MONOCLONAL ANTIBODIES TO THE C-TERMINAL DOMAIN OF HEMOLYSIN II *BACILLUS CEREUS*

A.V. Zamyatina[1], *N.V. Rudenko*[1*], *A.P. Karatovskaya*[1], *A.S. Nagel*[2], *A.V. Siunov*[2], *O.S. Vetrova*[1], *D.Sh. Dzhomikova*[1], *Z.I. Andreeva-Kovalevskaya*[2], *T.D. Ivanova*[2], *F.A. Brovko*[1], *A.S. Solonin*[2]

[1] Pushchino Branch, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 6 Prospekt Nauki, Pushchino, Moscow Region, 142290, Russia;

[2] FSBIS FRC Pushchino Scientific Centre of Biological Research, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, 5 Prospekt Nauki, Pushchino, Moscow Region, 142290, Russia.

* Corresponding author: nrudkova@mail.ru

Abstract. *Bacillus cereus* is an opportunistic Gram-positive anaerobic spore-forming bacterium found in various environmental conditions. One of the pathogenicity factors is hemolytic toxin II, which belongs to the group of β-barrel pore-forming toxins. HlyII B. cereus differs from other β- barrel pore-forming toxins by having a C-terminal extension of 94 amino acid residues. In this work, the binding sites of monoclonal antibodies obtained against recombinant HlyIICTD on the protein surface were determined. Using monoclonal antibodies, it was shown that HlyIICTD interacts with the erythrocyte membrane in a strictly defined manner. The localization of epitopes recognized by monoclonal antibodies suggested that the binding site of HlyIICTD with the membrane is localized in the C-terminal region of HlyIICTD molecule.

Keywords: pore-forming toxin, *Bacillus cereus* hemolysin II, C-terminal domain, monoclonal antibodies, epitope, enzyme immunoassay.

List of Abbreviations

HlyII – hemolytic toxin II *B. cereus*

HlyIICTD – C-terminal domain of hemolytic toxin II *B. cereus*

MAb – monoclonal antibody

NMR – nuclear magnetic resonance

PBS – phosphate buffered saline solution

BSA – bovine serum albumin

EIA – enzyme immunoassay

Introduction

Spore-forming Gram-positive bacteria *B. cereus* are characterized by varying degrees of pathogenicity and can exist in different environmental sites from soil to infection of higher organisms (Stenfors Arnesen *et al.,* 2008). *B. cereus* is capable of causing food poisoning (Thery *et al.,* 2022). In addition, *B cereus* contamination in wound infections is one of the common causes of nosocomial infections (Andreeva-Kovalevskaya *et al.,* 2008). It has been shown that *B. cereus* is the cause of severe diseases in immunodeficient patients and premature infants in 40% of cases (Cormontagne *et al.,* 2021). *B. cereus* secretes various pathogenicity factors (Enosi Tuipulotu *et al.,* 2021). Toxins secreted by the bacterium form nano-sized pores in target cell membranes, which leads to the loss of cellular components (Hu *et al.,* 2021). The pore-forming toxin hemolysin II is one of the main virulence factor of the opportunistic pathogen *B. cereus* (Ramarao *et al.,* 2013). This toxin is a homologue of *Staphylococcus aureus* α-toxin with 38% amino acid identity (Baida *et al.,* 1999), and also belongs to the group of β-barrel pore-forming toxins. HlyII, compared to *S. aureus* α-toxin, has a C-terminal extension of 94 amino acid residues, designated as HlyIICTD (Baida *et al.,* 1999). The deletion variant of HlyII lacking HlyIICTD possess 8 times lower hemolytic activity on rabbit erythrocytes compared to intact HlyII (Miles *et al.,* 2002). This fact indicates that HlyIICTD is important for the functioning of the toxin molecule. HlyIICTD has been shown to interact with the cell membrane (Rudenko *et al.,* 2020a).

The structure of HlyIICTD determined by NMR is a fold – a pseudo-barrel consisting of two α-helices surrounded by five β-sheets (Kaplan *et al.,* 2014; Kaplan *et al.,* 2017), these data indicate the uniqueness of this domain. No molecules with such a spatial structure have

been found to date (Kaplan *et al.,* 2017; Kaplan *et al.,* 2021).

