Enzymatic Properties of $\beta$-Lactamase Involved in the Hemolymph of Bombyx mori

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INTRODUCTION

To date, I have found that a $\beta$-lactamase might exist in the hemolymph of the domesticated silkworm, Bombyx mori, by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), in relation to the investigation of the B. mori humoral lectin related proteins. I.e., the identification result by using MALDI-TOF/MS homology by using the identification 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 guanosine 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 (Kato, 2007). I was able to obtain the very interesting results taking into consideration of the organism defense mechanism of the B. mori, because $\beta$-lactamase is known as the hydrolase which makes anti-bacterial characteristics become lost by cleaving to the $\beta$-lactam ring of the lactam medicine like penicillin (Lee, 1987; Sawai 1995). Also, assay of $\beta$-lactamase to confirm the actual existence of $\beta$-lactamase in the hemolymph of B. mori was estimated to be required in the process during metamorphosis of B. mori (Kato, 2008). In addition, the behavior of $\beta$-lactamase from B. mori was studied in the fractions of gel filtration or FPLC Mono Q ion exchange chromatography (Kato, 2009).

However, in previous reports, $\beta$-lactamase activity in the assay for the activity was in view of the poor, so the assay was misleading to compare.

The aim of the present study was to re-examine the enzymatic activity of $\beta$-lactamase on how to view and to continue to review the properties of $\beta$-lactamase and the reality, such as comparison of properties on chromatograms between silkworm-derived $\beta$-lactamase and microbial-derived $\beta$-lactamase. This investigation provides useful information for the understanding of the organism 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 was collected. The hemolymph was collected by cutting the pleopods and abdominal legs of the larvae. After centrifuging at 2,260 × g at 4°C for 15 min to remove the hemocytes, the resulting supernatant was lyophilized.
(2) 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.

(3) FPLC Mono Q ion exchange chromatography

Ion exchange chromatography was performed using a column of Mono Q HR 5/50 GL (5 x 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.

RESULTS and DISCUSSION

(1) Assay of $\beta$-Lactamase Activity

I studied the method of estimation of $\beta$-lactamase activity in detail. First, concerning the relationship between the concentration of iodine reagent and absorbance, the inspection result is shown in Fig. 1. The iodine reagent was prepared by adding 5 ml of the stock iodine solution to 95 ml of pH 4.0 acetate buffer, according to the procedure described in “MATERIALS and METHODS”. The horizontal shaft in the figure shows the quantity of ml of the iodine reagent which is included in 0.1 M phosphate buffer (pH 7.0). Getting the actual measurements to 5 ml of each tube so that plus the 0.1 M phosphate buffer, as shown in Table 1. As a result, the concentration of the iodine reagent and the absorbance at 540 nm indicates that there is a proportional relationship.

Figure 2 illustrates the relationship between the concentration of $\beta$-lactamase and absorbance. The horizontal axis in the figure shows the quantity of $\mu g$ of $\beta$-lactamase from Bacillus cereus 569/H9 which is included in 2.5 ml of 0.1 M phosphate buffer (pH 7.0). Regarding density 0 of $\beta$-lactamase in the phosphate buffer, iodine was not completely consumed and the reaction liquid showed high optical density value. When 160 $\mu g$ of $\beta$-lactamase was added in the phosphate buffer, a very low optical density value was shown. It was observed that the reaction product, which the $\beta$-lactam ring in the penicillin (Penicillin G Potassium Salt) as substrate was cleaved to by the action of $\beta$-lactamase, has consumed
iodine, as Sawai and Takahashi (1978) expressed. It was confirmed that the optical density was inversely proportional to the density of $\beta$-lactamase as shown in Fig. 2.

Figure 3 shows the relationship between the concentration of $\beta$-lactamase and the consumption of iodine reagent. The horizontal axis in the figure shows the quantity of $\mu g$ of
\(\beta\)-lactamase which is included in 2.5 ml of 0.1 M phosphate buffer. The consumption of iodine reagent could be obtained from the measurement results in Fig. 1 and Fig. 2. It was confirmed that the consumption of iodine reagent was proportional to the density of \(\beta\)-lactamase as shown in Fig. 3. Therefore, it is possible to guess \(\beta\)-lactamase activity from the consumption of iodine reagent.

