CHAPTER IV

RESULTS AND DISCUSSION

The proteins in *Pithecellobium dulce* seeds were analyzed using all methods as described in the previous chapter. The results will be shown and discussed in two major parts of this chapter. The first part is the result and discussion of protein profiling of *Pithecellobium dulce* seeds using 2-DE and MS/MS. The other is the result and discussion of purification of bioactive protein from *Pithecellobium dulce* seeds.

4.1 Protein Profiling of *Pithecellobium dulce* Seeds Using 2-DE and MS/MS

The protein profile of *P. dulce* seeds has been reported in this section. This protein profile showed the information about the individual proteins involved in specific biological responses such as several proteins have been reported as protease inhibitors which are discussed more detail.

4.1.1 Protein Extraction and Precipitation

The protein yield of the protein extraction using Tris-HCl buffer containing protease inhibitors and TCA-cold acetone precipitation process from 3 g mature *P. dulce* seed is 5.8%. Total amount of crude protein is 174.4 mg. In section 2.2.3, the dried mass seeds contain 17.6% protein which higher than over all yield of crude protein obtained from fresh seeds (5.8%).

4.1.2 2-DE of Crude Protein from *Pithecellobium dulce* Seeds

Protein extract from *P. dulce* seeds (3 mg) was used to generate 2-DE protein map (Figure 4.1). The first dimensional separation, i.e., isoelectric focusing, was performed using a 24-cm, pH 3-10 NL IPG strip. The use of a broad pH range allowed simultaneous separation and visualization of both acidic and basic proteins,

rendering a reference map of *P. dulce* seed protein. The coomassie stained 2-DE protein map revealed 317 protein spots and was dominated by about a dozen highly abundant proteins clustered in the region of p*I* 5-6 and with molecular masses of 55 to 97 kDa. These protein spots accounted for more than 50% of the total protein mass in the seeds as revealed by 2-DE. This is in contrast with other legume seed proteins such as soybean seeds and *M. truncatula* seeds (model legume plant) which do not contain such substantial protein cluster in the corresponding region in *M. truncatula* seed or soybean 2-DE maps.[119,120] Most of the *P. dulce* seed proteins were found in the weakly acidic region, especially within p*I* range 5 to 6, suggesting that future separation of the first IEF dimension into finer pH ranges may be beneficial.

