

Expression, purification, and characterization of a novel Ca^{2+} - and phospholipid-binding protein annexin B2

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Abstract Annexin B2 (AnxB2) is a novel member of the annexin family of Ca^{2+} - and phospholipid-binding proteins from *Cysticercus cellulosae*. To obtain highly pure AnxB2 with an easy and inexpensive purification approach, its cDNA was cloned into the prokaryotic expression vector pJLA503 and the translation initiation codon was immediately under the control of the inducible bacteriophage λ promoters P_R and P_L . After induction by shifting temperature, large amounts of non-fusion protein were produced in *Escherichia coli* in a soluble form. Then a novel purification method based on Ca^{2+} -dependent phosphatidylserine (PS)-binding activity was established, whereby the purity of AnxB2 was increased to 98.7%. Western blot analysis showed that recombinant AnxB2 was specifically recognized by serum of pigs infected with cysticercosis. In vitro test showed that, the recombinant AnxB2 had anticoagulant activity and platelet binding activity. The expression, purification, and initial characterization of AnxB2 set an important stage for further characterization of the protein.

Keywords Annexin B2 · Expression · Purification · Anticoagulant activity

Introduction

Annexins are an evolutionary conserved multigene family with Ca^{2+} and phospholipid-binding properties widely distributed in eukaryotes [1]. Each member of this protein family contains a conserved protein core characterized by high alpha-helix content that includes the calcium- and phospholipid-binding sites, and a variable N-terminal domain that is specific in sequence and length for each annexin. Although the structure of the protein core is highly conserved, different annexins exhibit a wide biochemical and functional diversity, e.g., membrane fusion and exocytosis, inflammatory response, ion channel regulation, angiogenesis and inhibition of blood coagulation, etc. [2, 3]. Despite an abundance of experimental evidence suggesting that annexins are associated with a plethora of biological processes, exact physiological functions of annexins remain to be identified.

Our group first isolated and characterized a novel annexin member from *Cysticercus cellulosae* by immunological screening designated as AnxB1 according to the new nomenclature of annexins [3, 4]. Primary investigation showed it can significantly inhibit animal thrombosis in vitro and in vivo [5, 6]. In addition, the platelet-binding specificity of annexin B1 may make it useful to detect and image arterial thrombi. Therefore, annexin B1 may have the clinical potential for the treatment of vascular diseases [7–9]. Recently, we isolated AnxB2, a novel member of the annexin superfamily from the cDNA library of *C. cellulosae* using degenerate PCR based on conserved structure core of annexins. Bioinformatics analysis revealed that it is a novel annexin subfamily member which shares the structural features of the annexin family in common. Due to difficulty in obtaining native AnxB2 protein from mammalian cells, we expressed it with pGEX5T- AnxB2

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as a glutathione *S*-transferase (GST) fusion protein and purified by affinity chromatography. A series of functional tests suggest that GST-AnxB2 possessed the common property of annexin family which binds acidic phospholipid in the presence of calcium [10].

However, the GST fusion system has several disadvantages in the production and application such as increased immunogenicity and thrombin contamination, which will be introduced when the GST tag is cleaved off. Thus, the GST system is not suitable for the large-scale protein production. For these reasons, we constructed a non-fusion-expression system producing soluble AnxB2 in *Escherichia coli* and investigated both the corresponding purification method and characterization of AnxB2.

Materials and methods

Chemicals, strains, and plasmids

All chemicals used for bacterial culture and protein purification were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified. The phospholipids 1, 2-dioleoyl-*SN*-glycero-3-phosphocholine (phosphatidylcholine, PC) and 1, 2-dioleoyl-*SN*-glycero-3-[phospho-l-serine] (phosphatidylserine, PS) were obtained from Avanti Polarlipids (Germany). Chromatographic resins and DEAE-Sepharose fast flow were obtained from Amersham Biosciences (Sweden). The mobile phase was prepared with Milli-Q water (17.6 M Ω). The filter system was from Millipore (Bedford, MA, USA).

