Peptide-tagged proteins in aqueous two-phase systems

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Abstract

This thesis deals with proteins containing peptide tags for improved partitioning in aqueous two-phase systems. Qualitatively the peptide-tagged protein partitioning could be predicted from peptide data, i.e. partitioning trends found for peptides were also found for the peptide-tagged proteins. However, full effect of the tag as expected from peptide partitioning was not found in the tagged protein. When alkyl-ethylene oxide surfactant was included in a two-polymer system, almost full effect of the tag was obtained. This indicates an improved exposure of the tag to the phase components in the surfactant-containing systems. The most efficient amino acid residue for partitioning towards a phase rich in random copolymer of ethylene oxide and propylene oxide (EOPO) was tryptophan. The second most efficient amino acids were tyrosine and phenylalanine. The tag efficiencies were higher for proteins with tyrosine/proline tags compared to proteins with tryptophan/proline tags. The reason can be that the tyrosine residues are more exposed to the solution and phase-forming components, since tyrosine is more hydrophilic than tryptophan.

The partitioning coefficient of cutinase wild-type was calculated from peptide partitioning data combined with surface studies with the computer program GRASP. The calculated partitioning coefficient agreed relatively well with the experimentally determined partition coefficient. Thus, it is possible to obtain an approximate partitioning coefficient of a protein before starting a purification procedure and thereby save time in finding an optimal partitioning system.

The fluorescence emission maximum wavelength for free peptide was longer than for peptide tag in tryptophan-tagged cutinase, indicating larger exposure of free peptides. However, all maxima were obtained at wavelengths corresponding to a polar environment and thus indicating solvent exposure of the tryptophan residues in both free peptides and tags. The emission maximum of the tryptophan tag was moved to longer wavelength when a spacer was introduced between protein and tag. This correlates with results obtained in aqueous two-phase partitioning where a spacer between tag and protein resulted in increased partitioning towards an EOPO copolymer phase.

Key words:

Aqueous two-phase system, peptide-tagged protein, tryptophan, cutinase, green fluorescent protein

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List of Papers

This thesis is based on the following papers[1] referred to in the text by their roman numerals.

I. Partitioning of peptides and recombinant protein-peptide fusions in thermoseparating aqueous two-phase systems: effect of peptide primary structure
Kristina Berggren, Anna Nilsson, Göte Johansson, Nina Bandmann, Per-Åke Nygren, Folke Tjerneld
Journal of Chromatography B 743 (2000) 295-306

II. Cutinase-peptide fusions in thermoseparating aqueous two-phase systems Prediction of partitioning and enhanced tag efficiency by detergent addition
Anna Nilsson, Maurice Mannesse, Maarten R. Egmond, Folke Tjerneld
Journal of Chromatography A 946 (2002) 141-155

III. Partitioning of peptide-tagged proteins in aqueous two-phase systems using hydrophobically modified micelle-forming thermoseparating polymer
Anna Nilsson, Hans-Olof Johansson, Maurice Mannesse, Maarten R. Egmond, Folke Tjerneld
Submitted

IV. Tryptophan-tagged cutinase studied by steady-state fluorescence for understanding of tag interactions in aqueous two-phase systems
Anna Nilsson, Maria Teresa Neves-Petersen, Hans-Olof Johansson, Jörgen Jansson, Karin Schillén, Folke Tjerneld, Steffen B. Petersen
Submitted

V. Partitioning and characterisation of tyrosine-tagged green fluorescent proteins in aqueous two-phase systems
Sara Fexby, Anna Nilsson, Gustav Hambraeus, Folke Tjerneld, Leif Bülow
Submitted

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Abbreviations

ASA	solvent accessible surface area
ATP	adenosine triphosphate
CCD	counter current distribution
C_mEO_n	alkyl-ethylene oxide surfactant
CMC	critical micelle concentration
CP	cloud point
D	aspartate (Asp)
DE	degree of exposure
E. coli	*Escherichia coli*
EO	ethylene oxide
EOPO	random copolymer of ethylene oxide and propylene oxide
G	glycine (Gly)
GRASP	graphical representation and analysis of surface properties
GFP	green fluorescent protein
HM-EOPO	hydrophobically modified random copolymer of ethylene oxide and propylene oxide
IgG	immunoglobulin G
LCST	lower critical solution temperature
NAD^+	nicotinamide adenine dinucleotide
P	proline (Pro)
PDB	protein data bank
PEG	polyethylene glycol
pI	isoelectric point
PO	propylene oxide
R	arginine (Arg)
S	serine (Ser)
S. cerevisiae	*Saccharomyces cerevisiae*
T	threonine (Thr)
Triton	polyoxyethylene alkyl phenol
W	tryptophan (Trp)
Y	tyrosine (Tyr)

1. Introduction

Proteins are important in most biological processes. To be able to study protein structure and function, pure proteins are often needed. Proteins are also used in the pharmaceutical industry and many other industrial applications. Recombinant proteins produced with genetic engineering techniques are often utilised in modern biotechnology industry. Thus, it is possible to slightly alter the protein primary structure by genetic engineering to simplify the purification. Many different purification steps are often combined in order to achieve a satisfying purification of a protein. In a primary recovery of the recombinant protein from cultivated cells, it is desirable to use a method where cell debris can be removed reasonably fast and sample volume can be reduced. It is also advantageous if the target protein is partly purified in this step. Moreover, it is favourable if the method is relatively straightforward to scale up from a small laboratory scale to a large industrial scale.

An aqueous two-phase system is formed when two structurally different polymers are mixed with water above certain concentrations. Each polymer is enriched in a separate water phase. Biomolecules, such as proteins, partition between the phases. Since the water content is high (80–95%) and the surface tension between the phases is low, aqueous two-phase systems offer a mild method for a primary recovery step [1, 2]. The partitioning in an aqueous two-phase system depends on many factors, such as the net charge of the biomolecule [3] or surface properties such as hydrophobicity [4, 5]. Thus, besides from large-scale extractions and purification in laboratory scale, aqueous two-phase systems can be used as an analytical tool to study protein properties or interactions between biomolecules.

The aim of the study presented in this thesis was to understand how the partitioning of a protein in an aqueous two-phase system is altered when a peptide tag is genetically fused to the protein, in order to make the extraction to a certain phase more selective. Several types of phase systems were studied to examine how the peptide tag affected the protein partitioning in different polymer or surfactant solutions. The composition of the tag was

evaluated and also the length of the tag. A spacer consisting of a few amino acids was introduced between tag and protein to investigate if interactions of the tag with phase components were facilitated when the spacer was present. Different methods have been evaluated to predict the partitioning of both tagged and wild-type proteins. The exposure of the tags towards different components in the phase systems was studied by steady-state fluorescence.

2. An introduction to aqueous two-phase partitioning

2.1 General concepts

Aqueous two-phase systems were first discovered in 1896 by Beijerinck who noticed the phase separation of gelatine and agar or starch in water solutions [6]. However, phase systems were not used until the nineteen-fifties when Albertsson rediscovered the phenomenon and developed the phase separation technique [1, 7-9].

Phase diagrams are used for characterisation of aqueous two-phase systems [1, 2, 10, 11]. A schematic phase diagram for a two-polymer system is shown in Fig. 1. The concentrations of polymers are expressed as weight percent (% (w/w)). The concentration of polymer P is plotted as abscissa and the concentration of polymer Q as ordinate. The binodal is the boundary line between the one and two-phase areas. Above the binodal a two-phase system is formed. When approaching the critical point (C) the difference between the two phases diminishes. The critical point is defined as the point in the phase diagram where composition and volume of the two phases are equal. The straight lines connecting two nodes at the binodal are called tie lines. Choosing an arbitrary mixing point (P) along the tie line, the polymer concentration values at the nodes correspond to the concentrations obtained in the two phases at equilibrium. Thus, it is possible to obtain the same partitioning coefficient with different volume ratios of the phases. The partitioning coefficient (K) of a substance is defined as the concentration of the substance in top phase divided by the concentration in bottom phase.

$$K = \frac{C_{\text{top phase}}}{C_{\text{bottom phase}}} \qquad (1)$$

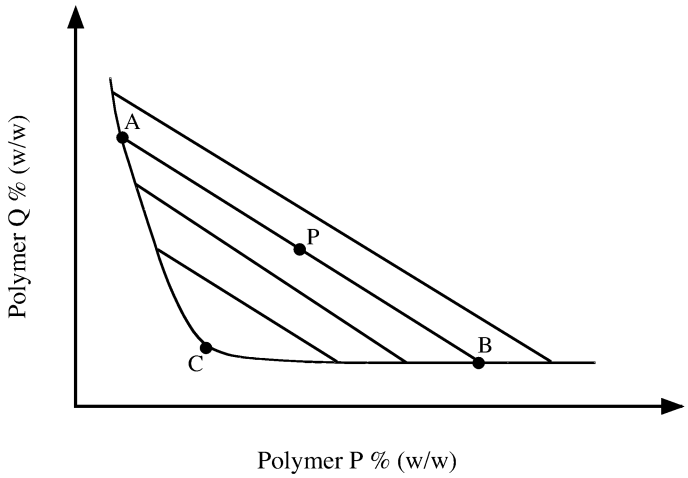

Fig. 1. A schematic picture of a phase diagram for a two-polymer system. P is an arbitrary mixing point resulting in the polymer compositions A and B respectively for the two phases. C is the critical point. Below the binodal curve is the one phase area and above the curve is the two-phase area.

2.2 Different aqueous two-phase systems

2.2.1 Polymer/Polymer systems

2.2.1.1 The PEG/dextran system

The most commonly used polymer/polymer system is composed of polyethylene glycol (PEG) and dextran. PEG constitutes the top phase forming polymer and dextran the bottom phase polymer. PEG is an unbranched polymer of ethylene oxide units (Fig. 2). Dextran is a bacterial polysaccharide obtained from *Leuconostoc mesenteroides* and consists of glucose units linked together with α-1,6 bonds. Approximately 5 % of the linkages are α-1,3 resulting in a branched polymer [12] (Fig. 3). An advantage of using two-polymer systems compared to the well established PEG/salt systems (see section 2.2.3) is the low salt content, and thus the risk of salting-out or precipitating proteins is smaller [13].

$HO\text{-}(CH_2\text{-}CH_2\text{-}O)_n\text{-}H$

Fig. 2. The structure of polyethylene glycol (PEG).

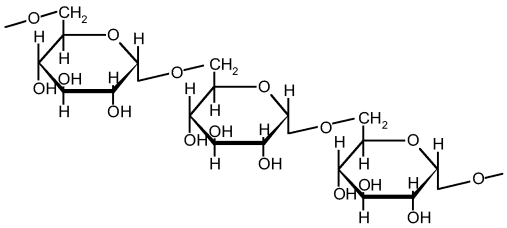

Fig. 3. The structure of dextran. Approximately 5 % of the glucose units are coupled with α-1,3 linkages; the rest is α-1,6 linked.

