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# Partitioning of hydrophobic amino acids and oligopeptides in aqueous two-phase system containing self-aggregating block copolymer

## Effects of temperature, salts and surfactants

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### Abstract

The partitioning of hydrophobic amino acids and oligopeptides in the Pluronic P105–dextran–water system has been studied. Pluronic P105 is a member of a family of triblock copolymers with the structure PEO–PPO–PEO, where PEO is poly(ethylene oxide) and PPO poly(propylene oxide). The partitioning was studied for tryptophan, phenylalanine and di- and tri-peptides composed of these amino acids at 5 and 40°C. These temperatures correspond to a unimeric (5°C) and a micellar (40°C) state of the P105 molecule. Partitioning depended strongly on the temperature which is attributed to the increased hydrophobicity of Pluronic P105 with increasing temperature. However, it appears that the presence of the micelles plays no major direct role. The effect of different pH, salts and surfactants (both cationic and anionic) on partitioning has also been investigated.

**Keywords:** Partitioning; Temperature effects; Amino acids; Peptides; Pluronic P105; Tryptophan; Phenylalanine

### 1. Introduction

When mixing two different polymers of certain concentrations in an aqueous solution they usually separate into two macroscopic isotropic phases. If the phase separation is segregative, which is the general case, one polymer is enriched in one phase and the other polymer in the other one. If biological materials, such as proteins, are added to such a

system they often tend to partition unevenly between the phases. The use of such an aqueous two-phase system (ATPS) in separation operations of biological substances has been studied extensively over the past decades [1–3]. Some major advantages achieved by using ATPS as a separation technique in biochemistry and biotechnology are for example: (i) the separated phases have a high content of water (80–95%). This offers, for example, a gentle environment for sensitive biomolecules; (ii) low interfacial tension [1,3,4]. This facilitates migration of biomole-

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cules, also cell particles, between the phases. It also minimises the risk of damaging the structure of proteins and cells; (iii) small relative difference in hydrophobicity between the phases enables very specific separation of biomolecule mixtures [1].

The properties of the phase forming polymers have of course, a large impact on the partitioning outcome. Molecular mass (at least for larger biomolecules), charges and hydrophobic residues on the polymers are examples of factors that affect partition behaviour [5]. Also the total polymer concentration in the phase system, affecting the tie line length, is an important parameter, leading to more extreme partition of the biomolecule between the phases when the polymer concentration is increased [6]. Salt has a large effect on the partitioning behaviour of molecules that possess a net charge. Although salt only slightly distributes unevenly between the phases, the difference is enough to create an interfacial electrostatic potential difference [7] which can be used to steer the desired biomolecule to any of the phases [1].

An aqueous solution of poly(ethylene oxide) (PEO) and dextran (a bacterial polysaccharide built up of  $\alpha$ -1,6-linked glucose units) is the most studied polymer system. Instead of using a polymer–polymer system it is also possible to use a polymer–salt system, e.g., PEO–salt systems. A system with larger relative difference in hydrophobicity [1] is then obtained. In recent years the use of thermo-separating (clouding) polymers in the partitioning technique has been investigated [8–11]. Such polymer phases separate at a certain temperature [cloud point temperature (CPT) or also referred to the lower critical solution temperature (LCST)] into one concentrated polymer phase and one almost pure water phase. An example of such a polymer is UCON, a random copolymer built up of ethylene (EO) and propylene (PO) units. The use of surfactants in partitioning processes has also been studied [12–14] and, not surprisingly, the effect is very strong when partitioning hydrophobic membrane proteins [15].

The use of block copolymers (e.g., Pluronic) has a great potential in the field of biomolecule partitioning. Phase systems containing Pluronic P105 as one of the phase forming polymers and a cellulose polymer (hydroxypropyl cellulose) as the other polymer component have been used by Skuse et al. [16].

Proteins and cells were partitioned and some physical characterisations were made (e.g., phase diagram, interfacial tension, separation time) of the investigated system.

We have recently investigated the phase behaviour of similar Pluronic–dextran–water systems [17]. As a first step to ascertain the partitioning power of such systems, we present here the partitioning of hydrophobic amino acids and some oligopeptides.

Since partitioning in systems containing Pluronic polymers is strongly controlled by the properties of the block copolymers, a brief description of some important features of the Pluronic (trademark of BASF) polymer is motivated. The Pluronic molecule is composed of three polymer blocks, PEO–PPO–PEO. The middle poly(propylene oxide) (PPO) block is more hydrophobic than the PEO-end blocks, due to the extra methyl group in the PO unit. By varying the length of the two different polymer blocks, one can achieve a Pluronic block copolymer with alteration in hydrophobicity and molecular mass. With the freedom in the molecular design a wide range of surfactant functions and physical properties are possible, which allows Pluronic to be used as detergents, emulsifiers, foamers, nonfoamers and lubricants [18]. The Pluronic copolymers are in many respects similar to low-molecular-mass non-ionic EO containing surfactants, showing clouding at high temperatures and self aggregation into micelles and liquid crystalline phases at lower temperature and higher concentration [19]. The phase- and aggregation behaviour are strongly dependent of temperature, concentration and molecular architecture [20]. However, in this work the concentration of Pluronic P105 is so low that no structures other than the unimer (solubilized single polymer chain) and the micellar state have to be considered [21].

