Human Interleukin-2 Production in Insect (Trichoplusia ni) Larvae: Effects and Partial Control of Proteolysis

Minh-Quan Pham,1,2* Susanna Naggie,1,2 Marjorie Wier,3 Hyung Joon Cha,1,2 William E. Bentley1,2

1Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland
2Department of Chemical Engineering, University of Maryland, College Park, Maryland 20742; telephone: (301)-405-4321; fax: (301)-314-9075; e-mail: bentley@eng.umd.edu
3Biotechnology Transfer, Inc., Columbia, Maryland

Received 16 January 1998; accepted 1 July 1998

Abstract: Many eukaryotic proteins have been successfully expressed in insect cells infected with a baculovirus in which the foreign gene has been placed under the control of a viral promoter. This system can be costly at large scale due to the quality of virus stock, problems of oxygen transfer, and severity of large-scale contamination. To circumvent this problem, we have investigated the expression of a foreign protein, human interleukin-2 (IL-2), in insect larvae, Trichoplusia ni, infected with the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV). The IL-2 gene was placed under control of the p10 promoter so that the polyhedra remained intact for efficient primary infection. From our results, it was clear that early infection limited larval growth and late infection delayed product production until near pupation, hence infection timing was important. Also, the harvest time was crucial for obtaining high yield, because IL-2 production had a sharp optimal peak with a time of occurrence dependent on both temperature and the initial amount of infection virus. Specifically, we found that, by raising the infection temperature to 30°C, we more than doubled the protein productivity. Furthermore, a significant concern of the larvae/baculovirus expression system has been the large amount of protease produced by the larvae, which adversely affects the protein yield. Therefore, we screened several protease inhibitors and characterized the larval protease specificity and timing to attenuate their impact. This report elucidates and delineates the factors that most directly impact protein yield in the larvae expression system, using IL-2 as a model. © 1999 John Wiley & Sons, Inc. Biotechnol Bioeng 62: 175–182, 1999.

Keywords: interleukin-2; baculovirus expression; protease degradation; insect larvae; Trichoplusia ni

INTRODUCTION

With the advances in recombinant DNA technology, proteins that are normally unattainable can be mass produced efficiently and cost effectively. Usually, genetically engineered proteins are expressed and produced in bacteria, yeast, mammalian cells, or insect cells. However, high cost and complexities in engineering and purification can still present problems in these systems. For example, in bacterial systems, the foreign proteins do not undergo posttranslational modification; hence, some remain biologically inactive. In mammalian and insect cell systems, posttranslational modifications are possible; however, expensive complex media are required and bioreactors must be run for extended periods, which can lead to contamination. In the insect cell/baculovirus expression system, a foreign gene is inserted into the genome of the Autographa californica nuclear polyhedrosis virus (AcNPV). The engineered baculovirus is added to suspended insect cell culture in the late exponential phase and, after ~3 days, the recombinant protein is expressed in large quantity. Because cost and bioreactor complexity can be a limitation, particularly for proteins having prices determined by market forces (e.g., veterinary vaccines, diagnostics), we have begun to examine the feasibility of insect larvae as a cost-effective alternative to insect cell culture.

Larvae have been used successfully for the production of foreign proteins at high levels of expression (Horiuchi et al., 1987; Korth et al., 1993; Kudora et al., 1989; Maeda et al., 1985; Medin et al., 1990; Miyajima et al., 1987). In these studies, a time-course evaluation of recombinant protein showed there was a peak protein concentration, after which time the protein was degraded. Protease activities have been documented in insect larvae; namely, sheep blowfly (Lucilia cuprina) larvae (Bowles et al., 1988; Elvin et al., 1994), Rhodnius larvae (Wigglesworth, 1970), flesh fly (Sarcophaga bullata) larvae (Bradley et al., 1989), and Galleria mellonella (Kucera et al., 1984), but not in Trichoplusia ni, which is a common host for baculovirus expression. Based on these observations, we hypothesized that protein production in insect larvae was subject to protease degradation in a manner similar to bacterial (Bentley et al., 1991; Gottes-
man and Maurizi, 1992; Harcum and Bentley, 1993b; Maurizi, 1992) and suspended insect cell expression systems (Wang et al., 1996), wherein intracellular proteases can rapidly degrade the desired proteins. In addition, however, larvae contain digestive proteases that potentially exacerbate the problem. Hence, proper timing and protease control is potentially critical in a large-scale larvae process.