2.2.1.2 Thermoseparating polymers

Other top phase forming polymers than PEG have been used in phase separations. One example is the random copolymers of ethylene oxide (EO) and propylene oxide (PO) [14-19] (Fig. 4).

$HO\text{-}(CH_2\text{-}CH_2\text{-}O)_m\text{-}(CH_2\text{-}CH\text{-}(CH_3)\text{-}O)_n\text{-}H$

Fig. 4. A schematic structure of a random copolymer of ethylene oxide (EO) and propylene oxide (PO). The EO and PO units are randomly distributed along the chain.

The EOPO copolymers are thermoseparating, like most ethylene oxide containing polymers (including PEG). The solubility of thermoseparating polymers in water decreases when temperature is increased and they have a lower critical solution temperature (LCST). The phenomenon has been explained by an increase in polymer segment hydrophobicity with increasing temperature [20, 21]. When temperature is increased above a certain temperature, the cloud point, a water solution of the polymer will become turbid and phase separate into one polymer-enriched phase containing typically 30-60 % (w/w) polymer and one polymer-depleted water phase with less than one percent polymer [21]. The cloud point temperatures for EOPO copolymers are in the range of 32 to 50 °C for a 10 % (w/w) solution [18, 21]. The EOPO copolymers with higher propylene oxide content have lower cloud point [22]. The cloud point of a polymer phase can be efficiently decreased by addition of salt, such as sodium sulphate [14]. Proteins have been found to partition exclusively to the water phase in thermoseparating

EOPO/water systems. The entropic driving force is important. Since the number of molecules is much higher in the water phase, partitioning towards this phase is entropically favoured [23]. Thus, the protein is obtained in a water phase from which it can be further purified and the polymer can be recycled [13]. The partitioning procedure in a system containing thermoseparating EOPO copolymer is presented in Fig. 5.

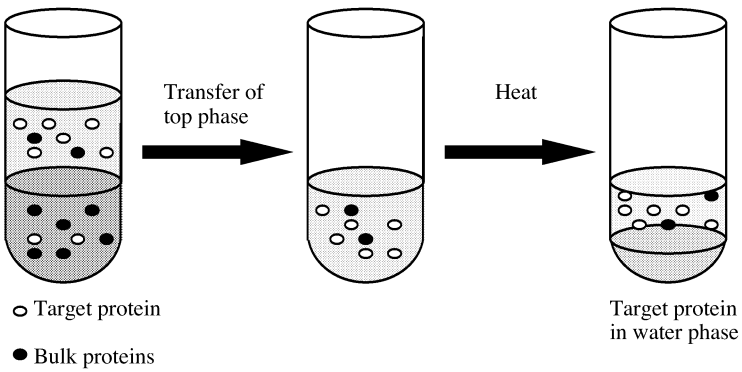

Fig. 5. Purification of a protein in an aqueous two-phase system followed by temperature-induced phase separation. The top phase (light grey) in the system to the left is enriched in an EOPO copolymer and water. The bottom phase (dark grey) contains hydroxypropyl starch or dextran and water. The top phase is transferred to an isolated vessel (picture in the middle) and temperature is increased to achieve a temperature-induced phase separation (picture to the right).

2.2.1.3 Pluronic polymers

Pluronic polymers have been used in a few cases in aqueous two-phase partitioning [24-27]. Pluronic consists of two blocks of ethylene oxide (EO) with one block of propylene oxide (PO) in between. The polymers form micellar-like structures above the critical micelle temperature, CMT [28]. Pluronic polymers are available in a wide range of molecular masses and EO/PO ratios [29]. Reverse Pluronics exist, with two blocks of propylene oxide and one block of ethylene oxide in between. Reverse Pluronics have lower cloud points than ordinary Pluronics with same EO/PO ratio [29].

2.2.1.4 Polymers used in large-scale extraction

In large-scale purification it is necessary to have as low process costs as possible. PEG/salt systems (see section 2.2.3) are the most commonly

utilised systems for large-scale extractions, since chemical costs are relatively low [30]. Dextran is an expensive polymer since it is produced from a bacterial organism and is further fractionated into different molecular masses prior use [12]. To make aqueous two-polymer extractions a possible alternative in large-scale purification, research efforts have been devoted to find cheaper alternatives to dextran. Different starch derivatives have been utilised [31] as well as unfractionated crude dextran [32]. Starch has been modified with hydroxypropyl ether groups in order to overcome gel-forming behaviour [31]. The structure of Reppal, a hydroxypropyl starch polymer, is presented in Fig. 6.

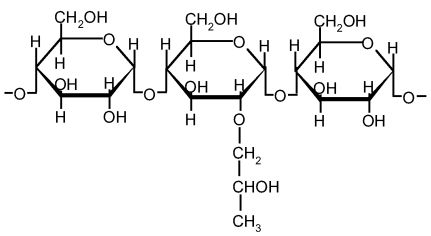

Fig. 6. The structure of hydroxypropyl starch, Reppal.

2.2.2 One-polymer systems

Thermoseparating polymers can be used in one-polymer extraction systems. In a thermoseparating system containing simple random copolymer of ethylene oxide and propylene oxide, the polymer phase is often too concentrated (typically 30-60 % (w/w) polymer) to be utilised for protein extraction [23]. This problem can be solved by using hydrophobically modified random copolymer of ethylene and propylene oxide, HM-EOPO (Fig. 7) for protein extraction in a one-polymer system. The polymer is hydrophobically modified with myristyl groups ($C_{14}H_{29}$) in both ends. The EOPO chains are linked together by an isophoronediisocyanate (IPDI) group. The HM-EOPO polymer has a cloud point of 12°C at 3 % (w/w) [Paper III]. The polymer phase-separates above the cloud point in one polymer-enriched bottom phase with 6-8 % (w/w) HM-EOPO and one water-rich top phase with less than 1 % (w/w) polymer. Thus, due to the relatively high water concentration in the polymer rich phase, this system can be used for protein partitioning at room temperature [33]. The

HM-EOPO polymer is like most hydrophobically modified polymers [34] strongly self-associating and forms micellar-like structures. The CMC is 12 μM [33]. The phases in a one polymer HM-EOPO system display large differences in hydrophobicity and target proteins for extraction to the HM-EOPO enriched phase should thus preferably be hydrophobically tagged or amphiphilic proteins [33, Paper III]. Block copolymers (Pluronics) have also been utilised for extractions in one-polymer systems [35]. The one-polymer systems have large similarities with surfactant-based cloud point extraction systems (see section 2.2.4), but the molecular masses of polymers are larger compared to surfactants.

$H_{29}C_{14}$-O-($EO_{66}PO_{14}$)-IPDI-($EO_{66}PO_{14}$)-O-$C_{14}H_{29}$

IPDI (isophoronediisocyanate)

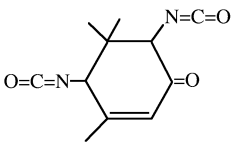

Fig. 7. The structure of HM-EOPO, a hydrophobically modified copolymer of ethylene oxide and propylene oxide. The EO and PO groups are randomly distributed on each side of the isophoronediisocyanate group. The polymer is hydrophobically modified with myristyl groups ($C_{14}H_{29}$) in both ends.

2.2.3 Polymer/Salt systems

Polymer/salt systems are most commonly composed of polyethylene glycol (PEG) and sodium or potassium phosphate. Other salts than sodium or potassium phosphate, such as sodium sulphate, magnesium sulphate or citrate, have also been utilised [36-39]. The higher the valencies of the ions the lower concentration of the salt is needed to achieve phase separation [36]. Citrate is advantageous since it is biodegradable [38]. The phase-forming salt affects partitioning of charged biomolecules, and phosphate or sulphate salts directs partitioning of negatively charged substances to the PEG rich top phase [40]. It is possible to alter the partitioning of proteins by addition of NaCl. Asenjo et al. obtained an increase of target protein partitioning towards the PEG phase with increasing concentration of NaCl, but bulk protein partitioning remained constant [41].

The hydrophobicity difference between the two phases is larger in PEG/salt systems compared to two-polymer systems [4] and thus more extreme partitioning can be obtained. The PEG-rich top phase usually contains 20-30 % (w/w) PEG plus salt. The salt-rich bottom phase contains 10-15 % (w/w) salt. Due to larger number of molecules in salt phase, partitioning towards this phase is entropically favoured [23].

2.2.4 Surfactant systems

The surfactant systems or cloud point extraction systems are similar to one-polymer systems since they both utilise the clouding behaviour of the phase component. The cloud point extraction systems were first studied by Bordier in the beginning of the nineteen-eighties [42] and have been extensively used for membrane proteins and relatively hydrophobic proteins [43-46]. The surfactant must have a cloud point since extraction is performed above the cloud point, often around 25-30°C [42, 44, 45]. Examples of surfactants that have been utilised for cloud point extraction are Triton X-114 and different alkyl-ethylene oxide surfactants with the general formula C_mEO_n [43]. The general structure of C_mEO_n surfactants is presented in Fig. 8. Recently, non-ionic $C_{10}EO_4$ has been used together with anionic sodium dodecyl sulphate (SDS) to form mixed-micelle extraction systems for protein partitioning [47].

2.2.5 Surfactant/Polymer systems

An alternative to surfactant-based cloud point extraction systems is surfactant/polymer systems. The systems can be compared with polymer/polymer systems with one of the polymers exchanged for a surfactant [48]. Since phase separation does not occur due to clouding of surfactant, the surfactant can be chosen in a broader range compared to surfactant-based cloud point extraction systems. It is also possible to perform the extraction at lower temperatures [48-50]. For instance PEG and dextran have been used together with C_mEO_n surfactants [50].

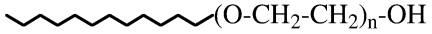

Fig. 8. A general structure of an alkyl-ethylene oxide surfactant, C_mEO_n.

2.3 System variables influencing partitioning

2.3.1 Polymer concentration and molecular mass

The partitioning in an aqueous two-phase system can be influenced by a set of system variables. The hydrophobicity of the polymers is a key variable for protein partitioning [17]. The partitioning depends on the chemical difference between the phases, and thus also the polymer concentrations. If a substance partitions towards one phase, partitioning towards this phase will be enhanced when polymer concentrations are increased (increased tie line length), since the chemical difference between the phases increases [1, 2, 23]. High molecular masses of polymers should in general be used to create a large difference between the phases. If the molecular mass of one of the polymers is lowered, partitioning towards the phase enriched in this polymer is often increased. With smaller polymer molecules in one of the phases the water content becomes higher in this phase. Thus, partitioning towards this phase is entropically favoured [23]. Partitioning of low molecular mass substances is not as affected of change in polymer molecular mass as substances with higher molecular mass [51].