Escherichia coli DH5 α [*supE44* Δ *lac* U169 (ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] and BL21(DE3) [F^- *lon* *ompT* r_B^- m_B^-] were used as the cloning and expression host cells, respectively. Plasmid pUC18-AnxB2, which contained the full cDNA sequence of AnxB2, was previously constructed and preserved in our laboratory. The plasmid pJLA503 was preserved in our laboratory as the expression vector for non-fusion expression of AnxB2 gene.

Plasmid construction

Plasmid DNA isolation and transformation of CaCl₂-treated *E. coli* cells were carried out by the procedure described before [11]. Restriction endonucleases and T4 DNA ligase were used according to the directions as recommended by the manufacturer (Promega). The AnxB2 full-length cDNA was obtained by PCR amplification on the plasmid pUC18-AnxB2 with primer 1 (forward): 5'-CCAT ATGGCAA AAAATACTCGCTCAC-3' (the *Nde*I site was underlined and the additional ATG for initiation of translation in italicized style) and primer 2 (reverse): 5'-GCGGATC CTAT

TAGGATTCATTCAGTAGTGCGAGC-3' (the *Bam*HI site was underlined and the complementary sequences of stop codons TAA and TAG were in italicized style). The resulting PCR product was gel purified and cloned into pJLA503 using *Nde*I and *Bam*HI restriction sites. The recombinant plasmid pJLA503-AnxB2 was transformed into *E. coli* DH5 α . and the transformants were confirmed by DNA sequencing (TakaRa).

AnxB2 expression and purification

The pJLA503-AnxB2 plasmid was transformed into *E. coli* BL21 (DE3) cells. Transformants were grown in 2 \times YTA medium (tryptone 16 g/l, yeast extract 10 g/l, NaCl 5 g/l, and ampicillin 100 μ g/ml). The high expression clone was obtained according to the results of SDS-PAGE analysis and cultured in 2 \times YTA medium overnight at 28°C for small-scale culture. The overnight culture (100 ml) was then inoculated into 900 ml of the fresh medium described above at 28°C until the optical density (A₆₀₀) reached 0.6–0.8, and protein expression was induced by shifting the temperature to 40°C. After induction for 5 h, the cells were separated from the culture medium by centrifugation at 8,000g for 15 min at 4°C, and then the harvested cellular pellet was resuspended in ice-cold calcium buffer (50 mM Tris-HCl, pH 7.2, and 10 mM CaCl₂). The suspension was sonicated on ice for 1 min \times 5 to disintegrate the cells and centrifuged at 10,000g for 20 min at 4°C. The supernatant was discarded, and the AnxB2 bound to bacterial membranes was released by resuspending the pellet in EDTA buffer (50 mM Tris-HCl, pH 7.2, and 20 mM EDTA) [12]. Shaken gently for 30 min, the cell debris were removed by centrifugation, and the supernatant containing the soluble recombinant AnxB2 was stored at 4°C for further purification.

The supernatant containing AnxB2 was dialyzed against buffer A (20 mM Tris-HCl, pH 8.0, and 20 mM NaCl) overnight at 4°C, and then applied to a DEAE-Sepharose fast flow column (25 ml bed volume) that was previously equilibrated with buffer A and eluted with a linear gradient of 0–1 M NaCl in buffer A. The void volume and eluted fractions from the chromatographic column were monitored by absorbance at 280 nm. Fractions were collected and subjected to SDS-PAGE analysis. For biological assay, The eluted fractions containing AnxB2 were pooled and dialyzed against buffer B (20 mM Hepes, pH 7.4, 100 mM NaCl), and stored at –20°C.

SDS-PAGE, immunoblotting, and protein determination

AnxB2 was analyzed by SDS-PAGE under reducing conditions using 12% gels and the Tris-glycine system of

Laemmli [13]. The separated proteins were stained with Coomassie brilliant blue R-250 or electroblotted on nitrocellulose for Western blotting analysis [14]. After being blocked with 5% non-fat milk in 0.05% Tween/PBS (PBS-T), the membrane was washed three times with PBS-T for 10 min and incubated further with a 1:100 dilution of serum from pigs infected with cysticercosis for 1 h at 37°C. The membrane was washed and incubated with a 1:1,000 dilution of HRP-conjugated goat anti-pig IgG (sigma) as second antibody, and revealed with DAB substrate (Amersham). Protein concentration was determined by modified Bradford method [15].

