Monoclonal Antibody HlyIIC-15 to C-End Domain HlyII *B. cereus* Interacts with the Trombin Recognition Site

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Abstract—Among the panel of monoclonal antibodies to the recombinant protein HlyIICTD *Bacillus cereus* an antibody was found capable of forming an immune complex with a thrombin recognition region, the amino acid sequence of which is located inside the recombinant HlyIICTD. Localization of the epitope was carried out using peptide phage display methods, as well as enzyme immunoassay and immunoblotting for interaction with recombinant proteins, either containing or not containing individual components HlyIICTD. The identified epitope is located in the region of the thrombin site and retains the ability to interact with the antibody after the proteolyotic attack of the protein by thrombin.

INTRODUCTION

The production of various recombinant proteins, individual subunits, and polypeptides leads to the development of biotechnological approaches that provide a quick and cheap method for their isolation in native form. In routine experimental research, various affinity sorbents for liquid chromatography are often used, which allow fast and efficient purification of recombinant proteins with subsequent removal of the "tags" necessary for chromatography of these proteins by specific proteases. One of the widely used options is the introduction of thrombin recognition sites. Thrombin (coagulation factor II) is a component of the mammalian blood coagulation system and functions as a site specific serine protease, insensitive to buffer solutions containing various detergents [1]. This protein recognizes the LVPR^GS site and introduces a gap between arginine and glycine [2]. To study the genesis of HlyII in bacterial cells and the interaction of the toxin with eukaryotic cells, an MA panel was created for the C-terminal domain of the pore-forming protein HlyII [3]. This work is devoted to the description of HlyIIC-15, which binds efficiently to the thrombin site of the fusion protein, but not to other peptide sites of the recombinant CTD.

RESULTS

Characterization of the amino acid sequence HlyIICTD. One of the virulence factors of an opportunistic microorganism *Bacilus cereus* is the pore-forming toxin hemolysin II (HlyII) [4]. Hemolysin II with a β-barrel type structure has high homology with alpha-toxin *S. aureus* and differs from it by the presence of a C-terminal amino acid excess of 94 residues [5]. This region in NMR analysis demonstrated a unique spatial structure not previously described [6]. The gene region was cloned and HlyIICTD was purified as described in the work of Rudenko et al. [3]. The amino acid sequence of HlyIICTD consists of the amino acid residues of the C-terminal redundancy of HlyII, a thrombin site, six histidine residues, and a linker (Fig. 1).

Features of the interaction of HlyIICTD with erythrocytes, detected by MA HlyIIC-15. MA number 15 (HlyIIC-15) was found in the panel of monoclonal antibodies against HlyIICTD, which is unable to recognize the antigen at the time of its interaction with

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DNQKALEEQMNSINSVNDKLNKGKGKLSLSMNGNQLKATSSNAG
YGISYEDKNWGIFVNGEKVYTFNEKSTVGNISNDINKLNIKGPYIEI
KQIGT**LVPR**/**GS**MAISDPNSSSVDKLAAAALE*HHHHHH*

Fig. 1. Amino acid sequence of HlyIICTD cloned from HlyII *B. cereus* ATCC 14579[T] in pET29b. The molecular weight of the cloned CTD is 13906.59 Da. The thrombin site is highlighted in bold letters, / indicates the site of proteolytic attack by thrombin. Italics indicate the area with six histidines.

the membranes of both erythrocytes [3] and liposomes [7]. The inability of HlyIIC-15 to interact with CTD bound to erythrocytes is apparently determined by the steric location of the epitope for this antibody and its inaccessibility relative to the membrane recognition point by the antigen.

