cDNA cloning, sequence analysis, and recombinant expression of akitonin beta, a C-type lectin-like protein from *Agkistrodon acutus*

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ABSTRACT

AIM: To clone the cDNA of a new member of snake venom C-type lectin-like proteins, to study its structure-function relationships and to achieve its recombinant production. **METHODS:** PCR primers were designed based on the homology and cDNA was amplified by RT-PCR using total RNA from snake venom gland as the template. The PCR products were cloned into the plasmid pGEM-T and sequenced. The deduced protein sequence was analyzed with some bioinformatic programs. A recombinant expression plasmid was constructed using pBAD-TOPO as vector and transformed into *E.coli* TOP10 competent cells. **RESULTS:** A novel cDNA sequence encoding akitonin β was found and accepted by GenBank (accession number AF387100). Akitonin β consists of a typical carbohydrate recognition domain (CRD) of C-type lectins, and it is homologous with other snake venom C-type lectin-like proteins. It was predicted to be a platelet antagonist. Upon induction with arabinose rAkitonin β expressing in *E coli* was achieved at a high level (superior to 150 mg/L). The recombinant fusion protein exhibited inhibitory activities on rat platelet aggregation *in vitro*. **CONCLUSION:** A new member of snake venom C-type lectin-like proteins was discovered and characterized, and an efficient recombinant expression system was established for its production.

INTRODUCTION

Snake venom C-type lectin-like proteins (CTL-like proteins) belong to the C-type lectin superfamily. They differ from some true snake lectins principally in two aspects: first, they are generally heterodimeric, ie, consisting of two chains (designated α and β chains) that are both homologous with the carbohydrate recognition domain (CRD) of C-type lectins, while the true lectins are generally homodimeric[1,2]. Secondly, unlike the true lectins, CTL-like proteins bind to protein ligands rather than saccharides. CTL-like proteins are found in venoms of several snake species of *Viperidae* and *Crotalidae*. They fall into four groups according to their binding characteristics: 1) IX/X-bp[3], X-bp[4] and IX-bp[5], which indirectly inhibit the conversion of prothrombin into thrombin; 2) Inhibitors of thrombin. For example, bothrojaracin inhibits thrombin activity[6], or binds to prothrombin to block its activation[7]. So far this type of CTL-like proteins is only found in *Bothrops*.

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Their entire amino acid sequences are not yet determined; 3) Platelet antagonists. Many CTL-like proteins from various species are in this group[8-10]. Some of them bind to GP Ib and function as receptor blockers of vWF, thereby inhibitig vWF-dependent platelet aggregation; 4) Platelet agonists. For instance, botrocetin binding to vWF changes the latter's conformation, activates its binding to GP Ib, and consequently induce platelet agglutination and aggregation[11].

Snake venom CTL-like proteins impact upon coagulation systems in various ways and exhibit anticoagulant or procoagulant effects, which makes them promising candidates for development of new drugs especially in antiplatelet therapy[12], and also makes them useful in fundamental medicinal research. However, in contrast to the distinct binding characteristics, the structural differences between different CTL-like proteins are relatively minor. Although the crystal structures of some snake venom CTL-like proteins have been resolved[13-17], the structure-function relationships remain to be elucidated in further details. In the present study we have determined the complete amino acid sequence of the β chain of a novel CTL-like protein, investigated its structure-function relationships, and established a highly efficient system for its recombinant production in *E coli.*

MATERIALS AND METHODS

Materials *Agkistrodon acutus* adult snakes were from Hu-nan Province, China. The vector pGEM-T was purchased from Promega. The Concert system for gel extraction of DNA was from Life Technologies. The vector pBAD-TOPO, TOP10 competent cells and the ProBond purification system were from Invitrogen. *Taq* DNA polymerase was from Sino-American Biotechnology Inc.

