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# Purification of recombinant annexins without the use of phospholipids

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**Abstract**

Due to their involvement in a variety of physiological and pathological processes, different isoforms of annexins are being utilized as markers of some human diseases and bio-imaging of tissue injury (due to apoptosis), and have been proposed as drug delivery vehicles. These, in addition to extensive biophysical studies on the role of annexins in organizing lipid domains in biological membranes, have necessitated development of an efficient protocol for producing annexins in bulk quantities. In this paper, we report a one-step purification protocol for annexin a5 without using lipid vesicles or involving any column chromatographic step. Depending on the growth and expression condition, a fraction of recombinant annexin a5 (cloned in pET3d vector) was sequestered into inclusion bodies. When these inclusion bodies were dissolved in 6 M urea, subjected to a 10-fold snap dilution in the presence of 5 mM $Ca^{2+}$ and stored overnight at 4 °C, annexin a5 was precipitated as a homogenous protein as judged by SDS–PAGE. This one-step purification protocol produced about 35 mg of highly purified annexin a5 per liter of bacterial culture. The annexin a5 purified from inclusion bodies exhibited similar properties to that obtained from the soluble fraction using the conventional lipid-partitioning approach. Our purification protocol for annexin a5 elaborated herein is equally effective for purification of annexin A2, and we believe, will serve as general protocol for purifying other annexins in bulk quantities for diagnostic as well as detailed biophysical studies.


*Keywords:* Annexin; Purification; Refolding; Apoptosis detection; Inclusion body

Annexins are a family of $Ca^{2+}$- and phospholipid-binding proteins, constituting ~2% of total eukaryotic intracellular protein [1]. Up to 160 unique annexins have been found present in 65 different plants and animals [2], and they are highly conserved across species [3]. Twelve members of this family have been found in humans [4]. Structurally, annexins are 35–40 kDa proteins, comprised of four α-helical domains that are conserved among different isoforms. A relatively unstructured amino terminal domain is found to vary from one isoforms of annexin to the other [4].

Annexins have been linked with several physiological processes, such as membrane organization [5], exocytosis [3], phagocytosis[5], and fibrinolysis [6]. In recent years, different annexins have been associated with a number of human disease states, viz., antiphospholipid syndrome [7], systemic lupus erythematosus [8], cancers [9–11], and diabetes [11]. Thus, detailed structural–functional studies on annexins are important from the point of view of understanding the pathophysiology of the above diseases.

Of the different annexins, Annexin 5 (Anx5) has found one of major biotechnological applications in "apoptotic" imaging. When cells undergo programmed cell death (apoptosis), they expose phosphatidylserine (PS)[1] on to the

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[1] *Abbreviations used:* GST, glutathione-*S*-transferase; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IPTG, isopropyl thiogalactopyranoside; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PMSF, phenyl methyl sulfonyl fluoride; Anxa*, non-human vertebrate annexin (natural or recombinant); AnxA*, human annexin (natural or recombinant); PS, phosphatidylserine; PC, phosphatidylcholine.

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outer membrane leaflets [12]. Due to its PS-binding specificity [13], Anx5 specifically localizes on the outer cell surfaces of cells undergoing apoptosis, and thus the apoptotic (vis a vis normal) cells are easily imaged using fluorescent-labeled protein. This approach has been widely used in cellular and molecular biology, and immunological research [14–18]. Radiolabelled recombinant human Anx5 is currently in clinical trials for imaging ischemic injury, transplant rejection, and tumor response to chemotherapeutic agents in cancer [19,20]. Anx5 has also been proposed for use as a drug delivery vehicle for the treatment of cancers [21]. With such diversity of its biotechnological and pharmaceutical applications, purified Anx5 and its other isoforms are in high demand.

Previously reported methods for annexin purification include isolation from human placenta [22,23], $Ca^{2+}$-dependent reversible binding to phospholipids vesicles [24] and expression of recombinant protein with GST tags and subsequent purification on glutathione–agarose column [25]. The latter approach is commonly employed in purification of recombinant Anx5 for apoptotic kits.

However, all these approaches for purifying annexins have some or the other drawbacks. For example, purification of annexins from human placenta raises several ethical issues, and thus, recourse is usually made to produce the recombinant forms of annexins which have been expressed in *Escherichia coli* systems, characterized by various techniques and found to have identical properties with their natural placental isoforms [26,27].

