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Purification and cytotoxic properties of *Bacillus cereus* hemolysin II

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Abstract

The hemolysin II from *Bacillus cereus*, HlyII, is a member of the β-barrel pore-forming toxin family of secreted microbial proteins that includes the *Staphylococcus aureus* α-toxin. Compared with other proteins of the family, hemolysin II has 90 extra amino acids at its C-terminus. To examine more closely the cytotoxic and pore-forming properties of the protein, we have cloned and expressed it in *Escherichia coli*. We developed a purification procedure for the matured HlyII protein from both culture media and cell extracts using a combination of cation exchange and affinity chromatography together with gel-filtration. In both cases, the fully processed HlyII protein was purified as confirmed by N-terminal sequence analysis. The HlyII protein exhibits cytolytic activity of different extent on erythrocytes from various kinds of mammals. The results presented here show for the first time that two types of human cells are sensitive to HlyII action. In view of its broad cytotoxic activity as well as the ability to interact with artificial membranes, we assume that HlyII needs no specific receptor to bind to cell membranes.

Keywords: Hemolysin; Pore-forming toxins; α toxins; *Bacillus cereus*; Pathogenicity; Dye affinity chromatography; Secreted proteins

Many bacterial pathogens produce toxins that kill and lyse host cells by interacting with their plasma membrane. The majority of these cytolytic toxins are pore-forming proteins, and several of them have been shown to represent important virulence factors of the corresponding bacteria [1].

Bacillus cereus is an opportunistic human pathogen found to be the infectious agent of endophthalmitis and food poisoning, sometimes lethal [2,3]. It is associated with a wide range of clinical symptoms and is well known as a food and cosmetic pollutant. In *B. cereus*, several different pore-forming cytolysins have been identified. One of them is the hemolysin II, HlyII,[1] which was originally cloned and characterized in our laboratory [4,5]. The protein is widely spread among bacteria which belong to the *B. cereus* group, including *Bacillus thuringiensis*, some subspecies of which are used as insecticide [5]. Based on its deduced amino acid sequence, HlyII is a member of the family of oligomeric β-barrel channel-forming toxins (β-PFT) which are common among gram-positive pathogens [6,7]. This family also includes a number of *Staphylococcus aureus* cytolysins (α-toxin, leukocidins, and γ-hemolysin) [8], the *Clostridium perfringens* β-toxin [9], and the *B. cereus* CytK toxin [10]. Studies of the β-PFT family are important in many aspects: (i) they are involved in serious pathologies of humans and farm animals, (ii) they are a good model system to investigate protein–membrane interaction, and (iii) they are the basic elements for the construction of nanopores with biotechnological applications in various fields [11].

HlyII has the longest polypeptide chain in the β-PFT family, with 412 residues, and contains a C-terminal 94 amino acid extension that has no homology with any other known β-PFT. The remainder of HlyII shares 30%

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[1] *Abbreviations used:* β-PFT, β pore-forming toxins; HlyII, hemolysin II; αHL, α hemolysin of *S. aureus*; IPTG, isopropyl-β-D thiogalactopyranoside.

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sequence identity with αHL. HlyII produced by in vitro transcription and translation forms a heptameric transmembrane pore in both red cell membranes and in planar lipid bilayers, which is resistant to SDS. Cytotoxic effects of HlyII against other cell lines have not yet been examined [7]. The involvement of HlyII in the development of infection in immune-compromised recipients or the enteric syndrome is unknown.

Studies of HlyII toxin properties were hindered by the absence of an efficient and reliable procedure for protein purification. In this paper, we report a procedure for expression and purification of hemolysin II from the culture medium of *Escherichia coli* cells. HlyII was purified to homogeneity with a high specific activity. The results presented here show, for the first time, that hemolysin II is cytotoxic for a number of mammalian cells.

Materials and methods

Materials

DNA oligonucleotides for PCR were from Syntol, Moscow. All restriction enzymes, *Taq* DNA polymerase, and T4 DNA ligase were from Fermentas. The Ni-CAM HC resin was from Sigma, the CM52-cellulose resin was from Whatman, Ultrogel AcA44 from IBF Biotechnics, and Superose columns were from Amersham. All other reagents were of the highest grade available. The purified chlorotriazinyl dyes [12] were the generous gift of Professor V.V. Karpov (Institute of Organic Semi-products and Dyes, Moscow, Russia).

