Endonuclease from *Micrococcus luteus* Which Has Activity Toward Ultraviolet-Irradiated Deoxyribonucleic Acid: Purification and Properties

W. L. CARRIER AND R. B. SETLOW

*Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830*

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An endonuclease purified ∼3,200-fold from *Micrococcus luteus* is active on native ultraviolet-irradiated deoxyribonucleic acid (DNA), but is inactive on unirradiated native or denatured DNA and has no activity toward irradiated denatured DNA. The major type of lesion for the nucleolytic activity is the cyclobutane pyrimidine dimer. The enzyme makes a number of single-strand breaks approximately equal to the number of dimers, but dimers are not excised. This endonuclease—a small molecular weight protein—therefore has all the attributes hypothesized for the first enzyme in the sequential steps in repair of DNA in vivo. Another paper shows that the endonuclease is able to reconvert ultraviolet-irradiated transforming DNA.

There have been a number of reviews (6, 7, 9, 19, 20, 23, 30) summarizing the evidence that microorganisms are able to repair damage to their deoxyribonucleic acid (DNA) in the dark. This phenomenon has been studied in detail for ultraviolet (UV) damage because one of the more important lesions, the pyrimidine dimer [cyclobutyl dimer between adjacent pyrimidines (22)], is easy to detect and hence easy to follow during the processes of repair. One of the major ways of repairing UV damage is by excision of pyrimidine dimers from the DNA, a process in which the dimers appear as components of oligonucleotides separable from the main DNA of the cell. The excision step, which is followed by others such as repair replication and rejoining of strands, requires at least two enzymes (5, 10, 25, 32).

It has been known for some time that extracts of *Micrococcus luteus* (*Micrococcus lysodeikticus*) are able preferentially to affect UV-irradiated DNA [see review by Strauss (30)]. Strauss (29) showed that such extracts reduce the biological activity of irradiated, but not unirradiated, transforming DNA of *Bacillus subtilis*. Rösch and his co-workers (18) showed that these extracts could increase the biological activity of the replicative (double-stranded) form of φX174 DNA which had been inactivated by UV irradiation, and Elder and Beers (3) reported that crude extracts could reconvert UV-irradiated transforming DNA of *Haemophilus influenzae*. Although the various extracts are similar, they may contain different proportions of specific and nonspecific nucleases; Strauss (30) suggested that the different features of the extracts (inactivation and reactivation) may be accounted for by the ability of the recipient cells to accept and maintain double-stranded DNA. In contrast to *H. influenzae* DNA (16), single-stranded *B. subtilis* DNA is found inside the recipient cell (1). Reduction in size of DNA decreases its transforming ability; hence, single-stranded breaks would reduce the size and inactivate *B. subtilis* DNA but not that of *H. influenzae*. Extracts of *M. luteus* selectively degrade irradiated DNA (2, 13, 31) and cause the excision of dimers in vitro (2). Several reports (5, 10, 15, 26, 27) have described properties of an endonuclease from *M. luteus* which acts on UV-irradiated DNA, and Kaplan et al. (10) have purified two enzymes whose combined action results in the excision of dimers from DNA. However, the relationship between the endonuclease and repair in vivo in *M. luteus* is not clear (5, 17, 32).

We report here some of the properties of a purified extract of *M. luteus* which has endonuclease activity toward UV-irradiated DNA. The nuclease is inactive toward unirradiated DNA and is most active toward native DNA containing pyrimidine dimers. It does not excise dimers. Many of its properties are similar to those observed by Kaplan et al. (10), who used a completely different assay. The accompanying paper shows that the nuclease is able to effect the reactivation of UV-inactivated transforming
DNA when the DNA is assayed on cells that are not able themselves to do dark repair.

MATERIALS AND METHODS

Materials. Spray-dried cells of *M. luteus* were purchased from the Miles Chemical Co., Elkhart, Ind. Calf thymus DNA, *Escherichia coli* alkaline phosphatase, and egg-white lysozyme were from the Worthington Biochemical Corp. 32P and 3H as carrier-free H3PO4, and 3H- and 14C-thymidine were from New England Nuclear Corp. DE-52 and CM-52 cellulose were obtained from Reeve Angel and Co., Inc., and Sepharose 4B from Pharmacia Fine Chemicals, Inc. Partially purified ribosomal ribonuclease acid (RNA) and transfer RNA were gifts from Mayo Uziel, and irradiated polyadenylate-polyuridydate (poly rA·rU) was a gift from Ronald Rahn.

