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Relationship of Water Activity to Prevention of Heat Injury in *Staphylococcus aureus*

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Cells of Staphylococcus aureus were exposed to sublethal heat stress in buffered solutions of selected salts, amino acids, sugars, or polyols at concentrations corresponding to a_w values ranging from 0.80 to 0.99. All solutes tested gave some protection against heat injury but with some striking differences among the individual compounds. No single class of compounds exhibited entirely consistent behavior. With six of the solutes, protection against heat injury decreased as the a_w value increased; six other compounds gave virtually complete protection at all a_w values tested. Maltose protected less well at all a_w values. The protective effect of xylose was anomalous in that protection increased as the a_w increased to 0.975. Ability to protect was clearly not determined by a_w per se, but by as yet undetermined interactions of solute and bacterial cell.

Introduction

Staphylococci can grow and synthesize enterotoxins at lower water activities (a_w) than other common food poisoning bacteria (28). The microorganism's lower a_w range (0.85 to 0.92) corresponds to the a_w of certain dried foods and intermediate moisture foods, and is equivalent to sodium chloride concentrations of 12.9% to 21.6% (2.2 to 3.7 M) or sucrose concentrations of 68.1% to 89.3% (2.0 to 2.6 M). Recently, HUGHES and HURST (16) and HURST *et al.* (18) have shown that addition of high levels of solutes (salts or sugars) to the growth medium of *Staphylococcus aureus* permitted both growth and enterotoxin production at temperatures at least 2°C higher than that of unsupplemented media. The addition of high concentrations of sugars or salts to the heating menstruum increased thermal resistance to killing (3, 8, 15) and prevented heat injury in *S. aureus* (26). Such effects may be of importance in food preservation when lowered processing temperatures are used in conjunction with elevated solute levels.

The mechanisms of solute protection against the adverse effects of increased temperatures has not been determined. Using a limited number of solutes, previous investigations have indicated that protection is not related to the a_w alone (2, 8, 13, 25, 26). In the present study, 15 compounds including salts, sugars, polyols, and amino acids were tested at a_w 's ranging from 0.80 to 0.99 to determine the relation of a_w to the protective effect against thermal injury to *S. aureus*.

Materials and Methods

Preparation of cells

S. aureus 196E was inoculated into 100 ml Tryptic Soy Broth (Difco*) and incubated on a reciprocating shaker (200 rpm)

at 35°C for 16 h. The contents of the culture flasks were centrifuged at 16,000 × g for 5 min at 5°C, washed three times with sterile potassium phosphate buffer (0.1 M, pH 7.2), and resuspended in a small volume of sterile buffer.

Heat injury

All flasks, experimental and control, contained 100 ml of sterile phosphate buffer (0.1 M, pH 7.2) and washed cells of *S. aureus* at a final concentration of approximately 10⁹ cells/ml. The experimental flasks contained the desired level of solute which was dissolved before addition of the washed cells. All flasks were equilibrated at 50°C in a constant temperature water bath and were continuously agitated during equilibration and the experimental run. Temperature was monitored with a thermocouple inserted below the surface of the heating menstruum. Temperature equilibrium occurred approximately 3 min after addition of the cells. One ml aliquots of the suspension were transferred to 99 ml sterile peptone (0.1%, Difco) water blanks after 0 and 45 min of heating.

Assay for injured cells

Appropriate dilutions of each sample were plated on Tryptic Soy Agar (TSA; Difco) plus 1% sodium pyruvate (TSAP) and on TSA plus 7% NaCl (TSAS). TSAP permits growth of both injured and noninjured cells, while TSAS allows growth of the noninjured cells only. Plates were counted after 2 days incubation at 35°C.

Calculation of a_w

All solutes were tested at a_w values of 0.900, 0.925, 0.950, and 0.975 except NaCl which was tested at a_w values ranging from 0.80 to 0.99. The salts (NaCl, KCl, or NH₄Cl) were dissolved in 100 ml sterile 0.1 phosphate buffer. The concentration of salts necessary for the appropriate a_w value was calculated according to the formula for nonideal solutes (28). The other solutes were dissolved in 50 ml of sterile double-strength buffer and diluted to 100 ml with sterile distilled water. The concentrations of sugars (glucose, sucrose, mal-

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tose, or xylose); polyols (glycerol or sorbitol); and amino acids (glycine, L-proline, β -alanine, L-lysine HCl, or L-ornithine HCl) required to give the desired a_w , were calculated by the method (5) of CHIRIFE *et al.* (9, 10).

Determination of the relative protective effect

The amount of protection against heat injury to *S. aureus* by a particular solute at the specific a_w was calculated by the method of SMITH *et al.* (26), and is reported as the relative protective effect (RPE). The RPE values range from 0.0 for no protection to 1.0 for complete protection. All data were analyzed statistically by regression analysis (27).

