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Application of aqueous two-phase partition to the production of homogeneous preparations of fluorescently labelled human serum albumin

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Abstract

The development of a traceable molecular probe was investigated for the monitoring of partition behaviour of biomolecules in aqueous two-phase systems. This work was based upon the selective labelling of the free thiol group of human serum albumin (i.e. Cys34) with the fluorophore N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid. The preparation of homogeneously labelled protein required purification operations. A succession of five processes was successfully applied, comprising two size-exclusion chromatographic operations by gel filtration and a series of three appropriately manipulated aqueous two-phase systems comprising PEG 1450 and phosphate salt. Aqueous two-phase partitioning is herein presented as an alternative to difficult separation and could be applied for 'fine' purifications.

Keywords: Aqueous two-phase systems; Partitioning; Human serum albumin

1. Introduction

Aqueous two-phase systems (ATPSs) have been increasingly applied to the separation of biomolecules since the pioneering experimental work of Albertsson [1]. The aqueous environment inherent to ATPSs is considered to provide mild conditions for the handling of biological materials such as protein [2]. However, it has been suggested that the transitions between top and bottom phases of loaded systems could affect the products in terms of modification of the native conformation, especially in the presence of a relatively dense interface [3]. Changes of tertiary structure have also been suggested for large proteins (i.e. molecular masses, $M_r > 20\,000$) partitioned in ATPSs characterised by various tie-line lengths (TLLs) [4]. Moreover, phase component chemicals such as PEG and salt are well-documented as having various effects on protein structures such as solubility and stability modulations [5,6], and PEG–protein associations [7,8]. Such interactions between proteins and phase component chemicals, together with any phase transition encountered in loaded ATPSs, may strongly affect not only the conformation and biological function of macromolecules but also their respective partition behaviour in terms of yield and quality of the products. It is therefore essential to evaluate the general impact that aqueous two-phase partitioning may have upon biomolecular structure and function.

This work necessitated a new approach compared to the common indexes and detection methods

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conventionally applied to aqueous two-phase partitioning. The focus of this work lies in the introduction of molecular probes in blank and loaded ATPSs. These carefully selected probes enable the collection of new experimental data having a signal independent from that of biomolecules, such as proteins, nucleic acids and cell debris, present in the system to circumvent any interference. Fluorescence spectroscopy appears suitable as a detection technique due to its potential specificity, which enables the detection of molecules at a specific excitation and emission wavelength and which is also commonly applicable to the conformational study of biomolecules [9].

The present work consequently focused on the development and construction of a defined traceable molecular probe by means of the specific derivatisation of a protein molecule, that is the specific labelling of HSA. N-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid (1,5-IAEDANS) was selected as reagent for the specific labelling of a free thiol group within a protein in order to generate a fluorescent functional moiety permitting conformational studies of protein molecules [10]. The characterisation of the resulting molecular probe was achieved by spectrophotometric methods, electrophoretic analysis and aqueous two-phase partitioning. The preparation of a homogeneous labelled protein was essential for its subsequent application as a molecular probe, and a suitable purification process was designed involving the appropriate manipulation of aqueous two-phase partitioning.

2. Experimental

2.1. Labelling reaction

The reaction was achieved by exposing the protein to a 10 fold molar excess of 1,5-IAEDANS (Molecular Probes, The Netherlands). Thus, 9 mg of 1,5-IAEDANS were added to 20 ml of 6 mg/ml human serum albumin (HSA) solution (HSA of approximate purity 99%, catalogue number A3782; Sigma, USA) in phosphate buffer 0.1 *M*, pH 7.5. The labelling reaction was conducted in a dark container for 1 h under stirring conditions at an optimum temperature of 37°C [11]. Samples of resulting products were gel-filtered through prepacked columns of Sephadex G25 (PD10 from Pharmacia, Sweden) equilibrated with deionised water to separate proteins from the excess of reagent, and to desalt the solution.