Preparation of labeled DNA species. The following labeled DNA species were prepared by the method of Marmur (12) from *E. coli* 1ST- were used: *E. coli* DNA grown on synthetic medium containing 3H-thymidine (3.8 μCi/μg), 160,000 counts/min per μg of DNA; grown on 14C-thymidine (0.22 μCi/μg), 16,000 counts/min per μg; grown (4) on medium containing cold thymidine plus 32P, 170,000 counts/min per μg; and grown on medium containing cold thymine plus 32P, 32,000 counts/min per μg. In some experiments, DNA isolated from T4 phage labeled with 3H-thymidine was also used. 3H- and 14C-DNA species were used over periods of 1 year or more. Because of radiation damage, the older preparations showed many more single-strand breaks not introduced by enzyme than the other labeled DNA forms.

UV irradiation. Radiation was obtained from a large quartz prism monochromator illuminated by a 500-w Philips mercury lamp. The incident intensity was measured with a calibrated photocell, and the values were corrected for absorbance by the sample. Native *E. coli* DNA irradiated in solution with 4 × 104 ergs/mm² at 280 nm was usually used for the assay for endonuclease activity. Such a DNA species contains about 1.3 × 10²⁷ pyrimidine dimers per nucleotide (24), from which we can calculate an average of 77 nucleotides (~24,000 daltons) between dimers on a single strand. For some purposes, smaller incident fluxes were used.

Enzymic reversal of dimers in irradiated DNA. Photoreactivation (done with the cooperation of Jane K. Setlow) was carried out with a purified preparation of yeast photoreactivating enzyme (14). The enzyme concentration was 300 μg/ml and the DNA concentration 1 μg/ml. The mixture was illuminated at 37°C for 1 hr by light from a "black light lamp," a procedure that monomerized all the cyclobutane pyrimidine dimers.

Molecular weight markers for gel-filtration chromatography. The average chain lengths of polynucleotides were measured on 32P- or 32P-labeled material by treating separated polynucleotides (about 1 μg/ml), obtained from endonuclease action, with alkaline phosphatase (50 μg/ml) in 0.1 M tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 8.2, for 15 min at 37°C. The reaction mixtures were chromatographed on diethylaminoethyl paper, and the average chain length was obtained from the ratio of the total radioactivity to that in inorganic phosphate.

Routine assay for endonuclease activity. The reaction mixture contained: DNA (usually 3H-thymine-labeled) irradiated in solution with 4 × 10⁴ ergs/mm² at 280 nm, 2.5 μg/ml; unirradiated DNA (usually 14C-thymine-labeled), 2.5 μg/ml; and unirradiated calf thymus DNA, 5 μg/ml, in 0.05 M potassium phosphate buffer (pH 6.5), 0.001 M ethylenediaminetetraacetate, and 0.01 M β-mercaptoethanol. The enzyme fraction was added, and the mixture was incubated for various times at 37°C. The reaction was stopped by adding NaOH to a concentration of ~0.1 M (addition of alkali also resulted in the separation of DNA strands); neutralization was with HCl. Portions of the mixture were applied to small columns of Sepharose 4B (0.6 by 10 cm) for separation of the DNA fractions which resulted from the enzyme treatment. Three fractions were collected (Fig. 1) and placed into vials. Ten milliliters of counting solution [125 g of naphthalene, 3. g of 2,5-bis-(2,5-terti-butyl-benzoxazolyl)-ibophene, and dioxane to 1 liter] was added per ml of fraction for scintillation counting.

