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Influence of protein and stationary phase properties on protein–matrix-interaction in cation exchange chromatography

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ABSTRACT

A large number of different stationary phases for ion-exchange chromatography from different manufacturers are available, which vary significantly in a number of chemical and physical properties. As a consequence, binding mechanisms may be different as well. In the work reported here, the retention data of model proteins (lysozyme, cytochrome c and two monoclonal antibodies) were determined for nine commercially available cation-exchange adsorbents. The linear gradient elution model in combination with a thermodynamic approach was used to analyse the characteristic parameters of the protein–stationary phase-interactions. Based on the pH dependency of the characteristic charge and the equilibrium constant for binding the differences between the standard Gibbs energies in the adsorbed and the solute state for the protein ΔG_P° and the salt ΔG_S° were calculated. The characteristic charge B of the proteins strongly depends on the molecular mass of the protein. For small proteins like lysozyme there is almost no influence of the stationary phase chemistry on B, while for the Mabs the surface modification strongly influences the B value. Surface extenders or tentacles usually increase the B values. The variation of the characteristic charge of the MABs is more pronounced the lower the pH value of the mobile phase is, i.e. the higher the negative net charge of the protein is. The standard Gibbs energy changes for the proteins ΔG_P° are higher for the Mabs compared to lysozyme and more strongly depend on the stationary phase properties. Surface modified resins usually show higher ΔG_P° and higher B values. A correlation between ΔG_P° and B is not observed, indicating that non-electrostatic interactions as well as entropic factors are important for ΔG_P° while for the B values the accessibility of binding sites on the protein surface is most important.

1. Introduction

A series of liquid chromatography steps are involved in separation and purification processes of biological products such as protein-based drugs [1–3]. Purification of recombinant proteins makes high demands on downstream processes as the target proteins have to be separated from very similar protein variants to very high purities. Amongst different separation modes such as size exclusion, hydrophobic interaction or affinity chromatography, ion-exchange chromatography (IEC) is commonly used and is therefore a major unit operation in purification processes of therapeutic proteins [2–6].

As design and optimization of IEC unit operations require consideration of many operating and chromatographic parameters one of the main challenges in biochromatography is predicting protein elution behaviour under various conditions based on a limited set of experimental data. In ion-exchange chromatography protein adsorption depends on the composition and concentration of the protein sample, on operating conditions such as buffer composition and pH, flow rate and sample load and on the physical properties of the adsorbent matrix [7].

Prediction is usually done using different mathematical chromatography models, which are based on the equilibrium model, the plate model and the rate model, for instance. They describe the retention behaviour of a protein dependent on the relevant separation parameters. For IEC several publications show the applicability of the models for different elution modes [4,8–14].

As IEC is widely used in protein drug purification a large variety of stationary phases is offered by different manufacturers. To obtain the benefits of ion-exchange chromatography, which are high selectivity, capacity and throughput, the evaluation and selection of a suitable adsorbent is very important [15]. Various resins, differing in chemical and physical properties, such as base matrix composition, stability regarding chemicals, pH and pressure, density of charged groups and ligand chemistry.

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In this study, several strong cation-exchange chromatography (CEX) resins were examined. Special attention was paid to the influence of surface modifications on the interaction between protein and matrix. Therefore several non-modified resins were compared to surface modified ones.

Source 30S, Toyopearl SP-650M and SP Sepharose FF are conventional resins whose functional groups are attached to the matrix by spacer arms. Source 30S and Toyopearl SP-650M have a polymeric base matrix while the bead of SP Sepharose FF consists of cross-linked agarose. Mean particle diameters range from 30 μm (Source 30S) to 65 μm (Toyopearl SP-650M) and 90 μm (SP Sepharose FF).

Toyopearl GigaCap S-650M is an improved ion-exchange resin consisting of the same polymeric base matrix as Toyopearl SP-650M except for a slightly larger average particle size of 75 μm, which leads to an improvement of pressure-flow characteristics. Furthermore, optimized ligand attachment chemistry leads to higher protein dynamic binding capacities, improved resin binding and desorption as the functional groups are preferentially placed in the larger pores which are more accessible for proteins.

Fractogel EMD SO_3^- (M) and Fractogel SE Hicap (M) have a similar base matrix as the conventional Toyopearl SP-650M but are surface modified by polyelectrolyte "tentacles". These long, linear polymer chains are attached to the beads and carry the functional ligands. As the tentacles are highly flexible the accessibility of the ligands without steric hindrance is improved and this results in a tighter binding of biomolecules. The polyelectric chains also allow additional interaction between proteins and ligands. Fractogel media was developed to afford high capacities at high flow rates and therefore allow higher yields at reduced throughput times compared to conventional resins. Due to the strong binding of proteins to the ligands higher salt concentrations in the sample have a minor effect on the binding capacity. As the Fractogel and Toyopearl resins are developed from the same beads, data for these materials allow immediate comparison to examine the effect of the tentacles.

Capto S consists of a highly cross-linked agarose matrix with similar bead and pore sizes as SP Sepharose FF. Dextran surface extenders link the functional group to the agarose matrix which increases capacities and mass transfer properties.

Poros 50 HS consist of polymeric particles with a mean diameter of 50 μm. The beads are coated with a proprietary hydrophilic polymer onto which the functional groups are covalently attached. Flow-through pores (500–10,000 Å) allow rapid mass transport.

S Ceramic HyperD F ion-exchangers combine characteristics of a soft, high capacity hydrogel with the stability of a rigid ceramic bead. The large pores of a rigid ceramic bead are filled with a functionalized hydrogel.

The selected set of strong cation-exchangers offers, apart from a variety of base matrices, particle and pore sizes, different ligand chemistries and this, therefore, allows the investigation of surface modified resins in comparison to conventional non-modified resins. Although many studies deal amongst other aspects with dynamic and static binding capacities, binding strength, ionic capacities, efficiency, resolution, adsorption isotherms, mass transfer, selectivity and protein recovery for conventional as well as surface modified resins [15–27], and several studies focus on properties of modified stationary phases [15,21,28–32], the impact of modifications is not yet well understood. Especially in the case of tentacle resins some studies show results regarding the influence of tentacles on the strength of protein retention which are contradictory to theoretical considerations. As tentacles offer an additional contact area between the charged sites of the proteins and the charged groups of the stationary phase this should lead to a significant increase in retention, which, without exception, was not reported for anion-exchange resins [33,34]. Contrary to these results, Bruch et al. observed increased numbers of interactions for tentacle modified resins [29]. DePhillips and Lenhoff tried to explain their sometimes ambiguous results for tentacle resins with differences in pore structure and tentacle length [15]. The examination of a broad variety of different stationary phases in this study should allow a better understanding of the influence of surface modifications on protein–matrix-interactions in ion-exchange chromatography and facilitate a target-oriented selection of chromatographic resins. GH-I_R-curves were calculated by the application of a linear gradient elution model [35,36]. Apart from data for the prediction of protein elution behaviour the applied model delivers information about stationary phase properties and electrostatic as well as non-electrostatic protein–matrix-interactions.

