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Effects of bovine serum albumin heterogeneity on frontal analysis with anion-exchange media

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Abstract

The presence of dimers in commercial bovine serum albumin (BSA) samples of nominal high purity is investigated along with their effects on the frontal analysis behavior of preparative anion-exchange media. Size-exclusion and analytical anion-exchange chromatography are used to determine the relative amounts of monomer and dimer for two samples of BSA. While the amount of dimer was generally low, its impact on adsorptive behavior is significant. Equilibrium experiments demonstrate that the anion-exchange media binds the dimer more strongly, leading to an unequal distribution of the monomer and dimer between the two phases. Analysis of the breakthrough behavior of BSA reveals that the monomer breaks through prior to the dimer. This leads to the characteristic tailing breakthrough curve often seen with BSA. Breakthrough experiments carried out using dimer-free BSA confirm that the extreme tailing observed with the commercial samples is curtailed by removing the dimer.

Keywords: Frontal analysis; Heterogeneity; Bovine serum albumin

1. Introduction

Bovine serum albumin (BSA) is the most frequently used protein as a model to study the adsorptive behavior of anion-exchange stationary phases for preparative chromatography because of its availability, stability, and low cost. BSA has been used extensively to compare the performance of different stationary phases based on frontal chromatography (e.g., Refs. [1–4]), as well as to study equilibrium uptake and mass transport kinetics under overloaded conditions (e.g., Refs. [5–16]). Moreover, other authors have relied on the shape of BSA breakthrough curves to determine transport parameters either by comparison with model prediction (e.g., Refs. [17–21]) or by calculating mass transfer fluxes from the derivative of the breakthrough curve [22]. In these studies, a commonly observed experimental feature of BSA is an extreme tailing behavior of the breakthrough curve as the effluent approaches the feed concentration.

Despite the central role of BSA in many published studies, however, the heterogeneity of the protein samples used and its effect on adsorptive behavior have not been investigated in detail. Although a majority of these studies have used BSA from commercial sources with nominal high purity (96–99+%), heterogeneity of these protein samples

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remains a concern. For example, bovine albumins (as well as other globular proteins) have long been known to exist in dimer and/or oligomer forms [23,24]. Obviously, the presence of these forms can affect the chromatographic behavior under overloaded conditions, as has been observed by Skidmore et al. [7] and Weinbrenner and Etzel [25].

In this work we have investigated how the heterogeneity of commercial BSA samples influences the frontal analysis behavior in packed columns. The relative amounts of dimer and monomer present in commercial samples were quantified using size-exclusion and analytical anion-exchange chromatography. Equilibrium and frontal analysis experiments with a preparative-scale anion exchanger are employed to demonstrate the manner in which these components affect adsorption. Finally, breakthrough experiments using as-received and purified samples of BSA are compared. Since BSA from Sigma (St. Louis, MO, USA) was used in the vast majority of the references cited above [3–7,9–22], BSA samples from this supplier were also used in this work.

2. Materials and methods

2.1. Materials

Two different BSA samples characterized as a ''Fraction V Powder'' were obtained from Sigma. The first of these samples (Sigma catalog No. A-3912) was described by the supplier as having a minimum purity of 96% with the remainder being mostly globulins. It was used without further purification, except for one of the experiments in this study, as described below. The second sample (Sigma catalog No. A-7638) was described by the supplier as having a minimum purity of 99% and being essentially globulin free. Other chemicals were obtained from Sigma and from Fischer Scientific (Pittsburgh, PA, USA).

Samples of BRX-Q, the anion exchanger used in this work, were obtained from Bio-Rad Labs. (Hercules, CA, USA). This material is based on water-soluble, hydrophilic and vinylic monomers polymerized to yield spherical particles. The properties of BRX-Q are given in Refs. [13] and [14]. The samples used in this work were washed in a gravity-fed column with alternating cycles of 500 m*M* NaCl and dilute buffer solutions and stored under refrigeration in buffer. All experiments were conducted at room temperature (22±2°C) in a 50 m*M* Tris–HCl buffer at pH 8.5.

2.2. Analytical chromatography

Size-exclusion chromatography (SEC) was conducted with a 30×1 cm Superdex 200 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The mobile phase consisted of 200 m*M* NaCl in Tris buffer at a flow-rate of 0.5 ml/min. Anion-exchange chromatography was conducted with 15 μm Source Q (Pharmacia) packed in a 0.5 cm diameter column (Pharmacia, Model HR 5/5) to a depth of 5 cm. The mobile phase consisted of Tris buffer at a flow-rate of 0.8 ml/min with a NaCl gradient from 150 to 400 m*M* in 20 min.

