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Journal of Chromatography B, 805 (2004) 361–363
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Affinity adsorbent based on combinatorial phage display peptides that bind α-cobratoxin

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Received 10 January 2003; received in revised form 10 March 2004; accepted 10 March 2004

Available online 10 April 2004

Abstract

Combinatorial phage display was used to discover peptides that selectively bind to the α-cobratoxin (neurotoxin) component of the multi-component venom of the Thai cobra, *Naja kaouthia*. Peptide sequences determined in this way were synthesized chemically and were covalently attached to agarose through the α-amino terminus. Such affinity chromatography supports selectively bound the α-cobratoxin component from crude venom, while passage of the crude venom over the support selectively depleted the venom of this component. The selective binding of α-cobratoxin to peptide-based solid-phase supports suggests that a limitless variety of peptides similarly obtained by combinatorial phage display can be used to craft specific analytical and preparative tools.
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Keywords: α-Cobratoxin; Combinatorial phage display peptides

1. Introduction

Snake venoms contain complex mixtures that consist of several toxic proteins with diverse biological activities. Venom from the Thai cobra *Naja kaouthia* contains paralytic "neurotoxin" activity, acting on nicotinic acetylcholine receptors, cytolytic "cardiotoxin" activity, acting on cell membranes, as well as phospholipase, and D-amino acid oxidase activities. For reviews of *Naja* spp. and their venoms, see [1–4]. The neurotoxin and cardiotoxin activities, respectively, represent families of structurally related peptides. These two classes are configured in a three loop or trefoil conformation maintained by pairs of disulfide bonds [5]. Cooper and Reich [6] separated the neurotoxin activity into four components, α, β, γ, and δ by phosphocellulose chromatography. Of these, α, the first to elute from the column, constitutes the major component that is present in the venom. Further resolution of α-cobratoxin into subfractions by FPLC has been reported by Quimin et al. [7]. Combinatorial phage display has been used in other published studies to identify sequences that bind to a similar neurotoxin (α-bungaratoxin) from the related *Bungarus multicinctus* [8,9]. Phages that bear sequences found at the interacting face of the nicotinic acetylcholine receptor were obtained. In the present studies we describe the use of peptides sequences obtained by phage display in crafting analytical tools based on their ability to bind to the α-cobratoxin.

2. Experimental

2.1. Reagents

Crude *Naja kaouthia* venom (cat. no. V-9125), α-cobratoxin (cat. no. C-6903), protein molecular weight markers (cat. no. M-3913), and *N*-hydroxysuccinimidyl-activated agarose (cat. no. H-8280) were purchased from the Sigma Chemical Company, St. Louis, MO. Microtiter plates, Costar EIA/RIA (cat. no. 3590) were purchased from Corning, Corning, NY.

2.2. Buffers

PBS: 0.02 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.155 M NaCl;

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doi:10.1016/j.jchromb.2004.03.019

PBST: PBS + 0.1% Tween-20;
EB: PBST + 0.1% sodium dodecyl sulfate (SDS), 0.01% bromphenol blue, 0.5% 2-mercaptoethanol, 10% glycerol.

2.3. Bacterial strains, phages, and plasmids

Bacterial strains used were: *Escherichia coli* DH5 α F' and JM109 (Promega. Madison WI). Phage libraries were all based on insertion of a random DNA sequence, as indicated, in a multiple cloning site at the N-terminus of phage M13 protein pIII. Library 1: X6PPX(Y/F)X6, where X6 consists of a series of six random amino acids, PP is ProPro, X is a random amino acid (Y/F) is Tyr or Phe, and X6 is again a series of six random amino acids. Library 2: X8, consists of eight random amino acids. Specific procedures involving phage adsorption, panning, and analysis were used as described by Kay et al. [10]. For a general review of phage display, see Smith and Petrenko [11]. Other methods of molecular biology used in this work were as described by Sambrook et al. [12].

2.4. Phages which bind to α-cobratoxin

α-Cobratoxin was pre-bound to a microtiter plate and used to pan the phage libraries through three cycles of adsorption, elution and amplification. Phages obtained after the third round of amplification were used to infect *E. coli* DH5 α F'. Replicative form DNA was prepared from infected cells and the combinatorial peptide display sequence was amplified by PCR with: 5′-ATT CAC CTC GAA AGC AAG CTG, as forward primer, and 5′- CTC ATA GTT AGC GTA ACG, as reverse primer. PCR conditions were: melting 94 °C for 1 min; annealing 55 °C for 1 min, and extension, 72 °C for 3 min; for 30 cycles, final extension 72 °C for 5 min. The respective replicative forms were used as template for sequence determination of combinatorial inserts by primer extension. The amino acid sequences of displayed peptides were inferred from their respective DNA sequence inserts and were prepared commercially on a 10–50 mg scale by solid-phase synthesis (Research Genetics, Huntsville, AL).

2.5. Fractionation of venom by affinity purification with peptide adsorbent

Chemically synthesized peptides were immobilized through their α–amino groups to *N*-hydroxysuccinimidyl-activated agarose. The crude venom sample (30 μg in 30 μl PBS) was applied to the affinity column (100 μl). The unadsorbed venom fraction was collected by centrifugation. The column was washed with PBST, and bound protein was eluted with EB. Protein samples from the affinity column were fractionated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) along with samples of crude venom and purified α-cobratoxin.

3. Results and discussion

3.1. Combinatorial peptide sequences

Sequences of seven peptides are shown in Table 1 along with their phage isolate index and the phage library (one of two) from which they were derived.

