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Process development in affinity separation of glycoconjugates with lectins as ligands

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Abstract

Process development for affinity separation is a crucial prerequisite for a successful biospecific isolation of biological active substances like glycoconjugates or enzymes. The functionalization of polymer and silica based adsorbents and their influence on the adsorption behaviour of the modified adsorbents are presented. Improvement of the immobilization conditions for different lectins lead to a stable binding of more than 90% within 4 h of ligands applied to the immobilization solution. The prepared adsorbents are characterized according to specificity, stability and capacity. The isolation of the glycoprotein fetuin from fetal calf serum with wheat germ agglutinin adsorbents and the purification of horseradish peroxidase with concanavalin A (Con A) adsorbent are described. The Langmuir model, using glucose oxidase as glycoprotein and Con A adsorbents, expresses the sorption behaviour. The fixed bed separation is represented by the dispersion model. The process simulation supports the process development evaluating design parameters and investigating and optimizing process conditions. The influence of the flow as well as the concentration of contaminants competing with the valuable product for the ligands on the separation performance are demonstrated and discussed.

Keywords: Affinity adsorbents; Process simulation; Glycoconjugates; Lectins

1. Introduction

Affinity separation techniques are based on biospecific molecular interactions. Thus they are extremely powerful tools for the isolation of valuable biological macromolecules. For the purification and analysis of glycoconjugates affinity separation using immobilized lectins has been described [1,2]. Lectins are proteins or glycoproteins, which display specificity for certain structural features of oligosaccharides. The ability of lectins to bind glycoconjugates specifically facilitates the purification of the target compounds from a complex mixture in a single chromatographic step. A further advantage of this technique is that the desired protein can be isolated with its natural glycosylation pattern. This is important as alterations in the saccharide structures often result in different physiological properties or can totally suppress the biological activity of the target glycoprotein [3,4]. Protein–carbohydrate inter-

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actions are involved in many important biological processes including signalling, recognition and catalysis. Most eukaryotic proteins are glycosylated but in many cases the biological functions of these modifications remain still unclear. In diseased cells, the relative proportions of carbohydrate structures are often characteristically different from sound cells, which may be useful for the assessment of the stage of the disease and for diagnosis. The knowledge of disease-specific glycoprotein structures and their functions can be applied in therapy [3,5,6].

Despite the advantages of affinity separation techniques their application is often restricted to analytical purposes and to laboratory-scale preparations. This is often due to agarose being applied as a support with its limited chemical, biological and mechanical stability. Additionally process parameters have to be thoroughly investigated and optimized for reproducibility and scalability of this modern specific separation technique. It is important to minimize unspecific adsorption during the selective separation process. On the other hand, the biological activity of the ligand and the molecules to be separated need to be preserved.

For the development of lectin affinity separation processes the important influence parameters have been investigated [7]. The work ranges from the preparation and characterization of the adsorbents over the development of the separation procedure to the modelling and simulation of the processes.

2. Experimental

2.1. Materials

The polymer based support Toyopearl AF-Tresyl-650M was obtained as a preactivated bulk material from Tosoh Biosep (Stuttgart, Germany). The inorganic support Silica XWP-I005 with a pore diameter of 500 Å was provided by Grace (Worms, Germany). Specific surfaces of Toyopearl and Silica were determined to be 42, and 78 m^2/g, respectively, using the method of nitrogen adsorption. The lectins wheat germ agglutinin (WGA), *Ricinus communis* agglutinin (RCA), concanavalin A (Con A) and the glycoprotein glucose oxidase (GOD) (EC 1.1.3.4.) from *Aspergillus niger* were purchased from Sigma (Munich, Germany); ready-to-use beaded lectin agarose was also from Sigma. Horseradish peroxidase (HRP) (EC 1.11.1.7.), 3-mercaptopropyltrimethoxysilane, 1,4-butanediol diglycidyl ether, pyridine, 2,2,2-trifluoroethanesulfonyl chloride and alcohol dehydrogenase (ADH) (EC 1.1.1.1.) from *Saccharomyces cerevisae* were purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical reagent grade.

