Adsorption of Myoglobin onto Hydroxyapatite Modified with Metal Ions†

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ABSTRACT: Optimization of the purification process remains a challenge in the production of proteins for pharmaceutical or industrial applications. In this context, the purpose of this work was to study the adsorption of myoglobin onto hydroxyapatite (HAp) modified with metal ions (Cu²⁺, Ni²⁺, Zn²⁺), with the aim of its purification from a complex medium. The influence of pH and ionic strength on the adsorption process was evaluated using experimental design methodology. Experiments for determining the kinetics and adsorption isotherms were undertaken, allowing the determination of important equilibrium parameters required for scale-up. High adsorption capacities (ca. 90 mg myoglobin/g modified HAp) and rapid adsorption kinetics were observed. These results indicate the applicability of HAp as a support for pseudo-affinity chromatography.

1. INTRODUCTION

The use of hydroxyapatite Ca₁₀(PO₄)₆(OH)₂ — HAp — for protein purification has been increasing lately due to its high selectivity and low cost. The framework of stoichiometric calcium hydroxyapatite can be described as a compact assemblage of tetrahedral PO₄ groups, where each PO₄ tetrahedron is shared by one column and delimits two types of unconnected channels. The first channel has a diameter of 2.5 Å and is bordered by Ca²⁺ ions [denoted as Ca(I)]. The second type plays an important role in the properties of apatites. It has a diameter of ca. 3.5 Å and is also delimited by oxygen and Ca²⁺ ions in a coordination of 7 [denoted as Ca(II)]. These channels host hydroxy groups along the c-axis to balance the positive charge of the matrix. The existence of two different calcium sites is of special interest because the material properties can be tuned by the specific site modified (Elliott 1994). The OH⁻ ions are located in columns perpendicular to the unit cell face, at the centre of the hexagon formed by groups of coplanar calcium ions (Narya-Sabo 1930; Beevers and McIntyre 1946).

Hydroxyapatite has two different adsorption sites, called C- and P-sites (Kandori et al. 2004; Resende et al. 2006). The C-sites contain positively charged calcium ions which bind to the acidic groups of proteins. The P-sites are negatively charged phosphate ions which bind preferentially to the basic groups of proteins. As a consequence, proteins can adsorb onto the C-sites through their carboxyl groups or onto the hydroxy groups through their amino groups. In addition, the hydroxy groups distributed on the crystal surface can also play a role in adsorption. Recently, the exchange of the calcium ions of HAp with different metal ions such as Cu²⁺, Ni²⁺, Zn²⁺, Co²⁺, Fe³⁺, Mg²⁺ and Cd²⁺ has been explored for use in the purification of proteins (Nordstrom et al. 1999; Suen et al. 2004). In this case, HAp can be employed as a pseudo-affinity adsorbent in IMAC.
(Immobilized Metal Ion Affinity Chromatography), with the advantages of lower cost and higher mechanical stability than conventional IMAC adsorbents.

The use of immobilized metal ions to purify proteins was first proposed by Porath et al. in 1975. IMAC is an adsorption method based on the affinity interaction between certain amino acid residues on the surface of proteins (tryptophan, cysteine and mainly histidine) and metal ions immobilized on a solid support. For histidine (His), the nitrogen atom of the imidazole ring is the electron-donor group that will form a coordination bond with metal ions (Wong et al. 1991). Essentially, any metal ion that interacts with proteins may be used in IMAC. However, the metal ions most used are Cu$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Fe$^{3+}$, Al$^{3+}$, Ca$^{2+}$ and Zn$^{2+}$.

Myoglobin, the protein used as a model in the present work, is an 18 kDa globular protein consisting of 153 amino acid residues and an iron-containing porphyrin group which is responsible for its intense red colour. The myoglobin amino acid sequence varies depending on the protein source and its isoelectric point lies between 7 and 8. Horse skeletal muscle myoglobin has a total of 11 His residues of which five are located on the protein surface. The choice of myoglobin was based on the fact that besides being a well-known molecule it also has a potential industrial application. Due to its efficiency in binding oxygen in the cells, myoglobin is being studied as an oxygen carrier in facilitated transport membranes (Ferraz 2003). This process is being evaluated as an alternative to the fractionation of industrial gases such as oxygen and nitrogen. These gases are among the most consumed chemical commodities in the world, with applications ranging from petrochemical industries to medical hospitals (Koros and Mahajan 2000). Thus, the production of myoglobin on a large scale is a key to the accomplishment of this technology.

