Enzyme–linked immunosorbent assay for the toxic fragment of bipyramidal δ-endotoxin produced by Bacillus thuringiensis kurstaki strain HD-1

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The liberation and decomposition of the toxic principle (P-59), a protein with a molecular weight of 59,000, of Bacillus thuringiensis kurstaki strain HD-1 bipyramidal δ-endotoxin in the larval gut juice of the silkworm, Bombyx mori, were investigated by using enzyme-linked immunosorbent assay (ELISA) and bioassay. The sensitivity limit of the ELISA for the quantification of P-59 was lower than 0.4 ng/ml. When estimated by ELISA, the amount of P-59 antigen produced reached its maximum value within 30 sec after the treatment of δ-endotoxin with gut juice (protease activity: 7.8 U/ml), and this value retained for 1 hr. After 2-3 hr incubation, the antigenic decomposition of P-59 was evident. No change was observed in the toxicity of δ-endotoxin to the silkworm after incubation with gut juice for 1 hr, but it decreased by half after incubation for 4 hr. When δ-endotoxin was incubated with each of the proteases (<0.1 U/ml) from gut juice of the silkworm, the reduction of either P-59 antigen or its toxicity was not observed even after incubation for 4 hr. From these results, P-59 seems to be unstable at a high concentration of protease, but stable in diluted one. This suggests that at a proper concentration of gut juice protease of the silkworm, ELISA for P-59 is an effective technique for the estimation of the activity of bipyramidal δ-endotoxin toward lepidopteran insect.

The sporeforming bacterium Bacillus thuringiensis kurstaki strain HD-1 produces two kinds of insecticidal inclusions, i.e., bipyramidal and cuboidal δ-endotoxins, during the sporulation (Sharpe and Baker, 1979; Samasanti et al., 1983). Former toxin is active preferentially to lepidopteran insect larvae perorally, and latter one has the toxicity to lepidopteran and dipteran insects.

The activation process of the bipyramidal δ-endotoxin in larval gut juice of the silkworm, Bombyx mori, was proposed in the preceding report (Tojo and Aizawa, 1983). Owing to the alkalinity (pH 10–11) of the gut juice, enzymatic dissolution of the toxin by gut juice protease(s) occurs, and the solubilized material is further degraded by the protease(s). Finally, 59,000-dalton toxic protein, designated P-59, is produced as the toxic principle. It was suggested that P-59 was further digested by the protease(s) into smaller nontoxic peptides. However, quantitative analysis on the process was not attempted.

In this paper, the liberation and decomposition of P-59 in the larval gut juice of the silkworm were quantitatively investigated by enzyme-linked immunosorbent assay (ELISA) and bioassay. Furthermore, the results suggested that ELISA for P-59 is an effective technique to estimate the activity of B. thuringiensis preparation toward lepidopteran insects.

Materials and Methods

Purification of parasporal inclusions: Parasporal inclusions of B. thuringiensis kurstaki strain HD-1 were purified by the method described previously (Tojo and Aizawa, 1983).

Larval gut juice of the silkworm: Gut juice
was obtained from fourth-instar larvae of the silkworm, *B. mori* (C124×J124), by electric shock and was centrifuged at 10,000×g for 10 min. The supernatant was allowed to stand in ice bath until use.

**Purification of gut juice protease.** Gut juice proteases designated P-I, P-II and P-III were purified by partially modified method of Sasaki and Suzuki (1982).

**Protease assay:** Protease activity was assayed by using casein as the substrate and expressed as the unit per milliliter (U/ml) (Tojo and Aizawa 1983).

**Purification of P-59:** After dissolution of inclusions in 0.2 M carbonate-NaOH buffer, pH 10.2, supplemented with P-III (0.06 U/ml), the toxic protein with 59,000 dalton designated P-59 was purified by using Sephadex G-200 and DEAE-cellulose column chromatographies (Tojo and Aizawa, 1983).

**Preparation of IgG against P-59:** A solution of P-59 (0.1%) was mixed with an equal volume of Freund's complete adjuvant (Difco Lab., Detroit Michigan). The rabbit received subcutaneous injection of 2 ml of the mixture 3 times at 7-day intervals and bled 7 days after the last injection. Antiserum was heated at 56°C for 30 min. The IgG was purified from the serum by using DEAE-cellulose column chromatography (Williams and Chase, 1967).

**ELISA:** Enzyme-linked immunosorbent assay (ELISA) was performed by the method of Voller et al. (1976). Each well of polyvinylchloride multwell plate (Sanko Junyaku Co. Ltd., Tokyo) was added with 200 µl of IgG solution (20 µg/ml) prepared in 50 mM carbonate-NaOH buffer, pH 9.6, and incubated at 4°C for 18 hr. After aspiration of the fluid, the well was added with 200 µl of phosphate buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.29% NaHPO₄, 12H₂O, 0.02% KH₂PO₄, 0.02% NaN₃, pH 7.4) containing 1% bovine serum albumin (BSA), and stored at 4°C until use. Prior to use, the well was washed three times with PBS containing 0.05% Tween 20. After incubation at 4°C for 18 hr with antigen solution in PBS, the well was washed 3 times, and added with 200 µl of alkaline phosphatase (Type VII: Sigma Chemical Co., St. Louis, Mo.)-conjugated IgG. Optimal concentration of the conjugate was determined in a preliminary test. After incubation at 37°C for 3 hr, the well was washed 3 times, added with 200 µl of substrate solution (0.1% 4-nitrophenyl phospho in 10% diethanolamine buffer, pH 9.8), and allowed to stand at room temperature for 20 min. Reaction mixture (175 µl) was then transferred to a test tube containing 350 µl of 1 N NaOH to terminate the reaction. Absorbance at 405 nm of the mixture was measured.

