Heat Inactivation of Catalase from *Staphylococcus aureus* MF-31

GAIL P. ANDREWS† AND SCOTT E. MARTIN*

Department of Food Science, University of Illinois, Urbana, Illinois 61801

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The effects of heat on catalase from *Staphylococcus aureus* lysates were examined. Catalase activity increased with increasing concentrations of potassium phosphate buffer, when heated at temperatures between 50 and 65°C for 10 min. Inactivation of catalase by NaCl during heating was demonstrated. Extended heating of *S. aureus* cells at 52°C resulted in a slight decrease in catalase activity of the resultant lysates. This decrease was more pronounced in the presence of salt. Heating at 62°C caused a decrease in catalase activity, but not complete inactivation. These results implicate the combined effects of heat and NaCl in the inactivation of catalase from *S. aureus*. The findings are consistent with the hypothesis that H₂O₂ may accumulate as a result of decreased catalase activity and be responsible for the decreased colony-forming ability of stressed *S. aureus*.

The enzyme catalase is produced by most aerobic microorganisms for the degradation of hydrogen peroxide (15). The result of normal respiration in most aerobically growing cells, hydrogen peroxide (H₂O₂), is highly toxic and can be bactericidal if not degraded (14). Catalase catalyzes the breakdown of H₂O₂ to molecular oxygen and water (5). The kinetics and mechanisms of this action have been studied in great detail (4, 7).

Recently, the addition of catalase to selective media for the enumeration of injured microorganisms has been shown to result in significant increases in counts (3, 9, 19, 21). Similar increases were seen when catalase was added to media utilized for the enumeration of anaerobic bacteria (10, 11). Injured microorganisms are those that have been subjected to a physical or chemical stress, but have not been killed. They become partially debilitated and may have different growth requirements than unstressed cells (1, 20). Although they may recover and grow, causing food poisoning and spoilage, their presence may not be detected by using normal procedures (24). Martin et al. (19) suggested that the decreased counts of stressed microorganisms was due to H₂O₂ accumulation. The addition of catalase degraded the H₂O₂, permitting growth of the injured cells. Flowers et al. (9) extended this hypothesis, stating that H₂O₂ accumulation was due to a partial inactivation of the catalase, possibly due to the synergistic effects between NaCl, the selective agent of various media in the isolation of *Staphylococcus aureus*, and heat.

Inactivation of catalase under varying conditions has been reported. Feinstein et al. (8) examined the heat stability of blood catalase in several species of mammals. Ashwood-Smith and Warby (2) examined the addition of various cryoprotective agents in the inactivation of catalase during freezing and thawing. Similar studies were performed during dehydration (6). High-molecular-weight agents such as polyvinyl-pyrolidone were found to exert more protection in both of these studies. Darbyshire (6) also reported a variation in catalase inactivation during dehydration using different buffers. Catalase in acetate buffer lost activity, whereas catalase in phosphate buffer exhibited no inactivation.

These studies present an examination of the heat inactivation of catalase from *S. aureus* lysates in phosphate buffer. The effects of NaCl and protein on catalase during heating were also examined. Supporting evidence for the theories of decreased counts of stressed *S. aureus* resulting from the action of NaCl and heat upon catalase are presented.

**MATERIALS AND METHODS**

**Growth of test organism and lysis.** *S. aureus* MF-31 was the test organism. Cultivation conditions were similar to those described by Iandolo and Ordal (12). A 12-h culture was grown in Trypticase soy broth at 35°C, and the cells were harvested by filtration. The cells were washed from the filters and pelleted by centrifugation (6,000 × g for 10 min), and the pellets were stored frozen (−20°C). Frozen cell pellets of *S. aureus* MF-31 were allowed to thaw and then sus-
suspended in TM4 buffer [10 mM tris(hydroxymethyl)-aminomethane, pH 7.6, 0.5 mM MgCl₂]. For lysis, 0.4 ml of a lyostaphin solution (0.5 mg per ml of TM4; Sigma Chemical Co.) per ml of cell suspension was added. The suspensions were incubated at 35°C for 15 min. Deoxyribonuclease (200 µg per ml of TM4; Sigma) was added to a concentration of 0.1 ml/ml of cell suspension. After incubation at room temperature for 1 min, the suspensions were centrifuged at 10,000 × g for 20 min. The supernatant was retained and stored in an ice bath.

