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Partial Purification of Hen Egg White Lysozyme by Ethanol Precipitation Method and Determination of the Thermal Stability of Its Lyophilized Form

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Abstract: Lysozyme was partially purified from hen egg white by precipitation of non-lysozyme protein impurities during incubation in the presence of ethanol. The thermal stability of the obtained partially purified enzyme was also characterized. The incubation of diluted egg white for 2-8 h in the presence of 20% ethanol was not very effective for the partial purification of lysozyme by precipitation of major egg white proteins; however, 4- to 6-h or 6- to 8-h incubation of diluted egg white in the presence of 30% and 40% ethanol could be employed more effectively for partial purification of lysozyme. Without applying the incubation period, the highest specific activity was obtained by the treatment of egg white with 40% ethanol. Thus, ethanol at this concentration could be used for a continuous process of partial purification. For batch lysozyme purification, on the other hand, incubation in the presence of 30% ethanol was more appropriate. The activities and protein contents of dialyzed and lyophilized enzymes obtained by 6 h-incubation in the presence of 20%, 30%, and 40% ethanol precipitations were 1878, 6669, and 6115 U/mg powder, and 0.98, 0.90, and 0.93 mg protein per mg powder, respectively. The ranges of thermal inactivation parameters, such as D ($D_{80\ °C}$ = 29.2-59 min, $D_{90\ °C}$ = 8.8-21 min) and z ($z_{80\text{-}90\ °C}$ = 17.4-22.3 °C) values of the enzyme, clearly indicated the moderate and variable heat stability of lyophilized lysozymes obtained from different batches of egg white.

Key Words: Lysozyme, hen egg white, partial purification, ethanol precipitation, thermal stability

Yumurta Akı Lisoziminin Etanol Çöktürme Metodu Kullanılarak Kısmi Olarak Saflaştırılması ve Liyofilize Formunun Isıl Stabilitesinin Belirlenmesi

Özet: Bu araştırmada yumurta akında bulunan safsızlık oluşturan başlıca proteinlerin etanolle farklı süreler inkübe edilerek çöktürülmesi yöntemiyle lisozimin kısmi saflaştırılması ve ısıl stabilitesinin incelenmesi çalışılmıştır. Lisozimin kısmi saflaştırılmasında seyreltilmiş yumurta akının 2-8 saat % 20 etanolle inkübe edilmesi etkinliği düşük bir işlemdir. Ancak, seyreltilmiş yumurta akının % 30 etanolle 4-6 saat, % 40 etanolle 6-8 saat inkübe edilmesi saflaştırma açısından daha başarılıdır. İnkübasyon uygulanmadan en yüksek saflık % 40 etanol kullanılarak elde edilmiş ve bu etanol konsantrasyonu sürekli yöntemle lisozim saflaştırılmasına ve üretimine uygun bulunmuştur. Kesikli yöntemle, inkübasyon uygulanarak lisozim saflaştırılması ve üretimi içinse en uygun etanol konsantrasyonu % 30 olarak belirlenmiştir. % 20, % 30 ve % 40 etanolle 6 saat inkübasyonla çökeltme, diyaliz ve liyofilizasyonla elde edilmiş lisozim örneklerinin aktiviteleri sırasıyla 1878, 6669 ve 6115 U/mg toz, protein miktarları ise sırasıyla 0.98, 0.90 ve 0.93 mg protein/mg toz düzeyindedir. Liyofilize enzimin ısıl inaktivasyon parametrelerinden D ($D_{80\ °C}$ = 29.2-59 dak ve $D_{90\ °C}$ = 8.8-21 dak) ve z değerleri ($z_{80\text{-}90\ °C}$ = 17.4-22.3 °C), lisozimin farklı yumurta partilerindeki ısıl direncinin beklenenin altında ve değişken olduğunu göstermiştir.

Anahtar Sözcükler: Lisozim, yumurta akı, kısmi saflaştırma, etanolle çöktürme, termal stabilite