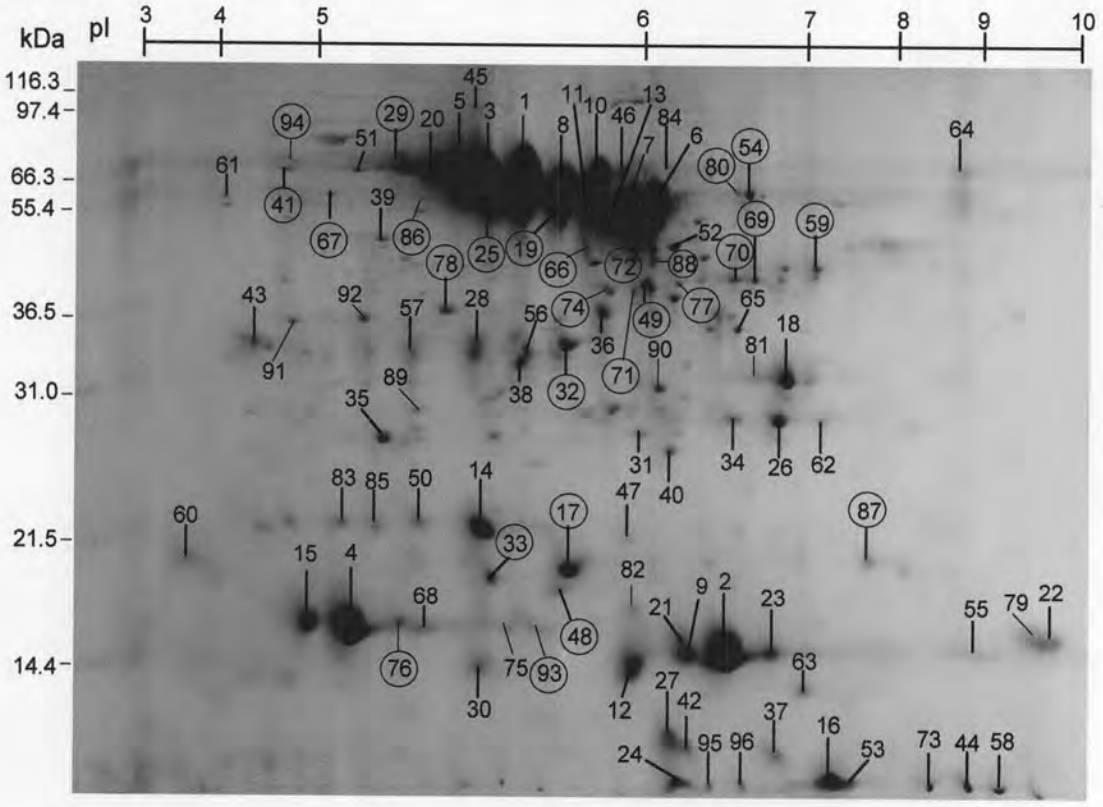

Figure 4.1 2-DE protein profile of *Pithecellobium dulce*. Proteins (3mg) were analysed by 2-DE on 24 cm, pH 3-10 non-linear gradient strips as the first dimension and 10% SDS-polyacrylamide gel as the second dimension and detected by coomassie blue staining. Numbers (1-96) indicate spots which were excised from the gel and subjected to LC-ESI-MS/MS. The numbers marked with circles indicate identified proteins spots from database with Mascot searching.

4.1.3 Protein Identification Using Tandem Mass spectrometry

Proteins in *P. dulce* seeds from the gel were identified. The 96 highly abundant protein spots representing 80% of the total protein mass were manually excised from the gel and digested with trypsin. The resulting tryptic fragments were analyzed using nano LC-ESI-MS/MS. Unfortunately, there is a lack of *P. dulce* genomic, Expressed Sequence Tags (EST), and protein sequences in the public databases. Under these conditions, cross-species of protein identification using mass spectral data has been demonstrated to be an alternative in the absence of genome sequences, although it is most reliable for closely related species and/or highly conserved proteins.[121]

4.1.3.1 Data Analysis and Mascot Searching

The MS/MS results of tryptic peptides were queried against the NCBI-nr database via the MASCOT program, and from the 96 proteins, 27 spots were 'positively' identified (Table 4.1, see also Appendix B), i.e. they had MOWSE scores higher than the significant threshold (≥90). In soybean seeds, the majority of the proteins observed were seed storage proteins such as β-conglycinin and glycinin.[119] However, these are not phylogenetically closely related species, and the substantial genetic distance between *P. dulce* and other legumes, including soybean, may explain why the identification success rate of *P. dulce* proteins using these model organism genomic and EST sequences were very low. Consequently, the unidentified but highly abundant cluster of some 12 proteins (p*I* 4–6 and MW 55–90 kDa) could be *P. dulce* seed storage proteins that share very low similarity with known legume storage proteins such as β-conglycinin and glycinin.

Identified *P. dulce* seed proteins were classified according to their putative (homologous annotation) functions in two general categories, disease/defence-related and general cell metabolismrelated (energy production, signal transduction, protein destination and storage, and secondary metabolism), as shown in Table 4.1. Nine *P. dulce* seed proteins were identified as likely disease/defence-responsive proteins. These include heat shock proteins, catalase and peroxidase. Heat shock proteins (HSP) are a group of proteins whose expression is increased in response to elevated

temperatures and other environmental stresses. They are involved in protein folding, assembly, translocation and stabilization of proteins and membranes under stress conditions.[124] They are traditionally classified into several families based on their molecular weights: HSP100 family, HSP90 family, HSP70 family, HSP60 family and sHSP (small HSP, 12–40 kDa) family. The four putative heat shock proteins identified in the *P. dulce* seeds were likely members of the 17 kDa sHSP (spots 17 and 48) and 70 kDa HSP70 (spots 93 and 94) heat shock protein families. While HSP70 are predominately chaperones involved in refolding heat denatured proteins, sHSP are thought to be incapable of refolding proteins, but instead bind and prevent the aggregation of misfolded proteins, allowing them to be correctly refolded by other HSP proteins such as HSP70 and HSP100.[122]

Another five proteins identified in this category were stress-responsive proteins, catalase: (spots 54 and 80), peroxidase (spot 33) and superoxide dismutase [Cu-Zn] 4A (spot 76) and thioredoxin-dependent peroxidase (spot 33). Catalase scavenges hydrogen peroxide, a harmful reactive oxygen species produced in normal metabolism as well as under stress conditions.[123] The elevated level of reactive oxygen species produced in stressed plants contribute to their defences, but if not controlled may also be detrimental or lethal to plant cells. To provide protection against oxidative damage caused by reactive oxygen species, plants produce a variety of antioxidants including metabolites such as ascorbate and glutathione and enzymes such as catalase and superoxide dismutases, to neutralize the reactive oxygen species. Copper/zinc superoxide dismutase which converts extremely reactive superoxide radicals (O_2^-) to hydrogen peroxide, while catalase further degrades the resulting hydrogen peroxide to water and oxygen, preventing oxidation of biological molecules. Thus, these two enzymes constitute an important part of an antioxidant defence system in plants.[124,125] Thioredoxin-dependent peroxidase also converts hydrogen peroxide to water and oxygen but unlike catalase it uses thioredoxin as proton and electron donor.[126] Thioredoxins are small thiol groups-containing proteins found in all living organism.[127]

