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Stability of Egg White Lysozyme in Combination with Other Antimicrobial Substances

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ABSTRACT

The effects of pH, ionic strength (I), and selected antimicrobial substances on the lytic activity of egg white lysozyme over an extended storage period of 30 d were studied by observing the rate of clearing of a cell suspension of *Micrococcus lysodeikticus*. During prolonged storage, lysozyme activity remained relatively stable at pH 7 and ionic strength <0.10, whereas lower activity ($P < 0.05$) was observed at pH 9 and ionic strength >0.14. Lysozyme activity was highly stable (i.e., maintained over 90% of lytic activity from day 1 to day 30) in solutions of 1.0% sodium chloride, 100 ppm sodium nitrite, 4.0% ethanol, and 100 ppm butylated hydroxytoluene. Lysozyme activity also was stable (i.e., retained over 80% of lytic activity) in solutions of 0.1% sodium benzoate and 100 ppm butylated hydroxyanisol. The lysozyme activity was relatively stable (i.e., retained over 70% of lytic activity) in solutions of 0.3% calcium propionate, 0.1% potassium sorbate, and 0.1% propyl paraben. About half (50%) of the lysozyme activity was retained in solution of 0.5% EDTA. Activity was lost when lysozyme was combined with 0.5% lactic acid, 4% acetic acid, and 100 ppm chlorine water.

The antimicrobial activity of egg white lysozyme against bacteria involved in food spoilage and foodborne disease has been reported by several researchers (*3,6,7,14,16,17,20*). Most investigations have been directed toward evaluating the effect of the lysozyme alone on growth of various bacteria. However, using egg white lysozyme in combination with other antimicrobial compounds in foods seems to have more potential. Currently, research on the effectiveness of such combinations is limited.

Lysozyme (or muramidase) is an enzyme that consists of 129 amino acids, cross-linked by four disulfide bridges (*8*). The lytic activity of this enzyme can be measured by visible lysis of *Micrococcus lysodeikticus* (*12,13*). Smolelis and Hartsell (*19*) indicated that lysozyme remains stable over the pH range of 5 to 10 and that lysis of *M. lysodeikticus* was observed at both alkaline and acidic pH values. Studies by Davies et al. (*4*) and Chang and Carr (*2*) indicated that the stability of lysozyme was sensitive not only to pH but also to ionic concentration, and that the optimum pH for lysozyme activity was strongly dependent on the salt concentration. Lysozyme was found to be stable and maintained 100% activity in mirin liquor (sweetened sake, containing less than 20% alcohol) at 37°C for a period of 20 weeks (*26*). Proctor and Cunningham (*15*) observed that lysozyme had good stability in solutions of boric acid, 4% sodium chloride, ethanol, and glycerol. Lysozyme activity in pharmaceutical products such as Visine® eye drops (*15*) was also maintained during a study period of 1 year. However, the activity of lysozyme decreased quickly in distilled water.

The results of combining egg white lysozyme with other common antimicrobial compounds and the subsequent effect on lysozyme stability and lytic action are unknown. No previous studies have dealt with the stability of lysozyme in various antimicrobial compounds commonly approved for food. Therefore, the objectives of this study were: (a) to determine the stability of lysozyme under different conditions of pH and ionic strength and, (b) to observe the effects of combinations with other antimicrobial compounds on the lytic activity of lysozyme.

MATERIALS AND METHODS

Materials

Egg white lysozyme (lysozyme chloride, Grade I, 54,000 units activity per mg of protein) and *M. lysodeikticus* (ATCC 4698) were obtained from Sigma Chemical Company (P.O. Box 14508, St. Louis, MO 63178). Antimicrobial compounds used to prepare solutions with lysozyme in this study were the following: potassium sorbate and sodium benzoate from Pfizer Inc. (Chemical Division, New York, NY 10017); butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), and n-propyl-p-hydroxybenzoate from Sigma Chemical Company; ethanol, sodium chloride, sodium nitrite, calcium propionate, EDTA, acetic acid, and lactic acid from Fisher Scientific Company (Fair Lawn, NJ 07410); chlorine from Best Choice® bleach (5.25% chlorine).

