Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography A, 1079 (2005) 221–228
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Hydrophobic interaction chromatography of proteins III. Unfolding of proteins upon adsorption

Alois Jungbauer*, Christine Machold, Rainer Hahn

Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria

Abstract

Hydrophobic interaction chromatography (HIC) exploits the hydrophobic properties of protein surfaces for separation and purification by performing interactions with chromatographic sorbents of hydrophobic nature. In contrast to reversed-phase chromatography, this methodology is less detrimental to the protein and is therefore more commonly used in industrial scale as well as in bench scale when the conformational integrity of the protein is important. Hydrophobic interactions are promoted by salt and thus proteins are retained in presence of a cosmotropic salt. When proteins are injected on HIC columns with increasing salt concentrations under isocratic conditions only, a fraction of the applied amount is eluted. The higher the salt concentration, the lower is the amount of eluted protein. The rest can be desorbed with a buffer of low salt concentration or water. It has been proposed that the stronger retained protein fraction has partially changed the conformation upon adsorption. This has been also corroborated by physicochemical measurements. The retention data of 5 different model proteins and 10 different stationary phases were evaluated. Partial unfolding of proteins upon adsorption on surfaces of HIC media were assumed and a model describing the adsorption of native and partial unfolded fraction was developed. Furthermore, we hypothesize that the surface acts as catalyst for partial unfolding, since the fraction of partial unfolded protein is increasing with length of the alkyl chain.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Protein; Unfolding; Pulse response; Langmuir–Freundlich isotherm; Hydrophobic interaction

1. Introduction

Hydrophobic interaction chromatography (HIC) for separation of proteins is an important separation method for separation of proteins in laboratory scale as well as for production of proteins in industrial scale [1–3]. In 1949, Shepard and Tiselius first reported on protein retention in a so-called salting out chromatography [4]. Proteins are retarded in a buffer containing cosmotropic salts. Hjerten called this method hydrophobic interaction chromatography [5]. The influence of cosmotropic and chaotropic salts on protein retention was further refined by the same group [6,7]. In 1977, Melander and Horváth introduced the solvophobic theory to describe the effect of salt-promoted adsorption [8]. They found a linear relationship between protein retention and molarity of salt in the mobile phase for high salt concentrations. The strength of retention depends on the surface tension increment of the salt dissolved in the mobile phase. To account for electrostatic interactions at low ionic strength, Melander and Horváth extended the solvophobic theory [9]. Several other attempts beside the solvophobic theory have been made to model adsorption of proteins on hydrophobic surfaces. These models are the preferential interaction analysis, the flicking cluster model, the random network model and the continuum model for liquid water [10]. The preferential interaction analysis is well suited to describe the effect of salt type on adsorption using experimentally estimated retention data [11–14]. Current understanding of salt-promoted interaction can be depicted as follows. A cavity in the liquid is formed and the protein molecule fills the cavity, fusion of cavities may lead to protein aggregation and precipitation. This process should be avoided in HIC. Water and ions surround the hydrophobic adsorption surface and the surface of the protein. Hydrophobic interactions between proteins and surface lead to adsorption. A structural arrangement of proteins may occur simultaneously along with the adsorption process. Rearrangement of water and ions in the bulk

* Corresponding author. Tel.: +43 1 360066226; fax: +43 1 3697615.
E-mail address: alois.jungbauer@boku.ac.at (A. Jungbauer).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.04.002

solution completes the process. Thus, hydrophobic adsorption of proteins is an entropy-driven process [15–18]. The driving force is reduction of surface area. Entropy gain by rearrangement of water may be superimposed the unfolding of proteins upon adsorption. In all aforementioned models, partially unfolding of proteins upon adsorption is not taken into account. In 1983, Saito and Wada [19] reported that a single protein with different conformation could be separated by chromatography. Goheen and Engelhorn [20] observed distorted peaks in linear gradient elution of albumin and β-lactoglobulin from HIC columns. They interpreted this effect as conformational change of the proteins. Benedek et al. [21] investigated the differential retention of unfolded and native proteins on reversed-phase columns. Then, their studies were extended to hydrophobic interaction chromatography [11,22] and the influence of salt and temperature was investigated. The model protein α-lactalbumin was extensively studied and at least two conformational variants were found [23]. A two-state model of unfolding of α-lactalbumin was proposed by Benedek [24]. The model system and the calculated thermodynamic values represent a useful method to estimate the contribution of the stationary and mobile phase to the protein denaturation processes. Additionally, a low recovery of enzyme activity from HIC columns was observed. This was interpreted as denaturation/unfolding on the column [25–27]. In 1989, Karger and Blanco [28] described the effect of on-column induced structural changes of proteins. They demonstrated the behavior by change of intrinsic fluorescence and provided strong evidence that the conformation of a protein may change upon adsorption or may change the conformation in the adsorbed state. Goheen et al. [29] has postulated that surfaces act like catalysts for protein unfolding. This has been exemplified using cytochrome *c*. McNay and Fernandez [30] and Buijs et al. [31] demonstrated by using the deuterium exchange technique that proteins are partially unfolded upon adsorption on hydrophobic surfaces. Hydrogen exchange detected by mass spectrometry was used to detect tertiary structure changes of calcium-free α-lactalbumin, a model protein with poor stability [32]. Two peaks were eluted from an HIC column. Hydrogen exchange measurement showed that the less-retained peak had solvent exposure similar to the native protein, while the more retained peak had increased solvent exposure. This is a strong indication that a fraction of the injected protein was unfolded upon exposure. For other proteins such as lysozyme, chymotrypsinogen A, and ovalbumin, the previous findings could be corroborated [33]. Protein adsorption was been also studied by various other physicochemical techniques and conformational change has been detected [34–36]. These methods work under conditions, which are not relevant for hydrophobic interaction chromatography. Shibata and Lenhoff [37,38] have measured protein adsorption under conditions relevant for HIC. Even under strong overloading conditions, they did not find multilayer adsorption. This confirms previous findings of adsorption isotherms. The isotherm shape did not indicate a multilayer adsorption [39].

