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# Cutinase–peptide fusions in thermoseparating aqueous two-phase systems
## Prediction of partitioning and enhanced tag efficiency by detergent addition

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**Abstract**

It is of increasing importance to develop efficient purification methods for recombinant proteins where the number of steps can be minimised. The aim has been to establish a method for predicting the partitioning of the wild-type target protein in an aqueous two-phase system, and with this as basis, develop fusion tags and optimise the phase system for enhanced partitioning of the target protein. The surface of the lipolytic enzyme cutinase from *Fusarium solani pisi* was investigated with a computer program, Graphical Representation and Analysis of Surface Properties (GRASP). The accessible surface areas for the different amino acid residues were used together with peptide partitioning data to calculate the partition coefficient for the protein. The separation system was composed of a thermoseparating random copolymer of ethylene oxide and propylene oxide, Breox PAG 50A 1000, as top phase forming polymer and a hydroxypropyl starch polymer, Reppal PES 200, as bottom phase polymer. The calculated partition coefficient for the wild-type protein ($K=1.0$) agreed reasonably well with the experimentally determined value ($K=0.85$). Genetic engineering was used to construct fusion proteins expressed in *Saccharomyces cerevisiae* based on cutinase and peptide tags containing tryptophan, to enhance the partitioning in aqueous two-phase systems. The partitioning of the cutinase constructs could qualitatively be predicted from peptide partitioning data, i.e. the trends in partitioning could be predicted. A spacer peptide introduced between protein and tag increased the partitioning of the protein towards the ethylene oxide–propylene oxide (EOPO) copolymer top phase. The aqueous two-phase system was modified by addition of detergent to increase the partitioning of the cutinase variants towards the EOPO copolymer phase. Triton and a series of $C_{12}E_n$ detergents selectively increased the partitioning of cutinase constructs with $(WP)_4$-based tags up to 14 times compared to wild-type cutinase. The protein partition could almost quantitatively be predicted from the peptide partition data. © 2002 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

In bioseparation processes it is desirable to have as few steps as possible. When working with recombinant proteins it is possible to genetically introduce a tag to achieve better separation. The main aim of this study was to investigate the effect of hydrophobic tags fused to a target protein. It would be advantageous to be able to predict the partitioning of the target protein and to design tags and modify the system for optimal separation.

An aqueous polymer two-phase system is formed when two structurally different polymers above certain concentrations are mixed with water [1]. The resulting two phases are each enriched in one polymer, but the main component in both phases is water. Due to the high water content in the phases (80–95%) and the low surface tension between the phases, aqueous two-phase systems constitute a mild method for separation of biomaterial. Rapid phase separation can be achieved and the systems can be scaled up for large-scale purification. Proteins and other biomaterial will partition between the polymer phases depending on interactions with the phase components. The surface hydrophobicity [2] and net charge [3] of the protein are important factors that determine the partitioning. The partition is also affected by the salt composition of the system [1,4–6].

The most commonly used aqueous polymer two-phase system is composed of poly(ethylene glycol) (PEG) and dextran. Since dextran is a rather expensive polymer, research efforts have been devoted on developing alternative polymers for large-scale applications [7–9]. Also other alternative polymers to PEG have been investigated. Thermoseparating EOPO copolymers have been studied for use in phase systems with dextran or hydroxypropyl starch [10–19]. The polymers are composed of ethylene oxide (EO) and propylene oxide (PO) units, which are randomly located along the polymer chain. The EOPO copolymers display inverse solubility, i.e. the polymers become less soluble in water when the temperature is increased [10–13]. Above a certain temperature, the cloud point, the polymer solution phase separates in one polymer-rich phase and one polymer-depleted (water) phase. Proteins have been found to partition almost exclusively to the water phase due to the entropic driving force, which favours partition to the water phase [20]. The polymer-rich EOPO phase is relatively more hydrophobic compared to the water phase and hydrophobic amino acids and peptides have been partitioned towards the EOPO phase [21,22].

In this study a thermoseparating EOPO copolymer, Breox ($EO_{50}PO_{50}$), has been used as top phase forming polymer and a hydroxyproyl starch polymer, Reppal PES 200, as bottom phase forming polymer. Both polymers are available in large quantities and low price and thus the aqueous two-phase system can be scaled up for industrial bioseparations. The protein investigated in this study was cutinase, which is a lipolytic enzyme, from *Fusarium solani pisi*. Cutinase catalyses the hydrolysis of the water-insoluble biopolyester cutin, which covers the surface of plants. The protein is also able to hydrolyse water-soluble esters [23].

Recently it has been shown that the partitioning of proteins could be correlated to their surface hydrophobicity [24]. Thus, it would be advantageous if it was possible to predict the partitioning of a protein before starting a separation procedure. In this study the surface of cutinase was investigated by the computer program Graphical Representation and Analysis of Surface Properties (GRASP) [25]. The calculations of the surface exposure of the various amino acid residues in the protein were used together with peptide partitioning data to calculate the partitioning coefficient of the protein.

Genetically engineered cutinase constructions expressed in *Saccharomyces cerevisiae* with tryptophan-rich peptide tags attached to the C-terminal were investigated in this study. The cutinase constructions were designed and produced within the European Union (EU) project ‘‘Integrated bioprocess design’’ (BIO4-CT96-0435) for developing effective recovery systems of recombinant proteins. The aim of the fusion tag was to alter the hydrophobicity of the protein and thereby direct the protein towards a more hydrophobic polymer phase, in this case the thermoseparating EOPO phase. The thermoseparating ability of the EOPO copolymer could be used to obtain the polymer in one phase and the protein in a polymer-depleted water phase, by increasing the temperature. The effect of introducing a spacer between the protein and the tag was also investi-

gated. A spacer between the protein and the tag should result in better exposure of the tag towards the polymer solution, resulting in increased effect of the peptide tag. Other fusion proteins with tryptophan containing tags have previously been studied [18,19,26–30].

The aqueous two-phase system was modified by adding detergents to increase the partitioning of the genetically engineered protein constructs by utilising hydrophobic interactions between tag and detergent micelles. A set of detergents with different lengths of polyethylene oxide chains was investigated. Previously it has been shown that the detergent could be recycled together with the thermoseparating EOPO copolymer [17].

## 2. Materials and methods

### 2.1. Peptides and proteins

The peptides $(WP)_n$=$(Trp–Pro)_n$, where $n$=2 and 4, $(W)_4$=$(Trp)_4$, (TGGSGG)=(Thr–Gly–Gly–Ser–Gly–Gly), $(Asp)_3$, $(Gln)_3$, $(Gln)_4$, $(His)_3$, $(His)_4$ and $(Ile)_4$ were synthesised by Synpep (Dublin, CA, USA). $(W)_n$=$(Trp)_n$, where $n$=1–3, $(Ala)_2$, $(Ala)_3$, $(Ala)_4$, $(Asp)_2$, $(Lys)_2$, $(Lys)_4$, $(Phe)_2$, $(Phe)_3$, $(Phe)_4$, $(Tyr)_2$, $(Tyr)_3$ and $(Tyr)_6$ were purchased from Sigma (St. Louis, MO, USA). (WP)=(Trp–Pro), $(Asp)_4$, $(Ile)_2$, $(Ile)_3$ $(Gln)_2$, $(His)_2$, $(Lys)_5$ and $(P)_n$=Pro, where $n$=1, 3 and 4, were purchased from Bachem (Bubendorf, Switzerland). Bovine serum albumin (BSA) was obtained from Sigma.

