Large-scale preparation technology of lysozyme from egg white liquid

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Commercial lysozyme is usually extracted from egg white by traditional methods such as crystallization, precipitation, and centrifugation. Those single separation and purification methods have the defects of low purity and recovery. Several methods could be combined reasonably to establish a continuous separation system to be used in the large-scale separation of lysozyme. In this study, a simple and fast extracting technology of lysozyme from waste egg white liquid was developed including resin adsorption and two ultrafiltration. The egg white liquid was diluted with water, and then filtered to remove impurities. The macromolecular protein was separated by ultrafiltration. The lysozyme in permeate was absorbed by X-5 resin, and then, washed with 0.01 mol/L NaCl solution. The eluent was desalted by ultrafiltration membrane with a molecular weight of 5,000 Da at 0.15 MPa. The purified lysozyme concentrate was obtained with lysozyme activity of 18,203.6±125.2 U/mL. The enzyme activity recovery was 86.47%. The technique can be used to extract lysozyme from egg white on a large scale, while the macromolecular proteins with high activity can be obtained at the same time of lysozyme preparation due to the mild conditions.

Keywords: lysozyme; egg white solution; ultrafiltration; resin adsorption; large-scale preparation.

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Introduction

Lysozyme, also known as muramidase, is an alkaline protein, which can be used as a potential substitute for antibiotics in food, biological engineering, medical treatment, animal feed, and other fields because of its antibacterial and non-toxic [1, 2]. Lysozyme is widely found in animals, plants, and microorganisms. Most commercially available lysozyme is extracted from egg white due to its abundant content and resources [3, 4].

The preparation methods of lysozyme mainly include direct crystallization, ion exchange, ultrafiltration, polyelectrolyte precipitation, and affinity chromatography, *etc*. The direct crystallization method is easy to operate, but it is not suitable for industrial production due to its long production cycle and low yield, as well as low purity and activity. The production cycle of polyelectrolyte precipitation method is long, and the reagent introduced in the extraction process make the product unsuitable for food and medicine. Affinity chromatography can quickly separate the object from other impurities, but it is easy to clog the column when the solution viscosity is high. Cationic exchange method is mainly adopted in industrial production. The relatively ideal yield and purity can be obtained through such steps as adsorption of cationic resin, high-salt elution, desalination, and spray drying [5, 6]. Most of the protein in egg white was destroyed in the process of adsorption and high-salt elution, making it impossible to carry out the subsequent processing and application of egg

white protein [7, 8]. Ultrafiltration is a common method of desalination and dehydration in industry. The high salt content of lysozyme eluent solution increased the burden of ultrafiltration, while the ultrafiltration membrane was easy to be polluted in a long time working.

Egg yolk is the main raw material of egg product such as mayonnaise, yolk powder, yolk liquid, and salted egg yolk. Egg whites is treated as waste during processing, which causes serious waste of resources. Egg white contains a variety of proteins with biological activities, such as ovalbumin, ovotransferrin, ovomucoid, lysozyme, and ovalbumin, *etc*. [9], which have attracted the strong attention of many scientific researchers. Eggs are also the main raw material of cold Tofu with Japanese dressing. To reduce manufacturing cost, many manufacturers begin to use egg white liquid as raw material instead of whole eggs. The egg white liquid needs to be preliminarily ultra-filtered to obtain macromolecular protein for the production, while the lysozyme exists in the low molecular permeate liquid. Therefore, the efficient recovery of lysozyme can turn the waste into treasure, extend the industrial chain, and further improve the efficiency of egg processing industry [10].

The main purpose of this study was to develop a simple and fast process with mild conditions, which could be used for the large-scale preparation of lysozyme from waste egg white liquid in egg yolk processing, while no complex equipment was required. In addition, we expected that this newly developed method could overcome the disadvantages in current technologies, and moreover, the other macromolecular proteins with high activity could also be obtained at the same time of lysozyme preparation.

Materials and methods

The procedures of lysozyme preparation from egg white liquid were divided into several units including pretreatment of egg white liquid, lysozyme extraction with resin, and ultrafiltration desalination.

