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Biochimica et Biophysica Acta 1284 (1996) 122–124

BBA Biochimica et Biophysica Acta

Rapid report

Mechanism of action of hemolysin III from *Bacillus cereus*

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Received 25 June 1996; revised 14 August 1996; accepted 14 August 1996

Abstract

Bacillus cereus hemolysin III activity was tested in crude extracts from *Escherichia coli* carrying the *hly*-III gene. It was concluded that hemolysin III is a pore-forming hemolysin with a functional pore diameter of about 3–3.5 nm. Hemolysis occurs in at least three steps: (i) the temperature-dependent binding of the *Hly*-III monomers to the erythrocyte membrane; (ii) the temperature-dependent formation of the transmembrane oligomeric pore; (iii) the temperature-independent erythrocyte lysis.

Keywords: Hemolysin III; Pore formation; (*Bacillus cereus*)

Bacillus cereus is known to produce several extracellular hemolysins considered as potential factors of virulence of the opportunistic pathogen [1]: cereolysin [2], sphingomyelinase [3], cereolysin AB [4], hemolysin II [5], hemolysin BL [6], 'cereolysin-like' hemolysin [7]. Recently we reported cloning and the primary structure of the gene encoding a new hemolysin of the microorganism, distinct from the above hemolysins and named 'hemolysin III (*Hly*-III)' [8].

Here we report on a study of the hemolysin III activity produced by recombinant *E. coli* cells carrying the *hly*-III gene. The results of this study suggest that hemolysin III acts as an oligomeric pore-forming hemolysin.

E. coli HB101cells [9,10] transformed with plasmid pBG92 bearing the *hly*-III gene from *B. cereus* [8] were used to produce hemolysin III. The cells transformed with vector plasmid pUC19 [11] served as a control. The recombinant cells were grown in LB medium supplemented with ampicillin (50 μg/ml), thiamine (1 μg/ml), $MgCl_2$ (1 mM) overnight at 37°C. Cells from one-liter cultures were harvested at 4°C, washed with saline, suspended in 10 ml of a 25 mM Tris-HCl buffer (pH 8.0) and treated with lysozyme in the presence of EDTA and sucrose, as described previously [12]. The lysates obtained were centrifuged at 40 000 × *g* for 40 min at 2°C, the supernatants were used as crude hemolysin preparations in hemolytic activity assays, as described below. Extracts from *E. coli* HB101/pUC19 were not hemolytic.

Human erythrocytes were washed with phosphate-buffered saline (PBS, pH 6.0) and adjusted to a concentration of 2% (vol/vol). The erythrocyte suspension (0.2 ml) was mixed with 0.2 ml of the *Hly*-III preparation diluted in PBS. The mixture was incubated for 30 min at 37°C, and unlysed erythrocytes were pelleted by centrifugation. Absorbance of the supernatant was measured at 540 nm in a colorimeter. One hemolytic unit (HU) was defined as a dose causing 50% hemolysis. For 100% hemolysis control, 0.2 ml of the erythrocyte suspension was mixed with 0.2 ml of distilled water containing saponin (0.04%) [13].

Each datum point in figures represents mean of 3 to 6 experimental determinations. Standard deviations are indicated in Figs. 2–4.

The temperature-dependent binding step. The *Hly*-III-induced hemolysis was temperature-dependent. It began after two minutes and was completed by 10–12 min of incubation at 37°C (Fig. 1, square). At 4°C no hemolysis occurred within 45 min (Fig. 1, circle). However, after heating the hemolysin-erythrocyte mixture up to 37°C hemolysis occurred similar to that which proceeded at 37°C from the beginning of incubation (Fig. 1, triangle). When the cells incubated with the hemolysin at 4°C were washed with cold PBS and resuspended at 37°C, no hemolysis was observed, indicating that the hemolysin did not bind to erythrocytes at 4°C (Fig. 1, turned triangle). To determine the length of incubation time at 37°C necessary

Abbreviations: *Hly*-III, hemolysin III; *hly*-III, hemolysin III gene; PBS, phosphate-buffered saline; HU, hemolytic unit.

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PII S0005-2736(96)00168-X

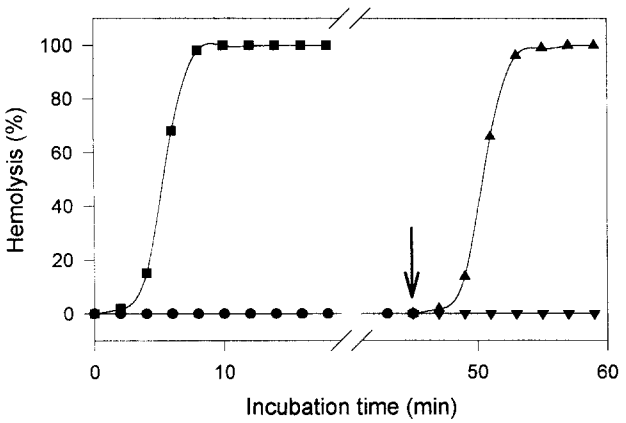

Fig. 1. Temperature-dependent hemolysis induced by hemolysin III. Erythrocyte suspensions (2%) mixed with equal volumes (0.2 ml) of hemolysin dilution (12.5 HU/ml) were incubated at 37°C (■) or at 4°C (●). Samples incubated at 4°C were either subjected to further incubation at 37°C (▲) or washed with PBS and incubated at 37°C (▼). The arrow indicates the point of the shift from 4°C to 37°C.

