Production of recombinant sarcotoxin IA in *Bombyx mori* cells

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A cDNA for sarcotoxin IA, an antibacterial protein of *Sarcophaga peregrina* (fleshfly), was inserted into a silkworm baculovirus vector and expressed in Bm-N cells, a line of *Bombyx mori* cells. When a cysteine proteinase inhibitor, *p*-chloromercuribenzenesulphonic acid, was present in the culture medium, a significant amount of recombinant sarcotoxin IA accumulated, but without this reagent the product seemed to be degraded in this system. The C-terminus of the recombinant sarcotoxin IA seemed to be glycine, not amidated arginine as found in authentic sarcotoxin IA. Probably, Bm-N cells lack the C-terminal α -amidation enzyme.

INTRODUCTION

Sarcotoxin I is a group of cecropin-type antibacterial proteins purified from the haemolymph of third-instar larvae of *Sarcophaga peregrina* (fleshfly) whose body wall has been pricked with a hypodermic needle (Okada & Natori, 1983, 1985). As sarcotoxin I shows a wide antibacterial spectrum, especially on Gram-negative bacteria, it seems important to investigate its precise mode of action, with special reference to the relation between its structure and function. However, the sarcotoxin I content of the haemolymph is normally low, and only about 70 μ g of sarcotoxin I (a mixture of three related proteins) was purified from 150 ml of haemolymph (Okada & Natori, 1983). Therefore, in order to study the function of this protein, a procedure had to be developed to obtain larger quantities of it.

We isolated a cDNA clone of sarcotoxin IA, which is a major protein of the sarcotoxin I family (Matsumoto *et al.*, 1986). *Escherichia coli* is highly sensitive to this protein, and so cannot be used to produce recombinant sarcotoxin IA. Therefore we tried to produce sarcotoxin IA in cultured *Bombyx mori* cells using a baculovirus expression system. This paper reports the production of sarcotoxin IA in *Bombyx* cells.

MATERIALS AND METHODS

Construction of transfer vector pBMSIA

The cDNA clone of sarcotoxin IA (pTO19) contains one *Pst*I site, one *Eco*RI site and two *Xmn*I sites (Matsumoto *et al.*, 1986). To integrate this cDNA into the baculovirus expression vector, we modified pTO19 to create a *Bgl*II site in the upstream region of the initiation codon. For this, we first ligated a synthetic 26-mer linker containing a *Bgl*II site to the *Pst*I site. Then we digested this DNA with *Xmn*I to separate three DNA fragments. Two large fragments were ligated to construct a plasmid containing the entire cDNA sequence of sarcotoxin IA between the *Bgl*II and *Eco*RI sites. Then the *Bgl*II–*Eco*RI fragment from this plasmid was inserted between the *Bgl*II and *Eco*RI sites of the baculovirus cloning vector pBM050 (Horiuchi *et al.*, 1987). The resulting subclone was named pBMSIA.

Cloning of recombinant nuclear polyhedrosis viruses carrying sarcotoxin IA cDNA

Sarcotoxin IA was produced in *Bombyx mori* Bm-N cells by the method of Maeda *et al.* (1985). *Bombyx mori* nuclear

polyhedrosis virus (T3 strain) DNA was prepared by treatment of the virus particles with proteinase K and then phenol extraction. Viral DNA and pBMSIA DNA were mixed in a molar ratio of 1:100 (2.5 μ g of viral DNA and 12.5 μ g of pBMSIA DNA), and transfected into 1×10^5 Bm-N cells by the calcium phosphate method. After overnight culture, the medium was changed, and culture was continued for 1 week. Infectious viral particles appearing in the culture medium were cloned by the limited dilution method (Liu & Henle, 1953), and each clone was infected into Bm-N cells. Recombinant virus clones that did not produce polyhedrins were selected and stored at -80°C .

Purification of sarcotoxin IA from the culture medium of Bm-N cells infected with recombinant virus

Sarcotoxin IA was purified from the culture medium of recombinant virus-infected cells by a modification of the method described previously (Okada & Natori, 1983). Briefly, culture medium was applied to a column of CM-cellulose. The sarcotoxin IA fraction recovered from the column was heated for 10 min at 100°C , and the resulting supernatant was further fractionated on a Sephadex G-50 column. The fraction containing sarcotoxin IA was subjected to reverse-phase h.p.l.c. on a Synchropak RP-P (C_{18}) column to obtain pure material. The amount of sarcotoxin IA obtained was determined by radioimmunoassay.

