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Pressure-induced effects in the heterogeneous adsorption of insulin on chromatographic surfaces

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Abstract

The effect of increasing the average column pressure (ACP) on the heterogeneous adsorption of insulin variants on a C_{18}-bonded silica was studied in isocratic reversed-phase HPLC. Adsorption isotherm data of lispro and porcine insulin obtained for values of the ACP ranging from 57 to 237 bar were fitted to the Langmuir–Freundlich and the Tóth equation. The resulting isotherm parameters, including the equilibrium adsorption constant and the heterogeneity index, were next used for the calculation of distribution functions characterizing the energy of interactions between the adsorbed insulin molecules and the stationary phase. It was observed that increasing the pressure by 180 bar causes a broadening of the distribution functions and a shift of the position of their maximum toward lower interaction energies. These findings suggest that, under high pressures, the insulin molecules interact with the stationary phase in a more diversified way than under low pressures. Additionally, the most probable value of the energy of the insulin–surface interactions becomes lower when the ACP increases. The pressure-induced changes in the interaction of insulin variants with the hydrophobic surface are attributed to a possible conformational flexibility of the molecular structure of this protein.

Keywords: Adsorption; Hydrophobic surfaces; Heterogeneous interactions; Insulin

1. Introduction

Interaction of proteins and peptides with chromatographic stationary phases plays a central role in the separation/purification processes that are commonly used in the biotechnology and the pharmaceutical industry [1–4]. Understanding the mechanisms that govern the retention of proteins in reversed-phase liquid chromatography is therefore of particular importance in the optimization and the control of various industrial processes involving large-scale chromatographic separations. This problem refers especially to the influence of external parameters, such as the column temperature, the pH or the composition of the organic-water mobile phases commonly used in the HPLC of bio-molecules. For example, a detailed knowledge of the molecular mechanism of protein

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retention under different conditions makes it possible to formulate mathematical models of the separation processes carried out on a preparative scale.

Despite recent considerable progress in experimental investigations into the mechanism of protein interactions with solid surfaces [5–11], theoretical predictions of protein adsorption in HPLC meet several difficulties. This is mainly because of the extremely complicated nature of the protein–surface interactions that are usually difficult to describe by means of idealized adsorption models. In such models proteins are often treated as rigid spheroidal or ellipsoidal structures of a fixed size [12–15], while in reality the spatial conformation of proteins can change easily under external forces [7,9,16,17]. Recent development in this field involves adsorption models that account for the possibility of adsorption-induced structural changes in a protein molecule. Models of such a type usually refer to (1) the irreversible adsorption of proteins (random sequential adsorption, RSA) [18–20] and/or (2) an adsorption process in which a protein molecule can adsorb reversibly and adopt a limited number (usually two) of different spatial conformations or can cover a different number of adsorption sites [21,22]. The first limitation (1) mentioned above is a major drawback in HPLC where the adsorption of solutes is assumed to be a reversible process. The application of reversible RSA-like models to chromatographic systems (2) is much more plausible since it gives correct results like, e.g. theoretical adsorption isotherms and breakthrough curves [22]. However, this approach involves usually the assumption that a protein molecule interacts with the surface in a homogenous way, i.e. that the energy of interaction is independent of the orientation/conformation of an adsorbed molecule having a fixed size. The above assumption can be questionable in some cases [23].

Proteins and peptides are relatively large molecules with many contact points accessible to the adsorbing surface. They are composed of a mixture of hydrophobic, hydrophilic, charged and uncharged residues. In consequence, the adsorption of proteins even on a homogeneous solid surface is intrinsically heterogeneous. This is because the adsorption energy is a strong function of the orientation of the adsorbed molecule. Also, in contrast with small molecules, proteins are flexible structures able to change their spatial conformation. This property complicates even more the description of the kinetics and equilibrium of protein adsorption on solid surfaces. The unfolding or spreading of protein molecules upon adsorption, leading to the exposition of their hydrophobic core, is a well-known effect observed experimentally [7,9,16,17,24] as well as modeled by means of analytical approximations [20] or by computer simulations including molecular dynamics [25] and Monte Carlo methods [18,19,21]. A general picture emerging from these studies indicates that the interactions of a single protein molecule with a solid surface can be largely diversified depending on the spatial orientation and conformation of the protein chain. Furthermore, interactions of proteins with chromatographic surfaces are strongly dependent on the external parameters mentioned previously. For example, changes in the composition and/or the temperature of the mobile phase can influence drastically the retention times of proteins as well as the elution order of the components of their mixtures. Suitable manipulations of the above parameters have been long recognized as powerful methods of performing chromatographic separations of complex mixtures of proteins and peptides [3,8]. On the other hand, the effect of pressure on the adsorption behavior of proteins and peptides in HPLC, hence on their interactions with the stationary phase, has attracted considerable interest only recently [26–31].

