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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 (BoMo15AIIc, 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 BoMo15AIIc cells or BmN cells. Recombinant viruses, into which the luciferase gene or the hGH gene was introduced, were also propagated on BoMo15AIIc cells.

Virus infection of *B. mori* larvae and collection of the haemolymph. BoMo15AIIc 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 injected 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 resulting from cutting off several abdominal legs from each 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, 3 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 A_{520} 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 Na_2CO_3 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 (Eguchi & Iwamoto, 1976). The following proteinase inhibitors were used in experiments on the inhibition of proteinase activity: iodoacetic acid, PMSF, *o*-phenanthroline, E-64 [*trans*-epoxysuccinylamido(4-guanidino)-butane], S-PI (Murao & Satoi, 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 *Bam*HI and fragments were separated by agarose gel electrophoresis. The 3·9 kb F fragment containing the cysteine proteinase gene was cloned into the pUC19 *Bam*HI 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 *Apa*I and *Xcm*I 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 *Xba*I and *Bam*HI, 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 pBmFCPdLZ. pBmFCPdLZ 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, pBmhGH) were purified by caesium chloride density-gradient centrifugation. The purified transfer vectors were mixed with purified CPd or wild-type viral DNAs (ratio of transfer vector to BmNPV DNA was 5:1). The mixture was co-transfected in BoMo15AIIc 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, CPdhGH 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

nitrocellulose membranes using a Trans-Blot transfer cell (Bio-Rad). The membrane was immersed in 5% skim milk in PBS for 90 min to block non-specific binding. Then, the membranes were incubated overnight at 4 °C with appropriately diluted antisera containing antibodies against the proteins of interest. The membranes were washed with PBS containing 0·1% Tween 20 and reacted with peroxidase-conjugated goat anti-rat or anti-rabbit IgG F(ab′)$_2$ fragment (1:1000 dilution, Wako) for 60 min. After washing, the immunoreactive bands were visualized with an ECL detection kit (Amersham).

■ **Lumino-assay.** Luciferase activity in haemolymph was determined using a luminometer (BLR-201, Aloka). Ten µl haemolymph, which was appropriately diluted in 0·25 M Tris–HCl (pH 7·8) containing 1 mg/ml BSA, was added to 350 µl of substrate mix (50 mM HEPES pH 7·55, 15 mM $MgSO_4$, 5 mM ATP). Each test tube was placed in the luminometer and 100 µl 1 mM luciferin solution was auto-injected. The quantities of light emitted in 10–30 s were integrated. Concentration of luciferase in the haemolymph was determined using purified luciferase (Sigma) as a standard.

Results

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, iodoacetic acid 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 acid 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 P6E 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 BoMo15AIIc 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 BoMo15AIIc 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. 5 *a*), 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

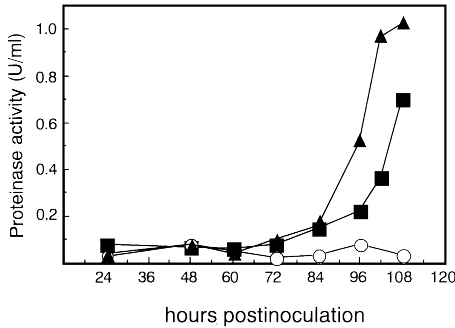

Fig. 1. Proteinase activity in haemolymph fluids of silkworm larvae infected with wild-type BmNPV (▲) and recombinant BmNPV (■), and in non-infected larvae (○). Proteinase activities were measured at pH 4·5 using azocoll as substrate. Silkworm larvae infected with BmNPVs had died by 110 h p.i.

