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Influence of Water Activity on the Production of Extracellular Enzymes by *Staphylococcus aureus*

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Two enterotoxigenic strains of *Staphylococcus aureus* were examined for their ability to produce a number of extracellular enzymes at various water activity (a_w) levels. Supernatant, dialyzed culture media were analyzed for total and relative levels of enzyme activity. With the exception of protease, enzyme activity was greatest in spent media obtained from cultures grown at 0.996 a_w, the highest level tested. Enzyme activity in spent media from an enterotoxin B-producing strain was generally more sensitive to a_w reduction than activity from an enterotoxin A-producing strain. Unlike the other enzymes assayed, acid and alkaline protease activities were greatest when the organism was grown at 0.94 a_w.

Staphylococcus aureus is an osmotolerant organism that may flourish in salt brines or in other low-water activity (a_w) conditions. Scott (18) first established the minimal a_w for growth of *S. aureus* when he determined that this organism will grow in media adjusted to a_w levels as low as 0.86. More recently, it has been reported (20) that staphylococcal growth will occur in enriched pork slurries at 0.83 a_w. Additional work, however, is required to substantiate these latter findings.

Because many staphylococci can produce relatively heat-stable enterotoxins in foods, a number of workers have attempted to determine whether reduced a_w levels influence enterotoxigenesis. These studies (7, 13, 21, 22) have shown that enterotoxin production is limited by low a_w in media and in foods and that both total and specific or differential levels of enterotoxin are suppressed. Growth suppression in response to reduced a_w is related to the toxin-producing strain involved, the type of humectant used to control a_w, and the interaction of a_w with other factors such as chemical inhibitors, temperature, and pH.

The staphylococci produce numerous extracellular products (1), which may have pathological significance and which, in some cases, may be used to identify this organism. The influence of lowered a_w levels on the production of many of these metabolites has not been investigated despite their importance. The objective of this study was to determine the influence of a_w on the production of several extracellular enzymes by enterotoxigenic staphylococci.

MATERIALS AND METHODS

Cultures and growth conditions. Stock cultures of *S. aureus* 196E and C243 were maintained on brain heart infusion (BHI) agar slants at 5°C. Preparatory to inoculating the fermentor vessel, 10 ml of BHI broth was inoculated from the slants. After 24 h of incubation at 37°C, the subculture was transferred to 200 ml of BHI broth in a 500-ml flask. Fermentor jars were inoculated from these flasks after further incubation. The initial and final bacterial counts in the fermentor vessel are shown in Table 1. A medium containing 3% (wt/vol) each of a partially hydrolyzed protein (Mead Johnson Company, Evansville, Ind.) and NZ amine NAK (Sheffield Chemical Division of National Dairy Products, Corp., Norwich, N.Y.) plus 0.00005% thiamine and 0.001% nicotinic acid was used in these studies. In all cases, a_w was adjusted to the indicated level (Table 1) by the addition of reagent-grade NaCl.

Growth occurred in a 10-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) vessel containing 4 liters of the medium described above. The culture was stirred at a rate of 100 rpm. Incubation was at 37°C, and pH was maintained at 6.8.

Fermentors were operated for varying time periods, depending upon the a_w (Table 1). Cells were harvested upon termination of the indicated incubation time by continuous centrifugation in a refrigerated centrifuge at 48,000 × *g*. The time of cell harvest corresponded to the attainment of maximal cell counts. The supernatant, spent medium was dialyzed against distilled water at 5°C for 24 h to remove NaCl, which might otherwise interfere with subsequent analyses. The medium was then concentrated 10-fold by dialysis against Carbowax 20M (Union Carbide Corp., Chicago, Ill.). This concentrate, stored frozen, was analyzed for extracellular enzymes.

Water activity. Water activity measurements were performed by using a Sinascope SJT read-out unit connected to EZFBA-4 sensors. The operation and calibration of this instrument are as described by Troller (23). Values were rounded to the nearest hundredth a_w unit except for the control cultures, which were reported as measured (0.996 a_w).