Miles et al. [8] demonstrated that native hemolysin II and a deletion variant lacking the C-terminal domain effectively oligomerize both in the presence of erythrocytes and liposomes. In this regard, the possibility of CTD oligomerization in the presence of erythrocytes and liposomes was tested. Figure 2 shows MA immunoblotting HlyIIC-15. As can be seen from this figure, HlyIICTD in the presence of rabbit erythrocytes and liposomes transforms into a tetrameric form. Before the addition of erythrocytes and liposomes, in addition to monomeric forms of HlyIICTD, weakly colored dimeric and tetrameric forms of HlyIICTD are
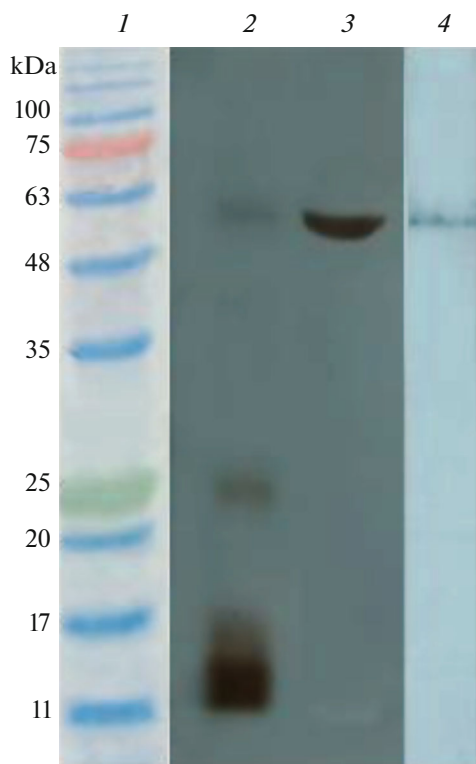

Fig. 2. Immunoblotting HlyIICTD before (*2*) and after (*3*) incubation with erythrocytes and liposomes (*4*). Painted with MA HlyIIC-15. (*1*) Molecular weight markers.

visible (Fig. 2, lane *2*). Incubation of HlyIICTD with erythrocytes MA HlyIIC-15 revealed tetrameric forms, while monomeric forms are not visible (Fig. 2 lane *3*). Similar results were obtained by incubating HlyIICTD with liposomes (Fig. 2 lane *4*). Thus**,** HlyIIC-15 is able to effectively interact with CTD, and the epitope recognized by this antibody is located on the surface and is accessible to MA HlyIIC-15 both in monomeric forms of the antigen and in its tetrameric forms.

Peptide phage display. Interaction features HlyIICTD with erythrocytes, identified using HlyIIC-15, set the task of determining the epitope recognized by this antibody. Antigenic determinant HlyIICTD recognized by MA HlyIIC-15 was determined by the method of peptide phage display. In this method, possible peptide variants are expressed on the surface of the phage virion. The selection of phages carrying peptide amino acid sequences specifically interacting with MA HlyIIC-15 was carried out according to their ability to interact with each other. There were three rounds of selection. The DNA of the selected individual phage clones was sequenced. Using the GeneRuner program, the nucleotide sequences were analyzed and the amino acid sequences of peptides recognized by MA HlyIIC-15 were determined and bacteriophages exposed in the pIII protein.

After three rounds of affinity selection, 30 phage clones were selected from the unamplified eluate of the last round. After analyzing their interaction with MA HlyIIC-15, 12 clones were selected, in which the sequence of the peptide displayed by the phage was determined. The amino acid sequences of all peptides were aligned and four consensus amino acid residues (a.a.) of LVPR were found. Moreover, in clones 7 and 9, this consensus sequence is directly adjacent to the glycine linker, using which the peptides are connected to the sequence of the structural protein pIII of the bacteriophage. Hence, the length of the HlyIIC-15 MA recognition site can include either four LVPRs or five amino acid residues—LVPRG. The glycine residue is a peptide fragment artificially introduced into the recombinant protein and the identified peptide almost completely corresponds to the thrombin recognition site LVPR^GS.

Experimental confirmation of the localization of the epitope recognized by MA HlyIIC-15 by enzyme immunoassay and immunoblotting. Using enzyme immunoassay, the interaction of MA HlyIIC-15 with the fol-

Table 1. Alignment of a portion of the amino acid sequence HlyIICTD from HlyII *Bacillus cereus* and sequenced peptides

Clone numbers	Fragment HlyIICTD VGNISNDINKLNIKGPYIEIKQIGT<u>LVPR</u>GSMAISDPNSSSVDKLAAAL
1, 4, 5	—————————L<u>QVPR</u>AQHHAIT————-
2	———————WSGA<u>LIPR</u>NSTF—————
3	————————NNAPPP<u>PL</u>LFYH—————
6,8	————————- IPSFPN<u>LIPR</u>YA——————
7	—————— IEPDVRGS<u>LIPR</u>———————
9	—————— YLSTPSGS<u>LVPR</u>———————
10	————————- SVQTLN<u>LIPR</u>QF——————
11, 12	————————— HL<u>LVPR</u>FDVHSM—————

