Direct Purification of Lysozyme from Chicken Egg White Using Weak Acidic Polyacrylonitrile Nanofiber-Based Membranes

Hsien-Tang Chiu,[1] Jian-Min Lin,[1] Tai-Hong Cheng,[2] Shin-Ying Chou,[2] Cheng-Chiang Huang[2]

[1]*Department of Materials Science and Engineering, National Taiwan University of Science and Technology, Taipei 10607, Taiwan*
[2]*Department of Products, Taiwan Textile Research Institute, New Taipei City 23674, Taiwan*

ABSTRACT: A highly efficient membrane process for direct purification of lysozyme from chicken egg white is reported. The ion-exchange nanofibrous membrane (AEA-COOH) comprises a polyethyleneterephthalate spunbond fabric as a supporting layer with upper and lower electrospun polyacrylonitrile nanofibrous layers treated with NaOH hydrolysis to transform —C≡N bond into —COOH functional group. SEM results demonstrate a porous structure with a fiber diameter ranged between 150 and 200 nm. Organic solvent residue analysis proves no significant dimethylacetamide release from electrospun polyacrylonitrile nanofibers. The lysozyme was directly purified from the chicken egg white using Millipore stirred cell reactor equipped with AEA-COOH membranes. Despite a protein recovery of only 22.3%, the purified lysozyme had an activity recovery as high as 80.5% with a 73.6-fold purification. SDS-PAGE confirms the purified lysozyme product. This study thereby verified that lysozyme protein can be isolated and purified directly from chicken egg whites using AEA-COOH membranes. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: electrospinning; ion exchange; lysozyme; polyacrylonitrile (PAN); protein purification

INTRODUCTION

Lysozyme is a soluble enzyme consisting of a single polypeptide chain containing 129 amino acids with the ability to break down bacterial cell walls. It has a molecular weight of ∼ 14.3 kDa and an isoelectric point value of 10.7. Lysozyme has many applications in the current biopharmaceutical industry including medicinal use, antibacterial, hemostasis, and the reduction of swelling and inflammation. It is also used as a preservative in the food industry and an important soluble agent for cell-free extracts in the fermentation industry.[1,2] Chicken egg white contains 3.5% lysozyme, the highest amount of any organic substance, making it the best source of purified lysozyme.[3]

Ion-exchange (IEX) column chromatography uses the charge difference between the desired molecules and the resin particles containing ionized or ionizable function groups to conduct the separation and purification. It is a well-developed method applied in biomolecules purification including proteins, polypeptides, nucleic acids, and polysaccharides.

The main drawback of this method is the compression and compaction of the chromatographic bed at high velocity, creating a higher-pressure drop over the column. The use of porous resin particles slows the molecular adsorption/desorption rate due to longer diffusion distances.[4,5] To solve these problems, IEX membrane chromatography has been developed to minimize some of the common limitations of chromatographic bed such as long process time, channeling, and intrabed diffusion. Another major advantage of membrane chromatography is their relative easy packing and scale-up.[6]

According to literature,[7,8] electrospinning is the only direct and efficient method to continuously produce nanofibers. More than a 100 polymer nanofiber types have been successfully developed. Electrospinning equipment typically includes three main parts: (1) a high-voltage supply capable of providing positively or negatively charged electricity; (2) a syringe providing a fixed supply at a fixed rate of delivery combined with a capillary tube or needle for feeding the solution; (3) collectors which is grounded in a variety of shapes including plates, drums, and rollers. Under high voltage, the charged polymers eject and rapidly swing and extend to produce nanometer-scale nonwoven fibers from the grounded collection plate.[9,10] The diameter of fibers prepared using electrospinning typically ranges
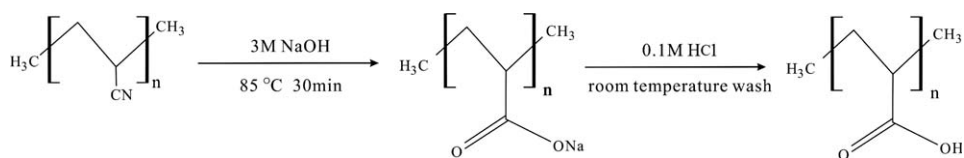
Figure 1 The alkaline hydrolysis mechanism of PAN nanofibrous membranes.

from tens of nanometers to hundreds of nanometers (nm). When compared with traditional membrane, nanofibrous membranes have a higher throughput, lower pressure drop, and higher adsorption capacity.[11,12] Polyacrylonitrile (PAN) has significant characteristics including high-mechanical strength, low cost, and high resistance to chemicals and sunlight.[13] In addition, PAN molecules carry modifiable cyanide functional groups that can be partially hydrolyzed to obtain carboxyl functional groups.[14]

In this study, we equipped a Millipore stirred cell reactor with a weak acid IEX nanofibrous membrane and operated continuously to directly purify lysozymes from chicken egg whites. The membrane was designed to explore the effects of nanofibrous membranes on the activity, adsorption behavior, specific activity, and purification efficiency of lysozyme.