2. Theoretical considerations

2.1. Electrostatic interactions in ion exchange chromatography

In ion-exchange chromatography the separation mechanism is based on electrostatic interactions between the sample proteins and the functional groups of the resins. The interaction between a protein P with z_P charged sites and the ion exchange group in the presence of a counterion S according to the law of mass action or the stoichiometric displacement model (SDM) is described in the following equation [37]:

$$P + Z_P \cdot \bar{S} \Leftrightarrow \bar{P} + Z_P \cdot S \tag{1}$$

where $\bar{S}$ and $\bar{P}$ represent the salt and protein in the stationary phase. The equilibrium constant K_{eq} based on activities is given by:

$$K_{eq} = \frac{a_{\bar{P}} \cdot a_S^{Z_P}}{a_P \cdot a_{\bar{S}}^{Z_P}} \tag{2}$$

Under the assumptions that the activity coefficients are constant and close to unity Eq. (2) can be rewritten into the following equation:

$$K_{eq} = \frac{C_q}{C}\left(\frac{I}{I_q}\right)^{Z_P} \tag{3}$$

with the concentration of bound protein C_q, the concentration of protein in the mobile phase C, the salt concentration in the mobile phase I and the salt concentration in the stationary phase I_q.

The ion-exchange capacity Λ is given by:

$$\Lambda = I_q + z_P C_q \tag{4}$$

The distribution coefficient K_q describes the ratio of protein bound to ion-exchange groups to unbound protein and is given by combining Eq. (3) and (4):

$$K_q = \frac{C_q}{C} = K_{eq}\left(\frac{I}{I_q}\right)^{-Z_P} = K_{eq} \cdot \Lambda^{Z_P} \cdot I^{-Z_P} \tag{5}$$

with $\Lambda = I_q$ valid for low protein concentrations.

2.2. Linear gradient elution model

Yamamoto et al. established a simple graphical model based on the equilibrium and the plate model for the determination of elution characteristics from linear gradient elution (LGE) experiments in IEC [35,36]. This model (here called LGE-model) delivers data concerning the salt concentration at the peak position and the peak width as a function of gradient slope or flow rate. This information can be used for the design and optimization of linear gradient elutions or step elutions and for scale-up. Further this model delivers information about stationary phase properties and protein–matrix-interactions.

By performing linear gradient elutions with different gradient slopes g [M/mL] and determining the ionic strength at the peak

position I_R [M], the LGE-model allows us to describe the distribution coefficient K as a function of the ionic strength I [7].

The slope of the salt gradient g is defined as:

$$g = \frac{I_F - I_0}{V_g} \quad (6)$$

with the initial salt concentration I_0, the final ionic strength I_F and the gradient volume V_g.

The normalized gradient slope GH is given by:

$$GH = (gV_o)\left(\frac{V_c - V_o}{V_o}\right) = g(V_c - V_o) \quad (7)$$

with the volume of the packed resin in the column V_c, the void volume V_0 and the phase ratio $H = (V_c - V_0)/V_0$.

Variation of the gradient slope results in the salt concentration at the peak position as a function of GH. The experimental data can usually be expressed by the following equation:

$$GH = \frac{I_R^{(B+1)}}{A(B+1)} \quad (8)$$

or as log (GH)-log I_R-plot with the linear correlation:

$$\log(GH) = (B+1)\cdot \log I_R + \log[A(B+1)]^{-1} \quad (9)$$

where $(B+1)$ is the slope of the GH-I_R-plot and $\log[A(B+1)]^{-1}$ is the y-intercept.

The meaning of the parameters A and B can be derived from the law of mass action. B is the number of charges involved in protein adsorption and is equal to z_p in Eq. (5) and A is related to K_{eq} as follows [7,37]:

$$A = (1 - \varepsilon_p)K_{eq} \cdot \Lambda^B \quad (10)$$

with the intraparticle porosity ε_p.

The ratio between the protein concentration in the stationary phase C_s and in the mobile phase C, K, can be derived with the parameters A and B as follows:

$$K = \frac{C_s}{C} = A \cdot I^{-B} + K_{crit} \quad (11)$$

with the distribution coefficient of the protein under non-binding conditions K_{crit}.

3. Materials and methods

3.1. Resins and columns

The following cation-exchange chromatography resins were used in this study: Fractogel EMD SO_3^-(M), FractogelSE Hicap (M) (Merck), Toyopearl SP-650M, ToyopearlGigaCap S-650M (Tosoh), Source 30S, SP Sepharose FF, Capto S (GE Healthcare), Poros 50 HS (Applied Biosystems) and S Ceramic HyperD F (Pall). All Fractogel resins, Toyopearl GigaCap S-650M and Poros 50 HS, are surface-modified to improve their binding capacities and recoveries at high flow rates, which makes them more suitable to meet the needs in high throughput purification of monoclonal antibodies and other proteins. The main characteristics of the resins are summarized in Table 1.

All resins were prepacked in MediaScout MiniChrom columns (Atoll) with an inner diameter of 0.8 cm and a length of 5 cm.

3.2. Buffers and samples

The following buffers were used for the different pH values: pH 4.5, 4.75, 5, 5.25, 5.5 and 5.75: 50 mM phosphate/acetate buffer consisting of 21.5 mM NaH_2PO_4, 3.4 mM Na_2HPO_4, 16.5 mM sodiumacetate and 0.48 mg/L acetic acid; pH 6: 30 mM Na-citrate; pH 7: 30 mM NaH_2PO_4; pH 9: 30 mM Tris, 20 mM NaCl; pH 10 and

Table 1
Characteristics of resins used in the study.

