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Partitioning of peptides and recombinant protein–peptide fusions in thermoseparating aqueous two-phase systems: effect of peptide primary structure

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Abstract

Genetic engineering has been used for fusion of peptides, with different length and composition, on a protein to study the effect on partitioning in an aqueous two-phase system. The system was composed of dextran and the thermoseparating ethylene oxide–propylene oxide random copolymer, EO30PO70. Peptides containing tryptophan, proline, arginine or aspartate residues were fused at the C-terminus of the recombinant protein ZZ-cutinase. The aim was to find effective tags for the lipolytic enzyme cutinase for large-scale extraction. The target protein and peptide tags were partitioned separately and then together in the fusion proteins in order to gain increased understanding of the influence of certain amino acid residues on the partitioning. The salt K_2SO_4 was used to reduce the charge dependent salt effects on partitioning and to evaluate the contribution to the partition coefficient from the hydrophobic–hydrophilic properties of the amino acid residues. The effect of Trp on peptide partitioning was independent of the difference in primary structure for (Trp)n, (Trp-Pro)n, (Ala-Trp-Trp-Pro)n and was only determined by the number of Trp. The effect of the charged residues, Arg and Asp, was dependent on the surrounding residues, i.e. if they were situated next to Trp or not. The partitioning behaviour observed for the peptides was qualitatively and in some cases also quantitatively the same as for the fusion proteins. The effect of the salts sodium perchlorate and triethylammonium phosphate on the partitioning was also studied. The salt effects observed for the peptides were qualitatively similar to the effects observed for the fusion proteins.

Keywords: Partitioning; Aqueous two-phase systems; Protein–peptide fusions; Peptides; Thermoseparating polymers

1. Introduction

The selectivity in a purification process for a target protein can be increased by genetic manipulation of the target protein. By molecular genetic techniques, properties of the protein such as charge or hydrophobicity can be changed or affinity-binding sites can be added. Aqueous two-phase extraction as a purification method has many desirable characteristics such as high water content in each phase which is suitable for biological material, ease of scale up and short processing times. An aqueous two-phase system consists of two phase forming components, e.g. polyethylene glycol (PEG) together with dextran or with a salt such as sodium phosphate. The phase forming components, separate into two phases above

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certain concentrations [1,2]. In a two polymer system, each phase is enriched in one of the polymers while in a polymer-salt system one polymer rich phase is in equilibrium with a salt-rich phase.

Partitioning of macromolecules, e.g. proteins, in aqueous two-phase systems depends on surface properties of the macromolecule such as net charge and relative hydrophobicity. It should be possible to understand and explain the partitioning behaviour of a protein from its exposed amino acid residues, which determine the surface properties of a protein. Genetic engineering can be used as a tool to understand more about partitioning but also to enhance the partitioning of a target protein to a desired phase. Our aim has been to use molecular genetic techniques in both ways: (1) to gain knowledge about the factors governing partitioning and (2) to study to what extent the partitioning of a target protein can be affected by altering its amino acid composition. It is also of interest if the effect of protein modification on partitioning can be predicted, e.g. to predict the partition coefficient of a fusion protein. A desirable general concept would be to study the partitioning of each fusion partner and from their partitioning behaviour predict the partitioning of the fusion protein. Different peptide tags could then be tested to find an optimal one. In this study, the target protein has been the lipolytic enzyme cutinase. The aim has been to develop effective peptide tags, which can be used for extraction of the recombinantly produced cutinase–peptide fusion in large scale.

The two-phase system used in this study was composed of dextran and a random EO–PO copolymer of 30% ethylene oxide (EO) and 70% propylene oxide (PO). Two-phase systems containing EO–PO copolymer, in combination with dextran and other polymers, have been investigated in a number of studies [3–9]. EO–PO copolymers are thermoseparating i.e., above a critical temperature aqueous solutions of EO–PO copolymers will phase separate into one concentrated polymer phase and one water phase almost free from polymer. The critical temperature where phase separation occurs (the cloud point) varies with PO-content, polymer concentration and polymer molecular weight [5,8]. In addition, the temperature can also be lowered by the addition of salts. Proteins have been found to partition exclusively to the water phase in such systems while small peptides and polypeptides partition partly to the polymer phase [10]. A two-stage process for the purification of a protein is thereby possible. In the first stage, for example with a dextran–EO–PO system, the target protein has to be partitioned strongly to the top EO–PO copolymer phase to achieve a separation of the target protein from contaminants. The EO–PO copolymer phase is then recovered and, as a second step, this phase is heated above the copolymer cloud point. The target protein is then recovered in a polymer free water phase, while the polymer can be recycled.

This study is a continuation of an earlier study with the aim of understanding and predicting the partitioning behaviour of fusion proteins [7]. In the former study, recombinant proteins, with fused peptides of different length and composition, were partitioned in the same two-phase system used here. The proteins were based on a synthetic IgG binding Z-domain derived from domain B in Staphylococcal protein A. Some of those fusion proteins and peptides have also been studied in PEG–salt systems [11–14], as well as in PEG–dextran and Ucon (EO50PO50)-dextran systems [9]. In order to gain increased understanding of the influence of certain amino acid residues on partitioning, the target protein and the fusion tags were partitioned separately and then together in the fusion proteins. The tags used in the present work have been designed to expose tryptophan residues, which have earlier been found to enhance the partitioning of peptides and proteins to the EO–PO copolymer phase [7,9]. In addition, charged amino acid residues were included in the tags to study the effect of charge together with added salt. The target protein in the present work, cutinase, was expressed in *E. coli* as a fusion protein, ZZ-cutinase. The two Z-domains constituted part of the proteins in order to make the purification of the different fusion proteins easier by affinity chromatography. A major difference from the earlier work [7] is that the lipolytic enzyme cutinase now constituted the other part of the protein making it significantly larger than that used previously, i.e. the molecular weight was increased from 17 to 36 kDa. Furthermore, the peptides had a different construct than before and the effect of charged amino acid residues have in this work been studied both with and without tryptophans.