PCR 1 and cloning of cDNAs of CTL-like proteins Venom gland total RNA was extracted and reverse transcription reaction was run as described by Zha *et al*[18]. The first-strand cDNA was used as template. Sense primer C_1 (5'-GATTGTCCTT CTGATTGGTCCT-3') and anti-sense primer C_2 (5'-TGCCTGGAACTCGCAGAC-5') were designed according to the coding sequence of agkisasin β cDNA[18]. The amplification reaction was catalyzed by *Taq* DNA polymerase and was carried out for 28 cycles under the following conditions: denaturation at 94 ºC for 1 min; annealing at 50 ºC for 1 min; extension at 72 ºC for 1 min. PCR products were cloned with the pGEM-T system. DNA sequencing was conducted by Sangon Company.

Bioinformatic analysis Multiple alignment and dot plot analysis were performed with Omiga 2.0; prediction of secondary structures was done using the PredictProtein program on the website http://cubic.bioc.columbia.edu/predictprotein/. Similarity search and conserved domain search were performed with the Blast program on http://www.ncbi.nlm.nih.gov/BLAST.

Akitonin β expression in *E coli* The akitonin β cDNA cloned in pGEM-T was reamplified by PCR 2 with the recombinant pGEM-T as template, while the other reaction conditions were the same as in PCR 1. The products of PCR 2 were gel-eluted with the Concert system especially for purpose of eliminating the templates, then were cloned into pBAD-TOPO and transformed into *E coli* TOP10 competent cells. Expression of akitonin β cDNA was induced with arabinose at concentrations of 0.0002 %-0.2 %. The fusion protein aggregates were dissolved in 8 mol/L urea solution. The fusion protein was purified with the ProBond purification system and was refolded by dialysis first against urea solutions step by step in decreasing the urea concentrations, then against the renaturation solution [composition in mmol/L: Tris-HCl (pH 7.0) 50; reduced glutathione 1, oxidized glutathione 0.2; arginine 100] , and finally against the stocking solution [10 mmol/L Tris-HCl (pH 8.0), 5 mmol/L $CaCl_2$ and 30 % glycerol].

Inhibitory activity on platelet aggregation Platelet aggregation inhibition activity of the recombinant akitonin β was measured as Xu *et al*[19].

RESULTS

Cloning of akitonin β cDNA Electrophoresis on agarose gel of the PCR products showed a band of nearly 400 bp. Cloning and sequencing of the PCR products revealed a mixture of cDNAs of CTL-like proteins (β chains). Among them a new cDNA sequence was identified and denominated akitonin β. It was accepted by GenBank (accession number AF387100). Its coding sequence and the deduced amino acid sequence are shown in Fig 1.

Akitonin β is homologous with other β chains of snake venom CTL-like proteins. The most similar chain is agkisacutacin β[20] with a sequence identity of 81 %. The sequence identity with akicetin β (79 %) ranks second. NCBI Conserved Domain Search detected in akitonin β the presence of the carbohydrate-recognition domain (CRD) of C-type lectins . Thus akitonin β can be considered as a new member of the CTL-like

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GATTGTCCTTCTGATTGGTCCTCCTATGAAGGGAATTGCTACCTGGTCGTCAAAGAAAAG
 D  C  P  S  D  W  S  S  Y  E  G  N  C  Y  L  V  V  K  E  K
AAGACCTGGGCCGAGGCACAGAAATTCTGCACAGAACAGCGCAAAGAATGCCATCTGGTC
 K  T  W  A  E  A  Q  K  F  C  T  E  Q  R  K  E  C  H  L  V
TCCTTCCACAGCGCTGAAGAAGTAGATTTTGTGGTCTCGAAGACCTTCCCAATTTTAAGT
 S  F  H  S  A  E  E  V  D  F  V  V  S  K  T  F  P  I  L  S
TACGATTTAGTCTGGATTGGACTGAAGAACATCTGGAACGGATGCTACTGGAAGTGGAGC
 Y  D  L  V  W  I  G  L  K  N  I  W  N  G  C  Y  W  K  W  S
GATGGCACCAAGCTCGACTACAAAGACTGGCGTGAACAATTTGAATGTCTCGTATCCAGG
 D  G  T  K  L  D  Y  K  D  W  R  E  Q  F  E  C  L  V  S  R
ACAGTTAATAACGAATGGCTAAGTATGGACTGCGGCACTACTTGCTCTTTCGTCTGCGAG
 T  V  N  N  E  W  L  S  M  D  C  G  T  T  C  S  F  V  C  E
TTCCAGGCA
 F  Q  A
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Fig 1. The cDNA sequence and the deduced amino acid sequence of akitonin β.

protein family. It contains 123 amino acid residues.

