

Production of biologically active recombinant annexin B1 with enhanced stability via a tagging system

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Received: 2 March 2009 / Revised: 8 June 2009 / Accepted: 8 June 2009 / Published online: 24 July 2009
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Abstract Annexin B1 is a novel Ca^{2+} -dependent phospholipid-binding protein from metacestodes of *Taenia solium* and has been shown to have many potential biomedical applications. Although annexin B1 has been produced successfully in *Escherichia coli*, the purified protein has poor stability at room temperature, which has hindered our attempts to further study its structure–function relationship. To increase the stability of the protein, the construction and purification procedures were examined and changed to hopefully increase its effectiveness. In this study, we describe a new recombinant annexin B1 expressed with a hexahistidine tag fused to its N-terminal end, which was purified to homogeneity in two steps using immobilized metal affinity followed by size exclusion chromatography. The final yield was approximately 23 mg/L of bacterial culture. Isoelectric focusing and mass spectrometry analysis showed that the protein purified by this method was quite stable at room temperature, even greater than 3 days later. A series of functional tests indicated that the recombinant protein had high anticoagulant activity, and fluorescence-labeled annexin B1 could bind to the outer membranes of apoptotic mammalian cells and efficiently detect them in the early stages of apoptosis.

Keywords Annexin B1 · Hexahistidine tag · Protein stability · Coagulation time · Apoptosis

Introduction

Annexins, which are distributed throughout the animal and plant kingdoms, are encoded by a well-known multigene family for Ca^{2+} -regulated phospholipid-binding proteins. Annexins interact with many targets and exert various biological functions, including regulation of membrane aggregation and trafficking (Gerke et al. 2005). They also have extracellular functions, for example, in anti-inflammation and anticoagulation (Gerke and Moss 2002). Despite abundant experimental evidence suggesting that annexins are associated with many biological processes, the exact physiological functions of annexins remain to be identified.

A novel member of the annexin family, annexin B1 (GenBank accession no. AF147955) was recently cloned from the *Taenia solium* metacestode cDNA expression library (Yan et al. 2002). Until now, only a few annexins in parasites have been isolated and studied. Interestingly, we found that annexin B1 is a protective antigen for vaccine development against cysticercosis (Guo et al. 2004). It also has anticoagulant activity and significant therapeutic potential due to its thrombus-targeting and antithrombotic properties (Zhang et al. 2002; Ding et al. 2006). To explore the biological activity of annexin B1, we initially expressed it in the pGEX5T expression vector, forming a glutathione S-transferase (GST) fusion protein (Zhang et al. 2002). However, the GST fusion system has several disadvantages. Since the GST carrier is as large as 26 kDa, it may interfere with the physiological function and activity of proteins. If the GST were cleaved by thrombin, a new protein

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contamination would be introduced. Other problems may also be encountered during the cleavage step, including low yield, tedious optimization of cleavage conditions, or failure to recover active or structurally intact protein (Baneyx 1999). In light of this, we subsequently produced annexin B1 using a nonfusion expression system (Zhang et al. 2003). Although recombinant nonfusion annexin B1 maintained biological activity after tedious purification procedures, it seemed that the purified protein had poor stability at room temperature (25°C) or at 4°C. After 24-h storage at room temperature, protein samples were subjected to polyacrylamide gel electrophoresis and isoelectric focusing analysis. The former displayed a single protein band while the latter showed many degradation bands. Such instability may have originated from copurified protease contaminants inducing degradation, as evidenced by N-terminal sequencing. Even though various protease inhibitors were added to prevent proteolysis during purification, the addition of these inhibitors did not completely prevent protein breakdown. In fact, recent attempts at crystallizing annexin B1 have been impaired by the instability of this protein in solution, which is one of the most important criteria for successful crystallization. Purification and storage of the protein at low temperatures (4°C or below) were also used to slow degradation, but attempts to crystallize annexin B1 at low temperature have not yet been successful. Therefore, a new protein expression–purification system for annexin B1 must be developed to increase protein stability for further characterization.

One of the simple and efficient methods for enhancing stability of recombinant proteins appears to use fusion expression partners. The affinity tags can be advantageous in terms of increased expression, enhanced solubility, protection from proteolysis, improved folding, and simplified purification procedure of their fusion partners (Baneyx and Mujacic 2004; Rozkov and Enfors 2004; Esposito and Chatterjee 2006). Unfortunately, crystal growth is hindered by the conformational heterogeneity induced by large fusion tags, such as maltose-binding protein or GST, requiring the tag to be removed with a potentially problematic cleavage step mentioned above. Accordingly, small affinity tags, such as the histidine tag (His-tag), are the tags of choice in structural biology as they do not increase the molecular size of the protein substantially, and cleavage of small tags is often not required to grow suitable crystals (Bucher et al. 2002).

Based on these considerations, a new recombinant annexin B1 with high stability and bioactivity was produced in this study. DNA sequence encoding a hexahistidine tag (6×His-tag) was fused next to the translation initiation codon of the annexin B1 gene. Then, the recombinant protein was expressed at high levels in a soluble form in *Escherichia coli* and purified via immobilized metal affinity chromatography under native conditions. Finally, we demonstrated that the

His-tagged annexin B1 was more stable than nonfusion annexin B1 at room temperature. The new recombinant protein could prolong coagulation time and detect apoptotic cells efficiently, thus showing high biological activity.

Materials and methods

Chemicals, strains, and plasmids

All chemicals used for bacterial culture and protein purification were purchased from Sigma (St. Louis, MO, USA). Chromatographic resins, Ni Sepharose Fast Flow, Sephadex G-200, and Sephadex G-25 were obtained from GE Healthcare (Piscataway, NJ, USA). The mobile phases were prepared with Milli-Q water (17.6 MΩ). The filter system was from Millipore (Billerica, MA, USA).