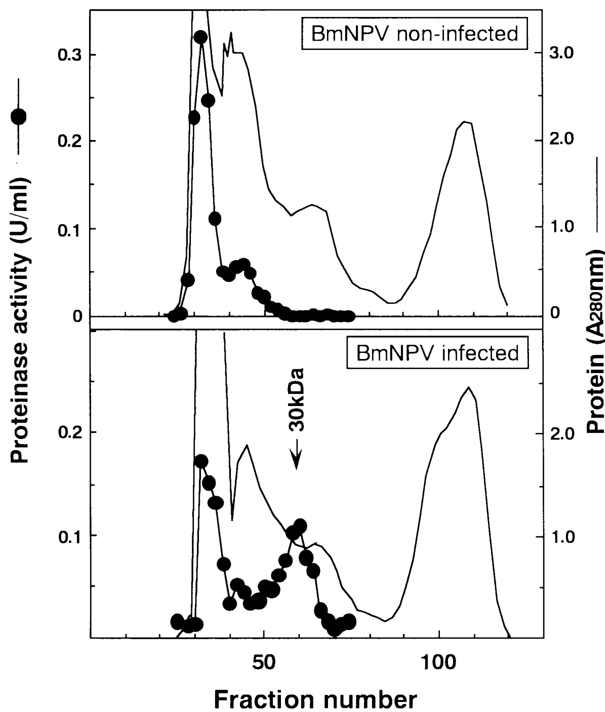

Fig. 2. Elution patterns of proteinases from the haemolymph of BmNPV-infected and non-infected silkworm larvae. Each haemolymph sample was applied to a Sephadex G-100 column [2 cm (internal diameter) × 85 cm] equilibrated with 5 mM sodium phosphate, 0·15 M NaCl buffer (pH 6·5). Proteinase activity (●)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

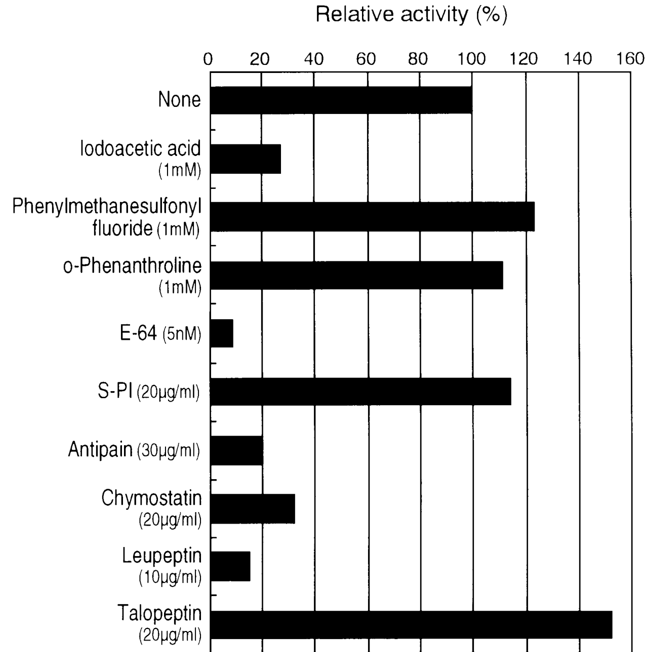

Fig. 3. Effect of inhibitors on partially purified haemolymph proteinase from silkworm larvae infected with BmNPV P6E.

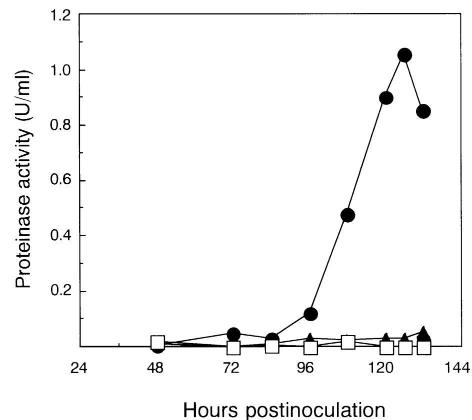

Fig. 4. Proteinase activity in haemolymph fluids of silkworm larvae infected with CPd (□) and wild-type (●) viruses. Haemolymph samples collected initially from silkworm larvae infected with CPd and wild-type viruses were assayed at pH 4·5 using azocoll as substrate. ▲, Haemolymph from non-infected larvae.