2.3.2. Temperature effects

The operating temperature affects the partitioning, since the phase composition is altered when temperature is changed. The closer the system composition is to the critical point, the more temperature will affect the partitioning. In order to have a robust system; it is thus advantageous to avoid operating too close to the critical point.

2.4 Protein properties affecting partitioning

2.4.1 The protein surface

The partitioning of a protein depends on the protein surface properties. The partitioning coefficient can be divided into different contributions [1]:

$$K = K^0 \cdot K_{el} \cdot K_{hphob} \cdot K_{biosp} \cdot K_{size} \cdot ... \qquad (2)$$

The different terms correspond to electrochemical effects (K_{el}, see 2.4.2), hydrophobic (K_{hphob}) respectively biospecific (K_{biosp}) contributions to the partitioning. The size of the biomolecule also affects partitioning (K_{size}). In the term K^0 other factors such as molecular mass or concentration of polymers, or temperature are collected. The hydrophobicity of proteins has been investigated by hydrophobic modification of PEG with saturated or unsaturated hydrocarbon groups [52, 53]. The partitioning in PEG/dextran systems containing these ligands was compared to partitioning in systems lacking ligands [52, 53]. If the partitioning towards the PEG-phase was enhanced in system containing ligand, hydrophobic interaction between protein and ligand occurred. The hydrophobic contribution to protein partitioning has also been studied by chemical modification of proteins with alkyl groups in order to make the proteins more hydrophobic [54]. The hydrophobicity of different PEG/salt and PEG/dextran systems was determined by plotting the logarithms of the partitioning coefficients for the modified proteins against their hydrophobicity determined by hydrophobic interaction chromatography, HIC [4]. The hydrophobicity of aqueous two-phase systems has also been determined by partitioning of homologous series of dinitrophenylated amino acids with aliphatic side chains containing different numbers of CH_2-groups [55, 56].

Recently it has been shown that partitioning of several monomeric proteins in an $EO_{30}PO_{70}$/dextran T500 system could be described by differences in surface exposed amino acid residues. Different hydrophobicity scales were used to evaluate the partitioning [5]. A linear correlation was found between logarithmic partitioning coefficients of the proteins and hydrophobicity of the protein surface. The hydrophobicity of the surface exposed amino acid

residues in the protein was determined by peptide partitioning. Aromatic residues preferred the EOPO-rich phase whereas charged amino acid residues enhanced the partitioning towards the dextran-rich phase. Larger partitioning effects of amino acid residues were obtained when polymer concentrations were increased, i.e. the system became more sensitive in detecting surface exposure changes at higher polymer concentrations.

2.4.2 Salt effects

2.4.2.1 General issues

Addition of salt, where anion and cation have different affinities for the two phases in an aqueous two-phase system, will influence the partitioning of a charged biomolecule [1, 3, 57-59]. One widely accepted explanation of the effect is that an electrochemical potential difference is created between the two phases [1, 58-61]. The requirement for electroneutrality forces anion and cation to partition together, but different affinities of the ions for the two polymer phases will generate an electrochemical driving force between the phases [23]. The anion or cation of a salt can use the protein as counter ion depending on the net charge of the protein, and thereby direct partitioning of the protein towards one of the phases in the system.

The higher the net charge of the protein the larger is the salt effect [3]. The pH is therefore a useful tool in protein partitioning since net charge of a protein can be modulated without major chemical changes of the system. If the concentration of a salt is ten times higher than the concentration of other solutes (ions) the effects of this salt is dominating in the system [3, 57]. The salt effect is independent of the salt concentration at concentrations of 50–100 mM [3]. Salt may change the polymer composition of the phases in the system [60, 62], however salts which have a relatively even partitioning between the phases affect the phase diagram for PEG and dextran only to a minor degree in concentrations up to 200 mM [63].

2.4.2.2 The Hofmeister series

In PEG/dextran and EOPO/dextran or hydroxypropyl starch systems, large ions with low charge, chaotrops, are efficient in directing proteins or other biomolecules of opposite charge to PEG or EOPO rich phases [17, 18]. The

order of decreasing efficiency of anions to direct a protein with a positive net charge to PEG or EOPO rich phases is $ClO_4^- \geq SCN^- \geq I^- > Br^- > Cl^- > NO_3^- > HPO_4^{2-} \geq SO_4^{2-}$ [17, 18]. The series follows the Hofmeister series of chaotropic water-structure breaking ions [64, 65]. The more hydrophobic the polymer used in the aqueous two-phase system the larger the effect of the chaotropic salt [17, 18]. High concentrations of chaotropic salts may denature proteins [66], but with the concentrations commonly used in aqueous two-polymer phase partitioning (50-100 mM) this is seldom a problem. The affinity of phosphate ions for the more hydrophilic dextran bottom phase increases when the charge of the ion increases [58]. The affinity of cations for the lower, dextran phase increases in the order $Li^+ < NH_4^+ < Na^+ < K^+$ [3]. In systems containing EOPO copolymers, triethylammonium ions (Et_3NH^+) together with phosphate ions have been shown to be efficient in directing proteins with a negative net charge to the EOPO phase [17].

2.4.2.3 Reference systems

If non-electrostatic effects such as hydrophobicity are to be studied it is necessary to minimise charge-dependent effects in the two-phase system. One way to achieve this is to perform the partitioning at the pI of the protein. However, some proteins have low solubility at the pI. Instead, it is possible to utilise a salt where the electrochemical driving force between the phases is minimised. Potassium sulphate (K_2SO_4) has been shown to have this ability in PEG/dextran systems [3] as well as in systems containing random EOPO copolymers [Paper I-III]. With K_2SO_4 as dominating salt, protein net charge will not affect partitioning (see Table 2 in Paper I), which thus can be carried out at pH values different from the pI.

2.4.2.4 Cross-partitioning

The partitioning of a protein with zero net charge is not affected by the type of salt used. This can be utilised to determine the pI of the protein [67-69]. Partitioning is carried out with two different types of dominating salts (e.g. Na_2SO_4 and NaCl). The protein is partitioned at different pH-values and the pI is found at the cross point of the K versus pH plot. The technique is called cross-partitioning and the pI-values have been found to correlate well with values obtained with other techniques [67-69].

2.5 Affinity partitioning

Affinity interactions are efficient to use in protein purification. In aqueous two-phase systems a substance for which the target molecule has affinity can be attached to one of the polymers [70, 71]. PEG has been modified with hydrophobic ligands such as palmitic acid to extract human serum albumin in a PEG/dextran system [72]. The ligand can also be a compound that resembles the enzyme substrate or a coenzyme. Triazine dyes such as Cibacron blue or Procion yellow resemble coenzyme NAD^+ and also ATP and have been used to purify dehydrogenases and kinases [73-76]. Inhibitors have also been utilised. P-aminobenzamidine, which is a strong inhibitor of trypsin, was attached to PEG to be able to extract trypsin in an aqueous two-phase system [77]. Different types of erythrocytes have been separated in aqueous two-phase systems by using IgG coupled to PEG [78, 79].

3. Applications of two-phase partitioning

3.1 Two-phase partitioning as an analytical tool

3.1.1. Interactions between biomolecules

Aqueous two-phase partitioning can be used to detect interactions between different kinds of biomolecules. If two biomolecules interact the partitioning will be affected when the biomolecules are partitioned together [80, 81]. In counter-current distribution (CCD) a large number of partitioning steps are carried out in order to separate substances having different partitioning coefficients [9]. The resolution of the partitioning is better than when a single separation is performed [1]. CCD has been used to study interactions of glycolytic enzymes and filamentous actin [82]. Interactions between molecules can be used to separate enantiomers in aqueous two-phase systems [83, 84]. If one of the enantiomers interact with a protein that has an extreme partitioning this can be utilised to collect the enantiomer in one of the phases [83, 84]. One example is the separation of L- and D-tryptophan. Bovine serum albumin partitioned towards the bottom phase in a PEG/dextran system and affected the partitioning of L-tryptophan and hence the enantiomers could be separated [83].

3.1.2 Protein surface studies

Since partitioning of a protein depends on protein surface properties, aqueous two-phase partitioning can be used to study changes on protein surfaces due to e.g. point mutations. The partitioning of a set of cutinase mutants has been characterised in an EOPO/dextran system [85]. The exposure of point mutated amino acid residues was studied with the computer program GRASP [86]. The change in partitioning coefficient of cutinase mutants compared to wild-type protein could be predicted from peptide partitioning combined with the exposure of the amino acid residues. Thus, aqueous two-phase systems can be used to probe protein surface changes caused by point mutations.

The formation of dimers and larger aggregates of proteins has been investigated in aqueous two-phase systems [80, 87]. Also, pH dependent conformation changes of proteins have been studied by aqueous two-phase partitioning [88]. Aqueous two-phase separation can be used to detect changes of the surface of cells. For instance, erythrocytes of different ages have been separated with counter-current distribution [89]. Pharmaceutical proteins can be modified with PEG or other neutral polymers in order to enhance the performance of the protein in vivo. Aqueous two-phase systems have been utilised to characterise PEG-modified proteins and a correlation between log K and weight fraction of PEG in the polymer-protein conjugate has been found [90].

3.2 Large-scale extraction

3.2.1 General issues

Aqueous two-phase systems can be utilised as a primary recovery step in a purification process, since it is possible to include cells or cell debris in the extraction. The same partitioning can be obtained both in small laboratory scale and in large-scale extractions and thus the method is relatively straightforward to scale up [40, 91]. When aqueous two-phase systems are loaded with biomass, lower concentrations of phase forming chemicals are usually needed to achieve phase separation [92]. By altering composition of the aqueous two-phase system along the tie line it is possible to change the volume ratio of the phases without changing the partitioning coefficient [1]. Thus, concentration of target protein can be achieved simultaneously with purification [93].

3.2.2 Isolation of protein from phase-forming chemicals

Different methods have been utilised to separate target protein from polymer phase after two-phase separation. Separation of protein and polymer can be achieved with ion-exchange chromatography, since the protein is charged and the polymer is often uncharged [94]. Ultrafiltration can be used to recycle both polymer and salt [95, 96]. The target protein can also been separated from a PEG phase by back-extraction. A fresh salt phase is added

to the collected PEG phase with target protein and a new phase system is formed. By changing system conditions target protein can be directed to the salt phase [97]. If thermoseparating polymers (e.g. $EO_{50}PO_{50}$) are used in the process, protein can be separated from polymer phase by an increase of temperature (see section 2.2.1.2). The polymer-phase separates into a polymer-depleted water phase containing the protein and a polymer-rich phase [22].

