Toxicon 208 (2022) 53–61

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

A new protein-coupled antigen of α-conotoxin MI displays high immunogenicity and can produce antiserum with high detoxification activity

Min Zhang [a,1], Shuo Yu [a,1], Xin Zhang [b], Qiuyuan Huang [a], Yue Huang [a], Min Luo [b], Yuanmei Wei [a], Wenwen Chen [a], Ze Chen [b], Xiaowei Zhou [a], Qiuyun Dai [a,*]

[a] Beijing Institute of Biotechnology, Beijing, 100071, China
[b] Shanghai Serum Biotechnology Co., Ltd., Shanghai, 200031, China

ARTICLE INFO

Handling Editor: Ray Norton

Keywords:
α-conotoxin MI
Antigen preparation
Antiserum
Neutralizing activity
Detoxification activity

ABSTRACT

α-conotoxin (α-CTX) MI is a small peptide toxin with 14 amino acids and two disulfide bonds. It potently inhibits muscle-type nicotinic acetylcholine receptors (nAChRs), and poses a threat as a toxin to tropical fishermen. However, there are currently no effective drugs for the treatment of MI envenomation due to the toxin's low immunogenicity. In this report, we generated neutralizing antiserum and F(ab')$_2$ to MI by synthesizing a new MI antigen through the coupling of alkynyl-modified MI and azide-modified bovine serum albumin (BSA), followed by immunization into mouse and horse. The new MI-BSA antigen generated high titers of mouse and horse antiserum (1:204,800 and 1:51,200, respectively), and both the antiserum as well as the horse F(ab')$_2$ displayed highly potent neutralization and detoxification efficacy. 12.5 μL of mouse or horse antiserum preincubated with MI could completely neutralize a lethal dose of the MI (0.4 μg, 1.7 × LD_{50}), while 6.25 μL (mouse) or 10.41 μL (horse) of the antiserum could exert complete detoxification of mice injected with 1.7 × LD_{50} of MI. Moreover, the mouse and horse antiserum exhibited medium cross-reactivity for highly toxic α-CTX GI. These results demonstrate that the integrity of MI's antigen epitope and carrier effect of BSA can improve MI's immunogenicity, and provides an effective detoxification treatment for highly toxic α-conotoxins as well as an effective method for the preparation of antiserum of small peptide toxins.

1. Introduction

α-conotoxin (α-CTX) MI (GRCCHPACGKNYSC-NH_2) is derived from the marine cone snail *Conus magus* (McIntosh et al., 1982; Gray et al., 1983). α-CTX MI adopts a type I cysteine framework (CC–C–C), with two disulfide bonds formed between Cys3-Cys8 and Cys4-Cys14. α-CTX MI potently inhibits muscle-type nicotinic acetylcholine receptors (nAChRs) (Jacobsen et al., 1999; Lebbe et al., 2014; Azam et al., 2009; Jin et al., 2019). It is the most toxic conotoxin identified to date, the median lethal dose (LD_{50}) in mice is 15–20 μg/kg (Gray et al., 1983; Kapono et al., 2003). α-CTX GI (ECCNPACGRHYSC-NH_2), derived from *Conus geographus*, possesses same disulfide bonds as MI, its LD_{50} in mice is 20–30 μg/kg (Cruz et al., 1978; Kaerner et al., 1999). α-CTX MI can cause muscle paralysis and numbness, difficulty in breathing, myocardial damage, cardiac arrest, and even death (Kapono et al., 2003; Cruz et al., 1978) and there have been reports of fishermen deaths caused by α-CTX MI envenomation (Chivian et al., 2003; Halford et al., 2015; Kohn et al., 2016, 2018; Anderson et al., 2012). However, there currently are no effective drugs for the treatment of MI envenomation, due to the low neutralizing potencies of antiserums and antibodies.

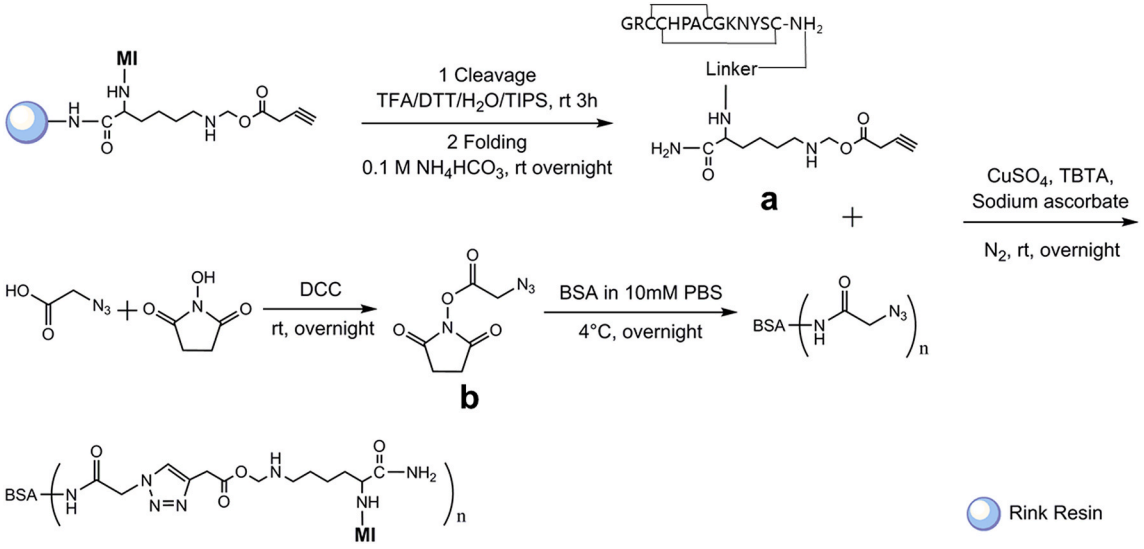
Several studies have reported preparation of antiserum or antibody for the detoxification of α-CTX GI. Generally, GI was coupled to protein carrier (keyhole limpet hemocyanin, ovalbumin and bovine serum albumin) by glutaraldehyde, and then immunized into mice, goats or

Abbreviations: α-CTX, α-conotoxin; BCA, bicinchoninic acid assay; BSA, bovine serum albumin; DMF, N,N-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; LD_{50}, the median lethal dose; PBS, phosphate buffer; TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine; TMB, 3,3,5,5-tetramethylbenzidine.

* Corresponding author.
E-mail addresses: qy_dai@yahoo.com, daiqy@bmi.ac.cn (Q. Dai).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.toxicon.2022.01.009
Received 1 November 2021; Received in revised form 26 January 2022; Accepted 26 January 2022
Available online 31 January 2022



Scheme 1. Synthesis route of the MI-BSA antigen.

rabbits (Stiles and Sexton, 1992; Tang et al., 2017; Ashcom et al., 1997). However, the antiserum prepared generally displayed low neutralizing potency , and a high dose of antiserum (100 μL or 200 μL) was required to neutralize a lethal dose of GI (Stiles and Sexton, 1992). The main reason is that the GI antigen used has low immunogenicity and high hydrophilicity. Furthermore, multiple basic amino acids are coupled to protein carrier with glutaraldehyde, which results in the change of the epitopes of GI and causes low immunogenicity. Our group has attempted but failed to prepare MI antiserum using MI multi-branched peptide antigen (Chen et al., 2019). While our MI antigen maintained integrity of its epitope and induced medium titer of mouse antiserum (1:25,600), its antiserum did not possess significant neutralizing potency.

