CHAPTER 4

RESULT AND DISCUSSION

The proteins in *Parkia speciosa* were analyzed using all methods as described in the previous chapter. The results have been shown and discussed in each part of this chapter, respectively.

4.1 Determination of protein concentration

Table 4.1 The amount of proteins from precipitate fraction

Crude protein	Amount of protein (mg)
25%	119.48
40%	205.2
60%	1,291.8
90%	779.4

4.2 Hemagglutinating activity and ∝-glucisidase inhibition

Crude proteins (crude 25%, 40%, 60%, 90%) were tested to define the hemagglutinating activity and ∝-glucisidase inhibition. The results are reported in the Table 4.2

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Table 4.2 Activity of crude protein

Crude protein (% ammoniumsulfate)	Activity	
	Hemagglutinating activity agglutinate with rabbit erythrocytes (IC_{50} μg/μl)	∝-glucisidase inhibitor
25%	6 (+)	+
40%	28.5 (+)	+
60%	2.51 (+)	-
90%	-	-

+ agglutinate and inhibit ∝-glucisidase

- no agglutinate and no inhibit ∝-glucisidase

4.3 Protein precipitating 60% ammonium sulphate saturation

60% ammonium sulphate of crude proteins was chosen for characterization because this fraction showed high amount of proteins and hemagglutinating activity with rabbit erythrocytes (IC_{50} = 2.51μg/μl).

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4.3.1 Characterization crude proteins by affinity chromatography and gel electrophoresis

The crude proteins presented hemaaglutinating activity as described in Section 4.2. The lectins from crude proteins were separated by ConA Sepharose column (Concanavalin A [5] is a tetrameric metalloprotein isolated from *Canavalia ensiformis*. Con A binds molecules containing ∝-D-mannopyranosyl, ∝-D-glucopyranosyl. Con A coupled to Sepharose is routinely used for separation and purification of glycoproteins). The chromatogram was shown in **Figure 4.1** and **Figure 8** in appendices.

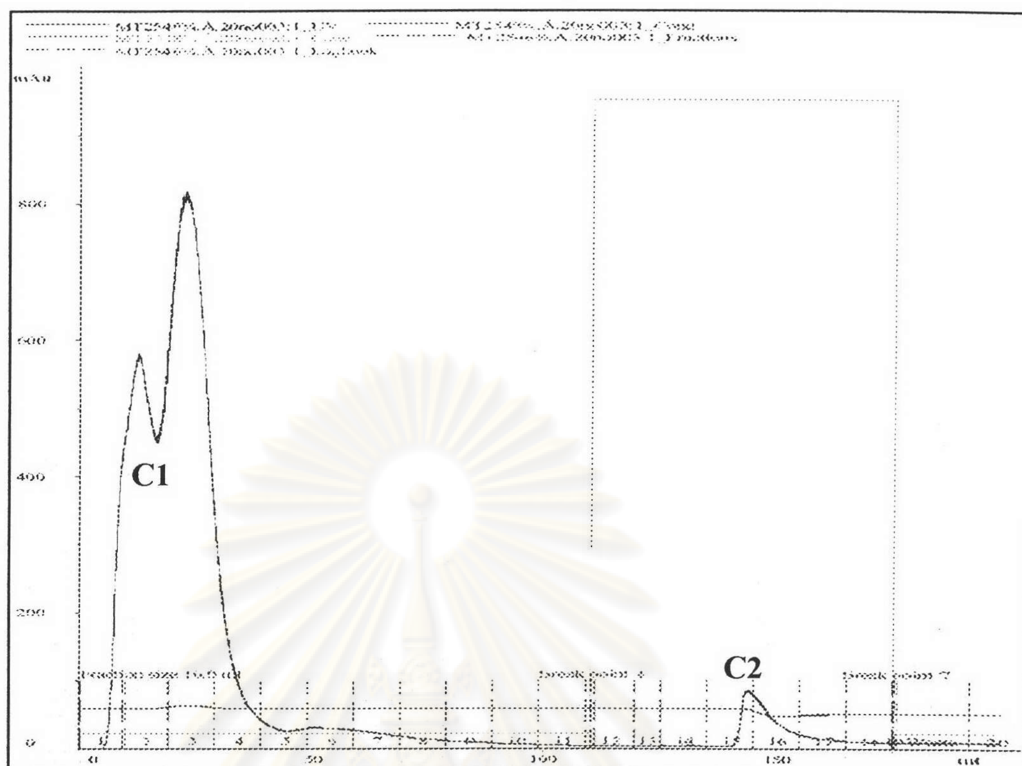

Figure 4.1 Affinity chromatography of crude protein from *Parkia speciosa* on the Con A sepharose column (1.6x5cm), Flow rate 1.5 ml/min.The equilibration buffer was 20 mM Tris-HCl pH 7.4 containing with 0.5 M NaCl, 1mM $CaCl_2{\cdot}2H_2O$, 1mM $MnCl_2$. The eluted lectin buffer was 20 mM Tris-HCl pH 7.4 containing with 0.1-0.5 M Methyl-∝-D-manopyranoside.

Table 4.3 The amount of proteins from Affinity chromatography

Fraction No.	Amount of protein (mg)
C1	42.792
C2	13.484

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From Figure 4.1, there were two peaks which were called C1 and C2. C1 was the unbound peak and C2 was the bounding peak. C1 fraction showed higher amount of proteins than C2 fraction because C1 fraction presented higher absorbance than C2 fraction. Con A sepharose is specific media for lectin, therefore the proteins which bind on Con A sepharose (fraction C2) should be lectin. So bring these lectins to test hemagglutinating activity and find the composition of C1 and C2 fraction by 1-D SDS-PAGE and then visualized with coomassie staining. The 1D gel was shown in **Figure 4.2**