3.2.3 Examples of large-scale extractions

PEG/salt systems have been extensively utilised in large-scale protein extractions since the phase chemicals are rather inexpensive [30, 91, 98]. Besides from PEG/salt systems, surfactant-based cloud point extraction systems have been used for large-scale purification. Cholesterol oxidase has been purified by surfactant-based cloud point extraction [99, 100]. The surfactant could be recycled by extraction to an organic phase of 2-methylpropan-1-ol and recovered by distillation of this phase [99, 100]. Thermoseparating EOPO copolymer has been utilised together with a starch derivative in a two-polymer system, to extract a recombinant peptide-tagged protein, zz-cutinase-$(WP)_4$ in large-scale. After extraction the protein was separated from the EOPO copolymer phase by thermoseparation [101].

4. Affinity tags for protein purification

To make a purification procedure efficient it is desirable to have a method to distinguish target protein from other proteins in the starting material. One way to generalise a purification procedure is to add an affinity tag to the target protein by genetic engineering and use this to perform the purification [102, 103]. Affinity tags have been utilised in chromatography [104], precipitation [105] as well as in aqueous two-phase partitioning [106, 107, 108, Paper I].

4.1 Different types of affinity tags

4.1.1 Peptide tags

Many different affinity tags exist. A tag consisting of six histidine residues is commonly used in chromatography. The histidine tag can interact with nickel, copper, zinc or cobalt ions coupled to iminodiacetate or nitriloacetic acid attached to a matrix [104]. Histidine-tagged proteins have been purified in aqueous two-phase systems by coupling metal ion-binding groups to one of the polymers [109]. Tryptophan-based tags have been utilised to a large extent in aqueous two-phase systems and are discussed in section 4.1.4 and chapter 5. Another example of an affinity tag is the FLAG-tag [110, 111]. The FLAG-tag consists of eight amino acids, (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and the tagged protein can be purified with help of monoclonal antibodies towards the tag. The binding of the FLAG-tag to antibodies is calcium dependent and thus can elution easily be performed with chelating agents such as EDTA. A streptavidin-binding peptide tag (the SBP-tag) consisting of 38 amino acids can be used to purify proteins with a matrix of immobilised streptavidin [112]. Also, a synthetic streptavidin-binding peptide of 8 amino acids exists and can thus be utilised as purification tag [113]. A thermosensitive tag based on elastin-like polypeptides can also be used [114]. Repetitive units of the sequence Val-Pro-Gly-X-Gly, where X is an arbitrary amino acid except proline, were added to a model protein. When temperature was increased the protein precipitated and could be separated from other proteins in the starting material [114]. Tags composed of cysteine

or phenylalanine have also been utilised to purify proteins with affinity (thiopropyl-Sepharose) respectively hydrophobic interaction (phenylalanine-Sepharose) chromatography [115].

4.1.2. Proteins as fusion tags

Proteins can be utilised as fusion tags. The z-domain binds immunoglobulin G (IgG) and is derived from domain B in staphylococcal protein A [116]. The IgG binding property can be used for affinity purification [117]. The streptococcal protein G binds to IgG and also to HSA. Thus, two different possibilities for affinity purification exist with protein G as fusion partner [118]. A cellulose-binding domain can act as fusion partner since it can interact with cellulose-based purification materials [119]. The maltose binding protein can also be used as a fusion partner since it interacts with cross-linked amylose chromatography material [120]. A protein with glutathione-*S*-transferase as purification tag has been purified by affinity chromatography with immobilised gluthatione [121]. β-Galactosidase has been used as fusion partner in aqueous two-phase systems due to a strong partitioning to PEG-rich phases and is discussed in section 4.1.4. Hydrophobin is a small hydrophobic protein from filamentous fungi [122]. By genetically fusing hydrophobin to a hydrophilic endoglucanase it was possible to separate the enzyme with aqueous two-phase partitioning from other types of endoglucanases in a culture filtrate from *Trichoderma reesei* [123, 124].

4.1.3 Charged fusion partners

A fusion partner that changes the pI of the protein can be utilised. Polyarginine tags have been added to the C-terminal of a protein to be able to use ion exchange chromatography for purification [125]. The z-domain, derived from protein A, has been genetically engineered to be utilised in cation exchange chromatography purification [126]. The pI of a protein with the z-domain as fusion partner was increased and the protein could be distinguished from other proteins using cation exchange chromatography [126]. β-Galactosidase has been genetically engineered with polyaspartate tags [127]. The partitioning in aqueous two-phase systems was altered

compared to wild-type protein and could be further affected by changing the pH [127].

4.1.4 β-Galactosidase and tryptophan tags

β-Galactosidase partitions strongly to a PEG-rich phase in an aqueous two-phase system [92]. Fusion proteins have been constructed with β-galactosidase as affinity partner for purification in aqueous two-phase systems [128, 129]. The strong partitioning of β-galactosidase towards the PEG phase was hypothesised to be due to the high tryptophan content in the protein. Peptides tags based on tryptophan were thus constructed to be utilised in aqueous two-phase partitioning [106, 130, 131]. The partitioning of the tryptophan-tagged proteins was increased compared to wild-type, and tryptophan tags have been extensively studied afterwards [108, 132-134, Paper I-IV]. Another reason for constructing proteins with tryptophan tags was the strong partitioning of tryptophan peptides towards the polymer phase in EOPO/water systems [135]. Diamond et al. [136] and Eiteman et al. [37] have also previously found that dipeptides containing tryptophan partitioned towards the PEG rich phase in PEG/salt systems.

4.2 Deletion of fusion tag

To be able to utilise a tagged protein for pharmaceutical applications it is generally necessary to cleave off the fusion tag. This can be achieved by introducing a cleavage site between protein and tag. The tag can either be chemically or enzymatically cleaved. Enzymatic cleavage is often advantageous due to higher selectivity and milder conditions compared to chemical cleavage [137, 138]. However, cleavage of tag introduces an extra purification step in the procedure. Thus, it can be more advantageous to use tags for extraction of proteins for diagnostic or industrial applications. If a protein should be utilised in e.g. a washing powder, the affinity tag could still be present in the final step as long as the tag does not interfere with the enzyme activity.

To chemically cleave off a tag, cyanogen bromide that cleaves after a methionine can be used [137-139]. Hydroxylamine can also be utilised and

cleaves the peptide bond between an asparagine and a glycine residue [137, 138, 140]. Many different enzymes can be utilised to cleave off the tag [137, 138]. Chymotrypsin cleaves at the carbonyl side of phenylalanine, tryptophan or tyrosine. Trypsin cleaves at the carbonyl side of arginine and lysine residues [141]. H64A subtilisin cleaves peptide bonds at the amino side of histidine [142]. Enterokinase cleaves after $(Asp)_4$-Lys- and is utilised in the FLAG-peptide [142].

5. Peptide-tagged proteins in aqueous two-phase systems

5.1 Aim of investigation

This thesis deals with proteins containing peptide tags for improved partitioning in aqueous two-phase systems. The purpose of the tag was to increase the hydrophobicity of the protein and thereby direct the protein towards a more hydrophobic polymer phase. The main focus has been on tryptophan-based tags [Paper I-IV], but also tyrosine-based tags have been studied [Paper V]. One aim was to understand the partitioning of tagged protein from peptide partitioning. It would be a great advantage to know the effect of the tag prior to genetic engineering of the protein, to be able to find an optimal tag. Besides from studying partitioning of the tagged proteins in different sorts of aqueous two-phase systems [Paper I-III, V], the exposure of tryptophan-based tags and interactions of the tags with phase components have been examined with steady-state fluorescence [Paper IV].

5.2 Genetically modified proteins

5.2.1 The model proteins

The model protein for studying tryptophan-based tags was cutinase from *Fusarium solani pisi*. Cutinase is a lipolytic enzyme and catalyses digestion of cutin that covers the surface of plants [143, 144]. Cutinase can also hydrolyse water-soluble esters [145]. Like most hydrolases cutinase has a triad of serine, histidine and aspartate in the active site of the protein. Cutinase lack a hydrophobic lid covering the active site, which is common for many lipases, but hydrophobic residues are enriched close to the active site [146]. The molecular mass of *Fusarium solani pisi* cutinase is 21 kDa and the pI is 7.8 [147]. Cutinase has been crystallised and the three dimensional structure has been determined to a resolution of 1 Å [148]. Cutinase was genetically engineered by adding peptide tags to the C-terminal. The cutinase variant used in Paper I had two z-domains (see section 4.1.2) attached to the N-terminal, i.e. zz-cutinase. Also, zz-cutinase

with tyrosine-based tags has been studied (unpublished results, see section 5.6.3). The z-domains were added to the protein in order to use affinity chromatography for purification [117].

Green fluorescent protein, GFP, was used when studying tyrosine-based tags in Paper V. GFP naturally occurs in the jellyfish *Aquorea victoria* [149]. This protein can generate light without need of any substrate or cofactor. Three amino acids, serine 65, tyrosine 66 and glycine 67 form the chromophore, p-hydroxybenzylideneimidazolinone [149]. The molecular mass of the GFP variants used in Paper V is approximately 30 kDa. The structure of GFP resembles a barrel. The barrel is formed by eleven β-sheets and a central α-helix containing the chromophore [150]. The GFP protein was genetically engineered by adding tyrosine-based tags in the N-terminal.

5.2.2 Composition of the tag

Tryptophan was chosen as main component of the tags in Paper I-III since peptides based on this residue previously have shown to partition strongly towards phases rich in random copolymers of ethylene oxide and propylene oxide (EOPO) or polyethylene glycol (PEG) in aqueous two-phase systems [133, 135, 151]. Proline residues were included in the tags to reduce proteolytic cleavage of the tag during cultivation. Proline residues are known α-helix breakers [152] and could thus prevent secondary structure formation that could diminish exposure of tryptophan residues. The secondary structure of the tagged proteins was predicted with PSIPRED [153]. In all cases the structure of the tag was represented by a coil, i.e. neither an α-helix nor a β-strand. Charged amino acid residues (arginine and aspartate) were included in some tags in order to study the effect of charged residues on the partitioning [Paper I]. The effect of introducing a six amino acids long spacer (TGGSGG) between protein and tag was also investigated [Paper II-III]. A spacer between protein and tag can result in better exposure of the tag towards the polymer solution, leading to increased partitioning effect of the peptide tag. Collén et al. have used a spacer of five proline residues in a $(WP)_4$-tagged endoglucanase I in an EOPO/dextran system. The partitioning increased 1.4 times when spacer was present compared to $(WP)_4$-tagged protein without spacer [107].

A compilation of the peptide-tagged proteins used in Paper I-V is presented in Table 1.