*S. cerevisiae* strains expressing cutinase wild type (MM01-pUR7320-9), cutinase-$(WP)_2$ (MM01-pUR807-1), cutinase-$(WP)_4$ (MM01-pUR806-6) and cutinase-TGGSGG-$(WP)_4$ (MM01-pUR817) were constructed at Unilever (Vlaardingen, The Netherlands) within the EU project ''Integrated bioprocess design''. Also purified cutinase wild type and cutinase-$(WP)_4$ were provided by Unilever.

The cutinase constructions were cultivated on agar plates in selective medium (0.67% yeast nitrogen base without amino acids, 2% glucose and 2% agar) for 48 h at 30 °C. Colonies from the agar plates were inoculated in 50 ml selective medium (same as for the plates, except for the agar) and cultivated for 48 h in 250 ml baffled shake flasks. The cultures were transferred and diluted 10-fold into rich medium (2% yeast extract, 1% bactopeptone, 2% glucose and 2.5% galactose) and cultivated for 48 h at 30 °C. The culture broth was cooled and centrifuged for 10 min at 4000 *g*. The main protein in the supernatant was cutinase. Before partitioning, the supernatant was desalted on a PD10 column from Amersham Pharmacia Biotech (Uppsala, Sweden).

The molecular mass of some of the cutinase constructions has been determined by electrospray mass spectrometry, to confirm the presence of the peptide tags (spectra not shown). For cutinase-$(WP)_2$ the calculated molecular mass was 21 175 and the mass spectrometry value was 21 172 and for cutinase-$(WP)_4$ the calculated value was 21 741 and the experimentally obtained value was 21 739. Thus, we conclude that the tags are preserved after production in *S. cerevisiae*.

### 2.2. Chemicals

The top phase polymer Breox PAG 50A 1000 [a random copolymer of 50% (w/w) ethylene oxide and 50% (w/w) propylene oxide] with a molecular mass of 3900 was obtained from Laporte Performance Chemicals (Southampton, UK). Reppal PES 200, which constituted the bottom phase polymer, was obtained from Carbamyl (Kristianstad, Sweden). Triton X-100 and $C_{12}E_9$ (Thesit) were purchased from Boehringer Mannheim (Mannheim, Germany) and Tween 20, Tween 80 and $C_{12}E_{23}$ (Brij 35) from Sigma. $C_{12}E_5$ was purchased from Nikko (Tokyo, Japan). Yeast extract, bactopeptone, yeast nitrogen base without amino acids and bactoagar were purchased from Difco Labs. (Detroit, MI, USA). All chemicals were of analytical grade.

### 2.3. Computer analysis (GRASP)

The computer program GRASP can be used to study protein surfaces [25]. Accessible surface areas of certain amino acids or a whole protein can be calculated. The input to the program is a protein data bank (PDB) file. The accessible surface area is defined as the area obtained by the centre of a probe when rolled on the Van der Waals surface of the protein. The default value of the probe radius is 1.4

Å, representing the radius of a water molecule. Cutinase has been crystallised and the structure has been determined with 1 Å resolution [31]. PDB-file:1CEX.

### 2.4. Two-phase diagrams

The binodal curve for the Breox–Reppal phase diagram was titrated, as well as the ones for systems containing an addition of 1% (w/w) of $C_{12}E_5$, $C_{12}E_9$ and $C_{12}E_{23}$. A set of two-phase systems with different composition were prepared. The systems were mixed and equilibrated and known amounts of water were added in small quantities until a one-phase system was obtained. The concentrations of polymer and detergent representing the transition from two-phase to one-phase system were calculated and the data points were represented in a two-phase diagram. Information about the detergents used in the study is compiled in Table 1.

Systems consisting of 7% (w/w) Breox and 9% (w/w) Reppal PES 200 with a total mass of 2 g, were made by weighting up appropriate amounts of a 100% solution of Breox and a 20% (w/w) stock solution of Reppal PES 200, in calibrated test tubes. The exact concentration of the Reppal PES 200 solution was determined by polarimetry, by using a standard curve. When detergents were added in the systems, stock solutions of 25% (w/w) were utilised.

Peptides were added to the systems from pre-made stock solutions, giving a final concentration of approximately 0.1 mg peptide/g of the system mass. $(W)_4$ and $(WP)_4$ as well as the phenylalanine containing peptides were dissolved in an aqueous solution of Breox (10%, w/w) due to their low solubility in water. This amount of Breox was included in the total Breox content in the phase system. Stock solutions of pure proteins were made giving a final concentration of 10–15 μg protein/g of the system mass.

Sodium phosphate buffer (NaP) was used at pH 7.0 and sodium acetate (NaAc) at pH 5.0. The effect of potassium sulphate ($K_2SO_4$) was investigated as well as triethylammonium phosphate ($Et_3NHP$). Triethylammonium phosphate was prepared by titration of triethanolamine with phosphoric acid to the desired pH. The concentration of buffer was 5 m*M* and the concentration of the salt was 50 m*M*. As the concentration of the salt was 10 times higher than the buffer, the salt was dominating in the system.

The partitioning of a substance is described by the partitioning coefficient, *K*, which is defined as the concentration of the substance in the top phase ($C_t$) divided by the concentration in the bottom phase ($C_b$). All partition coefficients are averaged values from at least duplicate experiments.

The systems were carefully mixed by inverting the test tubes for 2 min and equilibrated for 20 min at

Table 1
Information about the different detergents used in the study

| Detergent | *M* | *n* | CMC (m*M*) | Cp (°C) | Structure |
|---|---|---|---|---|---|
| $C_{12}E_5$ | 405 | 160 | 0.065 | 31.5 | $(O\,CH_2\,CH_2)_n$ - OH |
| $C_{12}E_9$ | 583 | 120 | <0.1 | 79 | |
| $C_{12}E_{23}$ | 1200 | 40 | <0.1 | | |
| Triton X-100[a] | 625 | 140 | 0.3 | 65 | $(O\,CH_2\,CH_2)_n$ - OH |
| Tween 20[b] | 1228 | <58 | 0.06 | | C-$(O\,CH_2\,CH_2)_w$ - O-$CH_2$-CH- ; $(O\,CH_2\,CH_2)_x$ - OH ; $(O\,CH_2\,CH_2)_y$ - OH ; $(O\,CH_2\,CH_2)_z$ - OH |
| Tween 80[c] | 1310 | 58 | 0.01 | | |

*M*, molecular mass; *n*, average number of monomers present in the micelle; CMC, critical micelle concentration; and Cp, cloud point. Data from Refs. [44,45].

