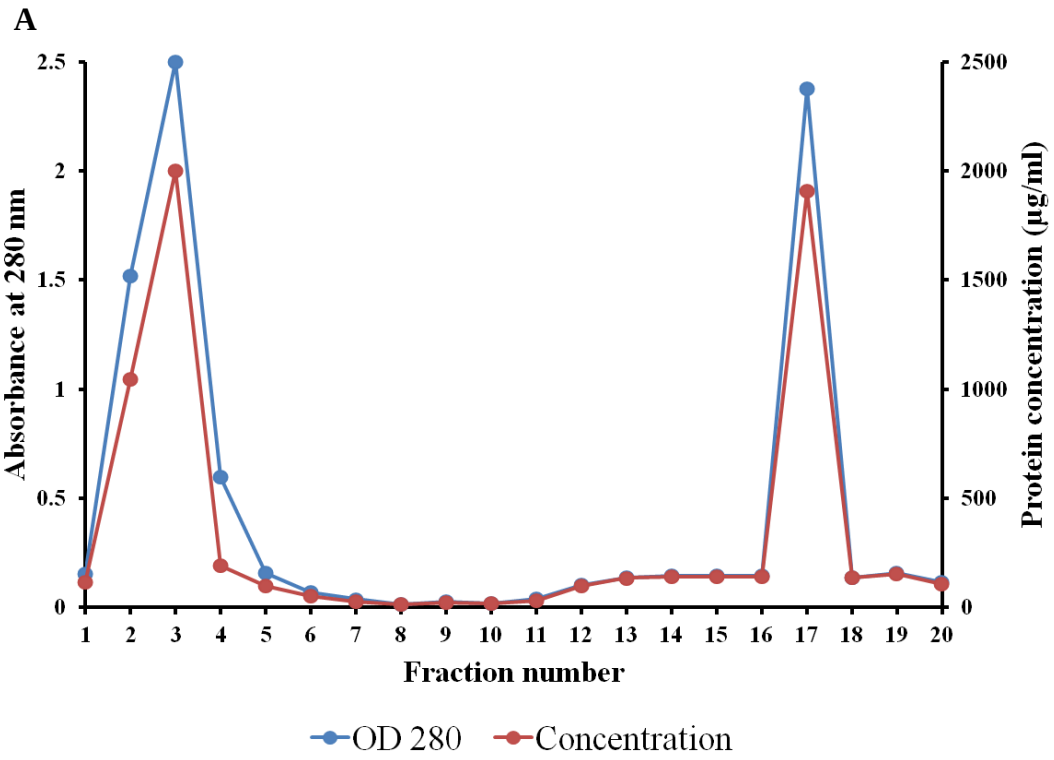

A
Absorbance at 280 nm
Protein concentration (µg/ml)
Fraction number
OD 280
Concentration
B
kDa
M
1
2
3
4
5
6
7
8
9
250
150
100
75
50
37
25
15

**Figure 5.4** (**A**) Partial purification of the r*Aci*$(His)_6$HBGase I by HisTALON™ gravity column chromatography. Protein concentration (μg/ mL), as calculated from the absorbance at 280 nm. (**B**) Protein profile (5 μg protein in each lane) after resolution by reducing SDS-PAGE (8% (w/v) acrylamide resolving gel). Lane M: protein MW markers; lane 1: non-induced culture; lane 2: total protein (25 mL culture); lane 3: total protein (1.25 L culture); lane 4: total soluble protein of cell lysate; lane 5: total insoluble protein of cell lysate; lanes 6 & 7: unbound fractions # 2 and 3; lane 8: fraction # 11; lane 9: elution fraction # 17.

The SDS-PAGE resolved protein profile pattern revealed that both glucosidase active fractions (unbound and unbound; peaks I and II, respectively) were heterogeneous with multiple protein bands (Fig. 5.4B). The bound fraction (fraction 17) was comprised of far fewer protein bands than the unbound fractions (fractions 2, 3 and 11) or unfractionated sample prior to HisTALON chromatography, but nevertheless was not enriched to homogeneity with several principal bands at 20-25 kDa as well as 45-50 and 75-80 kDa.

Moreover, the already mentioned glucosidase activity that was detected in the unbound fraction # 3 was supported by the presence of a single resolved band after glucosidase activity staining (Fig. 5.5). However, this protein had an apparent MW of 84 kDa on the reducing SDS-PAGE based zymograph, which is significantly higher than the predicted 67.4 kDa from the deduced amino acid sequence (plus six histidine residues),

which may then be due to bacterial post-translational N-glycosylation of the r$(His)_6$-*Aci*HBGase I enzyme.

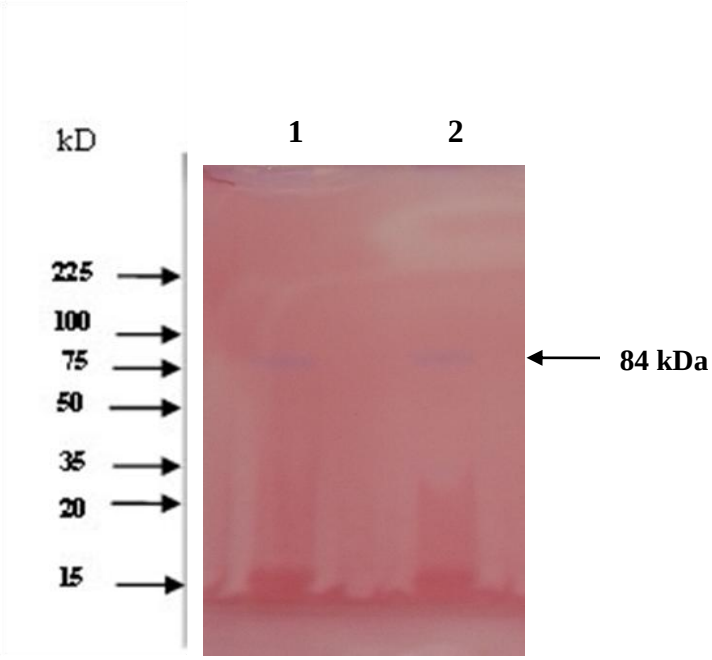



**Figure 5.5** Glucosidase activity stain (Zymograph) of the r*Aci*$(His)_6$HBGase I from the unbound fraction # 3 following resolution by reducing SDS-PAGE (8% (w/v) acrylamide resolving gel) and renaturation. Lanes 1 and 2 are the cell lysate prior to HisTALON chromatography and the unbound fraction # 3, respectively.