Here, we describe an approach to quantify partial unfolding of proteins upon adsorption on surfaces of HIC media. The data are valid for the linear range. Furthermore, we hypothesize that the surface acts as catalyst for partial unfolding.

2. Theory

Antia and Horváth [40,41] have approximated adsorption isotherms typical in reversed-phase chromatography by including the organic mobile phase modifier concentration (φ) into a conventional Langmuir adsorption isotherm. For the multicomponent situation, the isotherm writes as follows:

$$q_i=\frac{a_{0,1}\,\exp(-S_i\varphi)C_i}{1+\sum_{j=1}^{n}(a_{0,i}/\lambda_j)\exp(-S_i\varphi)C_i}\quad (i=0,1,2,\ldots,n) \tag{1}$$

where q_i is the elute in the stationary phase, $a_{0,1}$ is the initial slope of the multicomponent adsorption isotherm for component 1 without modifier in the mobile phase, S is 2.3 times the slope of the plot k' (for definition, see Eq. (3)) versus φ for component i, and λ_j is a parameter of the Langmuir type multicomponent adsorption isotherm for component i. The same equation can be used to describe adsorption isotherms in HIC, only the sign of S will change. In a single-component situation, Eq. (1) under linear conditions will be simplified to:

$$k'=k'_0+\exp\lambda m \tag{2}$$

where k' is the normalized retention expressed as:

$$k'=\frac{V_R-V_0}{V_0} \tag{3}$$

with V_R the retention volume and V_0 the void volume, m is the molality of salt concentration in the mobile phase and λ is an empirical parameter. The distribution coefficient (K) can be expressed as:

$$K=\frac{q}{C}=k'\phi \tag{4}$$

where q is the amount of protein in the stationary phase and C in the mobile phase after equilibrium is attained. ϕ is the phase ratio.

Convolution of Eqs. (2) and (3) leads to an expression for the protein concentration in the stationary phase as a function of concentration of protein and salt in the mobile phase:

$$q=C\left[\left(\frac{q_0}{C_0}\right)+\phi\,\exp\lambda m\right] \tag{5}$$

where q_0 is the stationary phase concentration in equilibrium with the feed concentration C_0.

Using this equation, the adsorption of proteins as a function of salt concentration can be approximated for linear conditions.

According to Kaltenbrunner and Jungbauer [42], this exponential equation can be convoluted with the

Langmuir–Freundlich adsorption isotherm and used for approximation of stationary phase concentration of proteins also under non-linear conditions:

$$q(C,m) = q_{\max}\frac{bC^n}{1+bC^n}\left[\left(\frac{q_0}{C_0}\right) + \Phi \exp\lambda m\right] \quad (6)$$

where b is an empirical parameter and n is the exponent in the Langmuir–Freundlich adsorption isotherm.

3. Methodology

3.1. Buffers and proteins

All buffer ingredients were from Merck (Merck, Vienna, Austria). The model proteins α-lactalbumin, β-lactoglobulin, bovine IgG, bovine serum albumin, ovalbumin, lysozyme and lactoferrin were purchased from Sigma–Aldrich (Vienna, Austria).

3.2. Instrumentation

All experiments were performed on an Äkta-Explorer 100 system (GE Healthcare, Sweden) consisting of a compact separation unit and a personal computer running a control system (UNICORN, version 3.1).

3.3. Stationary phases

Phenyl Sepharose high performance (HP), Phenyl Sepharose 6FF high substitution, Phenyl Sepharose 6FF low substitution, Octyl Sepharose 4FF, Butyl Sepharose 4FF, Hexyl-*S*-Sepharose 6FF, Butyl-*S*-Sepharose 6FF, Pyridyl-*S*-Sepharose 6FF, Methyl Sepharose 4FF and Butyl Sepharose high performance (HP) were a gift from GE Healthcare. Toyopearl butyl 35 μm, Toyopearl butyl 65 μm and Toyopearl phenyl 35 μm were purchased from TosoHaas (Stuttgart, Germany), Macro-Prep Methyl and Macro-Prep *t*-Butyl were purchased from Bio-Rad (Hercules, CA, USA).