[a] $C_8ØE_{9.6.}$

[b] $C_{12}$sorbitan$E_{20}$.

[c] $C_{18:1}$sorbitan$E_{20}$.

room temperature (21 °C). Then the systems were mixed again for 2 min and centrifuged at 800 *g* for 8 min. The top and bottom phases were separated and diluted for protein respectively peptide concentration determination. A standard activity assay comprising mixed micelles of detergent and substrate was used for determination of the concentration of the various cutinase constructions. The substrate, *p*-nitrophenylbutyrate (PNPB, from Sigma) was added as a stock solution (50 m*M* PNPB in acetonitrile) to the assay buffer (10 m*M* Tris–HCl pH 8.0, 10 m*M* NaCl and 50 m*M* taurodeoxycholate, TDOC, from Sigma) to a final concentration of 1 m*M*. The enzymatic activity was monitored spectrophotometrically at 400 nm at 21 °C, by measuring the release of *p*-nitrophenolate. The partition coefficient was calculated as the ratio of enzyme concentrations in the top and bottom phases. The polymers used in the two-phase systems affected the activity of the protein. To compensate for this a blank system was prepared (all components included except protein). When diluting the top phase the same amount bottom phase from a blank system, was added together with the top phase sample and vice versa when measuring the activity of the bottom phase. By this measure, all samples will have the same polymer concentration and the activity will be equally affected by the polymers.

The concentration of BSA, peptides containing tryptophan and Triton X-100 were determined by measuring the absorbance at 280 nm and subtracting the absorbance at 320 nm, as background absorbance. The contribution of the polymers was measured with blank systems diluted to the same extent as the samples. Peptides lacking tryptophan were analysed at 220 nm, by correcting with the absorbance at 320 nm.

### *2.5. Reference system*

In aqueous two-phase systems salts affect the partition in different ways. A salt where the anion and cation have different affinities for the two phases will influence the partitioning of a protein or peptide with a positive or negative net charge [1,5,6,32]. The mechanism of the salt effects on partitioning in aqueous two-phase systems has been much investigated. One widely accepted explanation of the effect is the creation of an electrical potential difference at the interface between the two phases [1,3,5,6,20,33–35]. The requirement for electroneutrality forces the anion and cation to partition together, but the different affinities of the ions for the two polymer phases will generate an electrical potential difference across the interface.

To investigate effects such as hydrophobicity and other non-electrostatic contributions, it is important to be able to uncouple the charge dependent effects of the partitioning. One way is to perform the partitioning at the isoelectric point (p*I*) of the protein, since the protein has no net charge. However, this could be a problem for many proteins, due to low solubility at this pH. Instead, a reference system, consisting of 50 m*M* of potassium sulphate ($K_2SO_4$), buffered with 5 m*M* sodium phosphate (NaP) at pH 7.0 has been used in this study. $K_2SO_4$ generates a potential difference close to zero in a PEG–dextran system [5] as well as in a thermoseparating $EO_{30}PO_{70}$–dextran system [19], and thereby minimising the contribution of charge-dependent salt effects. To investigate if the $K_2SO_4$ system could be used as reference even in a Breox–Reppal system partitioning of BSA was performed at pH 5.0 and 7.0. The net charges of BSA at pH 5.0 and 7.0 are approximately +4 and −10, respectively [36]. The partitioning coefficient differed only slightly between pH 5.0 and 7.0 with $K_2SO_4$ in the system, with *K* values of 0.38 and 0.44. When instead using $Et_3NHP$ as the dominating salt, the *K* value was 0.51 at pH 5.0 and 1.26 at pH 7.0. Hence, using $K_2SO_4$ as the dominating salt in the system resulted in a potential difference close to zero, and $K_2SO_4$ could be used in a reference system for minimising charge-dependent salt effects.

## 3. Results and discussion

### *3.1. Two-phase systems*

The binodal curve for the phase diagram containing Breox and Reppal is shown in Fig. 1 and has been published before [17]. Below the binodal curve all components are in a single phase and above the curve two separate phases are formed. In order to study the effect of detergent on the phase diagram, the system was studied with additions of 1% (w/w)

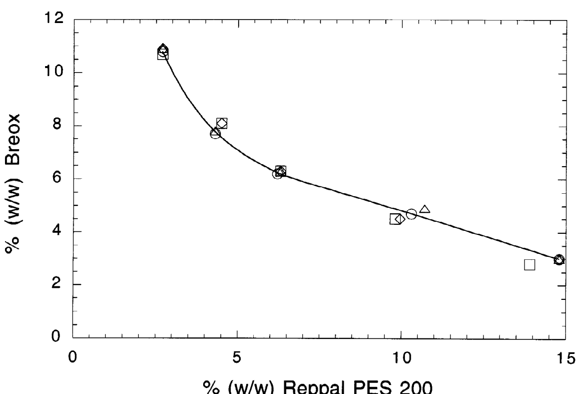


Fig. 1. The binodal curve for the Breox PAG 50A 1000–Reppal PES 200 system (○). The effect of addition of 1% (w/w) $C_{12}E_5$ (□), 1% (w/w) $C_{12}E_9$ (◊) and 1% $C_{12}E_{23}$ (△), respectively.

detergent. The points in the Breox–Reppal phase diagram showing the transition from one to two phases were determined for systems containing an addition of 1% (w/w) of the non-ionic detergents $C_{12}E_5$, $C_{12}E_9$ and $C_{12}E_{23}$ (Fig. 1). The transition points were all shown to be located on the binodal curve for the Breox–Reppal system (Fig. 1). Further on, the volume ratio in the two-phase system between the top and the bottom remained constant, 1.6±0.1 when the detergents were added. The volume ratio between the phases was also constant when 2% (w/w) of the detergent was added to the system. Thus, we conclude that the phase diagram for the Breox–Reppal system is not changed to any significant degree by addition of the alkyl-ethylene oxide detergents at concentrations of 2% and lower.

### *3.2. Prediction of the partitioning coefficient*

The partitioning of a protein depends on many factors. The most important factors are protein surface hydrophobicity [2], net charge [3–5] and size [20,37]. It would be advantageous if it was possible to predict the partitioning of a target protein before starting purification by aqueous two-phase partitioning. Time-consuming work of experimental testing of systems would be saved. Previously, it has been shown [24] that the partitioning of several monomeric proteins could be correlated to protein surface hydrophobicity. The best correlation was obtained based on peptide partitioning data in $EO_{30}PO_{70}$–dextran T500 systems.

The aim of this study has been to establish a method for predicting the *K* value of the wild type target protein, and with this as basis, design fusion tags and optimise the phase system for enhanced partitioning of the target protein. In order to develop separation technology suitable for large-scale separations, the hydroxypropyl starch, Reppal PES 200 was used instead of dextran as bottom phase forming polymer and the recyclable thermoseparating EOPO copolymer Breox PAG 50A 1000 as top phase polymer.

