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One-Step Purification of Lactoferrin from Crude Sweet Whey Using Cation-Exchange Expanded Bed Adsorption

Qiao-Yan Du,[†] Dong-Qiang Lin,[‡] Zhan-Sen Xiong,[†] and Shan-Jing Yao*[,†]

[†]Key Laboratory of Biomass Chemical Engineering of Ministry of Education, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China

[‡]State Key Laboratory of Chemical Engineering, Zhejiang University, Hangzhou 310027, China

ABSTRACT: In order to purify lactoferrin from crude sweet whey in one step, an expanded bed adsorption process was proposed with cation-exchanger Fastline SP. A series of experiments were carried out to assess the possibility of this process and optimize the operating conditions. First the adsorption equilibrium and kinetics of lactoferrin with Fastline SP were measured. The results indicated that Fastline SP had a fast adsorption of lactoferrin and was suitable for high-velocity operation. Then the expansion properties, liquid mixing, and breakthrough behaviors in the expanded bed were investigated, and the expansion factor was set at 2.0. In addition, the elution condition was optimized as 0.5 M NaCl in the phosphate buffer (pH 7.0). Finally, lactoferrin was successfully recovered with a high purity of 88.5% and a reasonable recovery of 77.1% in a single step. The purification factor reached 553.

1. INTRODUCTION

Sweet whey, a byproduct of the dairy industry, is a fluid obtained by separating the coagulum from whole milk, cream, or skim milk with rennet-type enzymes at about pH 5.6.[1] Historically, sweet whey was considered as waste stream and a nuisance by cheesemakers and casein manufacturers alike.[2] Currently, the composition and potential value of sweet whey has been recognized.[3,4] The most useful components in sweet whey are lactose and whey proteins, about 75% and 11% of total whey solids, respectively. Whey proteins contain mainly α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), immunoglobulins, lactoferrin, and lactoperoxidase.[5] Typical concentrations, molecular weights, and isoelectric points of whey proteins are given in Table 1.[6−9]

Table 1. Typical Concentrations, Molecular Weights, and Isoelectric Points of Whey Proteins

protein	approximate concn in whey (g/L)	molecular weight (kDa)	isoelectric point
β-lactoglobulin	3−4	18.4	5.35−5.49
α-lactalbumin	1.5	14.2	4.2−4.5
BSA	0.3−0.6	66.4	5.13
immunoglobulins	0.6−0.9	150−900	5.8−7.3
lactoferrin	0.03−0.1	87	8.0
lactoperoxidase	0.01−0.03	89	9.2−9.9

Among various valuable whey proteins, lactoferrin is of growing concern because of its biological functions, such as antimicrobial, antiinflammatory, anticarcinogenic, immunomodulatory, and bone growth factor.[10,11] Lactoferrin has a strong basic character with a pI of about 8.0, which allows it to be isolated from sweet whey by cation-exchange chromatography. The major impurity is lactoperoxidase, which has a pI of 9.2−9.9. The concentration of lactoperoxidase is usually very low and could be ignored.[12] Some authors have isolated lactoferrin from raw whole milk,[13] rennet whey,[14] and bovine colostrum[15] by strong cation-exchange chromatography. In addition, gel filtration,[16] chelating chromatography,[17] affinity chromatography,[18] ultrafiltration,[19] and membrane adsorption with a cation ligand[20] or an affinity ligand[21] were also used. However, for all of these separation techniques, preclarification of the feedstock is required; otherwise, the column and membrane will be fouled or even blocked by the solid content in the stock. As we know, the clarification procedure is time-consuming and also leads to high operation costs and low recovery. Currently, some new techniques have been adopted to directly capture the target protein from the particle-containing feedstock, in which the most common process is expanded bed adsorption (EBA).

With specially designed adsorbents and columns, EBA allows the adsorption of target proteins directly from an unclarified feedstock, such as fermentation broth, cell homogenate, or crude extract.[22] EBA technology integrates the clarification, concentration, and primary purification into a unit operation, which certainly increases the overall yield and reduces the operational time and the cost for capital investment and consumables.[23] It has been demonstrated that EBA is able to process a large volume of feedstock with a high operation flow that is very favorable in industry. EBA technology is widely used in rough extraction instead of accurate extraction. Fortunately, with a suitable resin, EBA may be used for refinement. There have been some applications in the purification of many proteins with multisteps, such as lysozyme, monoclonal antibodies,[24,25] and so on but few for lactoferrin. Lin et al.[12] separated lactoferrin from whey by EBA with a mixed-mode adsorbent (Streamline Direct HST). A mixed-mode adsorbent is a new resin with both ionic and hydrophobic ligands that enable one to bind proteins with a high capacity