A high-performance liquid chromatography (HPLC) apparatus from Waters (Milford, MA, USA) was used for the analytical determinations. The apparatus comprises two Model 510 pumps, a Model 717 Plus autosampler, a Model 481 LC UV–Vis detector set to 280 nm, and a Millennium 32 Chromatography Workstation for control and data analysis. The injection volume was adjusted to deliver approximately 20 μg of protein for SEC and 75 μg for anion-exchange chromatography.

2.3. Equilibrium experiments

The equilibrium experiments were conducted at different NaCl concentrations in a batchwise manner. Small samples of hydrated media (0.05 g) were placed in vials containing 6 ml of a solution containing a known initial protein concentration. The vials were sealed and rotated end-to-end at a few rpm for 6 h. As determined previously [13], this time is sufficient to attain an equilibrium state. The supernatant was analyzed using both size exclusion chromatography to determine the distribution of monomer and dimer and a UV spectrophotometer (Beckman-Coulter, Fullerton, CA, USA, Model DU-50) to determine the total protein concentration in solution. The initial protein concentration was chosen in such a way that the final concentration at equilibrium was approximately 1 mg/ml of protein

for each salt concentration examined. This was estimated using isotherms previously reported [13]. All equilibrium experiments used BSA Sigma catalog No. A-3912.

2.4. Frontal analysis experiments

Frontal analysis experiments were conducted in 0.5 cm I.D. glass columns packed to heights of 3.2 and 5.4 cm (Pharmacia, Models HR 5/2 and HR 5/5). The columns were slurry-packed and allowed to equilibrate at a flow-rate of 3 ml/min. An adapter was then lowered to minimize dead volume. Tris buffer and protein solutions were delivered using a Pharmacia Model P-500 pump. The flow-rate for all frontal analysis experiments was 1 ml/min, which corresponds to a superficial mobile phase velocity of 300 cm/h. Breakthrough curves were monitored using the UV detection module of a BioSepra ProSys Chromatography Workstation (Marlborough, MA, USA) at 280 nm. For some of the experiments 1 ml fractions were also collected for later analysis by SEC and anion-exchange chromatography. At the end of each column run, the feed protein solution was passed directly through the UV detector to determine the exact concentration of the feed solution.

3. Results and discussion

3.1. Analytical chromatography of BSA samples

SEC and anion-exchange chromatograms are shown in Figs. 1 and 2 for BSA samples A-3912 and A-7638, respectively. The protein solutions injected (2 mg/ml) were prepared using the mobile phase buffer. It can be seen that for both materials there is a major and a minor component, labeled α and β, respectively. Elution volumes for the major and minor components are 13.9 and 12.4 ml in the SEC column and 8.8 and 11.6 ml in the anion-exchange column. For both BSA samples, a shoulder on peak α appears in the anion-exchange chromatograms. A third early eluting peak, labeled γ, also appears for sample A-7638 in the SEC chromatogram. The percent of total area for each peak is given in Table 1. SEC and analytical ion-exchange chromatography were both highly reproducible in repeated runs.

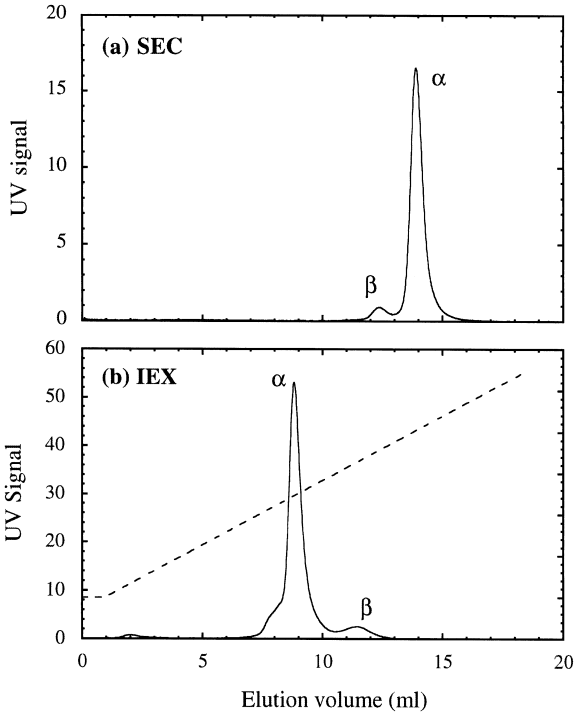

Fig. 1. Analytical chromatography of BSA sample Sigma catalog No. A-3912 (nominal purity >96%): (a) SEC and (b) anion-exchange chromatography. Dashed line shows NaCl gradient on a 100–450 m*M* scale. Conditions are noted in the text.