EXPERIMENTAL

Materials

PAN yarn and dimethylacetamide (DMAc) were purchased from Fortune Industries (Tao-Yuan, Taiwan) and I-Chang Chemical Co. (Taipei, Taiwan), respectively. Polyethyleneterephthalate (PET) spunbond fabric (basis weight 15 g/m², thickness 85 μm) was supplied from Freudenberg Far Eastern Spunweb Co. Sartobind ion-exchange membranes (S, Q, C, and D) with pore size >3 μm was obtained from Sartorius (Germany). Electrospinning device was purchased from Jyi Goang Enterprise Co. (Taipei, Taiwan). The stirred cell reactor was from Millipore for protein purification. Chicken egg whites were purchased from Chinyi Eggs Technology (New Taipei City, Taiwan); Lysozyme was from Sigma; all other chemicals were purchased from Merck. The reagents and solvents were of analytical grade and used without further purification.

Carboxylic acid IEX nanofibrous membranes

The preparation of carboxylic acid IEX nanofibrous membranes was described as follows: First, PAN yarn was dissolved in DMAc at a concentration of 15% (w/v) and an as-prepared PAN solution was placed in a syringe. A 21-gauge stainless steel needle was used as the nozzle, which was connected to a power supply and charged to 26.5 kV. The distance between the tip of the nozzle and the substrate was

15.8 cm, and the flow rate was 1 mL/h. The rotating collector (24 cm/s) and moving nozzle [along the y-axis (20 cm), frequency 12 times/min] were used. The PAN nanofibrous mat was collected on PET spunbond nonwoven (basis weight 15 g/m²), which was wrapped around the grounded steel sheet. After the heat pressing process (100°C, 10 min), PAN-PET-PAN membrane with a loose structure became dense and entangled together.

Then, the hot alkaline hydrolysis method was used to modify the PAN nanofibrous membranes under the conditions of 3M NaOH, an alkalized temperature of 85°C, and a hydrolysis time of 30 min. The alkaline environment caused ─CN bonds in PAN to oxidize into hydrophilic ─COO─ functional groups. Finally, the carboxylated electrospun PAN nanofiber mat was treated with 0.1M HCl to protonate ─COO─ into ─COOH, followed by drying in an oven, at 60°C. Figure 1 shows the alkalized hydrolysis reaction equation for the PAN membrane during the NaOH solution treatment.

Testing for organic solvent residue in electrospun nanofibrous membranes

Suitable organic solvents, such as DMAc, can help PAN polymer spin into filaments during the electrospinning process. However, DMAc is toxic to humans and harmful to the environment. Therefore, from a drug quality and consumer health viewpoint, the ability to detect DMAc content in nanofibrous membranes is crucial. Considering the strong UV absorption by DMAc in the spectrum of 190–230 nm, this study used deionized water to prepare 0.1–10 ppm of DMAc standard solution. Then, following electrospinning, hot press, and alkalized hydrolysis, the PAN nanofiber (1 g) was soaked in deionized water (20 mL) for 3 days before its residual DMAc concentration was determined quantitatively by ultraviolet spectrophotometry at 196 nm.[15]