Total protein. Protein concentrations of the spent

TABLE 1. *Growth characteristics*

S. aureus strain	NaCl (% wt/vol)	Hours incubated	CFU/ml[a]		Cell protein (mg/ml)
			Initial	Postincubation	
196E					
0.996[b]	0	24	2.5×10^6	1.2×10^{10}	1.64
0.97	5.3	24	2.1×10^6	3.7×10^9	1.28
0.94	9.5	30	2.05×10^6	1.9×10^9	.81
0.91	14.0	48	3.8×10^6	1.2×10^9	.81
C243					
0.996	0	24	2.5×10^6	2.0×10^9	1.22
0.97	5.3	24	2.9×10^6	2.1×10^9	1.01
0.94	9.5	30	2.5×10^6	1.4×10^9	.90
0.91	14.0	48	3.5×10^5	1.0×10^9	.69

[a] CFU, Colony-forming units.
[b] a_w of growth medium.

media and harvested cells were estimated by the method of Lowry et al. (12), with bovine serum albumin as the standard.

Enterotoxin. The single-diffusion method in agar as modified by Weirether et al. (24) was used to quantify enterotoxins A and B. Enterotoxins used in establishing standard plots and antisera were the gifts of M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison. The minimal level of enterotoxin A or B detectable by this method was 1.0 μg/ml.

Deoxyribonuclease (EC 3.1.4.5). The method of Kunitz (9) was used to analyze for deoxyribonuclease (DNase) activity in the dialyzed, spent fermentor medium. Deoxyribonucleic acid (type III, Sigma Chemical Co., St. Louis, Mo.) was reacted with a standard enzyme solution or the test solution at pH 5.0 and 25°C. Rate of change in absorbance at 260 nm was converted to Kunitz units after appropriate correction for dilution. No attempt was made to determine the heat stability of the measured DNase activity.

Lipase (EC 3.1.1.3). Lipase activity was determined by measuring reaction velocities with a Titrigraph-Titrator (Radiometer, Copenhagen, Denmark). Substrates consisted of a 100-μM concentration of either tributyrin or triolein emulsified ultrasonically in 0.003 M histidine buffer (pH 8.0) and 0.002 M $CaCl_2$. Activity rates were determined as the number of microequivalents of KOH required per minute to maintain the pH of the reaction mixture at 8.0. Rates routinely were followed for 5 min at 25°C.

Catalase (EC 1.11.1.6). Catalase activity was measured by following the disappearance of hydrogen peroxide spectrophotometrically at 240 nm (2). One unit was equal to 1 μmol of hydrogen peroxide decomposed per min at 25°C.

Alkaline protease. Quantification of alkaline protease was carried out by dissolving 0.5% Hammerstenquality casein in 0.1 M phosphate buffer (pH 7.6). Portions (200 μl) of the spent medium concentrate were then reacted with this substrate for 15 min at 37°C. The reaction was halted by the addition of 5.0% trichloroacetic acid followed by filtration through Whatman no. 40 filter paper. Tyrosine and tryptophan liberated by protease in the spent medium were analyzed by the method of Folin-Ciocalteau (6). A standard plot was established, using protease type VI (Sigma) as the standard enzyme source.

Acid protease (EC 3.4.23.5). A modified cathespin assay with hemoglobin (Worthington Biochemicals hemoglobin substrate powder) as substrate was used to quantify acid protease activity in the spent medium. The hemoglobin was prepared in 0.1 M acetate buffer (pH 4.0) and diluted with distilled water to obtain a concentration of 4.0%. A standard curve was prepared, using protease type VI (Sigma) as the standard enzyme source. Assays were reported as protease equivalents per milliliter.

Coagulase. Coagulase activity in the spent medium concentrate was determined, using rehydrated rabbit plasma plus ethylenediaminetetraacetic acid (Difco, Detroit, Mich.) as a substrate. The spent medium (0.1 ml), or an appropriate dilution thereof, was mixed with 0.5 ml of the plasma solution and incubated at 37°C for 120 min. The results of this assay are presented as the maximum dilution producing a clot that fulfilled the requirements for a positive reaction as described by Sperber and Tatini (19). In this determination, a firm clot that does not move when the tube is tipped is considered a positive reaction. Dilutions of 0 (no dilution), 1:1, 1:2, 1:4, and 1:8 were used.

