Affinity Partitioning of Proteins Tagged with Choline Binding Modules in Aqueous Two-Phase Systems

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Abstract

We present a novel procedure for affinity partitioning of recombinant proteins fused to the choline-binding module C-LytA in aqueous two-phase systems containing poly(ethylene glycol) (PEG). Proteins tagged with the C-LytA module and exposed to the two-phase systems are quantitatively localized in the PEG-rich phase, whereas subsequent addition of the natural ligand choline specifically shifts their localization to the PEG-poor phase by displacement of the polymer from the binding sites. The described procedure is simple, scalable and reproducible, and has been successfully applied to the purification of four diverse proteins, resulting in high yields and purity.

Keywords: Aqueous two-phase systems, protein purification, affinity chromatography, poly(ethylene glycol), choline-binding module, liquid-liquid extraction.

1. Introduction

Protein separation and purification represent biotechnological events of unquestionable importance, accounting in some cases for 50-90% of total production costs at the industrial level [1]. Affinity adsorption on a solid chromatography support constitutes the usual procedure for recombinant, tagged polypeptide separation, either aimed towards purification or to bioreactor setup [2-4]. However, the need for resin preparation and recycling, and other negative aspects and problems like column fouling or changes in the stability and enzymatic parameters of the adsorbed proteins, have fostered the search for alternative procedures. In this sense, aqueous two-phase systems (ATPSs) have been successfully used in the purification of many proteins of interest [5,6], and may be implemented in industrial downstream processes [7]. ATPSs are formed when two polymer solutions, or a polymer and a salt, are mixed at a concentration higher than a critical value, so that they separate into two phases at equilibrium. Most commonly used ATPSs involve the mixing of polyethylene glycol (PEG) or related polymers like thermoseparating ethylene-oxide-propylene-oxide copolimers [8,9] with dextran or phosphate salts. These systems present interesting advantages both for laboratory and industry processes. They are cost-effective, easy to scale up [10] and suitable for continuous operation [11]. Many variables can be manipulated to improve the partition, and compatibility with detergents allows the purification of membrane proteins [12]. Moreover, polypeptides partitioned in ATPSs are exposed to mild physical-chemical conditions as both phases consist mainly of water (70-90%) and the interfacial tension between them is very low [13], favouring mass transfer in enzymatic reactions. However, the use of ATPSs for protein purification on a routine basis, either at industrial or laboratory level has been hampered by the generally poor predictability of the partition coefficient of any given protein in a particular ATPS, as this parameter results from a complex interplay of macromolecular properties such as molecular weight, amino acid composition, hydrophobicity and electrostatic forces [13-16]. Several approaches take advantage of the affinity for a certain ligand in order to direct the localization of the protein of interest to a particular phase [17]. In many occasions it is necessary the use of translational fusions to different polypeptide tags, such as tryptophan and tyrosine-rich, hydrophobic sequences [18], poly-histidine tails [19] or combinations of both [20]. However, these affinity-enhanced

partitioning systems are not free of disadvantages, such as a decreased protein expression and solubility, or the need for derivatization of PEG [17].

The C-LytA module belongs to the choline-binding domain family (Pfam ID code PF01473: http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01473). C-LytA constitutes the C-terminal part of the LytA amidase from *Streptococcus pneumoniae* and is responsible for the attachment of this enzyme to the choline residues on the surface cell wall [21]. The C-LytA polypeptide is a 135-aa repeat protein, built up from six conserved β-hairpins that configure four choline-binding sites [22]. Each choline binding site is constituted by two aromatic residues from one hairpin and another one from the next, with the contribution of an additional hydrophobic side chain. The ligand is bound by hydrophobic and cation-π interactions, that significantly increase the stability of the protein [23,24]. The affinity of C-LytA for choline and choline structural analogs [25,26] allows its use as an affinity tag for single-step purification of hybrid proteins expressed in *Escherichia coli*, by specific adsorption to simple amine-containing chromatographic resins like DEAE-cellulose, followed by specific elution with choline [27-32]. We therefore decided to check whether the C-LytA tag might also be employed in affinity partitioning in PEG-containing ATPSs. Here we show that C-LytA may bind PEG molecules in the choline-binding sites, which can be used to accumulate both C-LytA and C-LytA-tagged proteins in the PEG phase, whereas addition of choline reverses this interaction and directs the protein to the PEG-poor phase. This allowed the purification of four diverse proteins and suggests that choline-binding polypeptide tags may be used in easily modulated systems for the predictable partitioning and easy purification of recombinant proteins in ATPSs.

2. Experimental

2.1.Materials

PEG8000, dextran DxT500 and choline chloride were purchased from Sigma (St. Louis, MO, USA)

2.2. Bacterial strains and plasmids

Escherichia coli strains REG-1 and REG-21 were supplied by Biomedal (Seville, Spain). Construction of plasmid vectors is represented in Fig. 1. Plasmid pALEX2-Ca-GFP (coding for the GFP-C-LytA protein) was constructed by insertion of the 718 pb *Sph*I-*Stu*I fragment containing the GFP coding sequence from pJBA111 [33] between the *Sph*I and *Sma*I sites of commercial vector pALEX2-Ca (Biomedal~~, Seville, Spain~~). For pALEXb-Lip36 construction (C-LytA-Lip36 protein), the 959 pb *Bam*HI-*Hin*dIII fragment of p36/LACK gene of *Leishmania infantum* [34] was inserted between the *Bam*HI and *Hin*dIII sites of commercial vector pALEXc (Biomedal~~, Seville, Spain~~). For pALEX2c-LacZ construction (LYTAG-β-Galactosidase), the 3116 pb *Bam*HI-*Hin*dIII fragment of the *E. coli lacZ* gene encoding β-galactosidase was inserted between the *Bam*HI and *Hin*dIII sites of commercial vector pALEX2c (Biomedal~~,~~) ~~Seville, Spain)~~. Finally, for pALEXb-ProtA (C-LytA-ProtA) construction, a 433 pb PCR product encoding two copies of the *Staphylococcus aureus* protein A IgG affinity domain Z was amplified with primers MAO55 (Forward): 5′-CGCGGATCCGAAAACCGCGGCTCTTGCGC-3′and MAO56 (Reverse): 5′-CGCGGATCCTCAGGTTGACTTCCCCGCGGAGTTCGCGTC-3′, digested with *Bam*HI and cloned in the *Bam*HI site of commercial vector pALEXb (Biomedal~~, Seville, Spain~~).

