Efficient protein production using a *Bombyx mori* nuclear polyhedrosis virus lacking the cysteine proteinase gene

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Infection by a baculovirus (*Bombyx mori* nuclear polyhedrosis virus, BmNPV) in silkworm (*Bombyx mori*) larvae is highly efficient as an expression system for the production of useful proteins. However, the amount of the protein of interest expressed tends to decrease in the later stages of infection presumably due, in part, to a proteinase produced in the larval haemolymph. The N-terminal amino acid sequence of a proteinase purified from the haemolymph of BmNPV-infected larvae was identical to the internal amino acid sequence of the viral cysteine proteinase gene of BmNPV, suggesting that the cysteine proteinase in the haemolymph originated from the BmNPV gene. We constructed a mutant virus (CPd) which had a deletion in the cysteine proteinase gene. No proteinase activity corresponding to this proteinase was detected in the haemolymph of silkworm larvae infected with CPd. The firefly luciferase and the human growth hormone genes were separately introduced into CPd under control of the polyhedrin promoter. These constructs produced these proteins very efficiently, because of a greatly reduced degree of degradation of these proteins. A BmNPV vector system using CPd enhances the stability of foreign expressed proteins, especially for those that are cysteine proteinase-sensitive.

**Introduction**

Baculovirus gene expression systems using nuclear polyhedrosis viruses (NPVs) have been used to produce many proteins. The gene expression vector of a baculovirus, *Bombyx mori* nuclear polyhedrosis virus (BmNPV), has been applied to the large-scale production of useful proteins in silkworm (*Bombyx mori*) larvae (Maeda, 1994). However, the foreign gene products are frequently degraded at a very late stage of BmNPV infection. We found a marked reduction in the efficiency of luciferase production during late stages of infection in the *in vivo* system using silkworm larvae (Suzuki et al., 1994). Similar reductions in productivity due to degradation have been reported for the production of hepatitis B virus surface antigen (Higashihashi et al., 1991), cat interferon (Sakurai et al., 1992) and human growth hormone (hGH) (Kadono-Okuda et al., 1995). This is a serious problem for the mass production of proteins of commercial interest.

Ohkawa et al. (1994) reported that BmNPV encodes a proteinase gene belonging to the papain superfamily. The putative amino acid sequence surrounding the active site of the BmNPV-derived cysteine proteinase (BmNPV-CP) is similar to the sequences conserved among cathepsin B, H, L and S, and papain (Ohkawa et al., 1994). A similar cysteine proteinase gene is encoded in *Autographa californica* NPV (AcNPV) (Rawlings et al., 1992; accession no. M67451) and *Choristoneura fumiferana* NPV (Hill et al., 1995; accession no. M97906). From deletion experiments of the viral cysteine proteinase gene, Ohkawa et al. (1994) and Slack et al. (1995) hypothesize that baculovirus cysteine proteinase participates in the destruction of insect tissues during the later stages of pathogenesis.

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In this study, we show that the BmNPV-CP appears in the silkworm haemolymph and is involved in degradation of foreign gene products. Moreover, we constructed a highly efficient protein expression vector in which the cysteine proteinase gene was deleted. The improved productivity of this virus vector was sufficient for the production of firefly luciferase or hGH at a commercial level.

Methods

Insect cell lines and viruses. Larvae of the silkworm, B. mori, were reared on an artificial diet at 27 °C. Only fifth-instar larvae were used in experiments. Cultured cells (BoMo15Allc, Kobayashi et al., 1992; BmN, Maeda, 1989) of the silkworm were maintained at 25 °C in an MGM448 medium containing 10% foetal bovine serum. The BmNPV P6E (wild-type; Kobayashi et al., 1990) and T3 (wild-type; Maeda et al., 1985) strains and CPd, a mutant which has the cysteine proteinase gene deleted, were propagated on BoMo15Allc cells or BmN cells. Recombinant viruses, into which the luciferase gene or the hGH gene was introduced, were also propagated on BoMo15Allc cells.

