Partition Mechanism of Protein Adsorption onto Bead 3-Phenoxy-2-hydroxypropyl Cellulose Role of External Surface of Cellulose Particles

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Adsorption of proteins onto 3-phenoxy-2-hydroxypropyl derivatives of bead cellulose (PHPC) was studied. Partition coefficient p characterizing the material balance of adsorption in equilibrium was found to show a tendency to increase with the increasing protein relative molecular mass. Affinities of protein—ligand interactions were found to follow a similar tendency because apparent dissociation constants of these interactions (K_d as a reciprocal parameter) decreased with the increasing protein relative molecular mass. Thus, size-exclusion properties of the original bead cellulose did not control adsorption onto hydrophobized PHPC. Fluorescence microscopy of PHPC particles after adsorption of fluorescein (as an amphiphilic fluorescent label) revealed localization of the label on the external surface of cellulose particles. The 3-phenoxy-2-hydroxypropyl (PHP) groups should be localized predominantly at the external surface of cellulose particles too, accordingly, steric problems of fluorescein (as a low-molecular mass label) in penetration into the internal space of bead cellulose macroporous structure are improbable. Open microenvironment around the PHP groups localized on the external surface of cellulose particles could not be an effective steric barrier in the contact with protein molecules. This may be considered as explanation for the lack of size exclusion properties in the control of protein adsorption onto the PHPC.

Hydrophilic adsorbents derived by hydrophobic functional groups are generally applicable for adsorption of proteins. Ability of these sorbents to interact with proteins represents the reason for their use in hydrophobic interaction chromatography and reverse phase chromatography [1]. Nonpolar alkyl or aryl groups and hydrophilic matrices of these sorbents enable hydrophobic interactions in the nondenaturing hydrophilic microenvironment. PHPC represents this type of sorbents [2, 3] and was used for calcium-dependent conformation chromatography of calmodulin [4] and for hydrophobic interaction chromatography of glycerol kinase [5].

In the experiments with perphenazine as a model amphiphilic substance, a constant ratio between the amount of adsorbed perphenazine under equilibrium and the equilibrium adsorptive concentration in solution was observed [2]. Analogical behaviour was found when proteins were used as the adsorptive [3]. This indicated the validity of a principle similar to the partition law, describing the distribution of substances in two phase systems formed by two immiscible solvents. Partition law was found to control the process of adsorption based predominantly on interactions of hydrophobic nature [6]. Thus the ability of the given protein to interact with hydrophobized sorbents may be characterized by a value of "partition coefficient". When different proteins were adsorbed on PHPC, partition coefficients increased with the increasing protein relative molecular mass [3] and ruled out the opposite sequence which should be expected on the basis of size-exclusion properties of cellulose matrix. Thus, size-exclusion properties of bead cellulose [7] are not involved in the control of protein adsorption on PHPC. The last fact may be explained by the interaction of proteins with the ligand localized in high density in the external surface of cellulose particles only. The aim of the current paper is to resolve the question if PHP groups are localized predominantly on the external surface of the cellulose particles.

EXPERIMENTAL

3-Phenoxy-2-hydroxypropyl derivatives of bead cellulose (PHPC) were prepared by alkylation of bead cellulose (Perloza MT 500 M, SECHEZA, Czech Republic, particle size 40—200 μm) with 1,2-epoxy-3-phenoxypropane (2 h at 80 °C) under acid catalysis [3]. Degree of cellulose substitution was estimated spectrophotometrically (λ = 495 nm) after hydrolysis of cellulose by concentrate sulfuric acid [2].

Proteins: Lysozyme (LYS, mucopeptide *N*-acetylmuramoylhydrolase, E.C. 3.2.1.17, M_r = 14 600) from chicken egg white, freed from salt and lyophilized (ICN Pharmaceuticals, USA); α-chymotrypsin type II (CHT, E.C. 3.4.21.1, M_r = 25 000) from bovine pancreas, recrystallized, freed from salt and lyophilized (Sigma, USA); trypsin (TRY, E.C. 3.4.21.4, M_r = 27 000) from bovine pancreas (Spofa, Czech Republic); ovalbumin (OA, M_r = 45 000) prepared by precipitation of egg albumin flakes (BDH, England) by ammonium sulfate [8]; bovine serum albumin (BSA, M_r = 67 000) lyophilized (Sevac, Czech Republic); immunoglobulin G (IgG, M_r = 155 000) stabilized by glycine (Imuna, Slovak Republic). Relative molecular mass set for calibration of bead cellulose packed column for size-exclusion chromatography was obtained from Pharmacia (Sweden). Fluorescein (C.I. 45350, $C_{20}H_{12}O_5$), crystallized, was purchased by Sigma (USA). All other chemicals were of anal. grade and were obtained from Lachema (Czech Republic) and Serva (FRG).