2.3. Protein expression

C-LytA protein was purified by affinity chromatography from the overproducing *E. coli* strain RB791 harbouring the pCE17 plasmid [21]. Optimized materials and protocols contained in the C-LYTAG Protein Expression and Purification System (Biomedal~~, Seville, Spain~~) were used. In order to remove the bound choline, the purified

proteins were subsequently applied onto a HiTrap desalting column (1.6 x 2.5 cm) (GE Healthcare) at 20 ºC equilibrated in 20 mM sodium phosphate buffer, pH 7.0, plus 50 mM NaCl, and stored at -80ºC. Protein concentration was determined spectrophotometrically as previously described [21] using a molar absorption coefficient at 280 nm of 62 540 M^{-1} cm^{-1}.

GFP-C-LytA protein was purified by affinity chromatography from the overproducing *E. coli* strain REG-21 harbouring the pALEX2-Ca-GFP plasmid, following instructions described in the C-LYTAG Protein Expression and Purification System (Biomedal~~, Seville, Spain~~).

For purification of C-LytA fusions in ATPSs, a 5-ml overnight culture of *Escherichia coli* REG-1 containing the pALEX2-Ca-GFP, pALEXb-Lip36, pALEX2c-LacZ or pALEXb-ProtA plasmids was grown at 37 ºC and then diluted 100-fold in 500 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 10g/l NaCl) containing 100 µg/µl ampicillin. The culture was grown at 37 ºC to an optical density at 600 nm of about 0.8-1.0, when gene expresion was induced with 2 mM salicylate and incubated overnight at 20 ºC. Cells were harvested by centrifugation at 4 ºC (5 000 *g*, 10 min) and resuspended in 30 ml of potassium phosphate pH 8.0, plus 10 mM $MgCl_2$ and DNAseI (50 U/ml). Cell suspension was passed through a French press and subsequently centrifuged 15 min at 10 000 *g*. Further purification is described in the text.

2.4. Protein characterization

Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). This procedure was employed for diluted samples of both extracts and pure proteins, as we found that small amounts of PEG and dextran interferred ~~Due to the interference of PEG~~ with the usual protein quantitation procedures either by UV absorbance or colorimetry. On the other hand, the concentration of GFP-C-LytA in each step of purification was specifically measured by fluorescence in a spectrofluorimeter (SFM 25, Kontron Instruments, Zurich, Switzerland) with excitation and emmision wavelengths of 475 nm and 515 nm respectively. Blanks were prepared using all components except protein. A calibration curve relating fluorescence intensity with protein concentration was created using GFP-

C-LytA previously purified by affinity chromatography (see above). ~~Partition constants (K) were calculated as the concentration ratios of protein in the upper (PEG) phase to the lower phase.~~

All stages of protein purification were followed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) [35]. Gels were stained with EZBlue (Sigma). Since PEG and DxT500 were found to produce very distorted lanes, samples were occasionally diluted 100-fold to decrease the load of polymer and gels were silver-stained using the Gel Code Silver SNAP Stain KitII (Pierce, Rockford, IL, USA).

2.5. Circular dichroism

Circular dichroism (CD) experiments were carried out in a J-815 spectropolarimeter (JASCO, Tokyo, Japan) equipped with a Peltier PTC-423S system. Isothermal wavelength spectra were acquired at a scan speed of 50 nm min^{-1} with a response time of 2 s and averaged over at least 6 scans at 20 ºC. Protein concentration was 6.3 µM and the cuvette path-length was 1 mm or 2 mm. Buffers were 20 mM sodium phosphate (pH 6.0 - 8.0), 20 mM sodium acetate (pH 3.5 - 5.5) or 20 mM glycine (pH 2.5 - 3.0), plus the corresponding additions in each case. Samples were centrifuged 5 min prior CD measuring. Ellipticities ($[\theta]$) are expressed in units of (deg cm^2 $dmol^{-1}$), using the residue concentration of protein before centrifugation. For CD-monitored temperature-scanning denaturation experiments the sample was layered with mineral oil to avoid evaporation, and the heating rate was 60 ºC h^{-1}.

2.6. Activity assays

β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopiranoside (ONPG) as described by Miller [36]. The unit of enzyme activity is definied as the amount that produces 1 nmol of *o*-nitrophenol per minute at 28 ºC and pH 7.0.