Table 2. Proteins used for immunoblotting

Protein	Recombinant plasmid (vector)	Gene source	Link/received from
HlyII	pBD170-EH2	*B. cereus* B771	[19]
HlyIICTD	pET29b	*B. cereus* 14579^T	[3]
HlyII 6His	pSWEET-bgaB	*B. cereus* 14579^T	Lab. Molecular Microbiology IBPM RAS
HlyII∆C	pET29b	*B. cereus* 14579^T	Lab. Molecular Microbiology IBPM RAS
PlcR	pET33b	*B. cereus* ATCC 4342^T	Ph.D. A. M. Shadrin
HlyIIR	pHR	*B. cereus* B771	[20]

lowing drugs was compared: HlyIICTD, a native HlyII∆C deletion variant (without a C-terminal region) containing a thrombin site, as well as with a native HlyII preparation that does not contain a thrombin site. HlyII∆C and HlyIICTD contained six histidine amino acid residues. HlyII contained an intact region corresponding to the C-terminal excess of the pore-forming toxin HlyII *B. cereus*. As a result of enzyme immunoassay, it was shown that with an effective interaction with HlyIICTD and HlyII∆C, MA HlyIIC-15 did not recognize native HlyII lacking a thrombin site, which confirms the results obtained by phage display that the epitope recognized by HlyIIC-15 includes a site recognized by thrombin.

To confirm the epitope of the recognized HlyIIC-15, immunoblotting, wherein the interaction of antibodies along with HlyIICTD with recombinant proteins PlcR [9], HlyII, the deletion variant HlyII∆C and HlyIIR (hemolysin II repressor) [10], as well as native hemolysin II *B. cereus* B771 cloned in *Bacillus subtilis* BD170 (Table 2), was used. In Fig. 3, it is shown that MA HlyIIC-15 is able to form an immune complex with samples containing a thrombin site and additional six histidine residues, but does not form such complexes with full-length wild-type HlyII, which does not contain either a thrombin site or six histidine amino acid residues. Thus, for the formation of an immune complex, the test antibody with the C-terminal region of HlyII in the recombinant protein, additional regions are required, including six histidine res-

idues, a peptide linker, and a site recognized by thrombin. This assumption was confirmed by the ability of MA HlyIIC-15 to recognize the recombinant transcriptional regulator PlcR *B. cereus* containing a thrombin site, a linker and a six-histidine site in the N-terminal portion of this protein. Further evidence of MA HlyIIC-15's recognition of an area outside the C-terminal domain of HlyII is the ability to form an immune complex of a recombinant protein lacking CTD, but containing a thrombin site, a linker and a six-histidine site in the C-terminal portion of this protein. Due to the fact that HlyIIR, containing six histidine residues in the N-terminal part, is unable to form an immune complex with MA HlyIIC-15, it can be concluded that the region of the thrombin site is required for MA to recognize its antigen.

Recombinant proteins containing a thrombin site (HlyIICTD and PlcR) were treated with thrombin. After that, the electrophoretic mobility of proteins changed towards a decrease in molecular weight. PlcR MA staining intensity HlyIIC-15 also decreased, which may indicate a decrease in the degree of binding of the antibody to the antigen when some of the amino acid residues included in the epitope are removed. Analysis of the possibility of interaction between recombinant proteins containing a thrombin site, a linker, and a six-histidine site at different ends of the recombinant proteins after thrombin treatment confirmed that the HlyIIC-15 MA recognition region is located in the region of the thrombin site. As can be
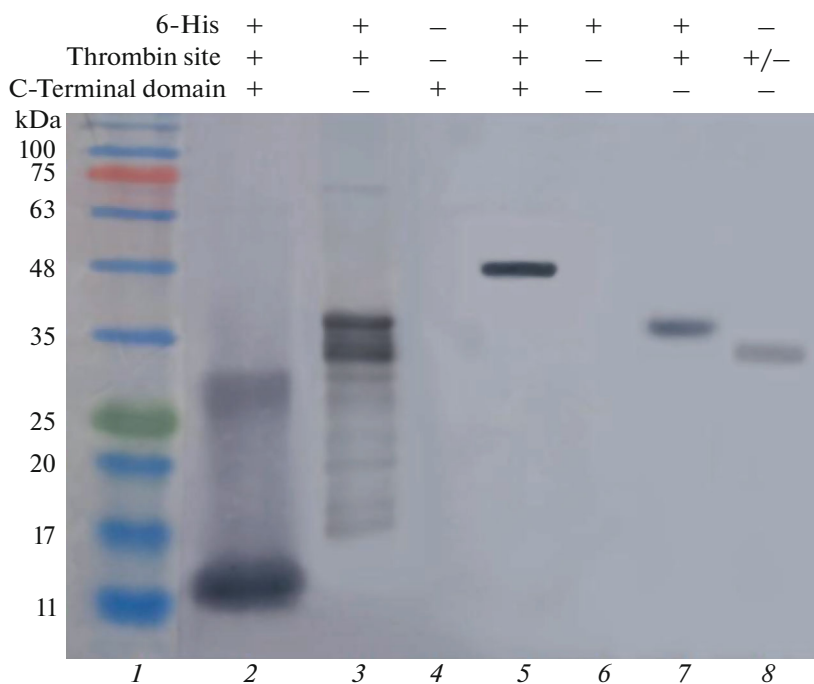
6-His	+	+	−	+	+	+	−
Thrombin site	+	+	−	+	−	+	+/−
C-Terminal domain	+	−	+	+	−	−	−

