CHAPTER V
DISCUSSIONS

Amylases have been used in many industries but their stability and activity are limited. In recent years, they are usually imported with high cost. Therefore, in the present research, new sources of amylase with high activity and stability from natural resources such as microorganisms, animals, especially in plants and fruits are under investigation. Amylase is the major enzyme that hydrolyzes starch into sugar, causing sweetness in fruits (Hulme et al., 1970); Edna et al. (1978) found the parallel between the increase in amylase and a decrease in the starch content of the avocado fruit pulp. In the same way, the concomitant increase of total sugars in the aril with the decrease in starch content was observed in durian fruit during ripening (Ketsa and Deuangkanit, 1998). The occurrence of such enzymes has been reported in avocado fruit pulp (Edna et al., 1978), mango (Luiz et al., 2001), apples (*Malus pumila)* (Kanwal et al., 2004), post-harvest *Pachyrhizus erosus* L. tuber (Noman et al., 2006) and banana (Do Nascimento et al., 2005)

However, Thai fruit amylases have not yet been reported especially in Mon Thong durians. Durian is a climacteric fruit and very favorite fruit of most of the Thai people (around 72.1%). They have preferred Mon Thong cultivar because of the sweet and buttery taste with soft and fine texture, rather than its mild odor (Somsri, 2007). Therefore, the Mon Thong durians are very interested to be a model for research because large amount of starch is stored in the pulp and converted into sugar by amylase, causing them to become sweeter during ripening. This ripening process might be expected from amylase activity.

Thus, to estimate ripening stage of fruits, the reducing sugar and starch content were used as marker because of increasing of sugar and decreasing of starch during ripening process are usually changed. Reducing sugar contents were increased during the ripening stage, featuring in avocado (Edna et al., 1978) and durian (Ketsa and Daengkanit, 1998). The results showed that starch and reducing sugar content in ripening durian pulp is 1.1% fruit pulp and 0.17% of fresh weight in crude durian

extract was found, respectively. Starch content in Mon Thong durian is in accordance with the previous report of starch content in banana ripening (1%). Ketsa and Daengkanit (1998) reported that the unripe durian cv. Chanee aril had a high starch content (approximately 24%) that decreased progressively to approximately 4-6% in ripe durians.

To study protein profile, 13% SDS-PAGE revealed that the protein bands with molecular weight between 28-97 kDa of which 38-55 kDa are major protein bands (Figure 4.5). During fruit ripening processes, synthesis of many proteins occur to control the process which depend on ripening phenotype of fruit (Siripanid, 2007). Moreover, fruits also produce high content of carbohydrates, vitamin, minerals and organic acids although they are usually very low in proteins and fats (Ornellas, 1998). Thus, there are many proteins synthesized during the ripening which depend on fruit phenotype as found in protein pattern of SDS-PAGE.

To test amylase activity and indicate molecular weight of amylase enzyme, Zymograhic method was performed by using crude protein solution (fruit extract). In non-reducing condition (without β-mercaptoethanol), crude durian exhibited amylase activity at around 45 kDa (Figure 4.6) according to protein band in coomassie stained gel. However, amylase activity of crude durian did not appear when treated with reducing agent, suggesting that disulfide bonds are essential for the enzyme function. Moreover, Iodine test demonstrated that the starch content in fruit pulp was decreased and Zymograhic detection showed clear transparent amylase band. This data might imply that the fruit pulp has activity of amylase in order to convert starch to sugar. These results are in accordance with those previously reported that molecular weight of amylases showed the same range (38–45 kDa) from other plant sources (Tripathi et al., 2007; Noman et al., 2006).

To obtain partial amino acid sequence of amylase, 2D-PAGE was used to separate protein solution. After that, the amylase spot was identified by using LC-MS/MS technique. The 7 cm IPG strip pH range 3-11 was used for separation of protein in ripe Mon Thong durian. The 40 major protein spots on 2D gel (Figure 4.7) were picked and identified by LC-MS/MS. Twenty seven spots of them were identified and organized into 9 groups; concerning carbohydrate metabolism (19.07%), protein metabolism (34.74%), lipid metabolism 1.47%. Some are

predicted to be protein in folding process (5.98%), secondary metabolism (1.50%), ripening process (0.96%), antioxidant enzyme (5.71%) and cell wall hydrolases (3.51%). The presence of other group (17.28%) which could not be identified. (Figure 4.8). The functional class is corresponding with proteome of *Citrus sinensis* L. (Osbeck) and strawberry. However, amylases could not be identified on 2D-PAGE possibly from low abundance of protein in combination with low resolution on 7 cm IPG dry strip and not be found in database. This data should be implied that amylase may be not major protein in fruit extract of Mon Thong durian.

