Biologically active and amidated cecropin produced in a baculovirus expression system from a fusion construct containing the antibody-binding part of protein A

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A synthetic antibody-binding part derived from protein A from *Staphylococcus aureus* was used as a fusion partner in a eukaryotic expression system employing *Autographa californica* nuclear polyhedrosis as a vector. This, in conjunction with an efficient signal sequence, facilitated the purification of the antibacterial peptide cecropin A from the medium of *Spodoptera frugiperda* cells infected with a recombinant virus. In order to increase further the concentrations of fusion protein, *Trichoplusia ni* larvae were used as host. Cecropin A could be obtained after cleavage of the fusion protein with CNBr. Biological activity as well as the correct structure including the C-terminal amide group was shown using electrophoresis with detection of antibacterial proteins and mass spectroscopy.

INTRODUCTION

Cecropins are antibacterial peptides from insects induced in response to a bacterial infection [for a review see Boman & Hultmark (1987)]. Cecropin A from the American moth *Hyalophora cecropia* is a strongly amphipathic peptide, 37 amino acid residues long (Steiner *et al.*, 1981; Steiner, 1982; Boman & Hultmark, 1987). It kills and lyses both Gram-positive and Gram-negative bacteria by attacking their membranes (Steiner *et al.*, 1988a). For full biological activity its C-terminus has to be amidated.

Peptides like cecropin are most conveniently synthesized on solid-phase supports (Andreu *et al.*, 1983). Modification, such as amidation of the C-terminus, is also possible by chemical means. For expression of larger peptides and proteins, DNA-recombinant-based techniques are often preferred. However, random-coil peptides are most often prone to degradation in such systems and their purification from complex growth media can be difficult. Fusion of the coding regions of two or more genes by recombinant techniques has been a means to facilitate synthesis and purification of heterologous protein products (Itakura *et al.*, 1977; Goeddel *et al.*, 1979). Protein A from *Staphylococcus aureus* has been extensively used as a fusion partner in bacteria because of its specific binding to the Fc part of immunoglobulins (Moks *et al.*, 1987a,b). We wanted to use this binding function to simplify the purification of recombinant cecropin A but its antibacterial nature and the post-translational amide modification made expression in a bacterial system impossible.

Recombinant baculoviruses have been engineered to express high levels of a large number of foreign proteins [for a review see Luckow & Summers (1988)]. The Sf9 insect cell most often used as host in this system is a clonal isolate of an ovary cell line from *Spodoptera frugiperda*. It produces proteins with a multitude of post-translational modifications such as glycosylation, phosphorylation, acylation, proteolytic processing and protein

secretion. For a few proteins, living larvae have been successfully employed as hosts for baculovirus-mediated expression.

We took advantage of these properties and constructed a fusion between the signal peptide from human tissue plasminogen activator (t-PA), a synthetic antibody-binding part (ZZ) (Nilsson *et al.*, 1987) of protein A from *Staphylococcus aureus* and cecropin A. By adding the codon for a C-terminal glycine we provided for amidation of the C-terminus. Baculovirus recombinants with this construct behind the strong polyhedrin promoter produced cecropin A as a fusion protein with ZZ that could easily be purified. Cecropin A liberated after cleavage was biologically active, and, furthermore, we now present conclusive evidence that, in larvae of the cabbage looper *Trichoplusia ni*, a proper amidation of the C-terminus takes place.

EXPERIMENTAL

Virus, cells and insects

Autographa californica nuclear polyhedrosis virus (AcNPV) was used to infect *S. frugiperda* cells (Sf9 cell line obtained from A.T.C.C.) at a multiplicity of infection of 10 plaque-forming units (pfu) per cell. Cells were grown in Hink's medium (Hink, 1970) obtained from NordVacc AB, Stockholm, Sweden. The medium was supplemented with 10% foetal calf serum, penicillin (50 units/ml) and streptomycin (50 µg/ml). *T. ni* were reared essentially as described previously (Andersons *et al.*, 1990; Shorey & Hale, 1965).