Our current effort examines the feasibility of protein production in insect larvae using human interleukin-2 (IL-2) as a model protein. IL-2 is a molecule of great interest, as it was the first of a series of lymphocytotrophic hormones (Gillis et al., 1978). Because of its role in tumor-attacking lymphocyte proliferation, IL-2 has been evaluated as a therapeutic agent in the treatment of cancer (Rosenberg et al., 1984). Recently, it has been shown to be an effective enhancer of immune function in HIV+ individuals, particularly at low doses (Jacobson et al., 1996). IL-2 is also used extensively as a tissue culture reagent due to its requirement in the survival of T lymphocytes in culture. In this work, the IL-2 is intended as a diagnostic reagent; hence, there is a desire to maintain low production cost. A human IL-2 gene was placed under control of the p10 promoter in AcNPV. This baculovirus was used to infect cabbage looper, *Trichoplusia ni*, larvae for the production of IL-2. IL-2 was previously shown to be sensitive to larvae proteases in in vitro extracts, although a characterization (origin, molecular weight, specificity) of the proteases was not demonstrated (Wier, 1995). To enhance yield and stability, we employed casein-PAGE to characterize and subsequently reduce protease activities in the larvae homogenates. Additionally, several infection experiments were performed and the optimal harvest time and virus loading were determined by monitoring IL-2 and in vivo protease activities during the infection process. The criteria that most strongly influence recombinant IL-2 yield in larvae are defined and discussed.

**MATERIALS AND METHODS**

**Baculovirus and Larvae**

The baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV), was used as the basis vector for this work. The virus (obtained from Biotechnology Transfer Inc.) was constructed by inserting a cDNA of human IL-2 downstream of the p10 promoter (*BglIII* site of pAeUW21 transfer vector; Pharmingen, Inc.). The recombinant virus was produced by transfection of SF-9 cells with Baculogold DNA (Pharmingen, Inc.) using calcium phosphate precipitation. Three rounds of amplification yielded high-titer virus stocks (Wier, 1995). The p10 promoter was chosen because efficient oral infection requires an intact polyhedrin gene and polyhedrin protein (the polyhedrin promoter has been a promoter of choice in baculovirus expression systems). Cabbage looper, *Trichoplusia ni*, eggs were obtained from Entopath, Inc. (Easton, PA). The eggs were hatched in our laboratory according to the directions provided by the manufacturer; and fourth instar larvae were used for infection.

**Infection**

Various amounts of AcNPV stock solution, consisting of occluded recombinant virus, (see Results) were spread on the larval diet, which was ordered premade in Styrofoam cups from Entopath, Inc (Easton, PA). The cups were covered and allowed to stand for an hour so that the virus was completely absorbed by the media. The fourth instar larvae were then placed into the cups (approximately 10 to 15 larvae per cup), and the cups were inverted. The larvae fed from the top (bottom of cup) so that fecal matter dropped onto the lid where it was discarded daily. The quantity of food was sufficient for at least 5 days of growth. Three to five larvae were collected daily for IL-2 and protease assays.

**Sample Preparation**

The infected larvae were collected and frozen at −60°C until the total protein, protease, and IL-2 assays were performed. The frozen larvae were thawed, and homogenized in phosphate-buffered saline (PBS) containing 60 mM dithiothreitol (DTT), and 0.5% Triton X-100 at pH 7.0. The samples prepared for the IL-2 assay also contained the following protease inhibitors: 0.02% (w/v) PMSF, 1 mM EDTA, and 0.2 mg/mL benzamidine. The homogenate was then centrifuged at 4°C to remove large debris. After centrifugation, the supernatant was further clarified using a 0.22-μm filter.