3.4. Pulse response experiments

Two milliliters of each sorbents were filled into HR5/10 columns (GE Healthcare) and packed at a flow velocity of 450 cm/h. The bed volume varied between 1.8 and 2 ml.

A 20 mM $NaPO_4$ buffer (pH 7.0) made by titration of a 20 mM Na_2HPO_4 and 20 mM NaH_2PO_4 to pH 7.0 was used as elution buffer. The salt buffer was $(NH_4)_2SO_4$ at various concentrations dissolved in 20 mM $NaPO_4$ buffer (pH 7.0). The buffers were filtered and degassed prior to chromatography. Isocratic runs were designed as follows: after equilibration of the columns at a flow velocity of 306 cm/h with $(NH_4)_2SO_4$ buffer of desired molarity for 3 CV, a 50 μl pulse of the protein sample was injected. The elution volume was 6 CV at a linear flow velocity of 100 cm/h. Regeneration was effected with 20 mM $NaPO_4$ buffer (pH 7.0). The desired salt concentration the eluent buffer was obtained by mixing 1.0 M $(NH_4)_2SO_4$ with 20 mM $NaPO_4$ (pH 7.0). The proteins were dissolved in the respective buffer with ammonium sulfate. Final protein concentration was 5 mg/ml for ovalbumin, 3 mg/ml for α-lactalbumin and BSA and 2 mg/ml for IgG, lactoglobulin, lactoferrin and lysozyme.

4. Results

With the intention of getting a general view of conformational change of proteins upon adsorption, we have selected a large variety of stationary phases and reference proteins, which have been extensively characterized as reported in previous papers [39,43]. The experimental set-up to determine the extent of partial unfolding of proteins upon adsorption on a hydrophobic surface consisted of two steps. First, breakthrough curves were determined at a fixed feed concentration but increasing ammonium sulfate concentration in the feed solution. Second, pulses of various model proteins were injected at increasing ammonium sulfate concentrations in the mobile phase and isocratically eluted. Complete elution was performed by a buffer with low ionic strength.

By using breakthrough analysis, the total amount of adsorbed protein as a function of ammonium sulfate concentration could be obtained. The experimental data were approximated by Eq. (5). The phase ratios were obtained from pulse response experiments under non-binding conditions. The plot (Fig. 1) represents the total amount of protein bound irrespective of the conformation of the protein. Then, pulse response experiments were performed (Fig. 2) and the chromatograms were evaluated as follows: the first peak was assumed to contain the native protein and the second peak contains the protein with partially changed conformation. This assumption was made based on the rational that in unfolded conformation, a protein exposes more hydrophobic surface area to the

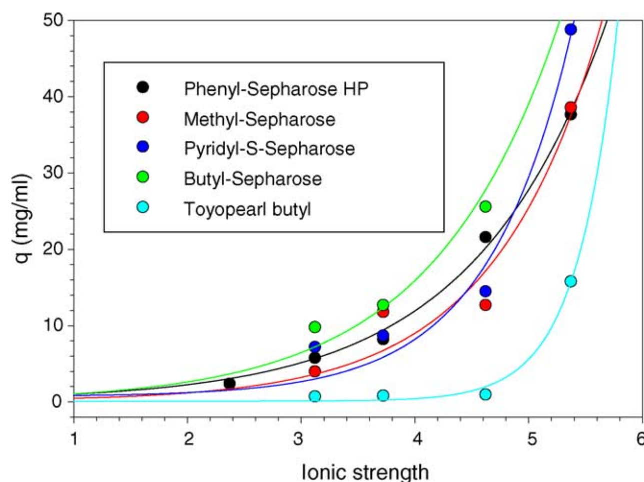

Fig. 1. Adsorption of BSA as a function of ammonium sulfate concentration in the mobile phase on different stationary phases.

stationary phase than in native conformation. The stronger retention of partially unfolded proteins was previously observed by various authors [11,21,24,28,32,33], but they never made an attempt to derive an isotherm for the native and partially folded proteins adsorbed on the stationary phase. Here, we refrain from additional experiments to confirm that the second peak has undergone partial unfolding. We assumed that there is enough experimental evidence about partial unfolding of proteins upon adsorption. In Fig. 2B, D, K, and L, a pronounced distortion or broadening was observed in the first peak. This is an indication that folding intermediates with slightly different retention are generated with increasing salt concentration. This was observed for the first time by Goheen and Engelhorn [20]. The progress of unfolding induced by the interaction with the stationary phase is clearly time dependent. For simplicity reasons, we kept residence time constant and we count the first peak as native protein, irrespective how distorted it was.