HPLC analysis

The homogeneity of the final purified protein was assayed by HPLC performed on a 300 × 7.8 mm Protein-Pak 125 column. The column was eluted with sodium phosphate (pH 7.4) for 30 min at a flow rate of 0.5 ml/min. Detection was measured at 280 nm.

Determination of N-terminal amino acids sequence

The purified AnxB2 was subjected to SDS-PAGE under reducing conditions and electrotransferred to polyvinylidene difluoride (PVDF) membranes. After the membrane was briefly stained by Coomassie Brilliant Blue, the band of protein was cut out and subjected to the N-terminal amino acid sequence analysis by Edman degradation method, using an Applied Biosystem 492 Precise Microprotein Sequencer.

Prothrombin time and activated partial thromboplastin time of AnxB2

Anticoagulant activity of the purified non-fusion AnxB2 was measured using the prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays. Fresh blood was gathered in polypropylene tubes contained sodium citrate. Blood coagulation was initiated by simultaneous addition of calcium and 50 pM tissue factor with GST-fusion AnxB2, GST-fusion AnxB1 and bovine serum albumin (BSA) as control. The fibrin formation was detected by Thrombolyzer RackRotor (Behnk Elektronik, Germany).

The platelet binding activity of AnxB2

Gel-filtered human platelet rich plasma (PRP) was prepared as previously described [16]. AnxB2, AnxB1 and urokinase were diluted, respectively with PBS to six different concentrations (0, 100, 200, 300, 400 and 500 nM). Flat-bottom 24-well plates were coated with 200 µl

samples at 4°C 12 h, followed by washing three times with PBS-Tween 20 buffer (140 mM NaCl, 10 mM Na₂HPO₄ · 12H₂O, 1.5 mM KH₂PO₄, 0.1% Tween 20, pH 7.4). Then the gel-filtered platelets in 2.5 mM CaCl₂ solution were added at final concentration of 2.3×10^5 platelets per well. Platelet were activated with adenosine diphosphate (ADP, 5 mM) at 37°C for 60 min, followed by washing three times with PBS-Tween 20. The bound platelets were counted under light microscope. Nonspecific binding was measured in presence of 5 mM EDTA.

Statistical assay

Data were expressed as the mean ± SD ($\bar{X} \pm S$). Statistical analysis was done using Student's paired *t*-test and SNK *q*-test. *P* < 0.05 indicated a significant difference.

Results

Non-fusion expression AnxB2 in *E. coli*

The entire protein-coding region of AnxB2 was cloned into pJLA503 to create pJLA503-AnxB2 using *Nde*I and *Bam*HI restriction sites (Fig. 1). The ATG translation initiation codon is located in the *Nde*I site and immediately downstream of the strong λ promoters *P_R* and *P_L*, which was regulated by a temperature-sensitive repressor *cIts857* and induced by a temperature shift from 28 to 42°C. *E. coli* strain BL21 (DE3) was selected as the host strain, which was deficient in the *Ion* protease and lacked the *ompT* outer membrane protease that degraded proteins during the

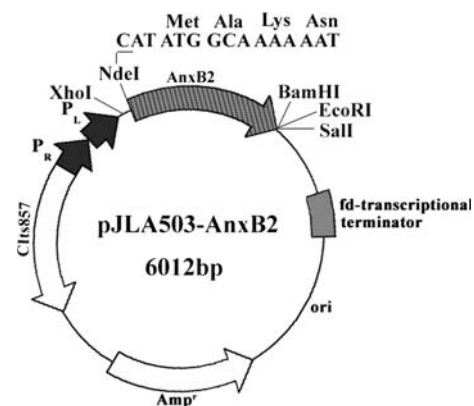


Fig. 1 Schematic representation of pJLA503-AnxB2 expression plasmid. The arrow with lines shows AnxB2 cDNA insert cloned in the *Nde*I and *Bam*HI sites in the pJLA503 expression vector. The black arrow shows the position of the λ promoters *P_R* and *P_L*. The higher part of the figure shows the nucleotide and corresponding amino acid sequences of the open reading frame

process of purification, thus proteins were more stable in the host. The resultant translation product was a full-length wild-type AnxB2.

