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THE RADIATION-RELEASABLE CELL WALL NUCLEASE OF *MICROCOCCUS RADIODURANS*

PURIFICATION AND PROPERTIES OF THE NATIVE ENZYME *

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Summary

Micrococcus radiodurans is known to possess a surface nuclease located in a mid-wall layer. Previous work showed that hydroxyl radicals, generated by sublethal doses of ionizing radiation, attack the cell wall and initiate release of the active enzyme into the external medium. The enzyme from unirradiated cells has now been purified to homogeneity by a simple 3-step process. The nuclease is a dimer of molecular weight about 260 000 and exonucleolytically degrades both DNA and RNA to 5′-mononucleotides. Single-stranded DNA is degraded at a rate about 200 times faster than double-stranded DNA. Oligonucleotides bearing a terminal 3′-phosphate are resistant to digestion. Dinucleotides must possess a 5′-terminal phosphate and a free 3′-terminal OH to be hydrolyzed. The enzyme has a pH optimum of about 9.0. A metal ion, possibly Ca^{2+}, appears to be tightly bound to the protein. Removal of this metal with EDTA inactivates the enzyme. Simple readdition of Ca^{2+} does not restore activity although partial function can be recovered by renaturation from urea in the presence of this ion.

The availability of pure enzyme should aid in the detection of radiation induced alterations which may be involved in the mechanism of its release from the cell wall.

Introduction

Micrococcus radiodurans is an extremely radiation resistant organism [1]. High radioresistance makes this bacteria a useful subject for the study of the

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Abbreviations: Ap, Adenosine 3′-monosphosphate; dpA, deoxadenosine 5′-monophosphate; d(NpN ... pNpN), a deoxyoligonucleotide containing 3′-5′ phosphodiester links but possessing to terminal phosphate.

effects of ionizing radiation on cellular structures, since complications due to degradative effects in dead and dying cells are eliminated. We have shown that hydroxyl radicals (OH ·) generated in the surrounding aqueous medium during a sublethal dose of γ-radiation attack the cell wall of this organism [2]. As a result of this damage a variety of proteins and at least one polysaccharide are immediately released from the cell [3,4]. These solubilized components originate from a membrane-like mid-wall layer [3,4] described as rich in lipids and containing the cell wall pigments [5]. The release is selective since a number of other proteins present in this layer remain bound [3].

One of the proteins freed from this wall structure is a nuclease [6]. Because the release mechanism appears to be due only to the action of free radicals [2,4] and not to an enzymatic hydrolysis [7], we consider that it may have general importance since ionizing radiation generates hydroxyl radicals wherever water is found inside the cell. In an attempt to understand this mechanism we wish to compare the nuclease extracted from unirradiated cells with that released by radiation. This paper describes the purification and properties of the native nuclease. Because of its ease of preparation, high activity and marked preference for single-stranded DNA, the enzyme should additionally be useful for a variety of nucleic acid studies.

Materials and Methods

Sources of materials and supplies were as follows: Tryptone and yeast extract from Difco Laboratories; glucose from Baker Chemical Company; sodium deoxycholate from Matheson, Coleman, and Bell; deoxyribonuclease I (EC 3.1.4.5), micrococcal nuclease (*Staphylococcus aureus* EC 3.1.4.7), β-galactosidase (EC 3.2.1.23), xanthine oxidase (EC 1.2.3.2), phosphorylase *a* (EC 2.4.1.1), alkaline phosphatse (EC 3.1.3.1), and venom phosphodiesterase (EC 3.1.4.1) from Worthington Biochemical Corporation; pepsin (EC 3.4.4.1) and aldolase (EC 4.1.2.13) from Nutritional Biochemical Corporation; bovine serum albumin, nucleosides, nucleotides, and salmon sperm DNA from Calbiochem; *Escherichia coli* ribosomal RNA from the British Drug House Limited; Sephadex G-200 and Sepharose 4B from Pharmacia Fine Chemicals; Whatman DEAE-cellulose DE-52 from Mandel Scientific. All chemicals were reagent grade.