Fig. 3. Immunoblotting using HlyIIC-15. (*1*) Proteins of molecular weight, (*2*) CTD, (*3*) HlyIIΔC, (*4*) hemolysin II *B. cereus* B771 cloned in *B. subtilis*, (*5*) HlyII6His, (*6*) HlyIIR, (*7*) PlcR, (*8*) PlcR treated with thrombin.

C-recombinant protein-gt**lvpr**/**gs**mainsd**p**nsssvdklaaaleHHHHHH

N-mgss**HHHHHH**ss**lvpr**/**gs**hmasr-recombinant protein

Fig. 4. Comparison of sections of amino acid sequences with their different localization: C-terminal localization of the thrombin site and N-terminal localization of the thrombin site. The thrombin recognition site is indicated in bold letters. / indicates the place of proteolytic attack by thrombin. Capital letters denote a region containing six histidine residues.

seen from the Fig. 3, PlcR, containing a thrombin site with a linker and six histidine residues at the N-terminus, after treatment with thrombin, decreases but retains the ability to stain with HlyIIC-15, which made it possible to conclude that MA HlyIIC-15 is able to recognize a wider region than is determined by the phage display. A similar result was obtained after treatment with thrombin HlyIICTD. Apparently, the key amino acid residues of the epitope are the constituents of the thrombin recognition site and, possibly, part of the region surrounding this site (Fig. 4). After proteolysis by thrombin of recombinant HlyIICTD containing a thrombin site with a linker and six histidine residues at the C-terminus, the protein interacted with MA HlyIIC-15, which may unambiguously indicate that this antibody recognizes at least four amino acid bases of LVPR that are part of the thrombin site (data not shown).

Thus, the region where the experimentally detected epitope is located confirms the determination of this epitope by a peptide phage display. Four amino acids of LVPR are sufficient for the recognition of MA HlyIIC-15.

DISCUSSION

For a long time purification, obtaining highly purified proteins was quite time consuming and costly, especially for proteins interacting with membranes. The use of modern genetic engineering technologies has partially solved this problem. To obtain recombinant proteins, numerous expression systems with promoters have been created that provide a high level of transcription of hybrid genes in various bacterial and fungal systems. Due to the fact that the question of both the potential toxicity of chemical expression inducers and the toxicity of the final sought products is still open, the previously described variants of the initiation of biosynthesis by means of an inductor or a temperature shift are widely used. In this case, the sequence of the target gene is placed under the control of effective phage promoters [11−13]. To increase the solubility, expression level, and effective purification by affinity chromatography, short amino acid marker sequences called "fusion tags" are attached to the N- or C-termini of recombinant proteins [14]. Sometimes it is possible to solve the production of a recombinant protein in a soluble form by attaching an auxiliary

sequence—a highly soluble partner protein [15]. However, in both the first and second cases, the final product is burdened with additional protein regions and is not a native product. The goal in the production of various recombinant proteins, individual subunits, as well as polypeptides, the restoration of the final products in their native form comes to the fore. This goal is achieved by the introduction of additional peptide regions, recognized by highly specific proteases, which are capable of cleaving off the used tags and restoring recombinant proteins to almost their native state. The development of a technology for the production of recombinant proteins or peptides using proteolytic cleavage is always a task that requires an individual solution for each product. One of the most commonly used highly specific proteases is thrombin. After enzyme-dependent proteolysis, thrombin can be easily removed by liquid chromatography to purify or remove serine proteases. However, the use of thrombin is limited to laboratory studies [16]. The most significant disadvantage of protease is the method for its production from mammalian blood plasma.

