This article was downloaded by:
On: *25 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK

Separation Science and Technology

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Superparamagnetic Cation-Exchange Adsorbents for Bioproduct Recovery from Crude Process Liquors by High-Gradient Magnetic Fishing

Anders Heebøll-Nielsen[a]; Sune F. L. Justesen[b]; Timothy J. Hobley[a]; Owen R. T. Thomas[c]
[a] Center for Process Biotechnology, BioCentrum-DTU, Technical University of Denmark, Kgs. Lyngby, Denmark [b] Institute of Medical Microbiology and Immunology, University of Copenhagen, The Panum Institute, Copenhagen, Denmark [c] Department of Chemical Engineering, School of Engineering, The University of Birmingham, Edgbaston, Birmingham, UK

Online publication date: 08 July 2010

To cite this Article Heebøll-Nielsen, Anders , Justesen, Sune F. L. , Hobley, Timothy J. and Thomas, Owen R. T.(2004) 'Superparamagnetic Cation-Exchange Adsorbents for Bioproduct Recovery from Crude Process Liquors by High-Gradient Magnetic Fishing', Separation Science and Technology, 39: 12, 2891 — 2914

To link to this Article: DOI: 10.1081/SS-200028791

URL: http://dx.doi.org/10.1081/SS-200028791

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

SEPARATION SCIENCE AND TECHNOLOGY
Vol. 39, No. 12, pp. 2891–2914, 2004

Superparamagnetic Cation–Exchange Adsorbents for Bioproduct Recovery from Crude Process Liquors by High-Gradient Magnetic Fishing

Anders Heebøll-Nielsen,[1,*] Sune F. L. Justesen,[2,*]
Timothy J. Hobley,[1,*] and Owen R. T. Thomas[3,†]

[1]Center for Process Biotechnology, BioCentrum-DTU, Technical University of Denmark, Kgs. Lyngby, Denmark
[2]Institute of Medical Microbiology and Immunology, University of Copenhagen, The Panum Institute, Copenhagen, Denmark
[3]Department of Chemical Engineering, School of Engineering, The University of Birmingham, Edgbaston, Birmingham, UK

Downloaded At: 10:03 25 January 2011

ABSTRACT

Different routes were screened for the preparation of superparamagnetic cation–exchange adsorbents for the capture of proteins using high-gradient magnetic fishing. Starting from a polyglutaraldehyde-coated

---

[*]These authors contributed equally to the experimental work in this study.
[†]Correspondence: Owen R. T. Thomas, Department of Chemical Engineering, School of Engineering, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK; E-mail: o.r.t.thomas@bham.ac.uk.

DOI: 10.1081/SS-200028791
0149-6395 (Print); 1520-5754 (Online)
Copyright © 2004 by Marcel Dekker, Inc.
www.dekker.com

Request Permissions / Order Reprints
powered by RIGHTSLINK
COPYRIGHT CLEARANCE CENTER, INC.

base particle, the most successful of these involved attachment of sulphite to oligomers of epichlorohydrin formed on the particle surface. The resultant cation-exchanger had a maximum lysozyme binding capacity of 272 mg g^{-1} and a dissociation constant of 0.73 μM. Using lysozyme as a model protein in small-scale studies, appropriate conditions were then selected for the capture of lactoperoxidase from sweet bovine whey. Subsequently, a high-gradient magnetic fishing process was constructed for the fractionation of whey, in which lactoperoxidase was purified 36-fold and concentrated 4.7-fold.

Key Words: Downstream processing; HGMF; Primary capture; Purification.

Downloaded At: 10:03 25 January 2011

INTRODUCTION

Adsorptive techniques capable of handling crude, unclarified feedstreams containing particulate contaminants have attracted much attention in recent years. Methods, such as expanded bed adsorption chromatography, intensify bioprocessing by capturing target macromolecules directly from crude feedstocks and thus bypass initial clarification steps. Recently, we have introduced a new process for direct product capture termed high-gradient magnetic fishing (HGMF).[1] This integrated process consists of (i) adsorption of a product of interest on to a superparamagnetic support having no magnetic memory, (ii) separation of the product-laden supports from the feedstock using high-gradient magnetic separation technology; and (iii) washing and recovery of bound target molecules from the adsorbents using multiple cycles of support capture and release.[1]

Several examples of magnetic affinity adsorbents have been described in the literature, such as metal chelators for immobilized metal ion affinity adsorption of proteins with exposed histidines,[2,3] use of cibacron blue F3GA for the recovery of dehydrogenases,[4] benzamidine-linked trypsin affinity supports,[5] bacitracin-derivatized broad range protease-binders,[1] and supports with dextran-tentacles for the capture of concanavalin A.[6] Surprisingly, only little work with superparamagnetic ion-exchangers designed for protein binding has been reported [e.g., Xue and Sun[7]]. The lack of interest in magnetic ion-exchangers may arise from the view that affinity interactions are needed if selectivity is to be achieved in a batch-adsorption-based process. However, high-adsorption selectivity might be achieved with ion-exchange adsorbents if the target is in a solution in which few other protein species carry a charge opposite to that of the ligand at the binding pH. One feedstock that seems ideally suited to fractionation by cation-exchange-based HGMF processing is cheese whey, which contains only few proteins with high pI values.

Rennet (sweet) whey is produced worldwide in huge quantities [$1.18 \cdot 10^{11}$ kg in 1997[8]] as a by-product from cheese manufacture, and has traditionally been used as a cheap source of low-value proteins for the food industry. However, there are a number of valuable proteins in whey, such as those with specific functions against microorganisms and viruses,[9–12] and fractionation of these is now becoming a topic of great interest. Two proteins in particular are lactoperoxidase (M_r 77,000; pI 9.8) and lactoferrin (M_r 78,500; pI 9.5), which have potential applications as natural additives to functional foods or improved infant milk formulas and may also have pharmaceutical applications.[11] Purification of these proteins from whey using traditional methods, such as packed bed chromatography, is problematic, as the proteins are present in low concentrations: $10–30\,\mathrm{mg\,L^{-1}}$ for lactoperoxidase and $20–350\,\mathrm{mg\,L^{-1}}$ for lactoferrin.[13] This necessitates the processing of large volumes of whey that contain high concentrations (total protein $\sim 7\,\mathrm{g\,L^{-1}}$) of other proteins, in particular β-lactoglobulin, α-lactalbumin and bovine serum albumin, all of which have pI values of ~ 5. Furthermore whey contains colloidal material, fats, and particulates which would rapidly foul a packed bed column. Extensive feedstock conditioning cannot be justified due to the modest value of lactoperoxidase and lactoferrin. Alternative unit operations such as expanded bed adsorption seem only partially suitable for the task of fractionating large-volume, dilute feedstocks such as whey, since the linear processing rates are limited to $\sim 300\,\mathrm{cm\,h^{-1}}$ or even less for viscous concentrates.[14,15] In contrast, HGMF-based processes can tolerate the application of crude feedstocks at very high rates.[1,15]

In light of the these considerations, we show here how high-capacity superparamagnetic cation-exchangers can be constructed and used to recover basic proteins from whey. A variety of derivatization routes were employed and the resulting adsorbents were tested for their ability to adsorb lysozyme, a model basic protein. Scouting experiments were then used to design a HGMF process, which was applied to the isolation and purification of lactoperoxidase.