For this reason, we turned to other ways of modifying the MI antigen to increase its immunogenicity for producing neutralizing antiserum and antibodies. The click reaction for coupling small molecules or peptides to protein or DNA caught our eyes due to its high yield and mild reaction conditions (Tang et al., 2014; Pickens et al., 2018; Boga et al., 2019; Parker et al., 2020). In the present study, a new MI antigen was synthesized by the coupling of alkynyl-modified MI to azide-modified bovine serum albumin (BSA-N_3), which produced a MI-BSA complete antigen. After the repeat immunization of MI-BSA complete antigen in mice and horses, we were able to prepare mouse and horse antiserum to MI and determined their detoxification activities. The results show that MI-BSA possesses high immunogenicity, with the titer of mouse and horse antiserums reaching 1:204,800 and 1:51,200, respectively. More importantly, a low dose (6.25 μL–12.5 μL) of mouse or horse antiserum could completely neutralize a lethal dose of MI (0.4 μg, 1.7 × LD_{50}) *in vivo*, and could fully protect mice from a lethal MI challenge. The potency observed for these antiserums are 10 times more potent than previous attempts. In addition, the mouse and horse antiserum exhibited medium cross-reactivity to the structurally similar α-CTX GI. This work provides an effective detoxification treatment for α-conotoxins as well as an effective method for the preparation of antiserum of small peptide toxins.

2. Materials and methods

2.1. Materials, reagents and animals

α-CTX MI and GI linear peptides were synthesized using Fmoc chemistry and standard side protection, then folded and purified to acquire final products with purity>95% (Liu et al., 2018; Chen et al., 2018; Ning et al., 2018). Bovine serum albumin (Purity≥98%), complete Freund's adjuvant and incomplete Freund's adjuvant were from Sigma-Aldrich (USA). Skim milk powder was from Oxoid Ltd (the United Kingdom). Phosphate buffer instant granules (pH 7.4) were purchased from Beckard Bioengineering Co., Ltd (Shaanxi, China). Rabbit anti-horse IgG-HRP monoclonal antibody was obtained from Biodragon Immunotechnologies Co., Ltd (Beijing, China). Goat anti-mouse IgG-HRP monoclonal antibody, Soluble 3,3,5,5-tetramethylbenzidine (TMB) Kit and SDS-PAGE Gel Kit were obtained from Cowin Biotech Co., Ltd (Beijing, China). Coomassie Blue Staining Solution was obtained from Solarbio Science & Technology Co., Ltd (Beijing, China). Acetonitrile, Page Ruler ™ Prestained Protein Ladder and F96 Maxisorp Nunc-Immuno Plate were purchased from Thermo Fisher Scientific (USA). Pepsin was obtained from (Sigma). All Fmoc-amino acids were obtained from GL Biochem Ltd (Shanghai, China). All chemicals and other reagents, unless otherwise indicated, were obtained from Sinopharm Chemical Reagent Sigma Co., Ltd (Beijing, China), Ouhe Technology Co., Ltd (Beijing, China) and Yinuokai Technology Co., Ltd (Beijing, China). All chemical reagents are of analytical grade.

BALB/c (16–18 g, 4–6 week old) and Kunming mice (18–20 g, 3–4 week old) were provided by SPF Biotechnology Co., Ltd (Beijing, China), the latter are derived from Swiss mice (Zhang et al., 1997). They were housed in groups of eight on a 12-h light–dark cycle (light cycle from 8 a.m. to 8 p.m.) at 23 ± 2 °C and a relative humidity of 50%. Food pellets and water were available *ad libitum*. All experiments were conducted in accordance with the guidelines of the Animal Research Advisory Committee of the Beijing Institute for Biological Sciences (Beijing, China) and conformed to European Community directives for the care and use of laboratory animals.

2.2. Synthesis and characterization of MI-BSA antigen

The MI-BSA antigen was synthesized according to Scheme 1.

2.2.1. Synthesis of alkynyl-containing MI

The linear alkynyl-containing MI was synthesized by Standard Fmoc method (Ning et al., 2018), in which the butynyl modified Fmoc-Lys (butynyl)-OH was synthesized according to the literature (Li et al., 2015). The folded alkynyl-containing MI (**a**) was synthesized as described previously (Chen et al., 2019). Briefly, the linear peptide (0.4 mg/mL) was then folded in 0.1 M NH_4HCO_3 buffer (pH 8.2) for 24 h at room temperature. The reaction was stopped by acidification with acetic acid to a pH of 3.0–4.0, and the folded solution was loaded on to a 25 × 250 mm preparative C18 column using a preparative high performance

liquid chromatography (HPLC) (Waters Delta Prep 4000). The absorbed peptide was then purified by preparative reverse phase (RP)-HPLC. The final products were assessed by analytical RP-HPLC.

2.2.2. Azide modification of BSA

Azide acetic acid (0.37 mL, 5.21 mmol) was added to a solution (15 mL) of N-hydroxysuccinimide (0.57 g, 5.21 mmol) and dicyclohexylcarbodiimide (1.17 g, 5.82 mmol). The reaction was allowed to continue at room temperature under stirring overnight. After excess acetonitrile was removed under reduced pressure, the active ester (**b**) is obtained by recrystallization with isopropanol. A dimethyl sulfoxide solution of active ester (15.7 mg, 0.08 mmol, 1 mL) was then added to BSA (0.53 g, 0.008 mmol, 10 mL) dissolved in PBS (phosphate buffer, 10 mM, pH 7.2), then stirred overnight at 4 °C. The resulting azide-modified BSA (BSA-N_3) was purified by HiTrap desalt column (General Electric Company) on the AKTA Prime purification system (Amersham Biosciences) to remove salts and small molecules. The mobile phase was 10 mM PBS (pH 7.4), with a flow rate of 5 mL/min. The coupling protein was further concentrated using an ultrafiltration tube (General Electric Company) with a molecular weight cut-off of 30 KDa and characterized by gel electrophoresis in 8% SDS-PAGE. Its concentration was determined by the BCA (Bicinchoninic Acid Assay) method, and the azide group was identified by infrared spectroscopy (Beijing LvPu Technology Co., Ltd) (Li et al., 2012).

2.2.3. Synthesis of MI-BSA via click reaction

Alkynyl-containing MI (49.95 mg, 27 μmol) was dissolved in a PBS solution (22.5 mL) of BSA-N_3 (72 mg, 1 μmol), followed by oxygen removal through nitrogen filling for 15 min. The following solutions were added in order to the deoxygenated MI-BSA solution: (1) DMF solution (80 μL) of tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 1.45 mg, 2.7 μmol); (2) water solution (15.2 μL) of copper sulfate (0.68 mg, 2.7 μmol), (3) water solution (37.9 μL) of sodium ascorbate (1.35 mg, 6.8 μmol) solution. After mixing, degassed phosphate buffer (10 mM, pH 7.4) was added to make reaction volume 50 mL and degassed again for 20 min, the mixed solution was then stirred for 24 h at room temperature. The coupled MI-BSA protein was purified by HiTrap desalt column and concentrated by using an ultrafiltration tube with a molecular weight cut-off of 30 KDa. Protein concentration was determined by the BCA method and characterized by gel electrophoresis in 8% SDS-PAGE gel with the stain Coomassie Blue.

2.3. Animal immunization with MI-BSA and preparation of antiserum

2.3.1. Mouse immunization and preparation of antiserum

For the initial priming, BALB/c mice received one subcutaneous and one intraperitoneal injection (20 μg) of MI-BSA in complete Freund's adjuvant. Four additional booster injections in incomplete Freund's adjuvant were administered (20 μg of antigen/dose) with a 14-day interval in between the injections. All doses were diluted in sterile saline buffer. Blood samples were collected 14 days after final booster injection through retro-orbital bleeding. After coagulation, samples were subjected to sedimentation at 4 °C for 6 h to isolate the antiserum. Finally, the samples were centrifuged at 12,000 rpm at 4 °C for 15 min and the supernatant containing MI antiserum was stored at −80 °C.