The experimental results obtained so far in our laboratory [28–31] confirm that the column pressure can have a substantial effect on the retention and separation of proteins in HPLC. For example, in the case of insulin variants adsorbed on a C_8-bonded silica it was observed that, under linear conditions, an increase of the average column pressure (ACP) by 150 bar causes increases of the retention time by up to 300% [28]. Similar observations were reported also by other authors for lysozyme [27] as well as, but to a lesser degree, for relatively small molecules such as derivatized fatty acids [32] or chiral drugs including hexobarbital and ibuprofen [33]. Moreover, variations of the column pressure were found recently to be responsible for the changes in the shape of insulin adsorption isotherms obtained in RPLC [31].

The examples cited above suggest that the pressure disturbs considerably the protein interactions with the chromatographic surfaces. This conclusion refers to the experiments performed under linear as well as under nonlinear conditions. In order to better understand

how the ACP influences the interactions between a protein molecule and a hydrophobic stationary phase, we study in this contribution the effect of the ACP on the adsorption of two insulin variants under nonlinear conditions.

2. Theory

Since a single protein molecule, depending on its orientation/conformation, can, in general, form largely diversified interactions with a solid surface, it seems reasonable to model the insulin adsorption as a heterogeneous process. Here, we emphasize that the term heterogeneous refers to the protein–adsorbent interactions, not just to the surface, which, itself, can be either energetically/geometrically homogeneous or heterogeneous. This interpretation renders the proposed approach somewhat different from the traditional concept of heterogeneous adsorption where the only source of heterogeneity is the adsorbing surface.

Among several isotherm equations accounting for heterogeneous interactions between a molecule and the surface [34,35] the Langmuir–Freundlich and the Tóth models were found previously [31] to be able to describe accurately the adsorption of insulin variants under different pressures. This encouraged us to apply and compare these two models in order to extract the qualitative changes in the insulin interactions with the C_{18}-bonded phase that are induced by pressure. Although the parameters associated with each of the applied models are different, they carry similar information about the adsorption equilibrium. Thus, the main trends in the adsorption behavior of insulin can be interpreted in the framework of one model and compared with predictions made with the other one.

The isotherm equations associated with the Langmuir–Freundlich and the Tóth models relate the concentration of the solute in the bulk liquid phase, c, to the concentration in the adsorbed phase, q, in the following way, with:

$$q = \frac{q^*(k_{\mathrm{LF}}c)^{\alpha}}{1+(k_{\mathrm{LF}}c)^{\alpha}} \tag{1}$$

and

$$q = \frac{q^* k_{\mathrm{T}} c}{[1+(k_{\mathrm{T}}c)^{\beta}]^{1/\beta}} \tag{2}$$

being the Langmuir–Freundlich and the Tóth equations, respectively. In the above equations q^* is the saturation capacity, k_{LF} or k_{T} is the equilibrium adsorption or binding constant and α and β are the heterogeneity parameters, varying from 0, for strongly heterogeneous interactions, to 1, for entirely homogeneous interactions.

In both models of adsorption, the energy of interaction between the molecule and the surface is characterized by an unimodal density probability function. In the case of the Langmuir–Freundlich model, the distribution function is a symmetrical, quasi-Gaussian curve, whereas for the Tóth model, one obtains an asymmetric Gaussian-like curve skewed towards low interaction energies. The shape of a particular distribution function depends on the value of the heterogeneity parameter, α or β, while the position of its maximum is related to the value of the equilibrium adsorption constant appearing in Eq. (1) or Eq. (2). For the Langmuir–Freundlich model, the maximum is located simply at $k_{\mathrm{m}} = k_{\mathrm{LF}}$, while for the Tóth model k_{m} is a complex function of k_{T} as well as of β. However, in both cases, the distribution function becomes broader as α or β tends towards 0, i.e. when the interactions between the molecule and the surface become more heterogeneous.