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Introduction

Due to the health concerns of consumers, producers are greatly interested in the use of biopreservatives in place of chemical preservatives used in foods. The antimicrobial substances used in the biopreservation of food include such bacteriocins as nisin, pediocin, and lacticin, and antimicrobial enzymes, such as lysozyme, lactoperoxidase, chitinase, and glucose oxidase (Labuza and Breene, 1989; Suppakul et al., 2003). Lysozyme is one of the few natural antimicrobial agents approved by regulatory agencies for use in foods. Thus, extensive studies have been conducted related to the antimicrobial effect and potential food applications of this enzyme (Padgett et al., 1998; de Roos et al., 1998; Chung and Hancock, 2000; Gill and Holley, 2000; Han, 2000; Marchal et al., 2000; Davidson, 2001; Nattress et al., 2001; Quintavalla and Vicini, 2002; Gill and Holley, 2003; Branen and Davidson, 2004; Delfini et al., 2004). Lysozyme mainly shows antimicrobial activity on gram-positive bacteria by splitting the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in their cell walls; however, when it is combined with EDTA, which destabilizes the protective outer membrane surrounding the peptidoglycan layer of gram-negative bacteria, the antimicrobial spectrum of lysozyme increases significantly (Padgett et al., 1998; Branen and Davidson, 2004).

In food applications, lysozyme is added directly to food products to prevent growth of undesirable bacteria. For example, in Europe, the enzyme is used in cheeses such as edam and gouda, and for the prevention of gas blowing caused by *Clostridium tyrobutyricum* (de Roos et al., 1998; Davidson, 2001). In Japan, it is used for the preservation of vegetables, seafood, pasta, and salads (Davidson, 2001). There are also extensive studies of the use of lysozyme in combination with nisin or EDTA for the preservation of meat and meat products (Chung and Hancock, 2000; Gill and Holley, 2000; Nattress et al., 2001; Gill and Holley, 2003), as well as lysozyme alone for the control of malolactic fermentation in wines (Marchal et al., 2000; Delfini et al., 2004). Another use of lysozyme in food technology involves the incorporation of the enzyme into plastic or edible films for the production of antimicrobial packaging materials. In different studies, lysozyme has been used in the production of antimicrobial films for such plastic materials as polyvinylalcohol (PVOH) and cellulose acetate, and for edible and biodegradable materials, such as zein, whey proteins, alginate, carrageenan, and chitosan (Appendini and Hotchkiss, 1997; Padgett et al., 1998; Cha et al., 2002; Buonocore et al., 2003; Park et al., 2004; Min et al., 2005).

In studies related to direct food applications of lysozyme and antimicrobial film production, most researchers used commercial lysozymes. The commonly used commercial lysozymes are quite pure and contain only 1%-6% (w/w) protein impurities (Judge et al., 1998). The classical method of lysozyme production involves the repeated salt crystallization of the enzyme in hen egg white, but this process requires a week for the enzyme to be obtained with high purity. There are also some other methods for the purification of lysozyme, such as ion-exchange and affinity membrane chromatography, and ultrafiltration (Hou and Lin, 1997; Grasselli et al., 1999; Ghosh and Cui, 2000; Jiang et al., 2001; Arıca et al., 2004). Nonetheless, for the widespread application of lysozyme in the food industry, the use of cheaper, partially purified lysozyme preparations obtained by faster and simpler methods may be economically more feasible. For this reason, some alternative partial purification procedures have recently been developed, based on selective precipitation or partitioning of lysozyme from hen egg white, or selective precipitation of major egg white proteins, such as ovalbumin and conalbumin, while maintaining lysozyme solubility in the egg white. Some examples of these simple strategies include partitioning of lysozyme by the polyethylene glycol/salt aqueous 2-phase system (Su and Chiang, 2006), selective precipitation and recovery of lysozyme with anionic surfactant di-(2-ethylhexyl) sodium sulfosuccinate (AOT) and acetone (Shin et al., 2003), selective precipitation of non-lysozyme proteins in the egg white by heat-induced denaturation and gelation applied at 70 °C (Chang et al., 2000), and by incubation in the presence of 30% ethanol (Jiang et al., 2001). Recently, we have modified the ethanol precipitation method given by Jiang et al. (2001) and used partially purified and lyophilized lysozyme successfully to obtain protein or carbohydrate-based antimicrobial packaging materials (Kandemir et al., 2005; Mecitoğlu et al., 2006). Partially purified lysozyme obtained by 6-h incubation in the presence of 30% ethanol and dialysis was very stable and lost almost no activity in lyophilized form stored at –18 °C for up to 8 months (Mecitoğlu et al., 2006). In the present study, we applied different ethanol

concentrations and incubation periods to improve the partial purification procedure of lysozyme and to determine the potential of the application of a continuous partial purification procedure with ethanol. The partially purified enzymes obtained at optimum conditions were also lyophilized and tested for activity, protein content, and thermal stability to determine their possible applications in the biopreservation of thermally and non-thermally processed food.