2.3.2. Horse immunization and preparation of antiserum

Horse immunization with MI-BSA and preparation of antiserum were performed as described previously (Zhou et al., 2019; Pan et al., 2020). Briefly, each horse received two initial injections (20 mg of antigen/dose) of MI-BSA in an immune adjuvant (lanolin and paraffin oil) with a 14-day interval in between the injections. After 14 days, five additional injections were administered (30 mg of antigen/dose) with a 7-day interval in between the injections. The horses were bled a week after the last injection. The plasma was collected and stored at 2–8 °C. After centrifuged at 9000 rpm for 10 min, 2 L of plasma were digested with pepsin (9 U/mL) at 30 °C and pH 3.0 to remove the Fc segment of IgG. The product is then precipitated twice with ammonium sulfate and adsorbed by alum. Finally, the obtained supernatant was concentrated and desalted using 50 KDa membrane ultrafiltration (Pall Corporation), purified by DEAE anion exchange chromatography (General Electric Company) and filtrated by nanomembrane (Bona Biological Technology Co., Ltd.). The protein concentration of the obtained F(ab')$_2$ stock solution was determined using Kjeldahl nitrogen determination and its purity was determined by SDS-PAGE and size-exclusion HPLC.

2.4. Indirect ELISA

Antiserum-based indirect ELISA was established for α-conotoxin MI and GI to determine the titer and evaluate specificity of horse and mouse antiserum. 96-well plates (Thermo Scientific) were coated overnight at 4 °C with 100 μL of a 10 μg/mL solution of antigen (MI or GI) in carbonate buffer (0.05 M) at pH 9.6. After washing three times with PBST (phosphate buffer with 0.1% Tween 20) and blocking with 5% skim milk (diluted in PBST) for 2 h at 37 °C, the plate was incubated with serial dilutions of specific sera for 1 h at 37 °C. The plate was washed three times with PBST, and 100 μL of horseradish peroxidase (HRP)-conjugated sheep-anti-mouse antibodies (1:20,000) or HRP-conjugated rabbit-anti-horse antibodies (1:2000) were added to each well and incubated for 30 min at 37 °C. After washing the plate three times with PBST, 100 μL peroxidase substrate TMB was added to each well and incubated for 15 min at 37 °C. The reaction was stopped by adding 50 μL of 0.5 M H_2SO_4 and absorbance values were determined at 450 nm and 600 nm using Thermo Scientific Microplate Reader. Absorbance values those with 2.1-fold higher than that of negative control (non-immunized mouse or horse serum) were considered positive.

2.5. Median lethal dose (LD_{50}) determination

Kunming mice were randomly divided into different dose groups with 10 animals in each group, half females and half males. Saline solution of MI or GI in different concentrations were prepared and injected (i.p.) (200 μL) into each mouse. After 24 h, the death rates of mice were recorded and LD_{50} was calculated by a non-linear regression with variable slope.

2.6. MI/GI neutralization test (pre-incubation of toxin with antiserum or F(ab')$_2$)

For evaluation of MI/GI neutralization potencies of the antiserums, lethal doses of MI or GI were pre-incubated with antiserum. Kunming mice were randomly divided into several dose groups with 10 animals in each group, half females and half males. Various volumes of antiserum or non-immunized animal serum (control group) were premixed with MI or GI, with the final concentration of MI and GI at 0.4 μg/100 μL and 0.8 μg/300 μL, respectively. The toxin/antiserum mixture was then incubated at 37 °C for 40 min. Subsequently, different concentrations of MI/antiserum mixtures (100 μL) or GI/antiserum mixtures (300 μL) were administered (i.p.) to mice. Control group received 100 μL or 300 μL of mixed solution containing 0.4 μg of MI (1.7 × LD_{50}) or 0.8 μg of GI (1.7 × LD_{50}) and non-immunized animals serum. Number and time of mouse deaths were recorded over a 24-h period.

Evaluation of neutralization potencies of horse F(ab')$_2$ follows similar protocols as the above neutralization test of antiserums. Briefly, different doses of horse F(ab')$_2$ were mixed with MI, with the final concentration of MI at 0.4 μg/100 μL, and the molar ratios of MI (0.4 μg) with horse F(ab')$_2$ at 1:3, 1:4, 1:5 and 1:6. After incubation at 37 °C for 40 min, 100 μL of mixture of MI with F(ab')$_2$ was individually administered (i.p.) to Kunming mice (10 animals each group, half females and half males). Control group only received 1.7 × LD_{50} of MI (0.4 μg/100 μL). Number and time of mouse deaths were recorded over a 24-h period.

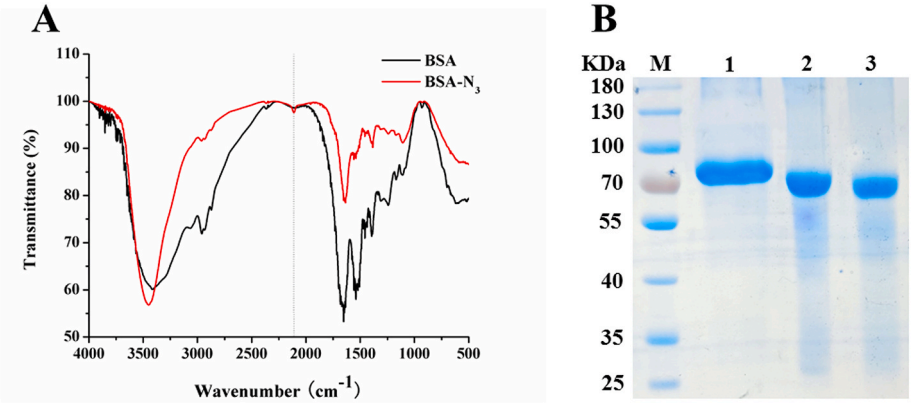


Fig. 1. Characterization of MI-BSA synthetic intermediates and final products. (A) Infrared spectra of BSA and BSA-N_3. (B) SDS-PAGE analysis of MI-BSA, BSA-N_3 and BSA. Lane 1: MI-BSA; Lane 2: BSA-N_3; Lane 3: BSA.

2.7. MI/GI detoxification test (rescue of mice envenomated i.p. with toxin)

The detoxification activities of MI-BSA antisera for MI or GI were assessed by observing survival of mice injected different doses of antisera 8 min after administration of the lethal dose of toxin. Groups of 10 mice (half females and half males) were administered (i.p.) with 1.7 × LD_{50} of MI or GI dissolved in 100 μL of saline. After 8 min, mice were administrated (i.v.) with 100 μL or 300 μL saline containing different amounts of antiserum, respectively. Control group was administrated with 100 μL or 300 μL non immunized animal serum. Number and time of mouse deaths were recorded over a 24-h period.

Evaluation of detoxification potencies of horse $F(ab')_2$ follows similar protocols as the above detoxification test of antiserums. Briefly, 8 min after mice (10 mice each group) were administered (i.p.) with 1.7 × LD_{50} of MI, different concentrations of $F(ab')_2$ (molar ratio of $F(ab')_2$ to MI at 3:1, 4:1, 5:1 and 6:1) were intravenously injected. Number and time of mouse deaths were recorded over a 24-h period.

