Purification and characterization of an insect haemolymph protein promoting \textit{in vitro} replication of the \textit{Bombyx mori} nucleopolyhedrovirus

Toshimichi Kanaya\textsuperscript{1} and Jun Kobayashi\textsuperscript{2}

\textsuperscript{1}Chuo-Sanken Laboratory, Katakura Industries Co. Ltd, 1548 Shimo-Okutomi, Sayama, Saitama 350-1332, Japan
\textsuperscript{2}Laboratory of Molecular Bioengineering, Faculty of Engineering, Mie University, 1515 Kamihama-cho, Tsu, Mie 514-8507, Japan

We have identified a novel protein that promotes \textit{Bombyx mori} nucleopolyhedrovirus (BmNPV) replication \textit{in vitro}. This protein was purified from heat-treated haemolymph of \textit{B. mori} larvae by gel filtration and ion exchange chromatography, and designated as promoting protein (PP). The molecular mass of native PP estimated by column chromatography and that of denatured PP estimated by SDS–PAGE were 9600 Da and 15 200 Da, respectively, suggesting that native PP is composed of a single polypeptide and may behave in the column as if it is a smaller protein because of its conformation and/or adsorptive nature. Addition of the PP to the culture medium of SES-BoMo-15A cells derived from \textit{B. mori} embryos resulted in the strong promotion of BmNPV replication. The promoting activity positively correlated with the amount of PP in the culture medium up to 1 \(\mu\)g/ml, above which maximum virus replication occurred and resulted in the highest budded virus production and polyhedrin promoter-mediated luciferase gene expression of 10 000-fold and 6000-fold higher than those without PP, respectively. A cDNA encoding the PP precursor (prePP) was successfully cloned and sequenced. Comparison between the amino acid sequence deduced from the nucleotide sequence of prePP cDNA and the N-terminal 18 amino acids determined for the purified PP indicated that the prePP (154 amino acids) consisted of a mature PP polypeptide (136 amino acids) with a signal sequence at the N terminus. Recombinant PP expressed from the cDNA using a baculovirus vector was similar in molecular mass, immunoreactivity and promoting activity to the native PP.

Introduction

Most of the recent studies on both basic and applied aspects of nucleopolyhedroviruses (NPVs) in the family \textit{Baculoviridae} have been performed by utilizing a variety of insect cell lines highly permissive to the NPVs. For these cell lines, the composition of the culture medium has been recognized as one of the important factors affecting gene expression and replication of the NPVs (Granados & McKenna, 1995). In the case of \textit{Bombyx mori} nucleopolyhedrovirus (BmNPV), its infection of the silkworm cell line Bm-N cultured in BML-TC/10 medium (Gardiner & Stockdale, 1975) was affected by the concentration of foetal bovine serum (FBS), suggesting that factor(s) promoting BmNPV infection were contained in FBS (Watanabe, 1987). Inoculation of BmNPV into another silkworm cell line, SES-BoMo-15A (BoMo; Inoue & Mitsuhashi, 1988), cultured in the inexpensive and simply formulated Mitsuhashi and Maramorosch (MM) medium (Mitsuhashi & Maramorosch, 1964) containing 3\% FBS, resulted in the production of only a negligible number of polyhedra. In contrast, the same cell line cultured in MGM-443 medium (Mitsuhashi, 1984) containing 3\% FBS produced a large number of polyhedra following BmNPV infection (Watanabe \textit{et al}., 1988). These results suggested that the MM medium was inadequate to support sufficient BmNPV replication and polyhedrin gene expression in BoMo cells. Further study revealed that even in MM medium containing 3\% FBS, when the medium was supplemented with 10\% heat-treated (60 °C, 1 h) silkworm haemolymph, a large number of polyhedra were formed in BoMo cells (Inoue \textit{et al}., 1989). In earlier works of lepidopteran tissue and cell culture, 5–10\%
heat-treated (60 °C, 3–5 min) silkworm haemolymph was customarily added to the medium because of its positive effects on cell growth and long-term survival (Wyatt, 1956; Grace, 1958, 1962, 1967). Thus, there must be factor(s) in silkworm haemolymph that promote insect cell growth and/or NPV replication, although none of them have been previously characterized. Recently we have started to isolate such factors to understand their functions in detail and utilize them for the improvement of baculovirus–insect cell culture systems.

