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Halophilic Nuclease of a Moderately Halophilic *Bacillus* sp.: Production, Purification, and Characterization

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A moderately halophilic bacterium, *Bacillus* sp., isolated from rotting wood on the seashore in Nauru, produced an extracellular nuclease when cultivated aerobically in media containing 1 to 2 M NaCl. The enzyme was purified from the culture filtrate to an electrophoretically homogeneous state by ethanol precipitation, DEAE-Sephadex A-50 column chromatography, and Sephadex G-200 gel filtration. The enzyme consisted of two charge isomers and showed both RNase and DNase activities. Molecular weight was estimated to be 138,000 by Sephadex G-200 gel filtration. The enzyme had marked halophilic properties, showing maximal activities in the presence of 1.4 to 3.2 M NaCl or 2.3 to 3.2 M KCl. The enzyme hydrolyzed thymidine-5′-monophosphate-*p*-nitrophenyl ester at a rate that increased with NaCl concentration up to 4.8 M. In the presence of both Mg^{2+} and Ca^{2+}, activity was greatly enhanced. The activity was lost by dialysis against water and low-salt buffer, but it was protected when 10 mM Ca^{2+} was added to the dialysis buffer. When the inactivated enzyme was dialyzed against 3.5 M NaCl buffer as much as 68% of the initial activity could be restored. The enzyme exhibited maximal activity at pH 8.5 and at 50°C on DNA and at 60°C on RNA and attacked RNA and DNA exonucleolytically and successively, producing 5′-mononucleotides.

Onishi et al. isolated a variety of halophilic bacteria from environments of various salinities by enrichment culture technique with a 4 M NaCl medium and classified them into nine types based on their growth response to salt concentration (12). The previous studies on enzyme production by the isolates showed that a moderate halophile, *Micrococcus varians* subsp. *halophilus*, produced a halophilic nuclease (7, 8), and two other moderate halophiles, *Micrococcus halobius* (14) and *Acinetobacter* sp. (13), produced amylases.

Subsequently, we found that another moderate halophile, *Bacillus* sp. N23-2, produced an extracellular nuclease in saline medium. Numerous papers have appeared concerning nucleases from microorganisms, animals, and plants (5). However, the activities of these enzymes are markedly inhibited by the presence of 0.5 to 1.0 M NaCl. Maeda and Taga (11) reported that an extracellular nuclease produced by a marine *Vibrio* sp. was activated by 50 mM Mg^{2+} ion and stabilized by 10 mM Ca^{2+} ion, which are the concentrations in seawater. However, there was no description on the effect of NaCl concentration on the enzyme activity. As for halophilic nucleases, only nuclease H of *M. varians* has so far been reported (7, 8). It seems interesting to compare the characteristics of the purified enzyme preparations of both moderate halophiles, *Bacillus* sp. and *Micrococcus* sp.

The present paper describes purification, halophilic properties, and the mode of action of the *Bacillus* sp. nuclease as well as the effect of salt concentration on enzyme production.

MATERIALS AND METHODS

Bacterial strain. The bacterium used in this study was obtained from rotting wood on the seashore in Nauru by aerobic enrichment culture technique in Sehgal and Gibbons complex medium (SGC medium) (15) containing 4 M NaCl. After colony isolation on a plate of this medium, the organism was identified as *Bacillus* sp. N23-2. The stock culture was maintained on 3 M NaCl SGC agar slants at 5°C.

Medium. SGC medium contains 0.75% vitamin-free Casamino Acids (Difco Laboratories), 1.0% yeast extract (Difco), 0.3% sodium citrate, 0.2% KCl, 2% $MgSO_4 \cdot 7H_2O$, and 2.3 mg of $FeCl_2 \cdot nH_2O$ (pH 6.6) per 100 ml. Nutrient broth (NB) consists of 1% beef extract (Difco) and 1% polypeptone (Daigo), pH 7.0. The bacterium was cultivated in SGC and NB to which designated amounts of NaCl or KCl were added.