Temperature is an important parameter when using Pluronic as one of the phase forming polymers in partitioning in ATPSS. An increase of the temperature causes an increase in the hydrophobicity of the different blocks which promote the polymer chains (unimers) to self-aggregate into micellar-like structures consisting of core dominated PO units surrounded by swollen hydrated PEO chains [22]. The temperature at which the first unimers begins to self-aggregate is referred to as the critical micelle temperature (CMT). The same self-aggregation will

of course occur if the concentration is increased to a certain degree (at constant temperature), which is well known as the critical micelle concentration (CMC).

The increase of the temperature also leads to a strong redistribution of water from the Pluronic phase to the dextran phase. This is driven both by the smaller amount of solutes (implying a smaller osmotic pressure since considering a micelle as one solute) and the increased hydrophobicity of the Pluronic polymers.

So by utilising this temperature triggered aggregation behaviour, it is possible to establish an aqueous two-phase system where, one phase consists of unimers in a water rich solution at low temperature, while at elevated temperature the unimers are aggregated into micelles in a more concentrated solutions (see Fig. 1). The creation of a more hydrophobic domain in the latter case, offering a favourable environment for hydrophobic and/or amphiphilic biomolecules, and the redistribution of water should offer additional possibilities to tailor aqueous two-phase systems.

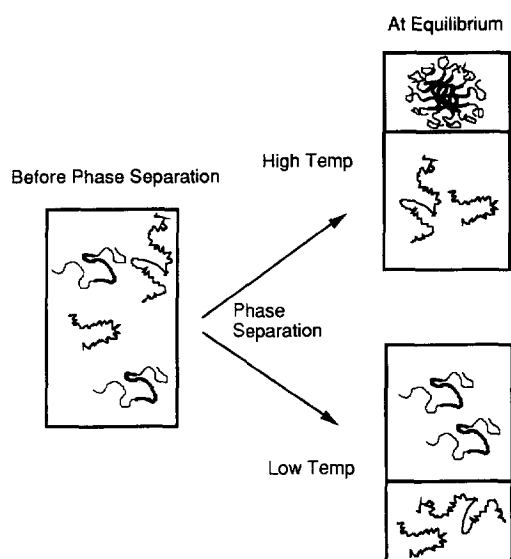


Fig. 1. Illustration of the phase separation of an aqueous polymer system with two polymers where one of them self-associates and creates a hydrophobic domain at high temperature. The volumes of the separated phases are drawn to scale. The minority component is not illustrated, for example, Pluronic micelles in bottom phase and dextran molecules in top phase at high temperature.

## 2. Experimental

### 2.1. Materials

Pluronic P105 was kindly supplied by BASF Corp., Parsippany, NJ, USA, and was used as received. According to the manufacturer [18] the total molecular-mass of Pluronic P105 is 6500 and the PPO block is 3250, which would correspond to the composition  $(EO)_{37}(PO)_{56}(EO)_{37}$ . No check of polydispersity or of impurities was performed. Dextran T500 with a molecular-mass of 500 000 was obtained from Pharmacia Bio Process Technology AB, Uppsala, Sweden and was used as received. The anionic surfactant, sodium dodecyl sulphate (SDS) was specially pure, 98%, and was purchased from BDH, Poole, Dorset, UK. N-Cetyl-N,N,N-trimethylammonium bromide (CTAB), a cationic surfactant with 99% purity, was purchased from Merck. DL-Tryptophan and tri-tryptophan were purchased from Sigma, USA, and Bachchem, Switzerland, respectively. Di-Tryptophan, D-phenylalanine, di-L-phenylalanine, tri-L-phenylalanine and tryptophan-phenylalanine were obtained from ICN Biomedicals, USA. Acids, bases and salts were all of analytical grade. Water of millipore-quality was used in all experimental preparations.

### 2.2. Partitioning experiments

The partitioning of the amino acids and the oligopeptides was determined at 5 and 40°C. The initial polymer concentrations (same for both temperatures) of 6.4 wt% Pluronic P105 and 5.6 wt% dextran T500 resulted in following phase concentrations when separated at 5°C (i) top phase: 9 wt% Pluronic P105 and <1 wt% dextran T500 and (ii) bottom phase: ≈1 wt% Pluronic P105 and 17 wt% dextran T500. Phase separation at 40°C changes the final phase concentrations drastically to (i) top phase: 17 wt% Pluronic P105 and <1wt% dextran T500 and (ii) bottom phase: <1 wt% Pluronic P105 and 8 wt% dextran T500. The large variation in phase compositions comes from the different distribution of water between the phases at the two different temperatures, and the associated changes in volumes of the phases (see Fig. 1). In all partition experiments we started with ca. 5 ml total sample

volume. After phase separation this resulted in the following approximative phase volumes (i) top phase: 3.1 ml and (ii) bottom phase: 1.9 ml at 5°C and (i) top phase: 1.8 ml and (ii) bottom phase: 3.2 ml at 40°C.