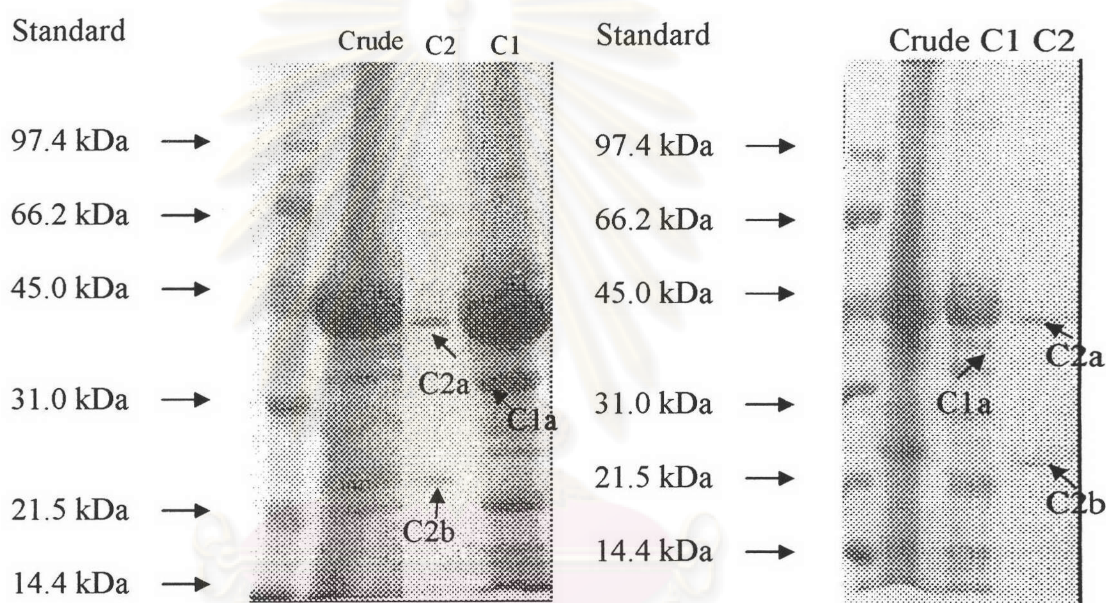

Figure 4.2 1-D SDS-PAGE of C1 and C2 fraction from affinity column

1-D gel of fraction C2 and C1 from affinity column were shown two individual protein spots. The crude proteins appeared seven spots and the band which had molecular weight about 45.0 kDa will have the highest amount of proteins. The C2 line in gel showed two spots which were called C2a and C2b.These spots were the shape band and showed small amount of proteins. The C1 fraction presented five spots and one of these spots was called C1a.The C2 fraction from affinity column agglutinated with rabbit erythrocytes (IC_{50} = 1.17μg/μl) but no showed activity with goat erythrocytes.

From 1-D SDS-PAGE C2a and C2b spot maybe separate by Gel filtration because C2a and C2b spot showed a different size and molecular weight. The chromatogram was shown in **Figure 4.3** and **Figure 9** in appendices.

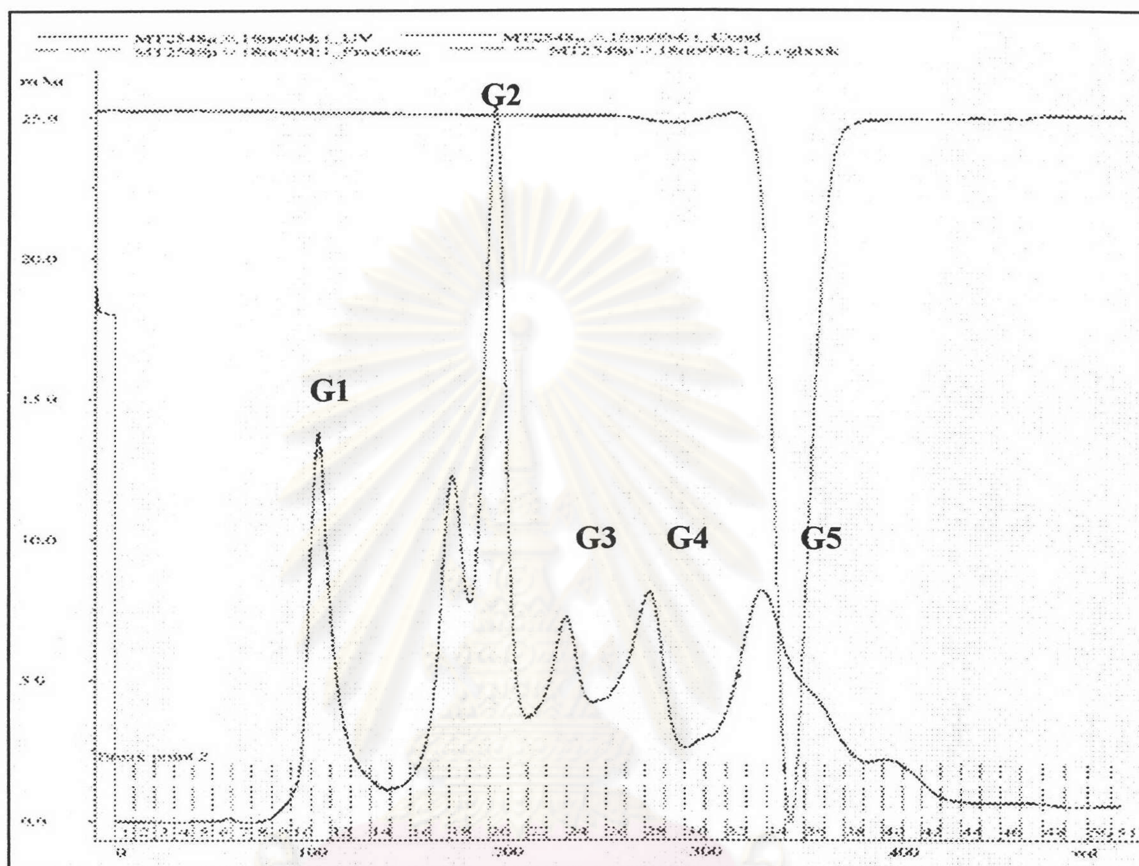

Figure4.3 Gel filtration chromatography of crude proteins on the superdex 200 column (1.6 x 60cm) in double distilled water, flow rate 0.5 ml/min.

From figure 4.3, there are five fractions G1, G2, G3, G4 and G5. The media in column was superdex G-200 that separate protein by size between 10^5 and 10^6 Da. G1 fraction was eluted in the first fraction so G1 fraction presented the highest molecular weight. Every fraction was tested hemagglutinating activity and determined the concentration of proteins. The results were reported in **Table 4.4, 4.5**

Table 4.4 Hemagglutinating activity of fractions from gel filtration chromatography

Fractions No.	Hemagglutinating activity agglutinate with rabbit erythrocytes (IC_{50} μg/μl)
G1	-
G2	1.20(+)
G3	±
G4	-
G5	2.63 (+)

\+ Agglutinated with rabbit erythrocytes

\- no agglutinated with rabbit erythrocytes

The agglutinating of rabbit red blood cell was shown in Figure 4.4

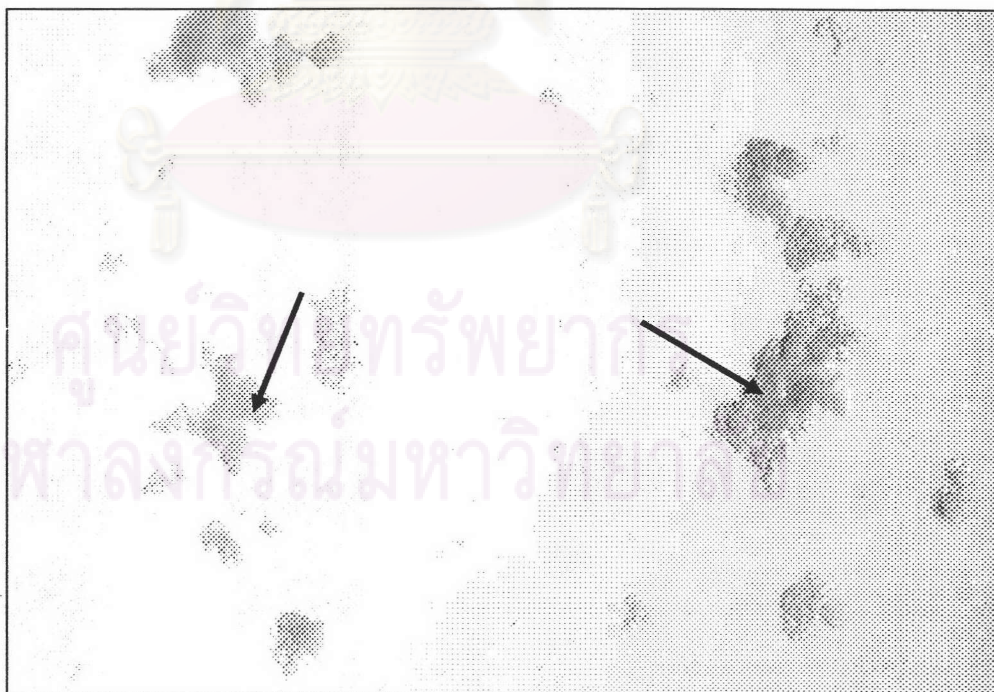

Figure 4.4 rabbit red blood cell agglutinating

The position at the narrow which showed the cluster of the rabbit red blood cells.