Resin	Matrix	Mean particle diameter [μm]	Surface functionality	Ionic capacity [meq/mL]
Fractogel EMD SO_3^- (M)	Methacrylate copolymers	65	$-C(CH_3)_2-CH_2-SO_3^-$	~0.078
Fractogel SE Hicap (M)	Methacrylate copolymers	65	$-CH_2-CH_2-SO_3^-$	~0.07
Toyopearl SP-650M	Methacrylate copolymers	65	$-O-R'-O-CH_2-CH_2-CH_2-SO_3^-$	0.13–0.17
Toyopearl GigaCap S-650M	Methacrylate copolymers	75	$-O-R-SO_3^-$	0.1–0.2
Source 30S	Polystyrene-divinylbenzene copolymers	30	$R-O-CH_2-CHOH-CH_2-OCH_2-CHOH-CH_2-SO_3^-$	0.08–0.12
SP Sepharose FF	6% highly cross-linked agarose	90	$-CH_2CH_2CH_2SO_3^-$	0.18–0.25
Capto S	highly cross-linked agarose with dextran surface extender	90	$-CH_2-CH_2-SO_3^-$	0.11–0.14
Poros 50 HS	Polystyrene-divinylbenzene copolymers, coated	50	$-CH_2CH_2CH_2SO_3^-$	Not reported[a]
S Ceramic HyperD F	Porous polystyrene-silica composites filled with functionalized hydrogel	50	Not reported	≥0.15

[a] The ionic capacity for Poros 50 HS was assumed to be 0.06–0.08 meq/mL.

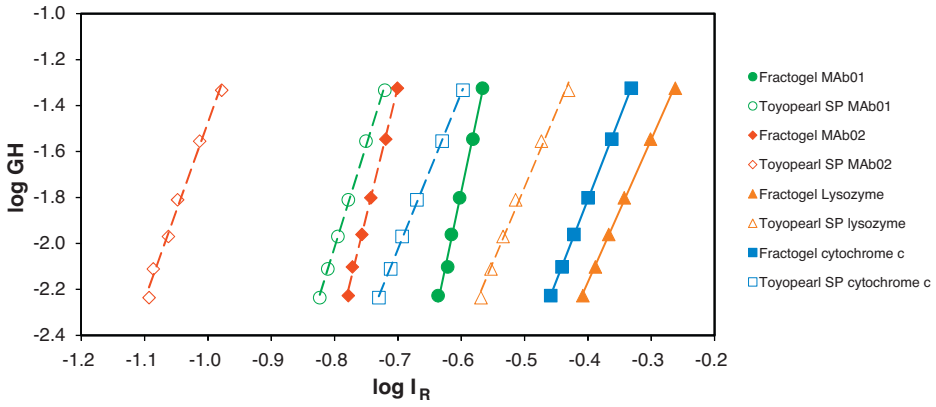

Fig. 1. GH-I_R-plots for Fractogel SO_3^- (M) and Toyopearl SP-650M at pH 5 for lysozyme, cytochrome c and the monoclonal antibodies MAb01 and MAb02.

11: 30 mM glycine, 10 mM NaCl. The pH was adjusted with either HCl or NaOH. All buffers were prepared either without or with little sodium chloride (buffer A) as described above and with additional 1 M NaCl (buffer B).

The monoclonal antibodies MAb01 and MAb02, lysozyme and cytochrome c were diluted and dissolved in buffer A. The protein load was 0.44 mg/mL packed resin.

3.3. Chromatography systems and performance of linear gradient elutions

Experiments were carried out with the liquid chromatography systems ÄKTA UPC 100, ÄKTApurifier 100 or ÄKTApurifier UPC 100 (GE Healthcare).

Linear gradient elutions (LGE) were performed to determine GH-I_R-plots for the different resins and proteins at different pH values. Therefore the samples were applied to the columns equilibrated in buffer A with either a sample pump or super loop. After a washing step a linear salt gradient from buffer A to buffer B was applied. The lengths of the gradients were in the range of 15–120 column volumes. The volumetric flow rate F was 1 mL/min and the linear flow rate $u = F/A_c$ was 119 cm/h.

The peak position was determined by fitting the elution curve with an EMG-Fit in TableCurve 2D. The ionic strength I_R [M] was calculated from the conductivity at this position.

4. Results

In this study a broad variety of different strong cation-exchange stationary phases was examined with special attention given to protein–matrix interactions and the impact of surface modifications on this aspect. The applied model introduced by Yamamoto et al. [35,36] delivers information about stationary phase properties and electrostatic as well as non-electrostatic protein–matrix-interactions. GH-I_R-plots were calculated according to this model from linear gradient elution experiments with different resins, proteins and buffers. Fig. 1 shows the GH-I_R-plots for Fractogel SO_3^- (M) and Toyopearl SP-650M.

GH-I_R-plots were also determined for the other surface-modified and non-modified stationary phases. The number of charges involved in protein binding, B, was derived from GH-I_R-plots and is shown in Fig. 2. At pH 5 the B values vary between 7 and 15 for MAb01 and between 6 and 12 for MAb02, while lysozyme and cytochrome c show B values between 4 and 6 and 4.5 and 5.5. The small variation of the B values for lysozyme and cytochrome c for the different stationary phases was observed for pH values up to pH 11. Although the B values are not identical to the net charge of the protein, the pH dependency of the B values follow the titration curve of the proteins. The surface modifying chemistry has only minor effects on the number of interactions between the protein and the stationary phase. Similar results are observed in other publications characterising binding and elution behaviour of small and mid sized proteins on anion- and cation-exchangers [29,31]. Pabst et al. [31] determined the B values for a set of various cation-exchangers, most of them also used in this study, for two monoclonal antibodies, lysozyme and cytochrome c. The numbers of interactions for the small proteins are very similar for all resins as observed here. Also for one of the tested antibodies, mAb *A*, the results are comparable to those achieved in this study. Only the observations made for the second antibody mAb *B* show some discrepancies at first view. B values for this protein are by trend smaller than those for mAb *A* and the two antibodies used in this study and show a smaller variation between the resins. Anyway Pabst et al. only determined B values at one pH while trends observed in this study are based on experiments at five or six pH values.

For the monoclonal antibodies the smallest numbers of interactions are observed for Toyopearl SP, Source and SP Sepharose for pH values between 4.5 and 5.75, while Fractogel SO_3^-, Poros and HyperD always show the largest B values. Fractogel SE Hicap, Capto S and Toyopearl GigaCap cannot be clearly included in one of the two groups and have by trend intermediate B values. For all of these resins a similar pH dependency of the B values was observed, except Ceramic HyperD, which shows a stronger pH dependency for MAb02. At low pH values of 4.5 up to 5.0 HyperD has the largest B values, but at pH 5.75 the B value is intermediate compared to the other materials. Regarding only the results for MAb02 at pH 5.75 the variation of B equals the variation observed by Pabst et al. [31] for mab *B* but taking into consideration the results at lower pH values the same trend as for MAb01 can be found.