Measurement of growth. A drop of a 2-day-old culture grown in 2 M NaCl SGC medium was added to

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Klett tubes containing 5 ml of medium containing 0 to 4 M NaCl or KCl. The tubes were shaken at 30°C on a reciprocal shaker operating at 140 rpm with a stroke of 7.5 cm. Turbidity was measured periodically by use of a Klett-Summerson colorimeter (no. 66 filter) against an uninoculated blank.

Cultural conditions for nuclease production. The SGC medium containing 0 to 4 M NaCl was used for enzyme production. Inocula (0.2 ml) of a 2-day-old culture grown in 1 M NaCl SGC medium were added to 500-ml flasks containing 80 ml of medium. The flasks were shaken as stated above.

Assay of enzyme activity. Nuclease activity was routinely determined by the following method (7). The assay mixture contained 0.5 ml of RNA or DNA (1 mg/ml), 0.4 ml of 0.1 M Tris-hydrochloride (pH 8.0) containing 4 M NaCl, 0.05 ml each of 0.2 M $MgSO_4$ and 0.2 M $CaCl_2$, and 0.1 ml of enzyme solution. After 2 h of incubation at 40°C, the reaction was terminated by the addition of 3 ml of 99.5% ethanol. After 10 min at 0°C, the precipitate was removed by centrifugation, and the absorbance of the supernatant fluid was measured with a spectrophotometer at 260 nm. One unit of the nuclease activity was defined as the amount of enzyme catalyzing an increase of 1.0 in absorbance at 260 nm under the above conditions.

Disc electrophoresis. Electrophoresis in polyacrylamide gel was carried out by the method of Davis (3). Standard 7.5% polyacrylamide gels (pH 8.0) were used. Samples were loaded on the column (0.5 by 8 cm) in glycerol and subjected to electrophoresis at 2 mA per gel column at 5°C. To observe isomer characteristics of the enzymes by the method of Hedrick and Smith (6), 6, 7, 8, 9, and 10% acrylamide gels (pH 8.5) were used.

Determination of protein. Protein concentration during most stages of enzyme purification was determined by the Folin phenol method (9) with bovine serum albumin as the standard. The protein concentration of each fraction during column chromatography was estimated by measuring the absorbance at 280 nm.

Action on synthetic substrates. Hydrolysis of synthetic substrates, thymidine 5'-monophosphate-*p*-nitrophenyl ester ammonium salt (*p*-nitrophenyl-pT), and thymidine 3'-monophosphate-*p*-nitrophenyl ester ammonium salt (Tp-*p*-nitrophenol) was measured spectrophotometrically by monitoring the release of *p*-nitrophenol (E_{410} = 1.62 × 10^4 M^{-1} cm^{-1}) at 30°C. The assay mixture contained 0.5 ml of substrate (3 mg/ml), 0.25 ml each of 0.2 M $MgSO_4$ and 0.2 M $CaCl_2$, 2 ml of 0.1 M Tris-hydrochloride (pH 8.0) containing various amounts of NaCl, and 0.1 ml of enzyme solution.

DNA digestion. To observe the time course of native DNA digestion, a reaction mixture containing 4 ml of calf thymus DNA (1 mg/ml), 4 ml of 0.1 M Tris-hydrochloride (pH 8.0) containing 4 M NaCl, 0.5 ml each of 0.2 M $MgSO_4$ and 0.2 M $CaCl_2$, and 1 ml of enzyme solution was incubated at 30°C. One-milliliter amounts of the reaction mixture were removed at appropriate time intervals and mixed with 3 ml of 99.5% ethanol, and the ethanol-soluble products were measured at 260 nm. The viscosity of the reaction mixture was measured separately with an Ostwald viscometer. Gel filtration of the enzymatic hydrolysate of heat-denatured DNA was performed on a column of Sephadex G-100 (1.5 by 22 cm) equilibrated with 0.01 M Tris buffer (pH 8.0) containing 2 M NaCl. One milliliter of the reaction mixture was periodically removed, heated for 5 min at 100°C to inactivate the enzyme, and cooled rapidly in an ice-water bath. The absorbance at 260 nm of the effluent was monitored.

