Egg white lysozyme activity in carbon dioxide solutions

K. KEENER[1*] and R. ROSALINE[2]

[1]Associate Professor, Department of Food Science, Purdue University, West Lafayette, IN 47907-2009, USA. [2]Undergraduate Student, Department of Food Science, Purdue University, West Lafayette, IN 47907-2009, USA.
[*]Corresponding author: kkeener@purdue.edu

Shell eggs contain high levels of carbon dioxide when layed. However, this is quickly lost within a few days after lay. Previous research using carbon dioxide gas for rapid cooling of shell eggs showed bactericidal effects beyond rapid cooling. It is hypothesized that lysozyme activity may be influenced by carbon dioxide concentrations and/or pH changes resulting from rapid cooling. In the United States, shell eggs are stored at 7 °C or less, and in Europe, shell eggs are not refrigerated. Thus, a study was undertaken to investigate the effect of temperature, carbon dioxide concentration, and pH on the activity of egg white lysozyme. A fresh shell egg has an ionic strength of approximately 0.06 mS and a carbon dioxide content of approximately 0.15 mg/g in the albumen. A standard microbial assay using lyophilized *Micrococcus lysodeikticus* ATCC 4698 was used to assess the lytic activity of purified egg white lysozyme. A 2x3x1 experimental design was selected consisting of two temperatures (5 °C and 22 °C), three pH's (4.5, 6.5, and 8.0) and one ionic strength (0.06 mS). Lysozyme lyses bacteria by hydrolyzing the glycosidic bond between two sugars found in the cell walls of Gram-positive bacteria. As with most biochemical processes, the effectiveness of lysozyme is highly dependent on the environmental conditions (pH, ionic strength, temperature, etc.). The activity was measured as the slope of the linear decrease in absorbance when measured by a spectrophotometer. Lysozyme activity was then normalized to the temperature of 22°C, pH 6.5, ionic strength 0.06 mS (control).

Statistical analyses were conducted using the statistical analyses package in Microsoft Excel ($P<0.05$). Results found that lysozyme activity for non-carbonated buffers, at 25°C and an ionic strength of 0.06 mS, as expected, was greatest at pH 6.5. Surpisingly, at pH 8.0 CO_2 enhanced buffer had equal lytic activity. Lytic activity at pH 6.5 decreased 25% at 5 ^oC and 9% at 22 ^oC, respectively when CO_2 was added . It was also observed that at pH 8.0, for temperatures of 5 ^oC and 22 ^oC, carbon dioxide addition significantly increased lytic activity. At pH 8.0, adding CO_2 resulted in a 155% increase in lytic activity at 5 ^oC and a138% increase in lytic activity at 22 ^oC, respectively. It is suspected that CO_2 is interacting with lysozyme and causing a conformational change. Since the egg system typically starts at pH 7.0 and increases to approximately pH 9.5 during storage this increased activity may be beneficial in further improving the safety of shell eggs. These results suggest that carbon dioxide addition may enhance egg white lysozyme activity and potentially improve shell egg safety.

Keywords: chicken eggs; egg white; lysozyme; carbon dioxide; pH

Introduction

Egg white constitutes about 60% of the whole egg by weight. It contains more than 40 different kinds of proteins, which represent about 11% of the egg white. Many of them have antimicrobial properties.

For example, ovotransferrin inhibits growth of microorganisms by binding iron, ovomucin inhibits hemagglutination used by viruses and lysozyme lyses the glycans in the cell walls of gram positive bacteria (Ibrahim, 1997).

Lysozyme in chicken egg white accounts for 3.4% of the proteins (Yi-Chan et al., 1995). It is a basic protein with an isoelectric point of pH 10.7-11.0. The optimal activity of lysozyme is found to be between pH 5.3 and 6.4 (Sim & Nakai, 1994). The role of lysozyme in egg white is to protect the proteins and fats that will nourish the developing chicken. Lysozyme has antimicrobial activity against some pathogens, including *Listeria monocytogenes* and *Clostridium botulinum*. Lysozyme also has inhibitory effects against certain spoilage organisms, including thermophilic, spore-forming bacteria and certain yeasts (Sim & Nakai, 1994); however, the antimicrobial activity of lysozyme appears limited to gram positive bacteria (Ibrahim, 1997).

The activity of lysozyme can be determined using a microbiological assay consisting of lyophilized *Micrococcus lysodeikticus* ATCC 4698. Spectrophotometric absorbance is measured at 450 nm for Micrococcus cells in a buffered suspension of lysozyme. As cell lyses occurs absorbance decreases.