Although the surface chemistry as well as the base matrix chemistry is different for the materials the results for the B values of the antibodies point to a correlation between surface properties and the number of interactions. The smallest B values are observed for the materials in which the charged groups are covalently attached to the surface through small spacer arms. Modifications of the surface tend to increase the number of interactions between stationary phase and large proteins. For small proteins little influence of the surface on B was observed. This conclusion is particularly confirmed by the results for Fractogel SO_3^- (M) and Toyopearl SP-650M. As both stationary phases have the same methacrylate copolymeric matrix with an identical mean particle diameter, differences in the

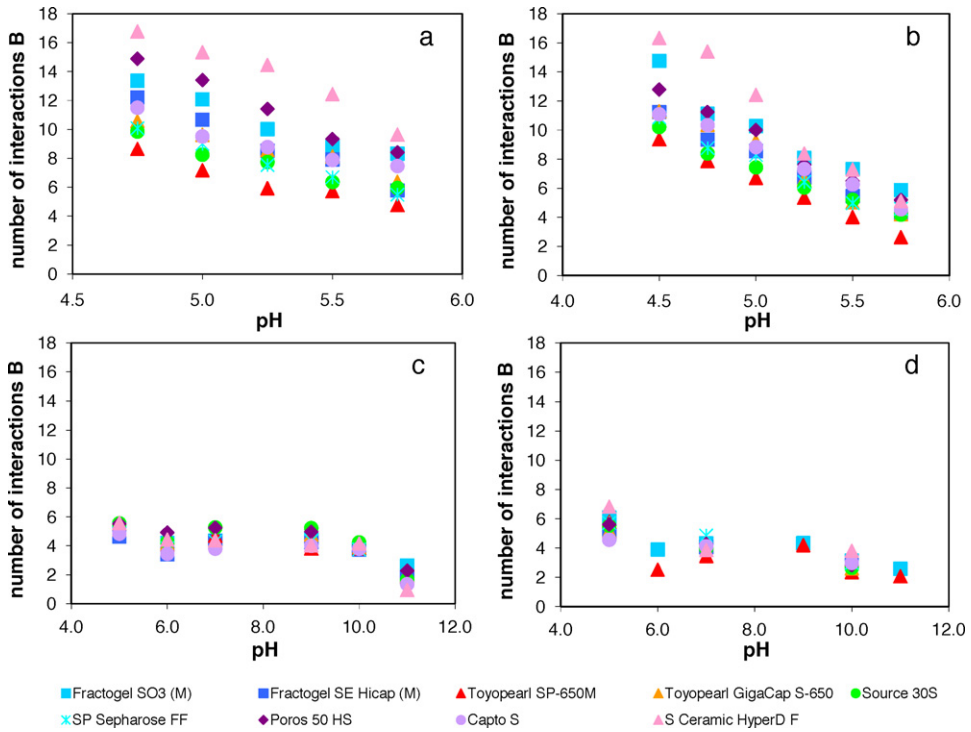

Fig. 2. *B* values for MAb01 (a), MAb02 (b), lysozyme (c) and cytochrome c (d) calculated from GH-I_R-curves for different stationary phases in dependency on the pH value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

interaction between the large proteins and the resin are results of the surface modification. A special characteristic of Fractogels is their surface modification by long, linear polymer chains, so called tentacles, which carry the functional groups. The tentacles improve the accessibility of the ligands without steric hindrance, which is likely to have a stronger effect on the binding of large biomolecules like antibodies than on the binding of small proteins like lysozyme or cytochrome c. In the case of the monoclonal antibodies the *B* values for the surface modified resin Fractogel SO_3^- (M) are always higher than those for the non-modified resin Toyopearl SP-650M. Similar results for weak anion exchangers were observed by Bruch et al. [29]. Suda and co-workers made similar observations for the dextran-grafted SP Sepharose XL in comparison to the non-modified SP Sepharose FF obtaining higher *B* values of an antibody for SP Sepharose XL [38].

Similar observations were made for the *A* values calculated from the GH-I_R-plots. In the case of the large proteins MAb01 and MAb02 the *A* value changes with pH while for the smaller proteins lysozyme and cytochrome c there is almost no variation of *A* with respect to pH as shown in Fig. 3. Remarkable is the difference between the *A* values for HyperD compared to the other resins for the two antibodies. For the two small proteins *A* values for HyperD and the other resins are in the same range.

A major difference between the antibodies and the small proteins is the absolute value of *A*. For all resins the *A* values of the antibodies are about 10^4 to 10^6 times smaller than for the small proteins, and for HyperD the difference is even greater.

The parameter *A* is a lumped parameter that varies with the *B* value, the ionic capacity and the equilibrium constant according to Eq. (10). Since *B* is small and does not significantly vary with pH for lysozyme and cytochrome c the small variation of the *A* value for these proteins is the result of a low pH dependency of K_{eq}. The smaller and pH dependent *A* values as well as the large *B* values for the antibodies point to a strong pH dependency of the K_{eq} for these proteins.

For further characterization of protein–matrix-interactions the equilibrium constant was calculated using the *B* and *A* values from Figs. 2 and 3 and the average ionic capacity (in mmol/mL stationary phase) reported by the manufacturers. Fig. 4 shows the equilibrium constants for the different resins and proteins. For MAb01 and MAb02 the variation of K_{eq} with respect to the stationary phase is stronger than for lysozyme and cytochrome c.

The absolute K_{eq} values change with the ionic capacity according to Eq. (10). As K_{eq} is proportional to Λ^{-B} the influence of the ionic capacity on the equilibrium constant is dependent on the number of charges involved in protein-binding. For low *B* values differences in Λ cause smaller differences of K_{eq} than for higher numbers of interactions.