Table 4.1 Protein identified in *P. dulce* seeds by LC-ESI-MS/MS analysis

Spot	Protein Identification	NCBI accession no.	MOWSE score	Peptides matched	Theoretical		Sequence coverage (%)
					MW	pI	
Disease/ defence responses							
17	17.8 kDa Class I heat shock protein	gi|232273	167	8	17.7	5.84	21
33	Thioredoxin-dependent peroxidase	gi|125620178	100	4	17.5	5.4	19
48	17.7 kDa heat shock protein	gi|37933812	185	4	17.7	6.4	13
54	Catalase	gi|33146311	211	8	56.9	6.73	13
76	Superoxide dismutase [Cu-Zn] 4A	gi|134597	102	2	15.1	5.65	12
78	Hypothetical protein -glyoxalase	gi|7488556	114	4	28.9	4.94	13
80	Catalase	gi|33146311	206	5	56.9	6.73	16
93	Heat shock protein Hsp70	gi|92868853	333	5	71.5	5.28	10
94	Heat shock protein Hsp70	gi|115456247	679	16	71.3	5.1	29
General metabolism							
19	ATP synthase subunit alpha, mitochondrial	gi|114408	132	2	55.6	6.23	5
25	Enolase (2-phosphoglycerate dehydratase)	gi|1169534	292	5	47.9	5.56	15
29	RuBisCO large subunit-binding protein subunit beta	gi|2506277	651	11	62.9	5.85	24
32	Seed maturation protein PM34	gi|9622153	101	2	31.7	6.6	12
41	Tubulin beta-2 chain	gi|81952	141	4	50.5	4.77	8
49	Cytosolic malate dehydrogenase	gi|78216493	366	10	35.6	6.01	23
59	Glyceraldehyde 3-phosphate dehydrogenase-like	gi|92873609	300	7	36.6	6.55	21
66	Alcohol dehydrogenase 1	gi|113361	139	2	41.1	6.09	5
67	ATP synthase subunit beta, mitochondrial	gi|231586	696	13	60.2	5.95	31
69	Fructose-bisphosphate aldolase, cytoplasmic isozyme	gi|113624	176	3	38.4	5.96	7
70	Glyceraldehyde-3-phosphate dehydrogenase	gi|462137	172	5	36.9	6.46	21
71	Cytosolic malate dehydrogenase	gi|10334493	145	5	35.5	5.92	15
72	Cytosolic phosphoglycerate kinase 1	gi|3738259	263	5	42.6	5.7	16
74	Malate dehydrogenase, mitochondrial precursor	gi|126896	234	3	36.2	8.88	11
77	Cytosolic malate dehydrogenase	gi|10334493	254	11	35.5	5.92	18
86	2-Phospho-D-glycerate hydrolase	gi|533474	166	2	48.3	5.62	6
87	Cyclophilin	gi|7649161	97	3	16.8	9.04	26
88	Alcohol dehydrogenase	gi|452767	256	2	40.9	5.88	14

Finally, the last of the nine identified plant defence-related protein in, *P. dulce* seeds was glyoxalase (spot 78). Glyoxalases are involved in detoxifying cytotoxic and reactive 2-oxoaldehydes into their corresponding 2-hydroxy acids in two consecutive

steps.[128] In the first step, glyoxalse isomerizes the non-enzymatic condensation product of glutathione and a 2-oxoaldehyde, mainly methylglyoxal which is an endogenous by-product of glycolysis, into D-lactoylglutathione. Glyoxalse II then catalyzes the hydrolysis of D-lactoylglutathione into reduced glutathione and D-lactate in the second step. Recent studies in plants showed that salt and metal stress resulted in enhanced expression of glyoxalase,[129] and transgenic tobacco plants over-expressing Gly I and II can tolerate high level of salt stress, presumably through detoxification of methylglyoxal and production of glutathione.[130] The protein was also found in the opium poppy latex[131], in the phloem sap of cucumber and pumpkin[125], and in the milky sap of greater celandine poppy.[132]

The majority of the remaining identified proteins (18 protein spots) are mainly involved in energy production. For example, Spot 29 was identified as ribulose-1,5-bisphosphate carboxylase/oxygenase or RuBisCO, a Calvin cycle enzyme that catalyzes the first step in the carbon fixation process. Spots 19 and 67 were identified respectively as alpha and beta subunits of mitochondrial ATP synthase that uses the transmembrane proton gradient as the energy source to produce ATP from ADP. Three enzymes involved in glycolysis to produce energy through the breakdown of glucose were also identified. These were enolase (spot 25 and 86), glyceraldehyde 3-phosphate dehydrogenase (spots 59, 69 and 70), and phosphoglycerate kinase (spot 72). Glyceraldehyde 3-phosphate dehydrogenase catalyzes the NAD^+-dependent oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate and NADH. 1,3-bisphosphoglycerate is then converted into 3-phosphoglycerate by phosphoglycerate kinase with the production of ATP. The resulting 3-phosphoglycerate is then converted into pyruvate by several enzymes including enolase that catalyzes the formation of phosphatepyruvate from 2-phosphoglycerate, the penultimate step in glycolysis. Malate dehydrogenase, another enzyme involved in energy production, was identified in four different spots (spot 74, 49, 71 and 77). It is an enzyme in the citric acid cycle in which the end product of glycolysis, pyruvate, is further oxidized into carbon dioxide with the production of $FADH_2$ and NADH which participate in oxidative phosphorylation to generate more ATP.

