[Full Paper]

Proteome analysis of silkworm 2. Hemolymph

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SUMMARY

Proteomic analysis of silkworm hemolymph at the fifth-instar day-3 larva stage was performed by combination of two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Prominent spots on 2D-gel were analyzed after trypsinization and proteins were identified by Mascot and Sequest using the amino acid sequence data obtained from the genome sequence of silkworm. The gel images were tracked from the fourth-instar day-1 larva stage to the stage before adult moth for twenty-one days. The results of mass spectrometry analysis and changes in gel images are summarized in the silkworm proteome database (http://kaiko2ddb.dna.affrc.go.jp/cgi-bin/search_2DDB.cgi).

Key words: hemolymph, mass spectrometry, proteome, silkworm (*Bombyx mori* L.), two-dimensional polyacrylamide gel electrophoresis.

INTRODUCTION

Silkworms (*Bombyx mori* L.) have been cultivated for centuries for their silk, and their protein synthesis ability has been used in the textile industry for traditional silk production. Recently, silkworms have also been used for the production of novel bioactive substances, particularly interleukins¹. As these substances usually accumulate in the hemolymph, it is important to understand the details of hemolymph proteins because useful substances must be purified from the crude hemolymph proteins. Additionally, peptidases and other modifying enzymes are considered to exist in the hemolymph, possibly affecting the quality of useful substances in industrial protein production by silkworms.

Hemolymph is very important for the metamorphosis of insects including silkworm. In other insect species, proteomic analysis of hemolymph was attempted in fruit fly (*Drosophila melanogaster*)², mosquito (*Anopheles gambiae*)³,

and cattle tick (*Boophilus microplus*)⁴. In classical experiments, silkworms have been laced with string to stop the flow of hemolymph through the body⁵: the posterior part of the body did not metamorphose into a pupa even though the anterior part had already become an adult moth. This was mainly due to the effect of hormones carried by hemolymph. Hemolymph also transports several metabolites. Vitellogenin, one of the main storage proteins in yolk, is considered to be synthesized in fat bodies and to travel to the eggs in the ovaries *via* hemolymph⁶.

Analyses of hemolymph at the fifth-instar day-3 larva stage were performed and the results compared with gel images from the fourth-instar day-1 larva stage to the stage before adult moth on the web. Two prominent electrophoresis profiles during metamorphosis are described in this article. We discuss the protein profiles based on the proteins identified by mass spectrometry (MS) and changes in two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) patterns of hemolymph.

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MALDI-TOF-MS.

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Table 1. Protein profile of silkworm hemolymph at the fifth-instar day 3 larva. Protein identification against 14,623 open reading frames which were found by the genome analysis of silkworm. Marked (*) ones were not showed significant score in the Mascot identification (scores <54).