To obtain enough soluble AnxB2, protein expression was performed at 40°C, instead of 42–45°C to minimize induction of heat shock protein and inactivate the repressor cIts857 for transgene expression. Another reason is that lower temperature induction may reduce thermal denaturation of the expressed protein and subsequent formation of inclusion bodies.

Production levels were analyzed by SDS-PAGE. It was observed that there existed a very intensely staining protein band with an apparent molecular mass of 38.6 kDa (Fig. 2a, lane 2), accounting for about 39% of the total cellular protein as indicated by scanning the protein density of the SDS-PAGE gel. This protein species was absent in control cells harboring the pJLA503 plasmid (Fig. 2a, lane 1). Its identity as AnxB2 protein was confirmed by immunodetection using a polyclonal antiserum against cysticercus (Fig. 2b, lane 2).

Purification of recombinant AnxB2

Based on Ca²⁺-dependent phosphatidylserine (PS)-binding activity, the reversible calcium-mediated binding ability of AnxB2 to PS was utilized for purification in our study. During the procedure of purification, 10 mM calcium and 20 mM EDTA were regarded as the optimal condition. A single-step ion-exchange chromatography with DEAE-Sepharose was performed for further purification. AnxB2 was eluted at about 250 mM NaCl. Fractions corresponding to the eluted peak were analyzed by SDS-PAGE gel. The single protein band exhibiting a molecular mass of 38.6 kDa was present (Fig. 2a, lane 3), which stained intensively with antiserum against cysticercus (Fig. 2b, lane 3). The purity of AnxB2 was also tested by HPLC showing >98% purity as estimated from the percentage of total peak area (Fig. 2c).

The purification of AnxB2 based on 1 l of the culture was summarized in Table 1. From approximately 339.7 mg of AnxB2 present in the initial 868.8 mg of total protein from a crude bacterial lysate, we finally isolated 39.2 mg of pure AnxB2 accounting for 11.5% of the yield.

N-terminal amino acids sequence

To identify the recombinant protein further, The N-terminal 13 amino acids residues sequence of the purified AnxB2 was determined as Met-Ala-Lys-Asn-Thr-Arg-Ser-Pro-Ser-Gln-Tyr-Phe-Asp in accord with the translated sequence from AnxB2 cDNA (GenBank accession number: AY998562).