Here, we report a study of the adsorption of myoglobin onto HAp modified with Cu$^{2+}$, Ni$^{2+}$ and Zn$^{2+}$ ions. The influence of pH and ionic strength on the adsorption process has been evaluated using experimental design technology. Batch adsorption experiments were carried out in order to construct the kinetics and isotherm curves. The results indicate the feasibility of using HAp as a pseudo-affinity matrix and its application to the purification of myoglobin from a complex source.

2. MATERIALS AND METHODS

2.1. Materials

Horse skeletal muscle myoglobin was purchased from Sigma-Aldrich (U.S.A.). The hydroxyapatite precursor was prepared using the co-precipitation method at the stoichiometric ratio, by slowly adding a 0.3 M (NH$_4$)$_2$HPO$_4$ aqueous solution to a 0.5 M Ca(NO$_3$)$_2$•4H$_2$O aqueous solution, both made alkaline by the addition of NH$_4$OH (pH = 10–11) at 80 ± 5°C under continuous stirring. The mother suspension was aged at the precipitation temperature for 2 h. The precipitate was washed several times with hot water (80°C) to eliminate any residual alkali. After filtration, the solid obtained was dried in an oven at 80°C for 24 h. HAp obtained by this method is a mesoporous solid with a specific surface area of 42 m$^2$/g and a medium-sized pore diameter of 174 Å. All other chemicals used were at least of analytical grade.

2.2. Methods

2.2.1. Preparation of HAp–Me$^{2+}$

Known amounts (50.0 mg) of HAp were contacted with 1.0 ml of 50 mM CuSO$_4$, NiSO$_4$ and ZnCl$_2$ solutions in 2.0 ml tubes. The suspensions were agitated end-over-end for 60 min at room
temperature and then centrifuged at 9300g for 5 min. The resulting HAp–Me\(^{2+}\) was washed with 1.0 \(m\ell\) of distilled water, centrifuged once more and then equilibrated with 1.0 \(m\ell\) of 20 mM sodium phosphate buffer (equilibration buffer). A final centrifugation step was undertaken before contacting HAp–Me\(^{2+}\) with protein solution.

2.2.2. X-Ray diffraction (XRD) characterization

The morphology and purity of HAp was determined by XRD methods using a Rigaku Miniflex TG diffractometer employing Cu K\(\alpha\) radiation (30 kV and 15 mA). The 2\(\theta\) range used was 20–80º with 0.05º steps and a counting time of 2 s. The preparation of the samples for XRD analysis followed the same procedure as described above up to the washing step. Subsequently, the excess water was removed by vacuum filtration and the samples dried at 100°C over 12 h.

2.2.3. Adsorption kinetics experiments

Known volumes of myoglobin solutions (1.0 \(m\ell\)) at an initial concentration of 1.0 mg/\(m\ell\) were added to tubes containing 50.0 mg of previously prepared HAp–Me\(^{2+}\) and the resulting suspensions agitated over increasing time intervals. After centrifugation, the amount of myoglobin remaining in solution was measured via UV absorption studies at 409 nm.

2.2.4. Adsorption isotherm experiments

The adsorption of myoglobin onto the HAp–Me\(^{+}\) samples was studied at 25°C using 2.0 ml sample tubes suspended in a stirred tank. Known volumes (1.0 \(m\ell\)) of myoglobin at different concentrations in 20 mM sodium phosphate buffer solution at a pH of 7.0 (equilibration buffer) were placed in the tubes which contained 50.0 mg of HAp–Me\(^{2+}\) that had been equilibrated previously with the equilibration buffer. In order to minimize mass-transfer resistance, tubes containing adsorbent and protein solution were agitated end-over-end for 1 h at 25°C, this time period having been established previously as sufficient to reach equilibrium after adsorption kinetics experiments. After this period, the protein concentration in the liquid phase was measured by UV absorption studies at 409 nm, the amount of protein adsorbed being determined from the difference between the initial protein concentration and the protein concentration remaining in solution after 1 h. Adsorption isotherms were then constructed by plotting the amount of myoglobin adsorbed by the HAp matrix (adsorption capacity expressed as mass of protein/mass of adsorbent) as a function of the concentration of unbound protein.