2.2. Surface modification

Irregular broken silica particles were covered with toluene, dried over a molecular sieve and mixed with 3-mercaptopropyltrimethoxysilane. The mixture was heated up to 90 °C. After 1.5 h the support and reagents were filtered, extracted 4 h with acetone and dried at 80 °C. Silica functionalized with 3-mercaptopropyltrimethoxysilane was covered with water and 1,4-butanediol diglycidyl ether was added. After 24 h at 45 °C the samples were filtered, washed with NaCl solution and treated at 60 °C with hydrochloric acid (pH 1.3). This material (diol derivative) was dried 24 h in a vacuum dryer at 65 °C. Dried material was covered with acetone; pure pyridine and 2,2,2-trifluoroethanesulfonyl chloride were added. The mixture was shaken 25 min at room temperature, filtered with a Buchner funnel and washed with acetone with three different mixtures of acetone–hydrochloric acid (pH 3) (ratios: 2:1, 1:1 and 1:2) and finally with hydrochloric acid (pH 3).

2.3. Adsorption isotherms

The sorption behaviour of the basic silica gel, of the diol derivative (immobilized spacer), of the activated support was investigated by adsorption isotherms of alcohol dehydrogenase. Ten solutions of different enzyme concentration in sodium phosphate buffer (pH 6.5) and one sample of pure buffer were prepared. Adsorbent in hydrochloric acid (pH 3) was added to each solution. The samples were shaken for 13 h at 20 °C. The protein concentrations were determined with a quantitative protein assay (Bradford reagent) with a multilabel counter (14020 VICTOR^2, Perkin-Elmer Wallac, Freiburg, Germany). The adsorbent's mass was determined by

drying the whole sample and weighing. The mass of the dried buffer was subtracted.

2.4. Preparation of affinity adsorbents and quantitative protein assay

The preparation of lectin affinity adsorbents was performed as described previously [7,14]. Immobilization of lectins onto tresylated silica and polymer supports was carried out within 4 h using phosphate buffer, pH 8.0 with gentle mixing. The remaining tresyl groups were blocked with Tris–HCl buffer, pH 8 and the coupling yield was determined from the decrease of protein in the supernatant as described elsewhere [8]. Protein concentrations were determined by a dye binding assay (Bradford reagent from Sigma) adapted to measurements in a microplate reader Spectra classic (Tecan, Crailsheim, Germany) with lectin standards.

2.5. Adsorption kinetics and equilibrium isotherms of lectin adsorbents

The adsorption kinetics of the glycoprotein glucose oxidase was determined for different Con A adsorbents. The used adsorbents are characterized in detail in Table 1. The kinetic measurements were carried out at 25 °C in a stirred vessel [17]. Equilibrium isotherms were investigated for the same adsorbents as listed in Table 1. The three Con A adsorbents were equililibrated with GOD solution and shaken for 20 h. Protein samples were taken from the supernatant and analyzed with the Bradford protein assay.

2.6. Affinity separation of fetuin and horseradish peroxidase

For affinity separations a low-pressure liquid chromatography system (Bio-Rad, Munich, Germany) consisting of a gradient pump, a UV detector (λ=280 nm) and a recorder was employed. The prepared adsorbents were packed into Bio-Rad glass columns (50×7 mm). Adsorption was performed in phosphate buffer, pH 7.4 for WGA adsorbents and acetate buffer, pH 6.0 for Con A adsorbents, respectively. Specific desorption was achieved with the same buffer containing *N*-acetylglucosamine for WGA adsorbents and methyl–D-mannopyranoside for Con A adsorbents. All separations were carried out at ambient temperature at a flow-rate of 0.8 ml/min. The recovery was determined as protein concentration in the resulting fractions as described above using fetuin or HRP as standards.

2.7. Fixed bed experiments

The fixed bed experiments were carried out using a chromatography system of Pharmacia (Pharmacia Biotech, Uppsala, Sweden). The Pharmacia HR-10 column (diameter 10 mm) with a thermostated jacket was used to maintain a constant temperature of 25 °C. The bed height of the column packed with ConA-agarose was 1.7 cm. A model mixture system consisting of glucose oxidase and bovine serum albumin (BSA) was investigated. Firstly, the Con A-agarose column was equilibrated with 0.1 *M* acetate buffer solution (pH 6.0) containing 0.1 *M* NaCl, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. In the adsorption step, the mixture solution (0.42 mg/ml glucose oxidase and 0.76 mg/ml BSA in the acetate buffer) was continuously fed to the bed at a velocity of 0.92 ml/min. Following the adsorption step, the feeding stream was switched to washing buffer at the same velocity. The washing step was conducted until the absorbance (at a wavelength of 280 nm) of the outlet stream reached the baseline. Then the elution