Comparison of the SEC elution volumes with those obtained for standard proteins as reported by the column manufacturer [26] as well as with the SEC results of Folta-Stogniew and Williams [27] based on laser light scattering detection confirms that the minor component has molecular mass around 130000 corresponding to a BSA dimer. For the nominally purer A-7638 sample (nominal minimum purity=99%), the minor component peaks comprise an even greater percentage of the total area. The early eluting peak previously mentioned for this sample has an elution volume of 11.4 ml corresponding to a molecular mass around 260000. Thus, this peak is likely BSA tetramer or a partially resolved mixture of oligomers as suggested by Folta-Stogniew and Williams [27]. It has been shown that BSA dimerization occurs via formation of disulfide bonds, is favored at low pH, and is catalyzed by

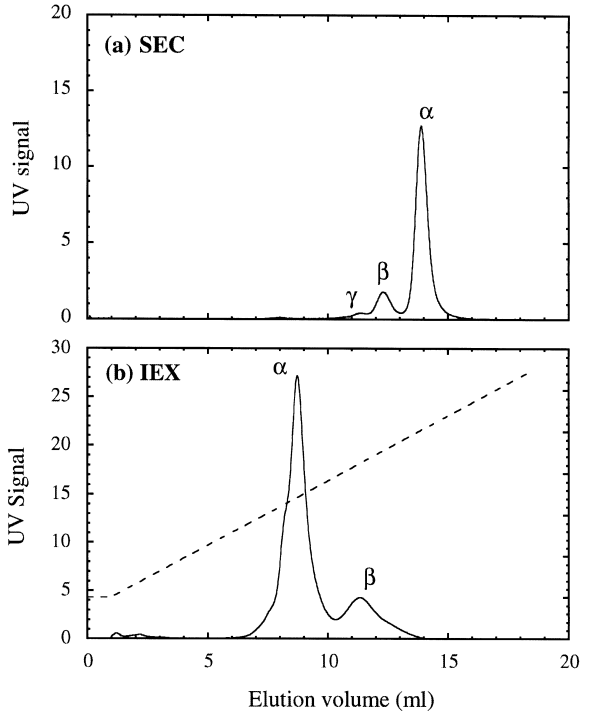

Fig. 2. Analytical chromatography of BSA sample Sigma catalog No. A-7638 (nominal purity ≥ 99%): (a) SEC and (b) anion-exchange chromatography. Dashed line shows NaCl gradient on a 100–450 m*M* scale. Conditions are noted in the text.

certain transition metal ions [24]. In our experiments, both BSA monomer and dimer forms appeared stable when dissolved in Tris buffer at 2 mg/ml and did not appear to undergo further reaction. SEC analyses conducted immediately after the protein solution was prepared yielded results identical to those conducted after the protein solution was allowed to rest at room temperature for 12 h. It was not possible to resolve the shoulder peak in the anion-exchange chromatograms suggesting that this peak corresponds to a component closely related to the BSA monomer, perhaps a BSA variant. This hypothesis is further reinforced by frontal analysis results that are discussed below.

Table 1
Percent contribution of peaks in commercial BSA samples

Sample (Sigma catalog No.)	SEC			IEX	
	α	β	γ	α	β
A-3912	93.6	6.4	–	90.2	8.4
A-7638	82.9	13.8	3.2	77.6	20.7

3.2. Equilibrium experiments

SEC chromatograms showing analysis of BSA solutions equilibrated with BRX-Q at 0, 100, and 150 m*M* NaCl are shown in Fig. 3. The solution concentration C and the total adsorbed protein concentration q are noted in the figures. The latter was calculated from a material balance as described in Ref. [13]. The reproducibility of these determinations was confirmed by duplicating the 100 m*M* NaCl experiment. As seen in this figure, the dimer is essentially completely removed from the solution at 100 m*M* NaCl, while only trace amounts remain at 0 and 150 m*M* NaCl. Presumably this behavior originates from a higher affinity of the BSA dimer for the resin under these conditions. We can rule out that this phenomenon is due to conversion of the dimer into monomer or irreversible binding since no change was noted during the time of these experiments in the absence of resin and BSA could be recovered quantitatively from BRX-Q when exposing it to 500 m*M* NaCl as shown in Ref. [28].

