Influence of carbon dioxide on the activity of chicken egg white lysozyme

P. Banerjee, K. M. Keener,1 and V. D. Lukito

Department of Food Science, Purdue University, West Lafayette, IN 47907-2009

ABSTRACT Rapid cooling of shell eggs by using liquid CO2 has shown increased bactericidal effects along with saturation of the egg albumen with CO2. Lysozyme is a bactericidal enzyme present in chicken eggs, and it lyses gram-positive bacteria. Newly laid chicken eggs have an initial pH of 7.6 to 8.5 and are saturated with CO2. During storage, the pH gradually increases to 9.7, accompanied by a loss of CO2. It is hypothesized that the lysozyme activity is influenced by either CO2 concentration or pH changes resulting from CO2 loss. The objective of this study was to determine the lytic activity of purified lysozyme and chicken egg white (unpurified lysozyme) under varying conditions of temperature, pH, and CO2 gas concentration. Lytic activity was determined by a standard microbial assay using lyophilized Micrococcus lysodeikticus. A 2 × 4 × 2 × 2 × 3 factorial design consisting of 2 temperatures (5 and 22°C), 4 pH (4.5, 6.5, 8.0, and 9.5), 2 treatments (with and without CO2), 2 types of lysozyme (purified and unpurified egg white), and 3 replicates was used. The highest lytic activity was found at pH 6.5 and 22°C. At pH 4.5 and 8.0, the addition of CO2 increased lytic activity by more than 50% at both temperatures. At pH 6.5, lytic activity was maintained with CO2 addition at both temperatures. At pH 9.5, lytic activity without CO2 addition was high; however, adding CO2 reduced lytic activity to zero. In conclusion, both pH and CO2 treatment influence lysozyme activity.

Key words: carbon dioxide, egg white, pH, temperature, lysozyme activity

INTRODUCTION

Egg albumen is approximately 60% by weight of the whole egg. The major solids content of the albumen is proteins, representing approximately 11% of the albumen (Stadelman and Cotterill, 1995). Thirteen major proteins are obtained from the chicken egg albumen. Many of these proteins have antimicrobial properties. For example, lysozyme is a bacterial inhibitor; ovotransferrin binds iron; ovomucin inhibits mucilaginous viruses; and flavoprotein and avidin bind biotin, rendering them unavailable for microbial growth (Burley and Vadehra, 1989).

Lysozyme (EC 3.2.1.17), also called as muramidase or N-acetylmuramichydrolase, is an ubiquitous enzyme found in humans, animals, and poultry (Huopalahti et al., 2007). The hen egg is the most abundant source of lysozyme (Mine, 2008), which constitutes approximately 3.4% of the albumen proteins (Stadelman and Cotterill, 1995). It is a basic protein consisting of 129 amino acids with an isoelectric point of pH 10.7 (Mine, 2008) and optimal activity between pH 5.3 and 6.4 (Sim et al., 1994). Lysozyme was discovered by Alexander Fleming in 1922. The lysozyme molecule is ovoid in shape (Yamamoto et al., 1997) and consists of 2 distinct lobes (C and N), with the active site lying between them (Huopalahti et al., 2007). The C-terminal lobe is composed of amino acid residues 1 to 39 and 89 to 129 in a mostly α-helix form (Mine, 2008). The N-terminal lobe is composed of amino acid residues 40 to 88 in a primarily antiparallel β-sheet form (Yamamoto et al., 1997).

Lysozyme is a natural antimicrobial that hydrolyzes the β(1–4) glycosidic linkage between N-acetylMuramic and N-acetylgulosamine found in the peptidoglycan layer of the bacterial cell wall and causing cell lysis (Stadelman and Cotterill, 1995). The bactericidal effect of lysozyme is primarily limited to gram-positive bacteria, including pathogens such as Listeria monocytogenes and certain Clostridium species (Mine, 2008) as well as some spoilage organisms, including thermophilic spore-forming bacteria and certain yeasts (Sim et al., 1994). The gram-negative bacteria are more resistant to lysozyme action because of their complex cell wall structure. The envelope of lipoproteins and lipopolysaccharides on the cell wall of gram-negative bacteria makes the peptidoglycan layer inaccessible to lysozyme (Ibrahim et al., 1991). However, some modified lysozyme structures have been shown to be active against gram-negative bacteria (Heath, 1977). Techniques used to manipulate the lysozyme structure and enhance its bactericidal activity against gram-negative
bacteria include thermal denaturation, covalent attachment of saturated fatty acids, and glycosylation and reduction of disulfide bonds (Mine, 2008).