In this paper, we describe the purification of a haemolymph protein of silkworm that promotes BmNPV replication and polyhedrin promoter-mediated gene expression in vitro. In addition, we report the successful cloning and sequencing of the cDNA encoding this novel insect haemolymph protein.

**Methods**

- **Insect cell culture and virus.** Larvae of the silkworm, *Bombyx mori*, reared on an artificial diet (Katakurabi Industries Co. Ltd) at 25 °C were used for collection of haemolymph. The silkworm cell line SES-BoMo-15A (BoMo), cultured in MM medium containing 5% FBS, was used to assay for promoting factor(s) in silkworm haemolymph. For virus titration and construction of the recombinant BmNPV, another silkworm cell line, NISES-BoMo-1507 (BoMo-II; Inoue et al., 1997), cultured in MGM-448 medium containing 10% FBS was used.

A recombinant BmNPV, CPd-luci (Suzuki et al., 1997), was used for estimating virus replication and the level of polyhedrin promoter-mediated gene expression by measuring luciferase activity. In this recombinant virus, the luciferase gene from the firefly, *Photinus pyralis* (de Wet et al., 1987), has been introduced just downstream of the polyhedrin promoter, and the viral cysteine protease gene (vcath) has been deleted.

- **Purification of the promoting protein (PP).** Several abdominal prolegs of the fifth instar larvae 2 days after ecysis were cut off, and haemolymph was collected into a tube on ice. After incubation in a water bath at 65 °C for 30 min, the haemolymph was centrifuged at 12,000 g for 20 min. The supernatant was filtered through a 0.45 µm mesh and stored at −80 °C. To separate protein components in the supernatant, gel filtration chromatography was carried out at 4 °C as follows. The stored supernatant (10 ml) was applied to a Sephadex G-200 column (ø 2.5 × 40 cm), pre-equilibrated with 10 mM phosphate buffer containing 200 mM NaCl, pH 7. Elution was performed with the same buffer at a flow rate of 20 ml/h and fractions containing 5 ml of effluent were collected. Those fractions that were positive in the assay for promoting activity were further separated by ion exchange chromatography performed at room temperature using an HPLC system (JASCO Co.) as follows. The positive fractions were applied to Biofine CM (JASCO Co.) pre-equilibrated with 10 mM phosphate buffer containing 200 mM NaCl, pH 7, at a flow rate of 1 ml/min. After washing the column with the same buffer for 15 min, the elution was performed with a linear gradient of 0–2 M NaCl for 25 min, and fractions containing 2.5 ml of effluent were collected. For each fraction, promoting activity was assayed and protein components were analysed by SDS–PAGE.

- **Assays for promoting activity.** During the purification of PP, promoting activities of (i) haemolymph, (ii) its chromatographic fractions and (iii) the purified PP were assayed using BoMo cells cultured in the medium containing one of the above and infected with recombinant BmNPV (CPd-luci) by measuring and comparing luciferase activities in the cells and/or virus titres in the culture supernatants as follows.

For assaying promoting activity of the silkworm haemolymph and its chromatographic fractions, BoMo cells were suspended at a density of 2 × 10⁶ cells/ml in MM medium containing 3% FBS, and 200 µl of the suspension was seeded in each well of a 96-well plate (Nunc). Then, 10 µl of heat-treated haemolymph or its fractions were added to the respective wells, and the cells in each well were inoculated with the recombinant BmNPV, CPd-luci, at an m.o.i. of 3. After 5 days of incubation at 25 °C, the cells were harvested from each well and used in the luciferase assay.

