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Purification of lactoferrin using hydroxyapatite

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ABSTRACT

Lactoferrin is an important nutriceutical with various physiological functions. It is present in whey at very low concentrations. This work describes a mixed-mode (hydroxyapatite) chromatography method for one-column fractionation of lactoferrin from whey. Lactoperoxidase, a protein with similar molecular weight and isoelectric point, was initially desorbed from the matrix under isocratic conditions. Lactoferrin was obtained in homogeneity without lactoperoxidase activity and free from other major whey proteins such as alpha lactoalbumin and beta lactoglobulin.

1. Introduction

Lactoferrin is a glycoprotein that belongs to the iron transporter or transferrin family. Lactoferrin contains 651 amino acids and has a molecular weight of 77 kDa as measured in dilute aqueous solutions [1]. It has antimicrobial properties associated with its iron chelating capacity. In pediatric clinical settings, it has been shown to have a number of significant biological functions which include ion transport, antifungal activity, antiviral activity, toxin binding properties, immunomodulation effects, wound healing and anti-inflammatory effects [2–4]. In related studies, peptide derivatives from pepsin digest of lactoferrin have shown enhanced antimicrobial action as compared to lactoferrin alone [5].

In recent years, increased demand for various whey proteins has come from a growing interest in high quality food supplements. These dietary supplements could include metals, vitamins, and proteins such as purified lactoferrin. At present, lactoferrin is isolated and purified on an industrial scale (approximately 20–30 tons annually [6]) from skim milk and cheese whey which contain two major whey proteins: alpha lactalbumin and beta lactoglobulin. These two proteins have acidic isoelectric points (pI) significantly different from that of lactoferrin (8.0). Therefore, by proper selection of pH and salt conditions, lactoferrin could be bound and eluted from ion exchange chromatography at high purity. Other purification methods include hydrophobic interactions, affinity chromatography, size exclusion chromatography and ultrafiltration [6–8]. Membrane ultrafiltration designed for diafiltration and concentration is presently used on an industrial scale [6]. However, the process requires extensive amounts of water to effectively separate large molecules. Product recovery is dependent on the volume of buffer used and this must be disposed. Product purity can be compromised, in part because ultrafiltration fails to distinguish molecules with similar hydrodynamic size. Additionally, the variable content of whey complicates the predictability of an ultrafiltration-based purification process. Another membrane-based technology that is attracting increasing attention is membrane adsorption. The feasibility of using a cation exchange module has been demonstrated [7]. However, as far as materials and module design are concerned, they have to be further optimized for industrial operation [9].

In this study we investigate mixed-mode chromatography–ceramic hydroxyapatite (CHT^{TM}), a unit operation that has been demonstrated to have all the performance characteristics needed for effective scale-up in the biotech industry. Previously, it has been exploited for separating whey proteins in general. To our knowledge, application of CHT for the specific purification of lactoferrin has never been attempted before. One challenge with regard to lactoferrin is that it can be difficult to obtain in high purity using conventional process chromatography. This is due to the presence of lactoperoxidase that is substantially similar to lactoferrin in both molecular weight and isoelectric point. In one study, two orthogonal column steps were required for the separation of the two proteins. In this communication we describe a CHT-based method for direct capture of lactoperoxidase, lactoferrin and other alkaline proteins from whey. Purified lactoferrin was then obtained by isocratic elution. The high purity obtained in a single step and the

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simplicity of the operation makes this technique highly attractive when compared to other alternative methodologies published in this field.

2. Experimental

2.1. Whey

Whey was obtained as a concentrate from Hilmar Ingredients, Hilmar, CA. The solution was aliquoted, stored frozen at −20 °C and thawed before the experiments. The whey concentrate was diluted tenfold, centrifuged at 5000 rpm for 20 min and filtered through a 0.45 micron Whatman PVDF filter to remove particulates prior to chromatography.

2.2. Purified proteins and reagents

Purified bovine proteins including alpha lactoalbumin, beta lactoglobulin, lactoperoxidase and lactoferrin were purchased from Sigma–Aldrich (St. Louis, MO, USA). The proteins were solubilized in equilibration buffer at a concentration of 2 mg/mL on the day of the experiments.

All chemicals used for the preparation of buffers were of analytical grade. The buffers were sterile filtered prior to use.

