

# Mass Spectrometric Analysis of *Bombyx mori* Humoral Lectin Related Proteins

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## INTRODUCTION

The recent development of mass spectrometric techniques, including in-gel digestion with trypsin and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), has enabled the rapid and sensitive identification of proteins separated by two-dimensional gel electrophoresis (2-DE) (Furuta *et al.*, 2002; Madi *et al.*, 2003; Abe *et al.*, 2004; Zhang *et al.*, 2004, 2005). Currently, 2-DE can reveal virtually all proteins present in various cells and tissue at any given time, and prepare respective proteins for protein identification and/or protein structural analysis. High-resolution 2-DE should be capable of preparing sufficient amounts of each protein for such structural analysis methods as amino acid sequencing or mass spectrometry (Ohishi, *et al.*, 2000).

To date, I have tried the novel technology to fractionate lectin related proteins in the hemolymph of the domesticated silkworm, *Bombyx mori*, by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), in relation to the investigation of the activation of the *B. mori* humoral lectin (Kato, 2004, 2005, 2006). Generally, biological activities elicited by lectins include cell agglutination, mitosis, apoptosis and so on. For these activities, animal lectins seem to play an important role in the defense mechanism of the living body, as removal of a foreign matter or a biopolymer which has lost its normal function in the living body (Kotani *et al.*, 1995; Fujita *et al.*, 1998; Koyama *et al.*, 2002; Ujita *et al.*, 2002). I showed that a humoral lectin (130K-glycoprotein) of *B. mori* played a physiological role in cellular communication throughout the metamorphosis of the *B. mori* (Kato *et al.*, 1994). I also emphasized the possibility that the humoral lectin was produced and activated in the fat body of the *B. mori*, and that it was secreted into the hemolymph (Kato *et al.*, 1998). Moreover, I reported that neuraminidase was related to humoral lectin activity and that galactosidase was involved in the inactivation of the lectin activity *in vivo* (Kato and Takeuchi, 2006).

My purpose in this paper was to approach proteomic analysis of *Bombyx mori* humoral lectin related proteins using 2-D PAGE, in-gel tryptic digestion, MALDI-TOF MS and database search. This investigation provides useful information for understanding of the original role of the lectin related proteins *in vivo*.

## MATERIALS and METHODS

### (1) Sample preparation

A hybrid race, Shunrei × Shogetu, of *Bombyx mori* was used. Larvae were reared on mulberry leaves. Hemolymph was collected by cutting the larval pleopods and abdominal legs, centrifuged at 2,260 g at 4 °C for 15 min to remove hemocytes, and the resulting supernatant was lyophilized.

### (2) Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

2-D PAGE was performed using the 2-D minislab system (Atto Corp.). The proteins were subjected to agar gel for isoelectric focusing (the first dimension) with a linear immobilized pH gradient. Electrophoresis was carried out for 210 min with a constant voltage of 300 V per gel tube. The proteins were then separated by SDS-PAGE (10%) in the second dimension, and electrophoresis was carried out for 90 min with a constant electric current of 20 mA per gel.

### (3) In-gel digestion with trypsin

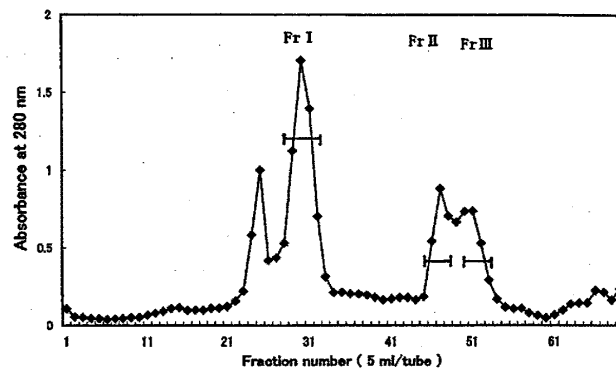
In-gel tryptic digestion of a protein in the piece of gel was done by the method of Shevchenko *et al.* (1996b), with minor modifications. After the gel pieces were excised and shrunk by dehydration in acetonitrile, which was then removed, they were dried in a vacuum centrifuge. A volume of 10 mM dithiotreitol (DTT) in 25 mM NH<sub>4</sub>HCO<sub>3</sub>, sufficient to cover the gel pieces was added, and the proteins were reduced for 1 h at 56 °C. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 25 mM NH<sub>4</sub>HCO<sub>3</sub>. After 45 min incubation, the gel pieces were washed by 25 mM NH<sub>4</sub>HCO<sub>3</sub>, dehydrated by addition of acetonitrile, swelled by rehydration in 25 mM NH<sub>4</sub>HCO<sub>3</sub>, and shrunk again by addition of the same volume of acetonitrile. The liquid phase was removed, and the gel pieces were completely dried in a vacuum centrifuge. Overnight digestion at 37 °C was done with a solution containing 0.1 µg trypsin (25 mM NH<sub>4</sub>HCO<sub>3</sub>). Peptides were extracted and dried.