Virus infection of B. mori larvae and collection of the haemolymph. BoMo15Allc cells were infected with wild-type and recombinant viruses at an m.o.i. of 0.5. Culture media supernatants from virus-infected cells were used as inocula for virus infection of larvae. On the first day of the fifth-instar, larvae were infected subcutaneously with 50 µl of a 10-fold dilution (in distilled water) of virus inoculum. Some larvae were infected by a peroral inoculation method (Okazaki et al., 1995). Infected larvae were reared at 27 °C. Haemolymph was collected at 48, 72, 84, 96, 102 and 108 h post-inoculation (p.i.) from the wound site in the abdomen by cutting off the abdomen of the larva. Phenylthiourea was added to the collected haemolymph to prevent melanization, and the haemolymph samples were stored at −20 °C.

Measurement of proteinase activity. Proteinase activity was measured using azocoll or acid-denatured haemoglobin as substrate. Using azocoll, proteinase activity was assayed according to the method of Kobayashi et al. (1985). Briefly, 5 mg azocoll was suspended in 1 ml 0.1 M succinic acid–NaOH buffer (pH 4.5) and was added to 20 µl haemolymph. The reaction was incubated at 37 °C for 3 h, after which it was stopped by adding 2 ml 10% SDS, and absorbance was measured at 520 nm. One unit of enzyme activity was defined as 1 A415 of azo dyes per 1 mg azocoll for 1 h. When haemoglobin was used as substrate, 70 µl 0.3% acid-denatured haemoglobin in 0.1 M succinic acid–NaOH buffer (pH 4.5) was added to 10 µl haemolymph. The reaction was incubated at 37 °C for 60 min and then was stopped by adding 80 µl 10% TCA. After the reaction tube was spun to pellet the precipitates, 100 µl of the supernatant was removed to a new tube and 625 µl 0.55 M Na2CO3 was added. After 5 min, 125 µl 1 M phenol reagent solution (Nacalai Tesque) was added, and the mixture was incubated at 37 °C for 30 min for colour development. Then, absorbance was measured at 660 nm. One unit of enzyme activity was defined as the generation of 1 µmol tyrosine per min (Iwamoto & Eguchi, 1976). The following proteinase inhibitors were used in experiments on the inhibition of proteinase activity: iodoacetic acid, PMSF, α-phenanthroline, E-64 [trans-epoxysuccinylamido(4-guanidino)-butane], S-1P (Murao & Sato, 1970), antipain, chymostatin, leupeptin and talopeptin (Murao et al., 1980).

Purification of proteinase in the haemolymph. Haemolymph collected from silkworm larvae infected with BmNPV P6E was centrifuged at 3000 g for 10 min to remove tissue debris. The supernatant was fractionated with 0–30% ammonium sulfate. The precipitate was dissolved in distilled water, and dialysed against 10 mM Tris–HCl buffer (pH 7.5) containing 10% ammonium sulfate. The dialysate was adsorbed onto a butyl-Toyopearl column (Toso), equilibrated with the above buffer, and the proteinase was eluted with a 10–0% ammonium sulfate gradient. The active fraction was further purified using preparative isoelectric focusing (Rotofor, Bio-Rad). Electrophoresis was performed for 4 h at 12 W using ampholytes (pH range 3–10). The active fraction was again purified by preparative isoelectric focusing.

Determination of the N-terminal amino acid sequence of haemolymph proteinase. Purified proteinase was separated by SDS–PAGE (Laemmli, 1970) and blotted onto a PVDF membrane (Bio-Rad). The protein was stained with Coomassie brilliant blue R250 (MERCK). The protein band was directly sequenced using a peptide sequencer model 476A (Perkin-Elmer).