Pretreatment of egg white liquid

The egg white liquid provided by Yantai Laoshaole Food Co., LTD. (Yantai, Shandong, China) with a small amount of visible yolk residue and 526.3±23.4 U/mL of lysozyme activity was diluted with water in ratios of 1:1, 1:2, 1:3, and 1:4, respectively. Water and egg white liquid were stirred and mixed well before sieved with a 0.85 mm sieve to remove the remaining yolk and other blocky impurities. The filtrate was ultrafiltered by using RO-NF-VF-400 membrane separation equipment for laboratory (Shanghai Mosu Science Equipment Co., LTD., Shanghai, China) with 20 kDa polysulfone ultrafiltration membrane. The operating pressure was set to 0.05 MPa, 0.1 MPa, 0.15 MPa, and 0.2 MPa, respectively, and was optimized with membrane flux, transmissibility, and interception of lysozyme as the indexes.

The membrane flux was calculated as follows:

$$J_w = \frac{V_1}{S_m t}$$

where J_w was the membrane flux (L/m^2•h). V_1 was the volume of permeate (L). S_m was the effective area of film (m^2). t was the ultrafiltration time (h).

The penetration rate of lysozyme was used to indicate the transmissibility of lysozyme and was calculated as follows:

$$PR(\%) = \frac{A_1 \bullet V_1}{A_0 \bullet V_0} \times 100\%$$

where A_1 and A_0 were the abilities of lysozyme in permeate and raw material solution, respectively. V_1 and V_0 were the volume of permeate and raw material solution.

The rejection rates of lysozyme and protein were

used to measure the interception characteristics of lysozyme. The rejection rate of lysozyme was calculated as follows:

$$R_L(\%) = \frac{A_2 \bullet V_2}{A_0 \bullet V_0} \times 100\%$$

where A_2 and A_0 were the abilities of lysozyme in retentate and raw material solution, respectively. V_2 and V_0 were the volume of retentate and raw material solution.

The rejection rate of protein was calculated as follows:

$$R_P(\%) = \frac{C_2 \bullet V_2}{C_0 \bullet V_0} \times 100\%$$

where C_2 and C_0 were the concentrations of protein in retentate and raw material solution, respectively. V_2 and V_0 were the volume of retentate and raw material solution.

Lysozyme extraction

Resin D401, X-5, D3520, AB-8, D301G, 732, D4020, Dg23, NKA-II, and NKA-9 obtained from Tianjin Nankai University Resin Co. LTD (Tianjin, China) were prepared according to the conventional method. Treated resins were added into the ultrafiltration solution for lysozyme adsorption, respectively, with the ratios of solid to liquid set as 1:3, 1:4, 1:5, 1:6, 1:7, and 1:8. After adsorption, resin was washed with 0.01 mol/L NaCl solution at volume ratio of 1:3 to wash out hetero proteins. The resin was then packed in a column (1 × 50 cm) with 30 mL bed volume (BV). The adsorbed resin was eluted with NaCl aqueous solution of equal volume of 0.01 mol/L, 0.05 mol/L, 0.10 mol/L, 0.15 mol/L, 0.20 mol/L, and 0.25 mol/L, respectively. The elution velocities were set as 1 BV/h, 2 BV/h, and 3 BV/h. The extraction conditions of lysozyme were optimized taking resin species, adsorption conditions, and elution conditions as investigation factors. The adsorption rate (R_A), residual rate (R_R), and elution rate (R_E) of resin adsorption were calculated as follows:

$$R_A(\%) = \frac{C_0 - C}{C_0} \times 100\%$$

$$R_R(\%) = \frac{C}{C_0} \times 100\%$$

$$R_E(\%) = \frac{C_E \times V_E}{C_0 \times V_0} \times 100\%$$

where C_0, C, and C_E were the abilities of lysozyme in raw material solution, residual liquid after adsorption, and eluent, respectively (U/mL). V_0 and V_E were the volume of raw material solution and eluent. The yield of lysozyme (Y) was calculated as follows:

$$Y(\%) = \frac{A_P \times V_P}{A_0 \times V_0} \times 100\%$$

where A_P and A_0 were the abilities of lysozyme in product liquid and raw material solution, respectively. V_P and V_0 were the volumes of product liquid and raw material solution.

Desalination

The eluent was desalted by ultrafiltration with a polysulfone membrane of 5,000 Da to obtain concentrated lysozyme. The effect of ultrafiltration pressure on desalting was investigated at 0.05 MPa, 0.1 MPa, 0.15 MPa, and 0.2 MPa, respectively.