2.8. Statistical analysis

ED_{50} is the dose of antiserum that induces 50% survival after mice injected with 1.7 × LD_{50} of MI. In this report, ED_{50} is expressed as μL/LD_{50} or μg/LD_{50}. LD_{50}, ED_{50} and 95% confidence intervals were determined by a non-linear regression using the software GraphPad Prism 8.0. The survival curves of mice and the titer curves of antiserum were plotted by using the GraphPad Prism 8.0 software. Kaplan-Meier survival analysis and Log-rank test were performed to analyze the difference in death times between the experimental groups and the control groups by using the IBM SPSS Statistics 22 software. In all cases,

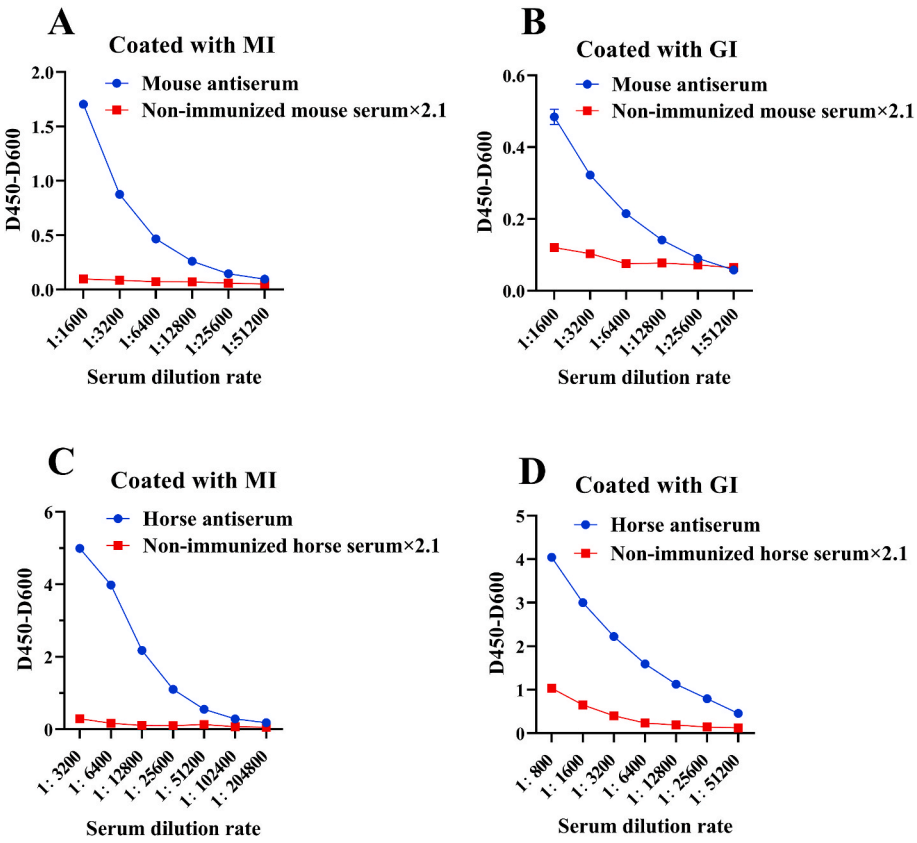


Fig. 2. The serum dilution rate plots of mouse or horse antiserum against MI or GI. (A) Mouse antiserum against MI. (B) Mouse antiserum against GI. (C) Horse antiserum against MI. (D) Horse antiserum against GI. Antiserum obtained after the final immunization of animals with MI-BSA. The OD450-OD600 values represent the means ± standards (SDs) of duplication measurements. Absorbance values those with 2.1-fold higher than that of negative control (non-immunized mouse or horse serum) were considered positive.

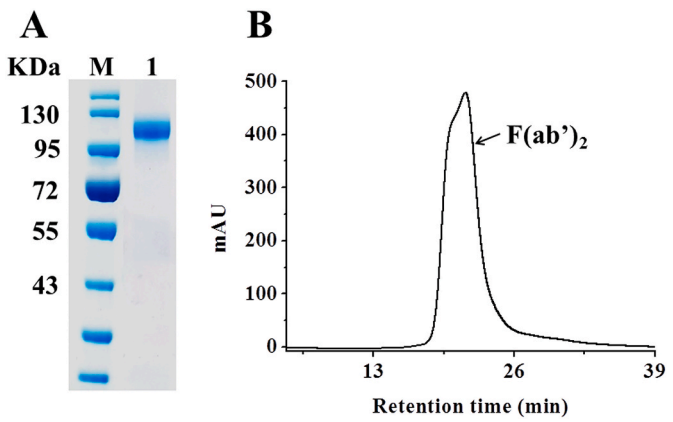


Fig. 3. Non-reduced SDS-PAGE identification and size-exclusion HPLC analysis of F(ab')$_2$. (A) SDS-PAGE identification of F(ab')$_2$. M, marker; 1, F(ab')$_2$. (B) size-exclusion HPLC analysis of F(ab')$_2$. Sample was applied to a XK16/40 column (General Electric Company, 16 mm × 40 mm) and eluted with PBS . Absorbance was monitored at 280 nm. The flow rate was 0.6 mL/min.

differences were considered significant at $p < 0.05$.

3. Results

3.1. Synthesis and identification of MI-BSA antigen

Click chemistry was used to synthesize MI-BSA from BSA-azide (BSA-N_3) and alkynyl-MI. BSA-N_3 was synthesized by the coupling reaction of BSA with azide acetic acid NHS ester (**b**). The infrared spectrum of BSA-N_3 shows a characteristic absorption peak of azide at 2100 wave number/cm^{-1} (Fig. 1A), indicating that BSA was successfully modified by azidoacetic acid. The coupling product (MI-BSA) of BSA-N_3 with the alkynyl-MI was purified using a HiTrap desalt column, and further concentrated by ultrafiltration tube. SDS-PAGE (Fig. 1B) analysis shows a single band of 80 KDa. According to the molecular weight of BSA and alkynyl-MI, about eight MI molecules are coupled to the BSA.

3.2. Immunogenicity of MI-BSA in mouse and horse

The titer and specificity of antiserum of the immunized mouse and horse after final immunization with MI-BSA were determined by indirect ELISA with MI/GI antigen. As shown in Fig. 2A and Fig. 2C, the titers of the mouse and horse antiserums to MI are 1:204,800 and 1:51,200, respectively. In addition, the antiserum also has a medium cross-reactivity to GI, with the titer of mouse and horse antiserum to GI reaching 1:25,600 (Figs. 2B) and 1:51,200 (Fig. 2D), respectively. These results indicate that MI-BSA exhibits very good immunogenicity and successfully produced high titers of antiserum to MI and GI in mouse and horse.

3.3. Preparation and characterization of F(ab')$_2$

Alternatively, we also prepared horse F(ab')$_2$ from the blood of animals immunized with MI-BSA. After horse plasma were digested with pepsin, the crude F(ab')$_2$ was purified by DEAE-sepharose-FF column, and protein purity was then determined using non-reduced SDS-PAGE (Fig. 3A) and size-exclusion HPLC (Fig. 3B). The results show that the molecular weight of F(ab')$_2$ is about 110 KDa and the purity is around 85%. The protein concentration of the F(ab')$_2$ stock solution (pH 6.99) is 10.7 mg/mL (Kjeldahl nitrogen method).