Materials and Methods

Materials

Fresh hen eggs used in the production of lysozyme were obtained from a supermarket in İzmir (Turkey). Batch 1, batch 2 and batch 3 discussed in the manuscript represent lysozymes obtained from different batches of eggs. *Micrococcus lysodeikticus* (ATCC NO 4698) and dialysis tubes (12000 MW, prepared as described in the product manual), were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Partial purification of lysozyme

For the partial purification of lysozyme, egg whites, carefully separated from the egg yolks, were diluted 3- or 3.3-fold with 0.05 M NaCl solution. To precipitate the egg white proteins other than lysozyme, the pH of this mixture was set to 4.0 by carefully adding several drops of 1 N acetic acid and it was diluted with an equal volume of 40%, 60%, or 80% (v/v) ethanol. After 0-, 2-, 4-, 6-, 7-, or 8-h incubation at room temperature in the presence of different concentrations of ethanol, the mixtures were centrifuged at 15,000 x g for 15 min at 4 °C; then the precipitates were discarded. The supernatants were analyzed for their lysozyme activity and protein content to determine the effects of different procedures on the efficiency of partial purification.

Dialysis and lyophilization of partially purified lysozyme

The lyophilized lysozymes were obtained by using supernatants from centrifugation of ethanol-treated (40%, 60%, or 80%) and 6-h (or 7-h) incubated samples. Before lyophilization, the supernatants containing lysozyme were dialyzed for 21 h at 4 °C by 3 changes of 2000 ml of distilled water. Lyophilization was applied by a freeze drier (Labconco, FreeZone, 6 liter, Kansas City, MO, USA) with –44 to –47 °C collector temperature, and 50 x 10^{-3} and 100 x 10^{-3} mbar vacuum. The sample container volume was 2-3 times the sample volume. The lyophilized enzymes were kept at –18 °C until they were used in different studies.

Protein content and enzyme activity of lyophilized partially purified lysozymes

The protein content and residual enzyme activity of lyophilized enzymes were determined by dissolving 20 mg of lyophilized preparation in 10 ml of distilled water. After centrifugation at 15 000 x g for 15 min at 4 °C, the residual lysozyme activity and protein content of the enzyme preparations were determined by the methods given below. Protein content was expressed as mg protein per mg of powder, while enzyme activity was expressed as U per mg of powder or protein.

Thermal stability of lyophilized partially purified lysozyme

Thermal inactivation studies were conducted at 60, 70, 80, and 90 °C with thermal inactivation time (TIT) tubes (i.d.: 9 mm; wall thickness: 1 mm). To minimize the lag phase, TIT tubes were heated to inactivation temperature. Then, 0.2 ml of the enzyme solution was prepared by dissolving lyophilized lysozyme in 0.05 M Na-phosphate buffer (pH 7.0), which was then pipetted into the TIT tubes. After heating for a given period the tubes were cooled in an ice water bath and their contents were immediately assayed for residual lysozyme activity.

The calculation of heat inactivation parameters of lysozyme was conducted by applying the standard methods that explain enzyme and microbial inactivation kinetics by a first order reaction (Holdsworth, 1997). The heat inactivation curves were formed by plotting log of percent remaining activity vs. heating time (min). The inactivation rate constants (k min^{-1}) were calculated by multiplying the slope of the heat inactivation curves by the factor of 2.303. The half-lives ($t_{1/2}$, min), which refer to the heating time at constant temperature to achieve 50% inactivation of the enzyme, were calculated from the formula $t_{1/2} = \ln (0.5)\ k^{-1}$. The decimal reduction times (D, min), which refer to the heating time at constant temperature to achieve 90% inactivation of the enzyme, were calculated from the negative reciprocals of the slopes of the heat inactivation curves. The z values (°C) that show the temperature dependency of heat inactivation was calculated from the equation $z = T_2 - T_1/\log (D_1/D_2)$, where T is temperature (°C).

Lysozyme activity

The activity of lysozyme was determined spectrophotometrically at 660 nm with a Shimadzu Model 2450 (Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 °C. The reaction mixture was prepared by mixing 2.9 ml of *Micrococcus lysodeikticus* cell suspension, which was brought to 30 °C just before measurement, and 0.1 ml of enzyme solution (incubated at 30 °C for 5 min). The *Micrococcus lysodeikticus* cell suspension was prepared freshly at the concentration of 0.26 mg ml^{-1} in 0.05 M Na-phosphate buffer (pH 7.0) and stored at 4 °C until it was used in activity measurements. The reduction in absorbance was monitored for 2 or 5 min and enzyme activity was calculated from the slope of the initial linear portion of absorbance vs. the time curve. Enzyme activity was expressed as unit (U) or percent initial activity. One unit was defined as 0.001 change in absorbance in 1 min. The average of 3 measurements was used to calculate activity.