The procedures for the growth, preparation and irradiation of *M. radiodurans* cells have been described [6]. Units of exonuclease activity were determined by measuring the rate of release of acid-soluble nucleotides from heat-denatured salmon sperm DNA as detailed elsewhere [6]. All digestions were in the range of linearity with respect to both time and enzyme concentration. Protein was assayed by the method of Lowry [8] except that the absorbance at 280 nm was used to monitor elution of protein from chromatographic columns. Inorganic phosphate was measured by the method of Chen [9].

The SDS polyacrylamide gel electrophoresis procedure used [3] was basically that of Weber and Osborn [10] except that prior to separation the samples were heated at 100° C for 3 min in their SDS/phosphate/mercaptoethanol buffer instead of being incubated at 37° C for 2 h.

Enzyme purification

Step 1. 4 g wet packed cells were suspended at 0°C in 20 ml of 5 mM Tris · HCl (pH 9.0) previously saturated at 0°C with *n*-butanol. The cells were removed by centrifugation and resuspended as before. The supernates from the two extractions were pooled and lyophilized.

Step 2. The lyophilized preparation from Step 1 was dissolved in 12 ml of 5 mM Tris · HCl (pH 9.0) containing 1% sodium deoxycholate, and chromatographed on a column of Sepharose 4B (2.5 × 85 cm) run in the same buffer/deoxycholate mixture. The elution profile is shown in Fig. 1 and the fractions indicated by the bar were pooled.

Step 3. The peak of activity from Step 2 was passed through a column of DEAE cellulose DE-52 (2.5 × 20 cm) equilibrated and eluted with 5 mM Tris · HCl (pH 9.0). The activity did not bind to the column.

Preparation of substrates

5'-Phosphate terminated di- and oligonucleotides. These were produced by the action of DNAase I on native DNA [11]. The digestion mixture was boiled for 15 min and applied to a column of DEAE-cellulose DE-52 (1.5 × 30 cm) carbonate form, in 10 mM $(NH_4)_2CO_3$. The column was eluted with a 1 l linear gradient of $(NH_4)_2CO_3$, 10 mM to 1.0 M. The dinucleotides were removed and all material eluting after them and prior to 0.6 M salt was pooled. Samples were evaporated to dryness under vacuum to remove $(NH_4)_2CO_3$.

Oligonucleotides lacking terminal phosphate. These were prepared from the 5'-phosphate terminated oligonucleotides by treatment of 50 A_{260} units with 200 μg of alkaline phosphatase at 37°C for 2.5 h. The 1 ml incubation mixture contained 100 mM Tris · HCl (pH 8.0) and 10 mM $MgCl_2$. The preparation was boiled and chromatographed on DEAE-cellulose under the same conditions as described for the DNAase I hydrolysis mixture.

Oligonucleotides bearing a terminal 3'-phosphate. These were produced by digestion of native DNA with *Staphylococcus aureus* nuclease under limiting conditions (4 mg/ml DNA, 0.4 μg/ml enzyme, 10 mM $CaCl_2$, 50 mM Tris · HCl (pH 9.0) 25°C 12 min). The hydrolysis was stopped with EDTA, the mixture boiled for 10 min and applied to a DEAE cellulose column (1.5 × 30 cm) carbonate form. The mono- and dinucleotides were eluted with a 1-l linear $(NH_4)_2CO_3$ gradient, 10 mM—300 mM. Longer oligonucleotides were removed with a 0.6 M $(NH_4)_2CO_3$ wash. Salt was removed from samples by drying under vacuum.