Some partition experiments at the low temperature were performed at a slightly higher temperature, at most 10°C. This does not change the final polymer concentrations or phase volumes compared to 5°C. The phase diagram for Pluronic P105–dextran T500–water system at 10°C is similar to the phase diagram at 5°C (result not shown).

The initial concentrations of the single amino acids were ca. 10 mM and the oligopeptides 1–2 mM. The partitioning experiments were performed with different salts [sodium acetate (NaAc), sodium chloride (NaCl) and sodium perchlorate (NaClO<sub>4</sub>)] at three different pH-regions (low, intermediate and high). The ionic strength throughout the experiments was held constant at 110 mM. To achieve the high pH, 10 mM NaOH was added together with 100 mM of the salt, giving a pH between 8 and 10. To reach the low pH around 2–3, 10 mM acid of the corresponding salt was mixed together with 100 mM of the salt. For example, NaOH (10 mM) and HClO<sub>4</sub> (10 mM) was used to establish high and low pH, respectively, in systems containing NaClO<sub>4</sub> (100 mM) salt. But in the HAc–NaAc system, 110 mM of HAc was used because 10 mM of HAc was not enough to reach pH values of ~3. For the approximate neutral system 110 mM of the salt was added and resulted in all cases of pH values around 5–6.

In those experiments where surfactants were added (SDS and CTAB), the surfactant concentration was 10 mM giving a total ionic strength of 120 mM. The partition coefficients of SDS and CTAB at the low temperature in the Pluronic P105–dextran T500–water system were determined by conductometric measurements of the top and bottom phase with a Metrohm 712 Conductometer. The partition coefficients for NaAc, NaCl and NaClO<sub>4</sub> in the Pluronic P105–dextran T500–water system were also determined conductometrically.

At 40°C, 1.5–2 h were enough to obtain a well-defined macroscopic interface as well as two almost clear isotropic phases. At 5°C, the samples were left overnight to phase separate due to the slower kinetics. Double samples and a blank sample (without

partitioned biomolecule) were prepared throughout. After careful separation and suitable dilution of the two phases, the concentration of the partitioned substrate was determined by spectroscopic measurement in the UV–Vis region. Partitioned biomolecules containing tryptophan and phenylalanine were analysed directly at 278 and 257 nm, respectively. The concentration of di-L-phenylalanine was determined with a Ninhydrin method [23]. In the case with tryptophan-phenylalanine, 278 nm was chosen due to a higher molar extinction coefficient. The spectroscopic measurements was performed with a Perkin Elmer UV–Vis spectrometer, Lambda 14 at room temperature.

### 3. Results

The partition results of tryptophan and its corresponding di- and tripeptides are shown in Table 1 and Fig. 2, where the *K* values are plotted on a logarithmic scale against the number of tryptophan amino acids. The panels differ only in the type of salt and the partitioning is given at three pH-regimes (low, neutral and high) at two temperatures (5 and 40°C). Tryptophan has previously been shown to be one of the most hydrophobic amino acids, especially in partitioning in ATPS [24–26]. This property is also reflected in this system containing amphiphilic block copolymers. The partition coefficient increased strongly as the number of tryptophan amino acids (and the molecular-mass) is increased for all cases. A fairly good linear relationship between log *K* and the number of trp units was obtained in most cases.

The effect of the temperature on the partition coefficient in systems containing micelle-forming Pluronic copolymers is clearly seen in Fig. 2. For example, at high pH the *K* value for tri-tryptophan is increased from 6.4 to 59 when the temperature is increased from 5 to 40°C (Fig. 2a). The temperature effect is more pronounced when the number of tryptophan amino acids is increased.

Fig. 2 also displays the effect of the type of salt. In Fig. 2a, with NaAc as salt, the partition coefficients were higher if the pH was high (8–10), lower if the pH was decreased to pH values around 2–3, and at intermediate *K* values at neutral pH values (5–6). In Fig. 2b the partitioning was per-

Table 1  
Partition coefficients obtained in P105–dextran T500–water system<sup>a</sup>

System	Temperature (°C)	Trp	Di-trp	Tri-trp	Phe	Di-phe	Tri-phe	Trp-phe
HAc–NaAc	5	0.9	1.5	2.9	1.0	1.0	– <sup>c</sup>	1.1
	40	1.1	5	15.9	1.0	1.6	– <sup>c</sup>	2.0
NaOH–NaAc	5	1.0	2.5	6.4	1.0	0.9	1.3	1.6
	40	1.2	11	59	0.9	1.4	5.1	4.6
NaAc	5	0.9	1.5	4.6	0.9	1.1	– <sup>c</sup>	1.1
	40	1.2	6.1	39	0.9	1.5	– <sup>c</sup>	2.7
HCl–NaCl	5	1.2	4.0	7.7	1.3	1.2	1.4	1.5
	40	1.5	15	38	1.1	2.1	4.6	4.5
NaOH–NaCl	5	1.1	2.5	6.1	0.9	1.0	1.4	1.4
	40	1.2	10	36	1.0	1.6	4.9	3.7
NaCl	5	0.9	1.3	4.0	0.8	1.2	– <sup>c</sup>	1.2
	40	1.1	4.1	22	0.9	1.1	– <sup>c</sup>	1.9
HClO <sub>4</sub> –NaClO <sub>4</sub>	5	1.4	8.5	– <sup>b</sup>	1.1	1.5	3.0	2.2
	40	2.3	33	53	1.4	4.2	14	12
NaOH–NaClO <sub>4</sub>	5	0.9	2.2	5.8	1.0	1.1	1.2	1.4
	40	1.1	4.9	42	0.9	1.5	3.2	2.1
NaClO <sub>4</sub>	5	1.1	1.6	3.8	1.0	1.0	– <sup>c</sup>	1.1
	40	1.2	3.8	38	0.9	1.7	– <sup>c</sup>	1.9