Heat inactivation procedure. To study the effects of heating at the same temperature over an extended time period, a 12-h staphylococcal culture was pelleted by centrifugation at 6,000 × g for 10 min. The cell pellet was suspended in 0.1 volume of 100 mM potassium phosphate buffer (PPB; pH 7.2) and added to 0.9 volume of PPB tempered to either 52 or 62°C with constant stirring. At appropriate intervals, 30-ml samples were removed, collected on membrane filters, washed from the filters, and transferred to centrifuge tubes for pelleting by centrifugation. The pellet was stored in an ice bath. When all samples were collected, the pellets were suspended and lysed. The amount of injury was estimated by the dual plating procedure of Landolo and Ordal (12). If NaCl was added after heating, the heated suspension was mixed with an equal volume of 20% NaCl to give a final concentration of 10% NaCl before the cells were collected on membrane filters (5 min).

Studies of the heat inactivation of staphylococcal catalase were performed at varying temperatures for constant time periods and for varying time periods at constant temperature. For studies using varying temperatures, 1.5 ml of cell lysate was diluted into 30 ml of PPB or PPB supplemented with NaCl or bovine serum albumin. All studies were done by using two different conditions per trial. For comparisons of more than two conditions, multiple trials (a minimum of 3) of each variation were run, and results were averaged. The mixtures were heated in 2-ml portions for 10 min and immediately cooled in an ice bath. In experiments involving increasing concentrations of potassium phosphate, each concentration was read with a similar concentration in the blank and the standards. This eliminated possible artifacts due to the formation of chromic phosphate. If NaCl was added after heating, the heated suspension was mixed with an equal volume of 20% NaCl to give a final concentration of 10% NaCl. As above, blanks and standards were prepared with 10% NaCl. The mixture was immediately cooled, and assays performed as quickly as possible. Temperatures examined ranged from 40 to 75°C.

Catalase assay. The colorimetric assay of Sinha (23) for catalase activity was utilized in these studies. This method is based on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of H₂O₂. The catalase reaction was allowed to proceed for different periods of time. The reaction was stopped by adding a portion of the reaction mixture to the dichromate-acetic acid mixture. Thus the remaining H₂O₂ could be determined by measuring the absorbance of the chromic acetate after the mixture had been heated to stabilize the color. The volume of lysate equivalent to one absorbance unit at 260 nm was determined and added to the reaction mixture.

Catalase activity was calculated at each time period, and the velocity constant of catalase (K₀) at zero time was determined by extrapolation. Activity was recorded as K₀ per absorbance unit of lysate at 260 nm. These values were also calculated to give activity in terms of K₀ per milliliter of lysate, or K₀ per milligram of protein. The protein concentration of the lysate was determined by using the Folin-Ciocalteau assay (18).

RESULTS

Previous studies have implicated decreased catalase activity as a result of the synergistic effects of NaCl, and heat as a major factor in the decreased colony-forming ability of heat-stressed S. aureus (9). The following studies present an examination of catalase under conditions of heat inactivation in an attempt to present further evidence in support of this hypothesis. The temperature (52°C) experimentally used for S. aureus injury was examined to determine its effect on the catalase activity over a period of time. Catalase activity at zero time was defined as 100%. Catalase specific activity was determined in units of K₀ per absorbance unit of the lysate at 260 nm (K₀/A₂₆₀). Other bases for catalase specific activity (K₀ per unit volume of lysate or K₀ per milligram of protein) were also determined. Results were similar using all three bases (data not presented). The basis of K₀/A₂₆₀ was used as the standard, since it was most quickly and accurately determined. The average range of error with this assay was determined to be 10%. The catalase activity in S. aureus MF-31 appeared to be stable throughout the 120-min heating period (Fig. 1), showing only a 7.8% decrease in catalase activity overall. These results disagreed with those of Flowers et al. (9) who found a 40% decrease in catalase activity over 20 min of heating at 52°C. Figure 1 also illustrates the results when the S. aureus cells were heated at 52°C for 20 min, then mixed with an NaCl solution to give a final concentration of 10% NaCl. A 50% decrease in catalase activity was seen through 20 min of heating. At this point, the catalase activity began to rise and increased to approximately 90% of the original activity. Possible explanations for this apparent rise will be discussed below.

