CHAPTER IV
RESULTS

4.1 Sample preparation

Mon Thong durian (*Durio zibethinus* Murr. cv. Mon Thong) was obtained from Tomandee durian orchard, Rayong province, Thailand, as shown in Figure 4.1. After exocarp peeling, fruit pulp was sliced into pieces and used to determine starch content. For fruit extract preparation, fruit pulp was ground by blender. Then, the sample was filtered by gauze and centrifuged at 10,000*g* for 15 minute. The supernatant was collected as fruit extract.

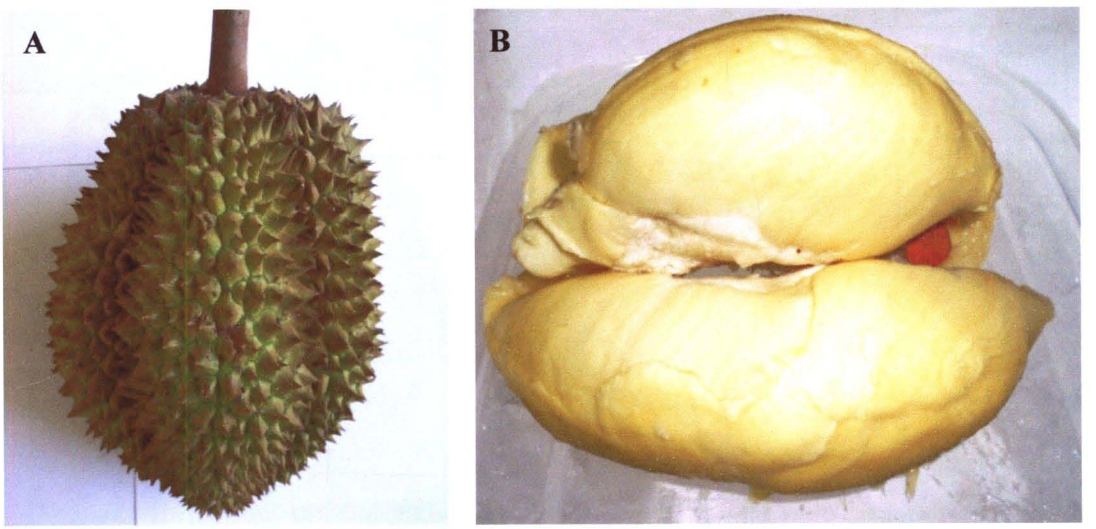

Figure 4.1 Mon Thong durian (*D. zibethinus* Murr. cv. Mon Thong) (A) and Mon Thong durian pulp (B).

4.2 Starch and reducing sugar content determination

Starch and reducing sugar content were determined for estimating ripening stage of Mon Thong durian. The starch content of durian pulp was determined by iodine test. Starch calibration curve was plotted (Table 4.1 and Figure 4.2) and used to calculate starch content of durian pulp. The starch content was calculated in the percentage value approximately 11.018 mg starch/g fruit pulp or 1.1 % starch of fruit pulp (Table 4.2).

Table 4.1 Absorbance at 620 nm of starch as standard by Iodine test.

Concentration (mg/ml)	Absorbance 620 nm			$\bar{X}$	SD
	1	2	3		
0.2	0.083	0.086	0.090	0.086	0.004
0.4	0.229	0.230	0.231	0.230	0.001
0.6	0.474	0.418	0.432	0.441	0.029
0.8	0.554	0.552	0.553	0.553	0.001
1.0	0.700	0.704	0.706	0.703	0.003

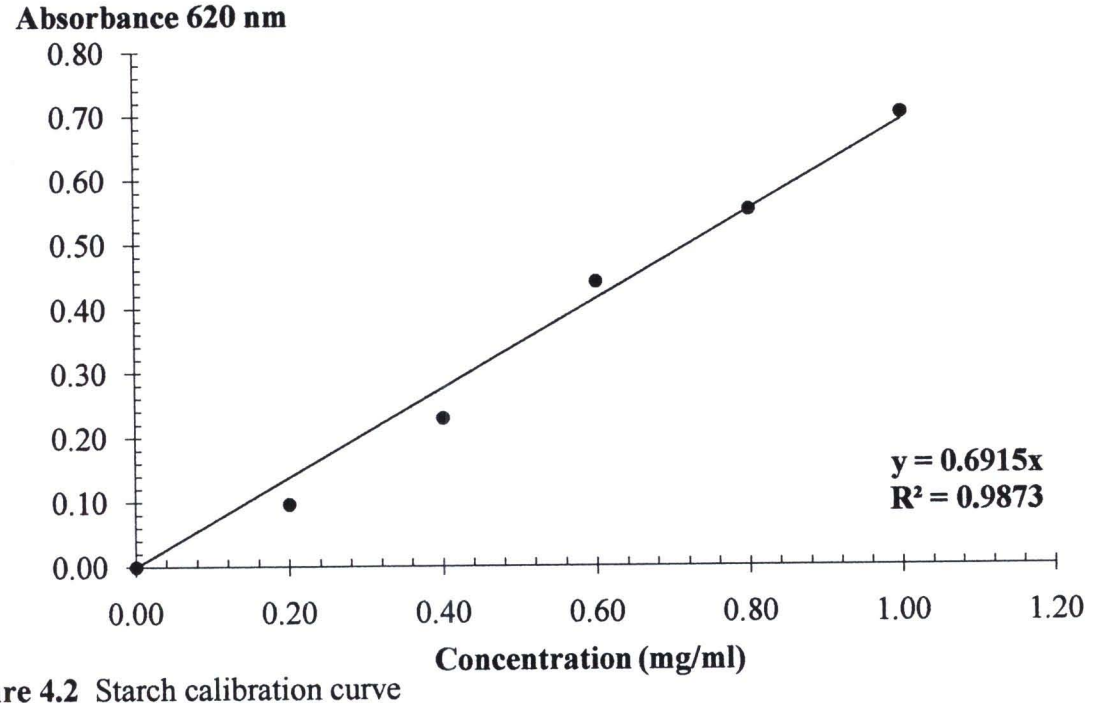

Figure 4.2 Starch calibration curve

Table 4.2 Determination of starch in fruit pulp.

Absorbance at 620 nm			$\bar{X}$	SD	Concentration	
1	2	3			mg Starch/ g fruit pulp	% Starch of fruit pulp
0.438	0.423	0.430	0.430	0.007	11.018	1.1

The reducing sugar content of fruit extract was determined by DNS method. Fruit extract was 100 times diluted in DDW. The amount of reducing sugar was calculated using standard curve of glucose (Table 4.3 and Figure 4.3). The reducing sugar content showed 0.17% of fresh weight (Table 4.4).

Table 4.3 Absorbance at 540 nm of glucose as standard by DNS method.

Concentration (mM)	Absorbance 540 nm			$\bar{X}$	SD
	1	2	3		
10	3.951	3.970	3.954	3.958	0.010
5	2.224	2.271	2.291	2.262	0.034
2.5	1.207	1.226	1.24	1.224	0.016
1.25	0.520	0.531	0.569	0.540	0.025
0.625	0.284	0.229	0.211	0.241	0.038

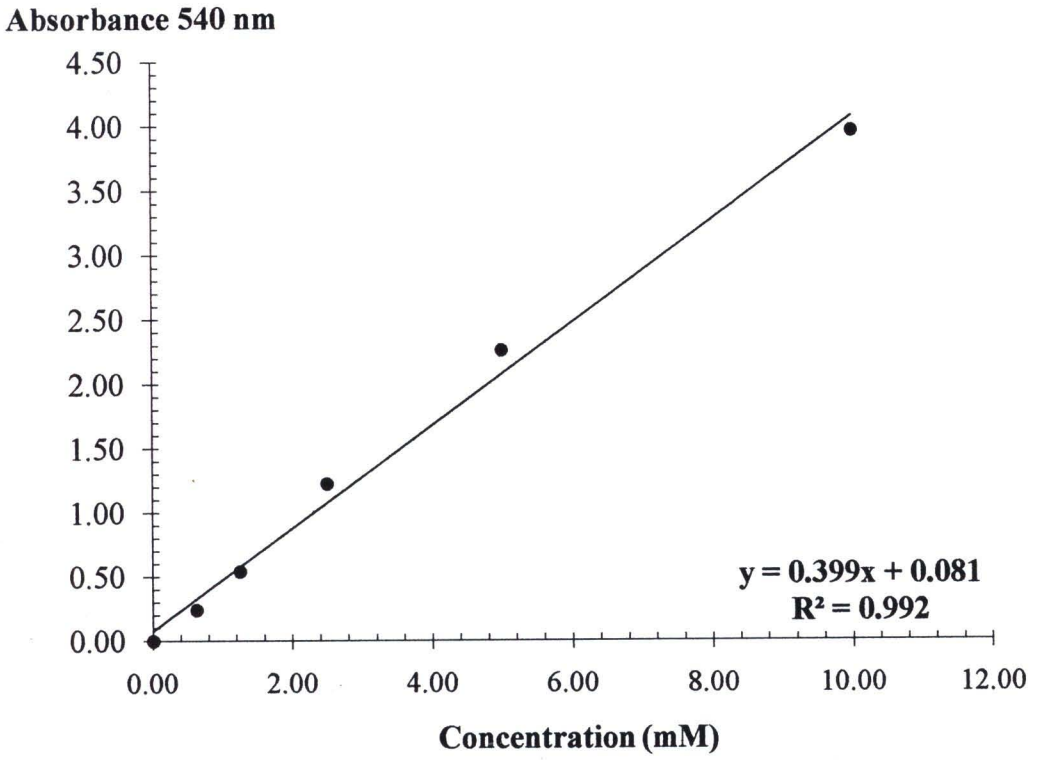

Figure 4.3 Glucose calibration curve

Table 4.4 The reducing sugar in fruit extract

Absorbance 620 nm			$\bar{X}$	SD	Concentration	
1	2	3			mM	% of fresh weight
0.699	0.563	0.589	0.617	0.072	134.34	0.17

4.3 Protein Determination using Bradford Method

Bradford method was applied to determine the protein concentration of durian extract. BSA calibration curve was plotted (Table 4.5 and Figure 4.4) and used for protein determination.

Table 4.5 Absorbance at 595 nm of BSA as protein standard by Bradford method.

BSA amount (µg)	Absorbance 595 nm			$\bar{X}$	SD
	1	2	3		
2.0	0.195	0.190	0.192	0.192	0.003
4.0	0.369	0.365	0.366	0.367	0.002
6.0	0.540	0.545	0.542	0.542	0.003
8.0	0.697	0.692	0.697	0.695	0.003
10.0	0.866	0.866	0.864	0.865	0.001
12.0	0.195	0.190	0.192	0.192	0.003

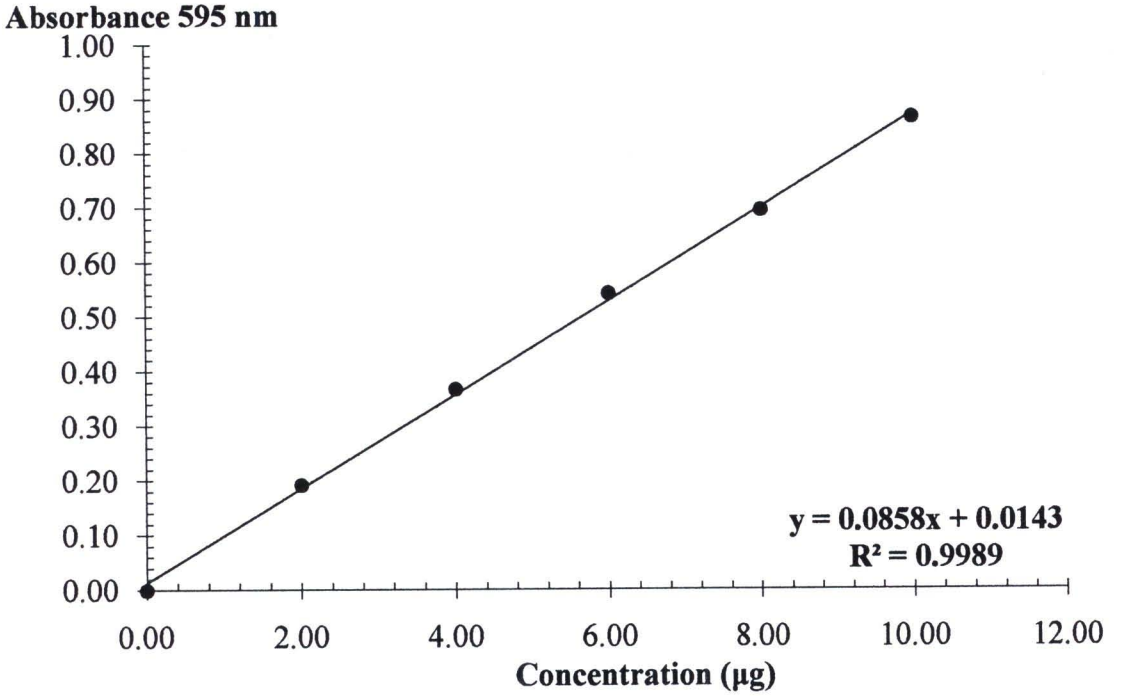

Figure 4.4 Bovine serum albumin (BSA) calibration curve

Protein concentration was determined using Bradford method and compared with BSA calibration curve. The results showed 14.019 µg/µl of total protein of crude durian or 0.97 mg protein/g fresh weight (Table 4.6).