Even when annexins are expressed in bacterial cells, their lipid vesicle-assisted purification leaves immunogenic phospholipid remnants in the final preparation, necessitating further purification by column chromatographic methods. Purification of annexins via lipid-partitioning method is also time-consuming, and thus is not amenable to large-scale purification. On the other hand, the GST-fusion based purification method often leaves immunogenic GST tag and/or thrombin (utilized to cleave the fusion tag) in the final purified preparation of annexins. With these limitations in mind, we sought to develop an alternative purification protocol for annexins which involves neither lipid-partitioning nor construction of GST-fusion proteins. In this endeavor, as observed by other investigators [28], we noted that a fraction of annexins A2 and a5 expressed in *E. coli* tend to be sequestered in inclusion bodies. Since refolding of proteins from inclusion bodies is often considered challenging, all previous purification protocols utilized the soluble fraction of the expressed annexin [24,28]. In an attempt to recover annexins from inclusion bodies, we realized that they could easily be purified to homogeneity in a single-step process as detailed in the following section. Our purification protocol is less time-consuming, highly reproducible, and it can be scaled-up to produce annexins in bulk quantities for biotechnological applications.

## Materials and methods

### *Materials*

#### *Strains and plasmids*

*Escherichia coli* strain BL21codon plus (DE3) were purchased from Stratagene® (La Jolla, CA). The expression vector pET3d containing full length coding region of rat Anxa5 and pSE420 containing full length coding region for human AnxA2 were gifts from Hitoshi Sohma and Harry Haigler, respectively.

#### *Chemicals*

All chemicals unless stated otherwise, were from VWR International® (West Chester, PA USA). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) were from Avanti® Polar Lipids (Alabaster, Alabama USA). 3-Morpholinopropanesulfonic acid (MOPS) was from Fluka Chemical Corp. (Ronkonkoma, NY). BCA Protein Assay kit was from Novagen® (La Jolla, CA).

#### *Expression and purification of annexin a5*

The coding sequence for Anxa5, cloned between *Bam*HI and *Nco*I sites into pET3d vector (containing an ampicillin antibiotic marker), was obtained as a gift from Hitoshi Sohma of Sapporo Medical University School of Medicine, Sapporo, Japan. The Anxa5 clone was transformed into chemically competent *E. coli* BL21 (DE3) cells, and the cells were grown in LB Broth (10 g/L tryptone, 10 g/L yeast, 5 g/L NaCl) at 37 °C with constant shaking at 250 rpm. The protein expression was induced when the OD of medium reached 0.6–0.8 by addition of IPTG up to 1 mM. The cells were allowed to grow overnight at 37 °C, and harvested next morning by centrifuging at 10,000*g* for 20 min. The cell pellet was resuspended in a lysis buffer (20 mM Hepes, pH 7.4 containing 100 mM NaCl, 5 mM $CaCl_2$, and 1 mM PMSF). The cells were lysed by sonicating for 8 min at 4 °C using a Branson probe sonifier set at 40% duty cycle. The lysate was centrifuged for 30 min at 12,000*g*. The pellet containing inclusion bodies was washed with 10 ml of 1 M urea, and centrifuged at 12,000*g* for 30 min. This approach was found to remove most of the protein impurities from inclusion bodies, leaving Anxa5 in the pellet fraction. The pellet containing Anxa5 was subsequently dissolved in resuspension buffer containing 6 M urea and 6 mM EDTA, and left to stir for 4 h at room temperature. The suspension was centrifuged at 12,000*g* for 40 min; SDS–PAGE analysis of the 6 M urea extract showed a highly prominent band at 36 kDa of Anxa5 (Fig. 1). The above suspension was subjected to a 10-fold "snap-dilution" in the refolding buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl and 5 mM $CaCl_2$. The mixture was left to stir overnight at 4 °C, producing a cloudy suspension of the protein. The presence of $Ca^{2+}$ in the refolding buffer was found to be essential for precipitation of Anxa5 in the cloudy form. The resulting cloudy suspension was centrifuged for 1 h at 16,000*g*, and