### (4) Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and database search

MALDI-TOF MS was done with a Voyager-DE PRO Bio-spectrometry Workstation MALDI-TOF (Applied Biosystems, Foster City, California, U.S.A). The workstation was operated in positive-ion linear mode with the following parameters: 20 kV accelerating voltages, 95% grid voltage, 100 ns extraction delay, and 500 m/z low mass gate. An aliquot (one µl each) from the gel tryptic digestion was mixed with 1 µl of 10 mg/ml cyano-4-hydroxy cinnamic acid (35%

acetonitrile and 0.1% trifluoroacetic acid). Measurements were calibrated externally and internally with a Sequazyme Peptide Mass standard kit and peptides by autoproteolysis of trypsin, respectively. Peaks detected by mass spectrometry were subjected to a SWISS-PROT database search by MS-Fit software (<http://prospector.ucsf.edu>). Proteins were identified by MALDI peptide mapping by the method of Shevchenko *et al.* (1996a).

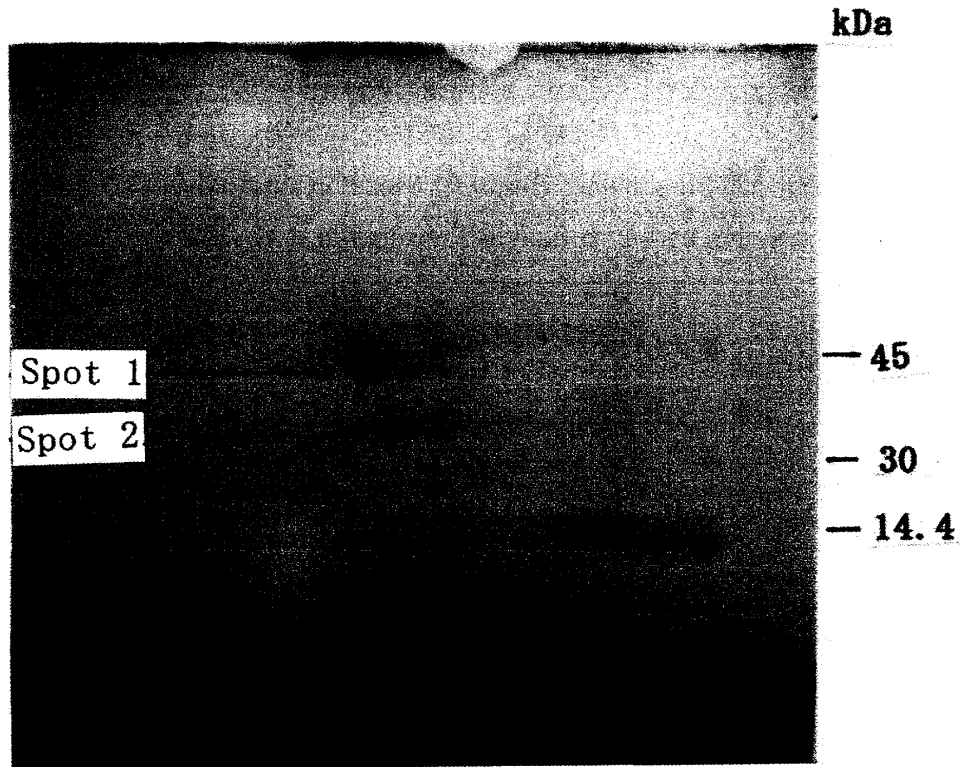
## RESULTS and DISCUSSION



**Fig. 1:** Fractionation of Larval Hemolymph on Day 10 of Fifth Instar by Gel Filtration through Superdex 200. Fraction II (tubes 47-49) was separately pooled, dialyzed and lyophilized.