E. coli DH5α (ATCC 53868) and BL21 (DE3; Novagen, Madison, WI, USA) were used as the cloning and expression host cells, respectively. The plasmid pET-28a (Novagen) was preserved in our laboratory as the expression vector and was used as the vector for DNA sequencing.

Plasmid construction

Plasmid DNA isolation and transformation of CaCl₂-treated *E. coli* cells were carried out by the procedure described before (Sambrook and Russell 2001). Restriction endonucleases and T4 DNA ligase were used according to the directions as recommended by the manufacturer (Promega, Madison, WI, USA). The full-length annexin B1 cDNA was originally cloned into the pUC18 vector (Sun et al. 1997). The resultant construct, pUC18-annexin B1, was used as a template for polymerase chain reaction. The oligonucleotide primers designed to amplify annexin B1 gene were primer 1 (forward): 5'-CATGCC***ATGGGCCATCATCATCATCACGCCTACTGTCGCTCCCTGGTTCATCTATAT***-3' (the *Nco*I site and the sequence for the 6×His-tag are italicized and the additional ATG for the initiation of translation is in bold italics) and primer 2 (reverse): 5'-GCGTCG***ACTATTATGCAGGGCCGATGAGTTTCAAG***-3' (the *Sal*I site is italicized and the complementary sequences of stop codons TAA and TAG are in bold italics). The recombinant plasmid pET28a-His-annexin B1, was constructed by inserting the amplified fragment into the *Nco*I and *Sal*I sites of pET28a and transformed into *E. coli* DH5α. The correct vector sequence was confirmed by DNA sequencing.

Expression and purification of His-tagged annexin B1

The pET28a-His-annexin B1 plasmid was transformed into *E. coli* BL21 (DE3) cells. Transformants were grown in 2×

YT–kanamycin medium (tryptone, 16 g/L; yeast extract, 10 g/L; NaCl, 5 g/L; and kanamycin, 50 µg/mL, pH 7.0) overnight at 37°C for small-scale culture. The overnight culture (100 mL) was then diluted into 900 mL of the fresh medium described above at 37°C until the optical density (A_{600}) of 0.6–0.8 was reached, at which time isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 500 µM to induce protein expression. Upon induction by different induction times (from 2 to 5 h), the production levels were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The cells were separated from the culture medium after induction by centrifugation at 8,000×g for 15 min at 4°C. The pellet may be frozen at this step and the protocol continued at a later time.

The harvested cell pellet was resuspended in buffer A (20 mM Tris–HCl, pH 8.0, 100 mM NaCl) supplemented with phenylmethyl sulfonyl fluoride (PMSF; to 1 mM), benzamide (to 0.5 mM), and aprotinin (to 10 µg/mL). The suspension was sonicated on ice four times for 1 min to disintegrate the cells and then centrifuged at 8,000×g at 4°C for 15 min. The supernatant was dialyzed against buffer B (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole) and applied to a Ni Sepharose Fast Flow column previously equilibrated with buffer B at a flow rate of 2 mL/min. The column was washed with five column volumes of buffer B and then eluted with a discontinuous gradient of imidazole (10, 20, 40, 60, 150, and 250 mM) in buffer B. The eluted fractions containing annexin B1 were pooled, dialyzed against buffer C (20 mM Tris–HCl, pH 8.0), and concentrated. This concentrated solution was then further purified by loading onto a Sephadex G-200 size exclusion column with buffer C. The void volume and eluted fractions from the two chromatographic columns were monitored at an absorbance of 280 nm. Fractions were collected and subjected to SDS–PAGE analysis.

SDS–PAGE, immunoblotting, and protein determination

Proteins were analyzed by SDS–PAGE under reducing conditions using 12% gels and the Tris–glycine system. The separated proteins were stained with Coomassie brilliant blue G-250 or electrotransferred onto nitrocellulose filters for Western blotting (Towbin et al. 1979). After blocking with 5% nonfat milk in 0.05% Tween–phosphate buffered saline (PBS–T), membranes were washed three times with PBS–T for 10 min and incubated further for 2 h with a 1:1,000 dilution of mouse anti-His-Tag monoclonal antibody (Novagen) or with a 1:100 dilution of serum from pigs infected with cysticercosis, followed by washes as described above. The membranes were incubated with a 1:1,000 dilution of horseradish peroxidase (HRP)–conjugated goat antimouse IgG (Sigma) or HRP–conjugated goat

antipig IgG (Sigma) as the secondary antibody, washed as described above, and revealed with 3,3'-diaminobenzidine substrate (Tiangen, Beijing, China). Protein concentration was determined using a modified Bradford method (Löffler and Kunze 1989).

Stability test by isoelectric focusing and mass spectrometry analysis

The stability of His-tagged and nonfusion annexin B1 was investigated by isoelectric focusing analysis as described before (Giulian et al. 1984). Protein samples of 2 mg/mL in 20 mM Tris–HCl (pH 8.0) were stored at room temperature (25°C) for different time periods (24, 48, and 72 h) and then subjected to isoelectric focusing analysis. The isoelectric point was determined in 5% polyacrylamide gel containing 2% ampholine with a pH gradient from 4.6 to 9.6.

Mass spectra of the samples were determined with a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI–TOF MS; Bruker Daltonics, Bremen, Germany). A sample solution (1 µL) at a final concentration of 2–3 pmol/µL was thoroughly mixed with a saturated matrix solution, α -cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 0.1% trifluoroacetic acid (1:4, v/v) before crystallization. Mass spectra were obtained in the reflex and positive ion mode. MALDI–TOF mass spectra were acquired with an average of 300 laser shots.

