# HYDROPHOBIC PARTITIONING OF PROTEINS IN A TWO-PHASE AQUEOUS SYSTEM OF POLY(OXYETHYLENE)–DEXTRAN ALTERNATIVELY DERIVATIZED BY 2-HYDROXY-3-PHENOXYPROPYL GROUP

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We have observed that in a two-phase aqueous system composed of poly(oxyethylene) and dextran, trypsin, ovalbumin, serum albumin and immunoglobulin G are preferentially partitioned into the dextran phase. The accumulation of proteins in this more hydrophilic phase increased with the increasing concentration of the polymers in both phases. This trend remained essentially unchanged even after the hydrophobization of the poly(oxyethylene) (POE) phase by 2-hydroxy-3-phenoxypropyl (HPP). The affinity of all four proteins for the dextran phase decreased after the hydrophobization of the dextran phase by the HPP ligand. The affinity decrease was uniform with all four proteins. The hydrophobic partitioning of all four proteins in the systems composed of (HPP–POE) and dextran and of POE and (HPP–dextran) was probably governed by an identical mechanism. The size and charge of the globular protein played an important role in this mechanism.

Liquid extractions offer an attractive alternative to traditional techniques of biopolymer separations. One of the most promising of the latter is the two-phase aqueous polymer separation technique, commonly referred to as aqueous partitioning. A typical feature of two-phase aqueous systems is a high water content (80–90% (w/w)) in both phases[1,2].

The partitioning of biomaterials in two-phase aqueous systems is insufficient for practical applications in most cases. The efficiency of the partitioning for purposes of separation of biomaterials can be improved by modification of the polymers which constitute the two phases. The substituents/ligands bound covalently to one of the polymers of the phases then preferentially interact with the biomaterial. This technique has been named conventionally affinity partitioning. Two affinity partitioning methods are usually used: site-specific ligand partitioning and partitioning employing a hydrophobic ligand (hydrophobic partitioning) (ref.[3]).

The latter and less usual method requires covalent attachment of a hydrophobic group to one of the polymers which form the phases. This polymer is usually poly-

(oxyethylene) (POE) and one of the fatty acids[2-4] is the hydrophobic group. Either one or both terminal hydroxy groups of POE are esterified by these fatty acids. The number of methylene groups in the ester determines the hydrophobicity of the chain terminus and hence also the strength of the hydrophobic interaction of the POE ester with the target biomaterial. Selective separation of blood serum proteins has been achieved[2-4] by optimal adjustment of the methylene chain length. On the other hand the differences in the strength of the interaction of proteins with the hydrophobic chain of the polymer reflect the composition of the surface of the protein molecule. The hydrophobicity of the proteins is then determined by the hydrophobicity of amino acid residues exposed on the surface (and in clefts) of their molecules as well as by their local distribution[5].

We have documented the hydrophobic character of the 2-hydroxy-3-phenoxypropyl (HPP) substituent in the form of its conjugate with cellulose in our previous studies[6-8]. The hydrophobic character of the HPP substituent manifested itself both during batch adsorption and also during zonal chromatography of proteins on HPP-cellulose. The time-concentration profile of the adsorption of proteins to HPP--cellulose demonstrated the corresponding adsorption mechanism. The strength of the interaction of proteins with HPP-cellulose was extremely high and signalized the existence of multipoint binding sites between the surface of the hydrophobic adsorbent (HPP-cellulose) and hydrophobic proteins (especially immunoglobulin G and serum albumin)[6-8].

The aim of this study was to investigate the partitioning of proteins in two-phase systems composed of aqueous solutions of POE and dextran, of hydrophobized POE (HPP–POE) and dextran, as well as of POE and hydrophobized dextran (HPP-dextran). We investigated mainly the effect of hydrophobization of both phase polymers, i.e. POE and dextran on the partitioning of proteins (trypsin, ovalbumin, serum albumin, immunoglobulin G) showing identical molecular shape but different molecular size, different net charge as well as different hydrophobicity.

## EXPERIMENTAL

### Materials

Poly(oxyethylene) (POE) was prepared by precipitation of an aqueous solution of commercial polyethylene glycol 6000 (CHZWP Nováky) with acetone. Dextran 70 SPOFA pulvis for the preparation of infusion solutions was provided by Biotika (Slovenská Ľupča). 1,2-Epoxy-3--phenoxypropane (EPP) was prepared by the reaction of 1-chloro-2,3-epoxypropane with phenol.