3.4. Median lethal dose (LD_{50}) of MI and GI

Before evaluation of the neutralization and detoxification potencies of antiserum and F(ab')$_2$, we first set out to determine a reasonable dose of MI/GI for animal tests. Various doses of toxins were injected through intraperitoneal (i.p.) route to different groups of mice (n = 10 each group), and deaths were recorded at 24 h. According to calculations,

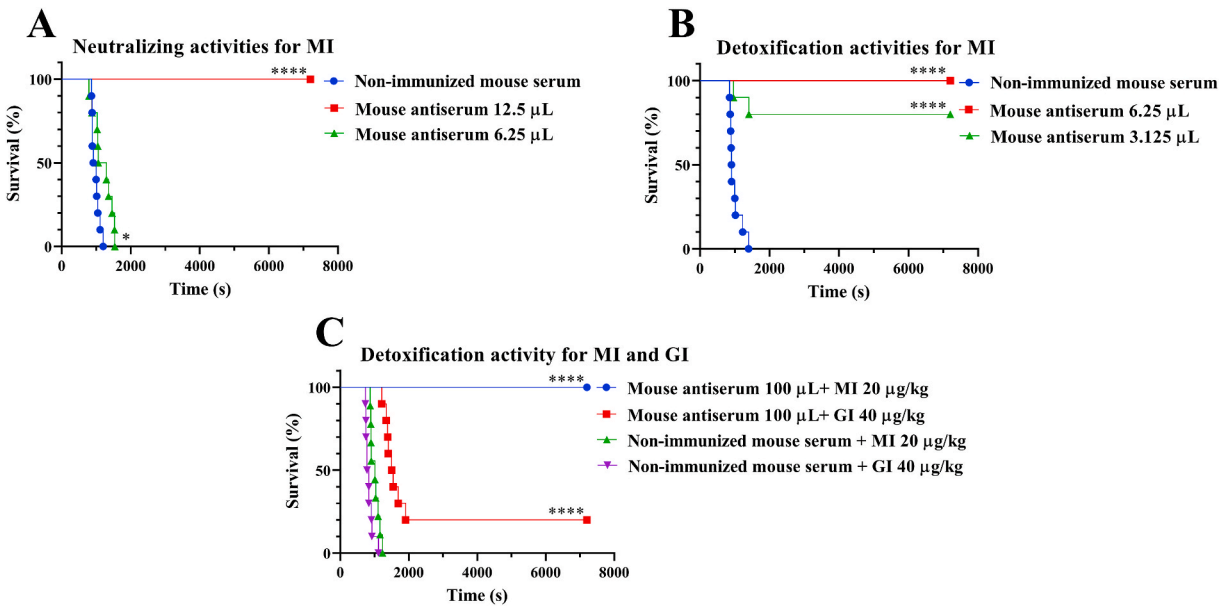


Fig. 4. The neutralization and detoxification activity of mouse antiserum for a lethal dose of MI or GI. (A) The neutralization potency for MI. MI-BSA antiserum was premixed with MI and diluted with saline and incubated at 37 °C for 40 min, the mixture of MI and antiserum (12.5 μL/100 μL or 6.25 μL/100 μL) or non-immunized mouse serum (99.8 μL/100 μL) was then administered (i.p.) to mice. *$p < 0.05$, 6.25 μL antiserum for MI vs. non immunized mouse serum. ****$p < 0.0001$, antiserum vs. non immunized mouse serum. (B) (C) Detoxification activity for MI or GI. Mice were administered (i.p.) with MI or GI dissolved in 100 μL of saline. After 8 min, mice were administrated intravenously (i.v.) with 100 μL or 300 μL saline solution containing different amounts of antiserum or non-immunized mouse serum, respectively. 10 mice were in each group, half females and half males. The lethal dose of MI and GI was 0.4 and 0.8 μg/mouse in each group, and is equal to 1.7 × LD_{50} for MI and GI, respectively. ****$p < 0.0001$, antiserum vs. non immunized mouse serum (B), ****$p < 0.0001$, antiserum/MI vs. non immunized mouse serum/GI (C).

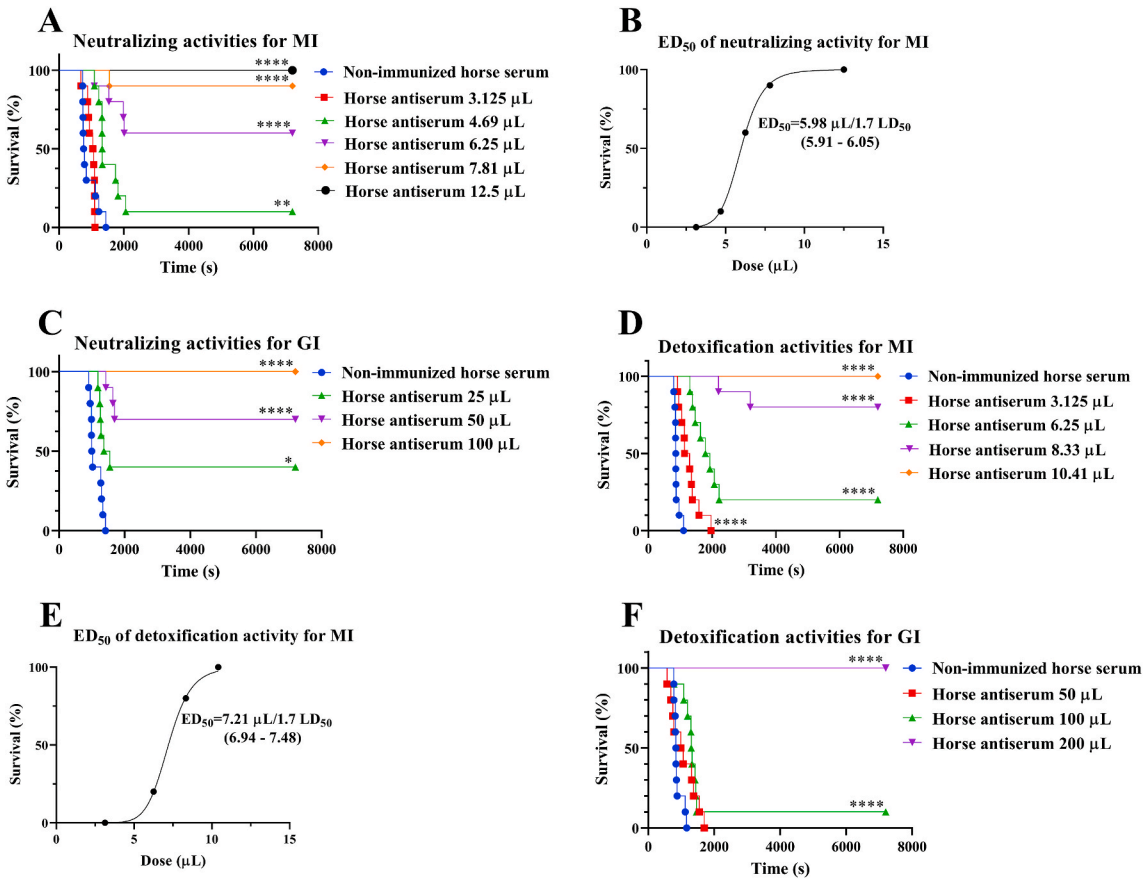


Fig. 5. The neutralizing and detoxification potency of horse antiserum for a lethal dose of MI or GI. (A) The neutralizing potency for MI. The antiserum was premixed with MI and diluted with saline and incubated at 37 °C for 40 min, after which 100 μL of the mixture of MI and antiserum (the content is 3.125, 4.69, 6.25, 7.81 and 12.5 μL, respectively) or non-immunized horse serum (99.8 μL/100 μL) were administered (i.p.) to mice. *$p < 0.05$, 25 μL antiserum for GI vs. non immunized horse serum. **$p < 0.01$, 4.69 μL antiserum for MI vs. non-immunized horse serum. ****$p < 0.0001$, antiserum vs. non-immunized horse serum. (B) ED_{50} of neutralizing activity for MI. 95% confidence intervals are shown in parenthesis. (C) Neutralizing activity for GI. Volume of administration is 300 μL, containing 25 μL, 50 μL and 100 μL of antiserum, respectively. (D) The detoxification activity for MI. Mice were administered (i.p.) with saline solution (100 μL) of MI, then administrated (i.v.) with 100 μL of saline solution containing different amounts of antiserum after 8 min. (E) ED_{50} of detoxification activity for MI. (F) The detoxification activity for GI. Similar experimental procedure as MI tests, with administration volume at 300 μL and the content of antiserum at 50 μL, 100 μL and 200 μL, respectively. The lethal dose of MI and GI was 1.7 × LD_{50}, 10 mice were in each group, half females and half males. No statistical significance unless marked.

