

Effect of Sodium Chloride on the Activity and Production of Staphylococcal Exonuclease

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Extracellular nuclease activity in *Staphylococcus aureus* was enhanced about fourfold by 1% (w/v) NaCl, KCl, CsCl or LiCl. The pH and concentration of Ca²⁺ for optimum activity varied with the NaCl concentration; with an increased NaCl concentration, a higher Ca²⁺ concentration and a lower pH were required. V_{max} but not K_m , varied with the concentration of NaCl. The addition of 3% (w/v) NaCl to growing cultures of *S. aureus* increased nuclease production fivefold.

INTRODUCTION

Although *Staphylococcus aureus* is not usually considered to be a halophilic bacterium, it is able to grow in a medium with a high salt concentration. Both the rate of synthesis (Kamekura & Onishi, 1974) and the catalytic activity (Lasen, 1962; Lanyi, 1969) of the enzymes of halophilic bacteria are increased by high salinity. In *S. aureus*, little is known about the effect of high salt concentration on either enzyme synthesis or activity.

The structure and catalytic properties of staphylococcal nuclease (EC 3.1.4.7) have been studied extensively by Anfinsen and his colleagues (e.g. Cuatrecasas *et al.*, 1967*a, b*, 1968*a*; Taniuchi *et al.*, 1967). Recently, we have found that staphylococcal extracellular nuclease and alpha-toxin synthesis may be closely associated with some metabolic aspects of the cell (Udou & Ichikawa, 1978).

This paper reports investigations of the effect of NaCl on the production and activity of staphylococcal exonuclease.

METHODS

Bacterial strain and growth conditions. *Staphylococcus aureus* Wood 46 (ATCC 10832) was maintained on nutrient agar slants at 4 °C with subculture at 6 to 8 week intervals.

Bacteria from cultures growing exponentially in nutrient broth (BBL) were washed twice and resuspended in fresh medium to give an A_{540} of 0.5 to 0.6 (equivalent to about 0.12 mg dry wt ml⁻¹). These cultures were incubated in flasks having three times the volume of the culture medium in a reciprocating shaker water bath (140 strokes min⁻¹) at 37 °C. Samples (1 ml) were removed at intervals and centrifuged at 18000 rev. min⁻¹ for 10 min; the resultant supernatant fluids were kept on ice. Growth was followed by measuring the absorbance at 540 nm, using a Shimadzu UV-200 spectrophotometer, and was converted to dry weight of bacteria by reference to standard calibration curves prepared for *S. aureus* in the presence and absence of salt.

Measurements of enzyme activity. Nuclease activity in the culture supernatant fluids was assayed using, as substrate, a solution of calf thymus DNA [3 mg ml⁻¹ in 10 mM-sodium borate buffer (pH 9.0) containing 10 mM-NaCl] which had been denatured in a boiling water bath for 20 min. The reaction mixture consisted of 10 µl enzyme solution, 5 µl 0.1 M-CaCl₂, 100 µl substrate solution and 100 µl 10 mM-sodium borate buffer (pH 9.0) containing NaCl at various concentrations. The mixture was incubated at 37 °C, usually for

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30 min, but for 20 min when enzyme kinetics were studied. The reaction was stopped by adding 0.5 ml ice-cold 10% (w/v) perchloric acid. The tubes were kept on ice for at least 15 min with occasional shaking, and 0.5 ml distilled water was added to each. Acid-insoluble nucleic acid was removed by centrifuging, and the extent of DNA hydrolysis was determined by measuring the absorbance of acid-soluble nucleotides at 260 nm. One unit of nuclease activity was defined as the amount of enzyme giving an increase in A_{260} of 0.2 units h^{-1} . The standard error in this assay was less than 10%.

Proteolytic activity was measured by a modification of the method of Nugent *et al.* (1974). Samples (0.5 ml) of culture fluid were incubated at 37 °C with 0.5 ml 1% Hammersten Quality Casein in 0.02 M-Tris/HCl (pH 7.8) containing 1 mM- $CaCl_2$ and cysteine. (The casein solution was denatured in a boiling water bath for 10 min before use.) After incubation for 60 min, 1.5 ml ice-cold 10% (w/v) trichloroacetic acid was added, and the mixtures were placed on ice for 10 min. The resulting precipitate was removed by centrifuging and the extent of hydrolysis was determined by measuring the absorbance of the supernatant fluid at 280 nm.