In the United States, shell eggs are stored at 7 °C or less, and in Europe, shell eggs are not refrigerated. A fresh shell egg has an ionic strength of approximately 0.06 mS and a carbon dioxide content of approximately 0.15 mg/g in the albumen. Previous studies have found that fresh shell eggs that are rapidly cooled with cryogenic CO_2 have reduced microbial levels and increased shelf life (Keener et al., 2000; Mermelstein, 2001). It is hypothesized that lysozyme activity may be influenced by carbon dioxide concentrations and/or pH changes resulting from rapid cooling. Thus, a study was undertaken to investigate the effect of temperature, carbon dioxide concentration, and pH on the activity of purified egg white lysozyme.

Materials and methods

Lysozyme assay

A 0.015% lyophilized *Micrococcus Lysodeikticus* (Sigma Aldrich, St Louis, MO) buffered solution was made. A 0.001% Purified egg white lysozyme (Sigma Aldrich, St Louis, MO) buffered solution was prepared at the same time. To measure the lysozyme activity, lysozyme solution is added into the cells solution in a 1:10 ratio. At each pH and temperature, a 3.3 mL cuvette sample was prepared from the stock solution of *Micrococcus* and lysozyme. This cuvette was placed in a HITACHI U-1100 (Hitachi, Chicago, IL) at 450 nm. For measurements of buffered solutions with CO2 added, the cuvette was covered with petrifilm immediately after preparation and during testing. Absorbance measurements were taken for 9 minutes at 30 seconds intervals. The linear rate of decrease in absorbance was calculated. This slope corresponds to lytic activity. A minimum three replicates were performed for each buffer-temperature combination with additional replicates for phosphate buffer solutions. Buffer pH was recorded at the beginning and end of each test.

Buffered solutions at pH 4.5, 6.5, and 8.0 were prepared as follows. For pH 4.5, 3.0761 g of sodium acetate (Sigma Aldrich, St Louis, MO) was mixed with 2.16 mL of acetic acid (Sigma Aldrich, St Louis, MO) and dissolved in 500 mL deionized water to make an acetate buffer of pH 4.50. For pH 6.5, potassium phosphate monobasic (Fisher Scientific, Fair Lawn, NJ) and potassium phosphate dibasic (Fisher Scientific, Fair Lawn, NJ) were used to create a buffered solution of 0.066 M at pH 6.5. For pH 8.0, bicine (Sigma Aldrich, St Louis, MO) and bicine sodium salt (Sigma Aldrich, St Louis, MO) were used to create a buffered solution with pH 8.0 and molarity of 0.33 M.

Temperature conditions were selected as room temperature (22 ^{o}C) and refrigeration temperature (5^{o}C). To collect the 5 ^{o}C data the stock solutions of lysozyme and *Micrococcus* were prepared and placed in a walk-in cooler for 24 hours. The spectrophotometer and related equipment were also placed in the walk-in cooler and allowed to equilibrate prior to testing. A minimum 1 hour equilibration time was required.

The addition of CO_2 varied with pH. For pH 4.50 and pH 6.50 CO_2 gas was bubbled into acetate and phosphate buffer system for 2 minutes at room temperature. The buffer was then stored in sealed glass jar

for 24 hours to equilibrate at testing temperature (5°C and 22°C). After 24 hours, *Micrococcus lysodeikticus*, dissolved in 1 mL of the respective buffer, was injected through the septum into another jar to make a 0.015% solution. The lysozyme was also injected to another jar to make 0.001% solution. The lysozyme assay was then performed. For samples in which CO2/ HCO3- was present, sealed jars with a rubber septum were used for sample preparation. Leak checking of all jars was performed prior to sample preparation. For pH 8.0, a 0.504% bicarbonate solution was prepared (initial pH 8.32) and ~1.5 mL of 0.1 N HCl was added to adjust the pH 8.0. The preparation required a covered container to minimize the loss of CO_2. Lysozyme assays were performed immediately for room temperature samples. Refrigerated samples were cooled to 5°C for about 2 hours and the lysozyme assay was performed.

CO_2 measurements of all samples were conducted following previously developed method (Keener et al., 2000). In brief, a 2 ml sample of CO_2 enhanced buffer solution is acidified in a closed container releasing CO_2 gas. This gas is absorbed into dilute NaOH solution. This NaOH is titrated with dilute HCl and amount of CO_2 present in sample determined.

Statistical analyses was performed using the data analyses package in Microsoft Excel[tm] (Microsoft, Redmond, WA). Statistical significance was indicated with a $P<0.05$.