LD_{50} of MI and GI are 11.63 (11.22–12.12) μg/kg and 24.27 (23.36–25.41) μg/kg after 24 h, respectively. We used MI/GI doses at 1.7 × LD_{50} for all subsequent animal experiments since this dose could induce death in the majority of the animals, but still retain sensitivity for antidotes.

3.5. MI-BSA mouse antiserum exhibits a high neutralizing and detoxification activity

The potencies of mouse antiserum were tested by two different assays, neutralization assay and detoxification assay, which reflects different properties of the antiserum. The toxin (MI/GI) is either pre-incubated with the antiserum (neutralization assay), or injected in mice 8 min prior to antiserum injection (detoxification assay).

For the neutralization assay, 1.7 × LD_{50} of MI was pre-incubated with mouse antiserum. The results show that 12.5 μL of mice (Fig. 4A) antiserum fully neutralized the toxin, and protected all mice from deaths.

For the detoxification assay, 1.7 × LD_{50} of MI was injected into mice, and varying concentrations of mouse antiserum were intravenously injected after 8 min. The results show that 6.25 μL of mice antiserum (Fig. 4B) completely rescued all mice. The mouse antiserum also shows some cross-reactivity with GI, with 100 μL of antiserum providing 20% protection to mice envenomated (i.p.) with 1.7 × LD_{50} of GI (Fig. 4C). Kaplan-Meier survival analysis and Log-rank test show a significant difference in the times to death between the experimental groups (antiserum) and the control groups (non-immunized mouse serum) ($p < 0.05$). In addition, the death times of mice injected with 100 μL antiserum differ significantly for MI compared to GI ($p < 0.0001$) (Fig. 4C).

3.6. MI-BSA horse antiserum exhibits a high neutralizing and detoxification activity

The neutralizing and detoxification activities of MI-BSA immunized horse antiserum were also determined with the same protocol as for the mouse antiserum. The results show that 12.5 μL of horse antiserum completely neutralizes 1.7 × LD_{50} of MI (Fig. 5A), and the ED_{50} (median effective dose) is 5.98 μL/1.7 × LD_{50} (Fig. 5B). Similar to the mouse MI antiserum, the horse antiserum also shows cross-reactivity with GI, albeit at a reduced potency: 100 μL of horse antiserum is required to neutralize 1.7 × LD_{50} of GI (Fig. 5C).

High detoxification activity for MI was also found with horse antiserum. 10.41 μL of antiserum (Fig. 5D) completely rescues mice envenomated (i.p.) with 1.7 × LD_{50} of MI, with an ED_{50} of 7.21 μL/1.7 × LD_{50} (Fig. 5E). Similar to mice antiserum, the detoxification of horse antiserum for GI is lower than for MI ($p < 0.0001$), with 200 μL of horse antiserum required to fully rescue mice through i.v. route (Fig. 5F). There were significant differences in the times to death between all

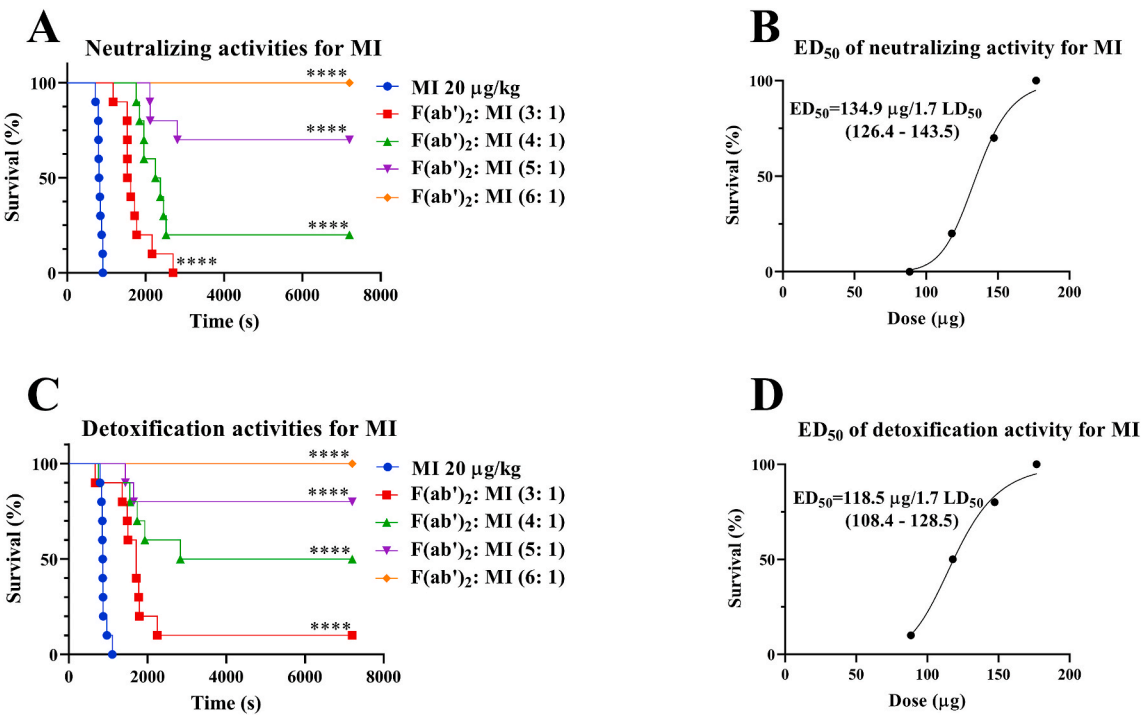


Fig. 6. The neutralizing and detoxification potency of F(ab')$_2$ for a lethal dose of MI. (A) The neutralizing potency for MI. 1.7 × LD_{50} MI (0.4 μg) with different molar ratios of F(ab')$_2$ (ratios of 1:3, 1:4, 1:5 and 1:6) in 100 μL saline were incubated at 37 °C for 40 min and further administered (i.p.) to mice. Control group only received 1.7 × LD_{50} of MI. ****$p < 0.0001$, experimental group vs. control group. (B) ED_{50} of neutralizing potency for MI. 95% confidence intervals is shown in parenthesis. (C) The detoxification potency for MI. Mice were administered (i.p.) with MI dissolved in 100 μL of saline. After 8 min, mice were administrated (i.v.) with F(ab')$_2$ with different molar ratios to MI (ratios of 3:1, 4:1, 5:1 and 6:1) by intravenous injection, respectively. (D) ED_{50} of detoxification activity for MI. The lethal dose of 1.7 × LD_{50} was for MI and GI, 10 mice were in each group, half females and half males. ****$p < 0.0001$, experimental group vs. control group.

experimental groups (antiserum) and the control groups (non-immunized horse serum) ($p < 0.05$ or lower); the exceptions are 50 μL horse antiserum for GI in the detoxification tests and 3.125 μL antiserum for MI in the neutralizing tests, which showed no statistical difference with the respective control groups.