Results

The variation in RPE with a_w values between 0.900 to 0.975 for salts, amino acids, sugars, and polyols is presented in **Tab. 1** and **Fig. 1**. These graphs represent the best fit computer-drawn curves obtained by regression analysis of duplicate RPE values with a_w as the independent variable and RPE as the dependent variable.

All three salts gave good protection (RPE > 0.9) at a_w values over the range tested while KCl gave a marked decrease in protection at 0.975 (**Tab. 1, Fig. 1B**).

Sodium chloride was tested for its ability to protect *S. aureus* against heat injury at a_w values ranging from 0.80 to 0.99. The data presented in **Fig. 2** indicate that NaCl conferred virtually complete protection (RPE > 0.95) at a_w values of 0.80 to 0.96. At a_w values ≥ 0.98 , the degree of protection decreased markedly. Once an a_w value ≤ 0.97 (corresponding to 5.2% w/v or a molarity of 0.89) has been attained with NaCl, little additional protection occurred with further decreases in a_w .

Cells of *S. aureus* were protected against heat injury by amino acids. The two di-amino acids (lysine and ornithine) and β -alanine were the most effective amino acids, having high RPE values at all a_w values examined. Glycine was less effective as a protective agent, and L-proline showed a statistically significant linear decrease in protection as the a_w approached 0.975 (**Tab. 1, Fig. 1A**).

Tab. 1 Effect of solute a_w on RPE in *S. aureus* 196E

Solute	RPE ^a at a_w of			
	0.900	0.925	0.950	0.975
KCl	1.00 ^b	0.99	1.00	0.66
NaCl	0.99	0.99	0.93	0.95
NH ₄ Cl	0.90	0.98	0.94	0.94
β -alanine	1.00	1.00	1.00	0.97
L-lysine HCl	0.99	1.00	0.93	1.00
L-ornithine HCl	0.99	1.00	0.97	0.99
L-proline	0.99	0.99	0.92	0.89
Glycine	0.94	0.90	0.97	0.93
Sorbitol	1.00	0.97	1.00	0.87
Sucrose	0.98	0.99	0.91	0.79
Glucose	0.97	0.92	0.62	0.33
Glycerol	0.93	0.91	0.89	0.73
Maltose	0.44	0.48	0.48	0.41
Xylose	0.00	0.00	0.40	0.94

^a RPE equals the number of injured cells in heated buffer (control) minus number of cells in heated buffer containing solute divided by the control.

^b Mean value of duplicate RPE determinations.

The uncharged sugars and polyols showed the greatest variation in protective effect (**Tab. 1, Fig. 1C**). Maltose was consistently less protective than the other solutes at all a_w values. Xylose was anomalous in that it protected well at a_w of 0.975, slightly at 0.950, and not at all at lower a_w values. Sorbitol was an effective protective agent even at a_w 0.975. Sucrose, glucose, and glycerol showed a quadratic increase in RPE values as the a_w decreased to 0.900.

Discussion

All of the compounds tested (amino acids, salts, sugars, and polyols) provided some degree of protection to *S. aureus* undergoing sublethal heat stress, but no consistent pattern of protection relative to a_w was evident. At any given a_w value (**Tab. 1**), the relative protective values (RPE) varied mark-

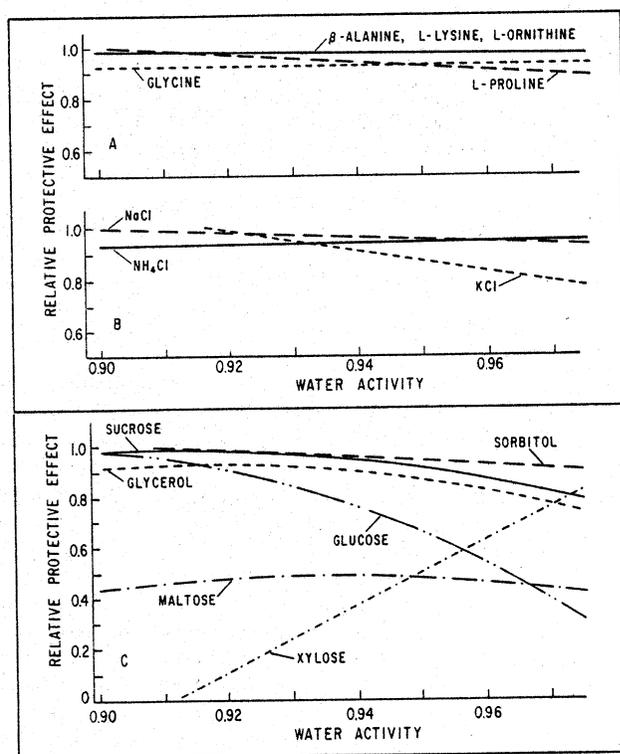


Fig. 1 The effect of amino acids (A), salts (B), and sugars and polyols (C) at various a_w values on protection (RPE) of *S. aureus* 196E against heat injury