Purification of enzyme: CM-52 cellulose chromatography. Spray-dried cells (12 g) were suspended by use of a Waring Blender in 0.01 M Tris buffer (pH 8.0) and collected by centrifugation. The cells were resuspended in 250 ml of 0.2 M sucrose and 0.01 M Tris (pH 8.0), and 50 mg of lysozyme was added. After 45 min at 37°C, 250 ml of cold water was added. The lysozyme was stirred vigorously until a uniform viscous gel formed, and was then sonically oscillated for 5 min at 50 kHz in a Biosonic sonic oscillator. The sonicated solution was centrifuged at 30,000 × g for 30 min, and the supernatant fluid was saved (fraction I). Ammonium sulfate was added to fraction I to a final concentration of 65%. The solution was stirred for 1 hr and centrifuged for 30 min at 30,000 × g. The precipitate was dissolved in 1,000 ml of 0.005 M potassium phosphate buffer, pH 7.5 (fraction II, ~4 mg/ml), and loaded onto a DE-52 cellulose column (2.5 by 40 cm) at 0.3 ml/min; 10-ml fractions were collected. Most of the endonuclease activity and about 10% of the protein was eluted by 400 ml of 0.005 M potassium phosphate buffer, pH 7.5 (fraction III). After the pH was adjusted to 6.5 with acetic acid, fraction III was loaded onto a CM-52 cellulose column (2.5 by 40 cm) at 0.3 ml/min, and 15-ml fractions were collected. The column was washed with 200 ml of 0.01 M potassium phosphate buffer (pH 6.5) before a linear gradient of 0.01 to 0.3 M was applied. The enzyme eluted in about 100 ml (<100 μg/ml of protein) in the 0.1 M region (fraction IV). Protein was determined by Folin phenol reagent (11) and by ninhydrin (8, 28).

RESULTS AND DISCUSSION

Characteristics of the assay for endonuclease activity. The general plan of the assay was to measure the number of single-strand breaks introduced by protein fractions into UV-in-
radiated DNA. Components of the reaction mixture are shown in Materials and Methods. (The enzyme does not require divalent ions for activity.) After reacting for measured periods of time, the mixture is made alkaline to separate individual strands and is then neutralized. The mixture now contains labeled polynucleotides, of which the distribution of lengths depends on the distance between lesions in the DNA and on the activity of the enzyme preparation. At the UV doses we used, the average distance between dimers corresponds to 24,000 daltons. Since the molecular weight of an untreated DNA is well over 10^6, it is a simple matter to use a small Sepharose column (0.6 by 10 cm) to separate the undegraded from the degraded material. Figure 1 shows the ability of such small columns to separate macromolecular from small polynucleotides and from mononucleotides. As many as 20 such columns may be run by one person at the same time. The use of these columns is a quick and easy way to monitor for endonuclease and exonuclease activity in purified and unpurified preparations. The use of irradiated and unirradiated DNA species with different radioactive labels in the reaction mixture allows one to detect the specificity of endonuclease activity toward irradiated DNA. For example, Fig. 2 shows that the amount of material that remains in the undegraded fraction (fraction A of Fig. 1) is a function of (i) the amount of enzyme used, (ii) the incubation time with a fixed amount of enzyme, and (iii) the amount of substrate (UV-irradiated DNA) in the reaction mixture. Thus, one can adjust the conditions of this simple reaction so as to measure relative enzyme activity or effective substrate concentration. The latter measurement allows one to assess the effects of competitors and, therefore, to ascertain whether lesions other than pyrimidine dimers are substrates for the enzyme. It is apparent from Fig. 2b that the optimal salt concentration is between 0.035 and 0.1 M. We did not extensively investigate the pH activity curve of the enzyme, but Fig. 3 shows that the optimum is near pH 6.

A number of agents are known to inhibit the excision of pyrimidine dimers in E. coli (25). The effect of some of these agents on endonuclease activity is shown in Fig. 4. Proflavin and KCN, both potent inhibitors of excision in vivo, are strong inhibitors of endonuclease activity. Caffeine, on the other hand, is not, even though it inhibits excision in vivo. Thus, we conclude that proflavin and KCN may block the first step in excision of dimers and that caffeine works on a later step, or that the E. coli and M. luteus enzymes are different.