Protein content

Protein content was determined according to the Lowry procedure by using bovine serum albumin as standard (Harris, 1987). The average of 5 measurements was used to calculate protein content.

Results and Discussion

Effects of ethanol concentration and incubation period on recovery and specific activity of lysozyme

The partial purification of lysozyme by ethanol precipitation was applied by Jiang et al. (2001) to increase the purity of crude lysozyme in diluted egg white before further purification of the enzyme by ion exchange chromatography. These researchers precipitated non-lysozyme egg white proteins in the presence of 30% ethanol by 8-h incubation. Before the addition of ethanol, the pH of the medium was set close to the isoelectric point of ovalbumin (pH 4.5) to achieve a more effective precipitation for this major egg white protein. In the present work, to determine the possibility of improving the efficiency of the ethanol precipitation method, we studied 20%, 30%, and 40% ethanol concentrations and 0 to 8 h incubation periods. The recovery and specific activity levels of the enzyme extracts treated with different concentrations of ethanol are given in Table 1. At the beginning of incubation, the use of 20% ethanol in the precipitation of non-lysozyme proteins gave almost 49% and 69% higher lysozyme activities than using 30% and 40% ethanol, respectively. This result is in line with Liu et al. (2004), who reported that ethanol concentrations between 0% and 20% (v/v) enhance

Table 1. Effect of ethanol concentration and incubation period on the recovery and specific activity of egg white lysozyme.

Incubation period (h)	Total activity (U)	Total protein (mg)	Recovery (%)	Specific activity (U mg^{-1})
Incubation in the presence of 20% ethanol				
0	2,883,400 ± 307,335	1604 ± 65	100	1798
2	2,932,600 ± 472,408	1607 ± 209	102	1825
4	3,642,100 ± 390,762	1550 ± 154	126	2350
6	3,145,450 ± 537,225	973 ± 120	109	3233
8	2,674,100 ± 19,1392	950 ± 140	93	2815
Incubation in the presence of 30% ethanol				
0	1,936,000 ± 293,409	526 ± 106	100	3681
2	2,263,200 ± 279,402	460 ± 112	117	4920
4	2,395,800 ± 31,6456	447 ± 123	124	5360
6	2,202,200 ± 59,1293	399 ± 144	114	5519
8	1,733,400 ± 169,857	334 ± 26	90	5190
Incubation in the presence of 40% ethanol				
0	1,704,000 ± 290,985	356 ± 53	100	4787
2	2,318,300 ± 338,841	379 ± 100	136	6117
4	1,081,000 ± 247,588	362 ± 35	63	2986
6	2,303,000 ± 177,421	415 ± 70	135	5549
8	235,4000 ± 37,066	318 ± 49	138	7403

lysozyme solution stability due to increased local hydrophilicity caused by exposed OH groups of the ethanol bound to lysozyme (Liu et al., 2004). However, the enzyme extract containing 20% ethanol had the lowest initial purity. At the beginning of incubation, the lowest protein content and highest specific activity levels in extracts were obtained at 40% ethanol concentration. The initial specific activity obtained at this ethanol concentration was 2.7- and 1.3-fold higher than those obtained initially at 20% and 30% ethanol concentrations, respectively. Thus, by using 40% ethanol and not applying an incubation period, the solution of partially purified lysozyme can be obtained with a continuous process. In such a process, it is necessary to use a continuous centrifuge following dilution of egg white with NaCl and ethanol solutions. The remaining processes are dialysis and lyophilization.