<sup>a</sup> Typical uncertainty from double samples were approximately 5% of *K*.

<sup>b</sup> Precipitation at the interface.

<sup>c</sup> The solute was insoluble in this type of system.

formed with NaCl as the added salt. The lowest *K* values were obtained with neutral pH, and when the partitioned solute was charged the partitioning towards the top phase was enhanced, with higher *K* values at low pH than at high pH. The partition results when NaClO<sub>4</sub> was used as the added salt are shown in Fig. 2c. High and neutral pH regimes were comparable to those determined in the NaAc and NaCl systems. At low pH, when trp and oligopeptides have positive net charge, slight deviation from previously found trends and linearity appeared. It can be seen in Fig. 2c that the *K* value for di-trp at 5°C and at low pH, *K*=8.5, exceeded the *K* values for the same solute determined at high and neutral pH at 40°C. Due to precipitation of the solute at the interface between the phases no determination of *K* was performed for the system: tri-trp, NaClO<sub>4</sub>, low pH, and at 5°C. To summarize, a strong temperature and molecular-mass dependence were obtained, for all salts and pH regimes.

The same partition experiments were performed with phenylalanine (phe), di-phe and tri-phe. These

results are shown in Fig. 3. However, in some of the systems (see Fig. 3 or Table 1) the tri-phe precipitated or was insoluble.

Fig. 3a shows the result with NaAc as salt. Again the *K* values were strongly dependent of the temperature. Partitioning at 5°C was almost unaffected by an increase of phe units, while at 40°C there was a significant effect, most clearly seen in the high pH system where the highest *K* value was reached, 5.1. Almost the same pattern was obtained when NaCl was used as added salt (see Fig. 3b). Increased molecular-mass or degree of polymerization did not seem to influence the partition at 5°C in contrast to the effect at 40°C. It is interesting to note that the *K* value for di-phe at 40°C, neutral pH, was in the same range as the corresponding values at 5°C. Moreover, in the case of tri-phe, the pH effect was small compared to the temperature effect. When NaClO<sub>4</sub> was used, the effect of pH changes was stronger (see Fig. 3c). High and neutral pH had the lowest *K* values and at low pH (when the solute possessed a positive net charge) the highest *K* values were

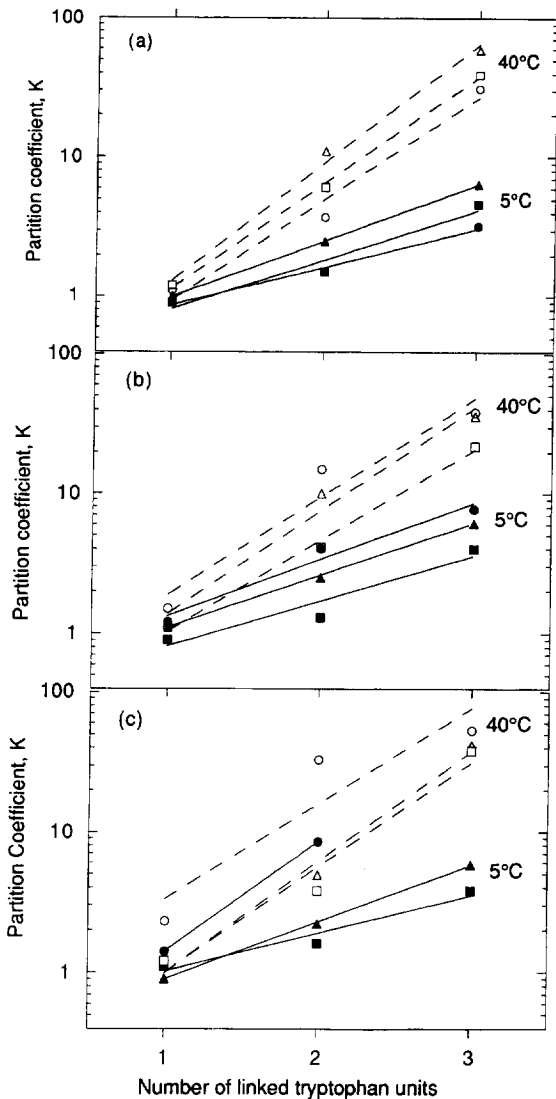


Fig. 2. Partition coefficient of tryptophan, di-tryptophan, and tri-tryptophan versus the number of linked tryptophan units in (a) sodium acetate (NaAc); (b) sodium chloride (NaCl); (c) sodium perchlorate ( $\text{NaClO}_4$ ), at 40°C (dashed lines and open symbols) and 5°C (solid lines and filled symbols), and at different pH regimes: low (circles), neutral (squares) and high (triangles). Ionic strength: 110 mM.

obtained.  $K$  values at high and neutral pH at 40°C were the same as at low pH at 5°C. The described trends in Fig. 3c for phe were qualitatively the same as those in Fig. 2c for trp.