Anticoagulant bioactivity assay

Prolongation of the clotting time activity of recombinant His-tag annexin B1 was assayed using modified kaolin partial thromboplastin time (KPTT) as previously described (Romisch et al. 1990). PBS, albumin, and nonfusion annexin B1 were used as controls. A 100-µL sample of standard human plasma was mixed with 10 µL protein sample and 100 µL kaolin active thrombofax. After incubation for 2 min at 37°C, 100 µL of 25 mM CaCl₂ was added. Fibrin formation was detected using a Thrombolyzer RackRotor (Behnk Elektronik, Germany).

Labeling recombinant annexin B1 with fluorescein isothiocyanate and detection of apoptotic cells

Fluorescein isothiocyanate (FITC) labeling was achieved by dialyzing purified recombinant annexin B1 against 25 mM sodium bicarbonate buffer (pH 9.0). Dialyzed annexin B1 (50 µM) was mixed with 50 µM FITC (Sigma) and incubated in sodium bicarbonate buffer for 24 h at 4°C. The labeling mixture was dialyzed against Tris buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid) and subsequently applied to a G-25 column

(GE Healthcare) to separate labeled protein from free FITC. The final FITC-labeled annexin B1 concentration was adjusted to 10 µg/mL and stored at 4°C.

For the assessment of FITC–annexin B1 binding to apoptotic cells, two apoptotic cell models were set up. Freshly prepared thymocytes from Balb/c mice were used for studies of cortisone-induced apoptosis (10^{-5} mol/L dexamethasone, Sigma). Topoisomerase inhibitor-induced apoptosis (10 µg/mL camptothecin; Sigma) in the Jurkat T lymphocytic cell line (ATCC TIB 152) was also investigated. All cell suspensions were cultured to a density of 1×10^6 cells per milliliter in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA, USA), 2 mM glutamine, 100 µg/mL streptomycin, and 100 IU/mL penicillin.

Double staining for FITC–annexin B1 or FITC–annexin A5 (Clontech, Mountain View, CA, USA) binding was performed as follows: after two washes in cold PBS, 1×10^6 cells were resuspended in binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl_2). Then, 5–20 µL FITC–annexin B1 (10 µg/mL) or 10 µL FITC–annexin A5 (10 µg/mL) were added to 100 µL of the solution (1×10^6 cells). To distinguish cells that had lost membrane integrity, 5 µL propidium iodide (PI; 50 µg/mL; Sigma) was added before analysis.

The mixture was incubated for 10 min in the dark at room temperature and then subjected to quantitative analysis by flow cytometry (BD FACS Calibur, San Jose, CA, USA) or qualitative evaluation by laser scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany).

Results

Construction, design, and expression of recombinant annexin B1

The sequence for the 6×His-tag fused to the entire coding region of annexin B1 was ligated into pET28a to create the expression plasmid (Fig. 1a). The translation initiation codon is located in the *NcoI* site and immediately downstream of a strong T7 promoter, which was induced by IPTG. The *SalI* site is located downstream of the translational stop codon and immediately before the T7 terminator. Therefore, the resultant translation product is a full-length annexin B1 with a His-tag linked to the N terminus of the protein. The plasmid pET28a-His-annexin B1 was confirmed by DNA sequencing and then transformed into *E. coli* BL21 (DE3).

Upon induction by IPTG, the protein expression increased gradually with the extension of induction time (Fig. 1b, lanes 3–6). After comprehensive analyses of time

and production factors, a 4-h induction was determined to be optimal. A very intense protein band with an apparent molecular mass of 39 kDa (Fig. 1b, lane 5) was observed in the pET28a-His-annexin B1-transformed bacteria. This protein accounted for approximately 37% of the total cellular protein as indicated by scanning the protein density of the gel. This protein species was absent in control cells, which were collected before IPTG induction (Fig. 1b, lane 2). Its identity as annexin B1 was confirmed by immunodetection using an anti-His-tag monoclonal antibody (Fig. 1c, lane 2) and serum from pigs infected with cysticercosis (Fig. 1c, lane 3). Most annexin B1 protein accumulated in the bacterial cytoplasm as demonstrated by SDS-PAGE analysis of *E. coli* pellet (Fig. 1d, lane 2) and soluble fractions (Fig. 1d, lane 3) after sonication. The soluble annexin B1 constituted over 47% of the total soluble proteins expressed in *E. coli* and was used for further purification.

Purification of recombinant annexin B1 to homogeneity

Purification of recombinant annexin B1 using 1,000 mL of the culture is summarized in Table 1.

The soluble *E. coli* extract was dialyzed against buffer B and loaded onto a Ni Sepharose Fast Flow column that had been previously equilibrated in the same buffer. A discontinuous gradient of imidazole (10, 20, 40, 60, 150, and 250 mM) in buffer B was chosen to elute the target protein. When 10 and 20 mM imidazole was running through the Ni Sepharose column, several hybrid proteins could be detected in the outflow (Fig. 2, lanes 2 and 3). When loaded with either 40 or 60 mM imidazole, there were scarce protein bands on the gel (Fig. 2, lanes 4 and 5). After 150 and 250 mM imidazole were added to the column, two clear and noninterfering stained protein bands could be seen on the gel (Fig. 2, lanes 6 and 7). One of the stained protein bands was the protein of interest. Buffer B containing 250 mM imidazole was chosen as the optimal elution buffer, since it provided better conditions for the next step: molecular sieve chromatography. Annexin B1 was revealed by SDS-PAGE, and at this stage, the recombinant protein appeared to be 85% pure. Using a Sephadex G-200 column in the next purification step, two obvious peaks were identified (Fig. 3). The annexin B1 product peak was pooled as the final purified product. A total of 23 mg of pure recombinant annexin B1 was isolated and sample homogeneity was confirmed by isoelectric focusing and MALDI-TOF MS (see below).