Dinucleotides lacking terminal phosphate. These were prepared from the

TABLE I

PURIFICATION OF *M. RADIODURANS* CELL WALL EXONUCLEASE

Purification step	Total units $\times 10^{-3}$	Specific activity units/mg · 10^{-3}	Yield (%)
1 *n*-butanol/buffer extract	252	6.5	100
2 Sepharose 4B chromatography	180	52.8	71.4
3 Passage through DEAE-cellulose	179	81.4	71

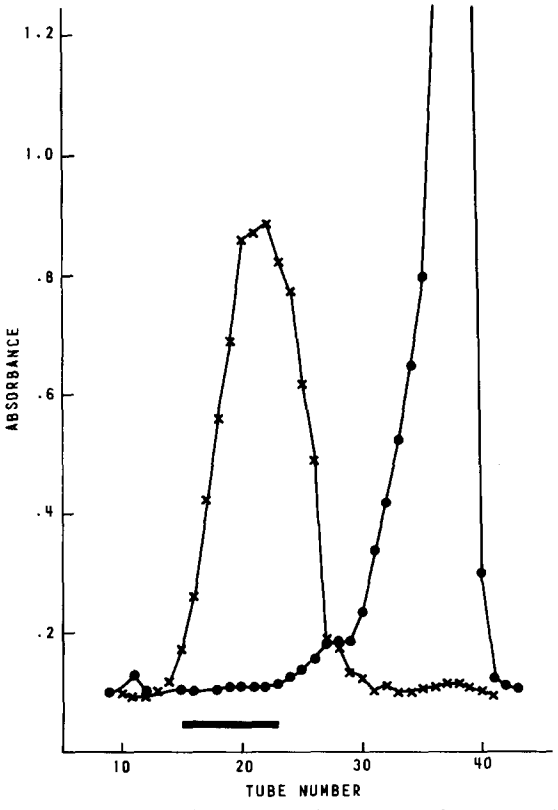


Fig. 1. Elution profile of *M. radiodurans* surface exonuclease chromatographed on Sepharose 4B in the presence of sodium deoxycholate. ●———●, A_{280} (protein) X———X, A_{260} (exonuclease activity).

S. aureus nuclease generated dinucleotide samples by treatment with alkaline phosphatase as described above followed by rechromatography on DEAE cellulose.

M. radiodurans exonuclease digestion of various substrates and analysis of products

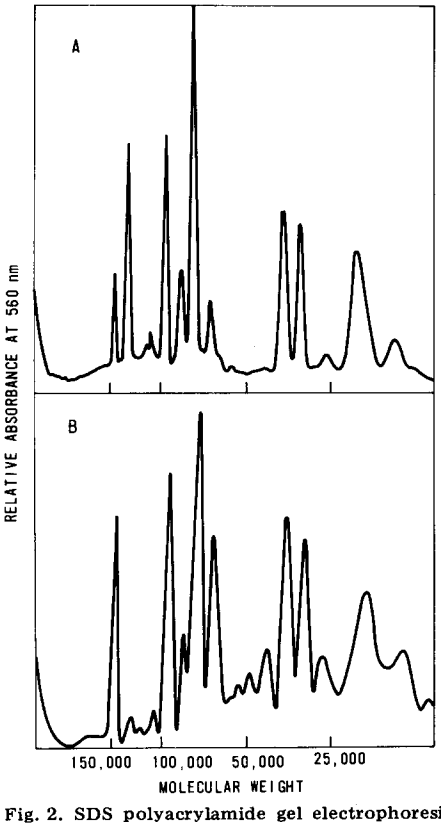
Digestions were done under the standard assay conditions of 0.1 M Tris · HCl (pH 9.0) and 10 mM $MgCl_2$ at 30°C. Samples were incubated for 2 h with 10—20 units of enzyme per A_{260} unit of substrate. To ensure complete hydrolysis some digestions were incubated overnight. The products of digestion were separated and identified by DEAE cellulose column chromatography, as described above, and by thin layer chromatography [12]. Dinucleotides and dinucleoside monophosphates were identified by hydrolysis with snake venom phosphodiesterase [13] followed by thin layer chromatography.