Heating at 62°C for 120 min (Fig. 2) resulted in an activation in catalase activity during the first 40 min of heating, followed by a sharp decrease. The decrease became more gradual at approximately 90 min. At this point, the catalase activity was approximately 35% of the initial activity. The activity decreased slightly after this point. The heated organisms showed no growth
Fig. 1. Effects of heating at 52°C followed by addition of NaCl on catalase activity in S. aureus MF-31. A stationary-phase culture (ca. 10^8 cells/ml) was heated in 100 mM PPB, pH 7.2, at 52°C. Samples were periodically removed, plated on TSA and TSAS, and collected on membrane filters. Additional samples were removed at the same time and mixed with an equal volume of 20% NaCl in 100 mM PPB, giving a final concentration of 10% NaCl. The cells were collected on membrane filters. Cells were transferred from membrane filters to centrifuge tubes and pelleted by centrifugation. Pellets were stored in an ice bath until all samples were collected. Cells were lysed as described in the text. Catalase activity was determined as K_v/A_260 and calculated to give percent activity. The catalase activity at zero time with no NaCl addition was defined as 100%. Symbols: ●, percent catalase activity of cells heated at 52°C; ○, percent catalase activity of cells heated at 52°C followed by NaCl addition to give 10% NaCl final concentration; ■, log number of colony-forming units per milliliter on TSA; □, log number of colony-forming units per milliliter on TSAS.

Fig. 2. Effects of heating at 62°C on catalase activity in S. aureus MF-31. A stationary-phase culture (ca. 10^8 cells/ml) was heated in 100 mM PPB, pH 7.2, at 62°C. Samples were periodically removed and plated on TSA and TSAS. Additional samples were collected on membrane filters. Cells were transferred to a centrifuge tube and pelleted by centrifugation. Pellets were stored in an ice bath until all samples were collected. Cells were lysed as described in the text. Catalase activity was determined as K_v/A_260 and calculated to give the activity as percent activity. The catalase activity at zero time was defined as 100%. Symbols: ●, percent catalase activity of cells heated at 62°C; ■, log number of colony-forming units per milliliter on TSA; □, log number of colony-forming units per milliliter on TSAS.

This activation is similar to that observed for whole cells (Fig. 2). A sharp drop occurred at 60°C, becoming more gradual at approximately 70°C. The temperature at which 50% of the activity was inactivated (T_50) occurred at approximately 67°C.

A comparison of the heat inactivation in various concentrations of PPB (Fig. 4) showed an increase in activity with an increase in the potassium phosphate concentration. The shapes of the curves were similar, and the T_50's were approximately the same (66 to 68°C).

The effects of NaCl on the heat inactivation of catalase from lysates were examined in the following series of experiments (Fig. 5). In this study, the lysates in 10 mM PPB were heated at the various temperatures and then mixed with NaCl solutions to give final concentrations of 10.0% (the concentration used in liquid selective medium). The catalase activity appeared to be

on the non-inhibitory medium, Trypticase soy agar (TSA), after 10 min of heating, indicating that no viable cells were present on this plating medium.

A representative heat inactivation curve of catalase from S. aureus MF-31 lysates is presented in Fig. 3. Catalase activity in 10 mM PPB, pH 7.0, was designated as the standard reference. The highest catalase activity in 10 mM PPB was arbitrarily defined as 100%. The heat inactivation curve demonstrated that the catalase activity remained unchanged from 50 to 60°C after a slight activation from 40 to 50°C.
followed by maintenance in mM PPB, pH described in reus was an unit as activity to inactivation of catalase from S. aureus MF-31 in 10 mM PPB. Cells were lysed as described in the text. The lysate was diluted in 10 mM PPB, pH 7.0. Portions were heated for 10 min, followed by maintenance in an ice bath. Catalase activity was defined as units of activity per absorbance unit of the lysate at 260 nm (Kₒ/A₂₆₀). The highest catalase activity in 10 mM PPB (50°C) was defined as 100%.

FIG. 4. Heat inactivation of catalase from S. aureus MF-31 in varying concentrations of PPB and in distilled water. Multiple trials (a minimum of three) were run for each variation, and the results were averaged. Cells were lysed as described in the text. Portions of each lysate suspension were heated for 10 min, followed by maintenance in an ice bath. Catalase activity was determined as Kₒ/A₂₆₀ and calculated to give percent activity. The highest catalase activity in 10 mM PPB (50°C) was defined as 100%. Symbols: □, 250 mM PPB; ■, 100 mM PPB; ○, 10 mM PPB; ▼, distilled water.

