Part II Partial Purification of Lectin from Hemolymph of *Penaeus merguiensis* with Antimicrobial Activity and Bacterial Clearance Activity

1. Characterization of the lectin

The lectin from hemolymph of *P. merguiensis* was purified by affinity chromatography on mucin-CNBr activated Sepharose 4B (Figure 8) and gel filtration on Sephacryl S-200 HR (Figure 9). The molecular weight of partial purified lectin results are summarized in Table10. The native molecular weight (Mr) of the lectin (PML) was 112 kDa and the protein consisted of 30.09 (PML1) and 28.01 kDa (PML2) subunits by SDS-PAGE (Figure 10). By using 2D-gel with immobilized pH gradients, the affinity purified lectin revealed two subunits of Mr 30.09 (PML1) and 28.01 kDa (PML2) with different isoelectric points (pI) as shown in Figure 11.

2. Partial amino acid sequences of lectin subunits

By using 2D-gel, the affinity purified lectin gave six high intensity spots as shown in Figure 11. Six protein spots of the affinity purified lectin were in gel digested and internal amino acid sequence analyzed by mass spectrometry. The internal partial amino acid fragmentation of spot 1, 2, 3, 4, 5 and 6 from affinity purified lectin by using 2D-gel as shown in Table 10. The purified lectin from hemolymph of banana prawn consisted of two bands with molecular weight of 30.09 (PML1) and 28.01 kDa (PML2) by SDS-PAGE while the protein spots number 2 and 3 gave molecular weight of 31.67 kDa and spots number 4 and 5 gave molecular weight of 29.39 kDa by 2D-gel. These results showed that PML1 gave the spot number 2and 3 while PML2 gave the spot number 4 and 5. The protein subunits spot 1 to 5 gave different amino acid sequences but not homologus to the proteins from invertebrate on database. However, the amino acid sequence of protein subunit spot number six was homologous to hemocyanin from *L. vannamei* (Figure21). A representative spot 1, 2, 3, 4, 5 and 6 are the spectra presented in Figure12-20.

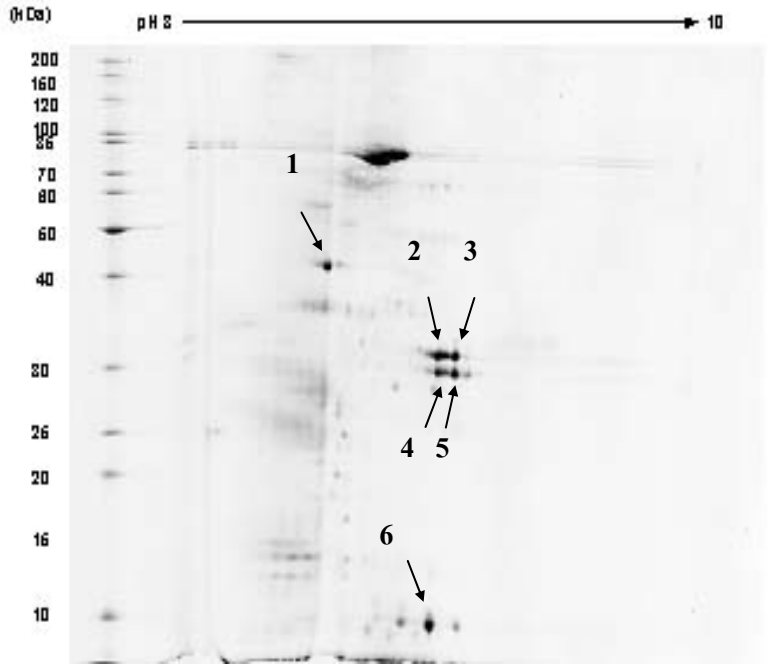
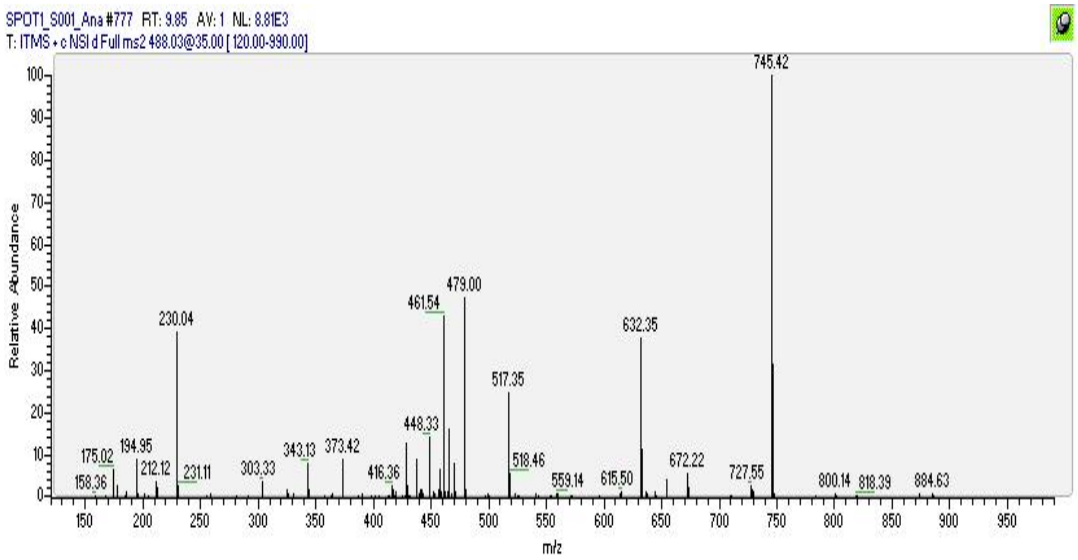


Figure 11 2-D gel performed with 200 μg of affinity purified lectin shows the seperation of subunits by using Immobiline DryStrip pH 3-10 (13 cm). Spots of subunits are indicated by arrow and number. Spot number 1 was observed at Mr 45.19 kDa. Spot number 2 and 3 were observed at Mr 30.09 kDa, spots number 4 and 5 at 28.01 kDa and spot number 6 observed at Mr 10.12 kDa.