Determination of lysozyme activity

Lysozyme activity was determined by the lysozyme detection kit (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, Jiangsu, China). Briefly, 5 mg of *M*. *Lysodeikticus* was suspended in 20 mL of phosphate buffer (0.2 mol/L, pH 6.2) to obtain the bacteria suspension. The lysozyme solution and the bacteria suspension were pre-heated at 37℃ for 15 min. Then, 2.0 mL of bacterial suspension was transferred into a 1 cm cuvette with 0.2 mL of lysozyme solution. After mixed well, the optical densities under OD_{530} were measured at 15 s and 75s. The enzyme activity was calculated as follows:

$$Lysozyme\ activity\ \ (U/mL) = \frac{\Delta E_{530}}{0.001 \times 0.2 \times C}$$

where ΔE_{530} was the difference between the absorbances at 15 s and 75 s. *C* was the content of lysozyme in solution, which was determined by the Coomassie method.

Statistical analysis

Software SPSS (version 11.0) (IBM, Ammon, New York, USA) was used for variance analysis. The data were analyzed statistically by one-way ANOVA. Significance of any difference between groups was evaluated using Student's t-test. All data were expressed as mean ± SD. *P* value less than 0.05 was considered statistically significant.

Results and discussion

Dilution and impurity removal of egg white liquid

1. Influence of dilution on pretreatment

The raw materials used in this study were commercial egg white liquid provided by food factory. A small amount of yolk and other blocky impurity contained in raw materials need to be removed before ultrafiltration because they could form complex compounds with lysozyme to affect the activity of lysozyme [11, 12]. Due to the high viscosity of egg white liquid, appropriately dilution was needed to ensure the smooth filtration and ultrafiltration.

In order to reduce the inactivation effect of residual egg yolk on lysozyme in the dilution process, low-speed mechanical stirring was adopted instead of high-pressure homogenization in this study. Low-speed mechanical stirring can effectively retain the activity of lysozyme, and at the same time, avoid the emulsification effect caused by the strong dispersive effect of high-pressure homogenization, and make the subsequent ultrafiltration smoothly. Obvious bubbling phenomenon could be observed with the increase of stirring speed and the extension of time, which should go against the subsequent filtration and ultrafiltration. The water and egg white liquid mixing uniformity without bubbling was observed at 200 rpm stirring for 5 minutes, which could further reduce the denaturation of lysozyme. It was difficult to mix the egg white liquid and water evenly in a short time when diluted in 1:1 ratio. The high viscosity of liquid made it difficult to carry out the filtration smoothly even at the initial stage. The filtering operation could be carried out smoothly when the dilution ratio reached 1:2 or above.

Ultrafiltration is considered as one of the most promising methods for protein separation based on the difference of molecular weights. The relative molecular weight of lysozyme was about 14,000 Da while the other proteins in egg white were above 30,000 Da [13, 14]. Therefore, polysulfone ultrafiltration membrane with a retaining molecular weight of 20 kDa was adopted to filter the diluted egg white liquid after filtration. The egg white liquid was diluted with water in volume ratios (V/V) of 1:2, 1:3, and 1:4, respectively. The diluted liquid was ultrafiltered at 0.10 MPa. The effects of dilution ratios on membrane flux and lysozyme were shown in Figure 1 and Table 1.

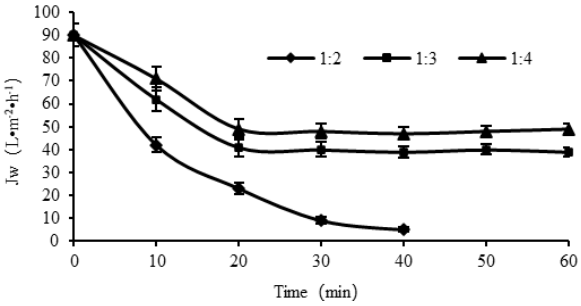

Figure 1. Effect of dilution on membrane flux.

In the initial 10-20 mins, the membrane flux decreased with the increase of ultrafiltration time (Figure 1) because a gel layer formed on the surface of the membrane with the progress of ultrafiltration, which increased the resistance of the liquid through the membrane [15, 16]. The membrane flux increased with the increase of the dilution at the same time, which might be due to

Table 1. Effect of dilution on transmissibility and interception of lysozyme.