**Purification of the endonuclease.** Table 1 shows a typical purification scheme for the enzyme. The greatest purification is effected in the last step in the elution from carboxymethyl cellulose. Figure 5 shows a typical chromatogram of such an eluate and indicates the endonuclease activity toward irradiated and unirradiated DNA. There is no exonuclease in the preparation as detected by chromatography on Sepharose 4B (see Fig. 7). We found it very difficult to observe UV absorbance peaks in the fractions corresponding to maximal activity because the protein in this peak contains little or no aromatic amino acids. There is a big difference between the amount of protein detected by the Lowry reaction (tyrosine) and by ninhydrin, the latter procedure always giving at least 10-fold higher values.

The purified activity is stable for several weeks at 4 °C. It loses 50% of its activity on freezing and is very unstable at higher temperatures. For example, it loses all its activity in 30 min at 37 °C. However, its stability is increased dramatically when unirradiated DNA, such as salmon sperm or calf thymus DNA, is added to the solution. (In the presence of 10 to 20 μg of DNA/ml, the enzyme is stable to freezing and at 63 °C
FIG. 2. Some properties of a partially purified UV-endonuclease as measured by the assay in Materials and Methods and chromatography on Sepharose 4B. The percentage undegraded is the fraction of label eluting from the column in the excluded volume (fraction A of Fig. 1). (a) Effect of enzyme concentration on UV-irradiated DNA (30-min incubation). (b) Effect of time and salt concentration. (c) Effect of substrate concentration. This experiment represents a 60-min incubation with a less active enzyme fraction than that used in (a) and (b). The radioactive UV-irradiated DNA suffered radiation damage, and, hence, the amount that is undegraded with no endonuclease action (plotted as the point at \( \infty \) concentration) is less than in (a) and (b).

FIG. 3. Activity of UV-endonuclease in 0.05 M phosphate or Tris buffer at various pH values. Assays were performed as described in Materials and Methods except for pH and buffer as indicated. Before assay, the UV-endonuclease used was dialyzed with unirradiated calf thymus DNA (5 \( \mu \)g/ml) against 0.005 M phosphate buffer, pH 6.5. Symbols: ●, ○, phosphate buffer; □, □, Tris-hydrochloride buffer.

has a half-life of 5 min.) It is inactivated by Pronase.

The enzymatic activity acts as a small molecular weight protein. The sedimentation of activity

in sucrose gradients (Fig. 6) is between those of cytochrome c (molecular weight, 13,000) and myoglobin (molecular weight, 16,000).

Substrates and competitors. UV-irradiated native DNA is a good substrate for the endonuclease; denatured DNA is not (Fig. 4). Pyrimidine dimers constitute the predominant photochemical change in DNA, and, therefore, it is natural to suppose that they are in the polynucleotide regions attacked by the endonuclease. There are several lines of evidence indicating that such dimers are the sites of attack of the endonuclease. The first is a numerical argument. The larger the number of dimers (and all other lesions, too), the smaller the resulting single-stranded
pieces should be after extensive endonuclease treatment. Figure 7 shows an analysis of endonuclease-treated DNA after 104 and 4 × 104 ergs/mm2 of 280 nm. The smaller dose is expected to give an average mass between dimers of 6.5 × 104 and the larger dose 2.4 × 104 daltons. In the experiment shown in Fig. 7, all three DNA species were incubated together; samples were removed at 0, 5, and 60 min and analyzed on Sepharose columns longer than those used in the standard assay to give somewhat better resolution. Figure 7a shows that unirradiated native DNA,

\[ \text{Fraction} \]

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Step</th>
<th>Total protein</th>
<th>Protein to degrade 50% of UV-irradiated DNA in 30 min</th>
<th>Purification ( ^a )</th>
<th>Relative recovery ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Lysate</td>
<td>7.0</td>
<td>1.00</td>
<td>1.00</td>
<td>0.93</td>
</tr>
<tr>
<td>II</td>
<td>Ammonium sulfate precipitate</td>
<td>4.0</td>
<td>1.6</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>DE-52 cellulose eluate</td>
<td>0.7</td>
<td>6.0</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>CM-52 cellulose eluate</td>
<td>0.001</td>
<td>3200</td>
<td>0.46</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Standard assay of Table 1.