The results of the partial purification of r(His)$_6$*Aci*HBGase are summarized in Table 3. Based on the much higher yield and enrichment obtained, the unbound fraction # 3 was used for characterization of the optimal conditions for glucosidase activity and the enzyme kinetics.

**Characterization of the partially purified r(His)$_6$*Aci*HBGase I enzyme**

*Optimal pH and temperature*

The effects of pH and temperature on the glucosidase activity of the partially purified r(His)$_6$*Aci*HBGase I enzyme were measured using PNPG hydrolysis. The enzyme preparation showed a clear thermal optimum at 40 $^0$C with > 50% residual activity in the range of 35-50$^o$C (Fig. 5.6A) and was stable to a 15 min incubation of up to 45 $^0$C, declining slightly to ~90% residual activity at 60$^o$C and then markedly with higher temperatures to essentially no activity after 15 minutes at 85$^o$C (Fig. 5.6B). In addition, the enzyme showed an optimum pH of 3.5 declining markedly at higher pH values to < 50% and < 5% residual activity at pH 7 and $\geq$ 10, respectively (Fig. 5.6C), and was stable in the pH range of 3.5-5.5 but declined markedly at higher pH values (Fig. 5.6D). Thus, it was neither active nor tolerant of alkaline conditions but rather was mildly acidophilic.

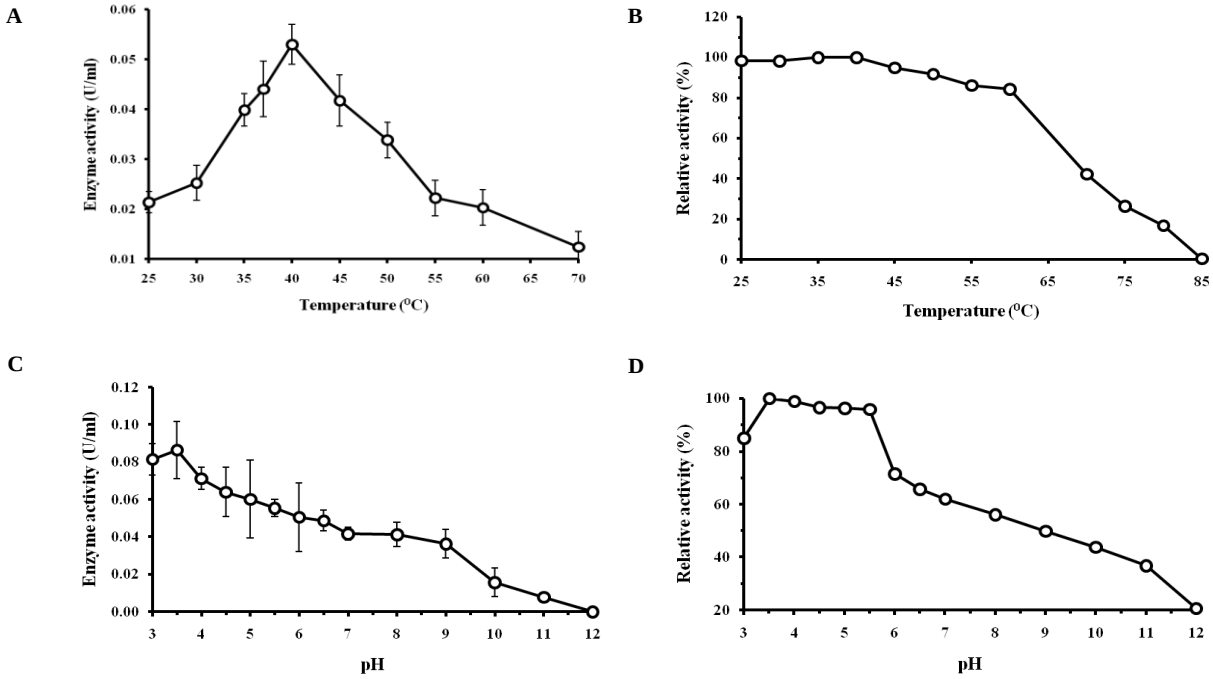



**Figure 5.6** The optimum (**A**) temperature and (**C**) pH conditions for glucosidase activity of the enriched r*Aci*$(His)_6$HBGase I, and its (**B**) thermal and (**D**) pH stability. Data are shown as the mean $\pm$ 1 SD, and are derived from three independent repeats.

*Substrate specificity and kinetics*

The substrate specificity of the partially purified r(His)$_6$*Aci*HBGase I was examined using six different carbohydrates plus PNPG as substrates at 5 and 10 mM. Of these, sucrose was hydrolyzed to the greatest extent at both 5 and 10 mM ($4.01 \times 10^{-2}$ and $12.35 \times 10^{-2}$ μM of liberated glucose, respectively), whilst isomaltose followed by soluble starch were the least utilized substrates at > 10-fold less than that for sucrose (Fig. 5.7). Because isomaltose was hardly hydrolyzed at all, it was omitted from the kinetics assay.