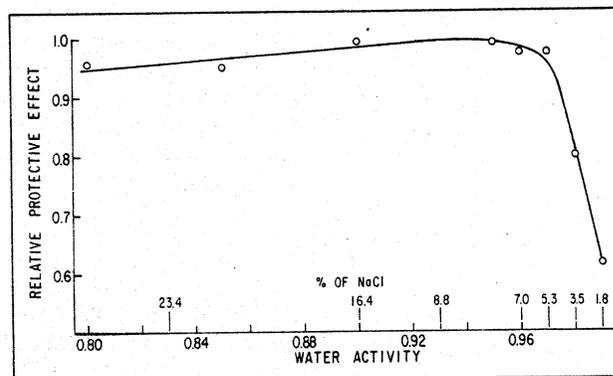


Fig. 2 The effect of sodium chloride at various a_w values on protection (RPE) of *S. aureus* 196E against heat injury

edly. Sodium and ammonium chlorides protected well at a_w of 0.975, but potassium chloride protection was markedly decreased at that a_w . Similarly, sorbitol, glycerol, and sucrose gave decreased protection as the a_w approached 0.975. These data suggest that the protective effect results more from the chemical and/or physical nature of the compounds used than from the a_w .

Maltose furnished considerably less protection than the other compounds (except xylose). Maltose might be expected to behave similarly to sucrose, but in these studies, regardless of the a_w value, maltose gave a consistently lower RPE over the a_w range studied.

Xylose behaved differently from the other compounds in that protection against heat injury increased with increasing a_w . No protection was observed at a_w values of 0.900 and 0.925. Lethality (approximately 1-log reduction in TSAP counts) was evident at the two lower a_w values. At a_w of 0.950, there was partial protection with no lethality, and at a_w of 0.975, protection was excellent (RPE > 0.9). The activity of xylose apparently depends on two opposing factors: protection versus cytotoxicity at high concentrations. The wide range of RPE's obtained in this and other studies (26) suggests that there may be more than one biological effect involved in protection by solutes. Various possible modes of action for a soluble component have been suggested. Removal of water from the cell's interior milieu, for example, might be expected to lead to cellular dehydration and shrinkage. GIBSON (12) and CORRY (11) have reported that addition of solutes to suspensions of salmonellae or yeast produced cellular shrinkage along with increased heat resistance.

Selected solutes may aid in stabilization of the microbial membrane. One of the earliest manifestations of heat injury in *S. aureus* is damage to the cytoplasmic membrane with ensuing leakage of intracellular materials (7, 17). When *S. aureus* cells were suspended in distilled water and subjected to sublethal heat, materials that absorbed at 260 nm were observed in the medium; such materials were not present when cells were heated under the same regimen in 5% sodium chloride solutions (26). LEE and GOEPFERT (22) noted a similar effect with sucrose and *Salmonella typhimurium*, and for that system suggested a stabilization of the bacterial membrane by sucrose similar to its osmotic effect on the bacterial protoplast membrane (23).

One effect of sublethal heat stress on *S. aureus* is the accumulation of metabolic hydrogen peroxide (6) resulting from the thermal inactivation of the microbial catalase and superoxide dismutase (1, 4). The latter enzyme converts the potentially toxic superoxide anion to peroxide and molecular oxygen, but in the presence of peroxide, the anion can form the more potent hydroxyl radical (5). Although addition of catalase or pyruvate (a peroxide decomposer) to TSA or TSAS led to increased counts of heat-stressed staphylococci (24), the addition of scavengers for superoxide anion, hydroxyl radical, singlet oxygen, or oxidizing free radicals showed no increase in count with the stressed cells. The protective relationship of the various sugars, polyols, salts, and amino acids to the deleterious effects of peroxides is unclear since it has been shown (1, 4) that solutes often potentiate destruction of peroxide decomposing enzymes.

HURST and coworkers (19, 20) showed that cells of *S. aureus*, during sublethal heating, lose magnesium and the ester-bound component of teichoic acid into the heating medium. One role of the cell wall teichoic acids appears to be that of a reservoir of magnesium ions (14, 21). HOOVER and GRAY (14) demonstrated that a teichoic acid-less mutant of *S. aureus* could not withstand the high temperatures tolerated by the parent strain (i.e., the mutant showed more injury) and did not grow in a magnesium-limited environ-

ment. SMITH *et al.* (26) showed that cells heated in 5% NaCl lost only $1/3$ of the magnesium of cells heated in the absence of salt. Selected solutes may thus protect the cell from thermal injury by stabilizing the teichoic acid moiety with respect to its magnesium binding function.

While one or more of these mechanisms may plausibly explain the general protective effect of solutes, none explain the concentration-dependence observed with KCl or glucose or the poor protection given by maltose.

The protection against heat injury in *S. aureus* provided by a solute is a phenomenon that should not be ignored. Food processors who manufacture intermediate moisture foods or foods containing high levels of solutes should be aware that under these conditions, thermal resistance of *S. aureus* may be significantly increased and that higher processing temperatures may be needed in order to be certain that contaminating *S. aureus* are destroyed.

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