Newly laid eggs are saturated with CO₂ (approximately 0.35%, wt/vol) most of which is present as bicarbonate (Sharp and Powell, 1931). However, most the CO₂ is lost quickly within a few hours of lay by diffusing out of the shell. Fresh eggs have a pH of approximately 7.6 to 8.5 (Heath, 1977), which increases to approximately pH 9.7 as the CO₂ escapes (Sharp and Powell, 1931) during storage. Previous research has indicated that using CO₂ for rapid cooling of shell eggs can reduce microbial levels and increase shelf life. (Hughes et al., 1999; keener et al., 2000). It is hypothesized that the antimicrobial activity of lysozyme may be altered by CO₂ addition. This activity change depends on pH, CO₂ molecules, or both being present. The goal of this study was to investigate the lytic activity of purified egg white lysozyme under varying conditions of pH and temperature in saturated solutions of CO₂.

MATERIALS AND METHODS

A 2 × 4 × 2 × 2 × 3 factorial design consisting of 2 temperatures (5 and 22°C), 4 pH (4.5, 6.5, 8.0, and 9.5), 2 treatments (with and without CO₂ gas), 2 kinds of egg white lysozyme (purified, lyophilized lysozyme and unpurified egg albumen), and 3 replicates was used to measure lytic activity. In addition, at pH 8.0, two CO₂ buffers were tested: CO₂ gas in bicarbonate and NaHCO₃ (solid) buffer.

Buffers

Buffered solutions at pH 4.5, 6.5, 8.0, and 9.5 were prepared as follows. Minor pH adjustments were done by using a few drops of 0.1 N HCl (Fisher Scientific, Fair Lawn, NJ) or 5 N NaOH (Sigma-Aldrich, St. Louis, MO) to achieve the target pH (Table 1).

**Table 1. Summary of CO₂ content of noncarbonated buffers**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Average CO₂ content (mg/mL of buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>4.5</td>
<td>5</td>
<td>0.308 ± 0.151a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>0.268 ± 0.141a</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.5</td>
<td>5</td>
<td>0.308 ± 0.21a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>0.269 ± 0.122a</td>
</tr>
<tr>
<td>Bicine</td>
<td>8.0</td>
<td>5</td>
<td>0.249 ± 0.084a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>0.289 ± 0.045a</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>8.0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>—</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>9.5</td>
<td>5</td>
<td>0.288 ± 0.109a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>0.169 ± 0.141a</td>
</tr>
</tbody>
</table>

*a*Means within the column having same letters are not significantly different (*P* < 0.05).

**Ionic Conductivity and pH**

The ionic conductivity and pH of the prepared buffers were measured using a conductivity meter (EC400, Extech Instruments, Waltham, MA) and a pH probe (IQ150, Spectrum Technologies, Plainfield, IL). Lytic activity also depends on ionic strength (Davies et al., 1969), which cannot be readily measured; however, ionic conductivity can. Ionic conductivity can be correlated with ionic strength. In these experiments, ionic conductivity was adjusted to the level found in egg white.

**Lysozyme Assay**

**Lysozyme Activity of Purified Lysozyme.** The procedure used for assessing the lytic activity is as described by Shugar (1952). Briefly, a 0.015% lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich) suspension was made with the buffer at the specified pH. A 0.001% purified egg white lysozyme buffered solution was prepared at the same time (Sigma-Aldrich). To measure the lysozyme activity under each condition of pH and
temperature, a 3.0-mL cuvette was prepared from the stock solution of Micrococcus and lysozyme in a 7:1 ratio. The absorbance was measured with a Hitachi U-1100 spectrophotometer (Hitachi, Chicago, IL) and the decrease in turbidity of the solution was determined at 450 nm for 9 min, at 30-s intervals. The linear rate of decrease in turbidity was calculated. This slope corresponds to the lytic activity of lysozyme. Three replicates were performed for each buffer-temperature combination. Measurement of pH was done at the beginning and end of each test. To compare the results, all activities were normalized to the maximum observed activity (pH 6.5, 22°C).