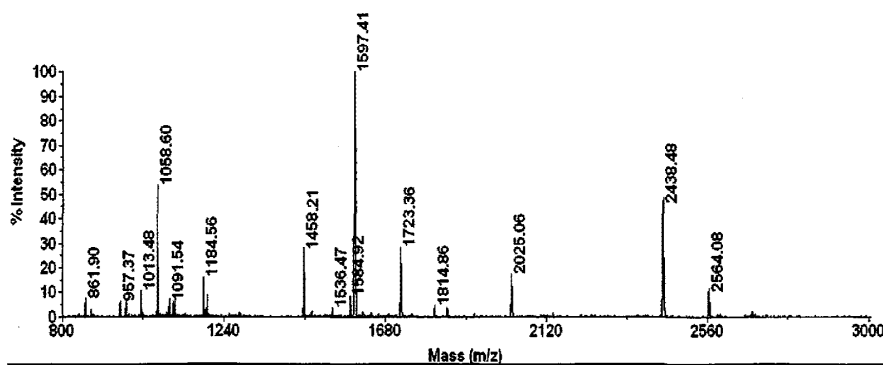
Initially, gel filtration of hemolymph from mature larvae on day 10 of the fifth instar through a Superdex 200 column (Fig. 1). The Superdex 200 column (2.6 × 60 cm, Amershan Pharmacia Biotech Ltd.) was equilibrated with 0.1 M Tris-HCl buffer containing 0.1M NaCl (pH 8.0) and eluted with the same buffer at a constant flow rate of 150 ml/h. The effluent was collected in 5 ml fractions and measured at 280 nm with a Shimadzu spectrophotometer type UV 1200. Three significant fractions, Fraction I, Fraction II and Fraction III were obtained by gel filtration in addition to the passed-through fraction (tubes 24-25). Among these, Fraction II (tubes 47-49) contained the  $\alpha$ - and  $\beta$ -galactosidase activities most abundantly as described in my former report (Kato, 2005). Accordingly, I tried to study the possibility about further separation of Fraction II using the 2-D PAGE method.

Fig. 2 illustrates the result of 2-D PAGE on Fraction II obtained by gel filtration from the larval hemolymph of the *B. mori*. 2-D PAGE was performed using the 2-D minislabs system (Atto Corp.) as described in MATERIALS and METHODS. After electrophoresis, the slab gel was stained with Rapid Stain Coomassie Brilliant Blue Kit (Nakalai Tesque Inc.) and then destained with distilled water. The Coomassie stained 2-D PAGE gel was interposed between two wet cellophane sheets to be dried at 80°C for 2 h under a vacuum in a Rapid Dry Mini type AE-3711 (Atto Corp.). The result showed that Fraction II gave two clear protein spots in the



**Fig. 2:** 2-D PAGE Pattern of Fraction II Obtained from Hemolymph by Gel Filtration. Arrows (Spot 1 and Spot 2) indicate protein spots used for further mass spectrometric analysis.

gel (Fig. 2). Arrows (Spot 1 and Spot 2) of the figure indicated protein spots used for further analysis. Both spots of these proteins were excised from the gel and subjected to in-gel tryptic digestion, mass spectrometric analysis, and a database search. Protein spots excised from dry gel were re-hydrated with water for 5 min at room temperature, and cellophane film was then detached with a forceps. In-gel tryptic digestion of a protein in the piece of gel was done by the method as described in "MATERIALS and METHODS" .



**Fig. 3:** MALDI-TOF MS of Spot 1. The MALDI-mass spectrum obtained after tryptic digestion of the corresponding spot 1 from Fraction II is shown.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) of spot 1 obtained by 2-D PAGE gave a highly clear spectrum (Fig. 3). MALDI-TOF MS was done with a Voyager-DE PRO Bio-spectrometry workstation MALDI-TOF, showing 15 peaks at 861.90, 957.30, 1013.48, 1058.60, 1091.54, 1184.56, 458.21, 1536.47, 1584.92, 1597.41, 1723.36, 1814.86, 2025.06, 2438.48 and 2564.08 m/z. Peaks detected by mass spectrometry