Table 4.6 Quantity of protein in fruit

Absorbance 595 nm			$\bar{X}$	**SD**	**Concentration**	
1	**2**	**3**			**(µg/µl)**	**mg protein/g fresh weight**
1.217	1.215	1.218	1..217	0.002	14.019	0.97

4.4 Crude durian protein pattern of Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed to estimate molecular mass of crude durian protein by plotting the migration distance of molecular weight standards and log molecular mass. Protein standard were α-lactalbumin (14.4), Trypsin inhibitor (20.1), Carbonic anhydrase (30.0), Ovalbumin (45.5), bovine serum albumin (66.0), phosphorylase b (97.0). After SDS-PAGE analysis of crude durian (with β-mercaptoethanol), the protein bands exhibited molecular weight between 28-97 kDa and 38-55 kDa of major protein bands (Figure 4.5). These results are in accordance with those previously reported of amylases from other plant sources (Tripathai et al., 2007; Noman et al., 2006).

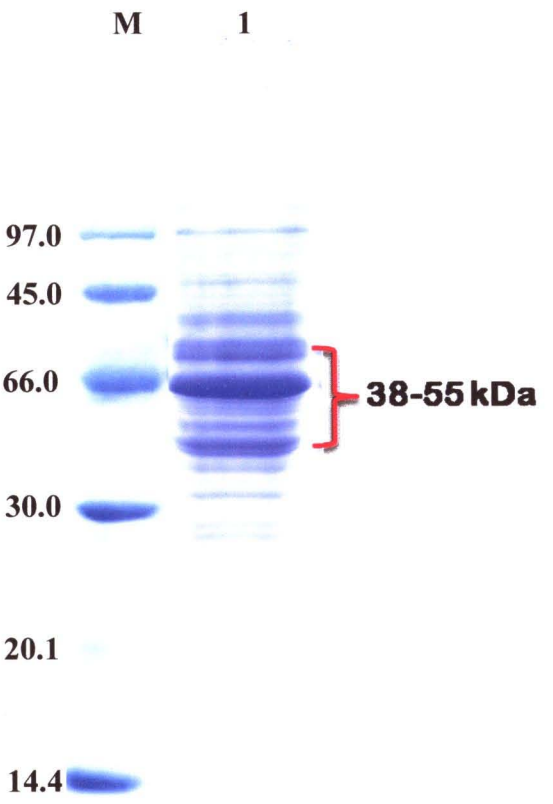

Figure 4.5 SDS-PAGE analysis of Mon Thong durians (*D. zibethinus* Murr. cv. Mon Thong) on 13% acrylamide gel. Crude durian was loaded 20 µg Protein/lane. Lane A: Low molecular weight marker; Lane B: crude ripe Durian.

4.5 Zymograhic method

The amylase activity of crude durian extract (with and without β-mercaptoethanol) was performed by Zymograhic method using the 13% acrylamide gel containing 0.1% starch soluble with constant voltage at 150 volt (Figure 4.6). The zymogram gel was cut into 2 pieces for staining with different staining solution. The gel stained with Coomassie brilliant blue was used to compare the migration of proteins. After that, the gel was washed with 1% triton X-100 to remove sodium dodecyl sulfate (SDS) for structure refolding of enzyme. Finally, the gel was incubated with activity solution (mixture of 0.2% starch with 10 mM Sodium acetate buffer pH 4.8, 2 mM $CaCl_2$) for 2 hr and stained with iodine solution. Native gel exhibited 28 to 97 kDa of protein bands and 38-55 kDa of the major bands (Figure 4.6, lane 1). For zymogram of crude durian exhibited the clear transparent band at 45 kDa (Figure 4.6, lane 4, indicated by arrow) comparing with standard protein marker in stained coomassie blue gel. However, amylase activity of crude durian did not detect when treated with reducing agent (Figure 4.6, lane 4, with β-mercaptoethanol treatment).

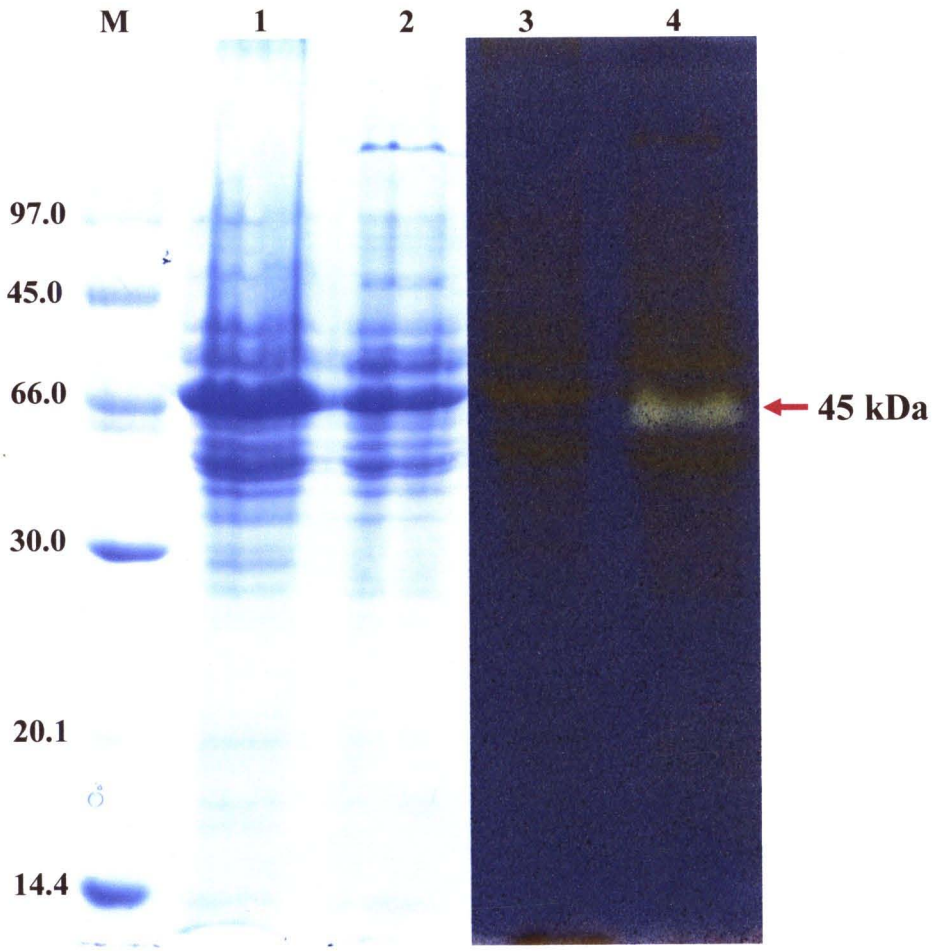

Figure 4.6 Zymograhic analysis of crude Mon Thong durian (*D. zibethinus* Murr. cv. Mon Thong) on 13% acrylamide gel containing 1% soluble starch. Crude durian was loaded 20 µg Protein/lane. Lane M: Low molecular weight marker; Lane 1: Crude durian with β-mercaptoethanol on native gel was stained with Coomassie brilliant blue; Lane 2: Crude durian without β-mercaptoethanol on native gel was stained with Coomassie brilliant blue; Lane 3: Crude durian with β-mercaptoethanol on activity gel was stained with iodine solution (1% acetic acid containing 10 mM I_2 and 14 mM KI); Lane 3: Crude durian without β-mercaptoethanol on activity gel stained with iodine solution. Arrow head: Clear zone was indicated at about 45 kDa.

4.6 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and Liquid chromatography tandem mass spectrometry (LC-MS/MS)

2D-PAGE and LC-MS/MS strategies were used to determine partial amino acid sequence of durian amylases. After cleaning up protein, crude durian extract was separated by IPG strip pH 3-11 size 7 cm at first-dimension. After separation with second dimension on 13 % SDS-PAGE, spots were analyzed for isoelectric point (pI) and molecular weight (MW) by using Image master 2D platinum program. 2D-PAGE analysis revealed that there were approximately 40 major protein spots after Coomassie brilliant blue staining (Figure 4.7). Most of protein spots were found at isoelectric point (pI) values ranging from 4.54 to 8.01 with molecular weight (MW) between 27 and 81 kDa (Table 4.7). Moreover, the protein spots were picked and identified by LC-MS/MS at Genome Institute, National Center for Genetic Engineering and Biotechnology Thailand. The ion spectra of peptides were searched against NCBI database using MS/MS Ion Search mode of MASCOT (http://www. matrixscience.com/search_form_select. html). The identified protein spots were shown on Table 4.8 and 4.9. There were organized into 9 groups (Figure 4.8 and Table 4.9). The first group was carbohydrate metabolism involving molecules such as enolase (spot no. 13), phosphoglycerate kinase (no. 23), fructose bisphosphate aldolase (no. 24 and 25), oxidoreductase (no. 36) and granule bound starch synthase (no. 31 and 32). The second group was protein metabolism involving molecules such as aminotransferase (spot no. 16-21), leucine aminopeptidase (no. 10), elongation fractor G (no. 8) and RNA polymerase C (no. 11 and 12). The third group were those concerning protein folding processes such as heat shock protein Hsp70 (spot no. 1) and 60-kDa chaperonin (no. 3). The fourth group was those in secondary metabolism such as flavonoid glycosyltransferase (no. 28). The fifth group was those in lipid metabolism such as S-malonyltransferase [acyl-carrier protein] (spot no. 33). The sixth group was antioxidant enzymes such as glutathione reductase (spot no. 15) and isoflavone reductase (no. 34). The seventh group was cell wall hydrolases such as endo-1, 4-beta-glucanase (spot no. 38) and glycosyl hydrolase family 10 protein (no. 5). The eighth group was ripening process enzyme such as ethylene responsive protein (no. 35). Moreover, The ninth group was other group (not identified) such as spot no. 4, 6, 7, 9, 14, 22, 26, 27, 29, 30, 37, 39 and 40. Interestingly, protein metabolism group

showed high proportions than other groups, followed by inferior carbohydrate metabolism (Figure 4.8). However, no spots were identified as amylase. It implied that amylase should not be major protein in Mon Thong durian extract.

However, 2D-PAGE and LC-MS/MS strategies were used to identify amylase spot and determine partial amino acid sequence of amylases. Crude durian was precipitated with ammonium sulphate at 70% saturation. Then, the precipitated protein was performed by 2D-PAGE technique. The protein spots were analyzed by Image master 2D platinum program. Seven protein spots at molecular weight around 45 kDa (Figure 4.9) were picked and analyzed by LC-MS/MS. They showed high homologies with known proteins. The identified protein spots were shown on Table 4.10. They were alpha amylase catalytic region (KP 1), fructose-bisphosphate aldolase (KP 2), malate dehydrogenase (KP 3), auxin-induced protein (KP 4), peroxidase precursor (KP 5), endochitinase 1 (KP 6) and heat shock cognate 70 kDa protein (KP 7).

To confirm the identification of amylase in Mon Thong durian, crude durian was performed by Zymograhic method. The clear transparent exhibited band at 45 kDa comparing with protein band in stained coomassie blue gel (Figure 4.10). Six protein bands showed molecular weight around 45 kDa (Figure 4.10, lane 1 and 2) and were picked to analyze by LC-MS/MS. The identified protein bands were shown on Table 4.11. They were phosphoglycerate kinase (KI 1), beta-amylase (KI 2), chloroplast biotin carboxylase (KC 1), alpha amylase (KC 2), phosphoglycerate kinase (KC 3) and glyceraldehyde-3-phosphate dehydrogenase (KC 4).

Interestingly, the alpha amylase (Figure 4.9, KP 1) was found in crude durian extract. The alpha amylase showed molecular weight 45 kDa and pI 6.51, with IATVLPDK as partial amino acid sequence. The clear transparent band from zymogram gel was identified as alpha amylase (Figure 4.10, KC 2) and beta amylase (Figure 4.10, KI 1). They both have molecular weight of 45 kDa.