**Lysozyme Activity in Chicken Egg White.** For measurement of lytic activity in egg white at each pH, temperature, and CO2 condition, eggs were randomly selected from a flat of eggs (2 dozen eggs) obtained from a local grocery store. To determine the amount of egg white to be added to obtain a 0.001% lysozyme concentration, it is documented that chicken egg white contains approximately 3.4% lysozyme (Stadelman and Cotterill, 1995). For determining egg white activity, 0.030 g of albumen was added to 100 mL of the buffered solutions. This equated to a concentration of approximately 0.001% lysozyme. In addition, the egg white contains other antimicrobial proteins that are naturally present, as mentioned in the Introduction section.

**Carbonation of the Samples**

Carbonation of 100-mL buffered solutions at pH 4.5, 6.5, and 8.0 was done by bubbling in CO2 gas at 9 mL/min for approximately 150 s to achieve saturated solutions, and then lysozyme was added. At pH 9.5, it was found that 12 s of bubbling was sufficient to create a saturated CO2 solution. It was noted that further bubbling of CO2 beyond 12 s significantly reduced pH. The resulting CO2 concentration was comparable with all other saturated CO2 buffers. Carbonated Micrococcus solutions were prepared similar to the lysozyme solutions. In addition, at pH 8.0, a 0.504% NaHCO3 solution (commercial baking soda, Kroger, Cincinnati, OH) was prepared (initial pH 8.32, adjusted with 0.1 N HCl). Both the solutions at pH 8.0 (bicarbonate and bicine) had the same amounts of CO2 (Tables 2 and 3).

After CO2 saturation, all samples were covered with Petri-film (3M, Maplewood, MN) to reduce CO2 loss. The CO2 loss rate was approximately 0.3%/min for the 5°C treatments and approximately 1%/min at room temperature. For measurement of the lytic activity of

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Average CO2 content2 (mg/mL of buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>4.5</td>
<td>5</td>
<td>2.518 ± 0.204ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.290 ± 0.334ab</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.5</td>
<td>5</td>
<td>2.688 ± 0.102ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.212 ± 0.375ac</td>
</tr>
<tr>
<td>Bicine</td>
<td>8.0</td>
<td>5</td>
<td>2.620 ± 0.113ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.188 ± 0.429ab</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>8.0</td>
<td>5</td>
<td>2.778 ± 0.060a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.296 ± 0.035ab</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>9.5</td>
<td>5</td>
<td>1.726 ± 0.182c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.324 ± 0.270ab</td>
</tr>
</tbody>
</table>

a-cMeans within the column having different letters are significantly different ($P < 0.05$).
1Average of 5 samples.
2Means ± SD.

**Carbonation of the Samples**

Carbonation of 100-mL buffered solutions at pH 4.5, 6.5, and 8.0 was done by bubbling in CO2 gas at 9 mL/min for approximately 150 s to achieve saturated solutions, and then lysozyme was added. At pH 9.5, it was found that 12 s of bubbling was sufficient to create a saturated CO2 solution. It was noted that further bubbling of CO2 beyond 12 s significantly reduced pH. The resulting CO2 concentration was comparable with all other saturated CO2 buffers. Carbonated Micrococcus solutions were prepared similar to the lysozyme solutions. In addition, at pH 8.0, a 0.504% NaHCO3 solution (commercial baking soda, Kroger, Cincinnati, OH) was prepared (initial pH 8.32, adjusted with 0.1 N HCl). Both the solutions at pH 8.0 (bicarbonate and bicine) had the same amounts of CO2 (Tables 2 and 3).

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<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Concentration of CO22 (mg/mL of buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>4.5</td>
<td>5</td>
<td>2.636 ± 0.118a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.478 ± 0.103ab</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.5</td>
<td>5</td>
<td>2.662 ± 0.122a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.249 ± 0.095b</td>
</tr>
<tr>
<td>Bicine</td>
<td>8.0</td>
<td>5</td>
<td>2.614 ± 0.199a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.228 ± 0.180a</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>8.0</td>
<td>5</td>
<td>2.692 ± 0.144b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.324 ± 0.071b</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>9.5</td>
<td>5</td>
<td>1.948 ± 0.125c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2.482 ± 0.036ab</td>
</tr>
</tbody>
</table>

a-cMeans within the column having different letters are significantly different ($P < 0.05$).
1Average of 5 samples.
2Means ± SD.
buffered solutions with CO₂ added, the cuvette was covered with Petri-film immediately after preparation and during testing. To document CO₂ loss, the CO₂ content in the buffers was quantified every 6 min. Total experiment time was 30 min. It was observed that lysozyme activity did not vary with analysis time in saturated buffers. The averages of these values are reported in Tables 1, 2, and 3.