#### *3.2.1. Peptide partitioning*

Data on peptide partitioning in the Breox–Reppal system was used for determining the contribution to the protein *K* value of amino acid residues. Oligopeptides with increasing number (*n*) of the same residue were studied. The slope of the log *K* vs. *n* plot was determined and represents the contribution of one (fully exposed) residue to the partitioning. Peptides consisting of aromatic amino acid residues have previously been shown to partition towards an EOPO phase [18,19,22,38]. Slope values, $\partial \log K/\partial n$, for peptides of the aromatic amino acids, phenylalanine, tryptophan and tyrosine were all determined separately, which was also done for histidine and proline. The remaining of the 20 amino acids used in proteins were divided in different groups with the data for one type of amino acid representing the entire group. The polar, uncharged amino acids, glutamine, asparagine, methionine, cysteine, threonine and serine were all represented by $\partial \log K/\partial n$ obtained from partitioning of glutamine peptides. The partitioning of alanine peptides represented the short aliphatic amino acids, alanine and valine. The partitioning of isoleucine peptides represented the longer aliphatic amino acids, leucine and isoleucine. Partitioning of lysine peptides represented positively charged lysine and arginine. Values for the negatively charged aspartate and glutamate were obtained from aspartate peptide partitioning. Glycine was assigned a $\partial \log K/\partial n$ value of zero. In an $EO_{30}PO_{70}$–dextran system a $\partial \log K/\partial n$ value of zero has previously been obtained for glycine [19].

In all cases a linear correlation between the

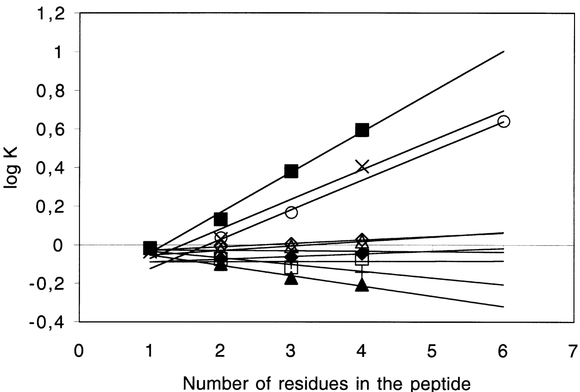


Fig. 2. The logarithm of the partition coefficient of peptides of different lengths containing only one type of amino acid residue. Partitioning was determined in the Breox–Reppal system with 50 m*M* $K_2SO_4$ as the dominating salt and 5 m*M* NaP, pH 7.0. Ala (◊), Asp (□), Lys (▲), Phe (×), Trp (■), Tyr (○), Ile (△), Gln (♦), His (+) and Pro (−). The experimental error was estimated to maximum 10% of the *K* value.

logarithm of the partition coefficient and the number of peptide residues were found (Fig. 2). The $\partial \log K / \partial n$ values for the various amino acid residues are collected in Table 2. The aromatic residues resulted in the largest increase towards the EOPO phase. Tryptophan was the most efficient amino acid to partition towards the EOPO phase, but it is important to note that six tyrosines were as effective as four tryptophans. Thus, tyrosine could offer an alternative to the tryptophan-based tags.

The aliphatic amino acids, alanine and isoleucine only slightly preferred the EOPO phase. The weak effect of aliphatic hydrophobic residues was obtained also in the $EO_{30}PO_{70}$–dextran system [38]. The lysine peptides preferred the Reppal phase. The positively charged peptides are hydrophilic and thus would be expected to prefer the more hydrophilic bottom phase. The negatively charged aspartates had a $\partial \log K / \partial n$ value of zero. This is consistent with earlier results found for $(DP)_n$ peptides in an $EO_{30}PO_{70}$–dextran system [19]. Several peptides showed an even partitioning between the phases (Pro) or with only slight preference for the Reppal phase (His), alternatively for the EOPO phase (Gln).

The propylene oxide content in the top phase polymer Breox is lower (50%, w/w) than in the

Table 2
Information compiled from surface analysis of cutinase

| Amino acid | Number of residues | *ASA* (Å$^2$) in cutinase | *ASA* (Å$^2$) Gly–X–Gly | *DE* | $\partial \log K / \partial n$ |
|---|---|---|---|---|---|
| Gln | 5 | 237 | 189 | 1.3 | 0.0142 |
| Asn | 10 | 576 | 158 | 3.6 | 0.0142 |
| Ser | 13 | 792 | 122 | 6.5 | 0.0142 |
| Thr | 14 | 826 | 146 | 5.7 | 0.0142 |
| Cys | 4 | 105 | 140 | 0.7 | 0.0142 |
| Met | 1 | 0 | 204 | 0 | 0.0142 |
| Pro | 9 | 470 | 143 | 3.3 | 0 |
| Gly | 24 | 419 | 85 | 4.9 | 0 |
| Ala | 27 | 711 | 113 | 6.3 | 0.0177 |
| Val | 9 | 222 | 160 | 1.4 | 0.0177 |
| Ile | 13 | 231 | 182 | 1.3 | 0.0234 |
| Leu | 17 | 567 | 180 | 3.1 | 0.0234 |
| Phe | 6 | 90 | 218 | 0.4 | 0.1531 |
| Trp | 1 | 23 | 259 | 0.1 | 0.2086 |
| Tyr | 6 | 24 | 229 | 0.1 | 0.1527 |
| His | 1 | 6 | 194 | 0.03 | −0.0347 |
| Lys | 6 | 399 | 211 | 1.9 | −0.0535 |
| Arg | 13 | 1380 | 241 | 5.7 | −0.0535 |
| Asp | 12 | 882 | 151 | 5.8 | 0 |
| Glu | 6 | 407 | 183 | 2.2 | 0 |

The number of each amino acid residue present in cutinase, the accessible surface area of each residue type in the protein and in a Gly–X–Gly peptide [39] and the degree of exposure of the different amino acid residues. The $\partial \log K / \partial n$ values obtained (from Fig. 2) for the various amino acid residue types are also included.

$EO_{30}PO_{70}$ (70%, w/w). Due to the lower propylene oxide content of Breox one can assume the system being less sensitive to hydrophobic amino acids. This is consistent with experimental findings, i.e. slightly lower slope values ($\partial \log K/\partial n$), were obtained for the peptides in the Breox–Reppal system (Fig. 2) than in the $EO_{30}PO_{70}$–dextran system [24,38].