For assaying promoting activity of the purified PP, 10 suspensions of BoMo cells at a density of 2 × 10⁶ cells/ml in MM medium containing 3% FBS and different concentrations of the purified PP (0, 0.001, 0.005, 0.01, 0.05, 0.1, 1, 5 and 10 µg PP/ml) were prepared. Then, CPd-luci was inoculated into each cell suspension at an m.o.i. of 3. After 2 h of incubation at 25 °C, the medium containing the virus in each suspension was removed by centrifugation at 3000 g for 10 min. The cells were washed twice with MM medium and resuspended in the same volume of fresh media containing respective concentrations of PP as those before virus inoculation. One ml of each cell suspension was transferred into a well of a 24-well plate. The plates were incubated at 25 °C. At 5 days post-inoculation, the cells and medium in each well were harvested together and separated by centrifugation at 3000 g for 10 min.

- **Luciferase assay.** The pellets of BoMo cells were lysed in cell lysis buffer (25 mM HEPES pH 7, 2 mM DTT, 2 mM 1,2-diaminocyclo-hematin, 10% glycerol, 1% Triton X-100) at room temperature for 20 min, and then centrifuged at 10,000 g for 10 min. One hundred µl of substrate mixture (50 mM HEPES pH 7.5, 15 mM MgSO₄, 5 mM ATP, 1 mM Luciferin) was added to 50 µl of supernatant of the cell lysate in a luminometer cuvette by an auto-injector, and the luciferase activity was measured using a luminometer (Rurmat, Berbol Japan Co.) as relative light units (RLU) per 10 s.

- **Virus titration.** BoMo-II cells (10⁴) suspended in 50 µl of MGM-448 medium containing 10% FBS were seeded in each well of a 96-well plate. Tenfold serial dilutions of the culture supernatant of BoMo cells infected with CPd-luci were prepared with the same medium and 50 µl of each dilution was added to a well (eight wells for each dilution). The plates were incubated at 25 °C for 7 days. The number of wells containing infected BoMo-II cells showing obvious cytopathic effects were counted by microscopic observation to calculate the TCID₅₀ of each culture supernatant according to Reed & Muench (1938).

- **Determination of protein concentration.** Protein concentration was determined using the DC protein assay kit (Bio-Rad) with BSA as a standard.

- **SDS–PAGE.** This was carried out according to Laemmli (1970) using a 15% polyacrylamide separating gel. The proteins resolved in the gel were detected by a silver staining kit (Wako).

- **Molecular mass determination of PP.** The molecular mass of the native PP was determined by comparing the relative mobility of the purified PP to marker proteins in the LMW Electrophoresis Calibration kit (Pharmacia) using Biofine PO-60 (JASCO Co.) column chromatography. The molecular mass of the denatured PP was estimated by SDS–PAGE.

- **Determination of the N-terminal amino acid sequence of PP.** The purified PP resolved in an SDS-polyacrylamide gel was blotted onto a PVDF membrane (Bio-Rad) using a Trans-Blot transfer cell (Bio-Rad) and stained with Coomassie brilliant blue R250 (Merck). The band of PP was cut out and used for direct sequencing of N-terminal amino acids by a peptide sequencer (ABI, model 473A).