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even in high ionic strength.[26,27] In whey proteins, there are many hydrophobic sites on the molecular surfaces of BSA, IgG, and so on. Therefore, all whey proteins except α-lactalbumin could be adsorbed to Streamline Direct HST at pH 4.7, and then a pH-step elution must be used to wash out the other proteins before 0.02 M NaOH was loaded to desorb lactoferrin. The recovery of lactoferrin was 87.8%, and the purification factor reached 10.5. Lihme and Otto[28] from Upfront Chromatography A/S invented a process of isolating lactoferrin from pretreated whey by a mixed-mode adsorbent. The feedstock with a pH of 4.5 was loaded onto a column packed with FastLine SALTO comprising an aromatic acid ligand. Because of the weak selectivity of the mixed-mode adsorbent, a two-stage elution must be used to obtain lactoferrin with a high purity of 90%. The bound impurities were eluted with 0.05 M boric acid, and then the lactoferrin was eluted with 0.02 M NaOH. As mentioned above, the selectivity of the mixed-mode adsorbent is too poor to capture lactoferrin specifically from the complex whey. As a result, a complicated elution process must be used to recover pure lactoferrin. To avoid the cumbersome elution procedure, a cation exchanger is an alternative. Because of the special high p*I* of lactoferrin, a cation exchanger might adsorb lactoferrin more specifically than a mixed-mode adsorbent. In addition, a cation exchanger is more common and cheaper for large-scale use.

The purpose of this study was to demonstrate that it is possible to purify a certain protein from an untreated mixture in only one step using a simple and effective method. EBA with a strong cation exchanger called Fastline SP was used for this purpose because EBA allows a crude feedstock with solid content while Fastline SP has a relatively stronger specificity. A series of experiments would be carried out to assess the possibility of this program.

2. EXPERIMENTAL SECTION

2.1. Materials. Fastline SP was purchased from Upfront Chromatography A/S, Koebenhavn, Denmark. The resin combines cross-linked agarose weighted by incorporating high-density particulates of tungsten carbide. The size distribution is at the range of 20−200 μm, and the average wet density is 2.44 g/mL. Sweet whey powder (SWP) was a gift from Beingmate Co. (Hangzhou, China). Lactoferrin from bovine milk (L9507) was ordered from Sigma (Milwaukee, WI). Other reagents were of analytical grade and were purchased commercially.

2.2. Adsorption Equilibrium. The adsorption equilibrium of lactoferrin on Fastline SP was measured using the stirred-batch adsorption method in 0.02 M, pH 7.0 phosphate-buffered saline (PBS) containing different salt concentrations (0, 0.2, and 0.4 M) at 25 °C as published previously.[29]

The adsorption isotherms were correlated with the Langmuir equation as follows:

$$Q^* = \frac{Q_m c^*}{K_d + c^*} \tag{1}$$

where Q^* is the equilibrium adsorption capacity and c^* is the equilibrium concentration of protein in the bulk solution. Q_m is the maximum adsorption capacity, and K_d is the apparent dissociation coefficient.

2.3. Adsorption Kinetics. The adsorption kinetics experiment of lactoferrin to Fastline SP was performed in a shaking incubator at 200 rpm and 25 °C. A total of 0.5 mL of drained gel was previously equilibrated by PBS and then mixed with a 50 mL lactoferrin solution (0.5 mg/mL) in the same buffer. The liquid was pumped through a K2600 spectrophotometer (Knauer, Berlin, Germany) to determine the protein concentration at 280 nm and then circulated back to the solution in a flask.

2.4. Expansion and Liquid Mixing in an Expanded Bed. A homemade column for EBA (0.02 m diameter and 1.0 m length) was used. A small amount of carbon steel beads (0.5 mm diameter, 3.6 g/mL density, and <5% total sedimented bed height) was added to improve the flow distribution at the column inlet. A movable adapter was employed to adjust the position of the liquid outlet to the top of the expanded bed. The fluid was transported using a peristaltic pump (Longer Precision Pump Co. Ltd., Baoding, China). Proper column vertical alignment was assured in all experiments. The experiments were performed at room temperature.

Determinations of the bed expansion and liquid mixing in an expanded bed were performed as described in our previous works.[30,31]

The sedimented bed height (H_0) was 15.9 cm in the present work. The bed height was measured after equilibrium expansion for 30 min, and then the bed expansion factor and bed voidage were calculated.