Results

Comparison of proteins and enzyme levels in extracts of irradiated and unirradiated cells

I have previously shown that the major protein released into the surrounding

medium during the γ-irradiation of *M. radiodurans* migrates during electrophoresis on SDS polyacrylamide gels with a mobility corresponding to a molecular weight of about 125 000 [3]. Isolation of the mid-wall layer from irradiated cells showed a loss of this protein and a corresponding loss in enzymatic activity [3]. I have also shown the release of the enzyme activity from whole cells by the *n*-butanol extraction procedure [2]. In Fig. 2 the proteins present in the *n*-butanol/buffer extracts (Step 1 of the purification scheme given in Materials and Methods and Table I) from normal and γ-irradiated cells are compared by SDS polyacrylamide gel electrophoresis. The irradiated cells, suspended in 5 mM Tris · HCl (pH 9.0), received 500 krad under N_2 at 0° C and were washed twice with buffer prior to the extraction procedure. A selective



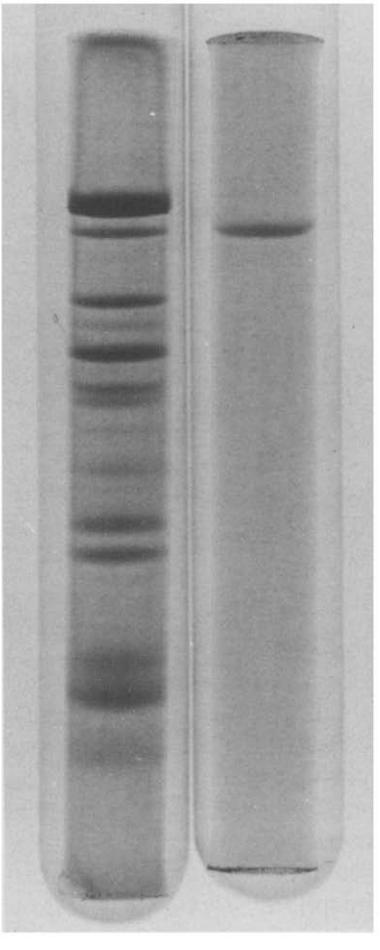


Fig. 2. SDS polyacrylamide gel electrophoresis patterns of the proteins present in extracts of cells prepared as in Step 1 of the purification procedure. A, normal cells; B, cells previously γ-irradiated under nitrogen to 500 krad. The peak at 125 000 daltons is absent. Approximately three times as much protein was placed on gel B as compared to gel A. Electrophoresis, as depicted, was from left to right.

Fig. 3. SDS polyacrylamide gel electrophoresis patterns of the proteins present after Step 1 (left) and Step 3 (right) of the purification procedure.

disappearance of the 125 000-dalton protein peak from the extract of irradiated cells can be seen. The exonuclease activity in the extract from irradiated cells was 5% of that in the extract from unirradiated cells. All these data suggested that the nuclease located in the mid-wall layer and released both by radiation-initiated hydroxyl radical attack [2] and by *n*-butanol/buffer extraction was a protein of molecular weight 125 000 when measured under SDS denaturing conditions.

Enzyme purification

The native enzyme was purified to apparent homogeneity by a three step process involving extraction from whole cells by Tris · HCl buffer saturated with *n*-butanol, chromatography on a column of Sepharose 4B in the presence of a detergent (Fig. 1) and final passage through a column of DEAE cellulose. The purification scheme is given in Table I along with the yield and specific activity after each step. Fig. 3 shows the SDS gel electrophoresis patterns of the extracted proteins and the purified enzyme. As shown previously [2], extraction of the cells with an *n*-butanol saturated buffer disrupts the mid-wall layer where this enzyme is located and allows its release to the outside of the cell. This procedure does not cause cell lysis and, as can be seen from Fig. 3, it releases a minimum of other proteins.

Chromatography of the extracted proteins on Sepharose 4B in the absence of deoxycholate caused the enzyme to be eluted in the void volume, as an aggregate with many of the other proteins present (data not shown). Removal of free lipids by $CHCl_3$/methanol extraction [14] did not prevent this aggregation. In the final step of the purification procedure, the enzyme did not bind to the DEAE cellulose. However, some other contaminating protein was removed, as was the deoxycholate remaining from the Sepharose 4B chromatography.

Stability

Rapid freezing in dry-ice/ethanol, lyophylization and rehydration of the *n*-butanol/buffer extract caused no apparent loss of activity. However, freezing at −20° C, storage for 5 days at that temperature and rethawing caused approximately one-third of the activity to be lost. The purified enzyme stored in 10 mM Tris · HCl (pH 9.0) at 3° C lost approximately 10% activity per week.