RNA digestion. A reaction mixture which consisted of 4 ml of RNA (1 mg/ml), 4 ml of 0.1 M Tris-hydrochloride (pH 8.0) containing 4 M NaCl, 0.5 ml each of 0.2 M $MgSO_4$ and 0.2 M $CaCl_2$, and 1 ml of enzyme solution was incubated at 40°C for 9 h. The RNA was precipitated with 75% ethanol; after centrifugation, the soluble fraction of the hydrolysate was evaporated to dryness in vacuo. The product was dissolved in distilled water, adjusted to pH 1.85 with HCl, and applied to a small column (0.6 by 3 cm) of a mixture of active charcoal and Celite 545 (2:3, wt/wt) equilibrated with 0.01 N HCl. After the column was washed with 0.01 N HCl, nucleotides were eluted with water–99.5% ethanol–25% ammonia (49:50:1, vol/vol), and the eluate was evaporated to dryness in vacuo. Nucleotides were dissolved in 50 μl of distilled water. Thin-layer chromatography of the reaction products was carried out with a solvent system composed of 80% saturated ammonium sulfate–*t*-butyl alcohol–1 N ammonia water (200:3:1, vol/vol). Compounds were detected by examining the cellulose thin layer under incident shortwave UV light (254 nm) in a dark room.

Chemicals. Calf thymus DNA, yeast RNA type XI, 2',3'-mononucleotides (mixed isomer), bovine serum albumin, and the synthetic substrates, thymidine 5'-monophosphate-*p*-nitrophenyl ester ammonium salt and thymidine 3'-monophosphate-*p*-nitrophenyl ester ammonium salt were purchased from Sigma Chemical Co. 5'-Mononucleotides were products of Kohjin Co., Japan.

Heat-denatured DNA was prepared by boiling calf thymus DNA type I (Sigma; 2 mg/ml) for 10 min, followed by rapid cooling in ice water. Plastic thin-layer chromatography sheets (cellulose F_{254}; 20 by 20 cm) were obtained from E. Merck AG.

RESULTS

Effect of NaCl and KCl on growth of *Bacillus* sp. N23-2. The best growth was obtained in SGC medium containing 2 M NaCl; this gave a maximum yield after 2 days of cultivation. In SGC media, growth was fairly good also in 1, 3, or 4 M NaCl, but little growth was obtained without added NaCl. In NB media, the growth was poor even in the presence of 1 to 4 M NaCl. On the other hand, the bacterium could not grow in SGC and NB containing 1 to 4 M KCl. Thus, *Bacillus* sp. N23-2 is a moderate halophile that specifically requires Na^+ for growth.

Effect of NaCl on nuclease production. The nuclease production was excellent in 1 and 2 M NaCl SGC media. Even after 5 days of cultivation in 3 and 4 M NaCl, nuclease production was poor, although growth was moderately good.

Enzyme purification. The procedures developed for the purification of nuclease are detailed in the following steps. All purification steps were carried out at 5°C. In all cases, Tris buffer refers to 0.01 M Tris-hydrochloride (pH 8.0).