The expansion characteristics were described by the well-known Richardson−Zaki correlation:[32]

$$U = U_t \varepsilon^n \tag{2}$$

The equation correlates the voidage of the expanded bed (ε) with the superficial liquid velocity (U) by two parameters: the terminal settling velocity of the particle (U_t) and the expansion index (n). The value of ε can be calculated from the following equation:

$$E = \frac{H}{H_0} = \frac{1 - \varepsilon_0}{1 - \varepsilon} \tag{3}$$

where the expansion factor (E) represents the ratio of the expanded bed height (H) to the sedimented bed height (H_0). The voidage of the sedimented bed ε_0 is usually appointed to 0.4.[24]

The test of the residence time distribution (RTD) was used to describe the liquid mixing behavior in the expanded bed. In each test, a 0.5 mL acetone solution (10%, w/w) was injected at the bottom inlet of the column as a tracer agent. The theoretical plate number (N), the Bodenstein number (Bo), and the axial dispersion coefficient (D_{ax}) are calculated to evaluate the liquid mixing in the expanded bed. According to the response curve of RTD measurement, N, Bo, and D_{ax} can be calculated from eqs 4−6.

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \tag{4}$$

where t_R and $W_{1/2}$ represent the retention time and half-peak width, respectively.

$$\frac{1}{N} = \frac{2}{Bo} + \frac{8}{Bo^2} \tag{5}$$

$$Bo = \frac{UH}{\varepsilon D_{ax}} \tag{6}$$

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2.5. Dynamic Binding Capacity in an Expanded Bed. The dynamic binding capacity was determined in packed and expanded beds, respectively. Lactoferrin (1 mg/mL) in PBS was used as the feedstock.

For a packed bed, a XK 16/20 column (GE Healthcare, Uppsala, Sweden) packed with 12 mL of Fastline SP adsorbent was used. After equilibrium with PBS, a lactoferrin solution was loaded onto the column at different flow velocities (30 and 76 cm/h) and detected by a UV detector at 280 nm. All of the procedures were executed with an ÄKTA Explorer 100 chromatographic system (GE Healthcare, Uppsala, Sweden).

For an expanded bed, the settled bed height of a Fastline SP resin was 17 cm. PBS was used as the mobile phase, and three expansions of 2.0, 2.2, and 2.4 were tested. After expansion equilibrium, a lactoferrin solution was loaded and the output signal was detected by a UV detector (WellChromfast K-2600 scanning spectrophotometer, Knauer, Berlin, Germany) and recorded.

The dynamic binding capacity, $Q_{10\%}$ (mg/mL of settled adsorbent), was calculated as[33]

$$Q_{10\%} = \frac{C_0 \int_0^V \left(1 - \frac{C}{C_0}\right) dV_{10\%}}{V_s} \quad (7)$$

where C and C_0 are the protein concentrations of the outlet and initial fluids, respectively, $V_{10\%}$ is the loading volume at a 10% breakthrough point, and V_s is the settled volume of the adsorbent.

2.6. Optimization of Elution Conditions in a Packed Bed. A C10/20 column (GE Healthcare, Uppsala, Sweden) was packed with 2 mL of Fastline SP adsorbent and was equilibrated with PBS. SWP was dissolved in PBS to a concentration of 50 g/L to simulate the natural sweet whey as the feedstock. Sweet whey was clarified by centrifugation (3000 rpm) and microfiltration (0.45 μm). A sample of 11 mL was applied to the column at 5 mL/min (382 cm/h). After sample loading, the column was washed with PBS, and then the linear gradient elution was performed by changing the PBS buffer or 0.02 mol/L, pH 10.0 sodium bicarbonate buffer (BBS) to the corresponding initial buffer containing 1 M NaCl in 10 CV. Finally, the column was cleaned by 0.5 M NaOH and reequilibrated by PBS. All of the procedures were executed with an ÄKTA Explorer 100 chromatographic system.

2.7. Isolation of Lactoferrin from Sweet Whey with an Expanded Bed. The expanded bed was filled with 50 mL of Fastline SP corresponding to a settled bed height of 17 cm. The bed was expanded and equilibrated with PBS at a flow rate of 34 mL/min (650 cm/h) until a constant bed height was obtained. The bed expansion was about 2.0. A total of 1 L sweet whey (50 g/L) was then loaded into the bed, followed by washing with PBS to remove unbound proteins. The elution was carried out with 0.5 M NaCl in PBS. The bed was cleaned by 0.5 M NaOH and regenerated by PBS.

