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Lactobacillus plantarum Exoribonuclease. Further Purification and Characterization*

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ABSTRACT: *Lactobacillus plantarum* exoribonuclease, a processive nuclease, has been purified to essential homogeneity. This has been achieved by elution of the enzyme from phosphocellulose columns with RNA and KCl. The purified enzyme gives rise to a single band on disc gel electrophoresis. The molecular weight of the enzyme measured by gel chromatography is 9.0×10^4. The enzyme apparently degrades RNA in the 3′ to 5′ direction.

Partial purification of the processive nuclease *Lactobacillus plantarum* exoribonuclease has already been published (Logan and Singer, 1968). Further studies on the processive mode of degradation and physical chemistry of the enzyme (Gardonio and Logan, 1971) have however required a more highly purified enzyme. In this paper we report on the further purification of the enzyme to essential homogeneity. Substantial purification has been achieved by the technique of substrate elution of the enzyme from phosphocellulose columns using yeast RNA as substrate. In addition the molecular weight and direction of degradation of the enzyme have been determined.

Materials and Methods

The methods of maintaining, growing, and harvesting *Lactobacillus plantarum* (ATCC8014) were described previously (Logan and Singer, 1968). Enzyme activity was also assayed as previously described with the exception that 0.25 M Tris (Fisher) was used in place of 0.25 M 2-amino-2-methyl-1,3-propanediol·HCl buffer.

For fraction IV a different initial assay was used.

The RNA which elutes with the enzyme from the phosphocellulose column was used as the enzyme's initial substrate in reaction mixtures supplemented with appropriate amounts of NH_4Cl and $MgCl_2$. Several incubation periods were used (0, 0.5, 1, 3, and 5 min) in each assay to determine at which times linear breakdown occurred. The reaction was stopped by addition of 0.1 ml of carrier RNA solution (Singer and Tolbert, 1965) and the addition of 0.4 ml of cold 95% ethanol. The reaction tubes were chilled to 0° and after 10-min incubation they were then centrifuged at 3500*g* for 10 min. The supernatant (300 μl) was diluted to 1 ml with water and the absorbance at 260 mμ was recorded. The column fractions showing activity were then incubated at 37° in the presence of 1.5 mM $MgCl_2$ and 0.4 M NH_4Cl until the accompanying RNA was degraded. (This degradation often required several hours.) The reaction mixture was then dialysed against water until free of RNA nucleotides and salts and an aliquot of the remaining enzyme was then assayed by the standard assay procedure.

Enzyme fractions I, II, and III were prepared as previously described with the exception of the initial cell lysis step. Frozen cells were thawed and mixed into a thick slurry by the addition of cold 0.02 M Tris·HCl (pH 7.8) (approximately one-fifth volume). This slurry was then frozen in a pressure cell and the cells were broken in a modified Hughes press. The lysate was suspended in twice its volume of the above buffer and the purification was carried out as described by Logan and Singer (1968).

$(pA)_3$* poly(A) in which the 5′-terminal trinucleotide is labeled with ^3H was prepared by Dr. N. G. Nossal and was generously supplied by Dr. M. F. Singer. Degradation of total polymer material and terminally labeled material was measured as described by Nossal and Singer (1968).

Protein was determined by the method of Lowry *et al.* (1951).

Polyacrylamide disc gel electrophoresis was performed by the method of Clarke (1964) as modified by Tobe and Loughton (1969). All samples were run on 7.5% polyacrylamide gels with a constant current of 3 mA/gel tube.

Sephadex G-100 (Pharmacia), DEAE (DE-32, Whatman), and phosphocellulose (P-11, Whatman) were prepared according to the manufacturer's instructions.

Molecular weight was determined from the position of elution of the enzyme from a Sephadex G-100 column (2.5 × 87 cm) as described by Andrews (1964; Andrews and Folley,

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TABLE I: Typical Purification of *L. plantarum* Exoribonuclease.