2.2. Aqueous two-phase systems

Phase component chemicals, e.g. poly(ethylene glycol) (PEG) and phosphate salt, were carefully weighed and simultaneously added to aqueous solution containing dissolved proteins, to reach the system concentration required. PEG preparations of three average nominal molecular masses have been investigated, i.e. PEG 1000, 1450 and 2000 (Sigma, USA). All phosphate concentrations resulted from the combination of dipotassium hydrogenorthophosphate (K_2HPO_4) and potassium dihydrogenorthophosphate (KH_2PO_4) in a mass ratio 18:7 respectively to buffer the resulting solution to an apparent pH value of 7.5 [11]. Each mixture was subsequently mixed for 30 min until total dissolution of the phase component chemicals. The phases were separated by centrifugation at 1700 *g* for 10 min.

A series of four discrete ATPS compositions (S1–S4) were selected for study, characterised by three discrete molecular masses of PEG associated with phosphate. The volume ratio (V_r) of each series of four ATPSs was calculated as the ratio of the top phase volume relative to that of the bottom phase, and the tie-line length was subsequently estimated [2]. The various parameters, characteristic of each ATPS, are listed in Table 1. The characteristics of the blank top and bottom phases of ATPSs comprising PEG 1450 and phosphate salt are reported in Table 2.

2.3. Partition coefficient (K_p and K_f)

In the present study, following the normal convention, the partition coefficient was expressed as the natural logarithm of K ($\ln K$), where K is estimated as the ratio of the top (C_t) and bottom (C_b) solute concentrations. When the protein concentrations were evaluated by Bradford assay [12], the partition coefficient was recorded as K_p. Partition coefficients determined by fluorescence measurements were recorded as K_f, i.e. the fluorescence intensity in the top phase divided by that of the bottom phase. The

Table 1
Compositions of PEG–salt ATPSs comprising potassium phosphate (K_2HPO_4–KH_2PO_4, 18:7) and PEG of three different average molecular masses, i.e. 1000, 1450 and 2000; for each of the three molecular masses of PEG, a series of four blank ATPSs (S1–S4) was characterised in respect of PEG and salt concentrations, tie-line length and volume ratio

M_r of PEG	System	TLL (%, w/w)	[PEG] (%, w/w)	[salt] (%, w/w)	Volume ratio
1000	S1	28.8	15.0	13.0	1.1
1000	S2	37.2	16.5	14.0	1.0
1000	S3	41.6	17.7	15.0	1.0
1000	S4	48.0	20.0	16.5	1.0
1450	S1	29.0	13.6	12.4	1.0
1450	S2	35.6	15.4	13.2	1.0
1450	S3	41.3	17.3	14.5	1.0
1450	S4	47.8	19.7	16.1	1.0
2000	S1	44.3	19.0	15.0	1.0
2000	S2	47.9	20.0	16.0	1.0
2000	S3	51.1	21.0	17.0	1.0
2000	S4	54.6	22.0	18.0	1.0

fluorescence measurements were conducted with excitation and emission wavelength of 340 and 470 nm, respectively.

3. Results and discussion

3.1. Characterisation of the labelled HSA

The labelling reaction was based on nucleophilic substitution between a thiol group (Cys34) and a halogen (iodine of 1,5-IAEDANS; [13]). A large proportion of the fluorophore underwent rapid hydrolytic decomposition to unreactive products that were subsequently eliminated from labelled samples by gel permeation. The products of the labelling reaction were subsequently analysed by UV absorbance, electrophoresis (both SDS-PAGE and isoelectric focusing) and partitioning in ATPSs.

Table 2
Composition of the phases in PEG1450–salt ATPS

Phase	[PEG] (%, w/w)	[Salt] (%, w/w)
S1-top	26.1	5.3
S2-top	30.3	4.0
S3-top	34.8	3.2
S4-top	39.5	2.4
S1-bottom	1.0	19.7
S2-bottom	0.2	22.7
S3-bottom	Nd[a]	25.9
S4-bottom	Nd	29.8

[a] Nd: the PEG concentrations were too low to be evaluated (below 0.2%w/w) and were considered not to have a significant effect upon the TLL.