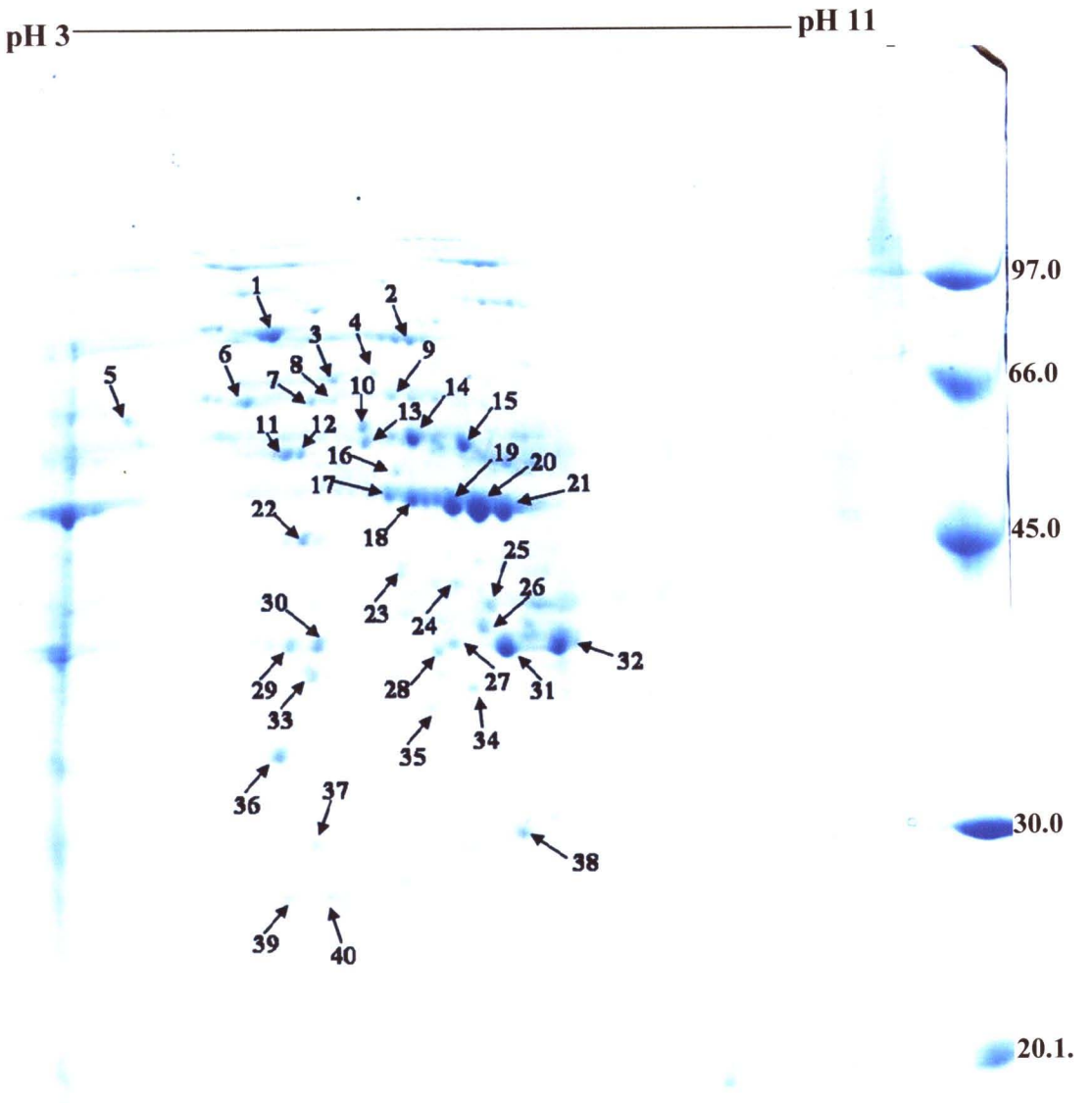

Figure 4.7 Representative 2D-PAGE profile of protein in Mon Thong durian (*D. zibethinus* Murr. cv. Mon Thong) (IPG strip pH 3-11 size 7 cm).

Table 4.7 Molecular weight (MW) and Isoelectric point (pI) of protein spots were obtained from Image master 2D platinum program.

Spot ID	pI	MW	Intensity	3D-View
1	5.72	81	61.8438	
2	6.84	80	41.6704	
3	6.20	69	25.6232	
4	6.53	71	18.1402	
5	4.54	62	21.3309	
6	5.50	65	39.6274	
7	6.03	65	25.2314	
8	6.21	65	15.8498	
9	6.67	66	22.7345	
10	6.45	60	31.9226	
11	5.82	51	40.2381	
12	5.94	57	30.1500	
13	6.47	58	34.7956	
14	6.86	58	68.3153	

Table 4.7 Molecular weight (MW) and Isoelectric point (pI) of protein spots were obtained from Image master 2D platinum program (Cont.).

Spot ID	pI	MW	Intensity	3D-View
15	7.28	58	63.7848	
16	6.73	54	18.8664	
17	6.66	51	43.0002	
18	6.85	51	62.2943	
19	7.18	50	83.0035	
20	7.40	79	97.8489	
21	7.58	49	84.9180	
22	5.94	46	33.8725	
23	6.73	43	15.0879	
24	7.18	43	17.4498	
25	7.46	41	25.9486	
26	7.40	40	24.9560	
27	7.15	39	24.2899	
28	7.04	39	21.8758	

Table 4.7 Molecular weight (MW) and Isoelectric point (pI) of protein spots were obtained from Image master 2D platinum program (Cont.).

Spot ID	pI	MW	Intensity	3D-View
29	5.84	39	24.0094	
30	6.06	39	31.9001	
31	7.59	39	79.2985	
32	8.01	39	73.3305	
33	6.02	37	21.4650	
34	7.32	37	19.7407	
35	6.99	36	14.0770	
36	5.74	33	32.9730	
37	6.04	29	12.9975	
38	7.72	30	30.0253	
39	5.83	27	12.5077	
40	6.15	27	15.3496	

Table 4.8 Protein identifications of protein in Mon Thong durian were obtained from LC-MS/MS analysis.

Spot ID	Protein annotation [organism]	Accession number	M_r/ pI/theor.	M_r/ pI/exp.	Peptide sequence	Mascot score
Carbohydrate metabolism						
13	Enolase [*Oryza sativa* Japonica Group]	gi|780372	48.30/ 5.42	58/ 6.47	K.FRAPVEPY.K	26
23	Phosphoglycerate kinase [wheat]	gi|129916	42.15/ 5.64	43/ 6.73	K.ELDYLVGAVANPK.K	76
24	Fructose-bisphosphate aldolase [*Oryza sativa* japonica cultivar]	ALF_ORYSJ	39.24/ 6.96	43/ 7.18	K.ARAAFLTR.C	16
25	fructose-bisphosphate aldolase, putative [*Ricinus communis*]	gi|255575381	38.90/ 6.57	41/ 7.46	K.VAPEVVAEYTVR.A	74
36	putative oxidoreductase [*Oryza sativa* Japonica Group]	gi|22093783	49.86/ 8.30	33/ 5.74	R.LPAGSVRLNSR.A	27
31	granule-bound starch synthase [*Capillipedium parviflorum*]	gi|3493003	27.84/ 5.38	39/ 7.59	K.KKFEGMLMSAEEK.Y K.KKFENMLMSAEEK.Y	31
32	granule-bound starch synthase [Capillipedium parviflorum]	gi|3493003	27.84/ 5.38	39/ 8.01	K.KKFENMLMSAEEK.Y	29

Table 4.8 Protein identifications of protein in Mon Thong durian were obtained from LC-MS/MS analysis (Cont.).

Spot ID	Protein annotation [organism]	Accession number	M_r/ pI/theor.	M_r/ pI/exp.	Peptide sequence	Mascot score
Protein metabolism						
16	Putative aminotransferase [*Oryza sativa* japonica cultivar]	Q65WV6_ORY	42.62/ 6.54	54/ 6.73	K.LQENAFVVGSYLK.E	81
17	Putative aminotransferase [*Oryza sativa* japonica cultivar]	Q65WV6_ORY	42.62/ 6.54	51/ 6.66	K.LQENAFVVGSYLK.E	81
18	Putative aminotransferase [*Oryza sativa* japonica cultivar]	Q65WV6_ORY	42.62/ 6.54	51/ 6.85	K.LQENAFVVGSYLK.E	91
19	Putative aminotransferase [*Oryza sativa* japonica cultivar]	Q65WV6_ORY	42.62/ 6.54	50/ 7.18	K.LQENAFVVGSYLK.E	143
20	Putative aminotransferase [*Oryza sativa* japonica cultivar]	Q65WV6_ORY	42.62/ 6.54	49/ 7.40	K.LQENAFVVGSYLK.E	94
21	Putative aminotransferase [*Oryza sativa* japonica cultivar]	Q65WV6_ORY	42.62/ 6.54	49/ 7.58	K.LQENAFVVGSYLK.E	80
10	leucine aminopeptidase [Solanum tuberosum]	gi|21487	58.60/ 5.75	60/ 6.45	K.GLTFDSGGYNLK.T	75

Table 4.8 Protein identifications of protein in Mon Thong durian were obtained from LC-MS/MS analysis (Cont.).

Spot ID	Protein annotation [organism]	Accession number	M_r/ pI/theor.	M_r/ pI/exp.	Peptide sequence	Mascot score
8	Elongation factor G, putative [Ricinus communis]	gi|255602381	46.48/ 5.75	65/ 6.21	R.QNVGKTVLVR.A	44
11	RNA polymerase C [Lonicera macranthoides]	gi|290585314	18.12/ 8.64	51/ 5.82	R.VAKSQIREK.E	28
12	RNA polymerase C [Lonicera macranthoides]	gi|290585314	18.12/ 8.64	51/ 5.82	R.VAKSQIREK.E	28
Secondary metabolism						
28	flavonoid glycosyltransferase [*Veronica persica*]	gi|226235168	50.51/ 5.84	39/ 7.04	K.GMFFR.H	9
Lipid metabolism						
33	[acyl-carrier protein] S- malonyl transferase [*Brassica napus*]	gi|5139348	37.10/ 7.08	37/ 6.02	K.SAMVSIIGLDSEK.V R.QVTSPVQWETTVK.T	150
Antioxidant enzymes						
15	Glutathione reductase [*Pisum sativum*]	GSHRC_PEA	54.20/ 6.59	58/ 7.28	R.TNIPSIWAVGDVTNR.L K.IASPNEVEVTQLDGTK.L	172

Table 4.8 Protein identifications of protein in Mon Thong durian were obtained from LC-MS/MS analysis (Cont.).

Spot ID	Protein annotation [organism]	Accession number	M_r/ pI/theor.	M_r/ pI/exp.	Peptide sequence	Mascot score
34	isoflavone reductase homolog 1 [*Glycine max*]	gi|6573169	33.98/ 5.75	37/ 7.32	K.ILVIGGTGYIGK.F	89
Ripening process enzyme						
35	ethylene-responsive protein 2 [Para rubber tree]	S71492	20.79/ 7.68	36/ 6.99	R.IPFVCGCR.N R.NMDDDEVFSFAK.K	133
Cell wall hydrolases						
5	Glycosyl hydrolase family 10 protein [*Oryza sativa* (japonica cultivar-group)]	gi|108706713	63.05/ 6.12	62/ 4.54	M.KVFTGK.L	13
38	endo-1,4-beta-glucanase, putative [Ricinus communis]	gi|255537593	57.36/ 8.14	30/ 7.72	K.FAGVQTLIAK.E	78

Table 4.8 Protein identifications of protein in Mon Thong durian were obtained from LC-MS/MS analysis (Cont.).

Spot ID	Protein annotation [organism]	Accession number	M_r/ pI/theor.	M_r/ pI/exp.	Peptide sequence	Mascot score
Protein folding						
1	Heat shock protein Hsp70 [*Medicago truncatula*]	gi|124360342	69.2/ 6.38	81/ 5.72	K.ATAGNTHLGGEDFDNR.T	75
3	60-kDa chaperonin [Arabidopsis thaliana]	gi|553036	19.84/ 4.75	69/ 6.20	R.GYISPYFVTDSEK.M	66
Other (Not identified) Spot ID 2, 4, 6, 7, 9, 14, 22, 26, 27, 29, 30, 37, 39 and 40						

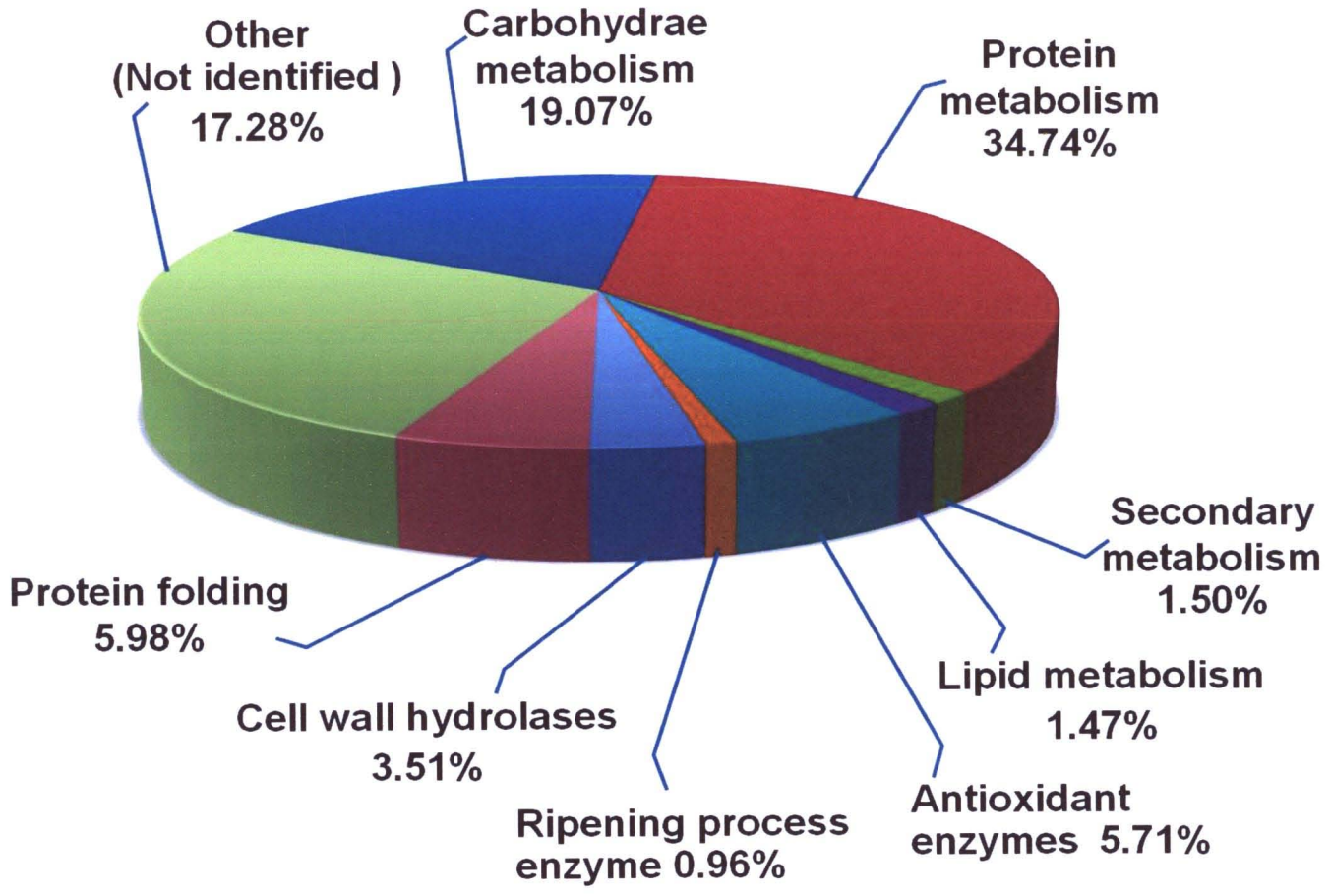

Figure 4.8 Functional classification and the correspondence percentage of each class were indicated.

