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# Oxalic Acid-Induced Modifications of Postglycation Activity of Lysozyme and Its Glycoforms

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The role of selected carboxylic acids and their potential to influence the glycation pattern and the enzymatic activity of lysozyme using glucose and ribose were investigated independently of the pH of the reaction medium. The model systems were incubated with and without selected carboxylic acids (maleic, acetic, oxalic, and citraconic) at 50 °C for 12 or 24 and 48 h at constant pH of 6.5. The effect of carboxylic acids on the glycation of lysozyme was studied by electrospray ionization mass spectrometry (ESI-MS) and by the measurement of the residual enzyme activity of lysozyme in the glycated samples. Of the carboxylic acids evaluated, oxalic acid showed the highest antiglycation activity. The residual lysozyme activity in both oxalic acid−glucose and oxalic acid−ribose systems was >80% compared with 46 and 36% activity in the controls of glucose and ribose systems, respectively. On the other hand, maleic, acetic, and citraconic acid containing systems with both sugars did not exhibit any enhanced enzyme activity relative to the controls. The results of this study show that oxalic acid was unique among the carboxylic acids evaluated with respect to its ability to interact with sugars and inhibit glycation.

**KEYWORDS: Lysozyme activity; glycation; glucose; ribose; maleic; acetic, oxalic, and citraconic acids; antiglycation**

## INTRODUCTION

Glycation involves a series of progressive chemical transformations that occur between reducing sugars and the amino groups of proteins, leading to the eventual formation of free or protein-bound dietary advanced glycation end products (AGEs) or Maillard reaction products (MRPs) (*1*, *2*). Glycation can lead to the formation of a complex mixture of protein−sugar adducts with various degrees of modifications depending on temperature, pH, and time of exposure (*3*). The number of sugar moieties that can be linked to a given protein molecule depends on many factors, including the number of lysine and arginine residues (*4*). It is difficult to estimate the critical sugar load needed before a protein can be considered to be functionally impaired or deactivated, in the case of enzymes, or is able to release free AGEs in a food system. Ingestion of dietary AGEs has been associated with cardiovascular and other age-related diseases and health complications of long-term diabetes (*5*). Developing strategies to alter the glycation profile of proteins during processing and hence to control their potential to deactivate enzymes or release AGEs may help reduce the risk factors associated with thermal processing of many foods. The objective of this research was to investigate the role of carboxylic acids in modulating the glycation profile of a model protein lysozyme. Carboxylic acids are commonly used in foods as preservatives and are also known to catalyze the first step of glycation (Amadori formation); however, by keeping the pH constant, the effect of different carboxylic acids can be studied on post-Amadori changes associated with AGE formation and deactivation of proteins.

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## MATERIALS AND METHODS

**Materials.** Chicken egg white lysozyme (LYZ) (purity, >96%; MW 14307 Da), D-glucose (purity, 99.5%), D-ribose (purity, 99%), maleic acid (purity, ≥99%), oxalic acid anhydrous (purity, ≥99%), and citraconic acid (purity, 98%) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Glacial acetic acid (glacial) was obtained from EMD Chemical Inc. (Gibbstown, NJ). *Micrococcus lysodeikticus* and potassium phosphate monobasic were purchased from Sigma-Aldrich Chemicals. Potassium phosphate dibasic was obtained from Fisher Chemical Scientific (Nepean, ON, Canada). All other chemicals and reagents were of analytical grade from Fisher Scientific. Ultrapure water from the Nanopure water purification system (Barnstead, Themolyne, Dubuque, IA) was used throughout the study.

**Sample Preparation.** Samples were prepared following the procedure previously described by Yeboah and Yaylayan (*3*) with slight modifications. Briefly, lysozyme (1.0 g), D-glucose (0.0757 g) or D-ribose (0.0632 g) was dissolved separately in fresh ultrapure water (50 mL). To these solutions were added oxalic acid (0.1067 g), maleic acid (0.0977 g), acetic acid (0.0503 g), and citraconic acid (0.1095 g) separately to give a protein/sugar/carboxylic acid molar ratio of 1:6:12 (approximately 1:1:2 molar ratio of lysine residue to sugar carbonyl to acid). All of the solutions were adjusted to a pH value of 6.5 with dilute HCl or NaOH and lyophilized. The lyophilized samples were then stored at −20 °C prior to incubation. Lysozyme (1.0 g) and D-glucose (0.0757 g) or D-ribose (0.0632 g) solutions were also prepared similarly as controls. All samples were prepared in duplicate.


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**Sample Incubation.** Portions of 100.0 ± 0.2 and 350.0 ± 0.2 mg of each freeze-dried stock sample were weighed into individual glass vials (10 mL). The vials were covered with perforated aluminum foil and incubated at 50 °C for 24 or 48 h in a desiccator placed in an oven. The relative humidity in the desiccator was maintained at 65% with saturated aqueous KI solution throughout the incubation period (*6*). After incubation, samples were stored at −20 °C until they were analyzed.

