Silkworm larvae at the 5th instar were injected with lipopolysaccharide from *Escherichia coli* and inducible polypeptides were examined within a pH range of 3–10 and a size range of 14–97 kDa by proteomics, including peptide mass fingerprinting. No polypeptides were induced in the midgut. FB1 and H1–4 polypeptides were significantly induced in fat body and hemolymph, respectively. FB1 and H1 were estimated to be antitrypsin and serpin-2 proteinase inhibitors respectively. H2 and H3 were novel polypeptides. H4 was estimated to be attacin antibacterial polypeptide with high coverage of sequence. The amounts of all the induced polypeptides decreased at 48 h after the injection.

**Key words:** proteomics; silkworm; peptide mass fingerprinting; lipopolysaccharide; attacin

Insects have specific defense systems to protect themselves from microbial invasion. Infection by bacteria induces a variety of antibacterial polypeptides and triggers proteinaceous interactions. Attacin, cecropin, lebocin, and moricin are antibacterial polypeptides induced in the silkworm, *Bombyx mori.* These polypeptides are induced when the silkworm larvae are injected with lipopolysaccharide (LPS) from *Escherichia coli.* In comparison with knowledge from genomic studies of antibacterial polypeptides, however, we still know little about the extensive changes in inducible polypeptides upon bacterial infection. To identify diverse polypeptides rapidly and to monitor changes in them, the proteomics by peptide mass fingerprinting (PMF) method using two-dimensional gel electrophoresis (2-DE), in-gel tryptic digestion, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), and database searching is one of the most powerful tools. Proteomic studies are also important to avoid over-looking unexpected changes due to researcher's *a priori* limitations. Traditional studies of antibacterial responses have tended to focus on the induction and transport of a specific polypeptide. In this study, we injected *E. coli* LPS into silkworm larvae and examined the changes in polypeptides in the hemolymph, fat body, and three portions of the midgut comprehensively by the PMF method. Over pH and molecular mass ranges between pH 3 and 10 and between 14 and 97 kDa, respectively, we compared changes in polypeptides under a challenged condition with those under a naive one. As described below, we found insignificant changes in the midgut and significant changes in the hemolymph and fat body.

The silkworms used were *B. mori* larvae (p50 strain) maintained and reared on mulberry leaves at the Institute of Genetic Resources of Kyushu University (Fukuoka, Japan). The LPS from *E. coli* serotype O55:B5 was purchased from Sigma (Tokyo, Japan) and injected into at least 10 female larvae at day 3 of the 5th instar. 20 μl each of 50 mM phosphate buffer saline (pH 7) containing 20 μg of LPS was injected. Hemolymph and tissues were sampled from the larvae immediately (0 h) and at given times (24 h and 48 h) after injection. All procedures to prepare samples were done at 4 °C. The hemolymph (about 40 μl from each larva) was collected with microcapillaries onto solid 1-phenyl-2-thiourea and hemocyte was removed by centrifugation at 2,000 × g for 5 min. Fat body and the anterior, middle, and posterior midguts were dissected from the larvae in 0.13 M NaCl and blotted on filter paper. All samples thus prepared were frozen with liquid N2 and stored at −80 °C until extraction of polypeptides. Buffer A was composed of 8 mM urea, 2 mM thiourea, 4% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 0.03 mM dithioerythritol, and 2% (v/v) pH 3–10 Pharmalyte (Amersham Biosciences). The frozen samples were thawed and suspended in buffer A which contained 20 mM Tris, 3 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 50–100 μg/ml of protease inhibitors.

Received February 16, 2004; Accepted June 24, 2004

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**Abbreviations:** LPS, lipopolysaccharide; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; PMF, peptide mass fingerprinting; 2-DE, two-dimensional gel electrophoresis