**Total Protein and Protease Activity Assays**

The Bio-Rad protein assay was used to determine the total protein in the crude extract according to the method described by Rodriguez and Tait (1983). The protein concentration was calibrated with known concentrations of bovine serum albumin (BSA). Protein concentrations were reported as grams of protein per liter (g/L).

Larval protease activity was monitored with a modification of the gel electrophoresis method developed by Harcum and Bentley (1993a). In this method, the SDS-polyacrylamide gel was polymerized with 6 mg of casein instead of 6 mg of gelatin (SDS-GPAGE; Harcum and Bentley, 1993a) and embedded within the matrix, unless otherwise noted. Each lane was loaded with 0.75 μL of larvae extract. The gel was then run at 180 V. At the completion of the electrophoresis, the gel was washed in Triton X-100 (2.5%) to remove the SDS and was then incubated with 100 mM glycine, 4 mM ATP, and 2 mM MgCl2 (pH 7.5) at 37°C for 24 h to regenerate protease activities. Subsequently, the gel was stained with amido black and destained yielding clear zones that indicated proteolytic activities. The protease activities were thus separated by relative molecular weight, and the intensity of the clear band was proportional to the activity of the protease (Harcum and Bentley, 1993a).
The IL-2 assay was a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; BioSource Cytoscreen IL-2 Kit, BioSource International). The kit contained microtiter strips coated with IL-2-specific monoclonal antibody, the required assay reagents, and detailed instructions. One hundred microliters of the diluted sample was pipetted into the wells on the microtiter strips where the IL-2 bound to the monoclonal antibody during incubation. After incubating for 2 h and washing, a biotinylated polyclonal antibody specific for IL-2 was added. This antibody, therefore, bound the IL-2 captured during the first incubation. After removal of the polyclonal antibody by washing, strepavidin-peroxidase was added, which bound to the polyclonal antibody, thus completing a four-member sandwich. After a 1-h incubation, excess enzyme was washed off and a substrate was added, producing a color change. The intensity of the color (λ_{450}) was directly proportional to the concentration of IL-2 in the original sample. A calibration data set was prepared each time IL-2 assays were performed. The assay is linear in the range of 0 to 1000 pg/mL. Throughout this work, we used these ELISA results to indicate quantity of IL-2 in the original sample. A calibration data set was prepared each time IL-2 assays were performed. The assay is linear in the range of 0 to 1000 pg/mL. Throughout this work, we used these ELISA results to indicate quantity of IL-2 as this most directly indicates active product yield to be used in the subsequent manufacture of diagnostic kits. Results from Western blot analyses were typically higher than ELISA (not shown).

**Protease Purification Techniques**

Extract proteases were separated by molecular weight using the BioRad Model 491 prep cell cylindrical preparative PAGE unit.

**Preparative Gel Preparation**

For proteins in the 80-kDa range, a 7% acrylamide gel was used, because the difference in molecular weight between the protease of interest and its nearest contaminant was assumed to be in the range of 2% to 10% and the protein loading was 3.3 mg. The monomer volume was 110 mL and the tube length was 10 cm. The resolving gel was cast and, after 2 h, the stacking gel was cast using twice the volume of the sample solution. The complete cylindrical gel was cooled (4°C) and left to stand overnight. This was accomplished by pumping chilled water through the cooling core.

**Sample Preparation**

Three milliliters of crude sample with a protein concentration of 1.11 mg/mL was diluted with 1 mL of standard SDS-PAGE sample buffer without β-mercaptoethanol to give a final concentration of 3.33 mg per 4 mL. The sample solution was slowly added to the top of the gel using a syringe affixed to a small-diameter rubber tubing.

