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Partitioning of recombinant *Fusarium solani pisi* cutinase in polyethylene glycol–aqueous salt solution two-phase systems

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Abstract

The partition behaviour of *Fursarium solani pisi* recombinant cutinase was studied in polyethylene glycol (PEG)–aqueous sodium or potassium phosphate solution biphasic systems. The partition coefficient of cutinase was enhanced with decreasing molecular mass of the PEG and increasing tie-line length and pH, and was also influenced by the type of cation (Na^+ or K^+) present in the system. Particular attention was paid to the influence of pH and the type of cation on the phase diagrams of PEG–phosphate systems.

1. Introduction

Liquid–liquid extraction using aqueous two-phase systems is a downstream processing technique that can be applied to the large-scale isolation of intracellular enzymes [1–3]. Besides being relatively easy to scale up, this purification technique offers mild conditions for the enzymes as both phases consist mainly of water and the interfacial tension between them is extremely low [4]. Further, aqueous biphasic systems show considerable versatility, allowing enzyme separations to be based on molecular mass, conformation, charge and/or hydrophobicity.

Lipases and other enzymes exhibiting lipolytic activity, such as cutinases [5], are particularly interesting catalysts for applications in the pharmaceutical, detergent and food industries [6]. The purification procedure for these enzymes could be improved by the introduction of liquid–liquid extraction in aqueous biphasic systems, which would avoid some drawbacks related to their hydrophobicity, namely non-specific adsorption on the surfaces of column supports and membranes [7]. A first approach to the optimization of PEG–sodium phosphate systems for the purification of some microbial extracellular lipases from fermentation broth was reported recently by Menge [7].

The aim of this work was to develop a polyethylene glycol (PEG)–sodium or potassium phosphate aqueous two-phase system for extraction of a cutinase from cell debris of a recombinant *Escherichia coli* strain. To define a successful purification system it is desirable to understand the main physico-chemical parameters that affect cutinase partitioning. Hence the influence of polymer molecular mass, polymer concentration, pH and type of cation on the enzyme partitioning properties was investigated using a purified cutinase preparation.

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2. Experimental

2.1. Chemicals

PEG 1000, 3350 and 8000 were supplied by Sigma. *Fusarium solani pisi* cutinase was produced and purified in our laboratory from an *Escherichia coli* recombinant strain [5], which was a kind gift from Corvas International (Ghent, Belgium).

2.2. Determination of binodials

Biphasic systems were prepared by the addition of appropriate amounts of NaH_2PO_4 or KH_2PO_4 and Na_2HPO_4 or K_2HPO_4 to PEG (50%, w/w) to obtain the required pH value. The systems were completed with water, leading to a total mass of 5.0 g. The mixtures were vortex mixed until complete solubilization of the salts. Fine adjustments of pH were made by adding small volumes of phosphoric acid.

The aqueous two-phase systems were diluted until they became clear after vortex mixing and the polymer and salt concentrations were calculated to determine the binodal. The assays were performed at 24 ± 1°C.

2.3. Preparation of aqueous two-phase systems for cutinase partition assays

The assays were carried out in 10-ml graduated centrifuge tubes with a conical tip. Stock aqueous solutions of PEG (50%, w/w) were measured by mass into the centrifuge tubes. Appropriate amounts of NaH_2PO_4 or KH_2PO_4 and Na_2HPO_4 or K_2HPO_4 were added to obtain the required pH. A 250-μl volume of an aqueous solution of purified cutinase (5 mg/ml) completed the system, which was made up to 5.0 g by addition of water. All phase systems were 10% (w/w) in phosphates. Biphasic systems at pH 5 and 6 were prepared with sodium salts while potassium phosphates were used for pH values ≥6, owing to the low solubility of Na_2HPO_4 and KH_2PO_4. The study of enzyme partitioning behaviour at different tie-lines was carried out by varying the PEG concentration as indicated in Table 1.