2. Materials and methods

2.1. Peptides and proteins

The peptides T*n*=(Ala-Trp-Trp-Pro)*n*, (WP)*n*=(Trp-Pro)*n*, (WPR)*n*=(Trp-Pro-Arg)*n*, (WPD)*n*=(Trp-Pro-Asp)*n*, (RP)*n*=(Arg-Pro)*n* and (DP)4=(Asp-Pro)4 where *n* was 2 or 4 were purchased from Synpep Corporation (Dublin, CA, USA).

The peptides containing glycine (Gly)*m* and tryptophan (Trp)*m* peptides; *m*=1–4 were obtained from Sigma (St. Louis, MO, USA).

The fusion proteins used in this study were based on the lipolytic enzyme cutinase. Cutinase was fused to a synthetic IgG-binding domain, Z, derived from the B domain of staphylococcal protein A in order to make the purification of the different fusion proteins easier by affinity chromatography. Different tags were then fused to the C-terminal of cutinase (Fig. 1). The cloning of the proteins ZZ-cutinase-wt, ZZ-cutinase-(WP)2, ZZ-cutinase-(WP)4, ZZ-cutinase-(RP)2, ZZ-cutinase-(RP)4, ZZ-cutinase-(DP)4, ZZ-cutinase-(WPR)4 and ZZ-cutinase-(WPD)4 is described elsewhere [17]. The production of the proteins was performed at 37°C in baffled shake flasks containing Tryptic Soy Broth (30 g/l), yeast extract (5 g/l) and ampicillin (100 mg/l). The cells were collected by centrifugation at 4000 *g* for 10 min and treated with osmotic shock (20% sucrose, 0.3 *M* Tris–HCl pH 8.0, 1 m*M* EDTA). The solution was centrifuged at 14 000 *g* for 10 min and an affinity chromatography procedure was performed with IgG-Sepharose equilibrated with 1×TST (25 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 1 m*M* EDTA, 0.05% Tween 20). The protein was eluted with 0.3 *M* HAc, pH 3.1. For further details see Ref. [17]. The purity of the isolated proteins was determined by reduced sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) on a Phast-system, Pharmacia (Uppsala, Sweden), stained with Coomassie brilliant blue R 350. The pI values of the proteins ZZ-cutinase-wt, ZZ-cutinase-(RP)2, ZZ-cutinase-(DP)4, ZZ-cutinase-(WPR)4 and ZZ-cutinase-(WPD)4 were determined by isoelectric focusing using a Phast-system, developed by silver staining. In addition, three proteins from another family of proteins based on the Z domain ZZT*n*=ZZ(Ala-Trp-Trp-Pro)*n*; *n*=0, 1, 2 were used in this study. Partition studies of the ZZTn-proteins in PEG–salt systems in addition to those in two polymer systems has been performed earlier [7,9,11,13,14,18]. The cloning, production and purification of the ZZTn-proteins have been described before [7,11,12]. BSA was purchased from Sigma (St. Louis, MO, USA).

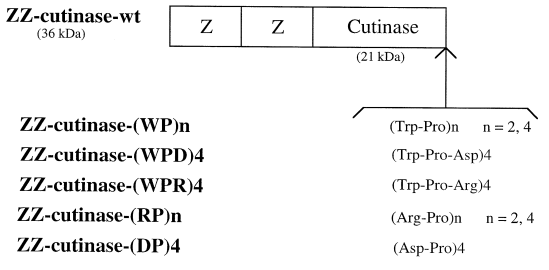

Fig. 1. Schematic outline of model peptides and proteins. The molecular weight of cutinase and ZZ-cutinase-wt are also given.

2.2. Chemicals

The bottom phase polymer dextran T500, with a molecular weight of 500 000, was obtained from Pharmacia Biotech (Uppsala, Sweden). The top phase polymer, EO30PO70, having a molecular weight of 3300, was obtained from Shearwater Polymers (Huntsville, AL, USA). All chemicals were of analytical grade. Triethylammonium phosphate was obtained by neutralising triethylamine with phosphoric acid in aqueous solution to the desired pH.

2.3. Two-phase systems for partitioning of peptides and proteins

Systems of a total weight of 2 g, containing 7.1% dextran and 6.8% EO30PO70 were made up by weighing appropriate amounts of a 25% polymer stock solution of dextran and a 100% solution of EO30PO70 in 10 ml graduated test tubes (calibrated by addition of known volumes of water). Phase diagram for the system has been published earlier [7]. Proteins or peptides were added in the form of pre-made stock solutions, giving final concentrations of 10–15 μg/g for the proteins and 0.01 mg/g for the peptides. Sodium acetate buffer (NaAc) of pH 5.0, sodium phosphate buffer (NaP) of pH 7.0 and triethylammonium phosphate buffer (Et_3NP) of pH

7.5 were used. The concentration of the buffer, based on the salt content was 50 m*M* when no additional salt was included. The effects of sodium perchlorate ($NaClO_4$) and potassium sulphate (K_2SO_4), on the partitioning, were studied at 50 m*M* of these salts, 5 m*M* buffer. This composition made $NaClO_4$ or K_2SO_4 to be the dominating salt in the system. Water was then added to give a final weight of 2 g. It has been shown for PEG–dextran systems [19] that the partitioning of a protein is independent of the salt concentration between 25 m*M* and 100 m*M*. The minor variation in salt concentration used here, 50–55 m*M*, should therefore have no effect on the partitioning.