**Preparation of Lysozyme Calibration Samples.** The incubated samples were reconstituted to a final concentration of 2 mg/mL using aqueous acetic acid (30%). The samples were diluted with the same solvent by transferring 5.0 mL portions into three 10 mL volumetric flasks. One of the flasks was left without spiking, and two other volumetric flasks were spiked such that the concentration of added lysozyme was between 0.05 and 0.20 mg/mL. In addition, a solution of aqueous acetic acid (30%) was used as blank.

**Sample Analysis and Quantification.** All calibration samples were prepared as described above, and the blank was analyzed by electrospray ionization mass spectrometry (ESI-MS). The ESI-MS spectra were obtained using a Sciex API 4000 triple-quadrupole mass spectrometer (Sciex, Concord, Canada). Samples were analyzed in the positive ionization mode using the following parameters. The turbo ion spray source was operated at 450 °C with ion spray voltage at 5500 V, curtain gas ($N_2$) at 10 psi, ion source gas 1 at 61 psi, and ion source gas 2 at 36 psi. During the experiment the entrance potential was set at 10 V, and the declustering potential was set at 100 V. Samples were infused into the electrospray ion source through a Cole Parmer Infusion pump model 74900 with a 1 mL syringe at a rate of 2 mL/h. The scan range was between 150 and 2500 amu. A plot of peak intensity versus added lysozyme concentration was generated for each sample set. A linear regression equation was determined for each plot and was used to calculate the lysozyme concentration of the unspiked sample. The calibration curve of each incubation system was determined at three different multiply charged ionization states (+10, +11, +12). The free lysozyme in the unspiked sample was calculated against each calibration curve and reported as the average of three calculations.

**Residual Lysozyme Activity Measurements Using *M. lysodeikticus* Cell Walls.** Samples were prepared essentially according to the method given in ref *7*b with minor modifications. Briefly, a 0.3 mg/mL suspension of *M. lysodeikticus* was prepared in water. To 2.9 mL of this suspension was added 200 μL of the 0.25 mg/mL of lysozyme-containing solution (lysozyme−glucose−carboxylic acid 48 h incubation samples and lysozyme−ribose−carboxylic acids 24 h incubation samples). In addition, the same concentration of oxalic acid solution was also prepared as a control.

**Preparation of Lysozyme Calibration Standards.** To 2.9 mL of a 0.3 mg/mL suspension of *M. lysodeikticus* were added 5, 50, 100, 150, or 200 μL of the 0.25 mg/mL of lysozyme standard solution and 200 μL of the 0.8 mg/mL of lysozyme standard solution, respectively. The total volume was adjusted to 3.1 mL by adding the 0.1 M potassium phosphate buffer solution, pH 7.0. Water was used as blank.

**Sample Analysis and Quantification.** All of the calibration standards, samples, control, and blank prepared above were analyzed by UV−vis spectrophotometry. The absorbance at 450 nm was measured every 5 s for 30 s. The slope of the linear part of the curve was recorded. According to Michaelis−Menten enzyme kinetics theory, a double-reciprocal plot of 1/initial velocities and 1/enzyme concentration will give a linear relationship. A plot of 1/initial velocities versus 1/added lysozyme concentration was generated for each sample set. A linear regression equation was determined for each plot and was used to calculate the lysozyme concentration of each incubated sample of lysozyme−glucose (LYZ−G) and lysozyme−ribose (LYZ−R) model systems. By multiplying the dilution factor, the mass of active lysozyme in each sample could be calculated. Furthermore, the percentage of remaining active lysozyme was quantified through the mass ratio of active lysozyme and total weight of sample.

**FTIR Analysis.** An equimolar mixture of ribose and oxalic acid in methanol was incubated for 10 h at room temperature in the presence of a catalytic amount of $AlCl_3$. One microliter samples of the solution were repeatedly applied and evaporated onto the ATR crystal and immediately scanned at time zero and after 10 h. Control experiments were conducted using oxalic acid alone in the presence of a catalytic amount of $AlCl_3$. The infrared spectra were recorded on a Bruker Alpha-P spectrometer (Bruker Optic GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector, a temperature-controlled single-bounce diamond attenuated total reflectance (ATR) crystal, and a pressure application device for solid samples. A total of 32 scans at 4 $cm^{-1}$ resolution were co-added. Processing of the FTIR data was performed using Bruker OPUS software.

## RESULTS AND DISCUSSION

The effect of selected carboxylic acids (maleic, oxalic, acetic, and citraconic) on the glycation of lysozyme was estimated by the amount of remaining unglycated lysozyme and its residual enzymatic activity in addition to the changes induced in the distribution pattern of different glycoforms in the incubation mixtures. The incubations were carried out in the presence and absence of the selected carboxylic acids at 50 °C for 12 or 24 and 48 h at a constant pH of 6.5. The effect of carboxylic acids on the glycation of lysozyme was studied by ESI-MS and by the measurement of the residual enzyme activity of lysozyme in the glycated samples. **Figure 1** represents a typical ESI-MS pattern of the incubated samples. Panel **A** shows the ESI-MS spectrum of untreated lysozyme, panel **B** represents the ESI-MS spectrum of lysozyme incubated with glucose (glucose control), showing the peak of unglycated lysozyme and other peaks associated with glycated lysozyme molecules containing various amounts of glucose moieties, and panel C shows the pattern for the glycoforms of lysozyme incubated with glucose in the presence of oxalic acid. **Figures 2** and **3** show the distribution of glucose- and ribose-induced glycoforms of lysozyme, respectively, in the different carboxylic acid systems. **Figure 1** clearly demonstrates that a mixture of glycoforms of lysozyme was generated in the incubation mixtures and that the sugar moieties of the glycoforms undergo dehydration reactions to produce a specific distribution of dehydrated glycoforms. Comparison of the glycoforms of lysozyme in the different lysozyme−sugar−acid systems shows that the distribution patterns were different in the different systems (see **Figures 2** and **3**).