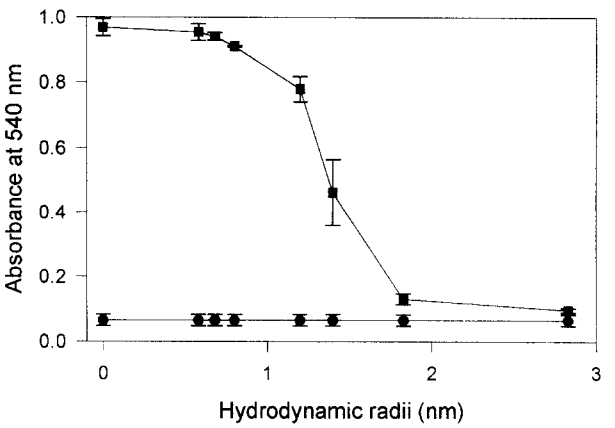

Fig. 3. Inhibitory effect of osmotic protectants on the *Hly*-III-induced hemolysis. The final concentration of the protectants was 30 mM, except for that of inulin, the final concentration of which was 15 mM because of its relatively low solubility. The osmotic protectants were assumed to have the following hydrodynamic diffusion radii [26]: PEG 300, 0.55 nm; PEG 400, 0.63 nm; PEG 600, 0.78 nm; PEG 1500, 1.2 nm; inulin, 1.4 nm; PEG 4000, 1.83 nm; PEG 6000, 2.83 nm. Symbols: ■, hemolysis in the presence of the hemolysin; ●, spontaneous hemoglobin release.

for the binding of the hemolysin to the erythrocytes, a mixture of 0.1 ml of erythrocytes (2%) and 0.1 ml of hemolysin (12.5 HU/ml) was incubated at 37°C for different time periods in the presence of 15 mM PEG 4000. Then, cold PBS (1.3 ml) was added to the mixture, which was immediately centrifuged at 3000 × *g* for 1.5 min. The washed erythrocytes were resuspended in the original volume of PBS and incubated for 30 min at 37°C. Five seconds of preincubation at 37°C was sufficient to induce 80% lysis of the erythrocytes, and 15 s – to induce complete hemolysis (Fig. 2, square). The data indicate that the binding of the hemolysin to the erythrocytes was temperature-dependent and at permissive temperature it occurred within seconds.

The temperature-dependent lesion-forming step and the temperature-independent lysis step. To test whether lysis of erythrocytes following the binding of the hemolysin is temperature-dependent, the above experiment was repeated with a single modification: after varying time periods of preincubation of the erythrocytes and hemolysin at 37°C, the washed erythrocytes were further incubated at 4°C, rather than at 37°C. Fig. 2 (circle) shows that complete hemolysis occurred at 4°C only after 3.5–4 min of preincubation at 37°C. No hemolysis was detected after 15–30 s of preincubation, sufficient time for complete binding of the hemolysin to the erythrocytes. These results indicate that after the binding of the hemolysin to the erythrocytes there is another temperature-dependent step necessary for the subsequent temperature-independent lysis step.

Osmotic protection experiments. The 4% erythrocyte suspension (0.1 ml) was mixed with 0.1 ml of hemolysin diluted in PBS (25 HU/ml), and 0.2 ml of an osmotic protectant solution. The hemoglobin released was measured after 60 min incubation of the mixture at 37°C. Low-molecular PEGs (300 to 1500) did not demonstrate

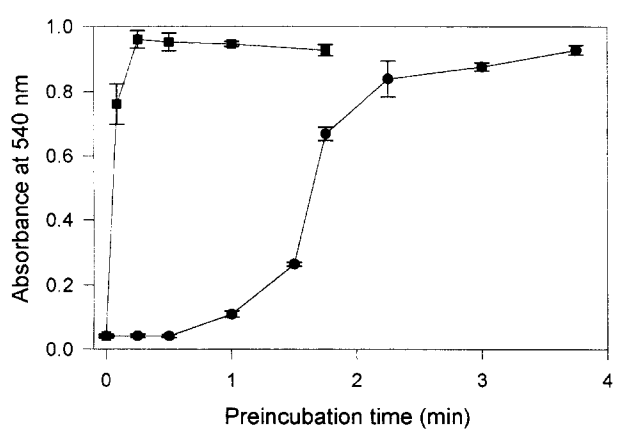

Fig. 2. Length of incubation time at 37°C required for the *Hly*-III-induced hemolysis in hemolysin-free buffer at 37°C (■) or 4°C (●).

