Applied Microbiology, Feb. 1968, p. 267–270
Vol. 16, No. 2
Printed in U.S.A.
Copyright © 1968 American Society for Microbiology

Influence of Catalase Activity on Resistance of Coagulase-positive Staphylococci to Hydrogen Peroxide[1]

V. M. AMIN and N. F. OLSON
Department of Food Science and Industries, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 29 September 1967

Catalase activities of intact cells and cell-free extracts of coagulase-positive staphylococcal cultures 105B and 558D isolated from milk, culture 25042 from a clinical source, and *Staphylococcus aureus* 196E were determined at 32.2 C. Cultures were treated with 0.025 and 0.05% hydrogen peroxide at 37.8 and 54.4 C and without hydrogen peroxide at 54.4 C to determine the relationship between catalase activity and resistance to these treatments. The relationship held true for cultures 105B and 196E; culture 105B had the lowest catalase activity and lowest resistance to H_2O_2 at 37.8 C, whereas *S. aureus* 196E possessed a high catalase activity and was most resistant at 37.8 C. Catalase activities of cell-free extracts of cultures 25042, 558, and 196E were similar, but resistance to H_2O_2 at 37.8 C was greater for culture 196E. The lower resistance of culture 25042 was related to low catalase activities of whole cells of this culture, which were only one-third that of whole cells of culture 196E. Culture 558 was least resistant to heat treatment at 54.4 C and showed the greatest sensitivity to added H_2O_2 at this temperature.

The overall resistance of a bacterial species to a chemical agent like hydrogen peroxide depends upon the inherent characteristics of the test organism and treatment parameters such as concentration and temperature. Increases in concentration of H_2O_2 have been shown to decrease the survival time of *Staphylococcus aureus* in sterilized milk at 30 C (13). Other studies have indicated that concentration of H_2O_2 is less important than time of treatment at 130 F (54.4 C) in effecting bactericidal action against *S. aureus* (12). These varied effects of concentration of H_2O_2 might have been caused by differences in the strains of *S. aureus* used in the studies. Von Ruden and co-workers have shown that cultures of coagulase-positive staphylococci from mastitic bovine milk and clinical sources varied in resistance to H_2O_2 (15). Interpretation of effects of H_2O_2 concentration in their study is complicated, since individual cultures did not have the same relative resistance to H_2O_2 at different treatment temperatures. Resistance to H_2O_2 at 54.4 C was related to heat resistance at this same temperature (15).

Resistance of staphylococci to H_2O_2, especially at lower treatment temperatures, might be related to catalase activity, since variability in catalase activity between strains has been demonstrated (8, 9). It has also been shown that staphylococci with higher catalase activities were able to grow in the presence of higher concentrations of H_2O_2 at 37 C than were cultures with lower activities (9, 10). To delineate the importance of catalase as a protective mechanism, the resistance of staphylococcal cultures to different levels of H_2O_2 (0.025 and 0.05%) at treatment temperatures of 37.8 and 54.4 C and catalase activities of the cultures were determined.

[1] Published with approval of Director of Wisconsin Agricultural Experiment Station.

Materials and Methods

Culture selection. Cultures of coagulase-positive staphylococci were selected for their unique pattern of resistance to H_2O_2 as shown in Table 1. *S. aureus* 196E was most resistant at both treatment temperatures. *Staphylococcus* culture 105B was most sensitive to H_2O_2 at 37.8 C, whereas culture 558D was most sensitive to H_2O_2 at 54.4 C. There was no significant difference between the resistance of cultures 25042 and 105B to H_2O_2 at 54.4 C. Hydrogen peroxide resistance of cultures at 54.4 C was directly related to heat resistance at this treatment temperature.

Preparation of cultures for catalase determination. Cultures were grown in a 2.0% peptonized milk broth with constant agitation for 16 hr at 37 C, cooled to 4 C, and the cells were recovered by centrifugation at 3,000 × *g*. The cells were washed twice in sterile

distilled water and once in sterile 0.049 M potassium phosphate buffer (*p*H 7.0).