*Proteins.* Trypsin (EC 3.4.21.4) ex pig pancreas, a salt-free freeze-dried powder, >300 NF U/mg of material and egg albumin (ovalbumin) were products of Koch–Light Ltd (Haverhill, U.K.) and Fluka AG (Buchs, Switzerland), respectively. Lyophilized bovine serum albumin and human gamma-globulin (gamma-globulin humanum normale "Norga"), 1·6 mg/ml stabilized with glycine, were from the Institute for Sera and Vaccines (Prague) and Imuna (Šarišské Michaľany), respectively.

*2-Hydroxy-3-phenoxypropyl poly(oxyethylene)* (HPP-POE) was prepared by acid-catalyzed ($HClO_4$) alkylation of polyethylene glycol 6000 at 60°C, 2 h, at a molar EPP/hydroxyl group of POE ratio of 0·25 : 1 (ref.[6]). The content of the HPP groups in 1 g of the dry product (180 μmol/g) was determined spectrophotometrically and was calculated from the value of $\varepsilon_{270} = 1\,723$ l. . $mol^{-1}$ $cm^{-1}$ (methanol, water) (ref.[6]).

*2-Hydroxy-3-phenoxypropyl dextran* (HPP-dextran) was prepared in a similar manner, i.e. by alkylation of dextran 70 SPOFA ($M_m$ 60 kD) at 80°C, 2 h, at a molar EPP/glucose dextran unit ratio of 0·67 : 1. The product was washed with dioxane and acetone and the content of the HPP-groups was determined spectrophotometrically (31·9 μmol/g). The value of $\varepsilon_{270} = 1\,723$ l. . $mol^{-1}$ $cm^{-1}$ (water) was used.

Partitioning of proteins. Stock aqueous solution concentrations of PEG 6000 samples (40% (w/w)) and of dextran 70 (23% (w/w)) were prepared. Stock aqueous solutions of HPP-POE and of HPP-dextran were obtained by mixing 40% aqueous solutions of HPP-POE and of POE at a ratio of 1 : 9 and by mixing 23% solutions of HPP-dextran and dextran at a ratio of 1 : 0·77. Hence, the hydrophobized polymer phase (HPP-POE, HPP-dextran) was employed in the form of a mixture with the original polymer at a volume ratio yielding a final hypothetical substitution ratio (content of HPP-groups) identical for both polymers, i.e. 18 μmol of HPP/g. The protein solutions were prepared in 0·1M phosphate buffer at pH 7 containing 1M-NaCl over the concentration range of 1 to 10 mg protein/ml solution.

Stock aqueous solutions of polymers and proteins were weighed as shown in Table I and made up to 4·0 g by 0·1M phosphate buffer at pH 7 containing 1M-NaCl. The system thermostated at (20 ± 1)°C was mixed repeatedly at 2-min intervals and was set aside for (20 ± 1) °C for 2 h. The volumes of the phases were read off and the protein concentration was determined[9].

The results of two-phase aqueous partitioning of proteins in this study are averages of 3—5 experiments.

*High performance liquid chromatography.* The concentration of the polymers in the phases was determined by HPLC. The HPLC analyses were performed with a high-pressure pump (HPP 5001, Laboratorní přístroje, Prague), an eight-port switching valve equipped with two

TABLE I

Weight composition of two-phase aqueous systems composed of POE–dextran, (HPP-POE)–dextran and POE–(HPP-dextran) for protein partitioning

| Components of two-phase system | Ratio of weight concentrations of polymers in phases, DXT/POE | | |
|---|---|---|---|
| | 9/6, mass/g | 8/6, mass/g | 7/5·3, mass/g |
| POE, HPP-POE | 0·6 | 0·6 | 0·5 |
| DXT, HPP-DXT | 1·6 | 1·4 | 1·2 |
| Protein | 0·5 | 0·5 | 0·5 |
| Buffer | 1·3 | 1·5 | 1·8 |
| Total mass | 4·0 | 4·0 | 4·0 |

DXT dextran.

100 μl loops (Model PK1, Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague), two stainless steel columns (250 × 8 mm i.d.) packed with Separon HEMA-BIO 1000 and Separon-BIO 100 (mean particle size 10 μm) connected in series (Tessek Ltd. Prague), and a flow differential refractometer (RIDK 102, Laboratorní přístroje, Prague). The analyses were carried out at 20°C. Deionized water served as the mobile phase. The flow rate of the eluent was kept constant at 0·7 ml $min^{-1}$. The sample volume was 100 μl and reference sample concentrations varied over the range 0·00125–0·01% (w/v). The samples withdrawn from the individual polymer phases were diluted 50 times. The concentration of the polymers (PEG 6000, dextran 70) in the phases was determined from a calibration curve constructed from peak heights of reference concentrations of polymer samples.

