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Liquid–liquid extraction of a recombinant protein, cytochrome b_5, with aqueous two-phase systems of polyethylene glycol and potassium phosphate salts

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Abstract

The partitioning of cytochrome b_5 in aqueous two-phase systems of polyethylene glycol (PEG) and potassium phosphate salts was investigated. Cytochrome b_5 partitioning is enhanced with decreasing polymer molecular mass and with increasing tieline length and pH. The effect of cytochrome b_5 mutation, by substitution of the glutamic acid at positions 56 and 92 of the polypeptide chain by a lysine, on protein partitioning was also studied. Partitioning of cytochrome b_5 mutants shows the same dependence on tieline length and pH, following the order cytochrome $b_5 >$ mutant $56 >$ mutant 92.

1. Introduction

The downstream processing of biological materials requires separation and purification techniques leading to a high degree of purification and high recoveries and to low operating costs. One of the bioseparation processes that fulfils these criteria is liquid–liquid extraction using aqueous two-phase systems.

This technique can be used in the early steps of the purification process (*e.g.*, separating proteins from cell debris), replacing difficult solid–liquid separations, and also for further purification [1]; the scale-up is relatively simple because it utilizes equipment common in the chemical industry. Aqueous two-phase systems contain a high proportion of water in both phases, providing an excellent environment for cells, cell organelles or biologically active proteins [2].

In order to find suitable conditions for the extraction of proteins with aqueous two-phase systems, it is necessary to know the partitioning behaviour of the desired proteins. Protein partitioning depends on the physico-chemical parameters of the systems, namely the type and molecular mass of polymers, tieline length (a function of the concentration of the system components), type and concentration of component or added salts, pH, temperature and addition of biospecific affinity ligands [2].

This paper reports the partitioning of cytochrome b_5 in polyethylene glycol (PEG)–potassium phosphate salt two-phase systems. The effects of polymer molecular mass, tieline length and pH on cytochrome b_5 partitioning were investigated. The influence of cytochrome b_5 mutation, by substitution of the glutamic acid at positions 56 or 92 of the polypeptide chain by a lysine, on protein partitioning was also studied, in order to evaluate the effect of genetic manipu-

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lation techniques on the downstream processing of proteins.

2. Experimental

2.1. Chemicals

PEG 400, 1000, 3350 and 8000 were supplied by Sigma and anhydrous potassium dihydrogenphosphate (KH_2PO_4) and dipotassium hydrogenphosphate (K_2HPO_4), both of analytical-reagent grade, by Merck.

2.2. Cytochrome b_5

The cytochrome b_5 used consists of the hydrophilic domain of cytochrome b_5 from mouse [3] that was cloned in a pUC 13 vector and expressed in *Escherichia coli* (TB1) [4]. This protein has a molecular mass of 13 600 and an isoelectric point (p*I*) of 4.4 [5].

This protein was mutated by site-specific mutagenesis in the glutamic acid at positions 56 or 92 of the polypeptide chain that was substituted by a lysine [5]. The p*I* of the mutants is 4.7 [5].

Cytochromes b_5 were produced by fermentation and purified using sequential chromatographic steps [5].

2.3. Preparation of aqueous two-phase systems

Aqueous two-phase systems of total mass 6 g were prepared by weighing appropriate amounts of concentrated solutions of PEG, KH_2PO_4 and K_2HPO_4 solutions, which were added until the required pH value was obtained, and water into 10-ml graduated centrifuge tubes. The contents of the tubes was intensively mixed on a vortex agitator and then 100 μl of purified cytochrome b_5 solution (2.8 mg ml^{-1}) were added. After vortex mixing, the phases were separated by centrifugation (10 min at 128 *g*).

The concentrations of cytochrome b_5 and cytochrome b_5 mutants in each phase were determined by measuring the absorbance at 411 nm (molar absorptivity $\varepsilon = 130$ l mmol^{-1} cm^{-1} [4]) using a Hitachi UV–Vis spectrophotometer.

Table 1
PEG–salt systems used in cytochrome b_5 partitioning studies

Polymer	PEG–potassium phosphates (%, w/w)		
	Tieline 1	Tieline 2	Tieline 3
PEG 400	16.7/14.8	17.7/15.7	19.7/17.7
PEG 1000	16.2/14.3	17.7/15.7	19.7/17.7
PEG 3350	14/11.8	17.7/15.7	19.7/17.7
PEG 8000	14/11.8	–	–

The pH in each phase was measured with a Metrohm pH meter.

The PEG–salt systems prepared are shown in Table 1.