**Running Conditions**

The sample was run at the constant power of 12 A. After the dye front ran off of the bottom of the gel (approximately 8 h) the elution pump was started; however, the effluent was not immediately collected. Sample collection was started, using the Model 2128 fraction collector, 3 h after the dye front ran off of the bottom of the gel. The flow rate for the elution pump was 1.0 mL/min and samples were collected every 1.5 min, thus 1.5-mL samples were collected in microcentrifuge tubes. Two hundred fifty-six total samples were collected over a period of 6.4 h. The cell prep apparatus was kept cool by placing it in an ice bucket and by circulating cold water through the cooling core.

**Protease Analysis Using SDS-GPAGE**

Every tenth sample was run on SDS-GPAGE to determine which samples contained protease activity. Nine well combs were used and the sample solution was mixed using a 4:1 ratio of sample to sample buffer. After running at constant voltage (200 V), the gels were washed in 2.5% (v/v) Triton for 1 h and then incubated in 200 mL of buffer consisting of 0.054 g KH₂PO₄ and 0.5 g ATP, at pH 9.0. Samples with similar protease activities were combined and concentrated using Centriprep-30 and Centriprep-50 (Amicon Centriprep), concentrators yielding the concentrated purified proteins separated via molecular weight.

**RESULTS AND DISCUSSION**

**In Vitro Stability and Protease Characterization**

Figure 1 depicts the IL-2 content in larvae extracts during overnight storage at three temperatures (adapted from Wier, 1995). In this experiment, a protease inhibitor cocktail consisting of 0.002% PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 0.1 mg/mL benzamidine was added to one set of samples, whereas the other was resuspended in PBS alone. It was readily apparent that IL-2 was rapidly degraded in the 4°C and room-temperature samples, both with and without protease inhibitors, although the inhibitors included increased stability three-fold. Subsequently, using the substrate gel electrophoresis developed here, we systematically examined the effectiveness of several inhibitors to reduce protease activity in T. ni larvae extracts. Several, but not all these results (which include other substrates such as gelatin), are shown here. Figure 2A shows a casein-PAGE of homogenized larvae extracts spiked with 0.02% (w/v) phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 mg/mL benzamidine. As evident by the size and intensity of the cleared bands in the casein-PAGE, PMSF was the only inhibitor that reduced protease activities in these gels, suggesting they may be serine proteases. This was not surprising, however, because serine proteases play an important role in an insect’s defense mechanisms (Sugumaran and Kanost,
1993). Note, however, only protease activities at the lower molecular weight (lower edge of smear) were inhibited by PMSF; the higher-molecular-weight protease activities were either not impaired or we could not determine the extent of inhibition using this assay. Dithiothreitol (DTT), urea, and 2-mercaptoethanol at high concentrations of 60 mM, 0.4 M, and 3.3% (v/v), respectively, were all found to be ineffective against these higher-molecular-weight proteases (not shown). We found that sodium dodecylsulfate (SDS) had an inhibitory effect, although significant reduction in activity occurred only at high levels of SDS (~24%, also not shown), which was impractical for protein processing. Through detailed analyses such as these, we concluded that PMSF (0.02%) was a principal protease inhibitor to be included in a resuspension buffer that minimized losses in extracts. This level of PMSF was tenfold higher than in the buffer depicted in Figure 1 and corresponded to the level shown in Figure 2A. In addition to PMSF, EDTA (1 mM), benzamidine, and Triton X-100 (0.5%) were added to further stabilize IL-2 and lyse cells, respectively.