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The non aggultinated rabbit red blood cell are shown in **Figure 4.5**

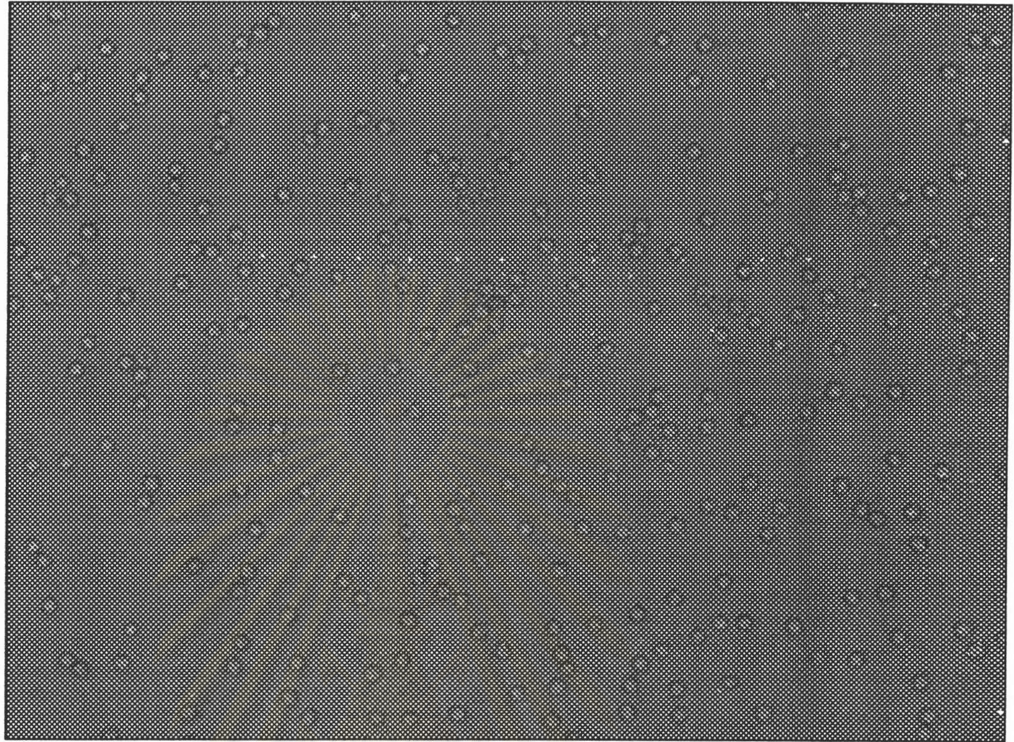

Figure 4.5 Blank of rabbit red cell shown the red blood cells are not the cluster

Table 4.5 The amount of proteins from gel filtration chromatography

Fraction No.	Amount of protein (μg)
G2	965.60
G3	216.40
G4	326.40
G5	526.50

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G1 fraction was not shown a signature protein because this fraction can not develop the color in Bradford test. G2 fraction has the highest amount of protein because it has highest peak area from gel filtration. After that, fraction G1, G2, G3, G4 and G5 were find the composition of protein by SDS-PAGE and visualized with coomassie staining. The 1D gel was shown in **Figure 4.6**.

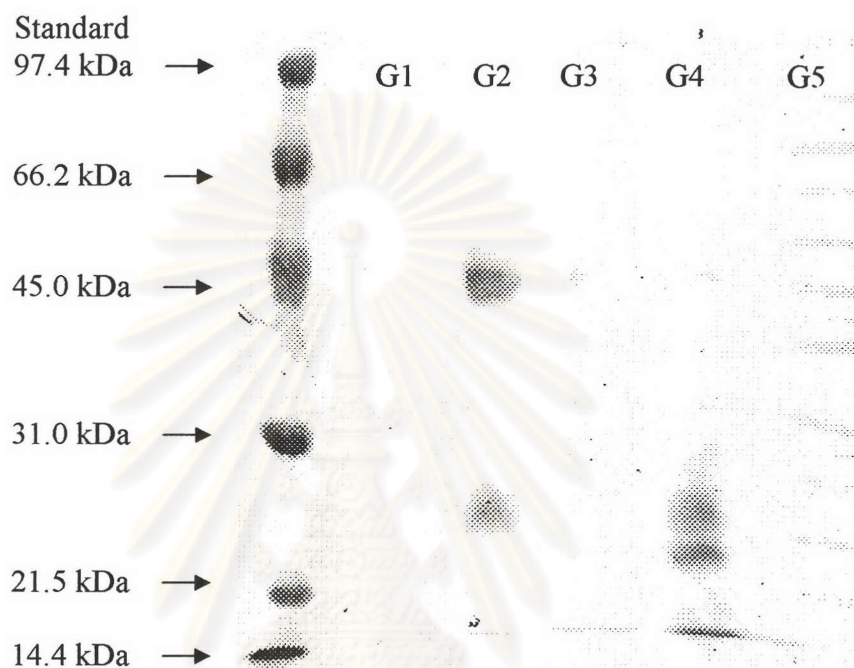

Figure 4.6 1-D SDS-PAGE of fraction G1, G2, G3, G4, G5 from gelfiltration chromatography

The protein patterns of fraction G2 from gel filtration chromatography and C2 fraction from affinity column were similar pattern. However, these proteins from gel filtration show that they can not separate G2 fraction by gel filtration chromatography. The G2 fraction also showed hemagglutinating activity and contain two spots similar as found C2 fraction from Con A sepharose column.