Results and Discussion

The results for lytic activity of purified, chicken egg white lysozyme are shown in Table 1. It can be seen that CO_2 content does influence lytic activity at pH 4.5, 6.5, and 8.0 with a slight decrease at pH 6.5. As expected, at 22 °C lytic activity was greatest for pH 6.5. Suprisingly, this was equal to the pH 8.0 CO_2 enhanced buffer at 22 °C. Lytic activity at pH 6.5 decreased 25% at 5 °C and 9% at 22 °C, respectively. It was also observed that at pH 8.0, for temperatures of 5 °C and 22 °C, carbon dioxide addition significantly increased lytic activity.

Table 1: Normalized lytic activity of chicken egg white lysozyme at different pH's and temperatures.

pH	5 °C[1,2]	5 °C with added CO_2/HCO_3^-	22 °C	22 °C with added CO_2/HCO_3^-
4.5	0.14 +/- 0.087[a]	0.44 +/- 0.098[b]	0.38 +/- 0.044[b]	0.40 +/- 0.15[b]
6.5	0.41 +/- 0.13[b]	0.27 +/- 0.28[d]	1.00 +/- 0.24[e]	0.91 +/- 0.21[f]
8.0	0.047 +/- 0.019[c]	0.12 +/- 0.078[a]	0.429 +/- 0.10[b]	1.02 +/- 0.010[e]

[1] +/- three standard deviations
[2] Statistical significance indicated by equivalent letters (a,b,c, etc.).

The impact of adding CO_2 at pH 8.0 is quite dramatic resulting in a 155% increase in lytic activity at 5 °C and 138% increase in lytic activity at 22 °C, respectively. It is suspected that CO_2 is interacting with lysozyme and causing a conformational change. Since the egg system typically starts at pH 7.0 and increases to approximately pH 9.5 during storage this increased activity may be beneficial in further improving the safety of shell eggs.

An important variable that will affect lytic activity is ionic strength. Egg white is a complicated solution, the ionic strength of which cannot easily be estimated. Thus, ionic conductivity was used to estimate ionic strength of the assay. The purpose of this measurement was to develop an ionic concentration in buffered solutions similar to those found in liquid egg white. Ionic conductivity is related to the number of ionized particles and, unlike ionic strength, can be directly measured. Table 2 shows the ionic conductivity of buffered solutions. The prepared buffered solutions had ionic conductivity similar to egg fresh egg white. Thus an ionic conductivity of 6.8 mS may be a fair approximation of the charges that exist in the liquid egg white.

Table 2: Conductivity of tested buffer solutions.

Buffer	pH	Ionic Conductivity (mS)

Acetic Acid/Sodium Acetate	4.5	6.7
Potassium Phosphate Monobasic/ Potassium Phosphate Dibasic	6.5	6.6
Bicine/Bicine Salt	8.0	6.5
Bicarbonate solution/HCl	8.0	6.5
Fresh Egg White (2 days old)	7.5	6.8
Aged Egg White (28 days)	9.2	7.1

At this time, it is unknown how CO_2/HCO_3^- influences lysozyme activity, but CO_2 gas applied in other food systems shows synergistic effects. Future research is focused on characterizing the conformational changes that might be occurring in the CO_2/HCO_3^-- lysozyme complex. With a better understanding of CO_2/HCO_3^-- lysozyme complex, one may be able to design a process to further enhance lysozyme activity in the egg system which may result in improved food safety.

References

IBRAHIM, H.R. (1997) Chapter 4. Insights into the structure-function relationships of ovalbumin, ovotransferrin, and lysozyme, in: *Hen Eggs: Their Basic and Applied Science* (Yamamoto, T., Juneja, L.R., Hatta, H., and Kim, M. eds), pp 37-52, Boca Raton, FL, USA, CRC Press.

KEENER, K.M., LACROSSE, J.D, CURTIS, P.A., ANDERSON, K.E., and FARKAS, B.E. (2000) The influence of rapid air cooling and carbon dioxide cooling and subsequent storage in air and carbon dioxide on shell egg quality. Journal of Poultry Science **79**:1067-1071.

MERMELSTEIN, N. (2000) Cryogenic system rapidly cools eggs. *Food Technology* **54**(6) 100-102

SIM, J.S., and NAKAI, S. (1994) *Egg Uses and Processing Technologies: New Developments* (J.S. Sim and S. Nakai, eds), pp 448. Cambridge, MA, USA, CABI Publishing.

YI-CHAN, E.C.Y., POWRIE, W.D., and NAKAI, S. (1995) Chapter 6. The Chemistry of Eggs and Egg Products, in: *Egg Science and Technology* (W.J. Stadelman and O.J. Cotterill eds), 4th edition, pp 105-176, Binghamton, NY, USA, Food Products Press.