The UV absorbance was monitored at 340 nm to evaluate the degree of substitution of the proteins (i.e. the average number of 1,5-AEDANS group per protein molecule) using an extinction coefficient of 6100 M^{-1} cm^{-1} for the fluorophore [14]. The degree of substitution was estimated at 1.14 mol of label per mol of HSA, although only one single free cysteine group per molecule of HSA was available [15]. It should be noted that the method of estimation of the average degree of substitution depended upon the assumption that the chemical environment of the fluorophore was identical for free and labelled states [14]. The extinction coefficient of free label applied to labelled protein could therefore artefactually yield a value >1.00. A second possible contribution could have been the existence of minor reactions competitive to that between the free thiol, Cys34, and the fluorophore (i.e. reaction of 1,5-IAEDANS with other amino acid residues or affinity adsorption of label by the protein). However, no competitive experimental labelling reaction has been reported (to the knowledge of the authors) between IAEDANS and amino acids other than cysteine.

Standard and labelled HSA were analysed by SDS-PAGE for estimation of protein molecular mass (data not shown; [11]). The standard HSA used for the labelling reaction was shown to be monomeric, i.e. free of dimers generated by oxidative reaction between free thiol groups. However, products of the labelling reaction, recovered from the gel filtration post-reaction with 1,5-IAEDANS, exhibited a limited dimerisation of HSA molecules. Inter-association between the reactive thiols of two HSA molecules were thus a minor side reaction in competition with the desired labelling reaction between Cys34 and 1,5-IAEDANS.

The modified HSA molecules were also characterised by isoelectric focusing in association with native HSA (data not shown; [11]). Native HSA was experimentally characterised by a number of discrete bands within the p*I* range of 4.80–5.00, demonstrating that HSA existed under distinct conforma-

tional forms. This heterogeneity of serum albumin could be due to post-translational modifications of the proteins such as deamidation and disulphide interchanges [16]. These modifications may have resulted from circulation in the bloodstream prior to the extraction operations, since the human serum albumin was purchased as a preparation having a high degree of purity. Such a series of bands characterising the native HSA molecules remained when the protein was labelled with the thiol reagent. Moreover, when HSA was labelled with the 1,5-IAEDANS group, isoelectric focusing of the products of the labelling reaction revealed the development of one specific band at an estimated p*I* value of 5.05, in addition to the range of bands corresponding to native HSA with isoelectric points varying from 4.80 to 5.00.

Products of the labelling reaction were also partitioned in ATPS comprising PEG 1450 and phosphate salt (refer to Table 1 and Section 2.2). The partition coefficients of native and labelled HSA were plotted as a function of tie-line length as shown on Fig. 1. The partition coefficient based on protein content of labelled HSA differed in a greater manner

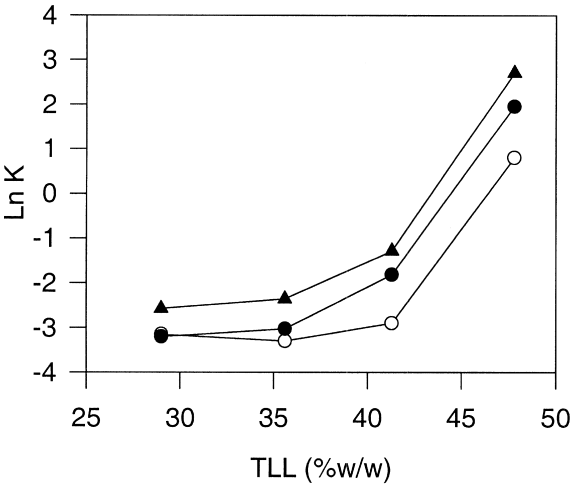

Fig. 1. Partitioning behaviour of native HSA and of two populations of labelled HSA. The products of labelling reaction conducted at 37°C were partitioned in a series of ATPSs comprising PEG 1450 and phosphate salt (see Table 1). Partitioned samples of native HSA were analysed by Bradford assay (○). For both temperatures of labelling reaction, samples of labelled HSA were analysed by both Bradford assay (●) and fluorescence measurements (▲).