Approximate mathematical expressions for the distribution functions are obtained by solving associated integral equations, using Stieltjes transform [35]. The results are given by:

$$\Gamma_{\mathrm{LF}}(k) = \frac{\sin(\pi\alpha)}{\pi}\frac{x}{x^2+2\cos(\pi\alpha)x+1} \quad \text{and} \quad x = \left(\frac{k}{k_{\mathrm{LF}}}\right)^{\alpha} \tag{3}$$

for the Langmuir–Freundlich model and

$$\Gamma_{\mathrm{T}}(k) = \frac{1}{\pi}y^{1/\beta}\sin\left[\frac{1}{\beta}\arcsin(xy\sin(\pi\beta))\right] \tag{4}$$

where

$$y = [x^2+2\cos(\pi\beta)x+1]^{-1/2} \quad \text{and} \quad x = \left(\frac{k}{k_{\mathrm{T}}}\right)^{\beta} \tag{5}$$

for the Tóth model.

As is seen from Eqs. (3)–(5), the only parameters required to evaluate the distribution functions, Γ_{LF} and

Γ_T, are the parameters k_{LF}, α and k_T and β appearing in Eqs. (1) and (2), respectively. These parameters can be easily found by fitting experimental adsorption isotherms to the corresponding models. Estimation of the above parameters under different external conditions (pressure, temperature, etc.) but in the framework of the same model enables one to deduce how the external factors influence the heterogeneous interactions between the adsorbate and the surface.

3. Experimental

A HP 1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) was used for all experimental determinations. This instrument was equipped with a multi-solvent delivery system, an automatic sample injector with a 100 μl loop, a diode-array detector, a high-pressure flow cell and a computer data station.

The adsorption isotherms of lispro and porcine insulin on a C_{18}-bonded silica used as the stationary phase in HPLC were determined by conventional frontal analysis at 25 °C. The column used in our experiments was a YMC ODS-A column (Waters, Milford, MA, USA), 3.9 mm × 150 mm. The particle size of the stationary phase was 5 μm with an average pore diameter of 12 nm. The mobile phase was a solution of 30% acetonitrile/water and 0.1% (v/v) trifluoroacetic acid (TFA). For the measurements of insulin adsorption at high pressures, a section of 0.0025 in. PEEK tubing, cut to the desired length was connected to the outlet of the detector, raising the ACP by approximately 135 bar/ft.

Detailed description of the experimental setup and all the experimental procedures including the preparation of the insulin samples and the generation of elevated average column pressures were reported elsewhere [30,31].

4. Results and discussion

In order to examine the influence of the pressure on the heterogeneous interactions of insulin with the C_{18}-bonded silica, we estimated the parameters k_{LF}, α, k_T and β at four different pressures. Namely, the parameters were found by fitting the experimental adsorption isotherm data of the insulin variants measured at 57, 118, 178 and 237 bar to the adsorption models described in Section 3 (Eqs. (1) and (2)). The fits obtained were characterized by values of R^2 varying typically from 0.9985 to 0.9999. The results of the fitting procedure are shown in Fig. 1. As is clearly seen in this figure, both models predict the adsorption behavior of insulin under different pressures with similar accuracy. The theoretical curves corresponding to the

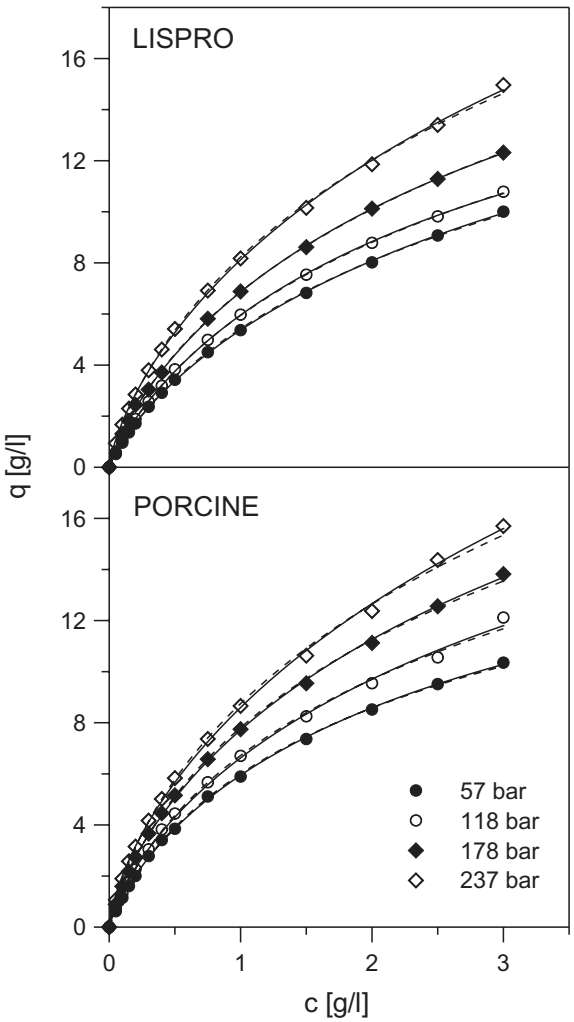

Fig. 1. Adsorption isotherms of insulin variants obtained for different average column pressures. The symbols denote experimental data, while the solid and dashed lines are their best fits calculated using the Langmuir–Freundlich and the Tóth equations, respectively.

