CHAPTER 4

RESULTS

4.1. Analysis of Viral Infection in Prawn Samples

M. rosenbergii of random size/weight (20-40g) grown in an aquaculture farm was obtained. These prawns were obtained from commercial growing farms. Prior to the collection of IHHNV infected serum, a sample set of 20 prawns were selected from a total of 300 prawns. These prawns were tested for infectious hypodermal and IHHNV by extracting DNA from the swimming legs and subsequent detection using appropriate commercial PCR detection kits (Promega). Six out of the twenty prawn samples showed positive results for viral infection when tested with the commercial kits**.**

4.2. IHHNV Screening

The final PCR product of the screened samples were run on a 1.5% agarose gel and then stained with ethidium bromide 1x solution for 30 minutes (Figure 4.1).

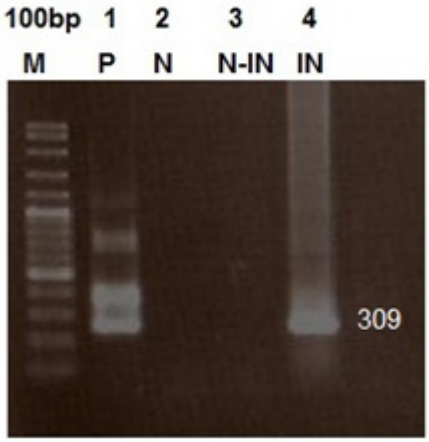


Figure 4.1, Detection of IHNNV in the infected hemocyte using specific primers. Crude IHHNV sequence was used as a positive control (P, Lane 1) and deionized water was used as negative control (N, Lane 2) .Non-infected samples (N-IN, Lane 3) did not show any PCR product. The 309bp PCR product, extracted from swimming leg DNA was observed in infected samples (IN, Lane 4).

4.3. Bovine Serum Albumin Protein Assay Standards

In order to quantify the total protein of a purified enzyme or specific protein, a sensitive, rapid, and consistent method for determining protein concentration is required. The Bradford dye binding assay is a well-known method to measure the relative concentration of purified proteins from the serum of *M .rosenbergii*. The BSA range used to develop two standard curves was from 0µg/ml to 100µg/ml. The absorbance was measured for each sample at 595 nm. The resulting line was fit by the linear least squared method. Pure serum was subjected to the Bradford assay in the same way. In theory, the relative protein concentrations in each sample must be determined by using the absorbance and equation for the generated line in the BSA standard curve for each sample.

Table 4.1, Bradford Assay Standard Curve

OD Values				
Serial Dilution BSA	Repeat 1	Repeat 2	Repeat 3	Mean
0	0	0	0	0
2	0.04	0.066	0.02	0.042
4	0.09	0.128	0.04	0.086
6	0.118	0.206	0.118	0.147333
8	0.136	0.308	0.136	0.193333
10	0.181	0.3212	0.181	0.227733

4.3.1. **Standard Curve**

For the colorimetric assay, the method developed by Bradford was employed using Coomassie blue dye and BSA as a standard. From this standard, a line was plotted directly from the absorbance values of the BSA to their corresponding known concentrations. As shown in Figure 4.2 24, the protein concentration was determined based on the standard curve.

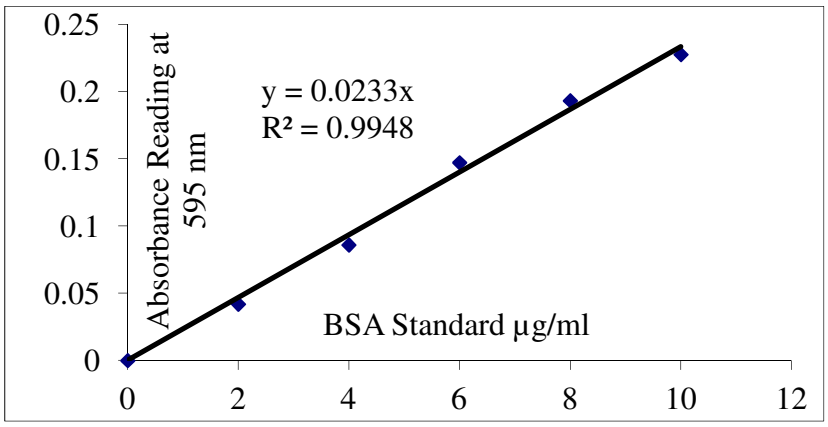


Figure 4.2, Standard Curves for Protein Determination Using BSA

4.4. Dimensional Gel Electrophoresis (2-DE)

4.4.1. **Construction of Proteome Maps from Extracted Prawn Serum**

Protein was extracted from the pooled serum of four prawns. A total of 450 µg of the soluble protein sample was subjected to 2-DE. The experiment was carried out as 13 independent 2-DE experiments using different prawn samples under the same 2-DE conditions. The serum proteins were optimized in the pH gradient of 4 - 7 and 12.5% SDS-PAGE. Approximately 300 protein spots on the silver stained gel were digitally detected in each experiment by PDQuest software. An example is shown in Fig 4.3 and 4.5(silver stained). Next from the Coomassie stained gels protein spots were chosen based on intensity and subjected to analysis MALDI TOF-TOF Mass spectrometry. Through database searches, 20 protein spots were categorized into 8 groups according to their function within the cell (Fig 4.3). These were proteins associated with cell function and physiology (14%), energy production and catabolism (14%), cell structure (5 %), antioxidants (5%), ATP-buffering and environmental stress (5%), calcium homeostasis (5%), oxygen transportation (19%) and immune system related proteins (33%) (Figure 4.5).

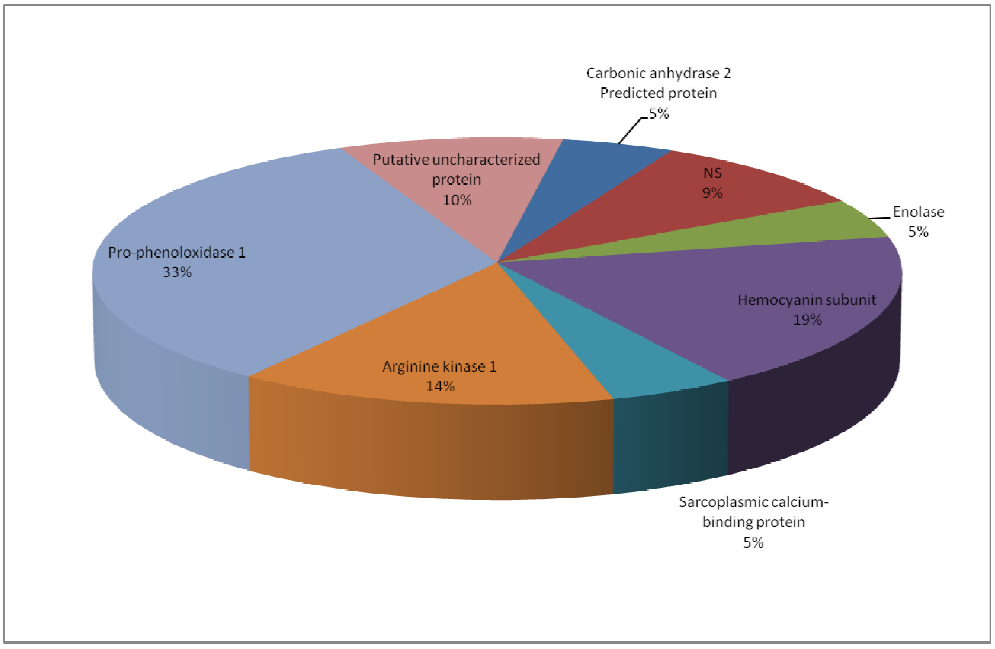


Figure 4.3, The grouping of the 20 Serum-protein spots from 2DE- gels after they were subjected to MALDI TOF-TOF Mass spectrometry followed by Data base searches. The 20 proteins were divided into 8 groups according to their functions within the cell.

