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

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Cytokines represent an essential part of the innate immune response in mammals. Recently, several studies have reported the presence of cytokine-like activities and molecules in the invertebrates such as echinoderms, tunicates, mollusks and insects. In our serial study, we investigated presence of cytokines in the silkworm, *Bombyx mori*, infected with several immune inducers. Western blotting analysis using rabbit anti-human cytokines showed the presence of IL-6-like molecule in the hemolymph collected at 8 and 24 hrs after infection with peptidoglycan and oligodeoxynucleotide, and the molecular weight of the proteins was ~45 kDa. We attempted to isolate the molecule by gel permeation HPLC, anion exchange chromatography, ultra centrifugation, and immuno-dot-blot assay, but until now the effort was not much successful yet. It, however, does not appear that the IL-6-like molecule in the silkworm larvae is a mere experimental artifact happened by Western blotting analysis. Instead, further experiment on this subject probably will provide us more fruitful result as detected in other invertebrates including insects.

Key words: Cytokines, Interleukin 6, IL-6-like molecule, Oligodeoxynucleotide, Silkworm, *Bombyx mori*, Invertebrate immunity

Introduction

Cytokines are small proteins important for humoral and

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cell-mediated immune response and involved in the prevention of disease, remedy, and homeostasis by controlling immune response and inflammation. For example, interleukine 6 (IL-6) is a macrophage-derived cytokine also responsible for mediation of nonspecific host defense in mammals (Akira *et al.*, 1990). IL-6 has multiple biological activities on different target cells such as activation of T cells and thymocytes, induction of acute phase proteins, and stimulation of haematopoietic precursor cell growth and differentiation in mammals (Dinarello, 1990).

In the molecular perspective, IL-6 was characterized from many mammals (Ohashi *et al.*, 1993; Kukielka *et al.*, 1994; King *et al.*, 1996; Takakura *et al.*, 1997; Hernández *et al.*, 2002). In the mouse, the gene coding for IL-6 consisting of 211 amino acid residues including typical 24 signal sequence (MW 21,710 Da) and showed 42% amino acid sequence identity to human homologue (van Snick *et al.*, 1988). From horse, Swiderski *et al.* (2000) cloned IL-6 consisting of 208 amino acid residues with a predicted 28 leader amino acid sequence (MW 20,471 Da) without post-translational modifications.

In contrast to an abundance of mammal-originated IL-6, the data on the molecular characterization of non-mammalian IL-6 are very scarce. One such non-mammalian IL-6 is that derived from chicken. The chicken IL-6 gene consists of 241 amino acid residues including a putative N-terminal signal peptide of 47 residues, and showed about 35% amino acid sequence identity to human protein, and induces proliferation of IL-6-dependent murine hybridoma cell line 7TD1 (Weining *et al.*, 1998; Schneider *et al.*, 2001). Except for chicken, no other paper has been reported for the molecular characterization of the molecule as far as we know.

However, cytokine research continuously reports the presence of cytokine-like molecules in the non-mammalian species, such as echinoderms (Beck and Habicht,

1986), tunicates (Beck et al., 1989), mollusks (Ottaviani et al., 1993), and insects (Franchini, 1996). The cytokines found in those organisms include IL-1, IL-2, IL-6, and tumor necrosis factor (TNF). Echinoderm IL-1-like molecule stimulates proliferation of mammalian fibroblasts, enhances fibroblast protein synthesis, induces the production of prostaglandin E2, and is cytotoxic for the human cell line A375 (Beck et al., 1989; Beck and Habicht, 1991). Tunica IL-1-like proteins also increase vascular permeability in rabbit skin and enhance the proliferation of tunicate and echinoderm cells in vitro (Beck et al., 1989; Raftos et al., 1991). Further, it has been demonstrated that the invertebrate IL-1-like molecules derived from echnoderms and tunicates enhance phagocytosis in the echinoderm and tunicate cells, demonstrating an involvement of the invertebrate IL-1-like molecules in the invertebrate host defense mechanisms (Beck et al., 1993). However, no molecular characterization of the molecules from such invertebrate has been reported as far as we know.

In this study, we attempted to identify the presence of cytokine-like molecules in the silkworm, Bombyx mori, by immunizing the silkworms with a typical immune inducer, peptidoglycan (PG), another immune inducer, oligodeoxynucleotide (ODN), and other a few other typical immune inducers such as Escherichia coli, Bacillus subtilis, and Candida albicans. In a previous study, we found that the synthetic nucleotides (~20 nucleotide length) containing varying number of CpG dinucleotides, even without CpG dinucleotide stimulate the immune response in the silkworm (Kim et al., 2004). Thus, we also utilized one of the ODNs as an immune inducer in this study. For the identification of the cytokines, SDS-PAGE and Western blotting analysis was performed using several rabbit anti-human cytokines as a first antibody. In this paper, thus, we report the results of Western blotting analysis, which shows a positive antigen-antibody reaction and the result of several attempts to isolate the molecule, although the effort was not much successful.