2.3. Electrophoresis

For sodium dodecyl sulfate polyacrymide gel electrophoresis (SDS-PAGE), samples were first mixed with an equal volume of loading buffer and heated at 95 °C for 5 min. Up to 30 μL of the mixture was loaded onto the wells of a 4–20% Criterion™ precast gels (Bio-Rad Laboratories, Hercules, CA, USA) and separated at 200 V for 1 hour. The gel was stained with Bio-Safe™ Coomassie stain (Bio-Rad) and scanned with a Molecular Imager® GS-800™ Calibrated Densitometer (Bio-Rad).

2.4. Column chromatography

All chromatography experiments were automated and executed using a BioLogic DuoFlow™ system and software (Bio-Rad). CHT™ Type II ceramic hydroxyapatite media was packed in a Bio-Scale™ MT2 column (Bio-Rad). To determine the elution profile of individual proteins or mixtures of proteins, chromatography was carried out with a sodium phosphate gradient or sodium chloride gradient at the target pH. For example, the column was equilibrated in 0.010 M sodium phosphate, pH 6. This pH is relevant since it is the mid-point between the pI of lactoferrin, the protein of interest and the pI of alpha lactoalbumin, the more acidic contaminant in whey. After protein injection, usually 50 μL, the column was washed with ten column volumes (CV) of equilibration buffer. The adsorbed protein was eluted using a 20-column volume linear gradient in sodium phosphate concentration to 0.8 M (buffer B). The column was cleaned with a sodium phosphate concentrate of up to 0.8 M prior to equilibration before each run.

Based on the gradient experiments, an isocratic elution was developed for the separation of lactoferrin and lactoperoxidase. After loading, equilibration and washing in 0.15 M sodium phosphate pH 7.0, the column was eluted with 10 CV of 0.01 M sodium phosphate, 0.4 M NaCl pH 7.0, followed by 10 CV of 0.01 M sodium phosphate, 0.6 M NaCl pH 7.0. pH 7.0 used in these buffers differed from earlier work performed at pH 6 since CHT is more stable at neutrality. The column was cleaned with a sodium phosphate concentrate of up to 0.8 M prior to equilibration before each run.

2.5. pH and A_{280} measurements

pH and A_{280} of the chromatography fractions were monitored by the DuoFlow system instrumentation. Total protein was determined by the Quick Start™ Bradford protein assay (Bio-Rad).

2.6. Western blot

Lactoferrin or proteins in the eluate pool were reduced by adding β-mercapethanol, and boiling for 5 min. Samples were then electrophoresed on 4–20% SDS–PAGE, 200 V, 50 min using a Criterion™ electrophoresis cell (Bio-Rad). Precision Plus Protein™ WesternC™ standards (5 μL, Bio-Rad) were loaded as markers. Proteins were transferred to polyvinyllidone difluoride (PVDF) membranes at 100 V for 30 min using cold Towbin buffer with 20% MeOH in a Criterion blotter. Membranes were blocked in a solution of 3% BSA in TTBS (Tris-buffered saline, 0.05% Tween 20) for 1 h. Membranes were washed in TTBS three times for 5 min each time. They were then incubated with a 1/1000 goat monoclonal anti-lactoferrin antibody (Bethyl Laboratories, Montgomery, TX) diluted in TTBS, then washed five times for 5 min each time. Detection was achieved by incubation for 1 h with a 1/50,000 goat-anti mouse antibody conjugated to horseradish peroxidase in TTBS as well as StrepTactin-HRP (1:10,000) to visualize the Precision Plus Protein WesternC standard bands. They were washed 6 × 5 min in TTBS. Excess liquid was removed from the membranes which were then incubated with 5 mL of ImmunStar™ WesternC™ Substrate on the surface of a sheet protector. Immunoreactive bands were revealed by imaging on a Molecular Imager® ChemiDoc™ XRS system. Precison Plus Protein WesternC standard was used for size estimations.

2.7. Dynamic binding capacity (Q)

Frontal loading studies to 10% breakthrough for a 0.3 mL CHT column were performed at 0.5 mL/h to give a residence time of 0.6 min. Feed material was the previously prepared phosphate eluate from a CHT column chromatography run of whey. The material was adjusted to a phosphate concentration of 0.15 M with water. After equilibration, the column was set off-line at the beginning of the run to determine the absorbance of the lactoferrin containing solution. The solution was loaded onto the column through the injector valve after attaining 100% breakthrough. Q at 10% breakthrough was calculated by multiplying the protein concentration in the load by the frontal volume.

