J. Biochem. 119, 1080-1085 (1996)

Expression of Bombyx Family Fungal Protease Inhibitor F from *Bombyx mori* by Baculovirus Vector

Thiên-Ngoc Pham,* Kaeko Hayashi,* Ryo Takano,* Hiroshi Nakazawa,† Hajime Mori,† Masatoshi Ichida,‡ Masanobu Itoh,† Masaharu Eguchi,† Fujiyoshi Matsubara,† and Saburo Hara*,[1]

**Department of Chemistry and Materials Technology, Faculty of Engineering and Design, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606; †Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606; and ‡Experimental Farms, Faculty of Textile Science, Kyoto Institute of Technology, Sagaippongi-cho, Ukyo-ku, Kyoto 616*

Received for publication, December 22, 1995

Fungal protease inhibitor F (FPI-F) from silkworm hemolymph is a novel serine protease inhibitor of the Bombyx family. The cDNA of FPI-F was introduced into a baculovirus vector and a recombinant virus was isolated and plaque-purified. The protease inhibitory activities increased in the culture medium of insect cells and in the hemolymph of silkworms infected with the recombinant virus. Judged from the behavior on ion-exchange and reversed-phase chromatographies, amino acid compositions, amino-terminal sequences, and CD spectra, the recombinant FPI-F was identical with native FPI-F. Infection with the recombinant virus caused inhibition of larval development of the silkworm. However, the degree of the effect was different in two strains, Shinryukaku and Taiheichoan, indicating that the selection of the strain of silkworm is important in using the baculovirus expression system.

Key words: baculovirus, Bombyx family inhibitor, expression, serine protease inhibitor, silkworm.

The hemolymph of the silkworm (*Bombyx mori*) includes several kinds of protease inhibitors (*1*). Among them, fungal protease inhibitor F (FPI-F), isolated from an inbred strain (C124) of the silkworm, is the smallest one, with a molecular weight of 6,100 (*2*). FPI-F inhibits proteases produced by fungi such as *Aspergillus melleus* and *Beauveria bassiana* as well as subtilisin, but does not inhibit bovine trypsin or chymotrypsin (*2*). *B. bassiana* is highly pathogenic to the silkworm. Since FPI-F is found not only in the hemolymph, but also in the integument, which is the first barrier against invading microorganisms, FPI-F seems to play a substantial role in the defense system of silkworms by the suppression of proteases of invaders.

The amino acid sequence of FPI-F has been established (*3*) and the positions of the reactive site and disulfide bonds of this inhibitor have also been determined (*4*). Based on the results, FPI-F was designated as a member of the Bombyx family of inhibitors. FPI-F consists of 55 amino acid residues, including eight cysteines forming four disulfide bonds, which seem to afford a rigid structure stable to heat and acid.

For further characterization, physicochemical studies and the development of applications of the inhibitor, a large-scale supply of FPI-F is necessary. Since purification of the inhibitor from the inbred strain C124 of the silkworm is not convenient because of the low content of FPI-F and the silkworm's small body size, we attempted to achieve high-level expression of FPI-F. In this paper, we describe the expression of FPI-F in insect cells and silkworm larvae by using a baculovirus gene expression system.

MATERIALS AND METHODS

Materials—*A. melleus* semi-alkaline protease from Amano Chemicals (Nagoya) and subtilisin BPN′ from Wako Pure Chemical Industry (Osaka) were used. Fetal calf serum (FCS) and the culture media, TC-100 and TC-900, were from Gibco BRL (Gaitherburg, MD, USA). A linearized DNA from AcNPV (Baculogold™) was purchased from Pharmingen (San Diego, CA, USA). AcNPV was kindly donated by Professor David Bishop (NERC Institute of Virology and Environmental Microbiology, Oxford, UK) and a hybrid virus (HyNPV) of AcNPV and BmNPV, which is infectious to both *Spodoptera frugiperda* and *B. mori* was prepared in our laboratory (*5*). Native FPI-F was purified from the hemolymph of inbred strain C124 of silkworm (*3*).