- **Cloning of PP cDNA.** The first-strand cDNA was synthesized using reverse transcriptase and oligo-dT from total RNA purified from the fat
bodies of fifth instar larvae using ISOGEN-LS (Nippongene). For the amplification of PP cDNA, a PCR reaction with degenerate primers 5’ gaStYaaYgtNgt3’ (S = c and g; Y = c and t; and N = a, c, g and t), corresponding to nucleotide sequences deduced from the N-terminal amino acid sequence of PP, and oligo-dT was performed using the TAKARA RNA LA PCR kit (Takara). The PCR products were ligated into pBluescript II SK — and sequenced by the chain termination method using a DNA sequencing kit (ABI) to identify the PP cDNA. The upstream region of the cDNA including the 5’ untranslated region and the coding region for the putative signal sequence of the PP was synthesized by the 5’ RACE method using three primers: 5’ tatgtggtcatacag3’, 5’ atacaatgtgagaagattta3’ and 5’ ggtgctgtgctgaacagaggg3’ (complementary to nt 530–550, nt 498–520 and nt 165–189, respectively, see Fig. 5). The DNA fragments obtained by the 5’ RACE method were ligated into pBluescript II SK — and sequenced to confirm the nucleotide sequence within overlapping regions. To exclude PCR-derived errors, the PP cDNA sequence was determined based on the predominant sequence among the 10 clones, which were sequenced completely in both directions.

**Construction of a recombinant BmNPV expressing PP cDNA.** A cDNA clone containing the whole coding region of PP was synthesized by RT–PCR using two oligoDNA primers, 5’ aagtagctagcagatgcgtc3’ (corresponding to nt 1–20 in Fig. 5) and 5’ atacaatgtgagaagattta3’ (complementary to nt 497–519 in Fig. 5), and inserted into a transfer vector plasmid, pBM050 (Maeda, 1989). The recombinant plasmid DNA was purified by a plasmid purification column (QIAGEN) and mixed with the linearized CPd viral DNA. The mixture was cotransfected to BoMo-II cells using lipofectin reagent (GIBCO-BRL). A recombinant virus producing PP was screened and purified by the endpoint dilution method on 96-well plates (Maeda, 1989). The structure of the recombinant virus was confirmed by both sequence analysis of the inserted DNA fragment and restriction analysis of the recombinant virus.

**Preparation of rat antiserum against PP.** Purified PP (600 µg) was emulsified with Freund’s complete adjuvant and injected subcutaneously into the leg of a rat. A booster injection with 500 µg of PP was performed 2 weeks after the first injection and blood was collected 2 weeks later. The serum was stored at —80 ºC.

**Immunoblot analysis of native and recombinant PP.** The purified native PP and the culture supernatant of BoMo-II cells infected with the recombinant BmNPV CPd-PP were resolved by SDS–PAGE and blotted electrophoretically onto a nitrocellulose membrane using a Trans-Blot transfer cell (Bio-Rad). After blocking with 5% skim milk in PBS for 60 min, the membrane was incubated for 60 min in appropriately diluted rat antiserum raised against the purified native PP as above, and then washed with PBS containing 0.1% Tween 20 (PBST). The membrane was further incubated in PBST containing peroxidase-conjugated goat anti-rat IgG (Fab)2 fragment (1:1000 dilution, Wako) for 60 min. After washing, the immunoreactive bands were visualized with an ECL detection kit (Amersham).

**Results**

**Isolation of a protein component of the silkworm haemolymph in the chromatographic fractions with promoting activity**

Components in the heat-treated haemolymph were separated by a Sephadex G-200 column for 360 min and divided into fractions containing 5 ml of the effluent. Each fraction was assayed for activity promoting luciferase gene expression by the recombinant BmNPV (CPd-luci), and obvious promoting activities were observed in three successive fractions from 195 to 240 min (Fig. 1). SDS–PAGE analysis revealed that molecular masses of the major protein components contained in the three fractions ranged from 10 to 40 kDa (see Fig. 3, lane 3). The fractions were then applied to a Biofine CM column, eluted with a 0.2–1 M NaCl linear gradient for 25 min and divided into fractions containing 2.5 ml of the effluent. Among eight fractions, five fractions (nos 4–8) showed strong promoting activities (Fig. 2A). SDS–PAGE analysis revealed that the five fractions contained a 15 kDa protein in common as a major or even the sole component, while no band or only a faint band of this protein was detected in the other fractions (Fig. 2B). This strongly suggested that the 15 kDa protein could be the factor responsible for the promoting activity.