3.3. Frontal analysis

Breakthrough of 1 mg/ml BSA in 150 m*M* NaCl on a BRX-Q column packed to a depth of 5.4 cm is shown in Fig. 4a. While the breakthrough curve has the characteristic S-shape expected for a favorable isotherm, a pronounced tailing behavior is also evident. In fact, the effluent concentration remains below 95% of the feed value even at 4000 s. This effect was found to be highly reproducible, with repeated runs using the same column yielding identical results. SEC analysis of fractions collected during the experiment revealed that the BSA dimer was being retained on the column and did not breakthrough with the monomer. Fig. 4b and c show representative SEC and anion-exchange chromatograms of a fraction collected near the end of the experiment (t=3600 s). By comparison with the chromatograms obtained for the feed solution in Fig. 1, it can be seen that no dimer was present in this fraction. Thus, it can be concluded that strong retention of the BSA dimer is responsible for the

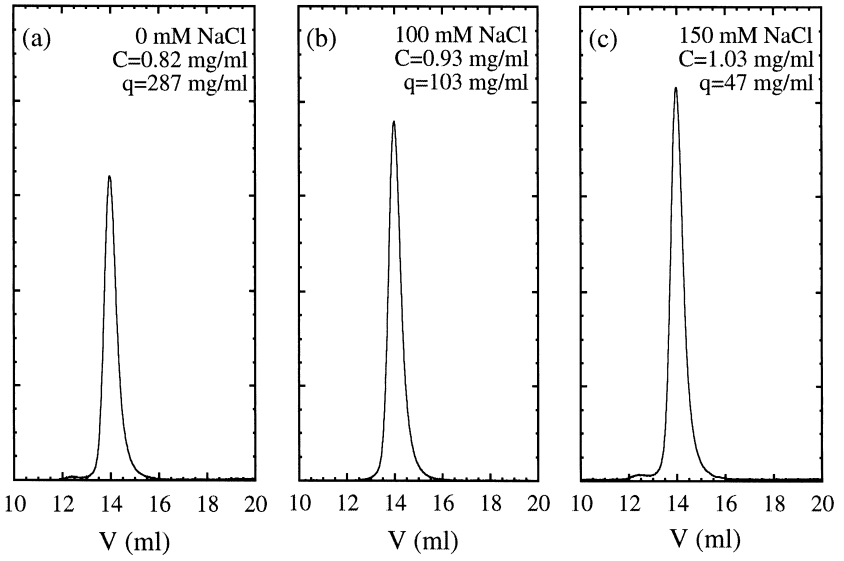

Fig. 3. SEC analysis of BSA solutions equilibrated with BRX-Q at: (a) 0 m*M* NaCl, (b) 100 m*M* NaCl, and (c) 150 m*M* NaCl. Protein concentrations in the fluid *C* and in the adsorbent *q* are noted in the figure for each salt concentration. Conditions are noted in the text.

tailing of the breakthrough curve. Lastly, it can be seen that the shoulder peak α shown in Fig. 1b is also seen in Fig. 4c, suggesting that BRX-Q is not able to discriminate between these closely related forms.