3. Results and discussion

PEG is an ubiquitous component in most characterized ATPSs [5,37]. On the other hand, the four choline binding sites of C-LytA contain solvent-exposed tryptophan and tyrosine residues [22] which might accommodate PEG molecules as described for the similar acetylcholine binding site of *Torpedo* acetylcholinesterase [38]. In this protein, the cation-binding site (formed by the faces of aromatic side-chains) is occupied by CH_2 groups of the inhibitor, establishing CH-π interactions that are similar to the cation-π interactions made by the choline moiety of acetylcholine. It should be pointed out that the choline-binding site of C-LytA is also formed by clusters of aromatic side-chains, so a similar interaction with PEG in the latter case is probable. Therefore, we decided to check the possible interaction of C-LytA with PEG by ~~circular dichroism~~ CD. As shown in Fig. 2A, addition of 10% PEG induced a moderate change in the CD spectrum of C-LytA at pH 7.0, similar to that induced by choline. Furthermore, the ellipticity of C-LytA displays a minimum around pH 5 (Figs. 2A and 2B), probably as a consequence of protein aggregation near the calculated isoelectric point (5.4). Nevertheless, this aggregation was prevented both by choline and PEG, that restored the native CD spectrum (Figs. 2A and 2B). Moreover, while unligated C-LytA undergoes a two-step thermal denaturation due to the accumulation of a folding intermediate [24] (Fig. 2C), addition of either choline or PEG induced a single-step transition due to destabilization of the intermediate, although the thermal stabilization induced by the natural ligand is clearly higher. Taken together, these results suggest that PEG might emulate, to some extent, the role of choline and occupy the same binding sites. This may also explain our previous finding that purification of C-LytA in DEAE-cellulose equilibrated with 10% PEG was accomplished with only a 10% efficiency with respect to the absence of the compound, as most of the protein contained initially in the extract was lost in the flow-through.

~~cannot be adsorbed onto a DEAE-cellulose column in the presence of 10% PEG (data not shown)~~

Given the C-LytA-PEG interaction, a preferential partition of C-LytA in the PEG phase of an ATPS based on this polymer could be anticipated. To check this hypothesis we employed the green fluorescent protein (GFP) fused to C-LytA because localization of this hybrid protein (GFP-C-LytA) can be easily monitored and quantified

by fluorescence. In this protein the C-LytA moiety is located in the C-terminal position, as in the whole LytA parental protein [21]. Cell extracts were prepared as described in the Materials and Methods section. The cleared supernatant (1.4 mg/ml of total protein) was partitioned in a PEG/salt mixture composed of 15 % PEG-8000 and 12.5 % di-potassium hydrogen phosphate (PEG/phosphate system) obtained by adding the corresponding amount of each solid component to the extract. After solubilization of all the components by gentle but thorough mixing, the aqueous two-phase system was generated by centrifugation (5 min at 10 000 *g*). Upon separation of the two phases, a strong accumulation of GFP-C-LytA was detected in the upper, PEG-rich phase (Fig. 3A). Partition coefficients in the ATPS was calculated by measuring fluorescence of the GFP moiety in each phase (Table 1). Moreover, the fusion protein was already more than 90% pure at this stage, as most of the extract proteins were localized in the bottom, salt-rich phase (Fig. 3B) and the ratio of GFP-tagged protein (specifically measured by fluorescence) to total protein content (measured by standard protein quantitation procedures) was close to 1 (Table 1). By contrast, a more uniform distribution of GFP was observed when a control experiment was carried out with untagged protein (Table 1). These results clearly show that the C-LytA module directs partitioning of the GFP-C-LytA fusion to the PEG-rich phase in an almost quantitative fashion, increasing substantially the partition constant (*K*). Partitioning was also very efficient even when highly concentrated cell extracts were used ~~(6 mg/ml) (data not shown).~~ (Fig. 4).

Although, as mentioned, the GFP-C-LytA protein is very pure at this stage, we decided to explore conditions leading to is localization in the salt-rich phase in order to achive a higher degree of purity. In the proposed C-LytA-PEG interaction model, choline should compete with PEG for C-LytA binding, disrupting C-LytA-PEG interaction and hence the partitioning pattern of GFP-C-LytA, and allowing recovery of the fusion protein by “elution” from the PEG-rich phase. To further test this possibility, the GFP-C-LytA-containing top phase was carefully removed and placed in a fresh tube. A similar volume of a “wash” solution containing 1 % PEG, 16 % potassium phosphate (a composition similar to that of the discarded bottom phase [1,13]) was added and mixed by inversion of the tube several times, followed by centrifugation 5 min at 10 000 *g*, without significant release of the GFP-C-LytA protein from the PEG-rich phase during this process (Figs. 3A, 3B, and Table 1). The top PEG-rich phase was again removed and placed in a new tube. Finally, a similar volume of a solution

containing 1 % PEG, 16 % potassium phosphate and 300 mM choline was added, mixed and centrifuged. This caused a change in the partition coefficient of the polypeptide ~~(Table 1)~~, resulting in the partial elution of the fusion protein to the bottom, salt-rich phase by displacement of PEG from the C-LytA moiety by choline, and which was completed after a second extraction (Figs. 3A and 3B, and Table 1). Total yield was 23.2 mg per liter of culture (A_{600} = 1.0), corresponding to more than 80% of the protein contained in the initial homogenate, and with a purification factor of near 4. These results are even better than those obtained~~, comparable to the yield~~ using solid chromatography in DEAE-cellulose in our laboratory (around 15 mg per liter, 52% yield, same purification factor~~ data not shown~~).

The promising results with GFP-C-LytA prompted us to set up a general scheme for protein purification (Fig. 5) that was subsequently tested for the purification of other fusions. First, we attempted the purification of a C-LytA fusion to the p36/LACK antigen from *Leishmania* (C-LytA-Lip36 protein) [34]. However, in this case, we observed the precipitation of the recombinant protein probably due to the relatively high ionic strength provided by the phosphate phase. As this might be the case for other proteins, we tried an alternative procedure using an ATPS composed of 6% PEG plus 6% dextran (DxT500) (PEG/dextran system) in 20 mM Tris pH 8.0. Use of Tris instead of phosphate improved significantly phase separation in this case. Washes were performed with solutions containing 0.5 % PEG + 16% DxT500, and final elution was carried out with a solution containing 0.5 % PEG + 16% DxT500 and 300 mM choline. As shown in Fig. 6, the C-LytA-Lip36 protein could be readily purified, suggesting that the nature of the bottom phase is not determinant in ruling the partition properties of C-LytA fusions. Yields were usually 3-5 mg per liter of culture. In order to better define the recovery of the C-LytA-Lip36 protein, 5 mg of purified C-LytA-Lip36 were spiked into 50 ml of an *E. coli* REG-1 cellular extract (1.5 mg/ml total protein) and subjected to partition in the PEG/dextran ATPS as described above. Recovery after the two elutions was 3.1 + 1.1 = 4.2 mg, *i.e.* 84% of total spiked protein, similar to the GFP-C-LytA case (Table 1).

