CHAPTER V

DISCUSSION

5.1 Expression level of *alpha glucosidase (AG)* in *Apis florea*

Expression profile of *AG* was studied in *Apis florea*. This experiment was at a transcription level. Three stages of development (egg, nurse bee, and forager bee) were chosen. Eggs were directly picked up from a honeycomb. Since duty of workers, nurse bee and forager bee, is flexible and can change depending on colony demands, therefore, nurse bees and forager bees were sampled due to their duties in a hive (Kubo, 1996). Total RNA from 3 stages mentioned above was isolated.

Quality of total RNA was firstly evaluated. Total RNA was measured at OD_{260} and OD_{280}. The purity was calculated by the ratio of OD_{260}/OD_{280}. It should be closed to 2.0. In this study, the ratio of total RNA was 1.9. It implied that total RNA were good quality.

Second, isolated RNA was visualized on native agarose gel; quick, easy, and non – hazardous technique, and formaldehyde gel; getting rid of secondary structure of RNA. Total RNA should appear as two bright discrete bands representing the 28S and 18S ribosomal species (Fig. 4.1 A). The amount of 28S rRNA is much higher than the amount of 18S rRNA so sometimes only one visible band of 28S rRNA on the gel is acceptable (Fig. 4.1 B). A band of rRNA looks sharper on formaldehyde gel since the secondary structure of RNA is inhibited.

Third, the quality of total RNA was checked by RT – PCR technique with control primers. The control primers designed from housekeeping genes that express throughout life. Elongation factor (*EF*) and *28S RNA* genes were used for the control primers. RT – PCR products amplified by *EF* (200 bp) and *28S RNA* (350 bp) genes were detected (Fig. 4.3). This result indicates that RNA in a reaction is in good condition. This result will be interpreted together with Figure 4.2.

The obtained expression profile of *AG* in *A. florea* (Table 4.1) is similar to that in *A. cerana* (Srimawong, 2003) and to that in *A. mellifera* (Kubo, 1996). Due to Figures 4.2 and 4.3, the result is obvious that there is no *AG* product in an RT – PCR

reaction containing egg RNA and small amount of *AG* product in the reaction containing nurse bee RNA. AG is highest expressed in forager bees. This brings to the decision that forager bees are AG source for total RNA isolation and purification.

5.2 cDNA sequence and phylogenetic trees

Various primers were designed according to the *AG* cDNA sequence of *A. mellifera* (Fig. 3.2). The nucleotide and amino acid sequences were compared to related genes in several organisms (Figs. 4.5 and 4.6). The nucleotide was similar to *AG* in *A. mellifera* 95%, to *AG* and *alpha – amylase* in fruit fly *Drosophila melanogaster* 46%, to *maltase* in mosquito *Culicoides sonorensis* 41%, to *maltose* 1 in *A. mellifera* 38%, to sucrose – specific enzyme II of the PTS (*Scr*A) and dextran glucosidase (*dex*B) genes in *Lactobacillus sakei* 19%. Relationship of *AG* among other organisms was shown in UPGMA and NJ trees (Figs. 4.7 and 4.8). The trees represented that *AG* in *A. florea* was mostly closed to *AG* in *A. mellifera*, to *AG* and alpha – amylase in *Drosophila melanogaster,* to maltase in *Culicoides sonorensis*, to maltase 1 in *A. mellifera*, respectively. Due to the above phylogeny, *A. florea* and *A. mellifera* were closed.

5.3 Major protein pattern of crude extract

Crude protein was extracted from head and honey crop in buffer insect saline. The SDS – PAGE showed different patterns (Fig. 4.9). Proteins of head crude contain higher MW than those in honey crop crude. The major protein band in head is 50 kDa and consists of minor protein at MW between 10 – 225 kDa while the major protein band in honey crop is 15 kDa and consists of minor protein at MW between 10 – 25 kDa. Previous study presented that MW of AG was higher than 50 kDa but from our research, SDS – PAGE of honey crop revealed no protein at the MW above 50 kDa. This may indicate that low quantity of AG is located in honey crop. The HPGs which are located on the head synthesized AG directly (Dade, 1994) but can be moved to function in honey crop (Kubota *et al.*, 2004). These data support our experiment in 3.4 that high AG is in a head.

5.4 Effect of ammonium sulfate

Whole protein was precipitated with 95% ammonium sulfate. The precipitation was performed according to a procedure of King (1972) and Takewaki *et al.* (1980). In this experiment, various percentages of AS were used and specific activity of AG was examined. The result showed that unprecipitated AS protein provided the highest specific activity (Fig. 4.10). Precipitation with 80 – 95% AS provided higher activity than other precipitation. Therefore, in our experiment, crude protein with 95% AS and without AS was loaded onto DEAE – cellulose. Later, AG activity was determined. The specific activity of AG was obtained from DEAE – cellulose at 0.171 u/ mg (with AS) and at 4.5 u/ mg (without AS). This result supports an idea that precipitated protein with AS have an effect to AG activity. AS may disrupt AG activity or AG was not able to be precipitated with AS. That is why AG activity in crude was higher. Our result is different from purification of AG in *A. mellifera*. Takewaki *et al.* (1980) reported that precipitated AG with 95% AS was suitable for purification.