Incubation for 2 h in presence of 20% ethanol did not increase the purity of the lysozyme extract considerably, but incubation for the same time period in the presence of 30% and 40% ethanol increased the purity of lysozyme extracts almost 1.3-fold (Figure 1). At all ethanol concentrations lysozyme activity increased slightly to moderately at the initial stages of incubation. In the presence of 20% or 30% ethanol, enzyme activity increased up to the fourth hour of incubation, but then gradually declined with extended incubation. Nevertheless, the specific activity of the extracts containing 20% and 30% ethanol increased up to the sixth hour of incubation due to the continuous reduction in their protein contents. At these ethanol concentrations, a reduction occurred in specific activities at the eighth hour of incubation due to the loss of part of lysozyme activity. Conversely, in enzyme extracts containing 40% ethanol a sharp decline occurred in lysozyme activity at the fourth hour of incubation and this reduced the specific activity considerably. At the sixth hour of incubation, the activity again increased and this caused fluctuation of enzyme activity and resulting purity. During the incubation period, the fluctuations also occurred in the protein contents and changes in both activity and protein content were similar up to the sixth hour of incubation in the presence of 40% ethanol. Thus, it seems that these fluctuations are due to the change in lysozyme and other proteins' solubility in the extract during incubation. The further increase to 8 h of incubation at the 40% ethanol concentration precipitated particularly non-lysozyme proteins and this increased the specific enzyme activity considerably. In addition to the change in protein solubilities, the fluctuations that occurred in lysozyme activity at 40% ethanol might also be related to change in enzyme conformation at this ethanol concentration during the incubation period. The effect of ethanol on lysozyme depends on its concentration (Bonincontro et al., 1997; Liu et al., 2004). For example, Bonincontro et al. (1997) reported that at very low ethanol concentrations, at which the mole fraction of ethanol in lysozyme solution is 0.06, the α-helix content of the enzyme increases. At higher mole fractions of ethanol (between 0.06-0.25) the α-helix content approaches that of its native form, but the enzyme becomes more tightly folded.

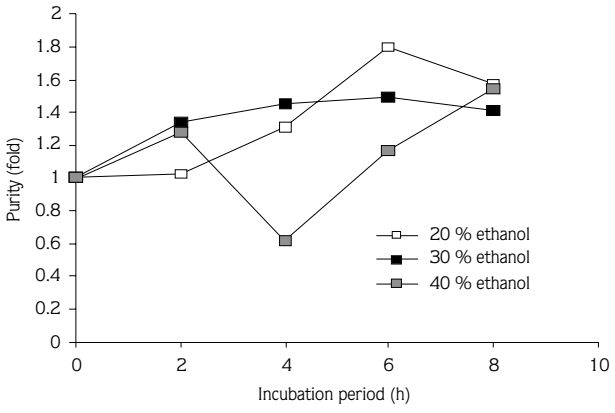

Figure 1. Effect of incubation at different ethanol concentrations on enzyme purity.

Partial purification parameters of lysozyme obtained by ethanol precipitation and dialysis

The partial purification parameters of lysozyme obtained by ethanol precipitation of egg white proteins and dialysis are given in Table 2. The recovery of lysozyme activity after precipitation of non-lysozyme egg white proteins at different ethanol concentrations and 6-h incubation ranged between 83% and 93%. These lysozyme recoveries following ethanol precipitation of egg white proteins were higher than those of lysozymes obtained by (a) heat precipitation of egg white protein impurities at 70 °C in the presence of ascorbic acid used as reductant (recovery: 78%) (Chang et al., 2000), (b) selective precipitation and recovery of the enzyme from hen egg white by anionic surfactant di-(2-ethylhexyl)

Table 2. Summary of the partial purification of lysozyme.

Step	V (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Recovery (%)	Purity (fold)
1. Dilution of egg white 3-fold with 0.05 M NaCl[a]						
	30	1,092,000	1326	824	100	1
2. pH adjustment + 6-h incubation in the presence of 20% ethanol						
	51	1,018,300	294	3464	93	4.2
3. 21-h dialysis						
	64	775,467	282	2750	71	3.3
4. Lyophilization Activity: 1878 ± 76 U mg^{-1} powder or 1916 ± 77 U mg^{-1} protein Protein content: 0.98 ± 0.05 mg protein per mg powder						
2. pH adjustment + 6-h incubation in the presence of 30% ethanol						
	52	960,267	89	10,790	88	13
3. 21-h dialysis						
	68	663,000	99	6697	61	8.1
4. Lyophilization Activity: 6669 ± 669 U mg^{-1} powder or 7410 ± 742 U mg^{-1} protein Protein content: 0.90 ± 0.02 mg protein/mg powder						
2. pH adjustment + 6-h incubation in the presence of 40% ethanol						
	55	902,000	96	9396	83	11.4
3. 21-h dialysis						
	81	757,350	212	3572	69	4.3
4. Lyophilization Activity: 6115 ± 608 U mg^{-1} powder or 6575 ± 653 U mg^{-1} protein Protein content: 0.93 ± 0.03 mg protein per mg powder						

[a] step 1 is the same for all samples

sodium sulfosuccinate (AOT) and acetone, respectively (recovery: 50%) (Shin et al., 2003), and (c) partitioning from diluted egg white by the polyethylene/salt aqueous 2-phase system (recovery: 70%) (Su and Chiang, 2006). On the other hand, at the end of the 6-h incubation period, the purifications achieved were 4.2-, 13-, and 11.4-fold in the presence of 20%, 30%, and 40% ethanol, respectively. These are the maximal purification folds achieved in the presence of 20% and 30% ethanol since specific activities obtained at these ethanol concentrations dropped with additional incubation of the enzyme extracts. However, in the presence of 40% ethanol, further increases may occur in the purification fold of lysozyme above a 6-h incubation period (see Table 1).