**Comparison of the Amount of Unreacted Lysozyme and the Residual Enzymatic Activity in Carboxylic Acid Treated Samples.** The amount of unreacted lysozyme remaining at the end of the incubation period was used as a measure of the inhibitory effect of the carboxylic acids on the glycation of lysozyme. The amount of unglycated or free lysozyme remaining in the incubated samples was estimated by the intensity of the peak of free lysozyme in the lysozyme−glucose model systems using the external calibration method described by Yeboah and Yaylayan (*3*). Although the peak intensities of the glycoforms of lysozyme, as measured by ESI-MS in the incubated samples, may correlate well with the relative amount of unglycated lysozyme in the reaction mixture, it is not known how the calibration will be affected by the presence of carboxylic acids and how the sample composition may affect the analytical signal. To address the possible signal suppression expected by matrix effects, the standard addition method was also chosen for the quantification of free lysozyme. In this method, known quantities of lysozyme were added to each model system, and from the increase in the signal intensity, the free lysozyme in each system was calculated. The results showed a linear relationship between the peak intensity and the concentration of lysozyme in lysozyme−sugar model systems with or without carboxylic acids. Both methods (standard addition and external calibration) yielded comparable data; however, values generated from the standard addition method are used in this study. **Table 1** lists the percentages of unglycated lysozyme remaining in the incubated samples and their corresponding residual enzyme activity values in different model systems. These values clearly show that under comparable conditions ribose glycated 98.8% of