Data for trp-phe are also given in Table 1. The

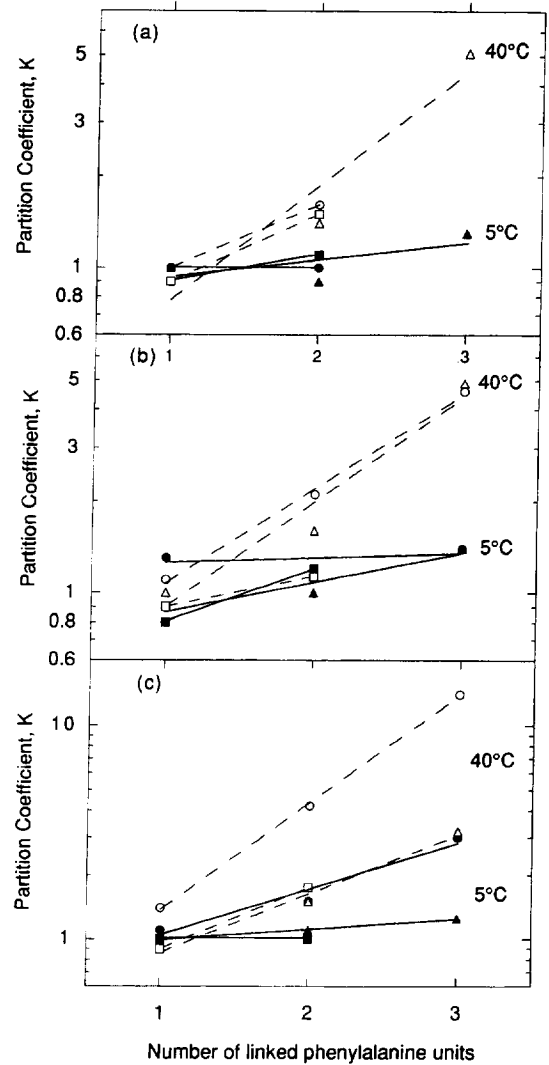


Fig. 3. Partition coefficient of phenylalanine, di-phenylalanine, and tri-phenylalanine versus the number of linked phenylalanine units in (a) sodium acetate (NaAc); (b) sodium chloride (NaCl); (c) sodium perchlorate ( $\text{NaClO}_4$ ), at 40°C (dashed lines and open symbols) and 5°C (solid lines and filled symbols) and at different pH regimes: low (circles), neutral (squares) and high (triangles). Ionic strength: 110 mM.

partition coefficient for trp-phe was always found to be between those for di-trp and di-phe and in most cases close to the geometrical mean of them.

The partition coefficients for the salts were determined, and obtained  $K$  values are listed in Table 2. The  $K$  value for  $\text{NaClO}_4$  was approximative unity,

Table 2  
Partition coefficients obtained for salts in Pluronic P105–dextran T500–water system

	5°C	40°C
NaAc	0.92	0.85
NaCl	0.93	0.89
NaClO <sub>4</sub>	1.01	1.03

Salt concentration: 100 mM.

whereas the partition coefficients for NaAc and NaCl were <1 and decreased with increased temperature.

The effect of surfactants, SDS and CTAB, on the partition behaviour for the di-trp peptide in the NaCl system versus pH can be seen in Fig. 4a (5°C) and Fig. 4b (40°C). The trends in both figures were similar. At intermediate pH no large effects of the surfactants were obtained. Nevertheless, a small increase of the *K* values were obtained when surfactant was present. At high pH, a significant effect

from surfactant addition was obtained. At 5°C (Fig. 4a) the *K* value for the NaCl+CTAB system was strongly increased as compared to the surfactant free system. The *K* values for the NaCl and NaCl+SDS systems were almost identical at 5°C. A similar effect for CTAB at high pH was found at 40°C (Fig. 4b). The addition of SDS at 40°C decreased the *K* value compared to the surfactant free system.

At low pH the surfactant effects were the opposite. Addition of SDS led to higher *K* values compared to the surfactant free system, whereas the *K* values for NaCl+CTAB were similar (Fig. 4a) or lower (Fig. 4b) compared to the surfactant free system. It is clear from the results in Fig. 4 that the charge of the di-trp peptide plays a dominant role in the partition behaviour. An explanation, from an electrostatic point of view, of the surfactant effects on the partition behaviour will be given in the discussion below.

We have also determined the partition coefficients for the ionic surfactants themselves in *salt free system* at low temperature. The *K* value for SDS (5°C) was 1.3. CTAB precipitated at 5°C but was solubilized at 7.5°C, and a *K* value of 1.8 was obtained. The temperature change (5 to 7.5°C) only causes very small changes in the phase volumes. At 40°C with surfactant present, these salt free systems become uniphase (discussed in Section 4.5).