Stability of recombinant annexin B1 at room temperature

Recombinant His-tagged and nonfusion annexin B1 were incubated for 24, 48, and 72 h at room temperature and then

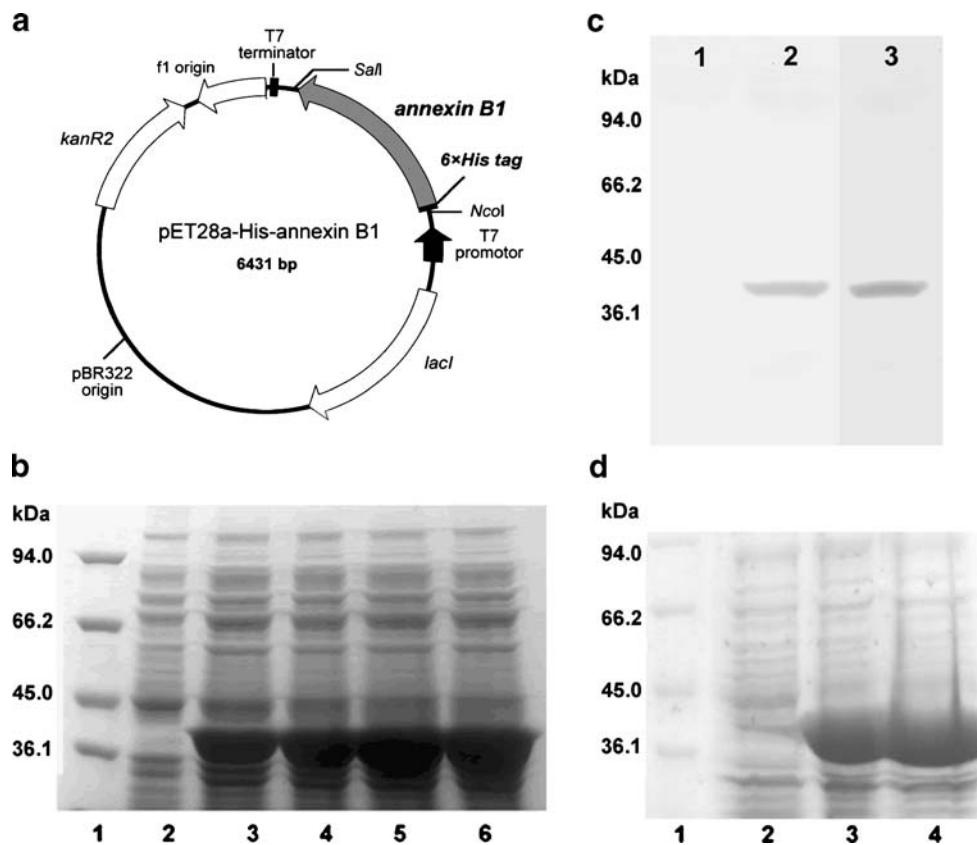


Fig. 1 **a** Schematic representation of pET28a-His-annexin B1 expression plasmid. The *gray line* depicts the annexin B1 cDNA insert cloned into the *SalI*–*NcoI* sites in the pET28a expression vector. **b** Analysis of *E. coli* extracts by SDS-PAGE after time shift by IPTG induction. Proteins were stained with Coomassie blue R250. Lane 1 protein markers, lane 2 total bacterial lysates before IPTG induction, lanes 3–6 total bacterial lysates after IPTG induction from 2 to 5 h, respectively. **c** Western blotting analysis of annexin B1 expressed in *E.*

coli transformed with plasmid pET28a-His-annexinB1. After IPTG induction, the bacteria were disintegrated and analyzed using mouse anti-His-Tag monoclonal antibody (lane 2) or serum from pigs infected with cysticercosis (lane 3) as primary antibody. The bacteria without IPTG induction were used as a negative control (lane 1). **d** SDS-PAGE analysis of *E. coli* extract after sonication. Pellet, soluble, and total fractions after lysis were loaded in lanes 2–4, respectively

subjected to isoelectric focusing analysis. As shown in Fig. 4a, protein degradation of nonfusion annexin B1 was visible after 24 h storage at room temperature and increased during long-term storage, while His-tagged annexin B1 was stable at room temperature more than 3 days later. The stability of His-tagged annexin B1 was further confirmed by MALDI-TOF MS analysis (Fig. 4b).

Anticoagulant activity of recombinant annexin B1

The anticoagulant activity of purified His-tagged annexin B1 was measured using KPTT clotting assays. As shown in Fig. 5, KPTT was significantly prolonged by His-tagged annexin B1 at a dose of 1 $\mu\text{mol/L}$. In comparison with PBS and albumin, His-tagged annexin B1 prolonged the clotting

Table 1 Purification summary

Purification procedures	Total protein (mg) ^a	Annexin B1 (mg) ^b	Fraction annexin B1/total protein	Yield (%)
Crude extract	658.7	243.7	0.370	100.0
Soluble extract	403.4	189.6	0.470	77.8
Ni Sepharose Fast Flow column	37.1	29.6	0.798	12.1
SephadexG-200 column	23.3	23.0	0.985	9.4

^a Total protein purified from Ni Sepharose Fast Flow column or Sephadex G-200 column represents the amount of total protein of eluted fractions having a high annexin B1 content. Total protein concentration is determined using a modified Bradford method

^b Recombinant annexin B1 concentration in extracts and partially purified samples is estimated by densitometric analyses after SDS-PAGE