Insect—Two strains of the silkworm, an inbred strain Shinryukaku and a hybrid strain Taiheichoan (Taihei × Choan), were used in this study. The larvae of strain Shinryukaku were reared on mulberry leaves at 25°C. The larvae of strain Taiheichoan were aseptically reared on an

[1] To whom correspondence should be addressed.

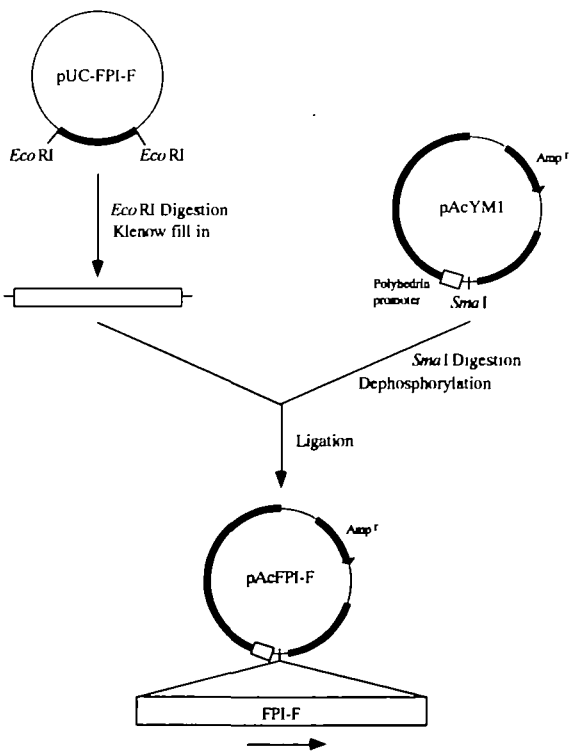
Abbreviations: AcFPI-F, recombinant AcNPV carrying the cDNA of FPI-F; AcNPV, *Autographa californica* nuclear polyhedrosis virus; BmNPV, *Bombyx mori* NPV; FCS, fetal calf serum; FPI-F, fungal protease inhibitor F from silkworm; HyFPI-F, recombinant HyNPV carrying the cDNA of FPI-F; HyNPV, hybrid virus of AcNPV and BmNPV; MCA, 4-methylcoumaryl-7-amide; NPV, nuclear polyhedrosis virus; PFU, plaque forming unit; PTH, phenylthiohydantoin; Sf21, *Spodoptera frugiperda* cell line IPLB-Sf21-AE; suc, succinyl; TFA, trifluoroacetic acid.

artificial diet (Nikko Marketing) by the method of Matsubara *et al.* (*6*).

Cell Line—The *S. frugiperda* cell line IPLB-Sf21-AE (Sf21) was used and maintained in TC-100 medium containing 10% (v/v) FCS in tissue culture flask (*7*).

Construction of Recombinant Baculovirus—A cDNA encoding FPI-F was isolated from a library which was constructed with poly(A)$^+$ RNA extracted from the fat body of day 8 fifth-instar larvae of strain C124 (*8*). The cDNA fragment encoding the entire coding region of FPI-F with the signal peptide sequence consisting of 22 amino acid residues was subcloned into the plasmid pUC19. The cDNA insert was excised from pUC19 vector by digestion with *Eco*RI, blunt-ended using a DNA blunting kit (Takara Biomedicals), and cloned into the *Sma*I site of a pAcYM1 vector (*9*). The recombinant transfer vector was constructed as shown in Fig. 1 to express FPI-F under the control of the polyhedrin promoter of baculovirus.

The orientation of the inserted cDNA in the pAcYM1 vector was confirmed by nucleotide sequence analysis (Applied Biosystems, model 373A). Sf21 cells cultivated in TC-100 medium containing 10% (v/v) FCS were transfected with 1 μg of a circular viral DNA from HyNPV and 14.9 μg of the recombinant transfer vector DNA, or 0.5 μg of a linearized viral DNA from AcNPV and 4.3 μg of the recombinant transfer vector DNA, using lipofectin (Gibco BRL). After incubation at 27°C for 5 days, the culture medium was collected and subjected to plaque assay. Recombinant HyNPV (HyFPI-F) and recombinant AcNPV (AcFPI-F) were selected by picking polyhedra-negative plaques, respectively. Three rounds of viral amplification were performed to obtain a high-titer stock solution (5×10^7 PFU/ml).



