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Selection of downstream steps by analysis of protein surface property: A case study for recombinant human lactoferrin purification from milk of transgenic cow

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ABSTRACT

Protein purification has long been regarded as an art rather than a science. Trial and error is the widely used practice for a protein to be purified from the crude extract. In this study, we carried out a rational approach by analysis of the protein surface properties before setting out to do experiments. The target protein was recombinant human lactoferrin (rHLF), to be purified from milk of transgenic cow. We need to overcome two major problems. One is its possible co-precipitation with casein during initial separation; the other is the difficulty in separating its homologous counterpart, bovine lactoferrin (BLF). By calculation of the surface hydrophobicity, an initial separation step was decided by calcium precipitation, which removed casein but left rHLF in the supernatant. Then the average surface hydrophobicity and electric potential of rHLF were compared with those of BLF. There was a more significant difference in the electric potentials than the average surface hydrophobicity (ASH) between the homologous pairs. Therefore, the purification step should be favored by ion exchange chromatography (IEC) instead of hydrophobic interaction chromatography (HIC). Experiments were performed to verify the prediction. After removing casein, one step cationic ion exchange chromatography realized complete separation of rHLF from BLF with rHLF recovery up to 83%, while the resolution of HIC process was very limited due to the small difference in ASH between them. The laboratory process was then successfully scaled up to pilot-plant scale of 500 l milk of transgenic cow per batch. Average rHLF recovery of 79%and purity of 98.9% were attained for five batches. The purified rHLF displayed bioactivities as good as the natural human-resource lactoferrin standard.

1. Introduction

Purification is a key step in bioprocess. On one hand, it is very important to remove impurities from complex materials to guarantee safe products for clinical or nutritional applications. On the other hand, purification affects, to a great extent, the economy of a bioprocess. In general, purification accounts for about 70% cost of the whole process [1]. The driving force is to obtain purest product at lowest cost. However, optimization of operating conditions and determination of downstream steps are still based on trial-and-error experiments, which are tedious and time consuming.

Mathematic tools, such as orthogonal design or uniform design, which are often called Design of Experiments (DOEs) [2,3] have been used to reduce the number of experiments and to optimize the whole purification process. This methodology still needs a number of experiments, especially when there are many parameters to be optimized. Experience prediction, on the other hand, is useful. For example, cationic exchange chromatography is proposed as the top priority in purification of basic or alkaline proteins which demonstrate high isoelectric points [4,5]. Size exclusion chromatography is often employed for virus-like particle purification because of its much greater size than other protein impurities [6,7]. However, experience prediction is rather limited when the physiological difference between the product and impurities is very small [8], especially when the two resembles each other such as purification of a human milk protein from a milk of transgenic cow.

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Experience prediction based on mathematical calculations of protein surface characteristics provides another useful approach to quantitatively predicting behavior of protein during separation. For examples, on the basis of a good linear correlation between surface hydrophobicity of different amino acid residues and the log of its partition coefficient in aqueous two-phase systems, partition coefficient of a protein can thus be predicted from its surface exposed amino acid residues [9]. Mathematical calculations of protein surface hydrophobicity were also found useful for predicting protein retention times in hydrophobic interaction chromatography after setting a predictive models based on statistics on a set of proteins [10–13]. However, the predictive capacities of these previous works were mostly evaluated by using some model proteins or in separation of protein from relative simple system. When proteins to be separated resembles each other, such as purification of a human milk protein from its homologous counterpart co-present in the milk from transgenic cow, such mathematical calculations of protein surface characteristics would be helpful for designing an effective purification strategy, but rarely reported so far.

Over the last few years, mammary gland bioreactor has become an active research field of bioreactors because of the relative low cost and high expression level [14]. Recombinant human lactoferrin (rHLF), which owns a wide variety of potential applications in human nutrition and healthcare [15], had been produced using cow mammary gland bioreactor [16,17]. However, purification of rHLF from the animal milks could be a big challenge. Firstly, there is a large quantity of casein in milk from transgenic cow, which could interact, even co-precipitate with the target recombinant proteins [18]. Until now, there is no report concerning the strategy for casein precipitation with less co-precipitation of recombinant protein. Secondly, the milk from transgenic cow contains an animal homologous counterpart to rHLF, namely bovine lactoferrin (BLF), which should be removed to guarantee the product safety. Nuijens [19] employed ion-exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC) to separate bovine milk artificially added with natural human lactoferrin. The simulated milk mixture was obviously different from a real transgenic system where the expression of genes is totally different, resulting in completely different components. Tillib [20] used immune-affinity chromatography with single-domain antibody as ligands to separate rHLF from goat lactoferrin. This method should be specific but was very expensive.