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After protein were separated by 1D SDS-PAGE and visualized with coomassie staining. The spots were excised and subjected to digestion with trypsin. The molecular weight of each tryptic fragment was analyzed by MALDI-TOF MS.C2a, C2b and C1a spot were chosen to analyze by mass spectrometry because C2a and C2b are the binding peak which presented hemagglutinating activity. C1a was the single band which showed one protein. The mass spectra are shown in **Figure 1-5** in

Appendices. And then, the mass spectra were used for performing of protein peptide mass mapping via Mascot Search Results (http://matrixscience.com). The search results were shown in **Table 4.6.**

Table 4.6 The search results of protein C1a, C2a and C2b

Band of protein	m/z	Matching peptides	NCBI Accession No.c	Molecular weight	Organism	Score	% protein covered
C1a	671.7727 521.9151 1207.9087 1031.1903	4(4)	gi | 54290632	20539	*Oryza sativa* (Japonica cultivar-group)	59	16
C2a	586.1463 633.5173 814.8358 946.8095 1016.4795 1125.5203 1157.2175 2600.5307	8(8)	Q69JW1_ ORYSA	14184	*Oryza sativa* (Japonica cultivar-group)	115	55
C2b	2597.4759 2673.9036 2000.6973 2728.6092 2641.4627 2688.6046	6(6)	Q8VXY3 _ARATH	43571	*Arabidops is thaliana* (Mouse-ear cress).	91	35

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Protein C1a

gi | 54290632

Matched peptides shown in **Bold Black**

1 MDDLRICTRA QPSAKCQHNS K**YLYGR**R**TTS** **GR**QSLASLLA ATAPDCRHPV

51 GEEAALAVAD GDGAALGEDV GEVGVLDKCV VAVVEGGAAE VVGDNLVVVD

101 GEGQAVGTLG EVLTATSLLA ATPGHRRDCH RARPPPRPGG R**LVPVAASPG**

151 **SPGR**RLAQPP PRPGGR**LAQA** **AASPFR**CLYR PPPPRPSRLR ELLREKR

Start - End	Observed	Mr(calc)	Delta	Sequence
22 - 26	671.7727	670.3438	0.4216	K.YLYGR.R
28 - 32	521.9151	520.2605	0.6473	R.TTSGR.Q
142 - 154	1207.9087	1206.6720	0.2294	R.LVPVAASPGSPGR.R
167 - 176	1031.1903	1030.5559	-0.3729	R.LAQAAASPFR.C

Protein C2a

Q69JW1_ORYSA

Matched peptides shown in **Bold Black**

1 MGR**VWGGLGW** **NGPAPNPRLL** **HPAPAPAR**RL TALALRCPAH RRVEKEGRGA

51 RGRR**LACATR** **SPARR**AR**TLP** **VALTVRR**SCA ACHAARPPPC EDRR**DWERGE**

101 **REIGGPEGR**R VEREMER**RTG** **VLATVAK**E

Start - End	Observed	Mr(calc)	Delta	Sequence
4 - 28	2600.5307	2600.3876	-0.8641	R.VWGGLGWNGPAP- -NPRLLHPAPAPAR.R
55 - 60	633.5173	633.3268	-0.8167	R.LACATR.S
61 - 65	586.1463	585.3347	-0.1956	R.SPARR.A
68 - 77	1125.5203	1124.7029	-0.1899	R.TLPVALTVRR.S
95 - 101	946.8095	946.4257	-0.6234	R.DWERGER.E
99 - 109	1157.2175	1155.5632	0.6471	R.GEREIGGPEGR.R
102 - 109	814.8358	813.3980	0.4305	R.EIGGPEGR.R
118 - 127	1016.4795	1014.6185	0.8537	R.RTGVLATVAK.E

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Protein C2b

Q8VXY3_ARATH

Matched peptides shown in **Bold Black**

```
  1 MDAAIFTSVY VCNIPKTKKA FFNPNPPALS SSSCWLCNSQ AKQIIKLRIR
 51 EGSNQGLLRV HALFNNEEAS SESEDKNGFG LLPADIFSLP QEKFGSNVSG
101 EKDSENIIDV ETSLAVPHGG GTRAGLYRTP ISGGVQSATS AHGLPRPALA
151 VRNLMEQARF AHLCTVMSKM HHRREGYPFG SLVDFAPDPM GHPIFSFSPL
201 AIHTRNILAE PRCTLVVQIP GWSCLSNARV TLFGDVYPLP EEQQEWAHKQ
251 YMLKHHQGPS QQWGNFHYFR MQNISDIYFI GGFGTVAWIN VNEYETLQPD
301 KIAVDGGEQN LKELNAIFSK PLRELLSSEA ELDDAAIISI DSKGIDIRVR
351 QGAQFKIQRL AFEESHGVET LEEAKSALWK VIEKGKLHNL QK
```

Start - End	Observed	Mr(calc)	Delta	Sequence
1 - 18	2000.6973	2000.0110	-0.3210	MDAAIFTSVY VCNIPKTK.K
19 - 42	2597.4759	2596.2202	0.2484	K.KAFFNPNPPALS SSSCWLCNSQAK.Q
103 - 128	2728.6092	2726.3623	1.2396	K.DSENIIDVETSL AVPHGGGTRAGLYR.T
206 - 229	2641.4627	2639.3675	1.0879	R.NILAEPRCTLVV QIPGWSCLSNAR.V
250 - 270	2688.6046	2688.2556	-0.6583	K.QYMLKHHQGPS QQWGNFHYFR.M
324 - 348	2673.9036	2672.3755	0.5208	R.ELLSSEAELDDA AIISIDSKGIDIR.V

4.4 Protein precipitating 25% ammonium sulphate saturation

Crude proteins were chosen for characterization because showed ∝-glucosidase inhibition and hemagglutinating activity. In addition, the crude protein agglutinated rabbit erythrocytes with a much lower activity (Table 4.2)

4.4.1 Characterization crude proteins by affinity chromatography and gel electrophoresis

Protein mixtures of *Parkia speciosa* fractions No. A1, A2 and A3 were collected from Affinity chromatography (Affi-Gel Blue Gel) .The affinity chromatogram was shown in **Figure 4.7** and **Figure 14, 15** and **16** in Appendices.

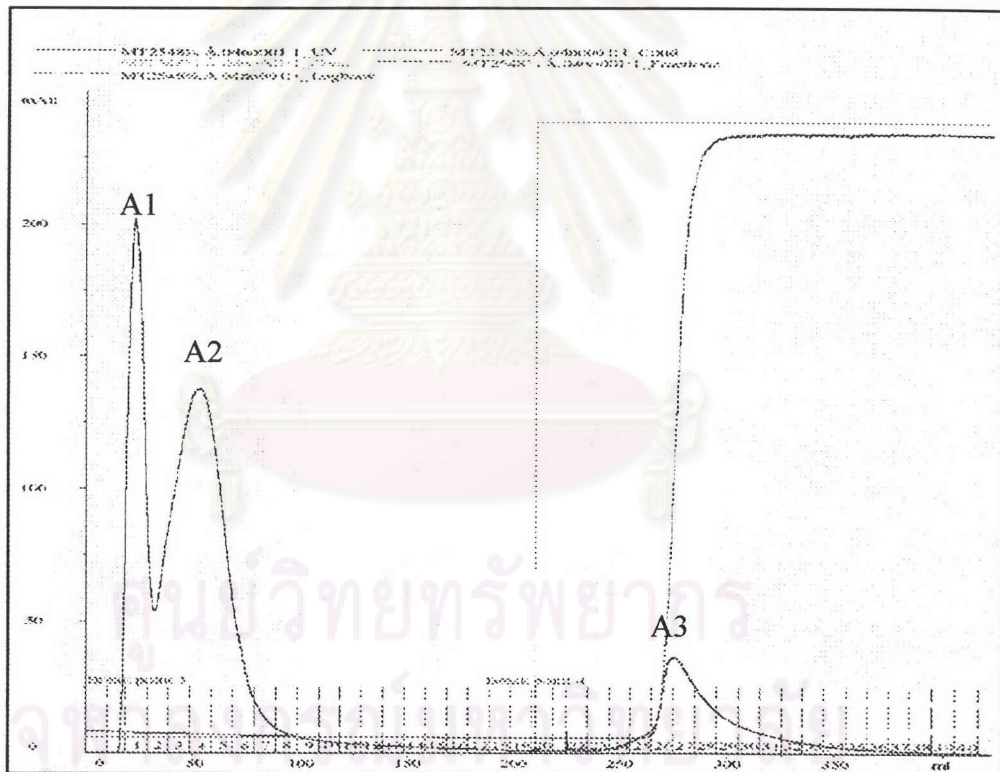

Figure 4.7 Affinity chromatogram of crude of *Parkia speciosa* on the Affi-gel blue gel column (1.6x10 cm) in binding buffer 1 mM Tris-HCl, pH 7.2. The dash line indicate using 0-100% linear gradient of 0.5 mM NaCl in binding buffer. Flow rate 1.5 ml/min.