Lysozyme activity assay

The lytic activity of lysozyme was determined based on the decrease in turbidity of *M. lysodeikticus* cells (*12*). Turbidity was monitored at 1-min intervals. Because effects of various pHs and ionic strengths on enzyme activity were determined, adding salt to the assay was omitted in the first experiment. The rate of turbidity change (i.e., percent change in transmission at 540 nm) in the linear region was used to express the activity of the enzyme.

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Experiment 1

A factorial design (9 × 6) was used to evaluate lysozyme stability and activity in solutions having different pHs (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) and ionic strengths (0.02, 0.06, 0.10, 0.14, 0.18, 0.22). Sodium phosphates (mono-, dibasic) were used to prepare the buffer solutions. The ionic strength (I) was calculated as a function of total concentration and pH (*11*). One milliliter of lysozyme (concentration of 0.20 mg/ml) was added to 50 ml of the prepared buffer solutions. The solutions were stored in glass jars at room temperature (25°C), and lysozyme activity was monitored over a period of 30 d.

Experiment 2

Solutions of antimicrobial compounds were prepared as follows: acetic acid, sodium benzoate, calcium propionate, potassium sorbate, n-propyl-p-hydroxybenzoate (paraben), sodium nitrite, ethanol, BHA, BHT, EDTA, chlorine water, lactic acid, and distilled water (control). The solution preparation used sterile distilled water. One milliliter of lysozyme (concentration of 0.20 mg/ml) was added to 50 ml of the prepared solutions. Lytic activity was determined over a period of 30 d.

Statistical analysis

The data analyses (i.e., Analysis of Variance, Means, Duncan's multiple range test, multiple regression) used the General Linear Model of the Statistical Analysis System (*18*). The interaction of the pH and ionic strength was analyzed, and three dimensional surface responses describing lysozyme stability were plotted using the SAS/GRAPH package (*18*).

RESULTS AND DISCUSSION

Experiment 1

Egg white lysozyme maintained high activity (above 80%) over a pH range of 6.5 to 9 at about 0.06-0.14 ionic strength (Fig. 1). The results are in agreement with findings of Smolelis and Hartsell (*19*) and Davies et al. (*4*). However, the results indicated that egg white lysozyme had relatively higher activities at pHs of 7 and 8 at ionic strength of 0.06, while Yashitake and Shinichiro (*26*) reported that lower activity was observed above pH 7. The difference was likely due to different salt concentrations between the studies. The ionic strength of the phosphate buffer solution had a significant effect on the lytic response of the lysozyme. At pH 6.5, either a lower ionic strength (I <0.04) or a higher ionic strength (I > 0.10) reduced lytic activity of lysozyme. Kravchenko et al. (*9*) fund that the enzymatic action of lysozyme towards *M. lysodeikticus* was inhibited by ionic strength higher than 0.1. The optimal pH for lytic response is dependent upon ionic strength as statistic analysis indicated an interaction ($P < 0.05$) between independent variables of pH and ionic strength. The highest lytic activity ($P < 0.05$) was observed at pH 8 when the ionic strength was about 0.06, and it dropped off to 6.5-7.0 when the ionic strength was increased to 0.14 (Fig. 1).

As is evident from Fig. 2, stability of egg white lysozyme decreased with extended storage time. This was expected because enzymes degrade over time. However, degradation of lysozyme was much more apparent at pH >8 and higher ionic strength >0.14.

Experiment 2

A summary of lysozyme activity in solutions combined with antimicrobial substances during 30 d is presented in Table 1. The lytic activity towards *M. lysodeikticus* of egg white lysozyme in solutions of 1.0% sodium chloride (NaCl), 100 ppm sodium nitrite ($NaNO_2$), and 4.0% ethanol was highly stable and was maintained over 90% of its original (day 1) value. The importance of adding NaCl to increase lytic action towards the bacterial cell wall is well-documented and was early reported by Fleming and Allison (*5*). Parry et al. (*12*) noted that NaCl is required for bovine milk lysozyme and is an activator for human milk lysozyme. Yashitake and Shinichiro (*26*) indicated that activity of lysozyme was stabilized against heat by NaCl. In the case of sodium nitrite, the salt concentration (100 ppm) seemed too low to contribute any ionic effect to lysozyme activity, but it was still maintained at a relative level of about 92.8% after 30 d. Such a result may have been caused by a minor reducing action of nitrite salt. Combinations of lysozyme and nitrate have been used in the manufacture of Edam