The proteases were further characterized for pH dependence. Our results show that the proteases did not require any cofactors (e.g., ATP, Mg²⁺) to be active (not shown). In both casein- and gelatin-PAGEs, the proteases were active while being electrophoresed, resulting in an intense white streak running vertically down the lane. Little protease separation was discernible on the gels (as seen in Fig. 2A). This problem was partially solved by using minute sample quantities (0.75-µL larvae sample per well) and a higher SDS content in the sample. Thus, we lowered the protease activity during the electrophoresis, then regained it upon incubation. Figure 2B shows casein-PAGEs incubated under this protocol at different pH levels, between 4 and 9. It was evident that the protease activity was most active under the alkaline condition of pH 9 and least active at low pH. Therefore, it was possible to reduce protease activity by simply lowering the pH of the larvae homogenate. This result in part explains an ancillary observation that recovery in downstream purification from lysates was increased 1000-fold (Wier, 1995). Although not part of this study, IL-2 immunoaffinity columns were eluted with a 0.1 M citrate buffer at pH 3.5, which disabled protease activities and enabled purification of active product (IL-2 was sensitive to pH below 3). By combining the protease inhibitor cocktail with the beneficial pH effects shown here, IL-2 instability in larvae homogenates was minimized.

The principal proteases from larvae extracts were then purified using the Bio-Rad Model 491 preparative cylindrical PAGE; fractions containing similar activities were pooled and concentrated. Two proteases with gelatin specificity also had significant IL-2 specificity. These were detected in fractions 60 to 70 (F60–70) and had molecular weights of ~72 and ~54 kDa. Figure 3 (inset) shows the purified proteases on SDS-GPAGE at the appropriate mo-
molecular weights; the 72-kDa protease was significantly more abundant, in terms of both activity and quantity (approximately threefold, SDS-PAGE not shown here). Also, additional GPAGE analysis showed that both these activities were inhibited by PMSF (not shown here). Finally, Figure 3 also demonstrates the activity of these proteases against IL-2. That is, Figure 3 depicts the results of incubating IL-2 with the 72- and 54-kDa proteases purified from larval extracts. It is clear that IL-2 was inherently unstable in PBS at room temperature (pH 7.4), because less than 25% of the initial IL-2 remained after 24 h. However, with the addition of the proteases, little or no IL-2 remained after 24 h. This observation confirmed our hypothesis that IL-2 was degraded by larval proteolytic activities as shown in Figure 1. Furthermore, according to the results presented in Figure 3, the purified 72- and 54-kDa proteases, as a group, had activity against IL-2. Finally, PMSF was shown to stabilize IL-2 as well as inhibit protease activities in the larval homogenates (Fig. 2).

In Vivo Protease Activity and IL-2 Expression

Because the 72- and 54-kDa proteases and the other PMSF-sensitive proteases depicted in the substrate-gels played a pivotal role in stabilizing IL-2 in extracts, we monitored their activity over the course of infection to determine whether they were temporally regulated in vivo; then, we assessed whether our infection timing can be coordinated to avoid the protease activity. Figure 4 depicts IL-2 production in T. ni larvae grown at 25°C as a function of time at two virus loadings (3.8 × 10⁷ pfu/cup and 7.50 × 10⁷ pfu/cup). The larvae were infected per os and were collected periodically for assays of IL-2 and protease. As expected, IL-2 maxima were observed for both cases. For the larvae infected with 3.8 × 10⁷ pfu/cup, the peak occurred at 5 days (120 h) postinfection (PI) with a value of 35 ng/larvae; for those infected with 7.5 × 10⁷ pfu/cup, the peak occurred at 4 days (96 h) PI with a value of 26 ng/larvae. Note that the higher maximum value was obtained at the lower virus concentration. The reason for this observation becomes apparent after evaluation of protease activities (Fig. 5). In the lower panel of Figure 4, the mass of larvae was depicted for each virus loading and the control. It was clear that the initial growth rates were the same for infected and uninfected samples; however, the growth of infected larvae stopped much earlier than that of uninfected larvae. Furthermore, final larvae mass was dependent on virus loading. Figure 5 shows protease production in the larvae grown at 25°C as a function of time. For control (uninfected larvae), protease activities increased progressively through the first 4 days of the fourth instar, evident by the clear bands on the SDS casein-PAGE. On days 5 and 6, however, no protease activity was detected that coincided with pupation (which started on day 5). For the AcNPV-infected larvae (3.8 × 10⁷ and 7.5 × 10⁷ pfu/cup), protease activities increased continuously, reaching a maximum on the fourth day PI; after which the protease activities decreased progressively over days 5 and 6 postinfection. Interestingly, an additional clear band was observed in infected day 5 and 6 samples at approximately 20 kDa. Because of the late timing and its absence in the control, this protease was likely due to the...
baculovirus infection. A 27-kDa protease was recently shown to be of BmNPV origin in the infected silk worm (Ohkawa et al., 1994). Also, Slack et al. (1995) demonstrated the presence a 27.5-kDa, cathepsin-L-like protease of AcNPV origin expressed in insect cells (SF-9) and T. ni larvae. In another report from our lab on SF-9 cells, 49-kDa protease activities were prominent and were shown to degrade heterologous proteins (Naggie et al., 1997). The molecular weights of those proteases were, however, significantly higher than the 20-kDa protease observed here in infected T. ni larvae. The higher-molecular-weight proteases in Figure 5 are clearly of larval origin because they appear in an identical pattern in the uninfected control samples.