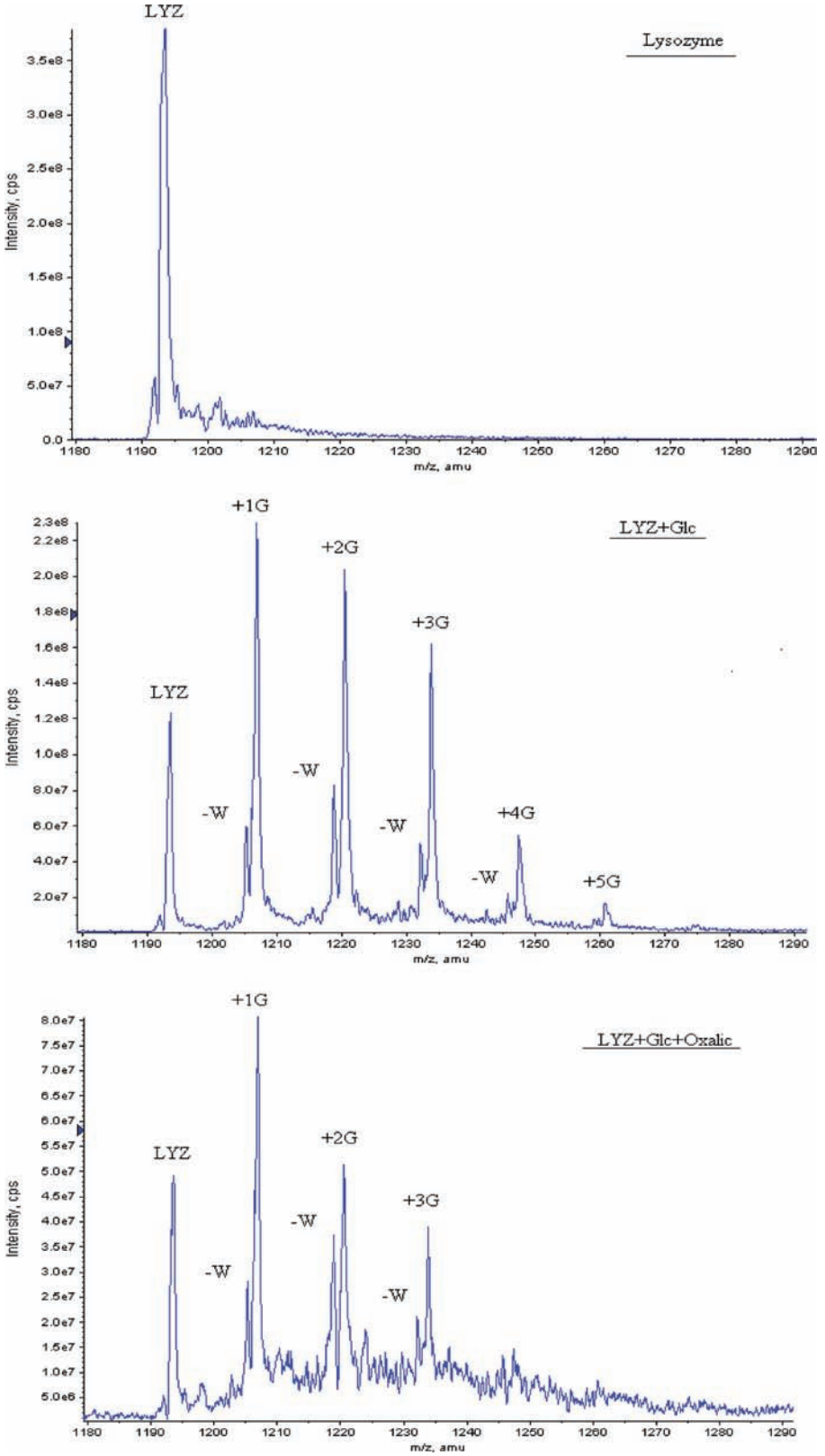


**Figure 1.** Typical ESI mass spectra of lysozyme, LYZ + glucose, and LYZ + glucose + oxalic acid (24 h of incubation). LYZ, lysozyme; G or Glc, glucose; W, water.

lysozyme in a 4 h period, whereas glucose needed 48 h to glycate only 94% of lysozyme. More importantly, **Table 1** also indicates that the addition of carboxylic acids to lysozyme had a sugar-specific effect on the resulting glycation profiles of lysozyme model systems. In the glucose model systems all of the added carboxylic acids, with the exception of oxalic acid, lowered the percentages of free lysozyme relative to the control (~6% free lysozyme in the control), whereas in ribose systems all of the added carboxylic acids increased the percentages of free lysozyme relative to the control (~1% free lysozyme). However, in both sugar systems oxalic acid induced the formation of the highest percentages of free lysozyme. According to **Table 1**, in glucose

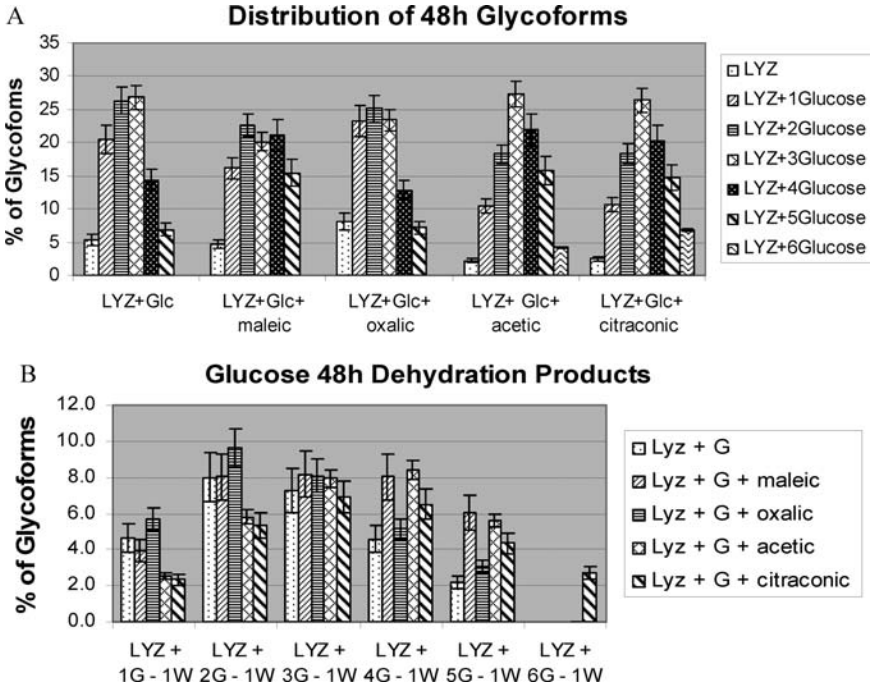


**Figure 2.** Percentage distribution of (**A**) intact glycoforms and (**B**) dehydrated glycoforms in the LYZ−G and LYZ−G−acid systems incubated for 48 h. The values are averages of two replicate measurements with relative standard deviation of 0.3−17.3%. LYZ, lysozyme; R, ribose; G or Glc, glucose; W, water.