Table 4.9 Protein identifications of protein were obtained from LC-MS/MS analysis.

Group	Protein name	Spot no.
Carbohydrate metabolism	1. Enolase 2. Phosphoglycerate kinase 3. Fructose bis phosphate aldolase 4. Oxidoreductase 5. Granule bound starch synthase	13 23 24, 25 36 31, 32
Protein metabolism	1. Aminotransferase 2. Leucine aminopeptidase 3. Elongation factor G 4. RNA polymerase C	16, 17, 18, 19, 20, 21 10 8 11, 12
Protein folding	1. Heat shock protein Hsp70 2. 60-kDa chaperonin	1 3
Secondary metabolism	1. Flavonoid glycosyltransferase	28
Lipid metabolism	1. S-malonyltransferase [acyl-carrier protein]	33
Antioxidant enzymes	1. Glutathione reductase 2. Isoflavone reductase	15 34
Cell wall hydrolases	1. Endo-1,4-beta-glucanase 2. Glycosyl hydrolase family 10 protein	38 5
Ripening process enzyme	1. Ethylene responsive protein	35
Other (not identified)	-	4, 6, 7, 9, 14, 22, 26, 27, 29, 30, 37, 39, 40

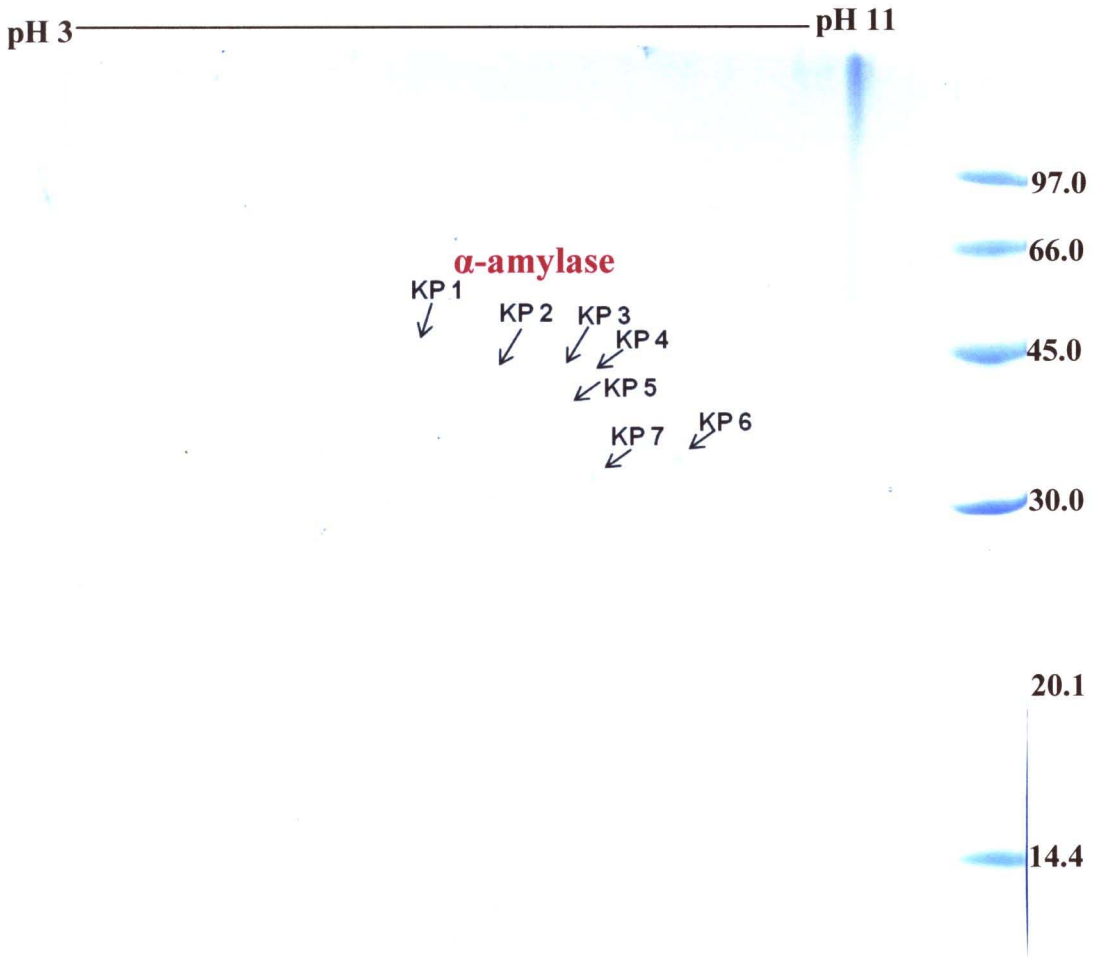

Figure 4.9 Representative 2D gel profile of precipitated protein in Mon Thong durian (*D. zibethinus* Murr. cv. Mon Thong).

Table 4.10 Protein identifications of precipitated protein in Mon Thong durian were obtained from LC-MS/MS analysis.

Spot ID	Protein annotation [organism]	Accession number	M_r/ pI/theor.	M_r/ pI/exp.	Peptide sequence	Mascot score
KP 1	Alpha amylase catalytic region [*Fervidobacterium nodosum* Rt17-B1]	gi|154248920	8.52 /55.24	6.51 /45	IATVLPDK	37
KP 2	Fructose-bisphosphate aldolase, putative [Ricinus communis]	gi|255575381	6.57 /38.90	7.19 /43	K.ANSEATLGTYK.G K.GILAADESTGTIGK.R K.VAPEVVAEYTVR.A R.LSSINVENVEENRR.A	251
KP 3	Malate dehydrogenase [*Citrullus lanatus*]	MDHM_CITLA	8.88 /36.41	7.76 /43	R.DDLFNINAGIVK.S K.ALEGSDVVIIPAGVPR.K	126
KP 4	Auxin-induced protein [*Vigna radiata*]	gi|1184121	5.35 /34.16	8.04 /43	K.FDVVYDAVGQSER.A	56
KP 5	Peroxidase precursor [*Raphanus sativus var. niger*]	gi|49609452	8.35 /38.80	7.78 /39	R.VFDIIR.R	43
KP 6	Endochitinase 1 [*Gossypium hirsutum*]	CHI1_GOSHI	6.28 /35.6	8.66 /35	R.GPMQLSWNYNYGQCGR.A R.GPMQLSWNYNYGQCGR.A R.VPGYGVITNIINGGLECGK.G R.AIGVDLLNNPDLLSSDPTISFK.S	155

Table 4.10 Protein identifications of precipitated protein in Mon Thong durian were obtained from LC-MS/MS analysis (Cont.).

Spot ID	Protein annotation [organism]	Accession number	M_r/ pI/theor.	M_r/ pI/exp.	Peptide sequence	Mascot score
KP 7	Heat shock cognate 70 kDa protein [*Vitis vinifera*]	gi|225434984	5.17 /71.59	7.96 /33	R.FSDSSVQSDIK.L R.VEIIANDQGNR.T K.DAGVIAGLNVMR.I R.MVNHFVQEFK.R R.EIAEAYLGSTVK.N R.MVNHFVQEFK.R R.TTPSYVAFTDTER.L R.IINEPTAAAIAYGLDK.K K.NQVAMNPINTVFDAK.R K.ATAGDTHLGGEDFDNR.M K.ATAGDTHLGGEDFDNR.M K.NQVAMNPINTVFDAK.R K.NAVVTVPAYFNDSQR.Q R.IINEPTAAAIAYGLDKK.A	733

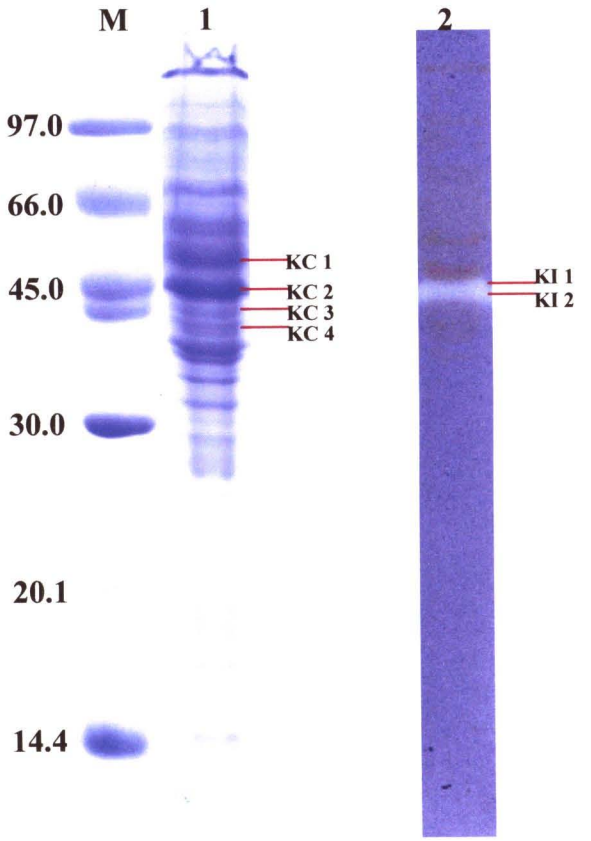

Figure 4.10 Zymograhic analysis of crude durian of Mon Thong durian (*D. zibethinus* Murr. cv. Mon Thong) on 13% acrylamide gel containing 1% soluble starch. Crude durian was loaded 20 µg/lane. Lane M: Low molecular weight marker; Lane 1: Crude durian with out β- mercaptoethanol on native gel was stained with Coomassie brilliant blue R 250, Lane 2: Crude durian without β-mercaptoethanol on activity gel was stained with activity staining solution (1% acetic acid containing 10 mM I_2 and14 mM KI

Table 4.11 Protein identifications of crude durian of Mon Thong durian were obtained from LC-MS/MS analysis.

Spot ID	Protein annotation [organism]	Accession number	M_r/ theor.	M_r exp.	Peptide sequence	Mascot score
KI 1	Phosphoglycerate kinase [*Gossypium hirsutum*]	gi|2257598	23.65	45	K.IGVIESLLAK.V K.FAAGTEAIAK.K K.GVTTIIGGGDSVAAVEK.A	160
KI 2	Beta-amylase [*Arabidopsis thaliana*]	BAM1_ARATH	64.18	45	R.KAMKASLQALK.S	46
KC 1	Chloroplast biotin carboxylase [*Gossypium hirsutum*]	gi|145910320	59.17	50	K.QEDIVLR.G K.LIVWAPTR.E K.LILDIEDFR.N R.GSFYFMEMNTR.I R.ITSYLPSGGPFVR.M K.SEAAAAFGNDGVYLEK.Y K.LLEEAPSPALTPELRK.A R.ALDDTVITGVPTTIEYHK.L	505
KC 2	Catalytic region:Alpha amylase [*Chloroflexus aurantiacus* J-10-fl]	Q3DYP7_CHLAU	66.17	45	R.FPSLRR.L	57

Table 4.11 Protein identifications of crude durian of Mon Thong durian were obtained from LC-MS/MS analysis (Cont.).

Spot ID	Protein annotation [organism]	Accession number	M_r/ theor.	M_r exp.	Peptide sequence	Mascot score
KC 3	Phosphoglycerate kinase [*Gossypium hirsutum*]	gi|211906450	42.29	44	K.IGVIESLLAK.V K.YSLKPLVPR.L K.KPFAAIVGGSK.V R.LSELLGVEVK.M K.LDLATSLLEK.A K.QLPGVLALNDA. K.SVGDLKEADLK.G K.ELDYLVGAVANPK.K K.MSHISTGGGASLELLEGK.Q	613
KC 4	Glyceraldehyde-3-phosphate dehydrogenase [*Ananas comosus*]	gi|312192239	36.69	42	R.VVDLIR.H K.LTGMAFR.V K.TLLFGEK.A K.LTGMAFR.V K.VLPALNGK.L K.VIISAPSK.D K.IGINGFGR.I K.AVTVFGIR.N K.AGIALNDNFVK.F K.DAPMFVVGVNEK.E K.DAPMFVVGVNEK.E R.AASFNIIPSSTGAAK.A K.EYKPDIDIVSNASCTTNCLAPLAK.V	655

4.7 Purification of amylase from Mon Thong durian

For further characterization of durian amylase, it is necessary to make the enzyme pure enough for minimize the confusion with other components in the extracts. Purification started from the easiest step, precipitation with ammonium sulphate and further with affinity column chromatography. The steps had been finished by ionic exchange chromatography. All purification steps were performed at 4 °C.