The effects of dialysis on the partial purification parameters are given in Table 2. The application of 21-h dialysis caused a partial loss of lysozyme activity and reduced the recovery of lysozyme to between 61% and 71%. In enzyme extracts containing 20% and 30% ethanol, the dialysis caused a slight increase or reduction in protein content. Yet, in extracts that contained 40% ethanol a considerable increase occurred in protein content with dialysis and this caused the reduction in specific lysozyme activity. It is interesting to note an increase in protein content by the use of dialysis; however, it seems that the removal of ethanol by dialysis increased the solubility of non-lysozyme protein micelles that were formed in the presence of ethanol, but could not be precipitated by centrifugation. This result clearly showed the solubility problems of egg white proteins in the presence of 40% ethanol. In general, it is observed that dialysis is not beneficial in reducing the proteins other than lysozyme; however, it is a practical procedure for removing ethanol before the lyophilization process conducted with a lyophilizer that is not equipped with a large-scale solvent trap.

Protein content and residual activity of partially purified lysozyme after lyophilization

In this study, following partial purification with ethanol precipitation, the enzyme extracts were dialyzed and then lyophilized. Thus, the obtained lyophilized preparations were also assayed for their soluble protein content and lysozyme activity. The results of protein assays showed that the partially purified enzyme obtained

by 20% ethanol precipitation was composed of 98% protein. The protein contents were 90% and 93% for enzymes obtained by 30% and 40% ethanol precipitations, respectively. Thus, it seems that the proteins in egg white treated with 20% ethanol retained their solubility to a greater degree than those in egg white treated with 30% or 40% ethanol. The results of activity measurements, on the other hand, suggested different responses of various lysozymes to lyophilization. For example, lyophilization applied following dialysis caused almost a 30% reduction in specific activity of lysozyme obtained by 20% ethanol precipitation, whereas it caused an almost 10% and 80% increase in specific activities of lysozymes obtained by 30% and 40% ethanol precipitations, respectively (Table 2). The slight change in specific activity of lysozyme obtained by 30% ethanol precipitation and dialysis clearly showed the minimal changes in this preparation by lyophilization. The lyophilized lysozyme obtained by 30% ethanol precipitation and dialysis also showed an excellent storage stability. In fact, we recently determined that the enzyme obtained by this method maintained 100% of its activity for 8 months at –18 °C (Mecitoğlu et al., 2006). In contrast, in lysozymes obtained by 20% or 40% ethanol precipitation and dialysis, the moderate reduction and considerable increase in specific activity, respectively, suggested some significant changes in these lysozymes by lyophilization. It is hard to explain the exact mechanism of these changes; however, it is likely that they are related to conformational changes in lysozyme molecules, or association-dissociation reactions among lysozyme molecules or lysozyme and other proteins.

Thermal stability of partially purified lysozyme after lyophilization

The thermal stability of lyophilized lysozymes obtained from different batches of egg white by 6- or 7-h incubation in the presence of 30% ethanol and a following dialysis were studied between 60 and 90 °C. As seen in Figure 2, the enzyme obtained in batch 1 showed activation during heating at 70 °C. At the same temperature, the inactivation rate of the enzyme obtained in batch 2 was initially fast, but it later slowed down due to initiated enzyme activation after 2.5 min of heating (Figure 3). The activation of enzymes during heating occurs mostly during the initial stages of mild heating (Yemenicioğlu et al., 1997; Rodriguez-Lopez et al., 1999; Yemenicioğlu, 2002) and is generally attributed to conformational changes in the enzyme active site (Rodriguez-Lopez et al., 1999). As seen in Figure 3, the