*3.2.2. Surface analysis*

The accessible surface area for the cutinase protein was calculated by the computer program GRASP [25,38]. The accessible surface area for each amino acid residue type was also calculated. The values are compiled in Table 2. Included in the table are the accessible surface areas for the different amino acids in a Gly–X–Gly peptide [39], where X represents the various amino acids. In the Gly–X–Gly peptide the amino acid residue, X, is fully exposed. By dividing the accessible surface area (*ASA*) in the protein for a residue X with the value achieved in the Gly–X–Gly peptide, the degree of exposure (*DE*) for each residue type is obtained:

$$DE = \frac{ASA_{\text{X in protein}}}{ASA_{\text{Gly-X-Gly}}} \quad (1)$$

*3.2.3. Calculation of K value*

In the partitioned peptides, the amino acid residues are assumed to be fully exposed. Thus, $\partial \log K/\partial n$ could be used as a measurement of how much one residue contributes to partitioning, when fully exposed. By taking the product of the degree of exposure with the respective $\partial \log K/\partial n$ value, the contribution to the partition coefficient for each type of amino acid residue in the protein can be obtained:

$$\log K_X = DE_X \cdot \frac{\partial \log K_X}{\partial n} \quad (2)$$

The estimated partition coefficient for the protein is obtained by:

$$\log K_{\text{protein}} = \sum \log K_X \quad (3)$$

The sum is over all residues in the protein. In earlier calculations [24] the effect of N- and C-terminals was not taken into account. Since these are fully solvent accessible and hydrophilic, they are expected to drive the partitioning towards the more hydrophilic Reppal bottom phase. Contributions of the N- and C-terminals could be calculated from the $\partial \log K/\partial n$ values obtained from the peptides:

$$\begin{aligned} \text{N-terminal } (NH_3^+) &= \frac{\partial \log K_{\text{Lys}}}{\partial n} - \frac{\partial \log K_{\text{Ile}}}{\partial n} \\ \text{C-terminal } (COO^-) &= \frac{\partial \log K_{\text{Asp}}}{\partial n} - \frac{\partial \log K_{\text{Ala}}}{\partial n} \end{aligned} \quad (4)$$

The accessible surface area (*ASA*) determined by GRASP depends on the value chosen for the probe radius. The program default value 1.4 Å corresponds to a water molecule, which could be too short considering the interaction between protein and polymer monomer units. It has in theoretical calculations earlier been shown that a depletion layer with lower polymer concentration is established around a protein in the top PEG phase in PEG–dextran systems [40]. This motivated a study of the effect of increasing the probe radius in GRASP.

In Table 3 the accessible surface area for the entire cutinase protein as well as the different residue types are represented at various probe radius values. The

Table 3
The effect of the size of the probe radius ($r$) used in GRASP on the calculated accessible surface area ($A$) for the amino acid residues in the protein and the total area ($A_{tot}$)

| Amino acid | $A$ (%) of $A_{tot}$ | | |
|---|---|---|---|
| | $r$=1.4 Å | $r$=2.8 Å | $r$=5.0 Å |
| Gln | 2.8 | 2.8 | 2.5 |
| Asn | 6.8 | 7.8 | 6.7 |
| Ser | 9.3 | 10 | 10 |
| Thr | 9.8 | 11 | 11 |
| Cys | 1.2 | 0.7 | 0.2 |
| Met | 0 | 0 | 0 |
| Pro | 5.6 | 5.3 | 5.4 |
| Gly | 4.9 | 4.1 | 3.5 |
| Ala | 8.4 | 9.2 | 10 |
| Val | 2.6 | 2.3 | 1.9 |
| Ile | 2.7 | 2.8 | 3.2 |
| Leu | 6.7 | 6.3 | 6.0 |
| Phe | 1.1 | 0.6 | 0.2 |
| Trp | 0.3 | 0.1 | 0 |
| Tyr | 0.3 | 0 | 0 |
| His | 0.1 | 0 | 0 |
| Lys | 4.7 | 4.3 | 3.7 |
| Arg | 16 | 18 | 19 |
| Asp | 10 | 11 | 11 |
| Glu | 4.8 | 4.4 | 4.1 |
| $A_{tot}$ (Å$^2$) | 8472 | 8343 | 9353 |

aromatic amino acids are not exposed to a large extent in the cutinase protein, but since they have the highest $\partial \log K/\partial n$ their contributions are significant in the calculated partition coefficient. When the probe radius is increased their accessibility decreases (see Table 3), which would result in a smaller contribution of these amino acid residues to the $K$ value.

There are only data available for the Gly–X–Gly peptides measured with a probe radius of 1.4 Å [39], so when calculating the estimated partition coefficients for other probe values, the Gly–X–Gly values obtained with a probe radius of 1.4 Å have been used. This is a reasonable assumption, since in a small peptide the residues are not involved in any secondary structure, thus allowing unrestricted interaction between residue and polymer. The calculated partition coefficients are collected in Table 4. The calculated $K$ values are 1.0–1.2, when the contribution from the N- and C-terminals are taken into account. By including the N- and C-terminals in the calculations, the prediction of the $K$ value is shifted closer to the experimental value ($K=0.85$). The experimental error is estimated to be maximum 10% of the $K$ value. The ASA for the Gly–X–Gly peptides are determined with an uncertainty of around 20%. The agreement between calculated and experimental value is satisfactory given the empirical basis of the model used. For instance, cutinase has two cystine bonds, which could not be included in the model and the contributions were thus calculated as free cysteins. A slightly better agreement with experimental $K$ value was obtained when the probe radius was increased to 5.0 Å.

By using the empirical model in Eqs. (1)–(4), it is possible to estimate an approximate partition coefficient for the target protein. Thus, if a protein has many surface accessible aromatic amino acid residues the partitioning is expected to be directed towards the top phase and a protein with many positively charges on the surface is expected to partition towards the bottom phase. Note, however, that the model developed here can only estimate the contributions from hydrophobic interactions to the $K$ value. Thus, it is necessary to use a phase system where the electrochemical contributions are minimised, as in the reference system with $K_2SO_4$ as dominating salt, in order to calculate the total protein $K$ value.

Table 4
The calculated partition coefficients for cutinase from Eqs. (2) and (3) with different probe radius ($r$) used in GRASP

| Probe radius (Å) | Calculated $K$ value | Calculated $K$ value N- and C-terminals included |
|---|---|---|
| 1.4 | 1.5 | 1.2 |
| 2.8 | 1.3 | 1.1 |
| 5.0 | 1.3 | 1.0 |

Values with N- and C-terminal contributions included using Eq. (4) are also presented.

