Proteomic study of lipopolysaccharide-induced immune response in hemolymph of the silkworm, *Bombyx mori*

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Lipopolysaccharide of *Escherichia coli* was injected into the 5th instar larvae to identify the proteins differentially expressed upon an immune challenge in the silkworm, *Bombyx mori*. Comparison of hemolymph proteins before and after the treatment was performed by using two-dimensional gel electrophoresis (2-DE) followed by peptide mass fingerprinting analysis. In the total of about 250 proteins resolved, 30 proteins were found to be differentially expressed upon the lipopolysaccharide challenge. Among of them, many storage proteins such as the 30 kDa lipoprotein, sex-specific storage-protein, and a vasa-like BmVLG were included.

Key words: *Bombyx mor*i, Hemolymph, Immune response, Lipopolysaccharide, Proteomics.

INTRODUCTION

Recently, we have examined several lipopolysaccharide (LPS)-inducible polypeptides in silkworm tissues such as the midgut, fat body, and hemolymph. Five polypeptides including the attacin were significantly induced in fat body and hemolymph (WANG et al., 2005). However, the extensive changes of overall proteins are not reported except the newly induced polypeptides by the injection of LPS in that study. Therefore, the primary aim of the present study was to characterize further the whole protein change in the hemolymph proteome by using the high resolution two-dimensional gel electrophoresis (2-DE) followed by peptide mass fingerprinting analysis. To this goal, we have injected *E. coli* LPS into silkworm larvae, and compared changes in polypeptides.

MATERIALS AND METHODS

We used silkworm larvae (p50 strain) that are maintained and reared on mulberry leaves at the Institute of Genetic Resources of Kyushu University (Fukuoka, Japan). At day 3 of the 5th instar, the larvae were injected by each of 200 µl LPS (1 mg/ml, *E. coli* serotype O55:B5, Sigma) solution in 50 mM phosphate buffer saline (pH 7). Hemolymph samples (about 0.4 ml from each larva) were collected from the larvae immediately (0 h) and at given times (24 h and 48 h) after the injection. The samples were homogenized in an extract buffer as described previously (WANG et al., 2005). Subsequently, lysates were sonicated in ice-cold bath several times for 20 sec (Elma Ultrasonic, Germany), and centrifuged at 15, 000×g for 15 min. The protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

The basic step for 2-DE was according to the method described previously (ZHANG et al., 2006). Image analysis was carried out using the PDQuest software (version 7.4.0, Bio-Rad). Analyses were performed using the quantitative and qualitative modes. Spot intensities were normalized using the PD-Quest software according to the protocols provided by Bio-Rad. To take into account experimental variations, 2-D gels were normalized by dividing each value spot volume data by the total volume of all the matched spots in the 2-D gel image to obtain a normalized spot volume value. The basic step for MALDI-TOF MS and peptide mass fingerprinting was according to the method described previously (ZHANG et al., 2006). Protein identification was performed by searching NCBInr.2005.01.06 and SWISS-PROT.2005.01.06 databases using MS-Fit (http://prospector.ucsf.edu/) in the ProteinProspector v.4.05 package (Protein Prospector, San Francisco, CA, USA).

RESULTS AND DISCUSSION

Samples before and after the challenge with LPS were analyzed in triplicate to confirm overall reproducibility of the protein spot patterns, loading 100 µg of proteins for all high-resolution (18 cm) 2-D gels (Fig. 1). The broad range IPG 3–10 gave a broad overview of hemolymph 2-DE pattern and the majority of hemolymph proteins was present in the p*I* range of 5–8 and in the molecular mass (*M*r) range of 25–100 kDa. Thirty proteins of about 250 proteins detected in this system were up- and/or down-regulated by at least two-fold change on expression level after the injection of LPS (Table 1). Of these, 13 proteins were up- regulated at 24h after the injection of LPS, while 4 proteins were down-regulated. With the time-dependent immune response, 8 proteins were up- regulated, while 11 proteins were down- regulated at 48h. The identification of these proteins was done by MALDI-TOF MS analysis and mass fingerprinting. Among the identified proteins, storage proteins such as 30 kDa lipoproteins (spots 3, 4,