Affi-Gel blue gel media purifies a large range of proteins from widely divergent origins. These fractions from Affi-Gel blue affinity chromatography were called A1, A2 and A3. Due to A1 and A2 can not completely separate, then A1 and A2 were combined together before further analysis. The hemagglutinating activity 3 times test have the results that shown in **Figure 4.8**. The A1 and A2 fraction agglutinated with rabbit erythrocytes but not showed activity in A3 fraction. The A1 and A2 fraction agglutinated rabbit erythrocytes with an IC_{50} of 0.58 μg/μl.

These fractions were analyzed the composition of proteins by gel electrophoresis.

The agglutinating of rabbit red blood cell was shown in **Figure 4.4**

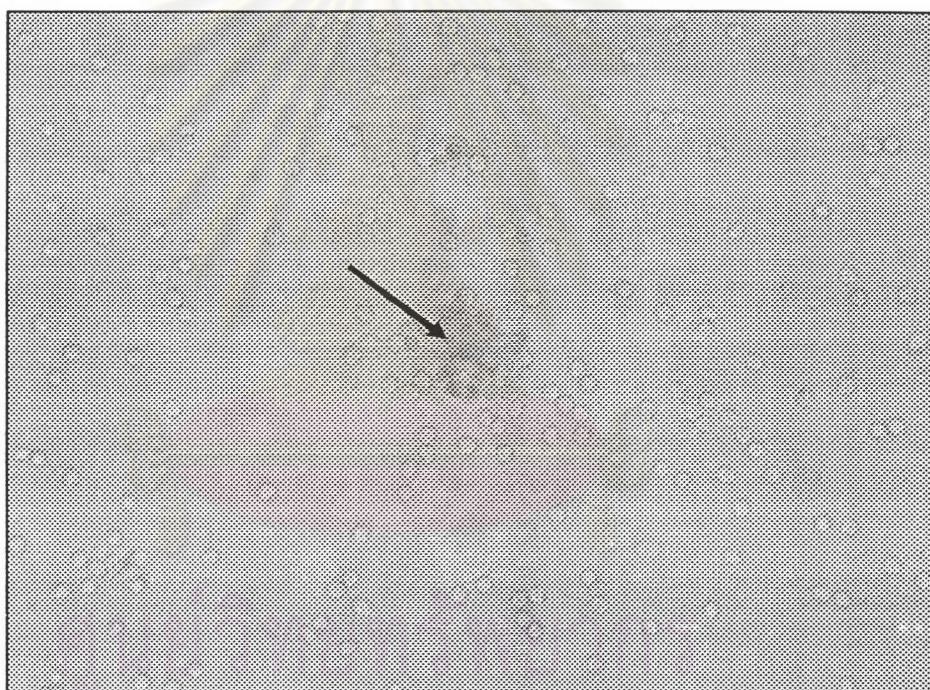

Figure 4.8 Rabbit red cell agglutinating

The position at the arrow shows the cluster of the red bloods cell of rabbit.

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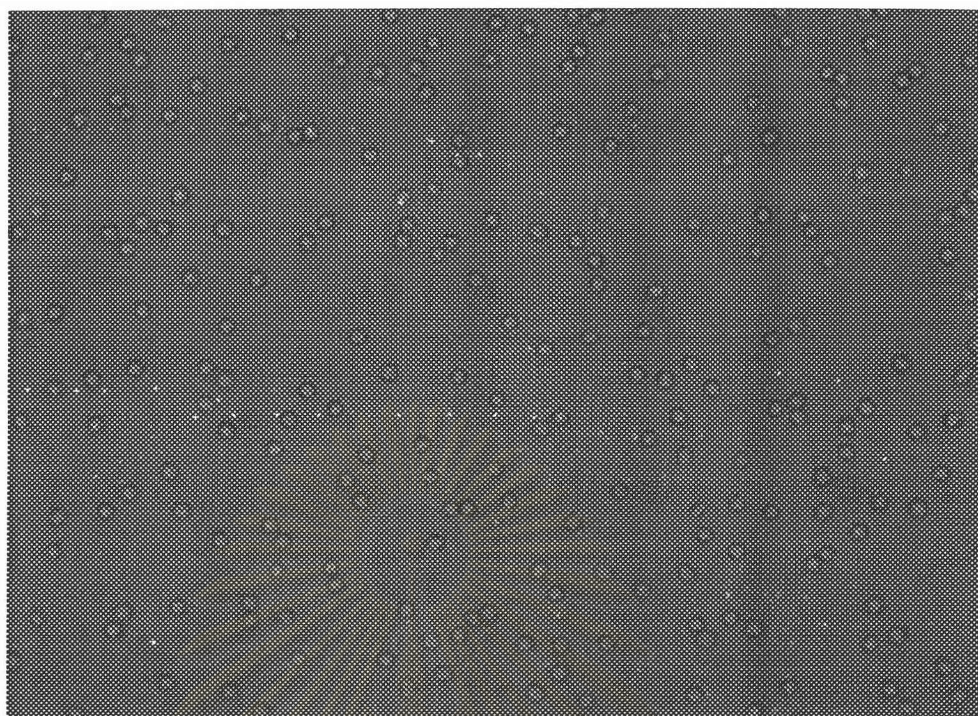

Figure 4.9 Blank of rabbit red blood cell Blank of rabbit red cell shown the red blood cells are not the cluster.

The determinations of amount of protein from Affi-Gel blue affinity chromatography were shown in **Table 4.7**.