Construction of transfer vector and purification of recombinant virus

To generate pAC610-ZZ-cecA, first the region coding for the mature part of t-PA was removed by digesting pAC610-t-PA (Steiner *et al.*, 1988b) with *Bgl*II and *Xba*I, leaving the vector with the signal-peptide-encoding sequence of t-PA. Secondly, the fragment encoding the IgG-binding protein, ZZ, a synthetic analogue of the B domain in staphylococcal protein A (Nilsson

Abbreviations used: AcNPV, *Autographa californica* nuclear polyhedrosis virus; pfu, plaque-forming units; t-PA, tissue-type plasminogen activator; ZZ, a synthetic antibody-binding part of protein A.

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et al., 1987), was excised from pEZZ18 (Löwenadler *et al.*, 1987) as a *Bam*HI–*Eco*RI fragment. Thirdly, a DNA sequence encoding cecropin A was synthesized and isolated as an *Eco*RI–*Xba*I fragment. A three-fragment ligation was carried out and the structure of the vector pAC610-ZZ-cecA was confirmed by sequence analysis.

To generate pVL941-ZZ-cecA (Fig. 1), the pAC610-ZZ-cecA was digested with *Sac*I and treated with T4 DNA polymerase. A phosphorylated *Bam*HI 12-mer linker was added and the fragment was then ligated into dephosphorylated *Bam*HI-digested pVL941 (Luckow & Summers, 1989). The proper orientation of the insert was confirmed by restriction analysis.

Co-transfection of pVL941-ZZ-cecA with AcNPV into *S. frugiperda* cells and purification of recombinant virus were according to Summers & Smith (1987). The ZZ-containing fragment was used as probe in two plaque hybridizations followed by visual screening in the last plaque purification to obtain the recombinant virus vVL941-ZZ-cecA. Hybridizations were done on Hybond-N filters (Amersham) according to the manufacturer's recommendations.

Expression of recombinant proteins *in vitro* and *in vivo*

Expression in cell culture was in bottles (75 cm²) with *S. frugiperda* cells infected with virus (10 pfu/cell). After 1 h the medium was changed to serum-free Hink's medium. For expression *in vivo*, day-1 last-instar *T. ni* larvae were injected with virus and haemolymph was collected as described earlier (Andersons *et al.*, 1990).

Purification of recombinant protein and cleavage with CNBr

Collected medium or haemolymph [diluted five times with equilibration buffer: 50 mM-Tris(pH 7.6)/150 mM-NaCl/0.05 % Tween 20] was passed through an IgG-Sepharose column (Pharmacia). The column was washed with 10 mM-ammonium acetate, pH 4.8, and fusion protein was eluted with 0.2 M-acetic acid, pH 3.3. Samples were freeze-dried, dissolved in 70 % (v/v) formic acid and cleaved with a 50 times molar excess of CNBr for 24 h at room temperature. The reaction was stopped by a 20-fold dilution with water and freeze-drying.

H.p.l.c. and m.s. analysis

After cleavage of the fusion protein, cecropin was further purified by reversed-phase chromatography using an Aquapore RP-300 column (30 mm × 2.1 mm) (Brownlee Labs) with 0.1 % trifluoroacetic acid and a gradient of acetonitrile. The flow rate was 0.4 ml/min. The column was fitted to f.p.l.c. equipment (Pharmacia) employing a home-made low-volume static mixer. The molecular mass of cecropin was determined by ²⁵²Cf plasma-desorption time-of-flight m.s. analysis (Sundqvist & Macfarlane, 1985) on a BIOION model 20 spectrometer (Bio-ion Nordic AB, Uppsala, Sweden).