Radioimmunoassay of sarcotoxin IA

Antibody was raised against synthetic sarcotoxin IA. Since sarcotoxin IA is a small protein consisting of 39 amino acid residues, we immunized albino rabbits with reverse-phase column-packing material (C_{18}) coated with sarcotoxin IA by the method of Flyg *et al.* (1987). Radioimmunoassay was performed essentially as described previously (Komano *et al.*, 1983) using sarcotoxin IA, radioiodinated by the method of Chisholm *et al.* (1969), and its antibody. Sarcotoxin IA could be radioiodinated because it contains a histidine residue. As competitor, we used synthetic sarcotoxin IA in which the C-terminal amino acid residue was amidated arginine, as in authentic sarcotoxin IA ($-\text{Arg-NH}_2$ form), and its derivative having an additional glycine at its C-terminus ($-\text{Arg-Gly}$ form) (Nakajima *et al.*, 1987).

Assay of antibacterial activity

Antibacterial activity was measured essentially as described previously (Okada & Natori, 1983). *E. coli* K-12 594 was grown

Abbreviations used: PCMBs, *p*-chloromercuribenzenesulphonic acid.

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in antibiotic medium (Difco). Cells in the exponential phase were collected and suspended in 10 mM-sodium phosphate buffer, pH 6.0, containing 130 mM-NaCl at a density of 2.5×10^8 cells/ml. A mixture of a test sample (200 μ l), antibiotic medium (190 μ l), and *E. coli* suspension (10 μ l) was incubated at 37 °C for 140 min with shaking to determine bacterial growth.

RESULTS

Construction of the recombinant virus and expression of sarcotoxin IA cDNA in Bm-N cells

For insertion of sarcotoxin IA cDNA into baculovirus cloning vector pBM050, a new *Bgl*II site had to be created in the upstream region of the initiation codon in pTO19. For this, we ligated a chemically synthesized 26-mer linker containing a *Bgl*II site to the *Xmn*I site located near the initiation codon in pTO19 and adjusted the frames. Then we isolated the *Bgl*II-*Eco*RI fragment containing the entire sarcotoxin IA sequence from this plasmid and inserted it into the cloning site of pBM050. The resulting subclone (pBMSIA) was co-transfected with DNA of a wild-type nuclear polyhedrosis virus (strain T3) into Bm-N cells, and recombinant viruses containing sarcotoxin IA cDNA in their polyhedrin gene were selected. Two virus strains termed BmSIA1 and BmSIA2 were established.

To determine whether cells infected with BmSIA1 or BmSIA2 produced sarcotoxin IA, we measured by radioimmunoassay the amount of sarcotoxin IA in the medium. As shown in Fig. 1, different competition curves were obtained on addition of increasing amounts of sarcotoxin IA (-Arg-NH₂ form) and its derivative (-Arg-Gly form) to reaction mixture containing radioiodinated sarcotoxin IA and its antibody. The results indicate that it is possible to determine the C-terminus of sarcotoxin IA added to the reaction mixture from the shape of the competition curve in this assay system.

When culture medium of Bm-N cells infected with BmSIA1 was tested in this radioimmunoassay system, no inhibition of the binding of radioiodinated sarcotoxin IA and its antibody was detected. This suggested two possibilities: sarcotoxin IA was not produced for some reason or sarcotoxin IA was produced but

Fig. 1. Competition curve for radioimmunoassay

Fixed amounts of radioiodinated sarcotoxin IA (¹²⁵I was incorporated into histidine) were incubated with increasing amounts of synthetic sarcotoxin IA or its derivative and diluted antiserum against sarcotoxin IA at 4 °C for 12 h. Then a suspension of *Staphylococcus aureus* Cowan I strain was added and the mixture was centrifuged to precipitate the antigen-antibody complex. The radioactivity of the precipitate was counted. ●, Sarcotoxin IA with -Arg-NH₂ C-terminus; ○, derivative of sarcotoxin IA with -Arg-Gly C-terminus.