Table 4.7 The amount of proteins from Affi-Gel blue affinity chromatography

Fraction No.	Amount of protein (μg)
A1,A2	4,481.40
A3	823.56

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A1, A2 were separated by 1-D gel electrophoresis and the results are shown in **Figure 4.10**

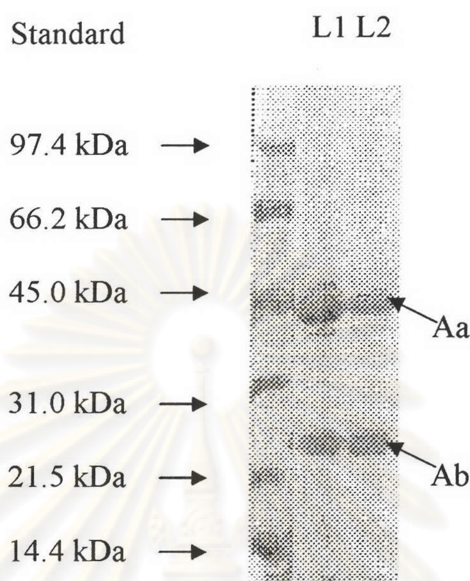

Figure 4.10 1-D SDS-PAGE gel stained by coomassie blue of fraction A1,A2 from Affi-gel blue gel chromatography. (L1 is crude proteins and L2 is A1 + A2 fraction)

Each spot in fraction L2 represented individual protein which was separated by molecular weight. L1 fraction also showed two spots which had the same molecular weight at the position of Aa and Ab spot in L2 fraction. The two spots have different molecular weight that can be separated by gel filtration chromatography (superdex 200 columns). The gel filtration chromatography was shown in **Figure 4.11** and **Figure 17** in Appendices.

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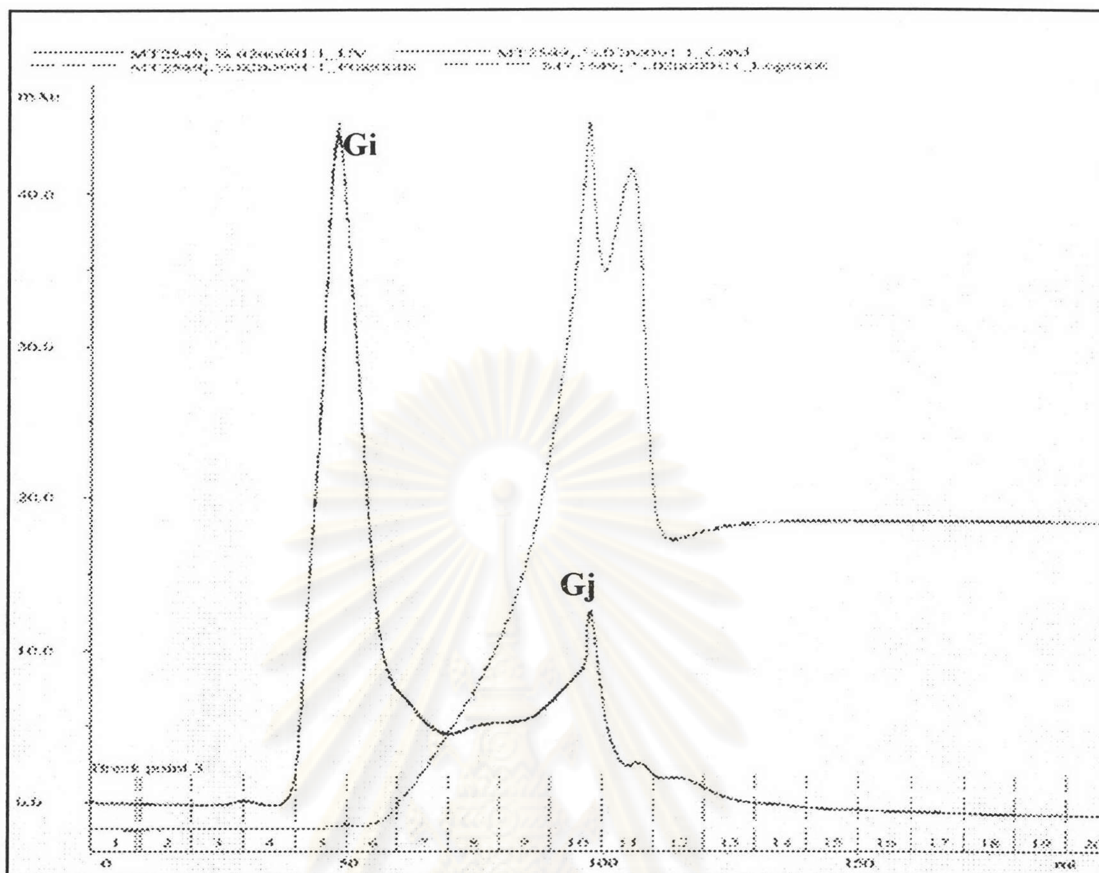

Figure 4.11 Gel filtration chromatogram of A1, A2 fraction from affi gel blue gel chromatography on superdex 200 column (1.6 x 60cm) in Double distilled water, flow rate 0.5 ml/min.

From this chromatogram showed two peaks which were called Gi and Gj fraction. The concentrations of proteins are 1036.70 and 31.02 μg, respectively. Gi fraction was the first fraction that presented higher molecular weight than Gj fraction. Consequently, Gi and Gj fraction were analyzed the component of protein by 1D SDS-PAGE. The result of the 1D gel was shown in the **Figure 4.12**.

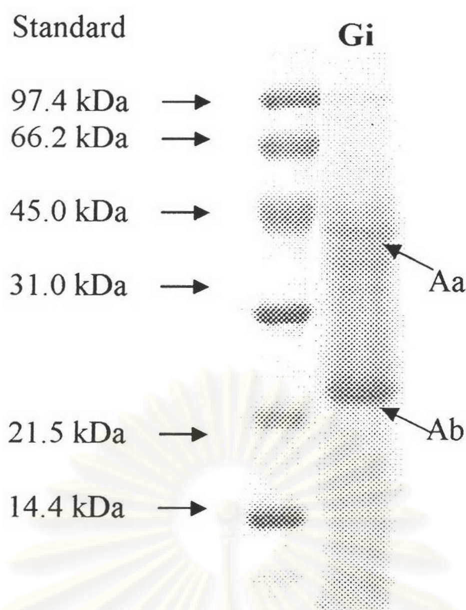

Figure 4.12 1D SDS-PAGE of fraction Gi from gelfiltration chromatography

This Gi fraction appeared 2 spots as the same position as Figure 4.10 which can not be separated by gel filtration chromatography. From the result, two proteins may be separated superdex 75 because this media separate proteins between 10^4 and 10^5 Da. For five times test, the peaks are different from another peak because of the superdex 75 column doesn't have efficiency to separate proteins Gi fraction. The chromatogram was shown in **Figure 4.13** and **Figure 10, 11, 12, and 13** in appendices.