Dilution ratio ($V_{egg\ white}$: V_{water})	Penetration rate of lysozyme (%)	Rejection rate of lysozyme (%)	Rejection rate of protein (%)
1:2	15.7±0.2	82.3±1.3	87.6±1.1
1:3	95.6±1.5	2.7±0.2	84.2±0.6
1:4	96.2±1.2	2.1±0.1	82.5±0.9

the decline of the concentration and viscosity of solution [17-19]. With a dilution ratio of 1:2, the membrane flux decreased rapidly with the ultrafiltration operation. There was basically no liquid penetration for about 40 mins, indicating that the membrane was seriously blocked at this time. Ultrafiltration could be carried out successfully at dilutions rates of 1:3 and 1:4. The membrane flux of the latter ratio was slightly higher than that of the former one. However, there was no significant difference (p>0.05) in the permeability and retention of lysozyme.

2. Effect of ultrafiltration pressure on pretreatment

After filtration, polysulfone ultrafiltration membrane with a molecular weight of 20 kDa was used for ultrafiltration of the filtrate. The operating pressure was set to 0.05 MPa, 0.1 MPa, 0.15 MPa, and 0.2 MPa, respectively. Figure 2 demonstrated the membrane flux under different operating pressures, while Table 2 showed the permeability and retention rate of lysozyme.

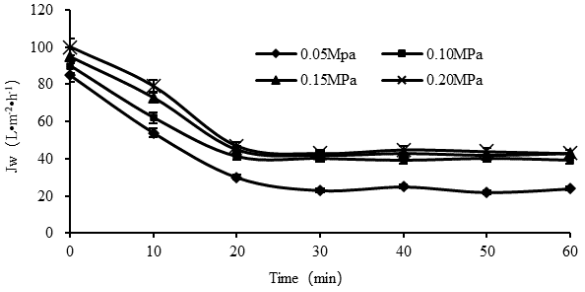

Figure 2. Effect of operating pressure on membrane flux.

As shown in Figure 2, increasing the operating pressure appropriately could increase the flow rate of liquid, thus increasing the membrane flux. However, when the protein concentration on the membrane surface reached saturation, concentration polarization would be intensified with the continuous increase of pressure, which led to the formation of "gel layer" on the membrane surface [20, 21] and reduced the permeability of the membrane, so that affected its separation performance. The membrane flux under the operating pressure of 0.10 MPa, 0.15 MPa, and 0.20 MPa showed no significant difference (p>0.05). However, it was significantly higher than that at 0.05 MPa (p<0.01). The protein rejection rate decreased with the increase of operating pressure, which indicated that higher operating pressure should push more proteins through the ultrafiltration membrane. Lower operating pressure was more beneficial to the membrane module, which could effectively reduce the damage and blockage of the membrane. In a comprehensive consideration, the operating pressure of 0.10 MPa was adopted for ultrafiltration.

Extraction of lysozyme

1. Resin selection

(1) Adsorption capacity of different resins to lysozyme:

Different types of resins were added into the ultrafiltration solution, respectively, at the ratio of solid to liquid 1:6. After shocked for 1 h, the residual lysozyme activity in the liquid was determined, and the adsorption rate of the resin on the lysozyme was calculated as shown in Figure 3. Among these resins, X-5 had the highest adsorption capacity for lysozyme with the adsorption rate reaching 92.15% followed by D4020 (90.12%), D401 (84.72%), D3520 (78.17%), and Dg23 (76.33%). The adsorption rate of 732 resin on lysozyme was only 20.27%.

(2) Elution effect of different resins:

The adsorbed resins were eluted with 0.2 mol/L NaCl aqueous solution. The elution rates of

Table 2. Effects of operating pressure on transmissibility and interception of lysozyme.

Operating pressure (MPa)	Penetration rate of lysozyme (%)	Rejection rate of lysozyme (%)	Rejection rate of protein (%)
0.05	83.6±1.6	15.7±0.2	86.3±1.5
0.10	95.6±1.5	2.7±0.3	84.2±1.3
0.15	96.4±1.7	2.6±0.5	82.1±1.4
0.20	96.5±1.8	2.7±0.3	80.7±1.3

lysozyme were shown in Figure 4. The elution rates of X-5, D4020, D401, D3520, and Dg23 were 80.15%, 7.98%, 78.95%, 82.82%, and 4.71%, respectively.