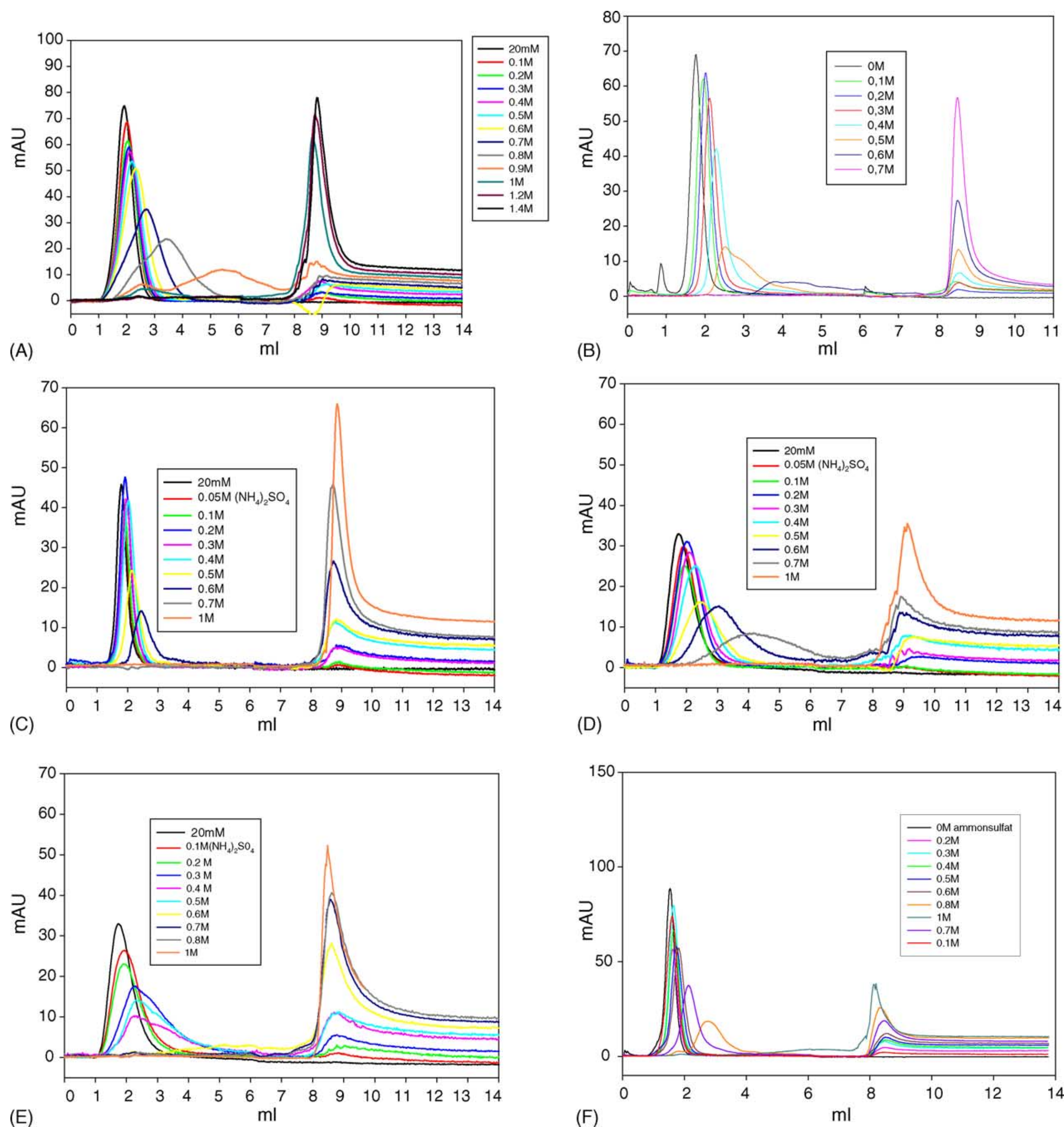

Fig. 2. Pulse response experiments of proteins with increasing concentration of ammonium sulfate in the mobile phase. (A) Methyl Sepharose FF, ovalbumin 5 mg/ml; (B) Phenyl Sepharose HP, BSA 3 mg/ml; (C) Butyl Sepharose HP, BSA 3 mg/ml; (D) Pyridyl-*S*-Sepharose 6FF, BSA 3 mg/ml; (E) Hexyl-*S*-Sepharose, BSA 3 mg/ml; (F) Toyopearl phenyl 35 μm, BSA 3 mg/ml; (G) Toyopearl butyl 35 μm, BSA 3 mg/ml; (H) Poros Phenyl 20 PE, BSA 3 mg/ml; (I) Macro-Prep Methyl, BSA 3 mg/ml; (J) Poros 20 PE, lactoglobulin 2 mg/ml; (K) Butyl Sepharose FF, IgG 2 mg/ml; (L) Butyl Sepharose HP, ovalbumin (5 mg/ml); (M) Tosoh phenyl 35 μm, lysozyme 5 mg/ml.