Next, we attempted the purification of a high-sized, tetrameric protein such as β-galactosidase. Here, we used as a tag an improved mutant of C-LytA (LYTAG), obtained from a 32-aa N-terminal deletion that do not affect its choline-binding

properties [24] while improving its solubility. Although both PEG/phosphate and PEG/dextran systems allowed the purification of the LYTAG-β-galactosidase protein, the best solubilities were obtained with the latter (Fig. 7). SDS-PAGE gels were somewhat distorted due to the high size of LYTAG-β-galactosidase (a tetramer of around 525 kDa) complexed with PEG, that affects the normal electrophoretical mobility of the samples, but the purity of the preparations is evident in any case. Table 2 depicts the characteristics of the subsequent purification steps in the PEG/dextran system. Around 70% of the expressed protein could be purified, with an specific activity on a small chromophoric substrate (ONPG) of 271-288 U/µg which is in close agreement to that calculated previously for the DEAE-cellulose purified protein (300 U/µg) [27]. It is noteworthy that in this case the choline-binding module is located in the N-terminal part of the fusion protein, showing that the localization of the tag is not relevant to dictate the partition properties. ~~Moreover, the protein conserved its enzymatic activity against small chromophoric substrates in both phases (data not shown).~~

Finally, we tested the partition properties of a C-LytA hybrid with a biotechnologically relevant polypeptide such as protein A from *Staphylococcus aureus* [39]. This protein is typically used as a reliable method for detecting/purifying total IgG from crude protein mixtures. Fig. 8 shows that the C-LytA-ProtA fusion can be efficiently partitioned and purified in both PEG/phosphate and PEG/dextran systems. Purity of the preparations was assessed by densitometry of the SDS-PAGE gels, yielding a 91% and 94% values for the first and second elution in PEG/phosphate , respectively (Panel A), and 91% in PEG/dextran (Panel B) The final yield was calculated as 80 mg per liter of culture (A_{600} = 3.7). Similarly to the C-LytA-Lip36 fusion, we measured the recovery of C-LytA-ProtA by spiking 10 mg of purified protein into 50 ml of an *E. coli* REG-1 cellular extract (1.5 mg/ml total protein). Upon two elutions with choline, recoveries in PEG/phosphate (7.9 mg, 79%) and PEG/dextran (7.2 mg, 72%) were very similar and showed a high yield in both cases.

4. Conclusions

We have developed a new aqueous two-phase partitioning system of proteins based in the affinity of C-LytA tag for PEG and choline. The most relevant aspect of this technology is that localization of the C-LytA fusion can be strongly modulated by the addition of choline. Therefore, as a proof-of-concept, we have shown the easy purification of four C-LytA-tagged recombinant proteins in PEG-containing ATPSs after an initial accumulation in the PEG-rich phase, followed by the subsequent elution to the PEG-poor phase upon addition of choline. The system is simple, rapid, cost-effective and scalable, and might constitute an attractive alternative to other protein purification methods based in solid-support procedures. It seems versatile enough for a variety of two-phase systems containing PEG or PEG-like polymers and, above all, it is highly predictable, a characteristic usually lacking in most ATPSs described so far. Other biotechnological applications that can be foreseen, besides the use for protein purification, include the use of C-LytA-tagged enzymes to catalyze a reaction in one of the phases (chosen at will, depending on whether choline is added) while recovering the product in the other, thus decreasing bioseparation costs. Moreover, addition of choline would separate the enzyme from the PEG phase after reaction, making possible the recycling of the polymer for a new extraction. Finally, this tool is amenable to be used at bench-scale for the test and set-up of enzymatic processes in ATPSs before scaling up to the industrial level.

Acknowledgments

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424

425 **Figure captions**
426
427 **Figure 1**. Plasmid diagrams. All the open reading frames were cloned under Pm
428 promoter control and fused in frame with C-LytA module, or its reduced and improved
429 version LYTAG (pALEX2c-LacZ).
430
431 **Figure 2**. Stability analysis of C-LytA by far-UV CD. (A) Wavelength spectra recorded
432 at 20 ºC at pH 7.0 (filled symbols) and pH 5.0 (open symbols) in the absence of
433 additives (circles), in the presence of 10% PEG (triangles) or in the presence of 140 mM
434 choline (squares); (B) pH titration monitored by CD at 223 nm in the absence of
435 additives (filled circles), in the presence of 10% PEG (open circles) or in the presence of
436 150 mM choline (filled squares); (C) Thermal stability of C-LytA monitored by CD in
437 the absence of additives (o), in presence of 10% PEG 10% (●) and in the presence of
438 150 mM choline (X).
439
440 **Figure 3.** Purification of GFP-C-LytA in PEG/phosphate from *E. coli* REG-1
441 [pALEX2-Ca-GFP]. (A) Photographs taken during the purification. (B) Analysis of
442 100-fold diluted samples by 12% SDS-PAGE and silver stain.
443
444 **Figure 4**. Effect of extract concentration on the partition properties of GFP-C-LytA in
445 PEG/phosphate. (A) 0.45 mg/ml; (B) 0.9 mg/ml; (C) 1.9 mg/ml; (D) 4.0 mg/ml.
446 Samples were illuminated with UV light in order to induce GFP fluorescence.
447
448 **Figure 5**. General procedure for purification of C-LytA fused proteins by PEG-
449 phosphate or PEG- dextran ATPSs.
450
451 **Figure 6.** Purification of C-LytA-Lip36 in PEG/dextran from *E. coli* REG-1 [pALEXb-
452 Lip36]. SDS-PAGE was Coomassie stained. (A) Partition of C-LytA-Lip36 in the
453 absence of choline. (B) Elution of C-LytA-Lip36 by choline.
454
455 **Figure 7.** Purification of LYTAG-β-galactosidase in PEG/dextran from *E. coli* REG-1
456 [pALEX2c-LacZ]. SDS-PAGE was Coomassie stained.
457