**Detection of P-59 antigen in gut juice of the silkworm:** Gut juice was allowed to stand at 27 °C for 5 min and added with purified inclusions at a concentration of 5 µg/ml. At specified intervals, 10 µl of the mixture was transferred to a test tube containing 990 µl of PBS supplemented with 1% BSA to terminate the reaction and was centrifuged immediately at 20,000 xg for 5 min to remove undissolved materials. Resulting supernatant was examined for the concentration of P-59 antigen by ELISA.

**Protein determination:** Protein concentration was determined by the micro-biuret method (ltzaki and Gill, 1964) using BSA as the standard.

**Assay of toxicity:** Oral toxicity to the silkworm, *B. mori*, was examined as described previously (Tojo and Aizawa, 1983).

**Results**

**ELISA for P-59:** Standard curve of ELISA for P-59 is shown in Fig. 1. Values in absorbance at 405 nm were linear to P-59 concentrations between 0.4 and 25 ng/ml. The multwell plate sensitized with antibody was able to be stored at 4°C for at least 6 months without any loss of activity.

**Liberation and decomposition of P-59 antigen**
Fig. 1. Standard curve of enzyme-linked immunosorbent assay for quantification of P-59 antigen. Equation ($Y=0.100+0.044$) and coefficient correlation ($r^2=0.994$) were determined by the method of least squares.

Fig. 2. Liberation and decomposition of P-59 antigen in the gut juice of the silkworm. Concentration of P-59 antigen was determined by enzyme-linked immunosorbent assay. Bars indicate 95% confidence limit of each value.

In the gut juice: As shown in Fig. 2, the concentration of P-59 antigen liberated from inclusions in the gut juice (protease activity: 7.8 U/ml) of the silkworm reached its maximum value of approximately 10 ng/ml within 30 sec. This value was equivalent to 29% of the total amount of the inclusion protein and was unchangeable for 1 hr. However, P-59 concentration after post-incubation for 2 and 3 hr was 81% and 62% of the maximum value, respectively.

Fig. 3. Liberation of P-59 antigen by proteases purified from gut juice of the silkworm. Reactions were conducted in 0.1 M carbonate-NaOH buffer, pH 10.2, supplemented with P-I (●), P-II (●), and P-III (○), respectively. Activity of each proteases was 0.04 U/ml.
Table 1. Effect of gut juice and purified gut juice proteases on the toxicity of δ-endotoxin

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>δ-endotoxin preparation</th>
<th>LD₅₀ (μg/g body weight)ᵃ⁻ᵇ⁻&lt;sup&gt;−&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Untreated</td>
<td>0.127</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gut juice-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>0.132</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>0.242</td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Untreated</td>
<td>0.422</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protease-treated</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>P-I</td>
<td>0.469</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-II</td>
<td>0.407</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-III</td>
<td>0.406</td>
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</tr>
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</table>

ᵃ Each sample was twofold serially diluted with PBS containing 1% BSA. Oral toxicity was assayed by direct inoculation of 5 μl of test sample into gut lumen of the fourth-instar silkworm larva using a glass capillary. The mean value of the body weight of the silkworm used in experiment 1 and 2 were 0.16 and 0.23 g, respectively.

ᵇ Purified inclusions were incubated in larval gut juice (protease activity: 7.8 U/ml) of the silkworm at 27°C for 1 or 4 hr.

ᵇ Purified inclusions were incubated in 0.1 M carbonate-NaOH buffer, pH 10.2, supplemented with P-I (0.060 U/ml), P-II (0.042 U/ml), and P-III (0.067 U/ml), respectively, at 27°C for 4 hr. Control was incubated in the carbonate buffer without protease.

Purified inclusions were suspended in the gut juice at a concentration of 1 mg/ml. After incubation at 27°C for 1 hr and 4 hr, respectively, the mixture was diluted with PBS containing 1% BSA and examined for the toxicity. In the control (BSA in PBS), silkworm larvae were normal. The toxicity of inclusions did not change after incubation with gut juice for 1 hr, but decreased by half after incubation for 4 hr (Table 1).

Effect of purified gut juice proteases on the liberation and toxicity of P-59: The liberation of P-59 antigen by purified proteases, P-I, P-II, and P-III, was investigated in the presence of each protease at a concentration of 0.04 U/ml. Other conditions were the same as that of the examination using intact gut juice. In all experiments with P-I, P-II, and P-III, the concentration of P-59 antigen reached its maximum value equivalent to 20% of total amount of protein within 5 min, and retained this value for 4 hr (Fig. 3).