3. Results and discussion

The effect of polymer molecular mass, tieline length and pH on cytochrome b_5 partitioning in PEG–potassium phosphate systems is shown in Fig. 1. The partition coefficient, K_p, was defined as the ratio between cytochrome b_5 concentration in the upper and lower phases and the yield, *Y*, as the ratio between cytochrome b_5 mass in the top phase and in the total system. Each K_p and *Y* value represents the average of at least two measurements.

Cytochrome b_5 partition coefficients and yields increase with decrease in polymer molecular mass. For aqueous two-phase systems of PEG 400 cytochrome b_5 is mainly in the PEG-rich phase under all the experimental conditions studied ($K_p > 2.7$ and $Y > 86\%$); for PEG 1000, depending on tieline length and pH, cytochrome b_5 may partition preferentially to the salt-rich phase or to the PEG-rich phase; for PEG 3350 cytochrome b_5 is mainly in the salt-rich phase except for the longest tieline at high pH ($K_p = 1.8$ and $Y = 61\%$); for PEG 8000, with only one tieline tested, cytochrome b_5 accumulates in the salt-rich phase at all pH values ($K_p < 8 \cdot 10^{-3}$ and $Y < 0.6\%$).

The effect of polymer molecular mass can be attributed to the increasing number of hydrophilic end groups on shorter PEG chains, which reduces the overall hydrophobicity [6], and to

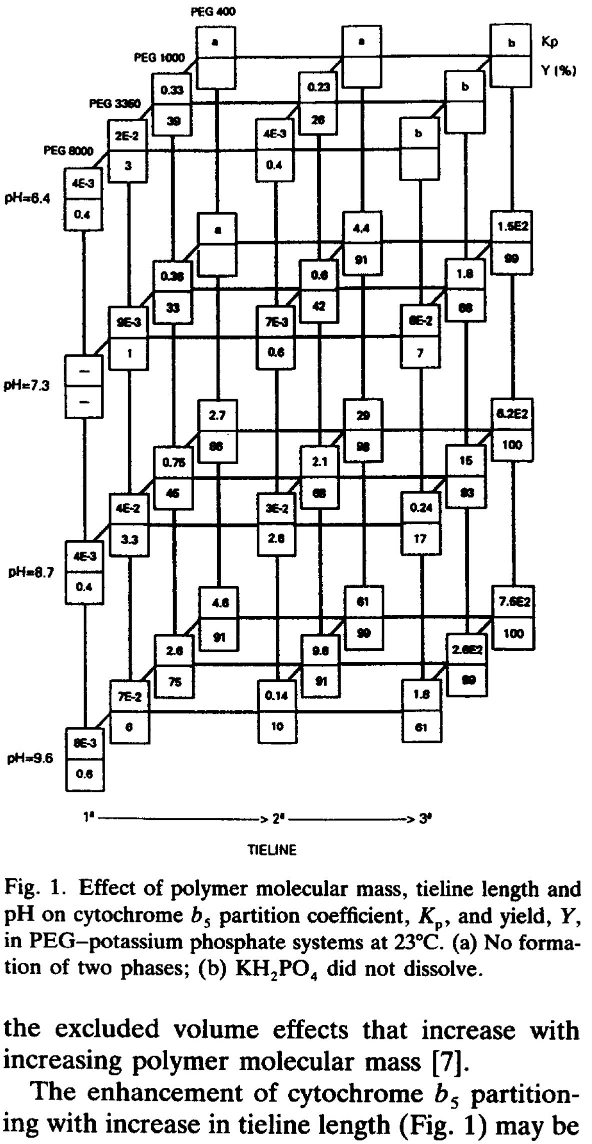

Fig. 1. Effect of polymer molecular mass, tieline length and pH on cytochrome b_5 partition coefficient, K_p, and yield, Y, in PEG–potassium phosphate systems at 23°C. (a) No formation of two phases; (b) KH_2PO_4 did not dissolve.

the excluded volume effects that increase with increasing polymer molecular mass [7].

The enhancement of cytochrome b_5 partitioning with increase in tieline length (Fig. 1) may be attributed to changes in the relative composition of the phases [8]. An increase in tieline length promotes an increase in phosphate concentration in the lower phase whereas in the upper phase it remains relatively constant and equal to its solubility limit in PEG. This results in the salting-out of proteins from the phosphate-rich phase to the PEG-rich phase mediated by the excluded volume effects of PEG. In the limit proteins will precipitate.