4.4.2. **Comparative 2-DE Analysis and Identification of Serum Proteins**

The serum protein profiles were obtained from non-infected and infected prawn. The protein profiles were compared to determine differential protein expression. Protein expression levels were determined through spot intensity calculations by software analysis. The changes in protein spot intensity levels for the 20 protein spots were presented as ratios (mean ± standard deviation from 8 independent experiments) which represent the protein expression levels in IHHNV infected and non-infected prawn. When 2-DE was performed it was found that the surviving prawn without any signs of IHHNV infection at the time of 50% cumulative mortality showed 10 protein spots that appeared to be up regulated and 10 protein spots that appeared to be down-regulated (Figure 4.3 and Figure 4.4) once infected with IHHNV.

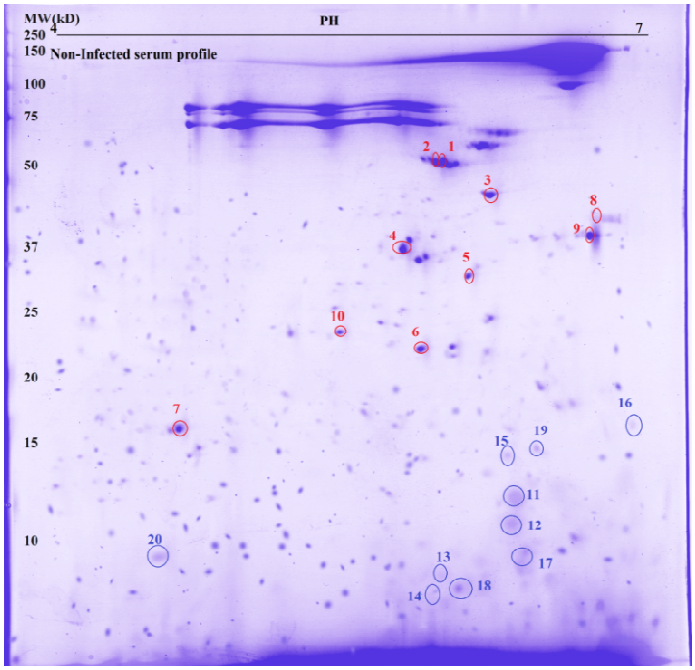


Figure 4.4, 2D gel view of non- infected samples stained with coomassie blue. Control sample is marked (A). The position of up-regulated protein spots (Blue circles) are numbered from 11-20. And the position of down-regulated protein spots (Red circles) is numbered from 1-10. Proteins are distributed within a pH range of 4 – 7 and a molecular weight range of 75 - 10 KD on 12.5% acrylamide gel.

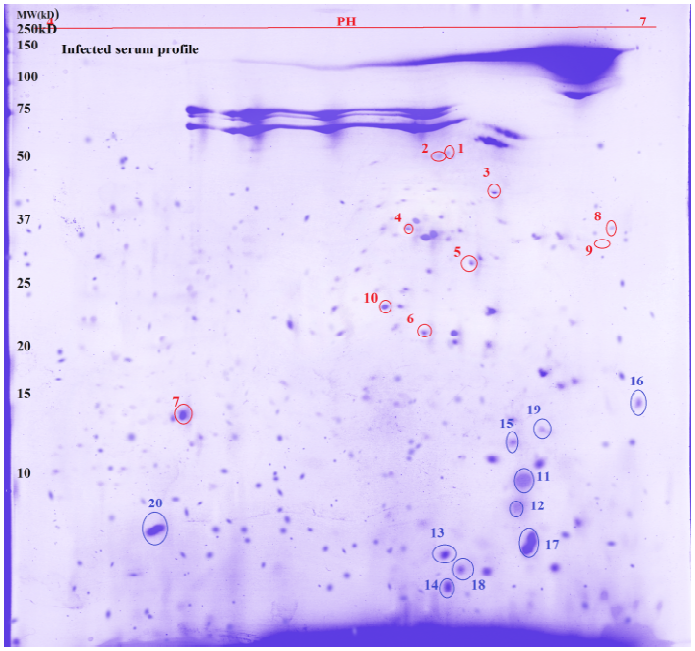
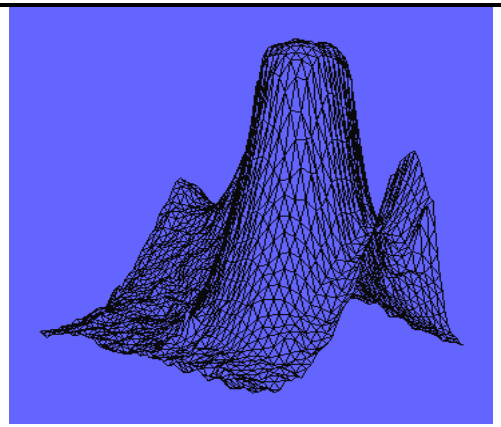
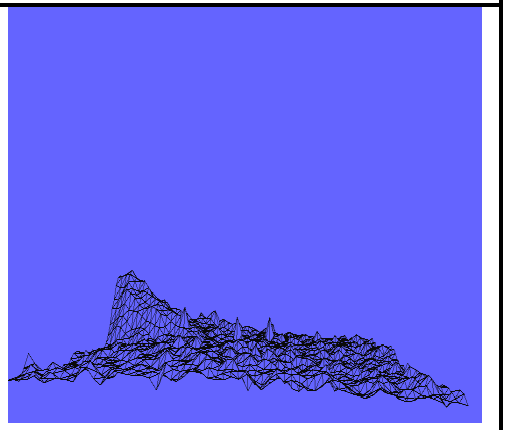
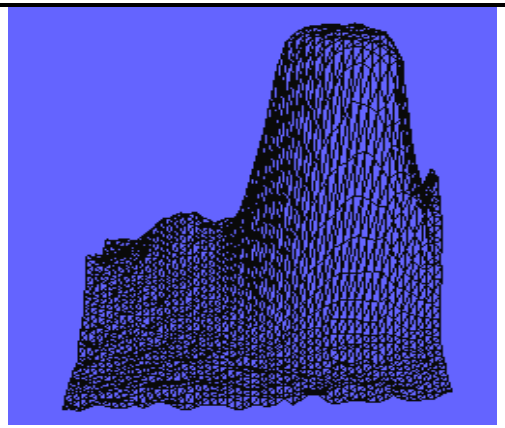
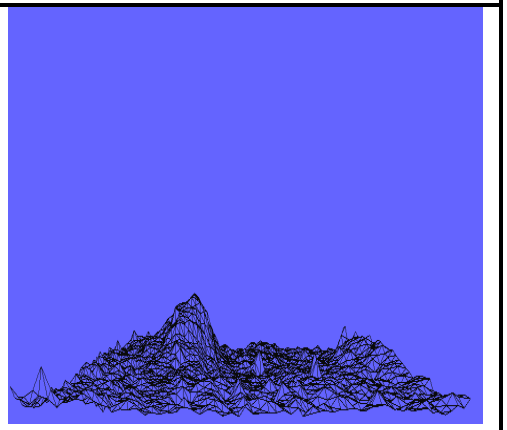
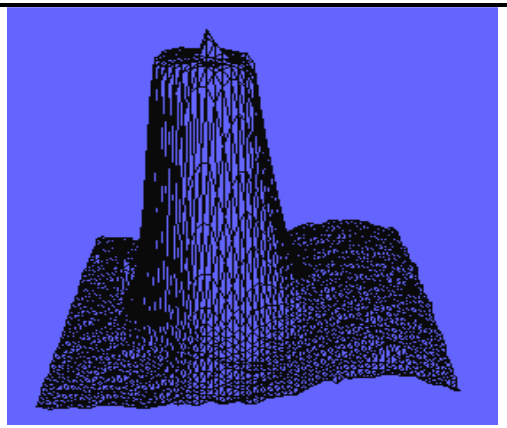
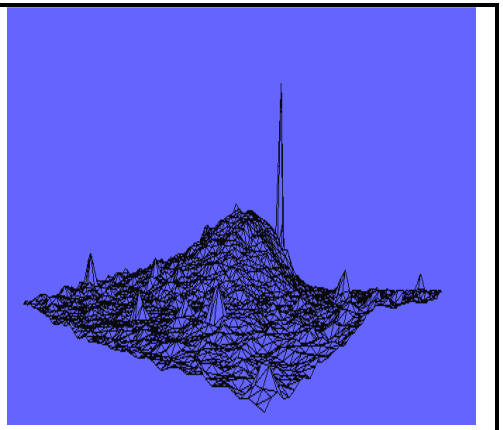
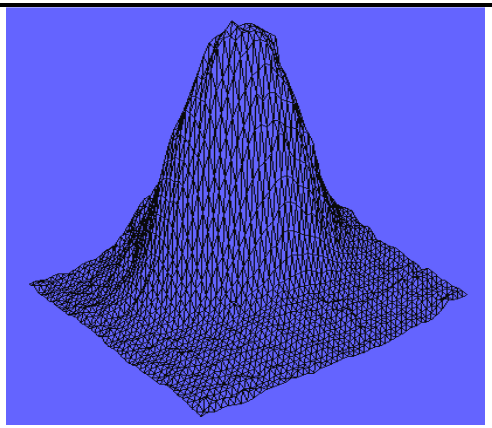
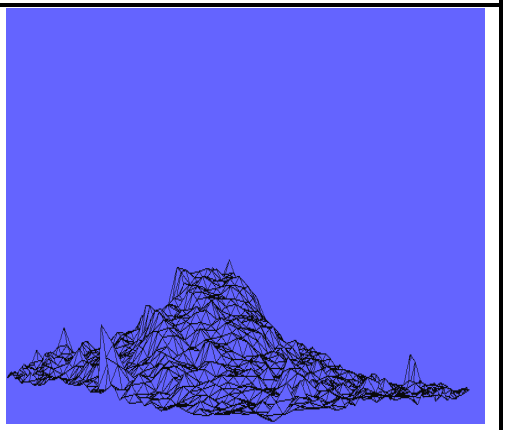