Materials and Methods

Insect, immunization, and sample collection

The silkworm, Bombyx mori, used was hybrid Jam-113 supplied by Department of Sericulture & Entomology, The National Institute of Agricultural Science & Technology, Korea. Silkworms were reared on fresh mulberry leaves at 26°C, 65 ± 5% of relative humidity, and a 12 hrs light: 12 hrs dark photoperiod as usual. On 3rd day of 5th instar, silkworm larvae were infected by an abdominal injection with several immune inducers such as E. coli

K112, B. subtilis, C. albicans, PG, and ODN. E. coli and B. subtilis injected were cultured to the concentration of 6×10^3 colony forming units and C. albicans to the concentration of 1×10^3 cores in tryptic soy broth (TSB, Difco, Detroit, USA) medium. Fifty μl of an ODN, dissolved in TE-buffer (10 mM tris, 1 mM EDTA, pH 8.0) was injected at a concentration of 1 mg/ml, based on the prior studies (Krieg et al., 1995; Kim et al., 2003). The sequence of the ODN is as follow: 5'-TCGTCGTTTTGT CGTTTTGTCGTT-3' (Bioneer, Korea). This ODN is 24 bp-long and contains four CpG dinucleotides, and is one of the strong immune inducers in the silkworm larvae (Kim et al., 2003). Ten μl of Fromstaphylococcus aureus-originated PG (Fluka, Switzerland), dissolved in the insect Ringers solution (2.3 mM $NaHCO_3$, 0.128 M NaCl, 1.4 mM KCL, 1.8 mM $CaCl_2$, pH 6.2) was injected at a concentration of 1 μg/ 10 μl. Fat body, midgut, hemolymph, and hemocytes were excised from the larvae 8 hrs and 18 hrs after infection. Non-injected larvae were used a control.

SDS-PAGE and Western blot analysis

A Model Mini-Protean 3 Cell (Bio-Rad) was used to perform tricine sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) (Schagger and Jagow, 1987). For tricine SDS-PAGE analysis, samples were subjected to shaking using Twist shaker TW-3 (Fine PCR, Korea) for 18 hrs and centrifugation for 30 min at 14,000 rpm, 4°C to obtain supernatants of each part. Tricine SDS-PAGE was conducted on 12% gel containing 0.1% of SDS at room temperature. After electrophoresis, gels were fixed and stained overnight with 0.1% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, USA). Molecular weights were estimated with SDS-PAGE size marker (Bio-Rad, Richmond, USA). Western blotting analysis was performed as described by Towbin et al. (1979). Briefly, samples were electrophoresed on tricine SDS-PAGE gels and transferred to nitrocellulose membrane in the Tris-Glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 100 volt for 1 hr. After transferred, the membrane was equilibrated in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and incubated in blocking solution (2% skim milk in TBS) for 1 hr. After washing briefly with TBS containing 0.05% Tween-20 (TTBS), the membrane was incubated in the primary antibody solution (1000-fold diluted antiserum with blocking solution) for 1.5 hrs. The primary antibodies utilized were C3c, α2-macroglobulin, IL-6α, IL-6, and TNF-α, which are rabbit anti-human cytokines (Sigma, St. Louis, USA). The membrane was washed twice with citrate-buffered saline (20 mM citrate, 500 mM NaCl, pH 5.5) containing 0.05% Tween-20 for 5 min at every time and then incubated for 1

in blocking solution containing a 3,000-fold diluted secondary antibody (GAR-HRP conjugated IgG; Bio-Rad, Richmond, CA). Finally, the membrane was washed twice with TTBS, once with TBS, and submerged in HRP color development solution (50 ml of TBS containing 30 ml of H_2O_2 mixed with 0.3% of chloronaphtol in 10 ml of ice cold methanol).

Sephadex G-100 gel permeation chromatography (GPC)

Samples were loaded on a 55 × 1.8 cm Sephadex G-100 GPC column, equilibrated with 20 mM Tris buffer (pH 8.3) with a flow rate of 1 ml/10 min. The eluates were collected in 1 ml fractions, concentrated by vacuum centrifugation (Centra Evaporator, Bioneer, Korea), and examined by Western blotting analysis. Fractions of interest were pooled for the next step, anion exchange high performance liquid chromatography (HPLC).

Anion exchange chromatography

Samples were applied to an anion exchange HPLC column (Vydac P/N 301 VHP575) connected to a Gilson HPLC unit, with a flow rate of 1 ml/min. Anion-exchange column was equilibrated with 50 mM sodium phosphate (pH 6.5). Unbound material was eluted by extensive washing with buffer at a flow rate of 1 ml/min. Bound material was eluted with a 50 mM sodium phosphate (pH 6.5) containing NaCl as follows: concentration of NaCl was linearly increased by 2%/min from 0 to 0.6 M for 30min and flow rate was 1 ml/min.