EXPERIMENTAL

Materials

KnitMesh type 9029, composed of $\sim 110\,\mu$m diameter stainless steel 430 wires, was a gift from C. Barnes (KnitMesh Ltd., South Croydon, Surrey, UK). $FeCl_3 \cdot 6H_2O$ was obtained from Merck (Darmstadt, Germany). $FeCl_2 \cdot 4H_2O$ was from Mallinckrodt Baker B.V. (Deventer, The Netherlands). $NaBH_4$, 3-aminopropyl triethoxy silane, potassium permanganate, hen egg white

Downloaded At: 10:03 25 January 2011

lysozyme (L6876), lactoferrin from bovine milk, lactoperoxidase from bovine milk, glutaraldehyde (50 %, photographic grade), anthrone, and epichlorohydrin were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Dextran (M_r 15,000–20,000) and bromoethane sulphonic acid sodium salt were purchased from Fluka Chemie AG (Buchs, Switzerland). The bicinchoninic acid (BCA) protein assay kit was bought from Pierce Chemicals (Rockford, IL). ^{14}C-methylamine hydrochloride was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Insta-Gel® Plus and scintillation vials were supplied by Packard BioScience B.V. (Groningen, The Netherlands). TMB ONE ready-to-use substrate was supplied by Kem-En-Tec A/S (Copenhagen, Denmark). Precast SDS-PAGE gels and protein stains were from Invitrogen (Groningen, The Netherlands). The sweet whey (kindly donated by E. O. W. Nielsen, The Royal Veterinary and Agricultural University, Copenhagen, Denmark) used throughout these studies was a calf stomach rennet digest of pasteurised bovine milk. Prior to use, the liquid portion was crudely separated from remaining casein precipitates and lipids by settling and aspiration. The resultant turbid liquid with a pH of approximately 6.5 had a conductivity of 5 mS cm^{-1}. All other chemicals were analytical grade.

Downloaded At: 10:03 25 January 2011

Support Separation

For separation of magnetic particles from liquid volumes of 25–2000 mL during particle preparation and derivatization, a powerful (~0.7 T) neodymium-iron-boron magnet block from Danfysik A/S (Jyllinge, Denmark) was used. Side-pull permanent (~0.15 T) magnet racks (PerSeptive Diagnostics, Cambridge, MA) were used for separation of magnetic supports during small-scale binding studies.

Base Matrix Preparation

Superparamagnetic iron oxides were prepared by alkaline precipitation of iron ion solutions followed by silanization with 3-amino propyl triethoxy silane as described by Hubbuch and Thomas,[5] and the amine-terminated supports were then coated with a thin layer of polyglutaraldehyde following the procedure of O'Brien et al.[2] Polyglutaraldehyde-coated particles were subsequently washed 10 times with distilled water, twice with 1 M NaCl, and finally twice with distilled water.

Activation and Coupling Methods

Epichlorohydrin Activation

Activation of polyglutaraldehyde-coated supports with epichlorohydrin (Fig. 1) was conducted using procedures modified from Hermanson et al.[16] A sufficient amount of particles to give a final concentration of $25\,g\,L^{-1}$ was suspended in a solution of 0.5 M NaOH, 19 mM $NaBH_4$. Epichlorohydrin was added to a concentration of 5% (v/v) or higher, and the reaction allowed to proceed for 6h at room temperature with sufficient mixing on an IKA VXR-S17 vibrating shaker (IKA Labortechnik, Staufen, Germany), or with an overhead stirrer (Heidolph Elektro KG, Kelheim, Germany) to keep the supports in suspension. The supports were then washed eight times with distilled water and twice with storage buffer (20 mM sodium phosphate, 1.0 M NaCl,

Downloaded At: 10:03 25 January 2011

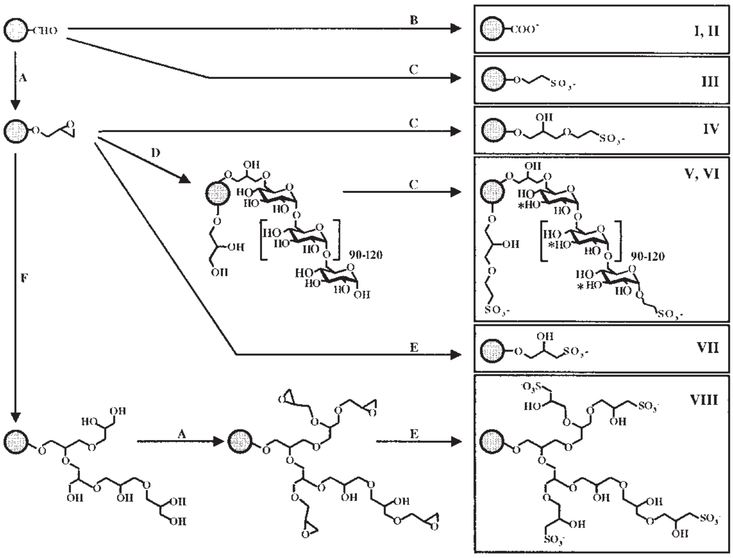

Figure 1. Idealized scheme for the preparation of superparamagnetic cation exchange adsorbents from polyglutaraldehyde coated supports. (A) NaOH, $NaBH_4$, epichlorohydrin (ECH), rt, 6 h; (B) $KMnO_4$, rt, 16 h or 60°C, 3 h; (C) bromoethane sulfonic acid (BESA), NaOH, $NaBH_4$, rt, 18 h; (D) Dextran, $NaBH_4$, Na_2CO_3, 60°C, 24 h; (E) Na_2SO_3, NaOH, rt, 2 h; (F) ECH, 60°C, 16 h. See text for further reaction details. *Indicates possible modification of secondary hydroxyl sites on the dextran backbone. The common order of reactivity of secondary hydroxyls on most hexopyranosides is 3–OH > 2–OH > 4–OH.

pH 6.8), in which they were kept at 4°C until use. This washing procedure was used for all finished adsorbents. Coupling to epichlorohydrin-activated supports was performed essentially as suggested by Hermanson et al.[16] and where necessary further details are given next for each of the ligands employed.

Oxidation of Aldehyde Groups

Ion-exchange adsorbents with carboxylate groups (support type I and II, Fig. 1) were prepared by treating 1 g quantities of polyglutaraldehyde-coated particles with 50 mL volumes of potassium permanganate (Fig. 1) for 16h at ambient temperature or 3h at 60°C, respectively. In the reactions, permanganate concentrations of 0.072 M or 0.152 M were examined.

Attachment of Ethane Sulphonic Acid

Ethane sulphonic acid functional groups were attached to polyglutaraldehyde-coated or epichlorohydrin-activated supports yielding support types III and IV, respectively (Fig. 1), by reacting with bromoethane sulphonic acid under reducing conditions. At the high pH employed, the epoxy groups were hydrolysed allowing reaction between the hydroxyls formed and bromoethane sulphonic acid. The polyglutaraldehyde or epichlorohydrin-activated particles (at a final concentration of $25\,\mathrm{g\,L^{-1}}$) were suspended in a solution of 0.5 M bromoethane sulphonic acid (sodium salt) in 1.0 M NaOH containing 26 mM $NaBH_4$, and the reactions were allowed to proceed for 18h at room temperature.

