

4. RESULTS

4.1 Mass Spectrometry of Protein Spots From Previous Studies

The first spots in this project to be analysed by MS, proceeding trypsin in gel digestion, were 201 (from susceptible 3 silver 23 November 2007 gel) and 594 (from susceptible 3 silver 23 November 2007 gel) chosen as Baker (unpublished, 2008) putatively identified them as manganese superoxide dismutase (MnSOD) and a possible member of FREP2 respectively, although their true identification remained to be determined empirically.

Baker predicted their MWs by creating a log graph (Figure 3) of the MW makers and distance the markers travelled (Table 2). The distance moved by spots 201 and 594 was measured and then those distances applied to the equation ($y=-0.0068x+5.0216$) for the line of best fit on the log graph (Figure 3). The anti log was converted back to a real number, which gave the theoretical MW of the spots which can be found in Table 3 along with their predicted *pI*.

Table 2: Log values of the molecular weight markers and the distance travelled through the gels (Baker unpublished, 2008).

MW of Molecular Weight Markers (Da)	Log (Da)	Distance Travelled (mm)
99000	4.995635	15
66000	4.819544	28
45000	4.653213	45
30000	4.477121	75
20100	4.303196	106
14300	4.155336	133

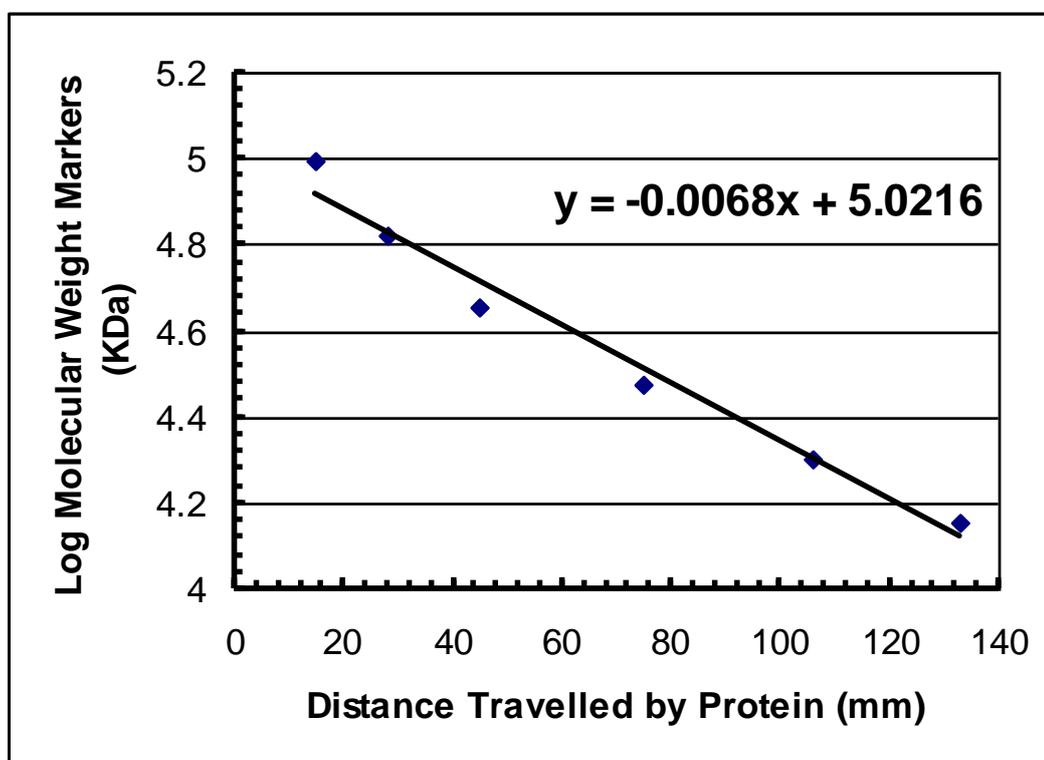


Figure 3: Molecular weight determination. Graph showing the log values of molecular weight markers (kDa) against the distance travelled by the molecular weight markers through the gel.

Table 3: Molecular weights of spots 594 and 201

Spot	Distance Travelled (mm)	Equation ($y=mx+c$)	Molecular Weight (kDa)
594 (Figure 4)	55	$y= (55) -0.0068+5.0216$	44
201 (Figure 5)	95	$y= (95) -0.0068+5.0216$	24

Spot 594 has a down regulated normalization volume of more than two fold (-2.16) on the susceptible average gel compared to the resistant gel (Baker unpublished, 2008) (Figure 4b).

Spot 201 has an up regulated normalization volume of more than two fold (2.56) on the susceptible average gel compared to the resistant gel (Baker unpublished, 2008) (Figure 5b).

Spot 594

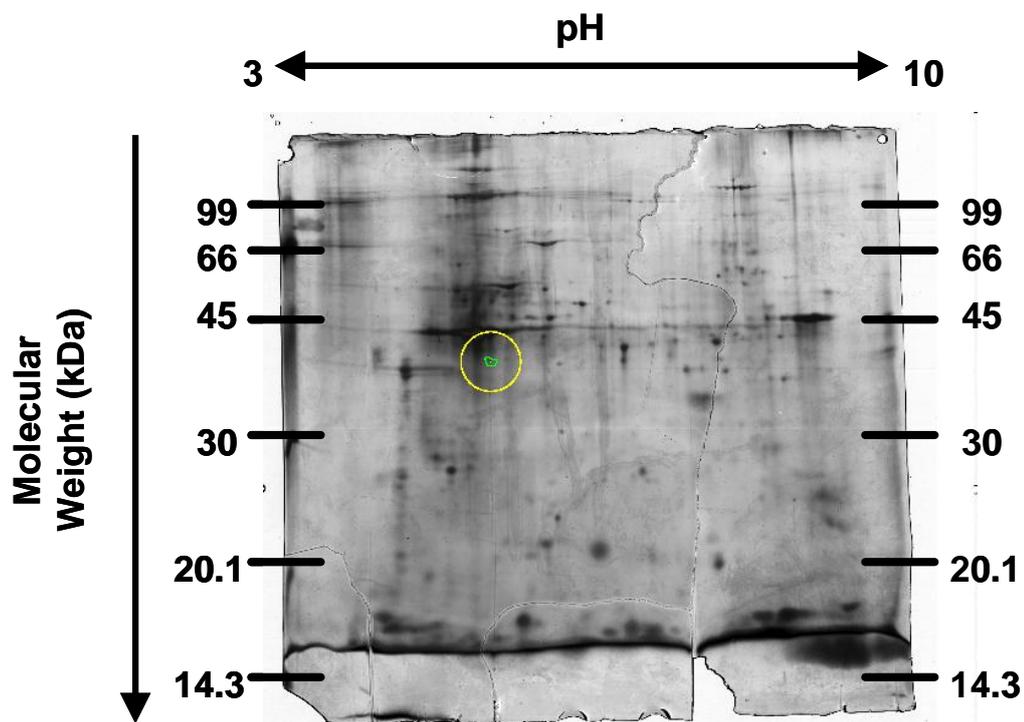


Figure 4a: Relative location of spot 594 on susceptible average gel indicated by a green outline in a yellow circle. Approximate molecular weight values and pI are shown (Murray unpublished, 2008).