Nucleotide sequence of the proteinase gene of BmNPV P6E. Virus particles of BmNPV P6E were purified by sucrose density gradient centrifugation (Maeda, 1989). Viral DNA was isolated by treatment with proteinase K and 1% SDS followed by phenol–chloroform extraction (Maeda, 1989). The purified viral DNA was cut with BamHI and fragments were separated by agarose gel electrophoresis. The 3.9 kb F fragment containing the cysteine proteinase gene was cloned into the pUC19 BamHI site, yielding pBmFCP. The nucleotide sequence of the viral cysteine proteinase gene in the F fragment was determined using a DNA sequencer, model 373A-36 (Perkin-Elmer).

Construction of a cysteine proteinase deletion mutant. pBmFCP was cleaved with the restriction endonucleases Apal and Xcrol to remove the part of the cysteine proteinase gene corresponding to amino acids 114–187. Then, plasmid DNA was made blunt-ended with T4 DNA polymerase and dephosphorylated with calf intestine alkaline phosphatase. pAcDZ1 (Kamita et al., 1993) was cleaved with Xhol and BamHI, and the β-galactosidase gene (lacZ) cassette containing the hsp70 promoter was excised. The lacZ cassette was made blunt-ended with the Klenow fragment of DNA polymerase I, and blunt end-ligated to pBmFCP, yielding pBmFCPBLZ. pBmFCPBLZ and BmNPV DNAs were co-transfected into BmN cells by the calcium phosphate method (Maeda, 1989). A cysteine proteinase gene-deficient virus (BmNPVCPd) which expresses β-galactosidase was obtained by screening for blue-coloured plaques in the presence of 5-bromo-4-chloro-3-indolyl β-d-galactoside (X-Gal, Wako) by plaque assay (Maeda, 1989). Plaque purification was repeated twice to obtain a purified clone.

Construction of recombinant viruses. Recombinant viruses, into which useful foreign genes were introduced downstream of the polyhedrin promoter, were constructed as follows. The luciferase gene from firefly, Photinus pyralis (de Wet et al., 1987), and the hGH gene (Yamakawa et al., 1989) were inserted into pBM050, a transfer plasmid vector (Maeda, 1989). The transfer vectors (pBmPL, pBmGH) were purified by caesium chloride density-gradient centrifugation. The purified transfer vectors were mixed with purified CPd or wild-type viral DNAs in a ratio of transfer vector to BmNPV DNA was 5 : 1. The mixture was co-transfected in BoMo15Allc cells using the CellPhect transfection kit (Pharmacia). Recombinant viruses were screened by the endpoint dilution method on 96-well plates (Maeda, 1989), and polyhedrin negative clones (CPdPL, WTPL, CPdGH and WTHGH) were obtained.

Immuno-blot analysis. Appropriately diluted haemolymph samples containing foreign proteins were analysed by SDS–PAGE (Laemmli, 1970). Proteins in gels were blotted electrophoretically onto...
Characterization of proteinase in the haemolymph of silkworm larvae infected with BmNPV

We measured proteinase activity in the haemolymph of silkworm larvae infected with BmNPV. Relatively high proteinase activity appeared at 84 h.p.i. and proteinase activity increased rapidly until the very late stage of infection (108 h p.i.) (Fig. 1). This high proteinase activity was detected in the haemolymph of larvae infected with either the wild-type or the recombinant virus carrying the luciferase gene, whereas extremely low activity was detected in the haemolymph of non-infected larvae.