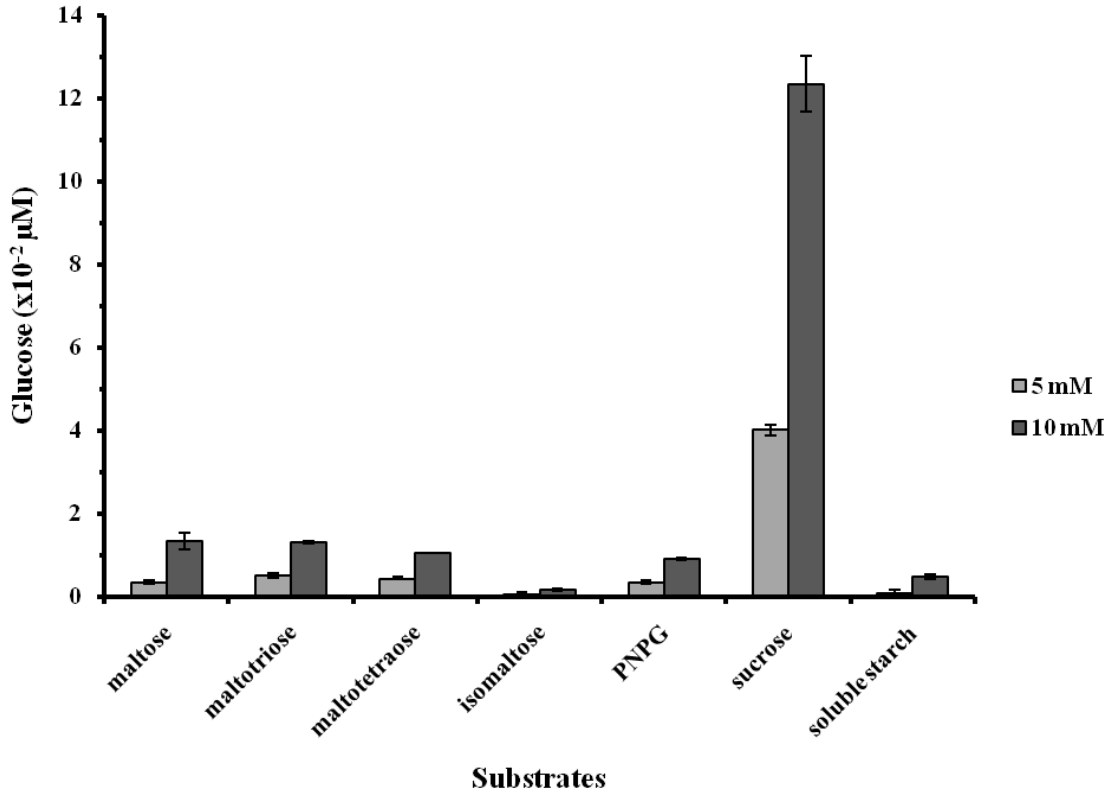



**Figure 5.7** Substrate specificity of the partially purified r(His)$_6$*Aci*HBGase I enzyme on maltose, maltotriose, maltotetraose, isomaltose, sucrose, PNPG and soluble starch as substrates at either 5 or 10 mM. Data are shown as the mean $\pm$ 1 SD, and are derived from 3 independent repeats.

Except for maltose as the substrate, the plots of the substrate concentration (*s*) versus the velocity (*v*) were consistent with hyperbolic Michaelis-Menten shaped curves, although not all substrates had a plateau at the tested range of concentrations (Fig. 5.8B-F). In contrast, although the plot for maltose appeared to be different in that no significant decline in the velocity with increasing substrate concentrations was noted (Fig. 5.8A), this may just reflect that much larger maltose concentrations are required.

The $K_m$, $k_0$ and $V_{max}$ values for the hydrolysis of those substrates were derived from Eadie-Hofstee plots, which give equal weight to the data points, and are reported in Table 5.3. Although the $K_m$ values for maltose (9.41 mM) and sucrose (10.82 mM) were very close, indicating a similar binding affinity to the enzyme, the other kinetic values were very different. For example, the $K_{cat}$, $K_{cat}/K_m$ and $V_{max}$ values for sucrose were 30-, 35.7- and 40-fold higher, respectively, than those for maltose, suggesting that more sucrose could be hydrolyzed (into glucose and fructose) per unit of time than maltose, perhaps because the enzyme encounters sucrose faster. Indeed, the $K_{cat}/K_m$ value for sucrose was the highest (25- to 35.7-fold) of all the substrates evaluated.

