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RESEARCH ARTICLE

Induction, selection and antibacterial activity of the antibacterial peptides from lepidopteran insect cultured cell lines

Rong PENG[1], Zhong YANG[2], Kaiyu LIU[1], Hanchao YAO[1], Hong YANG[1], Yanfang CUI[3], Huazhu HONG (✉)[1]

1 College of Life Science, Central China Normal University, Wuhan 430079, China
2 College of Bioengineering, East China University of Science and Technology, Shanghai 200237, China
3 College of Chemistry, Central China Normal University, Wuhan 430079, China

Abstract We induced 3 cell lines that were *in vitro* cultured from Lepidoptera with heat inactivated *Escherichia coli* $DH_{5\alpha}$ to stimulate the antibacterial peptide followed by antibacterial activity assay, induction dynamic research and Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE) experiment. The antibacterial activity of the induced BTI-Tn-5B1 cell line was the highest, and the antibacterial activity increased gradually to the highest level in 16 hours after stimulation. A new antibacterial peptide with a molecular weight of about 8000 Da was preferentially induced in *Trichoplusia ni* BTI-Tn-5B1 cells in 16 hours after stimulation. Antibacterial activity assays indicated that it had inhibition against *Staphylococcus aureus*, *Escherichia coli* $K_{12}D_{31}$ and *Salmonella derby*. It has especially strong inhibition against Gram-negative bacteria such as *Escherichia coli* $K_{12}D_{31}$ and *Salmonella derby*.

Keywords antibacterial peptide, BTI-Tn-5B1 cell line, Tricine SDS-PAGE, antibacterial activity

1 Introduction

In response to pathogen invasion, insects produce a battery of inducible antibacterial peptides, which are rapidly induced and synthesized in the fat body and hemocytes with their gene expression by intracellular signaling pathways and released into the hemolymph (Boman, 1991; Fearon and Locksley, 1996; Hoffmann, 1997). The antibacterial peptides are key components of the innate immunity response of insects. Since the first report of Cecropin (Boman et al., 1972; Steiner et al., 1981), which is an inducible antibacterial peptide induced from a lepidopteran insect (*Hyalophora cecropia*), a great number of antibacterial peptides of different kinds have been isolated and characterized from nearly every insect species that has been examined (Cociancich et al., 1994; Bulet et al., 1999; Lowenberger et al., 1995; Homma et al., 1996; Lemaitre et al., 1997; Viziole et al., 2000). In recent years, insect antibacterial peptides research has gradually attracted attention. These antibacterial peptides have many advantages, such as small molecular mass, good thermostability, and broad biological activity etc., which have demonstrated their great potential and application in medicine, pharmacy, agriculture, food industry and many other fields (Cociancich et al., 1994; Bulet et al., 1999).

The study of immune-responsive cells *in vitro* has provided the basis for our thorough understanding of the mammalian immunity. In comparison, the exploration of the innate immune response of insects using cultured cells is still to be developed. Actually, compared with the intact insects, there are more advantages and significances in using cell lines *in vitro* in many respects. The progress achieved world wide which used cultured cell lines supports the likelihood that these systems will be important in further biological and molecular studies of insect immunity (Gao et al., 1999; Sun et al., 1999; Fallon and Sun, 2001). In this paper, we attempt to study insect antibacterial peptides in this new direction.

2 Materials and methods

2.1 Cell lines and culture conditions

The 3 cell lines used were: *Spodoptera exigua* (SE), *Spodoptera litura* (SL), and *Trichoplusia ni* BTI-Tn-5B1 (5B1). The SE and SL cell lines were provided by

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E-mail: hzhong @mail. ccnu. edu. cn

Zhongshan University. The 5B1 cell line was kindly provided by professor Granados of Cornell University. Cells were maintained in Grace's medium containing 10% fetal bovine serum (FBS) at 26°C, and routinely subcultured every 3–4 days.

2.2 Bacteria strains and culture conditions

The bacterial strains used were: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* $DH_{5\alpha}$, *Escherichia coli* $K_{12}D_{31}$, *Salmonella derby*. The bacteria were cultured in Luria-Bertani (LB) broth at 37°C for 24 hours.

2.3 The induction and extraction of the antibacterial peptide

2.3.1 The preparation of the inducers

The bacterial cells of *Escherichia coli* $DH_{5\alpha}$ were harvested by centrifugation (2000 rpm,10 min), washed two times with normal saline solution (0.9% sodium chloride), and then sterilized (105 Pa) for 30 min.

2.3.2 Induction

We removed the old medium, collected the logarithmic growth phase cells (10^7/mL) and cultured in 100-mm tissue culture plates and allowed to reach 80%–90% confluency. After washing twice with phosphate-buffered saline (PBS), cells were incubated in Grace's medium without FBS with heat inactivated *Escherichia coli* $DH_{5\alpha}$ for 48 hours. About 1000 bacteria per cell were added into the medium.