Catalase activity of the whole staphylococcal cells. The washed cells were dispersed in phosphate buffer (*p*H 7.0) by shaking with glass beads and diluting to give an absorbance of 0.62 at 600 mμ. A 1-ml amount of the suspension of different cultures with an absorbance of 0.62 contained approximately 1.1×10^8 to 1.5×10^9 cells/ml by standard plate count (1). Based on preliminary experiments, 5×10^6 to 1.5×10^8 cells/ml were used to determine catalase activity of cells in the presence of 12.0 to 15.0 μmoles of H_2O_2/ml of phosphate buffer at 32.2 C. The cells were inactivated with trichloroacetic acid after specified intervals of contact with H_2O_2. Residual H_2O_2 was determined spectrophotometrically (17). Catalase activity constants (K) were calculated with the equation:

$$K = \frac{2.303}{tc} \log_{10} \frac{a}{a - x}$$

where t is the reaction time in minutes, c is the number of cells per milliliter, a is the concentration of H_2O_2 at the beginning of the reaction, and $a - x$ is the concentration of H_2O_2 at the time t.

Catalase activity of cell-free extracts. Washed cells were suspended in 11 to 12 ml of phosphate buffer (*pH* 7.0) and disintegrated by sonic oscillation using a 20-kc sonifier (model S-75) operated at 115 w for 30 min at −2 to 0 C. The buffer solution containing the disintegrated cells was diluted to 40 ml with additional phosphate buffer (pH 7.0) and centrifuged at $3{,}000 \times g$ for 30 min at 4 C. The supernatant was recentrifuged at $8{,}720 \times g$ for 30 min at 4 C. The protein content of the cell-free extract was determined colorimetrically by the quantitative biuret procedure of Gornell et al. (7).

TABLE 1. *Mean time intervals required for 99.9% destruction of coagulase-positive staphylococci subjected to heat and hydrogen peroxide treatments in reconstituted (11%) nonfat dry milk*[a]

Test culture and source of culture	Time required (min)		
	Treatment temp used with 0.05% H_2O_2		Treatment temp without H_2O_2
	37.8 C	54.4 C	54.4 C
Milk			
558D	75.3	0.9	7.4
105B	51.7	4.9	33.3
Clinical			
25042	115.0	3.6	13.7
196E (ATCC 13565)	140.3	6.9	46.8

[a] Adapted from original table (K. L. Von Ruden, V. M. Amin, and N. F. Olson. 1967. J. Dairy Sci. **50**:488).

TABLE 2. *Catalase activity of cell-free extracts of staphylococcal cultures*[a]

Reaction time	Specific activity[b]			
	S. aureus 196E	*Staphylococcus* 25042	*Staphylococcus* 558D	*Staphylococcus* 105B
min				
5	64.00	70.10	79.50	19.01
10	51.10	56.28	67.35	15.53
15	43.78	47.77	57.32	12.65
20	37.62	42.17	49.03	10.80
25	31.88	36.73	43.12	9.30
30	28.85	30.60	37.20	8.61

[a] Means of three replicate trials.

[b] Specific activity is defined as micromoles of H_2O_2 decomposed per minute per milligram of protein nitrogen.

Cell-free extracts of all cultures were diluted with phosphate buffer (*p*H 7.0) to give 0.004 mg of N/ml in a stock solution. A 5-ml amount of the stock solution was added to 3 ml of phosphate buffer (pH 7.0). The mixture was brought to 32.2 C and 1 ml of an aqueous solution containing 73.53, 147.06, or 294.12 μmoles of H_2O_2 was added. Decomposition of H_2O_2 was stopped after 5, 10, 20, 25, and 30-min intervals by adding 1 ml of 6 N HCl. Residual H_2O_2 was determined spectrophotometrically (17). The specific activity (K) of cell-free extracts was calculated with the equation used for intact cell activities, except that c is defined as the concentration of N (protein) of cell-free extract preparation in grams of N per liter of the reaction mixture.

H_2O_2 treatment. Stock and milk cultures of staphylococci were prepared according to the method of Amin and Olson before treatment with 0.025 (7.35 μmoles/ml) and 0.05% (14.71 μmoles/ml) H_2O_2 at 37.8 and 54.4 C (2). The cultures were heat-treated at 54.4 C without added H_2O_2. Numbers of survivors were determined by plate-count methods (1).

RESULTS

Catalase activity of cell-free extracts. Catalase activities of cell-free extracts of staphylococcal cultures are shown in Table 2. The order of catalase activity (K values) was 558D > 25042 > 196 E > 105B. The K values for culture 105B were one-third to one-fourth those of the other cultures. As the reaction time between catalase and H_2O_2 progressed, the K values for all cultures decreased at approximately the same rate, so that values at 30 min were 52 to 57% less than values obtained at 5 min. This decrease in activity probably was caused by inactivation of catalase by H_2O_2 (3, 6, 10, 11, 14, 16).