MAbs are an essential research tool in biochemistry, molecular biology and medicine. The success of MAbs is due to the direction against a specific unique site of the test substance – an antigenic determinant or epitope. Previously, the authors described obtaining a representative panel of MAbs against HlyIICTD *B. cereus* B14579^T (Rudenko *et al.,* 2020a). Since MA were obtained against a recombinant protein containing additional inserts, in this work, the binding sites of monoclonal antibodies on the surface of the recombinant protein were determined. The localization of epitopes recognized by monoclonal antibodies made it possible to presumably localize the binding site of HlyIICTD to the membrane.

Materials and Methods

Production and isolation of monoclonal antibodies

MAbs were isolated from culture fluids after cultivation of MA-secreting hybridomas. Affinity chromatography on protein A sepharose was used for purification (Mole *et al.,* 1989).

Enzyme immunoassay

Carried out as described in (Rudenko *et al.,* 2020a).

Immunoblotting

Carried out as described in (Zamyatina *et al.,* 2020).

Biotinylation of HlyIICTD

Carried out as described in (Rudenko *et al.,* 2022).

Inhibition of the interaction of HlyIICTD-bio with erythrocytes by MAbs

The experiment was carried out in round bottom plates (Greiner, USA). The volume of the experimental point was 100 μL. The reaction was carried out in PBS containing 5 mg/mL BSA (Diam, Russia). HlyIICTD-bio was added to each well to a concentration of 1 μM. The interaction of HlyIICTD-bio with erythrocytes in the presence of a tenfold excess of unlabeled HlyIICTD was used as a negative control. The final concentration of erythrocytes was 0.025%.

To test the effect of antibodies on the interaction of HlyIICTD with erythrocytes, monoclonal antibodies at a concentration of 5 μM were preincubated with HlyIICTD-bio for an hour at 37^0C, after which erythrocytes were added. The reaction mixture was incubated with stirring at 37^0C in a shaker–thermostat (Dynatech, Germany). The plates were centrifuged at 1000 rpm in a centrifuge (Beckman TJ-6, USA) for 5 min. 200 μL PBS, 5 μg/mL BSA was added to the pellet, the pellet was suspended and centrifuged again under the same conditions. Washing was repeated 3 times. Streptavidin conjugated with horseradish peroxidase (Thermo Scientific, USA) in PBS, 5 μg/mL BSA was added to erythrocytes according to the manufacturer's instructions. Incubated with stirring at 37 °C in a shaker–thermostat for an hour. Next, three washing steps were carried out. After the last centrifugation, the supernatant was removed and 4-mM solution of *ortho*-phenylenediamine (Sigma, USA) in citrate-phosphate buffer (26 mM citric acid, 50 mM Na_2HPO_4, pH 5.0) containing 0.003% (v/v) H_2O_2 was added to the pellet. After color development, the reaction was stopped with 10% (v/v) sulfuric acid. The supernatant was transferred to flat-bottom plates (Greiner, USA). The results were recorded by measuring the optical density at 490 nm on an iMark microplate photometer (Bio-Rad, USA).

Statistical analysis

The analysis was performed using Microsoft Excel. Statistical analysis was based on the Shapiro-Wilk normality test. An unpaired Mann-Whitney test was used to analyze statistical significance between experimental groups. Significance was achieved at $P < 0.05$. Data are resented as means ± standard error of the mean.

Results

Localization of epitopes recognized by MAbs on the surface of recombinant HlyIICTD

A representative panel of MAbs against recombinant HlyIICTD was obtained using hybridoma technology (Rudenko *et al.,* 2020a). The sequence of the protein used for immunization and selection of positive clones producing monoclonal antibodies included a region directly corresponding to HlyIICTD, a linker, a thrombin recognition site, and a 6-His tag at the C-terminus. To establish the antigenic determi-

nants recognized by the obtained antibodies, we used a set of recombinant proteins described in (Zamyatina *et al.*, 2020), which included proteins containing and not containing a linker, a thrombin site, and six terminal histidine amino acid residues. The recombinant proteins used are listed in Table 1.

EIA tested the interaction of all obtained antibodies with a set of recombinant proteins (Table 1), during which each of the MAbs was checked for interaction with each of the recombinant proteins from the set, immobilized on plastic for EIA at a concentration of 1 μg/mL. The results were also confirmed by immunoblotting, during which the interaction of each of the antibodies with the recombinant protein after electrophoretic separation and transfer to a nitrocellulose membrane was checked. The results of immunoblot interaction with HlyIICTD and HlyII 14579^T are presented in (Rudenko *et al.*, 2020a). Figure 1 shows that antibodies HlyIIC-12, HlyIIC-27, HlyIIC-34, HlyIIC-37 and HlyIIC-15 interacted with HlyIIΔCTD, which does not have a C-terminal domain, but contains the same linker peptide and 6-His tag. When immoblotting with the HlyII B771 preparation, which did not contain additional inserts, only antibodies directly interacting with HlyIICTD showed specific staining (Fig. 1). Only HlyIIC-15 interacted with PlcR (Zamyatina *et al.*, 2020).