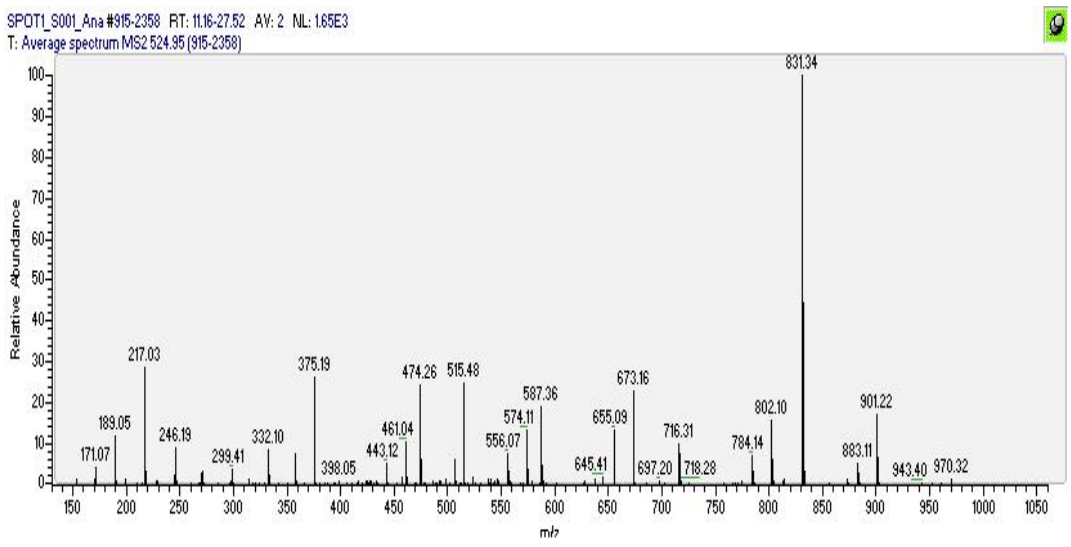
Table 10 Partial amino acid sequences of the affinity purified lectin subunits from hemolymph of *Penaeus merguiensis*

Spot	Rf	MW(kDa)	Amino acid sequence	Accession no.	Figure
1	0.36	45.19	(Q/K)(L/I)DT(L/I)(Q/K)R	No similarity	12
			DE(L/I)VEVK	No similarity	13
			DGV(L/I)S(Q/K)VK	No similarity	14
			(L/I)(Q/K)(F/MO)G(L/I)GDENSK	No similarity	15
2	0.51	31.67	MQTILYKANSR	No similarity	16
3	0.51	31.67	GG(L/I)ADSDCGASGSG-K	No similarity	17
			N(L/I)ADSDLGASGSG-K	No similarity	
			GGNAGGSDCGASGSG-K	No similarity	
			NNAGGSDCGASGSG-K	No similarity	
4	0.53	29.39	YEELQITAGR	No similarity	18
5	0.53	29.39	GDN(L/I)NGVYG-R	No similarity	19
6	0.94	10.12	HWFSLFNPR	gi 7414468 emb CAB85965.1 Hemocyanin (*Litopenaeus vannamei*) Mass: 76502.8 No. of Amino acids: 671 pI = 5.54	20



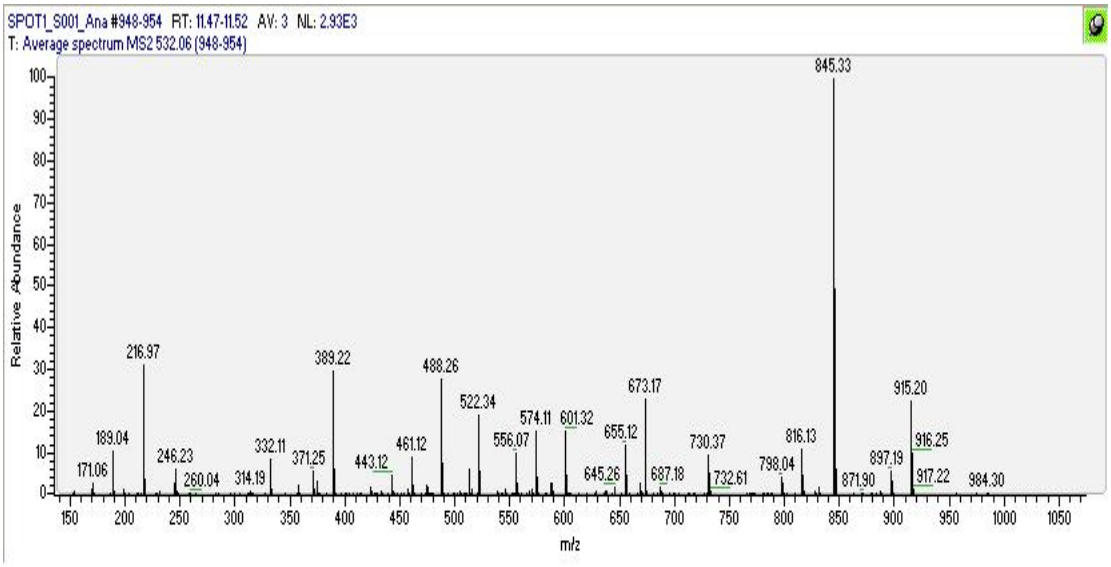
(Q/K)(L/I)DT(L/I)(Q/K)R

Figure 12 Mass spectrum of spot no.1 by LC-MS/MS; precursor mass 974.