Spot 594

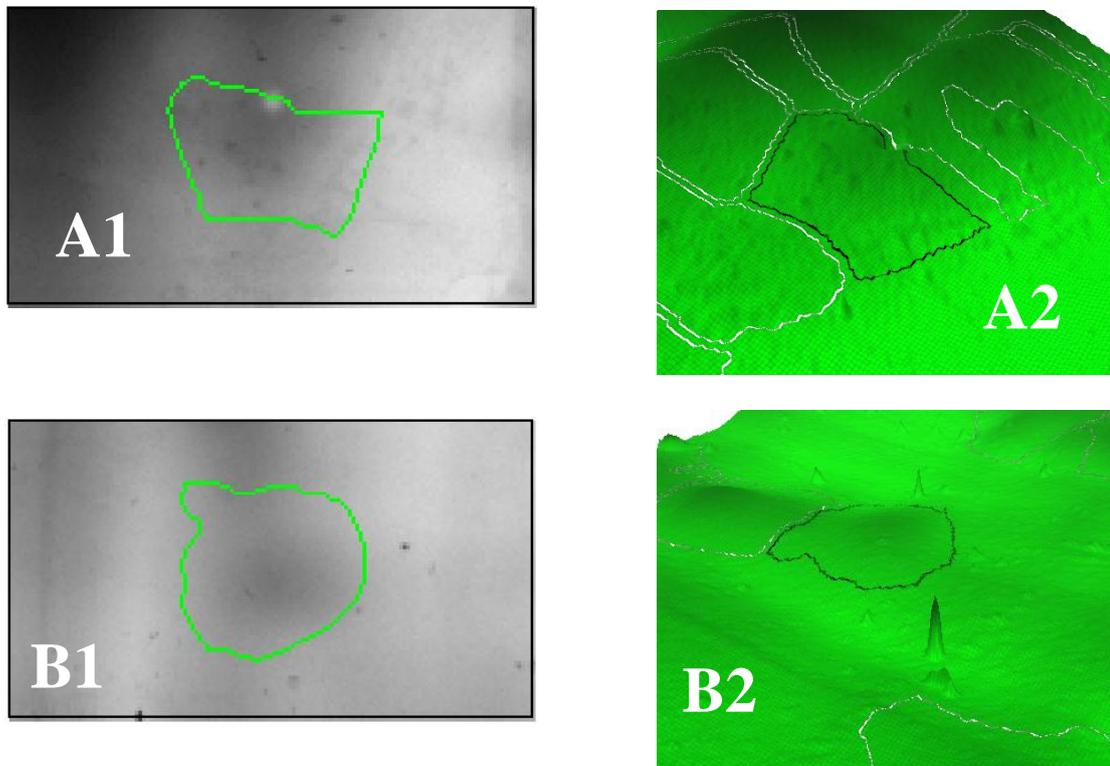


Figure 4b: Montage and 3D visualisation of spot 594 on susceptible average gel (A1 & A2) matched to spot 594 on resistant average gel (B1 & B2). Spot 594 has a down regulated normalization volume of more than two fold (-2.16) on the susceptible average gel compared to the resistant gel (Murray unpublished, 2008).

Spot 201

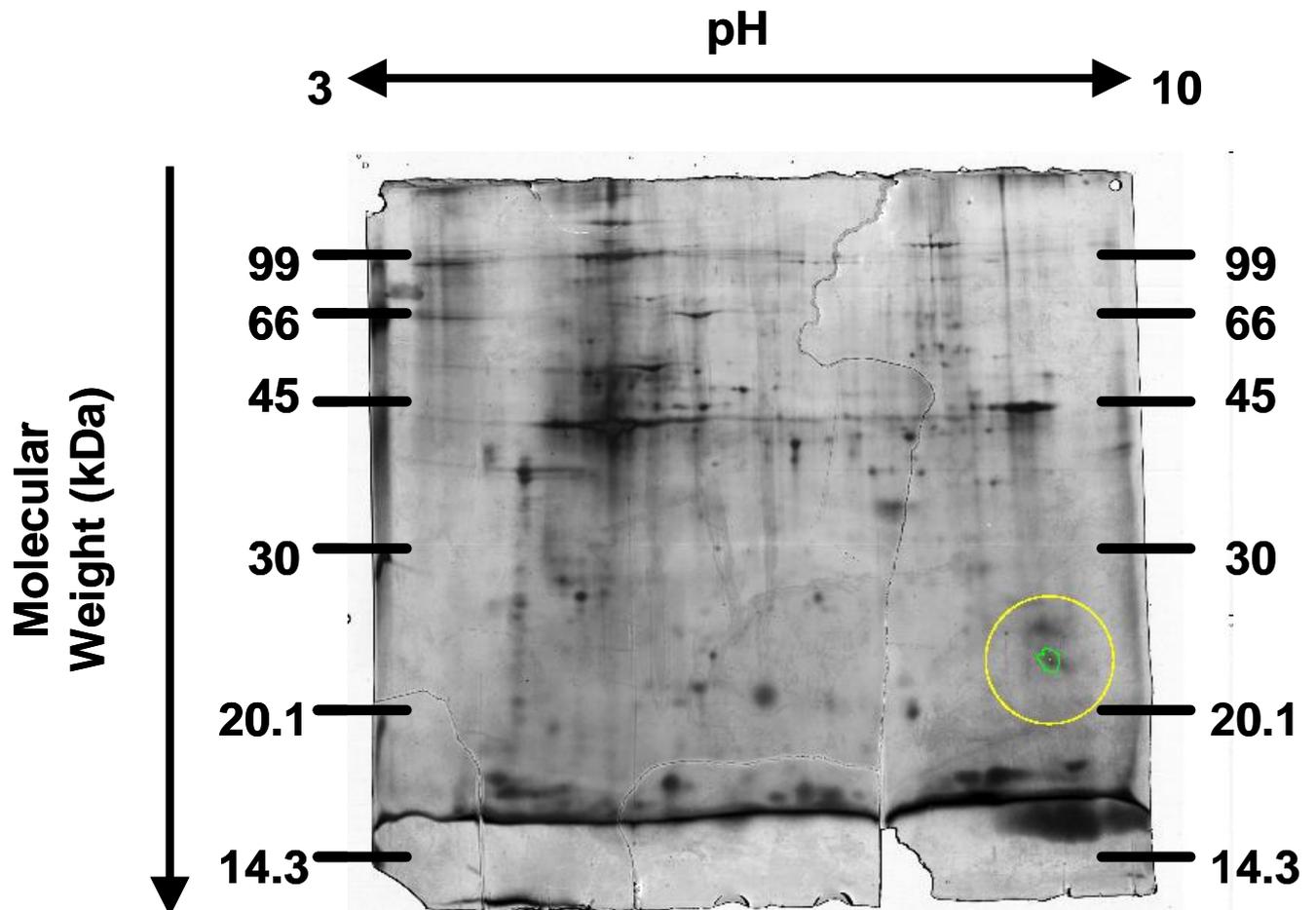


Figure 5a: Relative location of spot 201 on susceptible average gel indicated by a green out line in a yellow circle. Approximate molecular weight values and pI are shown (Murray unpublished, 2008).