The analysis revealed that the following MAbs interacted directly with HlyIICTD: HlyIIC-11, HlyIIC-13, HlyIIC-14, HlyIIC-16, HlyIIC-17, HlyIIC-19, HlyIIC-20, HlyIIC-21, HlyIIC-23, HlyIIC-24, HlyIIC-26, HlyIIC-30, HlyIIC-31, HlyIIC-32, HlyIIC-33, HlyIIC-38, HlyIIC-40. Outside HlyIICTD, antibodies HlyIIC-15 (recognized the thrombin site) (Zamyatina *et al.*, 2020), HlyIIC-12, HlyIIC-27, HlyIIC-34, HlyIIC-37 (recognized the linker) interacted. The results are summarized in Figure 2, which shows the localization of epitopes on the HlyIICTD molecule.

Inhibition of the interaction of HlyIICTD with erythrocytes by MAbs

To test the hypothesis that HlyIICTD itself is capable of binding to erythrocytes, biotinylated HlyIICTD labeled with biotin N-hydroxysuccinimide ester was used. The binding of HlyIICTD-bio to blood erythrocytes was detected with streptavidin conjugated with horseradish peroxidase. Binding of HlyIICTD-bio to erythrocytes, compared with a control experiment in which, in addition to HlyIICTD-bio, an excess of unlabeled HlyIICTD was present. The interaction of HlyIICTD-bio with erythrocyte membranes is evidenced by the data shown in Figure 3. Experiments were also carried out to inhibit this binding by monoclonal antibodies (the scheme is shown in Figure 4).

In these experiments, HlyIICTD-bio was incubated with erythrocytes in the presence of a 5-fold molar excess of MAbs. The results are shown in Figure 3. For brevity, Figure 3 shows only a few antibodies that do not inhibit the binding of HlyIICTD-bio to erythrocytes. It was shown that the interaction of HlyIICTD-bio with erythrocytes was prevented by MAbs HlyIIC-12, HlyIIC-15, HlyIIC-27, HlyIIC-30, HlyIIC-34, HlyIIC-37. The epitopes of antibodies HlyIIC-12, HlyIIC-27, HlyIIC-34, HlyIIC-37 are located in the linker part of the recombinant protein attached to the C-terminus of HlyIICTD. HlyIIC-15 recognizes a thrombin site also located in the C-terminal part (Zamyatina *et al.*, 2020). Therefore, it can be assumed that the HlyIIC-30 epitope is close to the C-terminal region of the molecule (Fig. 2). These results indicated that HlyIICTD possess the domain structure and is bound to erythrocytes by a strictly defined manner close to the C-terminal part of the domain.

Table 1

Recombinant proteins used to analyze sites interacting with MAbs

Protein \ Section of the recombinant protein sequence	Sequence HlyIICTD	Thrombin cutting site gtlvpr/gs	Linker	6His tag
HlyIICTD *B. cereus* 14579^T	+	+	+	+
HlyIIΔCTD *B. cereus* 14579^T	–	+	+	+
HlyII *B. cereus* 14579^T	+	+	+	+
HlyII B771 cloned in *Bacillus subtilus* BD170	+	–	–	–
HlyIIR *B. cereus* B771 (HlyII repressor)	–	–	–	+
PlcR *B. cereus* ATCC 4342^T	–	–	+	–