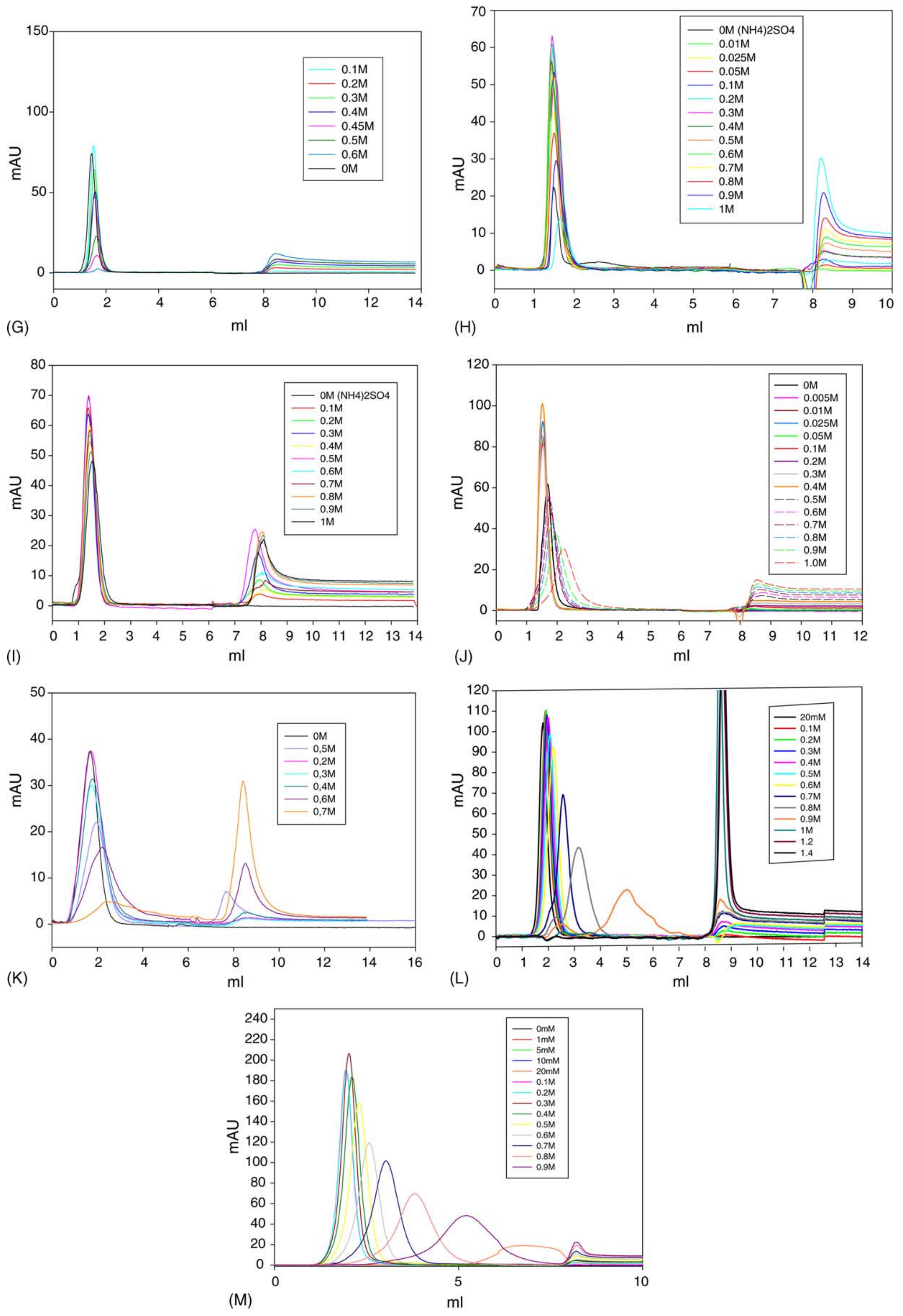

Fig. 2. (*Continued*).