Table 1
Adsorbents used for adsorption kinetics

Lectin adsorbent	Con A immobilized (mg/ml adsorbent)	Wet mass of adsorbent (mg)	GOD (mg/ml)	Volume (ml)
Con A-silica	10.7	305	0.50	20
Con A-polymer	8.4	405	0.50	20
Con A-agarose	18*	576	0.53	50

* Value given by the supplier.

buffer containing methyl-α-D-mannopyranoside was introduced to elute the glucose oxidase from the bed.

3. Results and discussion

The prepared lectin adsorbents were based on porous silica and porous polymer particles. The optimization of functionalization [8] resulted in a reproducible procedure delivering stable covalently bound ligands: silica particles were silylated with 3-mercaptopropyltrimethoxysilane. Butanediol diglycidyl ether was connected and after treating with hydrochloric acid, the supports were activated with tresylchloride [9]. The immobilization of the ligands (lectins) is the final step in the preparation of biospecific adsorbents, which is well investigated for the immobilization to tresylchloride-activated supports [10,11]. The stable covalent binding on the described tresylated supports could be synthesized at ambient temperature and mild conditions. The immobilization kinetics of different lectins show that a maximal yield of the bound ligands immobilized is reached within 4 h. The washing out effect of covalently bound lectins is negligible. Because of the valuable lectins a high yield of more than 90% is desirable. The adsorbent characterization includes several parameters such as ligand density, surface coverage and binding site accessibility [7].

3.1. Sorption behaviour of support materials and lectin adsorbents

The sorption behaviour of the basic silica gel, of the diol derivatives and of the activated support was investigated by adsorption isotherms (Fig. 1). The adsorption of ADH is used as a sufficient model for the binding of a protein ligand. It is well known that the unmodified silica gel adsorbs a high amount of protein unspecifically [12]. The shape of the isotherm is typical for unspecific adsorption. The adsorption of the diol derivatives remains very low. Consequently the functionalization procedure improves the separation specificity. Obviously a large portion of the surface is effectively covered and in this way protected against unspecific adsorption by the functionalization. The isotherm of the specific adsorbents shows typically high affinity isotherms

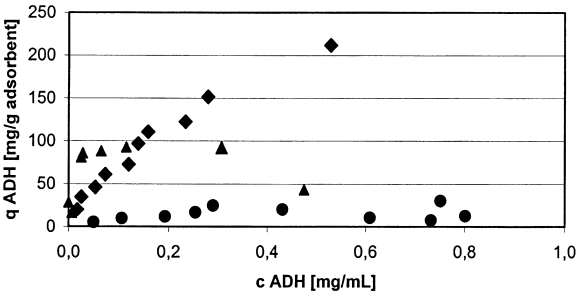

Fig. 1. Adsorption isotherms for different states of silica functionalization (♦) silica gel; (●) diol form; (▲) tresyl form.

[13]. The results prove that different states of surface modification have a strong influence on the unspecific adsorption of substances. Standard reaction conditions and washing procedures are prerequisites for a reproducible preparation of the adsorbents.

The results for the activated support were compared with commercial adsorbents based on agarose [14]. The adsorbents were characterized by kinetic and equilibrium investigations. The influence of the different support materials (silica, polymer, agarose) on the specific adsorption of different biological macromolecules was a specific focus.

Fig. 2 shows the adsorption kinetics of the glycoprotein glucose oxidase for Con A adsorbents based on silica, polymer and agarose as support material. It can be seen that equilibrium is reached within 100 min. There is no significant difference

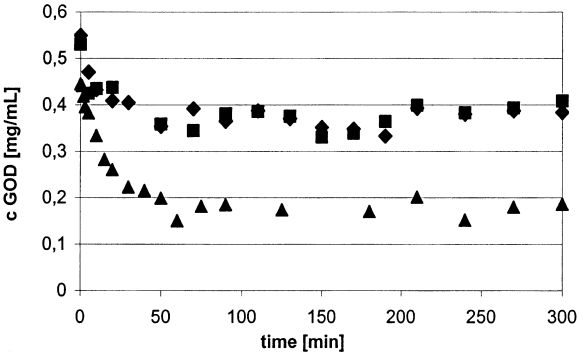

Fig. 2. Adsorption kinetics of glucose oxidase onto Con A adsorbents (▲) agarose; (♦) silica; (■) polymer.