Protein metabolism is a largest functional class (34.74%) which are aminotransferase, leucine aminopeptidase, elongation factor G and RNA polymerase C. These results suggested that the ripening process require of more enzymes for biosynthetic pathways involved at ripening time. The second largest functional class is constituted by protein involved in carbohydrate metabolism accounted for 19.07% of all identified protein. This group is dominated by enzymes of glycolysis, which are expected to provide the necessary energy metabolites for the ongoing ripening process (Bianco et al., 2009). Our analysis revealed the presence of enolase, phosphoglycerate kinase, fructose bis phosphate aldolase, oxidoreductase and granule bound starch synthase. Proteins devoted to protein folding are heat shock protein Hsp 70 and 60 kDa chaperonin. The heat shock protein is usually involved in stabilizing protein folding in response to different kind of stimuli, under non-stress condition and during plant development. Their functions also include the maturation of protein complexes and degradation of damaged or mis-folded peptide (Muccilli et al., 2009). Proteins devoted to antioxidant enzymes are glutathione reductase and isoflavone reductase. Isoflavone reductase is an enzyme involved in the isoflavone biosynthesis (Muccilli et al., 2009). Glutathione reductase is an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant. Two cell wall hydrolase enzymes, endo-1,4-beta-glucanase (belong to cellulase) and glycosyl hydrolase family 10 (xylanase) important for the cell-wall hydrolysis in fruit (Hulme, 1970) were identified. Moreover, Ketsa and Daengkanit (1998) reported that activity of cellulase was also high at harvest time and did not clearly increase during ripening. Within the secondary metabolism function class, flavonoid glycosyltransferase which is an enzyme involved in the

flavonoid biosynthesis was identified. Flavonoid are the most important plant pigments for flower, fruit coloration producing yellow or red/blue pigmentation. Flavonoids are most commonly known for their antioxidant activity. Recently, Haruenkit et al. (2010) reported that flavonoids level were the highest in ripe Mon Thong durian. Proteins devoted to lipid metabolism are S-malonyltransferase (acyl-carrier protein) which is an enzyme important for fatty-acid biosynthesis. The fatty acid was found in Mon Thong durian (Haruenkit et al., 2010). Protein devoted to ripening process enzyme is ethylene responsive protein which is responsible in part for mediating the response in plants to the ethylene. It is suggested ethylene is importance for ripening process of Mon Thong durian. Durian is a climacteric fruit. The rise in respiratory activity and ethylene production coincides with ripening (Ketsa and Daengkanit, 1998).

Therefore, to obtain partial amino acid sequence of amylase again, Crude durian protein was precipitated with 70% ammonium sulphate to obtain partially purified amylase. Then, the precipitated enzyme was separated by IPG strip pH 3-11, 7 cm in first-dimension step. After separation with 13% SDS-PAGE in second dimension step followed by coomassie blue staining. The picked protein spots were analyzed by LC-MS/MS and showed high homologies with known proteins. We found α-amylase with molecular weight of 45 kDa and pI 6.51, with IATVLPDK as partial amino acid sequence. These results are in accordance with molecular weight of the amylase by Zymograhic method. These result is in accordance with pI of amylases from malted barley (6.2-6.4) and malted wheat (pI 6.05-6.2) (Muralikrishna and Nirmala, 2005). Interestingly, the protein spot (KP 1, Figure 4.9) was matched with alpha amylase catalytic region from *Fervidobacterium nodosum* Rt17-B1 which is a thermophilic amylase, isolated from a New Zealand hot spring. Its optimal growth temperature is 65 to 70 °C. It is suggested that, amylase from Mon Thong durian may have some properties similar to alpha amylase from *Fervidobacterium nodosum* Rt17-B1 such as thermostability.

In addition, to confirm the amylase identification in Mon Thong durian, amylase was determined using Zymograhic method. The KC 1-4 and KI 1-2 bands (Figure 4.10) were cut and identified by LC-MS/MS. This approach revealed that KC 2 and KI 2 are α-amylase and β-amylase, respectively. Although KC 2 and KI 2

bands were identified as different group of amylase, but in this work we found amylase in Mon Thong durian displaying as thermostable enzyme which showed high stability at 60 °C which was not found in β-amylase (Muralikrishna and Nirmala, 2005). Thus, Mon Thong durian amylase may possibly be α-amylase rather than β-amylase.