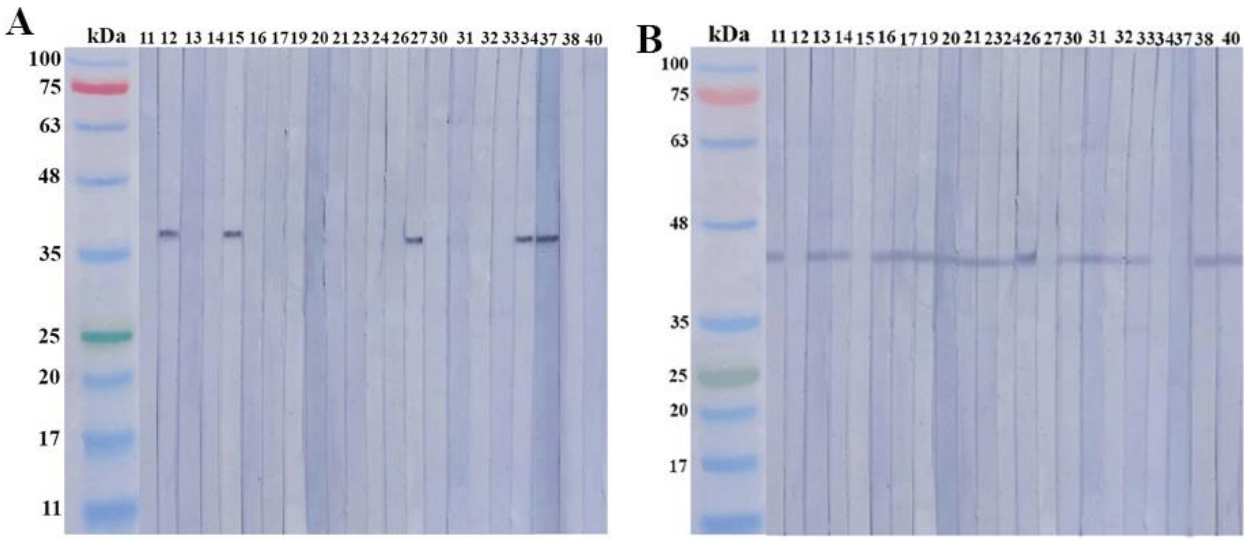

Fig. 1. Interaction of MAbs against HlyIICTD with HlyIIΔCTD (A) and HlyII (771) (B) in immunoblotting. Above the tracks are the names of MAbs. Left track, molecular weight standards

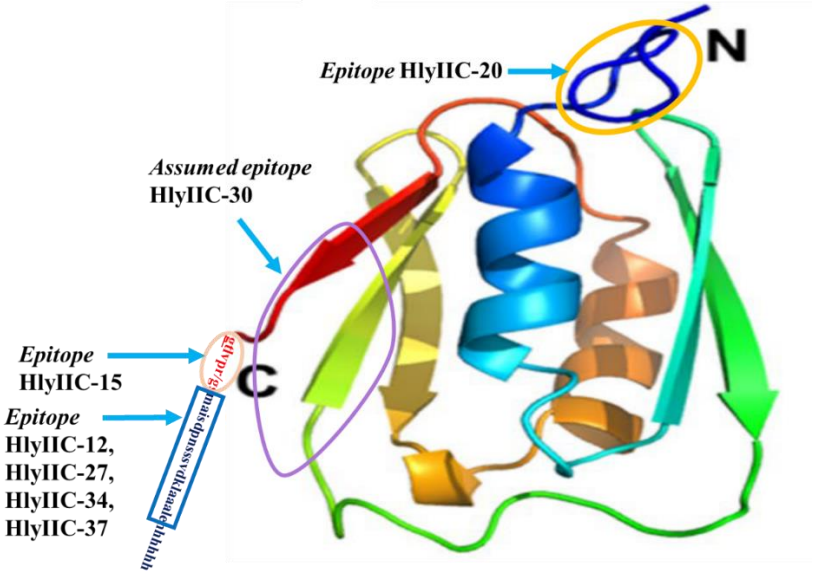

Fig. 2. Location of MAbs epitopes against HlyIICTD on the spatial structure of the domain according to (Kaplan *et al.*, 2017)