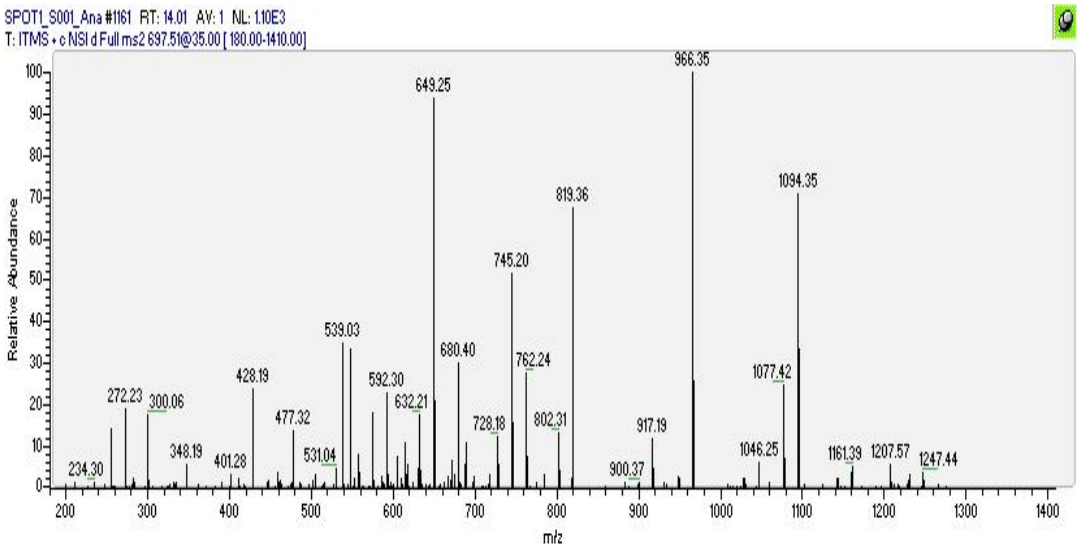
DE(L/I)VEVK

Figure 13 Mass spectrum of spot no.1 by LC-MS/MS; precursor mass 1047.



DGV(L/I)S(Q/K)VK

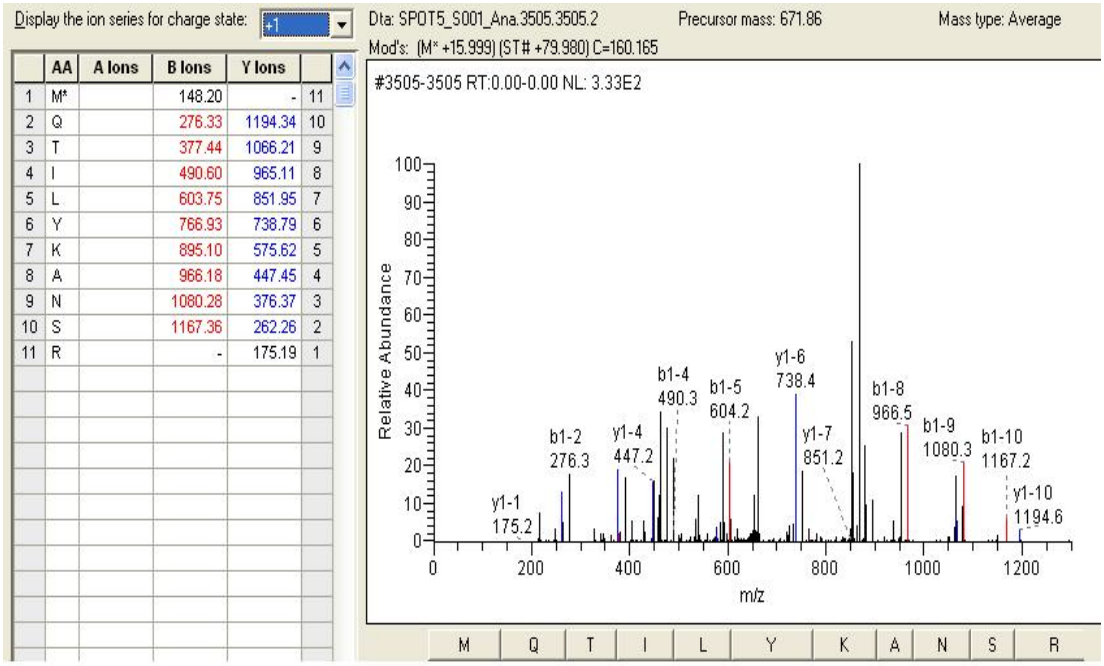
Figure 14 Mass spectrum of spot no.1 by LC-MS/MS; precursor mass 1062.



(L/I)(Q/K)(F/MO)G(L/I)GDENSK

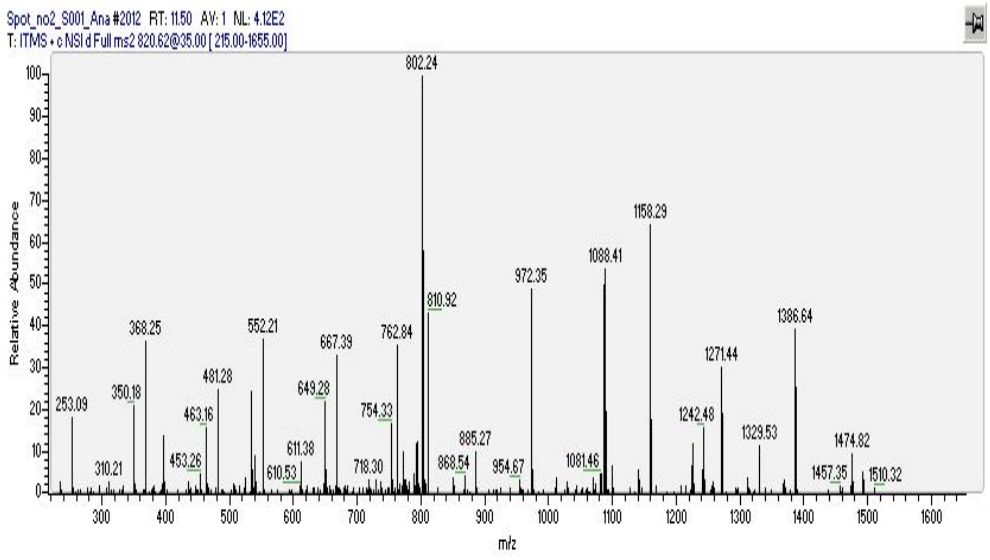
MO=Methionine oxidation

Figure 15 Mass spectrum of spot no.1 by LC-MS/MS; precursor mass 1393.