**Table 1:** Result of Sequence Analysis of Spot 1 by Database Search. Database search identified the protein as probable GDP-mannose 4,6 dehydratase (GDP-D-mannose dehydratase) (EC4.2.1.47)

m/z	MH <sup>+</sup>	Modifications	Start	End	Missed	Database
Submitted	Matched				Cleavages	Sequence
861.90	862.02		318	324	1	(R) EITWKGK (G)
957.37	956.89	2P04	200	206	0	(R) SPYACAK (M)
1058.60	1059.07	1P04	237	244	1	(R) RGENFVTR (K)
1101.75	1102.24		70	78	0	(K) DYEVHGIIIR (R)
1597.41	1597.58	1Met-ox 2P04	6	19	0	(R) LIAMSTSDGAPETK (K)
1849.25	1848.98		289	305	0	(R) ESPSDYVIATGETHSVR (E)

were subjected to a SWISS-PROT database search by MS-Fit software for protein identification. Table 1 summarizes the result of sequence analysis of spot 1. The analysis of spot 1 gave 6 matched peptide fragments. The matched fragment amino acid sequence for spot 1 is illustrated in Fig. 4. The result suggested that the probable identification of spot 1 was GDP-mannose 4,6-dehydratase. GDP-mannose 4,6-dehydratase (EC 4.2.1.47) is described as

Index Number: 150983  
 Acc. #: Q9VHW3 Species: DRONE Name: Probable GDP-mannose 4,6 dehydratase (GDP-D-mannose dehydratase) (Dm-gmd)  
 pI of Protein: 6.8  
 Protein MW: 44784  
 Amino Acid Composition: A29 C3 D22 E31 F13 G27 H10 I23 K27 L23 M15 N19 P14 Q10 R25 S27 T26 V27 W5 Y19

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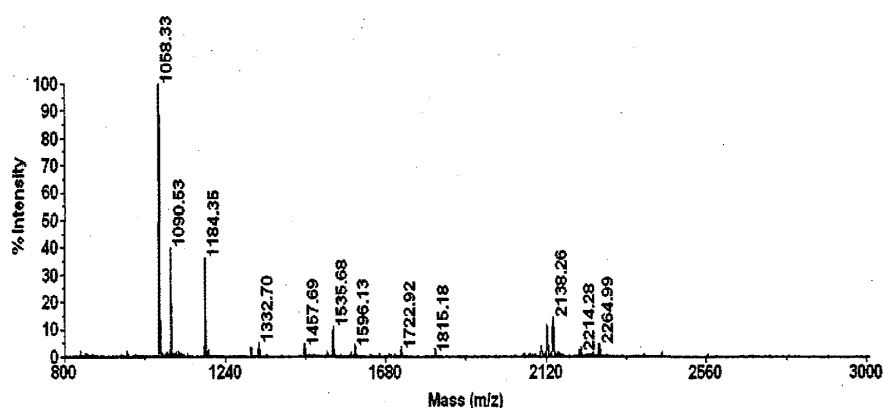
1      11      21      31      41      51      61      71
MLNRLIAMS YSDGAPETEK QRPSSSSNGS KDPNGTEAGA EGDSDKDEVAL ITGITGQDGS YLAEFLIKED YEVHGIIIRA
81      91      101     111     121     131     141     151
STFWTRIEH LYADPKAKHG GRMLHYGDR IDSSSLVKLI NNWVPEIYH LAAQSHVQVS FDLSEYTAEV DAVGTLRILD
161     171     181     191     201     211     221     231
AIRTCGEEKK VRFYQASTSE LYGVVETPQ NEQTFFYPRG PYACANWGF NIVENVREAY NMYACWILF NHESPRIGEN
241     251     261     271     281     291     301     311
FVTRKIVRSV AKIYHQMEY FELGNLDSKR DWGHASDYVE ANWDLQRES PSDYVIATGE IHSVRFVEA AFKHIDREIT
321     331     341     351     361     371     381     391
WKGKGVDEWG VENGIGIVRY RINPEYFRPT EVDLLGGDAS KANRELHWTP KVTFVELYSD NNEADIELNR KNPIA
  
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The matched peptides cover 15% (62/395 AA's) of the protein.

**Fig. 4:** Matching Amino Acid Sequence of Protein from Spot 1. The matched peptides cover 15% of the protein.

guanoshin 5'-diphosphoric acid-D-mannose dehydration enzyme and contributes to the formation of GDP-fucose from GDP-mannose.