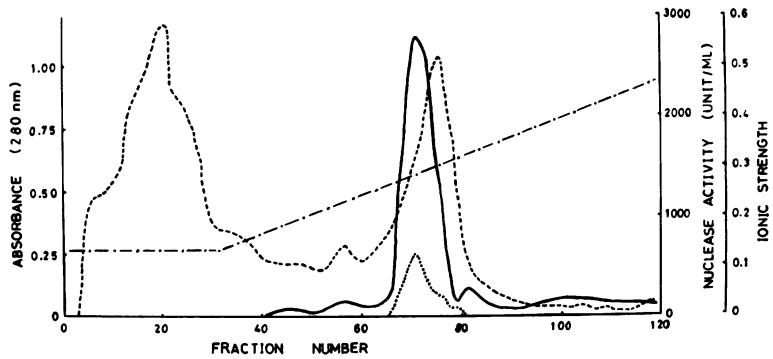

FIG. 1. Elution pattern of DEAE-Sephadex A-50 column chromatography of the nuclease. DEAE-Sephadex A-50 was hydrated, packed in a column (2.2 by 30 cm), and equilibrated with Tris buffer (pH 8.0) containing 40 mM each $MgSO_4$ and $CaCl_2$. The dialysate of the ethanol precipitate (160 ml) was passed through the column, and the enzyme was absorbed well in the column. For elution a linear gradient of 500 ml each of 0 and 0.4 M NaCl in the buffer was used. The elution flow rate was 20 ml/h, and 10-g fractions were collected. Symbols: ———, RNase activity; ·····, DNase activity; --------, protein; –·–·–·–, ionic strength.

(i) Cell-free filtrate. The 2-day-old culture broth of 1 M NaCl SGC was subjected to centrifugation (8,000 × *g*, 20 min) to remove cells, yielding about 5.3 liters of clear solution with a protein concentration of 2.58 mg/ml and a specific activity of 29.04 U/mg of protein (as RNase activity).

(ii) Ethanol precipitation. The cell-free filtrate was concentrated to 550 ml with a Diaflo ultrafiltration membrane PM-10. Cold ethanol was added with stirring to 40% final concentration. After standing overnight, the resulted precipitate was collected by centrifugation and dissolved in 500 ml of Tris buffer containing 40 mM each of $MgSO_4$ and $CaCl_2$.

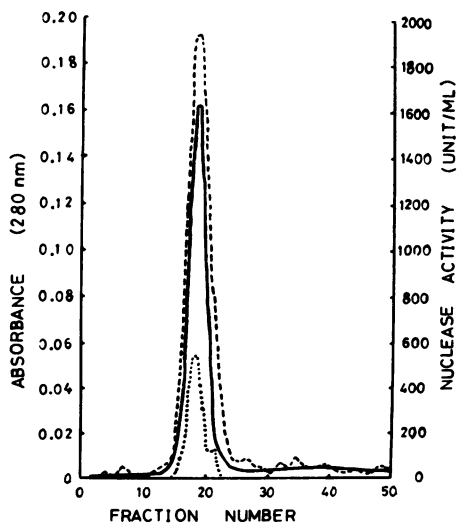

FIG. 2. Elution pattern of the third Sephadex G-200 column chromatography of the nuclease. Sephadex G-200 was hydrated, packed in a column (2.2 by 43 cm), and equilibrated with Tris buffer (pH 8.0) containing 40 mM $CaCl_2$. Elution was carried out at a flow rate of 6 ml/h with the same buffer used for equilibration, and 5-g fractions were collected. Symbols: ———, RNase activity; ·····, DNase activity; --------, protein.

(iii) DEAE-Sephadex A-50 column chromatography. The enzyme solution was concentrated to 160 ml with a Diaflo ultrafiltration membrane PM-10 and was dialyzed overnight against Tris buffer containing 40 mM each of $MgSO_4$ and $CaCl_2$. The dialysate was passed through a diethylaminoethyl Sephadex A-50 column (2.2 by 30.0 cm) equilibrated with the same buffer. Nuclease was absorbed into the column. A linear gradient elution was carried out in Tris buffer containing 40 mM each of $MgSO_4$ and $CaCl_2$ between 500 ml each of 0 and 0.4 M NaCl. Fractions of 10 g were collected, and the enzyme was eluted between 0.27 to 0.30 ionic strength (Fig. 1).

(iv) First Sephadex G-200 column chromatography. The active fraction was concentrated to 10 ml by Diaflo membrane and fractionated on a Sephadex G-200 column (2.2 by 43.0 cm) after the column had been equilibrated with Tris buffer containing 40 mM $CaCl_2$; fractions of 5 g were collected.

(v) Second and third Sephadex G-200 column chromatography. The active fraction was then successively fractionated on a second and third Sephadex G-200 column by the same procedure. In the peak fraction of eluted enzyme there was a coincidence of protein concentration and nuclease activity (Fig. 2).