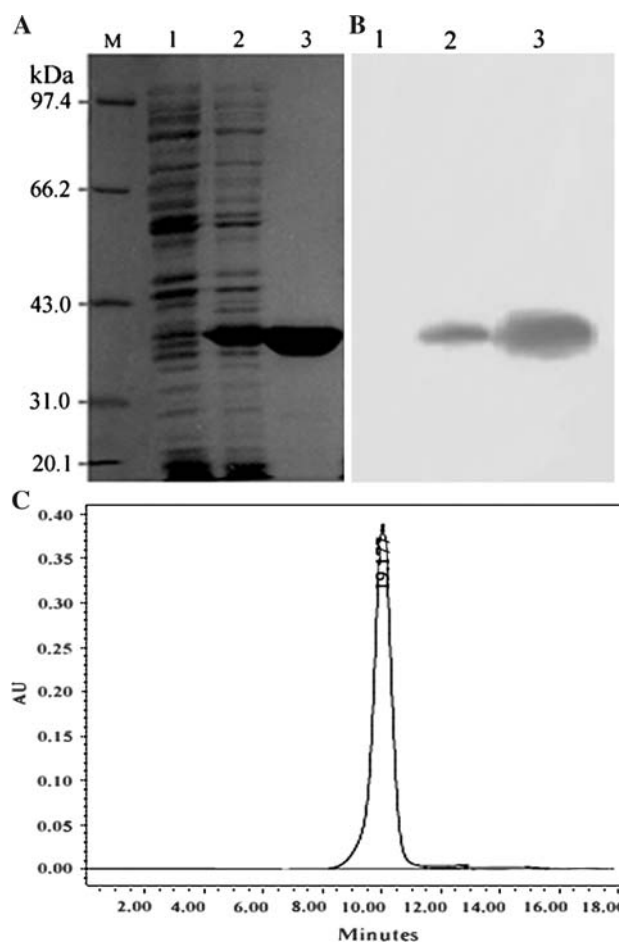


Fig. 2 a SDS-PAGE analysis of bacterial lysate and purified AnxB2. b Immunoblot analysis of AnxB2 using a polyclonal antiserum followed by HRP-conjugated secondary antibody. c HPLC analysis of recombinant AnxB2 on a gel filtration column. lane M, molecular weight markers; lane 1, total protein of *E. coli* before induction expression; lane 2, total protein of *E. coli* after induction by a temperature shift from 28 to 42°C; and lane 3, purified recombinant AnxB2

Anticoagulant activity of recombinant AnxB2

Anticoagulant activity of the purified AnxB2 was measured by PT and aPTT assays (Fig. 3). PT time was significantly prolonged by non-fusion AnxB2 at the dose of 40 µg/ml and higher ($P < 0.05$ vs. BSA control), similar to that of GST-fusion AnxB2 at the same dose. The clotting time was prolonged approximately 48 s at 120 µg/ml non-fusion AnxB2, but AnxB1 had not this activity. In comparison with AnxB1 and BSA controls, non-fusion AnxB2 and GST-fusion AnxB2 had not effect in anticoagulant activity in aPTT clotting assays. Therefore, there was some difference between the inhibition of coagulation pathways of AnxB1 and AnxB2 by PT and aPTT assays. The anticoagulant activities of non-fusion AnxB2 and GST-fusion AnxB2 are also compared in Fig. 3. There was some

Table 1 Purification summary

Purification	Total protein (mg) ^a	AnxB2 (mg) ^b	Fraction AnxB2/total protein	Yield (%)
Crude extract	868.6	339.7	0.391	100
Soluble extract	571.6	272.1	0.476	80.1
Column load sample	149.2	121.6	0.815	35.8
DEAE-Sepharose FF	39.7	39.2	0.987	11.5

^a Total protein concentration was determined by Bradford method

^b Recombinant AnxB2 concentration, in extracts and partially purified samples, was estimated by densitometric analyzes after SDS-PAGE

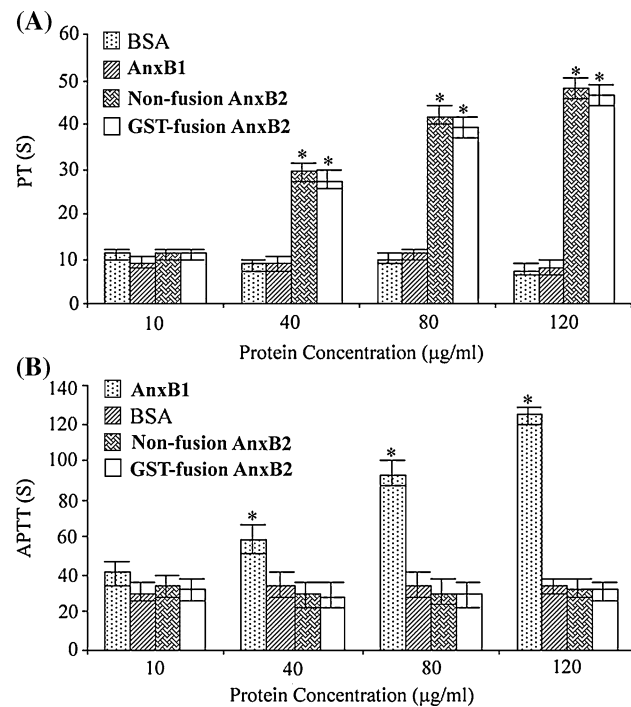


Fig. 3 Anticoagulant activity of recombinant non-fusion AnxB2 was assayed by PT and aPTT, BSA, AnxB1 and GST-fusion AnxB2 as control. Protein concentration of AnxB2, AnxB1 and BSA were 10, 40, 80, and 120 µg/ml. PT and aPTT were performed as described in materials and methods. Error bars indicate the standard deviation of three separate experiments. * $P < 0.05$ versus BSA

difference between the relative activity of non-fusion AnxB2 and GST-fusion AnxB2 in the figure, which suggested that the presence of the GST carrier had a little effect on the function.