2.8. Lactoperoxidase activity

50 μL of sample was combined with 50 μL of TMB substrate solution (Cygnus Technologies, Southport, NC) in a microtiter plate. 50 μL of stop solution (0.5 M H_2SO_4) was added to the well after a 15 min incubation period. Relative absorbance at 450/650 nm (450 nm for the test wavelength and 650 nm for the reference) was measured using the buffer solution as a blank.

2.9. Maximum production rate calculation

Similar to calculations for other biological compounds [10,11], the production rate (R in gm/h/cm^2) was calculated as the mass of lactoferrin obtained in one cycle divided by the time it takes to purify, divided by the column cross sectional area. Dividing by column sectional area makes production rate independent of scale. Thus, an equation relating the lactoferrin concentration in the feed (C in mg/mL), superficial velocity (U in cm/h), dynamic capacity (Q in mg/mL) and the column volumes (CV) for wash, elution and

equilibration (N) was obtained as follows:

$$R = \frac{1}{1000\left(\frac{1}{CU} + \frac{N}{QU}\right)}$$

3. Results and discussion

3.1. Cursory development of chromatography method

3.1.1. Examination of phosphate gradient elution

Purification procedures to isolate various proteins using CHT are well documented in the literature and in vendor instruction manuals [12]. Furthermore, a protocol for recovery of cheese-whey proteins using CHT already exists [13]. However, separation of several major protein species from lactoferrin has not been addressed in the literature or in protocols. Therefore preliminary development was needed.

Phosphate concentration is known to influence protein elution during CHT chromatography. A cursory examination was carried out to evaluate the separation of lactoferrin from the two major proteins (alpha lactoalbumin and beta lactoglobulin) that account for >80% of the proteins in whey. In the experimental results shown in Fig. 1, the profile of each individual protein was first identified by injecting the purified protein alone and then a mixture containing the three purified proteins. As expected, separation of the mixture paralleled that of the individual proteins. Analysis of the eluate pools by SDS-PAGE confirmed clear resolution of lactoferrin from the two proteins (data not shown). Lactoferrin eluted later in the gradient. It binds tightly to CHT, presumably due to charge interactions as well as the two Fe^{3+} ions associated with the molecule.

3.1.2. Examination of phosphate gradient method as applied to whey

Whey was subjected to CHT chromatography using conditions identical to those reported for the separation of lactoferrin from the two major marker proteins (lactoglobulin and lactoalbumin).

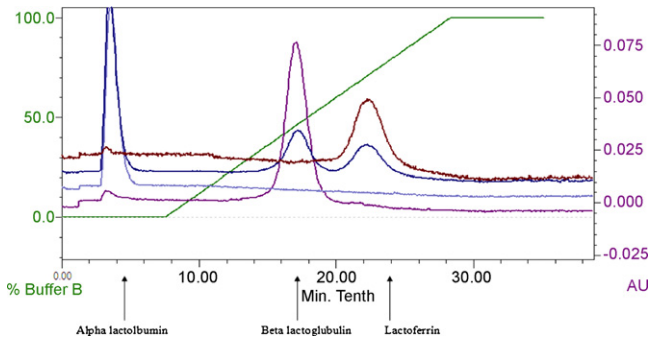

Fig. 1. Separation of alpha lactoalbumin, beta lactogloublin and lactoferrin by CHT ceramic hydroxyapatite chromatography. Chromatographic profiles of purified lactoferrin ( ), beta lactoglobulin ( ) and alpha lactoalbumin ( ) and a mixture of the three proteins( ). Elution buffer conductivity ( ).

As shown in Fig. 2 a major peak was obtained during the phosphate gradient elution step. Elution fractions were analyzed by SDS–PAGE followed by western blot (Fig. 2) and, in agreement with our preliminary experiment (Fig. 1), lactoferrin was found in the late fractions of the eluate.

3.2. Separation of lactoferrin from lactoperoxidase

Sodium or potassium phosphate buffers at near-neutral pH are used for the majority of hydroxyapatite chromatography applications used for the fractionation and purification of a wide variety of biologicals. In more recent instances, sodium chloride in low phosphate buffer has also been successfully used in the separation of host cell proteins, aggregates and other contaminants from monoclonal antibody [14]. The advantage of sodium chloride gradient over phosphate gradient was demonstrated in several case studies whereby aggregates levels up to 60% could be reduced to <1% [14–16]. This fact was used for attempting a simple strat-