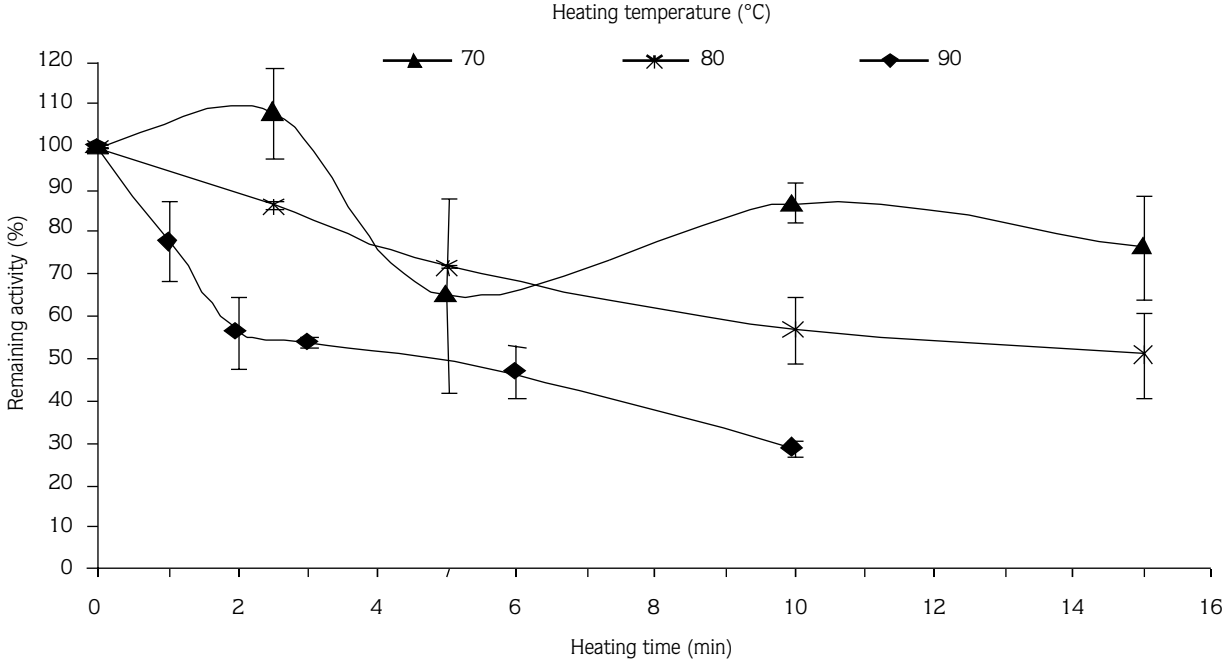

Figure 2. Heat inactivation curves of partially purified and lyophilized lysozyme (batch 1).

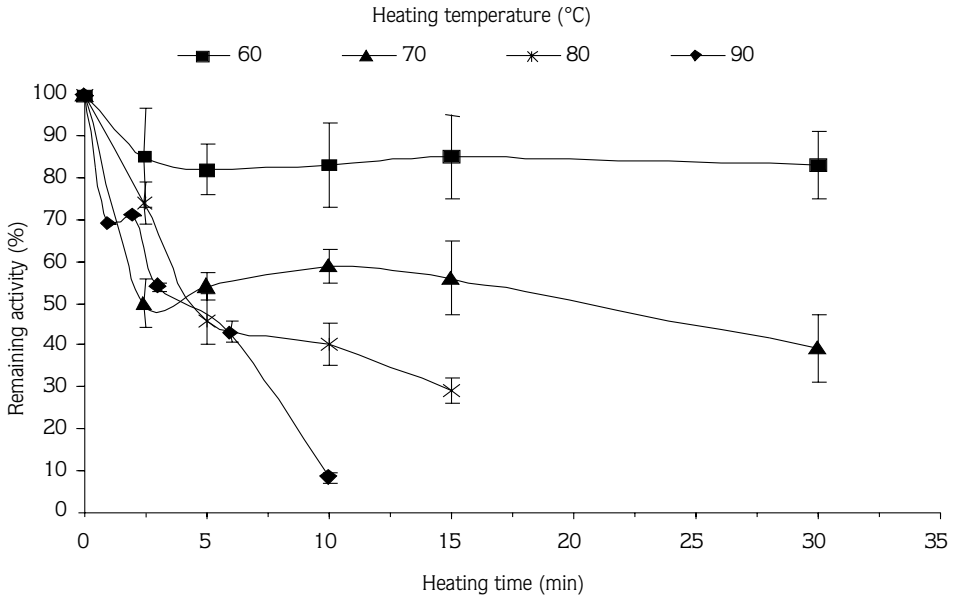

Figure 3. Heat inactivation curves of partially purified and lyophilized lysozyme (batch 2).