Batch protein adsorption test

This study used various 20 mM buffer solutions (acetate, pH 4–5; phosphate, pH 6–8; glycine-NaOH, pH 9–10; carbonate, pH 11–12) to prepare the lysozyme (2 mg/mL) and chicken egg white solution. The chicken egg white solution, with a buffer volume ratio of 1 : 10, was centrifuged twice (4°C,
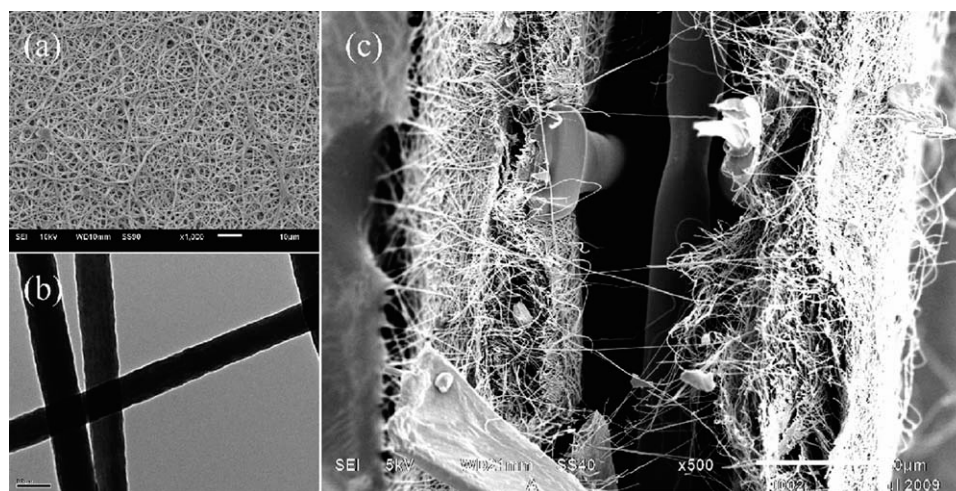
Figure 2 SEM and TEM micrographs of IEX AEA-COOH membranes. (a) Surface SEM image of AEA-COOH ($\times$1000). (b) TEM image of electrospun nanofibers ($\times$60,000). (c) Cross-sectional SEM image of AEA-COOH ($\times$500).

30 min, 10,000 rpm) to remove sediment. Then, the IEX nanofibrous membrane (AEA-COOH) and various Sartobind (C, D, Q, and S) IEX membranes were cut into size of 2 cm $\times$ 2 cm, and they were placed in a protein solution (pH 4–12) to begin the adsorption process under 100 rpm oscillation/vibration for 3 h. Total protein was measured by using the UV absorption method at 280 nm.[16]

$$\text{Lysozyme}(\text{mg/mL}) = \frac{A_{280}}{2.64}$$

This study dissolved Micrococcus lysodeikticus solution in 100 mM of pH 6.24 buffer solution (0.25 mg/mL) before pouring 1 mL of this bacterial liquid into a quartz tube and adding 200 μL of lysozyme solution. Upon mixing the solution, it was placed into the spectrophotometer and measured every 2 s for 30 s under a wavelength of 450 nm for changes in the absorbance value. A single unit of lysozyme activity (U) is defined as the amount of bacteria dissolved per minute that decreases the absorbance by 0.001. Lysozyme activity in the solution is defined as follows[17]:

$$\text{Lysozyme}(\text{U/mL}) = \frac{\Delta\text{OD}_{450}}{0.001 \times V_{\text{lysozyme}}} \times \text{Dilution rate}$$

Continuous membrane purification system test

First, the AEA-COOH membrane (6.7 cm in diameter) was placed into the Millipore stirred cell reactor before 100 mL of pH 9-adjusted fresh chicken egg white mixture was poured into the reactor. The resulting filtrate was collected at a fixed volume by gravity-flow purification. The adsorption process began by collecting ten 1 mL solutions, followed by nine 10 mL solutions. During the washing process of

100 mL pH 9 buffer solution, ten 10 mL solutions were collected. During the desorption process of 100 mL 1M NaCl/pH 9 buffer solution, ten 10 mL solutions were collected. Finally, the filtrate's protein concentration and lysozyme activity were measured by using the UV–Vis spectrophotometer. SDS-PAGE was used to confirm whether the indicated molecular weight of the purified product was that of lysozyme (14.3 kDa).

