Fractionation of $\beta$-Lactamase from *Bombyx mori*

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INTRODUCTION

To date, I have researched lectin related proteins in the hemolymph of the domesticated silkworm, *Bombyx mori*, in relation to the activation of the *B. mori* humoral lectin (Kato, 2006; Kato and Takeuchi, 2006). In the previous work (Kato, 2007), two spots obtained by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) on lectin related proteins from the larval hemolymph of the *B. mori* were excised from the gels and subjected to in-gel tryptic digestion, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI–TOF MS) analysis, and a SWISS–PROT protein database search (Madi et al., 2003; Zhang et al., 2005). The results identified one of spots obtained by 2-D PAGE as $\beta$-lactamase (EC3.5.2.6). I was able to obtain these very interesting results taking into consideration the defense mechanism of *B. mori*, because $\beta$-lactamase is known as a hydrolase which reduces anti-bacterial characteristics by cleaving to the $\beta$-lactam ring of the lactam medicine like penicillin (Lee, 1987; Sawai, 1995). Accordingly, I confirmed the existence of $\beta$-lactamase in the hemolymph of *B. mori* by an assay of $\beta$-lactamase activity, and guessed that $\beta$-lactamase should be necessary during the metamorphosis of *B. mori* (Kato, 2008).

The aim of the present study was to fractionate $\beta$-lactamase in the hemolymph and fat body from *B. mori* by means of gel filtration or Mono Q ion chromatography. This investigation provided useful information for the understanding of the defense system of *B. mori* in vivo.

MATERIALS and METHODS

(1) Preparation of samples
A hybrid race, Shunrei × Shougetu, of the domesticated silkworm, *Bombyx mori*, was used. In preparing the samples for this research, hemolymph and fat body were collected. The hemolymph was collected by cutting the pleopods and abdominal legs of the larvae, and by cutting the pupae. After centrifuging them at 2,260 $\times$ g at 4°C for 15 min to remove the hemocytes, the resulting supernatant was lyophilized. The fat body was
collected from the dissected larvae and pupae, washed carefully in a cold 0.7% NaCl solution to remove the hemocytes and homogenized in a glass homogenizer with a Teflon pestle. After centrifuging them at $2,260 \times g$ at 4°C for 15 min, the supernatant was lyophilized.

(2) Gel filtration

Gel filtration was performed using a Superdex 200 column (2.6 × 60 cm, Amersham Pharmacia Biotech Ltd.) equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl, at a constant flow rate of 150 ml/hr. The effluent was collected in 5 ml fractions and measured at 280 nm with a Shimadzu spectrophotometer type UV 1200.

(3) FPLC Mono Q ion exchange chromatography

Ion exchange chromatography was performed using a column of Mono Q HR 5/50 GL (5 × 50 mm, Amersham Pharmacia Biotech Ltd.) previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). Elution was performed with a linear gradient of 0 to 0.5 M NaCl in 0.05 M Tris-HCl buffer (pH 8.0) and the flow rate was 1.0 ml per minute. Column eluates were collected in 1 ml fractions and measured at 280 nm with the Shimadzu spectrophotometer UV-1200.

(4) Assay of $\beta$-lactamase activity

The activity of $\beta$-lactamase was assayed according to the method of Sargent (1968) as well as Sawai and Takahashi (1978), with slight modifications. A reaction mixture was prepared containing enzyme in 2.5 ml of 0.1 M phosphate buffer (pH 7.0). After equilibration at 30°C for 5 min, Penicillin G Potassium Salt (Nakalai Tesque Inc.) in 0.5 ml of the phosphate buffer was added. The reaction was stopped after incubation at 30°C for 10 min, by adding 5 ml of iodine reagent with rapid mixing. Iodine reagent was prepared by adding 5 ml of stock iodine solution to 95 ml of pH 4.0 acetate buffer (80 g of anhydrous sodium acetate adjusted to pH 4.0 with acetic acid and made up to 2 liters with distilled water). Stock iodine solution was prepared by dissolving 20.3 g of iodine and 100 g of KI in 500 ml of distilled water. After leaving at room temperature for 10 min, the mixture was measured for the iodine reagent by monitoring the absorbance at 540 nm with the Shimadzu spectrophotometer type UV 1200.