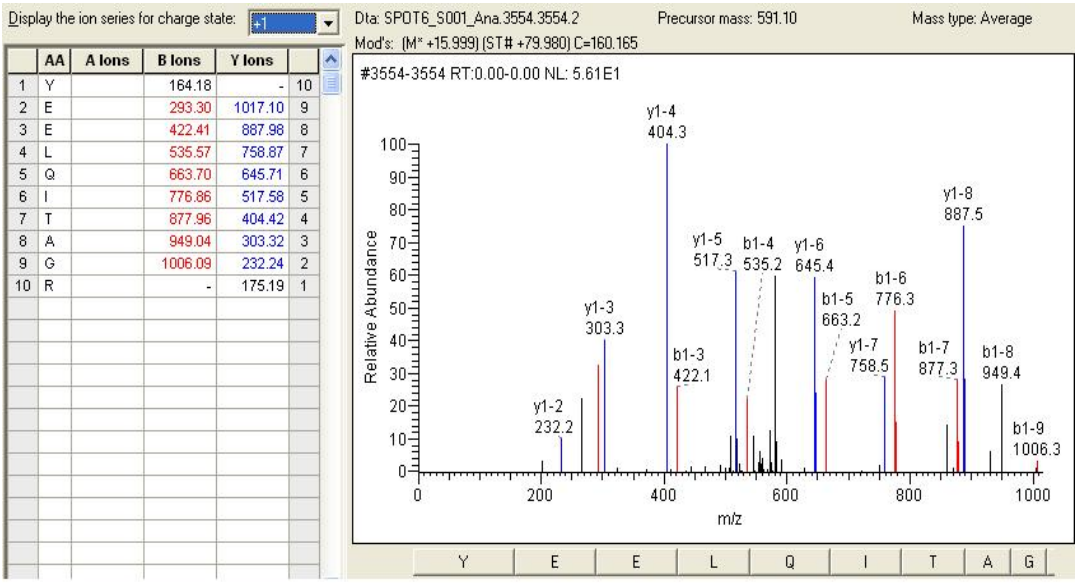
	AA	A Ions	B Ions	Y Ions	
1	M*		148.20	-	11
2	Q		276.33	1194.34	10
3	T		377.44	1066.21	9
4	I		490.60	965.11	8
5	L		603.75	851.95	7
6	Y		766.93	738.79	6
7	K		895.10	575.62	5
8	A		966.18	447.45	4
9	N		1080.28	376.37	3
10	S		1167.36	262.26	2
11	R		-	175.19	1

Figure 16 Mass spectrum of spot no.2 by LC-MS/MS; precursor mass 671.86.



Spot no.3 Mass 820.62
Amino acid sequence GG(L/I)ADSDCGASGSG-K
N(L/I)ADSDCGASGSG-K
GGNAGGSDCGASGSG-K
NNAGGSDCGASGSG-K

Figure 17 Mass spectrum of spot no.3 by LC-MS/MS; precursor mass 820.62.



	AA	A Ions	B Ions	Y Ions	
1	Y		164.18	-	10
2	E		293.30	1017.10	9
3	E		422.41	887.98	8
4	L		535.57	758.87	7
5	Q		663.70	645.71	6
6	I		776.86	517.58	5
7	T		877.96	404.42	4
8	A		949.04	303.32	3
9	G		1006.09	232.24	2
10	R		-	175.19	1

Figure 18 Mass spectrum of spot no.4 by LC-MS/MS; precursor mass 591.10.

