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THE EXTRACELLULAR NUCLEASE ACTIVITY OF *MICROCOCCUS SODONENSIS*

I. ISOLATION AND PURIFICATION

SHEILA A. BERRY* AND J. N. CAMPBELL
Department of Microbiology, University of Alberta, Edmonton (Canada)

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SUMMARY

1. The extracellular nuclease produced by *Micrococcus sodonensis* was isolated and purified 1400-fold by a sequence of $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography and Sephadex G-100 gel filtration. The activity was contained in a single peak which was homogeneous to electrophoresis and ultracentrifugation. Both phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) and phosphomonoesterase activities were associated with the protein and could not be separated by any of the purification steps used.

2. Sedimentation equilibrium calculations yielded a value of 103 000 for the molecular weight.

3. Enzyme production was strongly stimulated by the presence of NH_4^+ and was maximal during the logarithmic phase of growth.

INTRODUCTION

Intra- and extracellular nucleases have been demonstrated in a wide variety of microorganisms. Deoxyribonuclease (deoxyribonuclease oligonucleotidohydrolase, EC 3.1.4.5) production has been reported in *Pseudomonas aeruginosa*[1] and group A Streptococci[2,3], and ribonucleases (ribonucleate pyrimidinenucleotido-2′-transferase (cyclizing), EC 2.1.1.16) have been isolated from *Bacillus subtilis*[4]. All the above mentioned nucleases are released extracellularly into the culture medium and appear to be specific for the DNA or RNA substrate. Other extracellular nucleases, notably those from *Serratia marcescens*[5] and *Staphylococcus aureus*[6], are non-specific in their action and will attack both DNA and RNA. *S. aureus* nuclease has been well characterized and has been of great value in the elucidation of DNA structure. It has also

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been suggested by a number of workers[7,8] that there is a correlation between deoxyribonuclease production by this organism and pathogenicity and that, therefore, deoxyribonuclease production might be of value as a diagnostic tool. Since *Micrococcus sodonensis*, a non-pathogenic member of the family Micrococcaceae, has been shown to produce large amounts of extracellular deoxyribonuclease[9], it was therefore of interest to investigate this enzyme further in an attempt to characterize and compare it with that produced by *S. aureus*.

The first paper deals with the isolation and purification of the enzyme and a preliminary report on the control of production.

MATERIALS AND METHODS

Organism and culture conditions

The organism employed in these studies was *M. sodonensis*, ATCC 11880. For enzyme isolation and purification experiments, cells were grown in Trypticase soy broth (Baltimore Biological Laboratories) and for nutrient control studies, in the synthetic medium of CAMPBELL *et al.*[10]. Ammonia, if present, was added as NH_4Cl, 0.01 M.

Cultures were grown at 30° with vigorous aeration and growth was estimated turbidimetrically or by dry weight measurements of washed cells.

Assay of enzyme activity

Phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) activity was detected qualitatively by the diffusion plate method of BERRY AND CAMPBELL[11]. Quantitative measurements were made employing a modification of the method described by REDDI[12]. The reaction mixture consisted of DNA (salmon sperm, A grade, Calbiochem), 1 mg; Tris, 33.0 μmoles; $MgCl_2$, 13.0 μmoles; $CaCl_2$, 1.7 μmoles; $MnCl_2$, 1.7 μmoles; enzyme, 0.1 ml, in a total volume of 1.0 ml, final pH 8.8. The mixture was incubated at 37° for 16 h. One unit of diesterase activity was defined as that amount of enzyme causing an increase of 0.1 absorbance units at 260 mμ in the cold 10% trichloroacetic acid-soluble fraction.

Phosphomonoesterase activity was followed by measuring the release of inorganic phosphate using the method of FISKE AND SUBBAROW[13]. One unit of monoesterase activity was defined as that amount of enzyme releasing 1 μmole P_i per 16 h. Activities were calculated from that concentration of enzyme yielding 50% hydrolysis of the substrate after 16 h.

Chromatography

DEAE-cellulose (Eastman Kodak) was prepared by the method of PETERSON AND SOBER[14] and 1 cm × 10 cm columns were equilibrated with 0.01 M Tris (pH 8.8). 1 cm × 40 cm columns of Sephadex G-100 (Pharmacia) were prepared and equilibrated with 0.01 M Tris (pH 8.8).

Electrophoresis

Electrophoretic separation was carried out on 4 cm × 41 cm Whatman No. 1 paper strips or British Drug Houses Ltd. cellulose-acetate strips in a LKB electrophoresis apparatus at 200 V, 10 mA, for 10 h in 0.125 M veronal buffer (pH 8.6).