\( ^b \) Ratio of the amount of protein in the lysate to that in the other fractions to degrade 50% of UV-irradiated DNA in 30 min.

\( ^c \) Relative value of the ratio of total protein to that necessary to degrade 50% of UV-irradiated DNA in the standard assay.

FIG. 6. Sedimentation of UV-endonuclease in a sucrose gradient. Fractions were assayed for activity as described in Materials and Methods. Due to self-irradiation during storage, this irradiated DNA showed about 40% degradation with or without enzyme treatment. The enzyme (≈5 μg in 0.10 ml) was layered onto a 5 to 20% linear sucrose gradient (4.7 ml) in 0.01 M mercaptoethanol, 0.005 M ethylenediaminetetraacetate, and 0.05 M phosphate buffer (pH 6.5), and centrifuged at 4°C in the Spinco SW39 rotor for 17 hr at 37,000 rev/min. Symbols: ●, activity of the fraction on UV-irradiated DNA; ○, relative absorbance of cosedimented cytochrome c (0.25 mg); ◦, relative absorbance of myoglobin (2.5 mg) sedimented in a separate centrifuge tube.

even though badly degraded from tritium decays, is virtually unaffected by the endonuclease. Figures 7b and 7c indicate that the larger the dose, the smaller the molecular weight after endonuclease treatment; that there is little change between 5- and 60-min incubation; and that no mononucleotides appear. Within the uncertainties of these crude but simple methods, the average

FIG. 5. Chromatography of UV-endonuclease on a CM-52 cellulose column. A DE-52 cellulose column fraction containing UV-endonuclease (350 ml of 2 mg of protein/ml) in 0.01 M phosphate buffer, pH 6.5, was applied to a column (2.5 × 40 cm). After loading, the column was eluted with a linear potassium phosphate buffer gradient, pH 6.5. Protein was determined by ninhydrin by using alkaline-digested bovine serum albumin as the standard. Symbols: ●, protein; ○, UV-irradiated DNA treated with UV-endonuclease; □, unirradiated DNA treated with UV-endonuclease.
The fraction give should species These experiments the DNA would dimers two motion in At low dose, which is interpretation of the eluates (see Materials and Methods) and the position of pApTpT is from a separate experiment. The inset in (c) compares endonuclease-treated UV-DNA before and after denaturation.

molecular weight in the limit digests is about what is expected from the average distance between dimers. The inset to Fig. 7c shows that single-strand breaks and not double-strand breaks are produced by the enzyme, because, when the DNA is not denatured before a run through the column, the DNA is macromolecular. It is intriguing that the molecular weight distribution in the limit digests is so heterogeneous. An interpretation of the wide distribution is complicated by the fact that all pyrimidine dimers might not be substrates for the enzyme (for example, two dimers very close together) and by the fact that high doses of UV radiation make cross-links between strands. Such cross-links might not be substrates for the enzyme and therefore give rise to larger-molecular-weight pieces because they would prevent strand separation.

The simple assay, described in Materials and Methods, with a fixed amount of enzyme and DNA species irradiated with different doses should give a similar but more qualitative result. At low doses, the average distance between dimers is large, and an appreciable fraction of the DNA will always remain in fraction A (Fig. 1) and therefore will not be detected as degraded. The fraction degraded will then increase with dose, which is just what is observed in Fig. 8a. These experiments do not implicate dimers directly because all lesions increase with dose, and the average distances between them decrease with dose. However, when an irradiated DNA is subjected to enzymatic photoreactivation, subsequent endonuclease treatment has no effect on it (Fig. 8b). Since the only known substrates for enzymatic photoreactivation are the cyclobutyl pyrimidine dimers (21), this experiment is direct evidence that such dimers are the major lesions which are recognized by the endonuclease in DNA irradiated in solution.