Molecular weight

The molecular weight of the enzyme was determined by two procedures: agarose gel chromatography on Sepharose 4B in the presence of deoxycholate and polyacrylamide gel electrophoresis in the presence of SDS. Both systems were calibrated with proteins of known molecular weight [10,15]; the Sepharose 4B column with xanthine oxidase and aldolase, and the polyacrylamide gel with bovine serum albumin, β-galactosidase, phosphorylase *a*, ovalbumin and pepsin. The mobilities of proteins in the electrophoresis system were calculated relative to a Bromphenol Blue tracking dye and were linearly related to the logarithms of their molecular weight [10].

The molecular weight of the enzyme obtained by Sepharose 4B gel chromatography in the presence of deoxycholate was 260 000, and the value obtained by polyacrylamide gel electrophoresis in the presence of SDS was

125 000. This result suggests that the enzyme is normally a dimer, and can be disaggregated by heating in SDS into two subunits of 125 000 daltons each. The SDS-treated enzyme is inactive.

Effect of sulfhydryl reagents

As described in Methods, the samples prepared for SDS polyacrylamide gel electrophoresis were normally heated in the presence of mercaptoethanol. However, the absence of a mercaptan had no influence on the electrophoretic mobility of the purified enzyme. Likewise, the presence or absence of 10 mM dithiothreitol during chromatography on Sepharose 4B (normally run without mercaptan) had no effect on mobility. These results suggest the absence of intra-subunit disulfide bonds. Incorporation of 10 mM dithiothreitol or 1 mM *p*-chloromercuribenzoate into the assay mixture had no significant effect on the activity of the enzyme, suggesting that the active site does not involve a sulfhydryl group.

Effect of pH and buffer concentration

The influence of pH on the activity of *M. radiodurans* exonuclease is depicted in Fig. 4, and in Tris · HCl an optimum of 9.0 is indicated. Over the range shown, the linear release of acid soluble nucleotides with increasing digestion time indicated that the enzyme was stable for at least 60 min under the standard assay conditions of excess substrate. At pH values over 9.6 however, increasing instability was shown by a non-linear release with time. Because of this probable protein denaturation, activity measurements at pH values >9.6 are not meaningful under these conditions and are not included in the figure.

Exonuclease activity at various concentrations of Tris · HCl (pH 9.0) buffer was also examined. Approximately 20% stimulation was achieved by increasing

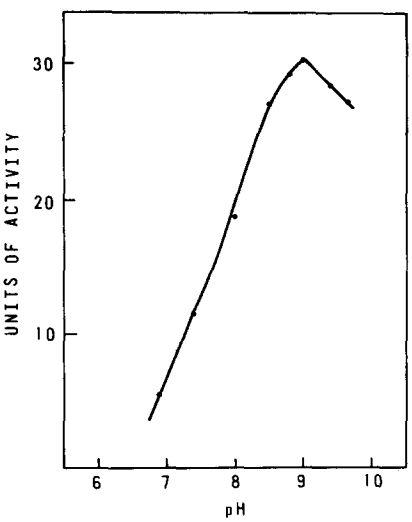


Fig. 4. Variation in exonuclease activity with pH. The enzyme was assayed under the standard assay conditions except that the pH of the Tris · HCl buffer was varied.

the buffer concentration from 0.02 M to 0.1 M; maximum activity was then sustained to 1.0 M.