458 **Figure 8.** (A) Purification of C-LytA-ProtA in PEG/phosphate; (B) Purification of C-
459 LytA-ProtA in PEG/dextran.

Figure 1

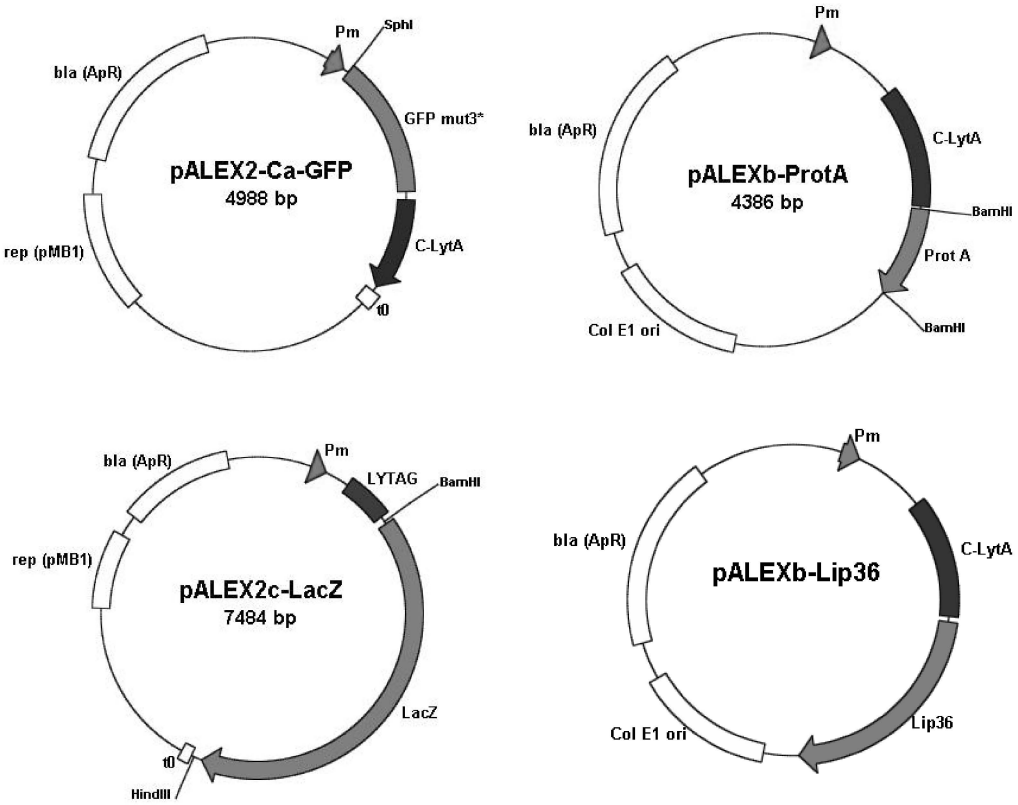

Pm
SphI
bla (ApR)
GFP mut3*
pALEX2-Ca-GFP
4988 bp
rep (pMB1)
C-LytA
t0
Pm
bla (ApR)
C-LytA
pALEXb-ProtA
4386 bp
BamHI
Prot A
Col E1 ori
BamHI
Pm
bla (ApR)
LYTAG
BamHI
rep (pMB1)
pALEX2c-LacZ
7484 bp
LacZ
t0
HindIII
Pm
bla (ApR)
C-LytA
pALEXb-Lip36
Col E1 ori
Lip36