Several proteins involved in other metabolism were also identified in the *P. dulce* seed proteome. Seed maturation protein and tubulin-beta-2-chain were found in the *P. dulce* seed proteins (spots 32 and 41). Alcohol dehydrogenase enzymes were identified in spots 66 and 88, which facilitate the interconversion between alcohols and aldehydes or ketones. Spot 87 was identified as cyclophilin, which are ubiquitous proteins present in all subcellular compartments, belong to a family of immunosuppressant receptors called immunophilins, catalyzes the isomerization of peptide bonds from *trans* to *cis* form at proline residues and facilitates protein folding, and are involved in a variety of cellular processes.[133]

4.1.3.2 *De novo* Sequencing and Sequence-Similarity Searches

The highly abundant proteins cluster (p*I* 4-6 and MW 55-90) failed to identify using Mascot searching. To overcome this problem, MS-BLAST homology searching has been employed to identify proteins via their known homologues in other species in order to try to identify these proteins via their known homologues in the other species. These proteins were further sequenced *de novo* and matched for homology-based identification with the modified MS-BLAST searching method[113] which uses an alternative scoring scheme based on threshold scores that are set conditionally on the number of retrieved high-scoring segment pairs (HSPs) and the total number of fragmented precursors. The four *P. dulce* proteins identified by this approach with significant identification scores, when the score of the high-scoring segment pair of the protein hit was compared with the threshold, are listed in Table 4.2 (see in Figures of Appendix C for the MS/MS spectrum data). Query amino acid sequence of peptide precursor using BLAST search of these four protein spots are shown in Tables of Appendix C.

The score of the top-ranked HSP (spot 25) in Table 4.2 is 92 (21 peptides used; threshold score 1HSP= 67). Spot 25 was positively identified as having similarity to enolase from *Medicago truncatula* in agreement with the protein identification produced by MASCOT searching. The second protein (spot 1) was identified as a peptide with similarity to growth-regulating factor 8 protein from *Oryza sativa* with the top-scoring HSP is 66 (17 peptides used; threshold score

1HSP= 66). The third protein (spot 8) was identified as a peptide having similarity to hypothetical C2H2-type protein from *Arabidopsis thaliana*, with a top-scoring HSP of 71, which significantly exceeds the threshold for a single match HSP that is 65 (10 peptides used). Finally, the forth protein (Spot 13) was identified as a peptide having similarity to Kunitz-type trypsin inhibitor alpha chain, with top-scoring HSP (11 peptides used; threshold score 1HSP= 65). The comparison of amino acid sequences of the query from MS-BLAST and from database is presented in Table 4.3.

Table 4.2 Protein Identification of 2D Gel Spots from *P. dulce* by MS-BLAST

Spot no.	NCBI accession no.	Protein	Organism	Protein MW (theor.)	Peptide monoisotopic mass (*m/z*)	Peptide sequence	HSP score[a]	% positive[b]
1	gi|115485875	Os11g0551900, Growth-Regulating factor 8	*Oryza sativa*	43 897	2510.1004	TMTGGNGGGALLGFCSPDGSAGALASGGK GG GGG L F SP + G AG GGGGGGQMLSFSSPNGTAG	66	68
8	gi|6554199	T23J18.16, Hypothetical protein, C2H2-type	*Arabidopsis thaliana*	75 587	2388.1003	NPGSDANPNPYYLPSHAGLYNK DANPNP + Y + P DANPNPFYIP	71	100
					2388.0642	NPSTANAPEMNYLPSHAGLYNK NPST N NPSTSN	37	83
13	gi|417176	Kunitz-type trypsin inhibitor alpha chain	*Prosopsis juliflora*	15 469	1261.6395	FECPLTVVQAR CPLTVVQAR CPLTVVQAR	66	100
25	gi|3023714	Enolase	*Medicago truncatula*	45 053	1791.8946	QLPSGASTGTYEALELR PSGASTG YEALELR PSGASTGVYEALELR	92	93
					2684.3466	LLAVSLAVCGK +LAVSLAVC I LAVSLAVC	57	100
					1355.767	LVLDYVLPLPSK VLP PS VLPVPS	34	83
					1355.767	LDPFMVLPLPSK +D FMV IDNFMV	34	83
					1937.8604	TVDACCLGFDVAADLLANK +G DVAA IGMDVAA	34	85

[a] MS-BLAST match was defined as statistically significant if the score of the High scoring pair (HSP) was higher than the threshold value that scoring scheme is described in Ref.[113]

[b] % positive was calculated from the number of matched amino acids in sequence divided by total number of query amino acids and multiply by 100.