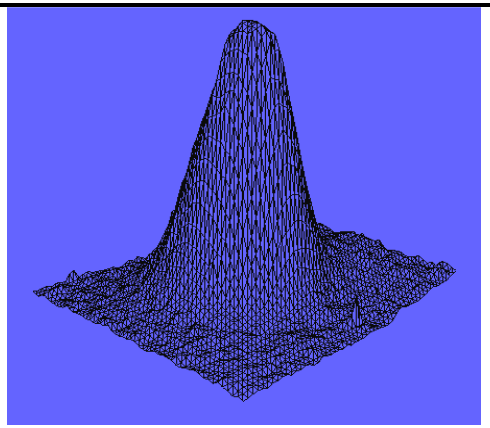
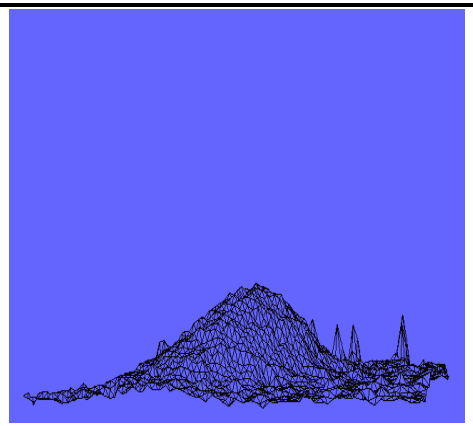
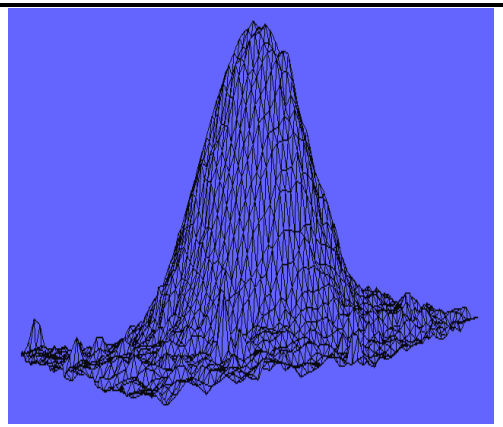
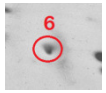
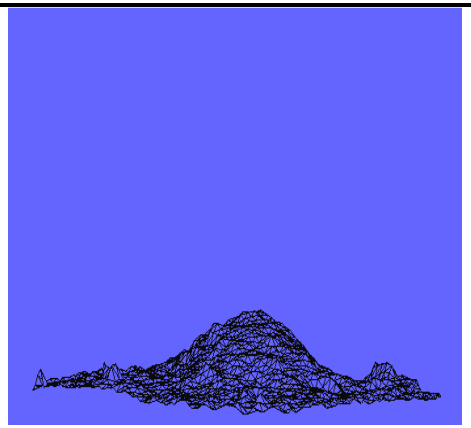
Figure 4.5, 2D gel view of IHHNV infected samples stained with Coomassie blue. Control sample is marked (A). The position of up-regulated protein spots (Blue circles) are numbered from 11-20. And the position of down-regulated protein spots (Red circles) is numbered from 1-10. Proteins are distributed within a pH range of 4 – 7 and a molecular weight range of 75 - 10 KD on 12.5% acrylamide gel. Blue circles show up-regulated proteins and Red circles indicate down-regulated proteins, based on expression levels.