Ultra-centrifugation

Potassium bromide gradient ultra centrifugation was performed as described by Entrala *et al.* (2000). Briefly, the gradient of potassium bromide (Sigma, St. Louis, USA) consisting of three solutions of 6, 16 and 28% (w/v) KBr in Tris-EDTA buffer. From bottom to top, 7 ml of ice-cold 28, 16 and 6% KBr solutions were carefully layered into 25 ml clear plastic centrifuge tubes. Three ml of the concentrated fecal suspension was carefully layered on top of the gradient, and the tubes were centrifuged in a Beckman GS-15R centrifuge equipped with a swing-out S4180 rotor at 3,000 g for 1 hr at 4°C. Each band was carefully aspirated using a pasteur pipette

Immuno-dot-blot assay

Aliquots of 10% from each fraction were dried in a vacuum concentrator and redissolved with 2 ul of distilled water. Samples were spotted onto nitrocellulose membranes and dried in room temperature. The membrane was equilibrated in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5; TBS) for 10 min. Subsequently, Western blotting analysis was performed as explained above.

Results and Discussion

Identification of IL-6-like molecule in the *B. mori* hemolymph infected with ODN and PG

Although we performed Western blotting analysis with several antibodies, which are rabbit anti-human cytokines such as C3c, α2-macroglobulin, IL-6α, IL-6, and TNF-α (data not shown), we only were able to detect signals from IL-6. In other invertebrates, detection of IL-1-like and TNF-α-like molecules in insects (*Galleria mellonella* and *Estigmene acraea*) (Wittwer *et al.*, 1999), IL-1-like protein in tunicate (*Styela clava*) and starfish cells (*Asterias forbesi*) (Beck *et al.*, 1993), IL-1β in protochordate (*Styela plicata*) (Pestarino *et al.*, 1997), and IL-1α, IL-2 and TNF-α-like molecules in fresh-water snails (*Planorbarius corneus* and *Viviparus ater*) (Ottaviani *et al.*, 1995) has been reported.

Fig. 1 shows the effect of several immune inducers for the induction of IL-6-like molecule. The SDA-PAGE and Western blotting analysis was performed to identify the IL-6-like molecule in the hemolymphs of the silkworms using the primary antibody, IL-6. Silkworm larvae at 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, PG, and hemocytes extracts of the larvae infected with *E. coli*. Although no detectable response was observed in most samples, the hemolymph obtained after infection with the ODN and PG showed a clear antibody-antigen reaction (Fig. 1; lanes 5 and 6).
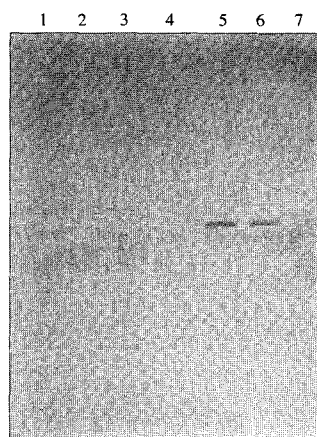

Fig. 1. Western blotting analysis of the hemolymph proteins collected at 16 hrs after infection with several immune inducers. Each sample was separated on 12% SDS-PAGE gel, transferred and probed with rabbit anti-human IL-6 antibody. Lane 1, hemolymph of naive larvae; lane 2, hemolymph of larvae infected 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*. Note that positive bands were detected only in the hemolymph of the larvae infected with ODN(lane 5) and PG (lane 6).

It is noteworthy that the positive reaction was detected only in the PG and ODN-immunized silkworms. In the case of *Galleria mellonella* and *Estigmene acraea*, lipopolysaccharide (LPS) was challenged to induce cytokines (Wittwer *et al.*, 1999), indicating that LPS is a proper cytokine inducer. In our study, we used several possible immune inducers, but we only were successful with non-live immune inducers such as PG and ODN. However, it has been well known that the PG unique to bacterial cell wall, LPS from the outer membrane of gram-negative bacteria, and β-1,3-glucans and β-1,3-mannans from fungal cell walls are all efficiently recognized by arthropods and even by the vertebrate (Gillespie and Kanost, 1997). Furthermore, the pathogenic microorganisms and eukaryotic parasites equipped with such immune inducing substances are *ad hoc* stimulating particle of the host defense system (Gillespie and Kanost, 1997), but the live-immune inducers such as bacteria and fungus in this study were not successful. The failure of the induction of positive reaction by bacteria and fungus is not obvious here. Thus, further investigation to clarify the role of immune-stimulating particles is required, otherwise the result was obtained simply by experimental error.

It has been known that bacterial DNA or synthetic ODNs containing unmethylated CpG dinucleotides in particular base contexts induce B cell proliferation, IL-6 and Ig secretion, apoptosis resistance, and activation of several immune cell types in the mammalian organisms (Krieg *et al.*, 1995, 1998; Ballas *et al.*, 1996; Klinman *et al.*, 1996). Furthermore, recently it has been known that ODN truly induce immune response in the silkworm, regardless of numbers of CpG motifs and methylation of CpGs in ODNs (Kim *et al.*, 2004). In this context, induction of immune response, particularly IL-6-like molecule by ODN appears to be reasonable within our knowledge of insect immunity.