The partition of a substance is described by its partition coefficient K, which is defined as $K = C_T / C_B$ where C_T and C_B are the concentrations of the substance in top phase and bottom phase, respectively. All partition coefficients are average values from at least duplicate experiments. The systems were equilibrated at room temperature (22°C) and were left for 15–30 min and then centrifuged for 5 min at 1600 *g*. The top and bottom phases were separated and diluted appropriately with water for the determination of protein or peptide content. The phases were analysed for their protein contents by enzymatic activity measurements of the cutinase variants. The enzymatic activity was determined by using a standard activity assay comprising mixed micelles of detergent and substrate. 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 final acetonitrile concentration in the assay solution was $\leq$5% (v/v). The enzymatic activity was monitored spectrophotometrically at 400 nm and 20°C by measuring the release of *p*-nitrophenolate. The partition coefficient was calculated as the ratio of the change in absorbance with time (dA/dt) in the top and bottom phase. The samples of each phase were mixed with equal amounts of opposite phase of a blank system to have the same polymer composition in the assay since the polymers are affecting dA/dt. The concentration of the ZZTn proteins and BSA as well as the peptide concentrations were determined from the absorbance at 280 nm corrected by subtracting the absorbance at 320 nm. For peptides lacking tryptophan the concentration was determined by the absorbance at 220 nm, corrected by subtracting the 320 nm value. The absorbance contribution from the polymers was taken into account by preparing a blank system.

3. Results and discussion

3.1. Characterisation of the fusion proteins

The isoelectric points determined by IEF can be seen in Table 1. As expected the insertion of aspartates lowered the pI while arginines increased it. Furthermore, four arginines, as in ZZ-cutinase-(WPR)4, increased the pI more than two arginines, as in ZZ-cutinase-(RP)2, while ZZ-cutinase-(DP)4 and ZZ-cutinase-(WPD)4, both containing four extra aspartates, had the same pI. ZZ-cutinase-(WP)2 and ZZ-cutinase-(WP)4 were analysed by mass spectrometry. The theoretical molecular weights were calculated to 36624.8 Da and 37191.5 Da for ZZ-cutinase-(WP)2 and ZZ-cutinase-(WP)4, respectively, while the experimental values were 36530.3 Da and 37211.0 Da, respectively (spectra not shown). Other peaks in the spectra corresponded to material lower than 21.6 kDa, which is the molecular weight of cutinase. Except for the full-length proteins, the highest molecular weight found in the ZZ-cutinase-(WP)2 and ZZ-cutinase-(WP)4 samples was 18.2 kDa and 18.6 kDa, respectively. No contaminating proteins were detected with a SDS–PAGE gel stained with coomassie brilliant blue (gel not shown). The detection limit for coomassie brilliant blue is 20–30 ng for each band and 6000 ng of

Table 1
The isoelectric points of the recombinant proteins determined with IEF

Protein	p*I*
ZZ-cutinase-wt	5.13
ZZ-cutinase-(RP)2	5.39
ZZ-cutinase-(WPR)4	5.91
ZZ-cutinase-(DP)4	4.91
ZZ-cutinase-(WPD)4	4.91

protein was loaded which means that contaminating proteins were less than 0.5%.

3.2. Reference system

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. Furthermore, the effect on partitioning is proportional to the net charge of the protein or peptide. The mechanism of the salt effects on partitioning in aqueous two-phase systems has been much studied. One widely accepted explanation of the effect is the creation of an electrical potential difference at the interface between the two phases [1,2,19–22]. 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. The electrical potential difference is due to the relative affinity of anions and cations for the phases and will increase with increased difference in anion and cation preference for the phases.

To study the effect of hydrophobic and other non-electrostatic interactions on partitioning of peptides or fusion proteins it is necessary to eliminate the salt effects. One way is to study the partitioning at the pI of the protein, which for several proteins is not possible due to low solubility at the pI. Instead, a reference system buffered at pH 7.0 with 5 m*M* NaP and with 50 m*M* K_2SO_4 as dominating salt has been used in this study. K_2SO_4 generates a potential difference close to zero in a PEG–dextran system [19] whereby charge-dependent salt effects are minimised. To evaluate the charge-dependent salt effects in the EO–PO–dextran system with K_2SO_4 as dominating salt the partitioning of BSA at pH 5.0 and 7.0 was studied (Table 2). The net charge of BSA at pH 5.0 and 7.0 is approximately +4 and −10, respectively [23]. The partition coefficients of BSA at pH 7.0 and 5.0 differed only slightly with K values of 0.39 and 0.41 respectively (Table 2). Thus, K_2SO_4 generates a potential difference close to zero also in the EO–PO–dextran system used in this study. The effect on partitioning of the hydrophobicity of a peptide or fusion protein can therefore be evaluated from the partition coefficient in the system with K_2SO_4 as dominating salt.

Table 2
The partition coefficients of BSA at pH 5.0 and pH 7.0 with 50 m*M* K_2SO_4 as dominating salt. Five m*M* NaAc or NaP was also included in the systems to keep pH 5.0 or pH 7.0, respectively. The experimental error was ±10 per cent of the *K* value

	K BSA	Net charge of BSA [23]
5 m*M* NaAc 50 m*M* K_2SO_4 pH 5.0	0.41	approx. +4
5 m*M* NaP 50 m*M* K_2SO_4 pH 7.0	0.39	approx. −10

3.2.1. Partitioning of peptides

The partitioning of the peptides used as tags in addition to the peptides (Gly)*m* and (Trp)*m* (*m* = 1–4) was investigated in the K_2SO_4 reference system (Fig. 2). Log *K* was found to be a linear function of the number of tryptophan units, as observed before [7]. Thus, the larger the tryptophan content in the peptide the larger the preference for the EO–PO-