Table 4.3 Comparison of the amino acid sequences of query peptides from *P. dulce* and sequences from NCBI-nr database

1	OS[a] 61 LGSTSSSCSGGGGGGQMLSFSSPNGTAGLGLSSGGSMQGVLARVRGPFTPTQWMELEHQA Query GGNGGGALLGFCSPDGSAG	
8	AT[b] 421 KLMIAISSDNSMWVLEKKAPVELVEGGKGIVGASHGWVATLKDGVVCLQDDLNPSTSNSD Query NPSTAN AT 601 FLSKSESFCVSATSSYKSVPSLVRNHVYVQAYDEYGFMDIAKPFDDSWVIPASPDANPNP Query DANPNP AT 661 FY IPPQTIEYTLSTMHPDL Query YYLP	
13	PJ[c] 1 QELLDVDGEILRNGGSYYILPAFRGKGGGLELAKTEGETCPLTVVQARSETDRGLPASIW Query CPLTVVQAR	
25	MT[d] 1 MVVTIKIVKARQIFDSRGNPTVEVDVTLSDGTFARAAVPSGASTGVYEALELRDGGSDYL Query PSGASTGTYEALELR MT 61 GKGVLKAVENVNSIIAPALLGKDPTKQTEIDNFMVQQLDGTVNEWGWCKQKLGANAILAV Query LDPFMV LLAV MT 121 SLAVCKAGALAKKIPLYKHIANLAGNKTLVLPVPSFNVINGGSHAGNKLAMQAVIKKKYG Query SLAVC VLP LPS MT 181 QDATNVGDEGGFAPNIQENKEGLELLKTAIAKAGYTGKVVIG MDVAASEFYDNKGKTYDL Query LG F DVAA	

[a] *Oryza sativa*, [b] *Arabidopsis thaliana*, [c] *Prosopsis juliflora*, [d] *Medicago truncatula*

Note: Identities are highlighted in dark grey and similarities in light grey.

4.2 Purification of Antifungal Protein from *Pithecellobium dulce* Seeds

4.2.1 Extraction and Precipitation

The protein yield of the protein extraction using Tris-HCl buffer, pH 8.0 and precipitation with 80% ammonium sulfate saturation from 100 g mature *P. dulce* seed is 3.26% and lower than 5.8% yield of the protein from the extraction using Tris-HCl buffer containing inhibitor and TCA-cold acetone precipitation. The drawback of the protein precipitation with TCA-cold acetone method is that the loss of biological activity of protein due to the denaturing of crude protein and the protein may be difficult to resolubilize and may not be resolubilize completely. Total amount of crude 80% saturation ammonium sulfate protein is 3.26 g (Table 4.4).

Table 4.4 Purification of antifungal protein from *P. dulce* seeds (100 g)

Purification step	Amount of protein (mg)	Protein yield (%)
Crude extract from ammonium sulfate precipitation (80% saturation)	3.26×10^3	3.26
Resource Q (first peak, Q1)	814	0.814
Superdex 200 (fourth peak, G4)	78.7	0.079

4.2.2 Biological Activity Screening of Crude Protein

The crude extract of the *P. dulce* seeds from 80% saturation ammonium sulfate precipitation were tested to define the biological activity. This crude protein possesses only antifungal activity. The crude protein was evaluated against three phytopathogenic fungi, *Macrophomina phaseolina* (Charcoal rot), *Phymtotrichopsis omnivora* (Cotton rot), and *Fusarium avicenariam* (see Figure 1D in Appendix D). The crude protein showed antifungal activity toward *Macrophomina phaseolina*. However, it had hardly any antifungal effect on *Phymtotrichopsis omnivora* and *Fusarium avicenariam*. For further antifungal activity assay, the protein fractions from chromatography column will be antifungal tested for inhibitory of the *Macrophomina phaseolina* fungus growth.

4.2.3 Purification of Antifungal Protein by Column Chromatography

The crude protein of *Pithecellobium dulce* seeds was first applied onto a Resource Q (anion exchange) column and eluted with 0–0.5 M NaCl gradient in 20 mM Tris–HCl buffer (pH 8.0). The anion exchange chromatogram represents a large unadsorbed fraction (Q1) and four smaller adsorbed fractions name Q2, Q3, Q4 and Q4 (Figure 4.2). All fractions were tested for antifungal activity toward

Macrophomina phaseolina (Figure 4.3). The unbound fraction (Q1) corresponded to the bulk of the antifungal activity as shown in Figure 4.3. Q1 was further analysised by SDS-PAGE. From Figure 4.4, there are broad protein bands at MW range from 14.4 to 21.5 kDa in polyacrylamide gel from Q1.