ATGGCTTCGAAAAATCTGTTTGTATTGTTTTTCATATTCGCTTTGTTCGCTGCGAATATTGCAGCTCTACAA
M A S K N L F V L F F I F A L F A A N I A A L Q
TGTCCGAAAAACAGTGAGGTTCGTAACAGCCCCTGCCCAAGAACTTGCAATGACCCTTATGGCCAGAACAGT
C P K N S E V R N S P C P R T C N D P Y G Q N S
TGCATAACGGTAATAAGAGAAACGTGCCACTGTAAAGGCGAACTGGTTTTCGATTCGGACAGTATCTGCGTT
C I T V I R E T C H C K G E L V F D S D S I C V
CCTATATCCCAATGCTGA
P I S Q C *

Fig. 1. **Construction of recombinant transfer vector.** The cDNA insert was excised from pUC19 by digestion with *Eco*RI and cloned into the dephosphorylated *Sma*I site of baculovirus transfer vector pAcYM1. The nucleotide sequence of the open reading frame of cDNA encoding FPI-F is indicated.

Expression of Recombinant FPI-F—Wild-type AcNPV-infected, AcFPI-F-infected, and mock-infected Sf21 cells were maintained in 4 ml of TC-900 media without FCS to avoid disturbance of the assay by inhibitory activity caused by the high protein concentration of the medium or inhibitors derived from FCS. After the appropriate period of incubation at 27°C, the culture media and cells were separated by centrifugation. The cells were lysed with 1% Triton X-100 and then the cell lysate was obtained by centrifugation. The culture media and cell lysate were used for assay of the inhibitory activity.

Silkworm larvae (strains Shinryukaku and Taiheichoan) were inoculated with 50 μl of the stock solution of wild-type HyNPV or HyFPI-F. The larvae were reared at 25°C for an appropriate period on mulberry leaves (strain Shinryukaku) or on artificial diet (strain Taiheichoan), then the legs of larvae were cut off and the hemolymph was collected.

Assay of Inhibitory Activity—The inhibitory activities of FPI-F against subtilisin BPN′ and *A. melleus* protease were measured. The reaction was carried out in 100 mM phosphate buffer containing 0.1 M NaCl and 0.05% Triton X-100 (pH 7.0). One milliliter of 15 nM subtilisin BPN′ or 10 nM *A. melleus* protease, of which the activity had been titrated preliminarily with a known amount of FPI-F, was incubated with 1 ml of the inhibitor solution at 30°C for 5 min to form the enzyme-inhibitor complex. The reaction was initiated by addition of 0.1 ml of 20 μM Suc-Ala-Ala-Pro-Phe-MCA (Peptide Institute, Minoh) as a substrate for both enzymes. The release of 7-amino-4-methylcoumarin (AMC) was monitored by measuring the fluorescence intensity at 440 nm with excitation at 350 nm. The inhibitory activity was estimated from the residual enzyme activity and one unit of the inhibitory activity was defined as the amount of the inhibitor which can inhibit 1 nmol of the protease. The dissociation constant (K_i) of the inhibitor-protease complex was determined by the method of Green and Work (*10*).

Purification of Recombinant FPI-F—Thirty-nine larvae of strain Shinryukaku infected with HyFPI-F were reared at 25°C for 4 days. From these larvae, the hemolymph (9.4 ml) was collected and proteins were precipitated with 30-65% saturated ammonium sulfate. After dialysis, the sample was heated at 65°C for 10 min to remove heat-labile proteins as precipitates. The supernatant was put on a Mono S HR5/5 column (Pharmacia Chemical, Sweden), which was equilibrated with 20 mM sodium acetate buffer (pH 4.0). The column was eluted with a linear gradient of NaCl from 0 to 0.3 M in the equilibrating buffer for 30 min at a flow rate of 0.5 ml/min. The eluate from the column was collected by a fraction collector (1 ml), and the absorbance at 280 nm and the subtilisin inhibitory activity of the fractions were measured. Hemolymphs from wild-type HyNPV-infected and mock-infected larvae of strain Shin-

ryukaku were also pretreated and chromatographed on a Mono S column HR5/5 under the same conditions. The inhibitor fraction was further purified on a Develosil ODS-5 (4.6×150 mm) (Nomura Chemical) by HPLC (Irica, model 852). The column was eluted with a linear gradient of acetonitrile from 0 to 60% in 0.1% TFA for 60 min at a flow rate of 1.0 ml/min. Proteins were monitored at 280 nm and the inhibitory activity was assayed for each peak.