To find out the possible location of the silkworm tissue/organ showing an induction of IL-6-like molecule infected with ODN and PG, Western blotting analysis was performed with samples of hemolymph, hemocytes, mid-gut, and fat body (Fig. 2). Positive signals were clearly detected in the immunized hemolymphs collected 8 hrs (lane 2) and 24 hrs (lane 3) after infection with PG and ODN, respectively, although the reaction in the normal hemolymph was weak. IL-6 is a macrophage-derived cytokine along with TNF and responsible for mediation of nonspecific host defense mechanisms in mammals (Gillespie and Kanost, 1997). In insect, it has been reported that two types of cells, plasmatocytes and granular cells, from the hemolymph of *G. mellonella* larvae react with antibodies raised against vertebrate IL-1α and TNF-α (Wittwer *et al.*, 1999). Also, in mollusks both IL-1 and TNF-like molecules have been
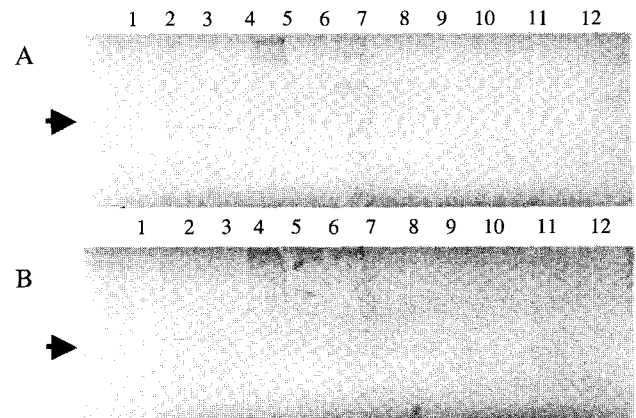

Fig. 2. Western blotting analysis of the hemolymph, hemocytes, mid-gut, and fat body collected at 8 and 24 hrs after infection with (A) PG and (B) ODN. Each sample was separated on 12% SDS-PAGE gel, transferred and probed with rabbit anti-human IL-6 antibody. Lane 1, normal hemolymph; lane 2 immunized hemolymph (8 hrs); lane 3 immunized hemolymph (24 hrs); lane 4, normal hemocytes; lane 5, immunized hemocytes (8 hrs); lane 6, immunized hemocytes (24 hrs); lane 7, normal mid-gut; lane 8, immunized mid-gut (8 hrs); lane 9, immunized mid-gut (24 hrs); lane 10, normal fat body; lane 11, immunized fat body (8 hrs); and lane 12, immunized fat body (24 hrs). Note that positive bands were detected only in the immunized hemolymphs collected 8 hrs (lane 2) and 24 hrs (lane 3) after infection with PG (A) and ODN (B).

found in the hemocytes with macrophage-like activity (Ottaviani *et al.*, 1993). Considering these previous reports it seems that macrophage-like cells are primary cytokine producers. In our study, however, antigen-antibody reaction was only detected in the hemolymph of the immunized silkworm larvae (Fig. 2). This probably was caused because our hemocyte sample is limited, except for a single hemocyte sample, obtained with the infection of *E. coli*. Therefore, it is difficult to extract any conclusion on the location of the primary producer of the IL-6-like molecule at this time. Thus, future study is required to expand our understanding on the producer of cytokine-like-molecules in the silkworm larvae.

Western blotting analysis only using the hemolymph collected at 24 hrs after infection with ODN and PG further conformed the induction of an IL-6-like molecule with the approximate molecular weight of 45 kDa (Fig. 3). The molecular weight of IL-6 in other organisms was 20,781 Da for human (Kishimoto, 1989) and ~30,000 Da for starfish *A. forbesi* (Beck and Habicht, 1996), but our IL-6-like molecule was larger. In the case of IL-1, much higher size variation has been reported. For example, Kimball *et al.* (1986) isolated a 35,000 Da form and a 17,000 Da form from human monocytes. Also, Auron *et al.* (1984) isolated three forms of IL-1 from human monocytes and larger
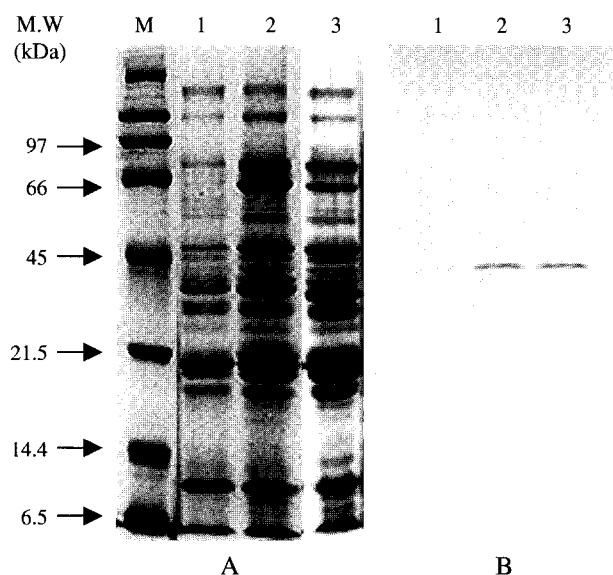
Fig. 3. SDS-PAGE (A) and Western blot analysis (B) of the hemolymph collected 24 hrs after infection with ODN and PG. Each sample was separated on 12% gel, transferred and probed with rabbit anti-human IL-6 antibody. M, molecular weight markers; lane 1, normal hemolymph; lane 2, hemolymph of 24 hrs after infection with PG; and lane 3, hemolymph of 24 hrs after infection with ODN.

form was speculated to be precursor of secreted IL-1. Considering these data, the 45 kDa of IL-6-like molecule of the silkworm larvae can be speculated to be a precursor.