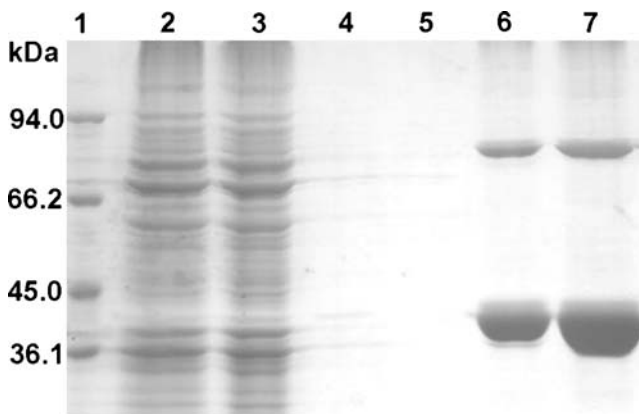


Fig. 2 SDS-PAGE analysis of the production of purified proteins from *E. coli* pET28a-His-annexin B1 transformant cells purified from a Ni Sepharose Fast Flow column with buffer A containing 10, 20, 40, 60, 150, and 250 mM imidazole, respectively (*lanes 2–7*). Protein markers were loaded in *lane 1*

times of control human serum in a concentration-dependent manner. At higher annexin B1 concentrations, a slowdown tendency towards prolonging KPTT was observed, which may reflect a depletion of the available partial thromboplastin. The anticoagulant activities of His-fused and nonfused annexin B1 were also compared in Fig. 5, which indicated that the degradation of the nonfusion form had a little effect on the protein activity.

Detection of apoptotic cells with FITC-labeled recombinant annexin B1

His-tagged annexin B1 was labeled with a detectable marker, such as the fluorochrome FITC. Mixed apoptotic cells with this conjugated form could then be measured by a variety of techniques like flow cytometry or laser confocal microscopy. However, phosphatidylserine (PS) exposure also takes place during cell necrosis. To facilitate the elimination of cells undergoing necrosis, PI was added to the cell suspension. Because cell membranes are impermeable to PI, only necrotic cells or apoptotic cells undergoing secondary necrosis will take up this dye (Krysko et al. 2008). Incubation of the FITC–annexin B1 (or FITC–annexin A5, green fluorescence) and PI (red fluorescence) with cells allowed for discrimination between intact cells (FITC–PI[−]), early apoptotic (FITC+ PI[−]) and necrotic cells, and apoptotic cells undergoing secondary necrosis (FITC+ PI⁺).

FITC–annexin B1 was titrated (0.5–2 μg/mL) to determine the optimum concentration for use in the detection of apoptotic cells. The final concentration of 1 μg/mL FITC-conjugated annexin B1 (1:1 stoichiometric complex) in HEPES binding buffer, corresponding to that of FITC-conjugated annexin A5, was finally used to assess cell binding. Typical flow cytometric dot plots of double-stained thymocytes in the time course of apoptosis were

shown in Fig. 6a (FITC–annexin B1/PI) and Fig. 6b (FITC–annexin A5/PI). At 0, 4, and 8 h after 10^{−5} mol/L dexamethasone treatment, the apoptotic cell populations (in the lower right quadrant of the cytograms, FITC+ PI[−]), as assessed by FITC–annexin B1 binding, were 7.9%, 36.4%, and 73.8%, respectively. Similarly, as assessed by FITC–annexin A5 binding, the apoptotic cells were 8.3% (0 h), 35.9% (4 h), and 71.0% (8 h). The other apoptotic model, camptothecin-induced apoptosis (10 μg/mL), was investigated in the human Jurkat T lymphocytic cell line. Following incubation of cells with FITC–annexins and PI, a time-dependent increase in annexin B1 (or annexin A5) binding to cells was observed via flow cytometry analysis (Fig. 6c, d). FITC–annexin B1/PI-labeled apoptotic thymocytes were also confirmed by laser confocal microscopy (Fig. 7). Early apoptotic cells, which had bound FITC–annexin B1, showed green staining in the outer membranes of cells (Fig. 7a), while necrotic or late apoptotic cells, which had lost membrane integrity, showed red staining (PI) throughout the nucleus and a halo of green staining (FITC) on the plasma membrane (Fig. 7c).

Discussion

Proteolytic degradation of recombinant proteins produced in heterologous hosts represents a major problem in the bioprocess industry. The results of proteolytic attack may vary from complete product hydrolysis to minor protein truncation without impairment of biological activity of the protein. The degree to which such partial proteolysis is a problem depends on the requirements of the ultimate use of the protein product. Annexin B1 is a novel protein and has been shown to have potential clinical applications. As there was negligible structural information about the novel

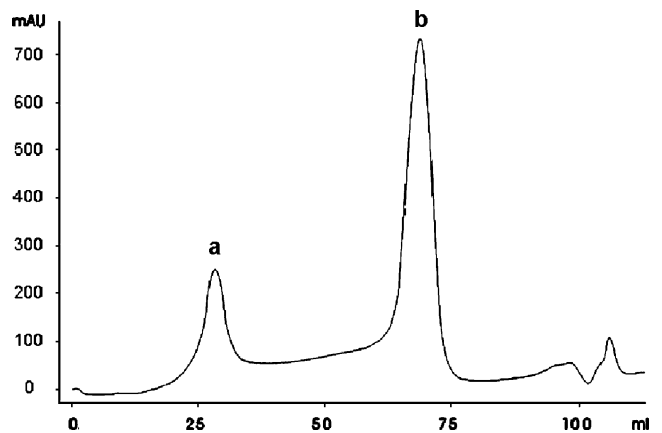


Fig. 3 Purification of annexin B1 recombinantly produced in *E. coli* pET28a-His-annexin B1 transformant cells with size exclusion chromatography packed with Sephadex G-200 on FPLC. *a* is the hybrid protein, while *b* is the protein of interest