Thus, this work describes the MA HlyIIC-15 revealing a thrombin site outside the structural portion of the cloned protein containing this site. The results of this work suggest an expansion of the possibilities of using thrombin and its recognition site not only for cutting off additional peptide regions, but also as a marker for identifying and tracking various recombinant proteins.

Given that thrombin activates clotting factors blood V, VIII, VII, XI, XIII, protein C, a thrombin-activated inhibitor of fibrinolysis [17], that is, they contain peptide regions recognized by this protein, MA HlyIIC-15, which binds to the cut site of thrombin, can be used for mass analysis of samples in the study of various pathologies of the blood coagulation system. Medical practice in recent months has shown that during infection with COVID-19, patients in severe form develop strange blood clots [18]. A number of patients showed signs of blood thickening and blood clots in various organs. Neurosurgeons note a sharp surge in strokes due to blood clots, and pathologists observed during autopsy lungs filled with hundreds of microthrombi. In this regard, the use of MA HlyII-15 may prove to be a successful tool in the study and understanding of the processes of blood coagulation during the COVID-19 infection.

EXPERIMENTAL

Plasmid strains and proteins. *E. coli* BL21 (DE3) (Novagen, Germany) was used to transform pET29b (+) (Novagen, Germany), *E. coli* ER2738 for affinity phage selection.

Media and solutions. Medium: 2YT (16 g/L bacto-tryptone, 1 g/L yeast extract, 5 g/L NaCl, pH 7.0), 1.5% agar and 0.7% agar based on 2YT. Solutions:

1000× IPTG/X-gal (1.25 g IPTG (isopropyl-β-*D*-thiogalactoside) and 1 g X-gal (5-bromo-4-chloro-3-indolyl-β-*D*-galactoside) in 25 ml DMF (dimethyl formamide), tetracycline 20 mg/mL in 50% ethanol, phosphate buffered saline (PBS), PBST—PBS containing 0.1% Tween-20, blocking solution of 1% gelatin based on PBS-T, PEG/NaCl precipitating solution (20% (w/v) polyethylene glycol-6000, 2.5 M NaCl). Protein markers (Abcam, England) and DNA electrophoresis markers (Fermentas, Lithuania), conjugate of goat antibodies with horseradish peroxidase (Thermo Scientific, United States), conjugate of streptavidin with horseradish peroxidase (Thermo Scientific, United States).

Phage peptide library. In work to determine the epitope on the molecule HlyIICTD we used a library of random peptides of 12 amino acid residues (New England Biolabs) displayed on the M13KE phage [21]. The twelve-dimensional phage peptide library has a repertoire of 10^9; peptides are exposed on the surface of bacteriophages in the composition of the minor protein pIII. Each clone in the library is represented by ~1000 copies.

Affinity selection of bacteriophages, specifically interacting with MA HlyIIC-15, was carried out in accordance with the instructions for the phage library with some modifications. To carry out all three rounds of selection, MA HlyIIC-15 was sorbed onto plates for enzyme-linked immunosorbent assay (Greiner, high binding, United States) at a concentration of 10 µg/mL in a volume of 100 µL in Na-carbonate buffer pH 9.6, overnight, at +4°C. For each round, eight wells were used. To prevent possible nonspecific sorption, immunoplates were incubated with blocking buffer for 1 h at room temperature. Then, 10 µL of the phage library with a particle concentration of 3.8×10^{12} particles/mL was added to the experimental wells and incubated overnight at +4°C. After incubation with phage particles, the immunoplates were washed at least 10 times with PBST. Elution of bound phages was performed by adding 100 µL of a suspension of *E. coli* cells (strain ER2738) in the exponential growth phase ($OD_{600} \sim 0.5$) to the wells followed by incubation at 37°C for 45 minutes; at this stage the cells were infected with bacteriophages. Further, *E. coli* were collected from the wells and transferred into flasks with 20 mL of the 2YT medium containing 20 µg/mL tetracycline and cultured overnight on a shaker at 200 rpm and 37°C to amplify the eluted bacteriophages. In parallel with this, the number of eluted bacteriophages was determined by growing in semiliquid agar. After the third round, the eluate was not amplified. To isolate individual phages, it was titrated on a sensitive culture and individual negative colonies of phage clones were transferred into tubes with 5 mL of 2YT medium containing diluted 1 : 100 night culture ER2738. Separate amplification of phage clones was carried out overnight on a thermal shaker at 37°C.