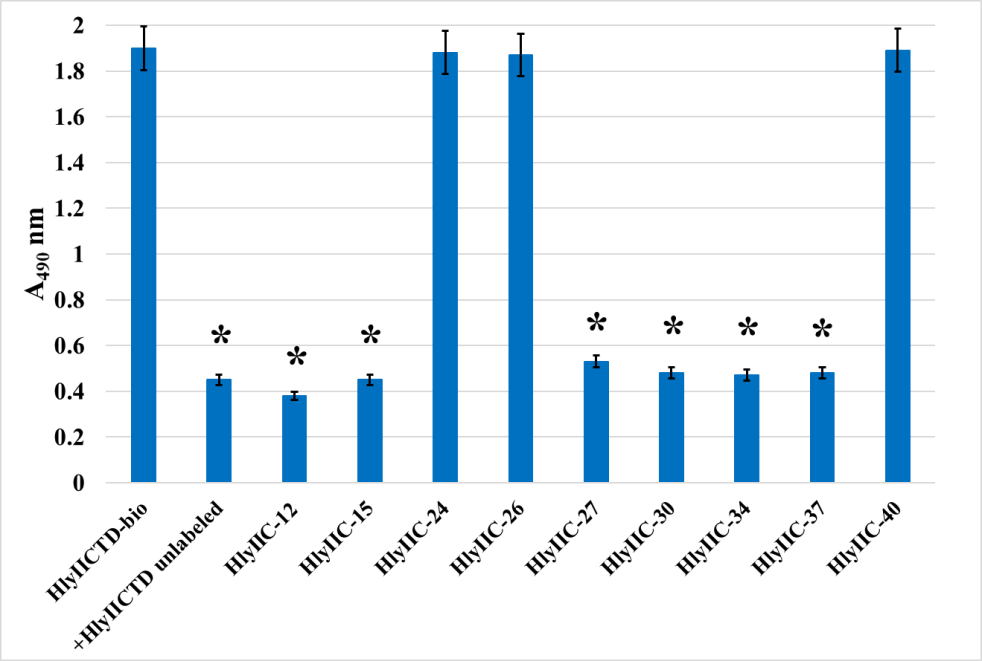

Fig. 3. Inhibition of the interaction of HlyIICTD-bio with erythrocytes by MAbs. Data are the means ± the standard errors of the means of 5 independent repeats. * – $P < 0.05$, compared with HlyIICTD-bio (Mann-Whitney test)

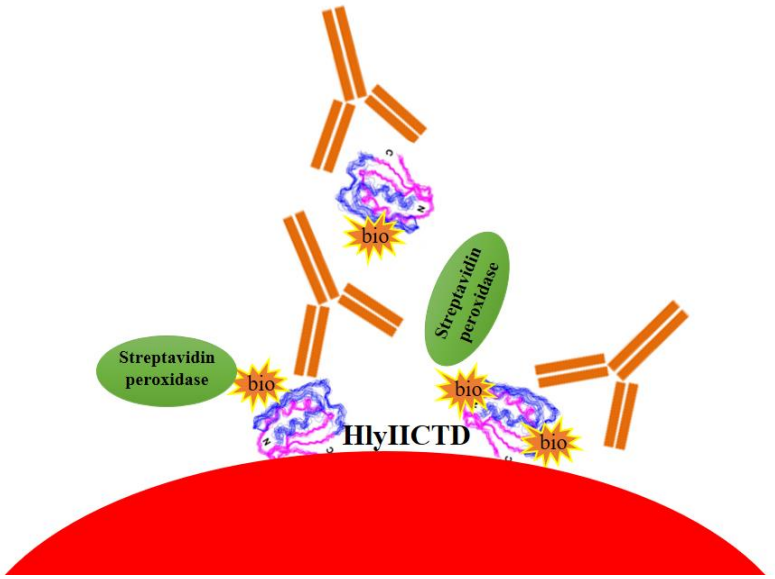

Fig. 4. Inhibition of the interaction of biotinylated HlyIICTD with erythrocytes by MAbs. Schema of the experiment

Discussion

Using HlyIICTD as an example, it was demonstrated that MAbs can be used as a tool for studying the structure and function of protein domains.

Among the panel of MAbs to the recombinant HlyIICTD protein, the HlyIIC-20 antibody was found to be able to inhibit the hemolysis of erythrocytes. HlyIIC-20 recognize the conformational epitope on the surface of the protein globule. Using the peptide-phage display method, it was found that the epitope is localized in the N-terminal part of HlyIICTD (Fig. 1). HlyIIC-20 interacted with the monomeric form of HlyII, thereby preventing toxin oligomerization and formation of a pore on the target cell membrane (Rudenko *et al.*, 2020b).

The ability of HlyIICTD to oligomerize in the presence of cell membranes was shown by using MAbs. In this case, stable oligomers are formed with an altered availability of antigenic determinants, which manifests itself in a change in binding to monoclonal antibodies (Rudenko *et al.*, 2022). These results suggested that the HlyII C-terminal domain increases the likelihood of pore formation by binding to the target cell membrane.

For further application of MAbs against HlyIICTD, it was necessary to determine the binding sites for each antibodies of the representative panel, which was done in this work using a set of recombinant proteins of various compositions. It has also been shown that HlyIICTD interacts with erythrocytes in a strictly defined manner, as evidenced by the fact that this interaction is inhibited only by certain MAbs whose epitopes are localized in the C-terminal region of the molecule. The results obtained suggest that the site of interaction between HlyIICTD and the membrane of the target cell is located within the C-terminal region.

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