to that of native HSA. Moreover, the partition coefficient evaluated by fluorescence measurements (K_f) was greater than K_p for the same labelled protein sample. Those results demonstrated competitive partitioning between distinct protein populations resulting from the labelling reaction; that is underivatised and labelled HSA molecules, the latter having stronger affinity for the top polymer rich phase. The thiol reagent 1,5-IAEDANS exploited in the labelling possessed a naphthalene ring, and was therefore a relatively strong hydrophobic molecule. Its reaction with HSA slightly modified the protein in terms of molecular mass, but more strongly in terms of partition properties in ATPS. Furthermore, it has been proposed that reaction with Cys34 could be accompanied by a 'flip-out' of that residue, resulting in enhanced exposure of the derivatised moiety to free solvent [17]. The covalent binding of the fluorophore would increasingly modify the surface hydrophobicity of the protein, promoting a higher affinity for the top PEG-rich phase as compared to the partition of native HSA molecules [18].

As a result, the labelling reaction between HSA and 1,5-IAEDANS, generated a mixture of variously modified proteins. Firstly, small amounts of HSA molecules did not react or were involved in dimerisation processes. Secondly, a small quantity was heavily derivatised by either competitive covalent binding or adsorption of the fluorophore at the surface of the protein. Thirdly and finally, the target modified HSA was found to be present in large quantities. Despite those discrete characteristics, the differences between the various protein populations generated by the reaction were very small and conventional methods of purification, such as hydrophobic interaction chromatography or size-exclusion chromatography, were not found to be sufficiently selective for product fractionation. Purification using aqueous two-phase partitioning appeared to be the most suitable and applicable technique in the present situation.

3.2. Purification process using ATPSs

Partition in ATPSs was designed to exploit the competitive affinity of these diverse protein molecules for the phases to achieve selective separation. The purification of labelled HSA was conducted

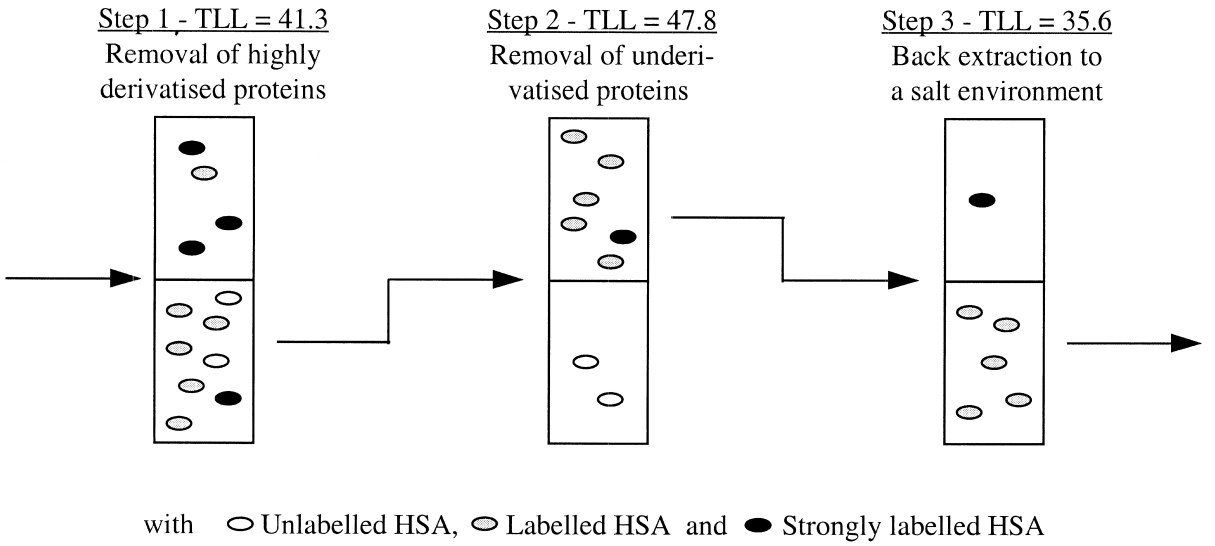

Fig. 2. Process used for the purification of labelled HSA. Such process was based on the application of aqueous two-phase systems comprising PEG 1450 and phosphate salt of various tie-line lengths.

using ATPSs comprising PEG 1450 and phosphate, and the process involved three steps as shown in Fig. 2. The three ATPSs were selected on the basis of their tie-line lengths (refer to Table 1 for their respective compositions).