Cytochrome b_5 partitioning also increases with increase in pH (Fig. 1). This is probably due to an increase of the $[HPO_4^{2-}]/[H_2PO_4^-]$ ratio, which promotes the shift of the phase diagram to lower polymer and salt concentrations. It is well known that small multivalent anions such as HPO_4^{2-}, used in conjunction with PEG, are more effective in inducing phase formation than monovalent anions [9] owing to the conflicting interaction between ether oxygens of PEG and small ions of high charge density [10]. The approach of salts, with these multivalent anions, to the polymer surface is constrained and a region of salt-depleted structured water is created at the polymer surface which might permit protein polyanion–PEG interactions [10]. The increase in the protein charge with increase in pH seems to be less important because cytochrome b_5 already has a high negative charge at the studied pH values.

Cytochrome b_5 partitioning in systems of PEG 1000 is strongly affected by the tieline length and pH. By changing the pH and/or the tieline length of the two-phase systems it is possible to manipulate cytochrome b_5 partitioning to the phosphate-rich phase or to the PEG-rich phase. This versatility of PEG 1000 systems for cytochrome b_5 partitioning makes them very attractive for separation and purification of cytochrome b_5 from an impure extract.

PEG 1000 was chosen for further partitioning studies with cytochrome b_5 mutants. Fig. 2 shows the effect of tieline length and pH on the partition coefficients and yields of cytochrome b_5 and cytochrome b_5 mutants in PEG 1000–potassium phosphate systems. Partitioning of the modified proteins (mutants 56 and 92) also increases with increasing tieline length and pH. The partition coefficients and yields obtained follow the order cytochrome $b_5 >$ mutant $56 >$ mutant 92.

The isoelectric point of cytochrome b_5 mutants (p*I* 4.7) is greater than the isoelectric point of cytochrome b_5 (p*I* 4.4), hence a decrease in modified protein partitioning would be expected due to the increase in their net positive charge. The differences in the partitioning behaviour of mutants 56 and 92 were attributed to the position of the mutated amino acids in the polypeptide chain. For mutant 92 the mutated amino acid is

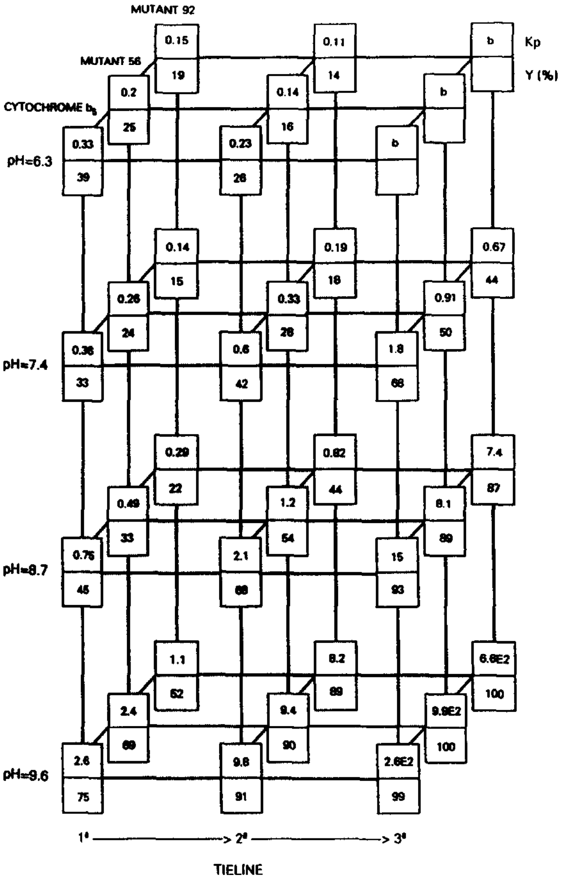

Fig. 2. Effect of tieline length and pH on the partition coefficients, K_p, and yields, Y, of cytochrome b_5 and cytochrome b_5 mutants (56 and 92) in PEG 1000–potassium phosphate systems at 23°C. (b) KH_2PO_4 did not dissolve.

situated at the C-terminus and is more accessible to interaction with both phases of the two-phasic system.

4. Conclusions

Cytochrome b_5 partitioning in PEG–potassium phosphate two-phase systems is enhanced with decreasing polymer molecular mass and increasing tieline length and pH. Partitioning of cytochrome b_5 mutants using PEG 1000 systems show the same dependence on tieline length and pH, following the order cytochrome $b_5 >$ mutant $56 >$ mutant 92. Owing to their versatility, PEG 1000–potassium phosphate systems seem to be suitable for application in more impure media.

The results of this work suggest that it is possible to change the partitioning behaviour of proteins by engineering the protein surface, for example by making it more positive or negative.

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