3.7. MI-BSA horse F(ab')$_2$ exhibits a potent neutralizing and detoxification activity

Finally, we determined the neutralization and detoxification activities of MI-BSA immunized horse F(ab')$_2$. The results of the neutralization activity assay show that all mice survived in a protective dose of F(ab')$_2$ of 176.7 μg, with the molar ratio of toxin to antibody was 1:6 (Fig. 6A). The ED_{50} of F(ab')$_2$ is 134.9 μg/1.7 × LD_{50} calculated by the survival rate-dose curve (Fig. 6B). The detoxification activity of horse F(ab')$_2$ was also assessed with the same procedure as for the antiserums. Administration (i.v.) of 176.7 μg of F(ab')$_2$ is enough to rescue all mice envenomated (i.p.) with 1.7 × LD_{50} of MI (Fig. 6C), with an ED_{50} of 118.5 μg/1.7 × LD_{50} (Fig. 6D). Kaplan-Meier survival analysis and Log-rank test show a significant difference in the times to death between the experimental group and the control group ($p < 0.0001$).

4. Discussion

In the present study, a new MI-BSA conjugate antigen was synthesized, and its mouse/horse antiserum and horse F(ab')$_2$ were found to display potent neutralization and detoxification activities for MI and GI. This stands in contrast with previously reported attempts at deriving MI/GI antiserum, which were less than satisfactory with either low antiserum titers or low neutralization potencies. We reasoned that previous failures to produce highly potent MI/GI antiserum may have been caused by either low immunogenicity of the MI/GI antigens used, or by a change in the exposed antigen epitopes that reduced neutralization potential. To solve this problem, we used click chemistry to conjugate MI antigen to BSA to increase its immunogenicity while preserving its correct epitopes, and subsequently validated the high neutralization and detoxification activities of the antiserums and F(ab')$_2$.

It is noted that the appropriate injection dose of toxins should be considered in the detoxification experiments. We found that the dose of 20 μg/kg (MI) and 40 μg/kg (GI) are a suitable lethal dose and can provide a rescue window of 8 min for envenomated mice.

In the present study, 12.5 μL of mouse or horse antiserum completely neutralized (preincubation) a lethal dose (1.7 × LD_{50}) of MI (0.4 μg/mouse, 20 μg/kg), and 6.25 μL of mouse antiserum or 10.41 μL of horse antiserum could completely detoxify (toxin injected before antiserum) mice envenomated (i.p.) with 1.7 × LD_{50} MI. Since F(ab')$_2$ had the advantages of better tissue permeability and lower immunogenicity due to the lack of Fc segment compared with IgG (Pucca et al., 2019; Pépin-Covatta et al., 1997), MI horse F(ab')$_2$ was also prepared. 176.7 μg of F(ab')$_2$ (toxin to antibody molar ratio was 1:6) exhibited a high neutralizing and detoxification against 1.7 × LD_{50} of MI.

The MI mouse or horse antiserum neutralizing potencies are significantly higher than previously reported GI antiserum, in which 200 μL GI mouse antiserum (GI-BSA as antigen) premixed with GI (25.8 μg/kg) only provided 75% protection for mouse (Tang et al., 2017), and 200 μL of goat antiserum immunized with GI-OVA and GI-KLH was required to completely neutralize 0.55 μg GI/mouse or 0.22 μg MI/mouse (Stiles and Sexton, 1992). To our knowledge, this is first report of successful preparation of MI antiserum and horse antiserum for conotoxins.

The high neutralizing potency of our MI antiserum is mainly from the integrity of MI's antigen epitope. In the reported GI mouse antiserum, basic amino acids of GI were coupled to protein carrier with glutaraldehyde, which resulted in the changes of epitopes and low immunogenicity (Stiles and Sexton, 1992; Tang et al., 2017; Ashcom et al., 1997). On the other hand, because α-conotoxins MI and GI are short and hydrophilic peptides with low immunogenicity, a protein carrier is needed

to elevate their immunogenicity. We previously found that MI multi-branched peptide antigen could not induce effective antibodies though it displays a medium titer in ELISA assay (Chen, R.F et al., 2019). Of course, peptide toxins with a large size, such as GVIA, MVIIC and MVIIA, may not significantly affect its epitopes when they are coupled to protein carriers (Tombaccini et al., 1990; Ichida et al., 2003; Nakao et al., 1999; Yang et al., 2014).

Interestingly, MI antiserum possesses medium cross-reactivity to α-conotoxin GI, 100 or 200 μL of horse antiserum could completely protect or rescue mice administrated by 1.7 × LD_{50} GI. These results are attributed to the similarity in their amino acid sequences and the disulfide bond connectivity, and similar cross-reactivity has been found in snake toxin antiserum (Ledsgaard et al., 2018; Arroyo et al., 2017; Ponce-López et al., 2020; Li et al., 2020).

In conclusion, α-conotoxin MI antigen MI-BSA synthesized by click reaction possesses high immunogenicity, its mouse and horse antiserum exhibit high neutralization and detoxification potency to MI and medium cross-reactivity to GI. This work provides an effective detoxification treatment for α-conotoxin MI and GI, as well as an effective method for the preparation of antiserum of small peptide toxins.

Ethical statement

I have read and have abided by the statement of ethical standards for this manuscript submitted to Toxicon. I confirm that all authors contributed to this manuscript and have approved the final article. The authors declare no conflict of interest.

Credit author statement

Min Zhang: Investigation, Formal analysis, Writing – original draft. **Shuo Yu:** Investigation, Formal analysis. **Xin Zhang:** Investigation. **Qiuyuan Huang:** Investigation. **Yue Huang:** Investigation. Min Luo: Investigation. **Yuanmei Wei:** Investigation. **Wenwen Chen:** Investigation. **Ze Chen:** Investigation. **Xiaowei Zhou:** Investigation. **Qiuyun Dai:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by National Key Research and Development Project of China (2019YFC0312605).