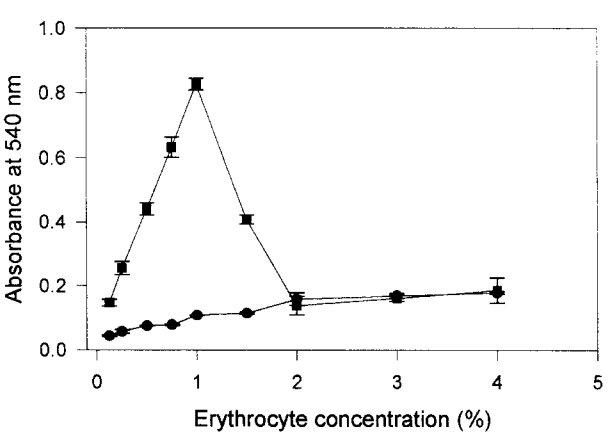

Fig. 4. Effect of the erythrocyte concentration on the *Hly*-III-induced hemolysis. Samples of 0.2-ml suspensions of erythrocytes were incubated with 0.2 ml of hemolysin III (1.3 HU) (■) or 0.2 ml of PBS (●).

marked protection. Inulin had a more significant inhibitory effect. PEG 4000 was almost as protective as PEG 6000, which inhibited hemolysis virtually completely (Fig. 3). When the erythrocytes treated with the hemolysin in the presence of PEG 4000 or 6000 were subsequently washed and resuspended in PBS, hemolysis proceeded within minutes. So, uncharged polysaccharides in the extracellular medium protected the erythrocytes against lysis and the inhibitory effect depended on their hydrodynamic radii.

Effect of erythrocyte concentration on the Hly-III activity. A constant amount of hemolysin (1.3 HU) was mixed with erythrocyte suspensions of varying concentrations in a constant volume of the assay mixtures. After 30 min at 37°C, the mixtures were centrifuged and the supernatant fluid was assayed for hemoglobin. This amount of hemolysin induced about 80% lysis of 1% erythrocytes. The hemoglobin release was limited by erythrocytes at lower erythrocyte concentrations (from 0.125 to 1%) and increased with an increase of the erythrocyte concentration. At erythrocyte concentrations of more than 1% the absolute amount of hemolysis decreased, and no hemolysis was observed at 2–4% erythrocytes (Fig. 4). The data indicate that hemolysin III acts by a multi-hit mechanism [14].

Thus, we have detected several peculiarities of the hemolysin III activity essential for identification of its mode of action. Based on the results on the temperature dependency of the hemolysin, we determined three steps of hemolysis: binding, formation of lesion, and lysis. This approach was employed previously to characterize streptolysin O [15], *Vibrio metschnikovii* cytolysin [16], *Gardnerella vaginalis* hemolysin [17], *V. parahaemolyticus* thermostable direct hemolysin [18], and *V. cholerae* EI Tor cytolysin [19]. Besides, the lytic step can be dissociated from the two preceding steps, if an osmotic protectant is present in the extracellular medium. Inhibition of cytolysis by osmotic protectants is considered a distinctive feature of pore-forming cytolysins and has been employed in studies of an increasing number of pore-forming cytolysins [16–25]. The *Hly*-III activity was inhibited almost totally by osmotic protectants with molecular diameters of 3.6 to 5.6 nm, and partially by that of 2.8 nm. Colloids of 1.1 to 2.4 nm did not protect against the *Hly*-III-induced hemolysis. These results suggest that hemolysin III acts as a pore-forming hemolysin with a functional diameter of pores of about 3–3.5 nm. There may be a minor population of pores whose size exceeds the value. The *Hly*-III-induced hemolysis occurred by a multi-hit mechanism, suggesting that the hemolysin binds to the erythrocyte membrane in a monomeric form and multiple monomers are required to lyse one cell. Previously this interpretation of the multi-hit mechanism was reported in the study of *V. metschnikovii* cytolysin [16]. The multi-hit mechanism of hemolysis has also been shown for streptolysin O and *Clostridium perfringens* theta-toxin, the well-studied oligomeric pore-forming hemolysins [14].

In conclusion, the collective data suggest that hemolysin III is a pore-forming hemolysin with a functional diameter of pores of about 3–3.5 nm. Hemolysis occurs in at least three steps: (i) the temperature-dependent binding of the *Hly*-III monomers to the erythrocyte membrane; (ii) the temperature-dependent formation of transmembrane pore by multiple molecules of the hemolysin; (iii) the temperature-independent erythrocyte lysis.

We thank Alexander Karpov for his help in preparing the manuscript.

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