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Peptide Mass Printing of Hemocyanin Protein of Spider, *Hippasa Agelenoides* From South India Using Maldi-TOF MS

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Abstract: ***In proteomics, MALDI is used for the rapid identification of proteins isolated by using SDS-PAGE, size exclusion chromatography, affinity chromatography, strong/weak ion exchange, isotope coded protein labeling (ICPL), and two-dimensional gel electrophoresis. The simplicity, robustness and sensitivity of MALDI have made it a fundamental technique of proteomics. Here we report a high throughput protein identification of hemocyanin protein from the spider Hippasa agelenoides by MALDI TOF MS, peptide mass fingerprinting.***

Keywords: SDS PAGE, MALDI TOF, *Hippasa agelenoides*, Hemocyanin, Peptide Mass fingerprinting.

1. Introduction

Peptide mass fingerprinting (PMF) (also known as protein fingerprinting) is an analytical technique for protein identification that was developed in 1993 by several groups independently (Pappin et al., 1993; Henel et al., 1993; Mann et al., 1993; Yates et al., 1993). In this method, the unknown protein of interest is first cleaved into smaller peptides, whose absolute masses can be accurately measured with a mass spectrometer such as matrix assisted laser desorption/ionization - time of flight (MALDI-TOF) or ESI-TOF (Clauser et al., 1999). Protein samples can be derived from SDS-PAGE and then subject to some chemical modifications. Disulfide bridges in proteins are reduced and cysteine amino acids are carbamidomethylated chemically or acrylamidated during the gel electrophoresis. Then the proteins are cut into several fragments using proteolytic enzymes such as trypsin, chymotrypsin or Glu-C. A typical sample:protease ratio is 50:1. The proteolysis is typically carried out overnight and the resulting peptides are extracted with acetonitrile and dried under vacuum. The peptides are then dissolved in a small amount of distilled water or further concentrated and purified using ZipTip Pipette tips and are ready for mass spectrometric analysis (Shevchenko, 1996). In the present analysis a high throughput protein (hemocyanin) from the spider Hippasa agelenoides has been identified using MALDI TOF MS. In future this technique can be used for the identification of proteins from other spiders because not much is done in Indian context with regard to spiders. The technique is simple and sensitive and hence can also be used for identification of any protein provided it is available in the database like NCBInr sequence database or Uniport KB/Swiss port

2. Materials and Methods

2.1 Sample collection

Hippasa agelenoides were collected from different regions of Karnataka, India. Collection was made by trapping method, in the early hours of the day during the study duration spanning from September 2008 to January 2012. A sample size of (N=10) was utilized for the present analysis. Hemolymph collection from adult spiders and processing was followed as described by Yigit and Benli (2008) and Jalal et al., (2010) with some modifications to suit experimental setup. The live spiders were anesthetized with alcohol; the fourth walking legs were separated from the coxa. The outward flowing liquid (20-25µl) was collected with micropipette. It is a pale, viscous and odorless liquid. The tubes were labeled and centrifuged at 12,000 × g for 10min. The resultant cell free hemolymph was refrigerated at approximately 3°C and analysed within 72h (Barron, 1999). It was diluted with distilled water (1:4) for further analysis.

2.2 SDS PAGE

The molecular weight of hemocyanin of *Hippasa agelenoides* was determined by SDS-PAGE. SDS-PAGE was carried out as described by Laemmli, 1970 using 5% (w/v) stacking gel and 12% (w/v) separating gel. Coomassie brilliant blue staining was used to visualize protein bands on the gel.

2.3 Excising the gel protein of interest

Protein bands/spots of molecular weight (70-75 KDa) of interest were excised from the stained polyacrylamide gel. The gel piece was cut into small particles (~1 mm 2mm) using a scalpel, and placed into a 0.5 mL siliconized tube (VWR *SuperSlik* microcentrifuge tubes). Also cut out and dice a gel piece from a protein free region of the gel, for a parallel control digestion to identify trypsin autoproteolysis products. The small gel particle size facilitates the removal of SDS (and Coomassie) during the washes, and improves enzyme access to the gel. For Coomassie stained proteins, one gel particle per tube is probably sufficient protein. About ~100 µL of 25mM NH4HCO3/50% acetonitrile (enough to immerse the gel particles) and vortexed for 35-40 min on a low setting

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(more like shaking). Use gel loading pipette tips to remove the solution (pale blue in the case of Coomassie staining) and discard. The washing/dehydration step was repeated up to ~3 times. Once all Coomassie has been removed, gel was dehydrated with acetonitrile (100 µL). At this point the gel pieces shrinks and becomes an opaque-white color. In case if they do not, remove the acetonitrile and replace with fresh. Remove acetonitrile and SpeedVac for 3-5 minutes.

2.4 Reduction and alkylation of cysteine residues

About 30 µL of 10 mM DTT solution was added to cover the gel pieces, and reduced for 30-45 min at room temperature. The DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide (30 µL). It was incubated for 45 min at room temperature in the dark. The iodoacetamide solution was removed and the gel pieces was washed with ~100 µL of 25 mM NH4HCO3 pH8, for 10 min while vortexing. The gel was dehydrated with ~100 µl acetonitrile. The gel pieces should shrink and become an opaque-white color. If they do not remove the acetonitrile, repeat the washing dehydration cycle until they do. The acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge for 3-5 minutes.