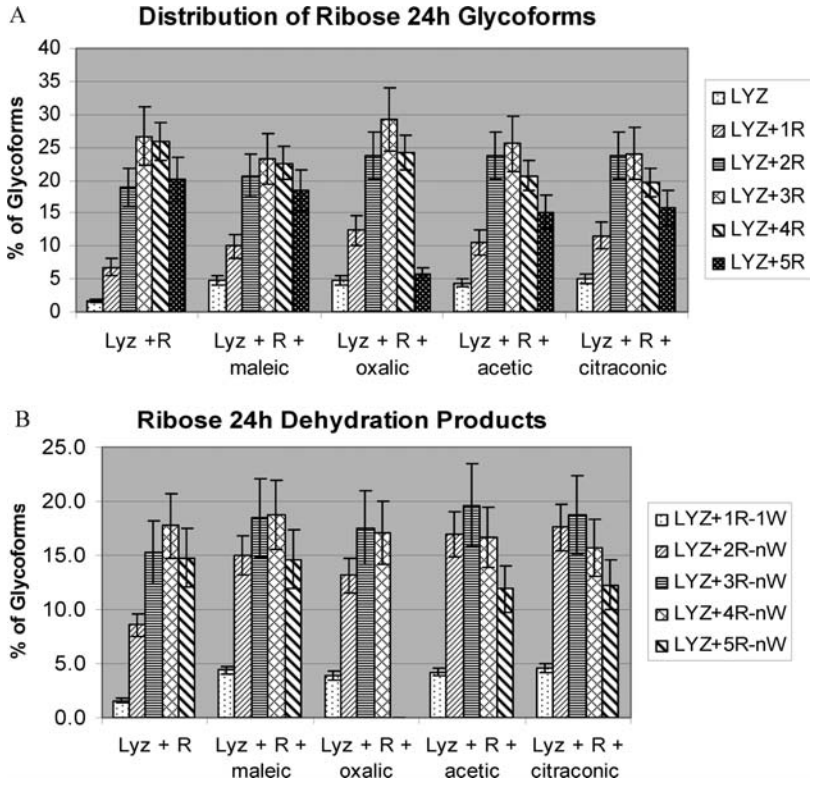


**Figure 3.** Percentage distribution of (**A**) intact glycoforms and (**B**) dehydrated glycoforms in the LYZ−R and LYZ−R−acid model systems incubated for 24 h. The values are averages of two replicate measurements with relative standard deviation ranging between 1.1 and 17.3%. LYZ, lysozyme; R, ribose; G or Glc, glucose; W, water.

systems this represented 9.4% (1.5-fold increase relative to the control) and in ribose 7.1% (5.9-fold increase relative to the control) of free lysozyme. The residual enzymatic activity was more or less correlated with the amount of free lysozyme remaining in the glucose model systems. Interestingly, when the enzymatic activity of the residual lysozyme was measured in ribose systems, it was found that not all of the remaining free lysozyme retained its activity to the same extent. For example, although we measured higher percentage of free enzyme remaining in citraconic acid model relative to the control (2.9 vs 1.2%), the control exhibited higher enzymatic activity (36.2 vs 11.8%). The question that arises, then, is "what glycation profile or extent

**Table 1.** Comparison of the Percentages of Residual Free Lysozyme in the Model Systems with the Amount of Remaining Active Enzyme

| | lysozyme−glucose (48 h) | | lysozyme−ribose (24 h) | |
|---|---|---|---|---|
| | % free LYZ[a] | % LYZ activity remaining[b] | % free LYZ[a] | % LYZ activity remaining[b] |
| control | 6.0 ± 1.8 | 46.4 ± 1.8 | 1.2 ± 0.3 | 36.16 ± 1.6 |
| maleic acid | 2.1 ± 0.5 | 16.9 ± 6.2 | 2.3 ± 0.6 | 8.5 ± 5.6 |
| oxalic acid | 9.4 ± 1.4 | 80.9 ± 0.8 | 7.1 ± 1.7 | 80.4 ± 2.0 |
| acetic acid | 2.3 ± 0. | 23.7 ± 3.7 | 1.7 ± 0.2 | 27.5 ± 2.7 |
| citraconic acid | 2.1 ± 0.4 | 18.0 ± 11.5 | 2.9 ± 0.4 | 11.8 ± 9.1 |

[a] Based on standard addition method. LYZ, lysozyme. [b] Residual enzyme activity based on absorbance assays using *Micrococcus lysodeikticus* cell walls and Michaelis−Menton enzyme kinetics.

**Table 2.** Percent Distribution of Free Lysozyme[a] versus Total Intact and Total Dehydrated Glycoforms in Lysozyme−Glucose Model Systems

| model system | glycoform type | control | maleic acid | oxalic acid | acetic acid | citraconic acid |
|---|---|---|---|---|---|---|
| LYZ−Glc, 24 h | free lysozyme | 12.9 ± 0.4 | 7.8 ± 0.6 | 15.6 ± 0.5 | 2.6 ± 0.0 | 4.0 ± 0.3 |
| | total intact glycoforms | 66.8 ± 0.4 | 63.7 ± 0.6 | 58.2 ± 0.5 | 69.4 ± 0.0 | 70.7 ± 0.3 |
| | total dehydrated glycoforms | 20.3 ± 0.9 | 28.5 ± 0.3 | 26.2 ± 1.1 | 28.0 ± 0.3 | 25.3 ± 0.5 |
| LYZ−Glc, 48 h | free lysozyme | 6 ± 1.8 | 2.1 ± 0.5 | 9.4 ± 1.4 | 2.3 ± 0.5 | 2.1 ± 0.4 |
| | total intact glycoforms | 68.1 ± 0.2 | 61.1 ± 0.2 | 60.2 ± 0.3 | 67.5 ± 0.1 | 69.4 ± 0.2 |
| | total dehydrated glycoforms | 26.7 ± 1.3 | 34.3 ± 0.8 | 31.6 ± 0.2 | 30.3 ± 0.6 | 28.1 ± 0.3 |

[a] Based on standard addition method. LYZ, lysozyme.