2.2.5. Experimental design

A full factorial design was used to evaluate the effect of two independent process variables, viz. ionic strength and pH, and their possible effect on myoglobin adsorption. The experimental design selected was a central composite design comprising 11 runs, corresponding to four cube points, four axial points and three central points, with the experiments carried out in a random order. The dependent variable (response) was the adsorption capacity, i.e. the amount of myoglobin adsorbed (in mg)/g HAp. Statistica software (Statsoft, version 5.0) was used for the analysis of the experimental data and the generation of the ANOVA (analysis of variance) data.
3. RESULTS AND DISCUSSION

3.1. X-Ray diffraction (XRD) characterization

XRD patterns were undertaken aimed at identifying the phases present in the HAp structure. Figure 1 presents the XRD patterns for the HAp–Cu$^{2+}$, HAp–Ni$^{2+}$ and HAp–Zn$^{2+}$ samples prepared. Analysis of the patterns indicated that all the adsorbents possessed a crystalline structure, thereby allowing the identification of the existing phases.

On the basis of the ICDD-PDF-09-0432 data bank, we have concluded that all the adsorbents presented a hexagonal structure which is characteristic of hydroxyapatites. This may be verified by the presence of the characteristics peaks of HAp in the various patterns depicted in Figure 1, viz. $2\theta = 31.773^\circ$ (2 1 1), $2\theta = 32.196^\circ$ (1 1 2), $2\theta = 32.902^\circ$ (3 0 0), $2\theta = 25.879^\circ$ (0 0 2), $2\theta = 49.468^\circ$ (2 1 3), $2\theta = 46.711^\circ$ (2 2 2), $2\theta = 34.048^\circ$ (2 0 2), $2\theta = 39.818^\circ$ (3 1 0), $2\theta = 50.493^\circ$ (3 2 1), $2\theta = 53.143^\circ$ (0 0 4). Analysis of the XRD patterns indicated that the inclusion of metal ions did not interfere significantly with the crystalline structure of the HAp nor create a new segregated phase. This suggests that either the metal ions were incorporated directly into the crystalline structure of HAp or the amount of metal ions employed was insufficient to produce a new phase.

3.2. Adsorption kinetics

Kinetic studies of myoglobin adsorption onto HAp–Me$^{2+}$ were carried out employing an initial protein concentration of 1.0 mg/mL (Figure 2). A reduction in the amount of myoglobin in solution occurred within the very first minutes of agitation for all the metal ions tested (Cu$^{2+}$, Ni$^{2+}$ and Zn$^{2+}$), attaining a constant value after 10 min. The data depicted in the figure indicate a difference in the binding affinity between myoglobin and the different metal ions: while virtually all the

![Figure 1. XRD patterns for the various HAp–Me$^{2+}$ samples. The characteristic hydroxyapatite peaks are identified by asterisks (*).](image-url)
myoglobin present in solution was adsorbed onto HAp–Cu$^{2+}$, the binding affinity with Zn$^{2+}$ ions was comparatively weaker than with Cu$^{2+}$ ions, and even weaker when Ni$^{2+}$ ions were employed. The adsorption was also less when HAp in its native form was used as the adsorbent. This was probably due to the use of phosphate buffer at a concentration of 20 mM, since this amount of phosphate ions in solution would be sufficiently high to compete with the protein for the HAp binding sites. Higher adsorption capacities for native HAp were achieved when 5 mM phosphate buffer solution was used (data not shown). However, as the focus of this work was to investigate the adsorption of myoglobin onto HAp modified with metal ions, we decided to use the 20 mM sodium phosphate buffer since this is commonly employed in IMAC chromatography. On the basis of the kinetic data obtained, an agitation time of 1 h was considered sufficient to allow the adsorption process to attain equilibrium conditions.

3.3. Effects of pH and ionic strength on adsorption

Protein adsorption onto IMAC adsorbents is usually carried out at a pH value where the electron-donor group, which is responsible for the interaction with the metal ion, would be partially protonated. Thus, for proteins possessing surface histidine groups, adsorption is favoured within the pH range 6–8. For this reason, we have studied the effects of pH on myoglobin adsorption onto HAp–Me$^{2+}$ within this pH range. A full 2$^2$ factorial design was used to investigate the interaction of pH and ionic strength on adsorption. Table 1 contains the experimental conditions and the results for the adsorption of myoglobin at an initial concentration of 1.5 mg/mL employing 50.0 mg of HAp.