Spot 201

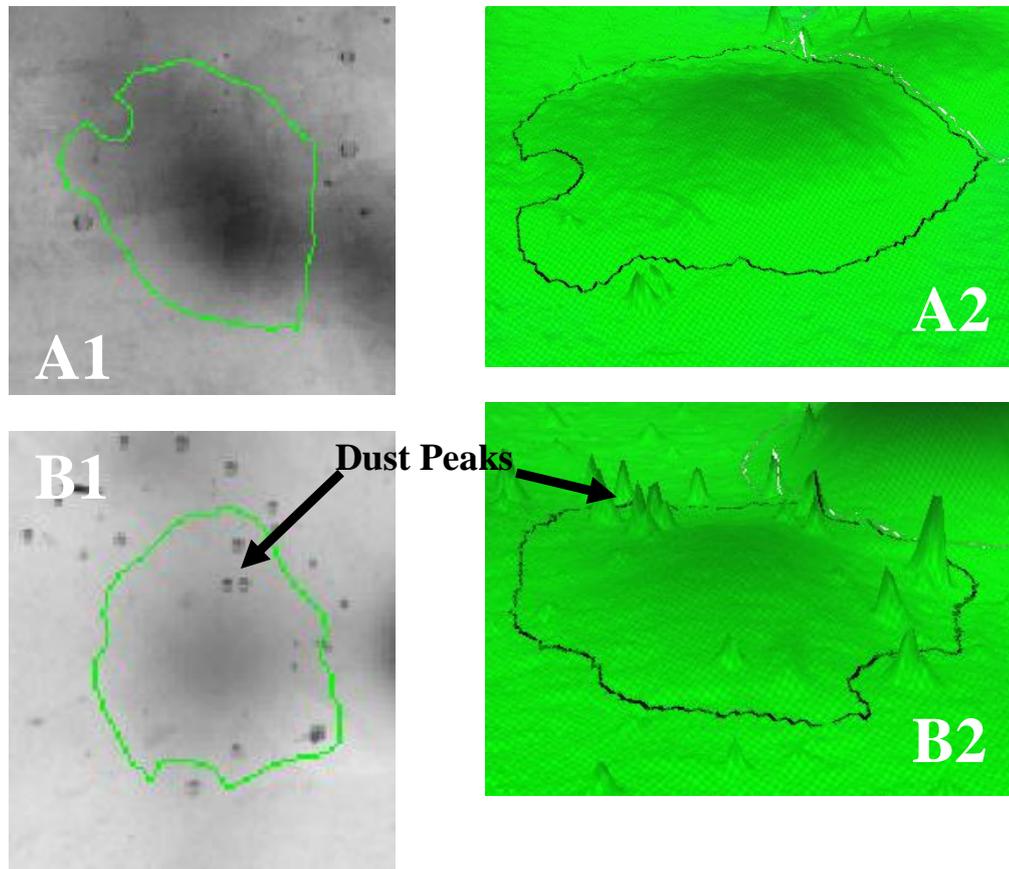


Figure 5b: Montage and 3D visualisation of spot 201 on susceptible average gel (A1 & A2) matched to spot 594 on resistant average gel (B1 & B2). Spot 201 has an up regulated normalization volume of more than two fold (2.56) on the susceptible average gel compared to the resistant gel. Note the dust peaks that can be observed in the montage image as black circles, and in the 3D image as abrupt peaks (Murray unpublished, 2008).

The results from MS for these two spots were poor, with initial visual observations suggesting only autolysis products from trypsin. It was possible to analyse the fragmentation spectra of spot 201, where sequence interpretation was initially conducted automatically using peptide mass sequencing within MassLynx v 3.5. yielding the sequence **V A T V S L P R** (Figure 6). The results of this sequence from a peptide sequence BLAST search can be seen in Table 4, suggesting the peptide detected is most likely trypsin.

Another fragmentation spectra was analysed automatically for spot 201 (Figure 7) generating the sequence **A A P/L S L P G/V**. This failed to provide accurate sequence information, so the fragmentation data was analysed manually (Figure 8). This generated the sequence **V S L P R** are of the sequence from Figure 6, preceded by other amino acids. When this spectrum was analysed manually from the other end (Figure 9) it gave the sequence **V A T V S L**, which when combined with the other manual sequence gives the sequence BLAST searched from Figure 6, increasing confidence in its identification as trypsin in spot 201.

V A T V S L P R (Figure 6)

V S L P R (Figure 8)

V A T V S L (Figure 9)

Trypsin Autolysis products are a sign of low protein sample, so new gels were run with a five times increase in protein concentration incorporated into the protocol.