Langmuir–Freundlich and to the Tóth isotherm equation nearly coincide for the same value of the ACP for a given insulin variant. This fact suggests that the two models, despite their different foundations, can be safely used to trace the qualitative changes in the adsorption behavior of insulin that are produced by pressure. On the other hand, the classical Langmuir model is much less useful for our purpose since it does not account for the heterogeneous interactions that seem present in the system. To support and explain this conclusion, we show in Fig. 2 the plots of q/c versus q (i.e. the Scatchard plots) drawn from the experimental data obtained for different ACPs. Note that, if the interactions between the surface and the insulin molecules were entirely homogeneous, one would observe that the data points in Fig. 2 form a straight line given by:

$$\frac{q}{c} = k(q^* - q) \tag{6}$$

All the curves in Fig. 2 are convex downward in the whole concentration range instead of being straight lines. This fact excludes definitely the Langmuir model from further discussion. However, it is noteworthy that heterogeneous interactions may not be the only possible source of the curvature in a Scatchard plot. Namely, other factors like attractive interactions in the adsorbed phase, multisite occupancy, or steric hindrance [22] alone can also produce the same curvature in a Scatchard plot that the one observed in our case. In practice, the separation of the contributing effects mentioned above is difficult, unless the detailed molecular mechanism of adsorption is known. This task is much easier for systems involving well-defined surfaces, like those of single crystal planes, and small rigid molecules, e.g. noble gases or light hydrocarbons. Obviously, in the present study, we are far from dealing with any such cases. For this reason, we limit ourselves to the discussion of the heterogeneous interactions viewed as a primary source of the observed deviations from the Langmuir model. Fine discrimination of all the effects accompanying the adsorption of insulin is beyond the scope of this paper.

Note first an important trend in the system behavior that is seen directly in Fig. 1. For both insulin variants, the amount adsorbed, q, at fixed protein concentration, c, increases systematically with increasing ACP. This means that the saturation capacity, q^*, also

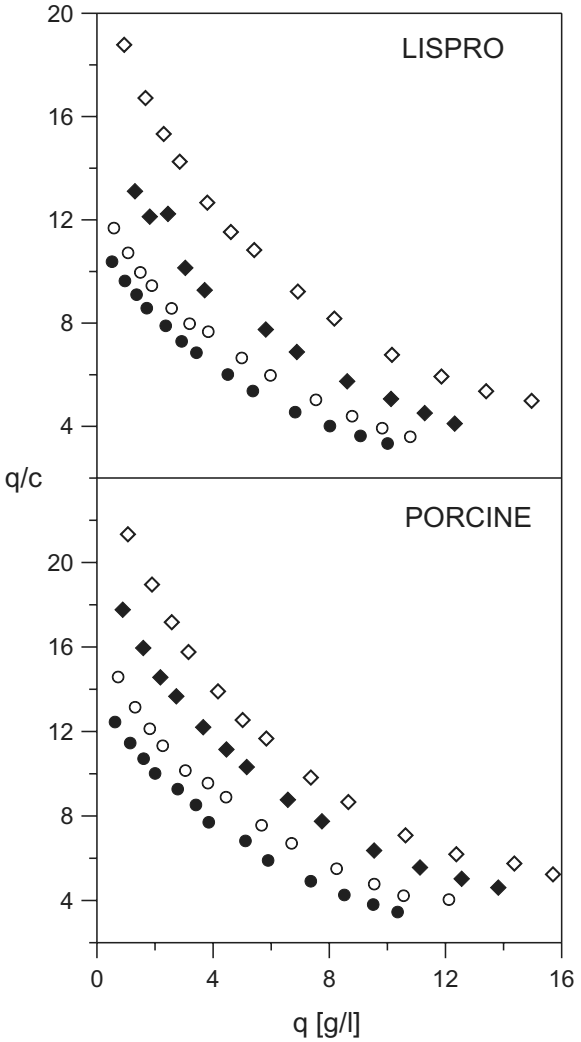

Fig. 2. Scatchard plot (q/c as a function of q) of the insulin variants on the packed C_{18} column with ACN-water 30/70 (v/v) + 0.1% TFA as the mobile phase. (●) 57 bar; (○) 118 bar; (◆) 178 bar; (◇) 237 bar.

increases with increasing column pressure. The practical consequences of this effect for chromatographic separations as well as its possible origin were previously described in more detail [28–31]. For the sake of brevity, we focus here exclusively on the effect of pressure on the nature of the insulin interactions with the immobilized alkyl chains.