2.3.3 Extraction of the antibacterial peptide

The collected cell culture supernatant was filtered (0.45 μm) and trichloroacetic acid (TCA) was added to a final concentration of 10%. This was followed by incubation on ice for 10 min. The sample was centrifuged at 10000 rpm at 4°C for 10 min and the protein precipitate was washed with acetone and dissolved in water (9:1). The final protein pellet was collected by centrifugation at 14000 rpm for 15 min and stored by vacuum freeze-drying.

2.4 Antibacterial activity assays

2.4.1 Liquid culture inhibition method

E. coli $K_{12}D_{31}$ as the indicator bacterium was cultivated in a shake flask over night. Then, we added 20 μL samples into each well of a 96-well plate. The control and repeat were done when necessary. The plate was dried by using a vacuum desiccator, then 100 μL of a suspension of bacteria per well was added. Cultivation in a shake flask was done overnight again. ELISA was used to assess OD_{595}. High OD_{595} value means high antibacterial activity.

2.4.2 Inhibition zone assay

The bacteria were cultured in 100-mm culture plates containing LB solid agar culture medium, wells of 5 mm in diameter were made in the solid agar medium, samples were assayed and the control samples were added into the wells. The plates were cultured at 37°C. Then the diameter of inhibition zone on the plates was measured.

2.5 Tricine SDS–polyacrylamide gel electrophoresis (Tricine SDS–PAGE)

2.5.1 Gel preparation

According to the system described by Schagger and Von Jagow (Schagger and Von, 1987), the 4% stacking gel and 16.5% separating gel were prepared. Buffer system using Tris-Tricine.

2.5.2 Sample application

The samples were incubated in the loading buffer for 5 min at 100°C and centrifuged at 8000 rpm for 5 min. Then the supernatant was used for electrophoresis.

2.5.3 Electrophoresis

The gel electrophoresis was done at 120 V until the tracking dye reached 2 cm at the bottom of the gel.

2.5.4 Rapid fixing

The gel was immediately fixed in a solution containing 50% methanol and 10% acetic acid for 1 h.

2.5.5 Staining

After fixing, the gel was stained in 0.25% (W/V) Coomassie brilliant blue R-250 for 1–2 h depending on the thickness and the composition.

2.5.6 Destaining

A complete background destaining was achieved by shaking the gel in 10% acetic acid for at least 2 h. The destaining solution was renewed every 30 min.

3 Results

3.1 Kinetics analysis of induction

3.1.1 Comparison of the inducible activity among the three cell lines

By liquid growth inhibition assay, the inducible antibacterial activities of the three cell lines were assayed. Activity

level was showed with H (%), H (%) = (A_0-A) /A_0 (A:OD of the induced cell supernatants, A_0: OD of the normal cell supernatants that were not induced). The higher the H was, the stronger the inducible activity was. The results (Fig. 1) showed that the inducible activity of the 5B1 cells was the highest, the activity of the SL cells was the lowest, and the activity of the SE cells was in the middle.

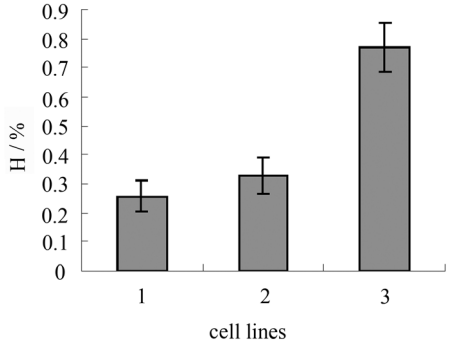

Fig. 1 Comparison of the inducible activity level of the three cell lines. Note: 1: SL cell line, 2: SE cell line, 3: 5B1 cell line

3.1.2 Inducible activity kinetics of 5B1 cells at different times after induction

Taking the samples of 5B1 cell supernatant at different times (4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 36 h) after induction, and using the liquid growth inhibition assay, the OD at different times was assayed respectively. The smaller the OD is, the higher the inducible activity is. The induction kinetics curve of the 5B1 cell line is determined in terms of OD (Fig. 2). As showed in Fig. 2, with the increase of time after induction, the OD decreased gradually and reached the lowest at 16 h after induction, afterward, the OD rose up gradually. That is, the inducible antibacterial activity of the 5B1 cells increased gradually and reached the highest at 16 h after induction, after which, the antibacterial activity reduced gradually. There was little change in the OD of the normal cells that were not induced.

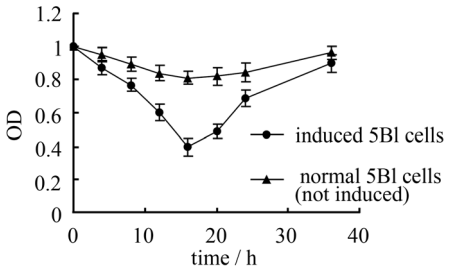

Fig. 2 Inducible activity kinetics analysis of 5B1 cells

3.2 Results of antibacterial activity assays

The results of liquid growth inhibition assay and inhibition zone assay indicated that the induced peptide had broad inhibitory activity against four tested species of *Staphylococcus aureus*, *Escherichia coli* $DH_{5\alpha}$, *Escherichia coli* $K_{12}D_{31}$, and *Salmonella derby*. Especially, it showed strong inhibitory activity against the Gram-negative bacteria: *Escherichia coli* $K_{12}D_{31}$, and *Salmonella derby* (Fig. 3).

