Purification and Properties of Single Strand DNA-binding Endo-Exonuclease of *Neurospora crassa* 

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Single strand DNA-binding endo-exonucleases purified from mitochondria, vacuoles, or a mixture of these organelles had the same high specific single strand DNase activity (910 μmol of nucleotides/min/mg), and each contained a polypeptide of M₀ = 31,000–33,000 which was found to be active by sodium dodecyl sulfate-DNA-gel electrophoresis. The properties of the three preparations were identical in all respects tested. The enzyme showed distributive endonuclease activity with single strand DNA, but processive exonuclease activity with double strand DNA. In the former case, 5'-phosphoryl-terminated fragments were released at early times, while in the latter case, short 5' oligonucleotides (n = 2–4) were released. Both activities were dependent on Mg²⁺ (or Mn²⁺), but to different extents. In 0.1 mM Mg²⁺, superhelical bacteriophage φX174 (replicative form (RF) I) DNA was converted to relaxed circular (RF II) DNA and, at higher enzyme concentrations, to unit length linear (RF III) DNA. In 10 mM Mg²⁺, these same conversions took place rapidly, and the RF III DNA which formed was degraded to pieces shorter than unit length. At very low enzyme concentrations, long single strand tails and gaps were detected in bacteriophage T7 linear double strand DNA molecules.

The major alkaline DNase activity of rapidly growing mycelia of *Neurospora* is associated with endo-exonuclease, an enzyme possessing both endonuclease activity with ssDNA and RNA and exonuclease activity with dsDNA (1, 2). Recently, the enzyme has been shown to be uniquely distributed in the organelles of mycelia (3). At least 90% of it is present in a ratio of 1:1.6 in mitochondria, where it is bound to the inner membrane, and in vacuoles, where it is not membrane-associated. The enzyme is also present in mycelia in an inactive (precursor?) form which is activated in vitro with trypsin (2, 4). This form of enzyme is distributed in a ratio of 1:2.5 in the inner membrane of mitochondria, where it is more tightly bound than the active enzyme, and in the cytosol (3).

A mutant-sensitive mutant of *Neurospora, uus-3*, which shows many pleiotropic effects, including effects on conidial survival, mutagenesis, and recombination (5, 6), shows a different distribution of endo-exonuclease than in the wild type (3). The vacuoles of the mutant are partially deficient in the enzyme, while the mitochondria are almost totally deficient in activity. On the other hand, the mitochondria contain a substantially higher level of the inactive form of endo-exonuclease than do wild type mitochondria (3). These and other observations on the uus-3 mutant (4, 7) were interpreted as indicating a possible role of endo-exonuclease in DNA repair, especially in mitochondria (3). With this possibility in mind, we have purified and characterized active ssDNA-binding forms of endo-exonuclease from both mitochondria and vacuoles and from a mixture of mitochondria and vacuoles which yielded a large amount of stable enzyme. All three preparations have very similar properties and act on DNA in a manner reminiscent of some known recombinases.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

Purification of ssDNA-binding Endo-Exonucleases of Mitochondria and Vacuoles—The endo-exonucleases of mitochondria, vacuoles, and the mixture of these organelles showed essentially the same chromatographic properties on DEAE-Sepharose and ssDNA-cellulose. About 90% of the single strand DNase activity recovered at each step was eluted in the gradient fractions in single peaks in the range 0.07–0.20 M NaCl from DEAE-Sepharose and in the range 0.5–0.8 M NaCl from ssDNA-cellulose (data not shown). The only exception seen was in the elution of a second, smaller peak of mitochondrial activity from ssDNA-cellulose in the range 0.2–0.4 M NaCl. This activity had many properties in common with that eluted at higher salt concentrations and increased in amount on aging the mitochondria *in vitro* before extraction, while the activity eluted from ssDNA-cellulose at higher salt concentrations showed a corresponding decrease in amount. These preliminary observations may indicate that the weak binding mitochondrial fraction was derived from the strong binding fraction, perhaps due to limited proteolysis (see below). Only the strong binding activity has been investigated further in the work reported here. The chromatography on ssDNA-cellulose acted as an affinity step, yielding in each case endo-exonuclease of high purity (see below) and with the same high specific activity of about 14,000 units/mg (Table I). This specific activity is equivalent to the release of
acid-soluble material at a rate of 910 μmol of nucleotide/min/mg. While the yields of activity from mitochondria and vacuoles were moderately high (each about 30%), the yield of activity from the mixture of the organelles was over 80% (Table I).