4.7.1 Ammonium sulphate fractionation

The crude durian extract was precipitated with varying percentage of concentration ammonium sulphate from 30 to 70 %. The precipitated fraction of 70% ammonium sulphate saturation showed higher specific activity (1.39 Unit/mg of protein) than 30-60% ammonium sulphate saturation (Table 4.12 and Figure 4.11). In addition, the protein pattern showed around 14.4-98 kDa (Figure 4.12). The amylase activity showed highest activity at 70 % ammonium sulphate saturation and clear transparent band exhibited at molecular weight 45 kDa in Zymograhic method (Table 4.12 and Figure 4.12).

Table 4.12 Analysis of amylase after ammonium sulfate fractionation

Ammonium sulphate (%)	Activity (Unit/ml)	Total activity (Unit)	Total protein (mg)	Specific activity (Unit/mg)	Purification fold
30	0.256	0.256	1.035	0.25	0.36
40	0.375	1.687	6.745	0.25	0.36
50	0.574	2.583	8.028	0.32	0.47
60	0.692	2.768	6.540	0.42	0.61
70	2.340	9.360	6.720	1.39	2.02

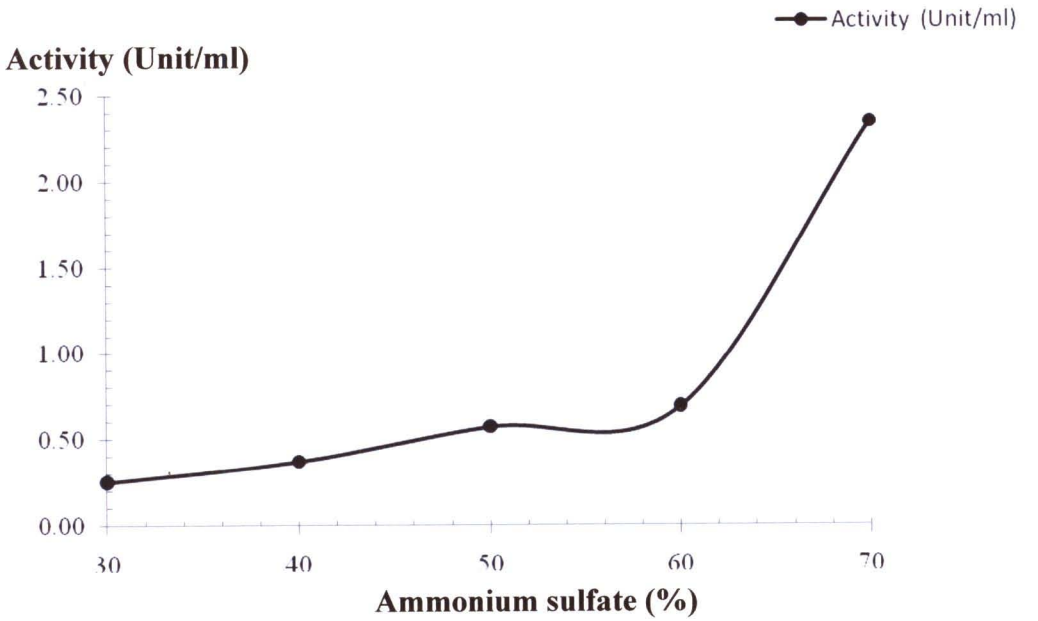

Figure 4.11 The ammonium sulfate fractionation of crude durian extract

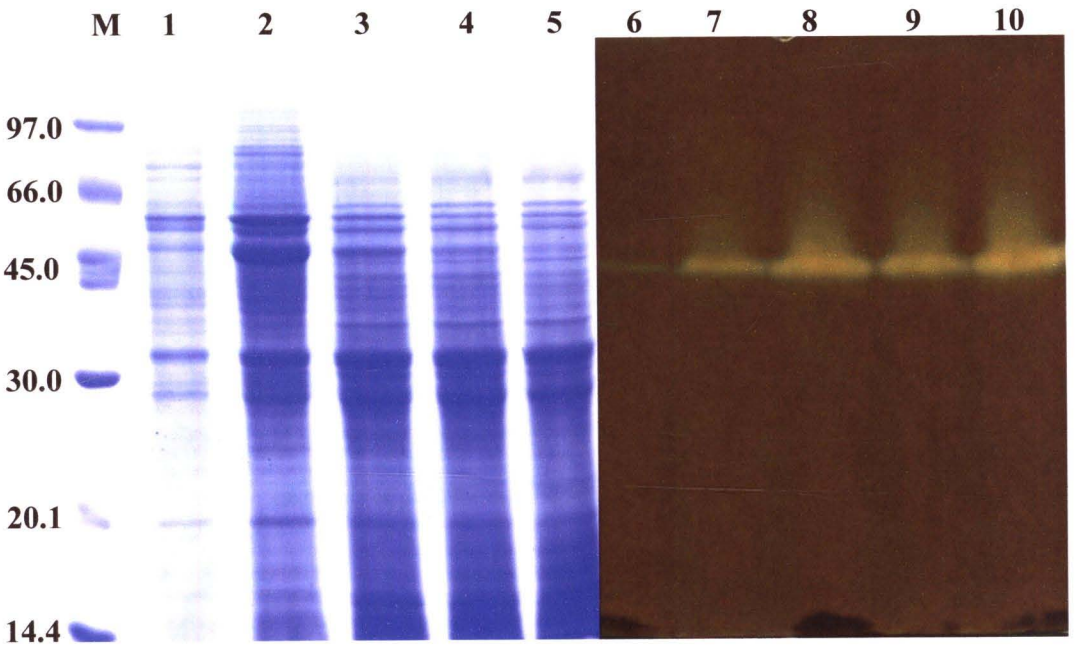

Figure 4.12 The protein pattern and amylase activity of ammonium sulfate fractionation (20 µg Protein) of crude durian extract. M = maker, Lane 1, 2, 3, 4 and 5 = 30, 40, 50, 60 and 70 % ammonium sulphate saturation were separated by 13% SDS-PAGE with Coomassie brilliant blue staining, Lane 6, 7, 8, 9 and 10 = 30, 40, 50, 60 and 70 % ammonium sulphate saturation were analyzed by Zymograhic method.

4.7.2 Purification of amylase from Mon Thong durian using Epoxy-activated Sepharose 6B affinity chromatography

Crude durian extract was purified by using Epoxy-activated Sepharose 6B affinity chromatography (epoxy-activated sepharose 6B ligated with β-cyclodextrin). This affinity column is specific to amylase, because β-cyclodextrin is competitive inhibitor of amylase. The specificity occurs between the interaction of cyclodextrin to active site and substrate binding site of amylase (Hamilton et al., 2000). The affinity of cyclodextrin could be explained by the fact that cyclodextrin structurally resemble the helical from of amylose.

After purification, chromatogram exhibited two protein peaks at absorbance 280 nm (Figure 4.13, black line). Then, the fraction was assayed amylase activity by DNS method. Chomatogram of amylase activity revealed single peak, after eluted by β-cyclodextrin (Figure 4.13 red line). Protein peaks at fraction no. 4, 29, 30, 31 and 32 separated by SDS-PAGE stained with coomassie brilliant blue and silver stain kit were shown in Figure 4.14 and 4.15, respectively. The fraction no. 2 contained several protein bands in Figure 4.14 and 4.15. The fraction number 29, 30, 31 and 32 were not appear protein peak in Figure 4.14, but exhibited four protein bands in Figure 4.15. Moreover, the fractions were determined amylase activity using Zymograhic method which was shown in Figure 4.16. The eluted fraction that had amylase activity was shown in protein peak at fraction number 29, 30, 31 and 32. The purity of enzyme showed 313.04 fold at 216.00 of specific activity (Table 4.13). Finally, the active peak (A1) was pooled and concentrated.

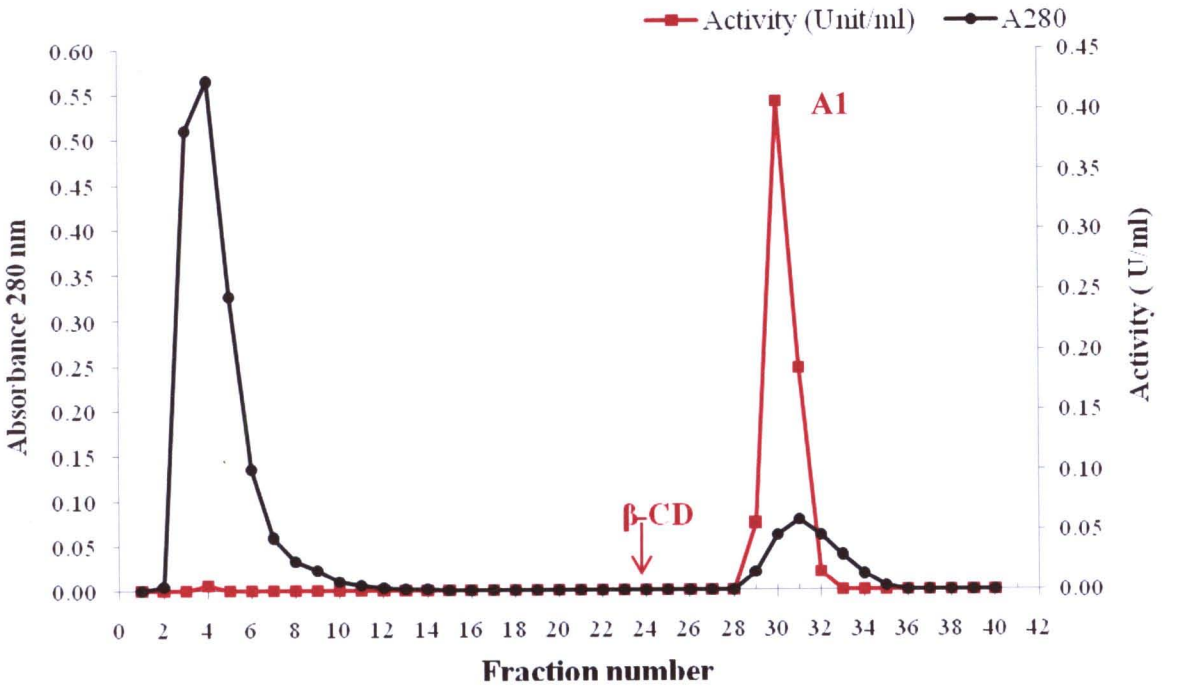

Figure 4.13 Chromatogram of crude durian purification using Epoxy-activated Sepharose 6B affinity chromatography. Symbols: ●Absorbance 280 nm; ■ Amylase activity.

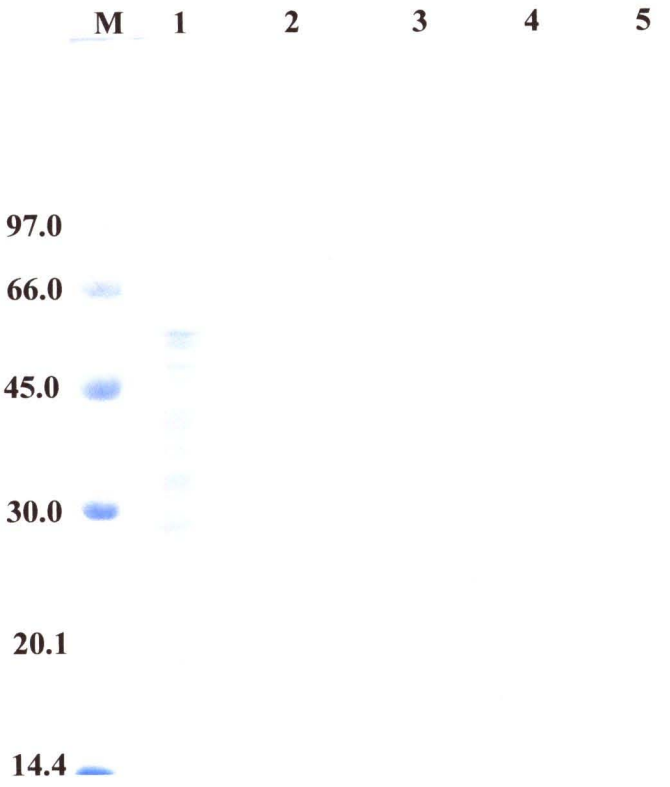

Figure 4.14 Protein bands by 13% SDS-PAGE, staining with coomassie brilliant blue of protein peaks at fraction number 4, 29, 30, 31, 32 (number 1, 2, 3, 4 and 5) from Epoxy-activated Sepharose 6B affinity chromatography. (M = maker)