2.8. Protein Determination. Protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), under nonreducing conditions, with 10% resolving and 5% stacking discontinuous gel conditions. The purity of lactoferrin was quantified by Bio-Rad GelDoc 2000 and Quantity One Quantitation software (Bio-Rad, Hercules, CA). An enzyme-linked immunosorbent assay (ELISA) kit was used to quantify lactoferrin specifically and a BCA protein assay kit for the total protein content. The bovine lactoferrin ELISA kit (E-d0223) was purchased from Ebioeasy Co. (Shanghai, China) and the BCA protein assay kit (A045-3) from Jiancheng Bioengineering Institute (Nanjing, China). All of the operations were in accordance with the instructions.

3. RESULTS AND DISCUSSION

3.1. Adsorption Equilibrium. Because the isoionic point of lactoferrin is about 8.0 and Fastline SP is a kind of cation exchanger, pH 7.0 was chosen for lactoferrin adsorption in the present work. The adsorption isotherms at different NaCl concentrations are shown in Figure 1. The maximum

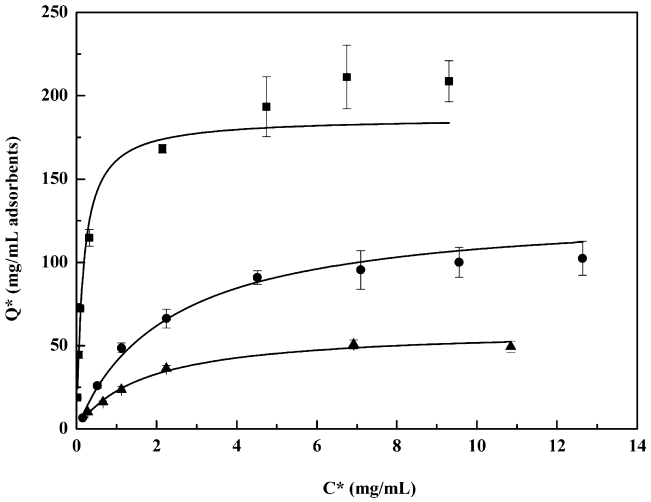

Figure 1. Adsorption isotherm curves of lactoferrin to Fastline SP at pH 7.0: (■) 0 M NaCl; (●) 0.2 M NaCl; (▲) 0.4 M NaCl.

adsorption capacity (Q_m) and dissociation coefficient (K_d) were obtained by fitting the experimental data with eq 1. Q_m of lactoferrin in PBS at pH 7.0 reached 186.87 mg/mL adsorbent. With an increase of the salt concentration, the adsorption capacity decreased significantly because of weakened electrostatic interactions between the protein and adsorbent. This result indicated that lactoferrin might be adsorbed with Fastline SP at pH 7.0 and eluted by a high NaCl concentration. In addition, it was interesting to find that K_d in PBS was 0.16 mg/mL, which meant that lactoferrin could be adsorbed effectively in quite low concentration. This should benefit the separation of lactoferrin from whey.

Table 2. Maximum Adsorption Capacity Q_m and Apparent Dissociation Coefficient K_d at 25 °C Correlated with the Langmuir Equation

	NaCl concn		
	0 M	0.2 M	0.4 M
Q_m (mg/mL adsorbent)	186.87	132.41	60.26
K_d (mg/mL)	0.16	2.28	1.66

3.2. Adsorption Kinetics. The adsorption kinetics of lactoferrin to Fastline SP was studied, and the results are shown in Figure 2. The experimental data were fitted with the pore diffusion model (PDM) model to assess the effective pore diffusivity (D_e).[34,35] The effective particle porosity ε_p of Fastline SP for lactoferrin was 0.35, determined by the method of Zhang and Sun.[36] The diffusion coefficient of lactoferrin in a free solution (D_{AB}) was set as 5.6×10^{-12} m^2/s.[37] It could be seen

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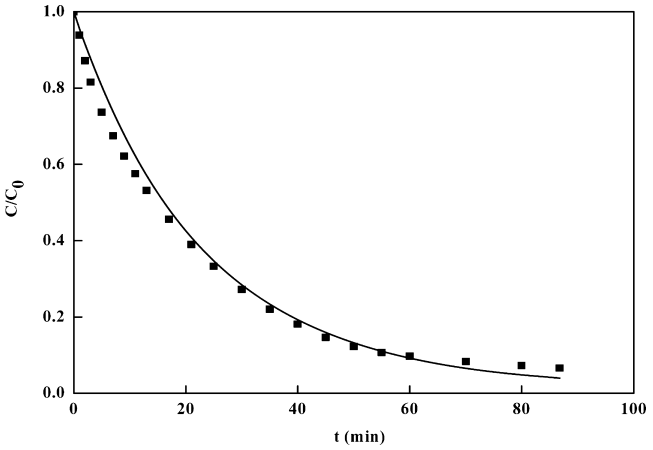