In order to elucidate the physiological meaning in the hemolymph of *Bombyx mori*. I inspected the time-dependent change of \(\beta\)-lactamase activity during the fifth instar of *B. mori*. The result is shown in Fig. 4. The enzymatic activity in the figure is expressed as the consumption of iodine reagent (Ogawara and Umezawa, 1975). Hardly any activity could be recognized on day 3 and on day 6 in the fifth instar, but activity could be recognized on day 8 (mature period), on day 10 (spinning stage) and on day 13 (pupation period). Activity could not be recognized again on day 17 (pupa period). This fact seems to suggest that the existence of \(\beta\)-lactamase is necessary for the living body of *B. mori* during one time of the

![Graph](image)

**Fig. 3** Relationship between the concentration of \(\beta\)-lactamase and the consumption of \(\beta\)-iodine reagent.

![Graph](image)

**Fig. 4** The time-dependent change of \(\beta\)-lactamase activity during the fifth instar of *B. mori*. The enzymatic activity in the figure is expressed as the consumption of iodine reagent.
metamorphosis period.

(2) The comparison of properties between silkworm-derived \( \beta \)-lactamase and microbial-derived \( \beta \)-lactamase

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 (Amershan Pharmacia Biotech Ltd.). 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 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 various salt concentration as shown in Fig. 5.

Figure 6 illustrates the result of the research of the activity of \( \beta \)-lactamase in the peaks obtained by Mono Q ion exchange chromatography from the hemolymph. The activity of each

![Fig. 5 FPLC Mono Q ion exchange chromatography of the hemolymph of *B. mori*.](image1)

![Fig. 6 \( \beta \)-lactamase activity in the peaks obtained by Mono Q ion exchange chromatography from the hemolymph of *B. mori*. The enzymatic activity in the figure is expressed as the consumption of iodine reagent.](image2)
peak (0.5 ml of solution) was assayed with Penicillin G Potassium Salt as a substrate. The enzymatic activity in the figure is expressed as the consumption of iodine reagent. The activity was highest on the peak (Peak IV) at the salt concentration of 0.25 M.

On the other hand, Fig. 7 shows the result of Mono Q ion exchange chromatography on standard β-lactamase from Bacillus cereus 569/H9 (Nakalai Tesque Inc.). The chromatogram revealed several peaks at various salt concentration as shown in Fig. 7. However, it revealed no peak at the salt concentration of 0.25 M. Namely, it seems that different elution pattern was obtained from the elution patterns of the silkworm-derived β-lactamase and the microbial-derived β-lactamase in Fig. 5 and Fig. 7.

Moreover, Fig. 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 microbial-derived β-lactamase. The β-lactamase activity was high on every peaks in Fig. 8 as a matter of course.

![Fig. 7 FPLC Mono Q ion exchange chromatography of the microbial β-lactamase from Bacillus cereus 569/H9.](image)

![Fig. 8 β-Lactamase activity in the peaks obtained by Mono Q ion exchange chromatography from the microbial β-lactamase. The enzymatic activity in the figure is expressed as the consumption of iodine reagent.](image)
In conclusion, I emphasize that \( \beta \)-lactamase might be actually present in the living body of \textit{B. mori}. In addition, it seems that \( \beta \)-lactamase is needed for the living body of \textit{B. mori} during one time of the metamorphosis period. Moreover, these results described above suggest that \( \beta \)-lactamase from the hemolymph of \textit{B. mori} and the microbial-derived \( \beta \)-lactamase were able to have fractionation by means of FPLC Mono Q ion chromatography. However, it seems that their elution patterns were different. Accordingly, the comparative study will be performed with known microbial \( \beta \)-lactamase in the future.

**SUMMARY**

In previous reports, \( \beta \)-lactamase activity in the assay for the activity was in view of the poor, so the assay was misleading to compare. Therefore, in this study, I tried to re-examine the enzymatic activity of \( \beta \)-lactamase on how to view. The result showed that \( \beta \)-lactamase activity was recognized in the hemolymph during the fifth instar of the domesticated silkworm, \textit{Bombyx mori}. The activity was higher during one time of the metamorphosis period. These results suggested the possibility that \( \beta \)-lactamase might be present actually in the living body of \textit{B. mori}. Moreover, I studied to fractionate \( \beta \)-lactamase by means of FPLC Mono Q ion exchange chromatography. The result suggested that \( \beta \)-lactamase from the hemolymph of \textit{B. mori} and the microbial-derived \( \beta \)-lactamase were able to have fractionation by means of FPLC Mono Q ion chromatography. However, it seems that their elution patterns were different.

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**REFERENCES**