When haemolymph collected from non-infected or BmNPV-infected larvae was applied to a Sephadex G-100 column, most of the proteinase activity was detected in the high molecular mass fractions (approximately 100 kDa). The BmNPV-infected haemolymph showed two peaks of proteinase activity. One corresponded to a high molecular mass protein (approximately 100 kDa) and the other corresponded to 30 kDa (Fig. 2). The high molecular mass proteinase was identified as an aspartic acid proteinase by its low optimum pH (pH 2–0) and by an inhibition study (data not shown). We partially purified and characterized the 30 kDa proteinase in infected silkworm haemolymph. The infected silkworm haemolymph, collected at 108 h p.i., was fractionated with 0–30% ammonium sulfate. More than 90% of the proteinase activity was recovered in the precipitated fraction. This fraction was further purified 160-fold by two successive separations with preparative isoelectric focusing using a Rotofor apparatus (Bio-Rad). The proteinase had an optimum pH of 4–0, and half of the maximum proteinase activity was attained at pH 6–8, the normal pH of silkworm haemolymph (data not shown). The proteinase activity was strongly inhibited by cysteine proteinase inhibitors, such as E-64, iodoacetate and leupeptin, whereas it was not inhibited by PMSF (a serine proteinase inhibitor), o-phenanthroline or talopeptin (metallo-proteinase inhibitors) or S-PI (an aspartic proteinase inhibitor) (Fig. 3). These results showed that the proteinase was a cysteine proteinase.

N-terminal amino acid and nucleotide sequence of the 30 kDa cysteine proteinase

Purified cysteine proteinase from haemolymph collected 108 h p.i. showed a single band at 35 kDa by SDS–PAGE (data not shown). The N-terminal amino acid sequence of the protein was determined to be NH$_2$ Tyr-Asp-Pro-Leu-Lys-Ala-Pro-Asn-. This sequence matched the predicted sequence of the proprotein of the BmNPV-CP gene (19–26 residues of BmNPV T3) (Ohkawa et al., 1994). In sequencing the BmNPV-CP gene of the BmNPV PoE strain, four nucleotide differences were found in the coding region, and one of the four differences resulted in an amino acid substitution of arginine for proline.

Construction and characterization of the CPd

A mutant virus lacking the cysteine proteinase gene was constructed using homologous recombination between viral DNA and the transfer plasmid with an incomplete cysteine proteinase gene, according to the method of Ohkawa et al. (1994).

Virus proliferation and polyhedron-forming ability of the constructed virus (BmNPVCPd) in BmN or BoMo15AIlc cells and silkworm larvae closely resembled those of the wild-type virus. All of the CPd-infected larvae had died by 130 h p.i. Prior to death, or shortly thereafter, the integument of larvae infected with the wild-type virus was fragile and easily torn when handled. In contrast, the larvae that died from CPd virus infection were intact and no melanization within the body was observed.

At 72 h p.i. proteinase activity in BoMo15AIlc cells infected with the CPd virus decreased markedly in comparison to that in cells infected with wild-type virus (data not shown). Fig. 4 shows the proteinase activities in the haemolymph of fifth-instar silkworm larvae infected with CPd and wild-type viruses. Proteinase activity in the haemolymph of larvae infected with wild-type virus increased dramatically after 96 h p.i., whereas proteinase activity in the CPd-infected larvae was not apparent; proteinase activity in CPd-infected larvae was similar to that in non-infected larvae. These results confirmed that the cysteine proteinase that appeared in the haemolymph during infection by the wild-type virus was expressed by the viral gene, and that the cysteine proteinase gene of CPd was not fully functional.

Histopathological observations of the tracheae of wild-type-infected larvae revealed that the tracheal epithelia were largely destroyed and were liberating polyhedra into the haemocoel (Fig. 5a), whereas epithelia of CPd-infected larvae appeared to be intact, even at a late stage of infection, and contained abundant polyhedra in the hypertrophied nuclei of
Fig. 1. Proteinase activity in haemolymph fluids of silkworm larvae infected with wild-type BmNPV (▲) and recombinant BmNPV (■), and in non-infected larvae (○). Proteinase activities were measured at pH 4–5 using azocoll as substrate. Silkworm larvae infected with BmNPVs had died by 110 h p.i.