2.5 In-gel trypsin digestion

The gel particles were rehydrated in 25 µL trypsin solution and placed on ice for 10-15 minutes. Excess trypsin solution was removed and the rehydrated gel particle was overlayed with 30 µL of 25 mM NH4HCO3 so as to keep them immersed throughout digestion. It was incubated for 12 to 16 hrs at 37ºC.

2.6 Recovery of peptides using a zip-tip

5 uL of 5% aqueous TFA was added to halt the digestion. The tubes containing gel pieces were shaken for about 10 minutes and centrifuged briefly to bring the liquid to the bottom of the tube. (Prepare a saturated solution of HCCA in 1:1 acetonitrile: acidified water (0.1% TFA). Dilute this matrix solution by a factor of 2 and place 3 µL in as many tubes as needed, one for each digestion). The pipettor was set to 10 µL and 1:1 acetonitrile: 0.1% TFA was aspirated through the Zip-Tip, dispensed to waste. The step was repeated twice with fresh solution. Binding of the peptides was done by performing 3-10 cycles of aspiration and dispensing the digested solution through the Zip-Tip. More the diluted solution, more the cycles may be required. The Zip-Tip was washed by aspirating with 10 uL of 0.1% TFA and dispensing to waste. It was repeated once with fresh solution. The pipettor was set to 3 µL and peptides eluted using 3 cycles of aspiration and dispensing the matrix solutions. On the fourth aspiration, 1 µL was dispensed directly on to the MALDI plate and allowed to dry.

3. Results and Discussion

3.1 SDS-PAGE

The SDS PAGE was used to estimate the molecular weight of the cell free extract (hemolymph). The gel showed a prominent band with molecular weight in the range of 70 to 75 Kda for *Hippasa agelenoides.*(Figure 1) as recorded from previous works (Markl and Decker, 1992; van Holde and Miller, 1995).

3.2 Matrix assisted laser desorption/ionization - time of flight (MALDI TOF) Primary sequence confirmation. Peptide Mass Fingerprinting

For the primary sequence confirmation and identification of the hemocyanin protein, the list of peptide masses (Table 1) generated by MALDI-TOF MS of peptides obtained from corresponding positions of coomassie stained gels (Figure 1) were searched with MASCOT search engines using the sequence database, NCBInr. During database searching, selected enzyme was trypsin. Other search parameters were set to allow up to 1 missed cleavage, methionins in oxidized form (variable modification), cysteins in carbamidomethylated form (fixed modification), average mass, peptide ions in protonated [M+H]+ form, mass tolerance was set to ±100 ppm. Protein molecular mass was not specified. The database searched in this study yielded peptides in an m/z range from 606.077 to 3701.688 (Table 1)

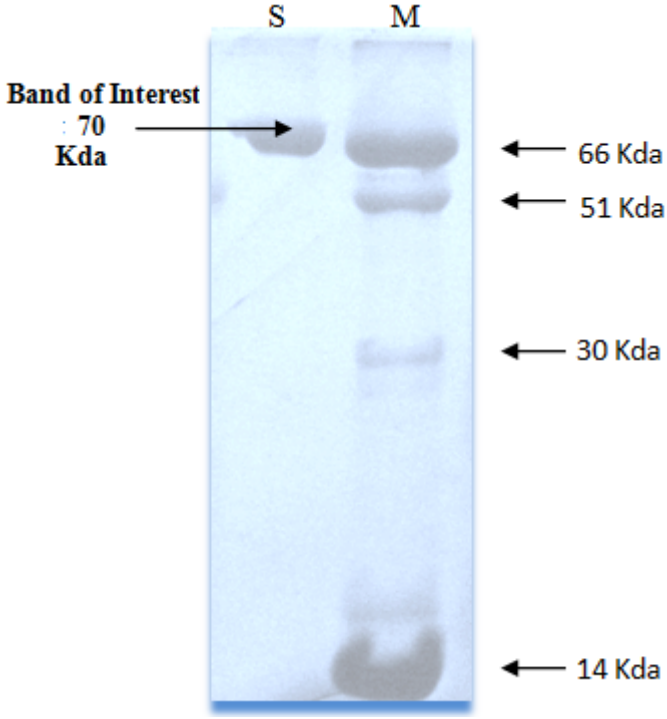

Figure 1: 12% SDS-PAGE showing the Band of interest (Hemocyanin)
S: Cell free extract of hemolymph of *Hippasa angelenoides*
M: Marker protein

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Table 1: List of peptide masses of *Hippasa agelenoides* obtained from MALDI TOF MS analysis.