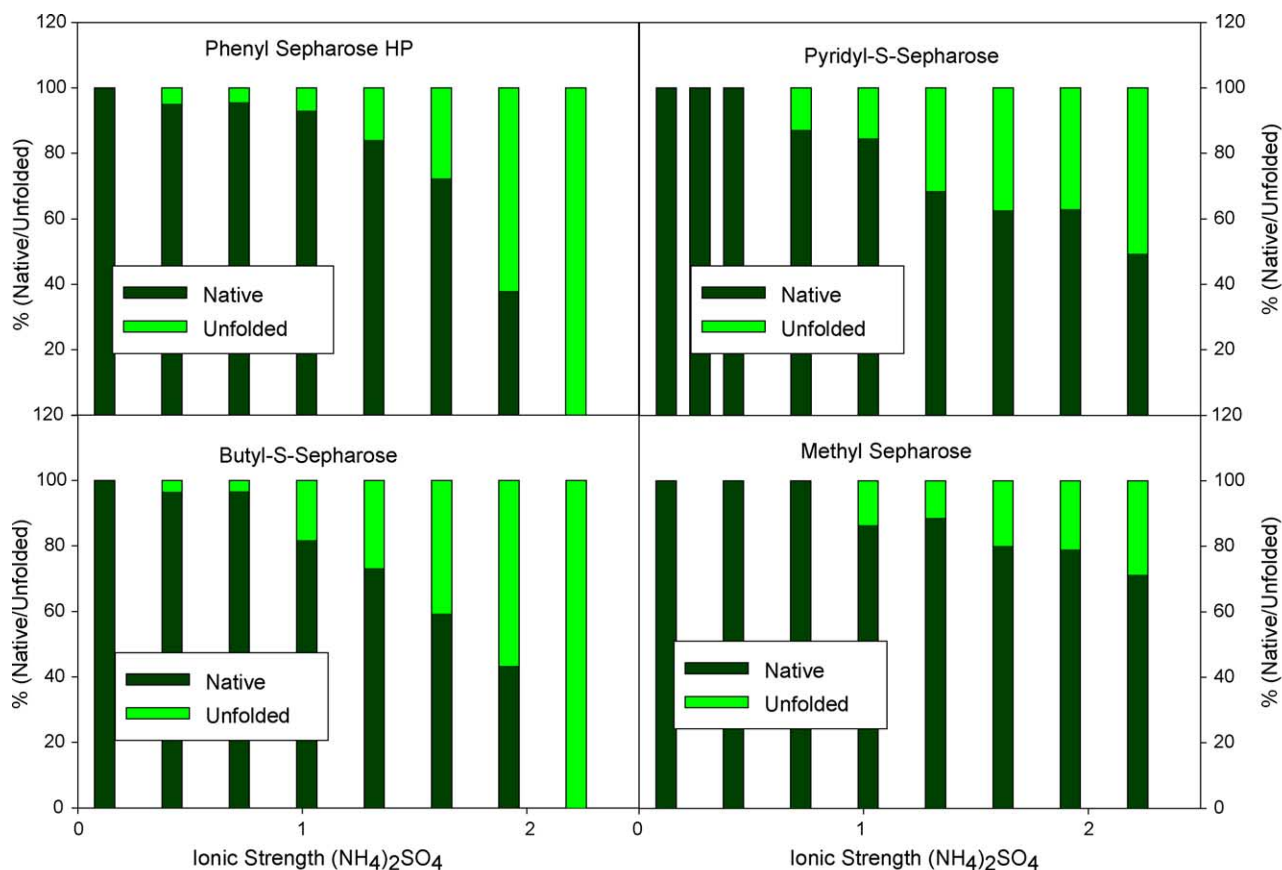

Fig. 3. Evaluation of pulse response experiments with increasing concentration of ammonium sulfate in the mobile phase injecting BSA at a concentration of 2 mg/ml. The ratios of the first, native peak area, and second, unfolded area, peak are plotted vs. increasing amount of ammonium sulfate in the buffer.

Lysozyme on a Tosoh phenyl 35 μm eluted almost quantitatively with ammonium sulfate. A linear $\log k'$ versus molarity relationship could be observed, similar to the work of Fausnaugh and Regnier [44]. A peak distortion at higher salt concentrations was also observed but neglected for simplicity reasons. The ratio of both peak areas, the first peak eluted with ammonium sulfate and the second peak eluted without ammonium sulfate, was plotted versus ammonium sulfate concentration (Fig. 3). Here, only a few examples were selected. The progress of unfolding with increasing ammonium sulfate concentration becomes evident. For the selected sorbents, it is shown that the ratio of areas of both peaks is different depending on ligand length. Thus, we hypothesize that the ligand promotes unfolding during adsorption and equilibrium is attained between proteins with partially unfolded and native conformation. Another interpretation of the data could be the presence of a non-homogenous surface. There could be sites with high and low affinity for proteins present. In this case, the ratio of both peaks should be constant and should not depend on ammonium sulfate concentration in the mobile phase. We cannot find such a behavior when we inspect Fig. 2 and compare it with Fig. 3. So, we conclude that proteins undergo conformational changes while or during adsorption on the hydrophobic surface. In order to get a quantitative picture of the unfolding process, we incorporated the ratios depicted in Fig. 3 into Fig. 1. We made also an attempt to quantify this effect. An empirical equation has been derived to describe the amount of partial unfolded species upon adsorption onto the stationary phase. The amount of native protein adsorbed as a function of ionic strength can be described as:

$$q(C, m) = \frac{q_{n,\max} b'}{\sqrt{c'^2 + b'^2}} \exp\left(-\frac{1}{2}\frac{(I - a')^2}{c'^2 + b'^2}\right) \times \left[1 + \operatorname{erf}\left(\frac{1}{2}\frac{\sqrt{2}d'((I - a')}{b'\sqrt{c'^2 + b'^2}}\right)\right] \quad (7)$$

where $q_{n,\max}$ is the maximal adsorbed protein, I is the ionic strength, and a', b', c' and d' are empirical parameters. The total amount of protein in the stationary phase is described by Eq. (5). The influence of salt type is not addressed in this work, but we assume that it will follow the trends described in the solvophobic theory [8,9]. Fig. 4 shows the influence of ligand type and length as well as the influence of ammonium sulfate concentration on the extent of unfolding. The longer the alkyl-chain length, the less ammonium sulfate in the buffer is required for structural rearrangement of the protein. This is well exemplified when comparing Butyl

Fig. 4. Influence of ligand type and length as well as of ammonium sulfate concentration on partial unfolding upon adsorption of protein in HIC. The stationary phase concentration of the respective protein was obtained from breakthrough curves and approximated by Eq. (5). The amount of native adsorbed protein was extrapolated from pulse response experiments and fitted by Eq. (7).