Table 1
Polyethylene glycol concentrations (%, w/w) in the phase systems studied

PEG M_r	Short tie-line	Long tie-line	Extra long tie-line
1000	20	25	30
3350	20	25	–
8000	6	12	–

The systems were vortex mixed until complete solubilization of the salts. The phases were separated by a 5-min centrifugation at 1000 *g* in a swing-out rotor. The volumes of the upper and lower phases were noted and a sample of each phase was taken with a pipette for the determination of cutinase activity. The assays were performed in triplicate.

2.4. Cutinase activity assay

The cutinase esterolytic activity was determined spectrophotometrically, following the hydrolysis of *p*-nitrophenyl acetate [8] or *p*-nitrophenyl palmitate [9]. For the former reaction, 5 μl of each phase, diluted 1:10 with distilled water, were added to 1 ml of a 0.5 m*M* *p*-nitrophenyl acetate solution in 50 m*M* potassium phosphate buffer (pH 8). The reaction was followed for 2–5 min at 400 nm. For the hydrolysis of *p*-nitrophenyl palmitate, 15 mg of this substrate were solubilized in 5 ml of 2-propanol and added to 45 ml of 50 m*M* potassium phosphate buffer (pH 8) containing 103.5 mg of sodium deoxycholate and 50 mg of gum arabic. A 40-μl volume of each phase diluted 1:10 with distilled water was added to 960 μl of the reaction buffer described above. The reaction was followed for 2–10 min at 410 nm. Both reactions were performed at 24 ± 1°C. A blank was prepared by the same procedure as described above, adding to the substrate solution a sample of top or bottom phase of a corresponding phase system that did not contain the enzyme. The total activity recovered in both

phases was compared with the initial preparation.

Preliminary studies in which the cutinase activity and stability were measured in the presence of high concentrations of PEG 1000, 3350, 8000 and phosphates were carried out. Under the conditions of the activity assays described above, no interference of these components of the phase systems on the enzyme activity was detected. Further, the cutinase maintained full activity for at least 3 h in the presence of polymer or salt.

3. Results and discussion

3.1. Effect of pH and type of cation on PEG–phosphate binodals

The influence of some physico-chemical parameters considered for the study of cutinase partitioning properties on the binodals of PEG–phosphate systems was analysed. Figs. 1 and 2 show the effect of pH and type of cation (Na^+ or K^+) on PEG 1000–phosphate and PEG 3350–phosphate binodals, respectively.

For both phase-forming polymers, decreasing pH values from 7.5 to 6.0 and from 6.0 to 4.5 increased the polymer and salt concentrations required for phase formation. This effect can be explained by the increase in the $H_2PO_4^-/HPO_4^{2-}$ ratio with decreasing pH. In fact, as the monovalent anion is less effective in salting out PEG [10], a higher salt and/or polymer concentration will be needed to obtain a biphasic system. At high pH values the displacement of binodals was almost negligible. The binodials obtained at pH 7.5 and 9.0 are almost coincident in systems formed by either PEG 1000 (Fig. 1) or PEG 3350 (Fig. 2).

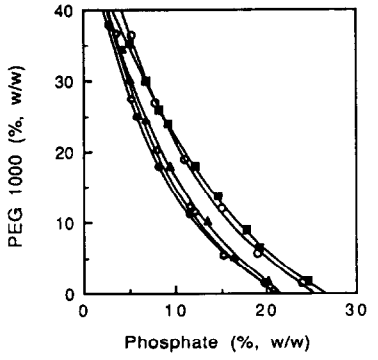

Fig. 1. Effects of pH and type of cation on the binodial of the PEG 1000–phosphate system at 24 ± 1°C. Sodium phosphate: ■ = pH 4.5; ● = pH 6.0. Potassium phosphate: ○ = pH 6.0; ▲ = pH 7.5; ◇ = pH 9.0.