Limited proteolysis plays a role in the recovery of ssDNA-binding endo-exonucleases from the mitochondria and vacuoles of Neurospora mycelia. The inclusion of 1 mM phenylmethylsulfonyl fluoride during the extraction almost completely abolished the appearance of ssDNA-binding activity that was seen when extracts made in the absence of phenylmethylsulfonyl fluoride were chromatographed directly on ssDNA-cellulose (data not shown). Proteolysis may be required either to dissociate the enzyme from other proteins which block a ssDNA-binding site, or it may act directly on a larger endo-exonuclease polypeptide to create a ssDNA-binding site. These possibilities are not mutually exclusive, but the latter is favored as our working hypothesis since there is other evidence of direct protease involvement. The inactive (precursor?) form of endo-exonuclease is a large single polypeptide with molecular weight about 90,000 (2) which is activated in vitro with a variety of endopeptidases and presumably activated in vivo to form the mitochondrial endo-exonuclease (3). Active polypeptides having endo-exonuclease activity with molecular weights ranging from 33,000 to 53,000 were recovered previously (2) from less pure endo-exonuclease preparations (1) by electrophoresis on 6 M urea-polyacrylamide gels. Recently, active polypeptides with molecular weights as high as 77,000 from Neurospora mycelia have also been detected (1) by SDS-DNA-gel electrophoresis (see below) in different types of preparations from mycelia, and an immunochemically cross-reactive M, = 70,000 nucl ease which binds to ssDNA-cellulose has recently been isolated from yeast. The ssDNA-binding endo-exonuclease characterized here thus may be derived from a larger polypeptide by limited proteolysis during extraction and purification. The procedures described to obtain large amounts of pure endo-exonuclease from the mixture of mitochondria and vacuoles have permitted the preparation of antibodies to endo-exonuclease. There are currently being used in various immunological tests to explore the role of proteolysis in vivo and in vitro in generating different forms of endo-exonuclease. A possible role for proteolysis in vivo has already been implied (see Introduction).

Characterization of the Proteins—A photograph of comigrating silver-stained SDS gels of endo-exonucleases derived from mitochondria, vacuoles, and the mixture of mitochondria and vacuoles is seen in Fig. 1A. Marker polypeptides (1 μg of total protein in lanes 1, 3, and 7) were loaded on the gel in a small volume (25 μl), but the samples containing endo-exonuclease were usually loaded on in the maximum volume of 100 μl which gave rise to two artifactual bands of silver-staining material running just ahead of the M, = 67,000 marker. These are not prominent in the channels which contain markers. This material is present in the denaturation mixture containing SDS and mercaptoethanol, but no protein (100 μl run in lane 8). The stain of the gel containing lanes 6, 7, and 8 was overdeveloped, but this does not obscure the results. When the amounts of protein present in the purified endo-exonuclease preparations are compared with that in the markers (see Fig. 1A, legend), it is apparent that the staining intensity of the former was much less than for marker proteins. This was also seen for Coomassie blue staining of the proteins.

The ssDNA-binding endo-exonuclease from all three

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2 M. J. Fraser and H. Cohen, unpublished results.
3 T. Y.-K. Chow and M. A. Resnick, personal communication.
4 T. Y.-K. Chow and M. J. Fraser, unpublished observations.

The reason for the lower amount of staining is not known. It is clear, nevertheless, that the major polypeptide present in the preparations from vacuoles (lane 6) and the mixture of mitochondria and vacuoles (lane 4) was that running slightly behind the M, = 30,000 marker, with an apparent molecular weight of 31,000, while that present in the preparation from mitochondria (lane 5) was slightly larger, about 33,000. These polypeptides were found to be the active ones by SDS-DNA-gel electrophoresis (Fig. 1B). In this case, the activity in each purified endo-exonuclease preparation had a mobility close to that of the pancreatic DNase I marker. The mitochondrial preparation especially showed small amounts of a number of contaminating polypeptides, but these were removed in a second round of chromatography on ssDNA-cellulose. This is illustrated for the preparation from the mixture of mitochondria and vacuoles in lane 2 of Fig. 1A. It should be noted that even though the mitochondrial and vacuolar polypeptides differed slightly in molecular weight (33,000 versus 31,000), only one polypeptide of M, = 31,000 is seen in lane 2 (10 μg of protein run) which has the same apparent molecular weight as the vacuolar enzyme. It is highly improbable that this preparation from the mixture of organelles did not contain endo-exonuclease of mitochondrial origin. From previous work (3) it is known that the sonication procedure used here releases 96% of the vacuolar enzyme and 60−70% of the mitochondrial enzyme. The data in Table I show that the activity recovered by purification of the enzyme from the mixture of organelles was 82%. Using the data from Ref. 3, it can thus be calculated that the ratio of mitochondrial to vacuolar enzyme should be about 0.5:1. This is very likely an underestimate since no phenylmethylsulfonyl fluoride was present during extraction to prevent the activation of inactive endo-exonuclease on the mitochondrial inner membrane (3). Because it is likely that limited proteolysis is required to obtain these ssDNA-binding enzymes (see above), it is possible that proteases have processed both the vacuolar and mitochondrial polypeptides in the same manner during the purification from the mixture of organelles. High levels of proteases are present in the light vacuoles which co-sediment with mitochondria (3). When attempts were made to purify ssDNA-binding endo-exonuclease from purified light vacuoles, only 8% of the extracted activity was recovered in this form, while the remainder of the single strand DNase activity was lost.