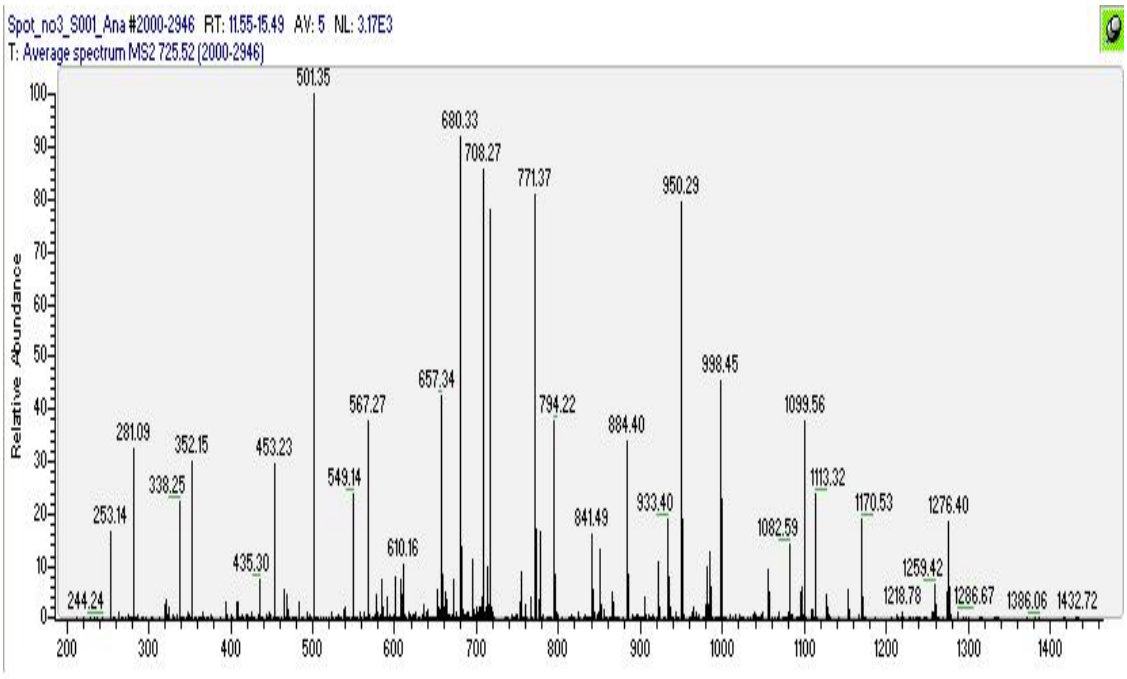
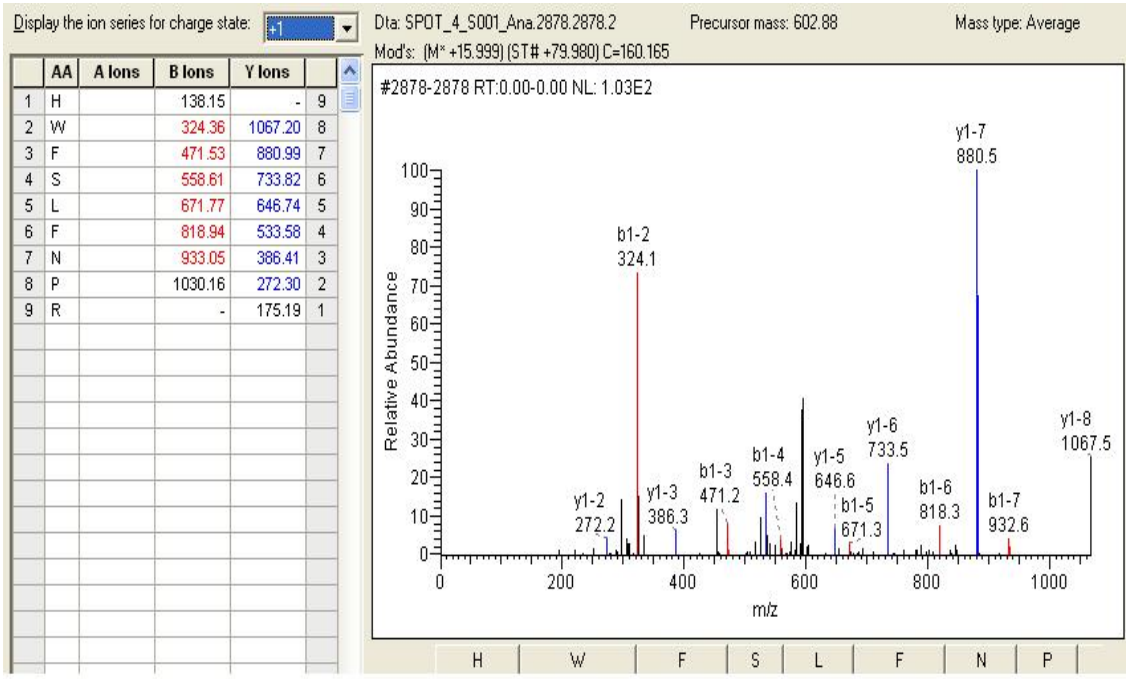
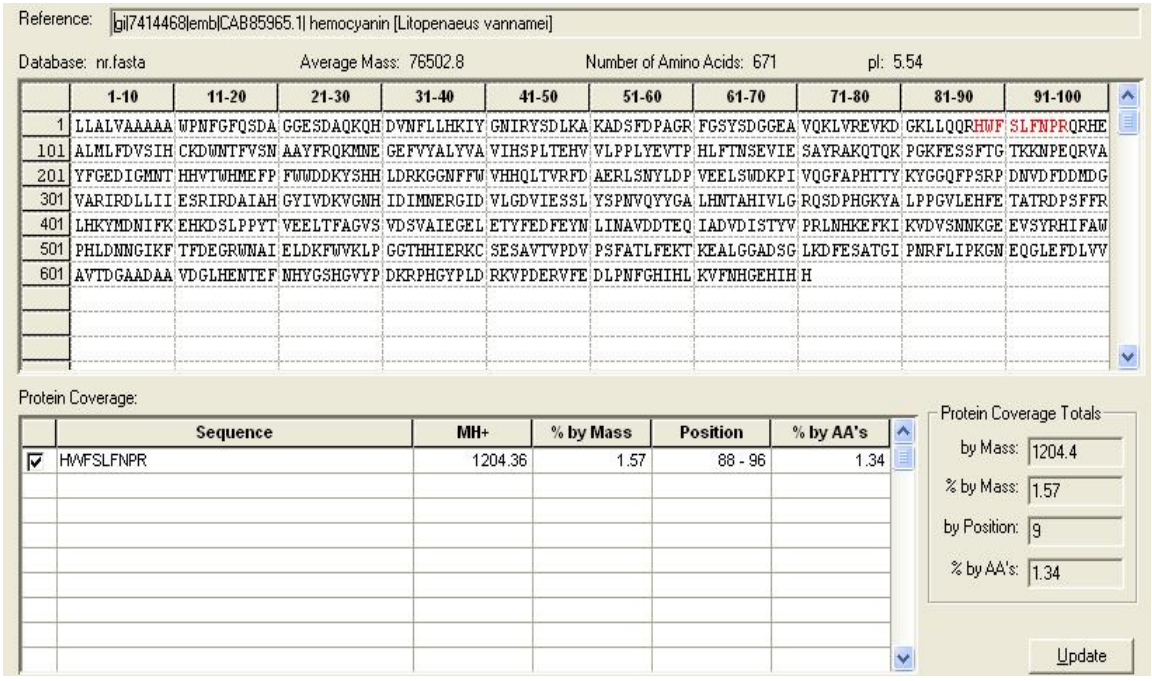


Figure 19 Mass spectrum of spot no.5 by LC-MS/MS; precursor mass 725.74.



	AA	A Ions	B Ions	Y Ions	
1	H		138.15	-	9
2	W		324.36	1067.20	8
3	F		471.53	880.99	7
4	S		558.61	733.82	6
5	L		671.77	646.74	5
6	F		818.94	533.58	4
7	N		933.05	386.41	3
8	P		1030.16	272.30	2
9	R		-	175.19	1

Figure 20 Mass spectrum of spot no.6 by LC-MS/MS; precursor mass 602.88.



	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100
1	LLALVAAAAA	WPNFGFQSDA	GGESDAQKQH	DVNFLLHKIY	GNIRYSDLKA	KADSFDPAGR	FGSYSDGGEA	VQKLVREVKD	GKLLQQRHWF	SLFNPRQRHE
101	ALMLFDVSIH	CKDWNTFVSN	AAYFRQKMNE	GEFVYALYVA	VIHSPLTEHV	VLPPLYEVTP	HLFTNSEVIE	SAYRAKQTQK	PGKFESSFTG	TKKNPEQRVA
201	YFGEDIGMNT	HHVTWHMEFP	FWWDDKYSHH	LDRKGGNFFW	VHHQLTVRFD	AERLSNYLDP	VEELSWDKPI	VQGFAPHTTY	KYGGQFPSRP	DNVDFDDMDG
301	VARIRDLLII	ESRIRDAIAH	GYIVDKVGNH	IDIMNERGID	VLGDVIESSL	YSPNVQYYGA	LHNTAHIVLG	RQSDPHGKYA	LPPGVLEHFE	TATRDPSFFR
401	LHKYMDNIFK	EHKDSLPPYT	VEELTFAGVS	VDSVAIEGEL	ETYFEDFEYN	LINAVDDTEQ	IADVDISTYV	PRLNHKEFKI	KVDVSNNKGE	EVSYRHIFAW
501	PHLDNNGIKF	TFDEGRWNAI	ELDKFWVKLP	GGTHHIERKC	SESAVTVPDV	PSFATLFEKT	KEALGGADSG	LKDFESATGI	PNRFLIPKGN	EQGLEFDLVV
601	AVTDGAADAA	VDGLHENTEF	NHYGSHGVYP	DKRPHGYPLD	RKVPDERVFE	DLPNFGHIHL	KVFNHGEHIH	H		

	Sequence	MH+	% by Mass	Position	% by AA's
☑	HWFSLFNPR	1204.36	1.57	88 - 96	1.34

Figure 21 Amino acid sequence which partial amino acid HWFSLFNPR blase to NCBI (Mass spectrum of Spot 6 by LC-MS/MS; precursor mass 602.88).