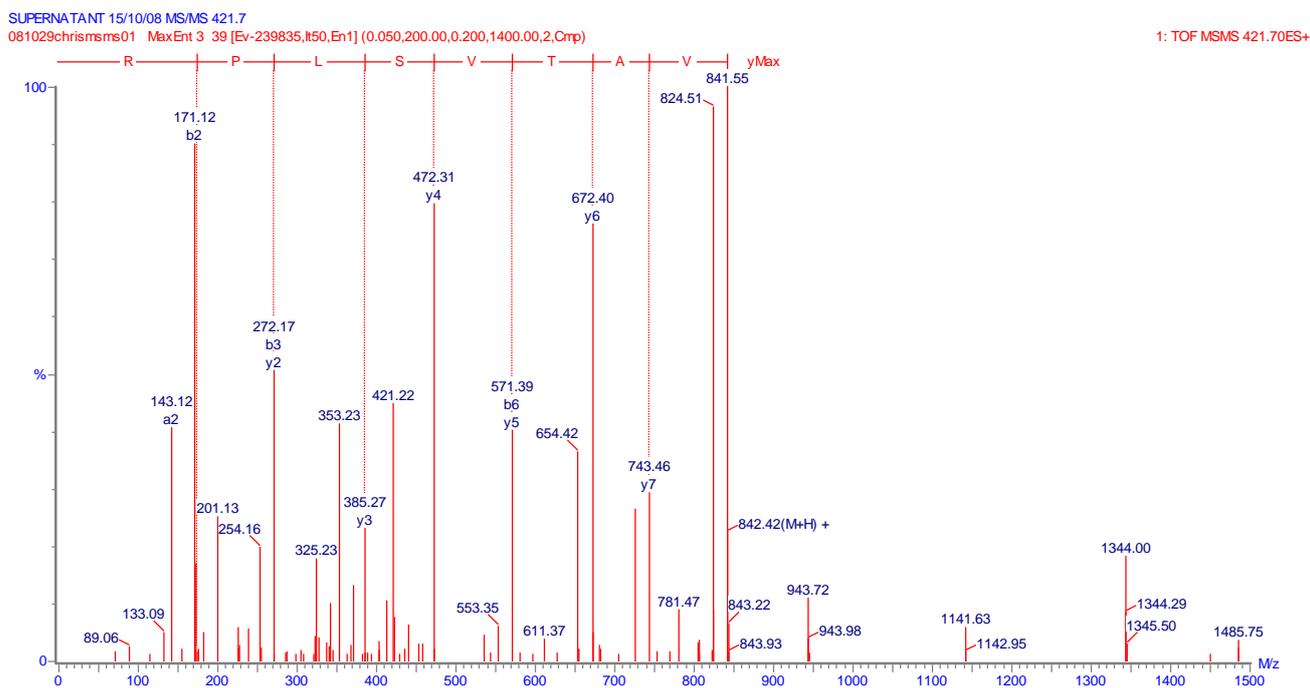


Figure 6: Spot 201 MS/MS Fragmentation Spectra (Automatic) for Mol Wt Ion

421.7. Y ions (red) giving the sequence **V A T V S L P R**.

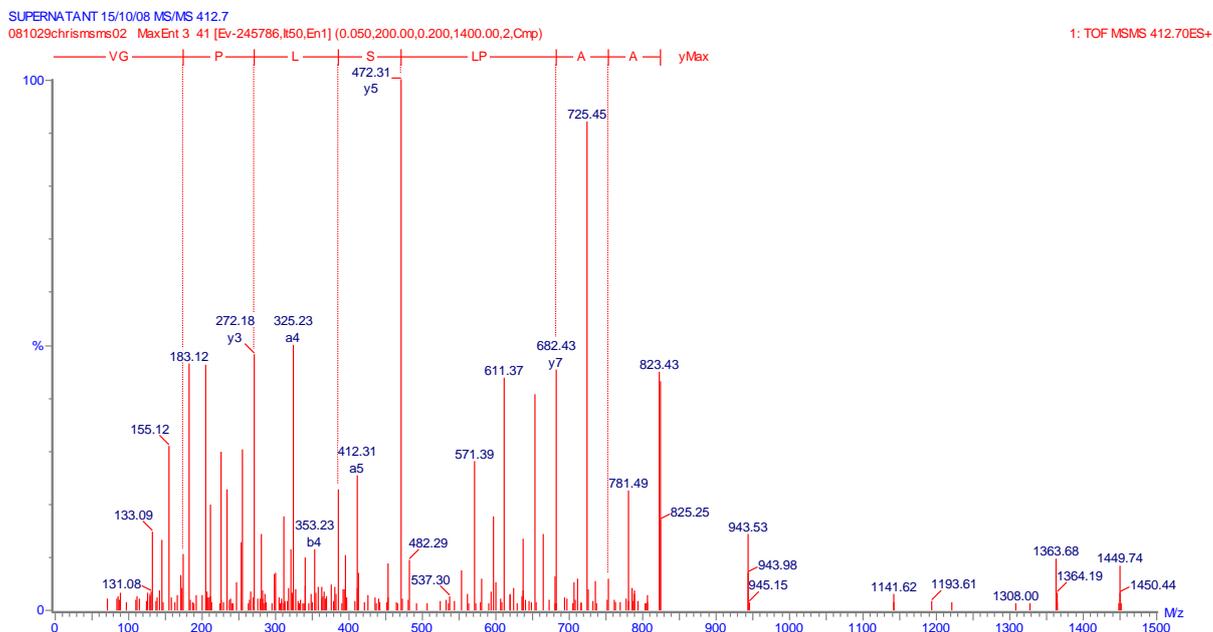


Figure 7: Spot 201 MS/MS Fragmentation Spectra (Automatic) for Mol Wt Ion

412.7. Y ions (red) giving the sequence **A A P/L S L P G/V**.

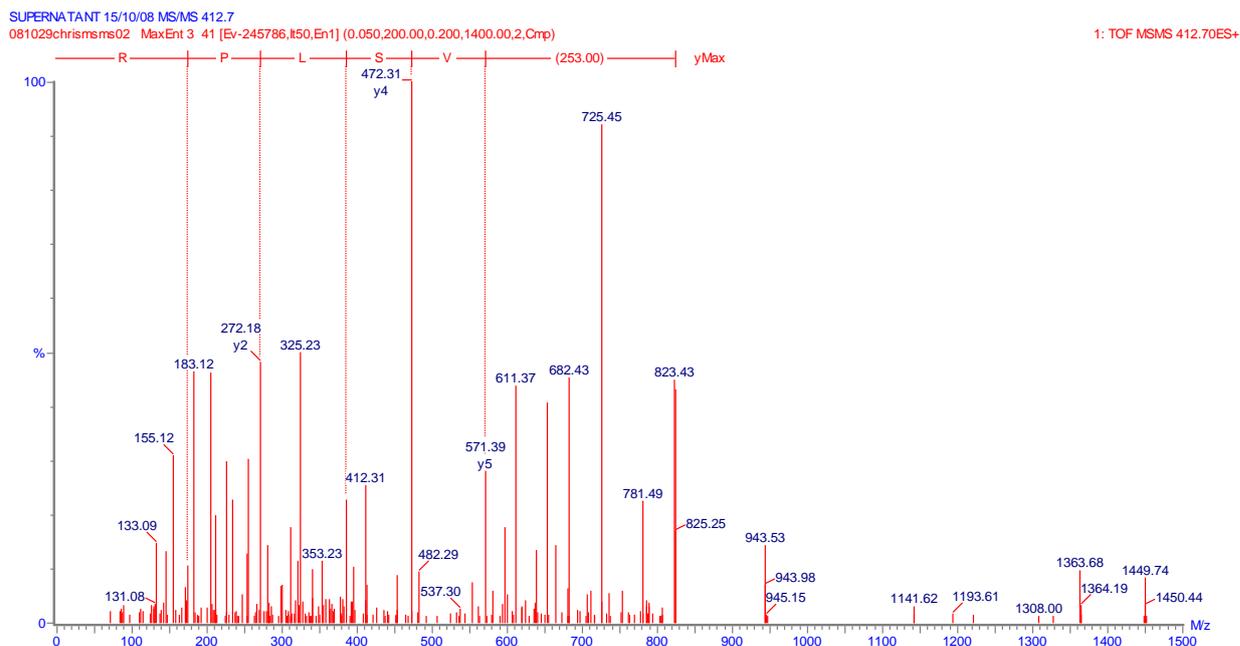


Figure 8: Spot 201 MS/MS Fragmentation Spectra (Manually Sequenced II) for Mol Wt Ion 412.7. Sequenced manually from the right. Y ions (red) giving the sequence **VSLPR**

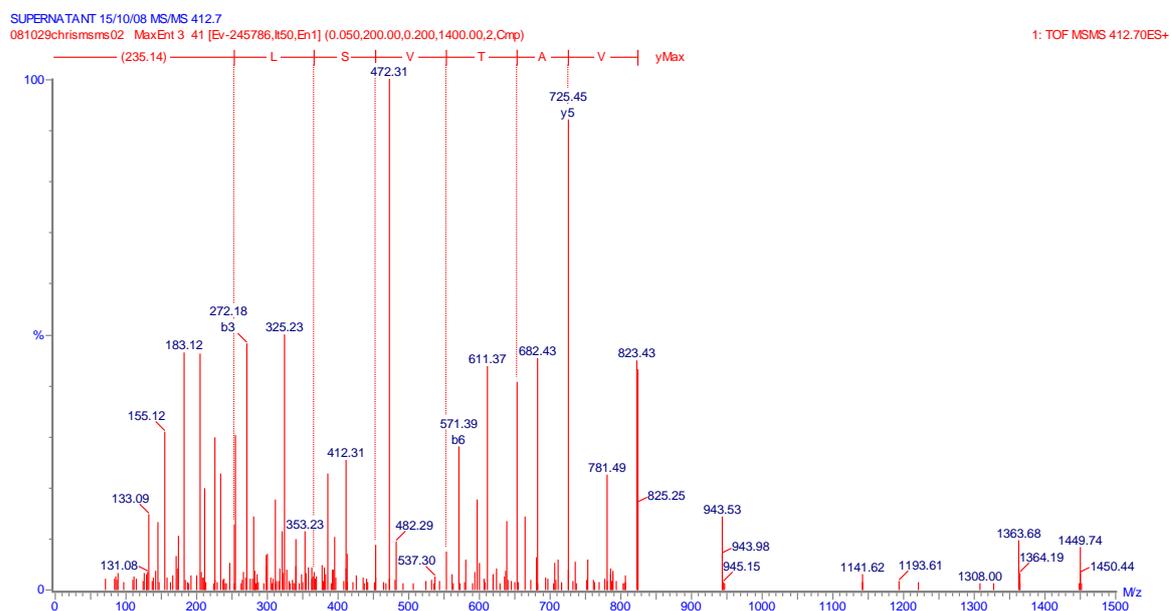


Figure 9: Spot 201 MS/MS Fragmentation Spectra (Manually Sequenced I) for Mol Wt Ion 412.7. Sequenced manually from the left. Y ions (red) giving the sequence **VATVSL**.

Table 4: Sequences producing most significant alignments from a peptideBLAST search for sequence: **V A T V S L P R**

Score	E- value	Identities	Description	Accession No.
26.9	177	8/8 (100%)	Chain E, Complex Of Eeti-Ii With Porcine Trypsin	1H9H_E
26.9	177	8/8 (100%)	Chain A, Trypsin In Complex With Borate	2A31_A
26.9	177	8/8 (100%)	RecName: Full=Trypsin; Flags: Precursor	P00761

4.2 Overview of Comparisons of Susceptible Gel Compared to Resistant Gel

The overall data obtained from the comparison of the new gel from susceptible snails to the new gel from the resistant snails using Progenesis (PG220, Nonlinear Dynamics, UK) are shown in Table 5.

Normalised volume of spots was used for the comparison of spots from the susceptible snails gel to the spots from the resistant snails gel. The value of one was given for the volume of the protein spots on the resistant snails gel, so the volume of the protein spots on the susceptible snails gel could be compared to the value of one.

In total, 302 protein spots from the susceptible snails gel were matched with spots on the resistant snails gel, of which 110 were up-regulated greater than

two fold in susceptible snails compared to resistant snails, and 105 were down-regulated greater than two fold in susceptible snails compared to resistant snails.