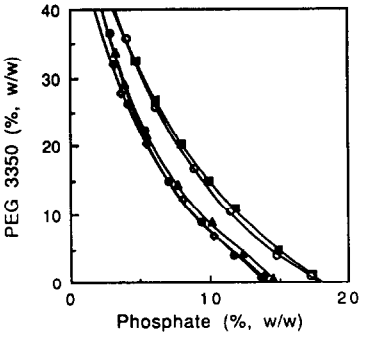

Fig. 2. Effects of pH and type of cation on the binodal of the PEG 3350–phosphate system at 24 ± 1°C. Symbols as in Fig. 1.

For the same pH (6), the replacement of sodium with potassium phosphates increased the polymer and/or salt concentrations required for phase formation (Figs. 1 and 2). The binodal obtained at pH 6 with sodium phosphates was almost coincident with that obtained at pH 9 with potassium phosphates and the binodals for the PEG 1000– or PEG 3350–potassium phosphate systems at pH 6 were almost coincident with those corresponding to PEG 1000– or PEG 3350–sodium phosphate systems at pH 4.5 (Figs. 1 and 2). These results suggest that Na^+ is more effective than K^+ in salting out PEG.

3.2. Effect of PEG molecular mass, pH and polymer concentration on cutinase partitioning

The influence of PEG molecular mass and concentration and pH on cutinase partitioning in PEG–sodium or potassium phosphate biphasic systems is shown in Tables 2–4. The partition coefficient, K, is defined as the ratio between cutinase activity per millilitre in the upper and lower phases. Each K value represents the average of three measurements. Eight independent

Table 2
Effects of pH and polymer concentration on the cutinase partition coefficient, K, and total enzyme yield from both upper and lower phases in PEG 1000–sodium or potassium phosphate (10%, w/w) biphasic systems at 24 ± 1°C

pH	PEG (%, w/w)	K	Yield (%)
5	–	–	–
	25	10	81
	30	48	97
6	20	6.8	97
	25	27	98
	30	91	100
8	20	20	100
	–	–	–
	30	141	100
9	20	41	100
	25	165	100
	30	303	100

measurements were carried out for one selected system to obtain the statistical significance of the results. The relative standard deviation of the partition coefficient was 11%. Results shown for pH 6 and 20% or 25% PEG are the average of the partition coefficients calculated for phase systems containing sodium and potassium phosphates.

In phase systems formed by PEG 1000, cutinase was mainly in the upper phase under all the experimental conditions tested (Table 2). When PEG 1000 was replaced with PEG 3350 (Table 3), pH and polymer concentration determined the predominance of the enzyme in either the upper or lower phase. Biphasic systems containing PEG 8000 led cutinase to accumulate preferentially in the lower phase under all the experimental conditions tested (Table 4). The decrease in protein partition coefficient with increasing PEG molecular mass in PEG–phosphate phase systems has been described previously and is probably a consequence of excluded volume effects [11].

Table 3
Effects of pH and polymer concentration on the cutinase partition coefficient, K, and total enzyme yield from both upper and lower phases in PEG 3350–sodium or potassium phosphate (10%, w/w) biphasic systems at 24 ± 1°C

pH	PEG (%, w/w)	K	Yield (%)
5	20	0.50	71
	25	0.75	63
6	20	0.36	81
	25	1.4	75
8	20	0.67	100
	25	3.9	98
9	20	2.8	100
	25	15	100

Table 4
Effects of pH and polymer concentration on the cutinase partition coefficient, K, and total enzyme yield from both upper and lower phases in PEG 8000–sodium or potassium phosphate (10%, w/w) biphasic systems at 24 ± 1°C

pH	PEG (%, w/w)	K	Yield (%)
6	–	–	–
	12	0.10	100
8	6	0.07	100
	12	0.06	100
9	6	0.11	100
	12	0.13	100

Polymer concentration and pH proved to be important factors for cutinase partitioning in biphasic systems formed by PEG 1000 and 3350 (Tables 2 and 3). The increase in enzyme partition coefficient with increase in PEG concentration may result from changes in the specific volume of the phases. As more PEG is added to the system the specific volume of the upper phase remains approximately constant while the specific volume of the lower phase decreases rapidly [12]. Hence the water molecules available for solute solvation in the lower phase decrease and cutinase reaches its solubility limit, being forced to partition to the upper phase.