Comparison of promoting activities per µg protein among heat-treated haemolymph and positive fractions obtained by gel filtration and ion exchange chromatography demonstrated that the sequential chromatographic steps effectively increased the specific activity per protein (Table 1). In fact, by adding 1 µg of protein components from heat-treated haemolymph, Sephadex G-200 fractions and Biofine CM fractions to the CPd-luci-infected BoMo cell culture, 227-, 322- and 541-fold increases in luciferase activities were obtained, respectively.

**Characterization of the purified PP**

Using the purification procedure established above, 1.75 mg of the 15 kDa protein, designated as PP (Fig. 3, lane 2), was successfully purified from 150 ml of heat-treated haemolymph from fifth instar larvae (day 2) to homogeneity.

![Fig. 1. Elution of the promoting factor(s) from a Sephadex G-200 column and analysis of the promoting activity of the fractions by the luciferase assay. Ten ml of heat-treated haemolymph was loaded on the Sephadex G-200 column, and proteins were eluted at a flow rate of 20 ml/h. The A280 profile of the effluent is shown by the solid line. The promoting activity of the fractions by luciferase assay (vertical bars) indicated that the promoting factor(s) eluted between 195 and 240 min.](image-url)
Fig. 2. Purification of the promoting factor(s) by ion exchange chromatography. (A) Elution of the promoting factor(s) from a Biofine CM column by an NaCl gradient. The fractions containing the promoting factor(s) in the first gel filtration step (Fig. 1) were loaded on the Biofine CM column, and the factor(s) were eluted using a 25 ml linear gradient of 0.2–1 M NaCl at a flow rate of 1 ml/min. The A280 profile of the effluent and NaCl concentration is shown by the solid and dashed lines, respectively. The promoting activity of the fractions by luciferase assay (vertical bars) showed that the promoting factor(s) eluted in the fractions between 4 and 8. (B) Analysis of the fractions by silver-stained SDS–PAGE showed that a putative PP of ca. 15 kDa (arrow) eluted in the fractions between 4 and 8 as the most abundant component.

Table 1. Comparison of promoting activity after each step in the purification process

<table>
<thead>
<tr>
<th>Purification process*</th>
<th>Luciferase activity (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>43511</td>
</tr>
<tr>
<td>A</td>
<td>98899</td>
</tr>
<tr>
<td>B</td>
<td>1402627</td>
</tr>
<tr>
<td>C</td>
<td>23520484</td>
</tr>
</tbody>
</table>

Luciferase activities in cell lysates of CPd-luci virus-infected BoMo cell cultures without additives (–), with heat-treated haemolymph containing 1 µg protein (A), Sephadex G-200 fractions containing 1 µg protein (B) and Biofine CM fractions containing 1 µg protein (C) were measured.

The molecular mass of native PP estimated by a Biofine PO-60 column was 9600 Da (Fig. 4), while that of denatured PP estimated by SDS–PAGE was 15200 Da (Fig. 3). This suggests that native PP is composed of a single polypeptide. However, the discrepancy between molecular masses of native and denatured PP was relatively large. As described later, the calculated molecular mass of mature PP polypeptide is 15266 Da, which is consistent with the molecular mass of the denatured PP estimated by SDS–PAGE. Thus, a possible explanation for the discrepancy is the influence of conformation and/or adsorptive nature on the behaviour of native PP in the column, which made us underestimate its molecular mass. In addition, PAS staining indicated that the purified PP was not glycosylated (data not shown). Using purified PP, the sequence of the N-terminal 16 amino acids, NH$_2$-EFNVVTTRLCRE-VDAS, was also determined.