Fraction	Total Protein (g)	Sp Act. (Units/mg)	Total Units	Recov (%)
I (Centrifugation)	14.5	4.6	66,600	100
II $(NH_4)_2SO_4$	6.62	12.0	79,200	118
III DEAE	0.800	42.0	33,600	50
IV Phosphocellulose[a,b]	0.0032	3870	12,400	19
V Phosphocellulose	0.0012	14880	17,860	27

[a] The activity measurement for fraction IV is hampered by contaminating carrier RNA and represents a probable minimum. [b] The reported recoveries of protein and activity in fractions IV and V are calculated by correcting the measured recovery as if all the previous fraction had been used; *i.e.*, 12% of fraction III was applied to the column to give fraction IV and one-half of this fraction was used as the input to fraction V.

1963). The standard proteins cytochrome *c* (horse heart), chymotrypsinogen A (beef pancreas), ovalbumin, albumin (bovine), γ-globulins (human), and apoferritin (horse) were purchased from Mann Research Laboratories. The standard proteins were run in combinations of three and the enzyme was run with two reference proteins. The void volumn, v_0, was determined with Blue Dextran 2000.

Results

Enzyme Purification. Unless otherwise stated all operations were performed at 0–4°. A summary of a typical purification is given in Table I. Fractions I, II, and III were prepared as previously reported (Logan and Singer, 1968). Fraction III protein (96 mg) in 6.5 ml was diluted 10-fold with buffer containing 0.02 M Tris·HCl (pH 7.8)–20% glycerol and applied (1 ml/min) to a phosphocellulose column (4 × 17 cm) equilibrated with the same buffer. The column was then washed with approximately two bed volumes of buffer and the enzyme was then eluted with 0.02 M Tris·HCl (pH 7.8) containing 20% glycerol and 100 μg/ml of carrier yeast RNA (Eley, 1969). Fractions (2 ml) were collected at a flow rate of approximately 1 ml/min.

The enzyme eluted with the RNA front. A typical elution profile can be seen in Figure 1. All fractions containing activity were pooled (fraction IV).

Fraction IV (122 ml) containing 0.38 mg of protein was then added at 15 ml/hr to a second phosphocellulose column (2.5 × 17 cm) which had been equilibrated with 0.02 M Tris·HCl (pH 7.8) containing 50% glycerol (v/v). The column was washed with the same buffer until the eluate was free of A_{260} millimicrons.

The enzyme was then eluted with 200 ml of 0.02 Tris·HCl (pH 7.8) containing 50% glycerol and 0.4 M KCl. Fractions (4 ml) were collected.

A typical elution profile can be seen in Figure 2. Contaminating RNA was always found to be present in the peak corresponding to enzyme activity. The peak fractions were pooled. dialyzed, and concentrated under vacuum to approximately 1 ml (fraction V).

Enzyme Purity. Fractions IV and V were analyzed by electrophoresis on 7.5% polyacrylamide gels (Figure 3), several minor bands in fraction IV having been eliminated between fractions IV and V. Fraction V shows a single protein band and a single band is also found after 8.0 M urea treatment of the enzyme suggesting the presence of a single protein species. Unstained gels were cut lengthwise into two sections. One section was stained for protein and the other was sectioned into pieces 2 mm long. These were assayed for enzyme activity by adding the gel fragment to an otherwise complete assay mixture and treating as a standard enzyme assay. In all cases the protein band and enzyme activity occur in the same gel position.

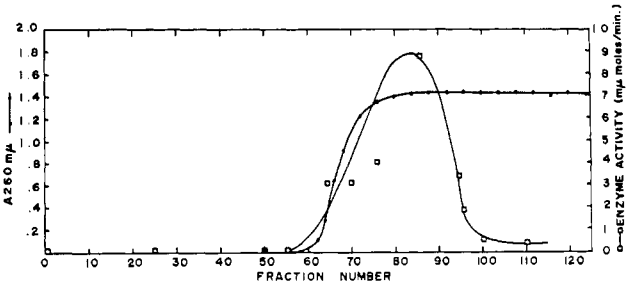

FIGURE 1: Elution of exoribonuclease from phosphocellulose with yeast RNA. The chromatography conditions are described in the text. The enzyme activity was assayed by the modified procedure described under Materials and Methods.