On the other hand, MALDI-TOF MS of spot 2 obtained by 2-D PAGE also gave a highly



**Fig. 5:** MALDI-TOF MS of Spot 2. The MALDI-mass spectrum obtained after tryptic digestion of the corresponding spot 2 from Fraction II is shown.

clear spectrum (Fig. 5), showing 12 peaks at 1058.33, 1090.53, 1184.35, 1332.70, 1457.69, 1535.68, 1596.13, 1722.92, 1815.18, 2138.26, 2214.28 and 2264.99 m/z. This result from the MALDI-TOF MS analysis was then searched in the SWISS-PROT protein database for protein identification. Table 2 summarizes the result of the analysis of spot 2. The analysis of spot 2

m/z	MH+	Modifications	Start	End	Missed	Database
Submitted	Matched				Cleavages	Sequence
1090.5287	1090.3793		3	13	0	(R) IVGSSMLAALK (R)
1184.3528	1184.1953	1PO4	197	205	0	(R) NLYENVHVK (I)
1310.3516	1310.4342		223	233	0	(K) GQMESSVWEEK (R)
1332.7039	1332.615		249	259	0	(K) IMENLNLPYPK (K)
1457.6939	1457.7456	1PO4	1	13	1	(-) MRIVGSSMLAALK (R)
1596.1348	1595.8263		170	183	0	(K) DQGCVFTGDTLLIR (G)
1596.1348	1595.8479		70	84	0	(K) NGQAVIIDPVLEQAK (R)
1722.9219	1722.7999	2PO4	118	133	1	(R) KLTGCQSVIAAASGAK (A)
2120.6992	2120.3223	1Cys-am	170	187	1	(K) DQGCVFTGDTLLIRGCGR (T)
2214.2768	2214.3292	2Cys-am	170	187	1	(K) DQGCVFTGDTLLIRGCGR (T)

**Table 2:** Result of Sequence Analysis of Spot 2 by Database Search. Database search identified the protein as  $\beta$ -Lactamase (EC3.5.2.6).

gave 10 matched peptide fragments. The matched fragment amino acid sequence for spot 2 is also illustrated in Fig. 6. The result suggested that spot 2 was identified as  $\beta$ -lactamase.  $\beta$ -Lactamase (EC3.5.2.6) is known as the hydrolase which makes anti-bacterial characteristic lose by cleaving the  $\beta$ -lactam ring of the lactam medicine like penicillin. Currently,  $\beta$ -lactamase is produced by a lot of kinds of bacilli.

In conclusion, I present here an analytical method for use with *B. mori* humoral lectin related proteins. The method comprising amino acid sequencing, MALDI-TOF MS is useful for the molecular study of spot 1 and 2 as described above. Spot 1 was identified as probably GDP-mannose 4,6-dehydratase (EC4.2.1.47) and spot 2 was identified as  $\beta$ -lactamase (EC3.5.2.6).

pI of Protein: 6.5  
 Protein MW: 31402  
 Amino Acid Composition: A18 C6 D18 E16 F13 G20 H8 I17 K16 L26 M7 N13 P15 Q10 R16 S18

1	11	21	31	41	51	61	71
NRITGSSNLA	ALRLSTNQF	PTLVSHIRYL	SSPGTMSLPE	QPFSPDFFF	QLFDGESST	YSTLLADLKW	QQAVIIDPVL
81	91	101	111	121	131	141	151
EQAKRDAQLV	EDLGRRLYA	INTHHADHI	TGSGWLRLY	GCQSVIAAAS	GAADRLNE	GDRIDFGTHV	IDALATPGHT
161	171	181	191	201	211	221	231
NGCHTYVID	QGCVPFGDIL	LIRGCGRTDF	QEGCPRLYE	NVHSEIFILP	HRFRIYPAED	YCGQHSVW	RRRYNPLTI
241	251	261	271				
KDIEEFVKIK	RHLNLPYFKK	IDASLPANRE	CGVYDIPKE				

The matched peptides cover 33% (93/279 AA's) of the protein.

Fig. 6: Matching Amino Acid Sequence of Protein from Spot 2. The matched peptides cover 33% of the protein

However, the reason why these enzymes exist in the silkworm's hemolymph is still unknown, and to elucidate it is a problem for the future.

### SUMMARY

Two spots of 2-D PAGE on Fraction II obtained by gel filtration from the larval hemolymph of the *Bombyx mori* were excised from the gels and subjected to in-gel tryptic digestion, MALDI-TOF MS analysis, and a SWISS-PROT protein database search. The result suggested that spot 1 obtained by 2-D PAGE was identified as probably GDP-mannose 4,6-dehydratase (EC4.2.1.47), which is described as guanoshin 5'-diphosphoric acid-D-mannose dehydration enzyme. On the other hand, spot 2 obtained by 2-D PAGE was identified as  $\beta$ -lactamase (EC3.5.2.6) which is known as a hydrolase to lose the anti-bacillus by rending the  $\beta$ -lactam ring. However, the reason why these enzymes exist in the silkworm's hemolymph is still unknown, and to elucidate it will be a problem for future study.

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