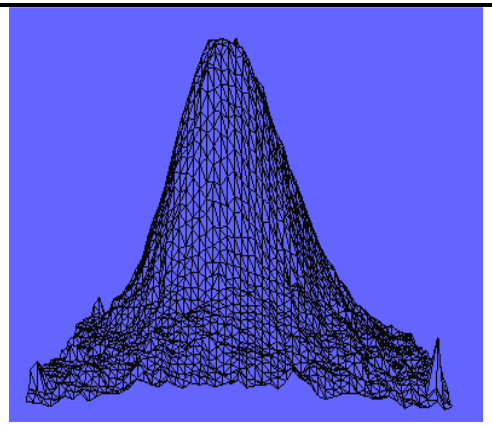
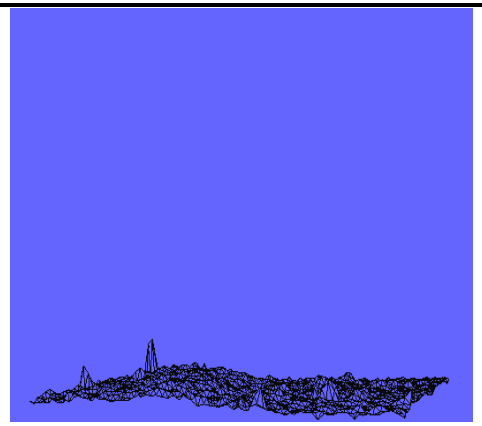
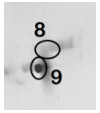
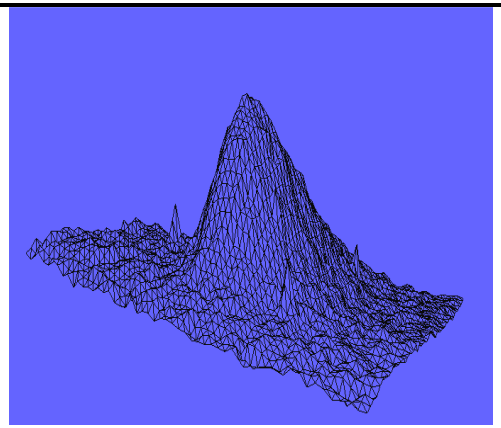
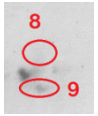
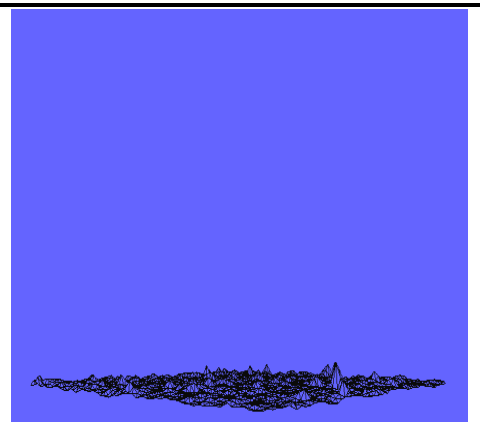
These protein spots confirmed the consistence of protein expression levels in 8 independent experiments from different IHHNV infected and non-infected prawn samples. Then the gel image is subject to contrast adjustment so the 3D view which is adjusted relative to the area surrounding the spot shows the scaled to the most intense spot. This means that if a spot of interest is next to a much more intense spot the 3D view is scaled to the most intense spot and would confirm the spot determination (Table 4.2).

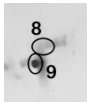
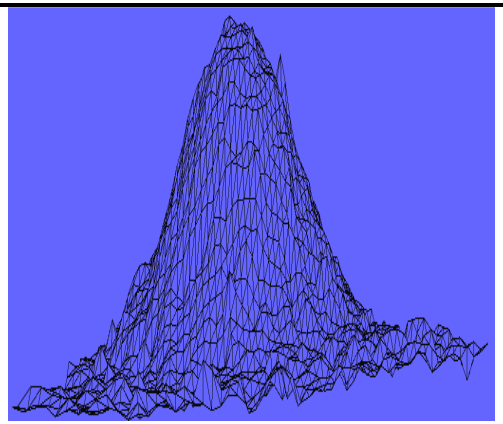
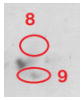
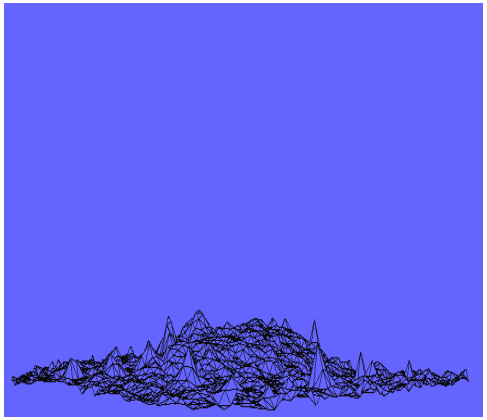
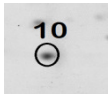
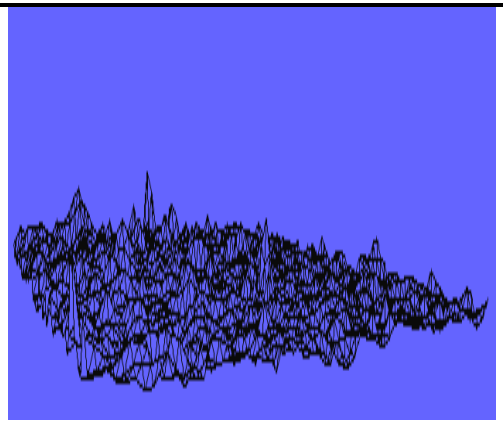
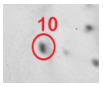
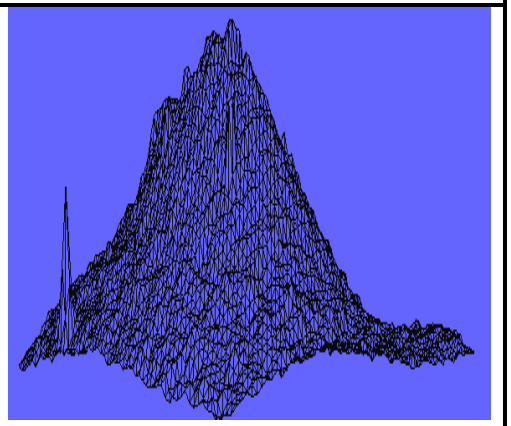
Table 4.2, 3D- view of infected and non-infected spot

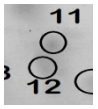
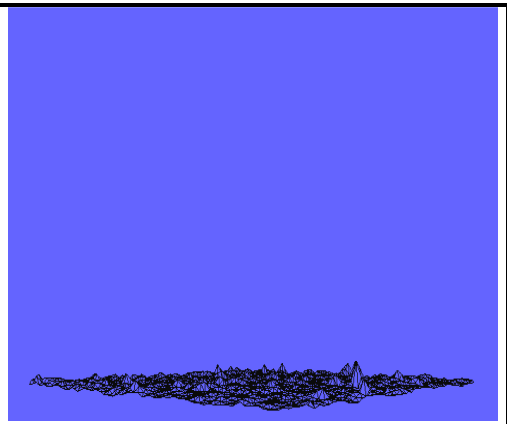
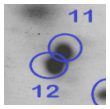
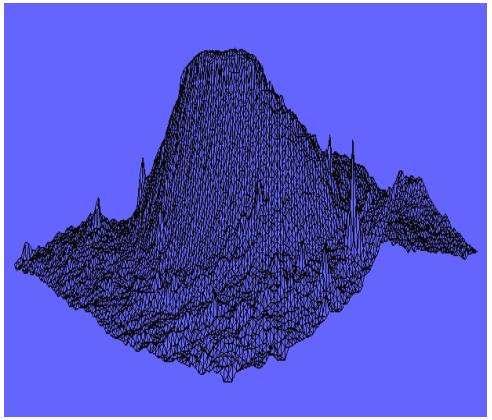
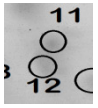
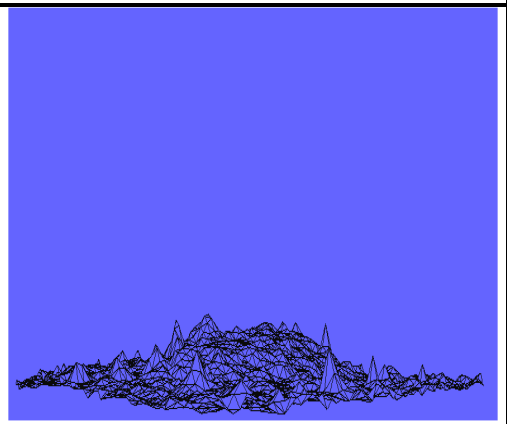
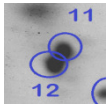
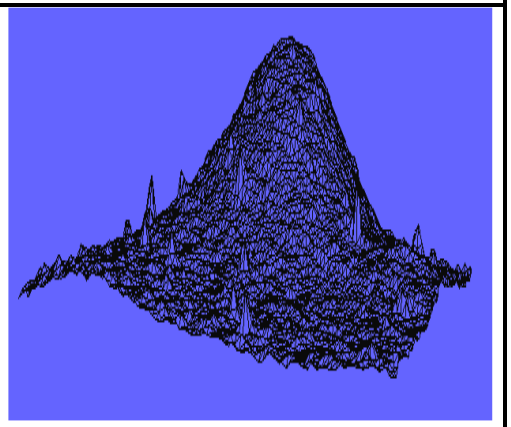
Spot	Protein name	IHHNV/Control Mean±SD	control	3D-view IHHNV Infected	IHHNV Infected	3D- view
1	Carbonic anhydrase 2 Predicted protein (Fragment)	0.51±0.00				
2	Predicted protein (Fragment)	0.75±0.2				