The ionic capacity is reported by the manufacturers as a range and can vary between different lots. The uncertainty of Λ and therefore of the equilibrium constant makes it desirable to find a parameter related to K_{eq} which is independent of Λ and more suitable to describe stationary phase characteristics. This can be realized by plotting the natural logarithm of K_{eq}^* (see Eq. 13) against *B*. The $\ln K_{eq}^*$-*B*-plots can be analysed according to the thermodynamic approach [39,40]

$$\mathrm{RT}\ \ln K_{eq}^* = -\Delta G_P^o + \upsilon \Delta G_s^o \qquad (12)$$

with the universal gas constant *R*, the temperature *T*, the difference between the standard Gibbs energy in the adsorbed and the solute state ΔG° for the protein (index P), the salt (index S) and the charge ratio $\upsilon = z_P/z_S$. υ corresponds to *B* for a single charged salt. The slopes of the $\ln K_{eq}^*$-*B*-plots represent $\Delta G_S^\circ/\mathrm{RT}$ and the *y*-intercepts give $-\Delta G_P^\circ/\mathrm{RT}$. ΔG_S° of the counter-ion depends on the media and the salt and is affected by variations of the ionic capacity. ΔG_P° of the protein depends on the media and the protein and is independent of Λ and pH. The charge ratio υ ($\triangleq B$) depends on media, protein and pH. A comparison of the evaluation of linear gradient elutions according to the model established by

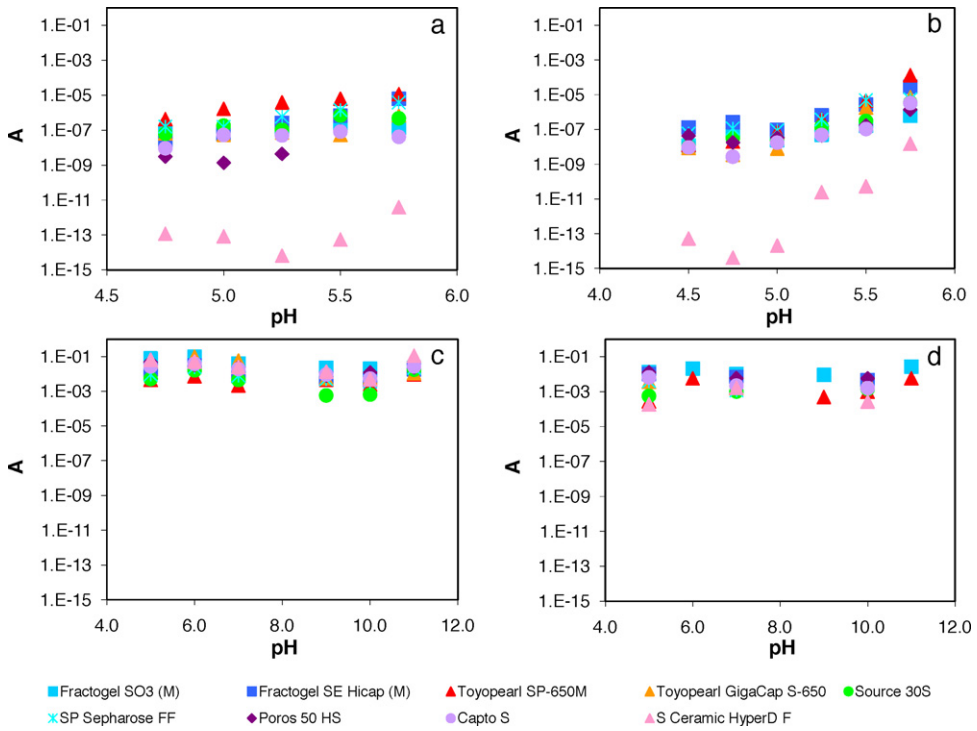

Fig. 3. A values for MAb01 (a), MAb02 (b), lysozyme (c) and cytochrome c (d) calculated from GH-I_R-curves for different stationary phases in dependency on the pH value.

Yamamoto and co-workers [7,35,36] to the evaluation described by Pedersen et al. [39] shows that the equilibrium constant K_{eq}* defined by Pedersen can be calculated from the parameters A and B determined according to Yamamoto by the following equation:

$$K^*_{eq} = A \cdot \Lambda^{*B} \cdot \varepsilon_p K_d \quad (13)$$

The intraparticle porosity ε_p represents the fractional void volume in the particle, the exclusion factor K_d represents the pore-accessibility for a protein and $\varepsilon_p K_d$ represents the fractional void volume in the particle available for a molecule. By consideration of $\varepsilon_p K_d$ the pore accessibility of the different stationary phases for the proteins is taken into account. Furthermore the ionic capacity Λ [meq/mL sedimented resin] has to be converted to Λ*[meq/mL

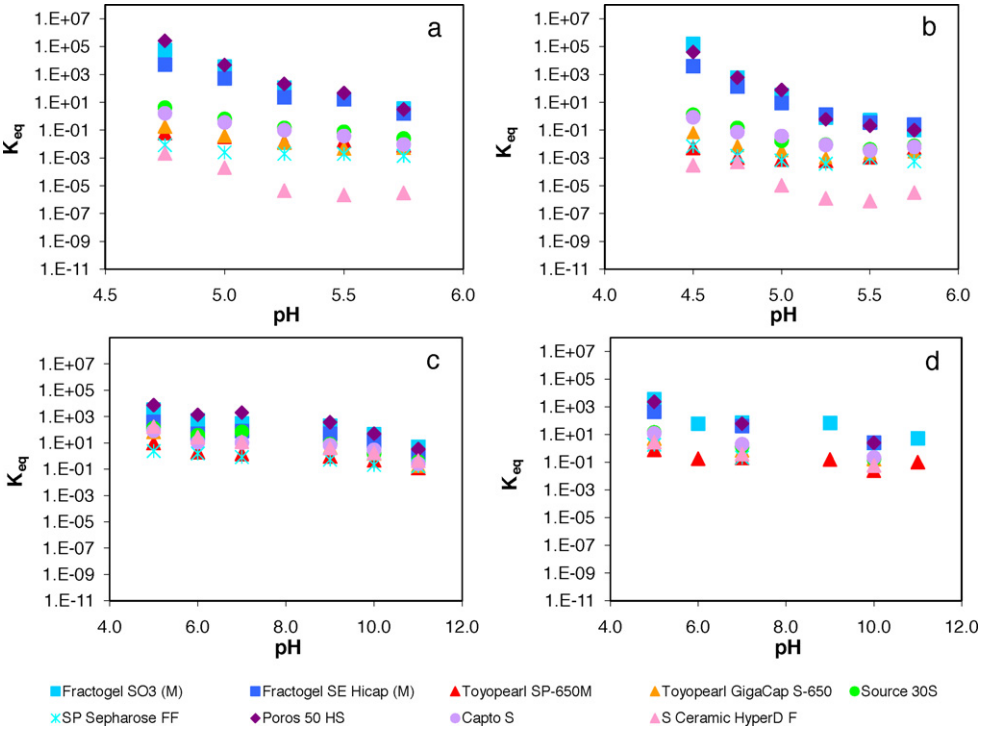

Fig. 4. K_{eq} calculated with A and B values from GH-I_R-plots for MAb01 (a), MAb02 (b), lysozyme (c) and cytochrome c (d) for different resins and pH values. K_{eq} was calculated with an average ionic capacity for each resin.