Sepharose with Methyl Sepharose. For Butyl Sepharose, a first peak was not obtained at an ionic strength of 1.7, i.e. the native protein was entirely converted to a partially denatured protein at this salt concentration. We explain this that due to the strong interaction and the influence of the surface the native structure could not be maintained. However, for others, a first peak was obtained until the ionic strength of the elution buffer was 3.

We observed also a qualitative difference between ligands. By comparing Toyopearl phenyl with Phenyl Sepharose (Fig. 4), the shape of the curve representing native protein in the stationary phase is smoother for Toyopearl phenyl than for Phenyl Sepharose. As ligand density, the way of immobilization of the ligand, and the contribution of the base matrix is different for those matrices, the catalytic effect of the matrix is different as well. Hence, alkyl-chain length is not the only reason for unfolding. The established model does not allow the inclusion of the effect of protein concentration. All this experiments were made under linear isotherm conditions. The only attempt we have made was to convolute Eq. (1) with the Langmuir–Freundlich isotherm. An algorithm as previously described for ion-exchange chromatography was used [42]. As an example, the three-dimensional plot of BSA on Butyl Sepharose is depicted in Fig. 5. This surface plot only describes the total amount of protein bound.

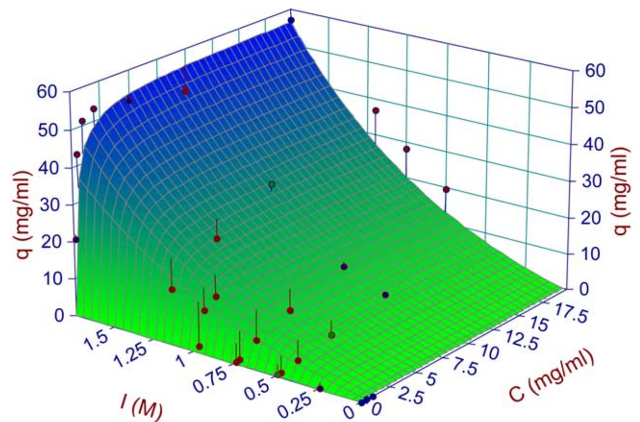

Fig. 5. Adsorption isotherm as a function of protein concentration and ammonium sulfate concentration for bovine serum albumin on Butyl Sepharose 4FF. Experimental data were approximated by Eq. (6).

A practical conclusion of our findings is to follow the concept of critical hydrophobicity proposed by Jennissen [45] for development of purification protocols. This approach allows retention of a protein under minimal salt concentration and shortest alkyl-length of the ligands. Our studies show that unnecessary high salt concentrations and length of alkyl chains lead to excessive partial unfolding. This partially unfolding may not affect the recovery of native protein, in case a very fast refolding process upon elution is taking place. For slow refolding kinetics, low recovery of native protein may be observed. In a previous paper, we have investigated the recovery of proteins from HIC columns [39]. In the majority, a satisfactory recovery was observed.

5. Conclusion

Interaction of the protein with ligands of HIC media may promote partial conformational changes of proteins upon adsorption. It is a combined effect between salt concentration in the mobile phase and ligand type. The extent of a conformational change cannot be assessed by the applied methodology, only the fraction of protein that undergoes structural modifications can be quantified. Conventional adsorption models do not distinguish between native and partially unfolded protein. Our approach is completely empirical but may serve as basis for further modeling. In light of our findings, the solvophobic model and preferential interaction analysis must be modified, if the effect of unfolding upon adsorption is also taken into consideration. The shortcomings of our current methodology are that folding intermediates and kinetics of unfolding promoted by interfacial surface contact are not known. The ligand may also penetrate into the core of a protein. This is the more complex interaction than pure adsorption. Our findings suggest that the concept of critical hydrophobicity [45] should be obeyed when an HIC method is developed.