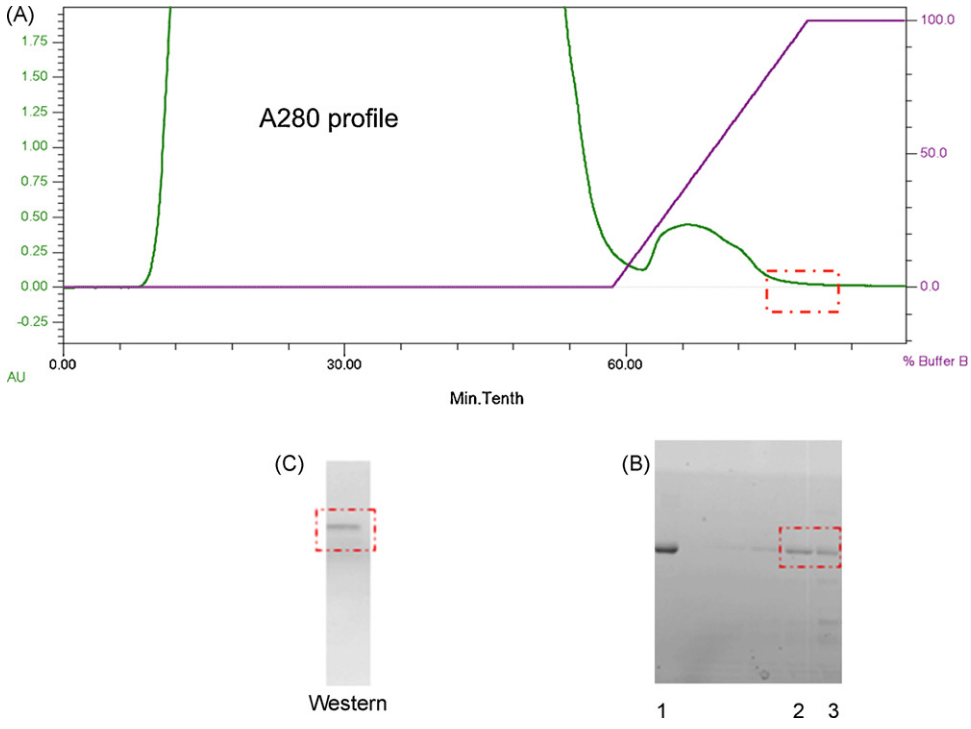

Fig. 2. Purification of lactoferrin from whey using phosphate gradient chromaography across CHT. (A) A280 profile (B) SDS-PAGE analyses of pools from the fractions. Lane 1—Lactoferrin standard, Lane 2 and 3—fractions in dashed rectangle (C) Western blot of lactoferrin in the pooled fractions

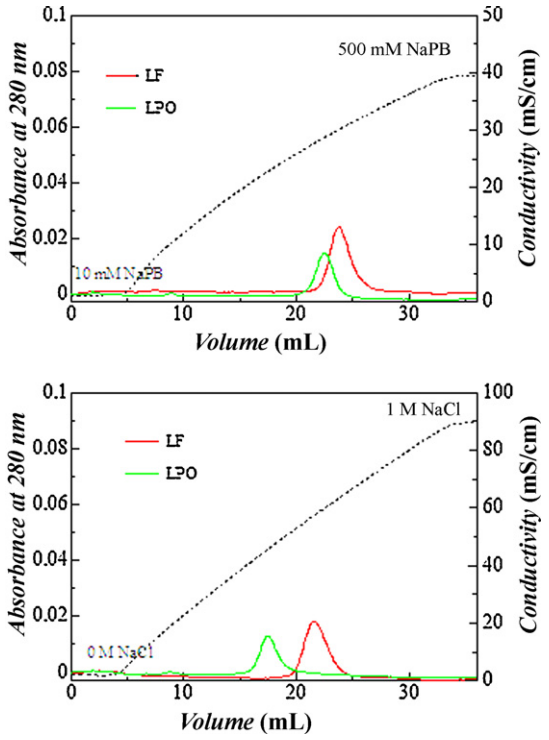

Fig. 3. (a) Sodium phosphate gradient chromatography of purified lactoperoxidase and purified lactoferrin. (b) Sodium chloride gradient chromatography of purified lactoperoxidase and purified lactoferrin

egy for improving the purity of lactoferrin as lactoperoxidase is co-purified with lactoferrin because of substantial similarities in molecular weight and isoelectric points. Accordingly, we compared the separation efficiency of a sodium chloride gradient to that of a phosphate gradient. Chromatographic profiles of the purified proteins are shown in Fig. 3. The peaks for lactoferrin and lactoperoxidase were mostly overlapping when using a phosphate gradient (Fig. 3a) whereas baseline separation was obtained across the sodium chloride gradient (Fig. 3b). On the basis of these results, a protocol using two isocratic elution steps was defined (see Section 2). Purified lactoferrin and lactoperoxidase were separated from a mixture of the two using sequential elution with 0.4 M NaCl and 0.6 M NaCl, respectively (Fig. 4). The peak positions matched well with those of the pure components. In separate experiments, low levels of peroxidase activities were revealed in the trailing fractions of the peroxidase peak (data not shown). To ensure adequate