Table 2
Intraparticle porosities ε_p and exclusion factors K_d for MAb01, MAb02, lysozyme and cytochrome c for the different stationary phases.

Resin	ε_p	K_d		
		MAb01	MAb02	Lysozyme, Cytochrome c
S Ceramic HyperD F	0.43	0.29	0.16	0.67
Fractogel EMD SO_3^- (M)	0.69	0.61	0.49	0.98
Poros 50 HS	0.57	0.79	0.75	0.99
Capto S	0.75	0.13	0.10	0.75
Toyopearl GigaCap S-650M	0.64	0.22	0.20	0.75
Source 30S	0.57	0.74	0.65	0.93
SP Sepharose FF	0.83	0.70	0.61	0.88
Toyopearl SP-650M	0.66	0.82	0.81	0.99
Fractogel SE Hicap (M)	0.72	0.56	0.58	0.82

pore volume]. The pore porosities and exclusion factors for MAb01, MAb02 and lysozyme and the different stationary phases are summarized in Table 2. K_d was calculated based on retention volumes of the proteins under non-binding high salt conditions. A very low accessibility of the pores under non-binding conditions, as found for S ceramic HyperD F, was reported previously for HyperD anion-exchange resins [39,41]. However, low values for K_d are not necessarily an indication for generally low pore accessibilities but indicate that proteins are excluded from the pores in the presence of high salt concentrations as observed by Bowes et al. for dextran-modified media [42]. Therefore under binding conditions the pore accessibility is expected to be higher supported by the high binding capacities observed under binding conditions.

Apart from all the considerations about the influence of the ionic capacity on the equilibrium constant, the *y*-intercept of the $\ln K_{eq}^*$-*B*-curve is independent of Λ as shown in Fig. 5 for Source 30S and MAb01. Fig. 6 shows some examples for $\ln K_{eq}^*$-*B*-plots for average ionic capacities for the two Mabs and lysozyme. For cytochrome c the analysis was only performed for Fractogel SO_3^- and Toyopearl SP-650M as data only exists for three pH values for the other resins. Very similar pH dependencies of *B*, *A* and K_{eq} for lysozyme and cytochrome c and the $\ln K_{eq}^*$-*B*-plots for Fractogel SO_3^- and Toyopearl SP-650M for these proteins lead to the assumption that changes in the standard Gibbs energy of cytochrome c is comparable to results for lysozyme rather than the Mabs.

Linear correlations between *B* and K_{eq}^* could not be observed over the whole pH-range for all stationary phases for MAb02. For high pH values K_{eq} increases for Capto S, Source 30S, Toyopearl SP and Toyopearl GigaCap (Fig. 4), which leads to a bend of the $\ln K_{eq}^*$-*B*-plots. This effect can be explained by insufficient binding strength of the protein at high pH values. This assumption is supported by the very low ionic strengths at peak position determined by linear gradient elutions for the resins mentioned above

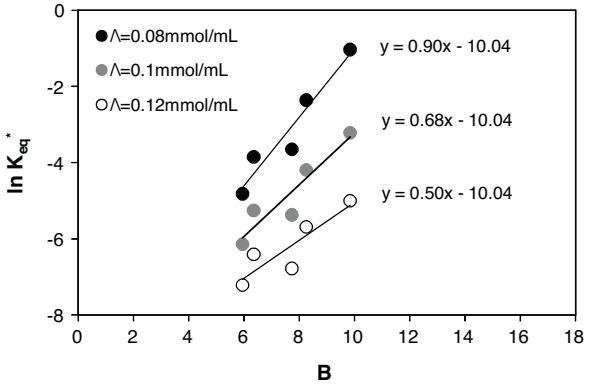

Fig. 5. $\ln K_{eq}^*$-*B*-plots for MAb01 for Source 30S. K_{eq}^* was calculated with the minimal, average and maximal values for the ionic capacity reported by the manufacturers.

for MAb02 at high pH values. Furthermore for resins with higher I_R values such as the two Fractogels or Poros, a decrease of K_{eq} with increasing pH and a linearity of $\ln K_{eq}^*$ versus *B* was observed for the whole pH-range. The unexpected increase of the calculated equilibrium constants indicate that the model used for analysis of the experimental data does not give reliable results for conditions that do not allow a sufficient binding of proteins to the stationary phase. Therefore ΔG_P° and ΔG_S° were calculated from the linear part of the $\ln K_{eq}^*$-*B*-curves. Ceramic HyperD was excluded from this analysis as ionic strengths at peak positions are low even for low pH values and the $\ln K_{eq}^*$-*B*-plots do not show a linear correlation.

The standard Gibbs energy changes for salt are almost identical for the two antibodies. A fit of the lysozyme data results in significantly differing slopes for some resins. As ΔG_S° values are independent on proteins [39,40] the slopes of the $\ln K_{eq}^*$-*B*-plots were set to the average value obtained for the Mabs for these resins. For a reliable calculation of standard Gibbs energies a wide range of *B* values is required. Though a wide range of pH was experimentally covered for lysozyme only a small number of interactions was covered due to the weak pH-dependency of *B* for this protein. In addition the binding of lysozyme at pH 10 and 11 is weak and for some stationary phases a linear correlation between $\ln K_{eq}^*$ and *B* is not given over the whole pH-range. While for MAb02 the analysis of the $\ln K_{eq}^*$-*B*-curves could be performed neglecting pH values at which no linearity was observed, this is hardly possible for lysozyme as there is almost no variation of *B* between pH 5 and 9. Again for resins allowing strong interaction between stationary phase and protein like Fractogels and Poros linearity of $\ln K_{eq}^*$ versus *B* is given for the whole pH range.