The remaining 87 revealing up regulation and down regulation of normalised volume by less than two fold were not counted towards analysis because the change in normalised volume was not significant enough for analysis. It has been suggested that there should be at least two fold difference when comparing the normalised volume of one matched spot to another protein spot on another gel for the results to be statistically significant (Barrett, Brophy & Hamilton, 2005). 215 matched spots were found to have more than two fold down regulation and up regulation (see Table 6).

Table 5: Gel report summary

Total Number of Protein Spots on Gel from Susceptible Snails	536
Total Number of Protein Spots on Gel from Resistant Snails	575
Number of Matched Spots Up Regulated > Twofold in Gel from Susceptible Snails Compared to Gel from Resistant Snails	110
Number of Matched Spots Down Regulated > Twofold in Gel from Susceptible Snails Compared to Gel from Resistant Snails	105
Number of Unique (Unmatched) Spots on Gel from Susceptible Snails	15
Number of Unique (Unmatched) Spots on Gel from Resistant Snails	273

Table 6: Matched spots in both susceptible and resistant snail proteomes. Fold change of normalised spot volume of susceptible snail proteome is given relative to resistant snail proteome. Figures highlighted in green indicate up regulation of normalised volume of >2 to infinite up-regulation. Figures highlighted in yellow indicate down regulation of >2 to infinite down-regulation. Spots up/down regulated <2 have been edited from the table.

Susceptible Gel		Resistant Gel	
Spot Number	Difference ('Norm. Vol.' v 'Resistant Gel) (Upregulation/ Downregulation of Spots (Fold Change))	Spot Number	Difference 'Norm. Vol' v 'Resistant Gel'
636	Infinite increase	636	1
682	Infinite increase	682	1
702	Infinite increase	702	1
717	Infinite increase	717	1
779	Infinite increase	779	1
871	Infinite increase	871	1
956	Infinite increase	956	1
975	Infinite increase	975	1
1198	Infinite increase	1198	1
1211	Infinite increase	1211	1
1451	Infinite increase	1451	1
1456	Infinite increase	1456	1
1505	Infinite increase	1505	1
1785	Infinite increase	1785	1
1803	Infinite increase	1803	1
608	326.368	608	1
604	306.952	604	1
670	214.85	670	1
879	173.78	879	1
713	160.831	713	1
1482	68.051	1482	1
280	58.112	280	1
593	46.447	593	1
1142	45.659	1142	1
1634	32.783	1634	1
980	28.616	980	1
275	28.581	275	1
1608	26.741	1608	1
610	25.573	610	1
643	23.079	643	1
1025	18.712	1025	1

519	16.185	519	1
1594	15.146	1594	1
1193	14.635	1193	1
616	14.365	616	1
1544	13.824	1544	1
1074	13.481	1074	1
581	12.495	581	1
277	12.291	277	1
952	11.434	952	1
1800	11.409	1800	1
1810	11.089	1810	1
1125	10.219	1125	1
1730	10.199	1730	1
831	10.038	831	1
623	9.947	623	1
1560	8.759	1560	1
1155	8.229	1155	1
522	8.196	522	1
606	8.189	606	1
276	7.673	276	1
603	7.629	603	1
592	7.528	592	1
495	6.931	495	1
819	6.752	819	1
1721	6.494	1721	1
312	6.445	312	1
502	6.349	502	1
1607	6.136	1607	1
1557	6.067	1557	1
272	5.895	272	1
458	5.811	458	1
1620	5.696	1620	1
612	5.66	612	1
1737	5.332	1737	1
1674	5.309	1674	1
1543	5.262	1543	1
490	5.253	490	1
1057	5.147	1057	1
987	5.133	987	1
1784	5.086	1784	1
449	5.039	449	1
1022	4.961	1022	1
355	4.846	355	1
1812	4.75	1812	1
1527	4.661	1527	1
1098	4.585	1098	1
625	4.582	625	1
332	4.367	332	1

481	4.366	481	1
1548	4.143	1548	1
1067	4.049	1067	1
1710	3.9	1710	1
1573	3.594	1573	1
328	3.583	328	1
1781	3.491	1781	1
748	3.438	748	1
1783	3.352	1783	1
1777	3.324	1777	1
1646	3.32	1646	1
267	3.156	267	1
1109	3.142	1109	1
1054	3.129	1054	1
1110	3.093	1110	1
1175	2.988	1175	1
841	2.965	841	1
1596	2.816	1596	1
1664	2.718	1664	1
853	2.588	853	1
1756	2.575	1756	1
421	2.557	421	1
704	2.491	704	1
566	2.308	566	1
1123	2.297	1123	1
703	2.269	703	1
851	2.26	851	1
631	2.243	631	1
513	2.208	513	1
279	2.139	279	1
800	2.079	800	1
1553	-2.009	1553	1
611	-2.016	611	1
660	-2.076	660	1
634	-2.089	634	1
928	-2.099	928	1
1701	-2.229	1701	1
1506	-2.288	1506	1
240	-2.415	240	1
525	-2.416	525	1
812	-2.435	812	1
1215	-2.489	1215	1
768	-2.57	768	1
689	-2.625	689	1
1643	-2.633	1643	1
294	-2.667	294	1
1627	-2.719	1627	1
666	-3.016	666	1

587	-3.116	587	1
1617	-3.242	1617	1
1129	-3.299	1129	1
738	-3.345	738	1
677	-3.39	677	1
806	-3.392	806	1
919	-3.524	919	1
737	-3.564	737	1
1597	-3.616	1597	1
1180	-3.65	1180	1
1775	-3.768	1775	1
1219	-3.797	1219	1
1028	-3.838	1028	1
846	-3.984	846	1
1742	-4.023	1742	1
324	-4.055	324	1
1728	-4.065	1728	1
573	-4.08	573	1
298	-4.245	298	1
1779	-4.437	1779	1
899	-4.471	899	1
839	-4.699	839	1
1554	-4.77	1554	1
1693	-5.277	1693	1
663	-5.493	663	1
1136	-5.535	1136	1
1153	-5.576	1153	1
874	-5.785	874	1
707	-6.377	707	1
1163	-6.649	1163	1
1156	-6.69	1156	1
430	-7.275	430	1
544	-7.699	544	1
1126	-7.885	1126	1
419	-8.114	419	1
1565	-8.316	1565	1
1669	-8.763	1669	1
674	-9.329	674	1
1050	-9.542	1050	1
859	-10.446	859	1
637	-10.468	637	1
614	-11.734	614	1
763	-15.546	763	1
678	-17.705	678	1
649	-18.451	649	1
772	-19.224	772	1
885	-19.449	885	1
441	-21.729	441	1

864	-22.335	864	1
639	-24.482	639	1
1088	-27.221	1088	1
974	-33.401	974	1
1526	-33.8	1526	1
487	-36.788	487	1
274	-43.206	274	1
943	-44.188	943	1
734	-46.285	734	1
910	-48.32	910	1
459	-49.231	459	1
1685	-52.982	1685	1
972	-59.386	972	1
669	-115.092	669	1
863	-196.707	863	1
887	-303.073	887	1
1708	-440.37	1708	1
1154	-604.903	1154	1
1192	-15,201.39	1192	1
245	Infinite decrease	245	1
697	Infinite decrease	697	1
735	Infinite decrease	735	1
749	Infinite decrease	749	1
755	Infinite decrease	755	1
757	Infinite decrease	757	1
783	Infinite decrease	783	1
813	Infinite decrease	813	1
852	Infinite decrease	852	1
884	Infinite decrease	884	1
909	Infinite decrease	909	1
926	Infinite decrease	926	1
938	Infinite decrease	938	1
955	Infinite decrease	955	1
993	Infinite decrease	993	1
995	Infinite decrease	995	1
1008	Infinite decrease	1008	1
1161	Infinite decrease	1161	1
1169	Infinite decrease	1169	1
1217	Infinite decrease	1217	1
1745	Infinite decrease	1745	1

4.3 Specific Spots of Interest

Unique spots 1880, 1906 and 2017 on the resistant gel were chosen for further analysis by mass spectrometry and bioinformatics as they had high normalised volumes (Table 7) providing relatively large quantities of protein for mass spectroscopy detection, and were in optimum positions on the gel where they could be picked minimising risk of cutting neighbouring spots.