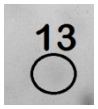
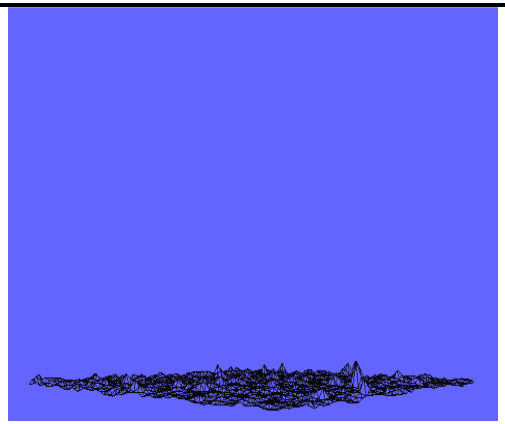
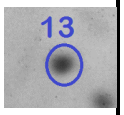
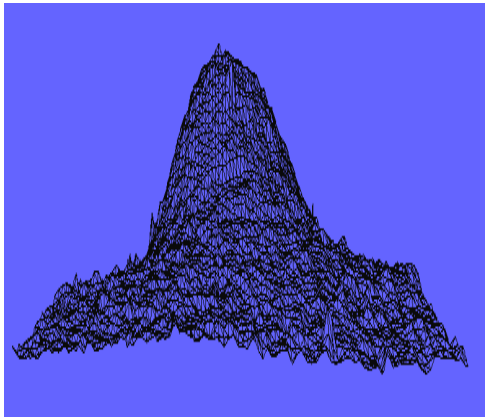
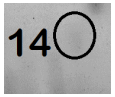
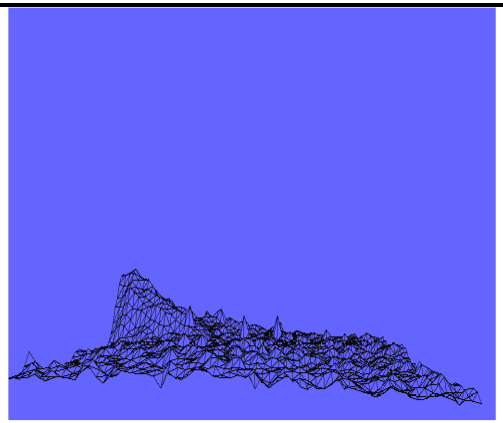
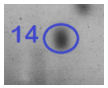
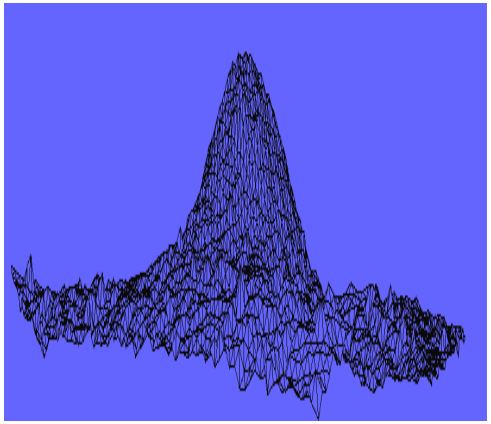
3	Enolase	0.34±/.00				
4	Hemocyanin subunit	0.19±.00				

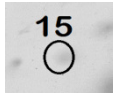
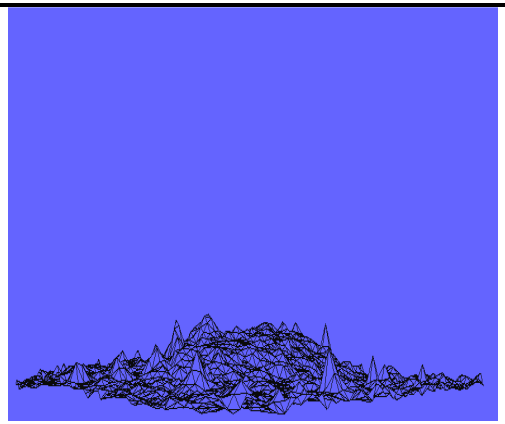
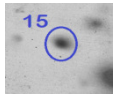
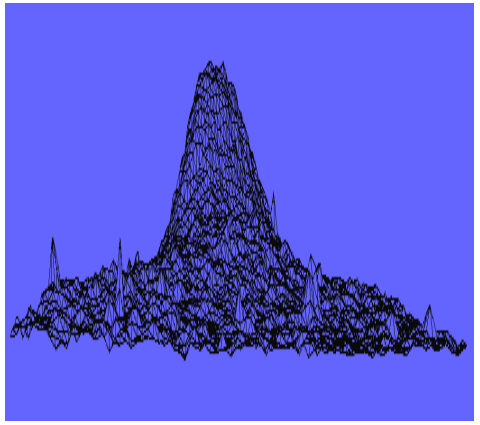
5	Hemocyanin subunit L	0.33±.03				
6	Hemocyanin subunit 1	0.31±0.01				

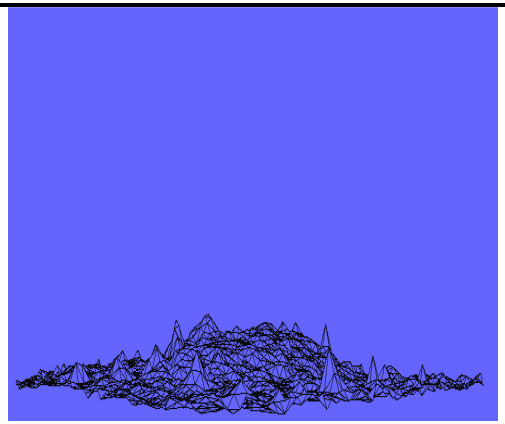
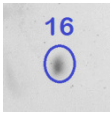
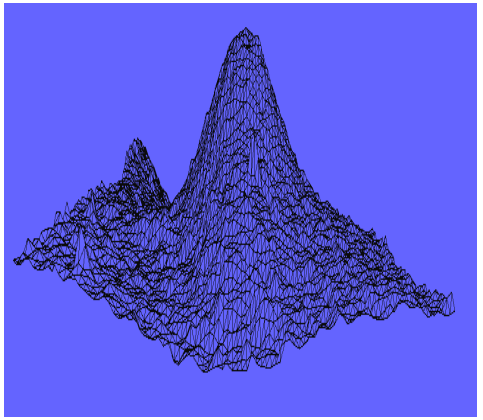
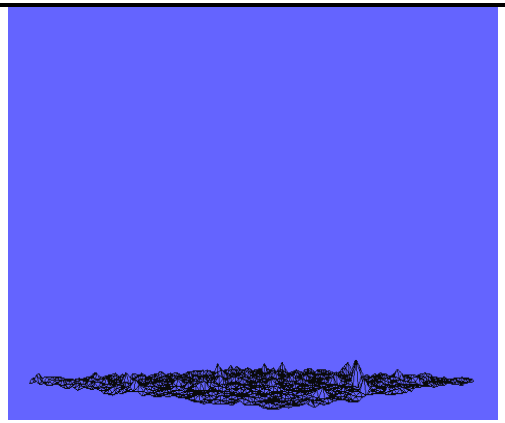
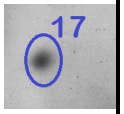
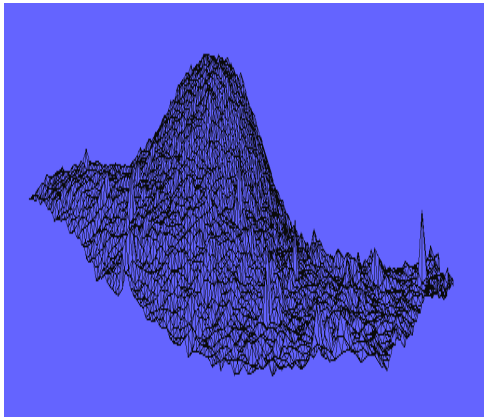
7	Sarcoplasmic calcium-binding protein	0.37±0.01				
8	Arginine kinase 1	0.49±0.00				