In this study, we carried out a rational approach by analysis of the protein surface properties before setting out to do experiments. The molecular properties of rHLF were analyzed and compared under three casein removal environments, aiming at designing an efficient initial separation with the lowest loss of rHLF. After that, the molecular properties of the rHLF and BLF were calculated and compared to select a suitable chromatographic step for separation of BLF from rHLF. The design was implemented to laboratory practice to verify the feasibility of step selection. Finally, the laboratory process was scaled up to a pilot-plant scale of 500 l milk per batch to test the reliability of the rational approach.

2. Materials and methods

2.1. Materials

All chemicals were analytical grade. Butyl Sepharose 4 FF, SP Sepharose FF and Superdex 200 (site packed in a 600 mm × 35 mm i.d. column) were purchased from GE Healthcare Bio-Sciences (GE, USA). Empty glass columns (200 mm × 10 mm i.d.) were obtained from Jinhua Chromatographic Equipment Factory (Shanghai, China). The standard HLF (human lactoferrin) and BLF (bovine lactoferrin) were purchased from Sigma (USA). The Elisa kits of BLF were from Bethyl Laboratory (Texas, USA). The milk of transgenic cow was provided by China Agricultural University (Beijing, China). rHLF used in analysis was pre-purified in our laboratory. All solutions were prepared using Milli-Q grade water (Millipore, USA).

2.2. Calculation and measurement of protein surface properties

Protein surface hydrophobicity measurements were made with ANS-binding fluorescence spectra [21] on F-4500 fluorescence spectrophotometer (HITACHI, Japan). The excitation wavelength was set as 380 nm.

The ASH of protein was calculated by the following equation [12]:

$$ASH = \frac{\sum_{k \in S} ASA(k)\varphi(k)}{\sum_{k \in S} ASA(k)} \quad (1)$$

where the surface of a protein (S) was coded by a set of points. Each point k ∈ S was a particular amino acid. For each of these amino acids $k \in S$, average surface area (ASA) corresponded to its accessible surface area. $\varphi(k)$ was a hydrophobicity scale for each amino acid [22]. The ASH of a protein was computed assuming that the contribution of each amino acid on the protein surface to the ASH value was proportionally to its abundance [23]. The ASA was calculated using the software STRIDE [24] from the protein three-dimensional structure, obtained from database.

The electrostatic potential of a protein was calculated by Graphical Representation and Analysis of Surface Properties (GRASP) program [25]. The internal and external dielectric constants of the proteins were set as 1 and 80, respectively. The salt concentration was set as 0.2 M.

2.3. Analytical methods

2.3.1. Determination of protein concentration

Protein concentration was determined using Bradford method [26] with slight modification. Briefly, a 96-well microplate was applied to provide the reaction wells and the absorbance was measured using the Microplate Reader 550 (Bio-Rad, USA) controlled by the Microplate Manager 4.0 PC data analysis software. The amount of protein was calibrated using bovine serum albumin (BSA) as reference standard.

2.3.2. Quantitative determination of contents of homologous proteins

rHLF was quantitatively analyzed by high-performance reversed phase chromatography (HPRPC) using Protein C4 column (Grace Vydac, USA) on an Agilent 1100 system, equipped with a degasser and a variable wavelength detector with UV monitoring at 280 nm. Start buffer was 0.1% acetic acid in water, and elution buffer was 0.1% acetic acid in acetonitrile. Samples was injected into the RPC column and eluted by linear gradient with a flow rate of 0.5 ml/min at 25 °C.

The BLF content in the milk of transgenic cow was quantitatively assayed by Elisa. Briefly, diluted sample containing BLF and antibody were added into 96-wellmicroplate pre-coated with antibody. Next, monoclonal antibody conjugated with peroxidase was added to each well and incubated for 30 min. Then 50 μl TMB substrate solutions were added. After incubation for 15 min, the reaction was stopped and the absorbance was measured at 450 nm by microplate reader 550 (Bio-Rad, USA). The amount of BLF was calibrated against a reference standard.

2.3.3. Purity analyses of the purified homologous proteins

Purities of the purified homologous proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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