The partition coefficient of cutinase increased strongly with increase in pH (Tables 2 and 3). These results may be partly explained by the displacement of the binodal towards lower polymer and salt concentrations with increasing pH (Figs. 1 and 2), which could be compared with the effect of increasing PEG concentration, as

described above. However, the binodals for phase systems at pH 7.5 and 9 (both containing potassium phosphates) are almost superimposable and the cutinase partition coefficients at these pH values are significantly different, particularly for the systems containing PEG 3350. In fact, the greatest increase in cutinase partition coefficient in these systems is observed when the pH is raised from 8 to 9.

In addition to changes in free volume, other physico-chemical factors seem to be involved in the enzyme partitioning behaviour with pH such as the charge variation of protein ionic groups and their interaction with polymer molecules [13].

3.3. Effect of type of cation on cutinase partitioning and yield

Table 5 shows the influence of the type of cation (Na^+ or K^+) on cutinase partitioning in phase systems formed by PEG 1000 or PEG 3350 and phosphates at the same pH (6). In biphasic systems containing PEG 1000 the enzyme partition coefficient appeared to decrease when Na^+ was replaced with K^+. The displacement of the binodal towards higher polymer and salt concentrations observed when potassium phosphates were used instead of sodium phosphates (Fig. 1) suggests that changes in the free volume of the phases are responsible for the effect of the type of cation on the cutinase partitioning pattern.

An opposite effect of the type of cation on the cutinase partitioning was observed for phase systems formed by PEG 3350, in which K increased when sodium phosphates were replaced with potassium phosphates. In these systems, sodium phosphates caused cutinase precipitation, only about 50% of the enzyme being recovered in both the upper and lower phases (Table 5). Protein precipitation did not occur when potassium salts were present or when PEG 1000 was the phase-forming polymer (Table 5). According to the influence of the type of cation on the binodial (Fig. 2), aqueous two-phase systems formed by PEG 3350 and sodium phosphates would possibly induce an increase in the cutinase partition coefficient, as happened for systems containing PEG 1000, if the enzyme did not reach its solubility limit in the upper phase.

Table 5
Effect of the type of cation on the cutinase partition coefficient, K, and total cutinase yield from both upper and lower phases in PEG (25%, w/w)–phosphate (10%, w/w) biphasic systems at 24 ± 1°C and pH 6

Cation	PEG M_r	K	Yield (%)
Na^+	1000	31	95
	3350	1.2	50
K^+	1000	22	100
	3350	1.6	100

4. Conclusions

The partition coefficient of cutinase was enhanced with decreasing PEG molecular mass, probably owing to PEG excluded volume effects. Increasing the polymer concentration promoted the salting out of cutinase, enhancing the partitioning of the enzyme to the upper phase. Increasing the pH also raised the cutinase partition coefficient, which may partly result from the displacement of the binodal towards lower polymer and/or salt concentrations. In fact, increasing the pH decreased the polymer and/or salt concentrations required for phase formation as the divalent anion HPO_4^{2-} is more effective in salting out PEG than the monovalent anion $H_2PO_4^-$.

For the same pH (6), the replacement of potassium with sodium phosphates displaced the binodal towards lower polymer and/or salt concentrations, suggesting that Na^+ is more effective than K^+ in salting out PEG. This shift of the binodal to lower compositions in the presence of Na^+ raised the tie-line length of PEG 1000 or PEG 3350 (25%)–phosphate (10%) phase systems, probably leading to an increase in the salting out of the enzyme. This effect resulted in the enhancement of the enzyme partition coefficient for the system formed by PEG 1000. In the PEG 3350–sodium phosphate phase system, cutinase apparently reached its solubility limit in

both phases, being subject to the excluded volume effects of PEG 3350, and was partially precipitated.

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