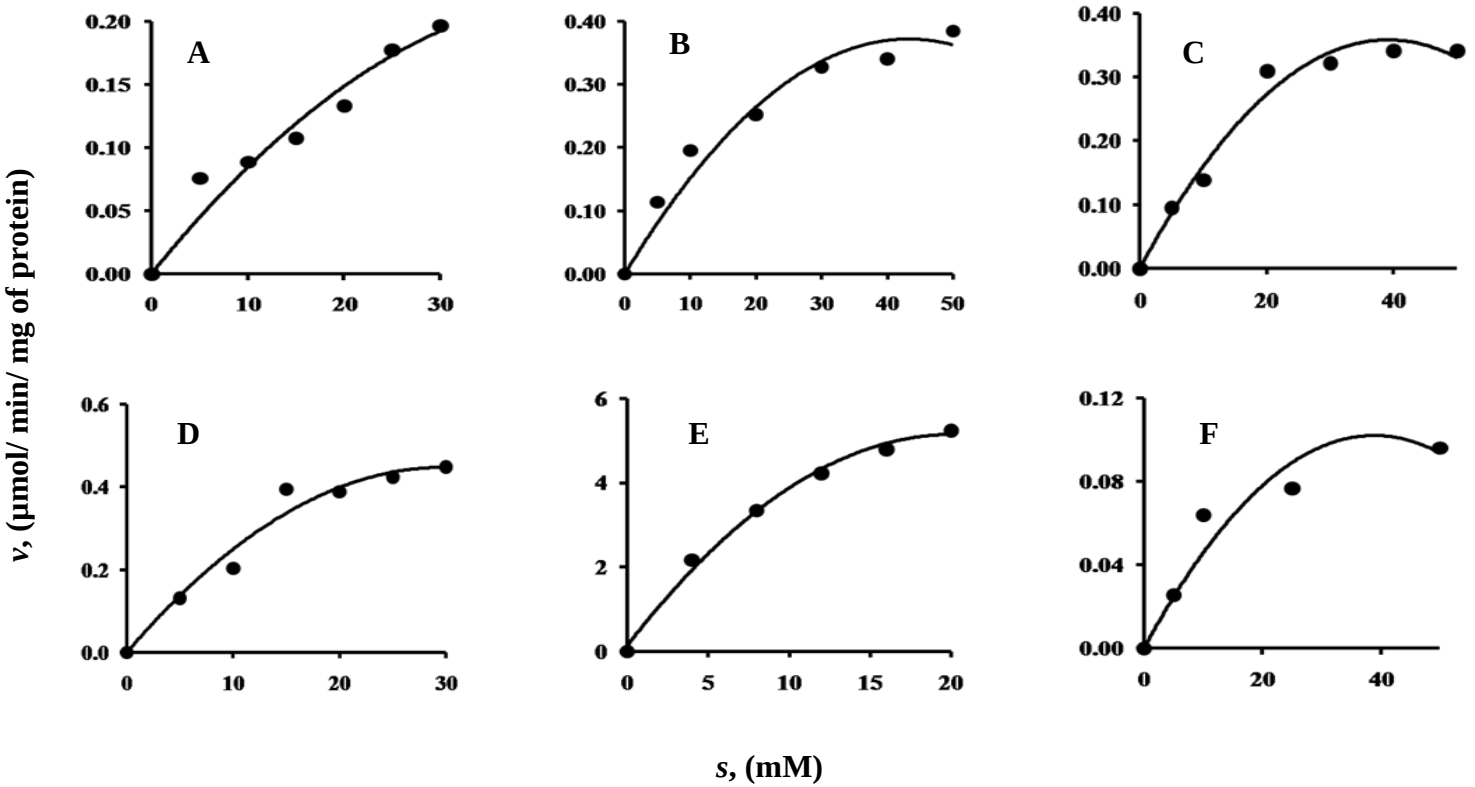



**Figure 5.8** Substrate (*s*) versus enzyme velocity (*v*) plots for the hydrolysis reaction with the partially purified r(His)$_6$*Aci*HBGase I preparation for (**A**) maltose (5-30 mM), (**B**) maltotriose (5-50 mM), (**C**) maltotetraose (5-50 mM), (**D**) PNPG (5-30 mM), (**E**) sucrose (4-20 mM), and (**F**) soluble starch (5-50 mM) as substrates. For PNPG, each molecule of *p*-nitrophenol released was equated to one glucose molecule.

**Table 5.3** Rate parameters[a] for the hydrolysis of various substrates by the partially purified r(His)$_6$*Aci*HBGase I preparation.

| **Substrate** | $K_m$[b] **(mM)** | $V_{max}$[c] **(μmol/ min/ mg of protein)** | $K_{cat}$ **($s^{-1}$)** | $K_{cat}/K_m$ **($mM^{-1}. s^{-1}$)** |
|---|---|---|---|---|
| Maltose | 9.41 ± 2.93 | 0.21 ± 0.01 | 0.28 ± 0.02 | 0.029 |
| Maltotriose | 16.70 ± 2.91 | 0.50 ± 0.03 | 0.69 ± 0.04 | 0.041 |
| Maltotetraose | 25.75 ± 3.34 | 0.57 ± 0.05 | 0.79 ± 0.08 | 0.031 |
| PNPG | 36.01 ± 4.35 | 1.07 ± 0.05 | 1.49 ± 0.07 | 0.041 |
| Sucrose | 10.82 ± 0.39 | 8.01 ± 0.67 | 11.21 ± 0.67 | 1.036 |
| Soluble starch | 23.41 ± 1.16 | 0.16 ± 0.01 | 0.22 ± 0.01 | 0.009 |

[a]Data are shown as the mean ± 1 SD and are derived from three independent repeats.

[b]The $K_m$ value is the substrate concentration giving one half of intrinsic $V_{max}$.

[c]Obtained from the calculated value as the glucose liberated from the reducing end residue of substrates. For PNPG, one mole of released p-nitrophenol is equated to one mole of glucose.

**DISCUSSION**

Whole foraging *A. cerana indica* bees were used as the source of total mRNA from which to isolate the *AciHBGase I* cDNA. These are the cast responsible for collection and processing of nectar (mainly sucrose into glucose and fructose) into honey and so are likely to be a suitable source for isolation of the *AciHBGase I* mRNA and to encode for a HBGase with a strong sucrose hydrolysis activity, even though we did not specifically use likely rich tissues (hypopharyngeal gland and honey stomach).

The *Am*HBGases and *Acj*HBGases have been reported to be glycoproteins (Wongchawalit *et al.*, 2006; Nishimoto *et al.*, 2007), raising the probability that the *Aci*HBGases, including the *Aci*HBGase I of this study, are glycoproteins as well. Consistent with this is that the deduced amino acid sequence of *Aci*HBGase I has 15 potential *N*-glycosylation sites (Fig. 5.1), and the recombinant enzyme appeared to be larger than that predicted from the primary amino acid sequence. Glycosylation, and especially asparagine (Asn or N)-linked oligosaccharides, is known to influence protein activities and to play important roles in numerous biological events (Freeze and Aebi, 2005; Varki, 1993). Considering the conserved *N*-glycosylation motif (Asn121-His122-Thr123) in the α-amylase family, Wongchawalit et al. (2006) reported that no sugar chain was linked to Asn121. Thus, it was highly possible that the active center of the catalytic reaction was charged and that the biochemical mechanism with the additional *N*-linked sugar chain would not be as efficient. However, whether the native protein in the bee is glycosylated, and on which sites, with what residues, and the function of such glycosylation upon its stability, activity and tissue location all remain to be evaluated.

Ideally, in the future the native form of *Aci*HBGase I should be enriched and then studied, but given the difficulty in obtaining sufficient amounts of the native enzyme for manipulative (such as deglycosylation) biochemical and enzyme kinetic studies, then alternatively the expression of this recombinant enzyme and genetically modified derivatives in the methyltrophic yeast *Pichia pastoris*, a robust expression system for eukaryotic glycoproteins (Daly and Hearn, 2005), may prove of worth in biochemical evaluations. However, N-glycosylation heterogeneity and heterologous expression of the same protein among hosts are different (Werner *et al*., 2007) and so such results without reference to the native form are equivocal.