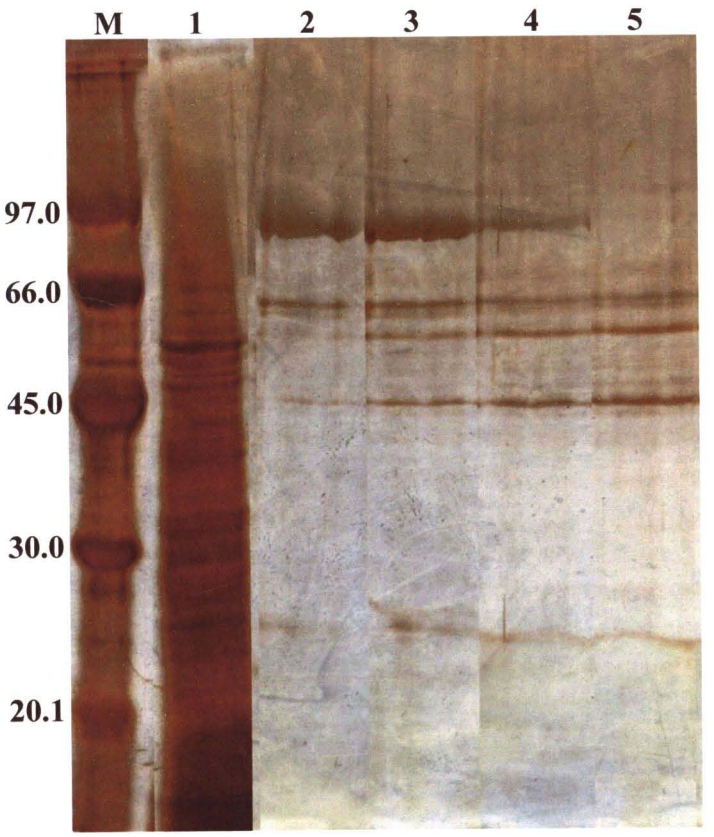

Figure 4.15 Protein bands by 13% SDS-PAGE, staining with silver of protein peaks at fraction number 4, 29, 30, 31, 32 (number 1, 2, 3, 4 and 5) from Epoxy-activated Sepharose 6B affinity chromatography. (M = maker)

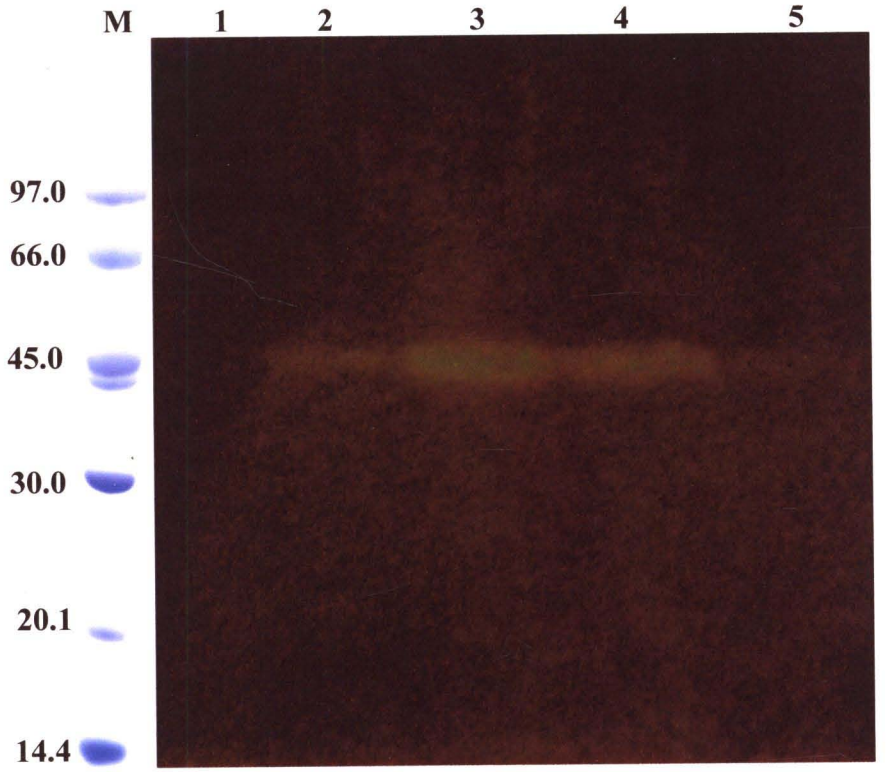

Figure 4.16 Amylase activity by Zymograhic method of protein peaks at fraction number 4, 29, 30, 31 and 32 (number 1, 2, 3, 4 and 5) from Epoxy-activated Sepharose 6B affinity chromatography. (M = maker)

Table 4.13 Purification of amylase from Mon Thong durian pulp (386 g)

Purification stages	**Activity (Unit/ml)**	**Total activity (Unit)**	**Total protein (mg)**	**Specific activity (Unit/mg)**	**Purification fold**	**Yield (%)**
Crude	5.42	271.00	394.00	0.69	1.00	100.00
Precipitation 70% ammonium sulfate	2.34	46.80	33.60	1.39	2.02	17.27
Affinity chomatography	0.216	2.808	0.013	216.00	313.04	1.04

4.7.3 Purification of amylase from Mon Thong durian using DEAE Toyopearl Anion Exchange chromatography

Although affinity column had been used, impurity still appeared on SDS-PAGE analysis. Therefore, the next step of purification using anionic column chromatography was necessary. The pooled active fraction from Epoxy-activated Sepharose 6B affinity chromatography was further subjected to DEAE Toyopearl Anion Exchange chromatography. Chromatogram revealed two protein peaks (Figure 4.17). The fractions were assayed amylase activity using DNS method and Zymograhic method which showed in Figure 4.17 (red line) and 33, respectively. Purified protein contained amylase activity peak was separated by SDS-PAGE and stained with silver stain kit (Figure 4.18). Fraction no. 18 was eluted at 0.16 M NaCl concentration which had high amylase activity, showed two bands approximately 65 and 66 kDa. No any interested band (45 kDa) appeared from SDS-PAGE, however, amylase activity exhibited on Zymograhic method. The purity of enzyme showed 340.04 fold at 234.85 of specific activity (Table 4.14). The active peak was pooled and concentrated for further investigation.

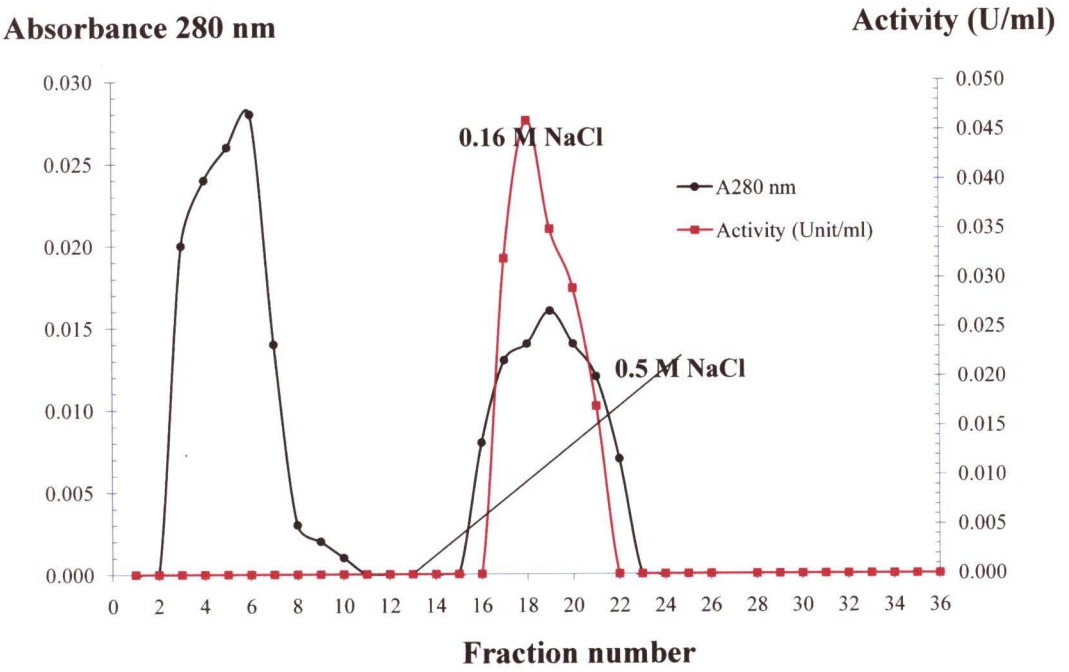

Figure 4.17 Chromatogram of crude durian purification using DEAE Toyopearl Anion Exchange chromatography. ● Symbols: Absorbance 280 nm; ■ Amylase activity

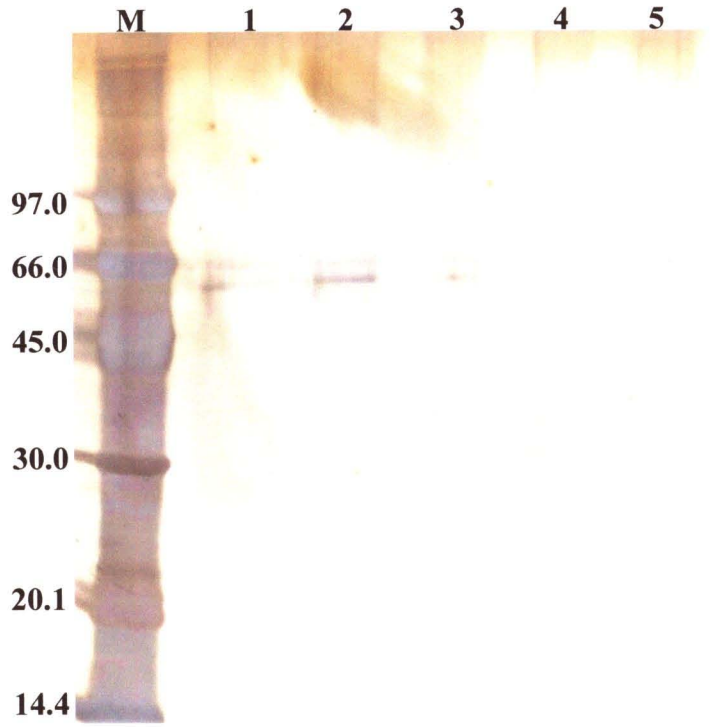

Figure 4.18 Protein bands by 13% SDS-PAGE, staining with silver of protein peaks at fraction number 17, 18, 19, 20 and 21 (number 1, 2, 3, 4 and 5) from DEAE Toyopearl Anion Exchange chromatography. (M = maker)

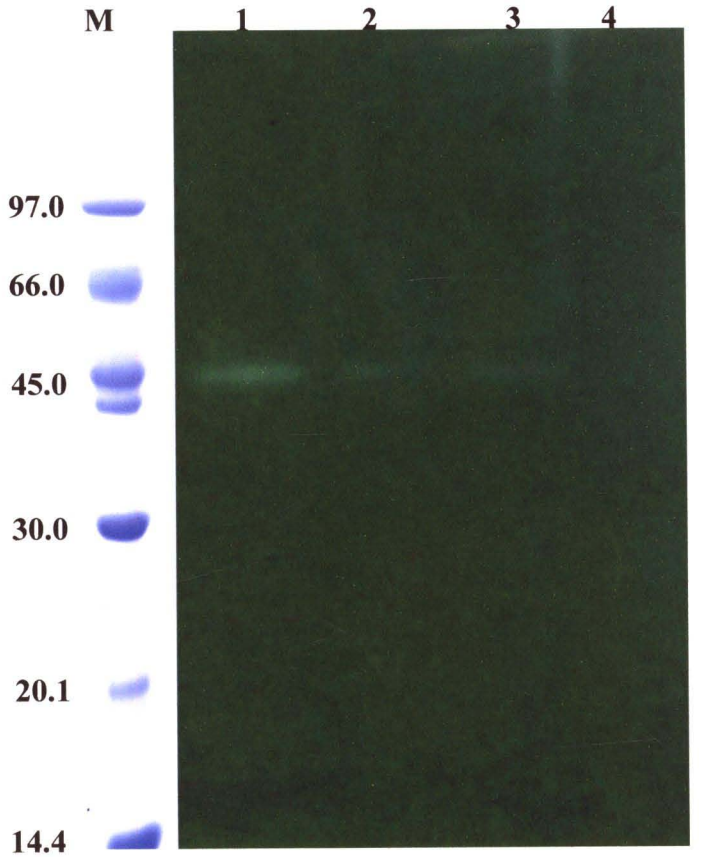

Figure 4.19 Amylase activity by Zymograhic method of protein peaks at fraction number 18, 19, 20 and 21 (number 1, 2, 3 and 4) from DEAE Toyopearl Anion Exchange chromatography. (M = maker)

Table 4.15 Summary purification of amylase from Mon Thong durian pulp (386 g)

Purification stages	**Activity (Unit/ml)**	**Total activity (Unit)**	**Total protein (mg)**	**Specific activity (Unit/mg)**	**Purification fold**	**Yield (%)**
Crude	5.42	271.00	394.00	0.69	1.00	100.00
Precipitation 70% ammonium sulfate	2.34	46.80	33.60	1.39	2.02	17.27
Affinity chomatography	0.216	2.808	0.013	216.00	313.04	1.04
DEAE Toyopearl Anion Exchange chromatography	0.155	0.16	0.00066	234.85	340.36	0.06

4.8 Enzyme characterization in durian crude using DNS method

4.8.1 Optimal pH determination

For further use, especially for application use in the industry, characterization of enzyme was unavoidable. In this study, optimal pH and optimal temperature were investigated. The optimum pH of amylase from durian crude extract was determined at various pH buffer at 37 °C, starch as substrate using DNS method. The pH activity profile was shown in Figure 4.20. The highest activity was observed at pH 7.0. The enzyme was highly active between pH 6.0 and 8.0. The relative activities at pH 6, 7 and 8 (phosphate buffer) were about 97.75, 100.00 and 86.07 %, respectively. However, the activity not only decreased gradually to 9.95 % at pH 10 (alkaline pH). But also decreased rapidly to 5.97 % at pH 4 (acidic pH).