The purification steps, summarized in Table 1, disclose 220-fold purification over the culture filtrate with a 8.0% recovery of activity.

The homogeneity of the purified enzyme prep-

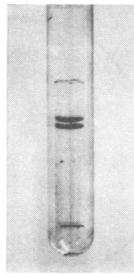

FIG. 3. Polyacrylamide disc gel electrophoretic pattern of the purified nuclease.

aration was examined by polyacrylamide disc gel electrophoresis, which revealed two bands on the gel (Fig. 3). Each slice of the two bands showed both RNase and DNase activities. The ratio of RNase and DNase activity of each band was almost the same. Polyacrylamide gel electrophoresis by the method of Hedrick and Smith (6) demonstrated that the purified enzyme consisted of two isomers of the same molecular weight, but different electric charges (Fig. 4).

Molecular weight. The molecular weight of the nuclease, determined by Sephadex G-200 gel filtration, was calculated to be 138,000 (Fig. 5).

Halophilic properties of the nuclease. The nuclease activity of the purified enzyme toward RNA was examined at various NaCl and KCl concentrations. The enzyme required 1.4 to 3.2 M NaCl or 2.3 to 3.2 M KCl for maximal activity. Also, the enzyme exhibited marked halophilic properties, showing increasingly high activity toward *p*-nitrophenyl-pT with increasing concentrations of NaCl up to 4.8 M. The effect

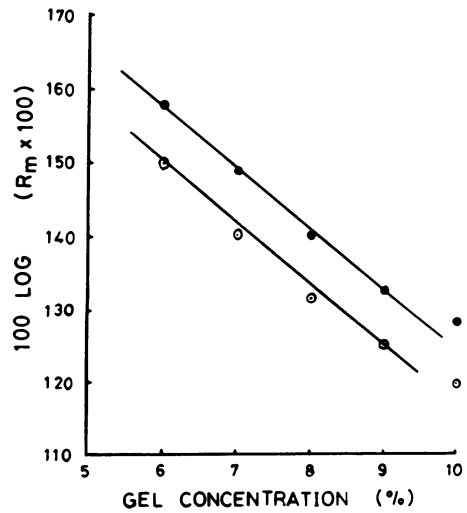

FIG. 4. Size and charge isomer separation of the nucleases by disc gel electrophoresis by the method of Hedrick and Smith (6).

TABLE 1. Purification scheme for the halophilic *Bacillus* sp. nuclease

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)		Sp activity (U/mg of protein)		Sp act RNase/ sp act DNase	Purification (fold)		Yield (%)	
			RNase	DNase	RNase	DNase		RNase	DNase	RNase	DNase
Culture filtrate	5,320	13,720	398,500	101,600	29.0	7.4	3.9	1.0	1.0	100	100
40% ethanol precipitate	470	493.5	267,000	62,040	541.0	125.7	4.3	18.7	17.0	67.0	61.1
DEAE-Sephadex A-50	100	51.0	82,410	22,110	1,616	433.5	3.7	55.7	58.6	20.7	21.8
First Sephadex G-200	18	15.7	55,720	18,090	3,549	1,152	3.1	122.4	155.7	14.0	17.8
Second Sephadex G-200	18	8.6	39,510	12,860	4,594	1,495	3.1	158.4	202.0	9.9	12.7
Third Sephadex G-200	18	5.0	31,890	9,860	6,378	1,972	3.2	219.9	266.5	8.0	9.7

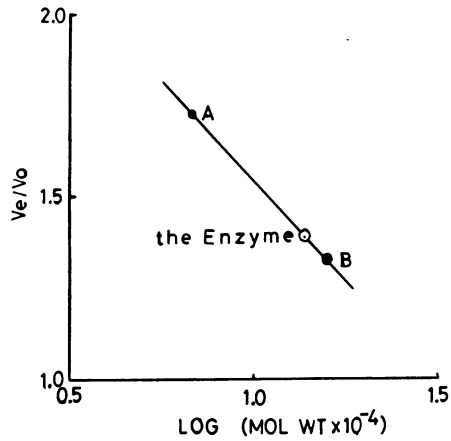

FIG. 5. Molecular weight estimation of the nuclease by Sephadex G-200 gel filtration. The reference proteins used were as follows: A, bovine serum albumin (molecular weight 68,000); and B, γ-globulin (molecular weight, 160,000).