Attempt to isolate IL-6-like molecule

The GPC (Sephadex G-100) was performed to isolate the IL-6-like molecule in the hemolymph proteins of *B. mori* larvae immunized with ODN (Fig. 4A). As shown in the Western blotting analysis (Fig. 4C), IL-6-like molecule was 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.

The pooled fractions, which showed positive signals in the Western blotting analysis, resulted in three separate peaks (P1, P2, and P3) in the anion exchange chromatography (Fig. 5). To detach the proteins binding to the resin, NaCl gradient was applied from the concentration from 0.1 M to 0.3 M for 1 hr. The three peaks were separately subjected to SDS-PAGE and Western blotting analysis (Fig. 6). Although the acid extracts of hemolymph proteins from *B. mori* larvae infected with ODN (Fig. 6B, lane 1) and the positive fractions (Fig. 6B, lane 2) obtained from GPC revealed positive signals, the three peaks obtained after anion exchange HPLC did not show any antigen-antibody reaction in the Western blotting analysis.

The GPC was also performed for the hemolymph proteins from *B. mori* larvae immunized with PG (Fig. 7). IL-
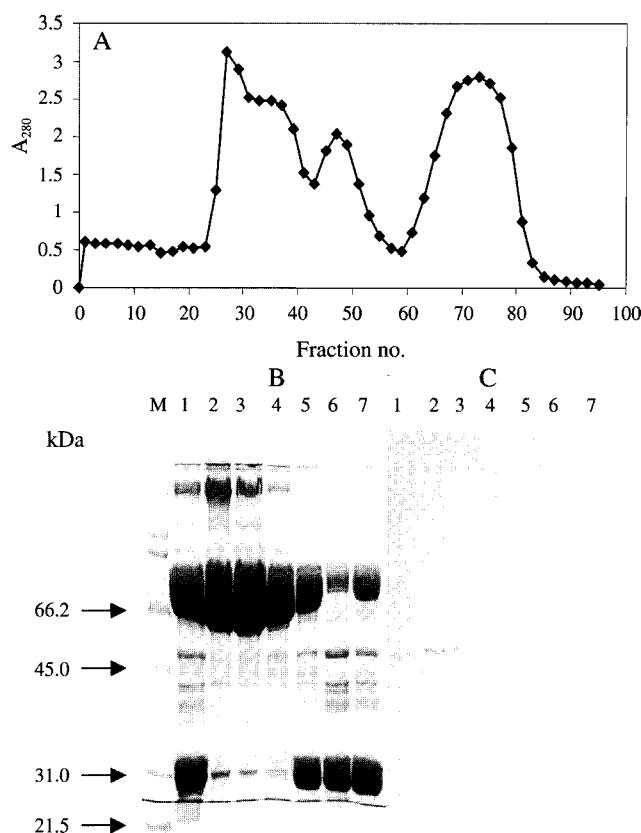

Fig. 4. The profile of gel permeation chromatography (GPC, Sephadex G-100) performed for the hemolymph proteins from *B. mori* larvae immunized with ODN (A), and SDS-PAGE (B) and Western blot analysis (C) of the fractions obtained from GPC. Fractions were eluted at 6 ml/h of flow rate. Only one % of every second fraction was loaded to each lane. M, molecular weight 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 the fraction no. 27, 29 and 31 (lanes 2, 3, 4, respectively). Thus, corresponding fractions were pooled together for the next purification step.