Catalase activity of whole cells. K values of washed whole cells of the cultures are shown in Table 3. *S. aureus* 196E and culture 558D had

TABLE 3. *Catalase activity of whole cells of staphylococcal cultures*[a]

Reaction time	Specific activity[b] (1×10^{-10})			
	S. aureus 196E	*Staphylococcus* 25042	*Staphylococcus* 558D	*Staphylococcus* 105B
min				
5	74.44	22.29	73.37	5.27
10	62.67	21.43	67.72	4.50
20	54.89	17.00	54.86	3.44
30	41.66	14.17	44.07	2.65

[a] Means of three replicate trials.

[b] Specific activity is defined as micromoles of H_2O_2 decomposed per minute per cell.

TABLE 4. *Time required for 99.9% destruction of staphylococcal cultures treated with 0.025 and 0.05% H_2O_2 at 37.8 and 54.4 C*[a]

Treatment temp	H_2O_2 concn	Time required (min)			
		S. aureus 196E	*Staphylococcus* 25042	*Staphylococcus* 558D	*Staphylococcus* 105B
C	%				
37.8	0.025	379.3	209.0	208.0	94.8
	0.05	177.1	101.1	97.5	71.7
54.4	0.00	73.3	16.8	9.8	39.9
	0.025	14.6	3.6	0.92	4.7
	0.05	11.6	3.4	0.85	3.5

[a] Means of three replicate trials.

the highest catalase activities per cell, whereas culture 105B had the lowest activity. Variations in catalase activities of intact staphylococcal cells have been observed among strains isolated from blood, abscesses, humans with septicemic and pyemic conditions, and boils and nasopharynx of healthy humans (8, 10).

K values of the whole cells decreased as the reaction between cells and H_2O_2 progressed in the same manner observed with the cell-free extracts. Similar behavior by intact cells of staphylococcal strains was observed by Molland (10).

H_2O_2 treatment. The time intervals required for 99.9% destruction of cultures by 0.025 and 0.05% H_2O_2 at 37.8 and 54.4 C and without H_2O_2 at 54.4 C are shown in Table 4. *S. aureus* 196E was most resistant to heat and both concentrations of H_2O_2 at the two treatment temperatures. Cultures 25042 and 558D had the same resistance to both levels of H_2O_2 at 37.8 C. Culture 558D was least resistant to H_2O_2 at 54.4 C.

The time required for 99.9% destruction of all cultures except 105B doubled with a decrease in concentration of H_2O_2 from 0.05 to 0.025% at 37.8 C; the time for culture 105B was increased by a factor of only 1.3. Time intervals for destruction at 54.4 C for cultures 105B, 196E, 558D, and 25042 were increased by a factor of 1.33, 1.26, 1.08, and 1.04, respectively, when H_2O_2 concentrations were reduced from 0.05 to 0.025%. The time required for 99.9% destruction of cultures 25042, 196E, 105B, and 558D increased by a factor of 4.7, 5.02, 8.56, and 10.7, respectively, when these cultures were treated at 54.4 C without H_2O_2 rather than 0.025% H_2O_2 at this temperature.

DISCUSSION

S. aureus 196E and culture 105B showed a positive relationship between catalase activities of whole cells and cell-free extracts and their resistance to H_2O_2 at 37.8 C. The positive relationship suggests that catalase played a predominant role in protecting the cell against H_2O_2 at this low treatment temperature. The protective effect of catalase is suggested also by the relative increase in resistance of the cultures when H_2O_2 concentrations were increased from 0.025 to 0.05%. Culture 105B, with low catalase activity, did not show as much increase in resistance as other cultures with higher catalase activities when H_2O_2 concentrations were reduced. Presumably, the catalase activities of cultures 196E, 558D, and 25042 were sufficiently high to give increased protection against the lower level of H_2O_2, whereas the catalase activity of culture 105B was too low to afford protection against even this level of H_2O_2.