**Table 3.** Percent Distribution of Free Lysozyme[a] versus Total Intact and Total Dehydrated Glycoforms in Lysozyme−Ribose Model Systems

| model system | glycoform type | control | maleic acid | oxalic acid | acetic acid | citraconic acid |
|---|---|---|---|---|---|---|
| LYZ−ribose, 12 h | free lysozyme | 2.2 ± 0.1 | 5.1 ± 0.2 | 5.2 ± 0.4 | 4.6 ± 0.2 | 5.2 ± 0.4 |
| | total intact glycoforms | 55.1 ± 0.1 | 36.5 ± 0.2 | 53.0 ± 0.4 | 39.8 ± 0.2 | 41.5 ± 0.4 |
| | total dehydrated glycoforms | 42.7 ± 0.3 | 58.4 ± 0.5 | 41.8 ± 0.2 | 55.6 ± 0.9 | 53.3 ± 2.3 |
| LYZ−ribose, 24 h | free lysozyme | 1.2 ± 0.3 | 2.3 ± 0.6 | 7.1 ± 1.7 | 1.7 ± 0.2 | 2.9 ± 0.4 |
| | total intact glycoforms | 40.5 ± 0.2 | 24.0 ± 0.4 | 43.5 ± 0.1 | 26.2 ± 0.1 | 26.2 ± 0.0 |
| | total dehydrated glycoforms | 57.9 ± 0.3 | 71.2 ± 0.3 | 51.7 ± 1.2 | 69.4 ± 1.4 | 68.8 ± 0.2 |

[a] Based on standard addition method. LYZ, lysozyme.

of glycation is necessary to inhibit the enzymatic activity of lysozyme" or the biological activity of a protein in general?

**Glycation Profiles of Carboxylic Acid Treated Lysozyme Samples Incubated with Glucose or Ribose.** As indicated above, the addition of carboxylic acids with the exception of oxalic acid decreased the residual enzymatic activity of lysozyme after incubation with glucose and ribose relative to the controls containing no acid (**Table 1**). Oxalic acid, however, played a protective role against glycative damage of lysozyme in both systems. The role of carboxylic acids was further studied relative to their effect on the distribution of different glycoforms of lysozyme. Lysozyme incubated with glucose generated free, glycated with up to 6 mol of sugar, and dehydrated glycoforms with loss of only 1 mol of water, consistent with the literature. Higher losses of water can occur on longer incubation periods (*3*, *4*). Ribose models, on the other hand, generated free, glycated with up to 5 mol of sugars, and dehydrated glycoforms with loss of up to 4 mol of water (see **Figures 2** and **3**; **Tables 2** and **3**). In the case of glucose incubated for 48 h (**Table 2**), in general, the addition of carboxylic acids decreased the percentages of free lysozyme and intact glycoforms at the expense of dehydrated glycoforms relative to the control, with the exception of the oxalic acid, which also increased the percentages of unreacted lysozyme. In the case of glucose models, residual enzymatic activity could be correlated more or less with the extent of free lysozyme remaining in the systems. Ribose model systems, on the other hand, exhibited a different behavior. When incubated for 24 h, the addition of carboxylic acids increased the percentages of free lysozyme and decreased the percentages of intact glycoforms at the expense of dehydrated glycoforms with the exception of the oxalic acid. The oxalic acid not only increased the percentage of free lysozyme but also decreased the percentage of dehydrated glycoforms. This behavior was not observed in the case of glucose. Although other carboxylic acids also increased the percentage of free lysozyme in ribose systems, they also significantly increased the percentages of dehydrated glycoforms and perhaps caused the observed reduced enzymatic activity relative to the control (see **Table 1**). More detailed glycation profiles of lysozyme incubated with glucose and with ribose are shown in **Figures 2** and **3**, respectively. Both figures indicate statistically significant changes in the distribution profiles of intact and dehydrated glycoforms in addition to specific effects such as acetic acid- and citraconic acid-induced hexaglycation products that were absent in the control and in the other carboxylic acid samples (**Figure 2A**). On the other hand, in the ribose systems, oxalic acid caused a dramatic decrease in the amounts of pentaglycated lysozyme and the complete absence of its dehydrated forms. In the case of ribose the enhanced residual enzymatic activity could be related to the lower percentages of dehydrated glycoforms in addition to the high percentage of free lysozyme remaining.