Number	Accession number**	Mascot score	Identified protein***
7	BGIBMGA000650	56	Hypothetical protein
9	BGIBMGA007893	65	Regulator of chromosome condensation, RCC1, β -lactamase-inhibitor protein II, Galactose-binding like, Cytochrome c region
10	BGIBMGA009692*	49	Pyridoxal-dependent decarboxylase
12	BGIBMGA007193	88	Dcp2, box A, NUDIX hydrolase
14	BGIBMGA001684	57	Zinc finger, C_2H_2 -type
17	BGIBMGA000061	58	Ribosomal protein L35
22	BGIBMGA003151	83	DNA topoisomerase I
23	BGIBMGA014288*	52	Hypothetical protein
30	BGIBMGA006919	56	Ribosomal protein L16
34	BGIBMGA000650	60	Hypothetical protein
39	BGIBMGA008331*	46	Hypothetical protein
40	BGIBMGA006463	54	RNA-binding region RNP-1
41	BGIBMGA012665*	49	F-actin capping protein, α subunit
42	BGIBMGA006463	66	RNA-binding region RNP-1
43	BGIBMGA001983	54	Proteinase inhibitor I4, serpin
49	BGIBMGA008170	55	Calponin-like
50	BGIBMGA009666	57	Hypothetical protein
51	BGIBMGA006456	64	Carboxylesterase, type B
54	BGIBMGA006456*	47	Carboxylesterase, type B
55	BGIBMGA003602*	45	RNA-binding region RNP-1, SWAP/Surp
57	BGIBMGA002985	54	Esterase, SGNH hydrolase-type
58	BGIBMGA010274	72	Hypothetical protein
62	BGIBMGA006463	65	RNA-binding region RNP-1
63	BGIBMGA007202	55	Pleckstrin-like, Ras guanine nucleotide exchange factor
69	BGIBMGA003151	66	DNA topoisomerase I
72	BGIBMGA00131*	41	Hypothetical protein
75	BGIBMGA009168*	43	Hypothetical protein
76	BGIBMGA005993*	44	Leucine-rich repeat
77	BGIBMGA009692*	47	Pyridoxal-dependent decarboxylase
88	BGIBMGA009238*	48	Hypothetical protein
91	BGIBMGA009777*	41	Hypothetical protein
94	BGIBMGA008760*	48	Zinc finger, C_2H_2 -type
95	BGIBMGA001066	56	Proteinase inhibitor I4, serpin
97	BGIBMGA009500*	50	UBA-like, Ubiquitin-associated
99	BGIBMGA008742*	49	Zinc finger, C_2H_2 -type
100	BGIBMGA011236*	53	Hypothetical protein
101	BGIBMGA001983	494	Proteinase inhibitor I4, serpin
103	BGIBMGA013693*	43	Class I peptide chain release factor
104	BGIBMGA001983	55	Proteinase inhibitor I4, serpin
105	BGIBMGA003518*	45	Nucleoside diphosphate kinase
106	BGIBMGA010568*	52	Ankyrin
108	BGIBMGA013192*	41	Short-chain dehydrogenase/reductase SDR, Glucose/ribitol dehydrogenase
114	BGIBMGA001351	111	Aldo/keto reductase
115	BGIBMGA003688	148	Hypothetical protein
122	BGIBMGA001222*	42	Protein of unknown function DUF812
127	BGIBMGA005684*	45	Ribosomal L38e protein
130	BGIBMGA011549*	52	Haemolymph juvenile hormone binding, Odorant binding protein
131	BGIBMGA010126*	43	Hypothetical protein
132	BGIBMGA004396	187	<i>Lepidopteran</i> low molecular weight lipoprotein
134	BGIBMGA004400	349	<i>Lepidopteran</i> low molecular weight lipoprotein
136	BGIBMGA004396	69	<i>Lepidopteran</i> low molecular weight lipoprotein
140	BGIBMGA011014*	41	Sin3 associated polypeptide p18
141	BGIBMGA004464	51	<i>Lepidopteran</i> low molecular weight lipoprotein
143	BGIBMGA008283*	49	Ca-dependent membrane targeting, Phosphatidylinositol-specific phospholipase C
150	BGIBMGA004394	209	<i>Lepidopteran</i> low molecular weight lipoprotein
152	BGIBMGA005928	55	Ribosomal protein L29
155	BGIBMGA011396*	47	Hypothetical protein
156	BGIBMGA012015	61	Hypothetical protein
159	BGIBMGA005363*	16	Hypothetical protein
160	BGIBMGA004398	193	<i>Lepidopteran</i> low molecular weight lipoprotein
169	BGIBMGA011620*	22	Ribosomal protein L18e
196	BGIBMGA000061*	41	Ribosomal protein L35
227	BGIBMGA000465*	49	Hypothetical protein
232	BGIBMGA004356*	32	Ribosomal protein S7E
233	BGIBMGA004356*	32	Ribosomal protein S7E
241	BGIBMGA008832*	44	Zinc finger, CCHC-type, Reverse transcriptase, RNA-dependent DNA polymerase
249	BGIBMGA000061*	31	Ribosomal protein L35

** Accession numbers on this table were correspond to the accession numbers in silkDB (<http://silkworm.genomics.org.cn/>).

*** In the case of more than two kinds of identified proteins were described, the gene encoding the analyzed spot had the partial homology against those proteins.

MATERIALS AND METHODS

Silkworms (*Bombyx mori* L., p50) were nurtured on mulberry leaves at 26°C, and the hemolymph of females from the fourth-instar day-1 larva stage to the stage before adult moth was used. Chemicals and details of the experimental procedure were described previously⁷. Hemolymph from fourth-instar day-1 (after about three weeks since oviposition) larva to the stage before adult moth was collected by cutting the posterior part of silkworm bodies⁸) and immediately mixing with an equal volume of lysis buffer. The mixtures were stored at -80°C until use.

RESULTS AND DISCUSSION

General comparison of gel images from fourth-instar day-1 larva stage to the stage before adult moth

Electrophoresis patterns at the fifth-instar day-3 larva stage were tracked from those of the fourth-instar day-1 larva stage (Fig. 1 and on the web). At the beginning of the fourth instar, there were fairly small spots on the gels. However, several of the spots grew bigger each day and started accumulating in the hemolymph. Based on the 2D-PAGE profiles, spot 132 (arrowed in Fig. 1 and Fig. 2) at the fourth-instar day-1 larva stage was 4.5% in spot volume against all of the spots in gel detected by image analysis. At the day-3 pupa stage, it reached a maximum spot volume of 23.9%. Accumulation of the protein significantly increased after the fifth-instar day-3 larva stage (Fig. 2). After the day-3 pupa stage, the volume of the spot gradually decreased. Although these values are from rough estimations on Coomassie stained gels, the accumulation of the protein is one of the distinguishable features of hemolymph proteome from others.