3. Antibacterial activity

The antibacterial activity of lectin from the hemolymph affinity and gel filtration purification (1.28 μg protein) from banana prawn is presented in Table 12. The affinity purified lectin exhibited strong antibacterial effect against all tested strains of inhibition ranging from 21.26-68.75%. The affinity purified lectin showed the highest antibacterial activity against *V. alginolyticus* approximately 68.75%. While, gel filtration purified lectin showed the highest antibacterial activity against *V. mimicus,* approximately 96.87%. The lectin from gel filtration purification showed more stronger antibacterial activity on *Vibrio* spp. than lectin from affinity purification except *V. cholerae, P. aeruginosa* and *S. aureus.* Serum of the banana prawn showed slight effect against some *Vibrio* spp., with inhibition ranging from 17.64-45.32% while inhibitory effect was not found on *V. harveyi, P. aeruginosa, S. aureus* and *M. luteus.*

Table 11 Antibacterial activity in serum, hemolymph affinity and gel filtration purified *Penaeus merguiensis* lectin incubated with *Vibrio* spp. for 1 h. Percentage of inhibition was determined from the appropriate serial dilution as colony forming unit (CFU).

Type of bacteria	% inhibition±SD		
	with serum	with affinity purified lectin	with gel filtration purified lectin
Vibrio fluvialis	34.44±10.05	61.16±6.28	76.02±2.41
V. alginolyticus	45.32±10.47	68.75±6.22	71.41±0.81
V. mimicus	29.66±7.96	33.90±2.05	96.87±1.37
V. harveyi	0	47.38±11.45	69.06±7.85
V. parahemolyticus	40.51±6.43	21.26±2.93	76.74±1.93
V. cholerae	17.64±7.15	62.05±0.29	0
Pseudomonas aeruginosa	0	21.97±2.70	0
Escherichia coli	18.12±3.95	13.01±4.58	75.69±5.36
Staphylococcus aureus	0	42.11±0.38	27.08±3.0
Micrococcus luteus	0	28.09±6.95	43.27±3.46

4. Protein profile of affinity purifed lectin from *Vibrio harveyi* infected *Penaeus merguiensis* hemolymph

The investigation on the relationship between the role of lectin to eliminate bacterial infection was carried out using *V. harveyi* as test organism. The amount of lectin in hemolymph after post injection with *V. harveyi* at 1, 15, 30, 45, 60 and 120 min gave hemagglutinating specific activity to be 1144.77, 1146.77, 1234.15, 1440.69, 1057.85 and 2143.33 titer/mg protein, respectively (Figure 22). In addition, the result showed that the rate or efficiency of bacterial clearance was decreased after 15 min and near zero at 60 min and increased again at 120 min. Hemagglutinating specific activity was high at 45 min instead of bacterial colony is very low. Until, at 60 min hemagglutinating specific activity and bacterial colony was lowest, it suggested that lectin might be agglutinated bacteria and some bacterial colony was not agglutinate and grown up again.

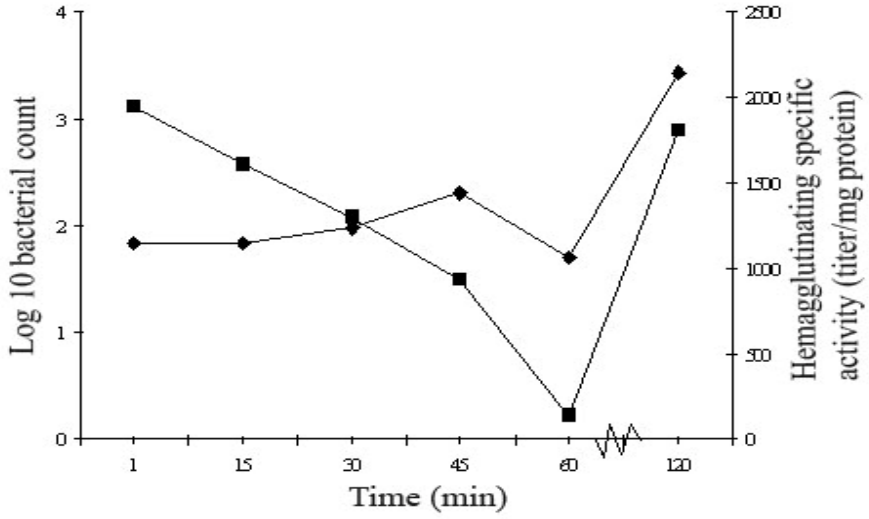


Figure 22 Bacterial clearance activity in banana prawn after challenge with *Vibrio harveyi*, ■ bacterial count, ♦ hemagglutinating specific activity

In order to study the lectin expression, PML1 and PML2 were separated by SDS-PAGE (Figure 23). The quantities of the protein bands were determined automatically by the ImageQuant TL software and presented in the intensity and the result was confirmed by 2-D gel electrophoresis. Using the 2-D gel, we were able to

detect five or six high intensity spots (Figure 24). A 2-D gel was used to investigate differences in the pattern of affinity purified lectin from hemolymph of banana prawn after challenged with *V. harveyi* for 1, 15 and 120 min (Figure 25). The results in Table 12 summarizes the hemagglutinating specific activity, the number of bacterial count, the lectin subunits intensity by SDS-PAGE and the different protein spot intensity by 2-D gel after banana prawn had been exposed to *V. haveyi*. For 2-D gel, the amount of spot 2 plus 3 refered to PML1 and spot 4 plus 5 refered to PML2.