**Role of Oxalic Acid.** If the oxalic acid effect observed in this study was due to its ability to interact with lysyl side chains of lysozyme and form oxalic acid monoalkyl amide (OMA), similar to the effect of ascorbic acid reaction (*8*), then no significant differences should have been observed between the responses of ribose and glucose models other than those corresponding to their controls. Furthermore, only activated oxalic acid derivatives can

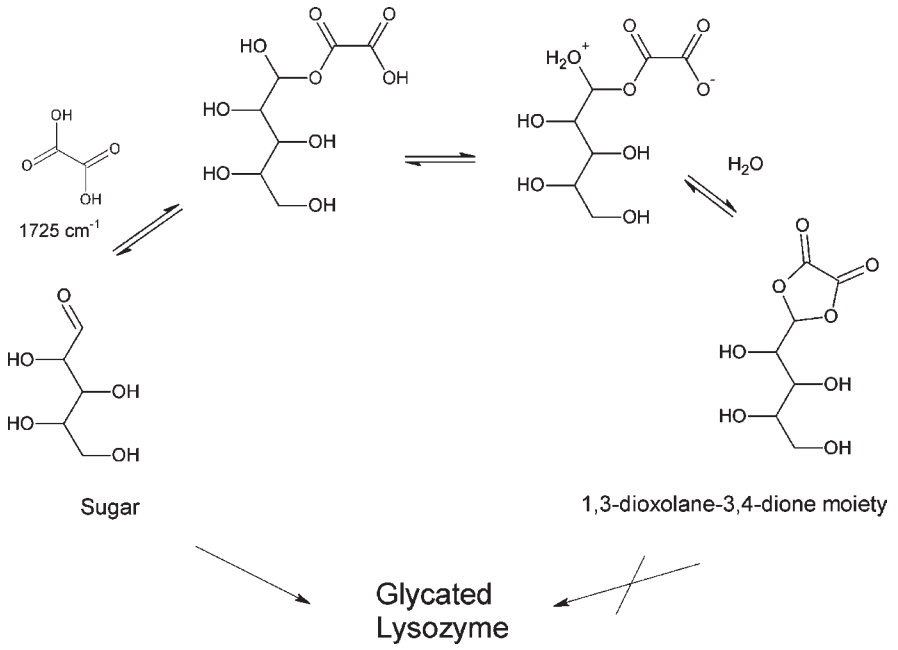


**Figure 4.** Proposed reaction of sugars with oxalic acid (based on ref *10*).

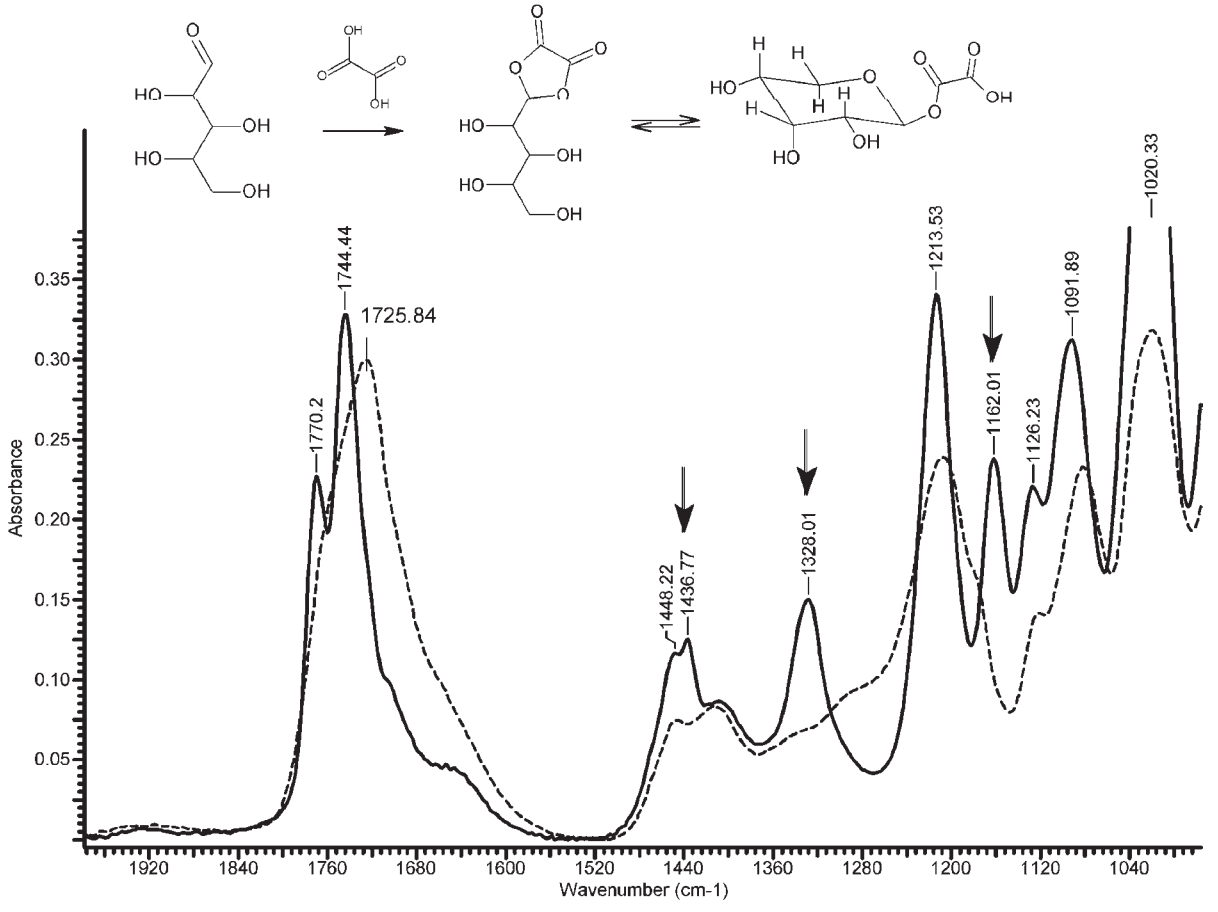


**Figure 5.** Partial FTIR spectra of ribose−oxalic acid (time zero dotted line) and ribose−oxalic acid (time 10 h solid line) incubated at room temperature. Arrows indicate appearance of new bands in the fingerprint region after incubation for 10 h at room temperature.

effectively form amide linkages with amino groups (*9*), especially under mild reaction conditions as in this study. Clearly, the observed oxalic acid effect was sugar-dependent, exhibiting different extents of interaction with the very reactive ribose (5.9-fold increase in measured free lysozyme) compared with the less reactive glucose (only 1.5-fold increase). In the less reactive glucose models, the amount of free lysozyme remaining could explain the residual activity of the enzyme in all of the carboxylic acid samples; however, in the more reactive ribose system, although the carboxylic acids increased the amount of

free lysozyme remaining in the different models, they also decreased the residual enzymatic activity with the exception of oxalic acid. This was rationalized on the basis of the observation of decreased percentages of dehydrated glycoforms in oxalic acid system relative to the control and relative to the other carboxylic acid containing models. Furthermore, the data may also indicate that unlike dehydrated glycoforms, intact glycoforms could retain some enzymatic activity depending on the extent of glycation and their sites. The sugar-specific effect of oxalic acid can be rationalized by its unique ability among other dicarboxylic acids to react with carbonyl groups in a similar fashion to that of 1,2-diols or 1,2-dithiols and form 1,3-dioxolane-4,5-dione moieties (*10*) as shown in **Figure 4**. The extent of blocking of the carbonyl group of the open form of the sugars with oxalic acid depends on their reactivity (percent of open forms). Ribose being more reactive than glucose, higher percentages will be blocked by oxalic acid, and hence less lysozyme will be glycated, as was observed. On the other hand, in dry state, free carboxylic acids can catalyze dehydration reactions through their ability to transfer protons in the absence of a solvent. Oxalic acid, being involved in 1,3-dioxolane-4,5-dione