their cells (Fig. 5 *b*). 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

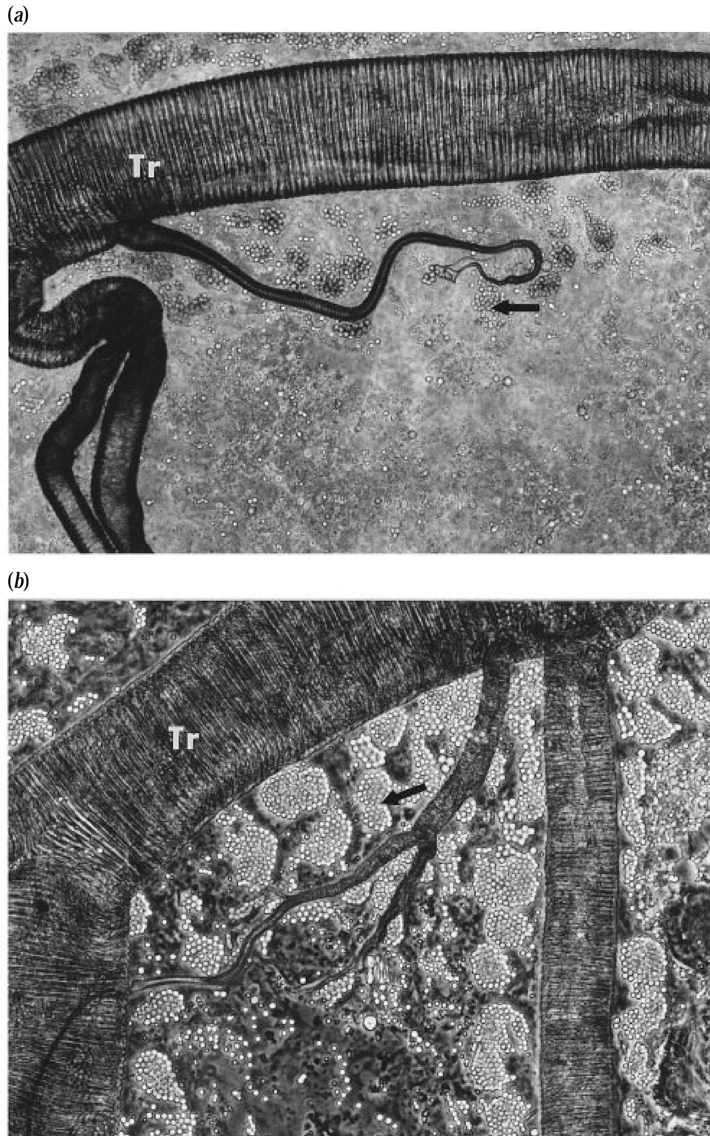

Fig. 5. Tracheal epithelia of silkworm larvae infected with wild-type virus (*a*) and CPd virus (*b*). Virus-infected fifth-instar silkworm larvae were dissected, and the tracheae attached to the midgut were observed under a light microscope. Tr, Trachea; arrow, polyhedra.

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. 6*a*). 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. 6*b*). 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. BoMo15AIIc cells were infected with CPdhGH or WThGH, and the hGH molecules, produced in the cells, were detected by anti-hGH serum (Fig. 7*a*). 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. 7*b*). 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.