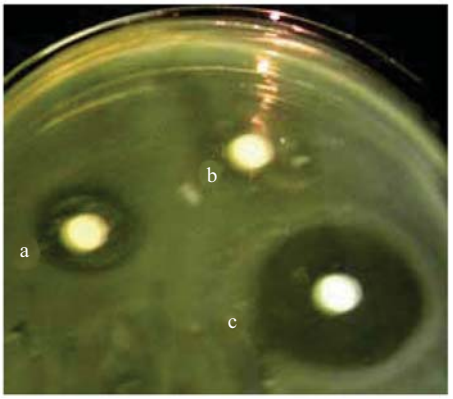

Fig. 3 Inhibitory effect on *Salmonella derby*. Note: (a): inhibitory effect on *Salmonella derby*; (b): the control from the normal cells; (c): the control with ampicillin.

3.3 Result of Tricine SDS– PAGE

The discontinuous Tricine SDS- PAGE analysis revealed the great difference between the induced 5B1 cells and the uninduced 5B1 cells in terms of the expression of protein. The main difference is that a new peptide with a molecular weight of about 8 KDa was expressed in the induced 5B1 cells (Fig. 4). That is, after stimulating with heat-inactivated bacteria, the 5B1 cells produced an antibacterial peptide that was responsible for the inducible antibacterial activity of the induced 5B1 cells.

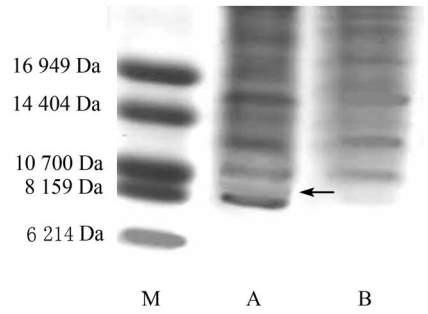

Fig. 4 Differential expression of the 5B1cells induced with heat-inactivated bacteria by Tricine SDS-PAGE analysis. Note: Lane A: 5B1 cells were induced with heat-inactivated bacteria; lane B: 5B1 cells were not induced as a control. The arrow indicates the prominent antibacterial peptide that was induced after induction; lane M: protein marker.

4 Discussion

So far, the study on antibacterial peptides by cultured cells has been carried out mainly in cell lines derived from Diptera and Coleoptera insects. In contrast, the cell lines from the *Lepidoptera* have received little attention (Gao et al., 1999; Sun et al., 1999; Fallon and Sun, 2001). However, the majority of cell lines *in vitro* have been established from members of the *Lepidoptera* (Fallon and Sun, 2001), which will provide rich experimental sources for studies on insect immunology. In this context, we chose three Lepidoptera cell lines to investigate the inducible activities. Results showed that these three cell lines exhibited various degrees of inducible antibacterial activities upon stimulation with heat-inactivated bacteria. It was interesting to note that the 5B1 cell line showed the strongest inducible activities. That is, the 5B1 cell line, an inducible competent cell line, was screened from the three cells in our present study. Furthermore, in the study of induction kinetics of the 5B1 cells, it was found that with the increase of time after induction, the inducible activity rose up gradually, and reaches the highest at 16 h, then reduced gradually. The normal 5B1 cells, as a control, did not show the antibacterial activity at all. The result of Tricine SDS-PAGE revealed that a new peptide with a molecular mass of approximately 8 KDa was induced in the 5B1 cells at 16 h after induction. Consequently, the antibacterial peptide was responsible for the inducible antibacterial activity of the induced 5B1 cells. Antibacterial activity assays indicated that it had broad inhibitory activity against four tested species of *Staphylococcus aureus*, *Escherichia coli* $DH_{5\alpha}$, *Escherichia coli* $K_{12}D_{31}$, and *Salmonella derby*. Especially, it showed strong inhibitory activity against the Gram-negative bacteria: *Escherichia coli* $K_{12}D_{31}$, and *Salmonella derby*. Therefore, this new antibacterial peptide would have potential value in application.

In our study, we concluded that the analysis of induction kinetics of cultured cell lines would facilitate the screening of the inducible cell line with high inducible antibacterial activity and provide a basis for finding new peptides with strong and broad activity spectrum. Considering the difficulties of practical manipulation, only three lepidopteron cell lines were studied in the present study. The investigation of other insect cell lines and even other animal cell lines are underway in our laboratory. We attempt to establish the generally applicable technique terrace *in vitro* that can be potentially extended to the other animal cell lines for screening new antibacterial peptides. This not only can be an important alternative to the present way of obtaining antibacterial peptides from the live animal or the animal tissues and organs, but also will accelerate the study of the inducible antibacterial peptides by using cells in culture.

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