5.5 Purification on column chromatography

In this experiment, suitable columns used to separate AG are DEAE – cellulose and Superdex 200. Crude protein precipitated with 95% AS was separated on DEAE – cellulose. AG activity was detected in unbound peak (Fig. 4.12). The unbound peak was collected and dialysed in sodium acetate buffer (pH 6.3). Then, the unbound peak was loaded onto Superdex 200 (Fig. 4.14). The highest fraction was concentrated and desalted by centrifugal filter (MWCO 10,000 Da) as in Fig. 4.15. The retentive solution was separated by SDS – PAGE. According to this data, we planned other experiment by using CM – cellulose instead of DEAE – cellulose.

Previously, AG of *A. mellifera* was purified on salting – out chromatography. The AG was separated into two components which were eluted in the range of about 60 to 80% saturation and about 30 to 50% saturation as component I and component II, respectively. The component I was separated on CM – cellulose and Sephadex G – 100. The component II was separated on DEAE – cellulose. Unbound active fraction of DEAE cellulose was separated on CM cellulose and Bio – Gel P – 150. Then purified enzyme, AGI and AGII, were confirmed on polyacrylamide disc gel electrophoresis. Both enzymes were migrated as a single protein band since only single protein band was detected on SDS – PAGE. The MW

of AGI and AGII is estimated be approximately 98 and 76 kDa, respectively (Takewaki *et al.*, 1980). Then, AGIII was purified from bounded active fractions of component II on DEAE – Sepharose CL - 6B. The fractions were separated on Bio – Gel P – 150 and CM – Toyopearl 650M. The preparation migrated as a single protein band in disc polyacrylamide gel electrophoresis, and also in SDS – disc gel electrophoresis on which the enzyme was confirmed to be electrophoretically a monomeric protein as well as AGI and AGII. The molecular weight of AGIII was estimated to be approximately 68 kDa (Nishimoto *et al.*, 2001).

The partial purified AG in *A. florea* was separated on SDS – PAGE. The SDS – PAGE of highest activity fraction from Superdex 200 (lane 5, Fig. 4.15 B) showed 2 distinguished bands at MW of about 52 and 30 kDa. After that the highest activity fraction from Superdex 200 was concentrated with centrifugal filter devices and was separated on SDS – PAGE. After SDS – PAGE, 6 bands at MW of about 73, 66, 60, 52, 30, and 18 kDa were appeared instead (lane 6, Fig. 4.15 B). This indicates that protein at MW of about 73, 66, 60 and 18 kDa should be composed in the activity fraction but in small amount.

Since many proteins were obtained after Superdex 200, other type of gel filtration was used. Protein was continued to be separated on Sephadex G – 150. The column was used to separate peak from unbound DEAE – cellulose under the same condition as Superdex 200. The SDS – PAGE represents only one band about 50 kDa while AG activity stain represents positive band of about 93 kDa (Fig. 4.21). There are 2 possibilities for this case. First, the 50 kDa band on SDS – PAGE may not be AG. Second, the condition for renaturation of AG is not suitable. The result of peptide analysis by MALDI – TOF MS also supports the first possibility because the peptide mass spectrum of the 50 kDa band shows that it is not AG.

In order to find out about the charge of AG in crude, CM – cellulose was used instead of DEAE – cellulose. The unbound from DEAE – cellulose was dialysed and loaded onto CM – cellulose. Although the protein was bound in the column, the AG activity was low (Fig. 4.18). Also, all fractions contain the activity.

In order to avoid the charge change during the process of DEAE – cellulose, precipitated crude in sodium phosphate buffer (pH 6.3) was loaded onto CM – cellulose equilibrated with sodium acetate buffer (pH 4.7). The chromatogram shows 2 unbound peaks (Fig. 4.19). The result revealed that protein can not bind to the column. This should be because the pH 6.3 in sodium phosphate buffer is far

different from the pH 4.7 in sodium acetate buffer. However, it may be possible that this condition was not suitable to purify AG because low AG activity were obtained.