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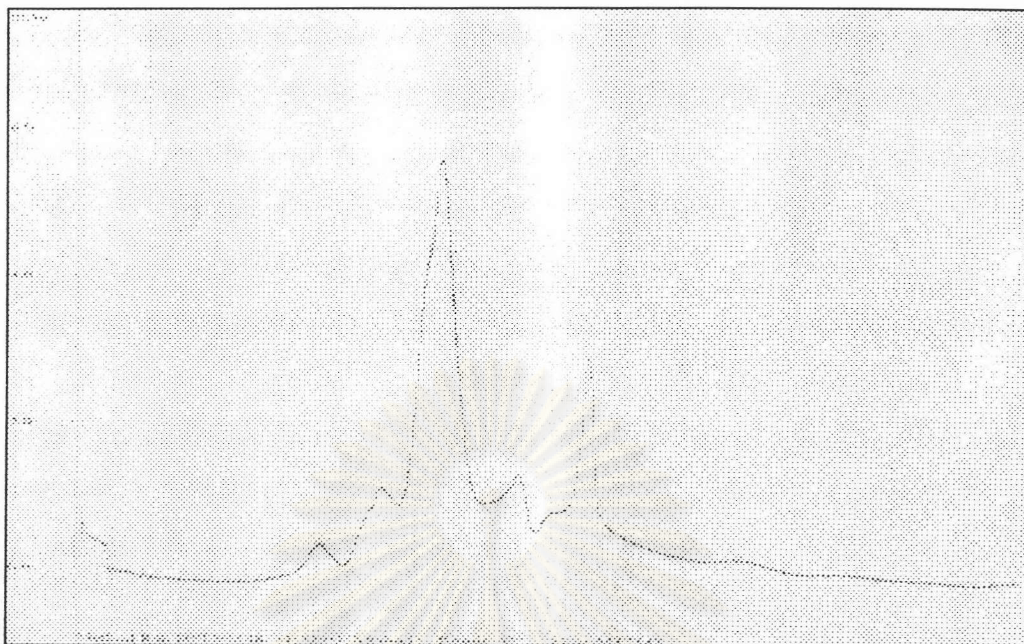


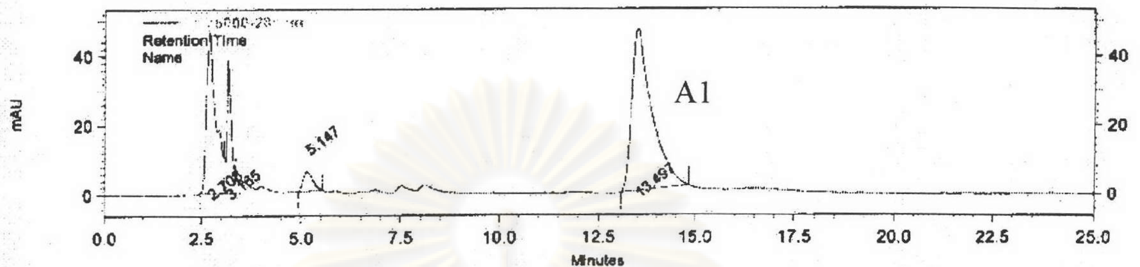
Figure 4.13 Superdex 75 gel filtration chromatogram of 25% A2 from affi gel blue gel chromatography, flow rate 0.5 ml/min.

High Performance Liquid Chromatography (HPLC) was used to separate Gi fraction which come from gel filtration chromatography. The suitable compositions of mobiles phase were analyzed by the ratios between acetone nitrile (ACN) and mili Q water. The ratios were 80:20, 40:60, 30:70, and 20:80. The results of analyst were shown in **Figure 18, 19, 20 and 21** in appendices, respectively.

From these chromatograms, the ratios between ACN and water, those are 80:20, 40:60 and 30:70 can not separate the proteins because the peaks had much lower resolution. So have to increase percentage of polar solvent (water) to 20:80 (ACN: water) which was a suitable ratio because the peaks result showed the highest resolution of A1 peak. The result was reported in **Figure 4.14,** A1 peak had the retention time at 13.497 min which was separated completely from other peaks.

The ratio between ACN and water is 20:80 was chosen to be the mobile phase for HPLC condition. The results are shown in **Figure 4.14** and **Figure 22, 23 and 24** in appendices.

uv 280 nm



uv 230 nm

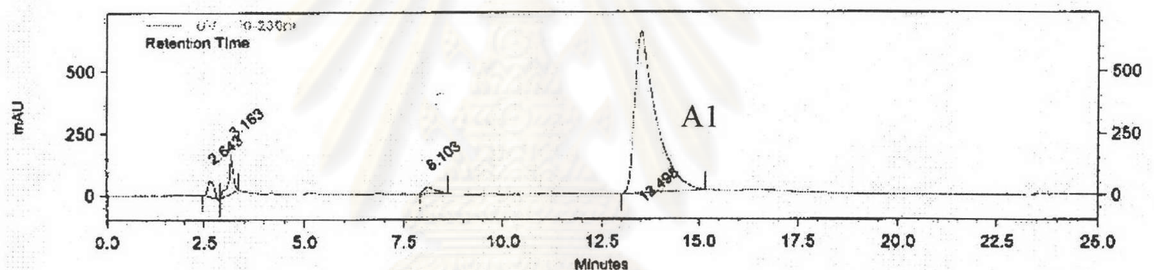


Figure 4.14 Chromatogram of Gi form HPLC mobile phase was ACN: water (20:80), flow rate 1.0 ml/min

A1 fraction showed ∝-glucosidase inhibition. After that, this fraction was found the component of proteins by 1D SDS-PAGE and the result was shown in **Figure 4.15**.

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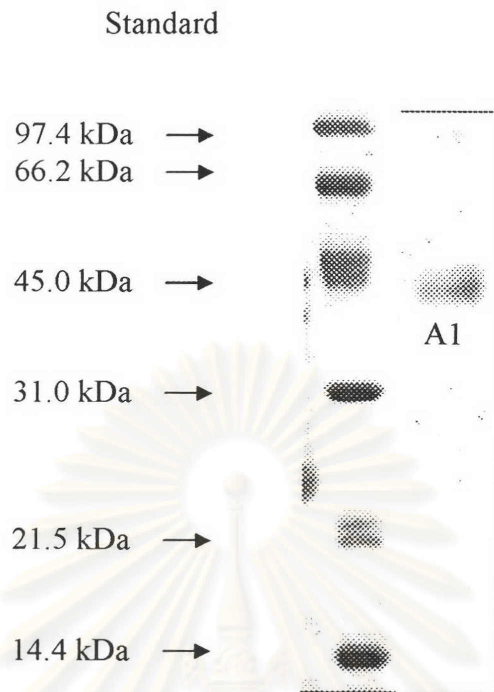

Figure 4.15 1D SDS-PAGE of fraction A1 from HPLC

From figure 4.15, there was one spot was called A1 which showed molecular weight about 45 kDa. This result of A1 fraction suggested that it has only one protein. After that, Gj fraction was analyzed by 1D-SDS-PAGE, the protein components were not be detected because this fraction has a small amount of protein. Furthermore, Gj was checked the component of proteins by MALDI-TOF MS. The result was shown in Figure 4.16.