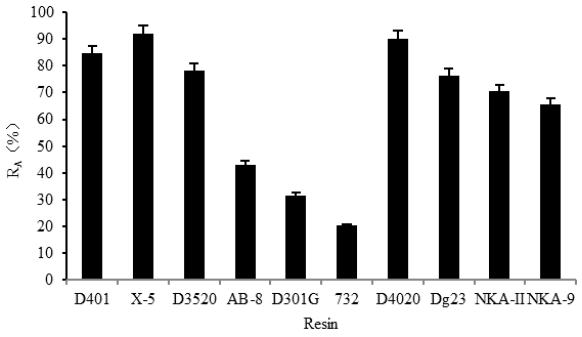

Figure 3. Adsorption capacity of different resins to lysozyme.

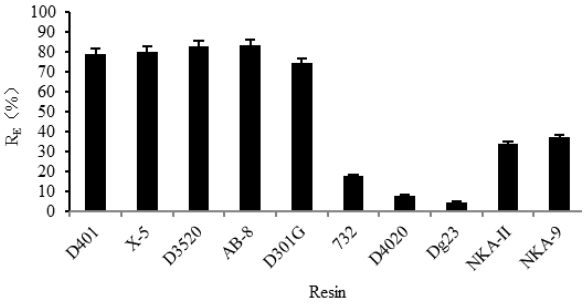

Figure 4. Elution effect of different resins.

Considering the adsorption capacity and elution effect of resin on lysozyme, X-5 was most suitable for the extraction of lysozyme from egg white liquid.

2. Effect of resin dosage on lysozyme adsorption

According to the current factory production mode, 25 g of X-5 resin was mixed with ultrafiltration permeate. After adsorbed for 2h, the residue rate of lysozyme was determined and shown in Figure 5. The residual rate of lysozyme decreased gradually with the increase of resin dosage, and tended to be saturated after the weight ratio of resin to liquid reached 1:5, as the residual rate of lysozyme in the liquid tended to be stable.

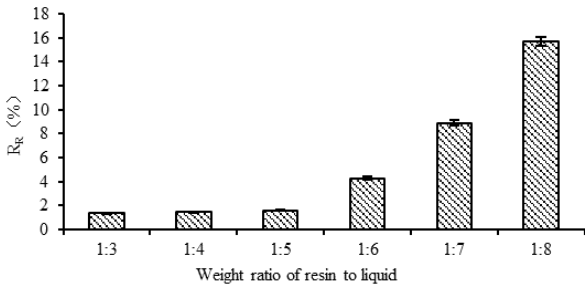

Figure 5. Effect of resin dosage on lysozyme adsorption.

3. Effect of adsorption time on lysozyme adsorption

The X-5 resin was mixed with the ultrafiltration permeate according to the mass ratio of 1:5. The residual rate of lysozyme decreased gradually with the extension of adsorption time (Figure 6), indicating that the lysozyme gradually adsorbed on the resin. After adsorption for 70 mins, the residual rate of lysozyme tended to be stable. From the perspective of industrial production, the adsorption time could be determined to be 60-80 mins.

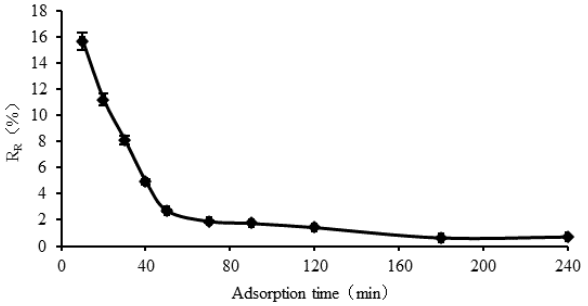

Figure 6. Effect of adsorption time on lysozyme adsorption.

4. Effect of NaCl concentration in desorption solution on lysozyme elution

The adsorbed X-5 resin was eluted for 60 mins with NaCl aqueous solution of equal volume of 0.01 mol/L, 0.05 mol/L, 0.10 mol/L, 0.15 mol/L, 0.20 mol/L, and 0.25 mol/L, respectively. The elution rate of lysozyme and protein were shown in Figure 7.