**CO₂ Quantification**

Measurements of CO₂ content were performed according to the method developed by Keener et al. (2001). In brief, a 5-mL sample of the carbonated buffer solution was acidified using 15 mL of acid phosphate solution inside a sealed jar, releasing CO₂ gas. The CO₂ was then absorbed into a dilute 10-mL NaOH solution inside a second smaller vial inside the sealed jar. The sealed jars were then stored at 37°C for 24 h to allow complete CO₂ absorption into the NaOH solution. After 24 h, the NaOH solution was removed from the sealed jar and titrated with dilute HCl. The amount of CO₂ in the sample could be determined.

**Refrigeration Conditions**

Experiments performed at 5°C were completed by placing the spectrophotometer, CO₂ cylinder, buffer solutions, and other related equipment in a walk-in cooler, maintained at 5°C, 24 h before testing. Stock solutions of lysozyme and *M. lysodeikticus* were prepared inside the cooler immediately before analysis.

**Statistical Analysis**

Statistical analysis was done by the GLM procedure of SAS (SAS 9.2, SAS Institute Inc., Cary, NC) using the 2-way ANOVA option. The main effects and interactions studied were temperature, pH, and CO₂ addition. Multiple mean comparisons were done using the Tukey-Kramer test. Statistical significance was indicated at *P < 0.05*.

**RESULTS AND DISCUSSION**

The lytic activities of purified lysozyme and egg white are shown in Figures 1 and 2, respectively. The concentrations of CO₂ in the buffered solutions are listed in Tables 1 and 2. For both purified and unpurified lysozyme, the maximum activity was obtained at pH 6.5 (phosphate), 22°C, without CO₂. It was observed that CO₂ had a pronounced effect on the lytic activity of lysozyme at different pH and temperatures. Lysozyme was most active at pH 6.5, and as pH deviated from 6.5, a decrease in activity was observed. Lytic activity was found to be greater at 22°C compared with 5°C. For all pH except pH 9.5, lytic activity was maintained or increased with CO₂ addition. At pH 9.5, the addition of CO₂ eliminated lytic activity.

For purified lysozyme (Figure 1) and egg white (Figure 2), the following interactions were significant (*P < 0.05*): temperature × pH, temperature × CO₂ treatment, pH × CO₂ treatment, and temperature × pH × CO₂ treatment. Replicate measurements did not have any effect. The effect of temperature was more pronounced than the effect of carbonation alone. By increasing temperature from 5 to 22°C, the activity of purified lysozyme increased by at least 75% (Figure 1; acetate buffer), whereas that of unpurified lysozyme increased by at least 210% (Figure 2; phosphate buffer).

As expected, the activity at pH 4.5 was low, compared with pH 6.5. However, CO₂ treatment increased the lytic activity of purified lysozyme at pH 4.5 by more than 50% at both temperatures. For egg white (unpurified lysozyme), carbonation increased the lytic activity by 25 and 48% at 5 and 22°C, respectively. Kato et al. (1984) have studied the reversible transition states of lysozyme in acetic acid solutions. Acetic acid solutions denature lysozyme by producing more β-sheet conformation than is found in the native state. This is brought about by forming new hydrophobic interactions between the amino acid residues and acetic acid instead of within the protein residues themselves.

When CO₂ gas is dissolved in water, it dissociates to form carbonate, bicarbonate, and carbonic acid in addition to dissolved gas. The concentration of these species is a function of temperature and pH (Butler, 1991). It is suspected that the amino acid residues of lysozyme interact with one of the CO₂ species (bicarbonate, carbonic acid, or both), reducing hydrophobic interactions. This results in a more active conformation of lysozyme. Kato et al. (1984) stated that in solutions, the conformation state of globular proteins is determined by an equivalence of hydrophobic and hydrogen bonding such that they undergo conformational change to reduce the solution free energy.