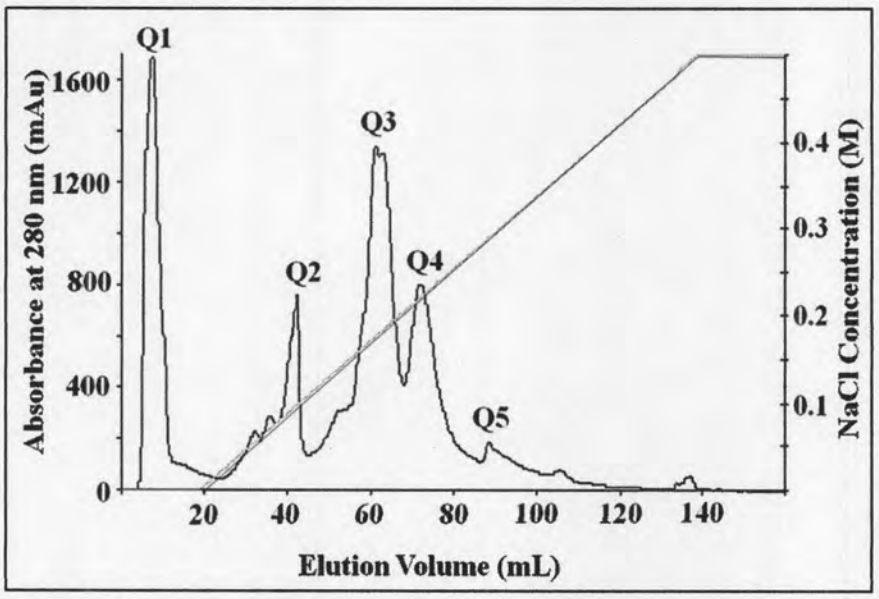

Figure 4.2 Anion exchange chromatography using Resource Q column of 80% saturation ammonium sulfate precipitated from *P. dulce* seeds extract.

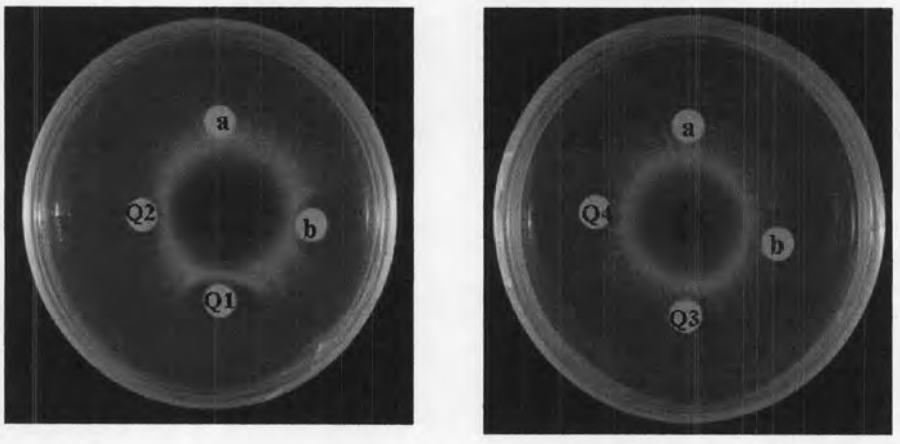

Figure 4.3 Inhibitory activity of the protein fractions from Resource Q column (Q1-Q4) toward *Macrophomina phaseolina.* (a) 20 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl, (b) 500 µg crude proteins from 80% sat. ammonium sulfate precipitation, 200 µg protein fractions of Q1, Q2, Q3 and Q4

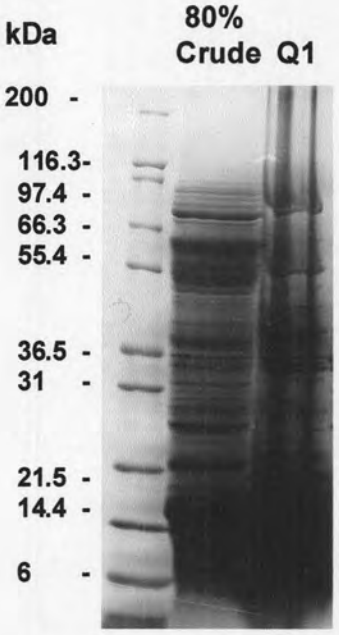

Figure 4.4 SDS-PAGE (12% gel) of fraction Q1 from Resource Q column.

The active fractions (Q1) were concentrated and purified further by gel filtration chromatography on Superdex 200 column. Four fractions (G1-G4) were eluted in this chromatographic step (Figure 4.5). The fourth peak (G4) from the column possessed antifungal activities (Figure 4.6). A SDS–PAGE pattern of G4 fraction was observed with a single protein band as shown in Figure 4.7. The molecular mass of this antifungal protein from *P. dulce* seeds obtained was estimated by SDS–PAGE to be 14.4 kDa.