Cation–Exchangers with Dextran Tentacles

Tentacles of dextran were coupled to epichlorohydrin-activated supports (Fig. 1) prior to functionalization with bromoethane sulphonic acid for the preparation of adsorbents type V and VI. The activated particles were suspended (at $10\,\mathrm{g\,L^{-1}}$) in a solution containing $20\,\mathrm{g\,L^{-1}}$ dextran and between 8 and 32 mM $NaBH_4$ in 1 M Na_2CO_3, pH 11.5, and the reaction suspension was then incubated for 24 h at 60°C. After washing, ethane sulphonic acid groups were attached to the dextran-linked supports by reacting with bromoethane sulphonic acid as previously described.

Sulphite Derivatization of Activated Particles

Type VII adsorbents were constructed by reacting epichlorohydrin-activated supports with saturated solutions of sodium sulphite adjusted to

Downloaded At: 10:03 25 January 2011

Downloaded At: 10:03 25 January 2011

pH 11.5 with NaOH (Fig. 1). The reactions took place at room temperature for 2 h with a final support concentration of 20 g L^{-1}.

In an alternate approach the polyglutaraldehyde-coated supports were first activated using an increased amount of epichlorohydrin (i.e., 20% v/v instead of 5% v/v). This suspension was then heated to 60°C for 16h to allow the hydroxyl groups of hydrolyzed epichlorohydrin to react with immobilised epoxy groups. The product obtained was washed and reactivated with (5% v/v) epichlorohydrin, and then finally derivatized with sulphite as described, yielding type VIII adsorbents (Fig. 1).

Support Characterization

Lysozyme Binding

In order to compare the supports resulting from the different preparation routes, binding of lysozyme was examined using small-scale magnetic rack studies. The adsorbents (1–3 mg aliquots) were first equilibrated by washing (three times) in a 10 mM sodium phosphate buffer, pH 7. Solutions (0.50 or 1.00 mL) of lysozyme in equilibration buffer (ranging from 0 to 4 g L^{-1} final concentration) were then added and allowed to bind for 15 min, before the adsorbents were separated in a magnetic rack and the supernatants recovered. The amount of bound enzyme was determined by measuring the total protein concentration in the supernatant following binding. The adsorbent concentration was determined by dry-weight measurement. The Langmuir equation (1) was used to model the binding of lysozyme to the supports.

$$Q^* = Q_{max} \frac{C^*}{C^* + K_d} \tag{1}$$

Here Q^* and C^* are the amounts of protein bound and in solution, respectively, at equilibrium; Q_{max} is the maximum binding capacity and K_d the dissociation constant. The values of Q_{max} and K_d were estimated using the nonlinear least-squares fitting function of Origin 4.1 software (Microcal Software Inc., Northampton, MA).

Capture of Whey Proteins

Prior to HGMF processing, the amount of adsorbent needed for removal of lactoperoxidase from sweet whey was determined in small-scale rack studies. The support types III and VIII (Fig. 1, Table 1) were equilibrated to pH 7 with a 10 mM sodium phosphate buffer as described and finally suspended to give a concentration of ~10 g L^{-1}. The dry-weight content of

Downloaded At: 10:03 25 January 2011

Table 1. Estimated parameters[a] for adsorption of lysozyme on various magnetic cation-exchangers.

Support[b]	Tentacle	Ligand	Q_{max} (mg g^{-1})	K_d (μM)	Q_{max}/K_d (L g^{-1})
I	—	—COO^{-}[c]	210	20	0.7
II	—	—COO^{-}[d]	170	5.0	2.4
III	—	—$(CH_2)_2$-SO_3^-	230	1.2	14
IV	—	—$(CH_2)_2$-SO_3^-	222	0.6	26
V	Dextran, 7.2 μmol g^{-1}	—$(CH_2)_2$-SO_3^-	184 (207)[e]	1.7	7.7 (8.7)[c]
VI	Dextran, 9.3 μmol g^{-1}	—$(CH_2)_2$-SO_3^-	172 (201)[e]	2.0	6.0 (7.0)[c]
VII	—	—SO_3^-	80.8	1.4	4.1
VIII	Epichlorohydrin-oligomer	—SO_3^-	272	0.7	26

[a] Figure 3 adsorption isotherm data were fitted to the simple Langmuir model (Eq. 1).

[b] See Fig. 1 and accompanying text.

[c] Type I supports were prepared by oxidation at room temperature.

[d] Type II supports were prepared at 60°C, permanganate concentration (0.072 M), same as for Type I.

[e] Numbers in parentheses indicate values based on the weight of magnetic material, where the contribution to the mass from the attached dextran has been accounted for.

— Not applicable.

Downloaded At: 10:03 25 January 2011

this suspension was then determined before aliquotting out into 2 mL eppendorf tubes to yield a series with 1–10 mg of adsorbent. Excess buffer was removed from the suspensions and 1.00 mL of whey was then added to each tube. The suspensions were incubated for 15 min at room temperature before separation of the supports on magnetic racks and aspiration of the supernatants. Added whey and the supernatants were subsequently analyzed for lactoperoxidase activity and total protein, and the recorded values used to estimate the amounts of bound protein and lactoperoxidase, and the specific activity of the adsorbed material.

High-Gradient Magnetic Fishing

HGMF System Design

The system employed for high-gradient magnetic fishing was a simplified version of that described by Hubbuch et al.,[1] and is illustrated in Fig. 2.

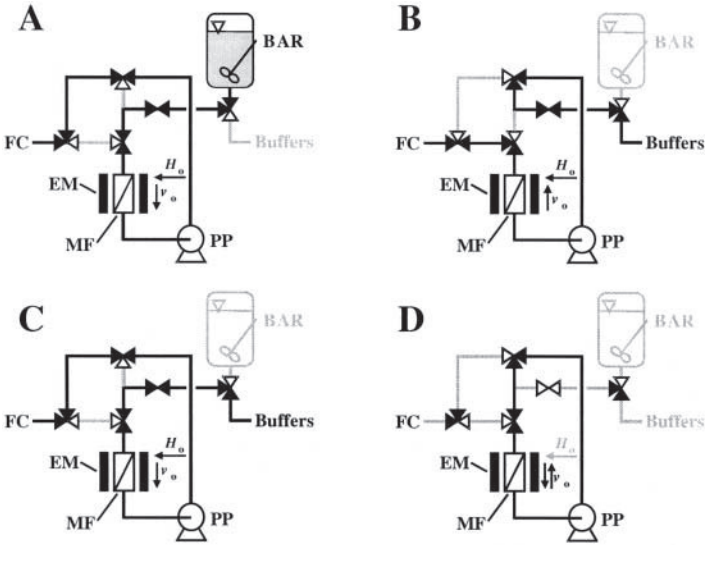

Figure 2. Schematic diagram of the HGMF system outlining the flow-paths and setting of the valves in each step of the process. (A) Application of adsorption suspension, (B) loading of washing buffer, (C) loading of elution buffer, (D) recycle mode for washing or elution. FC = fraction collector, EM = electromagnet, MF = magnetic filter, PP = peristaltic pump, BAR = batch adsorption reactor, H_0 = magnetic field, v_0 = fluid flow.