Northern blots

S. frugiperda cells were infected with virus (10 pfu/cell). Total RNA was isolated by guanidinium thiocyanate extraction (Chomczynski & Sacchi, 1987), treated with formaldehyde and resolved by electrophoresis in a denaturing 1 % agarose gel (Maniatis *et al.*, 1982). Transfer to Hybond-N filter (Amersham) in 3.6 M-NaCl/0.2 M-sodium phosphate/0.02 M-EDTA, pH 7.7, was overnight. Hybridization and washing were done at 42 °C according to the manufacturer's protocol.

SDS/PAGE and Western blots

Samples were separated on SDS/15 % (w/v) polyacrylamide gels (Laemmli, 1970) and electrophoretically transferred to Hybond-C filters (Amersham) and developed as described pre-

viously (Andersons *et al.*, 1990) except that only peroxidase-conjugated rabbit anti-peroxidase antibodies were used and that incubation was overnight. Molecular-mass markers were revealed on filters with 0.1 % Amido Black.

The antibacterial inhibition zone assay

Samples (2 µl) were placed in holes in thin plates seeded with *Escherichia coli* K12, strain D21, according to Hultmark *et al.* (1982), except that incubation was overnight at 30 °C and agarose was used instead of agar to increase the sensitivity. The inhibition zones were correlated to a standard curve calculated as described by Hultmark *et al.* (1982) with *H. cecropia* cecropin A as standard.

Acidic PAGE

Samples were separated on an acidic polyacrylamide gel (Hultmark *et al.*, 1980). Overlay of *E. coli* K12, strain D21, was also as described, but agarose was used instead of agar, and gels (3 mm thick) were washed in buffered Luria broth medium for 40 min with slight shaking. Synthetic non-amidated cecropin A-38-Gly (Boman *et al.*, 1989) used as a reference was a gift from Professor Hans G. Boman (Department of Microbiology, University of Stockholm).

RESULTS

Expression plasmid construct

An expression plasmid (pVL941-ZZ-cecA) was constructed containing the signal sequence from t-PA, ZZ and a synthetic fragment coding for the mature part of cecropin A plus a codon for a C-terminal glycine residue (Fig. 1). This construct should promote synthesis, export and easy purification of cecropin A and furthermore provide for C-terminal amidation of cecropin. The details of the construct are given in the Experimental section. To transfer this fusion gene into the baculovirus genome the pVL941-ZZ-cecA plasmid was co-transfected with *A. californica* nuclear polyhedrosis virus DNA into insect cells. Several recombinant viruses were isolated as described in the Experimental section and one, vVL941-ZZ-cecA, was chosen for a more detailed characterization.

Expression of t-PA-ZZ-cecropin A

The recombinant virus was used to infect *S. frugiperda* cells and the kinetics of gene expression were followed at the RNA level. Total RNA was extracted from cells harvested at various times after infection and analysed by Northern blotting (Fig. 2). A 1.4 kb transcript was detected 24 h after infection and its level increased until 72 h. The size of this transcript is compatible with one using the transcriptional start and stop signals present in the

Fig. 1. Construction of transfer vector pVL941-ZZ-cecA

DNA fragments coding for the signal sequence of t-PA, ZZ and a synthetic fragment coding the mature part of cecropin A, with the sequence shown, were ligated in the same reading frame and inserted into the transfer plasmid pVL941. Numbers are relative to the position of the A in the start codon of the original polyhedrin gene.

Fig. 2. Induction kinetics of mRNA

Total RNA was prepared from wild-type-infected and vVL941-ZZ-cecA-infected *S. frugiperda* cells (10 pfu/cell) at the indicated times after infection and resolved by electrophoresis in a 1% denaturing agarose gel. After blotting, RNA was hybridized to the oligo-labelled probe which was synthesized from a fragment at the 3'-end of the polyhedrin gene. This fragment is present in both wild-type and recombinant mRNA as polyhedrin signals are used for terminating transcription.

polyhedrin gene of the wild-type virus. The levels of transcription can be compared with the levels of the 1.25 kb transcript from wild-type AcNPV-infected cells as the probe hybridizes to identical 3'-regions of both transcripts. At 48 h after infection the amount of recombinant transcript is estimated to be half of that of the polyhedrin mRNA but at 72 h the levels are comparable. The mRNA level of the recombinant fusion protein is thus adequate for high-level protein synthesis.