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fluoride, 0.5 mM pepstatin, 0.5 mM leupeptin, 0.02 mM chymostatin, and 0.05 mM diisopropyl fluorophosphate. The suspension was sonicated several times for 20 s each time and centrifuged at 15,000 × g for 15 min. The supernatant was used as a polypeptide extract. The extract, containing 0.15 mg of polypeptides, was frozen and stored at −80 °C until 2-DE. Isoelectric focusing (IEF) was followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The 2DE was done essentially by the method of Goerg et al.8 The extract was thawed with buffer A to 0.35 ml and loaded onto an IPG strip (18 cm, pH 3–10; Amersham). Then IEF was done with an IPGphor system (Amersham) at 20 °C and with the following programs: 12 h at 30 V, 0.5 h at 100 V, 0.5 h at 200 V, 0.5 h at 500 V, 0.5 h at 1,000 V, 0.5 h at 2,000 V, 0.5 h at 4,000 V, 1 h at 6,000 V, and 10 h at 8,000 V. SDS-PAGE was done with 15% separating gel with 240 × 240 × 1 mm slab gel. Polypeptides were visualized using a SilverQuest Silver Staining Kit (Invitrogen). Detection, matching, and quantitation of polypeptide spots based on staining were done using PDQuest software (BioRad). The pIs and molecular masses of polypeptides in the gels were also determined with this software. The 2D-Protein pI Marker (pl 4–7) (Daiichi) and the Low Molecular Weight SDS Calibration Kit (97–14 kDa) (Amersham) were used as internal standards. Stained spots of polypeptides were excised from each gel and applied to in-gel tryptic digestion by the method of Shevchenko et al.,9 with minor modifications. Overnight digestion was performed at 37 °C with a solution containing 0.1 μg trypsin (50 mM ammonium bicarbonate). MALDI-TOF MS was performed with the Voyager-DE PRO Biospectrometry Workstation MALDI-TOF (Applied Biosystems), operated in positive-ion linear mode. Measurements were calibrated externally with the Sequazyme Peptide Mass Standard Kit (Applied Biosystems) and internally with peptides from the autoproteolysis of trypsin. Peaks detected by mass spectrometry were subjected to a SWISS-PROT database search with MS-Fit software (http://prospector.ucsf.edu/).

To examine the effects of LPS injection on polypeptides in the midgut, nine naive-challenged pairs of 2-DE gels for the anterior, middle, and posterior midguts after three different times were prepared and inspected. Although the size and charge distributions of polypeptides in the three portions of the midgut differed, an insignificant difference in 2-DE patterns between the naive and challenged conditions was detected (data not shown). Hence we concluded that injection of LPS through a cuticle does not induce any drastic change in polypeptides in a digestive organ. For both the extracts from fat body and the hemolymph, three naive-challenged pairs each of 2-DE gels were prepared and analyzed. We found that five polypeptides are induced within 24 h after injection of LPS: FB1 in the fat body, and H1, H2, H3, and H4 in the hemolymph (Fig. 1). As shown in Table 1, FB1 was slightly acidic and had a

![Fig. 1.](image-url)
Table 1. LPS-Induced Polypeptides in Fat Body and Hemolymph

<table>
<thead>
<tr>
<th>Source</th>
<th>Name</th>
<th>Mass (kDa)</th>
<th>pI</th>
<th>Relative amounts a (PPM)</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat body</td>
<td>FB1</td>
<td>15.0</td>
<td>6.1</td>
<td>n.d.</td>
<td>629</td>
</tr>
<tr>
<td></td>
<td>H1</td>
<td>21.8</td>
<td>6.1</td>
<td>n.d.</td>
<td>6760</td>
</tr>
<tr>
<td></td>
<td>H2</td>
<td>15.1</td>
<td>6.1</td>
<td>n.d.</td>
<td>7120</td>
</tr>
<tr>
<td>Hemolymph</td>
<td>H3</td>
<td>22.3</td>
<td>9.5</td>
<td>n.d.</td>
<td>6580</td>
</tr>
<tr>
<td></td>
<td>H4</td>
<td>22.5</td>
<td>9.6</td>
<td>n.d.</td>
<td>13300</td>
</tr>
</tbody>
</table>

a, Evaluated with PDQuest software (BioRad). n.d., not detected.

Amino acid sequence of silkworm (*Bombyx mori*) hemolymph antitrypsin10) and serpin-211) respectively. Both are proteinase inhibitors. Since the values of sequence coverage were relatively small in both cases, however, we cannot exclude the possibility that FB1 and H1 are novel polypeptides. H2 and H3 were identified as unknowns by database search and suggested to be novel polypeptides induced by injection of LPS. On the other hand, H4 was identified with *B. mori* attacin. It showed good sequence coverage (Table 1). Attacin is an immune polypeptide active against Gram-negative bacteria.12) LPS was confirmed to induce attacin in hemolymph. Using the PMF method, we observed LPS-induced changes in polypeptides in the midgut, fat body, and hemolymph, covering extensive ranges of charge and mass. We also found that the time-dependent changes in amounts of the induced polypeptides are similar. Very recently, the significance of the proteomic approach for the analysis of polypeptides induced with LPS in hemolymph of the fruit fly *Drosophila melanogaster* has been reported.13) Although there might be a difference between dipteran and lepidopteran insects, proteomic knowledge of insect innate immunity is still very limited in comparison with genomic knowledge of it. Further proteomic work is in progress in our laboratory.

Acknowledgments

We are grateful to Dr. Ningjia He (Southwest Agricultural University, Chongging, China) for helpful suggestions as to the LPS injection experiment. This work is supported by the National Bio-Resources Project (RR2002) of the Ministry of Education, Science, and Culture of Japan.

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