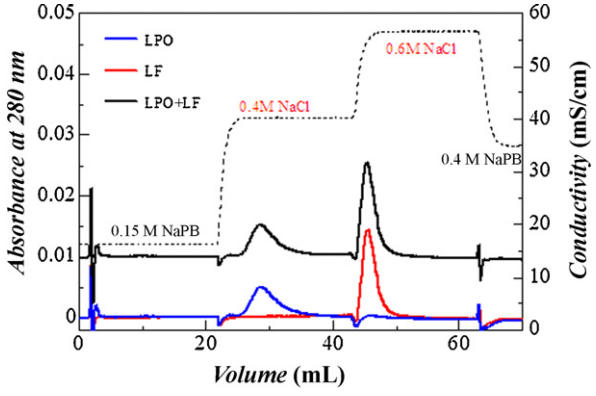

Fig. 4. Separation of lactoperoxidase and lactoferrin using isocratic elution – Chromatographic profiles of purified lactoperoxidase, purified lactoferrin and a mixture of both purified proteins.

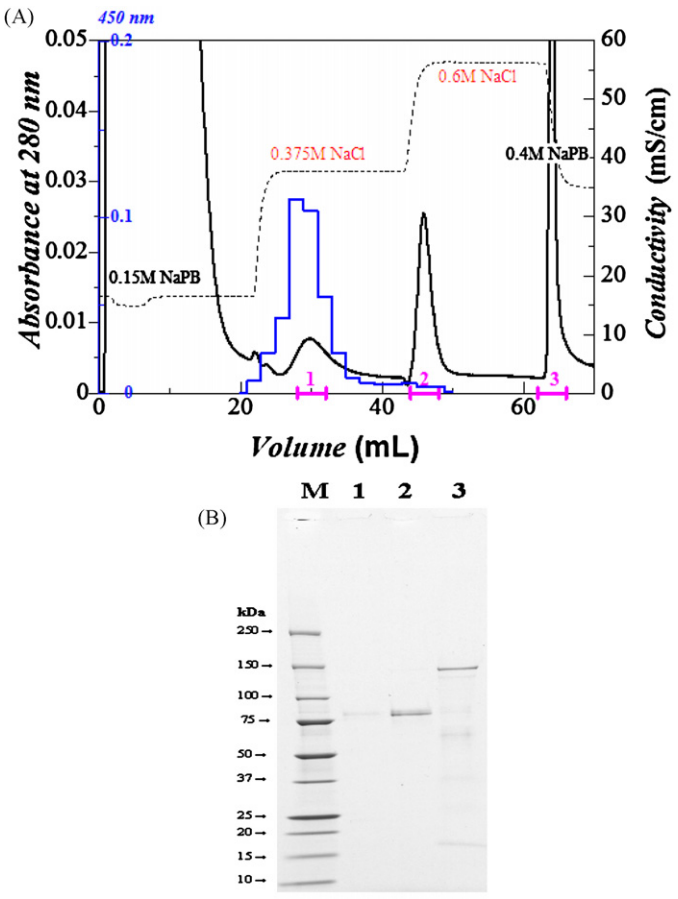

Fig. 5. Purification of lactoferrin from whey using sodium chloride step elution. Lactoperoxidase activities are shown by absorbance at 450 nm (blue line) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).
SDS-PAGE analyses of eluate pools:
M: Molecular weight standard.
1: Lactoperoxidase.
2: Lactoferrin.
3: Xanthine dehydrogenase.

removal of these residual activities from the lactoferrin eluate, NaCl concentration in the first elution buffer was adjusted from 0.4 M to 0.375 M.