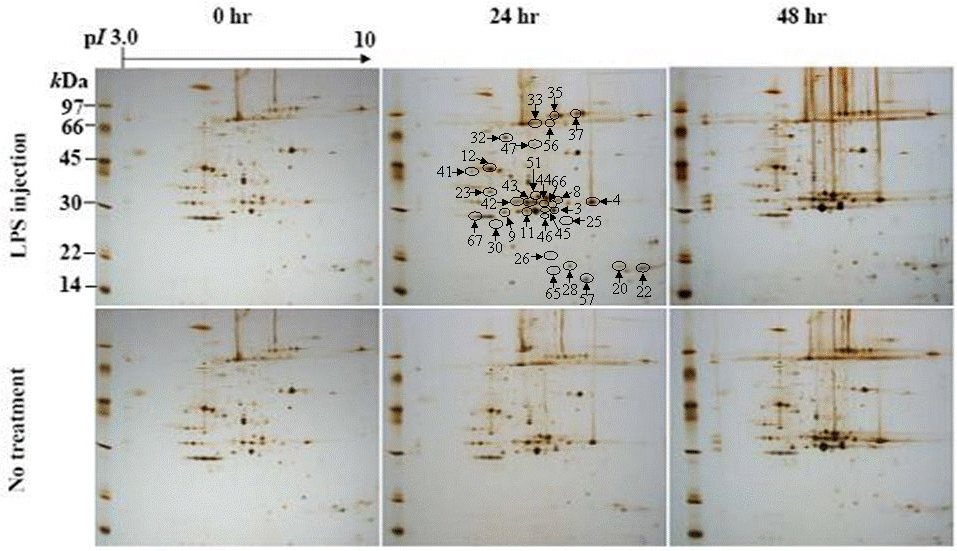

Fig. 1. 2-D map of silver stained proteins from hemolymph of the fifth instar silkworm larvae at 0 h, 24 h, and 48 h before and after challenge with LPS. Spots sampled at 24 h are numbered with 30 proteins that were up- and / or down-regulated by at least two-fold after the injection of LPS.

Table 1. List of up and down- regulated proteins in the hemolymph upon LPS

Spot no.	Protein name	Swiss-Prot/NCBI no.	kDa	p*I*	SC (%)	PM	0 h T/C	24 h T/C	48 h T/C
3	Low molecular mass 30 kDa lipoprotein	Q00801/266439	30.2	6.3	31.2	9	1.0	0.9	0.5↓
4	Low molecular 30 kDa lipoprotein	P09336/126417	29.2	6.9	17.0	9	1.0	0.5↓	0.8
8	Low molecular mass 30 kDa lipoprotein	Q00802/266438	30.9	7.3	44.3	5	1.0	2.4↑	1.1
9	Low molecular 30 kDa lipoprotein	P09336/126417	29.2	6.9	50.2	11	1.0	1.1	0.3↓
11	Low molecular 30 kDa lipoprotein 6G1	P09334/126415	31.5	6.4	30.5	6	1.0	2.0↑	1.3
12	Chymotrypsin inhibitor CI-8A	Q967V9/14028769	43.9	5.1	34.9	10	1.0	0.8	0.4↓
20	Late embryogenesis abundant protein 1	O49816/24418488	19.1	8.6	36.9	7	1.0	0.6	0.5↓
22	60S ribosomal protein L13	P41123/730529	24.2	11.5	26.6	6	1.0	2.6↑	75.0↑
23	Chymotrypsin inhibitor CI-8A	Q967V9/14028769	43.9	5.1	27.6	8	1.0	0.7	0.1↓
25	Pyruvate kinase	Q90WS6/15558810	23.5	7.0	40.9	9	1.0	0.5↓	0.7
26	Choline dehydrogenase	P54223/7404339	61.3	7.7	27.2	6	1.0	3.0↑	1.1
28	Hemoglobin beta chain	P18985/122577	15.9	6.9	41.0	6	1.0	2.2↑	1.5
30	Probable nucleolar GTP-binding protein	O44411/17367988	78.5	9.1	57.7	8	1.0	0.1↓	2.7↑
32	Stress-induced phosphoprotein	O35814/54036435	62.6	6.4	46.7	9	1.0	3.9↑	1.9
33	Sex-specific storage protein 2	P20613/1174445	83.5	6.0	40.0	5	1.0	8.3↑	0.6
35	BmVLG	O01378/74891742	68.9	6.3	24.3	12	1.0	1.7	0.5↓
37	Transferrin	O97158/74961091	74.8	6.7	14.7	7	1.0	2.0↑	1.1
41	Glycerol kinase	O66131/6016137	54.8	5.4	44.3	8	1.0	5.0↑	4.1↑
42	Low molecular 30 kDa lipoprotein	P09335/126416	30.0	6.8	36.3	9	1.0	1.3	0.5↓
43	Low molecular 30 kDa lipoprotein	P09335/126416	30.0	6.8	31.3	7	1.0	0.8	0.3↓
44	Zinc-alpha-2-glycoprotein	P25311/141596	33.9	5.6	31.7	9	1.0	1.2	0.5↓
45	Ubiquitin-conjugating enzyme	Q9Y385/52000881	35.2	6.3	24.0	5	1.0	0.9	5.6↑
46	Proteasome subunit alpha type 4	P18053/12643270	29.4	6.8	52.0	8	1.0	1.0	0.5↓
47	Eukaryotic translation initiation factor	P23116/6686292	161.9	6.4	23.0	7	1.0	2.1↑	1.8
51	6-phosphofructokinase	Q8ZJL6/21362630	35.4	6.0	24.0	5	1.0	2.0↑	3.5↑
56	Prophenoloxidase-1	Q9GU89/74824564	79.0	6.3	13.7	8	1.0	0.4↓	0.9
57	60S ribosomal protein L10	O61231/6093992	25.5	9.9	45.6	8	1.0	19.4↑	3.5↑
65	RNA replicase polyprotein	P35928/548563	193.9	8.2	47.2	6	1.0	22.2↑	2.1↑
66	Low molecular mass 30 kDa lipoprotein	Q00801/266439	30.2	6.3	20.8	6	1.0	0.8	0.04
67	Phosphoglyceromutase	Q8PST3/27151521	27.5	6.1	34.8	7	1.0	1.2	2.1↑