Analyses of Amino Acid Composition and Sequence—Samples were hydrolyzed at 110°C for 24 h in sealed, evacuated glass tubes with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Wako Pure Chemical Industry, Osaka). Amino acid analyses were done with an amino acid analyzer (Hitachi, model 835). The amino acid sequence analyses were performed with an automatic gas phase sequencer (Shimadzu, model PPSQ-10).

CD Spectra—Measurements of CD spectra were performed at 25°C with a recording spectropolarimeter (Jasco J-600) using a 1-mm light path length cell under a nitrogen atmosphere. Inhibitor solution of 0.1 mg/ml in 20 mM phosphate buffer (pH 7.0) was used.

RESULTS

Construction of Recombinant Viruses—Recombinant viruses, AcFPI-F and HyFPI-F, were constructed using the recombinant transfer vector (Fig. 1). Sf21 cells cultivated in TC-100 media containing 10% (v/v) FCS were infected with wild-type or recombinant viruses. After 72 h postinfection, polyhedra were observed in the cells infected with wild-type virus, AcNPV or HyNPV. However, in the cells infected with AcFPI-F or HyFPI-F, no polyhedron was observed. It suggests that polyhedrin gene of both viruses had been replaced successfully by cDNA encoding FPI-F.

Expression of FPI-F in Sf21 Cells—For confirmation of the expression of FPI-F, Sf21 cells infected with wild-type AcNPV or recombinant virus AcFPI-F, and mock-infected cells were maintained in TC-900 media without FCS. At appropriate intervals, the cells and the culture media were separated by centrifugation. The cells obtained were lysed by treatment with 1% Triton X-100 and the lysate was collected by centrifugation. The time course of expression of recombinant FPI-F was monitored by analyzing the inhibitory activity against subtilisin BPN′. As shown in Fig. 2, a marked increase of the inhibitory activity was observed in the culture medium of cells infected with AcFPI-F, while the medium of wild-type AcNPV-infected or mock-infected cells showed no increase of the inhibitory activity. Neither AcNPV-infected cell lysate nor AcFPI-F-infected cell lysate showed any increase of inhibitory activity against subtilisin BPN′.

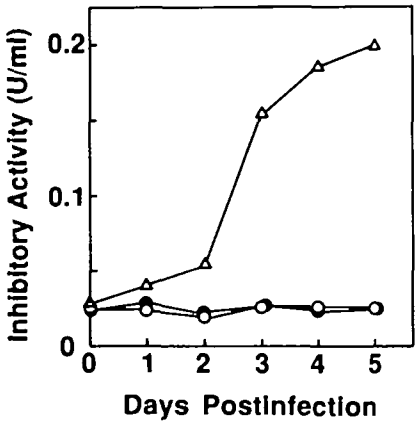


Fig. 2. **Inhibitory activity toward subtilisin BPN′ in culture media of mock-infected (○), wild-type AcNPV-infected (●), and AcFPI-F-infected (△) Sf21 cells.**

Expression of FPI-F in Silkworms—In order to attempt the expression of FPI-F in the silkworm, we used HyNPV as a baculovirus vector, because HyNPV is a hybrid virus of AcNPV and BmNPV and is able to replicate and express foreign gene products in Sf21 cells and silkworm (5). Early stage fifth-instar larvae of Shinryukaku and Taiheichoan were inoculated with wild-type HyNPV or recombinant virus HyFPI-F, or were mock-infected. The change of body weight of the silkworm larvae after injection of viruses is shown in Fig. 3. Due to the shorter period of the fifth instar stage of Shinryukaku, the weight of the mock-infected larvae of Shinryukaku reached maximum at 2 days postinfection and then decreased gradually. In Shinryukaku larvae infected with wild-type HyNPV and HyFPI-F, the weight increase was delayed. However, their weights became almost the same as that of mock-infected larvae at 4 days postinfection and no obvious differences of skin color