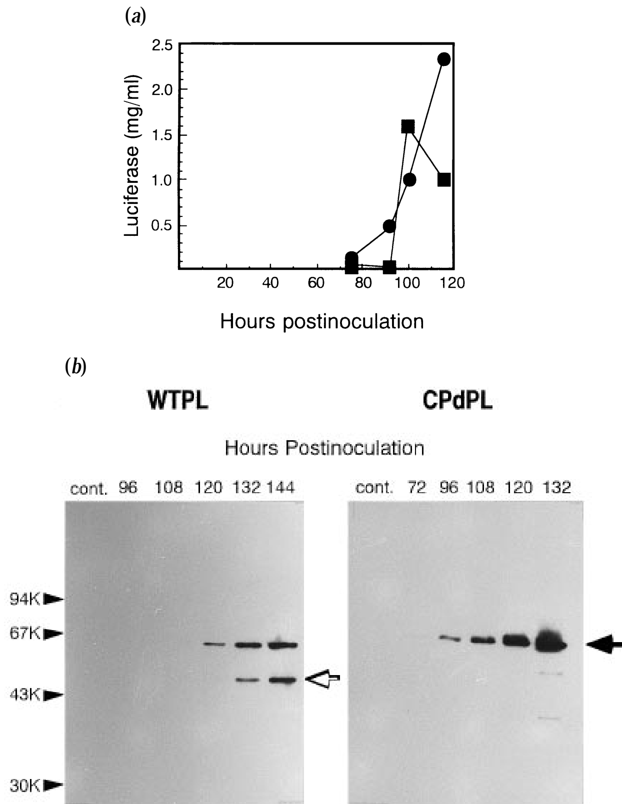

Fig. 6. Production of luciferase by the CPd (CPdPL) virus and the wild-type (WTPL) virus in which the luciferase gene from the firefly had been introduced. (*a*) Changes in the concentrations of luciferase in the haemolymph of larvae infected with CPdPL (●) and WTPL (■). Concentrations were determined with a luminometer (BLR-201, Aloka). In this experiment, both the CPdPL-infected larvae and the WTPL-infected larvae had died by 126 h p.i. (*b*) Immuno-blot analysis of luciferase produced by each recombinant virus. Native luciferase (65 kDa) and its degradation products (50 kDa) are indicated by black and white arrows, respectively. In this experiment, the CPdPL-infected larvae and the WTPL-infected larvae had died by 138 h p.i. and 150 h p.i., respectively.

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 lysosomal 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 silkworms. 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

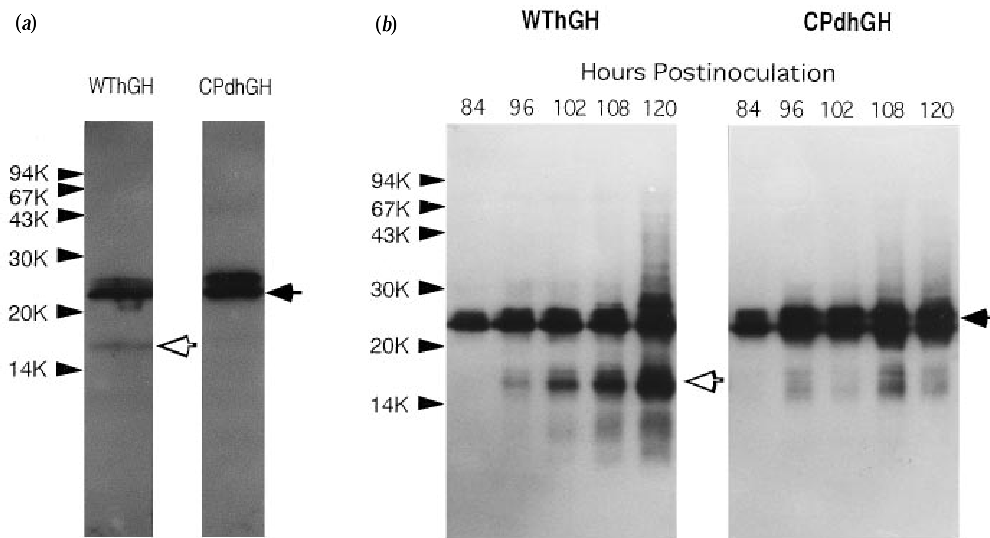

Fig. 7. Production of human growth hormone (hGH) using CPd (CPdhGH) and wild-type virus (WThGH) with the introduced hGH gene. (*a*) Immuno-blot analysis of hGH produced in BoMo15AIIc cells. At 96 h p.i. lysates of cells infected with each virus were separated by SDS–PAGE. Degradation products (17 kDa) 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.

haemolymph, contamination of the proteins of interest is minimal and can be easily eliminated during purification. On the other hand, in the production of cellular proteins which lack a signal peptide sequence, such as luciferase, the minimal destruction of CPd-infected tissues and cells might actually decrease the quantity of the foreign protein released into the haemolymph. However, our investigation into the production of firefly luciferase revealed no significant differences in the concentrations of luciferase in the haemolymph fluids of CPd-infected or wild-type virus-infected larvae.