Both the pH value and the ionic strength were statistically significant (within a confidence level of 95%) in the adsorption of myoglobin onto HAp–Me$^{2+}$, except for the effect of pH on Cu$^{2+}$ ions (Figure 3). The effects of both pH and ionic strength were negative, except for Ni$^{2+}$ ions. Such a negative effect means that an increase in any or both of these parameters would lead to a decrease in myoglobin adsorption. For native HAp, this behaviour with respect to ionic strength could be attributed to the reduction of negative binding sites on the HAp surface as the concentration of NaCl increased in the system. As a consequence, the contribution of interactions between P sites and the amino groups on the protein would be reduced. However, for HAp–Me$^{2+}$, the main type of interaction involving coordination bonds with metal ions should not be significantly affected.
by the ionic strength. With IMAC adsorbents, high salt concentrations (ca. 1.0 M NaCl) are usually added to the adsorption buffer in order to reduce non-specific interactions (such as electrostatic interactions) and increase selectivity, since the presence of salts helps to increase the stability of the protein–metal complex (Beitle and Ataai 1992). In addition, weakening of the forces between the metal ion and water induced by salts can also favour protein adsorption. Given that the increase in NaCl concentration resulted in a decrease in myoglobin adsorption onto HAp–Me\(^{2+}\) for all the metal ions tested, it must be assumed that other types of interactions, besides protein–metal interaction, were taking place in the system.

Besides the ionic strength, the pH is also an important parameter in IMAC matrix adsorption. For coordination (donor–acceptor) interaction bonds between metal ions and electron-donating...
amino acid residues at the protein surface, a reduction in the binding affinity would be expected with decreasing pH since there will be an increase in competition between protons and metal ions for the protein-binding sites (–NH₂, –S⁻ and –COO⁻). However, in the present study, this effect was only observed for Ni²⁺ ions. For Cu²⁺ ions, the effect of pH on adsorption was not significant, probably due to the strong interaction between Cu²⁺ ions and myoglobin. The pH value had a negative effect on HAp–Zn²⁺ and HAp in the native form, i.e. the adsorption rate decreased with an increase in the pH value. Over the pH range studied, the myoglobin molecule has a net charge which is close to zero and can thus adsorb either on the P- or C-sites of HAp. Moreover, since myoglobin behaves like a soft protein (Kandori et al. 2004), i.e. it is capable of undergoing conformational changes during the adsorption process, it is possible that the adsorption of myoglobin onto HAp could take place even when electrostatic repulsion conditions apply.

Only for myoglobin adsorption onto HAp–Ni²⁺ was the term relating to the interaction between pH and ionic strength statistically significant within a confidence level of 95%. The statistical significance of the interaction term is that the effects of pH and ionic strength cannot be interpreted separately, i.e. the combined effect of these factors cannot be explained in terms of the sum of their individual effects. The ANOVA analysis showed that the correlation coefficient and the F-test (Table 2) were satisfactory for the prediction of the models used to build the adsorption curves of Figure 4. This was achieved for each curve by fixing the pH on the model equation at the central point value (pH 7.0) and then calculating the adsorption capacity by varying the ionic

| Adsorbent   | R   | F_{cal} | F_{cal}/F_{tab} |
|-------------|-----|---------|-----------------|
| HAp–Cu²⁺    | 0.725 | 10.56   | 2.37            |
| HAp–Ni²⁺    | 0.801 | 6.03    | 1.33            |
| HAp–Zn²⁺    | 0.903 | 14.05   | 3.10            |
| HAp         | 0.864 | 14.76   | 3.39            |

Figure 4. Adsorption capacity of HAp towards myoglobin as a function of ionic strength for an initial protein concentration of 1.5 mg/ml in 20 mM sodium phosphate buffer (pH 7.0). The various curves were obtained using the statistical model. Data points correspond to the following: (△) HAp–Cu²⁺; (□) HAp–Ni²⁺; (◇) HAp–Zn²⁺; (●) HAp.
strength. It may be observed from Figure 4 that the effect of ionic strength was more pronounced in the HAp–Zn\textsuperscript{2+} adsorption system. However, as presented, the results do not allow any conclusions to be drawn regarding the difference in affinity between myoglobin and the various metal ions, since the initial concentration of protein varied as a result of experimental limitations. Nevertheless, analysis of these results in terms of the percentage of adsorbed protein (data not shown) corroborated the results achieved in the kinetic studies in terms of the amount of protein adsorbed: Cu\textsuperscript{2+} > Zn\textsuperscript{2+} > Ni\textsuperscript{2+}. In order to quantify these affinity differences, the adsorption isotherms for the various metal ions were determined as described below.