Figure 2. Adsorption kinetics curve of lactoferrin to Fastline SP at pH 7.0.

that the PDM could correlate the adsorption kinetics well. The value of D_e for lactoferrin was $(2.01 \pm 0.41) \times 10^{-12}$ m^2/s, similar to the previous data.[34] The results indicated that the diffusion of lactoferrin in the resin was fast, and Fastline SP might be used for the adsorption of lactoferrin under a high operation velocity.

3.3. Expansion Properties and Liquid Mixing in an Expanded Bed. In the experiments, it was found that some amount of adsorbents on the top of the expanded bed became unstable and dispersed when the expansion factor exceeded 2.8. This might be mainly due to the small particles (20−35 μm) of Fastline SP. Figure 3 shows the bed expansion as a function of

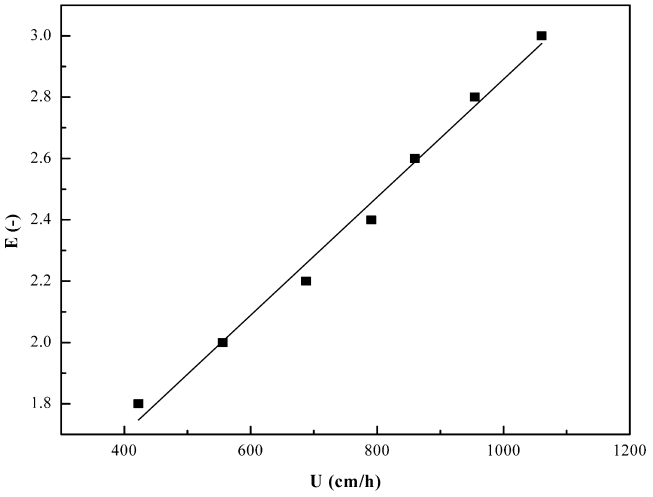

Figure 3. Bed expansion as a function of the aflow velocity for Fastline SP.

the flow velocity for Fastline SP. The bed expansion increased with an increase of the superficial velocity. Typically, for the expansion factor of 2.0, the superficial velocity was 556 cm/h, which was quite higher than that for Streamline series adsorbents.[38] The Richardson−Zaki equation was used to correlate the flow velocity and bed voidage, as shown in Figure 4. The values of n and U_t for Fastline SP were calculated to be 4.92 and 3181 cm/h, respectively, which were similar to other EBA absorbents with tungsten carbide as the densifier.[39]

Liquid mixing in the expanded bed is an important property for evaluating the bed stability and protein adsorption. As is

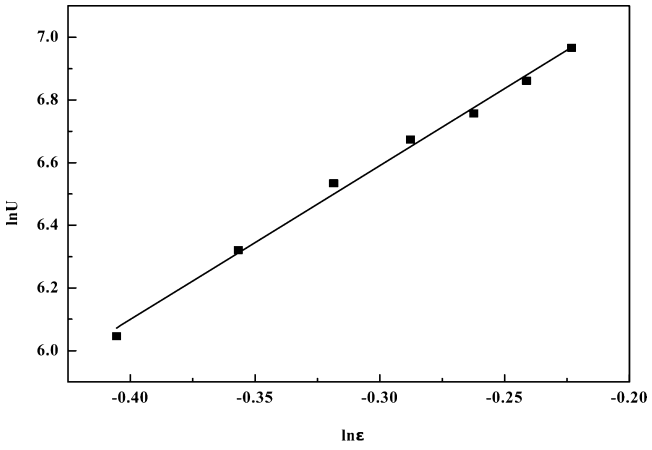

Figure 4. Richardson−Zaki correlation between the flow velocity and bed voidage for Fastline SP.

known, the *Bo* number relating the convective transport of liquid to the dispersion describes the possible influence of axial mixing on the performance of EBA operation. It is considered that the flow pattern is similar to plug flow when $Bo > 40$.[40] The *Bo* numbers under corresponding operation velocities are shown in Figure 5. All *Bo* values obtained were higher than 40.