3.3. One-column preparation of lactoferrin from whey

A protocol similar to that of the above-mentioned experiments using purified proteins was defined for purifying lactoferrin from clarified whey. A significant amount of protein (measured by A-280) was recovered in the flow through and washes. Similar to the previous results, two peaks of similar molecular weight were obtained (Fig. 5a) using a two-step isocratic elution protocol with 0.375 M NaCl, followed by 0.6 M NaCl. SDS-PAGE analysis (Fig. 5b) of the first peak (lane 1) showed a single protein band of about 80 kDa corresponding to lactoperoxidase, as confirmed by activity measurement (data not shown). The second peak (lane 2 in gel image) corresponds to lactoferrin without lactoperoxidase activities as shown by lack of absorbance at 450 nm in the lactoperoxidase activity measurement. The column was stripped using 0.4 M sodium phosphate and the generated fractions (lane 3) did not contain additional lactoferrin. The major band in the strip was identified as xanthine dehydrogenase (mass spectrometry, data not shown).

A protocol similar to that of the above-mentioned experiments using purified proteins was defined for purifying lactoferrin from

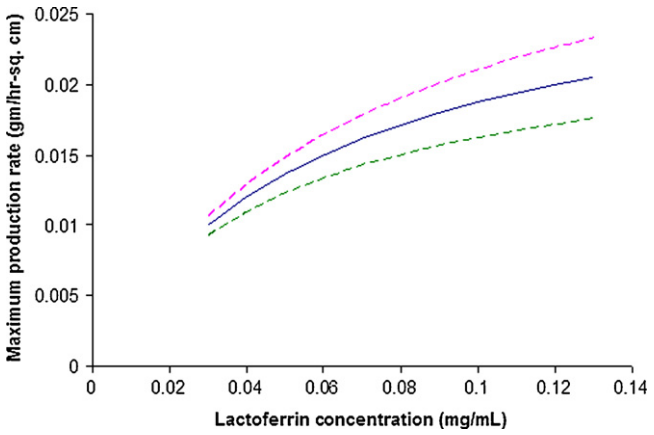

Fig. 6. Predicted maximum production rate in relation to lactoferrin concentration in whey. Solid line—binding capacity of 2.36 mg lactoferrin/mL. Upper dashed line—binding capacity of 2.9 mg lactoferrin/mL. Lower dashed line—binding capacity of 1.9 lactoferrin mg/mL.

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3.4. Production rate calculation

The lactoferrin concentration in the feed (C) depends on the source of whey. Literature suggested that the value could be 0.03–0.13 mg/mL [6,7]. This range is used in the production rate calculation.

Frontal loading studies to 10% breakthrough was shown to be 6.9 mg protein/mL. Of the total protein, protein determination showed that only 34% of it was lactoferrin. Hence, the dynamic binding capacity (Q) was adjusted to 2.36 mg/mL.

The number (N) of CV for wash/elution/regeneration is determined by purity and yield constraints. In the present study, we chose 10 CV of washes after load, 10 CV of the first elution buffer, 10 CV of the second elution buffer and 10 column washes of the strip buffer, for a total of $N = 40$.

For design consideration, we chose a shallow bed height of 5 cm which ensures minimum pressure drop across the column. The superficial velocity (U) is thus 500 cm/h which corresponds to a residence time of 0.6 min employed in the frontal loading study.

With these design constraints ($Q = 2.36$ mg/mL, $N = 40$, $U = 500$ cm/h), production rate could be calculated as a function of lactoferrin concentration in the feed. In Fig. 6, the solid line reveals changes in the maximum production rate relative to the feed concentration. In a scenario where an 80 L CHT column was used, a production rate of 0.32 kg/h was obtained with a lactoferrin concentration of 0.13 mg/mL. This value is qualitatively comparable to 0.39 kg/h projected for an 80 L cation exchange column reported in the literature [7].

Accounting for a deviation of ±20% from the measured dynamic capacity, a theoretical calculation of the maximum production rates is shown in Fig. 6. The upper dashed line represents a 20% increase in dynamic capacity and demonstrated higher production rate at higher feed concentration. Improvement could thus be achieved by enhanced dynamic capacity from further optimization.

4. Conclusion

We have determined chromatography conditions applicable to a one-column purification method of lactoferrin from whey using hydroxyapatite. We have demonstrated the successful removal of lactoperoxidase. This contaminant is presumably co-purified in other purification method due to similarities in molecular weight and isoelectric properties. The data is sufficiently encouraging in guiding the development of a commercial process. Using several measured values in the present study, an 80 L hydroxyapatite column would generate 0.32 kg of lactoferrin per hour. It is important that the process be high throughput and low cost. Finally, the capacity to produce large quantities of lactoferrin in a simple manner should facilitate its availability for therapeutic and preventative applications on infection, inflammation, and enhancing iron status in the nutriceutical field.

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