of metal ions on the nuclease activity was examined (Fig. 6). Mg^{2+} or Mn^{2+} stimulated the activity at optimal concentrations of 100 and 10 mM, respectively, whereas Ca^{2+} alone had no effect. In the presence of both Mg^{2+} and Ca^{2+}, the activity was enhanced, and maximal activity was observed in the presence of 100 mM Mg^{2+} and 10 mM Ca^{2+}. Inactivation of the enzyme occurred by removal of salt during dialysis against Tris buffer containing less than 0.5 M NaCl for 24 h at 5°C. From dialysis experiments,

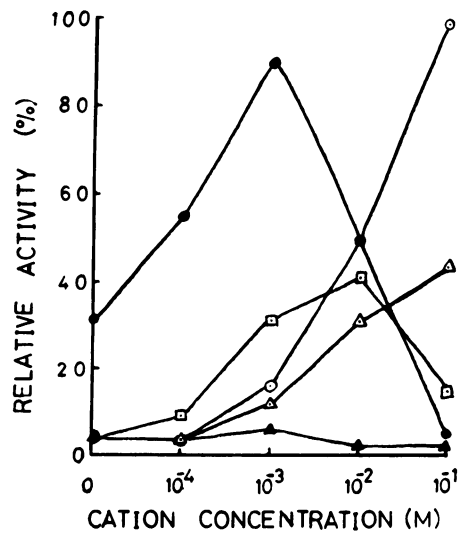

FIG. 6. Effects of divalent metal ions on the nuclease activity with RNA as the substrate. Symbols: △, Mg^{2+}; ▲, Ca^{2+}; □, Mn^{2+}; ⊙, Mg^{2+} plus 10 mM Ca^{2+}; ●, Ca^{2+} plus 10 mM Mg^{2+}.

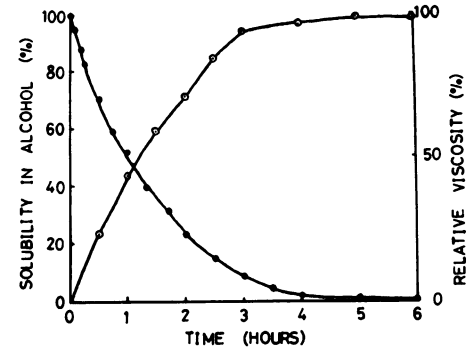

FIG. 7. Time course of the change of viscosity (●) and the appearance of 75% ethanol-soluble products (⊙) in a digest of calf thymus native DNA.

it was also apparent that Mg^{2+} and Mn^{2+} ions could not replace the NaCl requirement for stability. Activity was protected completely when 10 mM Ca^{2+} was added to the dialysis buffer. The loss of activity caused by exposure to low ionic strength could be partially reversed by slowly increasing the concentration of NaCl. As much as 68% of the initial activity was recovered when the inactivated enzyme with distilled water was dialyzed against Tris buffer containing 3.5 M NaCl for 48 h at 5°C. Thus, the purified nuclease showed marked halophilic properties.

Effects of pH and temperature on the activity. Optimal pH was 8.5 with RNase activity, and optimal temperature was 60°C with RNase activity and 50°C with DNase activity.

Mode of action on DNA. Figure 7 is a plot of the data from a digestion experiment in which a reaction mixture containing native DNA as the substrate was analyzed for a change in viscosity and the appearance of alcohol-soluble, 260-nm-absorbing products. A gradual decrease in viscosity, concurrent with the appearance of the alcohol-soluble products, was typical of the exonuclease. The exonucleolytic mode of action of this enzyme was also indicated by a gel filtration analysis of the hydrolysate (2). The reaction mixture at various stages of hydrolysis of heat-denatured DNA showed only two distinct peaks. A peak of unhydrolyzed substrate decreased with reaction time, and another peak, in the position expected for mononucleotides, increased with time. The results suggest also that the enzyme is progressive in action, i.e., each DNA molecule is completely degraded in turn by successive actions of the enzyme.