Acid phosphatase (EC 3.1.3.2). The method of Pan and Blumenthal (17) was used to analyze for acid phosphatase activity. In this test, *p*-nitrophenyl phosphate is hydrolyzed by the phosphatase under acidic (pH 4.8) conditions, with the production of *p*-nitrophenol and inorganic phosphate. Subsequent elevation of the pH converts *p*-nitrophenol to a yellow complex, which is measured at 410 nm. Phosphatase activity is proportional to the color intensity that develops after incubation of the reaction mixtures for 30 min at 37°C. Solutions of *p*-nitrophenol were analyzed directly to establish the standard plot. One unit of acid phosphatase was equal to 1 μg of *p*-nitrophenol released.

RESULTS AND DISCUSSION

Throughout most of these studies, the production of various extracellular enzymes is reported on both total and relative bases. In the latter case, extracellular products are related to cell protein recovered at the conclusion of the growth period stated in Table 1. Several means of measuring relative metabolic activity or growth were considered, including "maximal"

viable count attained, protein content of the spent medium, and total cell protein. The latter was selected as being most representative of extracellular metabolic activity and hence the most practical parameter to which enzyme activity could be related.

Enterotoxin. As noted above, the effect of a_w on the production of enterotoxins A and B has been described elsewhere (21, 22). The data in Table 2 generally agree with these findings. Enterotoxin A production appeared to be much less responsive to medium a_w adjustment than enterotoxin B production and appeared to be directionally, but probably not significantly, greater at 0.97 and 0.94 a_w than at 0.996. The recovery of 4.1 and 86.0 μg/ml of total enterotoxins A and B, respectively, at the highest a_w level tested (0.996) agrees with quantitative estimates of these toxins reported previously.

DNase. The production of a heat-stable nuclease has been related to enterotoxin production (11), and so a number of tests have been developed that determine the presence of this enzyme in foods (5, 10). Although staphylococcal DNase is remarkably heat resistant, some loss of activity occurs during heating. Therefore, to obtain more accurate assay results, spent medium concentrates were not heated before analysis, and the results are reported as DNase rather than thermonuclease.

DNase activity (Table 3) was strongly suppressed by a_w reduction; however, this reduction appeared to be more pronounced with the enterotoxin B-producing *S. aureus* C243. With this strain, the amount of DNase present in spent media was reduced below detectable levels at between 0.94 and 0.91 a_w, whereas 43 total Kunitz units were found in *S. aureus* 196E at 0.91 a_w, the lowest a_w level tested.

Lipase. Staphylococci produce enzymes that can hydrolyze a variety of fatty acid-containing substrates such as phospholipids, triglycerides, esters of fatty acids, and polyoxyethylene esters of sorbitan (Tweens).

Lipolytic activity could not be demonstrated at 0.91 a_w in spent medium concentrates obtained from either strain of *S. aureus* (Table 4). Only very limited hydrolysis of tributyrin and triolein was detected with the *S. aureus* 196E strain compared with the C243 strain. In all cases, triolein hydrolysis occurred at a slower rate than tributyrin hydrolysis. A decrease in a_w of the culture medium tended to reduce the rates

TABLE 2. *Enterotoxin production at various a_w levels*

S. aureus strain	a_w	Enterotoxin production	
		Total (μg/ml)	Relative (μg of toxin/mg of cell protein)
S. aureus 196E (enterotoxin A)	0.996	4.1	2.5
	0.97	3.5	2.7
	0.94	2.3	2.7
	0.91	0.85	1.0
C243 (enterotoxin B)	0.996	86	70.5
	0.97	16	16.1
	0.94	4.6	5.1
	0.91	2.0	2.8

TABLE 3. *DNase production at various a_w levels*

S. aureus strain	a_w	DNase activity	
		Total (Kunitz units)	Relative (Kunitz units/mg of cell protein)
196E	0.996	185	112.8
	0.97	106	82.8
	0.94	60	74.1
	0.91	43	53.1
C243	0.996	357	292.6
	0.97	271	268.3
	0.94	21	23.3
	0.91	NF[a]	

[a] NF, None found.