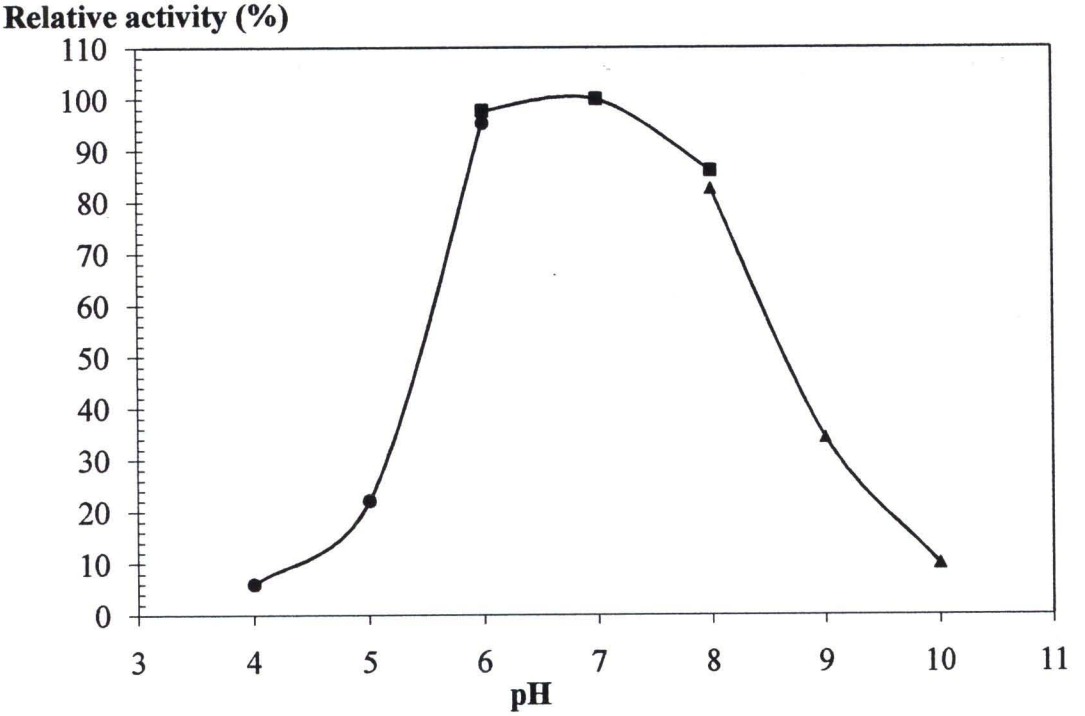

Figure 4.20 Effect of pH on the activity of crude durian. The amylase activity was determined in different pH from 4 to 10 by DNS method at 37 °C. Buffer used were; 0.1 M sodium acetate buffer (●), 0.1 M potassium phosphate buffer (▲), and 0.1 M Tris-HCl buffer (■).

4.8.2 Optimum temperature determination

The optimum temperature of amylase from durian crude determined at different temperatures (pH 7.0) starch as substrate using DNS method. The temperatures activity profile was shown in Figure 4.21. The enzyme was active at widely temperatures from 30 °C to 90 °C and had optimum activity at 40 °C. In addition, the amylase was highly active between 37 °C to 70 °C and highly relative activities in range 87.72 % to 90 % of that measured at 40 °C.

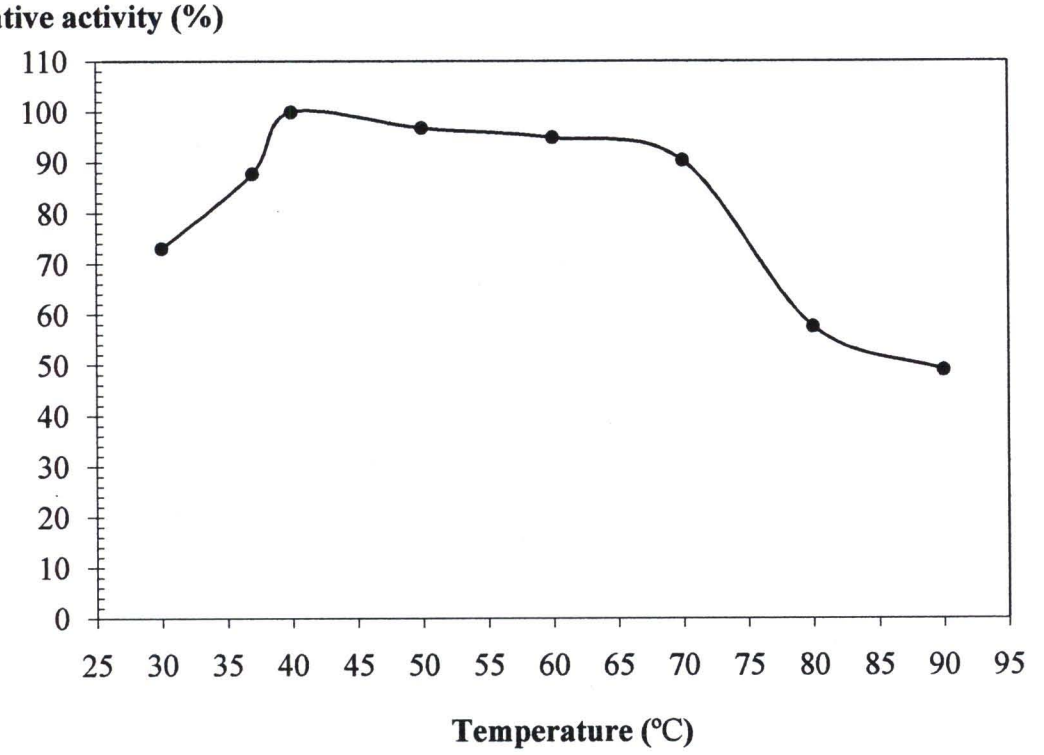

Figure 4.21 Effect of temperature on the activity of crude durian. The amylase activity was determined in different temperature from 30 to 90 °C by DNS method.

4.9 Enzyme characterization using Zymograhic method

4.9.1 Optimum pH determination

Although crude extract clearly showed amylase activity, crude extracts composed of many components that was unpredictable for any effects affecting enzyme activity. Therefore, the most reliable way was to determine the activity of amylase using Zymograhic method, that is quite specific for amylase activity determination. The effect of pH on partial purified active fraction was studied by using starch as a substrate at various pH values at 37 °C. The activity of crude durian exhibited the clear transparent band at 45 kDa. The partial purified amylase showed optimum activity at pH 7 (Figure 4.22, lane 5). The activity of enzyme increased when pH condition was increased from pH 4 to 7. On the other hand, the activity of enzyme was dramatically decreased when pH condition was increased from pH 8 to 10.

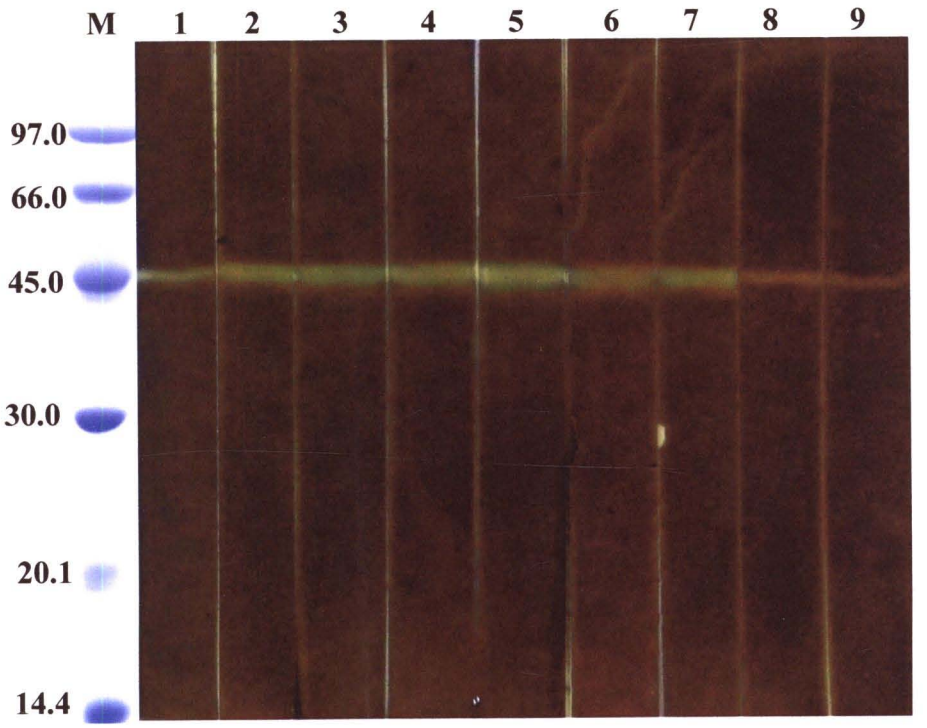

Figure 4.22 Effect of pH on the activity of partial purified amylase (0.01 µg Protein). The amylase activity was determined in different pH from 4 to 10 by Zymograhic method at 37 °C. Lane M: Low molecular weight marker; Lane 1,2,3 = sodium acetate buffer pH 4,5 and 6; Lane 4,5,6 = potassium phosphate buffer pH 6,7 and 8; Lane 7,8,9 = Tris-HCl buffer pH 8,9 and 10

4.9.2 Optimum temperature determination

The effect of temperature on partial purified active fraction was observed as shown in Figure 4.23. The partial purified amylase was highly active of assay over a wide range at 30 °C to 80 °C (incubated for 6 hr). The activity of enzyme was dramatically decreased when temperature condition was increased from 90 °C to 100 °C. In addition, the activity of amylase was determined by using incubating time for 15 min, 30 min, 1 hr, 3 hr and 6 hr at 90 °C and 100 °C (Figure 4.24). The result showed that amylase activity increased corresponding with incubation time at incubation time for 6 hr.

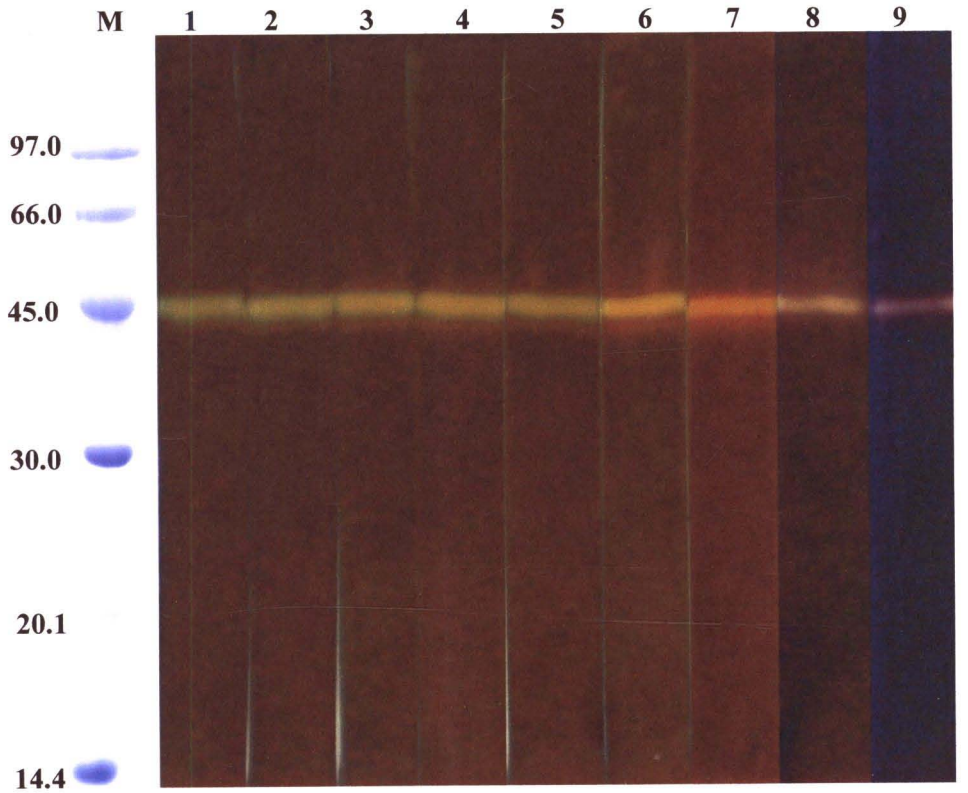

Figure 4.23 Effect of temperature on the activity of partial purified amylase (0.01 µg Protein). The amylase activity was determined in different temperature from 30 °C to 100 °C by Zymograhic method at pH 7.0. Lane M: Low molecular weight marker; Lane 1= 30 °C; Lane 2 = 37 °C; Lane 3 = 40 °C Lane 4 = 50 °C; Lane 5 = 60 °C; Lane 6 = 70 °C; Lane 7 = 80 °C; Lane 8 = 90 °C; Lane 9 = 100 °C