Ionic Requirements of Fraction V. Table II shows the results of experiments to test the ionic requirements of fraction V. The requirements for both Mg^{2+} and NH_4^+ ions found with less purified enzyme fractions remain unchanged.

Molecular Weight Determination. Figure 4 illustrates data obtained by chromatography of fraction IV enzyme and several molecular weight markers on Sephadex G-100. The relative elution position is intermediate between those for cytochrome *c* (12,400), chymotrypsinogen (25,000), ovalbumin (45,000), bovine albumin (67,000), and the higher molecular weight γ-globulins (160,000) and apoferritin (480,000).

From a regression line fitted to the data a molecular weight of 9.0×10^4 for the exoribonuclease can be interpolated.

Direction of Degradation. The direction of degradation of the enzyme was determined by a modification of the standard method, *i.e.*, the degradation of polymers selectively labeled in either the 3′ or 5′ end. With a random exonuclease,

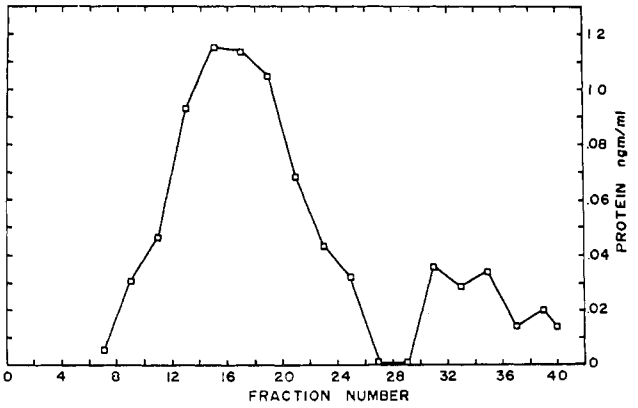

FIGURE 2: Elution of exoribonuclease from phosphocellulose with buffer containing 0.4 M KCl. Enzyme activity is confined to the leading peak but is not included because of the very low absolute activity prior to concentration and dialysis.

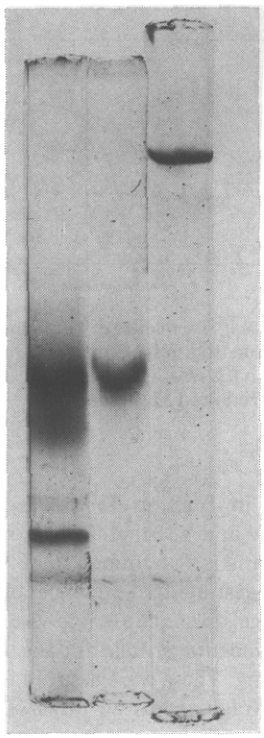

FIGURE 3: Disc gel electrophoresis of (left to right) fraction IV, fraction V, and fraction V electrophoresed in the presence of 8 M urea.

label on the end of the polymer which is degraded first will be released first, *i.e.*, before an equivalent amount of total polymer material has been released. However with a processive enzyme such as the Lactobacillus enzyme the release of terminal label is a linear function of total polymer degradation regardless of the site of labeling provided only that the relative number of enzyme molecules to polymer molecules is small. To overcome this problem, the incubation mixture as indicated in the legend to Figure 5 was adjusted first to give a large number of enzyme molecules and second to reduce the temperature sufficiently to slow down the reaction significantly. Figure 5 illustrates the degradation of $(pA)_3$*; at 38° the terminal label is released slightly slower

TABLE II: Ionic Requirements of Fraction V Enzyme.[a]