At pH 6.5, the untreated samples exhibited maximum activity, and thus an optimal conformation. With carbonation treatment, lytic activity dropped minimally (less than 10%) for purified lysozyme (Figure 1) and slightly more (approximately 20%) for unpurified egg white (Figure 2). It is hypothesized that the reduction of lytic activity in egg white resulted from the complexing of lysozyme with other albumen proteins such as ovomucin, which resulted in lysozyme being unavailable to lyse *Micrococcus* cells. This is further supported by the fact that at 5°C, the lysozyme activity for the unpurified lysozyme with and without CO₂ was approximately 33% less than the purified lysozyme with and without CO₂.

At pH 8.0, the lytic activity of lysozyme in the bicarbonate solution was statistically equivalent to that of the carbonated phosphate buffer system at pH 6.5 at both temperature conditions. In addition, we observed that both CO₂ treatments (bubbling CO₂ gas in bicarbonate buffer and NaHCO₃ solution) resulted in a significant increase in purified egg white lysozyme activity. The effect of CO₂ on proteins has received limited study, but
a study has been completed on casein stability and CO₂ treatment. Raouche et al. (2007) reported differences in casein protein micelle hydration when milk was carbonated and stated that it was independent of carbonation pH. They hypothesized that potential reorganization of the protein structure led to conformational change at the protein surface. The change in casein structure was attributed to increased hydration caused by exposure of

**Figure 1.** Normalized lytic activity of purified egg white lysozyme at different temperatures, pH, and CO₂ concentrations. Letters (a–l) indicate statistical significance at $P < 0.05 \pm 3$ SD. All data are reported relative to the standard (pH 6.5, 22°C, without CO₂). CHES = N-cyclohexyl-2-aminoethanesulfonic acid.

**Figure 2.** Normalized lytic activity of egg white (unpurified lysozyme) at different temperatures, pH, and CO₂ concentrations. Letters (a–i) indicate statistical significance at $P < 0.05 \pm 3$ SD. All data are reported relative to the standard (pH 6.5, 22°C, without CO₂). CHES = N-cyclohexyl-2-aminoethanesulfonic acid.
the protein to solvents. This was more pronounced for polar amino acid side chains, resulting in uptake of water into pockets that were earlier covered by hydrophilic surfaces (Gaucheron et al., 1996). The amino acids at the active site of lysozyme are polar, and lysozyme has sufficient hydrophilic cavities for a similar effect to occur. Increased lysozyme activity at pH 8.0 may result from increased water intrusion into hydrophilic cavities, opened by carbonate addition.

The purified lysozyme with bicarbonate addition showed a significantly greater increase in lytic activity than with CO2 gas addition: 102% higher at 5°C and 25% higher at 22°C, respectively. The difference in activity between bicarbonate and bicarbonate at the same pH 8.0 could be a result of a change in the active site chemistry. On dissolving in water, the bicarbonate produces carbonic acid and hydroxide ion, whereas CO2 dissolved in water initially exists as CO2 gas molecules and carbonic acid. It will eventually convert to bicarbonate and hydronium ion species, depending on pH. The 2 solutions are never in equilibrium, and the difference in the dominating species between the 2 systems might affect the active site conformation. At pH 8.0, the fraction of [HCO3−] is maximum, with traces of [H2CO3] or dissolved CO2 and [CO32−] (Butler, 1991). This suggests that the bicarbonate form could be more beneficial than free CO2. However, it is outside the scope of the current research to determine the fraction of each species and their relative benefits. Treatment with CO2 results in bicarbonate formation, so it would still result in increasing lysozyme activity. Freshly laid eggs contain primarily bicarbonate and CO2 (Healy and Peter, 1925; Brooks and Pace, 1938).

The CO2 treatment of egg white (Figure 2) at pH 8.0 had a significantly greater activity at 22°C, whereas at 5°C, the activities for both bicarbonate and bicine buffer were statistically equal. It is also noteworthy that in egg white at 5°C, both CO2-treated and untreated buffers had approximately the same lytic activity. This is quite the opposite of the purified lysozyme (Figure 1). This observed difference in lytic activity between lysozyme in egg white and purified lysozyme can be accounted for by the following factors. First, the egg white also contains other proteins (e.g., ovotransferin, ovomucin, ovalbumin). The CO2 may preferentially interact with some of these over lysozyme. Heath (1977) observed an increase in sulfhydryl content with storage time and attributed this to the breaking of disulfide bonds resulting from uncoiling of the albumen proteins. This denaturation of the egg albumen proteins occurs under increasing pH and CO2 loss (Heath, 1977). Second, for the current study, individual eggs were randomly selected for measurement of lytic activity of unpurified egg white at each buffer system. The lysozyme content in individual eggs will differ because of natural variability. One or both of these factors may account for the difference observed in the lytic activity trends for the purified and unpurified lysozyme (egg white).