3.4. Adsorption isotherm experiments

The various myoglobin adsorption isotherm experiments were carried out using 20 mM sodium phosphate buffer at pH 7.0, with the experimental data being fitted using the Langmuir model (Figure 5). The equilibrium parameters for the affinity constant (K\textsubscript{a}) and maximum adsorption capacity (Q\textsubscript{max}) are presented in Table 3.

The high adsorption capacity of myoglobin on HAp–Me\textsuperscript{2+} (ca. 90 mg/g HAp) is worthy of note. The data obtained via this set of experiments supported the observation that the affinity towards myoglobin was greatest in HAp–Cu\textsuperscript{2+}. Under the experimental conditions employed, the affinity towards myoglobin in HAp–Me\textsuperscript{2+} followed the order: Cu\textsuperscript{2+} > Zn\textsuperscript{2+} > Ni\textsuperscript{2+}. This order differs from that described by Sulkowski (1985) for the retention of proteins in the IDA–Me\textsuperscript{2+} system, viz. Cu\textsuperscript{2+} > Ni\textsuperscript{2+} > Zn\textsuperscript{2+}. However, the same behaviour as observed here for HAp–Me\textsuperscript{2+} was also described by Nordstrom \textit{et al.} (1999) and Suen \textit{et al.} (2004). These authors observed a lower efficiency towards the adsorption of His–Tag proteins on HAp–Ni\textsuperscript{2+} compared to the other metal ion systems tested. This could be due to the number of metal sites available for protein interaction.

![Figure 5. Adsorption isotherms of myoglobin onto HAp–Me\textsuperscript{2+} at 25°C in 20 mM sodium phosphate buffer at pH 7.0. Solid lines represents the fit obtained using the Langmuir model. Data points correspond to the following: (△) HAp–Cu\textsuperscript{2+}; (□) HAp–Ni\textsuperscript{2+}; (◇) HAp–Zn\textsuperscript{2+}; (✶) HAp.](image-url)
Wakamura et al. (1998) studied the surface composition of calcium hydroxyapatites modified with metal ions by ion-exchange methods and concluded that the exchange followed the order: \( \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} \). Sugiyama et al. (1996) also observed the order \( \text{Zn}^{2+} > \text{Ni}^{2+} \). Thus, the amount of \( \text{Ni}^{2+} \) ions per mass of HAp is probably less than that of \( \text{Zn}^{2+} \) ions. In addition to this fact, the accessibility of the metal ion to the HAp surface can also have an effect on protein adsorption.

### 3.5. Desorption experiments

The results for the desorption of myoglobin previously adsorbed onto HAp–Me\(^{2+}\) (using 20 mM sodium phosphate buffer at pH 7.0) with different elution solutions are presented in Figure 6. Usually the desorption of proteins in IMAC can be undertaken via three different procedures: (1) reducing the pH value, (2) adding a competing agent with similar characteristics to the amino acid involved in the binding, and (3) adding a chelating agent.

The first procedure, protonation, is that most commonly used. Increasing the amount of H\(^+\) ions in solution favours protonation of the electron-donor group on the protein surface; hence, its interaction with the metal ion is diminished and the protein is displaced into the solution. In the second method, the proteins are eluted at a pH close to 7.0 through the addition of a solute that

![Figure 6. Desorption of myoglobin previously adsorbed onto HAp–Me\(^{2+}\) in 20 mM sodium phosphate buffer at pH 7.0 by various elution solutions. These consisted of 125 mM sodium phosphate buffer (P0.1), 250 mM sodium phosphate buffer (P0.25), 500 mM sodium phosphate buffer (P0.5), 0.5 M NH\(_4\)Cl (N0.5), 1.0 M NH\(_4\)Cl (N1), 0.5 M imidazole (IMZ0.5), 1.0 M imidazole (IMZ1) and 50 mM EDTA solution (EDTA).](image-url)
has a higher affinity for the adsorption sites, such as histidine or imidazole. The third method, also used for matrix regeneration, removes the metal–protein complex from the adsorption matrix by adding a strong chelating agent such as EDTA. However, this type of regeneration is not selective, since the interaction that is destabilized is that between the metal ion and the matrix rather than that between the metal ion and the protein.