## 4. Discussion

### 4.1. General

The partition of amino acids and small peptides can be reasonably explained by an exponential function, which originates from Brønsted's formula [27],

$$K = \frac{c_t}{c_b} = \exp\left(\frac{\sum_i \lambda_i N_i}{kT}\right) \quad (1)$$

where *K* is the partitioning coefficient and *c<sub>t</sub>* and *c<sub>b</sub>* are the concentration of the partitioned solute in the top and bottom phase, respectively. *N<sub>i</sub>* and *λ<sub>i</sub>* are the discrete number of amino acid residues and a proportionality factor (in fact the free energy of

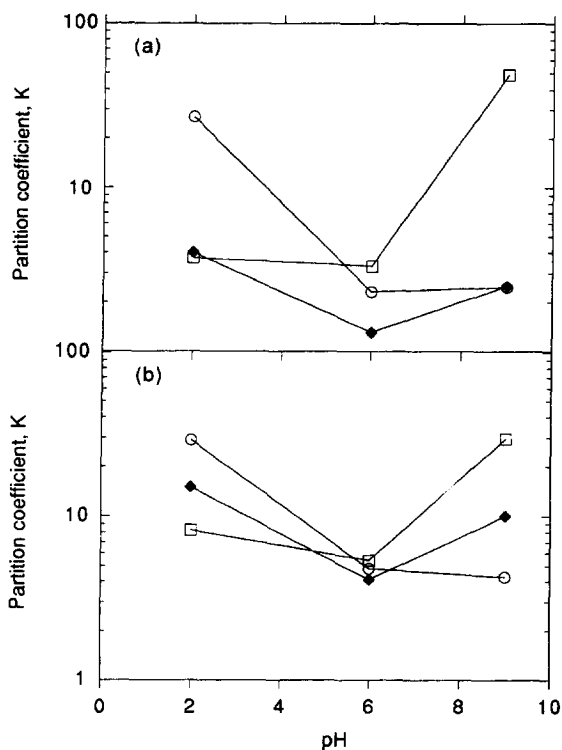


Fig. 4. Partition coefficient of di-tryptophan in the presence of ionic surfactants versus pH at (a) 5°C and (b) 40°C. System type: NaCl (◆), NaCl+SDS (○) and NaCl+CTAB (□). Ionic strength: 120 mM.

transfer) for residue  $i$ .  $T$  is the absolute temperature and  $k$  is the Boltzmann constant.

It was previously found that the difference in concentration, between the phases, for each polymer is an important parameter in experimental  $K$  values [1,28–30]. The logarithm of  $K$  has with Flory-Huggins theory been shown to be linearly dependent on the polymer concentration difference between the phases for each polymer [6,29,31–33]. This has also been experimentally verified [25,28,29]. By taking this into account, Eq. (1) can for the present system be modified to

$$K = \exp\left(\frac{(w_{P105}^t - w_{P105}^b) \sum_i \lambda'_i N_i}{kT}\right) \quad (2)$$

where  $w_{P105}^t$  and  $w_{P105}^b$  are the concentrations of the Pluronic polymer in top and bottom phase, respectively, and  $\lambda'_i$  is a new modified proportionality factor. A more detailed background on Eq. (2) can be found in ref. [29].

In Eq. (2) it is assumed that the partitioned solute is treated as a linear flexible chain, which in this case with small peptides is reasonable. The factor  $\lambda'$  can be said to comprise interactions in the system like solute–polymers, solute–solvent, and electrostatic interactions such as the influence of ions present and pH-dependence.

The charge of the partitioned molecule can be controlled by changes in pH. At low pH the amino acids and peptides are positively charged, whereas at neutral and at high pH they are zwitterionic and negatively charged, respectively. When salts or electrolytes are added to the system this will influence partitioning of charged molecules. This has been explained by an interfacial electrostatic potential between the phases [1,3,7,34]. The basis for this potential difference is the different affinities of the ions for the two phases, which is determined by the ions difference in interaction with the separating phases [1,3]. The influence of added salt is more pronounced when partitioning macromolecules, e.g., proteins with high net charge [7]. The partition coefficient is also dependent on the concentration of the added salt [7,34]. When increasing the salt concentration the logarithm of  $K$  asymptotically reaches a limiting value. From the work of Johan-

sson [7] an ionic strength of 110 mM, which is kept in these studies, is well above the concentration regime where small differences in salt concentration has strong influence.

#### 4.2. Molecular-mass effect

A fairly good linearity was found in most cases between  $\log K$  and the number of trp units (Fig. 2) in accordance with Eq. (1). Generally, the dependence on the number of amino acid residues was larger at 40°C than 5°C. The effect from an increase of the number of amino acid residues was smaller in the case of phe-containing solutes (see Fig. 3). Again, the residue number dependence was larger in the partition experiments at 40°C than in 5°C, and fairly good linearity was obtained in most cases between  $\log K$  and number of phe units. The larger dependence implies that trp is more hydrophobic than phe, which has been found previously in different aqueous two-phase systems [24,25,35].