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FIG. 5. Heat inactivation of catalase from S. aureus MF-31 lysates in 10 mM PPB followed by addition of NaCl. Cells were lysed as described in the text. The lysate in 10 mM PPB, pH 7.0, was divided into two fractions. Portions of each lysate suspension were heated for 10 min at the various temperatures. One set of suspensions was subsequently mixed with an equal volume of 20% NaCl in 10 mM PPB to give a final concentration of 10% NaCl. Sample size was adjusted for dilution. Suspensions were maintained in an ice bath. Catalase activity was determined as Kₒ/A₂₆₀ and calculated to give the catalase activity as percent activity. The highest catalase activity in 10 mM PPB (50°C) was defined as 100%. Symbols: ●, 10 mM PPB; ○, 10 mM PPB with 20% NaCl addition after heating to give 10% NaCl final concentration; □, 10 mM PPB with 10% NaCl present during heating.

inactivated by the salt, with the T₃₀ remaining at approximately 66°C. Even at the lower temperatures, the catalase activity of the lysates in NaCl was only 75% of the activity in the phosphate buffer solution alone. When heated in the presence of NaCl, similar results were observed (Fig. 5). Heat treatment of the lysate in 100 mM PPB followed by NaCl addition exhibited an inactivation of the catalase activity, but not to the same extent as when heated in 10 mM PPB (data not presented).

DISCUSSION

When S. aureus cells were subjected to heating at 52°C for 120 min, a minimal decrease in catalase activity from the resultant lysates was found. Cells enumerated on TSA continued to die, however, as evidenced by the decreasing counts. When the cell suspension was mixed with NaCl after heating at 52°C, to give a final concentration of 10% NaCl, a sharp decrease in activity was seen over the first 20 min. The activity increased again with increased heating. The time at which the catalase levels rose in the
lysates containing NaCl corresponded to the time at which death ceased, as found on the TSA + 10% NaCl (TSAS) plates. It has been suggested that a membrane-bound, inactive catalase may exist in *S. aureus* (13, 16). It is possible that this catalase was released as a result of lysis, caused by the prolonged heating and death of these cells. This released catalase may have protected those cells on the TSAS plates, permitting the low level of growth.

Heating the staphylococcal cells at 62°C showed a decrease in catalase activity after 40 min. Although the cells died quickly at this temperature, the catalase activity was not inactivated at the same rate. However, since both injury and death increased with heating, this catalase activity must not be useful to the cell, or must not be able to compensate for other lesions affecting the organism.

Studies of staphylococcal catalase from lysates under conditions of heat inactivation revealed that various factors influenced the activity. When heated in PPB, the catalase activity was elevated as a function of increased potassium phosphate concentration. Activation by one or both of the ions could have caused this increase in activity. Enzyme activation and inactivation have been demonstrated by several ions, both monovalent and divalent (22). In addition, both anions and cations have been attributed with this activation ability. Sodium chloride, a selective agent for enumeration of *S. aureus*, decreased catalase activity of lysates during heating at various temperatures. No data exist concerning the internal concentration of NaCl in heated cells recovering in its presence. Although it is possible that salt is concentrated in these cells, the use of 10% NaCl in lysates probably represents the maximum concentration possible, and was chosen for this reason. The possible inactivation of catalase by NaCl has been suggested previously (9) and is supported by these results. Litchfield (17) has observed an inactivation of the catalase activity by the chloride ion. Acetate, a monovalent anion, also has been implicated in the inactivation of catalase during dehydration (6). Bovine serum albumin was utilized to examine the effects of protein on heat stability of catalase (data not presented). A protective effect was demonstrated when the suspensions were heated at temperatures between 50 and 65°C for 10 min.

In all the heat inactivation studies, a T_{50} of 65 to 68°C was observed. Variations in potassium phosphate concentration or the addition of NaCl or bovine serum albumin resulted in no deviations from this temperature. This value is similar to the T_{50} of 67.1°C determined for residual catalase activity in human blood (8). The similarity between these two temperatures is coincidental, since *S. aureus* may be a human pathogen.

These findings demonstrate that several factors affect catalase activity during heating. Protein and potassium phosphate appear to activate or protect catalase, whereas NaCl inactivates it. These observations are consistent with the hypothesis that catalase is inactivated by the synergistic effects of NaCl and heat (9). They also support previous findings that suggested that decreased colony-forming ability of stressed *S. aureus* is a result of an accumulation of H_{2}O_{2} due to the decreased activity of catalase (9, 19). Additional studies are in progress to further implicate H_{2}O_{2} after sublethal heat injury.

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**LITERATURE CITED**

HEAT INACTIVATION OF CATALASE