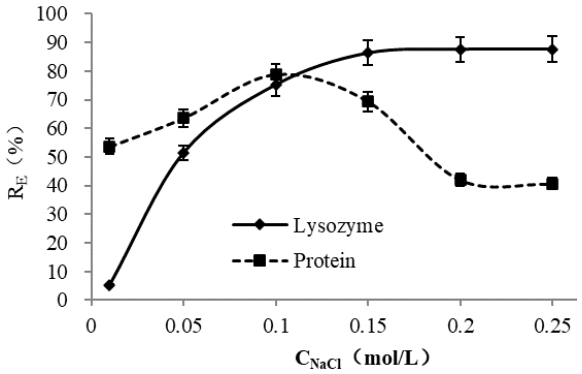

Figure 7. Effect of concentration of NaCl on lysozyme elution.

Low concentration of NaCl solution (0.01 mol/L) could wash down a certain amount of protein, but the elution rate of lysozyme was very low, indicating the major protein to be eluted was miscellaneous protein [22]. With the increase of NaCl concentration in the eluent, the elution rate of protein showed a downward trend after rising first. The elution rate of protein was lower with no significant difference ($p > 0.05$) when eluted with high concentration of NaCl solution (0.20 mol/L and 0.25 mol/L). The elution rate of lysozyme increased gradually with the increase of concentration of NaCl solution, and tended to be stable when the concentration of NaCl solution reached 0.15 mol/L, indicating that high concentration of NaCl was conducive to the elution of lysozyme and could properly inhibit the elution of other hetero proteins [23]. The elution rates of lysozyme showed no significant difference ($p > 0.05$) when higher concentrations of 0.15 mol/L, 0.20 mol/L, and 0.25 mol/L NaCl solution were adopted as elution. However, the elution rate of protein at the 0.15 mol/L NaCl solution was significantly higher than the others ($p < 0.01$), which indicated that 0.15 mol/L NaCl solution was optimal for simultaneous elution of lysozyme and protein, while 0.20 mol/L and 0.25 mol/L NaCl solutions were more helpful for the elution of lysozyme only. When NaCl solution of 0.20 mol/L and 0.25mol/L were used as elution, there were no significant difference in the elution rate of lysozyme, as well as protein ($p > 0.05$). Considering the aggravated pressure of desalting in subsequent processes, the concentration of NaCl solution should be reduced as much as possible on the premise of ensuring the elution of lysozyme. In order to improve the elution efficiency of lysozyme, it could be considered to wash out some hetero proteins with 0.01 mol/L NaCl solution first, and then elute lysozyme with 0.15 mol/L NaCl solution.

5. Effect of desorption velocity on lysozyme elution

After adsorption, resin was washed with 0.01 mol/L NaCl solution at volume ratio of 1:3 to wash out hetero proteins. The resin was then packed in a column (1 × 50 cm^2) with 30 mL bed volume (BV). 0.15 mol/L NaCl solution was adopted as elution by elution velocity of 1 BV/h, 2 BV/h, and 3 BV/h, respectively. The elution curves under the different flow rates were drawn as shown in Figure 8 after determining the lysozyme activity in eluent every 10 mL.

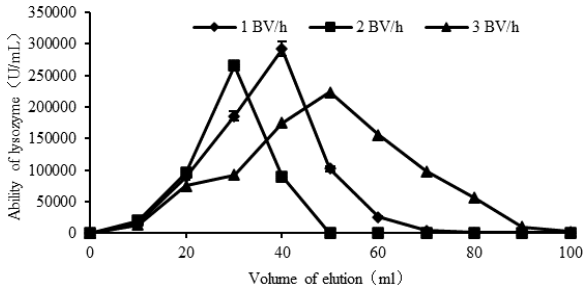

Figure 8. Effect of elution velocity on lysozyme elution.

As shown in Figure 8, the elution peaks were narrow and symmetrical under 1 BV/h and 2 BV/h elution rates. The highest lysozyme activity in eluent under 2 BV/h was slightly lower than that under 1 BV/h in the low volume. The elution peak obtained at the elution velocity of 3 BV/h was significantly wider than the former two, indicating that the elution at this rate was not

conducive to the enrichment of lysozyme [24, 25]. Although the highest concentration of elution solution obtained at 2 BV/h elution was slightly lower than that at 1 BV/h, the shorter elution time made the elution velocity of 2 BV/h more suited for selected industrial production.

Desalination technology

The eluent was ultrafiltered with a 5,000 Da polysulfone ultrafiltration membrane. The operating pressure was set as 0.05 MPa, 0.1 MPa, 0.15 MPa, and 0.2 MPa, respectively. Figure 9 showed the membrane flux under different operating pressures.