m/z	*S/N*	*Quality Fac.*	*Res.*	*Intens.*	*Area*
606.077	12.4	2028	5261	990.05	182
646.182	7.7	744	5095	609.18	125
669.231	15.9	3853	5127	1238.47	266
684.069	17.2	1316	4366	1338.13	336
1516.795	91.0	135986	5795	7452.43	5226
1567.753	93.9	191270	5713	7785.27	5814
1581.769	49.1	76318	5411	4086.63	3253
1876.779	15.5	20500	5494	1338.33	1484
1961.774	28.3	10429	4475	2382.34	3390
1993.832	8.2	2842	4142	682.32	1053
2156.959	6.5	6173	4653	506.14	813
2170.962	10.2	1633	4195	800.56	1351
2528.094	13.1	1790	4215	839.84	1758
2534.882	15.3	1311	3684	978.30	2315
2559.986	18.0	12441	5029	1140.47	2062
2573.191	6.9	387	4445	430.27	896
2864.395	42.9	2492	4164	2208.67	5635
2895.420	10.6	1125	4199	523.37	1383
3183.372	24.9	1288	3743	940.79	2998
3701.688	7.1	381	3986	165.93	669

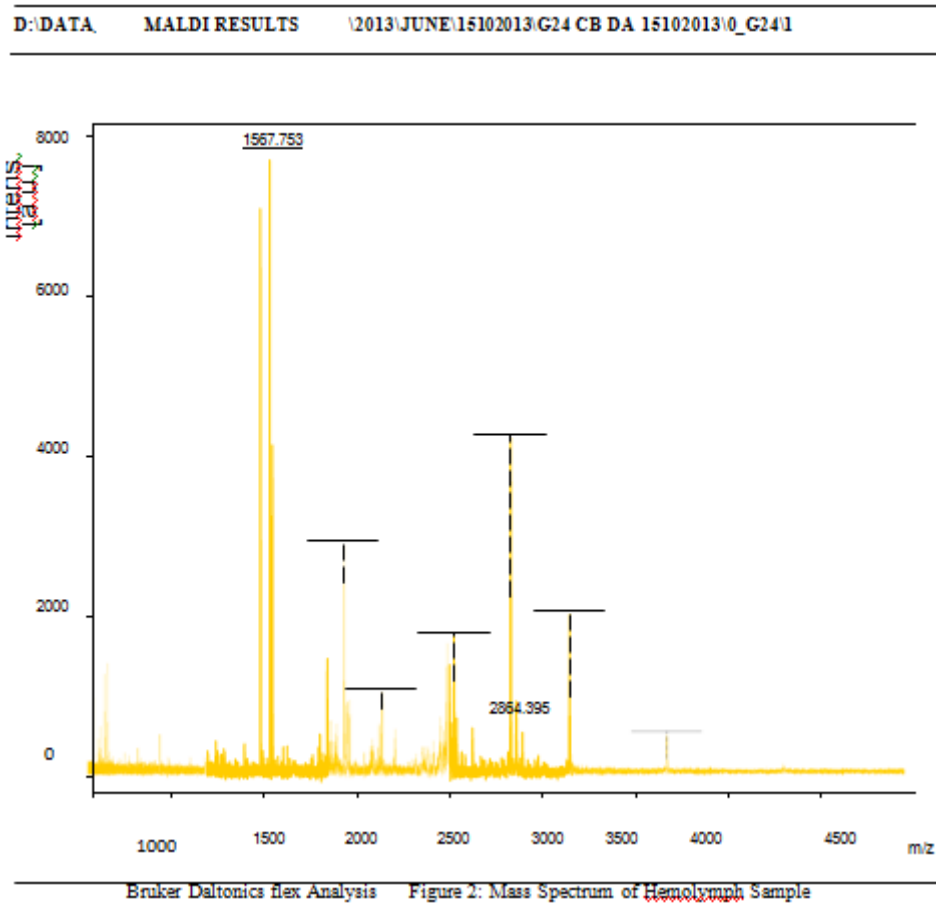

Figure 2: Mass Spectrum of Hemolymph Sample

3.3 MASCOT/NCBInr sequence database

The application of the MASCOT database led to the identification of hemocyanin subunit 6, 6' and 6" of *Cupiennius salei.* List of searched peptides in the search engine (NCBInr primary sequence database), generated the top total score of 99 for hemocyanin protein. The highest score mixture consisted of three identified proteins:

- **gi|15027028, hemocyanin subunit 6' [Cupiennius salei]**
- **gi|15027030, hemocyanin subunit 6" [Cupiennius salei]**
- **gi|15027026, hemocyanin subunit 6 [Cupiennius salei]**

4. Conclusion

The development of MALDI-MS and the rapid growth of databases have revolutionized the identification of proteins. PMF by MALDI-MS is the key approach for high

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throughput identification of well-separated proteins. Mass accuracy, sensitivity, and automatic measurement have been improved noticeably within the last decade for MALDI-TOF-MS, facilitating protein identification by PMF with high confidence. In the present findings on the South Indian Spider *Hippasa agelenoides* a high molecular weight protein of 70 Kda was isolated by SDS PAGE. The application of MALDI TOF and MASCOT search engine identified the protein as hemocyanin, a respiratory protein in Arachnids.

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