RESULTS AND DISCUSSION

Carboxylic acid IEX nanofibrous membrane structure and properties

This study combined electrospinning technology and nanofibrous surface carboxylation technology to develop a weak acid IEX nanofibrous membrane with high-specific surface area and porosity. As shown in the SEM graph in Figure 2, the carboxylic acid ion-exchange membrane (AEA-COOH) is formed by two layers of PAN nanofibrous membranes with one layer of PET microfibrous membrane in between [Fig. 2(c)]. The AEA-COOH membrane has a dense nanofibrous structure with a uniform pore size distribution [Fig. 2(a)] and an average fiber diameter of 150–200 nm [Fig. 2(b)]. The comparison between various physical properties of AEA-COOH and Sartobind® C membrane was described as follows: the AEA-COOH membrane's base weight (42.4 g/m^2) and thickness (167.1 μm) were $\sim$ 33% and 64% that of Sartobind® C membrane, respectively. In addition, it was discovered that the AEA-COOH membrane specific surface area (6.1768 m^2/g) was 7 times greater than that of Sartobind® C membrane (0.8873 m^2/g); the ─COOH functional group density was 3.3 times higher (Sartobind® C 135.1 μeq/g, AEA-COOH 439.8 μeq/g), and its porosity (84.4%)
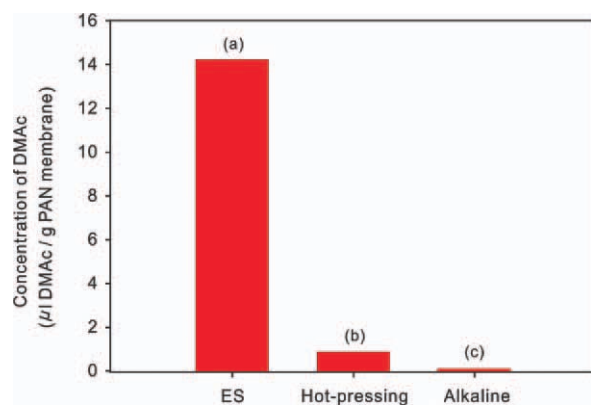
Figure 3 Evaluation of residual DMAc concentration in AEA-COOH membrane after different processes. (a) After electrospinning. (b) After hot pressing at 100°C. (c) After alkaline hydrolysis at 3N NaOH, 85°C, 30 min. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was also higher than Sartobind® C membrane (73.4%). These result confirmed that ion-exchange membrane formed by PAN nanofibers, was lighter and thinner, with a faster absorption rate and higher volume flux than that of Sartobind® C membrane.

Organic solvent residue test in electrospun nanofiber manufacturing process

According to the MSDS description, DMAc is harmful to the human body, with an 8-h time-weighted average of 10 ppm (on skin) and a short-term exposure limit of 15 ppm (on skin). Figure 3 shows the verification of organic solvent (DMAc) residue in PAN nanofibers. The results indicate that after electrospinning, the residual DMAc concentration is about 15 µL/g, $\sim$ 750 ppm. However, following the hot press process, it rapidly decreases to 25 ppm,

and after the alkaline hydrolysis and washing processes decreases to undetectable levels. This result proves that for the protein purification, the release of residual organic solvents is not an issue.

Evaluation of lysozyme adsorption using IEX nanofibrous membranes

An AEA-COOH membrane is compared with various Sartobind-based commercial membranes for protein adsorption in various pH environments (as shown in Fig. 4). Sartobind C membrane contains R-COO$^-$; Sartobind D membrane contains R-CH$_2$N$^+$(C$_2$H$_5$)$_2$; Sartobind Q membrane contains R-CH$_2$N$^+$(CH$_3$)$_3$; and Sartobind S membrane contains R-CH$_2$SO$_3^-$. The isoelectric pH of lysozyme is 10.7. In other words, when the pH value is 11, lysozyme protein becomes negatively charged; otherwise, it is positively charged.

The results showed that when the pH value is between 4 and 12, the commercially available positively charged ion-exchange membranes (Sartobind D and Q membranes) have no effect on lysozyme protein adsorption. The effect of Sartobind S membrane on lysozyme adsorption is not affected by changing pH values, which is about 52 mg/g. As to AEA-COOH and Sartobind C membranes, the adsorption curves of lysozyme shows the tendency in correlation with the value of pH. After the adsorption reaches the maximum, it decreases with the continued rise of pH. In terms of Sartobind C membrane, its adsorption capacity is at a maximum, $\sim$ 54.6 mg/g, when the pH reaches 10. When AEA-COOH is in a solution of pH 9, it reaches the highest adsorption amount at $\sim$ 105 mg/g. In other words, 1 g of AEA-COOH membrane in a pH 9 environment can adsorb $\sim$ 105 mg of lysozyme protein, which is approximately twice that of Sartobind C and Sartobind S membranes. This is because that AEA-
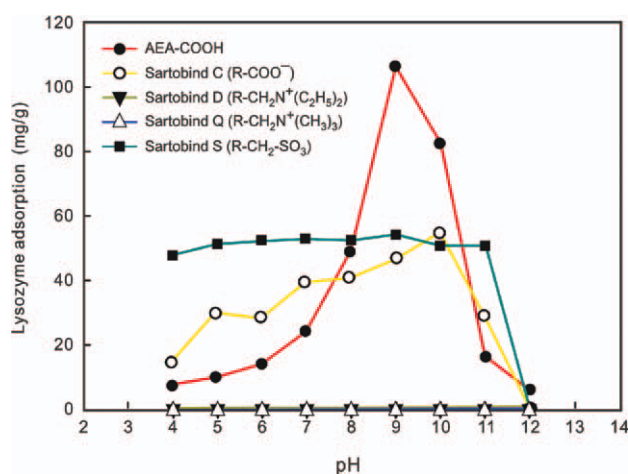