### 3.3. Peptide tags for enhanced partitioning

#### 3.3.1. Tag partitioning

Tags based on tryptophan were selected due to strong effect of tryptophan based peptides in partitioning (see Fig. 2). Prolines were added in the tags to increase the exposure of the tryptophans and to reduce proteolytic cleavage of the tag during cultivation [41]. The peptides used as fusion tags [$(WP)_n$] in the proteins were partitioned in the Breox–Reppal system, as well as peptides containing only tryptophan [$(W)_n$] and proline [$(P)_n$]. A linear correlation between $\log K$ and the number of amino acid residues in the peptide was observed (Fig. 3), which is consistent with earlier findings [18,19]. The larger the tryptophan content in the peptide, the

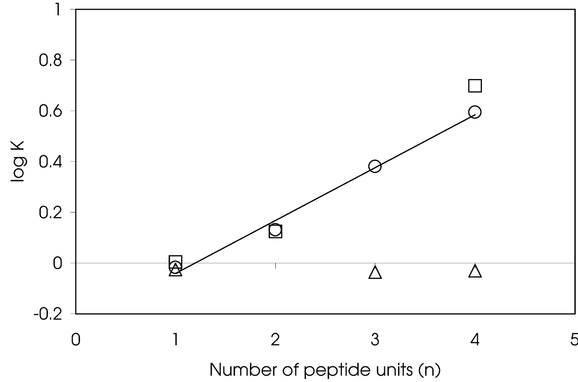


Fig. 3. The partitioning ($\log K$) of the peptides $(W)_n$ (□), $(WP)_n$ (○), $(P)_n$ (△) as a function of the number of peptide units ($n$), in the Breox–Reppal system. The system contained 50 m$M$ $K_2SO_4$ as the dominating salt and 5 m$M$ NaP, pH 7.0. The experimental error was estimated to maximum 10% of the $K$ value.

larger the preference for the EOPO copolymer top phase. The prolines included in the peptide tags did not affect the partitioning significantly (Fig. 3). Peptides consisting of only proline had an even partitioning between the phases.

Earlier studies have shown reduced effect of the tag, possibly due to incomplete exposure of the tag to polymer solution [19]. Thus, a spacer was introduced between the C-terminal and the tag. The peptide (TGGSGG) used as a spacer in cutinase-TGGSGG-$(WP)_4$, was partitioned in the system, resulting in a $K$ value of 0.85. The preference of the spacer for the more hydrophilic Reppal phase was weak, and would not significantly lower the partitioning of the target protein to the EOPO copolymer phase. Further on, the N- and C-terminals are not present when the peptide is used as a spacer in the protein. When the contribution of these is taken into account (according to Eq. (4)) we can calculate that the residues in the spacer peptide very weakly preferred the EOPO top phase ($K=1.1$).

### 3.3.2. Fusion protein partitioning

The partitioning results for the cutinase constructions are shown in Fig. 4. The partitioning of the protein was slightly increased when a $(WP)_2$ tag was added to the protein, compared with the wild-type protein partitioning. Just as in the peptide case, the partitioning to the EOPO copolymer phase increased, when increasing the length of the tag to $(WP)_4$. The spacer introduced between protein and tag in cutinase-TGGSGG-$(WP)_4$, resulted in a slightly higher partition coefficient than for cutinase-$(WP)_4$. A larger exposure is expected of the $(WP)_4$ tag towards the EOPO copolymer, due to the presence of the spacer.

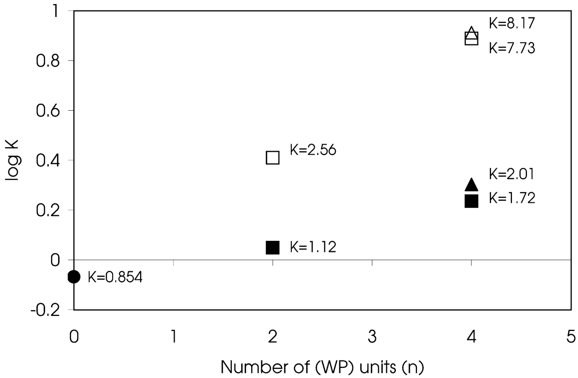


Fig. 4. The partitioning ($\log K$) of cutinase in the Breox–Reppal system shown as function of repeating WP units in the tag. The system contained 50 m$M$ $K_2SO_4$ as the dominating salt and 5 m$M$ NaP, pH 7.0. Cutinase wild-type (●), cutinase-$(WP)_n$ (■), cutinase-TGGSGG-$(WP)_4$ (▲). Calculated values (□) were obtained with Eq. (7). Calculated value for cutinase-TGGSGG-$(WP)_4$ is represented by △. The experimental error was estimated to maximum 10% of the $K$ value.

The tag effect can be defined as the ratio between partition coefficients for the fusion protein and the wild type protein [28]:

$$\text{Tag effect} = \frac{K_{\text{fusion protein}}}{K_{\text{wild-type}}} \qquad (5)$$

The tag effects for the constructs were rather low. Cutinase-$(WP)_2$ had a tag effect of 1.3, cutinase-$(WP)_4$ 2.0 and cutinase-TGGSGG-$(WP)_4$ 2.4.

### 3.3.3. Empirical formula for fusion protein partitioning

The following equation has previously been evaluated to predict the partitioning of a protein–peptide fusion [18] in an EOPO copolymer–dextran system:

$$\log K_{\text{fusion protein}} = \log K_{\text{wild-type}} + \log K_{\text{peptide}} \qquad (6)$$

The equation is based on the assumption of independent contributions from the fusion partner to the partition coefficient and could predict the partitioning of five of seven fusion proteins [18] of a molecular mass of around 17 000. In the earlier application of Eq. (6) [18,19], contribution from the C- and N-terminals of both the peptide and the wild-type protein is included, which introduces an error in $\log K_{\text{fusion protein}}$. To reach a better description of fusion protein partitioning, Eq. (6) has been modified by replacing $\log K_{\text{peptide}}$ with $\partial \log K_{(\text{WP})}/\partial n$, i.e. the slope obtained from the plot of $\log K$ for $(WP)_n$ vs. $n$, multiplied by the number of tryptophan residues ($n$) in the peptide (Eq. (7)). In this way the contribution to $\log K$ from the fused peptide will be represented by the added residues:

$$\log K_{\text{fusion protein}} = \log K_{\text{wild-type}} + \frac{\partial \log K_{(\text{WP})}}{\partial n} \cdot n \qquad (7)$$

The tag efficiency (TE) has been defined in order to express the effectiveness of a tag in promoting partitioning [42]. Following from Eq. (7) TE (%) can be given by:

$$TE = \frac{(\log K_{\text{fusion protein}} - \log K_{\text{wild type}})}{\frac{\partial \log K_{(\text{WP})}}{\partial n} \cdot n} \cdot 100 \qquad (8)$$

Peptide partitioning will give $\partial \log K/\partial n$ data, and *TE* of 100% indicates that the full effect of the peptide tag has been obtained in partitioning the fusion protein relative the wild-type protein.

Also shown in Fig. 4 are the calculated values for the fusion protein *K* values obtained with Eq. (7). The full effect of the tag was not achieved. Reasons for this could be that the tag is not fully exposed. When a spacer (TGGSGG) was introduced between the protein and the tag the partitioning was slightly increased. The tag efficiency, *TE* of the constructs was 25% for cutinase-$(WP)_2$, 32% for cutinase-$(WP)_4$ and 39% for cutinase-TGGSGG-$(WP)_4$. The experimental *K* values were low relative calculated values, which motivated modifications of the aqueous two-phase system to yield higher partitioning to one of the phases for effective extraction of the target protein.