Figure 2

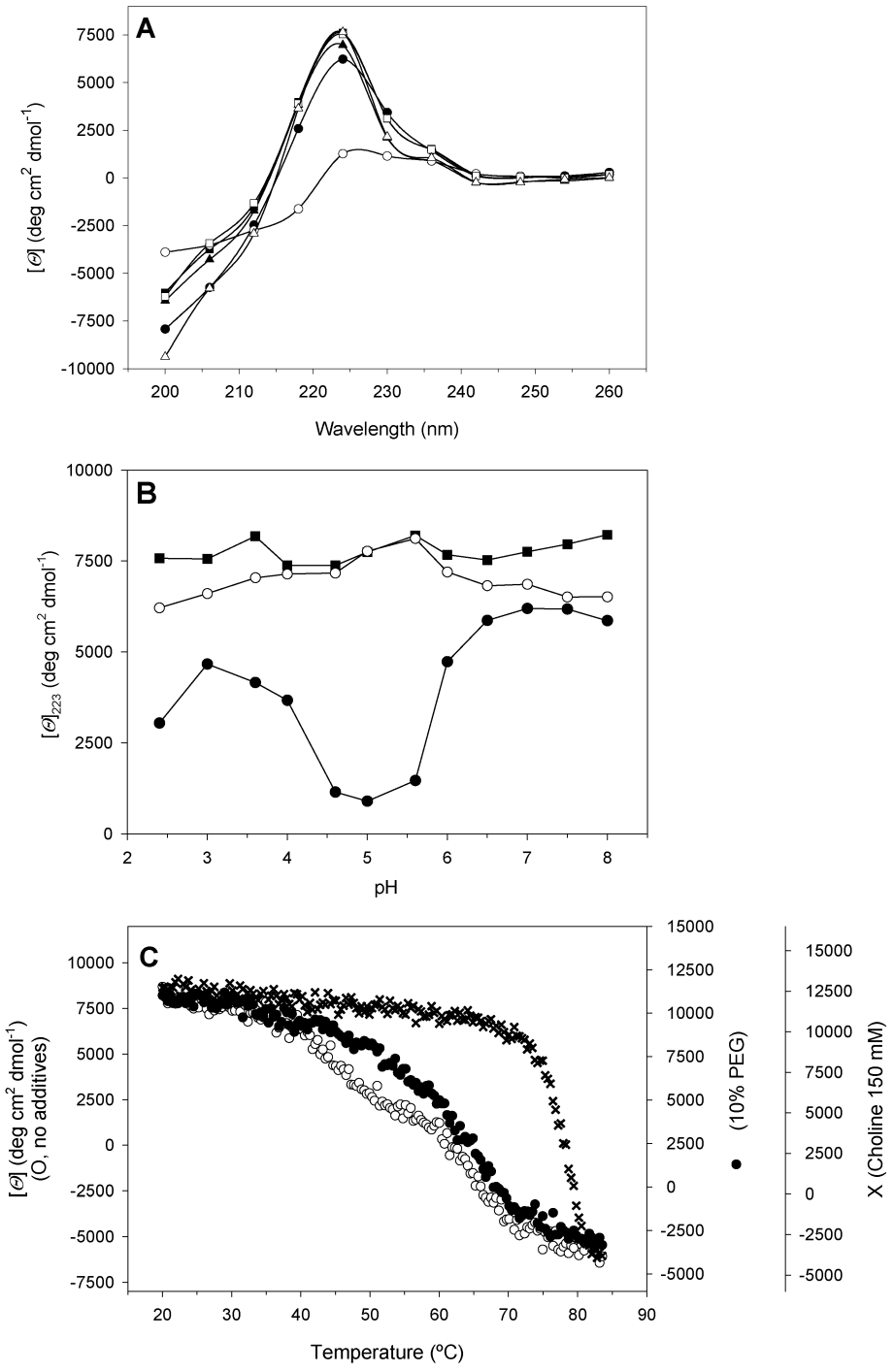

A
7500
5000
2500
0
-2500
-5000
-7500
-10000
200
210
220
230
240
250
260
Wavelength (nm)
[θ] (deg cm2 dmol-1)
B
10000
7500
5000
2500
0
2
3
4
5
6
7
8
pH
[Θ]223 (deg cm2 dmol-1)
C
10000
7500
5000
2500
0
-2500
-5000
-7500
15000
12500
10000
7500
5000
2500
0
-2500
-5000
20
30
40
50
60
70
80
90
Temperature (ºC)
[θ] (deg cm2 dmol-1)
(O, no additives)
(10% PEG)
X (Choline 150 mM)

Figure 3

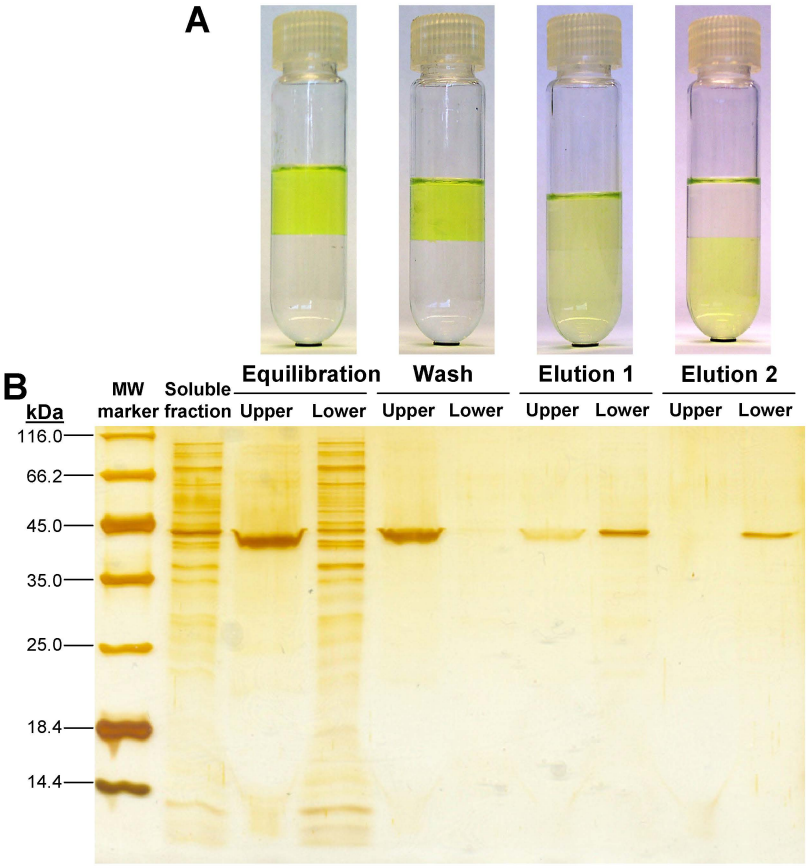

A
B
kDa
MW marker
Soluble fraction
Equilibration
Upper
Lower
Wash
Upper
Lower
Elution 1
Upper
Lower
Elution 2
Upper
Lower
116.0
66.2
45.0
35.0
25.0
18.4
14.4