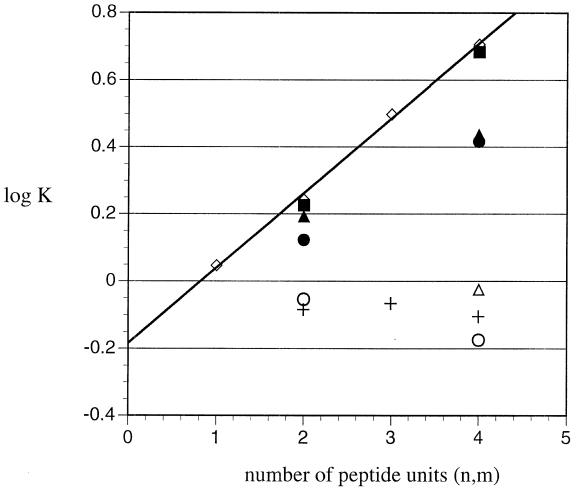

Fig. 2. The partitioning (log *K*) of the peptides (W)*m* (◇), (G)*m* (+), (WP)*n* (■), (WPD)*n* (▲), (WPR)*n* (●), (DP)*n* (△), (RP)*n* (○) in the EO30PO70-dextran aqueous two phase system as a function of number of peptide units (*n, m*). The system contained 50 m*M* K_2SO_4 as predominant salt and 5 m*M* NaP as buffer, pH 7.0. The error was ±10 per cent of the K value.

copolymer top phase. Furthermore, the proline residues introduced in the (WP)*n* peptides did not affect the partitioning significantly (Fig. 2). The log *K* values were only slightly lower for (WP)*n* compared with (W)*n* when it was increased from 2 to 4. The influence of the amino acid side chains on partitioning can be determined by comparing with peptides containing only glycine residues. The log *K* values for the glycine peptides were slightly below zero and log *K* gave no linear correlation with respect to the number of glycine residues.

The log *K* values for peptides which also contained the charged amino acid residues aspartate and arginine, (WPD)*n* and (WPR)*n*, increased less from $n=2$ to 4, than the corresponding (WP)*n* peptides (Fig. 2). The lower values for peptides with charged amino acid residues show their more hydrophilic nature and thus their preference for the more hydrophilic dextran-containing phase. This has also been seen in earlier studies [7,24]. The effect of the hydrophilic amino acid residues is also observed with (RP)*n* where (RP)4 has a lower log *K* value than (RP)2, both preferring the dextran phase (Fig. 2). However, (DP)4 has a log *K* value close to zero, which means that inserted aspartates would not affect the partitioning of a peptide. This contradicts the result of the (WPD)*n* partitioning where the (WPD)*n* peptides had lower partition coefficients than the corresponding (WP)*n* peptides (Fig. 2). Charged residues in a peptide have earlier been concluded to enhance partitioning to the dextran phase [7]. In that study, the charged residues (lysine and aspartate) were situated next to tryptophans in a tag similar to the (WPR)*n* and (WPD)*n* tags. Thus, the effects observed for the charged residues in combination with tryptophans correspond with earlier findings. One should notice that the order of the residues in the peptides containing both tryptophan and hydrophilic amino acid residues differs in comparison to the order in peptides lacking tryptophan. It has previously been shown that the order of the residues in a peptide might alter the partitioning in a PEG–salt system [24] but it has not been shown in two-polymer systems.

3.2.2. Partitioning of fusion proteins

The effect on partitioning by fusion of the peptide tags to the ZZ-cutinase proteins was studied in the

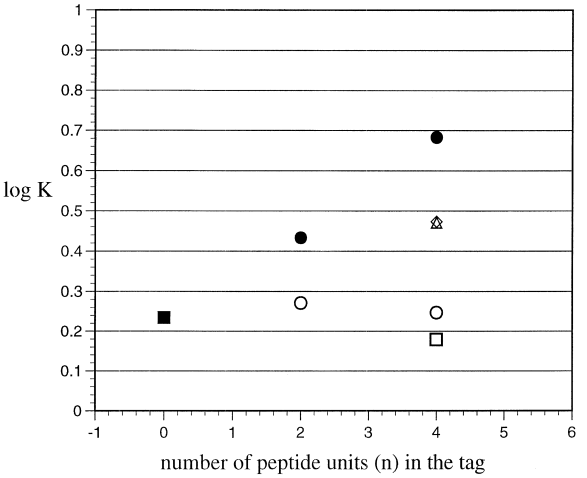

Fig. 3. The partitioning (log *K*) of the proteins ZZ-cutinase-wt (■), ZZ-cutinase-(WP)*n*, $n=2,4$ (●), ZZ-cutinase-(WPD)4 (△), ZZ-cutinase-(WPR)4 (◇), ZZ-cutinase-(RP)*n*, $n=2,4$ (○), ZZ-cutinase-(DP)4 (□) in the EO30PO70-dextran aqueous two phase system as a function of number of peptide units (*n*) in the tag. The system contained 50 m*M* K_2SO_4 as predominant salt and 5 m*M* NaP as buffer, pH 7.0. The error was ±10 per cent of the *K* value.

K_2SO_4-containing system (Fig. 3). The fused (WP)*n* peptides increased the partitioning of the protein to the EO-PO-copolymer phase as expected from peptide partitioning (Fig. 2). Charged residues together with tryptophans as in ZZ-cutinase-(WPD)4 and ZZ-cutinase-(WPR)4 decreased the partitioning to the EO–PO copolymer phase compared to ZZ-cutinase-(WP)4 in accordance with peptide partitioning data. The charged (RP)2 and (RP)4 tags were expected from peptide partitioning data to lower the partition coefficient of the respective fusion protein relative to the wild type protein. However, (RP)2, (RP)4 and (DP)4 as peptide tags had a negligible effect on the partitioning of the respective fusion protein compared to the wild type protein. Charged residues can therefore not simply be concluded to enhance the partitioning to the dextran phase. Firstly, the log *K* value of the (DP)4 peptide was around zero (Fig. 2) but was expected to be below zero since the log *K* value obtained for the (WPD)4 peptide was lower than the log *K* value for the (WP)4 peptide. Secondly, the partitioning of the ZZ-cutinase-(RP)*n* was expected to be lower than the wild type protein, however, very similar *K* values were obtained (Fig.