### 3.4. Addition of detergent for increased tag effect

Addition of non-ionic detergents was studied for enhanced partitioning of target protein with fused hydrophobic tag. A screening for effective detergents was performed and the Breox–Reppal system was modified by addition of 1% of Tween 20, Tween 80, Triton X-100, $C_{12}E_n$, where $n=5$, 9 and 23. The effects on partitioning of cutinase wild type and cutinase-$(WP)_4$ were studied. The results are compiled in Table 5. For both Tween detergents no significant alterations of the *K* values were obtained. When Triton X-100 was added to the system, the partitioning of cutinase-$(WP)_4$ to the thermoseparating EOPO-containing top phase was increased. The tag effect (Eq. (5)) reached was 6.5. Similar results were obtained with $C_{12}E_9$. Triton X-100 and $C_{12}E_9$ have basically the same structure except for the aromatic ring in Triton (Table 1). This indicates that the increased *K* value of cutinase-$(WP)_4$ was not predominantly due to the presence of the aromatic ring in Triton, which could interact with tryptophan by stacking interactions.

Table 5
A comparison of the partitioning effects when 1% (w/w) of Tween 20, Tween 80, Triton X-100 and $C_{12}E_n$, where $n=5$, 9 and 23, was added to the two-phase system

| Additive | *K* value | |
|---|---|---|
| | Cutinase (wt) | Cutinase-$(WP)_4$ (tag effect) |
| No additive | 0.854 | 1.72 (2.0) |
| 1% Tween 20 | 0.862 | 1.59 (1.8) |
| 1% Tween 80 | 1.05 | 1.63 (1.6) |
| 1% Triton X-100 | 0.671 | 4.35 (6.5) |
| 1% $C_{12}E_5$ | 0.849 | 6.23 (7.3) |
| 1% $C_{12}E_9$ | 0.854 | 4.71 (5.5) |
| 1% $C_{12}E_{23}$ | 0.823 | 2.68 (3.3) |

Partitioning of cutinase wild-type (wt) and cutinase-$(WP)_4$ was performed in the Breox–Reppal system with 50 m*M* $K_2SO_4$ and 5 m*M* NaP, pH 7.0. The error was maximum 10% of the *K* value. Tag effect was calculated with Eq. (5).

Of the $C_{12}E_n$ detergents, $C_{12}E_5$ increased the partitioning to a larger extent than $C_{12}E_9$. The effect of $C_{12}E_{23}$ was smaller, but still significantly larger than for the Tween detergents. The $C_{12}E_n$ detergents and Triton X-100 had the strongest effect on partitioning and these detergents are expected to partition strongly to the Breox top phase. Due to their ethylene oxide content they can interact favourably with Breox, which is the main polymer component of the top phase. The partitioning coefficient for Triton X-100 could readily be determined by spectrophotometry. Triton X-100 partitioned strongly to the EOPO copolymer phase, with a partitioning coefficient of 11.

#### 3.4.1. Peptide partitioning

To investigate if the tryptophan in the fusion tag interacted with the alkyl-ethylene oxide detergents, tryptophan containing peptides, $(W)_n$, where $n=1$–4, were partitioned with 1% (w/w) of $C_{12}E_5$, $C_{12}E_9$ and $C_{12}E_{23}$ respectively added in the Breox–Reppal system. All peptides showed an increased partitioning to the EOPO copolymer top phase, compared to two-phase systems without detergent (Fig. 5). The increase was similar for all detergents, and a linear correlation between $\log K$ and the number of residues in the peptide was found. A compilation of the $\partial \log K/\partial n$ values obtained for $(WP)_n$ peptides is

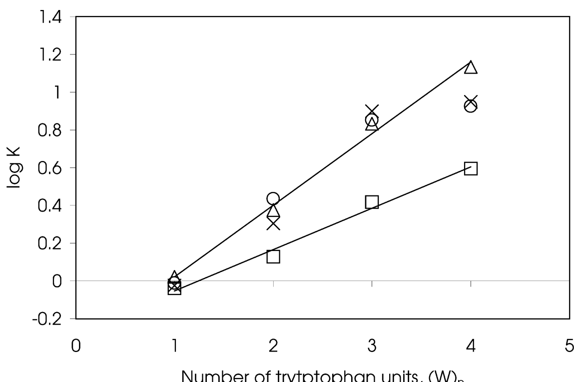


Fig. 5. The logarithm of the partition coefficient ($\log K$) of the $(W)_n$ peptides in the Breox–Reppal system with addition of detergent. The system contained 50 m$M$ $K_2SO_4$ as the dominating salt and 5 m$M$ NaP, pH 7.0. No detergent (□), 1% (w/w) $C_{12}E_5$ (○), 1% (w/w) $C_{12}E_9$ (△) and 1% (w/w) $C_{12}E_{23}$ (×). The experimental error was estimated to maximum 10% of the $K$ value.

shown in Table 6. The $\partial \log K/\partial n$ values were used to calculate the tag efficiency for the constructs in the systems containing detergent.

### 3.4.2. Effect of $C_{12}E_n$ detergents on fusion protein partitioning

The influence of the $C_{12}E_n$ detergents on partitioning of the cutinase-$(WP)_2$, -$(WP)_4$, and -TGGSGG-$(WP)_4$ constructions was investigated. The results are collected in Fig. 6a–c. For both the wild-type protein and cutinase-$(WP)_2$ the presence of $C_{12}E_n$ detergent in the phase system did not affect the partitioning. However, the $K$ value for the $(WP)_2$ peptide was increased when $C_{12}E_n$ detergent was included in the system. An explanation for this behaviour could be that the $(WP)_2$ tag is too short to interact with the detergent micelles. Similar results for cutinase-$(WP)_2$ have been observed in a detergent-based cloud point extraction system [43]. The partitioning of cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ was increased in a similar way in all systems with $C_{12}E_n$ detergents. The tag effect and tag efficiency was calculated for cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ in the various detergent systems (Table 6). The tag effect increased in all systems, as could be expected, since the peptides partitioned more to the top phase when the $C_{12}E_n$ detergents were added. However, the tag efficiency also increased when compared to a system without detergents. This means that the tag is better exposed to the to the phase components in the detergent system. In systems containing $C_{12}E_5$ and $C_{12}E_9$ tag efficiencies around 70–90% were found, compared with 32–39% in systems without detergent. The $C_{12}E_{23}$ detergent did not affect the partitioning to this extent. This detergent has a substantially longer EO chain compared to $C_{12}E_5$ and $C_{12}E_9$. The larger hydrophilic segment in $C_{12}E_{23}$ will lead to increased excluded volume (entropic) interactions, which will hinder the interaction of tryptophan with the micelle core.

