Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 341 (2005) 316–325
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Screening of plants containing *Naja naja siamensis* cobra venom inhibitory activity using modified ELISA technique

Sakda Daduang [a,*], Nison Sattayasai [a], Jintana Sattayasai [b], Pattara Tophrom [a], Achra Thammathaworn [c], Arunrat Chaveerach [c], Monruedee Konkchaiyaphum [a]

[a] *Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[b] *Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*
[c] *Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

Received 21 January 2005
Available online 9 April 2005

Abstract

Enzyme-linked immunosorbent assay (ELISA) has been modified for screening plants with antagonistic activity to *Naja naja siamensis* cobra venom. Aqueous extracts from plants were investigated for their inhibitory effects on the binding of anti-cobra venom antibody to antigen, cobra venom, fixed onto 96-well microtiter plates. Ingredients in extracts were allowed to react with immobilized venom before the subsequent addition of antivenom antibody. Venom components affected by exposure to the extracts, unable to interact with their specific antibody, were predicted to be unable to bind to their native destinations or natural receptors. *Curcuma* cf. *zedoaria*, an old Thai medicinal plant, showed clear inhibitory activity in the ELISA test. Neurotoxin and protein degradative enzymes, major components in venom, were identified as targets of this extract in Western immunoblotting analysis. Ingredients in the extract showed high affinity to the toxin in competition assay by immunoprecipitation. The extract attenuated toxin activity by extending contraction time of diaphragm muscle after envenomation and had a potency to protect cellular proteins from venom degradative enzymes. *Curcuma parviflora*, with less activity in ELISA, exhibited acceptable results in two experiments but negative results in two experiments, whereas *Curcuma longa*, having low activity in the ELISA test, never showed any favorable results. Screening of 36 samples could classify plants into an inhibition range of 0 to 86%. This modified ELISA is recommended as a preliminary screening method for inhibitors with a large number of samples.

Keywords: Cobra venom; Neurotoxin; Medicinal plant; ELISA

Venomous snakebites remain an important medical problem in both developing and developed countries [1–4]. Snake venom is a mixture of proteins, and neurotoxin and cardiotoxin are the most harmful compositions with life-threatening potential from their systematic attacks. The others, such as protein degradative enzymes, always cause patients to suffer from pain, inflammation, and necrosis at the local biting area. Using antivenom injection is the most effective method of treatment [5]. However, antivenom production and use are limited by many restrictions. Production of antibodies against neurotoxin of many species is very difficult due to their low molecular size and low immunogenicity [2]. For actual use in clinical work, snake species must be identified precisely before antivenom use. In many cases, time-consuming methods are unavoidably used and false positives frequently occur [6]. After use, many patients show various side effects of allergic symptoms, including anaphylactic and anaphylactoid reactions [7,8].

One alternative way of treating snakebite is to use chemicals. Many from synthetics [9,10] or naturals [4,11,12] are under investigation. From nature, plants are

* Corresponding author. Fax: +66 43 342911.
E-mail address: sakdad@kku.ac.th (S. Daduang).

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.03.037

the main sources. Extracts from plants have been used among traditional healers, especially in tropical areas where there are plentiful sources, as therapy for snakebite for a long time. Several medicinal plants, which appear in old drug recipes or which have been passed on by oral tradition, are believed to be snakebite antidotes [12–14]. In modern science, there have been many attempts to study these plants to clarify their effectiveness [12,15–20]. Many active ingredients have been purified. Their molecular structures have been identified and characterized in the most advanced laboratories [4,11,21–29]. Currently, however, no chemicals are reported to be effective for practical use clinically as medicative drugs for envenomed patients. Attempts to identify new agents with anti-snake venom activity are still very important for public health. Research studies requiring a large number of samples must be performed, but classical screening methods are laborious, are time-consuming, and/or need a large number of experimental animals [11,15–20,30,31].