Chemicals. Highly polymerized calf thymus DNA was obtained from P-L Biochemicals, Milwaukee, Wis., U.S.A.; Hammersten casein from Difco; micrococcal nuclease (Grade VI) from Sigma; and actinomycin D from Boehringer. Erythromycin was kindly supplied by Japan Upjohn Ltd (Tokyo, Japan). All other chemicals were analytical grade.

RESULTS

Effect of NaCl concentration on nuclease activity

Several enzymically active species of nuclease may be present in the supernatant of *S. aureus* cultures and could be the precursor of the major species (Taniuchi & Anfinsen, 1966; Wadström, 1967; Cohen *et al.*, 1971; Carpenter & Chesbro, 1974). Carpenter & Chesbro (1974) found that the amounts of individual species varied with the growth phase. Cultures were therefore first examined for enzyme activity at different growth phases. Samples were taken at 1.5, 3.5 and 7.0 h after the beginning of cultivation, when the corresponding cell densities, determined as A_{540} , were 1.50, 1.82 and 1.84, respectively. The effect of NaCl on the nuclease activity of the culture supernatants is shown in Fig. 1. The highest activity, observed when 1% NaCl (0.17 M) was added to the reaction mixture, was about three times greater than that in the absence of NaCl. Although the activity decreased subsequently with increasing NaCl concentrations, there was a small peak of activity at 4% (0.68 M). The activity of a 1.5 h culture supernatant was about twice that of a 7 h culture supernatant in the presence of 4% NaCl. A similar biphasic response pattern of nuclease activity against NaCl concentration was observed for a commercial nuclease preparation; this formed a homogeneous single band on 0.1% (w/v) sodium dodecyl sulphate–10% (w/v) polyacrylamide disc gel electrophoresis (data not shown).

Relationship between NaCl and Ca^{2+} concentrations

Ca^{2+} ions are required for the catalytic activity of staphylococcal nuclease. Cuatrecasas *et al.* (1967a) reported that the optimum Ca^{2+} concentration for enzyme activity was dependent on the pH of the assay system. We examined the effect of adding NaCl with the optimum Ca^{2+} concentration using the 7 h culture. The optimum NaCl concentration for enzyme activity was dependent on the Ca^{2+} concentration (Fig. 2). Surprisingly, the depression of activity at high Ca^{2+} concentration was less pronounced at high concentrations of NaCl. Thus, the enhancement of activity at 4% NaCl was dependent on the Ca^{2+} concentration, unlike the major peak of activity observed at 1% NaCl.

Relationship between pH and NaCl concentration

Although staphylococcal nuclease is more stable at acid than at alkaline pH, the optimum pH for the expression of enzyme activity is between 9 and 10 (Cuatrecasas *et al.*, 1967a). It is relevant, therefore, to consider both the active site and the spacer region surrounding the site for functional conformation of the enzyme molecule. As shown above, the optimum

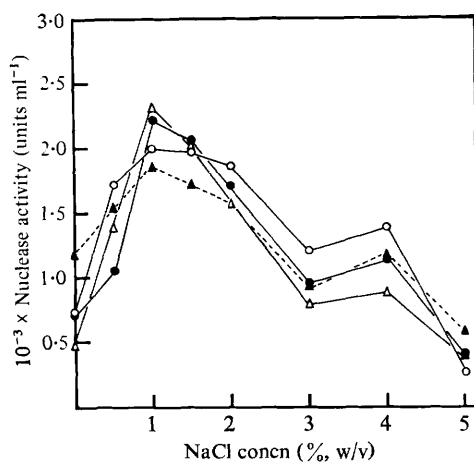


Fig. 1

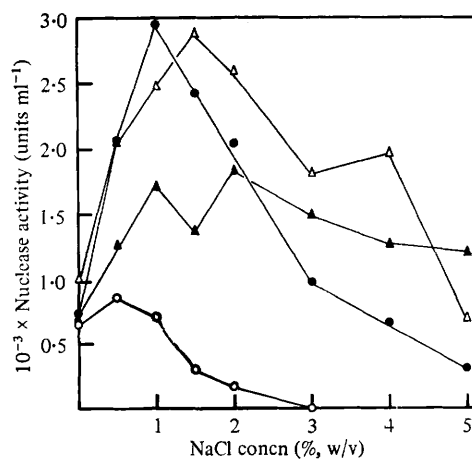


Fig. 2

Fig. 1. Effect of NaCl concentration on the nuclease activity of cultures grown for 1.5 (○), 3.5 (●) and 7.0 (△) h, and on a commercial enzyme preparation (▲).