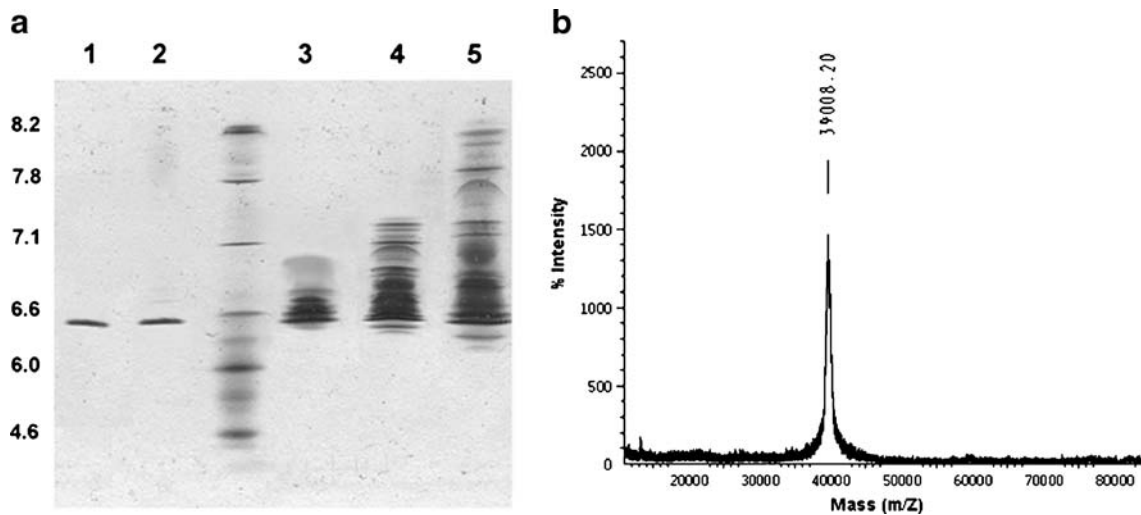


Fig. 4 Room temperature stability of His-tagged annexin B1 and nonfusion annexin B1 proteins. **a** Isoelectric focusing analysis of His-tagged and nonfusion annexin B1 protein samples stored at room temperature for different time intervals. *Lanes 3–5* nonfusion annexin B1 protein samples stored at room temperature for 24, 48, and 72 h, respectively. *Lanes 1 and 2* the His-tagged protein samples under the same conditions for different time periods (*lane 1* for 24 h, *lane 2* for

72 h). The gel was stained with Coomassie brilliant blue. The lane between *lanes 2 and 3* represents a pH marker, ranging from pH 8.2 (*top*) to pH 4.6 (*bottom*). **b** Identification of purified His-tagged annexin B1 (after 72-h storage at room temperature) by MALDI-TOF MS analysis. The molecular weight of His-tagged annexin B1 was 39,008 Da

protein, we produced a recombinant nonfusion annexin B1 and used X-ray crystallography to study its structure–function relationship. Although many approaches to optimize protein crystal screens were tried, our attempts to crystallize annexin B1 were not successful. We found that the instability of this recombinant protein may be the reason for its unsuccessful crystallization. After 24-h storage at room temperature, N-terminal sequencing revealed that the purified nonfusion annexin B1 proteins were missing several amino acids at the N terminus. The number of missing amino acids was variable (data not shown). Crystallization screens and experiments are generally

performed at room temperature or 4°C. Thus, to maximize the potential for crystal growth, annexin B1 stability at room temperature must be increased.

Recombinant DNA technology offers several strategies to improve the stability of expressed gene products, including the use of gene fusion partners, choice of host cell strain, and product engineering. In previous studies, many unstable target proteins were stabilized by adding flanking affinity tails (Rozkov and Enfors 2004; Murby et al. 1996). In this study, the His-tag system was applied to annexin B1. The *E. coli* strain BL21 (DE3) was selected as the host strain for the expression of recombinant annexin B1. This host is deficient in the Lon protease and lacks the ompT outer membrane protease that can degrade proteins during purification. Thus, proteins could be more stable in the host.

Purification of His-tagged annexin B1 was facilitated by high-level soluble expression of the protein. In the first chromatography step, it was found that the pH of the eluent had a significant influence on the resolution. Considering both the effect of pH on resolution and binding capacity, the optimal value of the eluent pH was 8.0. During the subsequent size exclusion chromatography step, the recombinant annexin B1 was purified to homogeneity. The stability of the final preparation of His-tagged annexin B1 was characterized by isoelectric focusing and mass spectrometry. The results showed that the His-tagged annexin B1 was more stable than nonfusion annexin B1 and it was quite stable at room temperature for more than 3 days, which sets an important stage for further characterization of the protein. In fact, we have recently achieved a single

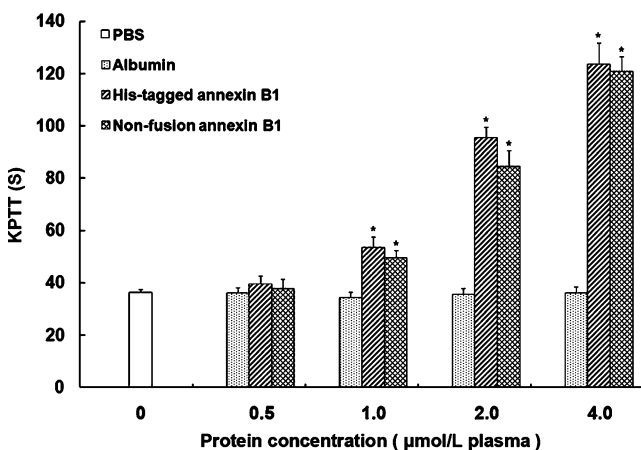


Fig. 5 Anticoagulant activity of recombinant His-tagged annexin B1 by KPTT using PBS, albumin, and nonfusion annexin B1 as controls. Values shown represent averages of duplicate analyses