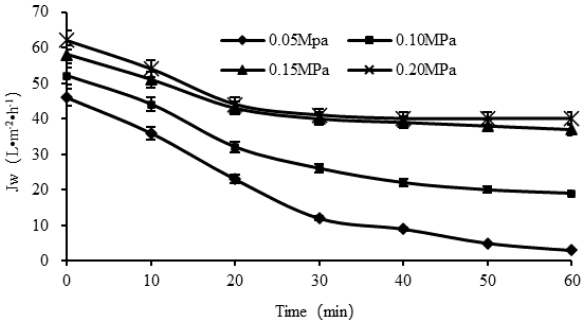

Figure 9. Effect of operating pressure on membrane flux.

The change trend of membrane flux with the operating pressure was similar to the first ultrafiltration, in which the material liquid flow rate increased with the raising of operating pressure, so as to increase the membrane flux. Membrane fluxes under 0.15 MPa and 0.20 MPa pressures were significantly higher than that of other pressures ($p < 0.05$) in the experiment with no significant difference between them ($p > 0.05$). The molecular weight of ultrafiltration membrane (5,000 Da) was significantly less than that of lysozyme (14,000 Da). The interception rate of lysozyme decreased slightly with the increase of operating pressure, while the transmissibility rate increased slightly ($p > 0.05$) (Figure 10). Taking all things into consideration, ultrafiltration was carried out at the operating pressure of 0.15 MPa on account of a relatively high processing speed on the premise of ensuring the retention rate of lysozyme.

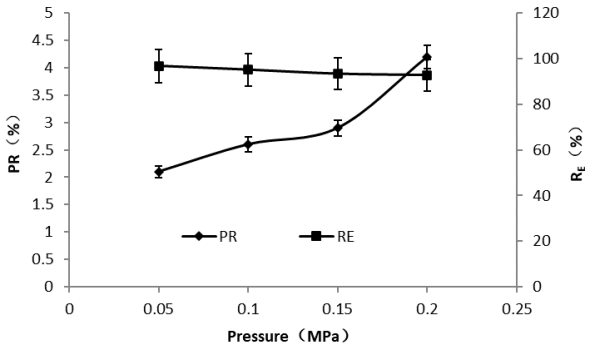

Figure 10. Effects of operating pressure on the transmissibility and interception of lysozyme.

In this study, NaCl solution was used to replace the traditional ammonium sulfate solution for the elution of lysozyme. Comparing to ammonium sulfate, NaCl solution could effectively reduce protein denaturation and precipitation caused by high ammonium sulfate concentration in the elution solution, which was more favorable for the preparation of lysozyme.

Amplification of lysozyme preparation

The lysozyme was extracted according to the optimization process determined above on a large scale. 20 m^3 egg liquid was diluted with threefold water. After stirred at 200 rpm for 5 minutes and followed by filtered with 0.85 mm sieve, the filtrate was ultrafiltered through 20 kDa polysulfone ultrafiltration membrane with a RO-NF-VF-400 ultrafiltration nano-filtration system. The effective membrane area was 20 m^2 and the operating pressure was 0.10 MPa. After the processing, 62 m^3 permeate was obtained, and then, added to an exchange tank filled with 12 m^3 treated X-5 resin for lysozyme adsorption in stirring state. The liquid was released after adsorption for 70 mins. The resin was then washed with 12 m^3 of 0.01 mol/L NaCl solution followed by dynamic elution at 2 BV/h flow rate. The eluent with lysozyme activity more than 5,000 U/mL was collected, and then was ultrafiltered with 5,000 Da ultrafiltration membrane at 0.15 MPa. 0.5 m^3 desalted lysozyme concentrate was obtained with lysozyme activity of 18,203.6±125.2 U/mL with the activity recovery of 86.47%. Comparing to the traditional ion exchange method, the specific

activity and the yield of lysozyme were both improved [8].

Conclusion

This study developed the extraction method of lysozyme by two-step ultrafiltration combined with ion exchange in order to realize the comprehensive utilization of waste egg white in food yolk processing. The simple and fast process could overcome the deficiency of lysozyme preparation process in the present and easy to realize large-scale production. Lysozyme could be obtained with high biological activity under mild conditions. The industrial feasibility of this method was confirmed by laboratory study and amplification experiment.

Acknowledge

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