3). Thus, the effect of charged residues on the partitioning seems to depend on the surrounding amino acid residues, i.e. the primary structure in the peptides such as the proximity to tryptophans as in the (WPD)4 and (WPR)4 tags. Genetic modifications on other proteins in order to understand the influence of charge in PEG–dextran systems have also been reported by others [15,16]. Studies in PEG–dextran systems [15] have shown an increase in affinity of β-galactosidase for the dextran phase by fusion of aspartate tags.

3.3. Empirical correlation for fusion protein partitioning

The qualitative effects of the fused peptides on protein partitioning have been discussed above. Furthermore, the tag effects must also be quantitatively determined in order to make it possible to predict the partition coefficient of a fusion protein. In our previous study for a family of proteins based on the Z domain, an empirical correlation was used to evaluate the efficiency of the fused peptides on the partitioning of the fusion proteins [7].

$$\log K_{\text{fusion protein}} = \log K_{\text{unmodified protein}} + \log K_{\text{peptide}} \quad (1)$$

In a reference system with the zwitterionic compound β-alanine the sum of log *K*-values of the peptide and unmodified protein gave the log *K*-value of the fusion protein for five of seven fusion proteins [7].

Independent contributions from the peptide and unmodified protein in the K_2SO_4 containing system had to be confirmed since another reference system (β-alanine) was used in the former study. To be able to compare the tag effects obtained in this study with previous results, partitioning experiments were performed in the K_2SO_4 containing system with the ZZTn proteins and Tn peptide tags used in the previous study (Fig. 4). First of all, the partitioning of the Tn peptides was similar to the partitioning of the (WP)*n* and (Trp)*m* (compare Figs. 2 and 4). This shows that despite the difference in primary structure of the Tn, (WP)*n* and (Trp)*m* peptides the same effect on partitioning of the tryptophans was obtained for all of them. Furthermore, the calculated

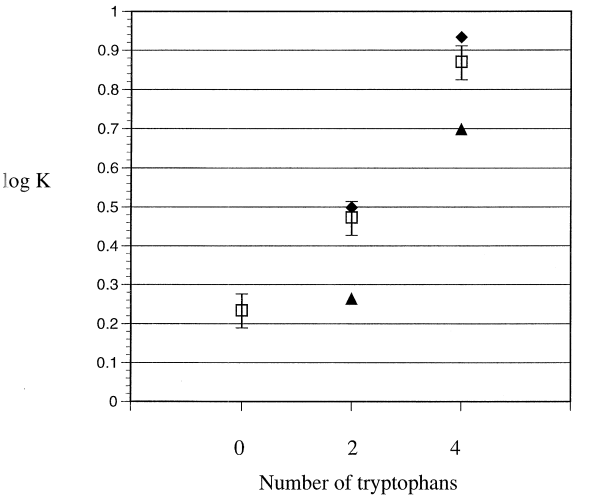

Fig. 4. The partitioning of Tn (▲) and ZZTn (□) in the EO30PO70-dextran system as a function of number of tryptophan residues. The system contained 50 m*M* K_2SO_4 as predominant salt and 5 m*M* NaP as buffer, pH 7.0. The symbol (◆) represents an empirical correlation based on independent contributions from each fusion partner ($\log K_{\text{fusion protein}} = \log K_{\text{ZZ-cutinase-wt}} + \log K_{\text{peptide}}$).

sum $\log K_{\text{peptide}} + \log K_{\text{unmodified protein}}$ corresponds reasonably well with the experimental data for the fusion proteins (Fig. 4). Thus, independent contributions from peptide and unmodified protein to the *K*-value of the ZZ fusion protein is obtained in the K_2SO_4-system, and we conclude that the system can be used to evaluate the tag efficiencies for the ZZ-cutinase-tag fusion proteins.

The sum $\log K_{\text{peptide}} + \log K_{\text{ZZ-cutinase-wt}}$ was calculated to evaluate the contribution to the partition coefficient of the tags in the fusion proteins. The calculated sum for ZZ-cutinase-(WP)2 (Fig. 5) approximately corresponded to the experimental value. A much larger discrepancy was obtained between the experimental and calculated values for ZZ-cutinase-(WP)4. Thus, the effect of the (WP)4 tag was lower than expected from the partitioning of the peptides. For the ZZTn proteins it was possible to quantitatively predict the effect on partitioning from four tryptophans from the peptide partitioning data (Fig. 4). However, the size of ZZ-cutinase-wt protein is about twice that of ZZT0, which might influence the result. Furthermore, a lower tag effect would be expected if the exposure of the tryptophans to the

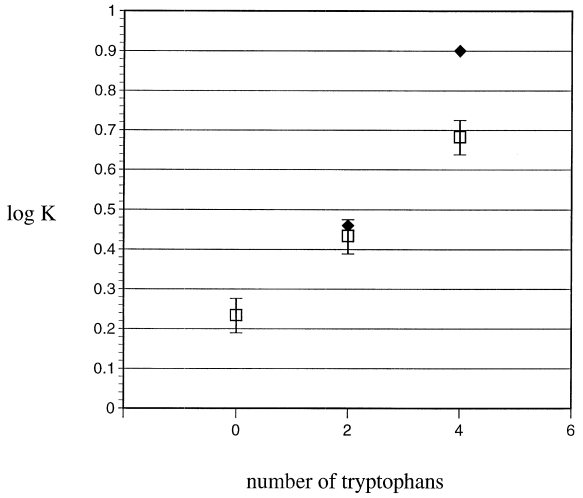

Fig. 5. The partitioning (log K) of ZZ-cutinase-wt and ZZ-cutinase-(WP)n (□) in the EO30PO70-dextran system as a function of number of tryptophan residues. The system contained 50 mM K_2SO_4 as predominant salt and 5 mM NaP as buffer, pH 7.0. The symbol (◆) represents an empirical correlation based on independent contributions from each fusion partner (log $K_{\text{fusion protein}} = \log K_{\text{ZZ-cutinase-wt}} + \log K_{\text{peptide}}$).