As with the results from the original gels (spots 201 and 594) the fragmentation spectra for the unique spot were poor.

It was decided to treat the single charged data obtained as if it had gone through MALDI ToF mass spectrometer, to manually generate a peak list of molecular masses of each peptide (Appendix 15) for PMF analysis, searching the MASCOT database. PMF's were obtained for spots 594, 201, 1880 and 1906, significance of identifications in MASCOT was made for each spot and for the most likely proteins the literature was searched. Figure 13 shows a representative PMF for spot 594, hitting a Relaxin-3 precursor. The Spots of interest with the most significant and interesting hits in MASCOT can be seen in Table 8.

Table 7: Unique unmatched spots of interest found on the resistant gel

Spot Number	Norm. Vol.
1880	1,295.43
1906	1,498.35
2017	2,088.25

Spot 1880

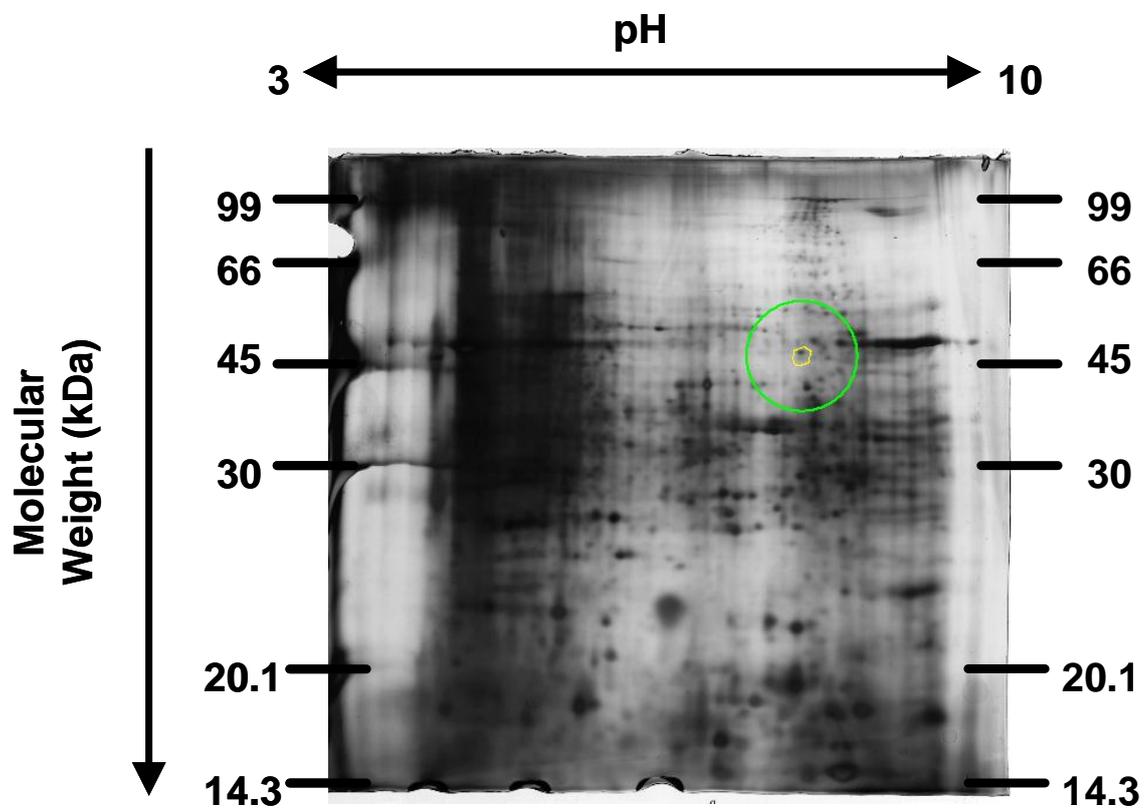


Figure 10a: Relative location of spot 1880 on the resistant gel indicated by a yellow out line in a green circle. Approximate molecular weight values and pI are shown.

Spot 1880

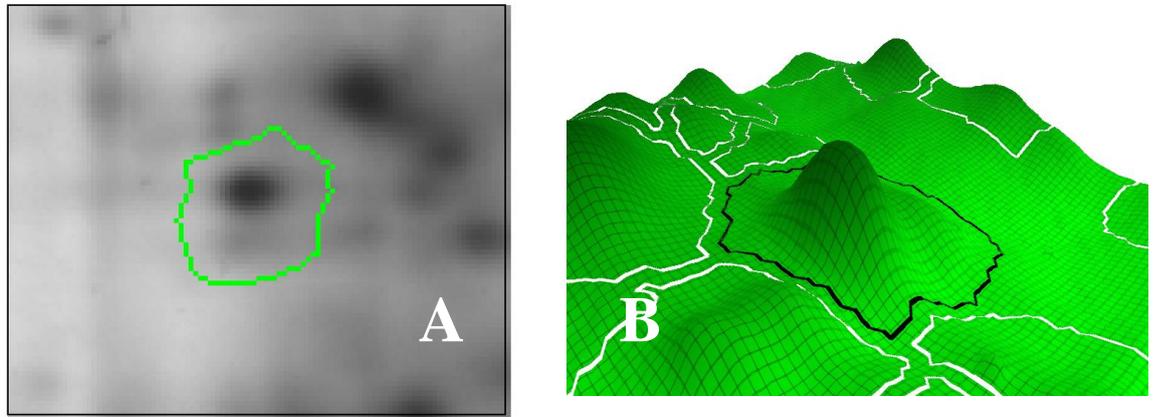


Figure 10b: Montage and 3D visualisation of spot 1880 on the resistant gel (A & B)

Spot 1906

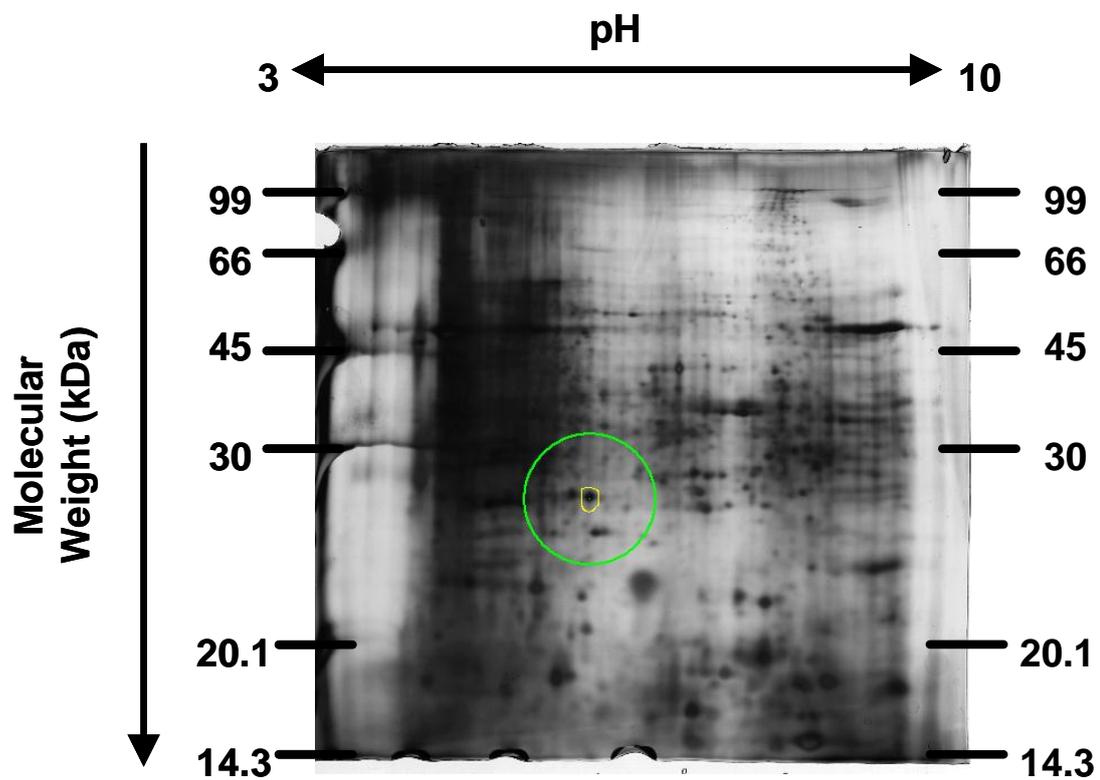


Figure 11a: Relative location of spot 1906 on the resistant gel indicated by a yellow out line in a green circle. Approximate molecular weight values and pI are shown.