Adsorption of Proteins on PHPC

Adsorption of proteins on PHPC was studied by batch procedure at room temperature. Suspension containing 20 mg PHPC (dry matter) and 100—600 μg of respective protein in 1 cm^3 of 0.1 M phosphate buffer solution (pH = 7.0) containing 1 M-NaCl was incubated for 5—240 min under intensive shaking. Amounts of bound proteins were ascertained from the decrease of protein concentration after incubation [2, 3]. Extrapolation of protein binding under equilibrium was computed from the following equation [6, 9]

$$B = B_e \frac{t}{t + \tau_{0.5}} \qquad (1)$$

where B represents the amount of bound protein after time of incubation t, B_e represents the extrapolation of amount of bound protein to the equilibrium, *i.e.* when t goes to the infinity, and $\tau_{0.5}$ represents the half-time of adsorption.

Mass fraction of the protein in the solution under equilibrium $w_{r,A}$ should be computed from the difference of total protein amount and B_e. "Partition coefficient" (p) characterizing the distribution of protein between solid (PHPC) and liquid phase was estimated as the slope of linear dependence between B_e and $w_{r,A}$ [9]. Apparent dissociation constants (K_d) for protein–ligand interaction [9] were computed from the equation

$$K_d = w_{r,A}\left(\frac{\beta_e}{B_e} - 1\right) \qquad (2)$$

where β_e represents binding capacity of PHPC for the respective protein given by extrapolation of the amount of adsorbed protein for total protein mass fraction $w_r \to \infty$ and time $t \to \infty$.

β_e was obtained from the equation

$$B = \frac{w_r t}{w_r t/\beta_e + t w_{r,B}/\beta_e + w_r/\bar{\omega}_0 + w_{r,v}/\bar{\omega}_0} \qquad (3)$$

where B represents the amount of bound protein from the solution at protein initial mass fraction w_r after time t, $\bar{\omega}_0$ represents extrapolation of the initial rate of adsorption for w_r going to infinity, $w_{r,B}$ and $w_{r,v}$ represent initial mass fraction of proteins when B_e = 0.5 β_e and initial velocity v_0 = 0.5 $\bar{\omega}_0$ [6, 9].

Parameters of eqns (*1*) and (*3*) were estimated by nonlinear regression using SigmaPlot 5.0 graphic software (Jadel corporation 1986—1990).

Size-Exclusion Chromatography of Protein Standards on Nonmodified Perloza MT 500 M

Samples (1 mg of respective protein per 1 cm^3) were applied on the column (28 mm × 300 mm, Pharmacia, Sweden) loaded with Perloza MT 500 M in 0.5 M phosphate buffered solution (pH = 7.6) containing 0.2 M-NaCl and 0.1 % sodium dodecyl sulfate as the mobile phase. A stable flow rate and spectrophotometrical monitoring of the column output were secured by a peristaltic pump SC4 and spectrophotometer UVM 4 (Workshop of the Academy of Sciences of the Czech Republic).

Relation between available distribution coefficients K_{av} and M_r of the respective proteins was fitted by nonlinear regression according to the following equation

$$K_{av} = 1 + k\,(M_r)^{1/3} \qquad (4)$$

where k represents a constant characterizing the matrix for size-exclusion chromatography [6].

Fluorescent Microscopy of PHPC with the Fluorescein as Fluorescent Label

200 mg PHPC (dry matter) was incubated for two days with 2 × 10^{-2} M fluorescein in 10 cm^3 of 5 × 10^{-2} M-tris-HCl buffer (pH = 7.0) containing 1.0 M-KCl. Sample was washed five times in 30 cm^3 of the same buffer solution but without fluorescein, dehydrated by equilibration with ethanol, acetone, and ether and dried in vacuum. Dry samples were embedded in paraffin wax, sectioned (mean section thickness was 4 μm), mounted on glass slides and viewed on a fluorescent microscope Nu2 (Zeiss, Germany) under excitation at λ = 495 nm. Nonmo-