Step 1 consisted of the partitioning of the products of the labelling reaction in ATPSs of tie-line length 41.3. Highly (i.e. non-specifically) labelled HSA molecules preferentially partitioned away from unlabelled and normally labelled HSA to the top phase, due to higher affinity for the top polymer rich phase (Fig. 1). On the other hand, the bulk of underivatised and target HSA molecules were fractionated to the bottom salt rich phase.

The second step involved an ATPS of higher TLL of 47.8. The bottom phase recovered from Step 1, having known composition as indicated by Table 2, was adjusted to form the aqueous two-phase system of required composition to obtain a TLL of 47.8% w/w (Step 2). At such a tie-line length, native HSA was known to preferentially separate to the top phase (Fig. 1). However, in respect of a competitive process between labelled and unlabelled HSA molecules, the labelled proteins preferentially separate to the top phase, while the underivatised proteins prefer the bottom phase environment.

Step 3 concerned the back extraction of the labelled material to the bottom salt-rich phase to facilitate further (i.e. polymer-free) processing. The top phase of the previous ATPS (corresponding to Step 2) was manipulated to construct an ATPS of lower TLL value, i.e. 35.6% (w/w). Labelled proteins were thus back-extracted to the bottom salt-rich phase, which enabled easier handling of the labelled material. The resulting bottom phase was gel-filtered on a PD10 column (Pharmacia), and the desalted solution of labelled HSA was subsequently freeze-dried.

Table 3
Protein recovery at various steps of the purification process of labelled HSA; the products of the labelling reaction of HSA with the thiol reagent were purified as described in Section 3.2

Step of the purification process	Goal	Step recovery (%)
Size-exclusion no. 1	Desalting + removal of excess fluorophore	86
ATPS no. 1, TLL 41.3	Removal of excessively labelled proteins	86
ATPS no. 2, TLL 47.8	Removal of unreacted proteins	60
ATPS no. 3, TLL 35.6	Back-extraction to a salt environment	88
Size-exclusion no. 2	Desalting	95
Total overall recovery		37