As described earlier, the abrupt halt of protease activity in the uninfected larvae coincides with pupation, where the larvae undergo many biochemical and physical changes; therefore, the sudden cessation of protease activity was not unexpected (Chapman, 1972). To our knowledge, however, this report is the first indication of these proteases in T. ni larvae. The protease activities are most likely developmentally regulated, because pupation occurs at the end of day 4 or on day 5. The infected larvae, however, did not undergo pupation, and protease activity persisted, but at a lower level. This evidence suggested that the AcNPV interfered with the normal life cycle of the T. ni larvae by inhibiting pupation. Therefore, unlike bacteria (Bentley et al., 1991; Gottesman and Maurizi, 1992; Harcum and Bentley, 1993b; Maurizi, 1992) and insect cells (Wang et al., 1996), in which proteolysis was induced by overproduction of foreign protein, the protease activities here (with the exception of the 20-kDa activity) were an intrinsic part of normal larval development.

Importantly, the 72-kDa and PMSF-inhibited protease activities shown previously to affect IL-2 in extracts also had an apparent in vivo effect on IL-2 level. For the case of larvae infected with 7.5 × 10<sup>7</sup> pfu/cup, maximal IL-2 occurred at the same time as maximal protease activity (4 days PI). For the lower virus concentration, maximal IL-2 occurred at 5 days, which corresponded to a much lower level of in vivo proteolytic activity. Previously, it was demonstrated that the dose of virus in the infection defines or regulates the overall rate of virus infection (Price et al., 1989; van den Heuvel, 1993). That is, the higher the virus inoculation, the more rapidly the virus spreads throughout the organism. These observations may explain the lower level of IL-2 expression in larvae infected with a higher amount of virus. By raising the amount of virus, the propagation rate of the virus was accelerated so that the IL-2 peak (which depends on the rate of virus propagation) coincided with the peak protease activity occurring at 4 days PI. It became clear that, to optimize the IL-2 production in insect larvae, it was necessary to strike a balance between the infection virus loading and the timing of protease production. Additionally, larval growth played an important role in that the rate of larval growth was stunted by virus infection. Thus, the primary factors contributing to IL-2 yield were virus propagation rate (virus loading), larval development cycle (protease), and larval growth rate (total mass). At 25°C, the optimal point was 4 days PI at the lower virus concentration.