In addition, in contrast to the pPICZαA expression vector in *P. pastoris* where the recombinant protein is secreted, in the pEcoli-Nterm-6xHN expression vector in *E. coli* Rosetta(DE3) cells the recombinant protein is found in the cell lysate along with all the host proteins making the subsequent enrichment more difficult (Cregg *et al*., 2009). In this study, the majority (95.4%) of glucosidase activity (assumed r(His)$_6$*Aci*HBGase I activity), as defined by the PNPG hydrolysis assay, unexpectedly did not absorb to the $Co^{2+}$-resin based HisTALON column, but was found in the unbound fraction. It is possible that the six N-terminal histidine residues were conformationally prevented from interaction with the $Co^{2+}$-resin, but this awaits clarification. Regardless this step was useful for the removal of the resin-adsorbed r*Aci*HBGase I (4.6%) and the other proteins present. The unbound fraction, which had a significantly higher specific and net glucosidase activity and enrichment level, was used as the partially purified enzyme fraction for further study. Following denaturing SDS-PAGE resolution, a single active

band in the zymograph (enzyme activity stained) was observed from the partially purified preparation (unbound fraction) (Figs. 5.4 and 5.5), with an apparent enzymatically active monomeric protein MW of ~84 kDa. This is significantly higher than the predicted 67.4 kDa based upon the deduced primary amino acid sequence plus the six histidine residues, and so may reflect bacterial post-translational N-glycosylation. Regardless, this apparent monomeric subunit MW of 84 kDa is very close to that reported for the related monomeric r*Acj*HBGase I (82 kDa) and the less related HBGase I of *Saccharomyces cerevisiae* (84 kDa) (Dhanawansa *et al*., 2002; Wongchawalit *et al*., 2006). The actual likely monomeric/multimeric nature of *Aci*HBGase I is unknown as the non-denatured native form was not studied, but the closely related *Acj*HBGase I is reported to be monomeric (Wongchawalit *et al*., 2006). However, across diverse taxa HBGases are not always monomeric. For example, the homologs of this enzyme are tetrameric in mammals (Shailubhai *et al*., 1991).

The recovery of most of the glucosidase activity in the unbound fraction was unexpected as the r$(His)_6$*Aci*HBGase I would be expected to bind to the $Co^{2+}$-resin and so be found in the $Ni^{2+}$ eluted fractions and not the unbound ones. We have not performed western blotting with anti-His antibodies on denatured samples, or N-terminal sequencing, to confirm the presence of the N-terminal six-histidine residue tag, so its cleavage could be one explanation whilst the enrichment of a bacterial HBGase homolog is a less likely explanation, especially given the very low glucosidase activity on the untransformed bacterial cells. Alternatively, as mentioned above, the r$(His)_6$-*Aci*HBgase I may have adopted a conformation that masked or prevented the histidine residues from

interacting with the $Co^{2+}$-resin and so the protein eluted in the unbound fraction. If so, how much the conformational change induced by either the bacterial N-glycosylation or the addition of the N-terminal $(His)_6$ tag itself also may have affected the substrate specificity, pH and thermal tolerance and optima and enzyme kinetics remains to be evaluated. However, it was previously reported in the purification of r*Acj*HBGase I that the majority of the recombinant enzyme (85%) unexpectedly did not absorb to the cationic exchange column (Toyopearl CM-650M) in 50 mM sodium acetate buffer (pH 4.7), suggesting the adopted confirmation had masked the electrostatic interactions between the protein and resin (Wongchawalit *et al*., 2006).

Although the primary amino acid sequence across the bee (Apoidea) HBGase I sequences appear to have been largely conserved during fairly recent evolution (Fig. 5.2), nevertheless some important parameters of the enzymes are different between closely related as well as more distantly related sources. For example, the closely related r*Acj*HBGase I and r*Aci*HBGase I homologs function optimally under relatively acidic conditions but with a different optimal pH (pH 5.0 and 3.5, respectively), while the more distantly related HBGase I homologs from yeast, mammals and plants are reported to function optimally at the less acidic to almost neutral conditions of pH 6.5-6.8 (Bause *et al*., 1986; Romaniuk and Vijay, 1997; Zeng and Elbein, 1998). In addition, HBGase I homologs from mammalian and plant sources were also different in sensitivity to *N*-ethylmaleimide and diethyl pyrocarbonate (Zeng and Elbein, 1998).

In terms of the six natural carbohydrate substrates tested (plus PNPG), the r*Aci*HBGase I showed a marked preference, although not absolute specificity, for sucrose

(1,2-*cis* glucosidic linkage), potentially in keeping with a role for the digestion of plant nectar (principally sucrose to fructose and glucose) to form honey. Alternatively, r*Aci*HBGase I could hydrolyze heteroside substrates (sucrose) more rapidly than holoside substrates (maltooligosaccharides). The obtained data coincided to the substrate specificity of r*Acj*HBGase I which was also sucrose (Wongchawalit *et al*., 2006). In contrast, the r$(His)_6$AciHBGase I did not favour isomaltose, which is the opposite to the native HBGase I from soil bacteria, *Bacillus licheniformis* (Nimpaiboon *et al*., 2011). However, HBGase I from the three above sources could all function well at the same temperature range of 40-45$^{o}$C.

Overall, the HBGase I homologs among three different *Apis* spp. (and within two subspecies of one of these species) and the three HBGase isoforms (I, II and III) within one *Apis* sp. (*A. mellifera*) differ in their apparent molecular size, pH stability, temperature stability and substrate specificity. This diversity in their enzyme properties between just a few related organisms (short evolutionary time scale), and so a potentially greater diversity in the animal kingdom alone (greater evolutionary time scale and diversity of selection), bodes well for the ability to find (or genetically engineer) enzymes with characteristics to suit specific but different applications. In addition, carbohydrate structures formed from different glycosidases and glycosyltransferases result in a wide variety of complex *N*-glycans and so functional diversity.

Furthermore, *A. mellifera* was revealed to contain the isoenzyme-system of *Am*HBGase I, II and III (Kubo *et al*., 1996; Ohashi *et al*., 1996; Kubota *et al*., 2004), and so it would be reasonable to expect a homologous isoenzyme-system of *Aci*HBGases given the close evolutionary and biological relatedness of these two honey bee species. An enhanced knowledge of these HBGases may reveal the relative importance to the bee and other related organisms.

