Rapid Separation of Lysozyme from Chicken Egg White by Reductants and Thermal Treatment

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Reductants (0.1−2.0% ascorbic acid, cysteine, or cystine and 0.04−1.0% β-mercaptoethanol) were added to 5-fold diluted, salted duck egg whites (commercially and laboratory prepared) and fresh egg whites (chicken and duck), and subsequently the mixtures were heated at 70 °C for 1−10 min. The maximal recovery and purification fold of lysozyme obtained from fresh chicken egg whites added with 1.0% ascorbic acid were 78% and 2.4, respectively. Storage tests showed that the obtained lyophilized lysozyme powder after dialysis was stable when refrigerated at 4 °C for 3 months.

Keywords: *Chicken egg white; lysozyme recovery; reductant; thermal treatment*

INTRODUCTION

Lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC3.2.1.17) is a unique enzyme with the ability to lyse certain bacteria by hydrolyzing the β-linkage between muramic acid and *N*-acetylglucosamine of mucopolysaccharides in the bacterial cell wall. The properties of lysozyme have long been known, and the lysozyme content in duck eggs has been determined to be 1.2% and in hen egg whites 3.4%. The primary structures of the lysozymes from hen and duck eggs have also long been known to be very similar (Hermann and Jolle's, 1970).

The antibacterial action of lysozyme is mainly limited to Gram-positive strains. However, certain Gram-negative strains, such as *Escherichia coli* and *Salmonella typhosa*, which can both cause food poisoning, are also sensitive to lysozyme. In addition, lysozyme can inactivate certain viruses by forming an insoluble complex with acidic viruses (Hasselberger, 1978). Thus lysozyme has been utilized as a preservative for processed food such as Kamaboko (Akashi and Ohno, 1972) and cheese (Akashi, 1972) and as an antiinflammatory drug for treatment of wounds and infections (Proctor and Cunningham, 1988).

Dickman and Proctor (1952) indicated that lysozyme activity increases with increasing NaCl concentration between 0 and 0.5 M at pH 4.6−5.0 but declines rapidly when NaCl concentration is higher than 0.5 M. The pH value also affects the activity of lysozyme. Matsuoka et al. (1966) found that lysozyme maintained almost 100% activity after thermal treatment at 100 °C for 3 min at pH 4.5, although an increase in pH decreased the enzyme activity. Moreover, heat resistance of lysozyme in phosphate buffer was 50 times higher than in egg white (Cunningham and Lineweaver, 1965). Thus, lysozyme was considered to be much more stable in diluted solution than in original egg white during thermal treatment. Disulfide-exchange reactions between proteins due to thermal treatment usually cause polymerization and gel formation (Peng et al., 1982; King, 1977). Lysozyme comprises four disulfides, and the polymerization between lysozyme and ovalbumin in egg white through disulfide-exchange reactions usually reduces lysozyme activity (Cunningham and Lineweaver, 1967).

Due to increasing demands for natural food preservatives, lysozyme has become increasingly important in food processing, and therefore, there is a need for developing efficient and simple techniques for lysozyme production. Some methods, such as gel permeation chromatography (Palladino et al., 1981), membrane separation (Peri and Feresini, 1972), affinity chromatography (Weaver et al., 1977), adsorption, and ion-exchange chromatography (Cherkasov and Kravchenko, 1969; Li-Chan et al., 1986), have been investigated. However, these methods suffer from major drawbacks, i.e., the high cost of lysozyme products, and they appear impractical for routine use in food items. The classic method for preparation of commercial lysozyme is direct crystallization from hen egg white in the presence of 5% NaCl at pH 9.5. This usually requires a week until the enzyme is efficiently recovered. Thus, a more efficient and rapid method for separation of lysozyme from egg white is needed.

Salted duck egg yolk, prepared by pickling whole eggs, is widely used in traditional Chinese cakes, while the egg white, separated from the yolk, is seldom used in the food industry due to its high salt content. Thus, the salted egg white is a waste and can cause severe disposal problems. In addition, duck egg white, which contains only about one-third of the lysozyme of hen egg, is also difficult to utilize as a lysozyme source by the conventional precipitate crystallization method.