Expt	Reaction Mixture	Act. (cpm)
1	Complete	1844 (100)
	$-NH_4$	184 (10)
	+0.1 M EDTA	72 (3.9)
2	Complete	1566 (100)
	$-NH_4$	90 (5.8)
	$-Mg^{2+}$	72 (4.6)

[a] All assays were carried out by the standard assay technique with the reaction mixture altered as indicated above. Activity is expressed as counts per minute of 5′-AMP released from [^{3}H]poly(A) (4.5×10^4 cpm/μmole of polymer nucleotide). Approximately 3.4 μg of fraction V enzyme was added in each case. The figures in parentheses are the residual activity as a percentage of that found with the complete system.

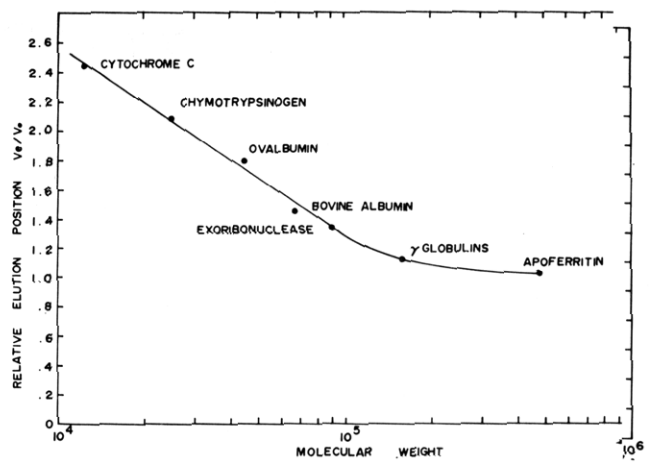

FIGURE 4: Relative elution position of exoribonuclease and molecular weight markers on Sephadex G-100. The apparent molecular weight of the exoribonuclease is 9.0×10^{-4}.

than total polymer material but the difference is essentially negligible. At 14°, however, considerable polymer degradation occurs before the labeled nucleotides (5′ end) begin to be released. It therefore appears that the enzyme degrades in the 3′ to 5′ direction.

Discussion

The processive mode of polymer degradation which is found with *L. plantarum* exoribonuclease makes this enzyme of particular interest. Further physical studies on the enzyme however have been hampered by the difficulty in obtaining sufficient amounts of highly purified enzyme. This difficulty has been overcome using the technique of substrate elution

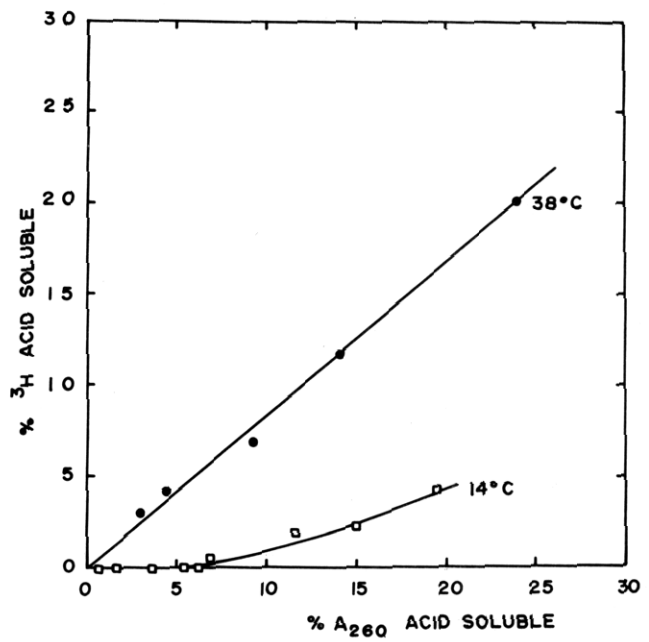

FIGURE 5: Degradation of $(pA)_3{}^{3H}$ poly(A) by exoribonuclease. Standard reaction mixtures were supplemented with 1.26 A_{260} units of $(pA)_3{}^{3H}$ poly(A) and sufficient fraction III enzyme to give 100% degradation in approximately 300 min at 38°. Samples were removed at intervals of 0–60 min and assayed for the release of acid-soluble A_{260} and acid-soluble counts per minute.

