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Cascade ultrafiltration systems—Integrated processes for purification and concentration of lysozyme

Mukesh Mayani, Carlos D.M. Filipe, Raja Ghosh*

Department of Chemical Engineering, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4L7

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ABSTRACT

This paper demonstrates the feasibility of using cascade ultrafiltration systems run at constant flux for efficient purification of lysozyme from chicken egg white. Integration of high-resolution purification with concentration is a key requirement in the purification of lysozyme, which is present at very low purity and concentration in egg white. A three-stage cascade ultrafiltration system, which focused on purification alone, gave a continuous diluted lysozyme product stream of 96% purity with over 75% recovery. Process variables such as the retentate to feed ratio as well as internal recycle rates within the cascade were found to have significant effects on the efficiency of separation. These findings could be explained using a material balance based mathematical model. A four-stage cascade system which integrated both purification and concentration attributes produced a continuous, 97% pure lysozyme product stream with over 71% recovery and with an almost twofold lysozyme concentration enrichment. This system, which utilized a high degree of buffer recycling, is likely to be attractive for economic large-scale protein purifications in general.

1. Introduction

Lysozyme is a bactericidal enzyme, widely used in the food and pharmaceutical industries. It has been investigated for important therapeutic applications such as the treatment of ulcers and infections, and in human cancer chemotherapy [1–5]. Purification of lysozyme from natural egg white is a commercially important process due to the significant value addition. Lysozyme is a low abundance protein, comprising about 3.5% of total proteins present in egg white. The conventional methods for lysozyme purification involve combination of techniques such as precipitation, centrifugation and crystallization [6]. These techniques have poor selectivity and therefore have to be repeated several times to obtain reasonably pure lysozyme. High-resolution purification techniques such as affinity [7,8] and ion exchange chromatography [9], and electrophoresis are also used but these are expensive and give low product throughput. These techniques also require ancillary operation steps such as dilution, buffer exchange, concentration and desalting, all of which contribute to high processing cost [10]. Other disadvantages of chromatographic techniques include (a) phase change (i.e. unbound to bound form and eventually to the unbound form), which could lead to product degradation, and (b) non-specific binding, which could have a significant impact in the purification of a low abundance protein such as lysozyme.

Ultrafiltration provides high throughput and is therefore desirable in large scale protein processing. In recent years it has been demonstrated that ultrafiltration processes could be optimized for high-resolution separations, particularly where the target protein has significantly dissimilar properties (e.g. molecular weight, isoelectric point) relative to its impurities. The low molecular weight (14.3 kDa) and high isoelectric point (p*I* 10.7) of lysozyme makes it suitable for purification by ultrafiltration [11–17]. In addition to obvious advantages such as high productivity and scalability, separation of lysozyme using ultrafiltration can be carried out under close to physiological conditions, thereby ensuring its stability. In an ultrafiltration process operated in the diafiltration mode, lysozyme is obtained in the permeate while the impurities are retained. The lysozyme thus obtained is free from pathogens such as bacteria and virus. However, diafiltration is better suited for purifying proteins that are selectively retained over impurities. Its use for purifying proteins that are selectively transmitted is limited by the following factors: progressively low yield, dilution and high buffer consumption. A continuous cascade ultrafiltration system operated with substantial buffer recycle therefore seemed to be an effective strategy for efficient lysozyme purification.

Cascade ultrafiltration systems have been found to be suitable for continuous protein purification and give excellent separation and recovery of product [18–21]. Precisely designed and operated cascade ultrafiltration systems make it possible to obtain purification factors comparable to chromatography. In such systems it is

* Corresponding author at: Department of Chemical Engineering, McMaster University, 1280 Main Street West, JHE-250, Hamilton, Ontario, Canada L8S 4L7.
Tel.: +1 905 525 9140x27415; fax: +1 905 522 1350.
E-mail address: rghosh@mcmaster.ca (R. Ghosh).

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not merely the number of stages but the manner in which the different flow streams within the cascade are handled that contribute towards efficiency of separation [18,21]. Indeed, a poorly designed and operated cascade system would be even less efficient than a single-stage system [18].

The current work examines the suitability and using cascade ultrafiltration systems run at constant flux for purification of lysozyme from natural chicken egg white (CEW). The main objective of this work was to combine high resolution and high recovery with concentration enrichment. This was achieved by efficient buffer recycling. CEW, initially isolated from commercial eggs was pretreated by microfiltration to remove particulate matter. This was then blended with appropriate buffers and used in subsequent experiments. The effects of permeate flux on sieving of proteins and selectivity was individually studied under steady-state conditions with the different membrane modules to be used in the cascade systems. Suitable membrane modules were identified based on these results. Lysozyme purification using batch as well as continuous single-stage diafiltration was carried out as control experiments. A three-stage cascade system was used to optimize operating conditions for high resolution and high recovery. The experimental data was compared with results obtained by simulation. A four-stage cascade ultrafiltration system based largely on the three-stage system was then developed for integrating concentration enrichment with high resolution and recovery. Purified lysozyme samples obtained were analyzed using appropriate methods such as ion exchange and size exclusion chromatography, electrophoresis and enzyme assay.