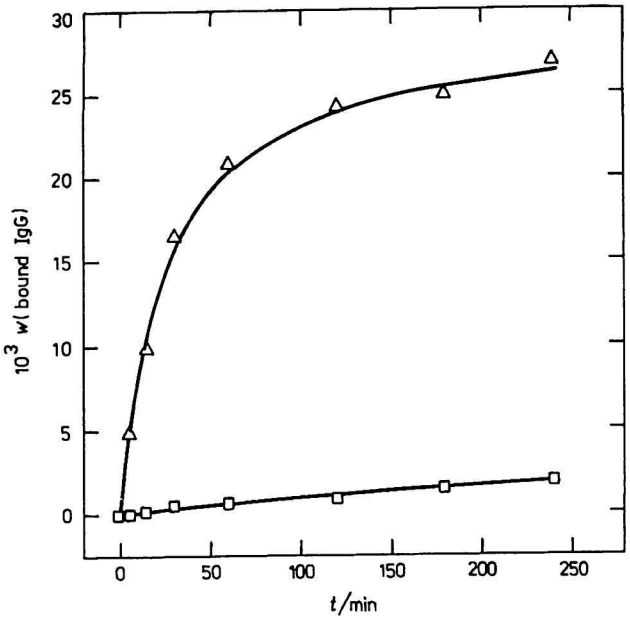

Fig. 1. Time dependence of IgG (initial mass fraction 300 mg cm^{-3}) adsorption onto PHPC (△) and nonmodified Perloza MT 500 M (□). Experimental data were fitted by nonlinear regression according to eqn (*1*).

dified Perloza MT 500 M was processed similarly and used as a control sample.

RESULTS AND DISCUSSION

PHPC was prepared by the reaction of Perloza MT 500 M with 1,2-epoxy-3-phenoxypropane at 80 °C under acid catalysis. This procedure yielded PHPC containing 0.276 mmol of PHP groups per gram (dry matter) of the cellulose carrier (molar degree of substitution D_s = 0.0448) with a high water content of 82.9 mass %. No considerable macroscopic or microscopic damage of cellulose particles was observed in spite of relatively hard conditions of sorbent preparation. Adsorption of protein on PHPC may be considered as a result of alkylation of cellulose by 1,2-epoxy-3-phenoxypropane, because original Perloza MT 500 M did not bind any considerable amount of protein under the conditions applied, as it was shown in Fig. 1.

Adsorption of protein onto PHPC may be considered as a result of a nonstoichiometric interaction of hydrophobic nature regulated by the partition law [2, 3, 6]. Indeed, the relationship of the equilibrium amount of protein adsorbed and the equilibrium adsorptive (protein) mass fraction in solution gave straight lines (Fig. 2) with slopes equal to the partition coefficients [9]. Values of partition coefficients are shown in Table 1. Ability of protein to be adsorbed onto PHPC parametrized by p value followed the sequence p(TRY) < p(CHT) < p(LYS) < p(OA) < p(BSA) < p(IgG) and showed a tendency to increase with the increasing protein M_r (Fig. 3a). Alteration in this behaviour may be observed for proteins with a lower M_r (LYS, CHT, TRY). Nevertheless, the dependence between p and M_r of proteins gave a statistically well determined relationship

$$p = a \exp [b(M_r)^{2/3}] \qquad (5)$$

where a and b represent the constants, the values of which amounting to a = 38.14 ± 3.95 and b = (1.54 ± 0.03) × 10^{-3} (mol g^{-1})$^{2/3}$ were obtained by nonlinear regression.