Sedimentation analyses

0.1% solutions of purified enzyme in 0.1 M Tris (pH 8.8) were analyzed in a Spinco Model-E analytical ultracentrifuge. Sedimentation velocity was measured with a rotor speed of 59 780 rev./min, bar angle 50° or 75°, rotor temperature 19.2°. Sedimentation equilibrium analyses employed a rotor speed of 12 590 rev./min, bar angle 50°, rotor temperature 19.2°.

Protein determination

Protein was estimated spectrophotometrically at 280 mμ or colorimetrically by the method of LOWRY *et al.*[15].

RESULTS

Enzyme production

Replicate flasks of Trypticase soy broth, synthetic medium, and synthetic medium + 0.01 M HN_4Cl were inoculated and incubated at 30°. At the designated time intervals, aliquots were removed, centrifuged, growth was measured and the supernatants assayed quantitatively for extracellular phosphodiesterase activity. The results (Table I) show that the addition of NH_4^+ to the synthetic medium resulted in an increase in growth, and also a marked increase in amount of enzyme produced per unit cell weight. Total enzyme production was highest in Trypticase soy broth, although the amount per unit cell weight was lower than in the synthetic medium + NH_4^+. Production of the extracellular enzyme was highest in young, rapidly dividing cells, falling off to a lower stable level as the culture entered the stationary phase.

Enzyme isolation and purification

The supernatant from a 48 h culture of *M. sodonensis* grown in Trypticase soy broth was brought to 0.55 saturation with $(NH_4)_2SO_4$ and held at 4° for 16 h.

TABLE I

EFFECT OF CULTURE MEDIUM AND AGE OF CELLS ON EXTRACELLULAR NUCLEASE PRODUCTION BY *M. sodonensis*

Growth medium	*Incubation time (h)*	*Cell dry weight (mg/ml)*	*Phosphodiesterase activity*	
			Units/ml culture supernatant	*Units /mg dry weight of cells*
Synthetic	24	0.360	0	0
	36	0.800	10	12
	60	1.600	210	131
	84	2.134	329	154
Synthetic + NH_4^+	24	1.164	775	666
	36	2.240	925	385
	60	2.560	925	361
	84	2.360	925	392
Trypiticase soy broth	12	1.190	688	578
	24	3.840	1143	298
	36	6.420	1500	234
	60	6.192	1463	236

TABLE II

PURIFICATION OF EXTRACELLULAR NUCLEASE FROM *M. sodonensis*

Fraction	*Phospho-monoesterase (μmoles P_i/mg)*	*Phospho-diesterase* (μmoles nucleoside/mg)*	*% recovery*	*Diesterase/monoesterase*
Crude culture supernatant	10	3	100	0.30
0.55 saturated $(NH_4)_2SO_4$ precipitate	26	22	48.5	0.84
Dialysis	42	47	48.5	1.12
DEAE-cellulose chromatography	566	513	48.5	0.92
Sephadex G-100 gel filtration	4900	4250	42.6	0.87

* 1 μmole of nucleoside is equivalent to an increase of 10 absorbance units at 260 mμ in the 10% trichloroacetic acid-soluble fraction.

The resultant precipitate was harvested, dissolved in 0.01 M Tris (pH 8.8) and dialyzed for 24 h against 0.01 M Tris (pH 8.8), which was 0.004 M with respect to both Mg^{2+} and Ca^{2+}. 5 ml of dialyzed material was chromatographed on DEAE-cellulose, and eluted with a linear gradient of 0.0 to 0.5 M NaCl in 0.01 M Tris (pH 8.8). 4-ml fractions were collected and assayed for phosphodiesterase and phosphomonoesterase activity and protein concentration. Both activities were eluted as a single peak between 0.15 and 0.25 M NaCl. Active fractions were pooled, concentrated and rechromatographed on Sephadex G-100. Activities were again recovered as a single peak which came off immediately after the void volume. The sequence and results of the purification procedures are summarized in Table II. The sequence effected a 1400-fold purification of the phosphodiesterase activity from the crude preparation with a recovery of 43% of the original total activity. At no step was it possible to obtain any separation of the mono- and diesterase activities.

The presence of contaminating phosphate in the crude culture supernatant resulted in a high value for monoesterase activity in this material. However, from the $(NH_4)_2SO_4$ fractionation step onwards, both activities were purified to the same extent (diesterase 192-fold, monoesterase 186-fold). Furthermore, with the exception of the crude material, the ratio of monoesterase to diesterase activity remained constant at each purification step.

Low-voltage electrophoresis of the purified enzyme was carried out using duplicate strips. One strip was stained for 16 h in 0.002% nigrosin in 2% acetic acid to detect protein while the duplicate was assayed for activity according to the procedure of BERRY AND CAMPBELL[11]. All the diesterase and monoesterase activity was restricted to a single band of protein which migrated 7 cm towards the anode.