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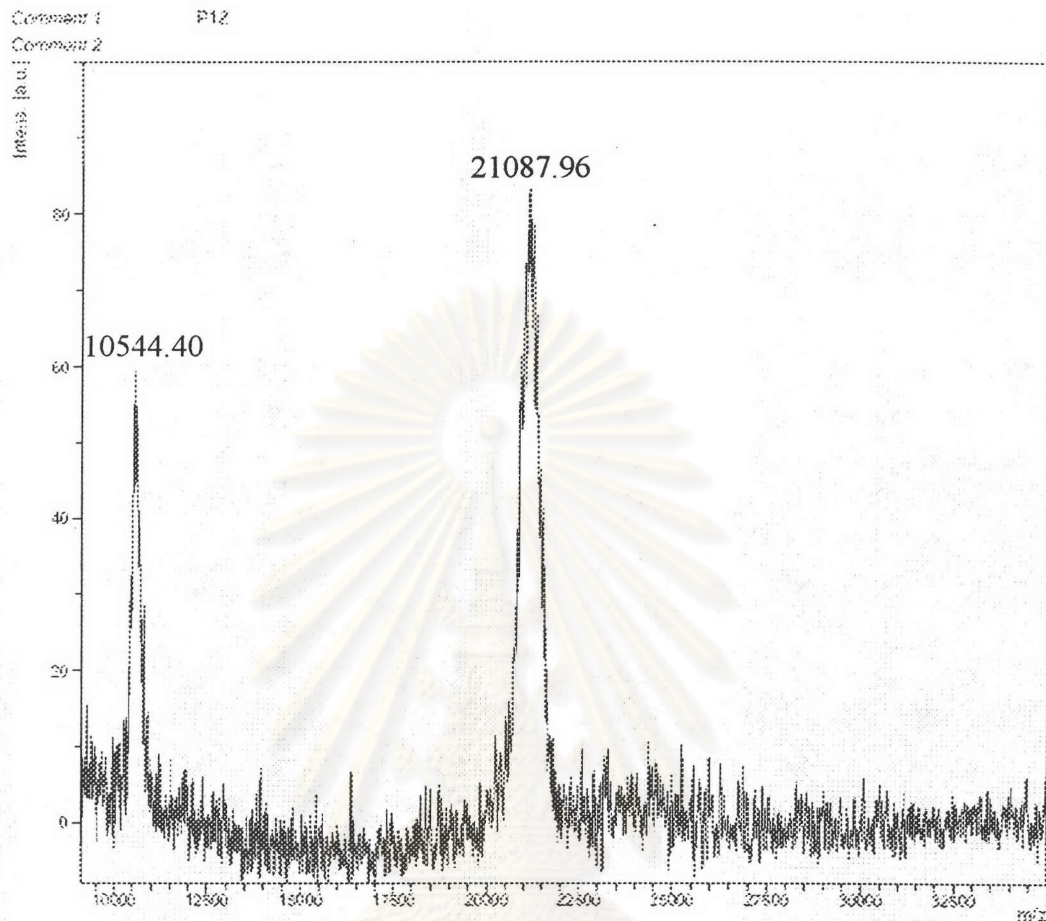

Figure 4.16 MALDI-TOF mass spectrum of Gj fraction from superdex 200 gel filtration

There were two peaks in Figure 4.16. The m/z of 21087.96 Da was singlet charged species and m/z of 10544.40 Da was doublet changed species of the same protein. Furthermore, Gj fraction showed hemagglutinatins activity with an IC_{50} of 0.39 μg/μl. From the result of fraction Gj suggested that it had only one protein which was easier for further analysis.

After proteins were separated by many steps, the A1 and Gj fraction were subjected to digestion with trypsin. The molecular weight of each tryptic fragment was analyzed by MALDI-TOF MS. A1 and Gj were chosen to analyze by mass spectrometry because they had only one protein. The mass spectra were shown in **Figure 6, 7** in appendices and the results were shown in Table 4.8

Table 4.8 The search results of protein A1 and Gj

Band of protein	m/z	Matching peptides	Molecular weight	NCBI Accession No.c	Organism	Score	% protein covered
A1	2791.5464 2728.5892 2524.5934 1279.5327 2673.9220 945.5638 2688.5641	7	55843	Q9LDD9_ ORYSA	*Oryza sativa* (Japonica cultivar group)	98	28
Gj	3860.9674 2037.6773 1249.4195 2280.6288	4	23232	Q8LN52_ ORYSA	*Oryza sativa* (Japonica cultivar group)	72	41

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Protein A1

Q9LDD9_ORYSA

Matched peptides shown in **Bold Black**

```
  1 MGWVRMRVRV RSPPVMQSKL LCLSLLYLLT TLPLALYVSF SDPASAASRC
 51 LVFLPFRSSA PSSAASAALF EYPREYGEHK HAIPATRALC SDPAVFSGRS
101 LSVREGHYKT VLEEINKFCR NLSASPYAKP ALRYQNGRRN SFAGNLSTVE
151 RRSFFNHTDS AVEIPCGFFK EFPVRESGDF FGQFAFFIEE KILVAHRLAM
201 EKCNGVVVAS AIFNDHDKIR QPKGLGSETL RTVCFFMFID DATHRVLASH
251 NILAGERGEA GTIGAWRVAR LVAGAGGDHR LPYENPAMNC VIVKYLLHRL
301 FPNARFSVWV DAKMQLTVDP LLLVHSFVAG KGADMAVSKH PFNLHTMEEA
351 IATARWRKWG DVDSIREQME TYCRNGLQPW SPIKLPYPSD VPDTAIIIRR
401 HGLASDLFSC LLFNELEAFN PRDQLAFAYV RDQMSPKVIM NMFDVEVFEQ
451 IAVEYRHNLK RGNGGAGGKQ GITRMASSGD IAGSSCERYL LKMWGETTE
```

(Bold in original: 50–74, 110–117, 153–175, 246–257, 271–294, 314–339, 375–399.)

Start - End	Observed	Mr(calc)	Delta	Sequence
50 - 74	2688.5641	2686.3577	1.1992	R.CLVFLPFRSSAPS SAASAALFEYPR.E
110 - 117	945.5638	944.5178	0.0387	K.TVLEEINK.F
153 - 175	2673.9220	2673.2685	-0.3538	R.SFFNHTDSAVEIP CGFFKEFPVR.E
246 - 257	1279.5327	1278.7044	-0.1790	R.VLASHNILAGER.G
271 - 294	2524.5934	2523.2725	0.3136	R.LVAGAGGDHRLP YENPAMNCVIVK.Y
314 - 339	2728.5892	2726.4498	1.1321	K.MQLTVDPLLLVHSF VAGKGADMAVSK.H
375 - 399	2791.5464	2789.5116	1.0276	R.NGLQPWSPIKLP YPSDVPDTAIIIR.R

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Protein Gj

Q8LN52_ORYSA

Matched peptides shown in **Bold Black**

1 MTLCWPLELE QQQQLR**WSSG SAPSAELIAI DDHPWSYGNY RCMPEGFMER**

51 **LDGLTNDVQE MLLHQRR**RQR **SSTASGGGGG ARER**MATVDH LKRLCIDHYF

101 QDEVDGAMDA HLEELAHGGD LLDATLAFRL MREAGHHVSP DEVLGRFTDG

151 NGDFSMAYSK DIRGLLSLQD ISHMNIGAEA SLYKAK**EFTS RNLQSAIDYL**

201 **EPGLAR**Y

Start - End	Observed	Mr(calc)	Delta	Sequence
17 - 50	3860.9674	3858.6864	1.2737	R.WSSGSAPSAELIAIDDH PWSYGNYRCMPEGFMER.L
51 - 67	2037.6773	2037.0425	-0.3725	R.LDGLTNDVQ EMLLHQRR.R
71 - 84	1249.4195	1248.5806	-0.1684	R.SSTASGG GGGARER.M
187 - 206	2280.6288	2279.1545	0.4670	K.EFTSRNLQSA IDYLEPGLAR.Y

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