RESULTS and DISCUSSION

Initially, gel filtration of the larval hemolymph (100 mg of lyophilizate) of Bombyx mori
on day 10 in the fifth instar was performed, because β-lactamase activity could be recognized as described in my earlier report (Kato, 2008). The Superdex 200 column was equilibrated with 0.1 M Tris-HCl buffer containing 0.1 M 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 the Shimadzu spectrophotometer type UV 1200. As shown in Fig. 1, gel filtration exhibited three major fractions, called Fraction I, Fraction II and Fraction III in addition to the passed-through fractions (tubes 24–25) as described in my previous report (Kato, 2007). Each fraction was separately pooled, dialyzed and lyophilized.

Figure 2 illustrates the result of the research of the activity of β-lactamase in the

![Fig. 1](image-url)  
**Fig. 1** Gel filtration of the larval hemolymph of *B. mori* on day 10 in the fifth instar.

![Fig. 2](image-url)  
**Fig. 2** Activity of β-lactamase in the fractions obtained by gel filtration from the hemolymph.
fractions obtained by gel filtration of the hemolymph on day 10 as shown in Fig. 1. The activity of each fraction (10 mg of lyophilizate) was assayed with Penicillin G Potassium Salt as the substrate, according to the procedure described in “MATERIALS and METHODS”. In this case, it was confirmed that the optical density was inversely proportional to the density of \( \beta \)-lactamase as described in my former report (Kato, 2008). Namely, \( \beta \)-lactamase activity is higher, absorbance of 540 nm is less. Therefore, among these fractions, Fraction II contained the \( \beta \)-lactamase activity most abundantly as shown in Fig. 2.

In order to elucidate the physiological meaning, I inspected the time-dependent change of \( \beta \)-lactamase activity in the fat body during the fifth instar of \( B. \) mori. The result is shown in Table 1. In Table 1, non-treated reaction mixture with the fat body was used as a "control". Hardly any activity could be recognized on day 6, on day 8 (mature period) and on day 10 (spinning stage) in the fifth instar, but activity could be recognized on day 13 (pupation period) and on day 17 (pupation period). This fact seemed to suggest that the existence of \( \beta \)-lactamase was necessary for the living body of \( B. \) mori during metamorphosis.

<table>
<thead>
<tr>
<th>Days</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>0.615</td>
</tr>
<tr>
<td>3</td>
<td>0.459</td>
</tr>
<tr>
<td>6</td>
<td>0.553</td>
</tr>
<tr>
<td>8</td>
<td>0.528</td>
</tr>
<tr>
<td>10</td>
<td>0.53</td>
</tr>
<tr>
<td>13</td>
<td>0.4</td>
</tr>
<tr>
<td>17</td>
<td>0.415</td>
</tr>
</tbody>
</table>

* Non-treated reaction mixture with the fat body was used as "control".

Next, gel filtration of the fat body of \( B. \) mori on day 13 in the fifth instar was performed, because \( \beta \)-lactamase activity could be recognized, as shown in Table 1. As shown in Fig. 3, four significant fractions, Fraction I, Fraction II, Fraction III and Fraction IV were obtained by gel filtration in addition to the passed-through fractions (tubes 23–24). Each fraction was separately pooled, dialyzed and lyophilized.

Figure 4 depicts the result of the research of the activity of \( \beta \)-lactamase in the fractions from the fat body of \( B. \) mori on day 13 in the fifth instar obtained by gel filtration. The activity of each fraction (10 mg of lyophilizate) was assayed with Penicillin
Fig. 3  Elution pattern of gel filtration of the fat body of *B. mori* on day 13 in the fifth instar.

Fig. 4  Activity of β-lactamase in the fractions obtained by gel filtration from the fat body.

G Potassium Salt as the substrate. The result showed that higher fraction of β-lactamase activity did not become clear.