ΔG° values for salt, MAb01, MAb02 and lysozyme are shown in Tables 3 and 4. Values for cytochrome c are only available for Fractogel SO_3^- and Toyopearl SP-650M. The changes in standard Gibbs energies obtained for MAb01 and MAb02 are almost the same for all resins except for Toyopearl SP 650M. In this case ΔG_P° for MAb02 is 1.5 times higher than for MAb01. ΔG_P° values for MAB01 are between 4.7 ± 0.2 times higher (Fractogel SE Hicap, Toyopearl SP-650M and Source 30S) and 19 times higher (Toyopearl GigCap) than those obtained for lysozyme and at least 6.2 times higher (Fractogel SE Hicap (M)) and up to 66 fold (Toyopearl SP-650M) higher than those for the salt. For MAb02 almost identical results are obtained for all resins except for Toyopearl SP 650M. Differences between the ΔG° values for lysozyme and salt are in the magnitude of factor 0.8 (Poros) and 14 (Toyopearl SP-650M). All changes in Gibbs energy for the protein as well as the salt are positive, which implies a shift of the equilibrium to the unbound state of the proteins and the salt. Values for the distribution coefficient $K_p = \exp(-\Delta G_P^\circ/RT)$ are shown in Table 4. As ΔG_P° values are calculated with Eq. (12) for $B = 0$ electrostatic interactions between the proteins and the stationary phases are excluded. Therefore K_p represents the distribution of protein in the stationary phase to

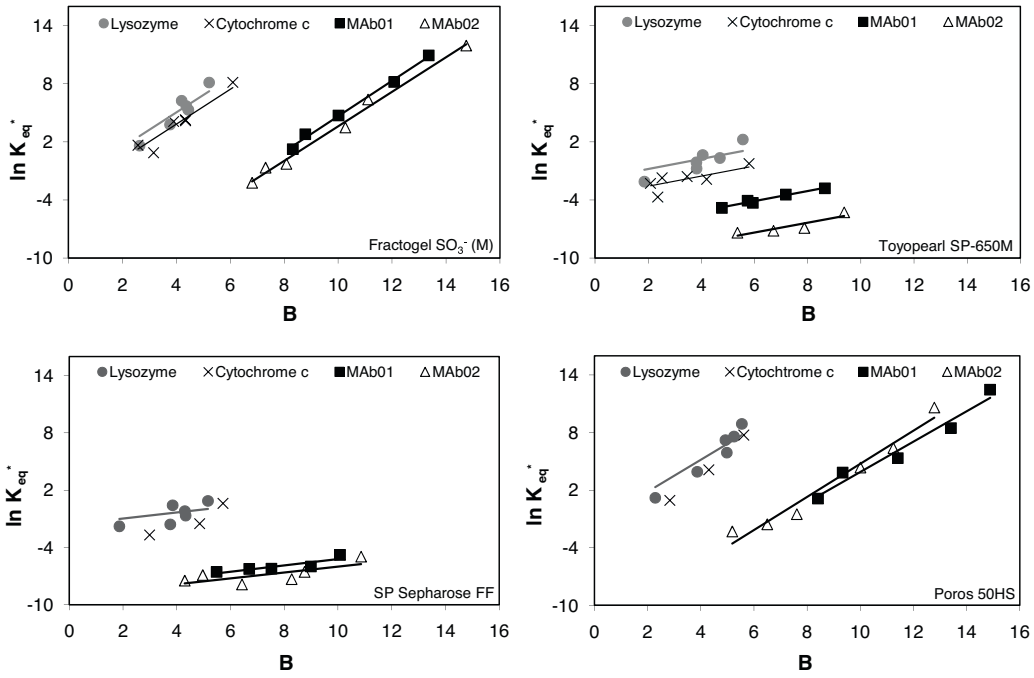

Fig. 6. ln K_{eq}*-B-plots for MAb01, MAb02, lysozyme and cytochrome c for different resins. K_{eq}* was calculated with the average ionic capacities.

protein in the mobile phase due to non-electrostatic interactions and entropic effects.

ΔG_P°/RT values are of similar magnitude to values observed by Pedersen et al. [39] for a Source 30 and Fractogel (S) resin. Although in the study mentioned anion-exchange materials were examined, it can be assumed that ΔG_P° values for the same or similar base matrices should be comparable as electrostatic interactions are excluded. All distribution coefficients are smaller than 1, i.e. the concentration of bound protein is very low as is expected in the absence of electrostatic interactions. While the variation of ΔG_P°/RT values for one protein with respect to the stationary phases for the Mabs and lysozyme do not exceed factor 4.4 the variation of the resulting distribution coefficients is much stronger, particularly for the antibody. K_P varies up to factor 10^3 for MAb01 and 10^2 for MAb02 compared to factor 5 for lysozyme.

As the distribution of bound and unbound proteins is unaffected by electrostatic interactions for $B = 0$ the amount of bound protein has to be dependent on non-electrostatic and therefore unspecific interactions. Müller [17] observed a considerable reduction of non-specific interactions between protein and support matrix resulting from grafted polymer chains which reduce the contact between protein and matrix. This effect can also be realized with cross-linked polysaccharide strands to which the ionic groups are bound, which could explain the results for CaptoS. The increased ΔG_P°/RT value of Source 30S compared to the other conventional resins could be due to the long carbon spacer arms which attach the functional groups to the surface. DePhillips et al. observed an increase in retention upon elimination of the hydrocarbon spacer arm of SP Sepharose FF. As a possible reason they mentioned an enhancement of non-electrostatic interactions that might be explained by an increased proximity of the protein to the adsorption interface [43]. Hydrocarbon spacer arms seem to have a similar effect on the influence of non-electrostatic interactions on the retention of proteins like tentacles. The lower ΔG_P°, and therefore higher K_q, values for lysozyme for all resins might be due to the increased contact with the support matrix and increased non-specific interactions as steric hindrance effects are less important in the case of a small protein.

Remarkable are the low values for Fractogel SE Hicap compared to Fractogel SO_3^-. The small ΔG_P° values for Fractogel SE Hicap show that the chemical structure of the surface modification has an influence on the stationary phase properties. Both Fractogel SE Hicap and Fractogel SO_3^- carry tentacles, but the resins differ in their surface functionality (see Table 1). Differences in the length and surface density of the tentacles can also be assumed as reported by DePhillips and Lenhoff [15] for Fractogel SO_3^- and COO^- resulting in differences in the number of non-electrostatic interactions and the extent of steric hindrance effects. However, as mentioned before, differences in the surface characteristics have minor influence on the distribution of protein in the stationary phase to protein in the mobile phase for small proteins while they affect the distribution of large proteins.

Table 3
Average ionic capacities and ΔG_S°/RT values calculated from ln K_{eq}*-B-plots.

	Fractogel EMD SO_3^- (M)	Poros 50 HS	Capto S	Toyopearl GigaCap S-650M
Λ [meq/mL settled resin]	0.078	0.07	0.125	0.15
Λ [meq/mL pore volume]	0.186	0.204	0.255	0.386
ΔG_S°/RT	1.4	1.1	0.7	0.4

	Source 30S	SP Sepharose FF	Toyopearl SP-650M	Fractogel SE Hicap (M)
Λ [meq/mL settled resin]	0.10	0.215	0.15	0.07
Λ [meq/mL pore volume]	0.281	0.413	0.382	0.162
ΔG_S°/RT	0.6	0.1	0.1	1.0

Table 4
$\Delta G_P^\circ/RT$ and K_p calculated from $\ln K_{eq}$*-B-plots.