TABLE 4. *Lipolysis at various a_w levels*

S. aureus strain	a_w	Tributyrinase		Trioleinase	
		Total (meq/min per mg)	Relative (meq/mg of cell protein)	Total (meq/min per ml)	Relative (meq/mg of cell protein)
196E	0.996	0.12	0.07	0.11	0.07
	0.97	0.04	0.03	0.06	0.05
	0.94	0.01	0.01	0.04	0.05
	0.91	NF[a]		NF	
C243	0.996	6.00	4.91	3.60	2.95
	0.07	2.60	2.89	1.30	1.29
	0.94	1.30	1.29	0.10	0.11
	0.91	NF		NF	

[a] NF, None found.

of lipid hydrolysis that occurred when spent, dialyzed medium concentrates were assayed. These results are similar to those of Mates and Sudakevitz (15), who reported a similar suppression of tributyrinase production at NaCl concentrations of 0.1 to 1.2 M. Although not reported, the a_w range of these NaCl concentrations is estimated to be 0.995 to approximately 0.96 a_w. Over this range, inhibition of lipase activity of from 8 to 86% was observed.

Catalase. In aerobic bacteria such as staphylococci, catalase hydrolyzes the toxic hydrogen peroxide that accumulates as a result of electron transport. Both of the test strains produced maximal levels of catalase at the highest a_w level tested, 0.996 (Table 5). The synthesis of catalase by *S. aureus* C243 was limited greatly by reduction in a_w. At 0.94 a_w, this enzyme could not be detected in the spent medium despite the presence of 10^9 staphylococci per ml.

The absence of detectable catalase production by the C243 strain at low a_w levels poses some questions about the mechanism by which H^+ ions are disposed of when the organism is grown at reduced a_w. One hypothesis could account for staphylococcal growth in the absence of catalase by an alteration in the normal aerobic, electron transport system that might resemble a metabolism more closely related to that of anaerobic bacteria. Certainly, staphylococci possess the ability to grow (and produce enterotoxin) anaerobically. In addition, Ingram (8) has shown that NaCl suppresses respiration of "salt-sensitive" bacteria, thus indicating that some effect on respiration may occur. This might account for the inhibition of extracellular enzyme synthesis and/or transport observed in these experiments.

TABLE 5. *Catalase production at various a_w levels*

S. aureus strain	a_w	Catalase production	
		Total (U)[a]	Relative (U/mg of cell protein)
196E	0.996	12.73	7.8
	0.97	2.75	2.1
	0.94	1.38	1.7
	0.91	0.34	0.4
C243	0.996	17.20	14.0
	0.97	1.38	1.4
	0.94	NF[b]	
	0.91	NF	

[a] Units/milliliter = $A_{240} \times 1{,}000 \times$ dilution $\times 3/43.6 \times 2$, where A_{240} is absorbancy at 240 nm and 43.6 is the molar absorbancy index for H_2O_2 at 240 nm in a 1-cm cuvette.

[b] NF, None found.

Protease. The proteases of *S. aureus* hydrolyze a variety of proteins. In these studies, two substrates, hemoglobin and casein, were poised at pH levels of 4.0 and 7.8, respectively. The data in Table 6 show a trend quite different from that of the other *S. aureus* metabolites observed in this work. Spent media from all strains exhibited optimal protease activity uniformly at 0.94 a_w. Furthermore, the control culture (0.996 a_w) spent medium contained less protease than either 0.97- or 0.94-a_w medium, and in one case (acid protease, *S. aureus* C243) exhibited less activity than the 0.91-a_w medium, the lowest a_w studied.

Coagulase. This enzyme is one of the extracellular products of *S. aureus* most closely associated with pathogenicity. Although numerous compounds such as tetracycline, streptomycin, and methocillin are capable of blocking coagulase activity in vitro (3), little is known about conditions that inhibit the synthesis of this enzyme.

The results of experiments intended to show the effect of medium a_w on the production of coagulase are shown in Table 7. With both *S. aureus* strains, the maximal dilution that clotted rabbit plasma occurred at the highest a_w tested, and minimal coagulase activity in the spent medium occurred at the lowest a_w, 0.91.

