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Possible Presence of an Interleukin-6-Like Molecule in the Immunized *Bombyx mori* L. (Lepidoptera)

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Objectives

Cytokines represent an essential part of the innate immune response in mammals. Recently, several studies have reported presence of cytokine-like activities and molecules in the invertebrates such as echinoderms, tunicates, molluscs and insects (Beck and Habicht, 1986; Beck *et al.*; 1989; Ottaviani *et al.*; 1993; Franchini, 1996). The cytokines found in those organisms include IL-1, IL-2, IL-6, and tumor necrosis factor (TNF).

In this study, we attempted to identify the presence of cytokine-like molecules in the silkworm, *Bombyx mori* by immunizing the silkworms with several typical immune inducers, such as peptidoglycan, bacteria, and fungus, and also another immune inducer, oligodeoxynucleotide (ODN). In a previous study, we found out that the synthetic nucleotides (~ 20 nucleotide length) containing varying number of CpG dinucleotides, even without CpG dinucleotide stimulate an immune response in the silkworm (Kim *et al.*, 2003). For an identification of the cytokines, SDS-PAGE and Western blot analysis was performed using several human cytokines as the probes. In this paper, we report the result of Western blot analysis showing a positive antigen-antibody reaction and a serial attempt to isolate the molecule, although the attempt was not much successful.

Materials and Methods

Materials - Insect: silkworm *Bombyx mori*

Methods - Immunization: immunization, and sample collection

Immune inducers: *Escherichia coli* K112, *Bacillus subtilis, Candida albicans*, peptidoglycan, and oligodeoxunucleotide (ODN)

SDS-PAGE and Western blot analysis

Sephadex G-100 gel permeation chromatography

Anion exchange HPLC

Ultra centrifugation

Immuno-dot-blot assay

Results and Discussion

Silkworm larvae at the 3rd day of 5th instar were injected with several sources of immune inducers such as

Gram-positive (*B. subtilis*) and -negative (*E. coli*) bacteria, fungus, ODN, and peptidoglycan (PG), and hemolymph were extracted 8 hrs after infection. For hemocytes, only silkworms infected with *E. coli* were subjected to extraction. Although no detectable difference was observed in the SDS-PAGE analysis among samples (Fig. 1a), the hemolymph infected with the ODN and PG showed a clear antibody-antigen reaction, indicating possible presence of IL-6-like molecule in the silkworm hemolymph (Fig. 1b). The molecular weight of the molecule was ~45 kDa.

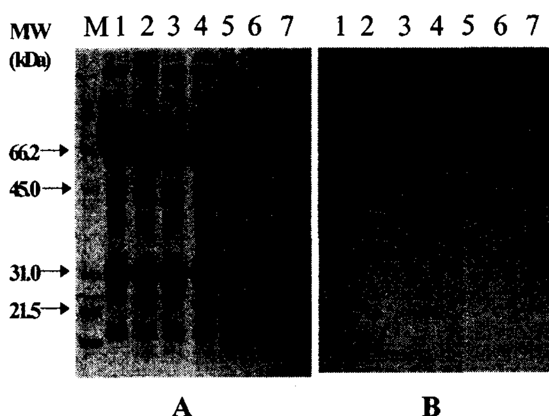

Fig. 1. SDS-PAGE analysis of hemolymph proteins of *B. mori* larvae after infected with several sources of immune inducers and Western blotting analysis of the hemolymph proteins probed with rabbit anti-human IL-6 antibody. (A) SDS-PAGE gel stained with coomassie blue. M, standard markers; lane 1, hemolymph of naive larvae; lane 2, hemolymph of larvae infected with *E. coli*; lane 3, with *B. subtilis*; lane 4, with *C. albicans*; lane 5, with ODN; lane 6, with peptidoglycan (PG); and lane 7; hemocytes extracts of the larvae infected with *E. coli*.(B) Western blotting analysis with duplicate gel of (A). Note that positive bands were detected only in the hemolymph of the larvae infected with ODN (lane 5) and PG (lane 6).

To find out the possible tissue/organ showing induction of IL-6-like molecules infected with ODN and PG, Western blot analysis was performed with samples of hemolymph, hemocytes, mid-gut, and fat body (data not shown). Positive signals were clearly detected in the immunized hemolymphs collected 8 hrs (lane 2) and 24 hrs (lane 3) after infection with PG (A) and ODN, respectively, although it was very week in the normal hemolymph (control) (Fig. 2). The reacted molecule was ~45 kDa and the difference of the molecules by the two immune inducers in the protein level cannot be mentioned yet.

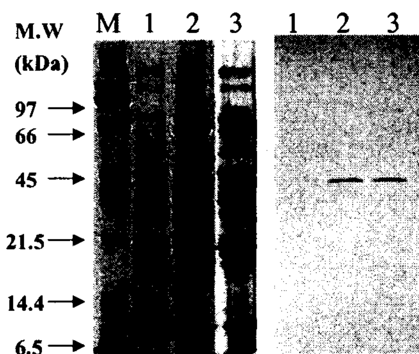

Fig. 2. SDS-PAGE (A) and Western blot analysis (B) of the hemolymph collected 24 hours after infection of ODN and peptidoglycan. Each samples was separated on 12% gel, transferred and probed with rabbit anti-human IL-6 antibody. M, molecular weight markers; lane 1, normal hemolymph; lane 2, hemolymph of 24 hrs after infection with peptidoglycan; and lane 3, hemolymph of 24 hrs after infection with ODN.