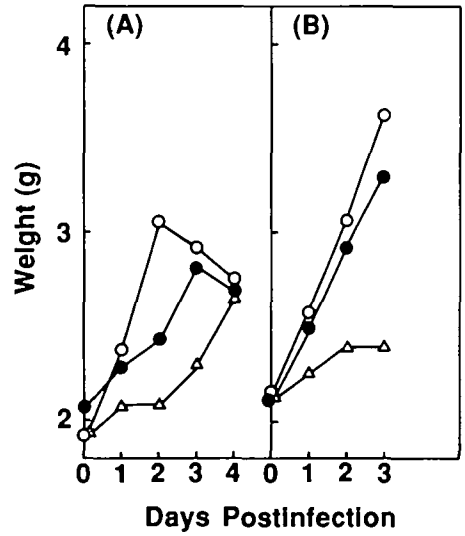


Fig. 3. **Effect of expression of FPI-F on body weight of larvae of Shinryukaku (A) and Taiheichoan (B).** ○, mock-infected; ●, wild-type HyNPV-infected; △, HyFPI-F-infected.

TABLE I. **Inhibitory activities toward subtilisin BPN′ in hemolymphs of mock-infected, wild-type HyNPV-infected, and HyFPI-F-infected silkworm larvae, strains Shinryukaku and Taiheichoan.** The hemolymphs of Shinryukaku and Taiheichoan were collected at 4 days postinfection and 3 days postinfection, respectively.

	Shinryukaku		Taiheichoan	
	No.	Activity (U/head)	No.	Activity (U/head)
Mock-infected	9	3.98	7	2.66
Wild-type HyNPV-infected	7	4.01	8	2.19
HyFPI-F-infected	9	10.50	8	5.38

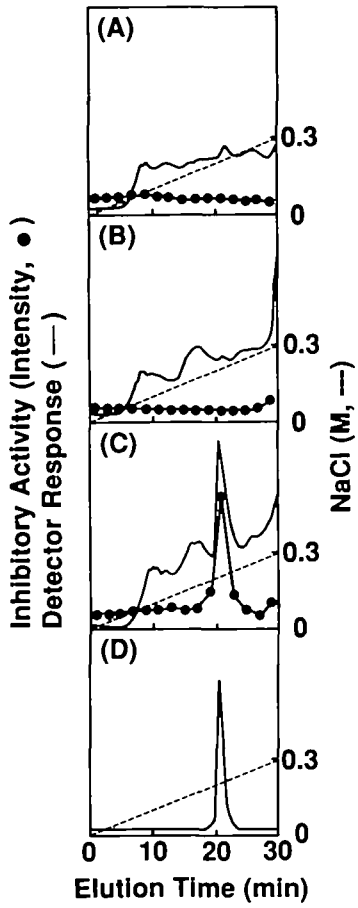


Fig. 4. **Mono S chromatography of hemolymph and native FPI-F.** Hemolymphs were collected from mock-infected (A), wild-type HyNPV-infected (B), and HyFPI-F-infected (C) larvae of Shinryukaku. After 30-65% ammonium sulfate precipitation and heat treatment of hemolymph, samples were put on a Mono S HR5/5 column equilibrated with 20 mM sodium acetate buffer, pH 4. The column was eluted from 0 to 0.3 M NaCl in the buffer for 30 min. Proteins were monitored at 230 nm. Fractions of 1 ml were collected. Native FPI-F (D) purified from larvae of strain C124 was chromatographed under the same conditions. Inhibitory activity toward subtilisin BPN′ was measured with a fluorogenic substrate as described in "MATERIALS AND METHODS."

or behavior were observed. On the other hand, the growth of the larvae of Taiheichoan infected with HyFPI-F was distinctly delayed in comparison with that of wild-type HyNPV-infected or mock-infected larvae. Furthermore, all larvae of Taiheichoan infected with HyFPI-F died at 3 days postinfection, while other larvae including strain Shinryukaku could live till 5 days postinfection. The larvae of Taiheichoan infected with HyFPI-F weakened and the skin color became slightly black, even though wild-type HyNPV-infected larvae showed no apparent change.