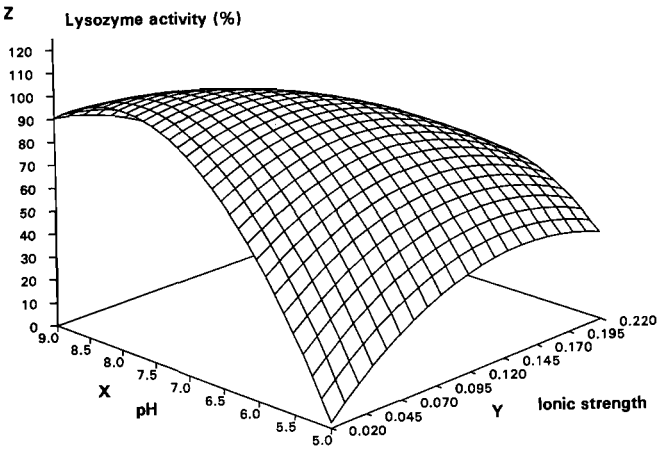

Figure 1. *Lytic activity of egg white lysozyme as a function of pH and ionic strength. Activities were expressed as a percentage of that observed at pH 6.5, I = 0.06.*
Response regression: $Z = -11.5X^2 - 2103.3Y^2 - 170.0XY + 185.9X + 1532.6Y - 653.3$.

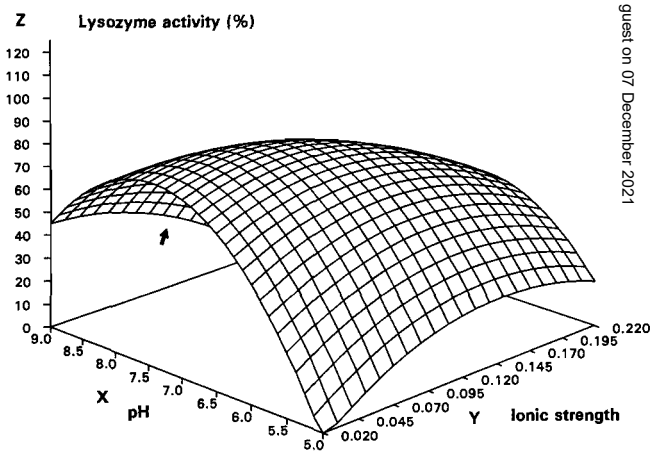

Figure 2. *Stability of egge white lysozyme as a function of pH and ionic strength during 30 d of storage. Activities were expressed as a percentage of that observed for lysozyme solutions freshly prepared at pH 6.5, I = 0.06.*
Response regression: $Z = -13.5X^2 - 1752.4Y^2 - 118.2XY + 203.1X + 1120.9Y - 690.8$. (arrow indicates significant decrease in the activity as compared to the normal enzyme degradation.)

TABLE 1. *Stability of egg white lysozyme in solutions combined with antimicrobial agents.*