from phosphocellulose columns as described by Eley (1969). By this technique we have obtained milligram amounts of essentially pure enzyme and there appear no technical limitations to scaling up the process for the production of larger amounts. The uses and future of phosphocellulose columns in the purification of enzymes which act on nucleic acids have been discussed by Eley; however one difficulty arises in the phosphocellulose chromatography of the Lactobacillus enzyme.

After elution of the enzyme with RNA from the first phosphocellulose column and throughout the second chromatography the presence of contaminating RNA interferes dramatically with the enzyme assay. In contrast to Eley's studies with chicken pancreas nuclease the RNA is not removed completely on the second (salt elution) column. We have resorted to extended incubation periods allowing degradation of the accompanying RNA followed by dialysis to eliminate the RNA. Unfortunately contaminating RNA (<2% by weight) is often still retained. However, when fraction IV or V enzymes are subjected to electrophoresis on acrylamide gels the enzyme which can be recovered from the gels is now free of detectable RNA. We anticipate that the use of preparative disc gel electrophoresis will provide us with completely pure protein for our future studies.

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Spectral Properties of the Chromophoric Material Associated with the Deoxyribonucleic Acid Photoreactivating Enzyme Isolated from Baker's Yeast*

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ABSTRACT: A DNA photolyase, heretofore referred to as a DNA-photoreactivating enzyme, was purified 70,000-fold from Baker's yeast. The last step in the procedure involved chromatography on far-uv-irradiated DNA noncovalently bound to cellulose. The final enzyme preparation absorbed near-uv light between 350 and 420 nm and exhibited maximum absorbance at about 380 nm. Maxima at 385 and 485–490 nm appeared in its excitation and fluorescence spectra, respectively. The molecular weight of the enzyme determined by gel filtration was 53,000. The photolyase was reversibly inactivated and reactivated by treatment first with ferricyanide and then with 2-mercaptoethanol. The fluorescent material was not removed from the enzyme by either chromatography on hydroxylapatite or Sephadex G-100, provided during the latter procedure the salt concentration in the buffer was maintained at 0.4 M. Partial dissociation of the fluorescent material occurred when salt-free buffer was used to elute the enzyme from the Sephadex column and loss of activity ensued. The spectral properties of the fluorescent materials associated with the DNA photolyase are those expected for the chromophore involved in DNA photoreactivation.

Photoreactivation may be defined as restoration of activity to biological material by illumination with longer wavelengths of radiation than that which engendered the inactivation. Significant contributions to our understanding of this phenomenon at the molecular level have been made during the past decade. Inactivation is caused principally by far-uv damage to deoxyribonucleic acid (DNA) or to ribonucleic acid (RNA) in those organisms such as most plant viruses and RNA phages where the genome is composed only of RNA. This communication is concerned only with DNA photoreactivation. In this case a substantial fraction of the inactivation by far-uv radiations is attributed to the formation of cyclobutane dipyrimidines[1] in DNA (Setlow, 1968) and the re-activation, to re-

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[1] The reasons for preferring the term cyclobutane dipyrimidines instead of pyrimidine dimers are discussed in another publication (Minato and Werbin, 1972). These compounds are engendered in deoxyribonucleotides (Cook, 1967), synthetic deoxypolynucleotides (Setlow *et al.*, 1965), and deoxyoligonucleotides (Setlow and Bollum, 1968) by far-uv-induced cycloaddition of the 5,6 double bond of a pyrimidine residue to the 5,6 double bond of an adjacent pyrimidine and are isolated by acid hydrolysis at 165° (Varghese and Wang, 1967). Because of their isolation by hot acid, it is conceivable that the cyclobutane dipyrimidines are derived rather than primary photoproducts.