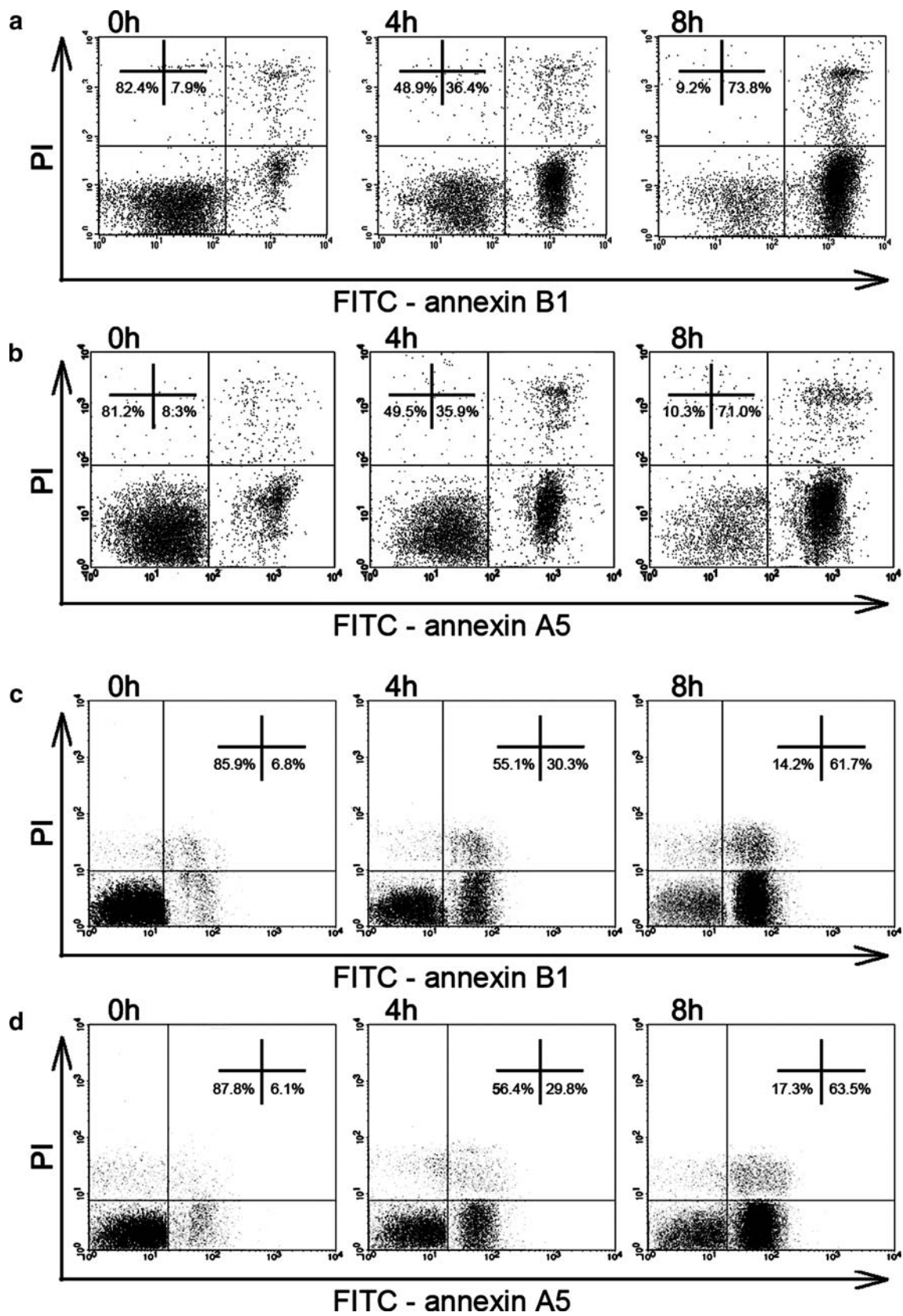


Fig. 6 Flow cytometric analysis of annexin B1 or annexin A5 staining. Representative dot plots of double-stained thymocyte apoptosis induced with dexamethasone, as assayed by FITC–annexin B1/PI (a) or FITC–annexin A5/PI (b) binding, at different time intervals. Jurkat cells were treated with camptothecin and apoptosis was assayed by FITC–annexin B1/PI (c) or FITC–annexin A5/PI (d) binding. The *bottom left* region of each quadrant shows the viable cells, which exclude PI and are negative for FITC–annexin B1 (or A5) binding. The *bottom right* region represents the apoptotic cells, FITC–annexin B1 (or A5)-positive and PI-negative, demonstrating cytoplasmic membrane integrity. The *top right* region contains the nonviable necrotic or late apoptotic cells, positive for FITC–annexin B1 (or A5) binding and for PI uptake