Therefore, our objective was to develop a method for rapid separation of lysozyme from egg whites, including salted duck egg white, for use in foods. Egg whites, from both hen and duck, were reacted with ascorbic acid, cysteine, cystine, and β-mercaptoethanol and then thermally treated. Supernatants were collected, and the

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10.1021/jf9902797 CCC: $19.00
Published on Web 01/21/2000

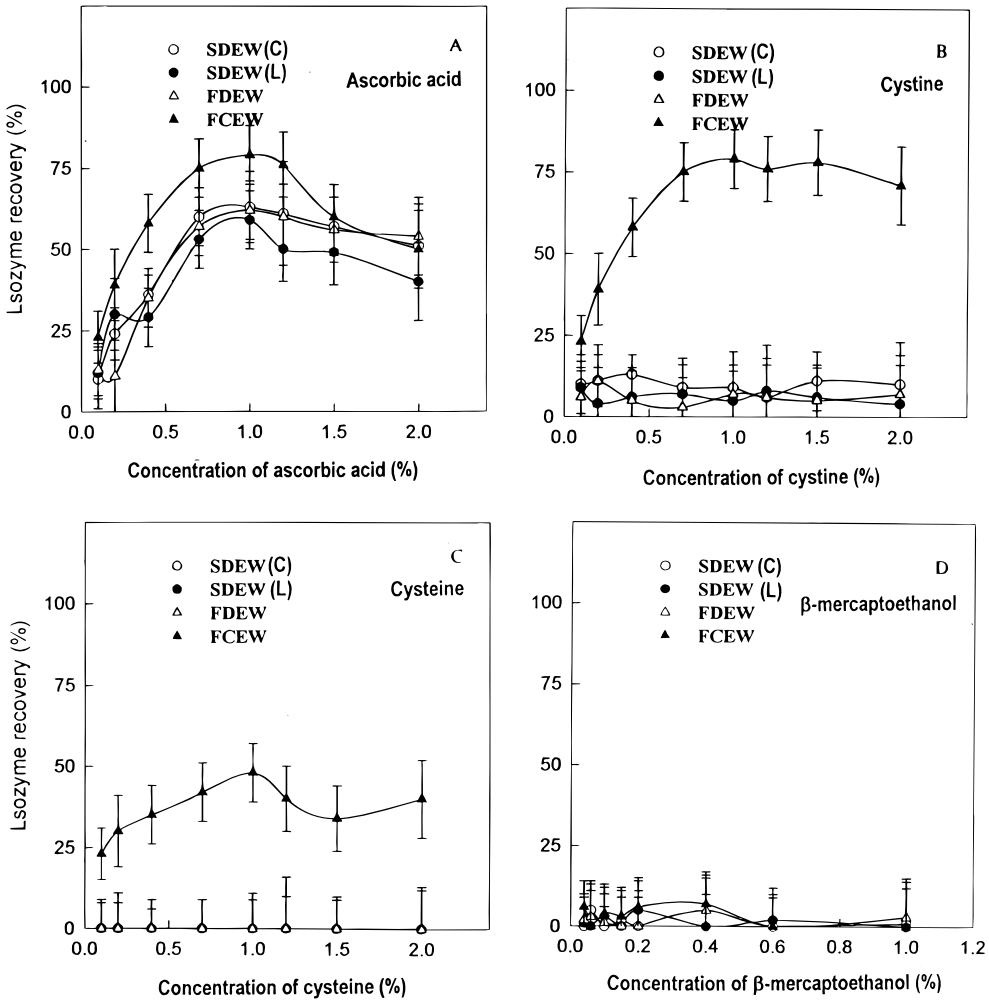

Figure 1. Effects of the addition of various levels of ascorbic acid (A), cystine (B), cysteine (C), and β-mercaptoethanol (D) on lysozyme recovery (%) [5-fold diluted commercial salted duck egg white (SDEW(C)), laboratory prepared salted duck egg white (SDEW(L)), fresh chicken egg white (FCEW), and fresh duck egg white (FDEW)]. Egg white samples were mixed with reductant and then heated in a water bath at 70 °C for about 5 min. Lysozyme activity in the supernatant was determined after centrifugation: lysozyme recovery (%) = (total lysozyme activity in supernatant)/(total lysozyme activity in corresponding starting egg white). Each value is the average of five replicates.

recovery, purification fold, and storage stability of the lysozyme thus obtained were investigated.