For purification of such enzyme in Mon Thong durian, three-step procedures were used. In the first step, the crude durian was precipitated with 30-70% ammonium sulphate. We found that protein precipitated with the 70% ammonium sulphate (w/v) showed higher specific activity (1.39 Unit/mg) than 30-60 % (w/v) and showed 2.02 purification fold. This method may help in removing α-amylase inhibitor, starch and sugar which are present in the crude extract (Kumari et al., 2010). After that, the precipitated protein was then subjected into Epoxy-activated Sepharose 6B affinity column, affinity chromatography, which has been widely used for purification of α-amylase from various sources (Tripathi et al., 2007; Elarbi et al., 2009, Kumari et al., 2010). The affinity chromatography showed highly purified enzyme with selective elution by β-cyclodextrin solution. After affinity purification, a single peak of amylase activity was obtained after elution with 10 mg/ml β-cyclodextrin solution. The durian amylase was purified more than 313.04 folds with specificity activity of 216.00 Unit/mg (Table 4.14). These results are in accordance with similar purification procedures in mung beans (Tripathi et al., 2007) and soybean (Kumari et al., 2010). Then, SDS-PAGE was used to check purity of fractionated proteins. We found four protein bands on SDS-PAGE (Figure 4.15). Next, the amylase fractions were pooled, concentrated and then loaded into DEAE Toyopearl Anion Exchange column. Proteins displaying high amylase activity were eluted at 0.16 M NaCl. The results of the purification procedure are summarized in Table 4.15. After completing purification step, the enzyme was purified 340.36 folds, 234.85 U/mg of protein of specific activity with 0.06% recovery. Generally, the decreasing in yield may be due to denaturation of the enzyme during purification (Noman et al., 2006). The purity of fractionated enzyme was analyzed by SDS-PAGE under reducing conditions (Figure 4.18). After silver staining, the two bands with molecular weight of approximately 65 and 66 kDa were observed, but the expected amylase band, 45 kDa, did not appear on SDS-PAGE. However, it showed

amylase activity at 45 kDa on Zymograhic detection, suggesting that the enzyme may be present in very low amount or cannot be stained with silver. Interestingly, the result indicated that amylase has very high activity. This data suggested that the 45 kDa band is amylase in Mon Thong durian which is confirmed by proteomic approaches. This molecular weight corresponded with α-amylases in mung beans (*Vigna radiate*) (Tripathi et al., 2007) and in the same rage 38-53 kDa as previously reported for α-amylase from other sources (Kanwal et al., 2004; Noman et al., 2006; Tripathai et al., 2007; Asoodeh et al., 2010; Souza and Perola, 2010).

In order to characterize the partially purified amylase, the optimum pH, optimum temperature, pH stability, heat stability, effect of metal ions and EDTA were analyzed. Firstly, crude durian was used in a preliminary study to analyze amylase properties using DNS method. The optimum pH of amylase was measured at various values of pH in buffer using starch as substrate at 37 °C. Maximum activity was observed at pH 7.0 in potassium phosphate buffer (Figure 4.19). The amylase was highly active between pH ranging 6.0 to 8.0. This optimum pH value is consistent with the previous reports for other plant α-amylase. Optimum pH of α-amylase from apple was found to be 6.8 (Kanwal et al., 2004); 7.3 for post-harvest *Pachyrhizus erosus* L. tuber (Noman et al., 2006). The optimum temperature of amylase was determined by varying temperatures from 30 to 90 °C. The enzyme showed optimum temperature at 40 °C. Interestingly, the enzyme showed a very wide range of optimum temperatures between 37 °C to 70 °C. The relative activities at 50, 60 and 70 °C are about 96.89, 94.94 and 90.41, respectively. This result showed that the enzyme is more stable with high temperature than α-amylase from apple (*Malus pumila*) (Kanwal et al., 2004) and *Pachyrhizus erosus* L. tuber (Noman et al., 2006) which had optimum temperature at 37 °C.

After preliminary study using DNS method, the partial purified amylase was characterized by using Zymographic method. The effect of pH on partially purified amylase was examined in the pH range 3-10 using starch as a substrate. The amylase from Mon Thong durian showed maximal activity at pH 7.0 in potassium phosphate buffer. Comparative study regarding pH effect on amylase revealed that the enzyme showed a broad activity in this pH 4-7, but it showed the highest activity at pH 7.0. And the activity of enzyme was dramatically decreased from pH 8-10.