To this purpose, we show in Fig. 3 plots of the equilibrium adsorption constant, k_{LF} and k_T, as a function

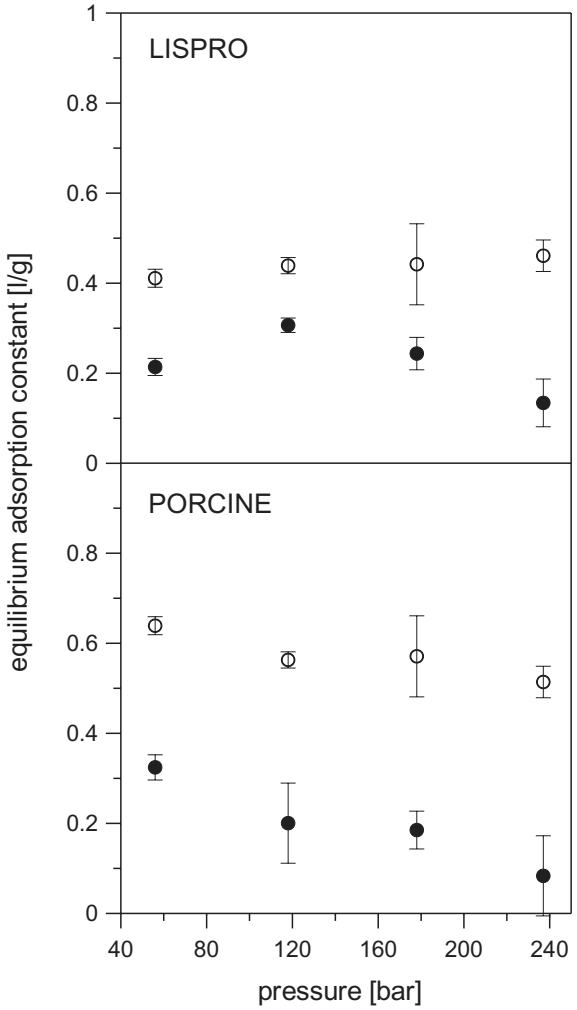

Fig. 3. Influence of the pressure on the equilibrium adsorption constant estimated using the Langmuir–Freundlich (●) and the Tóth (○) isotherm equation, k_{LF} and k_T, respectively. Note that the vertical lines across these symbols are error bars.

of the ACP. As seen in this figure, the pressure, in general, does not induce any dramatic changes in the estimated parameters. Nevertheless, the underlying trends in the behavior of k_{LF} and k_T can be indicated. For both variants, the values of the equilibrium constant predicted by the Langmuir–Freundlich model are lower than the corresponding values obtained with the Tóth model. Furthermore, k_{LF} and k_T exhibit slightly different relative trends for the same insulin variant. This refers particularly to lispro insulin. Namely, for lispro insulin, k_{LF} increases initially with increasing pressure then it starts to drop when the ACP exceeds 137 bar. On the other hand, k_T calculated for this variant remains practically constant. In the case of porcine insulin, the pressure influences both equilibrium constants in a very similar way. Here, we observe that both k_{LF} and k_T decrease nearly monotonically with increasing ACP. The only exception is the value of k_T estimated at 178 bar, which is slightly greater than the corresponding values obtained at lower ACPs.

Recall that only k_{LF} has a direct physical interpretation since this is the only parameter that is related to the most probable value of the equilibrium adsorption constant, k_m. For this reason, the observed changes in k_{LF} are a more direct manifestation of the system behavior than the corresponding changes in k_T, which together with β contributes to k_m. However, as we show later, qualitative changes in k_m predicted by both models are the same for porcine insulin or nearly the same for lispro. Taking into account the above remark, we may conclude at this stage that the strength of the interactions between each insulin variant and the stationary phase is, in general, lower at very high (237 bar) than at low (57 bar) pressures.