enzyme is very heat stable at 60 °C and it did not show a considerable activation at this temperature. In the 3 different batches of lyophilized lysozymes used in the inactivation studies, heat activation of lysozyme was not observed at 80 and 90 °C. The appearance of activation at mild heating temperatures and its disappearance with elevated heating temperatures was also observed by some other researchers who studied different enzymes, such as polyphenoloxidase activated at 68 and 73 °C (Yemenicioğlu et al., 1997), peroxidase activated between 65 and 75 °C (Vamos-Vigyazo, 1981), and pectin methyl esterase activated at 60 and 70 °C (Demirbüker et al., 2004). However, the data obtained in these studies failed to explain the exact mechanisms responsible for the disappearance of activation at high temperatures. It is likely that the very fast conformational changes at elevated temperatures skipped or prevented the formation of suitable conformational arrangements for the activation. It is also possible that the activation could not be monitored in our heat inactivation curves since it was completed at the initial stages of rapid heating.

The heat inactivation parameters of lysozyme at 80 and 90 °C were also calculated to compare the temperature stabilities of different batches of lyophilized lysozymes. At these temperatures the heat inactivation of lysozyme was fitted to the first-order reaction kinetic. As seen in Table 3, the D and $t_{1/2}$ values of lysozymes obtained in batches 2 and 3 were similar. In contrast, the lysozyme obtained in batch 1 was more heat stable than the enzymes from other batches. In particular, at 90 °C there is a 2.0- to 2.5-fold difference between the D value of enzymes from batch 1 and other lysozymes. At the studied temperature range, the greater z value of lysozymes from batches 1 and 2 indicated reduced temperature dependency of these enzymes' inactivation than the batch 3 enzyme. These results clearly showed the possible differences in the thermal stability of different batches of lyophilized lysozymes. Differences in the thermal stability of hen egg white lysozyme are also evident from data in the literature obtained by using pure commercial enzyme in heat inactivation tests. For example, the $D_{90\ °C}$ of pure commercial lysozyme (Sigma Chemical Co., St. Louis, MO, USA) calculated from the heat inactivation curves given by Okanojo et al. (2005) was almost 30 min at pH 6.5. Makki and Durance (1996) reported $D_{93\ °C}$ of the same commercial enzyme as 138 min at pH 6.2. The $t_{1/2}$ value calculated for pure commercial lysozyme (Sigma-Aldrich, USA) from heat inactivation curves given by Thammasirirak et al. (2006) was almost 150 min at 90 °C and pH 6.0. The comparison of the data obtained in the present study and

Table 3. Heat inactivation parameters of partially purified lyophilized lysozymes obtained from different batches of egg whites.

Batch # a	k (min^{-1})		D (min)		$t_{1/2}$ (min)		$z_{80\text{-}90}$ °C (°C)
	80 °C	90 °C	80 °C	90 °C	80 °C	90 °C	
1	–0.0394	–0.109	59.0	21.0	17.6	6.4	22.3
2	–0.0788	–0.232	29.2	9.9	8.8	3.0	21.3
3	–0.0696	–0.263	33.1	8.8	10.0	2.6	17.4

[a] batches 2 and 3 were obtained by 6-h incubation in the presence of 30% ethanol, whereas batch 1 was obtained by 7-h incubation at the same conditions.

those reported in the literature clearly shows that the commercial pure lysozymes are more thermostable than the partially purified lysozyme that we obtained in this study. It is possible that the lower thermal stability of partially purified lysozyme is related to conformational changes that occurred in the enzyme during the ethanol treatment. Further studies are needed to compare the properties of the partially purified lysozyme obtained in this work and pure commercial lysozymes.

Conclusions

The results obtained in this study clearly showed the importance of ethanol concentration and incubation period in the partial purification of lysozyme. The use of 30% and 40% ethanol was suitable for the partial purification of lysozyme. Ethanol at these concentrations precipitated the non-lysozyme protein impurities effectively when appropriate incubation periods were applied in partial purification. The most suitable ethanol concentration for a continuous partial purification process is 40% since ethanol at this concentration gave the highest specific activity without incubation. Our recent studies showed that the lyophilized partially purified lysozyme is very stable during frozen storage (Mecitoğlu et al., 2006), and it can successfully be used to produce antimicrobial protein and carbohydrate films that are effective against different bacteria (Kandemir et al., 2005; Mecitoğlu et al., 2006). Thus, the enzyme seems particularly suitable for use in functional packaging. Although the partially purified enzyme showed a considerable thermal stability at mild to moderate heating temperatures, it is less heat stable than the commercial pure lysozymes at pasteurization temperatures. Therefore, the enzyme should mainly be used for biopreservation of non-thermally processed food.

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