Fig. 2. Elution patterns of proteinases from the haemolymph of BmNPV-infected and non-infected silkworm larvae. Each haemolymph sample was applied to a Sephadex G-100 column [2 cm (internal diameter) × 85 cm] equilibrated with 5 mM sodium phosphate, 0–15 M NaCl buffer (pH 6–5). Proteinase activity (●) was measured with 0–75% acid-denatured haemoglobin as substrate. Solid line shows absorbance at 280 nm.

Fig. 3. Effect of inhibitors on partially purified haemolymph proteinase from silkworm larvae infected with BmNPV P6E.

Fig. 4. Proteinase activity in haemolymph fluids of silkworm larvae infected with CPd (□) and wild-type (●) viruses. Haemolymph samples collected initially from silkworm larvae infected with CPd and wild-type viruses were assayed at pH 4–5 using azocoll as substrate. ▲, Haemolymph from non-infected larvae.

their cells (Fig. 5b). When silkworm larvae were infected with the wild-type virus, the haemolymph became milky white due to the polyhedra and lipids released from tissues degraded by infection. On the other hand, the haemolymph of larvae infected with CPd was only slightly turbid and contained fewer polyhedra and less lipid than did the haemolymph of larvae infected with wild-type virus. Lipophorins (lipid transport proteins) in the haemolymph of larvae infected with wild-type virus were particularly degraded by cysteine proteinase, while lipophorins in the haemolymph of larvae infected with CPd showed little degradation on SDS–PAGE gels (data not shown), suggesting that very little lipid was released in CPd virus infection.

**Protein production using CPd**

We have introduced useful foreign protein genes under control of the polyhedrin promoter in CPd. Firstly, we constructed a recombinant CPd virus (CPdPL) with the luciferase gene from the firefly, *Photinus pyralis*. Luciferase
activity in the haemolymph of larvae infected with CPdPL or WTPL [a wild-type (CP⁺) virus that expresses luciferase] was measured with a luminometer and compared with a standard (Fig. 6a). The level of accumulated luciferase decreased at a late stage of infection in larvae infected with WTPL, whereas in CPdPL-infected larvae the level did not decrease until larval death. The level in the haemolymph of larvae infected with CPdPL was about twice that in the haemolymph of WTPL-infected larvae just before death (120 h p.i.). To investigate the degree of luciferase degradation, the quantity of luciferase was measured by immunological blotting using an anti-luciferase serum (Fig. 6b). In the case of WTPL, partially degraded products of about 50 kDa migrated further in the gel than did the native luciferase (65 kDa). In contrast, such degraded bands were not detected in the case of CPdPL.

Secondly, the hGH gene was introduced into CPd and P6E (wild-type), yielding CPdhGH and WThGH respectively, and the production of growth hormone was measured by immunological blot analysis. BoMo15AIlc cells were infected with CPdhGH or WThGH, and the hGH molecules, produced in the cells, were detected by anti-hGH serum (Fig. 7a). Only native hGH molecules of 22 and 25 kDa were detected in cells infected with CPdhGH. In contrast, in addition to native molecules, 17 kDa degraded products were detected in cells infected with WThGH. Also, large quantities of 17 kDa degraded products of hGH were detected in the haemolymph of silkworm larvae infected with WThGH, whereas very little of these degraded products was detected in the haemolymph of larvae infected with CPdhGH (Fig. 7b). Thus, production of the two proteins, luciferase and hGH, using the CPd expression vector was extremely stable and there was virtually no degradation of the expressed foreign proteins.
Discussion