Table 1.
The wild-type and tagged proteins used in Paper I-V.

Wild-type protein	Peptide tags
zz-cutinase-VD	$-(WP)_n$ n = 2 or 4
[Paper I]	$-(WPR)_4$
	$-(WPD)_4$
	$-(RP)_n$ n = 2 or 4
	$-(DP)_4$
Cutinase	$-(WP)_n$ n = 2 or 4
[Paper II-IV]	-TGGSGG-$(WP)_4$
MEFELGT-GFPuv	MEFEL-tag-ASGT-
[Paper V]	$-(Y)_n-$ n = 3 or 6
	$-(YP)_3-$
	$-(Y_nP_2)-$ n = 3 or 6

5.3 Tag effects in different aqueous two-phase systems

5.3.1 General issues

To be able to evaluate the contribution to the partitioning coefficient from hydrophobic or hydrophilic properties of amino acid residues, a reference system with K_2SO_4 as dominating salt has been used when investigating the partitioning of tagged proteins and peptides. In systems containing K_2SO_4 charge dependent salt effects are minimised (see section 2.4.2.3).

The concept "tag effect" is introduced to be able to compare partitioning of different tagged proteins. The tag effect is defined as the ratio between the partitioning coefficients of peptide-tagged protein and wild-type protein [132, Paper II]:

$$\text{Tag effect} = \frac{K_{\text{peptide-tagged protein}}}{K_{\text{wild-type}}} \qquad (3)$$

The tag effect is a measure of how many times a tag increases partitioning to a certain phase.

To be able to evaluate the effect of a peptide tag in a protein it is important to first study partitioning of the peptide separately. Prior to genetic engineering it is possible to evaluate the partitioning of a set of peptides in order to find an optimal peptide tag. Hassinen et al. partitioned a tetra peptide (Ala-Trp-Trp-Pro) in a PEG/salt system [130]. The peptide partitioned towards the PEG phase and was used as tag in a model protein consisting of two z-domains (see section 4.1.2). Berggren et al. studied the partitioning in an $EO_{30}PO_{70}$/dextran system of different peptides also used as tags in the zz-model protein [133]. The partitioning of the peptides could in both studies be correlated to increases of the partitioning for tagged proteins.

The partitioning results for tryptophan-containing peptides in the different systems used in Paper I-III shared some common trends. The larger the tryptophan content in the peptide, the larger the preference for the EOPO copolymer phase. The logarithm of the partitioning coefficient, log K, increased linearly with the number of tryptophan residues in the peptide. The proline residues included in the peptide tags did not affect the partitioning significantly. Peptides consisting of only proline had an even partitioning between the phases [Paper II-III]. A linear increase of log K with number of tryptophan residues in the peptide has also been found earlier in an $EO_{30}PO_{70}$/dextran system [133] and in an EOPO/water system [135].

5.3.2 Prediction and evaluation of peptide-tagged protein partitioning

The following equation has previously been used to predict partitioning of a protein-peptide fusion in an EOPO copolymer/dextran system [133, Paper I]:

$$\log K_{\text{peptide-tagged protein, calc.}} = \log K_{\text{wild-type}} + \log K_{\text{peptide}} \qquad (4)$$

Contributions from C- and N-terminal of both free peptide and wild-type protein are included in Eq. 4, which introduces an error in the calculated partitioning coefficient (log $K_{\text{peptide-tagged protein, calc.}}$) To reach a better description of tagged protein partitioning, Eq. 4 was modified. Log K_{peptide} was replaced

by $\partial \log K_{(WP)}/\partial n$, i.e. the slope obtained from the plot of log K for $(WP)_n$ versus n, multiplied by the number of tryptophan residues (n) in the peptide [Paper II-III]. Thus, the contribution to log K from the fused peptide will be represented by the added residues:

$$\log K_{\text{peptide-tagged protein, calc.}} = \log K_{\text{wild-type}} + \frac{\partial \log K_{(WP)}}{\partial n} \cdot n \qquad (5)$$

Eq. 5 can be used for prediction of peptide-tagged protein partitioning in a reference system where charge-dependent salt effects have been minimised. Even if it would not be possible to obtain a correct prediction of the protein partitioning coefficient, due to less exposure of tag, conclusions of protein partitioning trends can still be drawn from peptide partitioning and Eq. 5.

The tag efficiency (TE) has been defined in order to express the effectiveness of a tag [151]. The tag efficiency (in %) can be expressed as [Paper II]:

$$TE = \frac{\left(\log K_{\text{peptide-tagged protein, exp.}} - \log K_{\text{wild-type}}\right)}{\frac{\partial \log K_{(WP)}}{\partial n} \cdot n} \cdot 100 \qquad (6)$$

A TE of 100 % indicates that full effect of peptide tag, as expected from peptide partitioning, has been obtained when partitioning tagged protein ($\log K_{\text{peptide-tagged protein, exp.}}$).

5.3.3 $EO_{30}PO_{70}$/dextran system

Partitioning of zz-cutinase with tryptophan-based tags was studied in an $EO_{30}PO_{70}$/dextran T500 system in Paper I. All tryptophan-tagged proteins preferred the EOPO rich phase, as expected from peptide partitioning. The tag effects (Eq. 3) for zz-cutinase-$(WP)_2$ and zz-cutinase-$(WP)_4$ were 1.6 and 2.8 respectively.

The calculated partitioning coefficient (Eq. 4) for zz-cutinase-$(WP)_2$ corresponded approximately to the experimental value. The effect of the

$(WP)_4$ tag was lower than expected from peptide partitioning. The tag efficiency values (Eq. 6) for zz-cutinase-$(WP)_2$ and zz-cutinase-$(WP)_4$ were 44 and 50 % respectively. Thus, when contributions from end groups of the peptide tag were not included in the calculation by using (Eq. 6), also the partitioning coefficient of zz-cutinase-$(WP)_2$ was lower than expected from the calculated value.

Similar increases of partitioning coefficients towards EOPO phases have been obtained with other tryptophan-tagged proteins in EOPO/dextran systems. The tag effect was 2.9 for zz-model protein with four tryptophan residues in the tag [133]. For $(WP)_4$-tagged endoglucanase I the tag effect was 3.4 in a $EO_{50}PO_{50}$/dextran system [107].

It could be advantageous to utilise both salt effects and the hydrophobicity of tryptophan to direct a target protein in an aqueous two-phase system. Charged residues together with tryptophan residues, zz-cutinase-$(WPD)_4$ and zz-cutinase-$(WPR)_4$, resulted in less increase of partitioning to the EOPO copolymer phase compared to zz-cutinase-$(WP)_4$ in the K_2SO_4 reference system, in accordance with peptide partitioning data. The lower effects for tags with charged amino acid residues are due to the hydrophilicity of charged residues, which will lower the partitioning to the more hydrophobic EOPO-rich phase. Similar effects have been observed by Berggren et al. in a model protein consisting of two z-domains (see section 4.1.2) with tryptophan tags also containing charged residues [133].

To investigate the potential of the charged amino acid residues two different kinds of salts were used, triethylammonium phosphate (Et_3NHP) which directs negatively charged molecules towards the EOPO phase and sodium perchlorate ($NaClO_4$), where the perchlorate ion directs positively charged molecules towards the EOPO phase [17]. The effect of Et_3NHP on the logarithmic K-values for the peptides was directly proportional to the net charge of the peptides. $NaClO_4$ affected the partitioning as expected, i.e. positively charged peptides were directed to the EOPO copolymer phase and negatively charged peptides to the dextran phase. The effect of $NaClO_4$ decreased with increasing negative net charge of the peptides. The results obtained for peptide-tagged proteins corresponded qualitatively with the

effects observed for the peptides, but the salt effect was slightly lower for proteins than for peptides. Thus, it is possible to utilise both hydrophobicity and charge in the tag to affect partitioning of a peptide-tagged protein [133, Paper I].

5.3.4 $EO_{50}PO_{50}$/Reppal system

An industrially applicable system was used in Paper II, where hydroxypropyl starch (Reppal) was used instead of dextran as bottom phase-forming polymer. Similar effects of the $(WP)_n$-tags as found in the $EO_{30}PO_{70}$/dextran T500 system was found in the $EO_{50}PO_{50}$/Reppal PES 200 system [Paper II]. Partitioning of zz-cutinase-$(WP)_4$ (unpublished results) and cutinase-$(WP)_4$ resulted in same tag effects in the $EO_{50}PO_{50}$/Reppal system. All tagged proteins preferred the EOPO phase. Cutinase-$(WP)_2$ had a tag effect of 1.3, cutinase-$(WP)_4$ 2.0 and cutinase-TGGSGG-$(WP)_4$ 2.4 [Paper II]. The spacer TGGSGG, resulted in a slightly higher effect of the $(WP)_4$-tag, but the partitioning coefficients were modest. The tag efficiency was 25 % for cutinase-$(WP)_2$, 32 % for cutinase-$(WP)_4$ and 39 % for cutinase-TGGSGG-$(WP)_4$.

The system was modified by addition of 1-2 % (w/w) alkyl-ethylene oxide surfactant, $C_{12}EO_n$ (n=5, 9 or 23), to increase the partitioning of cutinase variants towards the EOPO copolymer phase [Paper II]. If a surfactant partitions strongly to one of the phases, a protein that interacts with the surfactant can be directed to this phase [154, 155, 156, Paper II]. Triton surfactants, which have a similar structure as alkyl-ethylene oxide surfactants, partition strongly to the EOPO phase [Paper II]. Since both EOPO copolymers and $C_{12}EO_n$ surfactants contain ethylene oxide groups the surfactants are expected to partition towards the EOPO copolymer top phase.

All peptides showed an increased partitioning to the EOPO copolymer/surfactant top phase, compared to two-phase systems without surfactant. The increase was similar for all $C_{12}EO_n$ surfactants, and a linear correlation between log K and number of residues in the peptide was found. The presence of $C_{12}EO_n$-surfactant in the phase system, did not affect the partitioning of wild-type protein and cutinase-$(WP)_2$. However, the K-value

for the $(WP)_2$ peptide was increased when $C_{12}EO_n$ surfactant was included in the system. An explanation can be that the $(WP)_2$ tag is too short to interact with the surfactant micelles when attached to the protein. The partitioning of cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ was increased in all systems with $C_{12}EO_n$ surfactants. The tag effects were larger for cutinase-TGGSGG-$(WP)_4$ than for cutinase-$(WP)_4$. The $C_{12}EO_n$ surfactants could selectively increase the partitioning of cutinase constructs with -$(WP)_4$-based tags up to 14 times compared to wild-type cutinase. The tag efficiency values were between 70 and 90 % and thus the partitioning of the tagged proteins could practically be predicted from peptide partitioning data when $C_{12}EO_5$ or $C_{12}EO_9$ surfactants were included in the system. Rodenbrock et al. also obtained a very low tag effect of cutinase-$(WP)_2$ in a cloud point extraction system (non-ionic surfactant/water) containing $C_{12\text{-}18}EO_5$, but cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ were efficiently extracted in the surfactant rich phase with tag effects of 45 and 105 [108].