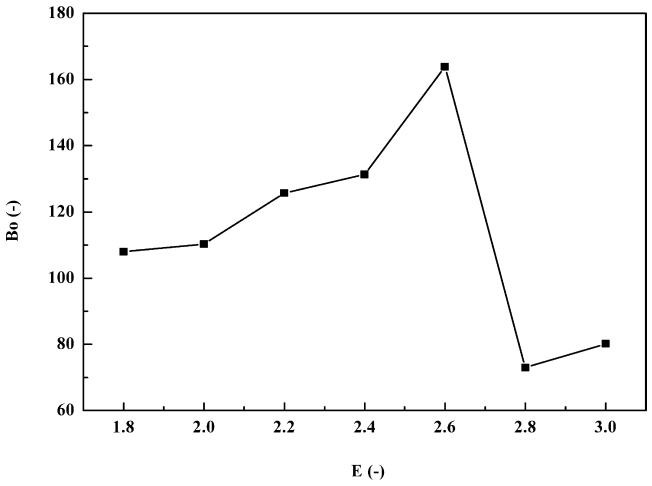

Figure 5. *Bo* number as a function of the expansion factor for Fastline SP.

It was also found that the value of *Bo* increased slightly with increasing expansion factor in a range from 1.8 to 2.6. This trend has also been reported by several researchers.[24,38,41] However, once the expansion factor exceeded 2.8, the *Bo* number descended dramatically because of the unstable bed disturbed by excessive flow rate.

The axial mixing coefficient (D_{ax}), representing the axial diffusion degree, was calculated. Small D_{ax} means lower back-mixing and a more stable bed. D_{ax} versus fluid velocity is shown in Figure 6. The value of D_{ax} increased with an increase of the fluid velocity. The motion of suspended particles in the bed appeared much disturbed at high fluid, resulting in enhanced axial mixing and weakened bed stability, especially for expansion by more than 2.8. It was reported that the value of D_{ax} should be kept at a level of 10^{-6} m^2/s to meet the requirement of expanded bed operation.[42]

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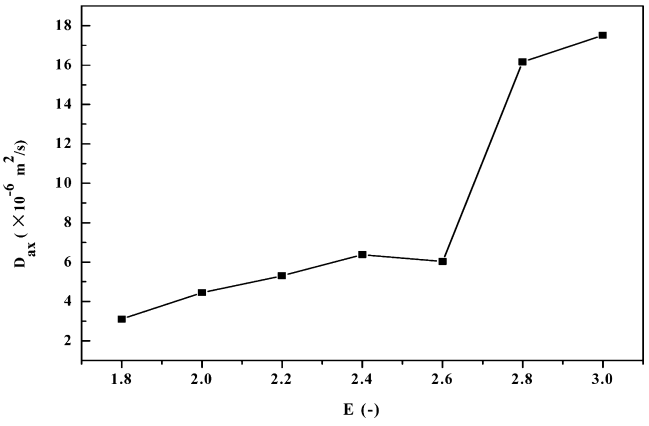

Figure 6. Axial mixing coefficient as a function of the expansion factor for Fastline SP.

On the basis of the results of expansion properties and liquid mixing, it could be concluded that Fastline SP might be more suitable for expansion in the range of 2.0−2.4.

3.4. Dynamic Binding Capacity. Frontal analysis of the breakthrough curve was used to determine the dynamic binding capacity in packed and expanded beds, respectively. In a packed bed, the velocity cannot be too high; otherwise, the pressure in the column will exceed the rated action value. At flow rates of 30 and 76 cm/h, the dynamic binding capacity $Q_{10\%}$ could reach 60.88 and 34.74 mg/mL adsorbent, respectively (Figure 7). For an expanded bed, $Q_{10\%}$ was 22.11, 18.22, and 15.10 mg/