Fig. 2. Effect of NaCl concentration on the optimum Ca²⁺ concentration for nuclease activity. Ca²⁺ concentrations in the reaction mixtures were 0.1 (○), 1.0 (●), 10.0 (△) and 50.0 (▲) mM. A 7 h culture was used as the source of enzyme.

NaCl concentration varied with the Ca²⁺ concentration, and this suggested that NaCl might play a similar role to pH in the activation of the enzyme. To test this possibility, the effect of NaCl on the optimum pH was examined. At a constant Ca²⁺ concentration of 2.5 mM, the optimum pH for enzyme activity was dependent on the NaCl concentration (Fig. 3); increasing the NaCl concentration from 0 to 2% caused a shift of the pH-activity curve to a lower range. The optimum pH in the absence of added NaCl was near 10, as found by Cuatrecasas *et al.* (1976*b*), and that in the presence of 2% NaCl was 8.7.

Effect of NaCl on the affinity of the enzyme for the substrate

To study further the promoting action of NaCl on nuclease activity, its effect on the affinity of the enzyme for substrate DNA was examined. The rates of hydrolysis of DNA, as expected, followed Michaelis-Menten kinetics in the presence or absence of NaCl. The K_m for DNA at every concentration of NaCl was 50 μ g per 215 μ l reaction mixture. However, the initial rate of hydrolysis varied with the concentration of NaCl added; the V_{max} values were 26.7, 145.5, 100.0 and 80.0 units min⁻¹ ml⁻¹ for 0, 1 to 2, 3 and 4% NaCl, respectively.

Effect of other monovalent cations on nuclease activity

The effect of other monovalent cations on nuclease activity was examined to ascertain whether the promoting effect of NaCl was specific. [High concentrations of Ca²⁺ or other divalent cations may inhibit nuclease activity (Cuatrecasas *et al.*, 1967*a*), possibly because different divalent cations can adequately serve the binding function; however, subsequent catalytic steps have more specific metal ion requirements (Cuatrecasas *et al.*, 1967*b*; Fuchs *et al.*, 1969).] Nuclease activity was enhanced by all of the metal ions tested (Table 1), with maximum activities between 150 and 300 mM. In addition, a linear relationship was observed between the relative activity at the optimum concentrations and ionic radius (Pauling, 1960) for these metal ions (Fig. 4). However, NH₄⁺, which has a greater ionic radius than K⁺, inhibited nuclease activity (Table 1). Both Cs₂SO₄ and CsCl were stimulatory. These findings therefore indicate that the stimulation of nuclease activity is characteristic of monovalent metal ions.

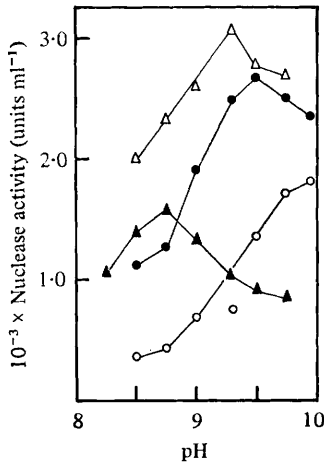


Fig. 3

Fig. 3. Dependence of the optimum pH for nuclease activity on NaCl concentration. All reaction mixtures were prepared with borate buffer. NaCl concentrations were 0 (○), 0.5 (●), 1.0 (△) and 2.0 (▲)% (w/v). A 7 h culture was used as the source of enzyme.

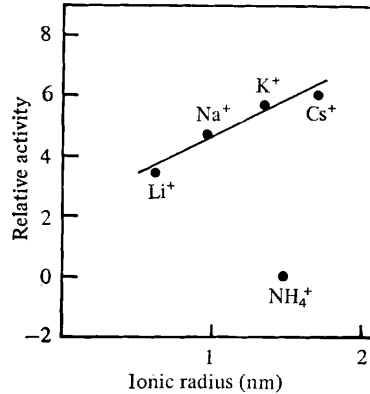


Fig. 4

Fig. 4. Relationship between nuclease activity and ionic radius for various monovalent cations. Relative activity was expressed as the ratio of the maximum activity with each cation tested to the activity in the absence of cations (see Table 1). Ionic radii were taken from Pauling (1960).

Table 1. Effect of various monovalent cations on the activity of staphylococcal exonuclease

A 7 h culture was used as the source of enzyme. Enzyme activity without added monovalent cation was 1.1×10^8 units ml^{-1} .