MATERIALS AND METHODS

Materials. Fresh duck and hen eggs were obtained from the local market. Ten duck eggs were pickled in saturated NaCl solution for 35 days at room temperature (24 °C) until the NaCl content in the egg white was 5−6%. The pickling solution was stirred daily during the pickling period in order to maintain homogeneous NaCl concentration. Fresh chicken egg white (FCEW), fresh duck egg white (FDEW), laboratory prepared salted duck egg white (SDEW(L)), and commercial salted duck egg white (SDEW(C)) were collected by straining through a nylon net (2-mm network) to remove the chalazae. To obtain homogeneous and less viscous samples for ease of operation, the egg whites thus obtained were diluted 5-fold with distilled water and treated as starting egg whites prior to mixing with the reductants. Cysteine, cystine, L-ascorbic acid, pure lysozyme, β-mercaptoethanol, and *Micrococcus leteus* powders were purchased from Sigma (St. Louis, MO).

Determination of Lysozyme Activity. The lysozyme activity in the collected supernatants as well as in the starting egg white was determined by the turbidimetric assay described by Li-Chan et al. (1986) and Weaver et al. (1977). *Micrococcus lysodeikticus* cells (Sigma Chemical Co., St. Louis, MO) were suspended in 0.067 M phosphate buffer (pH 6.24) at room temperature and absorbance at 450 nm (A 450) between 0.6 and 0.7 was measured. To 2.98 mL of this suspension, 20 μL of enzyme solution containing between 1000 and 2000 U/mL activity was added at zero time (the time hydrolysis was initiated) and was then thoroughly mixed with a vortex mixer. Absorbance at 450 nm vs reference buffer solution was recorded every 30 s for 2 min, using a spectrophotometer (JASCO 7800, Spectroscopic Co. Ltd., Tokyo, Japan). A decrease in absorbance at 450 nm (ΔA 450) of 0.001/min was taken as 1 unit of enzyme activity (U), and results were expressed as units/mL, calculated as:

$$\text{activity (U/min)} = (\Delta A\ 450/\text{min})/(0.001/\text{min} \times 0.02\ \text{mL}) \quad (1)$$

$$\text{specific activity (U/mg)} = (\text{U/mL})/(\text{protein mg/mL}) \quad (2)$$

Determination of Protein Content. Protein content was determined by the Biuret method (Gornall et al., 1949) at 540 nm. Bovine serum albumin (1−10 mg/mL) was used as a standard curve to calculate the protein content.

Addition of Reductants. To 50 mL of 5-fold diluted egg whites were added 0.1−2.0% (w/v) ascorbic acid, L-cysteine or L-cystine, and 0.04−1.0% (v/v) β-mercaptoethanol, and it was mixed thoroughly with a Vortex mixer for 5 min. The mixtures were then heated at 70 ± 1 °C in a water bath for about 5 min until the protein precipitation no longer proceeded. Shaking was conducted every 1 min during thermal treatment to maintain even temperature in the solutions. The pH value

Table 1. Comparison of Purification Fold and Lysozyme Recovered from Egg Whites by the Combination of 1% Ascorbic Acid (A), Cysteine (B), or Cystine (C) and Thermal Treatment[a]

	egg white[b] ($n^c = 5$)	total act. (U)	specific act. (U/mg)	purification (fold)	recovery[e] (%)
A					
	SDEW(C)	600000	671	1.0	100
	supernatant[d]	376200	1579	2.4	63
	SDEW(L)	470100	456	1.0	100
	supernatant[d]	282060	1346	3.2	60
	FDEW	399900	444	1.0	100
	supernatant[d]	257600	1455	3.3	64
	FCEW	1060200	1237	1.0	100
	supernatant[d]	829917	2996	2.4	78
B					
	SDEW(C)	500100	420	1.0	100
	supernatant[d]	44460	127	0.3	9.5
	SDEW(L)	470100	456	1.0	100
	supernatant[d]	21000	67	0.2	4.5
	FDEW	399900	444	1.0	100
	supernatant[d]	24400	69	0.2	6
	FCEW	1060200	1237	1.0	100
	supernatant[d]	795200	3203	2.6	75
C					
	FCEW	1060200	1237	1.0	100
	supernatant[d]	530100	2600	2.1	50