Reaction products. To identify the reaction products, thin-layer chromatography of a complete hydrolysate of RNA was carried out. The results showed that the products were 5'-AMP, 5'-GMP, 5'-CMP, and 5'-UMP. The fact that the

enzyme hydrolyzes *p*-nitrophenyl-pT, but not Tp-*p*-nitrophenol, to 5'-thymidylic acid and *p*-nitrophenol may also support that the nuclease is a 5' former.

DISCUSSION

There have been many reports on nucleic acid-hydrolyzing enzymes from various microorganisms (5). However, almost all of these enzymes are inhibited by the presence of relatively low concentrations of salts; rRNases of *Escherichia coli* (1) and *Staphylococcal* nuclease (4) are inhibited by more than 0.15 M and 0.2 M NaCl, respectively.

Maeda and Taga reported purification and properties of extracellular nuclease from a marine *Vibrio* sp. (10, 11). The enzyme exhibited high activity and strong stability when maintained in seawater. Seawater contains four major metal ions, namely, Na^+, K^+, Mg^{2+}, and Ca^{2+}, in concentrations of 0.5, 0.01, 0.05, and 0.01 M, respectively. Na^+, K^+, and Mg^{2+} did not increase enzyme stability, whereas Ca^{2+} ion was effective for the stability. It was not mentioned whether high concentrations of NaCl or KCl were required for the enzyme activity; moreover, the mode of action of the *Vibrio* nuclease is not known.

Nuclease H from a moderate halophile, *M. varians* subsp. *halophilus*, found by Kamekura and Onishi (7, 8), is the first report on a halophilic nuclease. During the course of subsequent studies on enzyme production by a variety of halophilic bacteria, the authors found that another moderate halophile, *Bacillus* sp. N23-2, produced an extracellular nuclease during growth in 1 to 2 M NaCl medium. The purification and properties of the *Bacillus* sp. halophilic nuclease are described here. The differences between the properties of purified nucleases of *Bacillus* and *Micrococcus* are shown in Table 2.

The *Bacillus* sp. nuclease resembles nuclease H in many respects such as halophilic properties, mode of action on RNA and DNA, and the reaction products. The *Bacillus* sp. enzyme required 3.5 M NaCl or 10 mM Ca^{2+} for stability and Mg^{2+} and Ca^{2+} (10:1) for maximal activity, whereas the nuclease H required 2 M NaCl, 10 mM Mg^{2+}, or Mn^{2+}, but not Ca^{2+}, for stability. The purified enzyme preparation from the *Bacillus* sp. consisted of the two isomers of the same molecular weight (138,000), but with different charges, yet the isomers were separated on gels and tested for enzyme activity.

TABLE 2. Differences between purified nucleases of the moderate halophiles *Bacillus* sp. and *Micrococcus* sp.

Nuclease source	Optimal salt concn for enzyme production	Mol wt	Isomer	Optimal salt concn for enzyme activity	Requirements of Mg^{2+}, Ca^{2+}, and Mn^{2+} for enzyme activity and stability	Optimal pH for activity	Optimal temp for activity
Micrococcus sp. (7, 8)	2 to 3 M NaCl or 3 M KCl	99,000 to 105,000	–	2.9 M NaCl or 2.1 M KCl	10 mM Mg^{2+} or Mn^{2+} effective for stability	8.0	43°C for DNase, 50°C for RNase
Bacillus sp. N23-2	1 to 2 M NaCl	138,000	Two-charge isomer	1.4 to 3.2 M NaCl or 2.3 to 3.2 M KCl	Presence of both Mg^{2+} and Ca^{2+} (10:1) required for maximal activity	8.5	50°C for DNase 60°C for RNase

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