Figure 4 Lysozyme adsorption capacity of different IEX membranes at different pH values. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
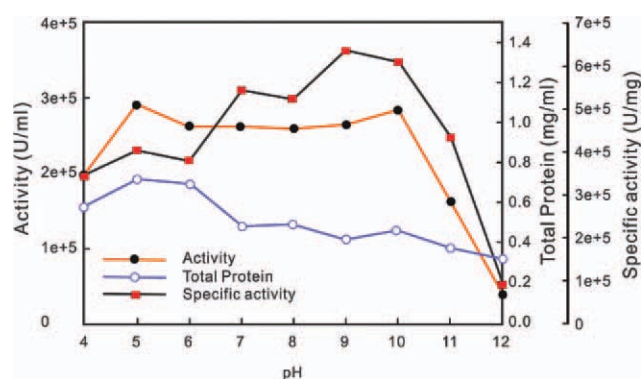

Figure 5 Direct lysozyme purification from chicken egg white using AEA-COOH IEX membranes at different pH values in a batch system. [Color figure can be viewed in the online issue, which is available at wileyonline library.com.]

TABLE I
Purification Performance of AEA-COOH

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Protein recovery (%)	Activity recovery (%)
Crude feedstock	305.8	$1.5e + 07$	$4.9e + 04$	1.00	–	–
Flow	288.8	$1.1e + 07$	$3.7e + 04$	–	–	–
Wash	13.3	$7.3e + 05$	$5.5e + 04$	–	–	–
Elution	0.8	$2.9e + 06$	$3.6e + 06$	73.6	22.3	80.5

Specific activity (U/mg) = total activity (U)/total protein (mg). Purification factor (fold) = specific activity of elution/ specific activity of crude feedstock. Protein recovery (%) = (protein of elution)/(protein of flow − protein of wash) × 100%. Activity recovery (%) = (activity of elution)/(activity of flow − activity of wash) × 100%.

COOH shows higher surface area and porosity than Sartobind C, resulting in dense ligand density after alkaline hydrolysis modification and high-protein binding capacity. Once pH exceeded 10, the adsorption trend for lysozyme declines.

IEX nanofibrous membrane for lysozyme purification from chicken egg white

The results in the previous study proved that AEA-COOH nanofibrous membranes with per unit membrane weight as the benchmark are more efficient for lysozyme protein adsorption than commercially available membranes (Sartobind C and S).[14] It showed the highest protein adsorption capacity in a pH 9 environment. Therefore, we choose AEA-COOH membranes to do batch purification of lysozyme from chicken egg white solutions under different pH values, exploring the interference from other complex proteins and impure substances. The experimental results are shown in Figure 5. With the pH value between 5 and 10, the lysozyme activity is maintained at ∼ 2.8e + 5 (U/ml). Because only lysozyme in the chicken egg white solution possesses cell lysis activity, the data prove that the impure protein adsorption on AEA-COOH decreases as increasing pH values, indicating that the charge of proteins in the chicken egg white solution can be changed by adjusting pH values. The experiment also shows that AEA-COOH membranes have the highest enzymatic specific activity at pH 9, indicating that lysozyme obtained in this condition has the highest purity.

Continuous lysozyme purification from chicken egg white solution

This study places surface-modified AEA-COOH IEX membranes in a Millipore stirred cell reactor. Under continuous operation, lysozyme protein is purified directly from commercially available fresh chicken egg whites. Table I shows the yield of each operation step throughout the entire purification process. Before purification, the commercial chicken egg white (100 mL) has a total protein of 305.8 mg, total

activity of 1.5e + 07 U, and specific activity of 4.9e + 4 U/mg. After adding ∼ 100 mL of chicken egg white solution into the reactor, the lysozyme is adsorbed by the AEA-COOH membrane with an adsorption amount of 17.0 mg and a protein activity of 4.0e + 06 U. When adsorption is completed, 100 mL of washing buffer is used to remove unbounded lysozyme and other impure proteins, during which ∼ 13.3 mg of the protein mass was washed off with protein activity at 7.3e + 05 U. Elution was the stage of desorption. Approximately 0.8 mg of protein is eluted and purified, however, protein activity is as high at 2.9e + 06 U. Although the protein recovery is only 22.3%, the activity recovery of lysozyme is as high as 80.5%, indicating that most of the lysozyme is desorbed by $1M$ NaCl and purification efficiency increases 73.6 times.