BmNPV-CP has an important function in proteolytic activity occurring in the final stages of infection. After virus replication, and at a late stage of infection, virions are occluded in polyhedra. Polyhedra are then released into the haemolymph from degraded tissues, and later disperse into the environment after breakdown and rupture of the larval integument. The p35 gene product, which inhibits virus-induced apoptosis, has been found in the genomes of AcNPV and BmNPV (Clem *et al*., 1991; Kamita *et al*., 1993). The p35 gene is believed to promote cell integrity until the completion of virus replication. BmNPV-CP, on the other hand, has an opposite function. Accordingly, BmNPV-CP is believed to function in the destruction of tissues, cells and the integument, and consequently plays a role in dispersion of polyhedra for horizontal and vertical virus transmission. This hypothesis is supported by our observations that very little destruction of the tracheal epithelium occurs and that few, if any, polyhedra are released from tissues of silkworm larvae infected with CPd. In addition, the dead body of a CPd-infected larva remains comparatively intact. The chitinase gene in the genome of NPVs (Hawtin *et al*., 1995) may fulfil a function similar to that of BmNPV-CP.

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References

Clem, R. J., Fechheimer, M. & Miller, L. K. (1991). Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science* **254**, 1388–1390.

Eguchi, M. & Iwamoto, A. (1976). Alkaline proteases in the midgut tissue and digestive fluid of the silkworm, *Bombyx mori*. *Insect Biochemistry* **6**, 491–496.

Hawtin, R. E., Arnold, K., Ayres, M. D., Zanotto, P. M., Howard, S. C., Gooday, G. W., Chappell, L. H., Kitts, P. A., King, L. A. & Possee, R. D. (1995). Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **212**, 673–685.

Higashihashi, N., Arai, Y., Enjo, T., Horiuchi, T., Saeki, Y., Sakano, K., Sato, Y., Takeda, K., Takashina, S. & Takahashi, T. (1991). High-level expression and characterization of hepatitis B virus surface antigen in silkworm using a baculovirus vector. *Journal of Virological Methods* **35**, 159–167.

Hill, J. E., Kuzio, J. & Faulkner, P. (1995). Identification and characterization of the *v-cath* gene of the baculovirus, *Cf*MNPV. *Biochimica et Biophysica Acta* **1264**, 273–278.

Kadono-Okuda, K., Yamamoto, M., Higashino, Y., Taniai, K., Kato, Y., Chowdhury, S., Xu, J., Choi, S. K., Sugiyama, K., Nakashima, K., Maeda, S. & Yamakawa, M. (1995). Baculovirus-mediated production of human growth hormone in the silkworm, *Bombyx mori*. *Biochemical and Biophysical Research Communications* **213**, 389–396.

Kamita, S. G., Majima, K. & Maeda, S. (1993). Identification and characterization of the p35 gene of *Bombyx mori* nuclear polyhedrosis virus that prevents virus-induced apoptosis. *Journal of Virology* **67**, 455–463.

Knepper, T. P., Arbogast, B., Schreurs, J. & Deinzer, M. L. (1992). Determination of the glycosylation patterns, disulfide linkages, and protein heterogeneities of baculovirus-expressed mouse interleukin-3 by mass spectrometry. *Biochemistry* **31**, 11651–11659.

Kobayashi, M., Mori, H. & Yaginuma, T. (1985). Stimulation of acid protease activity in the isolated pupal abdomens of the silkworm, *Bombyx mori*, infected with nuclear polyhedrosis virus. *Journal of Invertebrate Pathology* **46**, 202–204.

Kobayashi, J., Inoue, H., Katoh, I., Yoshinaka, Y. & Ikawa, Y. (1990). Construction of a new baculovirus vector system of the silkworm, *Bombyx mori*. In *Molecular Insect Science*, p. 325. Edited by H. H. Hagedorn, J. G. Hildebrand, M. G. Kidwell & J. H. Law. New York: Plenum.

Kobayashi, J., Imanishi, S., Inoue, H., Ohsuye, K., Yamaichi, K., Tsuruoka, N. & Tanaka, S. (1992). High level expression of a frog δ-amidating enzyme, AE-II, in cultured cells and silkworm larvae using a *Bombyx mori* nuclear polyhedrosis virus expression vector. *Cytotechnology* **8**, 103–108.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lebacq-Verheyden, A. M., Kasprzyk, P. G., Raum, M. G., van Wyke Coelingh, K., Lebacq, J. A. & Battey, J. F. (1988). Posttranslational processing of endogenous and of baculovirus-expressed human gastrin-releasing peptide precursor. *Molecular and Cellular Biology* **8**, 3129–3135.