Since the structure of hydroxyapatite is not stable at pH values below 5.0, an elution procedure involving protonation was not viable. Hence, we have only investigated the desorption of myoglobin from HAp–Me$^{2+}$ using the other two procedures mentioned above. Thus, imidazole and ammonium chloride at concentrations of 0.5 M and 1.0 M and a phosphate buffer at pH 7.0 and concentrations of 0.1, 0.2 and 0.5 M were used as competing agents. The use of a solution of 50 mM EDTA at pH 8.0 was also tested for elution purposes.

The higher affinity of myoglobin towards Cu$^{2+}$ ions could also be verified by the amount of protein recovered: only 60% of myoglobin adsorbed onto HAp–Cu$^{2+}$ could be eluted using 0.5 M phosphate buffer (cf. maximum protein eluted using EDTA was 74%). In the case of HAp–Ni$^{2+}$, a higher desorption rate (> 80%) was achieved using 0.25 M phosphate buffer. The recovery of myoglobin from HAp–Zn$^{2+}$ attained values > 90% when 0.5 M phosphate buffer was employed. Under these conditions, an ammonium chloride solution was also effective in desorption.

Desorption using EDTA was very similar for all the metal ions tested. However, as mentioned above, elution with EDTA is not selective and should only be used if there is no other alternative. In addition, the use of chelating agents with hydroxyapatites is not recommended, since Ca$^{2+}$ ions will also be removed from the crystalline structure, thereby damaging the crystal network.

The results obtained for the desorption experiments showed the negative influence of the strong interaction between myoglobin and Cu$^{2+}$ ions which was reflected in lower recovery rates than for the other metal ion complexes studied. Also, the strong interaction of Cu$^{2+}$ ions with proteins that have histidine residues could lead to a reduced selectivity when purifying myoglobin from a complex medium, such as the fermentation medium of *Escherichia coli* used in the production of recombinant myoglobin. It is most probable that the best results for myoglobin purification would be achieved using HAp–Ni$^{2+}$ or HAp–Zn$^{2+}$.

4. CONCLUSIONS

Adsorption studies of myoglobin onto hydroxyapatite (HAp) modified with metal ions demonstrated the feasibility of using HAp as a matrix for pseudo-affinity chromatography. The effect of pH on adsorption was less pronounced than the effect of ionic strength. The rate of adsorption decreased with increasing ionic strength of the adsorption buffer employed, leading to the conclusion that other mechanisms of interaction besides protein–metal may be taking place. Kinetics and adsorption isotherm experiments indicated a fast adsorption rate and a maximum adsorption capacity of ca. 90 mg myoglobin/g modified HAp. The results for HAp–Me$^{2+}$ indicated the potential of using HAp modified with metal ions for the purification of myoglobin from a complex medium.

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REFERENCES

Beevers, C.A. and McIntyre, D.B. (1946) *Mineral. Mag.* **27**, 254.
Beitle, R. and Ataai, M.M. (1992) *AIChE Symp. Ser.* **8**, 34.
Elliott, J.C. (1994) *Structure and Chemistry of the Apatites and Other Calcium Orthophosphates*, Elsevier Science, Amsterdam, The Netherlands.
Ferraz, H.C. (2003) *Ph.D. Thesis*, Universidade Federal do Rio de Janeiro, Brazil.
Kandori, K., Miyagawa, K. and Ishikawa, T. (2004) *J. Colloid Interface Sci.* **273**, 406.
Koros, W.J. and Mahajan, R. (2000) *J. Membr. Sci.* **175**, 181.
Naray-Szabo, S. (1930) *Z. Kristallogr.* **75**, 387.
Nordstrom, T., Senkas, A., Eriksson, S., Pontynen, N., Nordstrom, E. and Lindqvist, C. (1999) *J. Biotechnol.* **69**, 125.
Porath, J., Carlsson, J., Olsson, I. and Belfrage, G. (1975) *Nature (London)* **258**, 598.
Resende, N.S., Nele, M. and Salim, V.M.M. (2006) *Thermochim. Acta* **451**, 16.
Suen, R.B., Lin, S.C. and Hsu, W.H. (2004) *J. Chromatogr. A* **1048**, 31.
Sugiyama, S., Moriga, T., Goda, M., Hayashi, H. and Moffat, J. (1996) *J. Chem. Soc.* 4305.
Sulkowski, E. (1985) *Trends Biotechnol.* **3**, 1.
Wakamura, M., Kandori, K. and Ishikawa, T. (1998) *Colloids Surf. A* **142**, 107.
Wong, J., Albright, R.L. and Wang, N.H.W. (1991) *Sep. Purif. Methods* **20**, 49.