linkages, is less available to do so relative to other carboxylic acids that are unable to react with the carbonyl functionality of sugars to form stable end products and instead remain free to catalyze dehydration reactions as observed. Furthermore, other carboxylic acids may also undergo similar but reversible acetal formation with sugar carbonyls (first step of the reaction shown in **Figure 4**) but are unable to cyclize into stable products. The reversible acetal formation can explain the slight increases in the free lysozyme values observed in ribose models incubated with other carboxylic acids relative to the ribose control (see **Table 1**).

**FTIR Monitoring of Ribose Reaction with Oxalic Acid.** To confirm the ability of reducing sugars to interact with oxalic acid under the experimental conditions, an equimolar mixture of ribose and oxalic acid in methanol was monitored at room temperature by FTIR in the presence of a catalytic amount of $AlCl_3$. If oxalic acid indeed can react with ribose, this should induce significant changes in the frequency of carbonyl absorption band of oxalic acid centered at 1725 $cm^{-1}$ according to the findings of Serck-Hanssen (*10*), who reacted oxalic acid with formaldehyde and isolated 1,3-dioxolane-4,5-dione from the reaction mixture in 65% yield. IR analysis of this compound indicated the presence of two strong carbonyl stretching bands around 1845 and 1792 $cm^{-1}$, which were assigned to symmetrical and unsymmetrical vibrations of the 1,3-dioxolane-4,5-dione moiety. **Figure 5** clearly shows that the carbonyl region of ribose/oxalic acid mixture underwent significant changes after incubation for 10 h at room temperature. The free oxalic acid band centered at 1725 $cm^{-1}$ decreased dramatically, and two new bands appeared centered at 1744 and 1770 $cm^{-1}$. Although these two bands could be assigned to the symmetrical and unsymmetrical vibrations of the 1,3-dioxolane-4,5-dione moiety of the ribose oxalic acid adduct, due to the ability of ribose to cyclize through mutarotation, these two bands could also be attributed to the resulting ester (1770 $cm^{-1}$) and to the carboxylic acid (1744 $cm^{-1}$) absorption bands of the cyclized adduct as shown in **Figure 5**. Cyclization through the C-5 hydroxyl group can open the 1,3-dioxolane-4,5-dione ring and expose carboxylic acid and ester functional groups. In either case, the significant drop in the intensity of the oxalic acid band and the concomitant emergence of two strong bands in the carbonyl absorption region in addition to three new bands in the fingerprint region (1436, 1328, and 1162 $cm^{-1}$) clearly indicate the occurrence of oxalic acid sugar reaction, leading to blocking of the reactive carbonyl moiety as observed during incubation reactions.

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