*Calculation of partition coefficient K and of effective hydrophobicity* ($\Delta \log K$) *of selected proteins.* The partition coefficient was defined as $K = C_u/C_l$, where $C_u$ and $C_l$ are protein concentrations at equilibrium in the upper (POE) and lower (dextran) phase, respectively[5]. The value of $\Delta \log K$ was calculated from $\Delta \log K = \log K_1 - \log K_2$, where $K_1$ and $K_2$ represent partition coefficients in the polymer phase with and without HPP attached, respectively.

## RESULTS AND DISCUSSION

Table II shows a comparison of the values of partition coefficient of four proteins

TABLE II

Partition coefficients $K$ of proteins in two-phase aqueous systems POE-dextran (1), (HPP-POE)–-dextran (2) and POE–(HPP-dextran) (3)

| Protein | Molecular mass M[a] | Isoelectric point pI[a] | Phase polymers | Partition coefficient $K$ at various concentration ratios (w/v) | | |
|---|---|---|---|---|---|---|
| | | | | 9/6 | 8/6 | 7/5·3 |
| Ovalbumin | 44 000 | 4·6 | 1 | 0·07 | 0·1 | 0·3 |
| | | | 2 | 0·1 | 0·3 | 0·4 |
| | | | 3 | 0·4 | 0·7 | 2·3 |
| Serum albumin | 67 000 | 4·8 | 1 | 0·03 | 0·04 | 0·1 |
| | | | 2 | 0·03 | 0·06 | 0·1 |
| | | | 3 | 0·05 | 0·1 | 0·5 |
| Immuno-globulin G | 154 000 | 5·8 | 1 | 0·2 | 0·4 | 0·5 |
| | | | 2 | 0·1 | 0·3 | 0·4 |
| | | | 3 | 0·4 | 0·5 | 1·2 |
| Trypsin | 23 800 | 10·8 | 1 | 1·0 | 1·1 | 0·9 |
| | | | 2 | 1·2 | 1·3 | 1·0 |
| | | | 3 | 1·4 | 2·1 | 2·7 |

[a] Recorded data[11,12].

in experiments in which *a*) the concentration of the polymers in the phases was varied, *b*) the ratio of the polymer concentrations in the phases was varied, *c*) protein concentration in the polymer phases was varied, and *d*) each of the polymer phases was hydrophobized by the substituent, i.e. 2-hydroxy-3-phenoxypropyl (HPP).

The polymer concentration in the phases was monitored by high performance gel permeation chromatography (HP GPC). We observed for both higher polymer concentrations that the POE concentration in the dextran phase and vice versa was negligible. Hence, POE and dextran were completely partitioned at these dextran and POE concentrations (9 and 6% and 8 and 6%, resp.). In contrast, the partitioning was incomplete at the lowest concentrations (7 and 5·3%): the POE phase contained 4·3% (w/v) of dextran and the dextran phase 5·5% (w/v) of POE. A similar situation was encountered during the partitioning in the systems (HPP-POE)–dextran and POE–(HPP-dextran). Such a characteristic partitioning of the hydrophobized two--phase systems was achieved only on condition that the degree of polymer substitution was below or equal to 18 μmol HPP/g polymer. At a higher degree of substitution of any of the polymer phases the mixing of the latter was observed.

It also follows from the data given in Table II that the most of the proteins were preferentially partitioned into the phase of higher polymer concentration, i.e. into the dextran phase. The proteins became accumulated even more in the hydrophilic dextran phase when the concentrations of both polymer phases were decreased simultaneously.

Protein partitioning in the two-phase aqueous system POE-dextran is governed, in addition to the polymer dependent factors (polymer structure, polymer $M_m$, and polymer concentration), ion-dependent factors (ion types, ion concentration, pH of solution), also by protein-dependent factors[10]. To the latter belong protein net charge and the size and shape of the protein molecule[10]. The first two of these factors are given in Table II. All four proteins used in this study were of globular molecular shape.

The partition coefficients of these proteins were practically constant over the protein concentration range used (1 to 10 mg/ml).

The values listed in Table II were transformed to $\Delta \log K$ values and are shown graphically in Figs 1–4.