Role of metal ions

The purified enzyme was active in the absence of added metal ions but was stimulated approximately two-fold by the addition of 10 mM $MgCl_2$, the conditions used in the normal assay mixture. Stimulation by 10 mM $CoCl_2$ was less effective (1.5-fold stimulation). 10 mM $MnCl_2$ was partially inhibitory (36% activity remained) and the presence of 10 mM of either $CuCl_2$, $ZnCl_2$ or $CaCl_2$ was completely inhibitory. Removal of the added metal by dialysis reversed the above effects. However addition of 1 mM EDTA completely inactivated the enzyme. Unsuccessful attempts to reverse this inactivation included dialysis and/or the addition of the following metal ions (all as the chloride), at several concentrations (up to 100 mM): Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}. Activity could only be restored by allowing a urea-unfolded preparation of EDTA inactivated enzyme to renature in the presence of the appropriate metal ion. Inactivated samples were made 8 M in urea and then dialyzed out of the urea into 100 mM Tris · HCl (pH 8.0) containing either 10 mM $CaCl_2$, $MnCl_2$, $MgCl_2$, $CoCl_2$ or no added metal ion. Excess metal ions were then removed by dialysis against buffer only, and the samples assayed for activity. Only the samples dialysed in the presence of Ca^{2+} possessed active enzyme. Recovery of activity in these samples ranged between 10% and 55% in various experiments.

Mode of enzymatic hydrolysis

Fig. 5 shows the relative rates of hydrolysis of native and heat-denatured salmon sperm DNA and *E. coli* ribosomal RNA digested under the standard assay conditions. The rate of hydrolysis of double-stranded DNA was approximately 0.5% of that of single-stranded DNA. Ribosomal RNA was hydrolyzed at about 38% the rate of single-stranded DNA.

Fig. 6 shows the product distribution on Sephadex G-200 (0.9 × 55 cm, 100 mM Tris · HCl (pH 9.0)) after approximately 35% hydrolysis of single-stranded DNA. Digestion was stopped by boiling and the entire sample was applied to the column which was then eluted with 100 mM Tris · HCl (pH 9.0). Peak A, eluting at the column void volume, contained the residual undigested DNA. Peak B contained only deoxy-5′-mononucleotides. The absence of intermediate sized nucleic acid fragments suggests the enzyme acted as an exonuclease.

Table II shows the products obtained from the digestion of DNA, RNA, and various nucleotides and oligonucleotides prepared, digested and analyzed as described in Methods. The enzyme completely digested single-stranded DNA and RNA to 5′-mononucleotides. Oligonucleotides possessing free 3′-OH and 5′-phosphate terminal groups were also degraded to 5′-mononucleotides. Oligonucleotides without a 5′-terminal phosphate had the 5′-terminus released as a dinucleoside monophosphate, which was resistant to further hydrolysis. A terminal 3′-phosphate inhibited digesion. The purified enzyme was assayed for non-secific phosphatase and 5′-nucleotidase [12] activity with Ap (2′ + 3′) and dpA respectively as substrates. After incubation for 2 h under the exo-