Analysis of promoting activity of the purified PP

The luciferase activity in the CPd-luci-infected BoMo cells was clearly dependent on the PP concentration in the culture medium: the luciferase activity positively correlated with the PP concentration in the range of 0.001 to 1 µg/ml, reached its maximum level of 6000-times higher than that without PP at
A novel protein promoting BmNPV replication

Fig. 4. Determination of the molecular mass of native PP using a calibration curve obtained with standard proteins separated by gel filtration chromatography. The purified PP (10 µg) and standard proteins in 10 mM phosphate buffer (pH 7) containing 0.2 M NaCl were separated on a Biofine PO-60 column (JASCO Co.). Points 1, 2, 3 and 4 are ovalbumin (43 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13–5 kDa) and aprotinin (6.5 kDa), respectively. The molecular mass of native PP was estimated to be ca. 9600 Da (arrow).

1 µg/ml, and plateaued at greater than 1 µg/ml (Fig. 5). Virus titres in the culture supernatant of the CPd-luci-infected BoMo cells also showed a similar dependency on the PP concentration with the highest titres at 1 µg/ml or more of PP, approximately 10000 times higher than that without PP (Fig. 5).

**Nucleotide sequence of the cDNA encoding PP precursor and properties of recombinant PP expressed by a baculovirus vector**

Using RT–PCR and 5′ RACE methods, we obtained several partial cDNA fragments encoding the PP precursor. By combining sequence data from these fragments, we reproduced the 615 bp PP cDNA shown in Fig. 6. The cDNA contained a single long open reading frame (ORF) of 465 bp, 5′ and 3′ untranslated regions, and a poly(A) tail. In the deduced 154 amino acid sequence of the ORF, the sequence from amino acids 19 to 34 was completely consistent with the N-terminal 16 amino acids determined for the purified PP. The N-terminal 18 amino acids of the deduced sequence consisted of predominantly hydrophobic residues, such as alanine, leucine and phenylalanine. This suggests that the PP precursor encoded by the cDNA is composed of both a putative signal peptide of 18 amino acids at the N terminus and the mature PP polypeptide of 136 amino acids. The calculated molecular mass of 15266 Da for the mature PP was consistent with the estimated molecular mass of 15200 Da for the purified PP by SDS–PAGE. A putative N-glycosylation site (underlined), a putative N-glycosylation site (double underlined) and a translational stop codon (asterisk) are also indicated.

Homology searches on databases using both BLAST and FASTA indicated that PP precursor possessed relatively high similarities to several secretory protein precursors belonging to the E1 family, such as human and canine epididymal secretory protein E1 precursors, ecdysteroid-regulated 16 kDa protein precursor of Manduca sexta, group 2 allergen precursor of dust mite and bovine secretory protein E1 precursor (accession nos Q15668, Q28895, Q25481, O02380 and P79345, respectively) (data not shown).
Fig. 7. Immunoblot analysis of the native and recombinant PPs (A), and comparison of promoting activity between the native and recombinant PP by the luciferase assay (B). The recombinant PP produced by CPd-PP virus-infected BoMo-II cells and native PP purified from the silkworm haemolymph separated on a 15% SDS–polyacrylamide gel were transferred onto nitrocellulose and subjected to Western blotting using rat anti-PP serum and an ECL detection kit (Amersham). The recombinant PP (lane 1) shows the same mobility on the SDS–polyacrylamide gel as the native PP (lane 2). Luciferase activities in the cell lysates of CPd-luc-infected BoMo cells cultured without PP (a), with 1 μg recombinant PP (b) and with 1 μg native PP (c) were compared.

Discussion

In the present study, we could successfully purify a protein designated as PP (promoting protein) from silkworm haemolymph and demonstrate that both the luciferase activity and virus titre in the recombinant BmNPV (CPd-luc)–infected BoMo cell culture showed positive correlations with the PP concentration in the medium and similarly reached maximum levels at a relatively low concentration of PP (1 μg/ml). Because budded virus production occurred in the late phase of infection preceding the polyhedrin promoter-mediated luciferase gene expression in the very late phase of infection (O’Reilly et al., 1992), it is likely that the increased activity of the polyhedrin promoter is indirectly caused by the PP-induced promotion of virus replication in the BoMo cells by unknown mechanisms.