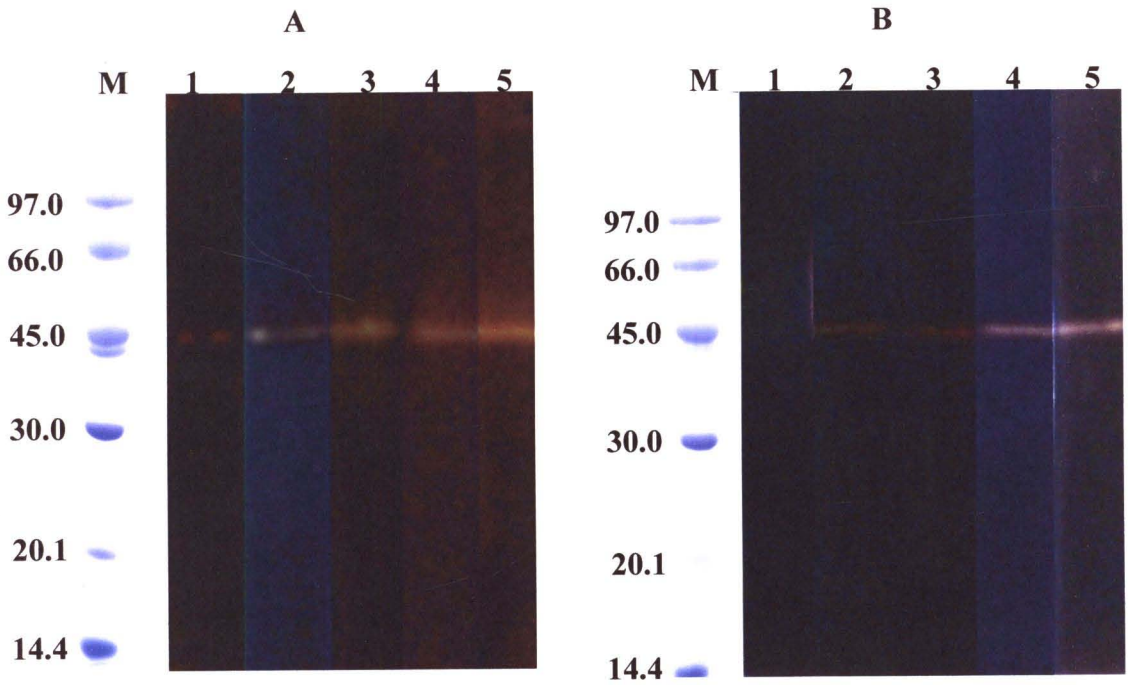

Figure 4.24 Effect of temperature on the activity of partial purified amylase (0.01µg Protein) at 90 °C and 100 °C. The amylase activity was determined at different time by Zymographic method at pH 7.0. Lane M: Low molecular weight marker; (A and B) Lane 1= 15 min, Lane 2 = 30 min Lane 3 = 1 hr; Lane 4 = 3 hr; Lane 5 = 6 hr

4.9.3 pH stability determination

The effect of pH on stability of partial purified amylase was studied by incubating enzyme for 30 min at 37 °C. The activity of enzyme remained highly activity after incubation with sodium acetate buffer pH 6, potassium phosphate buffer pH 6,7 and 8, tris-HCl buffer pH 8, 9 and 10. After incubation, the activity of enzyme showed clear transparent band equal to protein of control (Figure 4.25 Lane 1) However, the activity of amylase was not detected under strong acidic condition at pH 4 and 5 (Figure 4.25). The results show that this amylase was highly active over a wide range of pH 6 to 10.

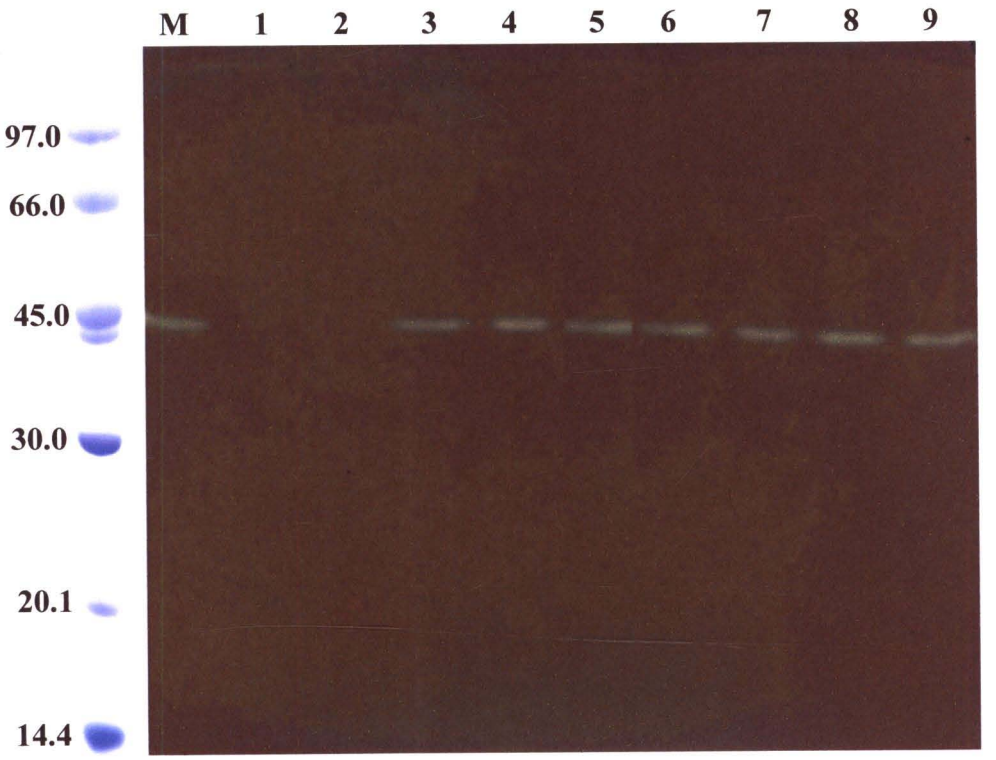

Figure 4.25 The pH stability of the partial purified amylase (0.005 µg Protein) was and the residual activity was measured by Zymograhic method at pH 7.0 and 37 °C. Crude enzyme was used as control (lane 1). Lane M: Low molecular weight marker ; Lane 1,2,3 = sodium acetate buffer pH 4, 5 and 6; Lane 4, 5 and 6 = potassium phosphate buffer pH 6,7 and 8; Lane 7,8,9 = Tris-HCl buffer pH 8,9 and 10

4.9.4 Heat stability determination

The thermal stability of the partial purified amylase was tested by incubating the enzyme for 30 min in the absence of substrate from 30 ºC to 100 ºC (Figure 4.26). The results showed that the activity of amylase was detected from 30ºC to 60 ºC. However, the activity was not detected when the temperature increased from 70 ºC to 100 ºC (Figure 4.26).

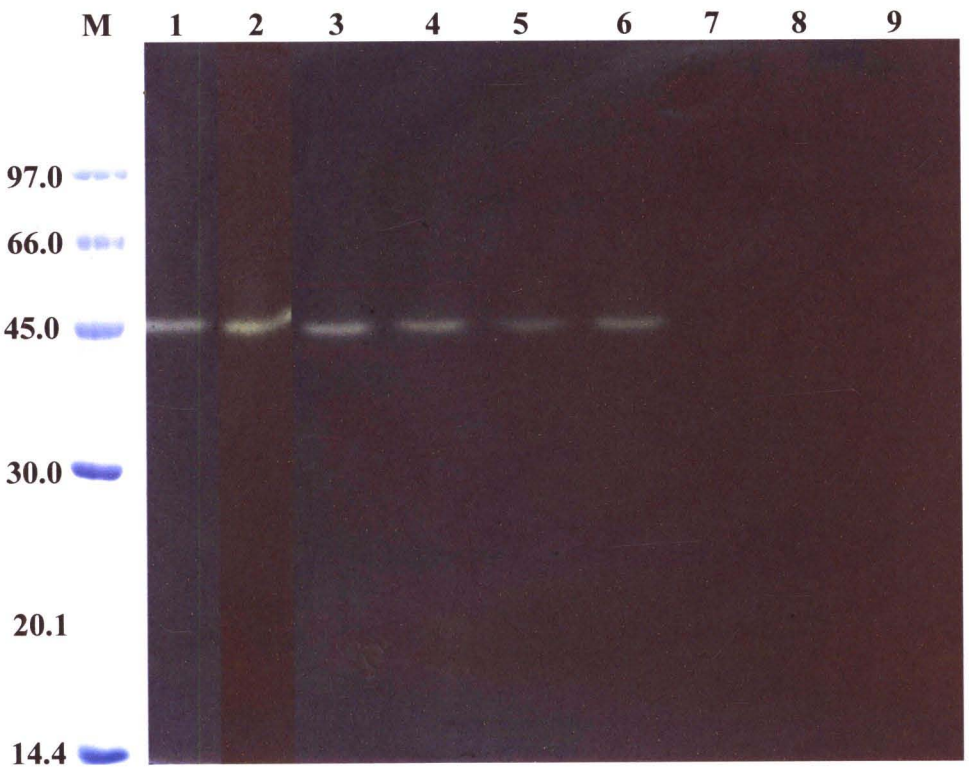

Figure 4.26 Effect of temperature on thermal stability of the partial purified amylase (0.005 µg Protein). The enzyme was pre-incubated in the absence of substrate from 30 ºC to 100 ºC and the residual activity was measured by Zymograhic method at pH 7.0 and 37 ºC. Crude enzyme was used as control. Lane M: Low molecular weight marker ; Lane 1= Control, Lane 2 = 30 ºC; Lane 3 = 37 ºC; Lane 4 = 40 ºC; Lane 5 = 50 ºC; Lane 6 = 60 ºC; Lane 7 = 70 ºC; Lane 8 = 80 ºC and Lane 9 = 90 ºC

4.9.4 Effect of metal ion and EDTA

For further application, especially in the industry, effects of metals affecting enzyme activity had to be investigated. Effect of mono and divalent metal ions on the partial purified amylase was determined by incubating the enzyme for 30 min at pH 7.0, 37 °C. The result shows that Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Na^{+} and K^{+} ions did not affect to the enzyme activity comparing with control (Figure 4.27). However, the amylase activity was completely abolished by EDTA (Figure 4.27), assuming of the important role of divalent ions for enzyme activity.

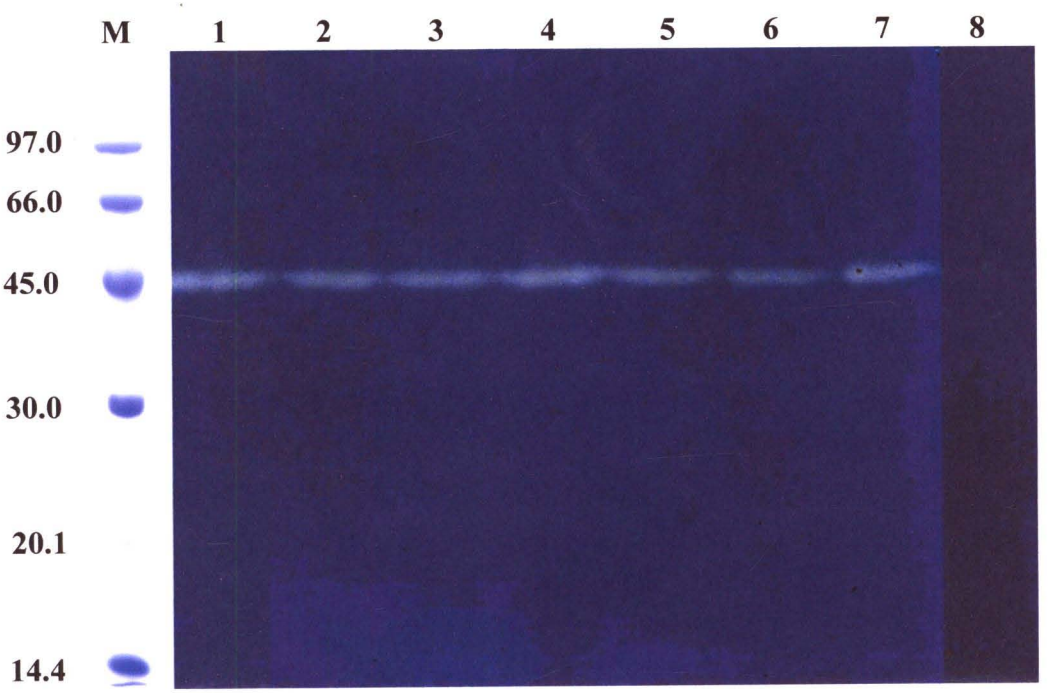

Figure 4.27 The effect of various metal ions and EDTA (5 mM) on the partial purified amylase activity. The activity of the enzyme was determined by incubating the enzyme in the presence of various metal ions and EDTA (5 mM) for 30 min at 37 °C and pH 7.0. All ions were used as chloride forms. The enzyme in the absence of various metal ions (5 mM) was as control. Lane M: Low molecular weight marker ; Lane 1= Control; Lane 2 = Ca^{2+}; Lane 3 = Mn^{2+}; Lane 4 = Zn^{2+}; Lane 5 = Co^{2+}; Lane 6 = Na^{+}; Lane 7 = K^{+}; Lane 8 = EDTA