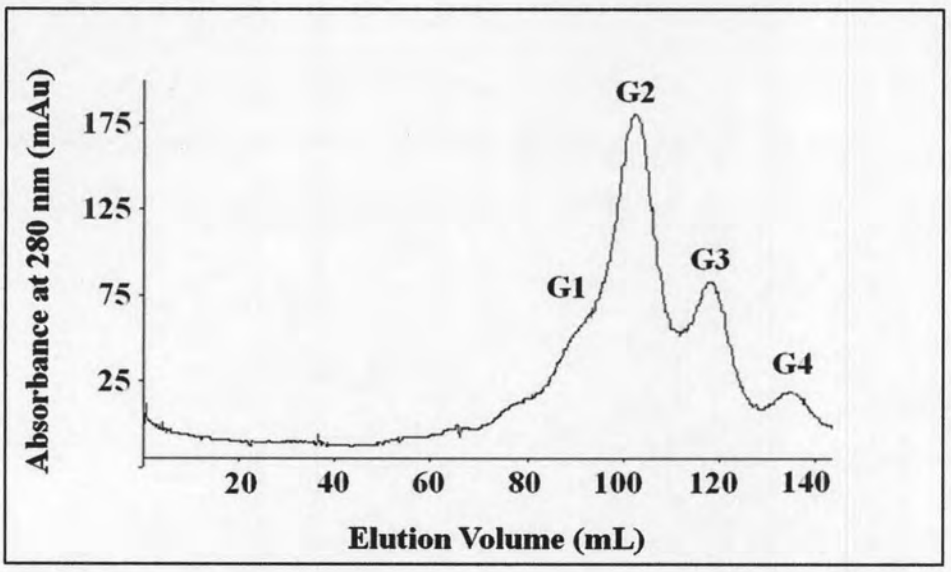

Figure 4.5 Gel filtration chromatography using Superdex 200 column of Q1.

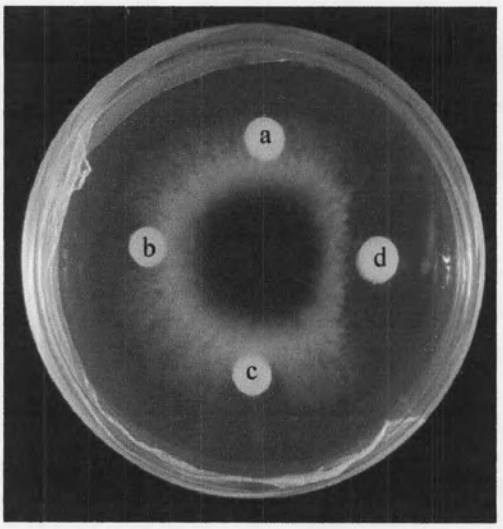

Figure 4.6 Inhibitory activity of the purified antifungal protein (G4) toward *Macrophomina phaseolina*. (a) 20 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl, (b) 500 µg, (c) 1 mg crude proteins from 80% sat. ammonium sulfate precipitation, respectively, and (d) 9.6 µg the purified antifungal protein (G4).

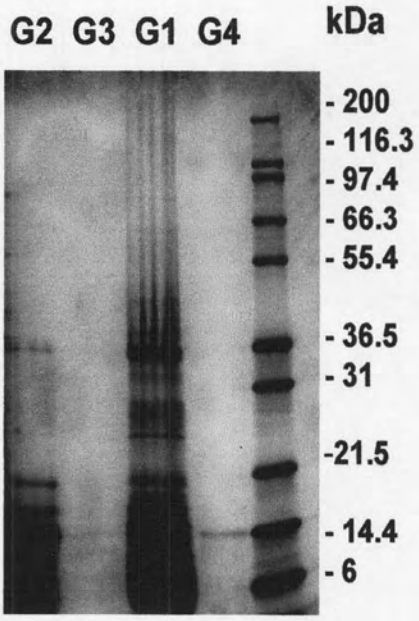

Figure 4.7 SDS-PAGE (12% gel) of the fractions (G1-G4) from Superdex 200 column.

The summary of antifungal protein purification step from *Pithecellobium dulce* seeds is presented in Table 4.4. From two step chromatography, yield of purified antifungal protein is 0.079% (from 100g *P. dulce* seeds). The result of antifungal activity assay shows that G4 inhibited the growth of *Macrophomina phaseolina* at in the minimum concentrations of G4 against this fungus was 0.06 mg/ml (Figure 4.8A). The thermal stability of antifungal protein from *P. dulce* was stable to heat at pH 8.0 with antifungal activity remaining even incubation at 80 °C for 15 min (Figure 4.8B).