A similar analysis of the pressure-induced effects was performed in the case of the heterogeneity parameters α and β. The results are shown in Fig. 4. In this case, in contrast with the relation observed between k_{LF} and k_T, the values of the heterogeneity parameter associated with the Langmuir–Freundlich model are higher than the corresponding values predicted by the Tóth model. Nevertheless, the tendencies displayed by α and β for a given insulin variant are quite similar. We observe that, in general, both α and β decrease as the pressure changes from 57 to 237 bar. In other words, both distribution functions, Γ_{LF} and Γ_T, become wider at high pressures. This finding suggests that an increase in the column pressure causes the insulin–adsorbate interactions to become more diversified.

Fig. 5 shows the distribution functions of the adsorption equilibrium constant and summarizes the results discussed above. First, consider the distribution functions calculated for lispro (Fig. 5, upper panel). The qualitative changes in the shapes of Γ_{LF} and Γ_T are similar. However, these changes are not monotonous with respect to those of the pressure. For example,

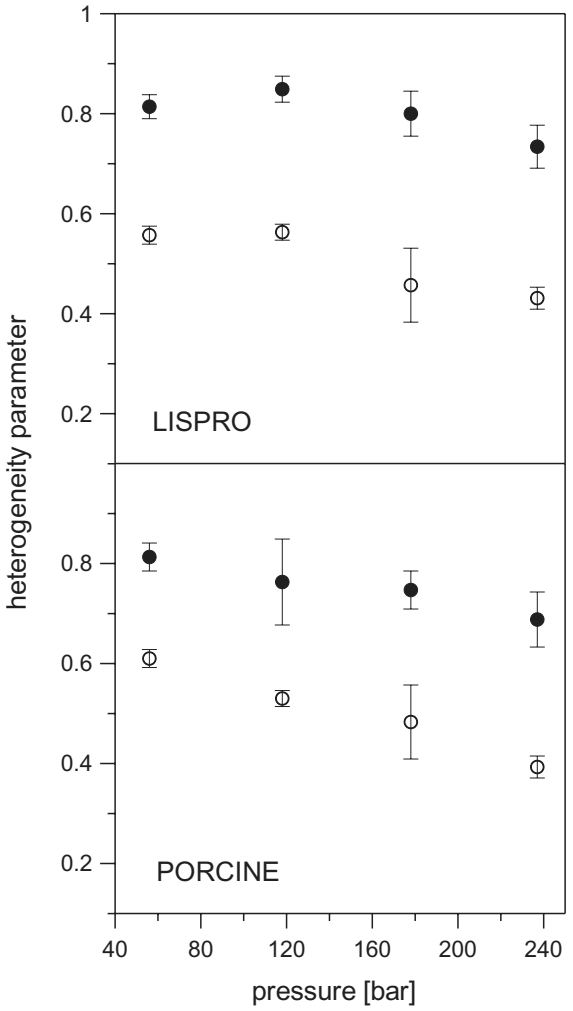

Fig. 4. Influence of the pressure on the heterogeneity parameter estimated using the Langmuir–Freundlich (●) and the Tóth (○) isotherm equation, α and β, respectively.

the height of Γ_{LF} decreases gradually for values of the ACP following the sequence 118, 57, 178 and 237 bar (b, a, c, d in Fig. 5). Consequently, the width of Γ_{LF} increases in the same order (see Fig. 5 and compare also with Fig. 4). In this case, the largest difference is observed between Γ_{LF} at 237 bar ($\alpha = 0.734$) and the three remaining functions obtained with the Langmuir–Freundlich model. Also, the functions Γ_{LF} calculated at 57 and 178 bar have very close widths ($\alpha = 0.8$ and 0.814, respectively) but are slightly shifted. Obviously, the shift in the position of the maximum of Γ_{LF} is consistent with the data shown in Fig. 3. In the case of the results predicted by the Tóth model, the qualitative behavior of Γ_T is similar to the behavior of Γ_{LF}. This refers especially to the distribution functions calculated at the ACP equal to 178 and 237 bar. In this case, the predictions of both models are consistent, i.e. k_m for Γ_{LF} as well for Γ_T shifts towards lower values when the pressure increases from 178 to 237 bar. Note that from Fig. 3 it follows that k_{LF} displays the tendency described just above, while k_T behaves in the opposite way, i.e. it increases with increasing pressure. As we mentioned before, this effect comes from the fact that k_m for Γ_T is a function of k_T as well as of β. Regarding the two lower ACP values, i.e. 57 and 118 bar, each of the adsorption models gives slightly different results. Namely, the amplitude of Γ_{LF} at 118 bar is considerably greater than the amplitude of Γ_{LF} at 57 bar, whereas Γ_T at 118 bar and Γ_T at 58 bar nearly overlap. Taking into account the above observations, we may conclude that, in general, the Gibbs free energy of adsorption becomes lower for lispro insulin when the ACP increases.