It consisted of a stirred batch adsorption reactor and a buffer container connected to a high-gradient magnetic filter via 2 mm inner-diameter teflon-tubes. Feedstock and buffers were transported via a single peristaltic pump (Masterflex® Easy Load® model 7518-00, Cole Parmer Instrument Co., Vernon Hills, IL), equipped with a 1.6 mm i.d. NorpreneA60F tube, and the fluid-flow paths in the separator were directed by three-way solenoid switching valves (Bürkert-Contromatic A/S, Herlev, Denmark) controlled by LabView software (Student Edition ver. 3.1, National Instruments Corporation, Austin, TX). Fractions were collected with a SuperFrac fraction collector (Amersham Biosciences AB, Uppsala, Sweden).

Magnetic separation was accomplished with a water-cooled electromagnet powered by a System 8000 Magnet Power Supply (100 A, 52 V) both from Danfysik A/S (Jyllinge, Denmark). A magnetic filter created from a 1.0 cm inner-diameter plastic tube, 7.0 cm long, filled with tightly rolled Knitmesh 9029, was placed between the pole shoes (9.0 cm diameter), which were set 5.0 cm apart. The matrix occupied 0.52 mL of the 5.5 mL volume of the filter. For all experiments the superficial fluid velocity in the filter during particle capture was 24 m h^{-1}, and the magnetic flux density was 0.4 T as determined with a LakeShore model 410 gauss-meter (Westerville, OH).

Downloaded At: 10:03 25 January 2011

Operation of the HGMF Apparatus

The flow paths and the flow directions used in the different steps of the HGMF process are outlined in Fig. 2 and explained next. Following initial batch adsorption (600 s) in a stirred reactor, the feedstock suspension was fed to the magnetized filter, and adsorbent-free filtrate was collected by the fraction collector (Fig. 2a). While the magnetic field was still on, residual feedstock was washed out of the system lines by pumping in equilibration buffer in the reverse direction (Fig. 2b). The lines were then filled by pumping in the original direction, with a volume (25 mL) of elution buffer that was only just sufficient to flush out the remaining washing buffer (Fig. 2c). Bound protein was then desorbed by recycling the suspension through the closed 25 mL loop (Fig. 2d) at zero magnetic field for 300 s using an increased flow velocity in the filter ($\sim$130 m h^{-1}). Following desorption, the magnetic field was switched on, and the suspension was recycled until all magnetic supports were recaptured (Fig. 2d). The flow was then directed to the fraction collector (Fig. 2c) and the eluted protein collected. In cases where multiple elution cycles were conducted this process (with eluent) was repeated. Adsorbent recovery was achieved by filling the loop, as for elution, and then recycling the suspension at 300 m h^{-1} with simultaneous manual vibration of the filter unit at zero magnetic field. The fluid velocity was lowered to 24 m h^{-1} before directing the flow to the fraction collector and collecting the adsorbents. Routinely, two adsorbent recovery cycles were performed in this manner.

Studies of the Dynamic Capacity of the HGMF Filter

The particle holding capacity of the high-gradient magnetic filter was determined by suspending amino silane-coated supports ($1-5\,\mathrm{g\,L^{-1}}$) in distilled water using overhead stirring, then pumping the suspension through the system with the field switched on. Fractions (5 mL) were collected, and the particle contents subsequently determined using a microtiter plate reader.

HGMF Capture of Purified Lysozyme

A 0.4 g aliquot of support type VIII was equilibrated to pH 7 with a 10 mM sodium phosphate buffer, mixed with 400 mL of a solution of lysozyme ($0.25\,\mathrm{g\,L^{-1}}$ in equilibration buffer) for 600 s at ambient temperature, and 375 mL of the suspension was processed as described (the loading time was 720 s). Washing was conducted using equilibration buffer, and desorption of bound lysozyme was effected using equilibration buffer supplemented with 1 M NaCl.

Capture of Basic Proteins From Whey Using HGMF

Cation–exchange support type VIII was employed to capture basic proteins from whey using HGMF. Whey, buffers, and the collected fractions were kept on ice at all times. The supports (0.92 g) were first equilibrated to pH 7.0 in a 10 mM sodium phosphate equilibration buffer, then settled magnetically and drained. The adsorbents were then stirred for 600 s with 380 mL whey, and 174 mL of this suspension was fed to the HGMF apparatus over 330 s and the flow-through fraction collected. A simplified wash cycle was used in which the feedstock remaining in the system was washed out with equilibration buffer (~25 mL) while the magnetic field was on. One elution cycle was conducted using equilibration buffer containing 1 M NaCl, and 40 mL of eluate in total was then collected over 77 s (additional buffer was used to ensure flushing out of eluted protein). Following particle recovery, the magnetic filter was removed from the HGMF system and flushed with tap water (at approximately $2000\,\mathrm{m\,h^{-1}}$) to remove any entrapped adsorbents. Whey, flow-through, and eluate were analyzed for total protein concentration, lactoperoxidase activity, and by SDS-PAGE.

Downloaded At: 10:03 25 January 2011

Analysis

The densities of the aldehyde groups on polyglutaraldehyde-coated particles and epoxy groups on epichlorohydrin-activated supports were examined in radio-labelling experiments. Both aldehydes and epoxides will form stable bonds with methylamines through formation of Schiff's bases[17] and amines,[16] respectively. A 27 mM aqueous solution of methylamine containing ^{14}C-methylamine hydrochloride corresponding to 74,000 Bq mL^{-1} was prepared, and 30 mg quantities of particles were each reacted with 3 mL of the methylamine solution for 6h at 60°C. Following the reaction, the labeled supports were washed five times in distilled water before being dissolved in 1.25 mL of 20% phosphoric acid at 60°C. The dissolved particles were diluted four-fold in distilled water and transferred to scintillation vials. The samples were made ready for counting by adding 10 mL Insta-Gel® Plus to each, and the number of counts per minute was recorded in a Tricarb 2100TR Liquid Scintillation Analyzer (Packard Instrument Company, Inc., Meriden, CT). Quenching effects from the magnetic matrix were studied by adding known amounts of labeled methylamine to unlabelled supports and analyzing the scintillation as above.

Dextran-densities were determined by reacting the polysaccharide bearing supports with anthrone under acidic conditions exactly as described by Heebøll-Nielsen.[6] A microtiter plate reader (Anthos Reader 2001, Anthos Labtech Instruments, Salzburg, Austria) was used for absorbance measurement.

The BCA protein assay was employed as recommended by the manufacturer and scaled for use with a Cobas Mira robot spectrophotometer (Roche Diagnostic Systems, Rotkreutz, Switzerland). All protein concentrations were expressed in milligrams of bovine serum albumin equivalents.

Lactoperoxidase activity was monitored using the TMB-ONE ready-to-use substrate containing 3,3′,5,5′-tetramethyl benzidine in an assay scaled for use with the Cobas Mira robot spectrophotometer. Product formation was followed at 37°C by measuring the change in absorbance at 600 nm after mixing three volumes of sample with 37 volumes of the reagent. Lactoperoxidase activity was expressed in units of ΔAU s^{-1} mL^{-1}.