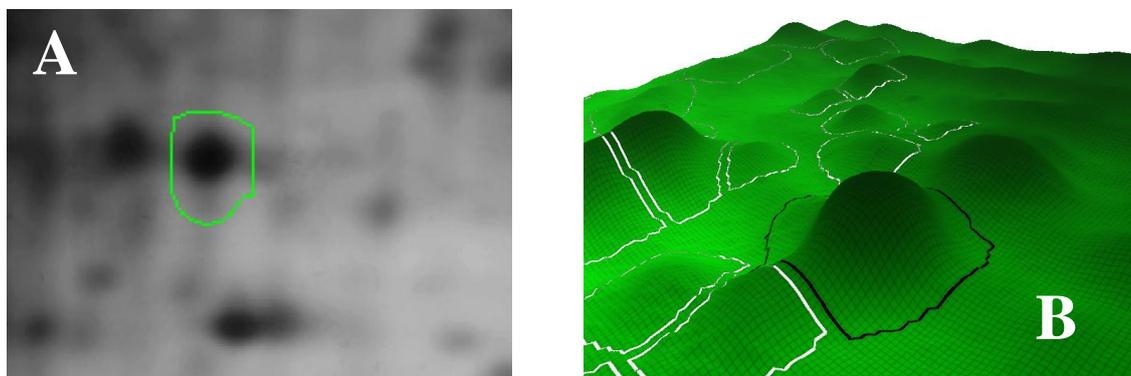
Spot 1906

Figure 11b: Montage and 3D visualisation of spot 1906 on the resistant gel (A & B)

Spot 2017

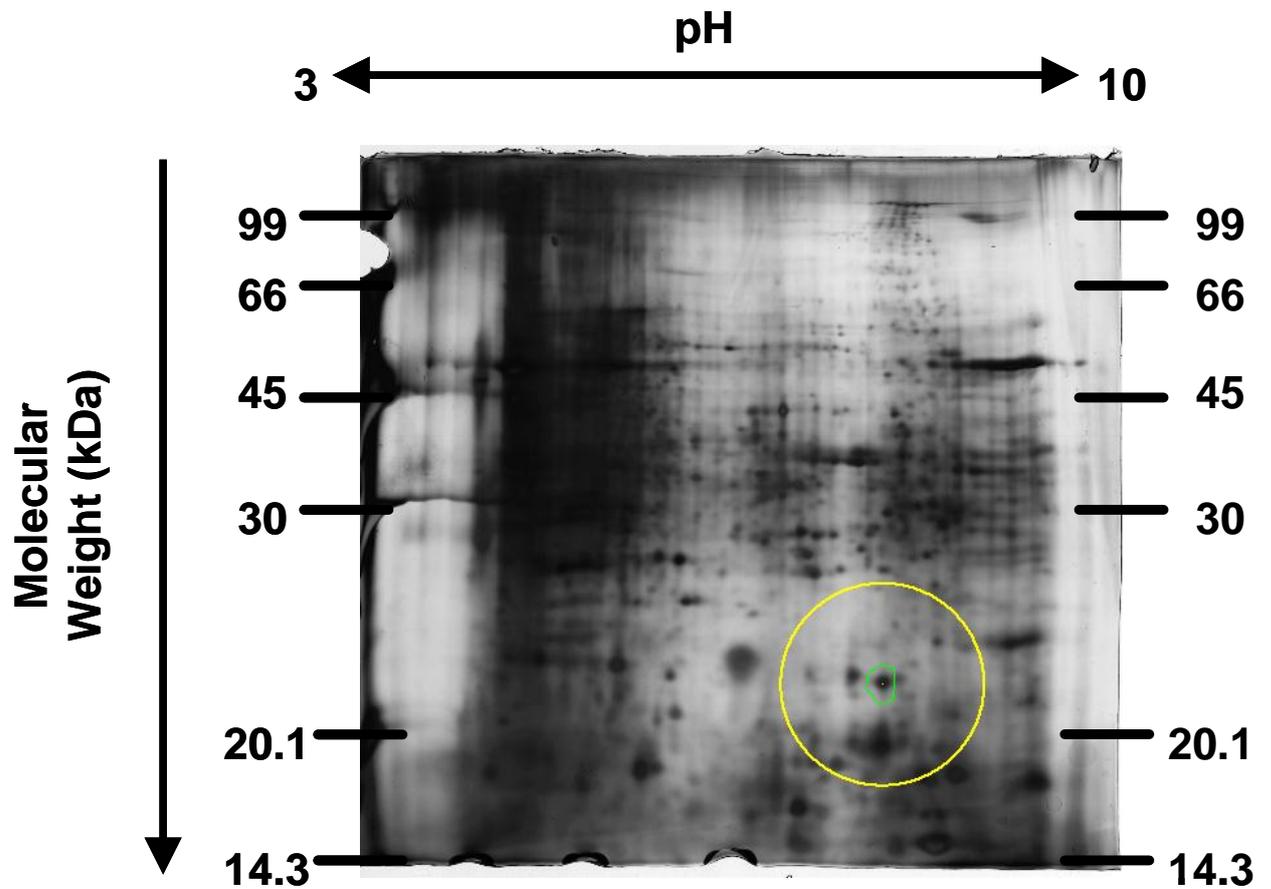


Figure 12a: Relative location of spot 2017 on the resistant gel indicated by a green out line in a yellow circle. Approximate molecular weight values and pI are shown.

Spot 2017

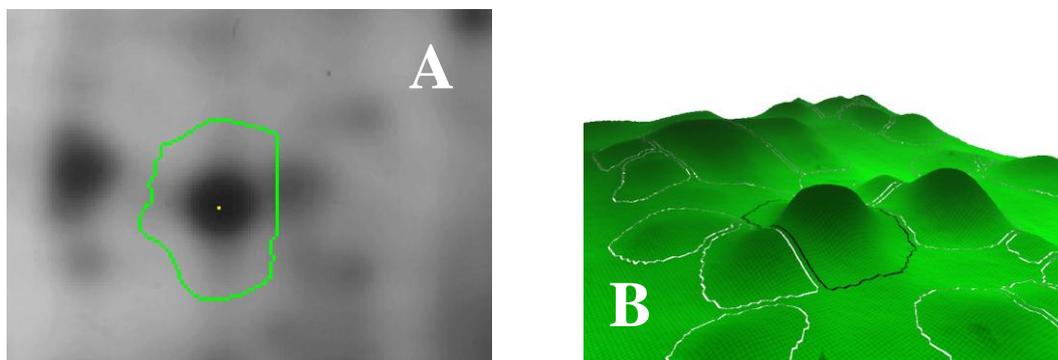


Figure 12b: Montage and 3D visualisation of spot 2017 on the resistant gel (A & B)