NOTE: T/C: the average ratio of the normalized spot volumes in challenged (T) and naive larvae (C) is presented; SC: percent of sequence coverage; PM: peptides matched; ↑: up-regulated proteins; ↓: down-regulated proteins.

8, 9, 11, 42, 43, and 66), sex-specific storage protein (spot 33), and a vasa-like BmVLG (spot 35) were included. The 30-kDa lipoproteins are a group of structurally related proteins, which are the most abundant plasma proteins of larvae and accumulate in a stage-dependent fashion in the larval hemolymph. Most of 30kDa lipoproteins were observed down- regulated, the 21G1 (spot 66) was totally suppressed to be undetected. However, two low molecular weight 30-kDa lipoprotein species 6G1 (spot 11) and 19G1 (spot 8),

increased in their relative abundances at 24 h upon the LPS injection. As similar up-regulated storage proteins are also seen in *Drosophila* larval hemolymph proteins upon LPS stimulation (Vierstraete et al., 2005), these two proteins might have an important role in defense against LPS infection besides fungal infection (Ujita et al., 2005).

On the other hand, sex-specific storage-protein 2 (spot 33) was observed to be 8.3-fold up- regulated at 24 h upon the LPS infection. This result is in agreement with the up-regulative induction of *Drosophila* larval serum protein 2 (LSP2) at 6 h and 24 h after LPS challenge (Guedes, et al., 2005). Despite there is species difference and different time point sampled, the highly up-regulative induction of silkworm SP2 indicates that it might act in the immune response induced by LPS. In addition, some protein metabolism-related proteins were also up- regulated, for example, the 60S ribosomal protein L13 (spot 22), L10 (spot 57), choline dehydrogenase (spot 26), ubiquitin-conjugating enzyme (spot 45). With respect to the saccharide metabolism, glycerol kinase (spot 41) and 6-phosphofructokinase (spot 51) that directly implicated in the Krebs cycle were found to be up- regulated, suggesting that the two enzymes may act in defense upon the LPS challenge.

In summary, the results of this study showed that extensive changes occurred in the hemolymph after the treatment of LPS. To know the mechanism of these changes, we need more detail information of genomics and proteomics.

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