This optimum pH value is consistent with the optimum pH from DNS method. These results confirmed that the optimum pH for Mon Thong amylase was pH 7.0. The effect of temperature on activity of partially purified amylase was examined in the temperature range of 30 to 100 °C using Zymograhic method. It exhibited the clear transparent band. Interestingly, the Mon Thong amylase showed broad activity at temperature from 30 to 70 °C. But when the temperature was increased from 80 to 90 °C, the amylase activity was slightly dropped. Furthermore, amylase activity of Mon Thong durian was confirmed by increasing temperature up to 100 °C (Figure 4.24). The result revealed that durian amylase is a thermostable enzyme. The formation of stable enzyme substrate complex that protects the enzyme from denaturation by heat (Kumari et al., 2010) is suggested. In addition, suggesting that amylase of Mon Thong durian has a high disulfide bond that may stabilize proteins more effectively at higher temperature (Stephen, 1993). The optimal condition of this amylase was nearly to the condition for thermal α-amylase from germinating soybean seeds and Ferdows hot mineral spring in Iran (70 °C) (Kumari et al., 2010, Asoodeh et al., 2010). The thermostable α-amylase from marine yeast *Aureobasidium pullulans* N13d had optimum temperature of the purified enzyme at 60 °C (Li et al., 2007). Moreover, optimum temperature of α-amylase from *Bacillus lichenifomis* NH1 was 90 °C. This optimum temperature corresponded with the optimum pH of amylase in Mon Thong durian on DNS method. The optimum pH and optimum temperature of amylase in crude durian and partially purified amylase was in accordance with the data in previous reports indicating that Mon Thong durian has a major α-amylase. Similar with this report, the main starch degrading enzymes in fruit was α- and β-amylase and starch phosphorylase (Seymour et al., 1993).

For testing of enzyme stability, the enzyme was pre-incubated at different pH, at 37 °C for 30 min. After that the residual enzyme activity was assayed using Zymograhic method. The results showed that the enzyme was stable and highly active over a wide range of pH from 6 to 10 when compared with control. In general, α-amylase was stabled in a wide pH range from 4.8 to 11. However, stability of α-amylases with a narrow range has also been reported (Brena, 1996). In addition, The enzyme was pre-incubated at different temperature from 30 °C to 90 °C, at pH

7.0 for 30 min. And then the residual enzyme activity was determined using Zymograhic method. The purified amylase was stable and highly active over a wide range of temperature from 30 °C to 60 °C. However, the enzyme was inactivated at higher temperature than 60 °C. From these results, the amylase seemed to have a considerable thermostability.

In order to study the effect of metal ion on amylase activity, we found the metal ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Na^{+} and K^{+} ions) did not have any effect on biological function of enzyme. However, amylase activity was almost completely abolished by 5 mM EDTA. According to a previous study, α-amylase from pea was completely inhibited by 5 mM EDTA. Tripathi et al. (2007) and Kumari et al. (2010) also reported the inhibitory effects of EDTA on the activity of α-amylase. The inhibition by EDTA confirmed that amylase of Mon Thong durian is a metalloenzyme and clearly revealed that it was α-type. This result is consistent with the partial amino acid sequence data of amylase in Mon Thong durian by 2D-PAGE and LC-MS/MS which showed that it was amylase α-type. Generally, α-amylases are more thermostable compared to β-amylase (Muralikrishna and Nirmala, 2005) which was in accordance with the optimum temperature of amylase in Mon Thong durian. In addition, α-amylases contain at least one calcium ion activating or stabilizing the enzyme. Therefore, one of the conserved regions of α-amylase probably is responsible for calcium binding. The calcium plays crucial role for maintaining and stabilizing the structure of α-amylase by interacting with negatively charged amino acid residues, such as aspartic and glutamic acid. Role of calcium may be in strengthening the binding between domains of enzyme. Calcium may also protects amylase against proteolytic degradation (Kumari et al., 2010).

Finally, the durian amylase showed a wide range of optimum temperature, good stability at pH 6-10 and good in the presence of stability metal ion which is very important. Thus, this enzyme should be considered to be used in industrial application. Therefore, α-amylase from crude Mon Thong Durian would be a good choice for various application in the future.