The real substrate might not be a dimer but just a local denatured or open region in a double helical stretch of polynucleotide. Therefore, we looked at the ability of other polymers, irradiated and unirradiated, to compete with UV-irradiated
DNA for the UV-endonuclease. Table 2 shows typical data. Transfer RNA, irradiated or not, does not compete nor does ribosomal RNA. On the other hand, poly rA·rU when heavily irradiated does compete. Such a polymer contains both dimers and hydrates. At the highest dose shown, approximately 5% of the uracil residues are in these two products. In view of this rather weak competition by heavily irradiated polyribonucleotide, it is not surprising that irradiated transfer RNA with its many fewer adjacent pyrimidines is not a competitor at all. The weak competition by what are presumably uracil dimers is to be compared to the relatively strong competition by irradiated polydeoxyadenylate-polydeoxythymidylate. The large number of dimers made in such a polymer by small doses (one dimer per $10^4$ daltons of the deoxythymidylate-strand; 22) is the reason it is so easy to observe its competitive ability. On the other hand, its competing ability per dimer is less than that of DNA. This result may reflect the different structure of this polymer compared to DNA, and it emphasizes the possibility that dimers in different parts of polynucleotides may be acted upon at different rates.

The strand cut by UV-endonuclease. The experimental evidence given above indicates that dimer-containing regions of double-stranded DNA are substrates for the endonuclease action, but they do not indicate whether the strand break is made only in the polynucleotide chain that contains the dimer. If it is only the distortion of the double helix that is recognized by the endonuclease, then there is an appreciable chance that the strand opposite the dimer is cut some of the time. Our measurements of chain breaks give no direct information on this point and therefore we performed the following experiments. A double-stranded DNA was constructed; one strand was labeled with $^3$H-thymidine and had pyrimidine dimers in it, and the other strand was labeled with $^{32}$P and had no UV lesions. This “hybrid” DNA was treated with endonuclease. The results of endonuclease action are shown in Fig. 9a, and the precise procedure in forming the hybrid DNA is described in the legend. It is clear that only the irradiated strand is degraded by the endonuclease. The $^{32}$P-labeled, unirradiated strand is unaffected. Figures 9b and 9c represent control experiments. Figure 9b indicates there is no degradation of either strand when neither has been irradiated. Figure 9c shows that, under the conditions of these experiments, the endonuclease acts both on irradiated native DNA and on $^{32}$P-containing DNA which was irradiated while denatured at low concentration and then had been annealed. Thus the endonucleolytic attack is preferentially on the strand which contains the pyrimidine dimer and is

**Table 2. Effects of various polynucleotides on the degradation of UV-irradiated DNA by endonuclease**

<table>
<thead>
<tr>
<th>Expt</th>
<th>UV-irradiated DNA</th>
<th>Competitor</th>
<th>Type</th>
<th>Concentration</th>
<th>UV: 280 nm (ergs/mm²)</th>
<th>Per cent UV-DNA undegraded</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$^3$H-labeled, 1.3 µg/ml</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>$^4$C-labeled, 0.8 µg/ml</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>$^{32}$P-labeled, 0.25 µg/ml</td>
<td>Poly rA·rU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Reaction mixtures were similar to that described in Materials and Methods, except for the use of smaller concentrations of UV-irradiated DNA (as indicated) and the addition of competitors as listed above. Assay incubation times, 60 min. Abbreviations: UV, ultraviolet; DNA, deoxyribonucleic acid; rRNA, ribosomal ribonucleic acid; tRNA, transfer RNA; poly dA·dT, polydeoxyadenylate-polydeoxythymidylate; poly rA·rU, polyadenylate-polyuridylate.

* Computed from the interpolated time that the normal system would take to reach the same level of degradation, assuming kinetics as in Fig. 2.
presumably close to the dimer. The preference for this strand seems to be at least 10-fold greater than for the unirradiated strand. The preference could be a kinetic one; for example, the phosphodiester bond near a dimer might be much more labile to enzymatic hydrolysis than those in the opposite side. Once hydrolyzed, this DNA is no longer a substrate. If indeed this were the explanation, then one would conclude that a warp in the DNA is what is recognized by the endonuclease, but that a more symmetrical warp, such as that produced by a mismatched base pair, would be necessary to demonstrate this type of enzyme specificity or lack of specificity.

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LITERATURE CITED