At pH 9.5, for both purified lysozyme (Figure 1) and egg white (Figure 2), lytic activity reduced to zero with CO2 gas treatment at both temperatures. Lysozyme is fairly stable to extreme pH, and results indicate that lysozyme is active at pH 9.5 at both temperatures when CO2 is absent. This suggests an inhibitory effect of CO2 on lysozyme at pH 9.5. The complete loss of lytic activity could be a result of misfolding (because of binding of a dissociated form of bicarbonate to lysozyme) that could prevent the active site from binding to the substrate. Conformational changes to lysozyme structure exposed to high-pressure CO2 have been documented (Striolo et al., 2003). Although the mechanism remains unknown, research suggests that addition of CO2 gas and its resultant dissociated species significantly modify the lysozyme structure so as to alter its biological activity at pH 9.5.

The CO2 content of noncarbonated buffers is shown in Table 1. Even though these buffers did not undergo a CO2 treatment, they contain minor amounts of dissolved CO2 from the atmosphere. The lytic activity does not solely depend on CO2 concentration. It is also affected by pH and temperature. For example, the CO2 concentrations in the carbonated solutions at pH 8.0 (bicarbonate) and pH 9.5 (NH4Cl) at 22°C are the same (Table 2). However the lytic activity at pH 8.0 is very high compared with that at pH 9.5 (Figure 1) because pH 8.0 is closer to the optimal activity pH of lysozyme (5.3 to 6.4). Similarly, at 5°C there is a difference in lytic activity of egg white with CO2 treatment (Figure 2) at pH 4.5 and 6.5. For both the instances cited, the activity is measured at the same temperature and saturated CO2 conditions.

Table 4 shows the ionic conductivity and pH of buffered solutions and egg white. The lytic activity of lysozyme is strongly affected by ionic strength (Davies et al., 1969) and has to be controlled. In the current study, ionic conductivity was used to estimate ionic strength of the assay. This was done to develop an ionic concentration in buffered solutions similar to those found in liquid egg white. Ionic conductivity can be directly measured, unlike ionic strength. The ionic conductivity ranged between 5.2 and 11.99 mS for all buffers, with egg white having an average of 8.11 mS. As seen upon buffer saturation with CO2 gas, there was a slight drop in pH for all buffers. However, it was observed that the decline in pH was largest at pH 9.5; thus, a larger amount of NaOH was needed to adjust the pH, resulting in higher ionic conductivity.

In summary, it was found that at all pH conditions, lysozyme was more active at room temperature (22°C) than at refrigeration (5°C). The carbonation treatment of purified lysozyme always showed greater activity than that for egg white. The CO2 gas treatment increased lysozyme activity of purified lysozyme by more than 50% at both temperatures at pH 4.5. In egg albumen, the activity increased by 25 and 48% at 5 and 22°C, respectively. At pH 6.5, a slight decrease in lytic
activity was observed for carbonated solutions at room temperature. However, no decrease in lytic activity was observed for carbonated solutions at 5°C. At pH 8.0, lysozyme activity increased significantly for both bicarbonate and CO2 gas addition at both temperatures. The largest increase in lytic activity with CO2 treatment was observed at 5°C for both purified lysozyme and egg albumen, with activity increasing by more than 100%. The addition of CO2 gas eliminated lytic activity at pH 9.5 for both egg white and lysozyme. In comparing CO2 gas and NaHCO3 (solid) at pH 8.0, the bicarbonate form was more effective in enhancing lysozyme activity.

These results document the potential for CO2 to alter lysozyme activity over a wide pH range. These findings have significant commercial value. However, the question remains of how dissociated species of CO2, such as bicarbonate, carbonate, or carbonic acid, influence lysozyme activity. With a better understanding of the dissolved CO2 speciation (HCO3−, CO32−) and lysozyme complex, it may be possible to further enhance lysozyme activity, leading to increased food safety in shell eggs and food products when lysozyme is used as an ingredient.

**ACKNOWLEDGMENTS**

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**REFERENCES**