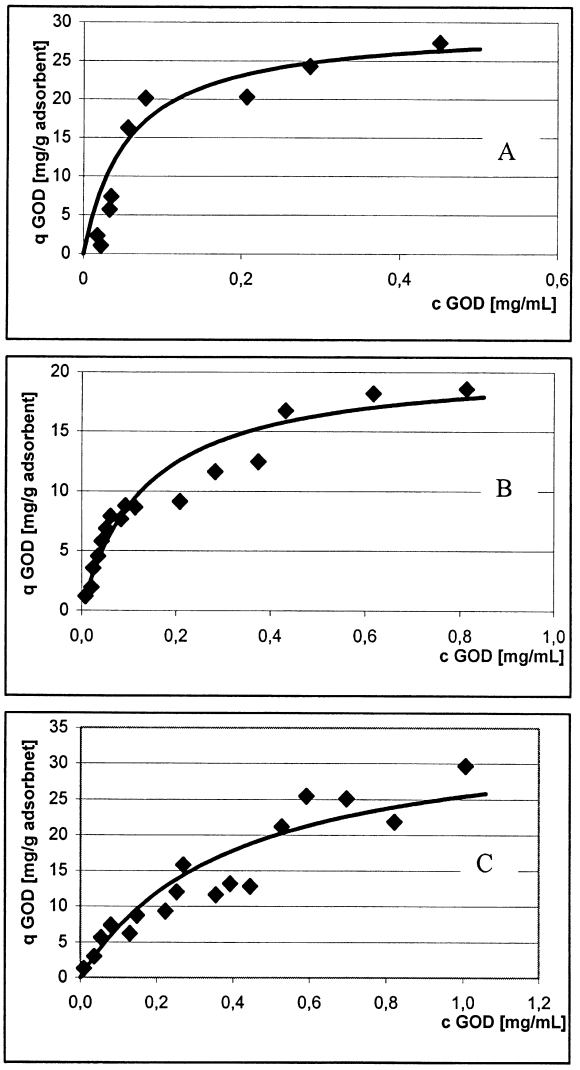

Fig. 3. Equilibrium isotherms for (A) Con A-agarose, (B) Con A-polymer and (C) Con A-silica; (♦) experimental data (—) data fitting according to Langmuir.

between the supports investigated. The greater decrease in GOD concentration for agarose is caused by the higher surface specific amount of ligands immobilized.

The results for equilibrium isotherms on Con A supports are presented in Fig. 3. From the data fittings according to the extended Langmuir model [15]:

$$q_{i,k} = \frac{a_k c_{i,k}}{1 + \sum_{l=1}^{N} b_l c_{i,l}} \quad (1)$$

where $a_k = q_{m,k}/K_{d,k}$, $b_k = 1/K_{d,k}$. For a single component adsorption, Eq. (1) reduces to the general Langmuir equation. The values for q_m and K_d for the single adsorbate GOD were determined and summarized in Table 2.

$q_{i,k}$ = adsorbed amount of the compound k (mg/g adsorbent); $q_{m,k}$ = maximum adsorbed amount of the compound k (mg/g adsorbent); $c_{i,k}$ = equilibrium concentration of the adsorbate k in the liquid phase (mg/ml); $K_{d,k}$ = dissociation constant of the compound k (mg/ml); l = number of compounds which exhibit competing adsorption onto the ligands.

The comparison of the experimental data and the Langmuir data fit shows a very good agreement and underlines the applicability of this model to specific affinity adsorption.

As a result it can be stated that all adsorbents show a high specific adsorption of GOD. The values for K_d were determined to be in the range of $K_d = 10^{-6}$–10^{-7} mol/l.