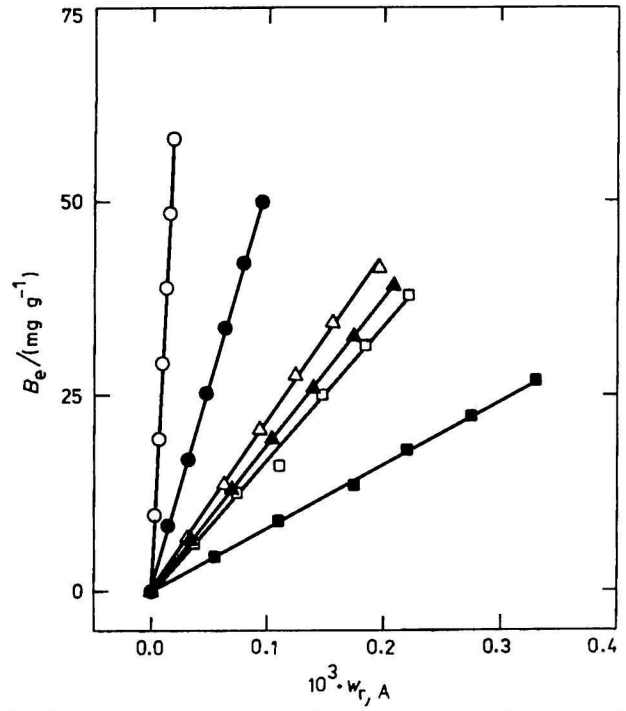

Fig. 2. Dependences between the amount of protein adsorbed in equilibrium and protein equilibrium mass fraction. Symbols: ■ trypsin, □ chymotrypsin; ▲ lysozym; △ ovalbumin; ● serum albumin; ○ immunoglobulin G. Slopes of straight lines equal to partition coefficients are shown in Table 1.

Table 1. Partition Coefficients p and Dissociation Constants K_d Characterizing the Interaction between PHP Groups and Protein

Protein	K_d / mmol cm^{-3}	p
Trypsin	0,402 ± 0.021	81.7 ± 3.6
Chymotrypsin	0.361 ± 0.025	171.9 ± 12.8
Lysozym	0.357 ± 0.009	187.5 ± 13.2
Ovalbumin	0.352 ± 0.012	215.9 ± 13.1
Serum albumin	0.289 ± 0.011	525.1 ± 32.1
Immunoglobulin G	0.237 ± 0.009	3272.6 ± 98.3

K_d were computed from eqn (*2*); p were established as the slopes of the straight lines from data in Fig. 2; Data represent means ± standard deviations for degrees of freedom equal to 5.

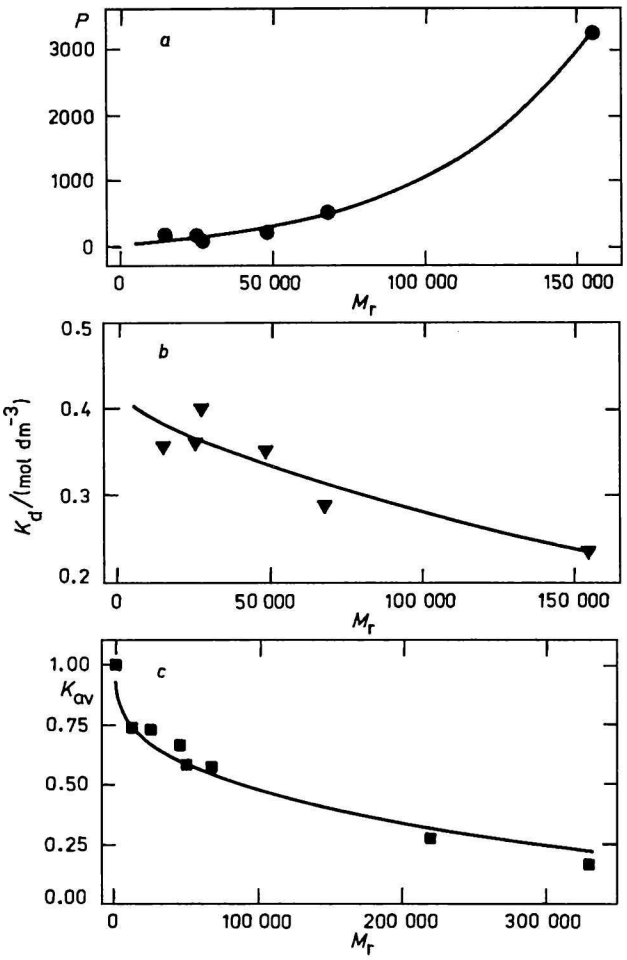

Fig. 3. Dependences between the relative molecular mass of proteins M_r and partition coefficient p of protein adsorption onto PHPC (*a*), dissociation constants K_d for protein PHP group interaction (*b*) and available distribution coefficients for size-exclusion chromatography on nonmodified Perloza MT 500 M (*c*). Experimental data were fitted according to eqns (4—6) by nonlinear regression.

Dissociation constants K_d (Table 1) for protein : PHP-ligand interactions, computed according to eqn (*2*) showed a tendency to decrease with increasing M_r of proteins (Fig. 3*b*). Thus, affinity of protein : PHP-ligand interactions follows an inverse sequence. Deviation from this behaviour was observed again in the cases of proteins with a lower M_r (LYS, CHT, TRY). Nevertheless, this dependence gave again a sufficiently determined relationship

$$K_d = a' - b'(M_r)^{2/3} \qquad (6)$$

where a' and b' represent the constants the values of which $a' = (0.421 \pm 0.010)$ mg g^{-1} and $b' = (64.8 \pm 6.6)$ (mol g^{-1})$^{2/3}$ were obtained by nonlinear regression.