Figure 6 shows IL-2 production in T. ni raised at 30°C. Our intent here was to take advantage of the presumed higher metabolic activity and virus propagation rate at high temperature. These results were similar to Figure 4 with two additional levels of infection, namely 1.9 × 10<sup>7</sup> pfu/cup and 1.5 × 10<sup>8</sup> pfu/cup. Again, the highest yield did not occur at the highest virus loading, instead an intermediate loading gave the best yield due to a proper balance between infection rate, larval growth, and protease activity. The yields were 135, 150, 60, and 35 ng/larvae for infection levels of 1.9 × 10<sup>7</sup>, 3.8 × 10<sup>7</sup>, 7.5 × 10<sup>7</sup>, and 1.5 × 10<sup>8</sup> pfu/cup, respectively. The trends were similar to those at 25°C; however, the overall process proceeded at a much faster rate. At 30°C, the peak IL-2 occurred at 2.6 days (63 h) PI for all levels of infection. This 30°C IL-2 peak developed in about half the time as for the 25°C IL-2, which occurred 4 or 5 days PI. Furthermore, the total levels of IL-2 expression were higher at 30°C than at 25°C. For example, larvae infected with 3.8 × 10<sup>7</sup> pfu/cup had a maximum IL-2 peak of 35 pg/larvae at 25°C, whereas for the same amount of virus at 30°C, the maximum IL-2 peak was 150 pg/larvae. Therefore, by increasing the temperature by 5°C, it was possible to increase the yield by more than 400%. Also, because the rate of larvae growth was increased as well (Fig. 6B), and because the final larval size was similar, the productivity was increased by 800%.

Increasing the temperature, unfortunately, did not alleviate the protease activity. Figure 7 shows larval protease at 30°C as a function of time. The trend was similar to that at 25°C, except that, at 30°C, the process occurred in a similarly compressed time. The maximum protease activity was reached at 1.6 days PI rather than 4 days. In addition, in the control, no protease activity was detected after the maxi-
mum peak, whereas diminished activity was found in the infected larvae. This observation was the same as described earlier for larvae grown at 25°C. At low virus loading (1.9 and 3.8 x 10^7 pfu/cup) the maximum in IL-2 production occurred after 2.6 days when the protease levels had begun to recede. At highest loading (1.8 x 10^8 pfu/cup), the protease profile was similar, but the AcNPV replication rate was increased so that the peak in IL-2 likely coincided with the protease activities. Hence, at this loading no distinct IL-2 peak was observed; instead, only residual levels were found.

It was apparent that both the rate of larval growth and the rate at which the larvae developed to pupation were strongly dependent on incubation temperature. Also, because of the rapid increase in IL-2 production at 30°C, we found the virus propagation rate also increased with temperature. By lowering the virus concentration in the diet, it was possible to slow the infection process so that the IL-2 production peak was delayed until a time when protease activity was at a minimum, resulting in higher specific yield. The initial virus concentration, however, must be sufficient to produce an infection (van den Heuvel et al., 1993). That is, there is a critical level of virus loading below which no infection will occur. This has not been determined precisely for the IL-2 virus; however, it appears to be below 1.9 x 10^7 pfu/cup, because the IL-2 yield was lower than at 3.8 x 10^7 pfu/cup.

Overall, proteolysis in the larvae/baculovirus expression system can be partially alleviated through the use of protease inhibitors and by lowering the pH of the larval homogenate. Furthermore, we have demonstrated that timing and virus loading can be coordinated as well to minimize the in vivo loss. A simple mathematical model of these factors is currently being developed. As similar results were obtained for the expression of green fluorescent protein, the relative importance of these factors in determining yield appears independent of the specific protein. Although much higher levels of IL-2 have been produced in continuous mammalian cell culture bioreactors (e.g., Kraitje and Wagner, 1992), the specific yield on a per-protein basis has been low (<0.1% by weight). Also, we have previously reported that a recombinant product comprised fully 26% of the total protein produced in T. ni larvae (Cha et al., 1997). Hence, the potential for heterologous protein expression in larvae is quite promising.

This article is dedicated to the memory of Dr. Marjorie Wier.

Figure 6. Profile of baculovirus infection experiments conducted at 30°C. (A) IL-2 time profile expressed per larvae basis (virus loading as indicated). (B) Larvae mass for same conditions.

Figure 7. Protease activities in larvae raised at 30°C and infected with IL-2 recombinant baculovirus at loadings indicated. Note compressed time-frame at 30°C relative to 20°C, which is the normal growth temperature.
References