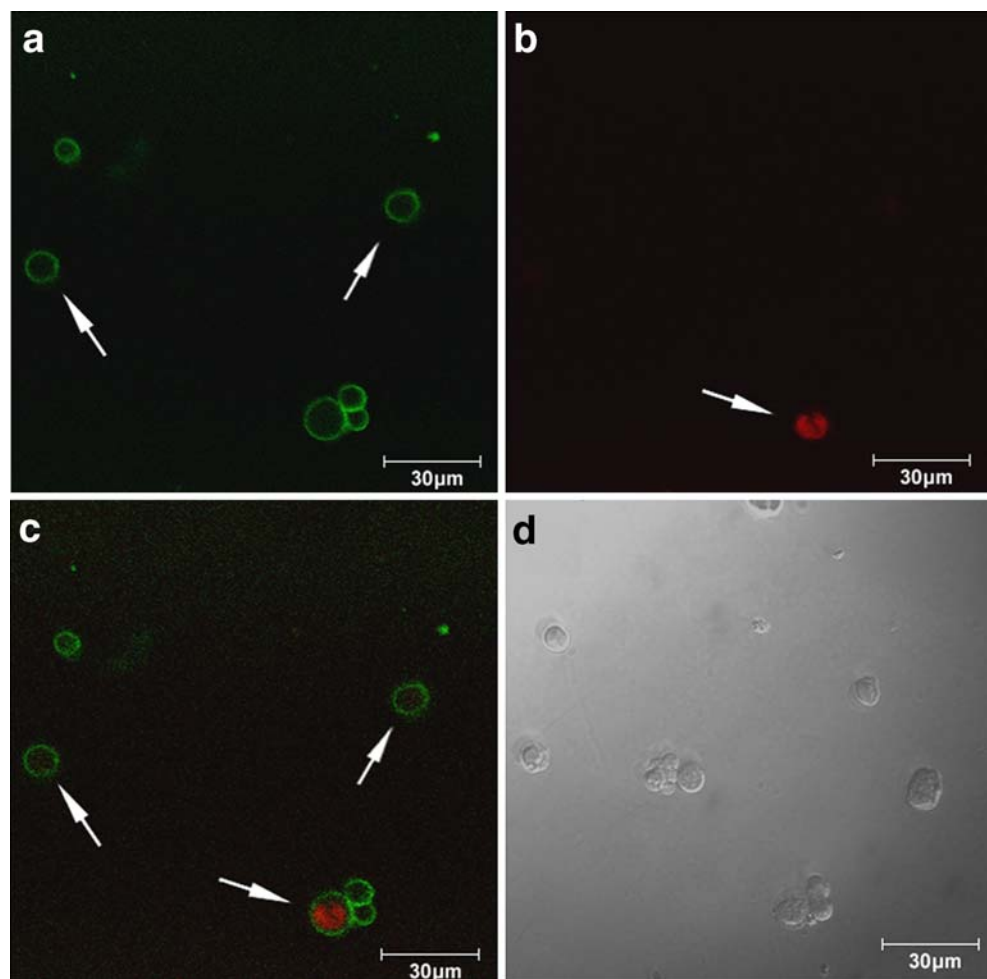
crystal of annexin B1 by the vapor diffusion method using this His-tagged protein.

The anticoagulant activity of the purified His-tagged annexin B1 was measured using the KPTT clotting assay. Anionic phospholipids (e.g., PS) are generally present in the inner leaflet of the lipid bilayer. When some agonists, like thrombin, activate platelets, PS moves from the inner to the outer leaflet of the lipid bilayer. The exposure of PS in activated platelets provides a surface upon which the coagulation factors gather to promote coagulation (e.g.,

activation of factors X by VIIIa and IXa) (Chang et al. 1993; Heemskerk et al. 2002). Annexin B1 possesses an anionic phospholipid-binding property; therefore, it can inhibit the activation of coagulant factors by shielding PS on the surface of the activated platelets and thereby is considered to possess anticoagulant activity. The KPTT assay showed that recombinant His-tagged annexin B1 has high anticoagulant activity.

PS exposure at the cellular surface is also one of earliest detectable molecular events in apoptosis. As is well documented, the externalization of PS is a general feature of apoptosis that occurs before membrane bleb formation and DNA degradation, which is an essential phenotype for the recognition and engulfment by phagocytes (Fadok et al. 1992; Wu et al. 2006). At present, the annexin A5-binding assay is routinely used for laboratory and clinical studies (Bossy-Wetzels and Green 2000; Kartachova et al. 2007), providing a very specific, rapid, and reliable technique to detect apoptosis. But in some cases, the cost of purchasing this reagent from commercial suppliers limits the application of this assay. For example, 100 μg of FITC–annexin A5 conjugate costs approximately 500 US dollars. Prelim-

Fig. 7 Analysis of FITC–annexin B1/PI staining by laser confocal microscopy in dexamethasone-induced thymocyte apoptosis. **a–d** The same scope under the different channels: **a** *green* fluorescent (FITC) channel, **b** *red* fluorescent (PI) channel, **c** overlay of **a** and **b**, **d** phase contrast panel. The *arrows* point to the representative cells that stain positive for FITC–annexin B1 and/or PI



inary studies have confirmed that annexin B1 has high PS-binding activity, similar to that of annexin A5 (Wang et al. 2004; Zhang et al. 2002), and this feature prompted us to investigate the binding of annexin B1 to apoptotic cells. In this study, we described a convenient expression–purification system to produce His-tagged annexin B1 that yielded milligram amounts of highly stable and bioactive protein. The method used for FITC conjugation of recombinant annexin B1 was also presented. From a series of unpublished experiments in our laboratory, we found that His-tagged-ANX B1-FITC conjugate is sensitive in detecting PS exposure in different apoptotic cells induced by various stimuli and is as effective as annexin A5, thereby providing a cost-effective tool to detect apoptosis at an earlier stage. This tagged protein still retains its high specificity for PS and is quite stable at 4°C for half a year.

Acknowledgements This work was supported by the National High Technology Research and Development Program of China (2006AA02A238 and 2007AA02Z408) and the National Natural Science Foundation of China (30530660 and 30370605).

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