5.3.5 The HM-EOPO systems

Aqueous two-phase systems based on a hydrophobically modified random copolymer of ethylene oxide and propylene oxide units, HM-EOPO, were studied in Paper III. Since the polymer has a cloud point below room temperature and does not form a concentrated polymer phase, it is possible to use HM-EOPO as the only phase-forming component to obtain a two-phase system (see section 2.2.2). By adding a few percent of hydroxypropyl starch polymer, Reppal PES 200, to the system, the densities of the phases were changed so the HM-EOPO enriched phase became the top phase and Reppal was enriched in the bottom phase. Thus, it is possible to choose whether the target protein should be extracted in the top or the bottom phase, which in each case would be the HM-EOPO rich phase. The Reppal content (3% (w/w)) is much less than what is needed in a traditional two-polymer system.

Tryptophan-based peptides had much stronger partitioning towards HM-EOPO rich phases compared to non-modified EOPO copolymer phases [Paper III]. Cutinase wild-type partitioned towards the water respectively Reppal phase. When a tryptophan-proline containing tag was introduced, the

partitioning of the protein towards the HM-EOPO phase was increased. The tag effect was 1.9 for cutinase-$(WP)_2$ and 2.2 for cutinase-$(WP)_4$ in the HM-EOPO/water system [Paper III]. In the HM-EOPO/Reppal system tag effects were larger, 2.5 and 5.1 respectively for cutinase-$(WP)_2$ and cutinase-$(WP)_4$. However, both the $(WP)_2$ and the $(WP)_4$ tagged proteins still preferred the water respectively Reppal phase. By adding a hydrophilic spacer, TGGSGG, between protein and $(WP)_4$-tag the partitioning was two-fold increased compared to cutinase-$(WP)_4$ and the protein was directed to the HM-EOPO phase. A two-fold increase of the partitioning coefficient has also been found for cutinase-TGGSGG-$(WP)_4$ relative to cutinase-$(WP)_4$ in a cloud point extraction (non-ionic surfactant/water) system [108].

The highest tag efficiency (around 50 %) in the HM-EOPO systems was found for cutinase-TGGSGG-$(WP)_4$. Peptides not attached to a protein can move more freely in solution and interact with the hydrophobic core of the HM-EOPO aggregates, to a larger extent than when the tag is attached to the protein. This is supported by the increased effect of the tag when spacer was present between tag and protein.

5.3.6 Comparison between different aqueous two-phase systems

The tag effects (Eq. 3) and tag efficiencies (Eq. 6) obtained in the different systems are presented in Table 2. Larger tag effects and tag efficiencies were obtained in the $EO_{30}PO_{70}$/dextran system compared to the $EO_{50}PO_{50}$/Reppal system. In both systems tags interact with non-associated chains of polymers, but since $EO_{30}PO_{70}$ has a higher propylene oxide content, and thus is more hydrophobic, tryptophan peptides partition stronger to a phase enriched in this polymer. Addition of $C_{12}E\,O_n$ surfactant in the $EO_{50}PO_{50}$/Reppal system introduced micelles that allowed interaction between tryptophan residues and surfactant micelles. The tag effects increased in the presence of surfactant and the highest tag efficiency values for cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ were found in these systems (see Table 2). The strong tag effects observed in non-ionic surfactant/water systems [108] may be explained by similar mechanism. Also in systems containing HM-EOPO, which is a micelle-forming polymer, large tag effects were found. In the HM-EOPO systems interaction could

occur between HM-EOPO aggregates and tryptophan peptides. The tryptophan peptides partitioned strongly towards the HM-EOPO phase, but tag efficiencies for cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ was lower than in surfactant containing systems. Tag effects were larger in the HM-EOPO/Reppal system compared to the HM-EOPO/water system but the partitioning trends were the same in both systems.

Table 2.
The tag effects (Eq. 3) and tag efficiencies (Eq. 6) obtained for cutinase-$(WP)_2$, cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ in different systems.

	Cutinase-$(WP)_2$	Cutinase-$(WP)_4$	Cutinase-TGGSGG-$(WP)_4$
	Tag effect (TE, %)	Tag effect (TE, %)	Tag effect (TE, %)
6.8 % $EO_{30}PO_{70}$ / 7.1 % Dextran*	1.6 (44)	2.8 (50)	-
7 % $EO_{50}PO_{50}$ / 9 % Reppal	1.3 (25)	2.0 (32)	2.4 (39)
7 % $EO_{50}PO_{50}$ / 9 % Reppal / 1 % $C_{12}EO_5$	1.3 (17)	7.3 (70)	9.0 (77)
4 % HM-EOPO / water	1.9 (42)	2.2 (26)	5.3 (47)
4 % HM-EOPO / 3 % Reppal	2.5 (42)	5.1 (38)	9.7 (54)

* Cutinase-$(WP)_n$ refers to zz-cutinase-$(WP)_n$.

In PEG/salt systems much higher tag effects have been obtained for cutinase tagged with $(WP)_2$ and $(WP)_4$ compared to two-polymer systems. The partitioning was increased 10 to 70 times towards the PEG phase when the tags were introduced [132]. Tryptophan-tag effects with zz-model protein in PEG/salt systems have also been strong [151]. This is consistent with the stronger partitioning of tyrosine-tagged GFP towards the PEG phase in PEG/salt systems relative to the EOPO phase in EOPO/dextran systems [Paper V]. An advantage of the EOPO-containing systems is the possibility to use thermoseparation in order to obtain the protein in a water phase suitable for further purification steps by chromatography.

5.4 Prediction of wild-type cutinase partitioning

Previously, partitioning of a set of monomeric proteins have been correlated to the hydrophobicity of the protein surface. The hydrophobicity of the surface exposed amino acid residues in the protein was determined by peptide partitioning (see section 2.4.1). The prediction method used for wild-type cutinase partitioning in Paper II is developed from the study by Berggren et al. [5].

The surface of cutinase from *Fusarium solani pisi* was investigated with the computer program Graphical Representation and Analysis of Surface Properties, GRASP [86, 148] in Paper II. The accessible surface areas for the different amino acid residues were used together with peptide partitioning data from an $EO_{50}P O_{50}$/Reppal system to calculate the partitioning coefficient for the wild-type protein.

The slope ($\partial \log K/\partial n$) obtained when logarithmic partitioning coefficients for a set of homopeptides were plotted versus number of amino acid residues in the peptide (n) represented the contribution of one fully exposed residue to partitioning. The degree of exposure (DE) for each residue type (X) in the protein was obtained by dividing the accessible surface area (ASA) obtained from GRASP with the value achieved in a Gly-X-Gly peptide [5, 157]:

$$DE_X = \frac{ASA_{X \text{ in protein}}}{ASA_{Gly-X-Gly}} \qquad (7)$$

By taking the product of the degree of exposure for X and the $\partial \log K_X/\partial n$-value, the contribution to the partitioning coefficient for each type of amino acid residue in the protein could be obtained:

$$\log K_X = DE_X \cdot \frac{\partial \log K_X}{\partial n} \qquad (8)$$

The estimated partitioning coefficient for the protein was obtained by adding together log K_X-values for the different amino acid residue types. However, effects of N- and C-terminal had not been taken into account. Contributions

of the N- and C-terminal could be calculated from the ∂log K/∂n values obtained from the peptides, assuming that log K was independent of the position of the amino or carboxyl group:

$$\text{N - terminal } (NH_3^+) = \frac{\partial log K_{Lys}}{\partial n} - \frac{\partial log K_{Ile}}{\partial n} \qquad (9\ a)$$

$$\text{C - terminal } (COO^-) = \frac{\partial log K_{Asp}}{\partial n} - \frac{\partial log K_{Ala}}{\partial n} \qquad (9\ b)$$

The size of the accessible surface area (ASA) determined by GRASP depends on the value chosen for the probe radius used to calculate the area. The program default value is 1.4 Å and represents a water molecule, which could be too short considering interaction between protein and polymer monomer units. Thus, the effect of different probe radii on the ASA was investigated. The only data available for the Gly-X-Gly peptides was measured with a probe radius of 1.4 Å [157], so this value has been used when the degree of exposure for other probe values was calculated. This is a reasonable assumption, since in a small peptide, residues are not involved in any secondary structure, thus allowing unrestricted interaction between residue and polymer. The calculated partitioning coefficient, using a probe value of 5.0 Å, was 1.0 when contributions from the N- and C-terminal were taken into account. The experimental value was 0.85 in the $EO_{50}PO_{50}$/Reppal system [Paper II]. The agreement between calculated and experimental values was satisfactory. The requirements for prediction of protein partitioning is the three dimensional structure of the protein which allows GRASP analysis, and peptide partitioning data in a system where electrochemical effects have been minimised (e.g. with K_2SO_4 as dominating salt).

5.5 Tag exposure studied by steady-state fluorescence

5.5.1 Steady-state fluorescence

The fluorescence emission of tryptophan depends on the polarity of the surrounding environment. Thus, tryptophan fluorescence can be used as a tool for studying interactions of tryptophan and phase components used in aqueous two-phase systems. When tryptophan is in a hydrophobic environment the fluorescence emission intensity maximum is approximately at 330 nm and in a polar environment, such as water, around 350 nm [158]. In order to selectively measure fluorescence from tryptophan, the excitation wavelength can be chosen to 295 nm [159, 160]. The fluorescence emission intensity of cutinase wild-type after excitation with light at 295 nm is very weak, since the single tryptophan of cutinase is highly quenched by a neighbouring disulphide bridge [161-163]. Thus, when using an excitation wavelength of 295 nm for tryptophan-tagged cutinase, the main fluorescence emission will be from the tryptophan residues in the tag.

5.5.2 Tag exposure

A reduced tag effect compared to what was expected from peptide partitioning has been found for different tryptophan-tagged proteins. Berggren et al. found a reduced effect of tryptophan tags in a model protein containing two z-domains in PEG/salt systems [151]. Collén et al. obtained reduced tag effect of a $(WP)_4$-tagged endoglucanase in an $EO_{50}PO_{50}$/dextran system [107] and reduced effects of $(WP)_4$-tags have also been found in the systems presented in this thesis [Paper I-III]. The reduced tag effects motivated an investigation of the exposure of tags both on protein surface and also towards phase components. The exposure study was performed with steady-state fluorescence.