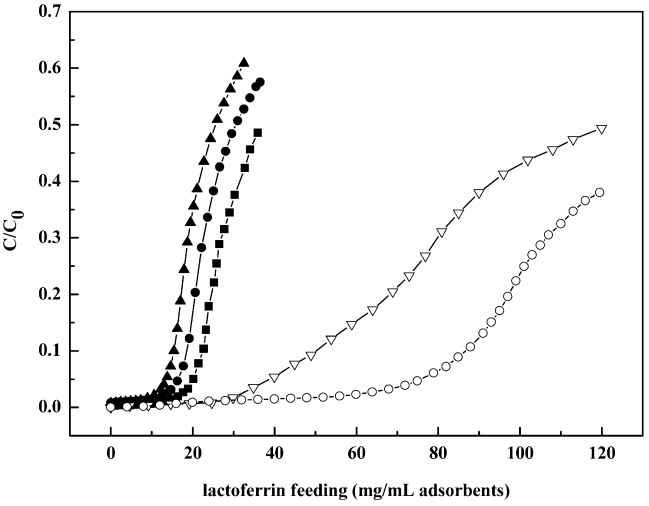

Figure 7. Breakthrough curve of lactoferrin to Fastline SP at different expansions in a bed: (■) 636 cm/h (expansion of 2.0) in an expanded bed; (●) 717 cm/h (expansion of 2.2) in an expanded bed; (▲) 802 cm/h (expansion of 2.4) in an expanded bed; (□) 76 cm/h in a packed bed; (○) 30 cm/h in a packed bed.

mL adsorbent for expansion of 2.0, 2.2, and 2.4, respectively, corresponding 636, 717, and 802 cm/h (Figure 7). The dynamic binding capacities in the expanded bed were lower than those in the packed bed, mainly because of the high flow rates for the expanded bed. The dynamic binding capacities were about 13.9−56.2% of the corresponding equilibrium adsorption capacity at same protein concentration. With an increase of the flow velocity, the resident time of protein in the bed was reduced, leading to a decrease of the dynamic binding capacity. Therefore, an expansion factor of 2.0 was chosen for the following separation experiments.

3.5. Separation of Lactoferrin from Sweet Whey by EBA. The component of the feedstock was first analyzed by SDS-PAGE. However, it was hard to distinguish lactoferrin and lactoperoxidase with SDS-PAGE because of their similar molecular weights (89 and 87 kDa). Thus, the band with a mobility of about 90 kDa was assumed to be a mixture of lactoferrin and lactoperoxidase. On the basis of the electrophoretogram of SDS-PAGE (Figure 9), the proportion of this mixture in whey proteins was about 1.8%; meanwhile, the total protein content of SWP was 9.39% determined by a Kjeldahl nitrogen method, so the content of lactoferrin and lactoperoxidase in SWP was calculated as 0.17%. On the other hand, with ELISA the content of lactoferrin was determined as 0.16%. As is known, ELISA has the advantage of high specificity and should not be affected by other whey proteins. The results of SDS-PAGE and ELISA indicated that the content of lactoperoxidase in SWP was quite low and could be ignored. The content of lactoferrin in SWP was set as 0.16%. Consequently, the lactoferrin concentration in the feedstock (50 g of SWP/L) was about 0.080 g/L. On the basis of the results of adsorption equilibrium and dynamic binding capacity, the sample loading was set to 1 L, corresponding to 0.080 g of lactoferrin.

The elution condition was optimized in a packed bed by comparing the recovery of lactoferrin in the elution fraction. Two elution strategies were tested, while other conditions were kept consistent. One was linear gradient elution from PBS to PBS containing 1 M NaCl, which is shown in Figure 8A. The other one was the linear gradient from BBS to BBS containing 1 M NaCl, which is shown in Figure 8B. The recoveries of two elution strategies were very similar, 94.3% and 95.6%, respectively. As shown in Figure 8B, the sudden change of the pH from 7.0 to 10.0 had some lagging effects and generated an asymmetrical elution peak. On the basis of the conductance of effluent at the elution peak of lactoferrin, the optimum eluting conditions were chosen as PBS containing 0.5 M NaCl for a simple and convenient procedure.

According to the above results, EBA was conducted under the following conditions: loading of 1 L of feedstock and elution by PBS containing 0.5 M NaCl. An elution fraction of 268 mL was collected. To determine the purity and recovery of lactoferrin, several methods were used including SDS-PAGE, ELISA, and BCA protein assay kits. The lactoferrin concentration in the eluent was not high enough for SDS-PAGE. Therefore, the eluent was concentrated by ultrafiltration with a cassette membrane with a molecular weight cutoff of 10 kDa (PALL Minimate system, PALL, Port Washington, NY). Figure 9 shows SDS-PAGE analysis of the concentrated protein fractions collected. It could be found that the main band of the elution fraction had the same mobility as that of lactoferrin. There was still some amounts of impurities with molecular weights higher than 200 kDa, which might be the aggregated IgG. Because of the insufficient resolution of SDS-PAGE, quantitative assays with the ELISA and BCA protein assay kits were adopted to determine the nonconcentrated eluent. The concentrations of lactoferrin and total protein were 0.23 and 0.26 mg/mL, respectively. The results indicated that the purity of lactoferrin could reach 88.5%, and the recovery was 77.1%. The purification factor reached 553 in a single EBA step.