The gel permeation chromatography (GPC, Sephadex G-100) was performed for the hemolymph proteins from *B. mori* larvae immunized with ODN (Fig. 3). As shown in the Western blotting analysis (Fig. 4c) IL-6-like molecules were detected at the beginning fractions of the larval proteins (fractions 27,

29 and 31). These fractions were pooled for the analysis with anion exchange HPLC column (Vydac P/N 301 VHP575).

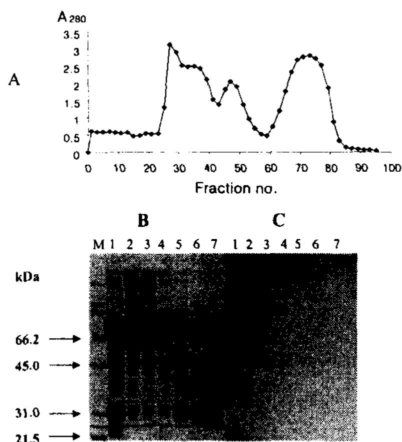

Fig. 3. The profile of gel permeation chromatography (GPC, Sephadex G-100) performed for the hemolymph proteins from *B. mori* larvae immunized with ODN (A), and SDS-PAGE (B) and Western blot analysis (C) of the fractions obtained from GPC. Fractions were eluted at 6 ml/h of flow rate. Only one % of every second fraction was loaded to each lane. M, standard markers; lane 1, acid extract of immunized hemolymph with ODN; lane 2, fraction no. 27 of GPC; lane 3, no. 29; lane 4, no. 31; lane 5, no. 33; lane 6, no. 35; and lane 7, no. 37. Note that positive bands were detected only in fraction no. 27, 29 and 31 (lanes 2, 3, 4, respectively). Thus corresponding fractions were pooled together for the next purification step.

The pooled fractions, which showed positive signals of an antigen-antibody reaction by GPC resulted in three separate peaks (P1, P2, and P3) in the anion exchange chromatography (Fig. 4).

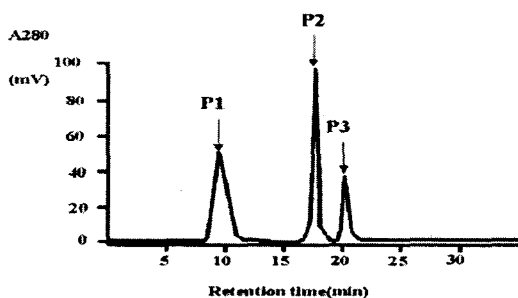

Fig. 4. Profile of anion exchange chromatography of the fractions (no. 27 - 30) obtained from GPC, which were confirmed to contain IL-6-like protein. Chromatography was performed in HPLC system equipped with anion exchange column (Vydac). Three peaks (P1, P2 and P3) were separately collected and subjected to Western blotting analysis.

The three peaks were separately subjected to SDS-PAGE and Western blotting analysis (Fig. 5). Although the acid extracts of hemolymph proteins from *B. mori* larvae infected with ODN (Fig. 5b, lane 1) and the positive fractions (Fig. 5b, lane 2) obtained from GPC revealed positive signals, the three peaks obtained after anion exchange chromatography did not show any an antigen-antibody reaction in the Western blotting analysis.

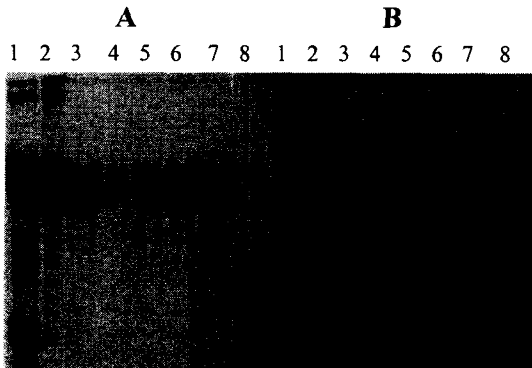

Fig. 5. SDS-PAGE and Western blotting analysis of the proteins od three peaks eluted from anion exchange HPLC. (A) SDS-PAGE gel stained with coomassie blue. Lane 1, acid extracts of hemolymph proteins from *B. mori* larvae infected with ODN; lane 2, fractions (no. 27 - 30) from GPC; lane 3, first-half part of P1 from anion change HPLC; lane 4, second-half part of P1; lane 5, first-half of P2; lane 6, second-half of P2; lane 7, first-half of P3; and lane 8, second-half of P3. (B) Western blotting analysis performed with duplicate gel of (A). No positive band reacted with antibody against human IL-6 was detected (lanes 3, 4 and 5).

We continued further purification steps such as Anion exchange HPLC, Ultra centrifugation, and Immuno-dot-blot assay to isolate the IL-6-like molecule, but until now it is not much successful yet. However, it does not appear that the IL-6-like molecule is mere experimental artifact happened by Western blotting analysis with human antibody. Thus, further experiment on this subject probably provides us more fruitful result as detected in other invertebrates including insects.

References

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