Protein identification and expression

After translation, unnecessary mRNAs are considered to decay by a NUDIX protein (12) in the hemolymph¹³) (Fig. 2). This protein was also found in the Malpighian tubes, an organ similar to the human kidney. The distribution of this protein in the hemolymph and the Malpighian tubes is considered to support the function of this protein. From the viewpoint of RNA binding, five RNA-binding proteins (40, 42, 55, 62, and 114) were found in both the hemolymph and the Malpighian tubes. Though RNA-related protein was also observed in fat bodies, the number of spots was limited. The expression of many RNA-related proteins in the hemolymph is one of the unique features in the silkworm hemolymph proteome. The presence of RNA-binding proteins in the hemolymph and Malpighian tubes suggests that they have a specific function in the hemolymph. Although there is no evidence of their function, one possible role in the hemolymph is protection against RNA viruses, since all harmful viruses for silk-

Fig. 1. Accumulation of proteins from the fourth-instar day1 larva stage to day-3 pupa stage.

(A) Gel image at the fourth-instar day-1 larva stage (IV1). (B) Gel image at the fifth-instar day-3 larva (V3). (C) Gel image at the day-3 pupa stage (P3). The spots indicated by the arrows were the example of newly appeared in hemolymph during the larva stage and pupa stage. All of the images including other developmental stages are in the silkworm proteome database (http://kaiko2ddb.dna.affrc.go.jp/cgi-bin/search_2DDB.cgi).

Fig. 2. 2D-PAGE profile of silkworm hemolymph at the fifth-instar day-3 larva stage (V3). Major spots observed on gel were analyzed by ion-trap MS.

worms are RNA viruses. In fact, it is well known that RNA interference is difficult in *Lepidopteran* including silkworms, and although the reason for this is not clear, it has been speculated that some mechanism rapidly inactivates foreign RNA in the silkworm body.

Several proteinase-related proteins were observed in the hemolymph. Serpins (43, 95, 101, and 104) are considered to be proteinase inhibitors. Class I peptide chain release factor (103) functioned to terminate protein synthesis⁹⁾ with the help of ribosomal proteins (17, 30, 127, 152, 169, 196, 232, 233, and 249).

Hemolymph juvenile hormone binding protein (130) binds to juvenile hormone to protect against non-specific hydrolysis by esterase¹⁰⁾. In fact, there were several types of esterase (51, 54, and 57) in the hemolymph. In our studies, proteinase was not found in the hemolymph, but several proteinases were observed in other tissues. Therefore, juvenile hormone could be distributed by hemolymph juvenile hormone binding protein without protein degradation in hemolymph.

A ubiquitin-like protein (97) was observed in hemolymph. One major function of ubiquitin is protein degradation by poly-ubiquitination¹¹⁾, but it is also known to have several other functions, such as cellular regulation. Although we have not yet determined the function of spot 97, one possible function of the ubiquitin-like protein might be protein quality control by circulating through the whole body.

Additionally, there are unique proteins in moth families. *Lepidopteran* low molecular weight lipoproteins (134, 141, 150, 160*, 132, and 136) were observed in every moth, but the asterisked protein was unique in the hemolymph. The function of these lipoproteins is not known, but one possi-

bility is as a carrier of lipids or for short-term storage of lipids because an abundant amount was observed in the midgut and fat bodies (see silkworm proteome database¹²⁾).

Several zinc finger proteins were found in the hemolymph (14*, 94*, 99*, and 241). Additionally, there were several hypothetical proteins (7, 23*, 30, 34, 39*, 50*, 58*, 72*, 75, 88, 91*, 100, 115*, 122*, 131*, 255*, 156, and 227*). These proteins still had no annotations. Understanding these hypothetical proteins and zinc finger proteins would clarify the nature of the hemolymph.

Previously, the gradients of protein distribution in the silkworm hemolymph were reported using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS¹³⁾. Hemolymph obtained from each sliced frozen silkworm body between the anterior and posterior regions was directly analyzed by MALDI-TOF-MS, then all of the peak intensities were compared between them. As a result, proteins in hemolymph were not distributed from the anterior to the posterior in equal concentrations. The concentrations of several proteins in the anterior region were higher than those in the posterior region, and *vice versa*. In that analysis, it was not possible to identify the proteins by MALDI-TOF-MS. Though we analyzed major proteins in hemolymph using the ion-trap MS system in this study, the distribution of each identified protein from the anterior to posterior region was not known because all of the hemolymph was collected for 2D-PAGE analysis. If hemolymph was collected from the anterior to posterior region by dividing the silkworm body into several sections, the change of spot volume could be observed by 2D-PAGE profile. Further analysis of this kind would contribute to understanding the classical lacing experiment which affects metamorphosis.

We believe that the information in the silkworm proteome database (http://kaiko2ddb.dna.affrc.go.jp/cgi-in/search_2DDB.cgi)¹²⁾ will be helpful for post-genome analysis. The results of further investigations will be added on the web.

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