Reducing SDS-PAGE was conducted according to Laemmli[18] using Novex® 4–20% Tris-glycine polyacrylamide gels followed by staining with Novex Colloidal Blue. Digital images of the preserved gels were created with a scanner connected to a personal computer.

Dry weight was determined by sample filtration through 0.2 μm pore size filters (PALL Corporation, Ann Arbor, MI) as has been described previously.[15] Fractions from the breakthrough experiment were analyzed for the concentration of magnetic supports by measuring optical density at 620 nm

Downloaded At: 10:03 25 January 2011

in the microtiter plate reader and the values compared to a standard curve of dry weight vs. OD_{620}.

RESULTS

Screening of Chemistries for Support Activation and Derivatization

Oxidation of aldehydes present on the surface of the magnetic supports was examined as a means for directly preparing cation-exchange adsorbents (type I and II). The results (Table 1, Fig. 3a) indicate that supports prepared using 0.072 M permanganate at 20°C had a lysozyme binding capacity of 210 mg g^{-1} adsorbents which was not improved by raising the reaction temperature to 60°C. Increasing the permanganate concentration to 0.152 M also had no effect on adsorbent performance. Coupling of cation-exchange groups was therefore examined in order to produce supports with higher capacities and lower K_d values.

Attachment of ethane sulphonic acid directly to reduced aldehydes produced an improved cation-exchanger (type III) with a higher lysozyme

Downloaded At: 10:03 25 January 2011

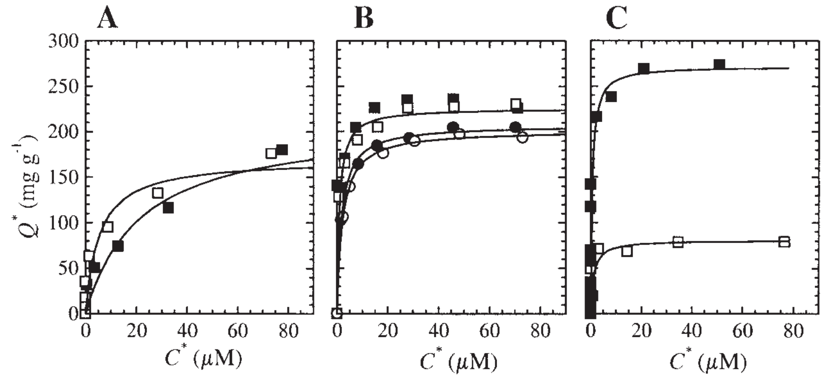

Figure 3. Equilibrium adsorption isotherms for lysozyme binding to superparamagnetic supports prepared by (A) oxidation of surface aldehydes at 25°C (support I, ■) and 60°C (support II, □), (B) functionalisation with ethane sulphonic acid showing support III (■), support IV (□), support V (●) and support VI (○); and (C) supports prepared by coupling sulphite after one epichlorohydrin-activation reaction (support VII, □), or multiple rounds of epichlorohydrin reaction and activation (support VIII, ■). The lines show the fit obtained using the Langmuir equation (Eq. 1), and the parameter values are quoted in Table 1.

Downloaded At: 10:03 25 January 2011

binding capacity and markedly lower dissociation constant than the supports prepared by direct oxidation (Table 1, Fig. 3b). For comparison, bromoethane sulphonic acid was reacted with an epichlorohydrin-activated matrix to produce the type IV adsorbent, which had a similar performance to the type III material (Fig. 3b). Coupling of sulphite to epichlorohydrin-activated supports, however, yielded an adsorbent (type VII) with much-lower maximum capacity (Fig. 3c). The reasons for this low capacity were examined by comparing the density of available aldehyde groups prior to activation with the epoxide density after activation. Reaction with ^{14}C-labeled methylamine indicated an aldehyde density of 495 μmol g^{-1} support, compared to only 73 μmol g^{-1} support for the epoxides. Coupling of bromoethane sulphonic acid requires nucleophilic groups, such as hydroxyls resulting from the reduction of aldehydes or hydrolysis of epoxy groups, for the reaction to take place. In contrast, sulphite coupling requires intact epoxy groups, and the similarity between support types III and IV as well as the low capacity of type VII can thus be explained by hydrolysis of the attached epoxy moieties.

In an attempt to increase the number of activation sites on the supports, dextran tentacles of 90–120 glucose residues were attached to the particles. Supports with two different densities of dextran, 7.2 and 9.3 μmol g^{-1} magnetic material, were produced. When these were derivatized with ethane sulphonic acid and the resulting adsorbents (type V and VI) were tested in batch binding experiments with lysozyme (Table 1, Fig. 3b), capacities were lower and the K_d values higher than those achieved by coupling bromoethane sulphonic acid directly to reduced aldehydes. A dextran density of 9 μmol g^{-1} corresponds to a utilization of approximately 2% of the superficial aldehyde groups when end-on attachment via the nonreducing end of the polysaccharide chain is assumed. The remaining surface aldehydes would thus be available for reaction with epichlorohydrin. Following hydrolysis of the epoxy groups so introduced, the resulting hydroxyls would in turn be available for modification with bromoethane sulphonic acid (Fig. 1). Nevertheless, approximately 3000 μmol g^{-1} extra hydroxyls can be expected to be added, as each glucose moiety of the polysaccharide will contribute three free secondary hydroxyls to the support. The poor performance delivered by supports V and VI suggests that the vast majority of secondary hydroxyls on the immobilized dextran chains did not react with bromoethane sulphonic acid under the conditions employed. It is likely that derivatization preferentially occurred at primary hydroxyl sites on the dextran chain (i.e., at the terminal glucose residue) and especially at the immediate support surface (see Fig. 1). The use of dextran chains was abandoned in favor of a preparation route employing multiple rounds of activation. Epichlorohydrin-activated particles were reacted again with epichlorohydrin under coupling conditions and then reactivated

Downloaded At: 10:03 25 January 2011

(with epichlorohydrin) prior to sulphite coupling. The resulting supports (type VIII) were superior to all those produced previously and had both a higher lysozyme binding capacity and a lower dissociation constant (Fig. 3c), giving the highest ratio of Q_{max}/K_d for any of the adsorbents (Table 1).

Optimization of Adsorbent Concentration for Removal of Lactoperoxidase from Whey

The optimal support concentration for the capture of lactoperoxidase from whey was determined in small-scale rack studies using support types III and VIII. Support III rather than IV was selected for comparison with support VIII, due to the simpler preparation of the former and therefore better potential for scale-up. Removal of lactoperoxidase from the crude whey feedstock was very similar for both supports and indicates that approximately 6.5 g L^{-1} of either adsorbent removed all lactoperoxidase (Fig. 4). However, support VIII was chosen for further work since at a concentration of ~3 g L^{-1}, a higher degree of purification in the binding step could be obtained than for support III (Fig. 4).