[a] Egg white samples were heated at 70 °C for about 5 min. [b] 50 mL of 5-fold diluted commercial salted duck egg white (SDEW(C)), laboratory prepared salted duck egg white (SDEW(L)), fresh duck egg white (FDEW), and fresh chicken egg white (FCEW) were used as egg white samples. [c] Number of samples. [d] Supernatant was obtained from heated egg whites by centrifugation (7000*g*, 20 min, 4 °C). [e] See legend to Figure 1.

of diluted egg whites with added ascorbic acid (0.1−2.0%) was between 3.5 and 4.5. To dissolve cystine powder, 0.1 N HCl (Merck, Germany) was added to reduce the pH to 2.0. Protein contents and lysozyme activity in the supernatants were determined after centrifugation (7000*g*, 20 min, 4 °C).

Lysozyme Recovery. The total lysozyme activity of the collected supernatant by centrifugation (7000*g*, 20 min, 4 °C) was divided by the total lysozyme activity of the corresponding starting egg white sample to calculate the recovery (%):

$$\text{lysozyme recovery (\%)} = \frac{\text{(total lysozyme activity in the supernatant)}}{\text{(total lysozyme activity in corresponding starting egg white)}} \quad (3)$$

Storage Test. One part of the crude lysozyme solution, separated from the egg white samples by the addition of ascorbic acid, cystine, or cysteine and subsequent thermal treatment after centrifugation (7000*g*, 20 min, 4 °C), was lyophilized (EYELA FD-5N, Rikakikai Co., Tokyo, Japan) without dialysis (control). The other part of the crude solution was lyophilized after dialysis against distilled water for 12 h. The products thus obtained were sealed in polyethylene bottles and refrigerated at 4 ± 1 °C for 90 days. Enzyme activity assay was conducted every 5 days.

RESULTS AND DISCUSSION

Lysozyme Recovery. Reductants were added to four kinds of 5-fold diluted egg white solutions and thermally treated at 70 ± 1 °C for about 5 min. Precipitates were removed by centrifugation, and the enzyme activity in the supernatants was determined. As shown in Figure 1A, lysozyme recoveries increased with increasing ascorbic acid concentration (0−1.0%) in the four kinds of egg white samples. The maximal recovery was about 78% when 1.0% ascorbic acid was added to the FCEW, while only around 60% recovery was observed when the same

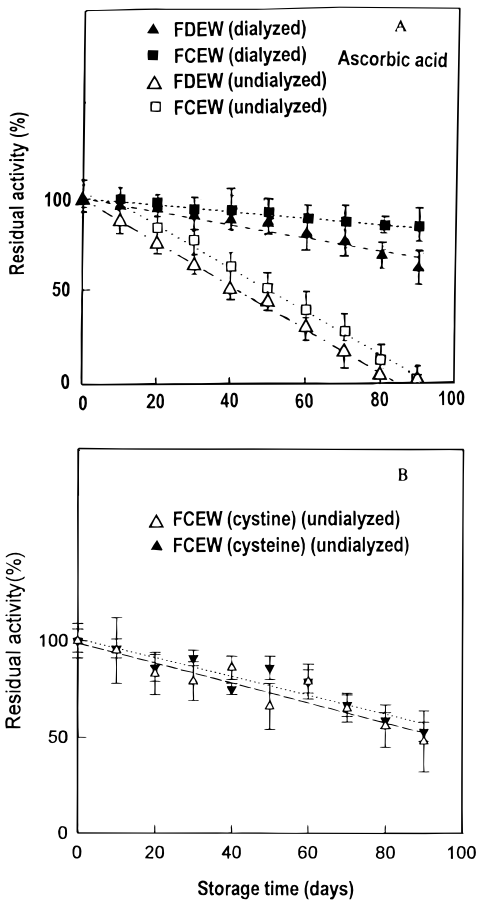

Figure 2. Stability of lyophilized lysozyme prepared with ascorbic acid (A) and cysteine and cystine (B) with or without dialysis. For definition of SDEW(C), SDEW(L), FCEW, and FDEW, see legend to Figure 1. Each value is the average of five replicates.