For porcine insulin, the influence of pressure on the distribution functions is much less complex than for the lispro variant. From the bottom panel of Fig. 5, it can be seen that Γ_{LF} and Γ_T display the same trend. In particular, their amplitudes decrease systematically with increasing pressure. At the same time the width of the distribution functions increases with increasing pressure, regardless of the applied adsorption model. This effect is less evident for Γ_{LF} at 118 bar and Γ_{LF} at 178 bar, whose widths are very close ($\alpha = 0.763$ and 0.747, respectively). For both types of functions, we observe also that k_m shifts systematically towards lower values when the ACP increases from 58 to 237 bar.

The results obtained so far show that both models of adsorption applied in our study give consistent predictions of the pressure-induced changes in the adsorption of insulin on a C_{18}-bonded silica. As we demonstrated the adsorption of insulin on the chromatographic surface under nonlinear conditions does not follow the Langmuir model. The observed deviation from the Langmuir model may result from conformational changes of the insulin molecule that are induced by its contact with the stationary phase. As it follows also from our previous studies [28,30], the pressure seems to enhance this effect. The structural

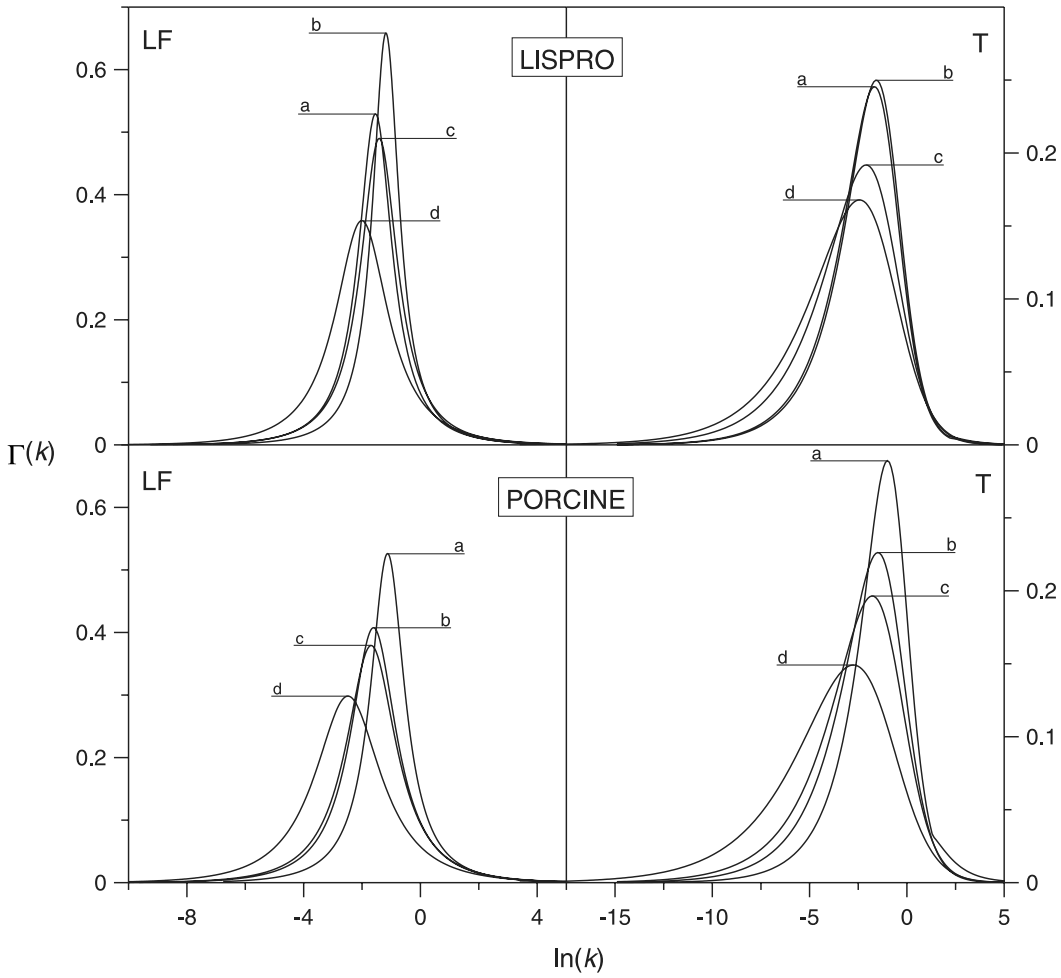

Fig. 5. Distribution functions of the equilibrium adsorption constant associated with the adsorption of the insulin variants under different pressures (a) 57 bar; (b) 118 bar; (c) 178 bar; (d) 237 bar. The curves in the left panel are the results obtained with the Langmuir–Freundlich model (LF), while the curves in the right panel correspond to the Tóth model (T).

perturbations (e.g. unfolding of the protein chain) can lead to an increase in a number of possible chain conformations and hence to a much more complex mechanism of interactions of the protein with the alkyl groups of the stationary phase. The same refers to the number of the contact points between a relatively large insulin molecule and the surface. In consequence, the protein–surface interactions may be much more diversified under high than under low column pressures. This interpretation seems supported by the observed broadening of the distribution functions at higher pressures (see Fig. 5).

While the effect of pressure on the width of Γ_{LF} and Γ_T can be associated with the conformational flexibility of an insulin molecule, the origin of the changes in k_m seems more difficult to explain. Since the equilibrium adsorption constant estimated here relates directly only to the Gibbs free energy of adsorption, ΔG, it is difficult to separate the enthalpic from the entropic contributions associated with this process and, accordingly, to assess which one of these two factors is the driving force for insulin adsorption. Namely, the pressure-induced decrease in k_m, hence in ΔG, may originate as well from a decrease

in the enthalpy as from an increase in the entropy of adsorption. Furthermore, a combination of these two factors, known as the enthalpy–entropy compensation (EEC) [36], may be also responsible for the observed decrease in k_m with increasing ACP.