The exponent of M_r (2/3) in eqns (*5*) and (*6*) was found to give the best agreement between experimental and computed data. M_r raised to this power should correlate linearly with the effective surface of protein coils on the basis of assumption that these proteins exist as hydrodynamic spheres [3].

Keshavarz and *Nakai* [10] and *Kato* and *Nakai* [11] have shown that effective hydrophobicity of proteins estimated by hydrophobic interaction chromatography did not correlate with average hydrophobicity calculated by *Bigelow* [12] as a sum of the side-chain hydrophobicity of the constituent amino acids. This may be explained by exposure of several hydrophobic residues in the interior space of protein coil. For effective interaction of proteins with PHPC the existence of areas with higher density of hydrophobic groups in external surface of protein coil should be awaited. Formation of these areas depends on flexibility and conformation of protein molecule [10, 11]. It may be speculated that for a protein with a higher relative molecular mass higher probability of formation of large hydrophobic surface areas should be expected. The last idea probably explains the observed correlation between p (K_d) and M_r shown in Fig. 3*a* and 3*b*. The deviations from this correlation for proteins with lower relative molecular mass were probably caused by the diversity in the physicochemical characteristics of these proteins (pI, net charge, *etc.*).

Nonmodified Perloza MT 500 M exhibits size-exclusion properties that may be documented by protein calibration curve (Fig. 3*c*). On the basis of these properties the opposite character of the dependences p *vs.* M_r and K_d *vs.* M_r, as observed in Fig. 3*a* and 3*b*, should be awaited. Indeed, a decrease of protein binding with increasing protein relative molecular mass was observed in experiments of protein chemisorption on dialdehyde of bead cellulose [13]. Thus the dependences shown in Fig. 3*a* and 3*b* indicate that the size-exclusion properties do not play a significant role in modulation of protein adsorption on PHPC. On the contrary, adsorption of proteins only on the external surface of cellulose particles may be considered as a simple explanation for the latter observation. This was verified by estimation of localization of fluorescein (as a model amphiphilic probe similar to perphenazine [2, 14]) adsorbed on PHPC (Fig. 4) by fluorescent microscopy. This experiment confirmed the localization of fluorescein predominantly in the external part of cellulose particles. In the control experiment with nonmodified Perloza MT 500 M no adsorption of fluorescein could be microscopically observed. The experiment with fluorescein indicated that substances may be hydrophobically adsorbed only on the external surface of PHPC particles and indeed the PHP groups are localized predominantly in this area. Inner space of cellulose particles remained relatively intact after alkylation of cellulose by 1,2-epoxy-3-phenoxypropane. Similar results were observed by *Brandts et*

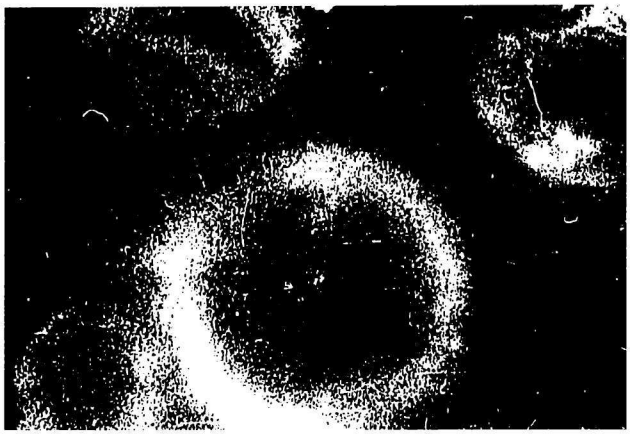

Fig. 4. Fluorescence microscopy of sections of PHPC after labeling of the binding areas by fluorescein. Nonmodified Perloza MT 500 M did not bind any detectable amount of fluorescein. Magnification 200 ×.

al. [15] for octyl Sepharose. This localization of PHP groups secures in spite of a low degree of substitution (0.044 mol per mol of glucose unit), a sufficient density of hydrophobic groups for formation of a coherent liquid-like film on the surface of cellulose particles. This liquid-like film simulates the phase with a lower polarity than the external environment of aqueous solution [15]. The latter idea should be assumed as an explanation of partition law validity described for adsorption of substances onto PHPC [2, 3, 6, 9, 14].

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