6-like molecules were detected at the beginning fractions of the larval proteins (fractions 21, 23 and 25) in the Western blotting analysis (Fig. 7C) and these fractions were pooled for the analysis with anion exchange chromatography. The pooled fractions, which showed positive signals in the Western blotting analysis, resulted in three separate peaks (P1, P2, and P3) in the anion exchange chromatography (Fig. 8), but no signal was detected in the Western blotting analysis (data not shown). Considering these results, it appears that the silkworm hemolymph infected with immune inducers (*e.g.*, ODN and PG) truly induce IL-6-like molecule, but undetectable in the Western blotting analysis. Possible cause of failure to detect IL-6-like molecule may include small quantity of the mol-
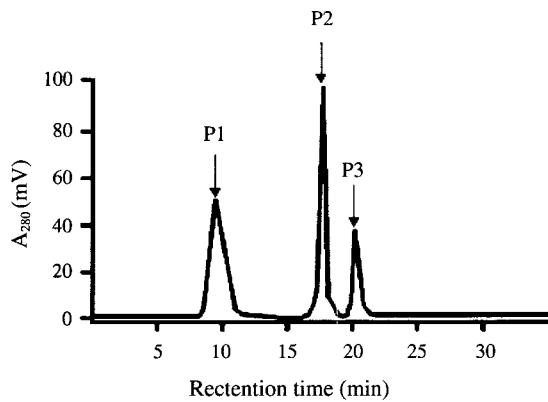
Fig. 5. Profile of anion exchange chromatography of the reacted fractions (no. 27 − 30) obtained from GPC. Chromatography was performed in HPLC system equipped with anion exchange column (Vydac). Three peaks (P1, P2 and P3) were separately collected and subjected to Western blotting analysis.
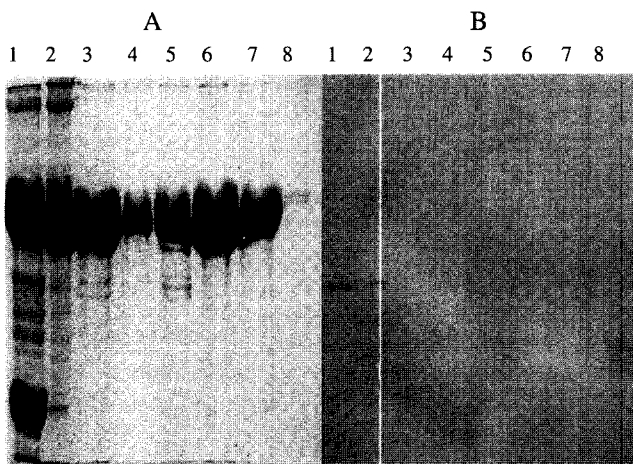

Fig. 6. SDS-PAGE and Western blotting analysis of the proteins of three peaks eluted from anion exchange chromatography. (A) SDS-PAGE gel stained with coomassie blue. Lane 1, acid extracts of hemolymph proteins from *B. mori* larvae infected with ODN; lane 2, fractions (no. 27 − 30) from GPC; lane 3, 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).

Fig. 7. The profile of gel permeation chromatography (GPC, Sephadex G-100) performed for the hemolymph proteins from *B. mori* larvae immunized with PG (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. Lane 1, acid extract of immunized hemolymph with PG; lane 2, fraction no. 21 of GPC; lane 3, no. 23; lane 4, no. 25; lane 5, no. 27; lane 6, no. 29; and lane 7, no. 31. As in the case of fractions from GPC performed with hemolymph proteins from larvae injected by ODN, positive bands were detected in the early fractions such as no. 21, 23 and 25 (lanes 2, 3, 4, respectively). Thus, corresponding fractions were pooled together for the next purification step.

ecule in the silkworm hemolymph or change of the molecule during purification process. Beck and Gabicht (1996) also reported difficulty to detect IL-6-like activity on crude coelomic fluid of *A. forbesi* supernatants. Thus, they performed dialysis of coelomic fluids by scrutinized steps including untra-centrifugation. In the case of silkworm, it seems that the portion of IL-6-like molecule in the hemolymph might be extremely low compared with
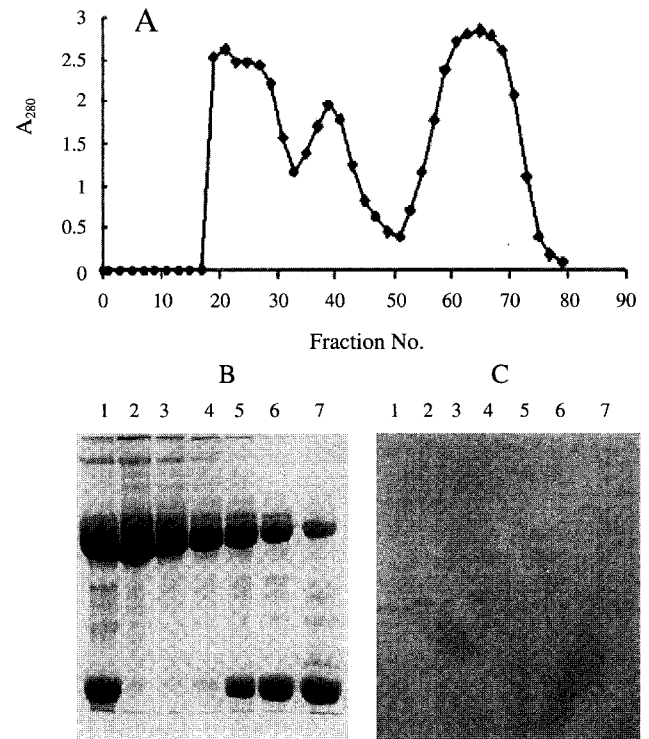
other similar sized-proteins. Another possible reason for the difficulty might be change of the protein structure during purification steps, particularly by anion exchange chromatography, which unavoidably requires salt.