The data obtained with cultures 558D and 25042 suggest that factors other than catalase activity are also important in determining the level of resistance to H_2O_2. Permeability of the cell to H_2O_2 or availability of catalase in the intact cell may have influenced the resistance of culture 25042. Catalase activities of the cell-free extracts of cultures 558D and 25042 were similar, whereas the activity of whole cells of 25042 was one-third that of culture 558D. The inability of catalase within the intact cells of culture 25042 to decompose H_2O_2 could minimize protection by the enzyme and be responsible for the lower level of resistance of the culture. The effect of cell permeability to H_2O_2 and lower catalase activity of intact cells, as compared to cell lysates, has been observed with other bacterial species. Whole cells and protoplasts of *Micrococcus lysodeikticus* exhibited about one-fourth of the catalase activity of the toluene-, chloroform-, and butanol-treated cells and cell lysates (5). Similarly, whole cells of *Rhodopseudomonas*

spheroides showed about one-third of the catalase activity of toluene-treated cells and cells disrupted sonically (4). Lipid solvents used in these studies may have altered the lipoproteins of the cytoplasmic membrane or cell wall and altered the permeability of the intact cell to H_2O_2.

Catalase activities of the cell-free extract and intact cells of cultures 196E and 558D were similar. It must be assumed that cells of culture 558D possessed characteristics which increased its sensitivity to H_2O_2 above that of culture 196E and that the high catalase activity of culture 558D was insufficient to overcome this effect. Culture 558D was the most sensitive culture to heat treatment at 54.4 C, and it also showed the greatest increase in sensitivity when treated with 0.025% H_2O_2 at 54.4 C, as compared to heat treatment at this temperature. It is possible that cell characteristics responsible for the increased sensitivity to added H_2O_2 at 54.4 C are operative also at 37.8 C.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant EF-00204 from the Bureau of State Services, Division of Environmental Engineering and Food Protection.

LITERATURE CITED

1. AMERICAN PUBLIC HEALTH ASSOCIATION. 1960. Standard methods for the examination of dairy products, 11th ed. American Public Health Association, Inc., New York.
2. AMIN, V. M., AND N. F. OLSON. 1967. Factors affecting the resistance of *Staphylococcus aureus* to hydrogen peroxide treatments in milk. Appl. Microbiol. **15**:97-101.
3. BONNICHSEN, R. K., B. CHANCE, AND H. THEORELL. 1947. Catalase activity. Acta Chem. Scand. **1**:685-709
4. CLAYTON, R. K. 1959. Permeability barriers and assay of catalase in intact cells. Biochim. Biophys. Acta **36**:35-39.
5. FEW, A. V., M. J. FRASER, AND A. R. GIBLY. 1957. The intracellular catalase of *Micrococcus lysodeikticus*. Biochim. Biophys. Acta **24**:306-314.
6. GEORGE, P. 1949. Effect of the peroxide concentration and other factors on the decomposition of hydrogen peroxide by catalase. Biochem. J. **44**:197-205.
7. GORNALL, A. G., C. J. BARDAWILL, AND M. D. MAXIMA. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751-766.
8. KEDZIA, W., M. MUSIELAK, B. KEDZIA, H. KONIAR, AND E. PNISUSKA. 1966. Enzymatic activity of coagulase positive *Staphylococcus aureus* strains isolated from patients and healthy carriers. Pathol. Microbiol. **29**:307-323.
9. MCLEOD, J. W., AND J. GORDON. 1923. Catalase production and sensitiveness to hydrogen peroxide amongst bacteria: With a scheme of classification based on these properties. J. Pathol. Bacteriol. **26**:326-331.
10. MOLLAND, J. 1947. Bacterial catalase. Acta Pathol. Microbiol. Scand. Suppl. **66**:1-165.
11. MORGULIS, S., M. BEBER, AND I. RABKIN. 1926. Studies on the effect of temperature on the catalase reaction. II. Loss of catalase activity. J. Biol. Chem. **68**:535-545.
12. OLSON, N. F., AND W. V. PRICE. 1960. Bactericidal effectiveness of hydrogen peroxide-catalase treatment on *Staphylococcus aureus*. J. Dairy Sci. **43**:852.
13. ROUSHDY, A. 1959. Studies on the efficiency of hydrogen peroxide as a preservative for raw milk. Neth. Milk Dairy J. **13**:151-152.
14. SIZER, I. W. 1944. Temperature activation and inactivation of the crystalline catalase-hydrogen peroxide system. J. Biol. Chem. **154**:461-473.
15. VON RUDEN, K. L., V. M. AMIN, AND N. F. OLSON. 1967. Variability in resistance of coagulase positive staphylococci to hydrogen peroxide. J. Dairy Sci. **50**:488-491.
16. WILLIAMS, J. 1928. The decomposition of hydrogen peroxide by liver catalase. J. Gen. Physiol. **11**:309-337.
17. WOLFE, W. C. 1962. Spectrophotometric determination of hydroperoxide in diethyl ether. Anal. Chem. **34**:1328-1330.