level of ascorbic acid was added to SDEW or FDEW. Further addition (2.0%) of ascorbic acid to the egg white samples reversibly reduced the recovery to about 50%. Comparing the results from SDEW and FDEW, it was clear that the presence of salt (approximately 1% NaCl) did not apparently affect the lysozyme recovery. Addition of 1.0−2.0% cystine to FCEW followed by thermal treatment also provided a high recovery of lysozyme (about 75%) (Figure 1B), although no apparent effects were observed when cystine was applied to SDEW and FDEW. Addition of 1% cysteine provided less lysozyme recovery (approximately 50%) from FCEW (Figure 1C) than ascorbic acid and cystine. However, β-mercaptoethanol was found to be least effective in recovering lysozyme from egg white samples.

Disulfide-exchange reactions usually occur between sulfhydryl or disulfide and disulfide-containing proteins, especially during thermal treatment (Peng et al., 1982; King, 1977). Such reactions usually cause the polymerization, denaturation, and gelation of proteins, resulting in protein precipitation. In egg whites, lysozyme containing four disulfide linkages is easily precipitated by such interactions with some proteins, such as ovalbumin, through disulfide-exchange reactions and displays thermal instability when heated at a temperature higher than 70 °C (Kato et al., 1975; Matsuoka et al., 1982). Reductants such as ascorbic acid, cystine, and cysteine in the egg white samples probably inhibited such exchange reactions to various extents and protected lysozyme from being denatured, thus leading to

significantly higher recovery of lysozyme (Figure 1). In addition, the presence of ascorbic acid also simultaneously provided the acidity to egg white samples to enhance the thermal stability of lysozyme in diluted egg whites (Cunningham and Lineweaver, 1967; Hayase et al., 1975). The recovery (78%), purification (2.4-fold), and specific activity of lysozyme by the use of ascorbic acid were satisfactory and are shown in Table 1A. In the case of the addition of 1% cystine to FCEW, 75% lysozyme recovery and purification of 2.6-fold were observed, while only 4−9% lysozyme recovery was determined in the other three kinds of egg white samples (Table 1B). Table 1C represents 50% lysozyme recovery and purification of 2.1-fold when cysteine was added to the FCEW.

Storage Test. Storage tests were conducted to investigate the stability of crude lysozyme prepared as above for possible commercial use. Lyophilized powders prepared with the dialysis process appeared to be stable at 4 °C for 90 days, while the samples containing ascorbic acid showed much lower residual activity (Figure 2A). The decrease in enzyme activity could be due to the presence of ascorbic acid in the lyophilized powder. Dehydroascorbic acid, derived from ascorbic acid, has been reported to cause the polymerization of proteins in the presence of dehydroascorbic acid reductase (Matz, 1972), thus leading to the reduction of lysozyme activity. Similar trends were observed in the residual activity of lysozyme powders containing cysteine or cystine (Figure 2B). The presence of sulfur-containing amino acids in the protein samples could have induced the reduction of proteins, also leading to a reduction in enzyme activity (King, 1977; Peng et al., 1982).

CONCLUSION

Lysozyme was recovered from egg whites with the combination of reductants and thermal treatment. Among the reductants tested, ascorbic acid was found to be most effective in recovering lysozyme from all egg white samples. By adding ascorbic acid to the FCEW to reduce the pH to about 4 and then heating the mixture to 70 ± 1 °C until no further precipitation occurred, crude lysozyme solution could be collected with a satisfactory yield (about 78%). The residual ascorbic acid in the lysozyme thus obtained should be removed to protect lysozyme from being denatured. The method developed in the present study is convenient and efficient and could be a helpful pretreatment either for the preparation of crude lysozyme for further purification or for the recovery of lysozyme from waste salted duck egg white, which contains about 5−6% NaCl and is currently a waste in food processing.

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Received for review March 18, 1999. Revised manuscript received October 27, 1999. Accepted November 11, 1999.

JF9902797