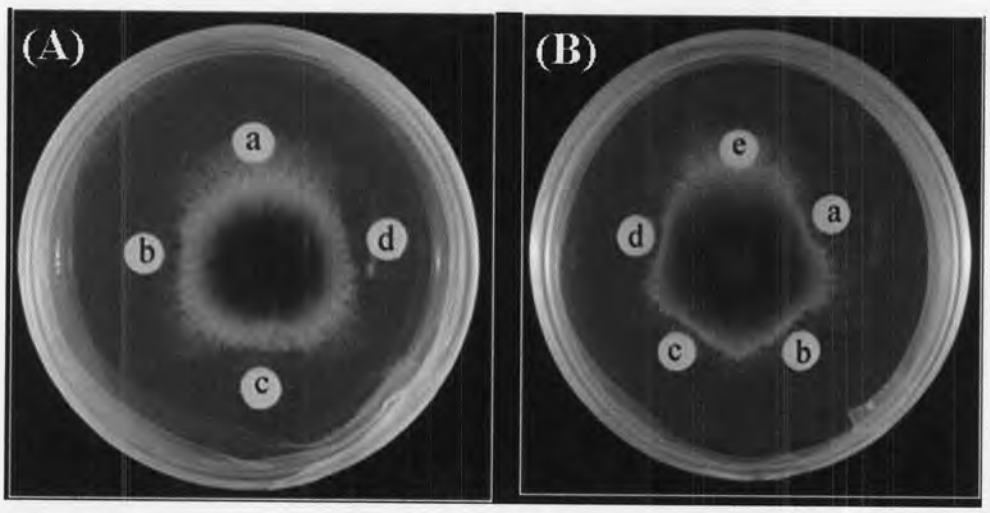

Figure 4.8 Determination of the minimum concentration and effect of temperature on antifungal activity toward *Macrophomina phaseolina* of G4.

(A) Determination of the minimum concentration of the antifungal activity of purified antifungal protein (G4) toward *Macrophomina phaseolina*, (a) 20 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl, (b) 0.06 µg/ µl, (c) 0.12 µg/ µl, and (d) 0.18 µg/ µl of purified antifungal protein (G4).

(B) Effect of temperature on antifungal activity toward *Macrophomina phaseolina* of 30 µg of purified antifungal protein (G4). Sample disks a, b, c, d, and e represent previously exposed to 20 °C, 40 °C, , 60 °C, 80 °C, and 100°C for 15 min, respectively.

4.2.4 Identification Antifungal Protein using LC-ESI-MS/MS

To identify this purified antifungal protein, a single band protein from SDS-PAGE was subjected to tryptic digestion. The resulting tryptic fragments were analyzed using nano LC-ESI-MS/MS. The MS/MS results of tryptic peptides were queried against the NCBI nr database via the MASCOT program (see Figure 2D and Table 1D in Appendix D). The antifungal protein, G4 matched with chicken egg white lysozyme (*Gallus gallus*) (129 amino acids, Mw 14290 Da, p*I* 9.46, NCBI accession no. **630460A**). The MOWSE scores of Mascot search is 144, which higher than the significant threshold (>52) and two matched peptides. This purified protein matched amino acids of 28 from 129 amino acids sequence and coverage percentage of 21. Moreover, molecular mass of a *P. dulce* seeds lysozyme, 14.4 kDa closes to molecular mass of chicken egg white lysozyme, 14.3 kDa. These are sufficient to specify that this antifungal protein is lysozyme. Amino acid sequences of purified lysozyme from *Pithecellobium dulce* seeds compared with chicken egg white lysozyme in bold underlined are shown below.

Chicken egg white lysozyme (*Gallus gallus*)

```
  1 KVFGRCELAA AMKRHGLDNY RGYSLGNWVC AAKFESNFNT QATNRNTDGS
 51 TDYGILQINS RWWCNDGRTP GSRNLCNIPC SALLSSDITA SVNCAKKIVS
101 DGDGMNAWVA WRNRCKGTDV QAWIRGCRL
```

Lysozymes, also known as muramidase or N-acetylmuramide glycanhydrolase, are a family of enzymes [EC 3.2.1.17] which damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. It is responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus it provides some protection against infection. The enzyme has been used for lysing *E. coli* and *Streptomycetes* for

extraction purposes[134] such as extracting group specific antigen.[135] Lysozyme is widespread distribution in animals and plants. Moreover, large amounts of lysozyme can be found in egg whites. Plant lysozyme is found in *Ficus carica* (fig) and papaya latex,[136] and chemically is distinct from the egg white enzyme.[137] In 2005, a novel plant lysozyme (14.4 kDa) from legume was firstly reported by Wang *et al.*[138] It was isolated from mung bean (*Phaseolus mungo*) seeds using a procedure that involved aqueous extraction, ammonium sulfate precipitation, ion exchange chromatography, and high-performance liquid chromatography. This lysozyme was identified by Edman degradation method and had 23% identity in N-terminal amino acid sequence with that of hen egg white lysozyme. This mung bean lysozyme exhibited antifungal activity against *Fusarium oxysporum*, *Fusarium solani*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, and *Botrytis cinerea*, with an antibacterial action against *Staphylococcus aureus*.