Accumulation of a fusion protein in cell culture medium and larval haemolymph

To follow the synthesis of a protein with antibody-binding capacity we subjected proteins from the growth medium of recombinant virus-infected cells to SDS/PAGE and blotting using an antibody with an Fc part that could bind to the ZZ part of the fusion protein. A protein with a molecular mass of 19 kDa accumulates in the cell supernatant (Fig. 3a, lanes 6–8). Thus the t-PA signal sequence used mediated export and cleavage of an antibody-binding protein with the expected size.

In order to exploit the potential of this fusion concept in a multitude of cell types we also employed living larvae as a host for baculovirus-mediated expression. *T. ni* larvae were injected with recombinant virus, and the haemolymph proteins were subjected to Western blotting as above. In this case also a protein with the expected size is detected (Fig. 3a, lanes 1–3). This is the

major band with antibody-binding properties except at the later time points in larval material where a strong band with molecular mass 15 kDa is seen occasionally. This corresponds to a ZZ protein without fused cecropin. These samples are from single animals. In Fig. 3(b), lanes 1 and 2, a more representative material pooled from 250 animals is shown. Weak protein bands of higher molecular mass are also present. It can be noted that these are always absent from wild-type-virus-infected haemolymphs or cell supernatants, thus excluding unspecific antibody binding. They are rather the result of inefficient protein termination.

Affinity purification of fusion protein and generation of native cecropin

One of the objectives with the fusion concept using the antibody-binding part of protein A was to facilitate the purification procedure for small proteins. From both haemolymph and cell medium the ZZ-cecropin fusion product was isolated in a single step using an antibody-conjugated Sepharose column (Fig. 3b, lanes 2 and 5). The high purity of the eluted protein is demonstrated in the Coomassie Blue-stained gel (Fig. 3c).

At the junction between the antibody-binding part and cecropin A, a methionine residue had been inserted in order to provide for cleavage with CNBr to create native cecropin A, which does not contain methionine internally. In the SDS/PAGE system used, cecropin migrates with the front but after cleavage with CNBr the antibody-binding material has a molecular mass of about 15 kDa, corresponding to the loss of the cecropin moiety (Fig. 3b, lanes 3 and 6). The equal intensity of the cleaved and uncleaved bands is misleading due to the fact that unfused ZZ protein stains very poorly with antibody (E. Holmgren, personal communication). The Coomassie-stained gel in Fig. 3(c) gives a better estimate of the amount of cleaved product, showing that the cleavage is nearly complete.

H.p.l.c. purification and characterization of recombinant cecropin A

It was obvious that biological activity was generated on CNBr cleavage of the affinity-purified fusion protein from both cell medium and larval haemolymph from tests using the inhibition zone assay (not shown).

To characterize the biologically active material further we used an acidic PAGE system with a bacterial overlay (Fig. 4). This system separates native proteins according to both size and charge differences. It easily resolved natural C-terminally amidated cecropin A (lane 2) and synthetic cecropin A-38-Gly (lane 1) with a free C-terminus differing by one net charge. In the recombinant material from cell medium (lane 4) the antibacterial spot clearly has the same mobility as the non-amidated precursor molecule but in the material from *T. ni* larvae (lane 3) the antibacterial spot has the same mobility as natural amidated cecropin A. A minor extension of antibacterial material into the region corresponding to non-amidated cecropin is implied. From lanes 5 and 6 it is clear that endogenous *T. ni* antibacterial proteins have not been induced by either the injection *per se* or injection of wild-type virus.