Fig. 2. Effects of various proteinase inhibitors on the concentration of sarcotoxin IA in the culture medium of virus-infected Bm-N cells

Increasing amounts of culture medium prepared in the absence of proteinase inhibitor (○) or in the presence of 0.25 mM-phenylmethanesulphonyl fluoride (▲), 1 μ g of pepstatin/ml (■) or 0.5 mM-PCMBS (●) were added to the radioimmunoassay system, and the percentage radioactivity of the antigen-antibody complex formed was determined.

was degraded rapidly. To test the latter possibility, we cultured Bm-N cells in the presence of various proteinase inhibitors, and examined the production of sarcotoxin IA.

As shown in Fig. 2, when virus-infected Bm-N cells were cultured in the presence of PCMBS, the culture medium significantly inhibited the binding of sarcotoxin IA and its antibody, indicating that it contained sarcotoxin IA. Two other proteinase inhibitors, phenylmethanesulphonyl fluoride and pepstatin, had essentially no effect on the production of sarcotoxin IA. As PCMBS is a cysteine-proteinase inhibitor, it is likely that some cysteine proteinase in Bm-N cells participates in the degradation of recombinant sarcotoxin IA.

Purification of recombinant sarcotoxin IA

As the culture medium of virus-infected Bm-N cells cultured in the presence of PCMBS contained sarcotoxin IA, we tried to

Fig. 3. Chromatogram of antibacterial activity on a column of CM-cellulose

Tenfold-diluted culture medium prepared in the presence of 0.5 mM-PCMBS was applied to a column (4.5 cm $\times$ 6 cm) of CM-cellulose, and adsorbed material was eluted with 10 mM-sodium phosphate buffer, pH 6.0, containing 520 mM-NaCl. The antibacterial activity of each fraction was measured. ○, A_{280} ; ●, relative bacterial growth.

purify sarcotoxin IA from the medium. Virus-infected Bm-N cells were cultured for 3 days. Then PCMBs was added to the medium to give a final concentration of 5 mM. Culture was continued for 1 more day, and then the medium was collected. The content of sarcotoxin IA in the medium determined by radioimmunoassay was about 1 mg/l. A procedure for purification of sarcotoxin IA from the haemolymph of *Sarcophaga* larvae has been established, involving CM-cellulose chromatography, heat treatment, gel filtration through Sephadex G-50 and reverse-phase h.p.l.c. (Okada & Natori, 1983, 1985). We used this procedure to purify sarcotoxin IA from 450 ml of culture medium. Antibacterial activity was monitored as growth inhibition of *E. coli*.

As shown in Fig. 3, antibacterial activity was recovered as a sharp peak from medium containing PCMBs. However, this

Fig. 4. Chromatogram of sarcotoxin IA on a column of Sephadex G-50

The active fraction from CM-cellulose was heated at 100 °C for 10 min, and the resulting supernatant was concentrated and applied to a column (1.2 cm × 120 cm) of Sephadex G-50. The column was washed with 10 mM-sodium phosphate buffer, pH 6.0, containing 130 mM-NaCl. Antibacterial activity and the amount of sarcotoxin IA in each fraction were determined. ○, A_{280} ; ●, relative bacterial growth; ▲, amount of sarcotoxin IA.

Fig. 5. PAGE of purified recombinant sarcotoxin IA

Recombinant sarcotoxin IA purified by reverse-phase h.p.l.c. was subjected to electrophoresis under denaturing conditions, and stained with Coomassie Brilliant Blue. Lane 1, recombinant sarcotoxin IA; lane 2, authentic sarcotoxin IA; lane 3, the derivative of sarcotoxin IA; lane 4, a mixture of molecular-mass markers. An arrow indicates the position of authentic sarcotoxin IA.

Table 1. Summary of purification of sarcotoxin IA from 450 ml of the culture medium of virus-infected Bm-N cells

Purification step	Total sarcotoxin IA* (µg)	Total protein (mg)	Specific content (mg/mg)	Yield (%)
Culture medium	511	2321	2.2×10^{-4}	100
CM-cellulose	210	22	9.5×10^{-3}	41.1
Heat treatment	189	9.5	2.0×10^{-2}	37.0
Sephadex G-50	169	7.7	2.2×10^{-2}	33.1
1st h.p.l.c.	24	0.035	6.9×10^{-1}	4.5
2nd h.p.l.c.	19	0.021	9.0×10^{-1}	3.7

* The amount of sarcotoxin IA was determined by radioimmunoassay.

peak was not clearly detected in medium containing no PCMBs (results not shown), consistent with the results of radioimmunoassay. Fractions containing antibacterial activity were pooled and fractionated further on a column of Sephadex G-50 after heat treatment. As is evident from Fig. 4, antibacterial activity was eluted as a single peak coinciding with the peak of sarcotoxin IA determined by radioimmunoassay. We subjected this peak fraction to reverse-phase h.p.l.c. and obtained sarcotoxin IA giving a single band on SDS/PAGE (Fig. 5). A typical purification is summarized in Table 1. About 20 µg of pure sarcotoxin IA was obtained from 450 ml of the culture medium.