Figure 5 shows the result of Mono Q ion exchange chromatography on the larval hemolymph of *B. mori* on day 10 in the fifth instar by means of FPLC system. The hemolymph sample (10 mg of lyophilizate) was applied on to the column of Mono Q HR 5/50 GL previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). Elution was performed with a linear gradient of 0 to 0.5 M NaCl in 0.05 M Tris-HCl buffer (pH 8.0). The chromatogram revealed four peaks, called Peak I, Peak II, Peak III and Peak IV, at
Fig. 5 Mono Q ion exchange chromatography of the larval hemolymph of *B. mori* on day 10 in the fifth instar.

Fig. 6 Activity of β-lactamase in the fractions obtained by Mono Q ion exchange chromatography from the hemolymph.

various salt concentrations as shown in Fig. 5.

Figure 6 illustrates the result of the research of the activity of β-lactamase in the peaks obtained by Mono Q ion exchange chromatography from the hemolymph. The activity of each peak (0.5 ml of solution) was assayed with Penicillin G Potassium Salt as the substrate. The activity was highest on the peak (Peak IV) at the salt concentration of 0.25 M.

Figure 7 shows the result of Mono Q ion exchange chromatography of the fat body of *B. mori* on day 13 in the fifth instar. The fat body sample (100 mg of lyophilizate) was
Fig. 7  Elution pattern of Mono Q ion exchange chromatography of the fat body of *B. mori* on day 13 in the fifth instar.

Fig. 8  Activity of β-lactamase in the fractions obtained by Mono Q ion exchange chromatography from the fat body.

applied on to the column of Mono Q HR 5/50 GL previously equilibrated with 0.05 M Tris–HCl buffer (pH 8.0). Elution was performed with a linear gradient of 0 to 0.5 M NaCl in 0.05 M Tris–HCl buffer (pH 8.0). Four significant peaks, Peak I, Peak II, Peak III and Peak IV, were obtained at various salt concentrations, as shown in Fig. 7.

Figure 8 illustrates the result of the research of the activity of β-lactamase in the peaks obtained by Mono Q ion exchange chromatography from the fat body. The activity of each peak (0.5 ml of solution) was assayed with Penicillin G Potassium Salt as the substrate. The result showed that higher peaks of β-lactamase activity did not be
become clear.

On the other hand, Fig. 9 shows the result of Mono Q ion exchange chromatography on standard $\beta$-lactamase from Bacillus cereus 569/H9 (Nakalai Tesque Inc.). The chromatogram revealed several peaks at various salt concentrations as shown in Fig. 9. However, it did not reveal a peak at the salt concentration of 0.25 M. Namely, it seems that a different elution pattern was obtained from the elution patterns of the hemolymph and fat body in Fig. 5 and Fig. 7. Moreover, $\beta$-lactamase activity was high on every peak in Fig. 9 as a matter of course.

In conclusion, these results described above suggest that $\beta$-lactamase from the hemolymph of $B.\ mori$ were able to fractionate by means of gel filtration or FPLC Mono Q ion chromatography. On the other hand, the higher fraction of $\beta$-lactamase activity from the fat body of $B.\ mori$ did not become clear by gel filtration or FPLC Mono Q ion chromatography. Accordingly, I will determine the various properties of fractionated $\beta$-lactamase and a comparative study will be performed with known microbial $\beta$-lactamase in the future.

**SUMMARY**

I examined the fractionation $\beta$-lactamase in the hemolymph and fat body from *Bombyx mori* by means of gel filtration or FPLC Mono Q ion chromatography. The result showed that Fraction II contained the most abundant $\beta$-lactamase activity, among the gel filtration fractions (Fr I, Fr II, Fr III) from hemolymph. Moreover, the $\beta$-lactamase
activity was highest for the peak corresponding to the salt concentration of 0.25 M, among Mono Q ion chromatography peaks from the hemolymph. They suggest that β-lactamase from the hemolymph of B. mori were able to fractionate by means of gel filtration or FPLC Mono Q ion chromatography. On the other hand, the higher fraction or peak of β-lactamase activity from the fat body of B. mori did not become clear by gel filtration or Mono Q ion chromatography.

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REFERENCES