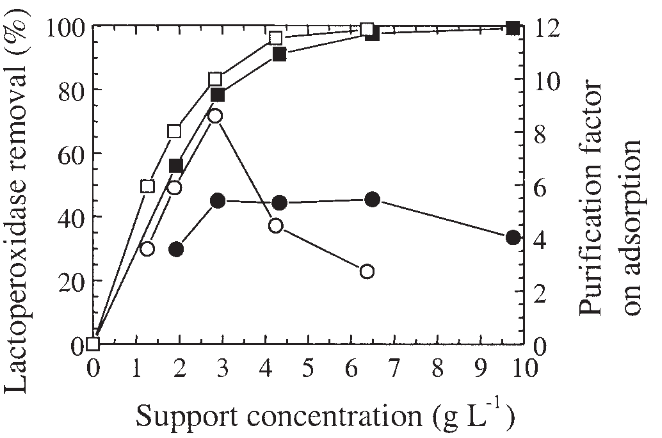

Figure 4. Effect of support concentration on binding of lactoperoxidase in whey. Key: Proportion of lactoperoxidase removed from whey using support III (■) and VIII (□). Apparent purification of lactoperoxidase due to adsorption to support III (●) and VIII (○), as determined by comparing the specific activity of the whey and of the supernatant following binding.

Characterization of the HGMF Apparatus

Prior to using the adsorbents in a HGMF process to fractionate whey, the characteristics of the apparatus were examined using model systems. The capacity of the magnetic filter for supports was determined by examining the breakthrough of amino silane-coated magnetic particles in water. Steep breakthrough of particles was observed (Fig. 5) after application of 350 mg of the supports and a filter capacity at 10% breakthrough of 65 g L^{-1} was determined using two different incoming particle concentrations (i.e., 1 and 4.7 g L^{-1}).

A model HGMF experiment with 1 g L^{-1} of support type VIII was conducted using lysozyme at a concentration of 0.25 g L^{-1} (similar to that of the two major basic proteins in whey, lactoperoxidase and lactoferrin). The results in Fig. 6 show that trace amounts of lysozyme were present in the flow-through ($C^* = 0.035$ g L^{-1}), which corresponds to a value for Q^* of 204 mg g^{-1} (~90% of Q_{max}) and is similar to that observed in Fig. 3c. No support breakthrough was visible after loading 375 mg of adsorbents, indicating a higher capacity than for the model amine terminated supports (Fig. 5). Batch-wise elution in the recycle loop led to the recovery of 81% of the bound material and a five-fold concentration of the lysozyme in the fractions pooled from the first elution cycle (Table 2).

Downloaded At: 10:03 25 January 2011

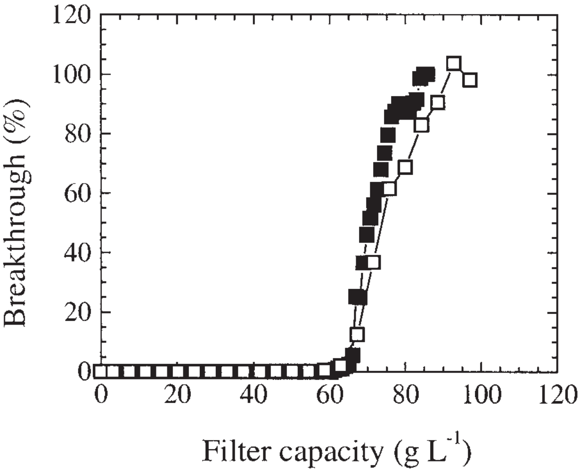

Figure 5. Breakthrough profile of amine-terminated superparamagnetic supports on the magnetic filter (total volume 5.5 mL) of the HGMF system. Support concentrations were 1.0 g L^{-1} (■) or 4.7 g L^{-1} (□).

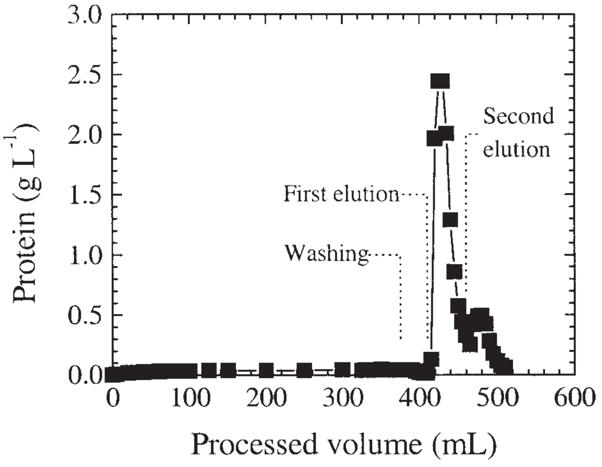

Figure 6. Protein concentration profile obtained during the HGMF recovery of lysozyme from a buffer solution.

Downloaded At: 10:03 25 January 2011

HGMF Purification of Basic Proteins from Whey

These results were used to design the HGMF process for lactoperoxidase purification from crude bovine whey. Support VIII was used at a concentration of ~2.5 g L^{-1} rather than 1 g L^{-1} in order to give greater selectivity for the target (Fig. 4). Filter capacity was maximized by loading until visible breakthrough of the adsorbents just began to occur, at which point 416 mg of supports had been applied. The results in Table 3 show that purification and concentration factors of 36 and 4.7, respectively, were obtained. Furthermore, only 8.2% of the lactoperoxidase was present in the flow-through,

Table 2. Recovery of lysozyme by HGMF using support VIII.

Step	Volume (mL)	Concentration (g L^{-1})	Concentration factor	Step yield (%)
Load	375	0.25	1	100
Flow-through	375	0.035	0.14	14.1
Wash	35	0.028	0.11	1.0
First elution	50	1.25	5.06	67.5
Second elution	50	0.28	1.13	15.1
Balance	—	—	—	97.7

— Not applicable

Downloaded At: 10:03 25 January 2011

Table 3. Recovery of lactoperoxidase from whey using HGMF with support VIII.

Step	Volume (mL)	Protein ($g\,L^{-1}$)	Lactoperoxidase ($U\,mL^{-1}$)	Purification factor	Concentration factor	Step yield (%)	
						Protein	Lactoperoxidase
Load	174	6.48	6.02	1	1	100	100
Flow-through	174	6.08	0.49	0.088	0.082	93.3	8.20
Elution[a]	40	0.84	28.4	36.4	4.72	3.1	111
Balance	—	—	—	—	—	96.3	119

[a] Prior to elution, whey in the system was removed using 25 mL of equilibration buffer.

—Not applicable

demonstrating that HGMF performance was better than predicted (~20% loss) by the scouting experiments (Fig. 4). Analysis by SDS-PAGE confirmed that the majority of lactoperoxidase and lactoferrin were removed from the whey during the batch binding step (Fig. 7, lane 4) and then concentrated and purified in the elution fraction (Fig. 7, lane 5). The protein mass balance (Table 3) closed to within ~4% and the unaccounted for matter was likely lost in the wash (not analyzed). In contrast, the mass balance for lactoperoxidase (119%) suggests that some activation of the enzyme occurred during the purification process.