Because we thought that the salt, which can be unavoidably added to the protein samples during anion exchange chromatography, might be one possible source of failure for the detection during SAD-PAGE, we reloaded the major peaks obtained by anion exchange chromatography to the gel GPC with the intention to avoid the chance of exposure of the protein to the salt during anion exchange chromatography. Fig. 9 shows the three peaks isolated by reloading the fractions obtained in the anion exchange chromatography and Fig. 10 shows the result of SDS-PAGE and Western blotting analysis of the each peak
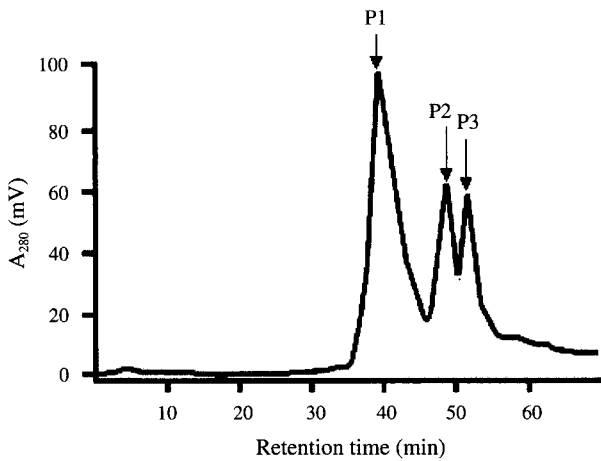
Fig. 8. Profile of anion exchange chromatography of the reacted fractions (no. 21 − 24) obtained from GPC. Chromatography was performed in HPLC system equipped with anion exchange column (Vydac). As in the case of fractions from GPC performed with hemolymph proteins from larvae injected by ODN, three peaks (P1, P2 and P3) were separately obtainable and subjected to Western blotting analysis.
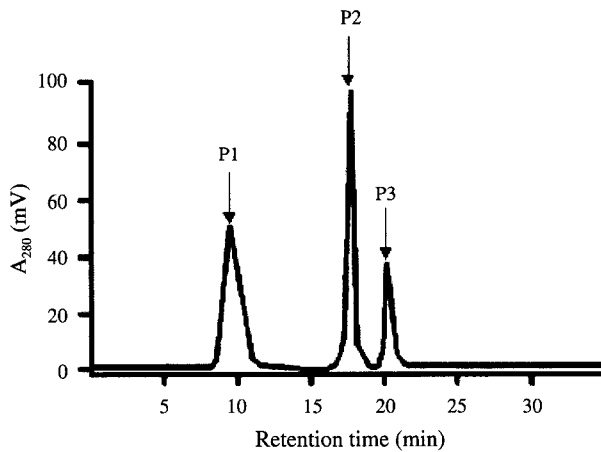

Fig. 9. Profile of GPC performed with proteins in peak 1 from anion exchange chromatography (Fig. 8). Three peaks were separately eluted and each one was subjected to Western blotting analysis.

(lanes 3, 4, and 5 in Fig. 10). Although the acid extracts of hemolymph proteins from *B. mori* larvae infected with PG (Fig. 10B, lane 1) and the positive fractions (no. 21 − 24) obtained from GPC (Fig. 10b, lane 2) revealed positive signals, the three peaks obtained after GPC showed no antigen-antibody reaction (Fig. 10B, lanes 3, 4, and 5), suggesting that the positive fractions are lost or degraded in this stage regardless of salt.

As a next experiment, we selected KBr ultra-centrifugation method. The immunized hemolymph of silkworm larvae was subjected to centrifugation at 50,000 g for 16 hrs and it provided a total of five separate fractions, con-
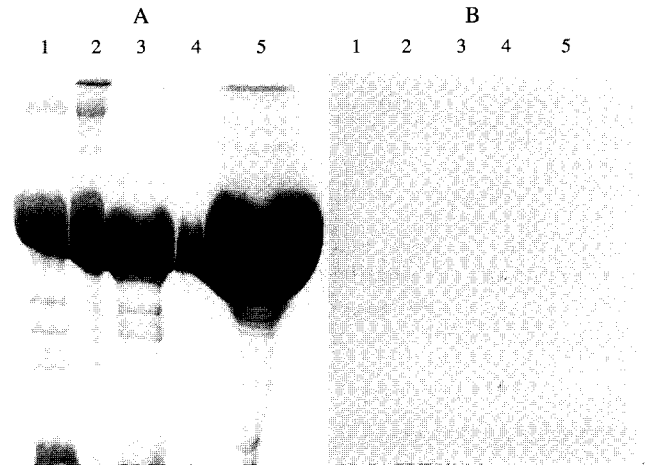

Fig. 10. SDS-PAGE and Western blotting analysis of the proteins of three peaks eluted from GPC. (A) SDS-PAGE gel stained with coomassie blue. Lane 1, acid extracts of hemolymph proteins from *B. mori* larvae infected with PG; lane 2, fractions (no. 21 − 24) from GPC; lane 3, P1 from GPC; lane 4, P2; and lane 5, 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).
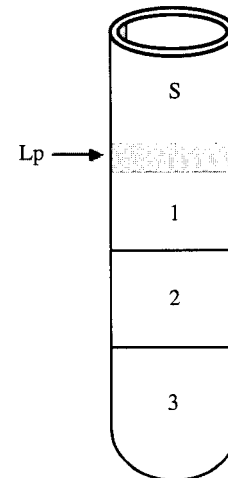