The most straightforward method to isolate pure cecropin from the cleaved fusion protein is to pass the material once more over the IgG affinity column to adsorb the ZZ moiety. In this study we wanted to separate different forms of cecropin and employed h.p.l.c. for the final purification. As shown in Fig. 5(a) this procedure resolved the material from larvae into three peaks out of which peak no. 2 had antibacterial activity and the same retention time as natural cecropin A from *H. cecropia* pupae. The material produced in cell culture (Fig. 5b) also contained a peak with antibacterial activity at this retention time. In addition,

Fig. 3. Protein accumulation in *S. frugiperda* cell culture supernatant and *T. ni* haemolymph after infection with ZZ-cecA recombinant virus

(a) Cells in monolayer culture were infected with virus (10 pfu/cell) and insects were injected with 1.25×10^6 pfu in a volume of 7 μ l. At the indicated times after infection, animals were killed or cell supernatants collected. Proteins were separated on SDS/polyacrylamide gels, blotted and probed for antibody-binding properties. Lanes: 1–3, vVL941-ZZ-cecA-infected haemolymph (1 μ l); 4, wild-type-virus-infected haemolymph (1 μ l); 5, wild-type-virus-infected cell culture supernatant (10 μ l); 6–8, vVL941-ZZ-cecA-infected cell culture supernatant (10 μ l). (b) Purification and cleavage of the recombinant protein ZZ-cecropin A followed by Western blotting. Lanes: 1, pooled haemolymph (1 μ l) from *T. ni*, 48 h after infection; 2, ZZ-cecropin A (400 ng) from haemolymph after elution from IgG-Sepharose; 3, IgG-Sepharose-purified ZZ-cecropin A (400 ng) from haemolymph after CNBr cleavage; 4, pooled cell culture supernatant (10 μ l); 5, ZZ-cecropin A (400 ng) from cell culture supernatant after elution from IgG-Sepharose; 6, IgG-Sepharose-purified ZZ-cecropin A (400 ng) from cell culture after CNBr cleavage. (c) Coomassie Blue-stained recombinant proteins purified from *T. ni* larval haemolymph, 48 h after infection. Lanes: 1, IgG-Sepharose-purified ZZ-cecropin A after CNBr cleavage; 2, ZZ-cecropin A after elution from IgG-Sepharose.

Fig. 4. Characterization of cecropins using acidic electrophoresis

After separation the gel was overlaid with *E. coli* K12, strain D21, to detect antibacterial activity. Lanes: 1, synthetic non-amidated cecropin A-38-Gly (0.6 μ g); 2, cecropin A (0.5 μ g) purified from *H. cecropia*; 3, CNBr-cleaved ZZ-cecA from *T. ni* haemolymph equivalent to 0.5 μ g of cecropin A from *H. cecropia*; 4, CNBr-cleaved ZZ-cecA from *S. frugiperda* cell culture supernatant equivalent to 0.5 μ g of cecropin A from *H. cecropia*; 5, *T. ni* haemolymph (10 μ l), infected with wild-type virus, 48 h after infection; 6, *T. ni* haemolymph (10 μ l), 48 h after injection of a Ringer solution; 7, peak no. 2 from h.p.l.c. fractionation of affinity-purified cecropin A from larvae; 8, peak no. 3 from h.p.l.c. fractionation of affinity-purified cecropin A from larvae.

peak no. 3 from larvae contained antibacterial activity. The electrophoretic zymograms for fractions no. 2 and no. 3 from larvae are shown in Fig. 4. Both peaks no. 2 and no. 3 contain an antibacterial substance with similar mobility to amidated cecropin A and thus the same net charge, as the corresponding molecular masses are close to that of natural cecropin A (shown below).