K_d as an inverse measure for specificity slightly decreases from the silica and polymer support to agarose. q_m is a measure of the maximum adsorption capacity. The high capacities of silica and polymer

Table 2
Comparison of parameters derived from data fitting according to Langmuir

Adsorbent	Con A immobilized (mg/ml adsorbent)	q_m GOD (mg/g wet mass)	Theoretical maximal capacity for GOD (mg/ml adsorbent)	K_d GOD (mol/l)
Con A-silica	10.7	35.5	59	$2.6 \cdot 10^{-6}$
Con A-polymer	8.4	20.8	47	$9.1 \cdot 10^{-7}$
Con A-agarose	18	29.6	100	$3.7 \cdot 10^{-7}$

supports are even more obvious comparing the value for q_m with the theoretical calculated capacities for GOD derived from the amount of ligands available.

3.2. Operational conditions and long term stability

For the optimization of the operational conditions the influence of pH on fractionation and recovery of various model glycoproteins was investigated for lectin adsorbents [14]. Experiments concerning the long term stability, solvent stability and affinity separation of glycoproteins were performed under these optimized conditions.

A major result was the proved long-term stability of the developed adsorbents. Over a period of 31, 50, and 84 weeks, respectively, no loss of binding capacity could be detected for adsorbents carrying RCA, Con A, and WGA as ligands [14,16]. For everyday separations RCA adsorbents showed no significant changes in separation performance and selectivity over a period of at least 10 months.

The polymer-based Con A adsorbent was used to determine the stability in organic solvents like methanol. There was no decrease in the adsorbent activity up to a methanol concentration of 20%. This result might be important for the regeneration of affinity adsorbents.

Examples for the application of the developed adsorbents in biospecific separation of glycoproteins are a one-step procedure for the purification of fetuin from the natural source fetal bovine serum using a WGA adsorbent and the purification of the enzyme HRP from a commercial available enyzme preparation by Con A-affinity separation. Under optimized conditions up to 21.6 mg fetuin per ml fetal calf serum could be recovered in a satisfactory purity as demonstrated as one single band in gel electrophoresis analysis [7]. The increase of HRP activity with an average factor of six showed not only the practicability of the procedure, but also the recovery of enzyme activity. The process development demonstrates that both materials, silica and polymer supports, provide suitable adsorbents for lectin affinity separation. The developed model enables the theoretical study of different influence parameters on the separation efficiency, to perform the process analysis as well as to carry out a scale-up [18].

3.3. Modelling and simulation of fixed bed experiments

The mathematical model describes affinity separations based on the fixed bed operations [18,19]. It considers film transfer, intraparticle diffusion, sorption rate integrated in a dispersion model [20]:

$$\frac{\partial c_{b,k}}{\partial t} = D_L \cdot \frac{\partial^2 c_{b,k}}{\partial x^2} - u_i \cdot \frac{\partial c_{b,k}}{\partial x} - H \cdot \frac{\partial \bar{q}_k}{\partial t} \quad (2)$$

The uptake rate (or desorption rate in washing and elution steps) of the component k into the adsorbent can be written as:

$$\frac{d\bar{q}_k}{dt} = \frac{3}{R} \cdot k_{f,k} \cdot (c_{b,k} - c_{i,k}|_{r=R}) \quad (3)$$

The intraparticle mass transfer of the proteins is described by a pore diffusion model:

$$\varepsilon_p \cdot \frac{\partial c_{i,k}}{\partial t} = \varepsilon_p D_{p,k} \cdot \frac{1}{r^2} \cdot \frac{\partial}{\partial r} \cdot \left(r^2 \cdot \frac{\partial c_{i,k}}{\partial r} \right) - \frac{\partial q_{i,k}}{\partial t} \quad (4)$$

A second-order equation of surface reaction was used to express the sorption rate [21]:

$$\frac{\partial q_{i,k}}{\partial t} = k_{a,k} c_{i,k} q_{m,k} \cdot \left(1 - \sum_{l=1}^{N} \frac{q_{i,l}}{q_{m,l}} \right) - k_{d,k} q_{i,k} \quad (5)$$

which leads to the extended Langmuir equation for multicomponent adsorption at equilibrium (Eq. (1)). Details on boundary and initial conditions are discussed in Refs. [20,22]. The numerical calculation was performed by finite difference using the Crank–Nicholson method [22,23]. The model was evaluated using parameters determined in independent experiments; it works for different stages of the separation process including adsorption, washing and elution as seen from Fig. 4. The course of the separation of GOD from BSA is demonstrated in graph (a). The detected protein outlet concentration increases fast just at the beginning of the adsorption step, which is caused by the nearly unretarded BSA (graph b). The increase at the plateau results from the beginning breakthrough of the valuable compound GOD. The comparison of the experimental and modelling data proves and provides satisfactory agreement for the whole course of the multicompound separation.