The platelet binding activity of AnxB2

Platelet binding activity of the purified AnxB2 was measured by platelet adhesion assays (Fig. 4). Both non-fusion AnxB2 and GST-fusion AnxB2 displayed calcium dependent affinity for ADP-activated platelets. The platelet binding activity of AnxB2 was more than that of AnxB1 at the dose of 100 nM protein concentration. The results

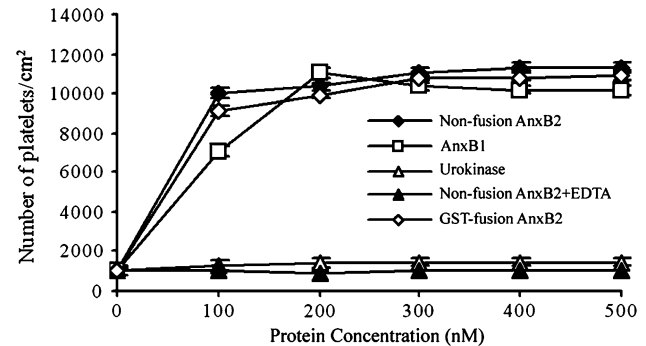


Fig. 4 Platelet binding activity of recombinant non-fusion AnxB2 was measured by platelet adhesion assay, BSA, AnxB1, GST-fusion AnxB2 and non-fusion AnxB2 (in present 5 mM EDTA) as control. The assayed was performed as described in materials and methods. Y-axis showed the number of platelets which were bonded by the proteins. Error bars indicate the standard deviation of three separate experiments

suggested that maybe the inhibitory effects of the AnxB2 are more potent than those of AnxB1. However, non-fusion AnxB2 was unable to bind inactivated platelets (in present 5 mM EDTA), because the calcium ions have been chelated by EDTA.

Discussion

AnxB2 is a novel member of annexin family and has common structural features of the family. AnxB2 contains four typical annexin repeats, each repeat with five-alpha-helices (A–E) and one type-II Ca^{2+} binding site [10]. Commonly, the anticoagulant properties of annexins are associated with its calcium-dependent phospholipid-binding activity [17, 18]. In the clotting cascade reaction, activated platelets provided a surface upon which the coagulation factors gather to promote coagulation (e.g., activation of factor X by VIIIa and IXa). During the procedure of reaction, the precise components of these surfaces were not well known, but anionic phospholipids constituted a major component of binding sites for these cofactors and proteases. Usually, anionic phospholipids

(e.g., phosphatidylserine) are present only in the inner leaflet of the lipid bilayer membrane. Phospholipids move from the inner to the outer leaflet of the lipid bilayer when platelets were activated by some agonists. Thus, the anionic phospholipids may play a key role in the blood coagulation [19, 20]. Previous studies reveal that AnxB2 has anticoagulant activity and platelet binding activity. Therefore, AnxB2 possesses anticoagulant activity through binding anionic phospholipids and preventing prothrombinase complex formation on phospholipid surfaces. Commonly, the intrinsic pathway is defined as a cascade that utilizes only factors that are soluble in the plasma, whereas the extrinsic pathway consists of some factors that are insoluble in the plasma, e.g., membrane-bound factors (factor VII). But biologic activity assay results suggested that maybe AnxB2 inhibited the extrinsic coagulation pathway but did not possess the endogenous anticoagulant activity which is opposite from AnxB1. Maybe AnxB2 is a specific antagonist of some coagulation factors [10]. We are further investigating the interaction of AnxB2 and special coagulation factors.

Recent studies revealed initial characterization of AnxB2, but we still do not know what the real role of AnxB2 is in *C. cellulosa* life cycle. To investigate the biochemical and biophysical properties of AnxB2 further, we reported here a non-fusion expression system in *E. coli* and a rapid purification method to obtain AnxB2 with high purity and bioactivity. This system provides a convenient source of recombinant AnxB2 for structure, function, and pharmacological studies.

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