Phosphatase. Table 8 shows the results of experiments on the acid phosphatase activity of *S. aureus* at various a_w levels. The extracellular

TABLE 6. *Protease production at various a_w levels*

S. aureus strain	a_w	Acid protease		Alkaline protease	
		Total (μg equivalents)	Relative (μg equivalents/mg of cell protein)	Total (μg equivalents)	Relative (μg equivalents/mg of cell protein)
196E	0.996	2,650	1,616	52	32
	0.97	2,600	2,031	78	61
	0.94	2,700	3,333	98	121
	0.91	850	1,049	9	11
C243	0.996	1,050	861	77	63
	0.97	1,450	1,435	135	134
	0.94	2,700	3,000	230	256
	0.91	1,090	1,580	11	16

TABLE 7. *Effect of a_w on coagulase production*

S. aureus strain	a_w	Dilution[a]
196E	0.996	1:8
	0.97	1:8
	0.94	1:2
	0.91	1:1
C243	0.996	1:8
	0.97	1:4
	0.94	1:4
	0.91	1:1

[a] Maximal dilution of spent medium that clots (4+) rabbit plasma after incubation for 2 h at 37°C.

TABLE 8. *Effect of a_w on acid phosphatase production*

S. aureus strain	a_w	Acid phosphatase	
		Total (μg of PNP/ml)[a]	Relative (μg of PNP/mg of cell protein)
196E	0.996	1.53	0.27
	0.97	0.75	0.22
	0.94	0.54	0.25
	0.91	0.09	0.05
C243	0.996	2.57	0.45
	0.97	1.60	0.68
	0.94	0.18	0.08
	0.91	0.25	0.16

[a] PNP, *p*-nitrophenol.

fraction of this enzyme has been characterized (14) as either free or loosely bound to the cell surface. The greatest proportion of phosphatase resides in the latter fraction, which may be eluted by an increase in ionic (KCl) strength. A similar release of bound phosphatase, if it occurred in the presence of increasing concentrations of NaCl (used in these experiments to adjust a_w), would increase the amount of the enzyme present as the a_w was lowered. In fact, the amount of total phosphatase activity generally decreased as a direct function of a_w, with highest activities occurring in the control cultures (0.996 a_w). The amount of enzyme per milligram of cell protein was not influenced by reduction in a_w until a_w was reduced below 0.94 in the case of the 196E strain and 0.97 with the C243 strain. Below these levels, relative acid phosphatase activities dropped steeply.

With the exception of protease and, to some degree, acid phosphatase, the suppression of many extracellular metabolites of the *S. aureus* test strains generally followed the pattern previously established for enterotoxin. In most cases, the production of these metabolites by the 196E strain appeared to be less sensitive to reduced a_w than in the C243 strain. The cause of these suppressions has not been determined, but the similarity of responses indicates that a common mechanism exists and that any hypothesis would have to account for the inhibition of metabolite production and concurrent synthesis of cellular protein.

Christian and Waltho (4) have demonstrated that staphylococci accumulate very high concentrations of proline when grown at reduced a_w levels, and it is this predominant pool which may, in some manner, restrict the availability of amino acids and/or energy for the synthesis of certain metabolites while allowing cellular synthesis to proceed, albeit at a somewhat reduced rate. Measures (16) has reported that organisms with a low tolerance for reduced a_w accumulate a high intracellular concentration of glutamate which, because it is negatively charged, requires high concentrations of a monovalent cation (usually K^+) to maintain neutrality. If, however, the glutamate is converted to a neutral amino acid such as proline, as is the case with the osmotolerant staphylococci, a buildup of intracellular and potentially harmful K^+ levels is avoided. This increase in the intracellular content of proline maintains the cell in a slightly hypertonic condition, and, while compatible with many of the metabolic processes occurring within the cell, the high intracellular proline levels could be responsible for the reduced synthesis of certain enzymes as seen in these studies. It is unlikely that the effects described herein are the result of altered membrane permeability. Gould and Measures (6a) have stated that membrane protein and lipid composition remains unaltered at low a_w levels and that cell permeability remains effectively unchanged. In the case of proline, transport of this compound appears to be even more efficient at low a_w levels despite the absence of specific proline-related "transport proteins."

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