#### 4.3. Temperature effect

The strong temperature effect on the  $K$  values in Figs. 2 and 3 can be attributed to the water transfer from the top phase when the P105 molecule begins to self aggregate. For instance, the concentration of P105 in the top phase is increased from 9 wt% to 17 wt% when the temperature is elevated from 5°C to 40°C. By taking this concentration difference into account it is possible to prove the validity of Eq. (2), viz. by plotting the logarithm of  $K$  versus the concentration difference of the P105 between top and bottom phase ( $w_{P105}^t - w_{P105}^b$ ), and by holding the number of residues of the partitioned solute fixed.

Such a plot for the three trp-containing solutes in the system NaOH–NaClO<sub>4</sub> is shown in Fig. 5. The values of  $w_{P105}^t - w_{P105}^b$  at 40°C has been normalised to 5°C (14.2 instead of 16.5) due to the temperature dependence in Eq. (2). As seen in Fig. 5 the linearity for all three trp-containing solutes between  $\ln K$  and  $w_{P105}^t - w_{P105}^b$  is fairly good. The same pattern was obtained with the other systems containing trp when examined in the same way as Fig. 5. As predicted by Eq. (2), the slope is increased when the number of residues are increased. Since we have micelles in both phases, albeit at different concentrations, at



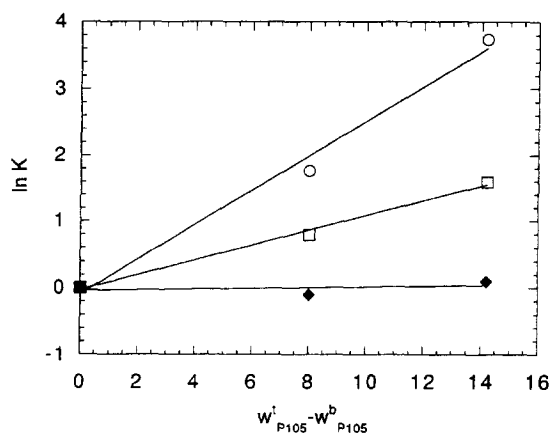


Fig. 5. Natural logarithm of partition coefficient of tryptophan (◆), di-tryptophan (□), tri-tryptophan (○) versus the concentration difference of Pluronic P105 between the top and bottom phase ( $w_{P105}^i - w_{P105}^b$ ) in the NaOH–NaClO<sub>4</sub>-system at high pH. Ionic strength: 110 mM. The point (0,0) is added since when  $w_{P105}^i - w_{P105}^b \rightarrow 0$ , the two-phase system approaches total miscibility and  $\ln K \rightarrow 0$ .

40°C and none at 5°C, we conclude that the micelle formation as such does not influence the partitioning of trp peptides. However, the affinity to the different phases of the hydrophobic di-trp and tri-trp is changed by the presence of the micelles but does so obviously in a parallel manner in both phases and hence the hydrophobic di-trp and tri-trp are partitioned to the Pluronic rich top phase.

In Fig. 6 the  $\ln K$  values for the three phe containing solutes for the NaOH–NaClO<sub>4</sub> system are plotted for the same conditions as in Fig. 5. The values for phe show fairly good linearity, whereas for tri-phe a slight deviation from linearity is obtained. This deviation might be attributed to an unequal effect of the micelle formation in the two phases. The described deviation was found in several other phe containing systems (result not shown).

#### 4.4. pH and salt effects

Different pH and salt also had an effect on the partitioning behaviour, but to a lesser extent compared to changes in number of amino acids and temperature.

Fig. 7 shows  $\ln K$  for the salts (see Table 2) versus  $w_{P105}^i - w_{P105}^b$ . A linear relationship was obtained for all three salts, i.e., in accordance with Eq.

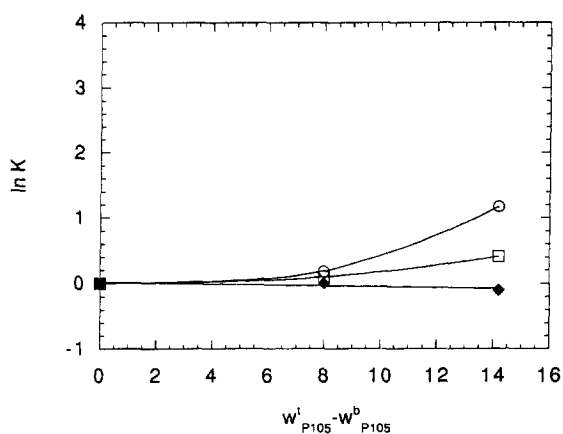


Fig. 6. Natural logarithm of partition coefficient of phenylalanine (◆), di-phenylalanine (□), tri-phenylalanine (○) versus the concentration difference of Pluronic P105 between the top and bottom phase ( $w_{P105}^i - w_{P105}^b$ ) in the NaOH–NaClO<sub>4</sub>-system at high pH. Ionic strength: 110 mM. The point (0,0) is added since when  $w_{P105}^i - w_{P105}^b \rightarrow 0$ , the two-phase system approaches total miscibility and  $\ln K \rightarrow 0$ .