By comparing tag effects and tag efficiencies obtained in these systems (Table 6) it is shown that $C_{12}E_5$ is the most effective of the $C_{12}E_n$ detergents. In the system containing $C_{12}E_{23}$ the tag efficiency

Table 6
Tag effect and tag efficiency (TE) for cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ for several detergent additives

| Additive | Tag effect: -$(WP)_4$ (*TE*, %) | Tag effect: -TGGSGG-$(WP)_4$ (*TE*, %) | $\partial \log K/\partial n$ $(WP)_n$ |
|---|---|---|---|
| 0% Detergent | 2.0 (32) | 2.4 (39) | 0.239 |
| 1% $C_{12}E_5$ (25 m$M$) | 7.3 (70) | 9.0 (77) | 0.311 |
| 2% $C_{12}E_5$ (49 m$M$) | 12 (67) | 14 (71) | 0.402 |
| 1% $C_{12}E_9$ (17 m$M$) | 5.5 (73) | 7.8 (88) | 0.254 |
| 2% $C_{12}E_9$ (34 m$M$) | 9.4 (68) | 12 (75) | 0.356 |
| 1% $C_{12}E_{23}$ (8 m$M$) | 2.6 (39) | 3.9 (55) | 0.265 |
| 2% $C_{12}E_{23}$ (17 m$M$) | 3.8 (46) | 6.8 (65) | 0.319 |

$\partial \log K/\partial n$ values for the $(WP)_n$ peptides obtained in the various systems are presented. Partitioning was performed in the Breox–Reppal system with 50 m$M$ $K_2SO_4$ as the dominating salt and 5 m$M$ NaP, pH 7.0.

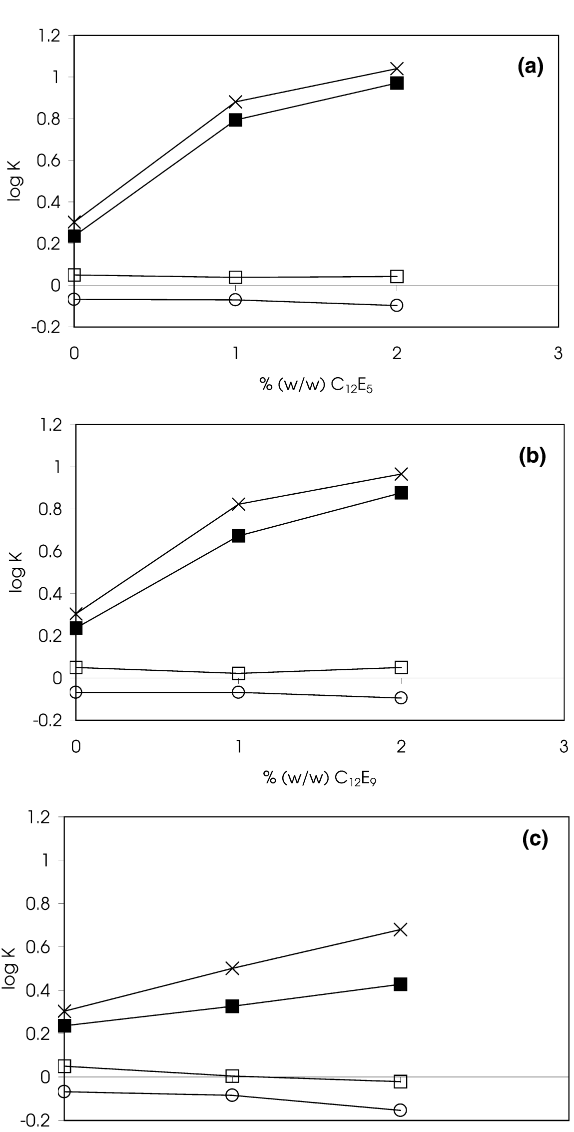


Fig. 6. The logarithm of the partition coefficient for the wild-type cutinase (○), cutinase-$(WP)_2$ (□), cutinase-$(WP)_4$ (■) and cutinase-TGGSGG-$(WP)_4$ (×) in the Breox–Reppal system. The system contained 50 m*M* $K_2SO_4$ and 5 m*M* NaP, pH 7.0. The experimental error was estimated to maximum 10% of the *K* value. (a) The effect of addition of $C_{12}E_5$. (b) The effect of addition of $C_{12}E_9$. (c) The effect of addition of $C_{12}E_{23}$.

for cutinase-TGGSGG-$(WP)_4$ was clearly higher than for cutinase-$(WP)_4$. The spacer effect was most pronounced in the $C_{12}E_{23}$ system, indicating that the spacer possibly could facilitate the interaction of the tag with relatively long EO chains.

## 4. Conclusions

Aqueous two-phase systems have large capacity in protein purification, i.e. high protein concentrations can be handled in the system. This has been clearly demonstrated in the works of Kula et al. for large-scale enzyme extractions with PEG–salt systems [46]. The high capacity has also been shown in systems with thermoseparating EOPO copolymers for extraction of apolipoprotein A-1 in a system of Breox and Reppal PES 100 [17]. The total protein concentration in the system was 38 g/l and the target protein concentration had a concentration of 4 g/l. The possibility to handle cell debris and high protein concentrations makes aqueous two-phase systems a strong candidate for a primary recovery step for extraction of target protein from fermentation solution. The phase separation is rapidly achieved and the phases can be separated with a separator.

The enzyme used in this study, cutinase, is a technical enzyme, i.e. a lipase for use in mixtures with detergents for laundry applications. Since the tag attached to the protein does not influence the activity of the enzyme it is not necessary to remove the tag after the purification. For a pharmaceutical protein, the tag needs to be removed which can be achieved by introducing a proteolytic cleavage site between the tag and the protein. However, after cleavage a second purification step is needed to separate the protein and the tag. Thus, it is more advantageous to use the tagged protein concept for technical enzymes. In biotechnology it is important to be able to design satisfactory purification procedures. The aim of the study has been to establish a method for predicting the partitioning of a wild-type target protein, and with this as basis, design fusion tags to increase the partitioning and modify the system with detergent addition for further increase of the partitioning of the target protein.

The calculated partition coefficient of the cutinase wild-type protein in the Breox–Reppal system, determined from peptide partitioning data combined with surface studies with the computer program GRASP, agreed relatively well with the experimen-

tally determined partition coefficient. Thus, it is possible to obtain an approximate partition coefficient before starting a purification procedure and thereby save time in finding an optimal partitioning system.

By fusion of tryptophan based tags to cutinase, the partitioning towards the EOPO copolymer Breox phase was increased. The partitioning of the fusion proteins could be qualitatively determined from peptide partitioning data. With longer tag, larger increase of the partitioning was obtained, as expected from peptide partitioning. By addition of non-ionic detergents $C_{12}E_5$ and $C_{12}E_9$ to the Breox–Reppal system, the partitioning coefficient of cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ constructs could be increased up to 14 times towards the thermoseparating EOPO phase compared to the wild-type protein. The tag efficiency also increased when the detergents were added indicating that the tag was better exposed to the phase components in the detergent systems.

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