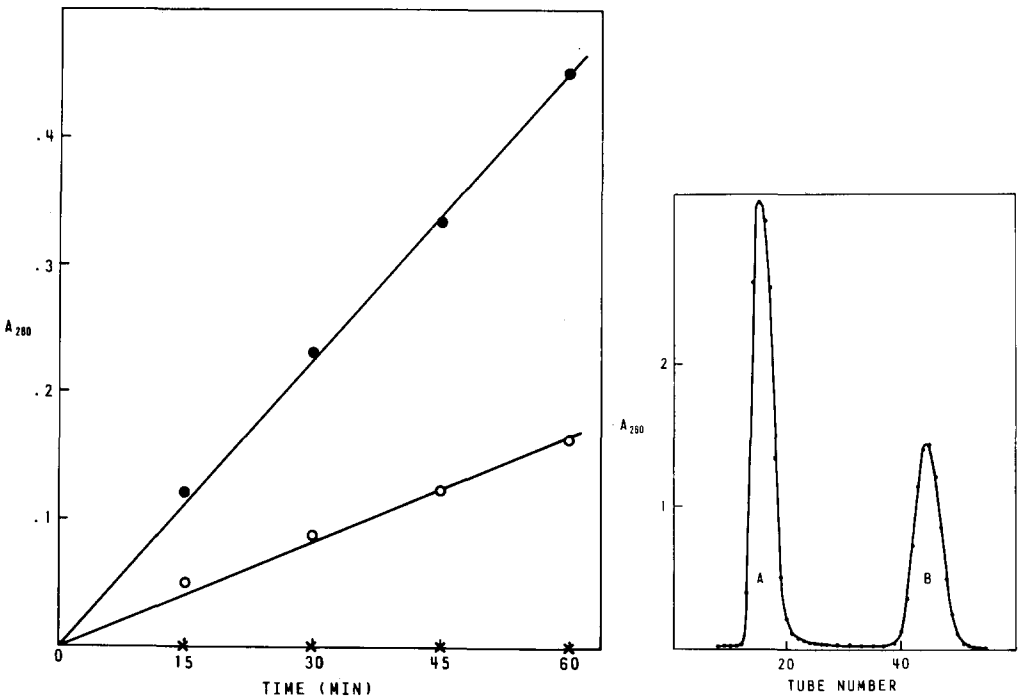


Fig. 5. Relative rates of hydrolysis of nucleic acids with *M. radiodurans* exonuclease. ●———●, single stranded DNA; X———X, double stranded DNA; ○———○, ribosomal RNA.

Fig. 6. Sephadex G-200 chromatography of the products of a partial digestion of single stranded DNA. Peak A contains unhydrolyzed DNA. Peak B contains deoxy 5′-mononucleotides.

TABLE II

PRODUCTS OF DIGESTION OF VARIOUS SUBSTRATES WITH *M. RADIODURANS* EXONUCLEASE

Substrate	Digestion products
single stranded DNA	dpN
ribosomal RNA	pN
d(pNpN...pNpN)	dpN
d(NpN...pNpN)	dNpN + dpN
d(NpN...pNpNp)	—
d(pNpN)	dpN
d(NpN)	—
d(NpNp)	—
Ap(2′ + 3′)	—
dpA	—

nuclease assay conditions using 15 units of enzyme per A_{260} unit of substrate, release of inorganic phosphate could not be detected in either case.

Discussion

The cell wall nuclease of *M. radiodurans* appears to be an exonuclease, highly specific for single strands, and which releases 5′-mononucleotides from both

RNA and DNA. The reduced rate of digestion of RNA suggests that a substrate bearing a 2′-OH may somewhat slow the enzyme. The presence of a terminal 3′-phosphate on oligonucleotides inhibited the digestion suggesting that hydrolysis proceeds in a 3′ → 5′ manner. The α-terminal dinucleotide can be hydrolyzed only if it possesses a 5′-phosphate. Oligonucleotides lacking this phosphate have their 5′-terminus released as a dinucleoside monophosphate. This property should make the enzyme useful in the identification of 5′-terminal nucleotides. The enzyme is free of phosphatase activity.

Added metal ions are not essential for activity although 10 mM $MgCl_2$ gave a 2-fold stimulation. Inhibition by EDTA appears to result from the removal of a tightly bound metal which may be required for the maintenance of tertiary structure, since activity could not be restored by the simple readdition of any of the metal ions tested. However, unfolding the inactive enzyme in urea and refolding in the presence of Ca^{2+} allowed a partial recovery of activity. Of the metal ions tested, renaturation depended specifically on Ca^{2+} suggesting that this may be the natural ion present. It is interesting to note that unbound Ca^{2+} is inhibitory. This result and the stimulating effect of Mg^{2+} suggest that a second metal binding site may be involved with catalytic activity or substrate binding.

Previous results showed that the enzyme was not released by aqueous extraction of the cell, only poorly released by detergents, but completely released in the presence of an organic solvent [2]. We have noted here that the extracted enzyme has a marked tendency to aggregate in the absence of detergents. These two observations suggest that the protein may have a hydrophobic character and be held in its cell wall binding site by hydrophobic interactions. Preliminary comparisons have been made between this enzyme as extracted from unirradiated cells and as released by ionizing radiation. Earlier data has suggested that after release by radiation the enzyme continues to have a monomer molecular weight of 125 000 [3]. There also appears to be no change in catalytic properties [6] but the radiation released nuclease is not amenable to the isolation procedure given here. For example it elutes as a very broad peak from Sepharose 4B and it is inactivated by exposure to DEAE cellulose. Attempts are being made to determine the changes responsible for these alterations in the enzymes physical properties. It is hoped that an understanding of these modifications will provide some insight into the mechanism of its radiation induced release from the cell wall.

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