Phage clones interacting with MA HlyIIC-15 in the best way were selected according to the results of enzyme-linked immunosorbent assay of their interaction with adsorbed MA HlyIIC-15. The interaction was revealed using rabbit polyclonal antibodies against M13 and goat antibodies against rabbit immunoglobulins conjugated with horseradish peroxidase. The bacteriophages of these clones were used for DNA isolation and sequencing.

Cultivation of bacteriophages in semiliquid agar. Five milliliters of 2YT medium with tetracycline was inoculated with 50 µL of overnight culture *E. coli* (strain ER2738) and cultured at 37°C to a suspension density corresponding to $OD_{600} \sim 0.5$. Petri dishes were prepared with agar medium (1.5% 2YT agar) with IPTG/X-gal and tetracycline. Semiliquid agar was melted in a water bath and poured into sterile culture tubes (3 mL), maintaining the temperature at 45°C. To 3 mL of semiliquid agar was added 20 µL of suspension *E. coli* in the exponential growth phase and 50 µL of the preparation containing bacteriophages (at the stage of the selection rounds, infected cells). If necessary, serial dilutions of bacteriophage preparations were pre-made. The resulting mixture was quickly poured out and spread over the agar medium. The plates were incubated overnight at 37°C, after which the number of negative colonies of phage clones—plaques—was counted.

Isolation of bacteriophage DNA. Night culture *E. coli* bacteriophages infected with individual clones were centrifuged for 10 minutes at 5000 *g* and 4°C. Bacteriophages were isolated from the supernatant by precipitation with polyethylene glycol MW 6000 in the presence of a high concentration of NaCl [21]. The concentration of phage particles in the supernatant was determined spectrophotometrically at a wavelength of 269 nm and calculated by the formula $[f.p.] = (2 \times 10^{14} \times A_{269})/30$. Single-stranded DNA of individual clones was isolated by phenol extraction followed by precipitation with ethyl alcohol [22].

DNA sequencing. Analysis of the DNA sequences of the descendants of phage clones to determine the sequence of the insertions was carried out at the Evrogen company. Sequencing results were processed using Gene Runner and ClustalW programs.

Liposome production. Liposomes were prepared from a solution of lecithin at a concentration of 1 mg/mL in a mixture of chloroform and methanol (molar ratio, 2 : 1). After drying under vacuum, the lipid film was resuspended in a buffer (10 mM Tris-HCl, pH 7.2, 100 mM KCl) to a total lipid concentration of 1 mg/mL. Liposomes were obtained by sonication for 30 min on ice [4]. Oligomeric forms of HlyIICTD were detected after electrophoretic separation of a mixture of HlyIICTD (9 µM) with liposomes preincubated for 1 hour at 37°C, and transfer to a nitrocellulose membrane, followed by MA staining with HlyIIC-15.

Immunoblotting. The electrophoretic separation of proteins in the preparations was carried out in the presence of β-mercaptoethanol as in [23]. Transfer to a nitrocellulose membrane was carried out for 15 h at a current of 20 mA in a buffer containing 25 mM Tris-HCl, 0.25 M glycine, 0.1% sodium dodecyl sulfate, 20% methanol, pH 8.3. The centers of nonspecific sorption were blocked by adding 1% (w/v) gelatin solution in PBST for 30 min. Then, the membranes were incubated for 2 h with MA HlyIIC-15 (10 µg/mL). After incubation with antibodies, the membranes were treated for 1 h with horseradish peroxidase conjugate with rabbit antibodies to mouse immunoglobulins diluted according to the manufacturer's recommendation (Thermo Scientific, United States) in PBST. At each stage, the nitrocellulose membranes were thoroughly washed with PBST. The membrane was stained with a solution containing 3 mM diaminobenzidine-3,3 tetrahydrochloride (Sigma-Aldrich, United States) and 0.03% hydrogen peroxide.

COMPLIANCE WITH ETHICAL STANDARDS

This work does not contain any research involving humans and animals as research objects.

Conflict of Interest

The authors declare they have no conflict of interest.

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