According to the prediction with the Protein-Prediction program, it seems not to be globular and is a mixed protein with a relatively high percentage of α helices. No glycosylation site was found. The *N*-terminal sequence is hydrophilic and there are two larger hydrophilic regions, the segment 70-95 and the segment 17-38, as well as a hydrophobic core ranging from 48 to 70 (Fig 2).

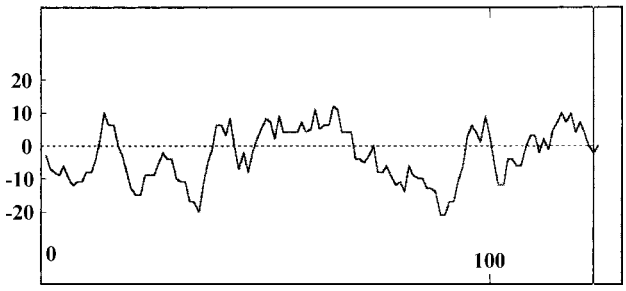

Fig 2 . Hydrophobicity profile of akitonin β.

Recombinant expression in *E coli* Since akitonin β is not a glycoprotein as predicted with Protein-Prediction, a prokaryotic recombinant expression system was chosen to produce akitonin β. *E coli* TOP10 cells harboring the recombinant plasmid pBAD-TOPO/akitonin β produced a fusion protein of about 3000 kD under induction, which was in accordance with the theoretical calculations. The yield of the fusion protein was stable at the different arabinose concentrations, accounting for approximately 40 % of the total cell proteins. The addition of 50 mg/L cysteine in the culture medium did not enhance the production (Fig 3). Different host strains were tested. The maximum yield was obtained with TOP10 as the host, while with other strains such as DH5α the productivity was greatly reduced. The fusion protein accumulated in TOP10 in the form of inclusion bodies, and after purification with the ProBond

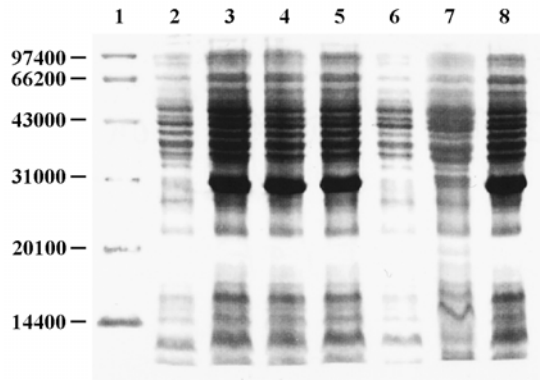

Fig 3. SDS-PAGE pattern of total cell lysate. (1) Molecular weight standard; (2,3,4,5) TOP 10 cells harboring the recombinant plasmid: uninduced, or induced for 4 h with 0.0002 %, 0.002 %, and 0.02 % arabinose respectively. (6,7) Top10 cells without the recombinant plasmid, uninduced and induced with 0.02 % arabinose for 4 h respectively; (8) TOP 10 cells harboring the recombinant plasmid treated with 50 mg/L cysteine, induced with 0.02 % arabinose for 4 h.

system the purity of the fusion protein attained 98 % as determined by RP-HPLC.

Platelet aggregation Akitonin β exhibited inhibitory activities on ADP-induced rat platelet aggregation *in vitro* (Tab 1).