A major problem in using the BmNPV vector system for the expression of foreign proteins is that protein productivity decreases markedly due to protein degradation during the later stages of infection. At first, we anticipated that this problem might be caused by lysozomal proteinases released from tissues destroyed during virus infection. Ohkawa et al. (1994), however, showed that induction of cysteine proteinase occurred by expression of a viral-encoded gene, and that host degradation after death occurred by means of this proteinase. In the present study, we have isolated a cysteine proteinase that appears specifically in the haemolymph of BmNPV-infected silkworm larvae. The N-terminal amino acid sequence of this proteinase was identical to the sequence at the 5’ end of the proprotein of the BmNPV T3 cysteine proteinase gene. When silkworm larvae were infected with a cysteine proteinase deletion mutant, viral cysteine proteinase activity in the haemolymph was not detected and degradation of expressed foreign proteins was suppressed. These results showed that cysteine proteinase activity in the haemolymph of silkworm larvae infected with BmNPV is derived from the cysteine proteinase gene of the virus genome and that the proteinase that accumulates in the haemolymph of infected silkworm larvae participates in the degradation of expressed foreign proteins.

The characteristics of the cysteine proteinase expressed by the cysteine proteinase gene of AcNPV were reported by Slack et al. (1995). Ohkawa et al. (1994) detected cysteine proteinase activity that was inhibited by E-64 in cultured cells infected with BmNPV and showed that no activity was detected in cultured cells infected with a mutant lacking the cysteine proteinase gene. In the present study, the cysteine proteinase found in the haemolymph was a 35 kDa proprotein (the signal peptide had been cleaved) rather than the mature form (28 kDa) speculated by Ohkawa et al. (1994). Only the activity of the 35 kDa proprotein form was detected, as the activity of the 28 kDa species, occurring by autolysis, had disappeared (Takahashi et al., 1997). Recovery of cysteine proteinase from the haemolymph seemed to be poor in our purification strategy due, presumably, to autolysis. Detailed characterization of the cysteine proteinase could be accomplished by studying enzymatic properties, such as substrate specificity, using an improved purification method and/or by overexpression of the proteinase under control of the polyhedrin promoter.

Cysteine proteinase in an insect infected with an NPV not only degrades many tissue-derived structural proteins, but also reduces the yield of foreign proteins, particularly those sensitive to cysteine proteinase. In addition, processing of glycoproteins of HIV-1 and bovine herpesvirus 1 produced using a baculovirus expression vector system (Wells & Compans, 1990; van Drunen Littel-van den Hurk et al., 1992) may be caused by a viral cysteine proteinase. The aberrant cleavages of mouse interleukin-3 and human gastrin-releasing peptide precursor, which differ from the native proteins (Knepper et al., 1992; Lebacq-Verheyden et al., 1988), may also be caused by a viral cysteine proteinase. These data indicate the necessity of analysing cleavage sites of cysteine proteinase.

We have constructed a mutant virus (CPd) which is deficient in the cysteine proteinase gene. We believe that this virus can be utilized as a very effective expression vector for the stable production of useful foreign proteins, especially proteins susceptible to cysteine proteinase.

At present, for efficient production of foreign proteins in silkworm larvae, it is important to collect haemolymph before degradation of foreign gene products begins (Suzuki et al., 1994; Kadono-Okuda et al., 1995). Using CPd as an expression vector, we were able to produce undegraded foreign proteins even in the last stages of infection. Another problem relates to degradation of proteins of interest during purification from the haemolymph. Using CPd, protein degradation during purification can be prevented. Furthermore, since fat-body tissues in larvae infected with CPd release very little lipid into the
Fig. 7. Production of human growth hormone (hGH) using CPd (CPdhGH) and wild-type virus (WThGH) with the introduced hGH gene. (a) Immuno-blot analysis of hGH produced in BoMo15AIIc cells. At 96 h p.i. lysates of cells infected with each virus were separated by SDS–PAGE. Degradation products (17 kDa) and native hGH molecules (22 and 25 kDa) are indicated by white and black arrows, respectively. (b) Immuno-blot analysis of hGH produced in larvae. Degradation products (17 kDa) and native hGH molecules (22 and 25 kDa) are indicated by white and black arrows, respectively.

We thank Professor Emeritus Y. Tanada, University of California, Berkeley, for critical reading of the manuscript.

References


Received 8 July 1997; Accepted 20 August 1997