Bacterial strains and cell lines

Escherichia coli Z85 [*thi*, Δ (*lac-proAB*), Δ (*srl-recA*), *hsdR*, *supE*, *Tn10*(*Tc*^{r}), (F^+*traD*, *proAB*, *lacI*, *DM15*)] and BL21 (F^-, *omp*T, *hsd* S_B (r_{B-} m_{B-}), *dcm*, *gal*, (DE3)) [13] were used as host strains for protein expression and in cloning. *E. coli* LEPI is a *pho*B^- derivative of the wild-type strain K10, it is defective in synthesis of the signal endopeptidase [14,15]. Plasmid pUJ2 has been described elsewhere [4], it contains the hemolysin II genetic determinant as a 2.9 kb *Eco*RI fragment of the *B. cereus* 771 chromosomal DNA cloned into vector pUC19 (Fig. 1A). The bacteria were routinely grown in a medium (20 g/L tryptic soy broth (Difco), 5 g/L yeast extract, and 10 g/L NaCl) supplemented with ampicillin (100 mg/ml).

For production of hemolysin II protein, *E. coli* Z85 (pUJ2) cells and culture supernatants were used. The cells were grown for 12 h at 37 °C. An overnight culture was diluted 100-fold, the bacteria were allowed to grow at 24 °C with vigorous aeration until the OD_{600} reached 0.4–0.6, and induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The growth was continued for 16 h after induction. The cell pellet was harvested by centrifugation, and both the culture medium and cells were used for hemolysin II purification.

The human neural cell line Paju was established from the pleural fluid of a patient with a widely metastasized neuroectodermal tumor [16]. The Paju cells were grown surface-adherent in the RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA, USA), penicillin G (6 μg/ml), streptomycin sulfate (10 μg/ml), and 1 mM glutamine. Caco-2 cells (ATCC # HTB-37) were cultivated in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% of fetal bovine serum (Gibco), non-essential amino acids, 2 mM glutamine, and penicillin–streptomycin (100 IU + 100 μg/ml).

Construction of pET29-HlyII-His6

To develop a high-throughput method for testing HlyII mutants, we prepared a construct for expression of the C-terminal His-tagged HlyII under regulation of a strong T7 phage promoter. Plasmid pET29b(+) (Novagen) was used as a cloning vector. The full HlyII gene was amplified using add-on PCR with primers *Nde*I-HlyII-for (GCGGAGCTC CATATGATGAAAAAAGCAAAGGGAAT) and HlyII-*Xho*I-rev (CGCAAGCTTCTCGAGGATTTTTTTAATC TCAATATAACG). The PCR product was cloned into the *Nde*I and *Xho*II sites of the pET29b(+)vector. The resulted plasmid details are shown in Fig. 1C.

Affinity adsorbent preparation and testing

Twenty-nine chlorotriazinyl dyes [12] were probed as pseudo-affinity ligands for HlyII purification. Generally, the same procedure was used for all dyes. 2.5 ml of 2% dye solution was mixed with 5 ml pre-washed Sepharose 4B (Amersham). The suspension was heated at 55 °C for 30 min prior to addition of 2.5 ml of 0.5 M sodium carbonate (pH 12). The incubation was continued at 37 °C for 24 h. To remove the non-reacted dye after completion of the reaction, affinity adsorbent was washed subsequently with excess of distilled water, 0.2 M ethylene diamine, and 1 M NaCl, and packed into 1 ml columns. Finally, the affinity columns were equilibrated with 20 mM K-phosphate buffer (pH 6.0) containing 1 M $(NH_4)_2SO_4$, 5% glycerol, and 2.5 mM phenylmethylsulfonyl fluoride (PMSF). Mini-columns were used in pilot experiments. The gold-yellow dye ICI Procion Yellow 4 [12] (Fig. 2A) was found to be the best and was used to prepare a 100 ml affinity column for preparative purification.

Purification of HlyII from the culture supernatant

The HlyII purification involved ion-exchange chromatography, affinity chromatography, and gel-filtration. All operations were carried out at 4 °C; 5% glycerol and 2.5 mM phenylmethylsulfonyl fluoride (PMSF) were added to all solutions used for purification. A culture supernatant (1 L) was concentrated 5-fold by ultrafiltration on a Biolar RIPOR-2 membrane filter (120 mm, Amicon). To decrease ionic strength of the concentrate, it was diluted to the