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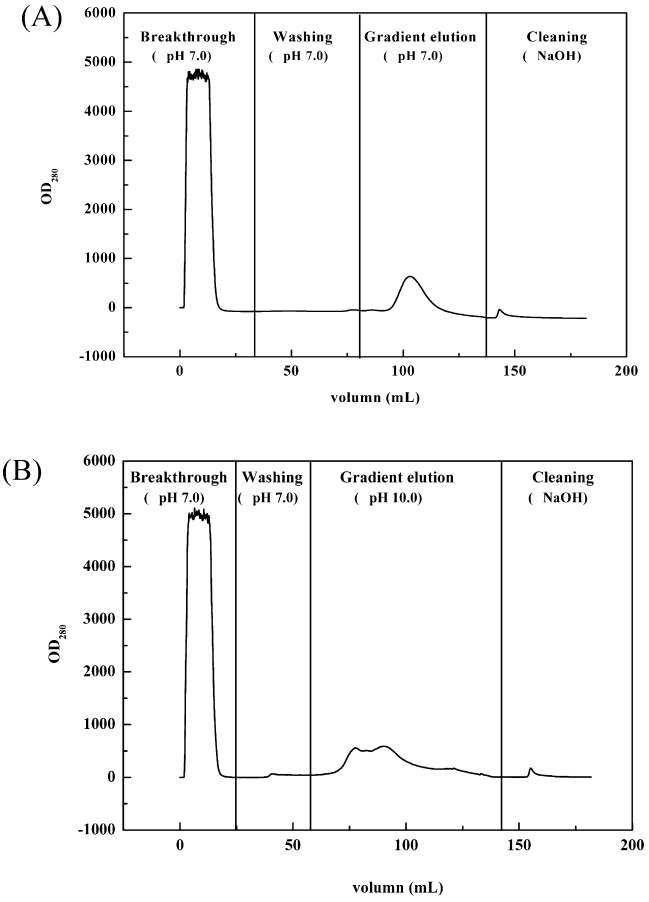

Figure 8. Optimization of the elution condition in a packed bed: (A) linear gradient elution from PBS to PBS containing 1 M NaCl; (B) linear gradient elution from BBS to BBS containing 1 M NaCl.

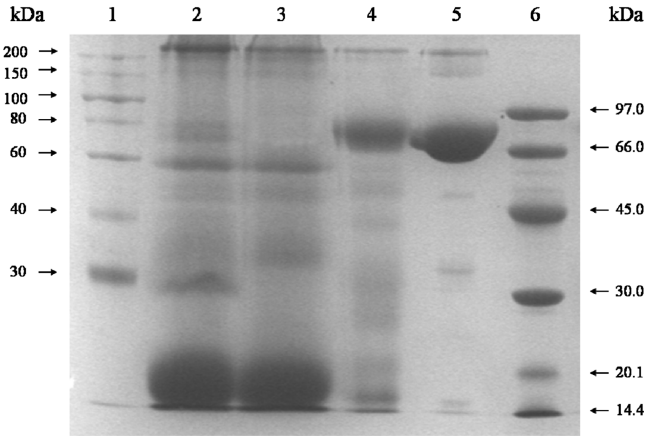

Figure 9. SDS-PAGE of protein fractions collected in EBA operation. Lane 1: ProteinRuler IV. Lane 2: feedstock (50 g/L of sweet whey). Lane 3: breakthrough. Lane 4: concentrated elution fraction. Lane 5: standard lactoferrin (1 mg/mL). Lane 6: LMW-SDS Marker Kit (GE Healthcare).

4. CONCLUSIONS

By a series of experiments, a simple and effective process was established as follows: 1 L of crude sweet whey (50 g/L) was loaded onto an expanded bed packed with Fastline SP with an expansion of 2.0 and washed by PBS to remove unbound proteins and lactoferrin was eluted by 0.5 M NaCl in PBS. With this process, lactoferrin has been successfully isolated from crude sweet whey. The purity of lactoferrin increased from 0.16% to 88.5%, corresponding to a purification factor of 553 in only a one-step separation. The results demonstrated that it is possible to purify a certain protein from an untreated mixture in a single step by robust technology with a suitable functional element.

AUTHOR INFORMATION

Corresponding Author

*Phone: 86-571-87951982. Fax: 86-571-87951982. E-mail: yaosj@zju.edu.cn.

Notes

The authors declare no competing financial interest.

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