2. Materials and methods

2.1. Materials

Medium size chicken eggs (Loblaws Inc., Canada) were purchased from the local market. Lysozyme (L-6876), ovalbumin (A-5503), conalbumin (C-0755), Bradford reagent (B-6916), *Micrococcus lysodeikticus* ATCC No. 4698 (M-3770), and other chemicals including sodium phosphate (mono- and di-basic), sodium chloride, sodium hydroxide and hydrochloric acid were purchased from Sigma–Aldrich, St. Louis, MO, USA. Filter cloth (Cascades Inc., Canada, Catalog No. 35000) was purchased from the local market. All buffers were prepared using ultrapure water (18.2 MΩ cm) obtained from a DIamond NANOpure water purification unit (Barnstead International, Dubuque, IA, USA, Catalog No. D50280). Glass fiber filter discs (2.0 μm, Catalog No. AP2504700) were purchased from Millipore Corporation, USA. Hollow fiber membrane modules (MiniKros 50 kDa MWCO PS; M11S-320-01P, 420 cm^2 membrane area) were purchased from Spectrum Laboratories, Inc., USA. Flat sheet membrane (3 kDa MWCO, Omega polyethersulfone PES, Catalog. No. OT003SHEET) was purchased from Pall Life Sciences, MI, USA. Rectangular sheet were cut out from this and fitted into a custom made tangential flow module having an effective surface area of 120 cm^2. Hollow fiber module (3 kDa MWCO polysulfone PS, 1400 cm^2 membrane area, Catalog. No. UFP-3C-4X2MA) was purchased from Amersham Biosciences Inc., USA. Each membrane module was wetted overnight using 70% ethyl alcohol followed by integrity test and water filtration to check for hydraulic permeability. If the module had been used previously, it was filled with 0.1 M sodium hydroxide and subsequently washed with plenty of pure water.

2.2. Preparation of feed solution

The eggs were broken and the contents were poured into a glass beaker. The yolks along with the thick gelatinous part were removed using a spatula. The remaining egg white portion which measured approximately 10–12 mL per egg was pre-filtered using two layers of filter cloth. The filtrate was diluted 1:18 with 20 mM sodium phosphate buffer (pH 6.6) containing 100 mM NaCl and further filtered through glass fiber filter pads (2.0 μm pore size). The solution pH was then adjusted to 6.6 for obtaining high selectivity [17]. The feed solution used in the four-stage cascade ultrafiltration system was prepared as above except for the dilution factor which was 1:7.

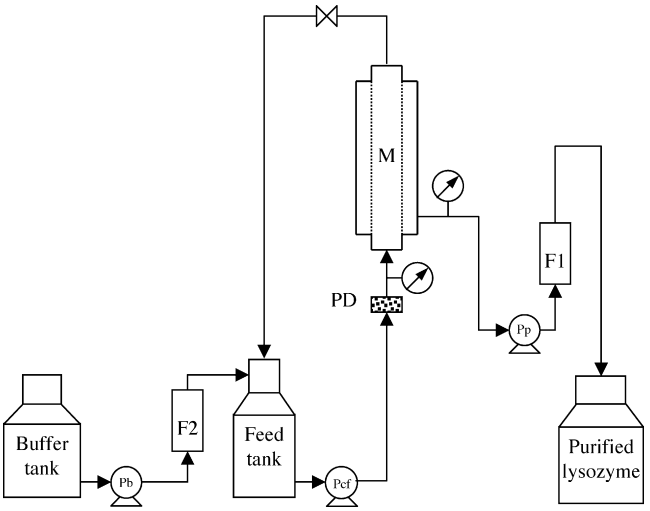

Fig. 1. Experimental set-up for diafiltration (M = membrane module; Pb = buffer pump; Pcf = cross-flow pump; Pp = permeate pump; PD = pulsation dampener; F_1 and F_2 are rotameters).

2.3. Ultrafiltration set-ups and experiments

All ultrafiltration experiments were carried out at room temperature (ca. 23 °C). The effects of cross-flow velocity (i.e. shear rate) and permeate flux were examined using individual membrane modules by separate steady-state ultrafiltration experiments operated in the total recycle mode. The batch diafiltration, single-stage continuous ultrafiltration, and three- and four-stage cascade ultrafiltration set-ups are discussed in the following paragraph using Figs. 1–4. In each of these set-ups, cross-flow was maintained using peristaltic pumps (Easy load II drive, Masterflex), whereas peristaltic pumps (Multi-channel drive, ISMATEC Cole-Parmer) were

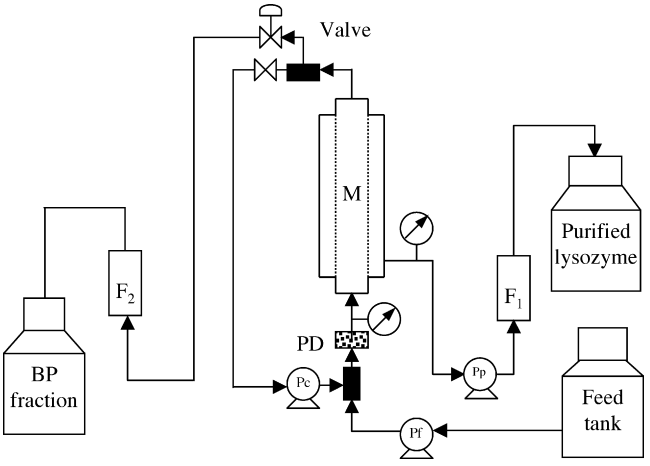

Fig. 2. Experimental set-up for continuous single-stage ultrafiltration (Pf = feed pump; Pc = cross-flow pump; Pp = permeate pump; PD = pulsation dampener; F_1 and F_2 are rotameters for measuring permeate and retentate flow rates, respectively).