Figure 1 illustrates the effect of hydrophobization of the POE phase on protein partitioning at three different concentrations of the two polymer phases. The hydrophobization of the more hydrophobic polymer phase, i.e. of the POE phase, is common[4] and the partition coefficient of the protein in the two-phase system (hydrophobized POE)–dextran can be recommended to serve as the hydrophobic coefficient of the protein[5]. As demonstrated from the plot in Fig. 1, the partitioning of most proteins was only negligibly altered after the hydrophobization of the POE phase ($\Delta \log K \leqq 0{\cdot}2$). Ovalbumin represented a partial exception since its partitioning into the POE phase increased after the hydrophobization of the latter. The

accumulation of ovalbumin in the hydrophobized POE phase increased with the increasing concentration of the polymers in the phases. All proteins including ovalbumin, however, were preferentially partitioned into the more hydrophilic, i.e. dextran phase. It also follows from the dependence shown in Fig. 1 that the plot of $\Delta \log K$ versus molecular mass of the four proteins used was not characterized by a monotonous function.

The affinity of the proteins studied for the dextran phase decreased only after hydrophobization of the latter (Fig. 2). A fact deserving interest is that the affinity of all four proteins for the dextran phase uniformly decreased after its hydrophobization (Fig. 3). This means that the hydrophobic partitioning of these proteins in two-phase aqueous systems (HPP–POE)–dextran and POE–(HPP-dextran) most likely proceeded via the same mechanism. The magnitude of this partitioning was obviously markedly affected by two protein-dependent factors[10]. Because of the identical (globular) shape of the protein molecules these factors were protein size (Figs 1–3) and protein net charge.

The sign of the protein net charge was derived from the difference in the isoelectric point (pI) values of the proteins investigated (Table II) and the pH-value used (pH 7·0). As follows from the data in Table II, ovalbumin, BSA and IgG should bear

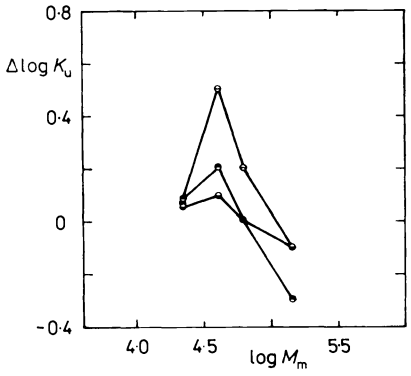


FIG. 1

Dependence of $\Delta \log K_u$ of proteins in hydrophobized two-phase aqueous system (HPP-POE)–dextran on molecular mass of partitioned protein. Ratio of concentrations of polymers in phases (% (w/w)): ◓ 9 : 6; ○ 8 : 6; ◒ 7 : 5·3. POE–dextran served as the reference two-phase aqueous system. Symbol u at $K(K_u)$ means that in this case the upper phase was hydrophobized

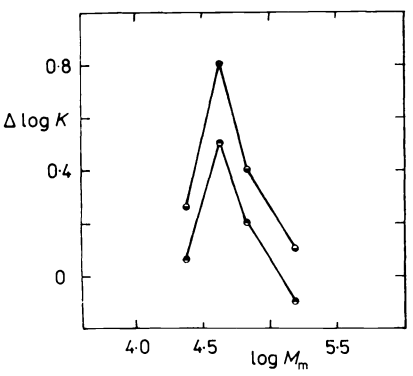


FIG. 2

Dependence of $\Delta \log K$ of proteins in hydrophobized two-phase aqueous systems (HPP-POE)–dextran (◓) and POE–(HPP-dextran) (◒) on molecular mass of protein partitioned. Ratio of concentrations of polymers in phases (% (w/w)) 8 : 6. POE–dextran served as the reference two-phase aqueous system

a negative and trypsin a positive net charge. The values of the net charge of the individual proteins should differ. This would be the case if the net charge values were proportional to the differences in pI-values of the proteins investigated (Table II) and the pH-value.

Examples have been reported[4] of proteins whose partition coefficient was exclusively dependent on the net charge of the protein. The following common empirical formula has been derived for ovalbumin (pI ~ 4·6), ribonuclease A (pI ~ 7·8–9·6) and lysozyme (pI ~ 10·9):

$$\log K = \log K_0 + z\gamma \qquad (1)$$

In this equation $K$ and $K_0$ are the partition coefficients for these proteins at pH ≠ pI and pH = pI, $z$ is the protein net charge and $\gamma$ the factor dependent on the composition of the system, salt and temperature used. Provided that all three proteins (ovalbumin, ribonuclease A and lysozyme) were characterized by discrete net charge values the plot of $\log K$ versus $z$ was a monotonous function[4].