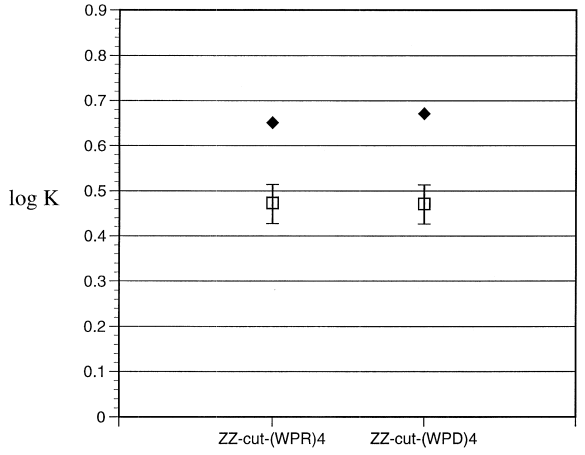

Fig. 6. The partitioning (log K) of ZZ-cutinase-(WPR)4 (□) and ZZ-cutinase-(WPD)4 (□) in the EO30PO70-dextran system. The system contained 50 mM K_2SO_4 as predominant salt and 5 mM NaP as buffer, pH 7.0. The symbol (◆) represents an empirical correlation based on independent contributions from each fusion partner (log $K_{\text{fusion protein}} = \log K_{\text{ZZ-cutinase-wt}} + \log K_{\text{peptide}}$).

solvent is less in the tag fused to the protein than in the free peptide.

The effect of the charged residues Asp and Arg in ZZ-cutinase-(WPD)4 and ZZ-cutinase-(WPR)4 compared to their effect in the peptides (WPD)4 and (WPR)4 can be observed by comparisons to ZZ-cutinase-(WP)4 and (WP)4, respectively (Table 3). The charged residues lower the partition coefficient in both peptides and fusion proteins as discussed before. Furthermore, the effect on partitioning of the charged residues in the peptides and fusion proteins was also quantitatively similar, which is in agreement with earlier findings [7]. Thus, in the tryptophan-containing tags, four aspartate or arginine residues lowered the partition coefficients to the same extent for the peptides as for the proteins (Table 3). However, a lower tag effect than expected from the empirical correlation Eq. (1) was observed for ZZ-cutinase-(WPR)4 and ZZ-cutinase-(WPD)4 (Fig. 6). The discrepancy between calculated and experimental K-values for ZZ-cutinase-(WPD)4 and ZZ-cutinase-(WPR)4 is due to the lower tryptophan effect also observed for ZZ-cutinase-(WP)4 (Fig. 5).

The empirical correlation Eq. (1) predicts that the (DP)4 tag has no effect on the fusion protein partitioning, which was also observed (Figs. 2 and 3). Furthermore, the qualitative tag effects observed for the ZZ-cutinase-(RP)n proteins (Figs. 2 and 3) were not as could be expected from peptide partition-

Table 3
The effect of the charged residues aspartate and arginines on partitioning of peptides and fusion proteins in the reference (K_2SO_4) system. The system contained 50 mM K_2SO_4 as predominant salt and 5 mM NaP as buffer, pH 7.0

The effect of four aspartates in the fusion protein Log $K_{\text{ZZ-cutinase-(WPD)4}}$ − Log $K_{\text{ZZ-cutinase-(WP)4}}$	The effect of four aspartates in the peptides Log $K_{\text{(WPD)4}}$ − Log $K_{\text{(WP)4}}$	The effect of four arginines in the fusion proteins Log $K_{\text{ZZ-cutinase-(WPR)4}}$ − Log $K_{\text{ZZ-cutinase-(WP)4}}$	The effect of four arginines in the peptides Log $K_{\text{(WPR)4}}$ − Log $K_{\text{(WP)4}}$
−0.21	−0.25	−0.21	−0.27

ing data and thus for the (RP)*n* tags Eq. (1) can not be used.

3.4. Effects of ions and charge on partitioning

Peptide and protein partitioning can be influenced by the salt composition in the system as described above. The salts Et_3NP and $NaClO_4$ have earlier been found to direct negatively and positively charged peptides and proteins respectively to the EO–PO copolymer phase [6–8]. The salt effect on peptide/protein partitioning can be isolated from the total value of the partition coefficient by representing the salt effect as the difference between partition coefficients in a system with the salt included and in the reference (K_2SO_4) system [7]:

$$\Delta \log K_{el} = \log K^{\text{salt system}} - \log K^{K_2SO_4} \quad (2)$$

The effect of a salt on the partitioning should be linearly dependent on the net charge of a peptide/protein [2], which has also been shown experimentally [7,19].