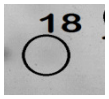
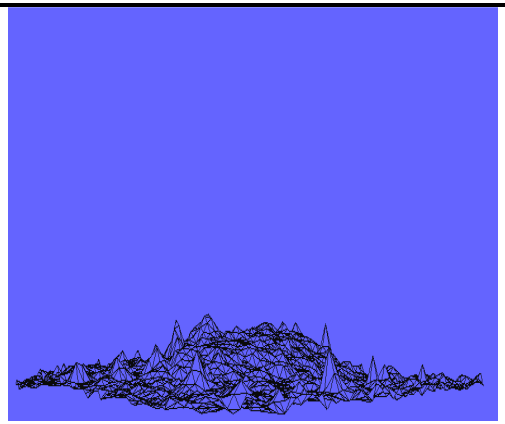
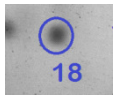
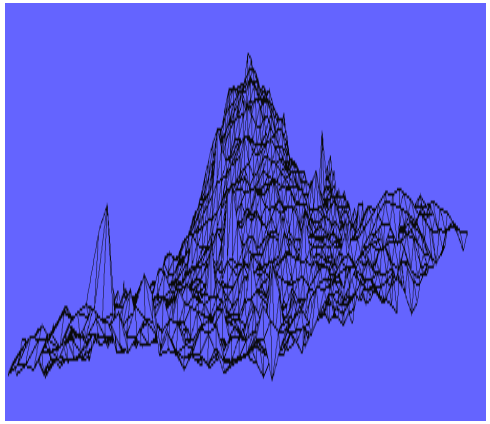
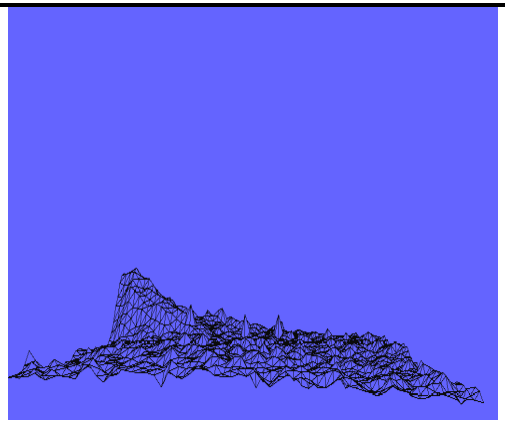
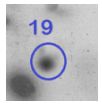
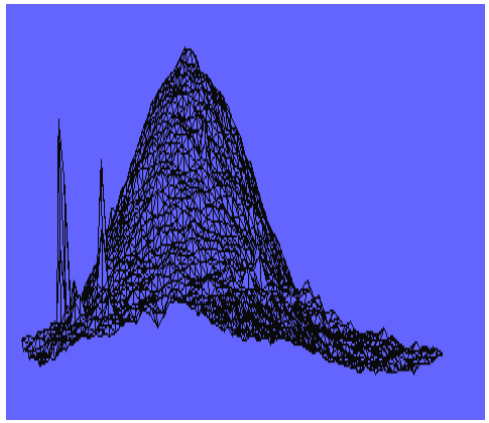
9	Arginine kinase 1	0.61±0.00				
10	Pro-phenoloxidase 1	0.48±0.00				

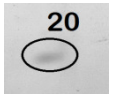
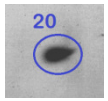
11	Pro-phenoloxidase 1	1.76±0.02				
12	Pro-phenoloxidase 1	2.11±0.02				

13	Pro-phenoloxidase 1	2.17±0.02				
14	Pro-phenoloxidase 1	1.26±0.00				

Spot	Protein name	IHHNV/Control Mean±SD	Control Serum	3D-view IHHNV Infected	IHHNV Infected	3D- view
15	Pro-phenoloxidase 1	1.64±0.02				

16	Putative uncharacterized protein	1.91±0.03				
17	Putative uncharacterized protein	1.89±0.03				

18	Pro-phenoloxidase 1	2.99±0.09				
19	Hemocyanin	1.97±.02				

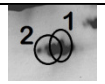
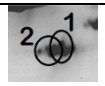
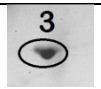
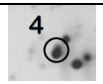
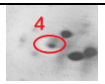
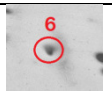
20	NS	1.65±0.02				

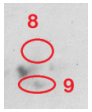
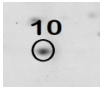
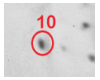
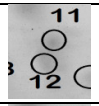
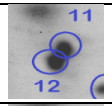
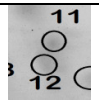
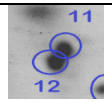
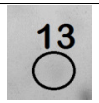
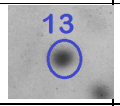
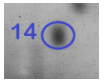
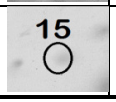
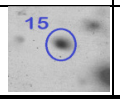
These twenty protein spots were analyzed by MALDI TOF-TOF Mass spectrometry and compared for peptide matching through public databases.

Some identified proteins spots matched proteins revealed in an analysis of proteins in the stomach of *P. vannamei* after WSSV challenge like the up-regulated proteins (ratio intensity more than 0.5) (Table 4.4), hemocyanin (spots 18*), prophenoloxidase (spots 10, 11, 12, 13,14and 17)*. The dual spots observed for both hemocyanin may be the result of protein modification. Up-regulated proteins involved in other cellular functions included hemocyanin (spot18), NS and 2 other putative uncharacterized proteins. All of the up-regulated spots increased more than 0.5 fold in intensity when compared to the same proteins from non-infected prawn. Significantly down-regulated proteins (ratio intensity less than 0.5 when compared to the (non-infected) control group involved in cellular functions included sarcoplasmic calcium binding proteins (SCPs) (spots 7), carbonic anhydrase2(spot 1), predicted protein (fragment) (spot X).

In addition, 22 other protein spots of unchanged level (compared to the control) from the serum of IHHNV challenged prawn was identified by PDQuest software. However they were not subjected to MALDI TOF-TOF Mass spectrometry. Figure 20 shows the spots which were not submitted.