The computer simulation was carried out to analyze the separation process and to evaluate suitable

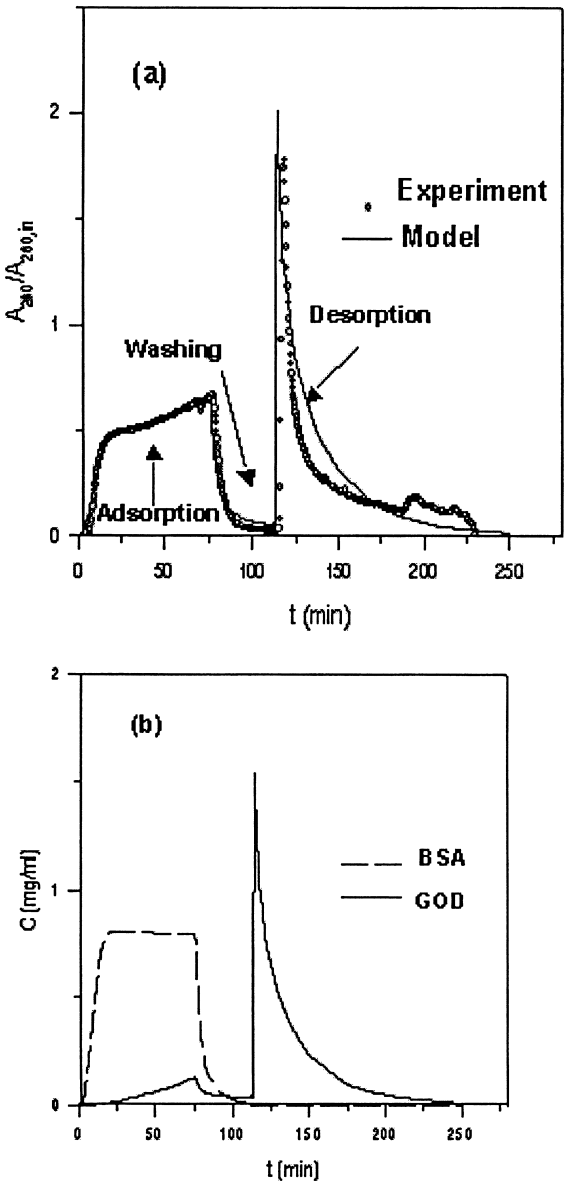

Fig. 4. Course of affinity separation: (a) course of the total protein outlet concentration; (b) simulation of the course concentrations of the desired (GOD) and contaminant (BSA) proteins.

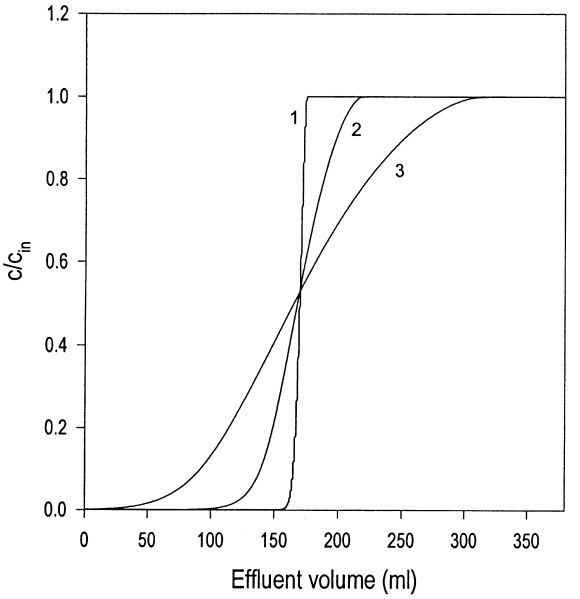

Fig. 5. Simulation analysis: effect of velocity on breakthrough curves of glucose oxidase. The value of c/c_{in} in the column outlet is plotted against the effluent volume. Modelling conditions: bed diameter is 1 cm, the length of the column is 5 cm, bed void fraction = 0.34, the protein concentration in the inlet stream is 1 mg/ml. The velocity is varied as: curve 1 of 0.1 ml/min; curve 2 of 1 ml/min; and curve 3 of 3 ml/min.