Several proteins enhancing NPV infection in vivo have been isolated from viral occlusion bodies. A protein component in the occlusion bodies of *Pseudaletia unipuncta* granulovirus (PuGV) called the synergistic factor (SF) enhanced *P. unipuncta* NPV (PuNPV) infection in the larvae (Tanada et al., 1973). The PuGV SF can also enhance NPV infections in both *P. separata* and *Spodoptera littoralis* (Hukuhara et al., 1987). The SF is a lipoprotein with a molecular mass of 126 kDa (Tanada, 1985; Uchima et al., 1989; Hukuhara & Zhu, 1989). Similarly, occlusion bodies of *Trichoplusia ni* GV contain a protein factor named enhancin that can enhance NPV infection in *T. ni* larvae (Derksen & Granados, 1988; Gallo et al., 1991). Furthermore, Lepore et al. (1996) confirmed that the 98 kDa enhancin is a metalloprotease that can digest the peritrophic membrane protein. An enhancing factor which enhanced NPV infection in larvae of the armyworm, *P. separata*, was purified from the spheroid of a *P. separata* entomopoxvirus, and characterized as a homogeneous glycoprotein of 38 kDa (Xu & Hukuhara, 1994).

The PP isolated and purified from silkworm haemolymph in this study was clearly different in molecular mass and in biochemical nature from those enhancing or synergistic proteins derived from viral occlusion bodies. The PP was identified as a single polypeptide of 15-2 kDa. In the silkworm larvae, the PP is probably synthesized in the fat body, although we have not yet examined other tissues, secreted into the haemolymph and maintained at concentrations of more than 10 μg/ml throughout the fifth instar larval stage. Thus, it is reasonable to assume that PP may play a specific role in silkworm physiology as an important ubiquitous component of haemolymph. Sequence similarities to several secretory proteins belonging to the E1 family indicate that PP may also share a function with these proteins, although none of their functions have been revealed.

The mechanism by which PP promotes virus replication in BoMo cells cultured in MM medium remains to be solved. A preliminary experiment showed that the addition of PP to BoMo cell culture induced cell agglutination (data not shown). Ohba & Tanada (1983, 1984) reported a similar agglutination of several insect cell lines induced by PuGV SF, which also enhanced PuNPV infection in cultured insect cells. Specific binding of SF to the surface of *Spodoptera frugiperda* cells and to the envelopes of PuNPV and *T. ni* NPV was visualized by immunoelectron microscopy (Hukuhara & Zhu, 1989). These results suggested that SF enhanced NPV infection in vitro by increasing virus adsorption to the cell membrane and facilitating virus entry. Thus, PP may act like SF to facilitate virus entry to BoMo cells and, as a consequence, increase the number of infected cells, although further studies are required to demonstrate this hypothesis.

We also succeeded in producing sufficient amounts of recombinant PP which is similar to native PP in promoting activity, molecular mass and immunoreactivity. This recombinant PP production system enables us to purify PP more quickly and easily than trying to use silkworm haemolymph as the starting material. Taking advantage of the recombinant PP, we are investigating the mode of PP action on BmNPV replication in BoMo cells, as well as in other baculovirus–insect cell systems. Studies aimed at identifying the natural role of PP in silkworm physiology are also in progress.
The authors thank Professor H. Watanabe (Tokyo University of Agriculture) for critical reviews of the manuscript, and Dr S. Imanishi (National Institute of Sericultural and Entomological Science) for supplying both SES-BoMo-15A and NISES-BoMo-15Alc cell lines. The authors are also indebted Mr K. Ogawa, Mr A. Usami and Dr T. Nagae in Katakura Industries Co. Ltd for their support and encouragement during this study.

References


Lepore, L. S., Roelvink, P. R. & Granados, R. R. (1996). Enhancin, the granulovirus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. *Journal of Invertebrate Pathology* 68, 131–140.


Received 23 August 1999; Accepted 17 December 1999