# CHAPTER VI

# CONCLUSIONS

α-Glucosidase (HBGase; EC 3.2.1.20) can hydrolyze the α-glucosidic linkage of non reducing end of substrates such as carbohydrate polymers and other glucosides including phenyl α-glucoside to liberate α-glucose (Takewaki *et al*., 1980). The enzymes are classified into 3 isoforms of HBGase I, II and III which are based on their substrate specificity (Giannesi *et al*., 2006; Nishimoto *et al*., 2007). HBGases are useful in biotechnological, clinical, and microbiological applications. These enzymes come from various sources of microorganisms, plants, mammals and insects (Nakai *et al*., 2007; Nimpiboon *et al*., 2001; Nishimoto *et al*., 2007; Okuyama *et al*., 2001). Different isoforms of enzymes can display marked differences in their kinetics especially in their substrate specificities, in tissue or developmentally dependent control of their expression levels or response to stimuli (Harzer *et al*., 2012).

In this research, it was focused on finding a new source of HBGases in the form of recombinant *Aci*HBGases. To obtain the *Aci*HBGase I and II transcriptional expression pattern, *A. cerana indica* at different developmental stages (egg, larvae, pupae and forager bee) were collected. RNA was extracted and reversed to be cDNA by RT-PCR by using primers designed from *Am*HBGase I and II. The amount of amplified cDNA was quantitated and analysed. After that, the full length cDNA of *Aci*HBGase I, II and III were synthesized by RT-PCR by using primers designed from the close nucleotide sequences of *A. mellifera* and *A. cerana japonica*. *Aci*HBGase I was cloned into pEcoli while *Aci*HBGase II and III were cloned into pPICZαA as expression vectors. To enrich the recombinant enzymes, *P. pastoris* GS115 was

selected as a host for r*Aci*HBGase II and III while *E. coli* Rosetta (DE3) was a host for r*Aci*HBGase I. Then, all three r*Aci*HBGases were purified by one-step chromatography and characterized.

### 6.1 *Aci*HBGase transcriptional expression pattern

Previously, *Aci*HBGase III transcriptional expression pattern was already reported (Chanchao *et al*., 2006). Like *Aci*HBGase III, *Aci*HBGase I was the highest expressed in forager bee. While *Aci*HBGase I was not expressed at all in eggs, larvae and pupae, the expression of *Aci*HBGase III trended to be gradually increased in eggs, larvae and pupae. For *Aci*HBGase II, the highest expression level was found in larvae and pupae but less expression level was found in eggs. Neither was in forager bee.

It was reported that only *Am*HBGase III was likely to play a role in honey synthesis through the hydrolysis of sucrose from nectar in the honey crop (Kubota *et al*., 2004). Thus, the function of this isoform coincided to the expression of the gene since the highest expression of this gene was found to be in forager bee but not *Aci*HBGase II. Although the expression of *Aci*HBGase I was found only in forager bee but this isoform was not found in honey (Kubota *et al*., 2004). Thus, the function and role of this isoform will be further found out.

It is highly possible that *Aci*HBGase I and II involved in the development of this honeybee. Since 1999, Hercovics (1999) 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. Even though, we do not know the exact function of

*Aci*HBGase I and II in *A. cerana indica* development, but it is suggested if HBGase is defective, it can lead to the negative effect on cellular functions.

### 6.2 The full length cDNA of *Aci*HBGase I, II and III

In this research, forager bees and pupae were selected to obtain the full length cDNA of *Aci*HBGase I, III and II, respectively due to the highest expression of the gene in the mentioned development stage. Primers to obtained the full length cDNA of *Aci*HBGase I and II were designed from conserved regions of *A. mellifera* and *A. cerana japonica*. The expected RT-PCR products were obtained on 1.2% agarose gel electrophoresis and then direct sequencing. Clustal X and Sequencher 4.1.4 program were used to obtain the full length cDNA of *Aci*HBGase I and II.

In the previous research (Chanchao *et al*., 2008), it was found that the full length cDNA of *Aci*HBGase III was 1,704 bp with the predicted polypeptide of 567 amino acids (GenBank, accession # EF441271). In this research, we found that the full length cDNA of *Aci*HBGase I and II were 1,734 and 1,740 bp with the predicted deduced amino sequences of 577 and 579 residues. They were recorded in Genbank as accession # KC149921 and JX468895, respectively.

When searching the NCBI GenBank database using the megaBLASTn algorithm (http://www.ncbi.nlm.nih.gov) revealed that the *Aci*HBGase I, II and III cDNA nucleotide sequences were mostly identical to that of *Acj*HBGase I, II and III, respectively. Moreover, the deduced amino acid sequences of *Aci*HBGase I and II were aligned to the homologous amino acid sequences in other organisms, we found

that both *Aci*HBGase I and II had four highly conserved regions of α-amylase (Fig. 5.2 and Fig. 4.3). Considering the nucleotide and predicted amino acid sequences, it could be implied that *Aci*HBGases could be divided into three isoforms (*Aci*HBGase I, II and III) like *Am*HBGases and *Acj*HBGases.

### 6.3 Expression of r*Aci*HBGase I, II and III

To allow post-translational glycosylation, we used yeast *P. pastoris* as a host due to a suitable system mentioned before (Chen *et al*., 2010). We found that r*Aci*HBGase II and III were successful to be inducible expressed in *P. pastoris* but no production of r*Aci*HBGase I was found which was similar to r*Am*HBGase I (Nishimoto *et al*., 2007).

An expression plasmid of r*Aci*HBGase II and III was pPICZαA and transformed into *P. pastoris* GS115. The optimal conditions of these two recombinant clones were different. The optimum condition of r*Aci*HBGase II was induced by 0.5% MeOH for 96 h while the optimum condition of r*Aci*HBGase III was induced by 1% MeOH for 144 h. Although, both enzymes were different in inducible conditions, their activity in supernatant was much higher than in cell lysate. It was due to α-factor signal in pPICZαA expression vector which led a recombinant enzyme to secret into culture media during MeOH induction. Although, the full length cDNA of r*Aci*HBGase I was not successfully expressed in *P. pastoris*, a construct of this cDNA and pEcoli Nterm-6xHN expression vector, could be successfully expressed in *E. coli* Rosetta (DE3) cells. The optimal condition for induction was 1 mM IPTG for 3 h. The enzyme activity of r*Aci*HBGase I was found in cell lysate as soluble protein.