Our previous findings suggest that the effects discussed above are more likely to originate from the associated changes in the adsorption enthalpy alone. As we have shown, at moderate temperatures, the column temperature, T has only a negligible effect on the volume change, ΔV, of the insulin molecule that is induced by the adsorption [30]. Since from the thermodynamics it follows that:

$$\left(\frac{\partial \Delta V}{\partial T}\right)_P = -\left(\frac{\partial \Delta S}{\partial P}\right)_T \quad (7)$$

we may conclude that the pressure, P, exerts a marginal effect on the entropy of adsorption. As a result, changes in the Gibbs free energy of adsorption seem to be dominated by the changes in the enthalpy. This is, of course, a hypothesis that has to be verified by further experimental studies supported by, e.g. spectroscopic investigations into molecular structure of insulin adsorbed under different pressures.

5. Conclusions

The adsorption of insulin on hydrophobic stationary phases is a complex phenomenon that is much affected by the column pressure. In addition, it seems that the adsorption process is intrinsically heterogeneous since the observed Scatchard plots of insulin on a C_{18}-bonded silica deviate much from linear behavior. This suggests that adsorption models accounting for heterogeneous interactions between a protein molecule and the hydrophobic surface are more useful tools for studying the influence of pressure than the classical Langmuir model. Two popular models of such type including the Langmuir–Freundlich and the Tóth models proved to be particularly helpful in our studies. Namely, the approximate distribution functions of the equilibrium adsorption constant associated with these models have relatively simple mathematical forms that can be readily used in order to calculate the spectrum of protein–surface interactions.

The results obtained with both models show that the pressure affects the strength as well as the number of possible interactions between the protein molecule and the stationary phase. Moreover, the applied models predict the pressure-induced changes in the adsorption behavior of both insulin variants in a consistent way. In principle, it was observed that, when the column pressure increases, the distribution functions of the equilibrium adsorption constant become wider and tend to shift towards lower values. This would mean that the protein–surface interactions become more diversified (heterogeneous) when the pressure increases. Such an effect can be attributed to the conformational changes in the protein chain, that are produced by adsorption and it seems enhanced by the pressure. The results suggest also that the strength of the interactions between insulin molecules and the stationary phase is lower under high than under low pressures. This conclusion comes from the observed pressure-induced shifts in the most probable value of the equilibrium adsorption constant. In this case, however, a clear explanation of the observed trend is difficult at this stage, due to the very complex energetics of the interactions formed between a large protein molecule and the surface of an adsorbent. Further experimental studies seem indispensable to clarify this point.

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