Table 4.3, Comparative level of expression for protein spots from IHHNV-infected vs. normal prawn determined using PDQuest image analysis software Biorad

Spot	Protein name	species	ACC. No.	Exp. Mw(KDa) /PI	Theor. Mw(KDa)/ PI	MS/mps	control	IHHNV Infected	IHHNV/Contr Mean±SD
1	Carbonic anhydrase 2	Bos taurus	P00921	29.096 / 5-6	29.78/ 6.41	54			0.51±0.00
2	Predicted protein (Fragment)	Nematostella vectensis	A7RVD2	13.9716 /5-6	16.97/10.4	44			0.75±0.2
3	Enolase	Callinectes sapidus	Q6QWP6	39.852 /6-7	85.39/5.89	42.6			0.34±/.00
4	Hemocyanin subunit	Gammarus roeseli	Q571R4	76.304 /5-6	74/ 5.27	45			0.19±.00
5	Hemocyanin subunit L	Penaeus japonicus	B0L611	77.184 /6-7	74/ 5.27	52			0.33±.03
6	Hemocyanin subunit 1	Gammarus roeseli	Q571R4	76.304 /5-6	74/ 5.27	42			0.31±0.01
7	Sarcoplasmic calcium-binding protein	Procambarus clarkii	Q2XT28	21.761 /4-5	21.97/4.58	44			0.37±0.01

Spot	Protein name	species	ACC. No.	Exp. Mw(KDa) /PI	Theor. Mw(KDa)/PI	MS/mps	Control	IHHNV Infected	IHHNV/Contr Mean±SD
8	Arginine kinase 1	Neocaridina denticulata	Q6QWP6	39.520 / 7	40/6.5	59.6			0.49±0.00
9	Arginine kinase 1	Neocaridina denticulata	Q6QWP6	39.520 / 7	40/6.5	59.6			0.61±0.00
10	Pro-phenoloxidase 1	Locusta migratoria	C0LV9	80.766 / 5-6	39.514/6.17	33.5			0.48±0.00
11	Pro-phenoloxidase 1	Locusta migratoria	C0LV9	80.766 / 6-7	39.514/6.17	31.5			1.76±0.02
12	Pro-phenoloxidase 1	Locusta migratoria	C0LV93	80.766 /6-7	39.514/6.17	34			2.11±0.02
13	Pro-phenoloxidase 1	Locusta migratoria	C0LV93	80.766 /6-7	39.514/6.17	33.5			2.17±0.02
14	Pro-phenoloxidase 1	Locusta migratoria	C0LV93	80.766 /6-7	39.514/6.17	32.5			1.26±0.00
15	Pro-phenoloxidase 1	Locusta migratoria	C0LV93	80.766 /6-7	39.514/6.17	36			1.64±0.02

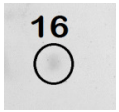
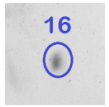
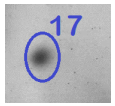
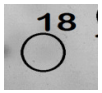
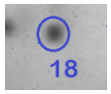
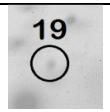
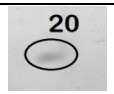
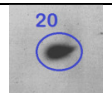
Protein name	species	ACC. No.	Exp. Mw(KDa)/PI	Theor. Mw(KDa)/PI	MS/mps	control	IHHNV Infected	IHHNV/Contr Mean±SD
Putative uncharacterized protein	Ixodes scapularis	B7Q192	38.314 /6-7	26.92/9.38	16			1.91±0.03
Putative uncharacterized protein	Trichoplax adhaerens	B3S8W0	36.166 /6-7	26.92/6.5	20			1.89±0.03
Pro-phenoloxidase 1	Locusta migratoria	B9VR33	80.766 / 6-7	39.514/6.17	24.5			2.99±0.09
Hemocyanin	Fenneropenaeus chinensis	B9VR33	77.538 / 6-7	74/ 5.27	20.5			1.97±.02
NS	----------	----------	10-20/4-5	----------	------			1.65±0.02

Table 4.4, **Identification** of Up-regulated and Down-regulated Protein upon IHHNV Infection from 2D-gels by MALDI TOF-TOF Mass spectrometry analysis

No	PI	Approximate M.W/ Mass (KD)	Result	Species	Partial Sequence
1	5-6	50-75	Carbonic anhydrase 2	*Bos taurus*	1-K.DGPLTGTYR.L 2-K.AVLKDGPLTGTYR.L 3-K.YAAELHLVHWNTK.Y
2			Predicted protein (Fragment)	*Nematostella vectensis*	1- K.IGQDISQR.E 2- IGSAFMLAHPYGIIR + Oxidation (M
3	6-7	40-60	Enolase	*Callinectes sapidus*	1-R.MGSEVYHHLK.A + Oxidation (M) 2-K.IEIGMDVAASEFYK.G + Oxidation (M)
4	5-6	30-40	Hemocyanin subunit	*Gammarus roeseli*	1-K.AHVDRLNHK.D
5	6-7	25-37	Hemocyanin subunit L	*Penaeus japonicus*	1-R.LHKYMDNIFK.E + Oxidation (M) 2-K.YMDNIFKEHK.D + Oxidation (M)
6	5-6	20-30	Hemocyanin subunit 1	*Gammarus roeseli*	1-K.AHVDRLNHK.D
7	4-5	15-20	Sarcoplasmic calcium-binding protein	*Procambarus clarkii*	1-R.NTLIEGR.G

8	7	37-50	Arginine kinase 1	*Neocaridina denticulata*	1-R.SIDGFGLSPGITKEQR.V 2-R.QQLVDDHFLFMSGDR.N + Oxidation (M)
9	7	37-50	Arginine kinase 1	*Neocaridina denticulata*	1-R.SIDGFGLSPGITKEQR.V 2-R.QQLVDDHFLFMSGDR.N + Oxidation (M)
10	5-6	20-30	Pro-phenoloxidase 1	*Locusta migratoria*	1-R.SMGYPFDR.V + Oxidation (M) 2-R.RSMGYPFDR.V + Oxidation (M)
11	6-7	10-20	Pro-phenoloxidase 1	*Locusta migratoria*	R.SMGYPFDR.V + Oxidation (M)
12	6-7	10-20	Pro-phenoloxidase 1	*Locusta migratoria*	R.SMGYPFDR.V + Oxidation (M)
13	6-7	10-20	Pro-phenoloxidase 1	*Locusta migratoria*	R.SMGYPFDR.V + Oxidation (M)
14	6-7	8-15	Pro-phenoloxidase 1	*Locusta migratoria*	R.SMGYPFDR.V + Oxidation (M)
15	6-7	15-20	Pro-phenoloxidase 1	*Locusta migratoria*	R.SMGYPFDR.V + Oxidation (M)
16	6-7	15-25	Putative uncharacterized protein	*Ixodes scapularis*	----------

17	6-7	10-15	Putative uncharacterized protein	*Trichoplax adhaerens*	1- R.TQRLGDK.K 2-K.MVDTTENIK.E + Oxidation (M)
18	6-7	10-15	Pro-phenoloxidase 1	*Locusta migratoria*	1-R.SMGYPFDR.V + Oxidation (M)
19	6-7	15-20	Hemocyanin	*Fenneropenaeus chinensis*	Unigene56219_All Unigene63672_All 1-K.VFNHGEHIHHH.- 2- R.TQRLGDK.K 3- K.MVDTTENIK.E + Oxidation (M)
20	4-5	10-20	NS		1-AQLDQYYR 2-KDLFSTNHK 3-GEGSVMESLAK + Oxidation (M)

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4.5. Results of Proteomic Expression Level Compression by Transcriptome Data

Poly-A mRNAs, previously extracted from both IHHNV-infected and non-infected prawns, were purified using beads with oligo(dT) and cleaved into smaller RNA fragments(Kwong Qi Bin *et al.*, 2011). The RNA fragments were reversely transcribed into cDNA using Invitrogen reverse transcriptase. This was followed by second strand cDNA synthesis using Invitrogen RNase H and DNA polymerase I by New England BioLabs. The cDNA libraries were constructed according to the Illumina GA platform sequencing protocols. The detailed results are documented in the paper by (Kwong Qi Bin *et al.*, 2011). Transcriptome data was obtained from the IHHNV infected and non-infected hepatopancreas' of *M.rosenbergii* and shows some expressions of this protein at the genomic level (Kwong Qi Bin *et al.*, 2011). The comparison between proteomic data and transcriptomic data confirmed that both Arginine kinase and sarcoplasmic calcium-binding protein were down regulated in infected individuals. It was also confirmed that both Pro-phenoloxidase 1 and Hemocyanin are up/down regulated in infected individuals. This further confirms the existence of these protein isomers and their structural differences before and after infection (Table 6.1). This data also confirms that the protein down regulation would occur in other organs just as in the hemocyte.