Determination of molecular mass

To determine the molecular mass of the active components accurately the fractions from the reversed-phase chromatography were subjected to m.s. The plasma desorption m.s. (Fig. 6) showed that peak no. 2 from larvae contains a molecular ion at m/z 4003.0. As the precision is about 0.1 %, this is in accordance with the calculated molecular mass for MH^+ of amidated cecropin A which is 4004.8 Da. It also shows that the C-terminal glycine

Fig. 5. Reversed-phase h.p.l.c. purification of recombinant cecropin A

The applied material was from affinity-purified and CNBr-cleaved fusion protein. (a) Cecropin from *T. ni* larvae injected with recombinant virus. (b) Cecropin from the medium of *S. frugiperda* (Sf9) cells in culture infected with recombinant virus. The arrow indicates the elution position of natural cecropin A from *H. cecropia* (Cec A). Also synthetic cecropin A-38-Gly was eluted close to this position.

residue must have been removed from the precursor. This fact, in conjunction with a similar net charge to that of amidated cecropin A (shown above), suggests that the recombinant cecropin A produced in larvae is indeed amidated. In peak no. 3 from the h.p.l.c. separation of the larval material the only molecular ion present in the spectrum appeared at m/z 4032.5. The identity of this material is not immediately evident. The antibacterial

Fig. 6. Plasma desorption m.s. of recombinant cecropin A from *T. ni* larvae and Sf9 cell medium

The spectra contain the number of counted ions as a function of mass over charge (m/z). Analysis of peak no. 2 (a) and peak no. 3, (b) from the h.p.l.c.-purified material from *T. ni* larvae. (c) Material from peak no. 2 from Sf9 cell medium. All spectra were obtained after the foil had been washed with 0.1% trifluoroacetic acid.

material from cell culture only contained a molecular ion at m/z 4060.1, close to 4062.9 calculated from the amino acid composition of non-amidated cecropin A-38-Gly.

Yield of protein

We tried to estimate the amount of fusion protein produced by comparison with Western blots of a dilution series of ZZ-IgF-I (Moks *et al.*, 1987b), assuming that ZZ-cecropin A and ZZ-IgF-I have the same colour to mass ratio. This gave a concentration of fusion protein of about 10 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ in cell culture and larval haemolymph respectively. Cecropin amounts to approximately one-fifth of the fusion protein. To estimate the final yield of cecropin we compared the antibacterial activity obtained with a dilution series of cecropin A in the inhibition zone assay. From 96 ml of cell medium we obtained a total of 33 μg of cecropin A. The yield is 16%. From 17 ml of haemolymph we obtained an activity equivalent to 160 μg of cecropin A if we, in this case, assume that all the antibacterial activity is derived from fully active amidated cecropin A. The total yield of cecropin amounts to 45% of the amount of cecropin contained in the unpurified fusion protein.

DISCUSSION

This work represents the first report of expression in a eukaryotic system of a fusion protein containing the antibody-binding function from the bacterial protein A. Apart from being a high-level expression system, the baculovirus system was advantageous for our purpose because insect cells in culture have no absolute requirement for serum supplementation. This made it feasible to use an immunoglobulin-binding protein as a fusion partner in this system. The fact that insects lack immunoglobulins made the same concept practicable in living insect larvae. The humoral insect immune protein, haemolin (formerly protein P4) (Sun *et al.*, 1990), which recently was shown to be a member of the immunoglobulin superfamily, is obviously not binding to protein A.

Peptides like cecropin with a random coil structure in water solution have been difficult to express in heterologous systems owing to degradation. In the present approach we have tried to minimize degradation by fusing cecropin to a hydrophilic globular domain and also by using a vector that mediates excretion. However, the most important factor for stability was the choice of expression system, as a similar construct used in bacteria

merely resulted in ZZ protein with the cecropin part removed, presumably by proteolysis (E. Holmgren, personal communication). The importance of the ZZ part for efficient concentration and purification is best recognized from our expression studies using an unfused cecropin gene (Hellers *et al.*, 1991). In cell culture this approach did not produce enough material to assay biological activity. The 16–45% yield of cecropin obtained with the present fusion approach is also high compared with the 2.6% yield from the natural source, immune haemolymph from *H. cecropia* (Hultmark *et al.*, 1982).