Molecular nature of recombinant sarcotoxin IA

Analysis of recombinant sarcotoxin IA suggested that its C-terminus was not amidated arginine, like that of authentic sarcotoxin IA, but glycine. As described above, it is possible to deduce the structure of the C-terminus of sarcotoxin IA from the profile of the competition curve obtained by radioimmunoassay. The profile of the competition curve of recombinant sarcotoxin IA coincided with that of the derivative of sarcotoxin IA having the -Arg-Gly structure at its C-terminus, not with that of authentic sarcotoxin IA, as shown in Fig. 6. Furthermore, the mobilities of authentic sarcotoxin IA and its derivative on PAGE are a little different when compared under non-denaturing conditions. We subjected recombinant sarcotoxin IA to electrophoresis and located its activity by the method of Hultmark *et al.*

Fig. 6. Competition curve of recombinant sarcotoxin IA in radioimmunoassay

Purified recombinant sarcotoxin IA was used as a competitor for radioimmunoassay. The competition curve (●) was compared with those of authentic sarcotoxin IA (Δ) and its derivative (○).

Fig. 7. PAGE of recombinant sarcotoxin IA under non-denaturing conditions

PAGE was performed under non-denaturing conditions. Then soft agar containing *E. coli* was layered on top and the gel was incubated for 18 h at 37 °C to locate spots of antibacterial materials according to the method of Hultmark *et al.* (1980). Lane 1, authentic sarcotoxin IA; lane 2, recombinant sarcotoxin IA; lane 3, the derivative of sarcotoxin IA.

(1980). As is evident from Fig. 7, the mobility of recombinant sarcotoxin IA coincided with that of the derivative of sarcotoxin IA, again suggesting that the C-terminus of recombinant sarcotoxin IA is glycine.

DISCUSSION

This paper describes the production of sarcotoxin IA using the baculovirus system of *Bombyx mori*. When PCMBS was added to the culture medium of Bm-N cells, intact sarcotoxin IA was recovered from the culture medium. However, without this reagent, no appreciable sarcotoxin IA was detected. This finding suggested that sarcotoxin IA was degraded by a cysteine proteinase in the Bm-N cells. Using exactly the same baculovirus system, we were able to produce eukaryotic transcription factor S-II in the absence of PCMBS (Hirashima *et al.*, 1989). Although recombinant S-II stayed in the cells, recombinant sarcotoxin IA was secreted into the culture medium, as cDNA of sarcotoxin IA contains a signal sequence (Matsumoto *et al.*, 1986). However, secretory protein is not necessarily unstable in this system, since a high level of interferon- α was reported to be produced in the culture medium of Bm-N cells (Maeda *et al.*, 1984). Probably, sarcotoxin IA is an extremely unstable protein in this system, and PCMBS is a useful reagent to protect such an unstable protein from attack by this proteinase.

The C-terminus of recombinant sarcotoxin IA seemed to be different from that of authentic sarcotoxin IA. Probably, Bm-N cells do not have an amidating enzyme that cleaves C-terminal glycine to amidate adjacent amino acid residues (Glembotski

et al., 1984; Kizer *et al.*, 1984). This is important because various biologically active peptides, including cecropins (van Hofsten *et al.*, 1985; Qu *et al.*, 1982), have amidated amino acid residues in their C-termini. From the present results, we propose that these peptides are produced as precursor peptides with glycine in their C-termini when this production system is used.

About 20 μ g of pure sarcotoxin IA could be obtained from 450 ml of culture medium. Isolation of sarcotoxin IA using this system was much easier than its isolation from the haemolymph of injured *Sarcophaga* larvae, because collection of haemolymph is very time-consuming. However, the C-terminus of the recombinant sarcotoxin IA produced must be subjected to α -amidation to obtain authentic sarcotoxin IA.

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