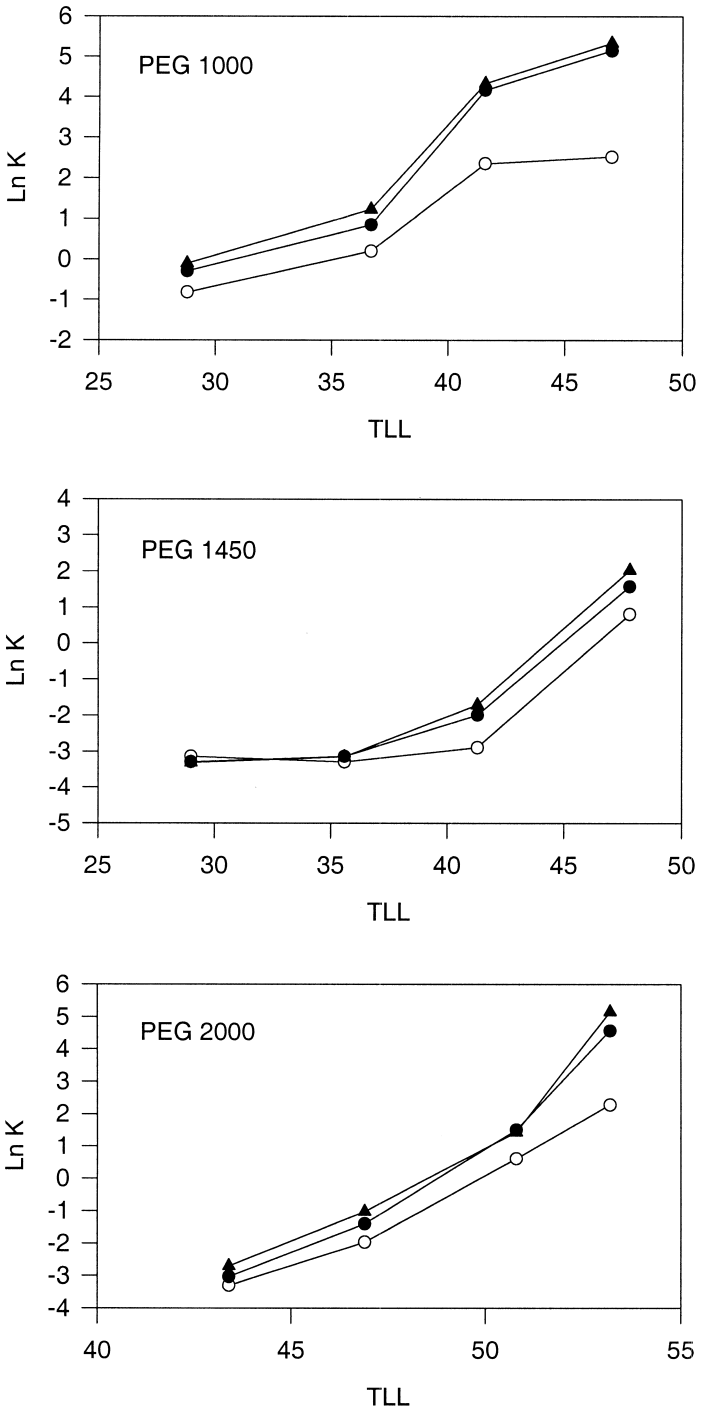

Fig. 3. Partitioning behaviour of native and purified labelled HSA in ATPS comprising PEG of various molecular masses, i.e. 1000, 1450 and 2000, and phosphate salt. The products of the labelling reaction of HSA molecules with 1,5-IAEDANS were purified according to the process illustrated in Fig. 2. The partition coefficient of native HSA was estimated by Bradford assay (○), while partition coefficients of purified labelled HSA were evaluated by both Bradford assay (●) and by fluorescence measurements (▲; refer to Section 2.2).

At each process step, losses of protein were observed and quantified. The protein recovery was evaluated for each step of the purification (i.e. step recovery) as the amount of protein recovered divided by the amount of protein processed, based on protein content estimated by Bradford assay. The results are shown in Table 3. As a result, the overall recovery of protein was of 37% of the total amount of HSA introduced into the labelling reaction.

3.3. Characterisation of the purified products by partitioning study

The molecular probe obtained was characterised by its partition behaviour in aqueous two-phase systems comprising potassium phosphate and poly-(ethylene glycol) of three different molecular masses, i.e. M_r of 1000, 1450 and 2000. The partition behaviour of purified labelled HSA was compared with that of native HSA in the same ATPS as shown in Fig. 3. Increasing the molecular mass of PEG promoted an increasing bottom phase preference for native HSA. Such a phenomenon was previously reported as the effect of molecular mass upon the partition behaviour of proteins in ATPSs [18]. Labelled HSA partitioned in a similar manner to native HSA, but its extraction to top polymer-rich phase occurred best at lower TLL, regardless of the molecular mass of PEG. As explained in Section 3.1, the addition of the thiol reagent to the protein involved a mild modification of the overall property of the protein but sufficient to observe a discrete partition behaviour as compared to that of underivatised proteins. In addition the partition coefficients based on protein or fluorophore content, K_p and K_f, respectively, were very similar. Such results demonstrated the achievement of the purification with the generation of a homogeneous preparation of labelled HSA irrespective of the ATPS.

4. Conclusion

This work has described the development of a homogeneous preparation of derivatised protein, further applied as molecular probes for the monitoring of the partition behaviour of biomolecules in aqueous two-phase systems [19]. Aqueous two-phase systems were first used to characterise the products of the labelling reaction in respect of its partitioning behaviour. Then, ATPSs were successfully applied to recover the target molecules from a mixture of comparable protein molecules (in terms of size, charge) by means of appropriate manipulation of a series of ATPSs comprising PEG 1450 and phosphate salt. ATPSs were shown to be highly sensitive to slight modification of the protein surface and to enable a fine purification where other conventional methods failed.

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