Tab 1. Inhibitory effects of the recombinant protein akitonin β on ADP-induced rat platelet aggregation. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

Concentration of the recombinant protein/mg·L^{-1}	Platelet aggregation/%	Inhibition/%
Control	65.7±1.2	
0.5	63.5±0.5	3.3
1	60.0±2.8^{b}	8.7
2	51.6±0.6^{c}	21.5
5	25.3±1.2^{c}	61.5
10	20.1±1.7^{c}	69.4

DISCUSSION

The multiple alignment of the amino acid sequences of some CTL-like proteins showed that most of the β chains contained 123 amino acid residues (Fig 4), with the exception of botrocetin which has 125. The seven cysteine residues are invariant in all the β chains, indicating their important role in the stabilization of

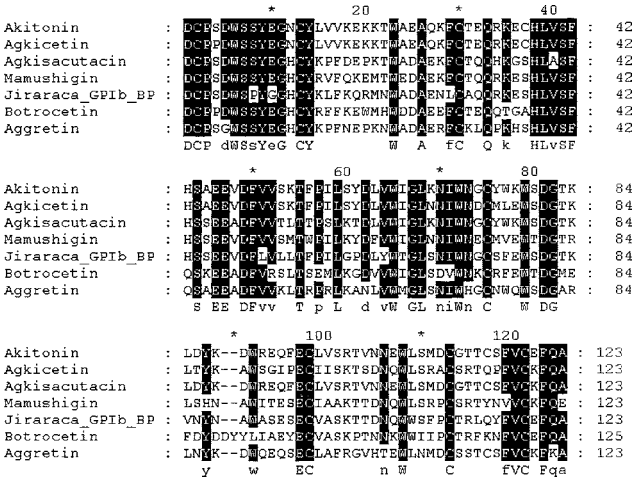

Fig 4. Multiple alignment of the β chains of some C-type lectin-like proteins.

conformation. Among all the proteins, botrocetin is the only one that binds to vWF. In reality, botrocetin has some peculiar amino acid residues such as H22, R52, R76, E84, L90, P101, I108, and I109. These residues, especially the polar ones, may contribute much to the ligand-binding specificity. For example, at the position 84 only botrocetin possesses an acidic residue while all the others have basic ones, K or R. Furthermore, the segment 70-95 generally represents a principal hydrophilic region, and therefore the residues in this region are likely exposed on the surfaces of the molecule. Besides, there exists a small hypervariable segment ranging from 89 to 94, where the two additional residues of botrocetin, D and Y, are just located. As for the β chains of the three GP Ib binding proteins: agkicetin[21], agkisacutacin, and mamushigan[22], their amino acid residues are identical at the above-mentioned positions, and akitonin β is consistent with them. Dot plot analysis (Fig 5) also demonstrated that akitonin β can be viewed as a combination of two segments: the segment 1-61 identical with the counterpart in akicetin β, and the segment 62-123 identical with the counterpart in agkisacutacin β. All the analytical results strongly suggested that akitonin acted as a GP Ib binding protein.

Stitching of different entities into a protein of new function constitutes an evolutionary shortcut[23]. Drikamer[24] supposed that the CRDs evolved from more general binding domains, and relatively minor changes near the sugar-binding site allowed the CRDs to accommodate a variety of ligands, some of which probably had nonsaccharide structures. The snake venom CTL-like proteins are structurally homologous compared with the respective chain of each other, and there exists a certain degree of homology between the α and β subunits as well, which indicates that the snake venom CTL-like proteins should have evolved from a common ancestral protein[25]. Nevertheless, to a great extent the evolutionary divergency and functional coordination of the two subunits remains to be a puzzle. While some dimeric or tetrameric structures facilitate the binding to the receptors as is the case in Alboaggregins A and B[26], the cooperative relationships between the α and β subunits seem more complex in the other heterodimeric proteins. In IX-bp the two subunits are disposed to form a central concave for the binding of IX factor, losing the lectin active site and acquiring a new function[27]. In some GP Ib binding proteins the division of work between the two subunits appears more explicit and imbalanced, as Kawasaki [28] demonstrated that the