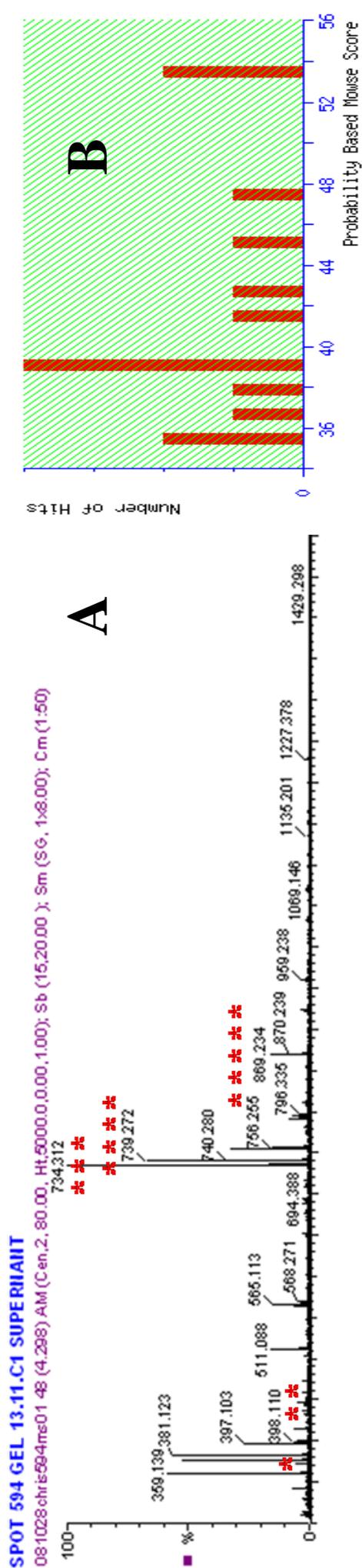


Figure 13:

A) A representative PMF from spot 594; hitting REL3_PANTR a Relaxin-3 precursor (Prorelaxin H3) (Accession No. Q5CZK2) with labelled peptides as below: * -.MAR.Y, ** R.LCGR.E, *** R.AAPYGV.R.L, **** K.SEISSLC., ***** K.SPQAFYR.G

B) MASCOT identification of significance. Protein score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 71 are significant ($p < 0.05$).

C) Sequence of Relaxin-3 precursor (Prorelaxin H3). Matched peptides shown in **Bold Red**

Table 8: Summary of possible protein identifications from PMF using MASCOT, ranked by E-value (MOWSE score) for each spot.

Spot No.	E-value (MOWSE)	Sequence Coverage (%)	No. Matched Peptides	Predicted MW (Da)	Predicted pI	Score	Accession No.	Description
594ms01	3.2	5	8	66238	9.03	54	Q25239	Transposase.- <i>Lucilia cuprina</i> (Greenbottle fly) (Australian sheep blowfly).
	6.8	19	5	15338	6.56	50	Q5CZK2	Relaxin-3 precursor (Prorelaxin H3) [Contains: Relaxin-3 B chain; Relaxin-3 A chain].- <i>Pan troglodytes</i> (Chimpanzee).
	8.3	7	6	31692	5.71	49	Q8BYB4	3 days neonate thymus cDNA, Transmembrane protein VEZATIN homolog, full insert sequence.- <i>Mus musculus</i> (Mouse).
	85.0	45	3	2613	8.42	39	AAA02731	Antigen, T-Cell Receptor (fragment) - <i>Homo sapiens</i> (Human).
	120.0	20	4	12396	9.82	32	AAD30680	Immunoglobulin Heavy Chain Variable Region (fragment)- <i>Homo sapiens</i> (Human).
594ms02	62.0	29	5	10761	8.94	41	CAD33332	<i>Homo sapiens</i> (Human) Sequence 59 from Patent WO0218424.
201ms01	7.4	14	7	25404	8.37	50	Q8C2Z6	2 days pregnant adult female oviduct cDNA.- <i>Mus musculus</i> (Mouse).
1880ms01	6.8	100	3	1085	7.98	50	Q7Z5A2	Hypothetical protein.- <i>Homo sapiens</i> (Human).
	390.0	16	3	6625	9.65	33	Q16R17	Hypothetical protein.- <i>Aedes aegypti</i> (Yellowfever mosquito).
1880ms02	13.0	5	7	55666	6.11	47	Q2PQR0	Gram negative binding protein 1-like protein.- <i>Glossina morsitans morsitans</i>
	87.0	28	4	7071	9.85	39	Q6TKS0	Transcription factor Pax-5 (Fragment).- <i>Raja eglanteria</i> (Clearnose skate).
	110.0	20	5	12959	9.19	38	Q5DDM6	<i>Schistosoma japonicum</i> (Blood fluke). SJCHGC07047 protein
1880ms03	180.0	9	6	38653	8.81	36	Q17A92	Ras association domain protein, putative.- <i>Aedes aegypti</i> (Yellowfever mosquito).
	15.0	16	5	15470	5.65	47	Q86DC9	Smn (Survival of motor neuron) protein interactor protein 1, isoform b. <i>Caenorhabditis elegans</i> .
	15.0	16	5	15470	5.65	47	Q86DC9	Smn (Survival of motor neuron) protein interactor protein 1, isoform b. <i>Caenorhabditis elegans</i> .
	17.0	4	6	39145	8.81	46	Q76B94	Blue sensitive cone opsin.- <i>Eulemur fulvus</i> (brown lemur).
1906ms01	270.0	3	5	39082	8.71	34	Q645K1	Short-wave sensitive type 1 opsin.- <i>Isoodon obesulus</i> (Southern brown bandicoot).
	12.0	15	5	10917	9.52	48	Q9VQG2	<i>Drosophila melanogaster</i> (Fruit fly). CG17734-PA, isoform A (RE71331p).
1906ms03	210.0	5	4	31705	8.13	35	Q16WN5	TRNA delta(2)-isopentenylpyrophosphate transferase.- <i>Aedes aegypti</i> (Yellowfever mosquito).
	830.0	3	4	53379	5.94	29	Q9JJT1	Glucocorticoid-attenuated response gene 16 product.- <i>Rattus norvegicus</i> (Rat).
	1400.0	3	4	53701	7.24	27	B55508	interferon alpha Ifi56 - mouse
	1400.0	3	4	53673	7.24	27	Q3U5K8	Bone marrow macrophage cDNA. product: interferon-induced protein with tetratricopeptide repeats 1, full insert sequence.- <i>Mus musculus</i> (Mouse).
	1400.0	3	4	53717	7.24	27	Q3U5N2	Bone marrow macrophage cDNA. product: :interferon-induced protein with tetratricopeptide repeats 1, full insert sequence.- <i>Mus musculus</i> (Mouse).
	1400.0	3	4	53689	7.24	27	Q3UNG3	CRL-1751 WEHI 164 cDNA. product: interferon-induced protein with tetratricopeptide repeats 1, full insert sequence.- <i>Mus musculus</i> (Mouse).
	1400.0	3	4	53704	6.97	21	Q8BN43	Activated spleen cDNA. product: interferon-induced protein with tetratricopeptide repeats 1, full insert sequence.- <i>Mus musculus</i> (Mouse).
	1600.0	3	4	53702	7.92	27	Q3U7A2	Bone marrow macrophage cDNA. product:interferon-induced protein with tetratricopeptide repeats 1, full insert sequence.- <i>Mus musculus</i> (Mouse).
	300.0	9	3	15243	7.97	34	A29391	hemoglobin alpha-1 chain - Brazilian free-tailed bat
	300.0	9	3	15930	7.23	34	Q6F3D2	Minor alpha globin chain.- <i>Ambystoma mexicanum</i> (Axolotl).
	340.0	12	3	10917	9.52	33	Q9VQG2	CG17734-PA, isoform A (RE71331p).- <i>Drosophila melanogaster</i> (Fruit fly).
360.0	8	3	16662	8.37	33	AAB84320	T-Cell Receptor Beta Chain (Fragment).- <i>Capra hircus</i> (Goat).	