DISCUSSION

The performance of HGMF is limited by two general constraints: First, the adsorbents' protein binding properties, which affect both the concentrating power and the purification factors that can be achieved; and second the system design and setup. In the latter, the flow rates and field strengths used determine the capacity of the magnetic filter and therefore process productivity, and the volume of the recycle loop affects the degree of concentration that can be achieved. The availability of advanced HGMS separators and filters in general and those designed specifically for biotech applications[19] makes

Downloaded At: 10:03 25 January 2011

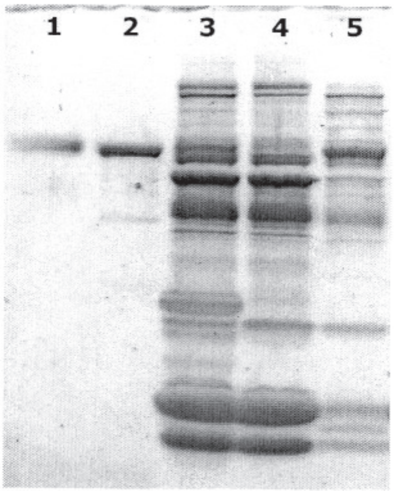

Figure 7. Reducing SDS-PAGE of fractions obtained during HGMF processing of sweet whey with support VIII. Lane 1: lactoperoxidase marker, lane 2: lactoferrin marker, lane 3: whey feedstock, lane 4: unbound material, lane 5: eluate.

system design an engineering problem. The first of the two general constraints thus appears to present the biggest challenge to design of HGMF processes for biotech applications. Suitable types of cheap adsorbents are not available in large quantities. Therefore in the current work we have focused on the chemistries involved in activation and functionalization, as a step toward development of cheap generic adsorbents (i.e., ion exchangers) with high capacities and low K_d values.

The amino silanized iron oxide crystal agglomerate that is the support employed for constructing the adsorbents used here has an amine group density of $\sim 100\,\mu mol\,g^{-1}$ particles.[5] In the present study we have found that polyglutaraldehyde coating of this starting material yields a five-fold increase in the density of active groups. However, epichlorohydrin activation lowered this density to only $73\,\mu mol\,g^{-1}$, a value consistent with the findings of Hubbuch and Thomas[5] who reported $\sim 50\,\mu mol\,g^{-1}$ when a similar material was activated with epichlorohydrin. This decrease in active group density to approximately 20% of the number of aldehydes is probably caused by premature hydrolysis of the attached epoxy groups. Attachment of surface structures to increase the number of sites for ligand coupling is an attractive prospect. We have previously demonstrated how attachment of dextran tentacles to superparamagnetic supports could improve the binding capacity for a glucose-specific lectin.[6] However, in the present work no advantage was found by using dextran tentacles, most likely due to the low reactivity of the secondary hydroxyls present. Thus attachment of prederivatized tentacles or coupling of more reactive surface features may yield more successful results.

Another approach to increase the ligand density is to "grow" tentacular structures on the support surface. It is hypothesized that a branched tentacle or tree-like structure resulted during the multiple rounds of reaction with epichlorohydrin, which ultimately yielded the best support (VIII). Under the alkaline conditions employed, both the epoxy and chlorine groups of the epichlorohydrin are reactive and a variety of hydrolysis products, and thus structures can be expected to form.[20] However, due to the large excess of water present, very limited reaction between the hydrolyzed epoxy groups and the chlorinated carbons is envisaged[21] and thus polymerization per se, leading to linear tentacles, is not expected. Rather, it is speculated that hydroxyls would be formed by hydrolysis of immobilized epoxy groups during the first activation step (Fig. 1) and that during the long 16h reaction with epichlorohydrin, these hydroxyls permitted attachment of more epoxy groups, which in turn would hydrolyse to generate yet further reactive hydroxyls. Such a process is envisaged to lead to a branched oligomeric intermediate somewhat akin to that depicted in Fig. 1, which following secondary activation with epichlorohydrin, and coupling of sulphite is expected to produce a tree-like cation exchange structure. The success of in situ formation

Downloaded At: 10:03 25 January 2011

Downloaded At: 10:03 25 January 2011

of branched tentacle tree-like structures merits further examination. There is reason to believe that improved cation-exchangers can be prepared simply by reacting such supports with bromoethane sulphonic acid. Moreover, branched epichlorohydrin-oligomers bearing particles may possibly be employed as a starting material for creation of superparamagnetic adsorbents with binding interactions based on other principles, such as affinity.

The highest lysozyme binding capacity of the supports produced without tentacles was $>200\,\mathrm{mg\,g^{-1}}$ for support III, which is very similar to the capacities found by O'Brien et al.[2] using metal chelators ($\sim 200\,\mathrm{mg\,g^{-1}}$) or Hubbuch et al.[1] for protease adsorbents ($90-250\,\mathrm{mg\,g^{-1}}$). The specific surface area of polyglutaraldehyde-coated supports identical to those used in the preparation of the cation-exchangers has been determined to be $110\,\mathrm{m^2\,g^{-1}}$,[4,22] and the lysozyme Q_{max} values found for supports type III and VIII thus corresponded to $2.1\,\mathrm{mg\,m^{-2}}$ and $2.5\,\mathrm{mg\,m^{-2}}$, respectively. A lysozyme monolayer on a negatively charged polystyrene surface has been reported to give a protein loading of $2.3\,\mathrm{mg\,m^{-2}}$ when the adsorption took place at pH 7.[23] Thus it appears that there was good utilization of the available surface area for support type III where ethane sulphonic acid groups were attached directly to the surface aldehydes. This evidence further supports our contention that the higher Q_{max} value for the type VIII adsorbents is due to the increase in activation sites on the tentacles arising from the complex epichlorohydrin activation.

The importance of adsorbents with a high protein binding capacity was highlighted in the HGMF separation of lactoperoxidase from whey. A support concentration of $\sim 2.5\,\mathrm{g\,L^{-1}}$ led to capture of 90% of the lactoperoxidase (as well as the majority of the lactoferrin) and a five-fold concentrated eluate. The concentrating power of HGMF is governed largely by the ratio of the adsorbent concentration during elution in the recycle loop (fixed by the filter capacity) to that in the binding suspension. The latter is determined by the capacity of the supports for the protein of interest, and if the binding capacity is low, little protein will be captured, as was observed by Zulqarnain.[4] In that work it was not possible to concentrate native malate dehydrogenase from a yeast homogenate using $1.7\,\mathrm{g\,L^{-1}}$ cibacron blue F3GA functionalized adsorbents in a HGMF process. The maximum binding capacity was only $120\,\mathrm{mg\,g^{-1}}$, and an adsorption yield of only 47% was observed.[4]

Additional criteria for the success of a support for use in batch adsorption operations are a low K_d value and selectivity for the target of interest. The dissociation constants observed here generally indicated very tight interactions between lysozyme and the ion-exchange supports. The K_d values (as low as $0.6\,\mu\mathrm{M}$) were in the same order as those reported for affinity-based magnetic supports such as the benzamidine-trypsin system of Hubbuch and Thomas[5] ($K_d \sim 0.3\,\mu\mathrm{M}$) and the yeast dehydrogenase-biomimetic cibacron blue system

Downloaded At: 10:03 25 January 2011

(K_d values of 0.1–1.2 μM) of Zulqarnain.[4] Furthermore, when the cation-exchangers were added directly into sweet whey, highly selective adsorption was found and lactoperoxidase was purified 13-fold in that step. The peak in purification of lactoperoxidase in the binding step at $3\,g\,L^{-1}$ adsorbents (Fig. 4) was not expected, but is likely due to competition for the binding sites from lactoferrin, and possibly lysozyme, at low support concentrations. At high support concentrations, an increase in the amount of protein bound nonspecifically probably occurred.