At 4 days or 3 days postinfection, the hemolymphs of larvae of Shinryukaku and Taiheichoan were collected and the inhibitory activity against subtilisin BPN′ was measured. The result is included in Table I. About 2.6- and 2.0-fold higher inhibitory activities than those of mock-infected silkworms were detected in the hemolymphs of Shinryukaku and Taiheichoan infected with HyFPI-F, respectively.

TABLE II. **K_i values of native and recombinant FPI-F toward subtilisin BPN′ and *A. melleus* protease.**

	K_i value (M)	
	Subtilisin BPN′	*A. melleus* protease
Native	2.3×10^{-10}	1.4×10^{-10}
Recombinant	2.3×10^{-10}	2.3×10^{-10}

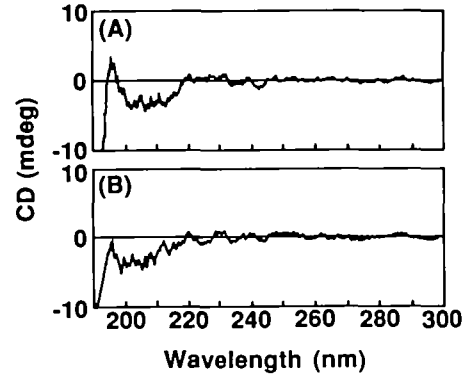


Fig. 5. **CD spectra of native (A) and recombinant (B) FPI-F.** CD spectra were measured in 10 mM potassium phosphate buffer pH 7.0 containing the inhibitor (0.1 mg/ml) at 25°C with a 1-mm light path length cell.

Identification of Recombinant FPI-F—After ammonium sulfate precipitation and heat-treatment, the hemolymph of wild-type HyNPV-infected, HyFPI-F-infected, and mock-infected larvae (strain Shinryukaku) were chromatographed on a Mono S column. The elution patterns are illustrated in Fig. 4. Because the strain Shinryukaku of the silkworm has no endogenous FPI-F (*11*), the hemolymph of mock-infected and wild-type HyNPV-infected silkworms had no inhibitory activity against subtilisin BPN′ at the same elution position as that of native FPI-F. However, the hemolymph of the Shinryukaku larvae infected with HyFPI-F showed an active peak at the same position as that of native FPI-F.

For the analyses of the amino acid composition and sequence, recombinant FPI-F obtained in Fig. 4 was further purified by C18 reversed-phase chromatography. The elution position of recombinant FPI-F on the reversed-phase chromatography was identical with that of native FPI-F. At the final step of purification, 10.8 μg of FPI-F was obtained from one head and the yield was 16.9%.

The amino acid compositions and sequences from the N-terminal to the 15th residue of the native and recombinant FPI-F coincided well (data not shown). The K_i values of native and recombinant FPI-F against subtilisin BPN′ and semi-alkaline protease from *Aspergillus melleus* are summarized in Table II. No marked difference of K_i values between native and recombinant FPI-F was observed for inhibition of subtilisin BPN′ or *A. melleus* protease.

CD spectra of native and recombinant inhibitors were almost identical, as shown in Fig. 5. These results indicate that the folding of recombinant FPI-F had been successful and that the processing site of recombinant FPI-F was the same as that of native FPI-F.

DISCUSSION

Nuclear polyhedrosis viruses are members of the subfamily *Eubaculovirinae* (family *Baculoviridae*) (*12*) and have genomes of approximately 130 kb of double-stranded circular DNA. After infection of insects, virus particles are embedded within a protein capsule (polyhedron) composed almost wholly of a single protein known as polyhedrin. At a very late stage of infection, polyhedrin constitutes 20% or more of the total protein of the infected cells, but it is not essential for virus replication. Thus, the baculovirus expression vector system is convenient for large-scale expression of foreign gene products owing to the strong polyhedrin promoter (*13*). Furthermore, this system achieves proper folding, disulfide bond formation, oligomerization, and post-translational modification of overexpressed recombinant proteins (*14*).