We have not analysed the ZZ protein obtained after cleavage of the fusion product but as the material in peak no. 1 in the h.p.l.c. separation lacked antibacterial activity and bound rabbit IgG (not shown) it is understood to be the ZZ part. It can be noticed that the ZZ material from larvae and cell culture did not co-migrate in this chromatography (peaks no. 1 in Fig. 5). We do not know the reason for this difference.

To our knowledge three attempts have earlier been made to obtain amidation in baculovirus systems. Human gastrin-releasing peptide [GRP(1–27)] was expressed as its precursor in *S. frugiperda* cells (Lebacqz-Verheyden *et al.*, 1988). In this case proteolytic processing took place in such a way that the potential amide donor (Gly-28) was separated from the GRP(1–27) peptide resulting in secretion of GRP(1–27) with a free C-terminus. The second attempt was the expression of a diuretic hormone from *Manduca sexta* (Maeda, 1989). Larvae of *Bombyx mori* were used as a host. C-Terminal amidation was inferred to have taken place from the high biological activity of the peptide obtained. However, no data were given regarding the extent of biological activity of the acid form. The third attempt concerned *Sarcophaga peregrina* sarcotoxin IA, a protein analogous to cecropins, which was recently expressed with a *Bombyx mori* baculovirus vector (Yamada *et al.*, 1990). These authors employed expression in cell culture and reported low levels of expression in the presence of a proteinase inhibitor, without amidation of the C-terminal, however.

In the present work we present conclusive results on C-terminal amidation using electrophoresis to monitor charge differences and m.s. to determine molecular mass accurately. With these methods we now show that in *T. ni* larvae the amidation reaction is operative on products synthesized from a gene introduced by a baculovirus infection. The degree of modification is high. The small amount of non-amidated product that is suggested from the acidic gel could have a number of explanations. The high strength of the polyhedrin promoter used could lead to an over-expression which saturates the protein-modification machinery. Alternatively, a subpopulation of the infected cells in the animal could lack the potential to amidate peptides. We have earlier noticed that cecropin A induced as a response to a bacterial infection in *H. cecropia* was secreted in both the amidated form and the non-amidated form (designated cecropin C), albeit at a low level (Hultmark *et al.*, 1982). Apart from the correctly processed form, we obtained a peptide with the same net charge as natural cecropin A and with molecular mass 4032.5 Da. As this is intermediate between the glycine-extended precursor and the amidated form we postulate that the peptide lacks glycine, is amidated and possibly contains a modified side chain. From the acidic electrophoresis it is clear that this form has the same net charge as natural cecropin A. Addition of a formyl group to the single tryptophan residue during the CNBr cleavage in formic acid would not change the net charge but it would add a mass of 28 Da and explain the observed difference of 29.5 Da.

We have not been able to detect amidation of cecropin produced in cell culture but instead the precursor with the additional glycine residue accumulates in the medium. In the

light of our positive results from larvae, the most likely explanation of the lack of modification would be an inability of the Sf9 cells to perform this reaction. The lack of amidation of sarcotoxin IA reported by Yamada *et al.* (1990) is another example of the inability of insect cells in culture to perform this modification reaction during a baculovirus infection.

In conclusion the fusion concept presented should be generally applicable for the synthesis and purification of peptides. The system has the added advantage that the ZZ fusion proteins are immunogenic (Löwenadler *et al.*, 1987), and it is thus suitable for the production of antibodies against any gene product, also those containing post-translational modifications. The transfer vector used could easily be modified to facilitate in-frame cloning and to contain additional targets for cleaving agents downstream of the ZZ part, as has been done in bacteria-based systems.

Financial support for this work was obtained from the Swedish Natural Science Research Council (grant B-BT/BU 4027-102) and The Swedish National Board for Technical Development (grant 512-87-00430).

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Received 25 March 1991/30 May 1991; accepted 6 June 1991