The situation during the hydrophobic partitioning of the proteins is different, however: it may be assumed that the individual proteins are not only characterized

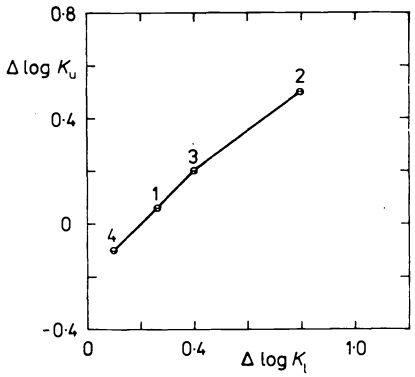


FIG. 3

Correlation of values of Δ log $K$ of four proteins determined from hydrophobic partitioning in system (HPP-POE)–dextran ($K_u$) (ordinate) with values determined in system POE–(HPP-dextran) ($K_l$) (abscissa). Proteins: *1* trypsin, *2* ovalbumin, *3* serum albumin, *4* immunoglobulin G. Ratio of concentrations of polymers in the phases (% (w/w)) 8 : 6. Symbol 1 at $K$ ($K_l$) means that the lower phase was hydrophobized

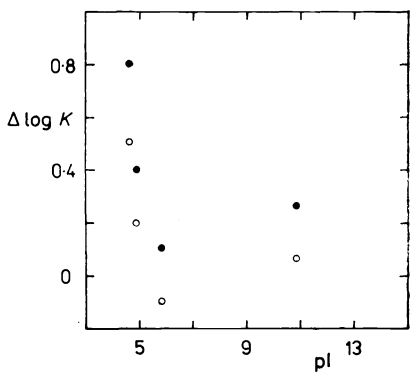


FIG. 4

Correlation of values of Δ log $K$ of four proteins determined from hydrophobic partitioning in system (HPP-POE)–dextran (○) and POE–(HPP-dextran) (●) with values of isoelectric points (pI) of proteins[11,12] (see Table II). Ratio of concentrations of polymers in the phases (% (w/w)) 8 : 6

by different net charge values yet also by different $K_0$-values. The parameter of $K_0$ should then account for the hydrophobic surface of the protein molecules.

The relationship between the isoelectric point of the protein (pI) and its effective hydrophobicity ($\Delta \log K$) is shown in Fig. 4. The absence of the monotonous course of this function well corresponds to the presence of another variable in addition to net charge, i.e. to the hydrophobicity of the surface of the protein molecule $K_0$.

The protein molecule surface hydrophobicity has been implicated in its chromatographic behavior[6]. Hydrophobic protein–protein interactions appear to be enhanced in some solutions causing precipitation of protein molecules when salt concentration of the solution is increased. In contrast, the large differences in the potential of the two phases are significantly moderated by the salt present in the system. Even 100 mM salts can nullify polymer generated potentials[13]. Nevertheless, because of the polyionic nature of proteins and the fact that they can bear large net charges, they will become strongly partitioned in a system with any potential differences between the two phases[13].

In view of the high salt concentration (0·1M sodium phosphate and 1M sodium chloride) of our two-phase system (Fig. 4) we may expect the elimination of potential differences during hydrophobic partitioning and hence also a decrease of the effective protein hydrophobicity bearing a minimal net charge (IgG). In contrast, the effective hydrophobicity of proteins with a large net charge (ovalbumin) may increase in systems with any or low potential difference between the phases (Fig. 4).

We may conclude that the two-phase system POE-dextran containing a high salt concentration ($c \leqq 1$ mol $l^{-1}$) was sensitive to hydrophobization by the HPP substituent. We observed that the concentration of hydrophobic groups of 18 μmol of HPP/g of dry polymer was limiting for the complete exclusion of one polymer from the phase containing the other polymer. This limitation prevented us from systematically investigating the effect of the hydrophobization degree of one or the other polymer phase over a sufficiently wide range. It is likely that it was the limitation of the hydrophobization degree of either of the two polymer phases which did not allow us to achieve the higher partitioning degree ($\Delta \log K_{max}$) than one. The results presented here show that the maximal partitioning effect in the system described can be achieved by controlling the net charge of the proteins rather than their size-exclusion effect.

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