Maeda, S. (1989). Gene transfer vectors of a baculovirus, *Bombyx mori*, and their use for expression of foreign genes in insect cells. In *Invertebrate Cell System Applications*, pp. 167–181. Edited by J. Mitsuhashi. Boca Raton, Fla.: CRC Press.

Maeda, S. (1994). Expression of foreign genes in insect cells using baculovirus vectors. In *Insect Cell Biotechnology*, pp. 1–31. Edited by K. Maramorosch & A. H. McIntosh. Boca Raton, Fla.: CRC Press.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Sato, Y. & Furusawa, M. (1985). Production of human α-interferon in silkworm using a baculovirus vector. *Nature* **315**, 592–594.

Murao, S. & Satoi, S. (1970). New pepsin inhibitors (S-PI) from *Streptomyces* EF-44-201. *Agricultural Biological Chemistry* **34**, 1265–1267.

Murao, S., Katsura, M., Hukuhara, K. & Oda, K. (1980). New metallo proteinase inhibitor (MK-I) produced by *Streptomyces mozunensis* MK-23. *Agricultural Biological Chemistry* **44**, 701–703.

Ohkawa, T., Majima, K. & Maeda, S. (1994). A cysteine protease encoded by the baculovirus *Bombyx mori* nuclear polyhedrosis virus. *Journal of Virology* **68**, 6619–6625.

Okazaki, H., Kanaya, T., Nishimura, S., Ogawa, K. & Watanabe, H. (1995). Peroral inoculation of baculovirus vector to the silkworm, *Bombyx mori*, treated with a low temperature. *Journal of Sericultural Science of Japan* **64**, 504–508.

Rawlings, N. D., Pearl, L. H. & Buttle, D. F. (1992). The baculovirus *Autographa californica* nuclear polyhedrosis virus genome includes a papain-like sequence. *Biological Chemistry Hoppe-Seyler* **373**, 1211–1215.

Sakurai, T., Ueda, Y., Sato, M. & Yanai, A. (1992). Feline interferon production in silkworm by recombinant baculovirus. *Journal of Veterinary Medical Science* **54**, 563–566.

Slack, J. M., Kuzio, J. & Faulkner, P. (1995). Characterization of *v-cath*, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *Journal of General Virology* **76**, 1091–1098.

Suzuki, T., Ogawa, K., Usami, A., Mori, H., Matsubara, F., Takahashi, S. & Oda, K. (1994). The cysteine proteinase in the hemolymph of silkworm larvae infected with BmNPV. In *Program of the Abstracts of the Annual Meeting of the Japanese Society for Sericultural Science*, Abstract. 523 (in Japanese).

Takahashi, S., Ushiyama, S., Suzuki, T., Ogawa, K. & Oda, K. (1997). Purification and characterization of cysteine proteinase originated in the baculovirus gene. *Bioscience Biotechnology and Biochemistry* **61**, 1507–1511.

van Drunen Littel-van den Hurk, S., Parker, M. D., Fitzpatrick, D. R., van den Hurk, J. V., Compos, M., Babiuk, L. A. & Zamb, T. (1992). Structural, functional, and immunological characterization of bovine herpesvirus-1 glycoprotein gI expressed by recombinant baculovirus. *Virology* **190**, 378–392.

Wells, D. E. & Compans, R. W. (1990). Expression and characterization of a functional human immunodeficiency virus envelope glycoprotein in insect cells. *Virology* **176**, 575–586.

de Wet, J. R., Wood, K. V., Deluca, M., Helinski, D. R. & Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Molecular and Cellular Biology* **7**, 725–737.

Yamakawa, M., Sugisaki, K., Morimoto, M., Tanaka, M., Yamamoto, M., Ichikawa, T. & Nakashima, K. (1989). Effects of gene dosage on the expression of human growth hormone cDNA in *Escherichia coli*. *Biochimica et Biophysica Acta* **1009**, 156–160.

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