In comparison of lectin expression to the number of bacterial count, the result showed that the intensity of PML1 and spot no.1 were the highest at 15 min which the bacterial count was decreasing. At the same time, the intensity of spots no. 2 to spot no. 5 were in the range of 0.045028-0.133672. On the other hand, after 15 min the number of bacterial count decreased but hemagglutinating specific activity, PML2, intensity of spot 2 plus 3 and spot 4 plus 5 tended to increase. After post injection 60 min, the bacterial count increased again which was similar to the hemagglutinating specific activity and the volume of spots no.2 to spot no. 5.

The result suggested that lectin might agglutinate bacteria and some bacterial colonies were not agglutinated and could grow up again. In this result, bacterial clearance ability was not due only to lectin, it might dependent on other immune substances.

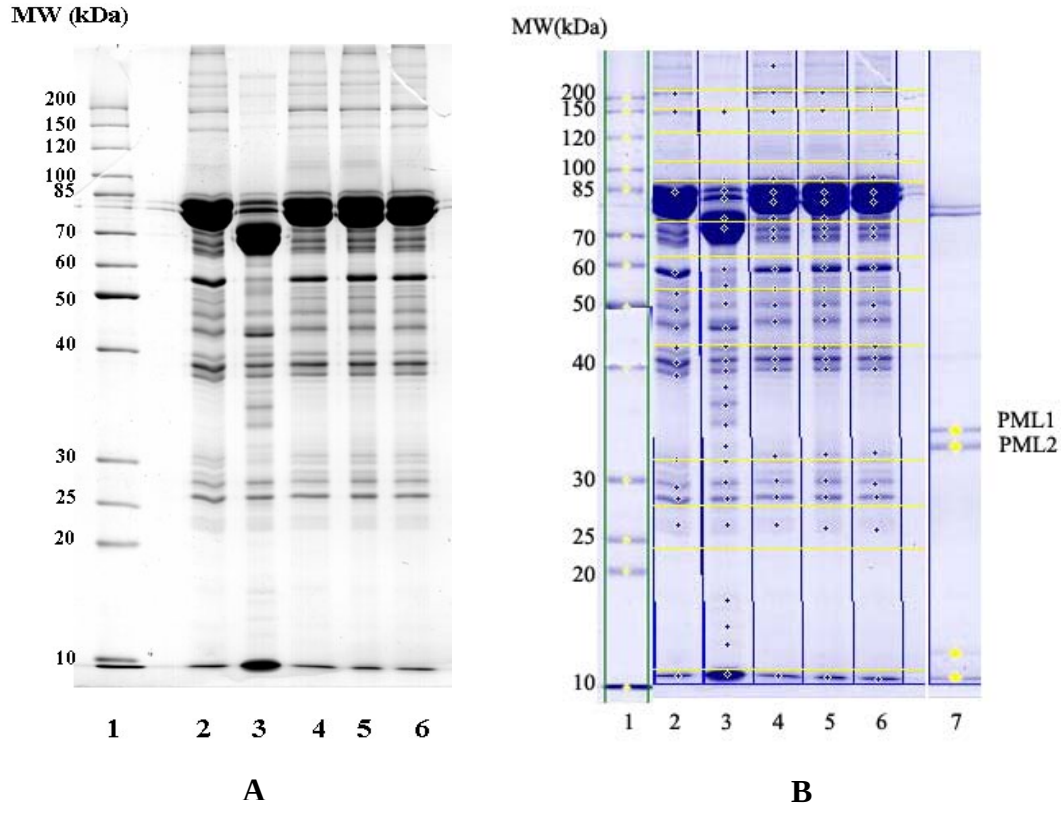


Figure 23 Protein profile of hemolymph from the *P. merguiensis* in 10% SDS-PAGE (13 cm,) after challenged with *V. harveyi*. Molecular weight markers (lane1), hemolymph (20 µg protein) after post injection with *V. harveyi* at 1, 15, 30, 45 and 60 min, lane 2, 3, 4, 5 and 6 respectivly (A and B) and affinity purified lectin, lane7(B). Figure 23-B, The protein bands were analyzed by ImageQuant TL V2005 (Amersham Bioscience, USA).

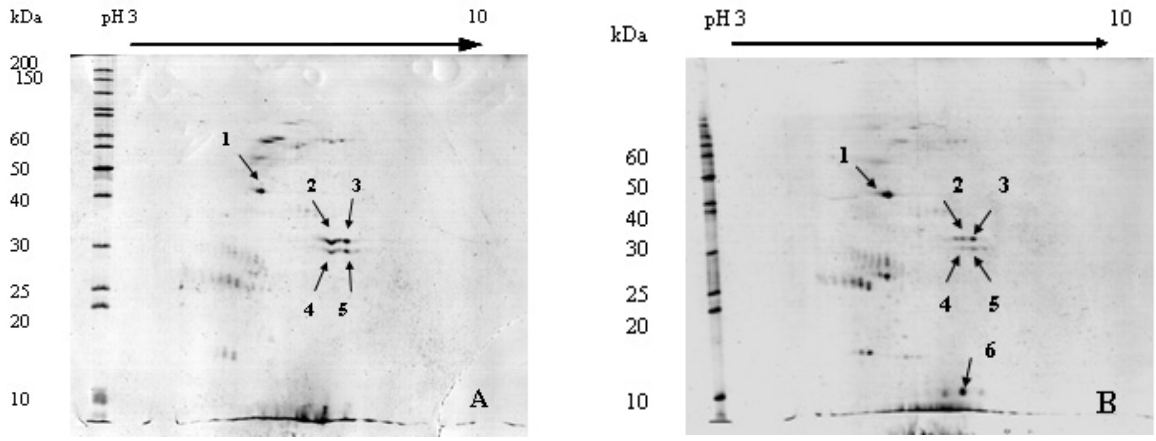


Figure 24 2-D maps of colloidal Coomassie brilliant blue G-250 stained proteins of the affinity purified lectin after post injection with *V. harveyi* at 1 min (A) and 120 min (B). The protein was separated by 2-Dimensional Gel Electrophoresis. Isoelectric focusing (IEF) was perfomed in the Immobiline™ DryStrip pH3-10, 13 cm, IPG strips in a disposable cassette. Protein volumes were adjusted in order to analyze the same amount of 200 μg for each set of injected samples. Maps were analyzed with ImageMaster 2D Platinum software. Labeled spots are described previously on Figure 3. A comparison was made between the affinity purified lectin after post injection with *V. harveyi* at 1 min and 120 min. Labeled spots (1-6) are responsed to *V. harveyi* infection.