The system utilizing AcNPV and Sf21 cells is widely used for the production of many heterologous proteins, because of the ease of maintaining a large-scale culture of Sf21 cells in serum-free medium. Another baculovirus gene expression system, utilizing BmNPV, is advantageous for convenient, inexpensive, high-level production of heterologous proteins in silkworm larvae. Sf21 cells are favorable to prepare viruses because of their faster growth compared with silkworm cells. However, AcNPV can not be adapted to the baculovirus expression vector system using silkworms, since this virus is not infectious to the silkworm. Therefore, the use of HyNPV, which can infect both *S. frugiperda* and the silkworm is preferred. The advantageous character of the gene expression system utilizing HyNPV is supported by the rapid preparation of recombinant viruses in Sf21 cells and the inexpensive, high-level production of the protein in silkworm larvae.

We succeeded in the expression of FPI-F both using AcFPI-F in Sf21 cells and using HyFPI-F in silkworm larvae. The silkworm infected with HyFPI-F produced and secreted recombinant FPI-F into the hemolymph. Although several kinds of fungal protease inhibitors are generally included in the hemolymph of the silkworm, the strain Shinryukaku lacks FPI-F (*11*). This is the reason for the choice of the strain Shinryukaku for the expression of FPI-F. As shown in Table I, the hemolymph of the mock-infected larvae of strain Shinryukaku showed almost the same amount of subtilisin BPN′-inhibitory activity as that from larvae infected with wild-type AcNPV. These activities might be caused by fungal protease inhibitors other than FPI-F. In the hemolymph of strain Shinryukaku infected with HyFPI-F, extra inhibitory activity against subtilisin BPN′ was observed. This extra inhibitory activity reached about 6.5 units/head, which corresponds to 39.7 μg of FPI-F per head at 4 days postinfection. Maeda *et al.* reported that 4×10^7 units/ml, which corresponds to 60 μg/head, of interferon α was produced in the hemolymph of silkworm infected with recombinant baculovirus at 5 days postinfection (*15*). This indicated a production level of 100-fold more than that in the eukaryotic expression system (4×10^5 units/ml for mouse cells with a bovine papillomavirus vector) for interferon α (*15, 16*). Our result with FPI-F using the baculovirus expression system is comparable.

As the hemolymph includes several kinds of inhibitors other than FPI-F, native and recombinant FPI-F was purified from the hemolymph of the strains C124 and Shinryukaku infected with HyFPI-F, respectively. At the last step, C_{18} reversed-phase chromatography, of the purification, 1.8 and 10.8 μg of native and recombinant FPI-F were obtained from one head of the strains C124 and Shinryukaku, respectively. Thus, almost six times more recombinant FPI-F was obtained from each larva as compared with strain C124.

Judged from the amino acid compositions, the amino-terminal sequences, the inhibitory activities against subtilisin and *A. melleus* semi-alkaline protease and the CD spectra, recombinant and native FPI-F can be regarded as identical. These findings indicate proper formation of the four disulfide bonds and folding of the peptide chain. The existence of recombinant FPI-F in hemolymph of silkworm and the culture medium of Sf21 cells indicates that the secretion of FPI-F from the cells is also successful. The processing site of recombinant FPI-F was identical with that of the native molecule as judged from the results of amino-terminal sequence analyses. These results indicate that the signal peptide of 22 amino acid residues functioned correctly in the baculovirus expression system.

The infection of HyFPI-F caused growth inhibition of the larvae of both strains, Shinryukaku and Taiheichoan. However, the degree of the effect was different in the two strains. All larvae of Taiheichoan died at 3 days postinfection, while no Shinryukaku died during the 4-day observation, regardless of growth inhibition. Expression of FPI-F in the silkworm might cause a lack of balance between endogenous protease and inhibitor. Further investigation is required to test this. These results indicate the importance, in producing protein with the baculovirus expression system, of selecting the optimum strain of the silkworm, timing of the inoculation, rearing conditions and so on.

Insects are always exposed to many pathogenic microorganisms. Among them, *B. bassiana* is highly pathogenic to insects, including the silkworm. Such a fungus first germinates on the surface of the silkworm and then invades the body. Yoshida *et al.* showed that FPI-F suppresses the growth and development of conidia of *B. bassiana* (*17*). FPI-F, which exists not only in hemolymph, but also in skin, is considered to be important in the defense system of the silkworm, against pathogenic fungi.