	MAb01	MAb02	Lysozyme	Cytochrome c
Fractogel EMD SO_3^- (M)				
$\Delta G_P^\circ/RT$	12.8	13.2	2.2	3.4
K_p	2.8×10^{-6}	1.9×10^{-6}	1.1×10^{-1}	3.4×10^{-2}
Poros 50 HS				
$\Delta G_P^\circ/RT$	11.2	11.7	0.9	
K_p	1.4×10^{-5}	8.6×10^{-6}	4.1×10^{-1}	
Source 30S				
$\Delta G_P^\circ/RT$	10.8	10.0	2.2	
K_p	2.0×10^{-5}	4.4×10^{-5}	1.1×10^{-1}	
Toyopearl GigaCap S-650M				
$\Delta G_P^\circ/RT$	9.5	10.5	0.5	
K_p	7.6×10^{-5}	2.8×10^{-5}	6.1×10^{-1}	
Capto S				
$\Delta G_P^\circ/RT$	9.5	9.7	1.1	
K_p	7.6×10^{-5}	6.4×10^{-5}	3.3×10^{-1}	
SP Sepharose FF				
$\Delta G_P^\circ/RT$	8.0	8.4	1.31	
K_p	3.3×10^{-4}	2.2×10^{-4}	2.7×10^{-1}	
Toyopearl SP-650M				
$\Delta G_P^\circ/RT$	6.6	9.8	1.4	3.2
K_p	1.3×10^{-3}	5.6×10^{-5}	2.5×10^{-1}	4.3×10^{-2}
Fractogel SE Hicap (M)				
$\Delta G_P^\circ/RT$	6.3	7.6	1.4	
K_p	1.9×10^{-3}	5.0×10^{-4}	2.5×10^{-1}	

ΔG_P° and ΔG_S° allow the calculation of the distribution coefficient K as a function of ionic strength at different pH values (through the pH dependency of the B value) and therefore allow a prediction of the pH-dependent protein elution behaviour. In addition the calculation of K at a fixed pH value for varying B values is possible. This approach represents the situation of different binding orientations for a protein with different numbers of interaction sites. As an example the K–I dependencies for MAb01 on Fractogel SO_3^- at pH 5 for B values from 1 to 12 are calculated and shown in Fig. 7. Even under strong binding conditions (10 mM NaCl) only binding orientations with more than five interaction sites lead to large K ($\geq$200) values equivalent to a strong binding of the protein. For Toyopearl SP-650M and MAb01 about four interactions are necessary whereas for lysozyme on Toyopearl a B value of 2 is sufficient (data not shown). These minimal B (B_{min}) values correlate well with the ΔG_P° values of the proteins and the stationary phases. The larger the ΔG_P° values, the more orientations with small B values are excluded from binding.

For B values above B_{min} the distribution coefficients are large but vary significantly for the different B values. For MAb01 and Fractogel K is equal to 3900 for $B = 5$ and 8.5×10^{16} for $B = 12$. Whether protein orientations with lower B values convert to high B value orientations may strongly depend on the binding and dissociation kinetics of the protein.

Elution of MAb01 ($B = 12$) in a linear sodium chloride gradient occurs in the range of 230–270 mM NaCl on Fractogel. All binding orientations with B values between 5 and 10 show much lower salt concentrations necessary for elution, indicating that this binding orientation does not contribute to the elution behaviour of the antibody on Fractogel. Only binding orientations with B values close to the experimentally determined B value are relevant for elution. The experimentally measured B value is therefore a mean value of a small number of binding orientations with similar but high B values. The same results are obtained for the other stationary phases as well as the other proteins.

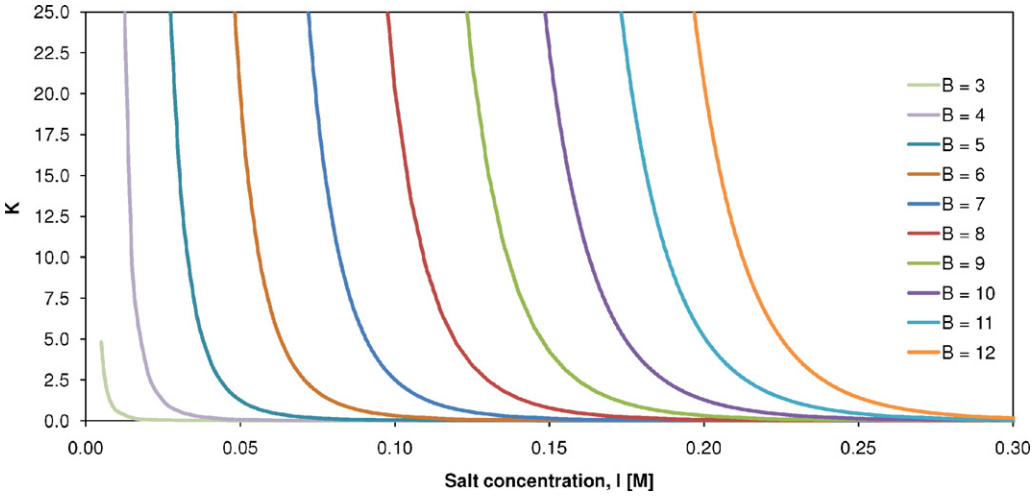

Fig. 7. Theoretical K–I-curves for Fractogel SO_3^- and MAb01 at pH 5 for calculated from ΔG_P° and ΔG_S° values for varying B values. $B = 12$ was determined experimentally.

5. Conclusion

The effect of surface modifications on the interaction between several strong cation exchangers and different proteins was characterized by linear gradient elution experiments. For the large monoclonal antibodies MAb01 and MAb02 an influence of the surface structure on the binding behaviour was found resulting in a strong variation of the parameters B, A and K_{eq}. In contrast only little variation of these parameters was determined for the smaller proteins lysozyme and cytochrome c.

It was also possible to calculate the changes in standard Gibbs energy with the data obtained from the LGEs. Values for the Mabs are between fourfold and tenfold higher than those for lysozyme. This indicates a stronger contribution of non-electrostatic interactions and steric hindrance effects on the distribution of the antibodies compared to lysozyme.

Theoretical calculations for different binding orientations showed an exclusion of low affinity binding orientations especially on the stationary phases which allow a high number of electrostatic interactions. Furthermore the calculations show that the experimentally observed B value is the result of only a few high affinity binding orientations and not a mean value of all possible orientations.

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