References

Anderson, P.D., Bokor, G., 2012. Conotoxins: potential weapons from the sea. J. Bioterrorism Biodefense 3, 1–4.
Arroyo, C., Solano, S., Segura, A., Herrera, M., Estrada, R., Villalta, M., Vargas, M., Gutiérrez, J.M., León, G., 2017. Cross-reactivity and cross-immunomodulation between venoms of the snakes *Bothrops asper, Crotalus simus* and *Lachesis stenophrys*, and its effect in the production of polyspecific antivenom for central America. Toxicon 138, 43–48.
Ashcom, J.D., Stiles, B.G., 1997. Characterization of α-conotoxin interactions with the nicotinic acetylcholine receptor and monoclonal antibodies. Biochem. J. 328, 245–250.
Azam, L., McIntosh, J.M., 2009. Alpha-conotoxins as pharmacological probes of nicotinic acetylcholine receptors. Acta Pharmacol. Sin. 30, 771–783.
Boga, S.B., Krska, S.W., Lin, S., Pissarnitski, D., Yan, L., Kekec, A., Tang, W., Pierson, N. A., Strulson, C.A., Streckfuss, E., Zhu, X., Zhang, X., Kelly, T., Parish, C.A., 2019. Site-selective synthesis of insulin azides and bioconjugates. Bioconjugate Chem. 30, 1127–1132.
Chen, J., Liang, L., Ning, H., Cai, F., Liu, Z., Zhang, L., Zhou, L., Dai, Q., 2018. Cloning, synthesis and functional characterization of a novel α-conotoxin Lt1.3. Mar. Drugs 16, 112.
Chen, R.F., Yu, S., Zhou, L.Y., Zhang, X.R., Dai, Q.Y., 2019. Synthesis and Immunogenicity of α-conotoxin MI branched peptides. Lett. Biotech. (Chin) 30, 768–774.
Chivian, E., Roberts, C.M., Bernstein, A.S., 2003. The threat to cone snails. Science 302, 391.
Cruz, L.J., Gray, W.R., Olivera, B.M., 1978. Purification and properties of a myotoxin from *Conus geographus* venom. Arch. Biochem. Biophys. 190, 539–548.
Gray, W.R., Rivier, J.E., Galyean, R., Cruz, L.J., Olivera, B.M., 1983. Conotoxin MI. Disulfide bonding and conformational states. J. Biol. Chem. 258, 12247–12251.
Halford, Z.A., Yu, P.Y., Likeman, R.K., Hawley-Molloy, J.S., Thomas, C., Bingham, J.P., 2015. Cone shell envenomation: epidemiology, pharmacology and medical care. Diving Hyperb. Med. 45, 200–207.
Ichida, S., Abe, J., Sugimoto, W., Uematsu, M., Komoike, K., Imoto, K., Mori, M., Wada, T., Minami, T., Kakutani, K., 2003. Antigen selectivity characteristic of polyclonal antibodies against omega-conotoxin GVIA and N-type voltage-dependent calcium channels. Neurochem. Res. 28, 789–796.
Jacobsen, R.B., DelaCruz, R.G., Grose, J.H., McIntosh, J.M., Yoshikami, D., Olivera, B.M., 1999. Critical residues influence the affinity and selectivity of alpha-conotoxin MI for nicotinic acetylcholine receptors. Biochemistry 38, 13310–13315.
Jin, A.H., Muttenthaler, M., Dutertre, S., Himaya, S.W.A., Kaas, Q., Craik, D.J., Lewis, R. J., Alewood, P.F., 2019. Conotoxins: chemistry and biology. Chem. Rev. 119, 11510–11549.
Kaerner, A., Rabenstein, D.L., 1999. Stability and structure-forming properties of the two disulfide bonds of alpha-conotoxin GI. Biochemistry 38, 5459–5470.
Kapono, C.A., Thapa, P., Cabalteja, C.C., Guendisch, D., Collier, A.C., Bingham, J.P., 2003. Conotoxin truncation as a post-translational modification to increase the pharmacological diversity within the milked venom of *Conus magus*. Toxicon 70, 170–178.
Kohn, A.J., 2018. Conus envenomation of humans: in fact and fiction. Toxins 11, 10.
Kohn, A.J., 2016. Human injuries and fatalities due to venomous marine snails of the family Conidae. Int. J. Clin. Pharmacol. Ther. 54, 524–538.
Lebbe, E.K., Peigneur, S., Wijesekara, I., Tytgat, J., 2014. Conotoxins targeting nicotinic acetylcholine receptors: an overview. Mar. Drugs 12, 2970–3004.
Ledsgaard, L., Jenkins, T.P., Davidsen, K., Krause, K.E., Martos-Esteban, A., Engmark, M., Rørdam Andersen, M., Lund, O., Laustsen, A.H., 2018. Antibody cross-reactivity in antivenom research. Toxins 10, 393.
Li, J.Q., Lv, J.G., Wen, L., Miao, Y.F., Gao, J.F., Lin, C.X., Du, Y., Ji, X., 2020. Preparation and detection of sea snake antisera raised in rabbits. Toxicon 186, 168–174.
Li, N., Ramil, C.P., Lim, R.K., Lin, Q., 2015. A genetically encoded alkyne directs palladium-mediated protein labeling on live mammalian cell surface. ACS Chem. Biol. 10, 379–384.
Liu, Z., Bartels, P., Sadeghi, M., Du, T., Dai, Q., Zhu, C., Yu, S., Wang, S., Dong, M., Sun, T., Guo, J., Peng, S., Jiang, L., Adams, D.J., Dai, Q., 2018. A novel α-conopeptide Eu1.6 inhibits N-type (CaV2.2) calcium channels and exhibits potent analgesic activity. Sci. Rep. 8, 1004.
Li, X.Y., Li, T.H., Guo, J.S., Wei, Y., Jing, X.B., Chen, X.S., Huang, Y.B., 2012. PEGylation of bovine serum albumin using click chemistry for the application as drug carriers. Biotechnol. Prog. 28, 856–861.
McIntosh, M., Cruz, L.J., Hunkapiller, M.W., Gray, W.R., Olivera, B.M., 1982. Isolation and structure of a peptide toxin from the marine snail *Conus magus*. Arch. Biochem. Biophys. 218, 329–334.
Nakao, Y.K., Motomura, M., Suenaga, A., Nakamura, T., Yoshimura, T., Tsujihata, M., Mori, M., Itoh, M., Nagataki, S., 1999. Specificity of omega-conotoxin MVIIC-binding and -blocking calcium channel antibodies in Lambert-Eaton myasthenic syndrome. J. Neurol. 246, 38–44.
Ning, J., Li, R., Ren, J., Zhangsun, D., Zhu, X., Wu, Y., Luo, S., 2018. Alanine-scanning mutagenesis of α-conotoxin GI reveals the residues crucial for activity at the muscle acetylcholine receptor. Mar. Drugs 16, 507.
Pan, X., Wu, Y., Wang, W., Zhang, L., Xiao, G., 2020. Development of horse neutralizing immunoglobulin and immunoglobulin fragments against Junín virus. Antivir. Res. 174, 104666.
Parker, C.G., Pratt, M.R., 2020. Click chemistry in proteomic investigations. Cell 180, 605–632.
Pépin-Covatta, S., Lutsch, C., Grandgeorge, M., Scherrmann, J.M., 1997. Immunoreactivity of a new generation of horse $F(ab')_2$ preparations against European viper venoms and the tetanus toxin. Toxicon 35, 411–422.
Pickens, C.J., Johnson, S.N., Pressnall, M.M., Leon, M.A., Berkland, C.J., 2018. Practical considerations, challenges, and limitations of bioconjugation via Azide-Alkyne Cycloaddition. Bioconjugate Chem. 29, 686–701.
Ponce-López, R., Neri-Castro, E., Borja, M., Strickland, J.L., Alagón, A., 2020. Neutralizing potency and immunochemical evaluation of an anti-Crotalus mictlantecuhtli experimental serum. Toxicon 187, 171–180.
Pucca, M.B., Cerni, F.A., Janke, R., Bermúdez-Méndez, E., Ledsgaard, L., Barbosa, J.E., Laustsen, A.H., 2019. History of envenoming therapy and current perspectives. Front. Immunol. 10, 1598.
Stiles, B.G., Sexton, F.W., 1992. Immunoreactivity, epitope mapping and protection studies with anti-conotoxin GI sera and various conotoxins. Toxicon 30, 367–377.
Tang, W., Becker, M.L., 2014. Click" reactions: a versatile toolbox for the synthesis of peptide-conjugates. Chem. Soc. Rev. 43, 7013–7039.
Tang, Y.F., Hu, X.B., Yu, S., Dai, Q.Y., 2017. Preparation of conotoxin GI antiserum and determination of neutralization activity. Mili. Med. Sci. (Chin) 41, 338–341.
Tombaccini, D., Adeyemo, O.M., Pollard, H.B., Feuerstein, G., 1990. Monoclonal antibodies against the presynaptic calcium channel antagonist omega-conotoxin GVIA from cone snail poison. FEBS Lett. 261, 71–75.

Yang, Y., Ma, Y., Li, H., Wang, S., Zhuang, Z., 2014. Preparation and identification of monoclonal antibodies against ω-conotoxin MVIIA. Monoclon. Antibodies Immunodiagn. Immunother. 33, 254–260.

Zhang, G.M., Yao, G.H., 1997. Investigation on genetic background of Kunming mice in China. Chin. J. Lab. Anim. Sci. (Chin) 7, 246–250.

Zhou, Z.J., Yu, S., Liu, Z.G., Sun, J.R., Dai, Q.Y., 2019. Preparation and antitoxic activity of horse anti - toxin from neoverrucotoxin. Mili. Med. Sci. (Chin) 43, 112–115.