Three common motifs, SWW (or TWW) and VYE were seen. SWW (or TWW) occurs in f02, f10, f13, and e18, while VYE occurs in f05, f17 and e18. It is noteworthy that e18 was derived from an unconstrained library which differed in structure from that of the f-series, nevertheless, had the two consensus motifs SWW and VYE.

3.2. Combinatorial peptides as specific adsorbents

The peptides were chemically synthesized, and bound to NHS activated agarose through their respective N-terminal primary amino groups. Crude venom was applied to the affinity resin and the run-through was collected and fractionated by polyacrylamide gel electrophoresis suggest that peptides f02, f05, f17, and e18 selectively depleted the venom of the α-cobratoxin component.

A detailed analysis was performed with an affinity column based on peptide f14. Crude venom was applied to a peptide f14 affinity column and the run-through was collected. After washing with PBST, bound protein was eluted with EB. Protein samples were fractionated by PAGE and results are shown in Fig. 1a in which the gel was stained with Coomassie Blue (low sensitivity), and in Fig. 1b, in which the same gel was re-stained with silver nitrate (high sensitivity). The band pattern in Fig. 1b, Lane 4, indicates that f14 depleted the venom of both a 6.5 kDa and a 14.2 kDa component; however, only the α-component, together with contaminating faster-moving ca. 6 kDa component were eluted. The 14 kDa component that is present in the venom is apparently also adsorbed, together with the α-cobratoxin, but in contrast to the α-cobratoxin, it is bound much more strongly and is only partially eluted by SDS-containing solution.

Peptides obtained by phage display have yielded useful structural information and specific ligands capable of modulating macromolecular interactions. The application of these peptides to the fabrication of specific chromatographic adsorbents has been reviewed by Lowe [13]. In the present

Table 1
Peptides that bind α-cobratoxin

Deduced peptide	Phage isolate	Phage library
SWWRHAAVYEWD	e18	2
YSGSWWPPTYNNEVPL	f02	1
HTWWYNPPSYMGLEAS	f10	1
GTWTWWPPTYAGMDHL	f13	1
TLWGLFPPVYEDSFGL	f05	1
PWTSWWPPVYEGSTTN	f17	1

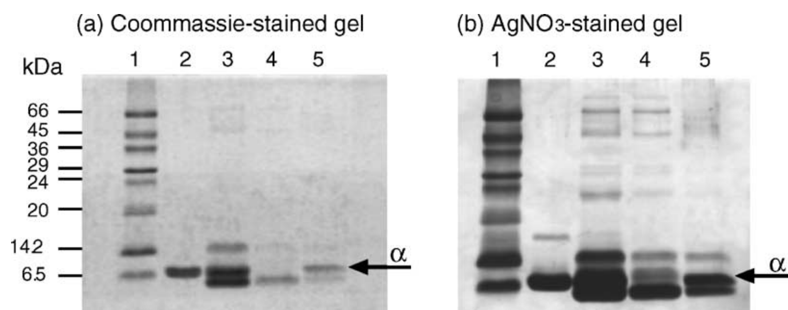

Fig. 1. Fractionation of venom by affinity purification with peptide adsorbant. Peptides were immobilized through their alpha amino groups to *N*-hydroxysuccinimide-activated agarose. Crude venom (30 μg in 30 μl PBST) was applied to the column (100 μl). And the unadsorbed venom protein fraction was collected by centrifugation. The column was washed with PBST, and bound protein was eluted with EB. Protein samples were fractionated by PAGE with 15% SDS-PAGE along with samples of total venom and purified toxin. Panel a—polyacrylamide gel stained with Coomassie (low sensitivity). Lane 1, molecular weight marker; Lane 2, α-cobratoxin; Lane 3, crude venom; Lane 4, peptide adsorbent run-through; Lane 5, eluted sample. Panel b—the same gel stained with silver nitrate (high sensitivity). Lane 1, molecular weight marker; Lane 2, α-cobratoxin; Lane 3, crude venom; Lane 4, peptide adsorbent run-through; Lane 5, eluted sample.

preliminary studies we have shown that phage display peptides can be used to bind selectively to individual components of the venom of the Thai cobra *Naja kaouthia*. By attachment of a phage-derived combinatorial to a solid support, a limitless variety of specific adsorbents can be conveniently crafted, and applied to problems of protein purification.

Of particular interest today are sensors for the detection of toxins. Only a small modification of present techniques, i.e., attachment of the peptide to a firmer support such as activated silica, instead of agarose, would be needed to extend these findings to the construction of peptide affinity-based biosensors. Goldman et al. [14] have constructed peptide affinity-based sensors capable of detecting Staphylococcal enterotoxin B. Phage display technology, thus likewise, makes it potentially possible to construct a limitless variety of toxin biosensors whose application to contemporary problems has been reviewed by Paddle [15].

The binding specificity of peptides obtained in this work by phage display was not absolute and the observed signal to noise ratio may limit their usefulness in some applications. Overcoming this limitation may be achieved by: (1) further evolving the binding peptide by affinity maturation to higher affinity and specificity, (2) use of a related technology, e.g., selection using a single-chain combinatorial antibody fragment (scFv), or (3) tandem use of the described peptide adsorbent with an orthogonally binding adsorbent from a different combinatorial phage display library.

Acknowledgements

We thank Brian Kay for the use of samples of his phage libraries, for helpful advice, and for numerous other kindness in connection with these studies.

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