According to the result in Figure 4.10, AS precipitation may disturb the charge of protein which affects the specific activity. Alternatively to the procedures of purification, unprecipitated crude was loaded onto DEAE – cellulose. The result shows both bound and unbound peaks but unbound peak contains high AG activity (Fig. 4.20). Considering Figure 4.21 (A), SDS – PAGE of unbound peak of crude protein without AS on DEAE – cellulose represents many bands but major bands were located at the position of about 35 and 50 kDa (lane 3). On the other hand, the SDS – PAGE of unbound peak of crude protein precipitated with AS on DEAE – cellulose represents high MW (lane 1). This is because there is no AS which can get rid of small proteins. Considering activity staining in Figure 4.21 (B), only activity band was observed in unbound without AS precipitate (lane 3), not in unbound with AS precipitate (lane 1). Although the loaded protein in lane 1 (3 mg protein) is more than in lane 3 (1 mg protein). This confirms the data in Figure 4.10 that AS precipitate was affected to AG. Moreover, the activity stain of protein on Sephadex G – 150 is higher than unprecipitated protein on DEAE (Fig. 4.21 B, lane 2 and 3). From our research, partial purified AG was obtained by being precipitated with 95% AS, separated on DEAE – cellulose, and Sephadex G – 150. In contrast, crude without AS precipitation and separated on DEAE – cellulose was less purified.

5.6 Molecular weight of AG in *A. florea*

According to the graph of log MW and R_f value, the activity band on renatured gel coincides to a band at MW of 93 kDa on SDS – PAGE (Fig. 4.22). It can be assumed that MW of AG in *A. florea* is 93 kDa. In *A. mellifera*, MW of purified honeybee sucrase was 70 kDa (Huber, 1975). Then MW of purified AGI and AGII was estimated to be approximately 98 and 76 kDa, respectively (Takewaki *et al.*, 1980). Then, MW of AGIII was estimated to be 68 kDa (Nishimoto *et al.*, 2001). In addition, by the same method, MW of AG from crude protein in *A. cerana* was estimated to be 96 kDa (Srimawong, 2003). From the above data, MW of AG in *A. florea* is not greatly different from AGI in *A. mellifera* and AG in crude of *A. cerana*.

5.7 MALDI - TOF mass spectrometry

The partial purified AG was separated by SDS – PAGE. Target bands locating in the range of MW of 55 and 52 kDa were excised because they are common bands which are always found from all columns. Furthermore, a target band was selected because of the result of activity band at the MW of 93 kDa (Fig. 4.21). In addition, SDS – PAGE shows an outstanding band of 73 kDa (Fig. 4.15). This band shows the vicinity to AGII in *A. mellifera* (76 kDa). That is why this band was excised and went on being analysed by MALDI - TOF.

The protein complement was partially identified by MALDI – TOF MS. The MALDI – TOF MS of Af1 and Af2 (55 and 52 kDa, respectively) does not match to AG at all. It reveals that they are not AG. Since these proteins are always found in all steps of purification, it indicates that they are in abundant amount. If we had got rid of these proteins before our procedures, it might have helped. In contrast, the MALDI – TOF MS of Af3 (73 kDa) shows six peptide masses which are matched to those of AG in *A. mellifera* (Q17058), score 70 [significant 67 ($p < 0.05$)], with 12% coverage (based on the M_r of 65523 Da). This result represents that the protein at MW of 73 kDa is AG in *A. florea*. For this reason, it implies that AG in *A. florea* was mostly closed to AGII in *A. millifera* (76 kDa).

5.8 Comparison of amino acid sequences between deduced amino acid sequence from cDNA and amino acid from MALDI – TOF MS

After comparison, one of amino acids, was different. It is Leucine (L) in deduced amino acid sequence from cDNA but is Valine (V) from MALDI – TOF MS. The 2 amino acids have close MW. MW of Valine is 117 Da while Leucine is 131 Da.

5.9 Two – dimensional electrophoresis

Two – D electrophoresis of crude protein represents a lot of spots. The result of 2 – D was useful in performing AS precipitation. The result showed large amount of small MW proteins. Due to MALDI – TOF MS, it can imply that AG protein should be at MW of 73 kDa. The AG is probably located on a circle mark in Figure 4.26. Considering protein scattering, AG seems to be in much less amount

than other proteins. Lots of contaminated proteins may cause a problem in AG purification. That is why low activity was assayed in partly purified AG.

5.10 Optimum conditions

Optimum pH of partial purified AG in *A. florea* was pH 5 (Fig. 4.27). This means that AG in *A. florea* can work well in an acidic condition. This pH is not greatly different from optimum pH of purified AG in *A. mellifera*. The pH optimum of purified sucrase was 5.5 (Huber, 1975). The pH optimum of purified AGI, AGII, and AGIII was 5, 5, and 5.5, respectively (Takewaki *et al.*, 1980 and Nishimoto *et al.*, 2001).

The optimum temperature of partial purified AG in *A. florea* was 55°C (Fig. 4.28) while the activity of purified AG in *A. mellifera* was completely lost at 60°C (Huber, 1975; Takewaki *et al.*, 1980; Nishimoto *et al.*, 2001). From this result, it can be assumed that AG in *A. florea* is stable at high temperature.

Considering other conditions, the optimum substrate concentration was 80 mM sucrose while the highest AG activity was for 40 min incubation (Figs. 4.29 and 4.30).