Antimicrobial agents	pH	Relative lysozyme activity (%)[1] Day 1	Day 5	Day 10	Day 20	Day 30
0.1% Na benzoate	6.6	111.1[a]	98.3[bc]	101.7[ab]	92.0[bc]	90.1[c]
0.3% Ca propionate	6.5	109.0[a]	99.0[ab]	93.8[ab]	87.6[b]	82.4[b]
0.1% K sorbate	6.7	99.5[a]	98.1[a]	87.5[a]	86.3[a]	70.1[b]
100 ppm $NaNO_2$	6.8	100.6[ab]	100.6[ab]	108.8[a]	96.2[b]	93.4[b]
1.0% NaCl	6.0	117.7[a]	111.7[a]	116.3[a]	107.9[a]	107.9[a]
4.0% ethanol	6.8	97.1[ab]	103.1[a]	96.4[ab]	94.0[b]	97.1[ab]
100 ppm BHA	6.4	96.8[a]	98.1[a]	93.3[a]	90.1[a]	78.6[b]
100 ppm BHT	6.4	85.7[a]	78.8[a]	86.1[a]	81.0[a]	81.6[a]
0.1% propyl paraben	6.0	96.8[a]	96.3[a]	90.1[b]	73.6[c]	67.1[c]
0.5% EDTA	10.7	109.4[a]	106.7[a]	86.9[b]	82.0[b]	69.2[c]
0.5% acetic acid	2.4	3.6	-[2]	-	-	-
0.5% lactic acid	2.4	-	-	-	-	-
100 ppm clorine water	7.7	-	-	-	-	-
Distilled water	6.8	82.4[a]	81.0[a]	73.3[a]	72.2[b]	49.2[c]

[abc] Means (three replicates) within a row with no common superscripts are significantly different ($P < 0.05$).

[1] Reported as a percent of that for lysozyme activity in 1.067 M sodium phosphate buffer (pH = 6.2) freshly prepared.

[2] Lysozyme activity was not detected.

cheese to eliminate the development of acetic- and butyric-acid producing bacteria (*10,23*). The stability of lysozyme in the ethanol solution was excellent and was in agreement with the findings of Yagima et al. (*25*) and Proctor and Cunningham (*15*). Yashitake and Shinichiro (*26*) reported that lysozyme maintained 100% activity in mirin liquor (sweetened sake containing less than 20% alcohol) even after 20 weeks of storage.

The antimicrobial activities of BHA and BHT have been reported in several studies (*1,22*) and were thought to be more applicable in foods because of their additional function as antioxidative agents. Lysozyme activity in the solution of 100 ppm BHA was stable and was maintained at over 80% of its original (day 1) level. Lysozyme activity in the solution of 100 ppm BHT was only 85.7% at day 1; however, the activity was unchanged ($P < 0.05$) over the storage time. The stabilizing effect contributed by BHA and BHT was likely due to their antioxidative activity. Lysozyme in solutions of 0.1% sodium benzoate, 0.3% calcium propionate, 0.1% potassium sorbate, and 0.1% propyl paraben maintained 81.1, 75.6, 70.5, and 69.3% of its original (day 1) lytic activity, respectively. This suggests that these chemicals did not contribute to the decrease of lysozyme activity, and the loss was likely due to the storage time and enzyme degradation.

Lysozyme was unstable and activity diminished about 50% in solution of 0.5% EDTA. A high pH of 9.4 is likely a contributor to this instability. Reports (*21,24*) indicated that the combination of EDTA and lysozyme was quite effective against gram-positive and gram-negative bacteria. However, EDTA was found to have only the effect of partially disrupting the outer cell wall structure of the bacteria and allowing lysozyme to penetrate to the peptidoglycan (*6*).

Lysozyme was denaturated and lost its activity upon immediate contact with 100 ppm chlorine water. This result was expected because of the strong oxidizing action of chlorine. Trace lytic activity was detected when lysozyme was combined with 4.0% acetic acid at day 1. However, in the study by Yashitake and Shinichiro (*26*), 10 μg/ml lysozyme in vinegar had 40% of the original activity after 2 weeks and 5% after 10 weeks. The difference suggests that other substances in the vinegar solution may have exerted some protective effect on lysozyme activity. Acetic acid is naturally present in vinegar at a level of 4.0%, whereas pure acetic acid may cause lysozyme to be denatured rapidly. A similar result was also observed from the combination of lysozyme with 0.5% lactic acid solution with a pH of 2.4.

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resulted in a maximum yield of 73% and an aflatoxin content of 10 ppb.

Response surface methodology is a very useful tool for determining the optimum hydrogen peroxide treatment condition necessary for removing aflatoxin-contaminated Florunner kernels from sound medium-sized kernels. Additional experiments will be required to determine the usefulness of this treatment for separating contaminated and sound kernels of other peanut cultivars. The efficacy of the method as affected by kernel size and temperature of the hydrogen peroxide solution must also be investigated.

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