To test the hypothesis that strong retention of the dimere causes the tailing behavior, we purified a sample of BSA to obtain pure monomer and used this solution as the feed for a subsequent breakthrough experiment. To accomplish this a 1.5 mg/ml BSA solution (Sigma catalog No. A-3912) was prepared in 100 m*M* NaCl and an amount of BRX-Q media was added such that the concentration at equilibrium would yield approximately a 1 mg/ml BSA solution. The mixture was agitated for 4 h after which the media was removed by filtration. The protein solution was analyzed by SEC and found to be dimer free, as seen in Fig. 3b. The concentration of the solution was determined to be 0.97 mg/ml. This solution was then immediately used as the feed for a frontal analysis experiment. The results are shown in Fig. 5. For comparison purposes the figure also shows the results of frontal analysis experiments carried out under identical conditions except using unpurified BSA (i.e., the samples whose analyses are shown in Figs. 1 and 2) as the feed solution. Since slightly different column lengths were used, the results are presented as a function of elution volume divided by the column volume. It is evident that the extreme tailing behavior observed when using the unpurified BSA samples as the feed is largely eliminated when dimer-free BSA is fed to the column. After 190 column volumes the effluent reaches 99.5% of the feed concentration using dimer-free BSA, but only 96% for the A-3912 BSA sample and only 90% for the A-7638 BSA sample. It should be noted that since the position of the breakthrough front must satisfy an overall material balance, the entire breakthrough pattern is skewed by the tailing behavior. Thus modest shifts in time and slope can be seen throughout when comparing unpurified and dimer-free feeds.

4. Conclusions

We have studied the effects of the heterogeneity of commercial BSA samples on frontal analysis for a preparative scale anion exchanger. The presence of BSA dimer in commercial samples of high nominal purity was found to be the principal cause of the extreme tailing breakthrough curves frequently observed with BSA. This behavior has previously been observed for the breakthrough of BSA with many

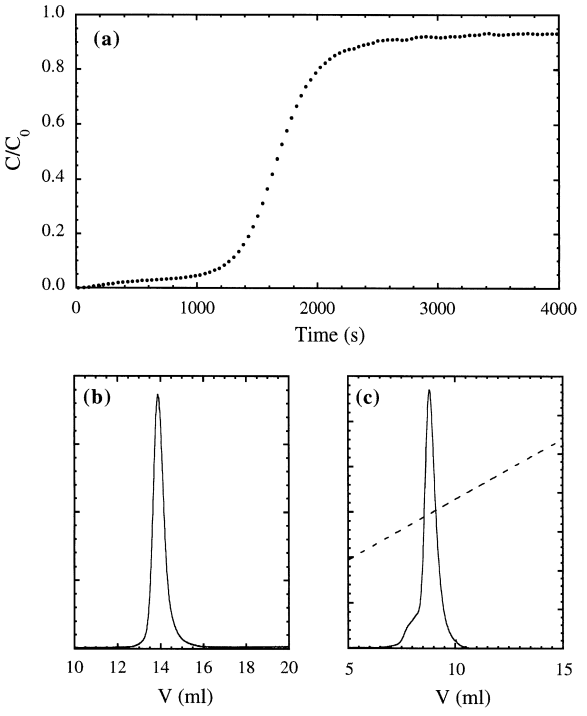

Fig. 4. Breakthrough curve for 1 mg/ml BSA on BRX-Q at 150 m*M* NaCl: (a) breakthrough curve in 5.4×0.5 cm column at 1 ml/min, (b) SEC analysis of fraction collected at $t=3600$ s, and (c) anion-exchange chromatography analysis of fraction collected at $t=3600$ s. Dashed line shows NaCl gradient on a 100–450 m*M* scale.

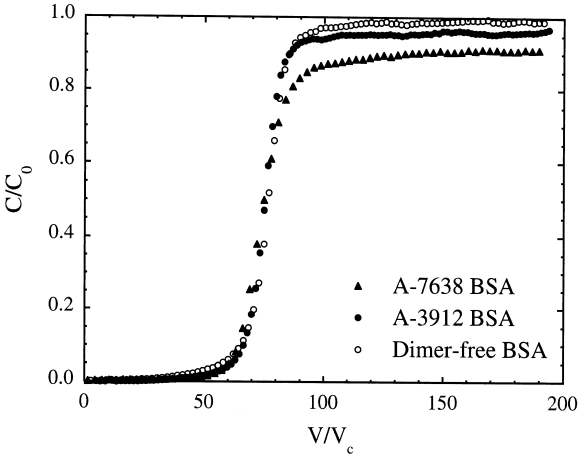

Fig. 5. Breakthrough curves for 1 mg/ml BSA on BRX-Q at 100 m*M* NaCl for commercial and dimer-free BSA samples in 3.2×0.5 cm columns at 1 ml/min. Note that the extreme tailing is largely absent for the dimer-free BSA feed.