Because of the feasibility of convenient large-scale production, the system established in this study should be useful in physicochemical investigations which require large amounts of samples and may lead to applications of FPI-F in the medicinal or agricultural fields.

We wish to thank Drs. Kenji Soda and Tohru Yoshimura of Kyoto University for measurements of CD spectra.

REFERENCES

1. Eguchi, M. (1993) Protein protease inhibitors in insects and comparison with mammalian inhibitors. *Comp. Biochem. Physiol.* **105B**, 449-456
2. Eguchi, M., Itoh, M., Chou, L.-Y., and Nishino, K. (1993) Purification and characterization of a fungal protease specific protein inhibitor (FPI-F) in the silkworm haemolymph. *Comp. Biochem. Physiol.* **104B**, 537-543
3. Eguchi, M., Itoh, M., Nishino, K., Shibata, H., Tanaka, T.,

Hayashi, K., and Hara, S. (1994) Amino acid sequence of an inhibitor from the silkworm (*Bombyx mori*) hemolymph against fungal protease. *J. Biochem.* **115**, 881-884
4. Pham, T.-N., Hayashi, K., Takano, R., Itoh, M., Eguchi, M., Shibata, H., Tanaka, T., and Hara, S. (1996) A new family of serine protease inhibitors (Bombyx family) as established from the unique topological relation between the positions of disulfide bridges and reactive site. *J. Biochem.* **119**, 428-434
5. Mori, H., Nakazawa, H., Shirai, N., Shibata, N., Sumida, M., and Matsubara, F. (1992) Foreign gene expression by a baculovirus vector with an expanded host range. *J. Gen. Virol.* **73**, 1877-1880
6. Matsubara, F., Sang, Q., Sugimori, H., Ishiko, S., Sumida, M., and Matsumoto, T. (1988) New method of silkworm rearing on an artificial diet (Two feedings throughout the larval period). *J. Seric. Sci. Jpn.* **57**, 118-122
7. Brown, M. and Faulkner, P. (1977) A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *J. Gen. Virol.* **36**, 361-364
8. Itoh, M., Takenaka, T., Ashikari, T., and Eguchi, M. (1996) cDNA cloning and expression of a novel type protease inhibitor (FPI-F) from the silkworm, *Bombyx mori*. *J. Seric. Sci. Jpn.* **65**, in press
9. Matsuura, Y., Possee, R.D., Overton, H.A., and Bishop, D.H.L. (1987) Baculovirus expression vectors: The requirement for high level expression of proteins, including glycoproteins. *J. Gen. Virol.* **68**, 1233-1250
10. Green, N.M. and Work, E. (1953) Pancreatic trypsin inhibitor. *Biochem. J.* **54**, 347-352
11. Yamashita, M. and Eguchi, M. (1987) Comparison of six genetically defined inhibitors from the silkworm haemolymph against fungal protease. *Comp. Biochem. Physiol.* **86B**, 201-208
12. Francki, R.I.B., Fauquet, D.L., Knudson, D.L., and Brown, F. (1991) *Classification and Nomenclature of Viruses*, Springer-Verlag, Vienna and New York
13. Maeda, S. (1989) Expression of foreign genes in insects using baculovirus vectors. *Annu. Rev. Entomol.* **34**, 351-371
14. O'Reilly, D.R., Miller, L.K., and Luckow, V.A. (1992) *Expression Vectors, A Laboratory Manual*, W.H. Freeman & Co., New York
15. Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Sato, Y., and Furusawa, M. (1985) Production of human α-interferon in silkworm using a baculovirus vector. *Nature* **315**, 592-594
16. Fukunaga, R., Sokawa, Y., and Nagata, S. (1984) Constitutive production of human interferons by mouse cells with bovine papillomavirus as a vector. *Proc. Natl. Acad. Sci. USA* **81**, 5086-5090
17. Yoshida, Y., Yamashita, M., Yonehara, S., and Eguchi, M. (1990) Properties of fungal protease inhibitors from the integument and haemolymph of the silkworm and effect of an inhibitor on the fungal growth. *Comp. Biochem. Physiol.* **95B**, 559-564