Table 4.5, Data evaluation based on Transcriptome Data

Protein name	Transcriptome ID	Expression value at gene level	Expression at protein level	E.value	Protein	Protein	Prosite
					Proteomincs	Transcriptom	
Sarcoplasmic calcium-binding protein	Cluster695_Consensus1	-0.8416	down	4.00E-13	R.NTLIEGR.G	QDGEVTLDEFKQAVKNVCTGKAYDSFPQ AFKAFIANQFKTIDVNGDFNKDGEVSDDE FKQAVQKNCSGKGFADFPNAFKSFITTQF KTIDVNGNQDGEVSGEEFKQAVKNACLG KKFEEFPNAFRVFIINQFKTVDVNG	DFNKDGEVSddEF - EF-hand calcium-binding domain :
Arginine kinase 1	Cluster15921_Consensus1	1.26	down	3.00E-73		MGEPFPDIKSKHSLVAKHVTKERWEKLS GHKTATSGFTLKQAIACAVEFDNQHCGIY AGDWDSYKDFKDVFDPIIQEYHGISPDAV HTSDMEVEKIKGNINAEVPVHSVRIRVGR SIDGFGLSPGITKEQRIGVENLMKSAFGKL SGDLAGNYYPLTGMDEKVRQQLVDDHF LFMSGDRNLQVAGMERDWPEGRGIYHN AEKTFLVWVNEEDQLRIISMQMGGDVRG VFERLARGIKAVGDSVKAESGKDFMLDP KYGFVHSCPTNLGTGMRASVHVDLPGW TKEGLDALKKRCEELKVQPRGTRGESGG QTGHTYDISNKHRLGYSEVELVQCMIDG VNTLYAEDVALQKKHGI	CPTNLGT - *ATP:guanido phosphotransferases active site*

Pro-phenoloxidase 1	Cluster125_Consensus1	-0.3939	down/up	0	RRSMGYPFDR	MLLPRGMPQGMDFQLFVMLTDHAVDKV EQVIRGRPCTNAVSYCGILDSKFPDARPM GFPFDR	
Hemocyanin	Singletons43466, not in muscle			2.00E-17	KVFNHGEHIHHH	LPNRFLLPKGNHNGMKFDLFVCVTDGAA DAAIADLHSKDEFLHYGANGVYPDKRPH GYPFDRHVQDERLFNQVTNFHHIQVKVF NHGEHIHHH	nil
SAC domain-containing protein 3 (NS)		no changes	up	2.00E-13	GEGSVMESLAK	ETEQLVFDAHVPSIHSGRYTSFVQIRGSIP CHWSQDVSKMVPKRRSCCYAGTRFLKR GA	ETEQLVFDAHVPsihsgryTSFVQI RGSIPCHWSQDVSKMVPK - Sac phosphatase domain profile :

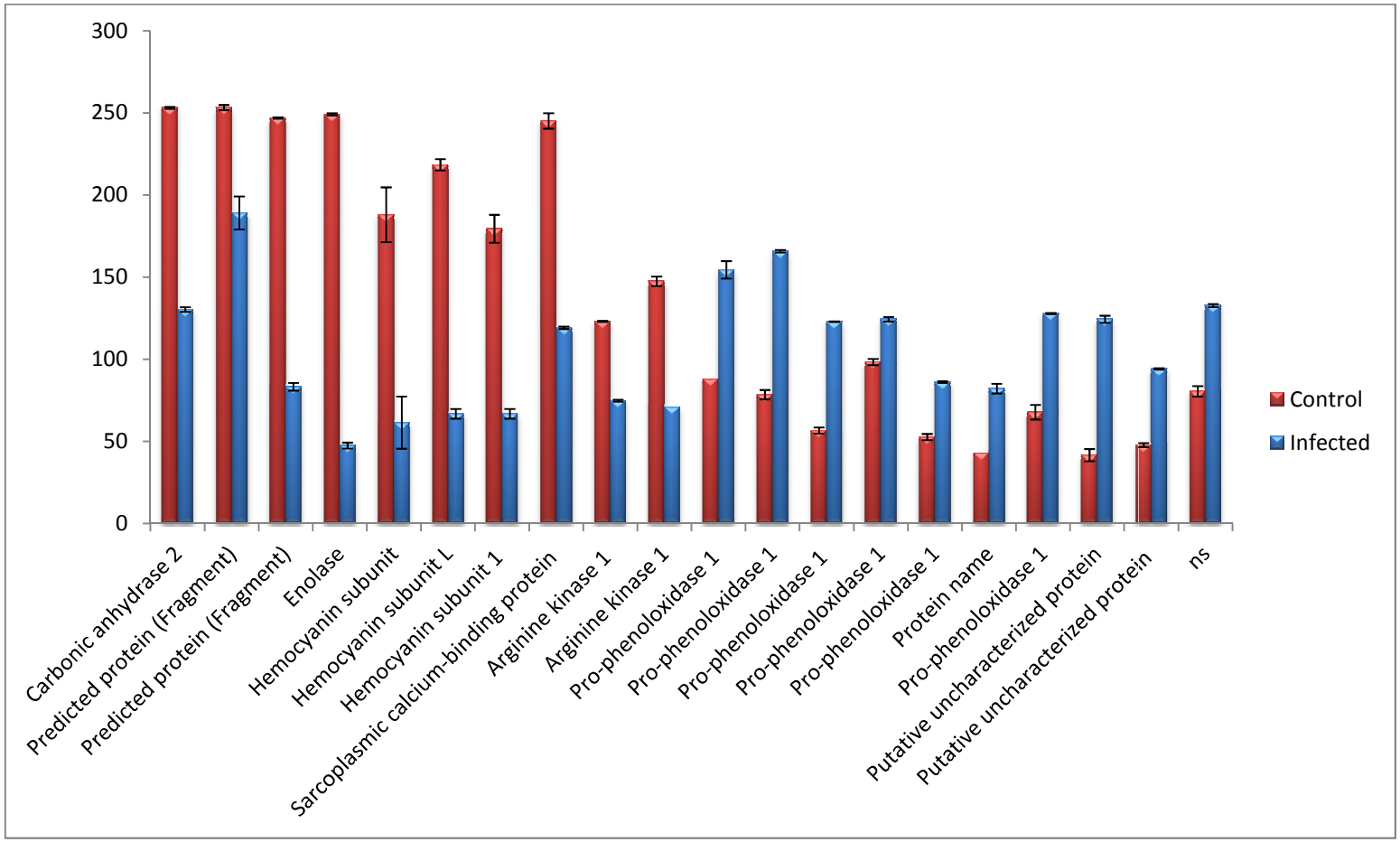

Figure 4.6, Intensity comparisons between Prawn IHHNV-Infected and control samples.