(2). The slopes can be taken as a measure of the hydrophobicity of the salts, i.e., NaClO<sub>4</sub> > NaCl > NaAc. This hydrophobicity scale is in agreement with the partitioning results in Figs. 2 and 3. The effect of changing the anion at low pH, with positively charged solutes, leads to lower  $K$  values in the order: ClO<sub>4</sub><sup>-</sup> > Cl<sup>-</sup> > Ac<sup>-</sup>. This order is in agreement with previously found trends concerning salt effects on protein partitioning [1,3,7].

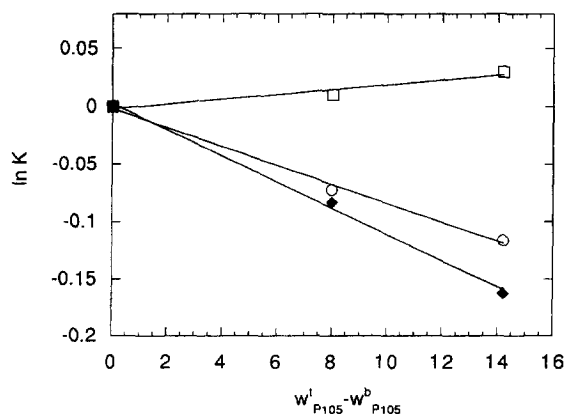


Fig. 7. Natural logarithm of partition coefficient of NaAc (◆), NaCl (○), NaClO<sub>4</sub> (□) versus the concentration difference of Pluronic P105 between the top and bottom phase ( $w_{P105}^i - w_{P105}^b$ ). Salt concentration: 100 mM.

#### 4.5. Ionic surfactants

The influence of ionic surfactants on the partitioning behaviour of di-trp in the NaCl containing system was investigated with anionic surfactant SDS and cationic surfactant CTAB. The critical micelle concentrations (CMC) for SDS and CTAB are 8.3 and 0.9 mM [36], respectively, and of the total concentration used 10 mM of the surfactants should be sufficient to induce micelle formation of the charged surfactants in absence of polymers. The partition coefficients for the surfactants were determined (see Section 3), and showed that SDS and CTAB have a tendency to partition to the Pluronic rich phase.

When adding SDS or CTAB to the Pluronic P105–dextran T500–water system at 40°C without salt, the macroscopic phase separation disappeared which resulted in a clear one-phase solution. When NaCl was added to the one-phase system, the macroscopic phase separation with same phase volumes reappeared. It has been shown that SDS can form mixed micelles with Pluronic micelles [37,38], and in this case we obtain an uneven distribution of the counterions of the surfactants between the two phases. The transition from a two-phase to a one-phase system releases this entropic penalty since the counterions now becomes evenly distributed over the whole system [39,40]. Thus we have strong indications that the surfactants (both SDS and CTAB) to a large extent partition to the top phase at 40°C.

As seen in Fig. 4 the net charge of partitioned solute plays an important role when surfactants participate. The trends can qualitatively be explained with purely electrostatic considerations. In Fig. 4a, for example, at low pH the solute (di-trp<sup>+</sup>) can serve as a counterion to the negative sulphate part in SDS, which will enhance the partitioning toward the top phase compared to the system without surfactant. CTAB had no effect in this pH-regime. However, the opposite effect was obtained at high pH (di-trp<sup>-</sup>), where SDS had no effect, but a higher *K* value is reached with CTAB, due to a favourable TAB<sup>+</sup>–di-trp<sup>-</sup> interaction. At neutral pH, when di-trp possess zero net charge the effect of surfactants was much smaller. The slightly higher *K* values as compared to surfactant-free systems may be due to a hydrophobic interaction between di-trp and the hydrophobic part of the surfactant. In this case, the higher *K* value for

di-trp+CTAB than for di-trp+SDS at neutral pH can be related to the higher *K* value for CTAB compared to SDS, 1.8 and 1.3, respectively.

Similar effects of surfactants were found in the partitioning experiments performed at 40°C (see Fig. 4b). While there were almost no effects at neutral pH, at low and high pH the effects from surfactant were significant. SDS increased the *K* value at low pH and decreased the *K* value at high pH compared to the system without surfactant. Again, the opposite effect was obtained in the presence of CTAB.

#### 5. Conclusions

The partition behaviour of hydrophobic amino acids and oligopeptides in the Pluronic P105–dextran T500–water system has been studied. Tryptophan and phenylalanine partitioned almost evenly between the phases, but when they were linked together into small oligopeptides the logarithm of the partition coefficient increased linearly with the number of amino acid residues. The partition behaviour depended strongly on the difference in the concentration of P105 between the two phases, which here was controlled by the temperature. Partitioning results from a variation of salts and pH were in agreement with a scale of the anions with respect to hydrophobicity; ClO<sub>4</sub><sup>-</sup> > Cl<sup>-</sup> > Ac<sup>-</sup>. When ionic surfactants were added to the system, large effects were found when the partitioned solute was oppositely charged to the surfactant, indicating strong attractive electrostatic interaction between the two species.

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