In addition to the protein binding properties of the support, the capacity of the filter for adsorbents influences concentration factors. In the current work, filter capacities ($65\,g\,L^{-1}$) were equivalent to those reported by Hubbuch[24] for very similar types. Furthermore, for the recovery of lysozyme using $1\,g\,L^{-1}$ supports in the batch-binding reaction used here, the maximum concentration factor was 15 for the system set-up employed. However, the highest concentration factor observed in the elution fractions was 10 and the overall factor was 5 for the pool of the first elution cycle. The low overall concentration factor results from a combination of variables, including incomplete adsorption of the lysozyme (86%) and only ~80% elution of the bound enzyme in each cycle. However, the volume of the elution cycle (50 mL) was double that of the recycle loop, since extra liquid was used to flush out the system lines in order to increase product recovery. Thus optimization of the system parameters and operation merits tackling in future studies.

CONCLUSIONS

Superparamagnetic cation exchange adsorbents can be applied to the task of selective capture and concentration of lactoferrin and lactoperoxidase present in minute concentrations in whey, using HGMF processing. When coupled with the high feeding rates possible during adsorbent capture, HGMF is thus an ideal adsorptive technique generally applicable to feedstocks with low concentrations of target protein. Furthermore, it is an important advantage of HGMF that the technique permits direct treatment of unclarified feedstreams. For optimal application of HGMF, very high protein binding capacities are required in order to achieve good yields and high purification factors. It is therefore important to fully utilize the large specific surface area of the base matrix, in order to yield adsorbents with protein capacities $>200\,mg\,g^{-1}$. At such capacities, tightly packed monolayers of protein are postulated to exist on the support surfaces used and thus an increase of adsorbent capacity requires enhanced surface features. Such surface features can be generated through multiple rounds of reaction with epichlorohydrin prior to activation and coupling.

ACKNOWLEDGMENTS

We thank the Danish Technical Research Council (Statens Teknisk-Videnskabelige Forskningsråd, STVF) for supporting the work carried out here. The assistance of Lektor Klaus Bukhave with the labeling studies is also gratefully acknowledged.

Downloaded At: 10:03 25 January 2011

REFERENCES

1. Hubbuch, J.J.; Matthiesen, D.B.; Hobley, T.J.; Thomas, O.R.T. High gradient magnetic separation versus expanded bed adsorption: a first principle comparison. Bioseparation **2001**, *10*, 99–112.
2. O'Brien, S.M.; Thomas, O.R.T.; Dunnill, P. Non-porous magnetic chelator supports for protein recovery by immobilised metal affinity adsorption. J. Biotechnol. **1996**, *50*, 13–26.
3. O'Brien, S.M.; Sloane, R.P.; Thomas, O.R.T.; Dunnill, P. Characterisation of non-porous magnetic chelator supports and their use to recover polyhistidine-Tailed T4 lysozyme from a crude. *E. coli.* extract. J. Biotechnol. **1997**, *54*, 53–67.
4. Zulqarnain, K. Scale-up of Affinity Based Separation Based on Magnetic Support Particles; University College London: London, 1999; Ph. D. Thesis.
5. Hubbuch, J.J.; Thomas, O.R.T. High-gradient magnetic affinity separation of trypsin from porcine pancreas. Biotechnol. Bioeng. **2002**, *79*, 301–313.
6. Heebøll–Nielsen, A.; Dalkiær, M.; Hubbuch, J.J.; Thomas, O.R.T. Superparamagnetic adsorbents for high-gradient magnetic fishing of lectins out of legume extracts. Biotechnol. Bioeng. **2004**, *87*, 311–323.
7. Xue, B.; Sun, Y. Fabrication and characterization of a rigid magnetic matrix for protein adsorption. J. Chromatogr. A **2002**, *947*, 185–193.
8. Geberding, S.J.; Byers, C.H. Preparative ion-exchange chromatography of proteins from dairy whey. J. Chromatogr. A **1998**, *808*, 141–151.
9. Ekstrand, B. Antimicrobial factors in milk—A review. Food Biotechnol. **1989**, *3*, 105–126.
10. Wolfson, L.M.; Sumner, S.S. Antibacterial activity of the lactoperoxidase system: a review. J. Food Prot. **1993**, *56*, 887–892.
11. McIntosh, G.H.; Royle, P.J.; Leu, R.K.L.; Regester, G.O.; Johnson, M.A.; Grinsted, R.L.; Kenward, R.S.; Smithers, G.W. Whey proteins as functional food ingredients. Int. Dairy J. **1998**, *8*, 425–434.
12. Shimazaki, K. Lactoferrin: a marvellous protein in milk. Anim. Sci. J. **2000**, *71*, 329–347.

13. Vasavada, P.C.; Cousin, M.A. Dairy microbiology and safety. In *Dairy Science and Technology Handbook*; Hui, Y.H., Ed.; 2 Product Manufacturing; VCH Publishers Ltd.: Cambridge, UK, 1993.
14. Thömmes, J. Fluidized bed adsorption as a primary recovery step in protein purification. Adv. Biochem. Eng. Biotechnol. **1997**, *58*, 185–230.
15. Heebøll-Nielsen, A.; Choe, W.S.; Middelberg, A.P.J.; Thomas, O.R.T. Efficient inclusion body processing using chemical extraction and high gradient magnetic fishing. Biotechnol. Progr. **2003**, *19*, 887–898.
16. Hermanson, G.T.; Mallia, A.K.; Smith, P.K. *Immobilized Affinity Ligand Techniques*; Academic Press: London, UK, 1992.
17. Monsan, P.; Puzo, G.; Mazarguil, H. Étude du mécanisme d'établissement des liaisons glutaraldéhyde-protéines. Biochimie **1975**, *57*, 1281–1292.
18. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **1970**, *227*, 680–685.
19. Hoffmann, C.; Franzreb, M.; Höll, W.H. A novel high gradient magnetic separator (HGMS) design for biotech applications. IEEE Trans. Appl. Superconduct. **2002**, *12*, 963–966.
20. Yoneda, G.S.; Griffin, M.T.; Carlyle, D.W. A kinetic study of the reaction between sulfite ion and propylene oxide. J. Org. Chem. **1975**, *40*, 375–377.
21. Royappa, A.T. On the copolymerisation of epichlorohydrin and glycidol. Int. J. Appl. Poly. Sci. **1997**, *65*, 1897–1904.
22. Hoffmann, C. Einsatz Magnetischer Separationsverfahren zur Biotechnologischen Produktaufarbeitung; Forschungszerntrum Karlsruhe: Germany, 2002; Ph.D. Thesis.
23. Haynes, C.A.; Sliwinsky, E.; Norde, W. Structural and electrostatic properties of globular proteins at a polystyrene–water interface. J. Colloid Interf. Sci. **1994**, *164*, 394–409.
24. Hubbuch, J.J. Development of Adsorptive Separation Systems for Recovery of Proteins from Crude Bioprocess Liquors; The Technical University of Denmark: Denmark, 2001; Ph. D. Thesis.

Downloaded At: 10:03 25 January 2011