Sedimentation analyses

Fig. 1 is a photograph of the schlieren diagram obtained after centrifugation at 59 780 rev./min. Photographs were taken at 16-min intervals and a calculated $s_{20,w}$ of 1.92 was obtained. A single peak was obtained which, upon further magnification, revealed a slight distortion of the leading edge indicating the presence of a small amount of contaminant, representing a maximum of 5% of the total protein. A sedimentation equilibrium analysis using the ARCHIBALD[20] approach was also carried out

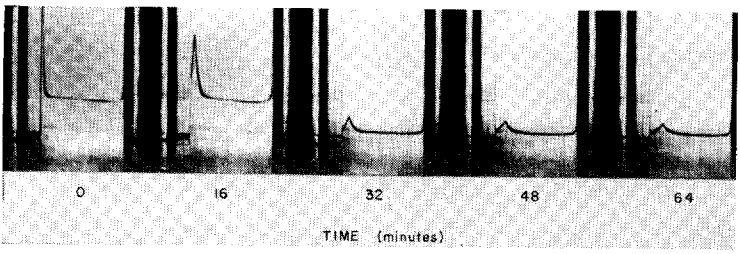

Fig. 1. Sedimentation pattern of purified *M. sodonensis* nuclease after 0, 16, 32, 48 and 64 min at 59 780 rev./min, rotor temp. 19.2°. Initial bar angle of 50° was changed to 75° after 16 min to reduce peak height. Enzyme concentration 0.1% in 0.1 M Tris (pH 8.8).

and the molecular weights at 48 and 64 min were calculated to be 104 692 and 102 600, respectively. This decrease in molecular weight with time is to be expected if in fact a heavier contaminant were present. However the fact that this decrease is small, almost within the limits of experimental error, is indicative that the contaminant must closely approximate the enzyme in weight.

DISCUSSION

M. sodonensis nuclease can be classed as an extracellular enzyme since maximun production per unit dry weight of cells occurs when the cells are in the logarithmic growth phase, at a time when autolysis is not a major factor. HIGNETT[16] reported a similar situation in *S. aureus* where nuclease production parallelled the growth curve in the early stages and declined rapidly with the onset of the stationary phase.

M. sodonensis has a specific requirement for NH_3 which can be met from an exogenous supply or endogenously by the deamination of glutamic acid[10]. It was postulated that NH_3 is required ultimately for nucleic acid biosynthesis and this has been supported by the demonstration of an NH_3-activated XMP aminase (xanthosine-5′-phosphate:ammonia ligase (AMP), EC 6.3.4.1) as well as the rapid incorporation of label from $^{15}NH_4Cl$ into the acid-soluble nucleotide pool in this organism (CAMPBELL unpublished results). From these data it is seen that NH_4^+ also stimulates enzyme production. However, increased growth-rate alone cannot be the only factor involved since, although the growth rate is highest in Trypticase soy broth, enzyme production per unit cell is lower than in the synthetic medium + NH_3. SHORTMAN AND LEHMAN[17] demonstrated that the level of endonuclease I in *Escherichia coli* is dependent upon the nature of the nitrogen source and that in its absence the level of enzyme dropped even though the cells remained in the logarithmic phase. They suggested that enzyme production might better be related to DNA synthesis in the cell. The correlation of enzyme production with the period of maximum nucleic acid biosynthesis in *M. sodonensis* leads to the postulation that the observed stimulatory effect of NH_3 on enzyme production is involved with its demonstrated role in nucleotide biosynthesis. These control mechanisms are the subject of a separate investigation.

The 1400-fold purification and 43% recovery by the purification procedures compares favourably with results achieved with other microbial nucleases[5,18,19]. The single active peak obtained on gel filtration, electrophoresis and in ultracentrifugation, the inability to separate the phosphodiesterase and phosphomonoesterase activity

at any step in the purification, and the fact that these two activities were purified to the same extent by, and maintained a constant ratio at each step of the purification, suggest the possibility of a single enzyme protein possessing 2 activities. This possibility is further considered in the next paper.

The discrepancy of the $s_{20,w}$ value of 1.92 and the calculated molecular weight of 103 000 deserves comment. The behaviour of the enzyme on Sephadex G-100 supports the calculated molecular weight value. *S. aureus* nuclease has a comparable $s_{20,w}$ value (1.8) but a smaller molecular weight (12 000) than the nuclease of *M. sodonensis*. In the latter case, the low $s_{20,w}$ value, as well as the sharpness of the peak observed in the sedimentation velocity experiments suggests an asymmetric molecule.

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