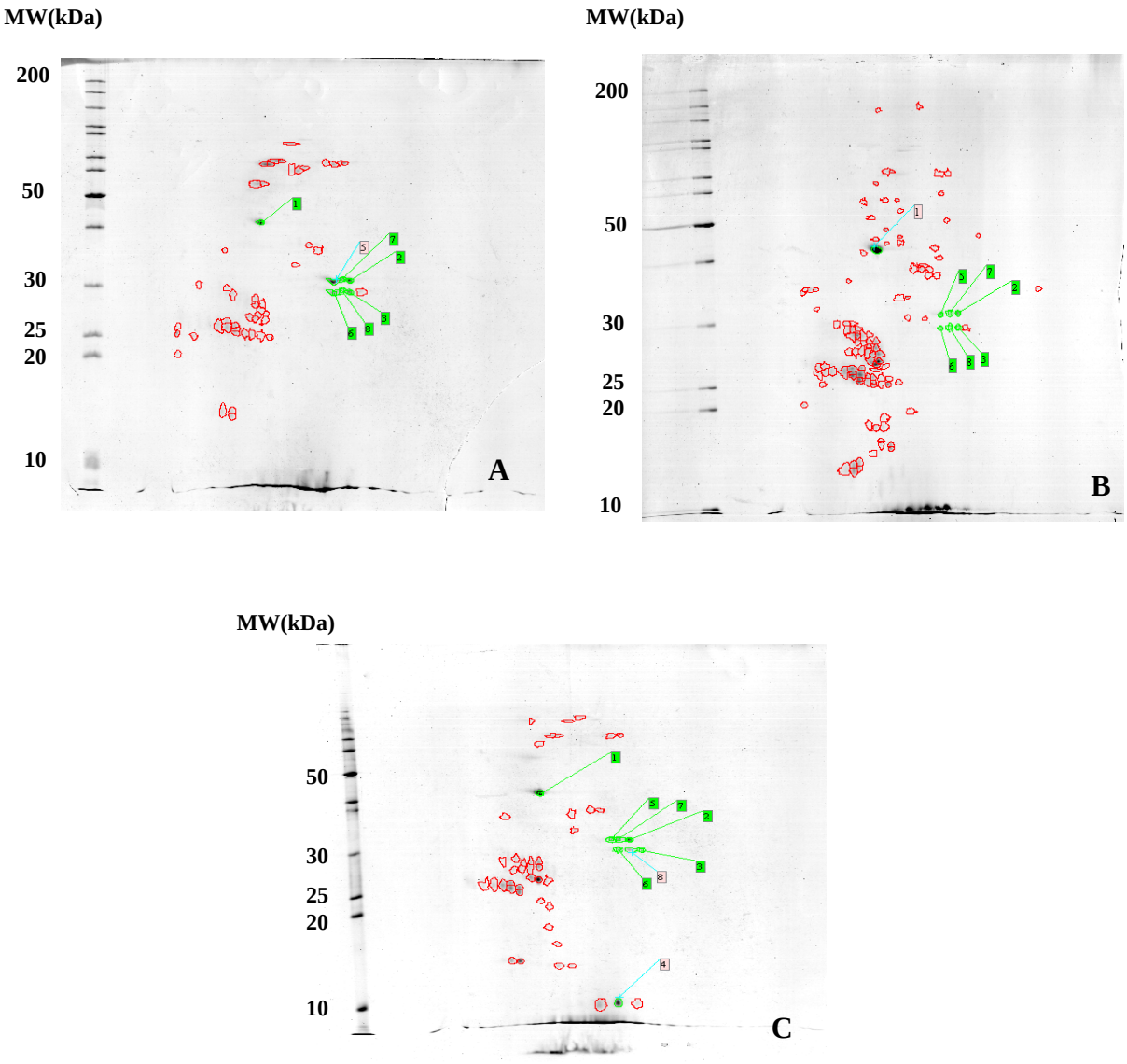


Figure 25 2-D maps of colloidal Coomassie brilliant blue G-250 stained proteins of the affinity purified lectin after post injection with *V. harveyi* at 1 min (A) 15 min (B) and 120 min (C). Protein volumes were adjusted in order to analyze the same amount of 200 μg for each set of injected samples. Maps were analyzed with ImageMaster 2-D Platinum software. A comparison was made between the affinity purified lectin after post injection with *V. harveyi* at 1, 15 and 120 min (green labeled spots (1-8)). Green and red label spots are responsed to *V. harveyi* infection. (Figure 25A, 25B and 25C as shown in appendix)

Table 12 Protein expression in hemolymph of *Penaeus merguiensis* after challenging with *Vibrio harveyi.*

Time after bacterial injection (min)	Hemagglutinating specific activity (titer/mg protein)	Bacterial Count (n=10) mean (CFU)±SD	SDS-PAGE Intensity×10^5 PML1 band	PML2 band	Spot Intensity from 2D-gel Spot 2+3	Spot 4+5	Spot Intensity from 2D-gel 1	2	3	4	5	6
1	1144.77	1318±0.43	49.9	39.25	1.121417	0.573962	0.535281	0.669488	0.451929	0.317006	0.256956	0.239643
15	1146.77	372±0.49	132.66	41.67	0.213166	0.084577	2.00586	0.079494	0.133672	0.039549	0.045028	0.841620
30	1234.15	117±0.38	74.47	38.90	-	-	-	-	-	-	-	-
45	1440.69	32±0.29	41.05	48.34	-	-	-	-	-	-	-	-
60	1057.85	2±0.31	44.75	52.69	-	-	-	-	-	-	-	-
120	2143.33	776±0.27	44.84	74.22	0.4712425	0.137864	1.200329	0.168125	0.3031175	0.078455	0.0594086	0.680408