The purified solution was checked using SDS-PAGE to determine whether the molecular weight of the purified product was that of lysozyme (14.3 kDa). As shown in Figure 6, M is the protein marker; Lane 1 is crude feedstock sample comprising a fresh raw egg white solution containing many complex proteins
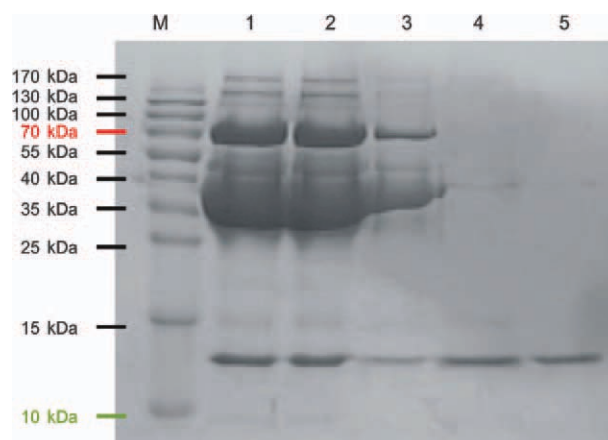

Figure 6 SDS-PAGE of lysozyme protein after ion-exchange purification by the AEA-COOH membrane. M is the protein marker; Lane 1 is crude feedstock sample; Lane 2 is the adsorption stage; Lane 3 is the cleaning stag; Lane 4 is the elution stage; Lane 5 is a commercial lysozyme standard. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II
Comparison of Different Lysozyme Purification Systems

Purification process	Type of ligand	Purification factor (fold)	Activity recovery (%)	References
PAN nanofiber membrane adsorption	PAN nanofibers modified with carboxylic group	73.6	80.5	This work
Stirred fluidized bed adsorption	Streamline SP	10.9	97.8	18
Poly(hydroxyethylmethacrylate)/chitosan (pHEMA-chitosan) composite membrane	Reactive Green 19	25.4	82.0	19
Glass fiber-based cation-exchange membranes	Glass fibers modified with sulfonic acid	38.6	68.8	20
Polysulfone-based cation-exchange membranes	Sulfonated polysulfone	20.7	51.1	21

together with the target protein lysozyme; Lane 2 is the adsorption stage, where the target protein and other complex proteins were present in the filtrated protein solution, indicating excessive chicken egg whites; Lane 3 is the cleaning stage, where a small amount of the target product, along with a large amount of hybrid proteins, were washed off; Lane 4 is the elution stage, where the lysozyme adsorbed on the AEA-COOH membrane was eluted, with the figure showing minimal amounts of unwanted proteins indicating effective purification; Lane 5 is a commercial lysozyme standard product. By comparison with lysozyme standard and protein marker, the molecular weight of purified protein was confirmed to be 14.3 kDa, which is that of lysozyme protein. Purification factor and activity recovery of different lysozyme purification systems were compared and shown in Table II. The results further validated that AEA-COOH membrane can be used to separate highly pure lysozyme protein from chicken egg whites.

CONCLUSIONS

In this study, a combination of electrospinning technology and alkaline hydrolysis process was used to prepare a weak acid IEX membrane with a high-surface functional group density, high porosity, and 3D nanofibrous structure. The membrane, when placed in a pH 9 environment, was able to adsorb $\sim$ 105 mg of lysozyme protein, which is approximately twice that of any commercially available product. By placing the membrane in a stirred cell reactor, the feasibility of using continuous operation methods to directly separate and purify lysozymes from chicken egg whites was confirmed and protein purification efficiency was improved by 73.6 times. This method can effectively reduce subsequent processing steps, shorten overall purification time, and lower the cost

of separation and purification. Therefore, the weak acid AEA-COOH ion-exchange membrane developed in this study, when used in a protein purification system, has a wide range of commercial value.

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