3.4.1. Partitioning of peptides

A compilation of the log *K* values which were determined with Et_3NP and $NaClO_4$ as dominating salts are shown in Table 4. As expected, for peptides without charged residues, (G)*m*, (W)*m*, and (WP)*n*, the partitioning differed only slightly with Et_3NP or $NaClO_4$ in the system. Values for $\Delta \log K_{el}$ Eq. (2) were plotted against peptide net charge (Fig. 7a and b) to enable a comparison of the salt effects on the free peptides with the effects when the peptides were fused to the protein. For Et_3NP (Fig. 7a) the uncharged peptides (WP)*n*, $n=2$–4, all resulted in $\Delta \log K_{el}$ values close to zero while negatively charged peptides were directed to the EO–PO copolymer phase and positively charged peptides to the dextran phase, as expected. Furthermore, the effect on peptide partitioning was directly proportional to the net charge of the peptides (see fitted line). However, a small deviation from the fitted line was obtained for the tryptophan containing peptides also containing four charged amino acid residues (WPD)4 and (WPR)4 compared to peptides with four charged amino acid residues and no tryptophans, (RP)4 and (DP)4 (Fig. 7a). The effect of $NaClO_4$ on partitioning was as expected (Fig. 7b), i.e. the salt directed positively charged peptides to the EO–PO copolymer phase and negatively charged peptides to the dextran phase. However, the linear behaviour observed for Et_3NP was not observed for the peptides containing the negatively charged aspartates. A fitted line to the uncharged and positively charged peptides shows the expected effects on the negatively charged peptides. The effect of $NaClO_4$ on partitioning seems to level out with increasing negative net charge on the peptides.

Table 4
The partition coefficients of the peptides with Et_3NP or $NaClO_4$ as dominating salt. The concentration of the salts was 50 m*M* in all cases. Five m*M* Et_3NP was also included in the $NaClO_4$ system to keep pH 7.5. The experimental error was ±10 per cent of the *K* value

	Log *K* Et_3NP pH 7.5	Log *K* $NaClO_4$ pH 7.5
(G)2	−0.0459	−0.0600
(G)3	−0.0519	−0.0822
(G)4	−0.0749	−0.0489
(W)	0.0107	0.00260
(W)2	0.300	0.191
(W)3	0.488	0.480
(W)4	0.694	0.732
(WP)2	0.237	0.236
(WP)4	0.640	0.640
(WPD)2	0.262	0.0934
(WPD)4	0.580	0.264
(WPR)2	0.102	0.407
(WPR)4	0.175	0.845
(RP)2	−0.123	0.124
(RP)4	−0.311	0.301
(DP)4	0.206	−0.163

3.4.2. Partitioning of fusion proteins

The log *K* values for the ZZ-cutinase-tag constructs with Et_3NP and $NaClO_4$ included in the systems are collected in Table 5. The $\Delta \log K_{el}$ values Eq. (2) for the proteins with $NaClO_4$ or Et_3NP as dominating salt plotted against the change in protein net charge due to the fused peptides are shown in Fig. 8. The effects of the salts on protein partitioning corresponded qualitatively with the effects observed for the peptides. Thus, $NaClO_4$ increased the partitioning of the fusion proteins with positively charged tags to the EO–PO copolymer top phase while

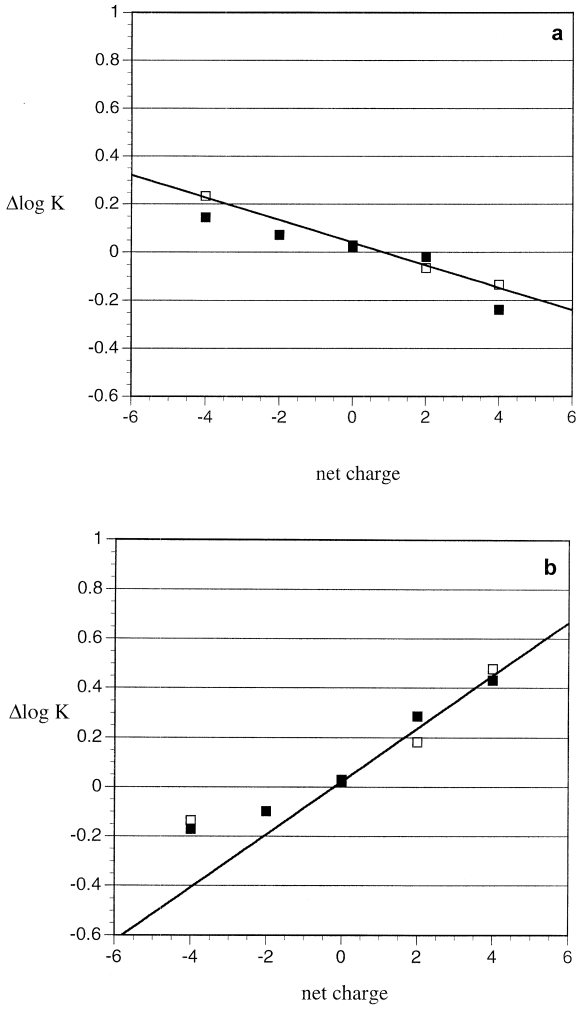

Fig. 7. The salt effect ($\Delta\log K_{el} = \log K^{salt\ system} - \log K^{K_2SO_4}$) on the partitioning of the peptides as a function of net charge, pH 7.5, (a) system with Et_3NP, the line has been fitted to the partitioning of the peptides without tryptophan residues, and (b) system with $NaClO_4$, the line has been fitted to the partitioning of the positively and uncharged peptides. Tryptophan containing peptides (WP)*n*, (WPD)*n* and (WPR)*n*; $n=2,4$ (■); peptides without tryptophan residues (RP)*n*; $n=2,4$, (DP)4 (□). The concentration of the salts was 50 m*M*, and 5 m*M* Et_3NP was also included in the $NaClO_4$ system to keep pH 7.5.

Et_3NP increased the partitioning of the fusion proteins with negatively charged tags to the EO–PO copolymer phase.

The trends observed for the effect of Et_3NP on protein partitioning were the same as for peptide partitioning. Thus, a rather linear behaviour of the salt effect on protein partitioning was seen. The slope value of the fitted line was approximately 85% of the slope value observed for the peptides, i.e. a slightly lower salt effect for the proteins than for the peptides. Furthermore, as for the peptides, the $\Delta\log K_{el}$ values for the fusion proteins containing both four tryptophan residues and four charged amino acid residues (ZZ-cutinase-(WPD)4 and ZZ-cutinase-(WPR)4) were lower compared to the fusion proteins containing only four charged amino acid residues (ZZ-cutinase-(RP)4 and ZZ-cutinase-(DP)4).