The fluorescence behaviour of wild-type and tryptophan-tagged cutinase, free peptide tag and tryptophan was investigated in Paper IV. Cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$, showed emission spectra similar to free peptides and tryptophan indicating solvent exposure of the tag. The maximum fluorescence intensity for cutinase-$(WP)_4$ was observed

at 352 nm and for cutinase-TGGSGG-$(WP)_4$ at 354 nm. The maximum fluorescence intensity for the peptides was obtained at 356-357 nm. The observed difference in wavelengths at maximum fluorescence intensity for cutinase-$(WP)_4$, cutinase-TGGSGG-$(WP)_4$ and peptides was correlated with larger solvent accessibility of tryptophan residues in peptides compared to the exogenous tryptophan residues of cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$. The difference in wavelengths at maximum fluorescence for cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ was correlated with larger solvent accessibility for tryptophan residues in tag when spacer was present.

5.5.3 Interaction with phase components.

The influence of polymers and surfactants on fluorescence of tagged proteins was examined [Paper IV]. Dynamic light scattering was used to determine the apparent hydrodynamic radii ($R_{H,app}$) of EOPO copolymers. From the size of the radii it was concluded that the polymers molecules did not aggregate at concentrations used in aqueous two-phase systems.

When wild-type cutinase or tryptophan was exposed to polymers and surfactants no significant change in emission spectra could be observed. When tryptophan-containing peptides, cutinase-$(WP)_4$ and cutinase-TGGSGG-$(WP)_4$ were exposed to polymer the emission maximum was slightly shifted to a shorter wavelength. The shift was the largest for the most hydrophobic polymer $EO_{20}PO_{80}$ and the second largest shift was obtained for $EO_{30}PO_{70}$. The smallest shift was obtained for PEG (EO_{100}). Larger shifts of emission maximum compared with polymers were observed when $C_{12}EO_n$ surfactants (n=5, 9 or 23) were utilised. Since the emission maximum changed for both peptides and tagged proteins in presence of polymer or surfactant it can be stated that the tag is still able to interact with the phase components when attached to the protein. However, the shift in wavelength was smaller for tagged proteins compared to free peptides, indicating that free peptides can interact with phase components to a larger extent. The large shifts to shorter wavelengths obtained for $C_{12}EO_n$ surfactants might be correlated to the strong partitioning effects in

$C_{12\text{-}18}EO_5$/water systems [108] and when $C_{12}EO_n$ surfactants were added in $EO_{50}PO_{50}$/Reppal systems [Paper II].

The fluorescence emission intensity increased when polymer or surfactant was present in solution. The fluorescence intensity is known to become larger with increasing hydrophobicity of the solution [164, 165]. The intensity increase was larger for surfactants than for polymers. The increase of fluorescence intensity was larger for cutinase-TGGSGG-$(WP)_4$ than for cutinase-$(WP)_4$ both with polymers and surfactants included in the sample. This indicates that the hydrophobic interaction between tag and hydrophobic groups on surfactant or polymer is facilitated when a spacer (TGGSGG) is introduced between protein and tag. This is consistent with results obtained in aqueous two-phase systems, where the presence of the spacer causes larger partitioning effects of the tag [Paper II-III].

5.6 Tyrosine tags

5.6.1 Tyrosine partitioning

Low expression of tryptophan-tagged endoglucanase I and proteolytic cleavage of the tag have been found with expression in *Trichoderma reesei* [107]. Proteolytic cleavage of proteins in *E. coli*, induced by tryptophan tags has also been reported [166]. An alternative to fusion tags with tryptophan can be tyrosine tags. Tyrosine is more common on protein surfaces than tryptophan, and extra tyrosine residues could thus be easier for the host organism to tolerate [157]. Tyrosine is together with phenylalanine the second most efficient amino acid in partitioning towards an EOPO copolymer phase after tryptophan [85, Paper II]. In a study by Bandmann et al. fusion tags containing tyrosine peptides were selected by phage display technique for partitioning towards a PEG phase in an aqueous two-phase system [167]. When proteins with tyrosine-based tags were partitioned in a PEG/salt system partitioning towards the PEG rich top phase was increased compared to wild-type protein [167]. The partitioning increase of a tag with four tyrosine residues was slightly less than for a tag with four tryptophan residues. Fexby et al. have altered the partitioning of LDH in an $EO_{30}PO_{70}$/dextran systems by addition of tyrosine-based tags [168].

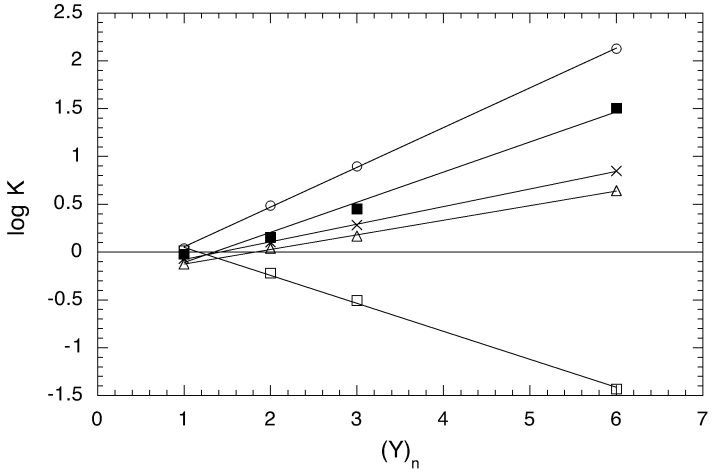

Fig. 9. The logarithms of the partitioning coefficients for tyrosine homopeptides are plotted versus number of residues in peptide tag, $(Y)_n$. A set of different systems has been used with the following compositions: 10.3 % (w/w) PEG 4000/11.0 % (w/w) potassium phosphate, pH 6.8 (○), 4 % (w/w) HM-EOPO (□), 4 % (w/w) HM-EOPO/3 % (w/w) Reppal PES 200 (■), 7 % (w/w) $EO_{50}PO_{50}$ (Breox)/9 % (w/w) Reppal PES 200 (Δ) and 7 % (w/w) $EO_{50}PO_{50}$ (Breox)/9 % (w/w) Reppal PES 200 with 1 % (w/w) $C_{12}EO_5$ (×). All systems except the PEG/salt system contained 50 mM K_2SO_4 and 5 mM sodium phosphate buffer, pH 7.0. The partitioning results in the PEG/salt system are obtained from Paper V; the rest of the data are unpublished results. The HM-EOPO phase is the bottom phase in the HM-EOPO/water system.

The partitioning of a set of tyrosine peptides in different aqueous two-phase systems is presented in Fig. 9 (unpublished results and Paper V). A linear correlation between the logarithm of the partitioning coefficient and the number of tyrosine residues was found in all systems. The partitioning towards a PEG or EOPO copolymer phase was enhanced with increasing length of the peptide. Thus, tyrosine-tagged proteins have the potential to be used in different kinds of aqueous two-phase systems. The increase in partitioning per residue is smaller relative to tryptophan peptides. Approximately, the same partitioning coefficient is obtained for a peptide containing four tryptophan residues and a peptide of six tyrosine residues [85].

5.6.2 GFP partitioning

Green fluorescent protein, GFP, was genetically engineered with tyrosine tags and the partitioning in aqueous two-phase systems was studied in Paper V. The partitioning was investigated in two different sorts of aqueous two-phase systems, one two-polymer system composed of $EO_{30}PO_{70}$ and dextran and a PEG/salt system with potassium phosphate. GFP was engineered with three respectively six tyrosine residues in the N-terminal. Tags with three tyrosine residues resulted in stronger partitioning towards $EO_{30}PO_{70}$ respectively PEG rich phases compared to wild-type protein. The partitioning of proteins with six tyrosine residues was similar to or only slightly higher than for non-tagged GFP. The tag may have been partially cleaved off by proteases. Alternatively, the tag might interact with the interior of the β-barrel of GFP and thus not be sufficiently exposed.

The effect of proline residues in the tag was investigated. Besides from increased expression of the protein, tag effects (Eq. 3) and tag efficiencies (Eq. 6) increased when proline residues were included in tags with three tyrosine residues. Similar results have previously been found by Fexby et al. for LDH with Y_nP_2-tags [168]. The Y_3P_2- and the $(YP)_3$-tagged GFP had similar partitioning coefficients. Since proline peptides earlier have shown even partitioning between the phases in an EOPO/Reppal system [Paper II], the proline residues are suggested to cause increased exposure of tyrosine residues in the tag. Partitioning of tagged proteins in the PEG/salt system showed larger effects on the K-values relative the $EO_{30}PO_{70}$/dextran system, as expected from peptide partitioning. The tag effects (Eq. 3) and tag efficiencies (Eq. 6) are presented in Table 3. The tag effects found for GFP proteins with three tyrosine residues were similar as for the LDH constructs investigated by Fexby et al. [168]. However, since LDH consists of four subunits it was tagged with four tags, i.e. a total of 12 tyrosine residues. The molecular mass of LDH (140 kDa) is approximately 4 times larger than for GFP (30 kDa). The size of the protein could influence the effect of the tag, thus making it necessary with 4 times as many tyrosine residues on LDH relative GFP.

Table 3.
The tag effects (Eq. 3) and tag efficiencies (Eq. 6) for the GFP variants.

	6.8 % (w/w) $EO_{30}PO_{70}$ / 7.1 % (w/w) Dextran T 500		10.3 % PEG 4000 / 11.0 % potassium phosphate	
Construct	Tag effect	Tag efficiency, TE (%)	Tag effect	Tag efficiency, TE (%)
Y_3-GFP	1.7	34	3.7	45
$(YP)_3$-GFP	2.3	57	6.1	63
Y_3P_2-GFP	2.5	62	6.3	64

5.6.3 zz-cutinase partitioning

Partitioning of tyrosine-tagged proteins, zz-cutinase-$(Y)_4$ and zz-cutinase-$(YP)_4$ (constructs obtained from Bandmann et al. [167]) was compared with partitioning of zz-cutinase wild-type and zz-cutinase-$(WP)_4$ in a 7 % (w/w) $EO_{50}PO_{50}$/9 % (w/w) Reppal PES 200 system. A reference system containing 50 mM K_2SO_4 and 5 mM sodium phosphate buffer, pH 7.0 was used. The partitioning effect of addition of 1 % (w/w) $C_{12}EO_5$ was also examined. The partitioning results are presented in Fig. 10 (unpublished results). All tags resulted in higher partitioning compared to wild-type protein with the largest effect for zz-cutinase-$(WP)_4$. When no surfactant was included, the effect of zz-cutinase-$(WP)_4$ was only slightly larger than for zz-cutinase-$(YP)_4$. However, when surfactant was used the difference between the two proteins was larger, as expected from peptide partitioning (see Fig. 9, section 5.6.1). The tag efficiency (Eq. 6) of zz-cutinase-$(WP)_4$ was 36 % in the $EO_{50}PO_{50}$/Reppal system containing no surfactant and the corresponding value for zz-cutinase-$(YP)_4$ was 51 %. The tag efficiency for zz-cutinase-$(Y)_4$ was 36 %. Thus, as observed with the GFP proteins in Paper V the proline residues in the tag had positive effect on partitioning. Tag efficiency for zz-cutinase-$(WP)_4$ in system with surfactant was 65 %. The increase in tag efficiency due to surfactant addition was comparable with the increase found for cutinase-$(WP)_4$ in the same system (see Table 2).