References

[1] M.T.W. Hearn, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, Marcel Dekker, New York, 2002 (Chapter 5).
[2] A. Jungbauer, W. Feng, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, Marcel Dekker, New York, 2002.
[3] J.A. Queiroz, C.T. Tomaz, J.M.S. Cabral, J. Biotechnol. 87 (2001) 143.
[4] C.C. Shepard, A. Tiselius, Discussion of the Faraday Society, Hazell Watson and Winey, London, 1949, p. 275.
[5] S. Hjerten, J. Chromatogr. 87 (1973) 325.
[6] S. Pahlman, J. Rosengren, S. Hjerten, J. Chromatogr. 131 (1977) 99.
[7] J. Rosengren, S. Pahlman, M. Glad, S. Hjerten, Biochem. Biophys. Acta 412 (1975) 51.
[8] W.R. Melander, C. Horvát, Arch. Biochem. Biophys. 183 (1977) 200.
[9] W.R. Melander, C. Horvát, J. Chromatogr. 317 (1984) 67.
[10] M.T. Hearn, in: S. Ahuja (Ed.), Handbook of Bioseparation, Academic Press, New York, 2000 (Chapter 3).
[11] S.L. Wu, A. Figueroa, B.L. Karger, J. Chromatogr. 371 (1986) 3.
[12] B.F. Roettger, J.A. Myers, M.R. Ladisch, F.E. Regnier, Biotechnol. Prog. 5 (1989) 79.
[13] T.W. Perkins, D.S. Mak, T.W. Root, E.N. Lightfoot, J. Chromatogr. A 766 (1997) 1.
[14] F. Xia, D. Nagrath, S.M. Cramer, J. Chromatogr. A 989 (2003) 47.
[15] A. Vailaya, Cs. Horváth, Ind. Eng. Chem. Res. 35 (1996) 2964.
[16] M.A. Esquibel-King, A.C. Dias-Cabral, J.A. Queiroz, N.G. Pinto, J. Chromatogr. A 865 (1999) 111.
[17] H.-M. Huang, F.-Y. Lin, W.-Y. Chen, R.-C. Ruaan, J. Colloid Interface Sci. 229 (2000) 600.
[18] F.-Y. Lin, W.-Y. Chen, R.-C. Ruaan, H.-M. Huang, J. Chromatogr. A 872 (2000) 37.
[19] Y. Saito, A. Wada, Biopolym. Pept. Sci. Sect. 22 (1983) 2105.
[20] S.C. Goheen, S.C. Engelhorn, J. Chromatogr. 317 (1984) 55.
[21] K. Benedek, S. Dong, B.L. Karger, J. Chromatogr. 317 (1984) 227.
[22] S.-L. Wu, K. Benedek, B.L. Karger, J. Chromatogr. 359 (1986) 3.
[23] P. Oroszlan, R. Blanco, X.-M. Lu, D. Yarmush, B.L. Karger, J. Chromatogr. 500 (1990) 481.
[24] K. Benedek, J. Chromatogr. 458 (1988) 93.
[25] J.L. Fausnaugh, L.A. Kennedy, F.E. Regnier, J. Chromatogr. 317 (1984) 141.
[26] M. De Frutos, A. Cifuentes, J.C. Díez-Masa, J. Chromatogr. A 778 (1997) 43.
[27] S.C. Goheen, B.M. Gibbins, J. Chromatogr. A 890 (2000) 73.
[28] B.L. Karger, R. Blanco, Talanta 36 (1989) 243.
[29] S.C. Goheen, B.M. Gibbins, J.L. Hilsenbeck, J.V. Edwards, ACS Symp. Ser. 792 (2001) 20.
[30] J.L. McNay, E.J. Fernandez, J. Chromatogr. A 849 (1999) 135.
[31] J. Buijs, C. Costa Vera, E. Ayala, E. Steensma, P. Hakansson, S. Oscarsson, Anal. Chem. 71 (1999) 3219.
[32] T. Tibbs Jones, E.J. Fernandez, J. Colloid Interface Sci. 259 (2003) 27.
[33] T.T. Jones, E.J. Fernandez, Biotechnol. Bioeng. 87 (2004) 388.
[34] C.A. Haynes, W. Norde, Colloids Surf. B: Biointerfaces 2 (1994) 517.
[35] C.A. Haynes, W. Norde, J. Colloid Interface Sci. 169 (1995) 313.
[36] P.B. Welzel, Thermochim. Acta 382 (2002) 175.
[37] C.T. Shibata, A.M. Lenhoff, J. Colloid Interface Sci. 148 (1992) 469.
[38] C.T. Shibata, A.M. Lenhoff, J. Colloid Interface Sci. 148 (1992) 485.
[39] R. Hahn, K. Deinhofer, C. Machold, A. Jungbauer, J. Chromatogr. B 790 (2003) 99.
[40] F.D. Antia, Cs. Horváth, J. Chromatogr. 484 (1989) 1.
[41] F.D. Antia, Cs. Horváth, J. Chromatogr. 550 (1991) 411.
[42] O. Kaltenbrunner, A. Jungbauer, J. Chromatogr. 734 (1996) 183.
[43] C. Machold, K. Deinhofer, R. Hahn, A. Jungbauer, J. Chromatogr. A 972 (2002) 3.
[44] J.L. Fausnaugh, F.E. Regnier, J. Chromatogr. 359 (1986) 131.
[45] H.P. Jennissen, Int. J. Biochromatogr. 5 (2000) 131.