### 6.4 Purification of *Aci*HBGase I, II and III

Since we designed the recombinant enzymes to contain six histidine-tagged, we could use only a few purification steps to purify our recombinant enzymes. In this research, HisTrap affinity column (GE Healthcare) was used to purify r*Aci*HBGase II (C-terminal $(His)_6$ tag) and r*Aci*HBGase III (N-terminal $(His)_6$ tag). Also, HisTALON™ gravity column (Clontech) was used to purify r*Aci*HBGase I (N-terminal $(His)_6$ tag). Both columns (as immobilized metal ion affinity chromatography) contained metal ions ($Ni^{2+}$ ion for HisTrap affinity column and $Co^{2+}$ ion for HisTALON$^{TM}$ gravity column) which were efficient in purifying his-tagged proteins.

After purification, the activity of both r*Aci*HBGase II and III was found in the bound and eluted fractions (Figs. 4.4 and 3.3). The activity of r*Aci*HBGase I was appeared in both unbound and bound fractions but unbound fractions had higher enzymes activity (Fig. 5.4). However, it was previously reported that, for the purification of r*Acj*HBGase I, the majority of the recombinant enzyme (85%) was unexpectedly not absorbed to the cationic exchange column (Toyopeal CM-650 M) in 50 mM sodium acetate buffer (pH 4.7). It was suggested that the adopted confirmation had masked the electrostatic interactions between the protein and resin (Wongchawalit *et al.*, 2006). For commercial scale production of r*Aci*HBGase III, where the carbohydrate and $(His)_6$ tag might also affect the purification procedure (Nishimoto *et al.*, 2001), fractionation over DEAE-Sepharose CL6B, Bio-Gel P150, CM- Toyopeal 650 M and Sephacryl S-100 may be considered in order to avoid minor bands (Takewaki *et al.*, 1993; Nishimoto *et al.*, 2001; Nishimoto *et al.*, 2007).

Considering the mass weight (MW) of recombinant protein, r*Aci*HBGase III was ~ 68 kDa which was close to the MW of 68 kDa for native *Aci*HBGase III and *Am*HBGase III. For r*Aci*HBGase II, the single active band indicated the ~ 73 kDa which was close to the 76 kDa of native *Am*HBGase II (Takewaki *et al*., 1993). However, the MW of native *Aci*HBGase II has not been known yet.

For r*Aci*HBGase I, it had an apparent MW of 84 kDa on the reducing SDS-PAGE based zymograph which was significantly higher than the predicted 67.4 kDa from the deduced amino acid sequence (plus six histidine residues). Nevertheless, it coincided to the 82 kDa of native *Acj*HBGase I.

According to the above data, this research showed that *Aci*HBGase not only expressed *in vitro* in a yeast expression system but it also expressed in a bacterial system.

### 6.5 Characterization of r*Aci*HBGase I, II and III

Our data (Table 6.1) supported that HBGases contained 3 isoforms which were different in their expression pattern, MW, and enzyme properties such as optimal pH, optimal temperature, pH stability, thermal stability, theirs enzyme kinetics and substrate specificity (Giannesi *et al*., 2006; Harzer *et al*., 2012; Nishimoto *et al*., 2007).

From our work, three r*Aci*HBGases were different in their enzyme properties. r*Aci*HBGase III showed the same optimum pH as native *Aci*HBGase III which was 5.0. In contrast, both r*Aci*HBGase I and II could function well in the acidic

environment (optimal at pH 3.5 and >90% and 80% activity at pH 3.5-5.5 and at pH 3.5-5.0, respectively). Other than enzyme production for industry, heterogeneous expression of both genes in *P. pastoris* may be useful for curing Pombe disease, an autosomal recessive metabolic myopathy in the glycogen storage disease type II category. This disease was caused by the deficiency of acidic HBGase activity in human. Due to Moreland et al. (2012), it was reported that human placental lysosomal acidic HBGase could hydrolyze glycogen to glucose.

More than the above, r*Aci*HBGase I, II and III were different in optimal temperatures (40, 45 and 37°C, respectively). Comparing to native *Aci*HBGase III which the optimal temperature was 50°C, the optimal temperature of r*Aci*HBGase III was lower (37°C). However, different methods were used to assay the activity. This could make the result different. The native form was assayed by Momose's method (Momose and Inaba, 1961) while our recombinant form was assayed by PNPG as the substrate (Nishimoto *et al*., 2007).

Since the substrate specificity of HBGase was reported to differ greatly with the enzyme source (Chiba, 1988), this became one of the reasons to find a new source of enzyme with the hope to get a challenging isozyme with novel properties. Our results were somehow satisfying because three isoforms of r*Aci*HBGases were different in substrate specificity.

The enriched r*Aci*HBGase III showed normal Michalis-Menten type reaction kinetics on all tested substrates including the complex polysaccharide soluble starch. This was in agreement with previous reports (Chiba 1997, 1998). The substrate specificity was relatively high for maltose ($K_m$ of 4.5) and PNPG ($K_m$ of 4.4 mM).

Comparing to r*Am*HBGase III (Nishimoto *et al*., 2007), the recombinant enzyme in our research showed a higher substrate specificity for maltose, maltotriose and PNPG.

Furthermore, the enriched r*Aci*HBGase I and II showed a reasonably wide substrate specificity encompassing α-(1,1), α-(1,2), α-(1,4) and α-(1-6) glucosidic linkages. *Aci*HBGase II rapidly hydrolyzed PNPG α-(1,6) and sucrose α-(1,2) while *Aci*HBGase I rapidly hydrolyzed maltose α-(1,4) and sucrose α-(1,2). However, the $K_{cat}/K_m$ value for sucrose of both enzymes was the highest among all evaluated substrates.

Our three isoforms could hydrolyze soluble starch (very well hydrolysis for r*Aci*HBGase III but less hydrolysis for r*Aci*HBGase I and II). Thus, it is interesting that r*Aci*HBGase III could cleave not only small α(1,4) and α(1,6) linkage glucose units but also large polymers like starch.