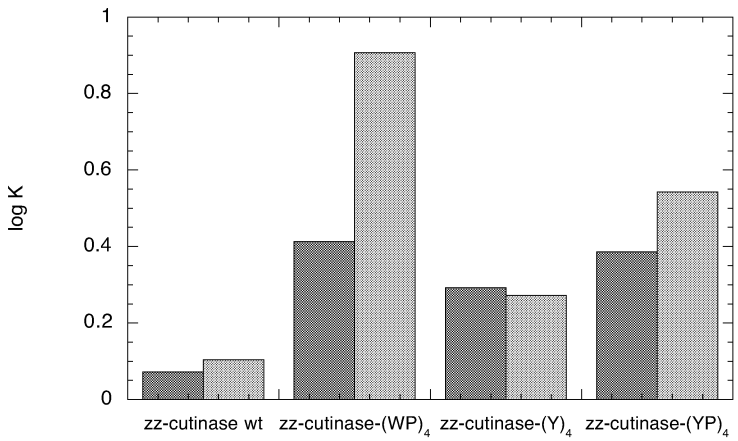

Fig. 10. The logarithmic values for the partitioning coefficients for different zz-cutinase constructs (unpublished results). The columns in dark grey to the left for each construct correspond to partitioning in a system composed of 7 % (w/w) $EO_{50}PO_{50}$ (Breox) and 9 % (w/w) Reppal PES 200. The light grey columns correspond to partitioning in a system also containing 1 % (w/w) $C_{12}EO_5$. All systems contained 50 mM K_2SO_4 and 5 mM sodium phosphate buffer, pH 7.0.

Even though the partitioning coefficients of tryptophan peptides are higher compared to tyrosine peptides of the same length, the effects in tagged proteins need not differ to that extent. Tyrosine is more hydrophilic than tryptophan and is also present to larger extent at protein surfaces. Thus, tyrosine residues might be easier to expose to the solvent, and hence result in higher tag efficiencies.

6. Concluding remarks and future perspectives

The studies presented in this thesis show that it is possible to alter the partitioning of a protein in an aqueous two-phase system by adding a peptide tag to the protein by genetic engineering. Qualitatively, tagged protein partitioning could be predicted from peptide data, i.e. partitioning trends found for peptides were also found for peptide-tagged proteins. However, full effect of tag as expected from peptide partitioning was not found in the tagged protein. When alkyl-ethylene oxide surfactants were included in two-polymer systems [Paper II], almost full effect of the tag was obtained, indicating a larger exposure of the tag to phase components in surfactant-containing systems.

Peptides of tryptophan partitioned most efficiently towards an EOPO copolymer phase. The second most efficient residues were tyrosine and phenylalanine. Tag efficiencies (Eq. 6, section 5.3.2) were higher for proteins with tyrosine/proline tags compared to proteins with tryptophan/proline tags. The reason could be that tyrosine residues are more exposed to water and phase components in an aqueous two-phase system, since tyrosine is more hydrophilic and easier to solvate in water compared to tryptophan.

The calculated partitioning coefficient of cutinase wild-type in an $EO_{50}PO_{50}$/Reppal system [Paper II] agreed relatively well with the experimentally determined partitioning coefficient. Thus, it is possible to obtain an approximate partitioning coefficient for the target protein before starting a purification procedure and thereby save time in finding an optimal partitioning system.

The emission maximum wavelength for free peptide was longer than for peptide tag in tryptophan-tagged cutinase, indicating larger exposure of free peptides [Paper IV]. However, all maxima were obtained at wavelengths corresponding to a polar environment and thus indicating solvent exposure of the tryptophan residues in both free peptides and tags. The emission maximum of the tryptophan tag was moved to longer wavelength when a spacer was introduced between protein and tag. This correlates with results

obtained in aqueous two-phase partitioning where a spacer between tag and protein resulted in increased partitioning towards an EOPO copolymer phase. The residues in a tag can be exposed on the protein surface but not able to interact properly with components in the aqueous two-phase system. A spacer between protein and tag may facilitate the interaction.

As a continuation of the work presented in this thesis it would be interesting to model interactions between tag and protein. Before constructing tags with genetic engineering it would be advantageous to examine possible interactions between tag and protein surface. Moreover, modelling of interaction between peptide tag and phase components could give information on the effectiveness of different tags in aqueous two-phase partitioning. Experimentally it would be interesting to study proteins with same fusion tag but different spacers between protein and tag. A small hydrophilic spacer has been used in some of the studies presented in this thesis, but the effect of a more rigid spacer would be interesting to examine. The spacer effects would be especially interesting to investigate in HM-EOPO systems. Both tryptophan and tyrosine peptides display a strong partitioning towards the phase enriched in this polymer, but full potential of the tag is not transferred to the tagged protein [Paper III]. The simplicity of the system containing only one polymer makes it an attractive system to use, but so far it has been difficult to extract proteins to the HM-EOPO phase.

It would also be interesting to mix tyrosine and tryptophan residues in fusion tags. Tyrosine-based tags are not as efficient as tryptophan tags to use in two-polymer systems containing alkyl-ethylene oxide surfactant. On the other hand, tyrosine-based tags have resulted in a higher protein expression for the tagged protein compared to non-tagged protein [Paper V]. By combining tryptophan and tyrosine residues it might be possible to utilise the benefits of both types of amino acids.

7. Populärvetenskaplig sammanfattning på svenska

När en dressing tillreds används ofta olja, vinäger och kryddor. Även om dressingen skakas ordentligt kommer den efter ett tag att dela upp sig i två lösningar, en med olja och en med vinäger. Ett tvåfassystem har bildats där kryddorna fördelar sig mellan olje- och vinägerfasen. Inom biokemiforskning används tvåfassystem av vatten och polymerer. Polymerer är stora molekyler som består av mängder av små enheter, som är sammankopplade till långa kedjor. Genom att blanda två olika sorters polymerer med vatten, kan vattnet på liknande sätt som oljan och vinägern i dressingen dela upp sig i två faser. Polymererna löser sig i vattnet, men lösningen separerar i två faser med i stort sett bara en typ av polymer i varje fas. På motsvarande sätt som kryddorna i dressingen fördelas mellan vinägern och oljan, kan biologiskt material fördelas mellan vattenfaserna.

Ett stort problem inom biokemiforskning är att isolera biomolekyler (t. ex. proteiner, celler eller DNA) på ett milt och effektivt sätt. Ofta krävs en kombination av flera metoder i många olika uppreningssteg. En metod som kan minska antalet steg i ett tidigt skede är separationstekniken vattenbaserade tvåfassystem. Tvåfasseparering går snabbt att utföra och det behövs ingen avancerad utrustning. Vidare är tvåfasseparering relativt enkelt att skala upp från laboratorie- till industriell skala. Ett sätt att förbättra fördelningen av ett protein till en viss fas i ett tvåfasssystem är att sätta på en fusionspartner, exempelvis en peptid, på proteinet med genteknik. En peptid är en kedja av aminosyror (byggstenar i proteiner). Beroende på aminosyrainnehållet i peptiden kan fördelningen av fusionsproteinet riktas mot önskad fas. Den här avhandlingen handlar om sådana fusionsproteiner.

En del av polymererna som använts i detta avhandlingsarbete är termoseparerande. Med termoseparerande menas att polymererna blir mindre lösliga i vatten då temperaturen höjs. Ovanför en viss temperatur, grumlingspunkten, kommer en vattenlösning av polymeren att separera i en vattenfas och en koncentrerad polymerfas. Eftersom proteiner då fördelar sig till vattenfasen kan polymeren återvinnas. I artikel I undersöktes hur aminosyrasammansättningen hos peptiden i fusionsproteinet påverkade fördelningen av proteinet i vattenbaserade tvåfassystem. Den aminosyra som

fördelade sig starkast till fasen med termoseparerande polymer var tryptofan. Peptider som innehöll tryptofan användes därför i fusionsproteinerna i artikel I-IV. Om längden av peptiden ökades fördelade sig proteinet starkare till fasen med termoseparerande polymer.

I artikel II och III studerades bland annat effekten av en länk av aminosyror mellan proteinet och peptiden i fusionsproteinet. Länken fick fusionsproteinet att fördela sig bättre mot fasen med termoseparerande polymer. För att förutsäga fusionsproteinets fördelning i tvåfassystemet jämfördes proteinfördelningsdata med hur fria peptider fördelade sig. Full effekt av peptiden erhölls inte i fusionsproteinet. Då detergent (tvålämne) tillsattes i tvåfassystemet blev fördelningen mot den önskade polymerfasen starkare och fusionsproteinet fördelade sig nästan som förväntat från peptidfördelningarna (artikel II). I artikel III användes en hydrofobt (vattenskyende) modifierad termoseparerande polymer. Polymeren termoseparerade vid lägre temperatur än rumstemperatur. Polymeren bildade en mindre koncentrerad polymerfas efter termoseparering än ickemodifierade polymerer och kunde därför ensam användas för tvåfasfördelning av proteiner, d.v.s. ett enpolymersystem.

Med hjälp av fluorescensstudier undersöktes i artikel IV peptidens exponering dels på fusionsproteinytan och dels mot polymerer och detergenter som används i tvåfassystem. Fusionsproteinerna fluorescerade på liknande sätt som de fria peptiderna, vilket tyder på att peptiderna i fusionsproteinerna var exponerade. Vidare ändrades emissionsmaxima för både fusionsproteiner och fria peptider då polymer respektive detergent var närvarande, vilket tyder på en växelverkan mellan peptiden och faskomponenterna.

I artikel V undersöktes fusionsproteiner med peptider som innehöll aminosyran tyrosin. Tyrosinpeptider kan fungera som ett alternativ till tryptofanpeptider, eftersom fusionsproteiner med tryptofanpeptider i en del studier varit svåra att uttrycka vid odling av värdorganismen. Fria tyrosinpeptider fördelade sig något mindre än fria tryptofanpeptider mot en fas som innehöll termoseparerande polymer, men tyrosinpeptider påverkade fördelningen av fusionsproteinet på motsvarande sätt som tryptofanpeptider.

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