Fig. 11. KBr density gradient ultracentrifugation with hemolymph of *B. mori* larvae infected with PG. Gray band indicates lipophorin (Lp)-containing part. Five parts including lipophorin fraction were separately removed into each tube: supernatant fraction (S) over Lp part, Lp, 2 ml fraction (part 1) below Lp, 2 ml fraction (part 2) below part 1 and lower fraction (part 3) below part 2.

sisting of one above Lipophorin (Lp) and three below Lp (Fig. 11). The five parts including lipophorin fraction were separately removed into separate tube and subjected to dot-blotting analysis with an antibody against human IL-6. Among these, the second below Lp (part 2 in Fig. 11; number 4 in Fig. 12) showed clear positive reaction. This part of fraction (part 2) was subjected to GPC (Fig.
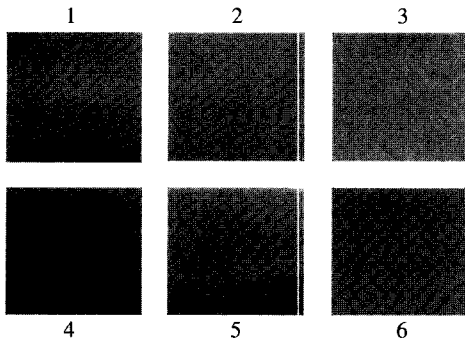
Fig. 12. Dot blotting analysis with Ab against human IL 6: sheet 1, hemolymph proteins; sheet 2, Lp fraction removed from KBr density gradient ultracentrifugation; sheet 3, part 1; sheet 4, part 2; sheet 5, part 3; and sheet 6, supernatant part.
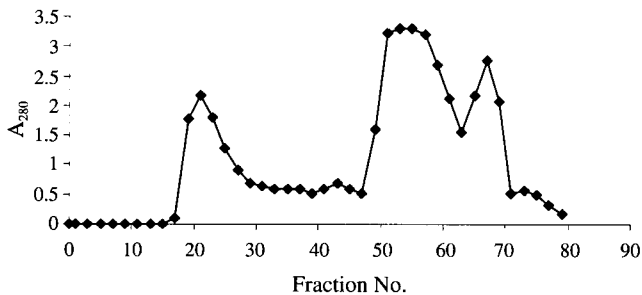

Fig. 13. The profile of gel permeation chromatography (Sephadex G-100) performed with fraction of part 2 from KBr gradient ultracentrifugation tube.
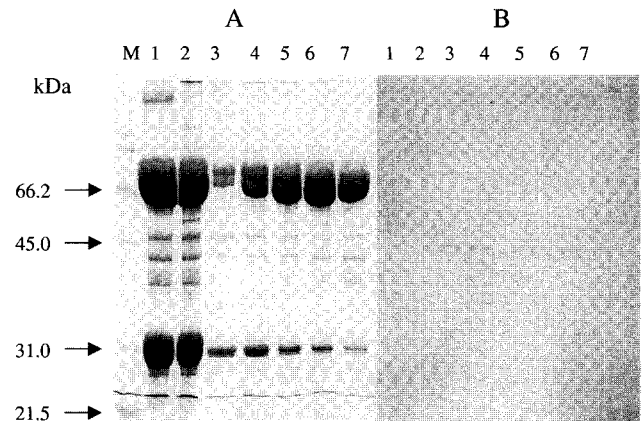

Fig. 14. SDS-PAGE and Western blotting analysis of the fractions from gel permeation chromatography (GPC). (A) SDS-PAGE gel stained with coomassie blue. Only one % of every second fraction was loaded to each lane. M, molecular weight markers; lane 1, acid extract of immunized hemolymph with PG; lane 2, part 2 from ultra-centrifugation; lane 3, no. 21 from GPC; lane 4, no. 23; lane 5, no. 25; lane 6, no. 27; and lane 7, no. 29. (B) Western blotting analysis with duplicate gel of (A). Note positive bands were detected only in the fraction no. 21, 23, and 25. Still we were not able to discern a corresponding band on SDS-PAGE gel to positive band confirmed in the Western blotting analysis.

13), and the fractions were analyzed for antigen-antibody reaction by Western blotting analysis as the same manner before. As shown in the previous experiment, positive signal was also detected at early fractions (Fig. 14B). However, we were unable to identify the reacted band in the SDS-PAGE gel (Fig. 14A), which can be utilized for the subsequent experiment such as N-terminal amino acid sequencing and MALDI-Top Mass.

In summary, we attempted to isolate the molecule by several purification methods such as anion exchange chromatography, GPC, anion exchange chromatography, ultracentrifugation, and immuno-dot-blot assay, but until now the effort was not much successful yet. However, it does not appear that the IL-6-like molecule is a mere experimental artifact happened by Western blotting analysis with human antibody. Thus, further experiment on this subject probably will provide us more fruitful result as detected in other invertebrates including insects.

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