Figure 4

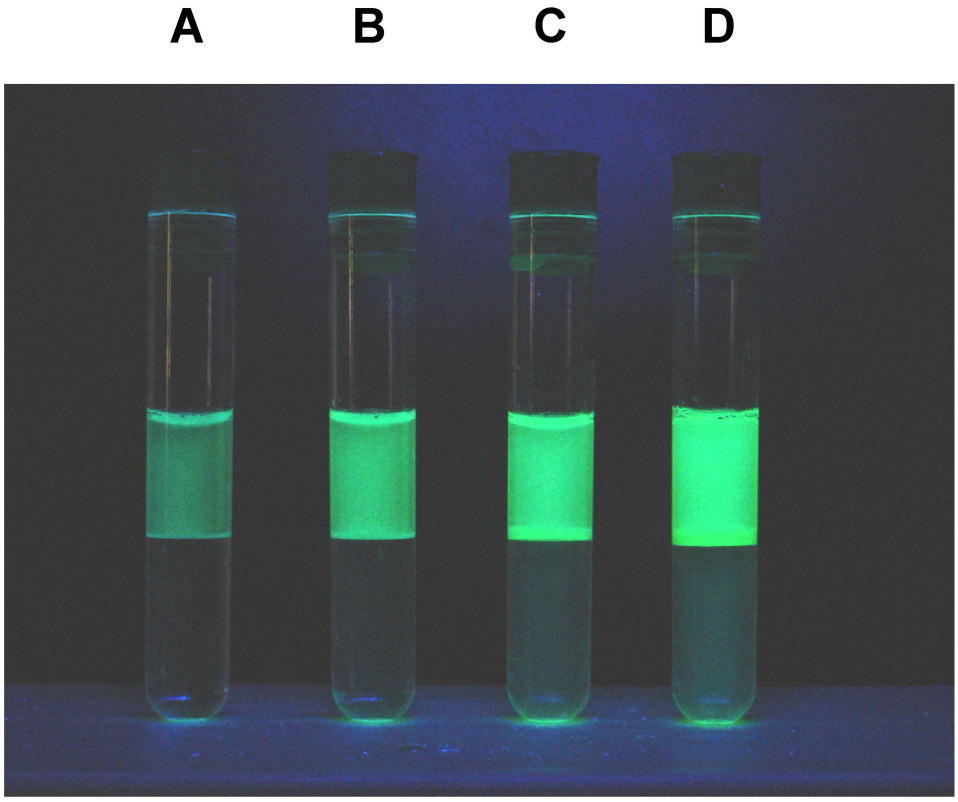


Figure 5

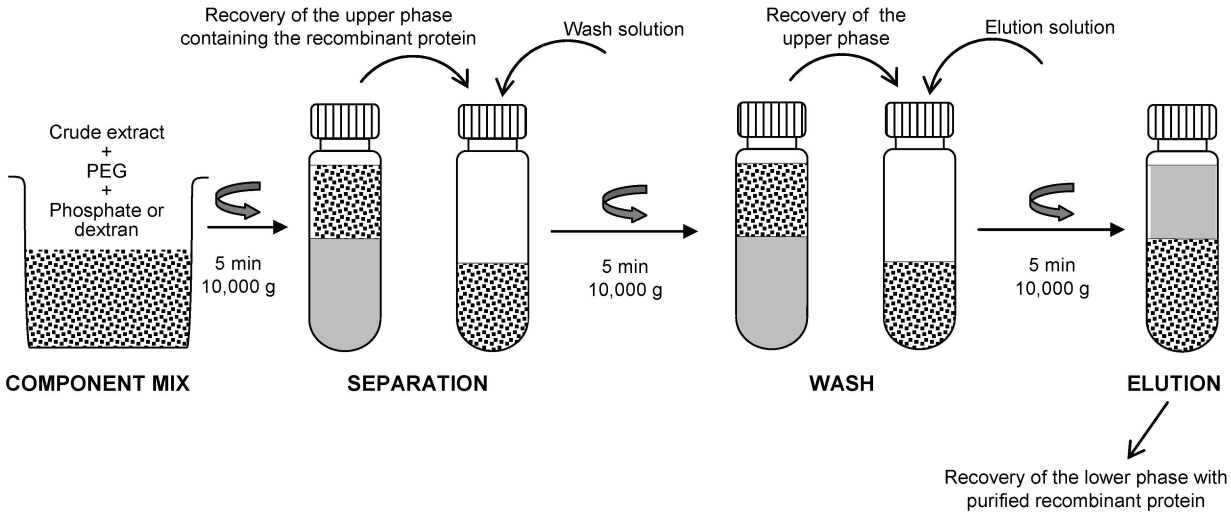

Recovery of the upper phase containing the recombinant protein
Wash solution
Recovery of the upper phase
Elution solution
Crude extract
+
PEG
+
Phosphate or dextran
5 min
10,000 g
5 min
10,000 g
5 min
10,000 g
COMPONENT MIX
SEPARATION
WASH
ELUTION
Recovery of the lower phase with purified recombinant protein