Salt added	$10^{-3} \times$ Nuclease activity (units ml^{-1})					
	Cation concentration (mM)					
	37.5	75	150	300	500	800
NaCl	2.3	3.1	4.9	5.1	4.3	2.3
KCl	2.5	3.4	4.5	6.2	4.9	2.8
CsCl	3.3	4.2	5.4	6.0	4.7	3.0
LiCl	2.2	2.2	3.3	3.6	2.1	0.8
NH_4Cl	1.1	1.0	0.75	0.4	0.2	0.0
Cs_2SO_4	4.6	5.4	6.5	6.0	4.0	1.2

Effect of NaCl on nuclease production of *S. aureus*

The addition of NaCl to the culture medium causes decreased production of enterotoxins and other extracellular proteins in *S. aureus* (Genigeorgis & Sadler, 1966; Jarvis *et al.*, 1973). The effect of NaCl on cell growth and nuclease production was therefore studied; the optimum concentration for both was 3% (Table 2). Growth in the absence of NaCl probably reached the stationary phase after 3 h incubation, whereas the exponential growth phase was prolonged by the addition of NaCl.

Nuclease production at the optimum NaCl concentration (3%) was about five times greater than that in the absence of NaCl, and no direct correlation was observed between the extent of growth and enzyme production. This was a true increase in production as increased enzyme activity persisted after removal of NaCl by dialysis. No detectable proteolytic activity was found in any of the samples assayed for nuclease activity, regardless of the presence or absence of NaCl in the culture medium (data not shown). Therefore it is unlikely that the stimulation of nuclease production by NaCl was due to enhancement of production or to the activation of proteolytic enzyme caused by the addition of NaCl. The concentrations of Na^+ and K^+ in the nutrient broth were negligible.

Table 2. *Effect of NaCl on exonuclease production in S. aureus*

Cultures were grown as described in Methods with different concentrations of NaCl in the medium, and samples were taken after 1, 3 and 7 h. Nuclease activities in the cell-free medium were determined with 1% (w/v) NaCl (final concentration) in the assay mixture.

NaCl concn in culture medium (%, w/v)	Growth (mg dry wt ml ⁻¹)			10 ⁻³ × Nuclease activity (units ml ⁻¹)		
	1 h	3 h	7 h	1 h	3 h	7 h
0	0.20	0.44	0.40	0.12	1.8	1.9
1	0.21	0.48	0.63	0.80	4.5	5.1
2	0.22	0.48	0.65	0.88	6.6	9.1
3	0.22	0.49	0.65	1.20	8.5	11.0
5	0.20	0.43	0.67	0.72	6.9	10.0
8	0.16	0.31	0.52	0.86	1.4	5.1

DISCUSSION

The catalytic properties of staphylococcal nuclease have been investigated by Cuatrecasas *et al.* (1967*a, b*), who found that the enzyme had an optimum pH of 9, a requirement for Ca²⁺ for binding with substrate or for the subsequent catalysis, and that it degraded both DNA and RNA. These authors also reported that changes in ionic strength produced by adding NaCl or by increasing the buffer concentration had no effect on nuclease activity.

Our experiments indicate that this activity is affected by monovalent cations. However, the presence of NaCl or other monovalent cations may not be essential for expression of catalytic activity of the enzyme, because some activity was also observed in the absence of NaCl. Since the monovalent cations did not apparently act directly on the active site in the enzyme, it is possible that their role is to change the enzyme molecule into a suitable form for approach to, or binding with, the substrate by associating with the enzyme, as proposed by Lanyi (1974) for halophilic bacteria. If one assumes that a few pairs of hydrogen bonds formed between tyrosine residues and certain carboxyl groups are present in the active site (Sokolovsky *et al.*, 1966; Cuatrecasas *et al.*, 1968*b*) and that the 'door' corresponding to the active site is closed at low ionic strength, then this 'door' may be open at the optimal ionic strength, but any excess cations could hinder the binding of substrate to the enzyme by contracting the enzyme molecule.

Sodium chloride also stimulated nuclease production in this bacterium. Since the addition of NaCl did not reverse the inhibition of nuclease synthesis by actinomycin D or erythromycin (data not shown), NaCl is probably not involved at the transcriptional and translational levels of protein synthesis. In addition, Hayashi *et al.* (1978) reported that there was no significant change in respiratory function or energy metabolism in staphylococcal membrane fractions prepared from cultures grown at different salinities.

The nature of the stimulatory effect of NaCl on nuclease production remains unexplained. Possibilities might be the facilitation of membrane transport out of the cell following nuclease synthesis or of permeation into the cell of an inducer of nuclease.

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