other stationary phases [5–7,11,14,16,17,22], including monoliths and membrane adsorbers [2,25], where mass transfer effects are generally considered to be absent. Various explanations and theories have been advanced in the literature for this effect including extracolumn contributions, hindered diffusion, heterogeneous particle size distribution [16], as well as steric hindrance [29,30]. In practice, however, it appears that a dominant cause is the heterogeneity of the protein samples commonly used in these studies. Furthermore, it follows that since the breakthrough curve is significantly affected by the presence of these multiple forms, attempts to elucidate mass transfer mechanisms by matching breakthrough curves to theoretical models or taking derivatives are futile unless highly pure protein feeds are employed. On the other hand, as previously shown by several authors (e.g., Refs. [7,11,14,16]), an approximate prediction of the breakthrough curve for commercial BSA samples can still be obtained if mass transfer rates are measured independently and correlated by a suitable model.

Acknowledgements

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References

[1] P.R. Levison, C. Mumford, M. Streater, A. Brandt-Nielsen, N.D. Pathirana, S.E. Badger, J. Chromatogr. A 760 (1997) 151.
[2] G. Iberer, R. Hahan, A. Jungbauer, LC–GC 17 (1999) 998.
[3] A. Staby, I.H. Jensen, I. Mollerup, J. Chromatogr. A 897 (2000) 99.
[4] A. Staby, I.H. Jensen, J. Chromatogr. A 908 (2001) 149.
[5] E.E. Graham, C.F. Fook, AIChE J. 28 (1982) 245.
[6] H. Tsou, E.E. Graham, AIChE J. 31 (1985) 1959.
[7] G.L. Skidmore, B.J. Horstmann, H.A. Chase, J. Chromatogr. 498 (1990) 113.
[8] H. Yoshida, M. Yoshikawa, T. Kataoka, AIChE J. 40 (1994) 2034.
[9] A.E. Rodrigues, J.M. Loureiro, C. Chenou, M. Rendueles de la Vega, J. Chromatogr. B 664 (1995) 233.
[10] M.A. Fernandez, G. Carta, J. Chromatogr. A 746 (1996) 169.
[11] M.A. Fernandez, W.S. Laughinghouse, G. Carta, J. Chromatogr. A 746 (1996) 185.

[12] M. Rendueles de la Vega, C. Chenou, J.M. Loureiro, A.E. Rodrigues, Biochem. Eng. J. 1 (1998) 11.
[13] A.K. Hunter, G. Carta, J. Chromatogr. A 897 (2000) 65.
[14] A.K. Hunter, G. Carta, J. Chromatogr. A 897 (2000) 81.
[15] J.R. Conder, B.O. Hayek, Biochem. Eng. J. 6 (2000) 215.
[16] J.R. Conder, B.O. Hayek, Biochem. Eng. J. 6 (2000) 225.
[17] H. Guan-Sajonz, P. Sajonz, G. Zhong, G. Guiochon, Biotechnol. Prog. 12 (1996) 380.
[18] P. Sajonz, H. Guan-Sajonz, G. Zhong, G. Guiochon, Biotechnol. Prog. 13 (1997) 170.
[19] G.A. Heeter, A.I. Liapis, J. Chromatogr. A 776 (1997) 3.
[20] K. Miyabe, G. Guiochon, Biotechnol. Prog. 15 (1999) 740.
[21] K. Miyabe, G. Guiochon, J. Chromatogr. A 866 (2000) 147.
[22] E. Hansen, J. Mollerup, J. Chromatogr. A 827 (1998) 259.
[23] J. Janatova, J.K. Fuller, M.J. Hunter, J. Biol. Chem. 243 (1968) 3612.
[24] J.F. Foster, in: V.M. Rosenoer, M.A. Oratz, M.A. Rothschild (Eds.), Albumin Structure, Function and Uses, Pergamon Press, Oxford, 1977, p. 53.
[25] W.F. Weinbrenner, M.R. Etzel, J. Chromatogr. A 662 (1994) 414.
[26] Biodirectory Product Catalog, Amersham Pharmacia Biotech, 2001.
[27] E. Folta-Stogniew, K.R. Williams, J. Biomol. Techn. 10 (1999) 51.
[28] A.K. Hunter, G. Carta, J. Chromatogr. A 930 (2001) 79.
[29] X. Jin, J. Talbot, N.-H.L. Wang, AIChE J. 40 (1994) 1685.
[30] J. Talbot, G. Tarjus, P.R. Van Tassel, P. Viot, Coll. Surf. A 165 (2000) 287.