When purified inclusions were treated with P-I (0.060 U/ml), P-II (0.042 U/ml), and P-III (0.067 U/ml), respectively, at 27°C, no alteration in the toxicity was observed for 4 hr (Table 1).

Discussion

There are several reports (Wie et al., 1982, 1984; Smith and Ulrich, 1983) on the application of ELISA to the detection of B. thuringiensis δ-endotoxin. However, all of them prepared the alkali-solubilized δ-endotoxin as the antigen by using 0.1 N NaOH. Under such an alkaline condition, the activity of δ-endotoxin to the silkworm is decreased markedly (Nishitsutsuji-Uwo et al., 1977), and antigenic denaturation of this toxin is occurred (unpublished data).

In this study, the liberation and decomposition of P-59, the primary toxic principle of bipyramidal δ-endotoxin (Tojo and Aizawa, 1983), were investigated from the viewpoints of antigenic integrity and toxicity to the silkworm. Although subsp. kurstaki strain HD-1 produce another insecticidal inclusion which is cuboidal shaped (Iizuka and Yamamoto, 1983) than bipyramidal one, the activity of this toxin to the silkworm is lower than that of bipyramidal toxin, and the toxicity of HD-1 preparation to the silkworm is mainly associated with the bipyramidal inclusion (Samasanti et al., 1998).

The toxicity of δ-endotoxin was reduced during the incubation in gut juice of the silkworm (Tojo and Aizawa, 1983). In the present study, the lethal activity of the toxin to the silkworm was
gut juice was more than 3 hr. In contrast, the median time for the liberation of P-59 antigen in gut juice was less than 15 sec, when estimated by ELISA. The results with ELISA also revealed that the decrease in the amount of P-59 antigen began 1 hr after treatment. This corresponds to the results with bioassay.

When inclusions were treated with purified gut juice proteases, the toxicity did not alter even after incubation for 4 hr, and the concentration of P-59 antigen retained its maximum value for 4 hr in the test with each protease solution. The activity of these proteases used was less than 0.1 U/ml. This value was much lower than that (7.8 U/ml) of the fresh gut juice. Therefore, it is very likely that the difference between the results with gut juice and purified proteases is caused by the difference in protease activity. From these results, P-59 seems to be unstable at a high concentration of protease, but stable in diluted one. This suggests that ELISA for P-59 liberated from the bipyramidal inclusion with proper concentration of gut juice protease of the silkworm B. thuringiensis preparation toward lepidopteran insects. In addition, as shown in Fig. 1, ELISA for P-59 is extremely sensitive (sensitivity limit: <0.4 ng/ml).

Several intermediate-products have been detected in the process of P-59 liberation (Tojo and Aizawa, 1983), and such products could react to the antibody against P-59, suggesting that the P-59 antigen detected by ELISA in the protease solution contained that of the intermediaries. Therefore, the results shown in Fig. 2 mean that solubilization of the inclusions, instead of P-59 production, was terminated within 30 sec in the gut juice. However, production of P-59 is surmised to be terminated within at least several minutes in the gut juice, because it was concluded within 30 min even under the protease activity of 0.0067 U/ml (Tojo and Aizawa, 1983).

The maximum amount of P-59 antigen liberated in the gut juice or in carbonate buffer supplemented with each protease was equivalent to 20% of the total amount of inclusion protein. This indicates that 20% of the whole inclusion protein is P-59. As mentioned above, strain HD-1 produces bipyramidal and cuboidal inclusions and P-59 is apparently derived from bipyramidal one which is composed of subunit protein with 130,000 daltons. When single subunit protein is digested with gut juice protease, one molecule of P-59 and nontoxic peptides are produced (Tojo and Aizawa, 1983). Therefore, the residual 80% of the total protein is thought to consist of the cuboidal inclusions and the nontoxic region of the bipyramidal ones.

References

東條昭彦：*Bacillus thuringiensis kurstaki* HD-1 株の産生する菱形毒素の活性成分の酵素抗体法による定量

*Bacillus thuringiensis kurstaki* HD-1 株の産生する菱形毒素の活性フラグメント (P-59) のカイコ消化液中での生成と分解過程を酵素抗体法と生物検定法で調べた。酵素抗体法による P-59 抗原の測定限界は 0.4 ng/ml だった。菱形毒素をカイコ消化液（ブラサーゼ活性：7.8 U/ml）に殺虫すると遊離する P-59 抗原量は30秒以内に最大値に達し、その後1時間維持された。しかし2 〜 3時処理すると減少し、3時間後には最大値の約60%になった。カイコに対する毒性は消化液で1時間処理しても変化しなかったが、4時間処理で半分に低下した。精製した消化液ブラサーゼ (≤0.1 U/ml) で処理すると4時間後でも、P-59 抗原量および毒性の低下はみられないかった。以上の結果から、適当な濃度のブラサーゼのもとで酵素をした P-59 抗原を酵素抗体法で定量することによって、種々の菱形毒素標品の殺虫活性を推定することが可能であると考えられた。