## 6.6 Perspective

The full length cDNA, the predicted ORF and the conserved regions of *Aci*HBGase I and II could be analysed in this research. The different transcriptional expression pattern was observed. The former enzyme was found only in forager bees and the latter one was found to be restricted from eggs to pupae, but not forager bees in the developmental stages. After that, we constructed the expression construct containing the full length cDNA of those three isoforms (*Aci*HBGase I, II and III). The r*Aci*HBGase I was over-expressed in *E. coli* Rosetta (DE3) while r*Aci*HBGase II and III were over-expressed in *P. pastoris* GS 115. Considering the characteristics of

those enzymes, they showed the optimal pH of 3.5, 3.5, 5.0 and was stable (>90%) in the pH range of 3.5-5.5, 5.0-7.0, 5.0-7.5, respectively.

In addition, the r*Aci*HBGase I and II showed a thermal optimum at 40 and 45°C, respectively. They preferred the acidic environment and could be acid tolerant. Furthermore, their activities still remained at the relatively high temperatures (35-50 and 40-55°C, respectively). In contrast, r*Aci*HBGase III showed the lower optimal activity at 37 °C with no thermotolerance. Thus, it was suggested that r*Aci*HBGase I and II could be interesting for enzyme applications and in biotechnological processes when the conformational stability of the enzymes to heat and pH are required. Moreover, the substrate specificity of r*Aci*HBGase I and II was sucrose but the substrate specificity of r*Aci*HBGase III was maltose and PNPG. However, the native enzyme of *Aci*HBGase I and II should be purified and characterized in the future in order for comparison.

**Table 6.1** Summary of r*Aci*HBGase I, II and III

| Properties | r*Aci*HBGase I | r*Aci*HBGase II | r*Aci*HBGase III |
|---|---|---|---|
| **Stage of highest expression** | Forager bee | Larva and Pupa | Forager bee |
| **Nucleotide length** | 1,734 bp | 1,740 bp | 1,704 bp |
| **Deduced amino acid length** | 577 residues | 579 residues | 567 residues |
| **Induction condition** | 3 h, 1 mM IPTG | 96 h, 0.5% MeOH | 144 h, 1% MeOH |
| **Host of expression** | *E. coli* Rosetta (DE3) | *P. pastoris* GS115 | *P. pastoris* GS115 |
| **pH optimum** | 3.5 | 3.5 | 5.0 |
| **pH stability (>90%)** | 3.5-5.5 | 5.0-7.0 | 5.0-7.5 |
| **Optimum temperature** | 40 ℃ | 45 ℃ | 37 ℃ |
| **Thermal stability (>80 °C)** | 35-50 ℃ | 40-55 ℃ | <50 ℃ |
| **Molecular weight** | ~84 kDa | ~73 kDa | ~68 kDa |
| **Specific substrate** | Sucrose | Sucrose | Maltose, PNPG |

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## BIOGRAPHY

Miss Jirattikarn Kaewmuangmoon was born on January 6, 1981 in Lamphun, Thailand. She graduated her Bachelor's Degree of Science in Biology and Master's Degree of Biotechnology from Chulalongkorn University in 2003 and 2006, respectively. At present, she is a graduate candidate in Ph.D. Program in Biotechnology, Faculty of Science, Chulalongkorn University.

**Article publications**

1. **Kaewmuangmoon, J**., Kilaso, M., Leartsakulpanich, U., Kimura, K., Kimura, A. and Chanchao, C. 2013. Expression of a secretory α-glucosidase II from *Apis cerana indica* in *Pichai pastoris* and its characterization. *BMC Biotechnology*. 13: 16. (ISI, IF=2.35)

2. **Kaewmuangmoon, J.,** Yoshiyama, M., Kimura, K., Okuyama, M., Mori, H., Kimura, A. and Chanchao, C. 2012. Characterization of some enzymatic properties of recombinant α-glucosidase III from the Thai honeybee, *Apis cerana indica* Fabricus. *African Journal of Biotechnology*. 11: 16220-16232. (ISI, IF = 0.57)

3. **Kaewmuangmoon, J**. and Chanchao, C. Over-expression and characterization of recombinant alpha-glucosidase I from *Apis cerana indica* in *E. coli*. *Journal of Apiculture.* Accepted.

4. **Kaewmuangmoon, J.,** Nonthapa, P., Rattanawannee, A., Winayanuwattikun, P. and Chanchao, C. 2012. Preliminary screening for various bioactivities in honey and propolis extracts from Thai Bees. *European Journal of Medicinal Plants.* 2(2): 74-92.

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6. **Kaewmuangmoon, J.,** Suwanvijitr, T., Cherdshewasart, W. and Chanchao, C. 2010. Leaf morphometric and genetic variation of *Butea superba* in Thailand. *ScienceAsia*. 36: 180-186. (ISI, IF = 0.34)
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2. **Kaewmuangmoon, J.,** Chanchao, C., Winayanuwattikun, P. and Kimura, A. 2010. Characterization and expression of recombinant alpha-glucosidase III and partial cDNA of alpha-glucosidase I and II in the Thai honeybee *Apis cerana indica*. Abstract. The 15$^{th}$ Biological Sciences Graduate Congress, Kuala Lumpur, Malaysia. 113. (December 15-17, 2010)
3. **Kaewmuangmoon, J.,** Chanchao, C., Winayanuwattikun, P. and Kimura, A. 2010. Recombinant alpha-glucosidase I, II, and III in Thai honeybee *Apis cerana indica*. Abstract. The 10$^{th}$ Asian Apicultural Association Conference & Api Expo, Busan, Korea. 80. (November 4-7, 2010)

4. **Kaewmuangmoon, J.,** Chanchao, C. and Kimura, A. 2008. Partial cDNA sequence of alpha-glucosidase I (HBGI), expression pattern of HBGII, and cloning of HBGIII of *Apis cerana indica*. Abstract. The 13th Biological Sciences Graduate Congress, NUS, Singapore. 86.

5. **Kaewmuangmoon, J.,** Chanchao, C. and Cherdshewasart, W. 2006. Leaf morphometry, genetic variation and phylogeny of red Kwao Krua *Butea superba* in Thailand. Abstract. Proceeding of Faculty of Science, Chulalongkorn University, Bangkok, Thailand. 119.

6. **Kaewmuangmoon, J.,** Chanchao, C. and Cherdshewasart, W. 2006. Leaf morphometry, genetic variation and phylogeny of red Kwao Krua *Butea superba* in Thailand. Abstract. Proceeding of KMITL International Conference on Science and Applied Science, Bangkok, Thailand. 31.