Table 5
The partition coefficients of the proteins with Et_3NP or $NaClO_4$ as dominating salt. The concentration of the salts was 50 m*M* in all cases. Five m*M* Et_3NP was also included in the $NaClO_4$ system to keep pH 7.5. The experimental error was ±10 per cent of the *K* value

	Log *K* Et_3NP pH 7.5	Log *K* $NaClO_4$ pH 7.5
ZZ-cutinase-wt	0.589	−0.056
ZZ-cutinase-(WP)2	0.778	0.055
ZZ-cutinase-(WP)4	1.032	0.275
ZZ-cutinase-(RP)2	0.545	−0.032
ZZ-cutinase-(RP)4	0.491	0.057
ZZ-cutinase-(DP)4	0.706	−0.256
ZZ-cutinase-(WPD)4	0.853	0.111
ZZ-cutinase-(WPR)4	0.655	0.319

For proteins with a tag containing four tryptophan residues (ZZ-cutinase-(WPD)4, ZZ-cutinase-(WP)4 and ZZ-cutinase-(WPR)4) the effect of $NaClO_4$ on partitioning showed larger salt effects for positively charged residues fused to the protein (ZZ-cutinase-(WPR)4) than for a negatively charged fusion tag (ZZ-cutinase-(WPD)4). The same was observed for the peptides, as discussed above. The effect of $NaClO_4$ on fusion protein partitioning (Fig. 8) was lower than that observed for the isolated peptides. Values of $\Delta\log K_{el}$ ranged from approximately +0.4 to −0.2 for the peptides (Fig. 7b) and from approximately −0.2 to −0.4 for the proteins (Fig. 8).

It would be advantageous to utilise both salt effects and the hydrophobicity of Trp to direct a target protein to the EO–PO copolymer phase. In order to include both tryptophans and charged res-

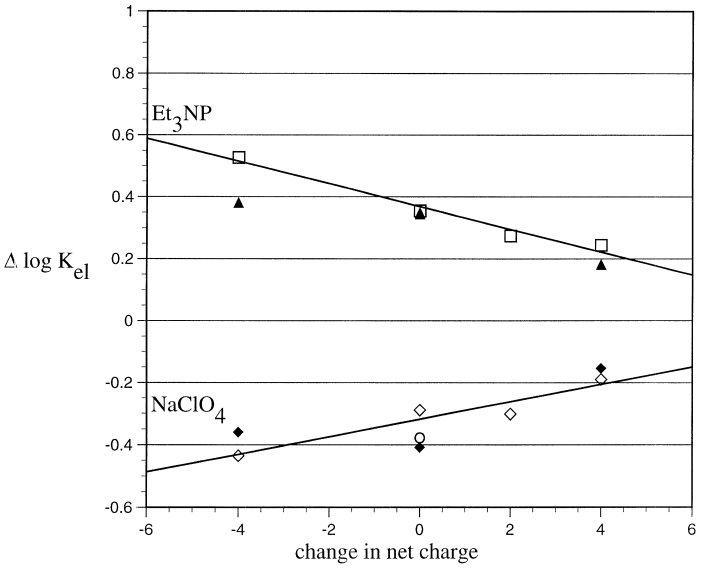

Fig. 8. The salt effect ($\Delta\log K_{el} = \log K^{salt\ system} - \log K^{K_2SO_4}$) on the partitioning of the proteins as a function of change in net charge due to the fused tag, pH 7.5. The salts are indicated in the figure. For Et_3NP, (▲) and (□) indicate tags with and without tryptophan residues, respectively. For $NaClO_4$, (◆) and (◇) indicate tags with and without tryptophan residues respectively, and (○) indicates ZZ-cutinase-(WP)2. The lines have been fitted to the partitioning of proteins without fused tryptophan residues. The concentration of the salts was 50 m*M*, and 5 m*M* Et_3NP was also included in the $NaClO_4$ system to keep pH 7.5.

idues in a fusion tag another primary structure of the tag than used here is needed. A spacer between the charged residues and Trp could be an alternative.

4. Conclusions

The partitioning of ZZ-cutinase in an EO–PO–dextran system could be affected by fusion of peptide tags to the C-terminal. A system with K_2SO_4 as predominant salt has been evaluated and used as a reference system to isolate the partitioning from charge dependent ion effects. This system made it possible to evaluate the contribution to the partition coefficient by fused amino acid residues depending on the relative hydrophobicity of the residues. The effect of Trp was to enhance the partitioning to the EO–PO phase. The effect on peptide partitioning was independent of the difference in primary structure for (Trp)*n*, (Trp-Pro)*n*, (Ala-Trp-Trp-Pro)*n* and only determined by the number of Trp. The enhancement by Trp in the cutinase fusion protein could be qualitatively but not quantitatively predicted from peptide partitioning. The effect of the charged residues, Arg and Asp, was dependent on the peptide primary structure e.g. the partitioning was lowered with Asp and Arg residues situated next to tryptophans. The lowering effect in the fusion proteins could be both qualitatively and quantitatively predicted from the peptides. However, in peptides and tags without tryptophans, the effect on partitioning of the charged residues was somewhat different. Aspartates had no effect on partitioning while arginines showed preference for the dextran phase in the peptides but had no effect when fused to the protein. The salt effects for the fusion proteins could be qualitatively predicted from peptide partitioning. The most effective fusion partner, for directing a protein to the EO–PO copolymer phase, judged from peptide partitioning was (WP)4. The same was observed for the fusion proteins i.e. ZZ-cutinase-(WP)4 in a system with Et_3NP included gave the highest partitioning to the EO–PO copolymer phase i.e. the most effective peptide tag could be predicted from peptide partitioning.

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