Figure 6

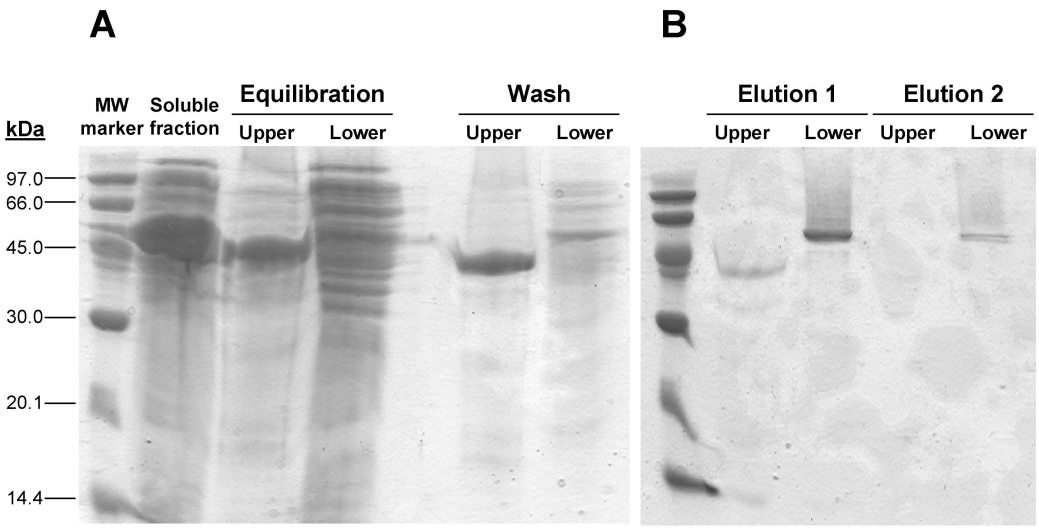

A
B
kDa
MW marker
Soluble fraction
Equilibration
Upper
Lower
Wash
Upper
Lower
Elution 1
Upper
Lower
Elution 2
Upper
Lower
97.0
66.0
45.0
30.0
20.1
14.4

Figure 7

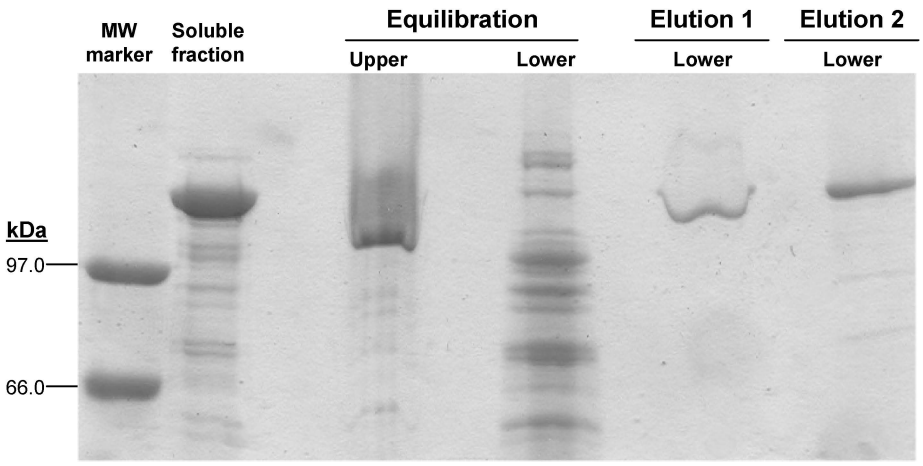
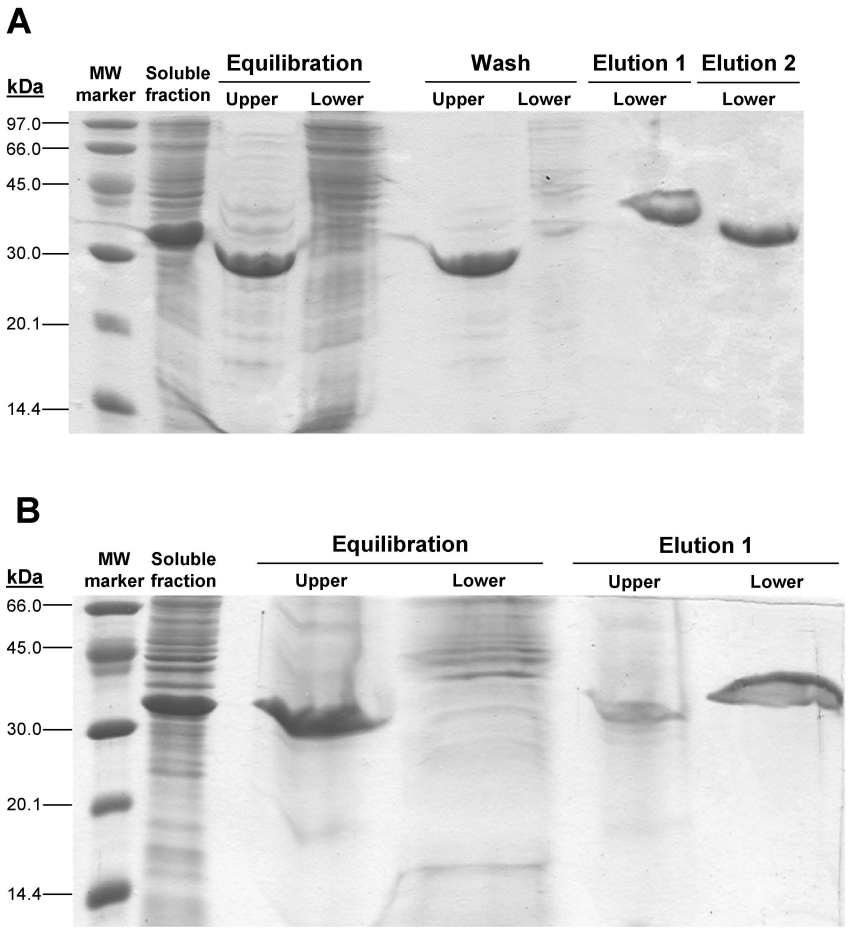

MW marker
Soluble fraction
Equilibration
Upper
Lower
Elution 1
Lower
Elution 2
Lower
kDa
97.0
66.0

Figure 8


A
kDa
MW marker
Soluble fraction
Equilibration
Upper
Lower
Wash
Upper
Lower
Elution 1
Lower
Elution 2
Lower
97.0
66.0
45.0
30.0
20.1
14.4
B
kDa
MW marker
Soluble fraction
Equilibration
Upper
Lower
Elution 1
Upper
Lower
66.0
45.0
30.0
20.1
14.4