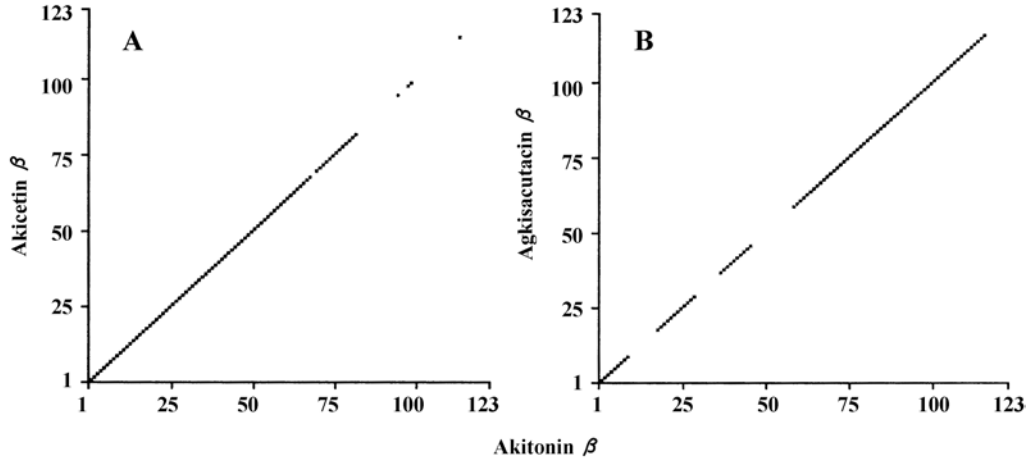

Fig 5. Dot plot analysis of akitonin β with akicetin β and agkisacutacin β. (A) With akicetin β; (B) With agkisacutacin β.

platelet GP Ib-binding site resided on the beta and not on the alpha subunit based on an enzyme-linked immunosorbent assay (ELISA) using biotin-labeled jararaca GP Ib-BP and competing ligands. Another case in point is that reduced echicetin retained its binding activity and its inhibitory effect on the agglutination of fixed platelets[29]. These facts, taken together, imply a less stringent conformation requirement and a subsidiary role of α chains in GP Ib binding proteins. In the present study the functional prediction of akitonin as platelet antagonist was preliminarily testified by the *in vitro* assay of the renatured recombinant fusion protein, which exhibited inhibitory activities on ADP-induced rat platelet aggregation. But its activity of binding to GP Ib should be directly demonstrated in further studies and whether it boasts some unique functional features needs more detailed research.

Sequence analysis revealed some other interesting rules in the primary structures and structure-function relationships. However, the significance of some highly conserved tryptophan residues, is yet neglected and unknown. Besides, the results of dot plot analysis seemingly support Drickamer's hypothesis that the diversity in protein architecture derives from exon shuffling[24]. But in our opinion, the possibility of gene rearrangement at DNA levels in somatic cells should not be excluded unless the genomic structure of CTL-like proteins is totally unveiled.

GP Ib binding proteins only interfere with the binding of vWF to GP Ib, but they do not affect the platelet agglutination and aggregation under normal circumstances. Therefore GP Ib binding proteins have great medicinal potential particularly in the prevention and treatment of arterial thrombosis. Through reverse-genetics techniques we have identified a new member of snake venom CTL-like proteins, predicted its function, and expressed it at high level in prokaryotic system. The maximum expression yield was superior to 150 mg/L and the recovery after the simple one–step purification by the ProBond system was about 75 %-80 %. The recombinant production system was easy and cost-effective. It can also be used for the study of structure-function relationships. For instance, because other cDNAs of snake venom CTL-like proteins can be expressed with satisfactory yields (data not shown), different subunits can be produced separately and then all possible combinations of the recombinant subunits can be tested *in vitro*. This provides an opportunity to determine if different combinations may impart some new functions. Moreover, directed point mutations could be conveniently introduced using the system. In conclusion, this work laid a good foundation for the exploitation and study of snake venom CTL-like proteins.

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