In this study, an enzyme-linked immunosorbent assay (ELISA)[1] technique was modified for screening plants with inhibitory activity on the binding of anti-*Naja naja siamensis* cobra venom antibody to its antigen, *N. naja siamensis* cobra venom. Plant aqueous extracts were allowed to react with venom, immobilized on 96-well microtiter plates, before the subsequent addition of antivenom antiserum. Loss of affinity of antigens, in venom, to their specific antibodies, in antiserum, was predicted to have a tendency to lose affinity for their native sites of action. Western immunoblotting analysis was used to reveal target molecules in venom, to which inhibitors in plant extracts reacted. The binding affinity of ingredients to targets in venom has been examined by competition assay using the immunoprecipitation method. For more reliability, two methods were further investigated. The modified ELISA was used for screening 36 plants for their inhibitory activity against venom action to present applicable uses of the technique.

Materials and methods

Plant samples, chemicals, reagents, and instruments

Plant samples, listed in Table 1, were weighed, chopped into small pieces, ground in double distilled water at a ratio of 1 g/ml, and filtered through many layers of gauze to remove big particles. The cloudy filtrates were stored at −65 °C as aqueous plant extracts. Before use, they were thawed in a water bath at 37 °C for 3 to 5 min. Plant grinding in water was the main method for sample preparation in this study, mimicking the most convenient protocol used among traditional healers. To make solutions clearer, in rat phrenic nerve/hemidiaphragm in vivo assay, absolute ethanol was used for extract preparation. *Curcuma* cf. *zedoaria*, *Curcuma longa* L., and *Curcuma parviflora* Wall. were mainly used throughout the study.

Freeze-dried *N. naja siamensis* cobra venom and globulin-refined horse anti-cobra venom antibody were donated by the Queen Soavabha Memorial Institute (Thailand). Freeze-dried purified *N. naja siamensis* cobra neurotoxin-3 was kindly provided by Kavi Ratanabanangkoon (Department of Microbiology, Faculty of Science, Mahidol University, Thailand). Other chemicals and instruments were obtained from commercial sources: Rec–protein G–alkaline phosphatase and alkaline phosphatase–goat anti-mouse immunoglobulin G (IgG, H + L, Zymed, USA), ELISA polystyrene plate (Nalge Nunc International, Denmark), Alkaline Phosphatase Conjugated Substrate Kit and Mini-Protean II Dual Slab Cell (Bio-Rad, USA), Hybond ECL nitrocellulose membrane and Protein G sepharose beads (Amersham Pharmacia Biotech, UK), Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), bovine serum albumin (BSA), rabbit anti-BSA antibody, collagenase type XI and porcine pancreas trypsin (Sigma, USA), NP1 protein transfer apparatus (product of N. Sattayasai et al.), Grass model S-48 and S-4 stimulator (Grass Medical Instruments, USA), and ELISA plate reader (Labsystem Multiskan, USA).

Enzyme linked immunosorbent assay

Each well of the polystyrene microtiter plate was coated with 1 μg/50 μl antigen in 50 mM carbonate buffer (pH 9.5) and incubated overnight at 4 °C. The wells were washed with Tris-buffered saline (TBS: 10 mM Tris–HCl, pH 8.0, and 150 mM NaCl) containing 0.05% Tween 20 (TBST) and were blocked with 5% skim milk in TBST for 60 min at 37 °C. After washing, they were incubated with various dilutions of the plant extracts in phosphate-buffered saline (PBS: 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 135 mM NaCl, and 2.5 mM KCl, pH 7.4) for 60 min at 37 °C. After three washes, specific primary antibody, diluted in TBST, was incubated for 60 min at 37 °C. The wells were washed with TBST and incubated with recombinant protein G or secondary antibody coupled with alkaline phosphatase diluted in TBST. Following incubation at 37 °C for 60 min, the wells were washed with TBST and TBS and were visualized using freshly prepared chromogenic substrate (1 mg/ml of *p*-nitrophenyl phosphate, 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 50 mM

[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; TBST, TBS containing 0.05% Tween 20; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HBSS, Hank's balance salt solution; BSA, bovine serum albumin; nAchR, nicotinic acetylcholine receptor.