operation parameters. A simulation example is given in Fig. 5 in which breakthrough curves at different velocities are compared. With an increase in the flow from 0.1 to 3 ml/min, the breakthrough curve becomes shallow. This is due to two reasons. Firstly, the residence time of the adsorbates decreases at a higher velocity. Thus less time is allowed for the diffusion of the compounds into the particles to reach the ligands, and to interact with them, respectively. Secondly, at a higher velocity, the dispersion coefficient has a larger value, which decreases the efficiency of the column from the hydrodynamic point of view. In contrast, operation at a velocity of 0.1 ml/ml offers a sharp breakthrough curve. However, this lower velocity demands a long operation time. Thus, the choice of the flow has to be optimized according to economical aspects for each biotechnological system to be purified. For especially high valuable products a low flow has to be chosen.

The influence of the concentration of contaminants competing with the valuable product at the ligands on the separation performance is demonstrated in Fig. 6. BSA as contaminant exhibits a small affinity to the immobilized Con A ligand in contrast to the valuable product (GOD). The simulation results were plotted in a diagram with the protein load in the effluent of the column printed on the ordinate against

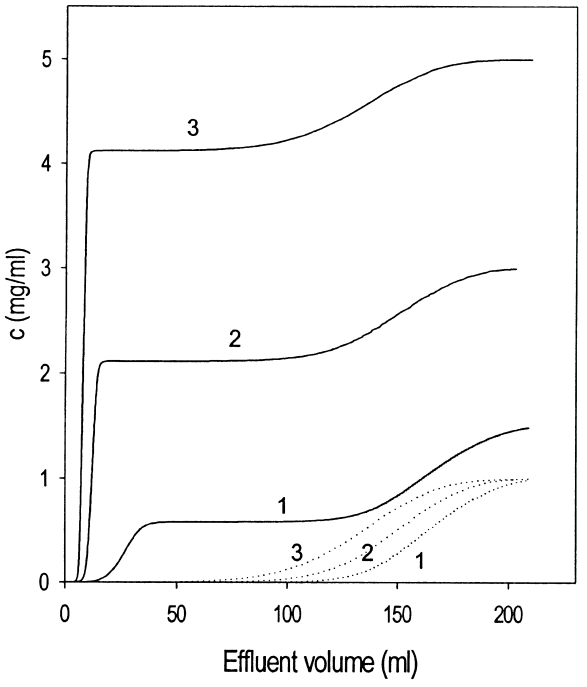

Fig. 6. Simulation analysis: effect of the concentration of the competing contaminant BSA on the breakthrough of the valuable compound GOD. Modelling conditions: concentration of the glucose oxidase in the inlet stream is fixed (1 mg/ml); BSA concentration varied as indicated; solid lines present the total protein concentration (BSA+GOD); dotted lines present the concentration of the GOD. The column conditions and other calculation parameters are as in Fig. 5.

the effluent volume (abscissa). The full lines represent the total protein load of BSA and GOD. The dotted lines indicate the course of the GOD. The amount of GOD was kept constant, while the amount of BSA increased. The breakthrough of the BSA occurred nearly instantaneously after the application of the protein mixture. Furthermore the higher the contaminant load the earlier the breakthrough of the valuable product, which probably is due to occupied ligands by the contaminants. The modified Langmuir model (Eq. (1)) describes this effect by the sum the products of the dissociation constants of the compounds adsorbed ($K_{d,l}$) and the equilibrium concentrations of the adsorbates ($c_{i,l}$). Each adsorbing compound results in an additional value in the nominator which lowers the adsorbed amount of the desired compound ($q_{i,k}$). The optimization of the process has to consider the concentrations and affinities of the impurities towards the ligands of the adsorbent. Furthermore economical aspects decide whether additional separation processes have to be employed for reasons of a maximized product recovery.

Using the developed model, an optimization of the recovery of the desired compound against operational parameters such as flow-rate, concentration of contaminant and the desired protein as well as multistage discontinuous operations were investigated [22].

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