When the purified endo-exonuclease from the vacuoles and mitochondria were electrophoresed in nondenaturing disc gels, only one protein of relatively low mobility (Rf = 0.18) was detected by Coomassie blue staining with up to 200 μg of protein loaded on the gel (data not shown). This protein was found to contain all of the single strand DNase, double strand DNase, and RNase activities detected in parallel unstained gels (data not shown). The single strand DNase, double strand DNase, and RNase activities of these two enzyme preparations not only co-electrophoresed, but also co-sedimented in sucrose density gradients slightly faster than the hemoglobin marker (data not shown). Assuming that the proteins are spherical molecules of normal density, the calculated apparent native molecular weights were both about 80,000. The purified mitochondrial enzyme (from one round of chromatography on ssDNA-cellulose) sedimented considerably faster than hemoglobin and showed signs of aggregation, i.e. asymmetrical sedimentation profiles (data not shown). An apparent native molecular weight of 80,000 for ssDNA-binding endo-exonuclease is consistent with the native protein being a dimer.

The ssDNA-binding endo-exonuclease from all three

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Neurospora Endo-Exonuclease
sources were quite stable when stored at concentrations of protein exceeding 100 μg/ml. They could be stored in buffer A containing 10% glycerol at 0-4 °C or frozen at -20 °C for at least 5 months without loss of single strand DNase activity or binding to ssDNA-cellulose. The presence of 1 mM dithiothreitol had no effect on the stability of the enzymes. The purified enzyme from the mixture of organelles did not have any detectable activity with p-nitrophenyl phosphate (tested at pH 8.0), bis-p-nitrophenyl phosphate, either of the 3’- or 5’-p-nitrophenyl esters of thymidine, or 5’-AMP. It was therefore not contaminated with alkaline phosphatase, phosphodiesterase, or 5’-nucleotidase activities. It also did not have any detectable DNA polymerase activity.

**Substrate Specificity**—In addition to degrading linear ssDNA, linear dsDNA, and RNA as described above, the actions of ssDNA-binding endo-exonuclease purified from the mixture of mitochondria and vacuoles were tested on a variety of potential substrates (data not shown). The enzyme rapidly and completely degraded 3H-labeled bacteriophage fd single strand circular DNA to acid-soluble material, but did not degrade DNA-RNA hybrid (poly(dA)-poly(rU)) or dsRNA (poly (rA)-poly(rU)). It was shown previously (1) that a relatively crude preparation of *Neurospora* endo-exonuclease derived from sonicates of mycelia did not degrade Reovirus dsRNAs, but the termini of those RNAs are modified (25), so the lack of activity on Reovirus RNA could have been due to...
Neurospora Endo-Exonuclease

The actions of this endo-exonuclease on superhelical bacteriophage φX174 DNA are shown in Fig. 2 where, in this case, the products of the reactions were separated by electrophoresis on 1% agarose slab gels and then the gels were stained with ethidium bromide (18). Untreated RF I DNA, seen as the front running (lower) band in lanes 1 and 10, contained a small amount of relaxed circular (RF II) dsDNA which migrated more slowly on the gel. Experiments were performed at two levels of Mg"+ concentration above the concentration of EDTA present: at 0.1 mM Mg"+ in which little or no activity was seen on linear dsDNA (see Miniprint) and at 10 mM Mg"+ in which both single strand and double strand DNase activities were maximal. It can be seen that in 0.1 mM Mg"+ (lanes 2-5), the superhelical DNA was nicked efficiently by increasing concentrations of enzyme to produce relaxed circular dsDNA. The molar ratio of enzyme to DNA at the highest enzyme concentration was close to 1:1. At this concentration of enzyme, a small amount of linear (RF III) dsDNA formed, as seen by the presence of a faint band of mobility slightly greater than that of RF II DNA. In 10 mM Mg"+, with the same enzyme concentrations (lanes 6-9), all of the RF I DNA was converted to RF II DNA (major product) and RF III DNA (minor product), and at 40 and 80 units/ml of enzyme, there was also a fast running smear of DNA in the gel, presumably shorter fragments of linear dsDNA. These results demonstrate that the main action of endo-exonuclease on φX174 RF I DNA in 0.1 mM Mg"+ is endonucleolytic and indicate that the enzyme senses single strand-like regions in superhelical DNA. Neurospora single strand-specific endonuclease (26, 27), which may be a proteolytic derivative of endo-exonuclease lacking exonuclease activity (1), also nicks unirradiated and UV-irradiated φX174 RF I DNA (28), and the former reaction is enhanced by pretreating the DNA with formaldehyde to "trap out" or "fix" the single strand regions (29). The nicking showed an S-shaped response with respect to enzyme concentration, indicating some cooperativity in the process (29). The presence of two or more enzyme molecules in a single strand-like region may induce a double strand break. Thus, Neurospora endonuclease, like endo-exonuclease, also converts RF I DNA to the linear RF II form. In 10 mM Mg"+, endo-exonuclease degraded φX174 RF I DNA to small fragments (Fig. 2) presumably through its rapid exonucleolytic action on the intermediate RF III form.

Products of Digestion—Digests of ssDNA, dsDNA, and RNA made in the presence of high concentrations of purified endo-exonuclease from the mixture of mitochondria and vacuoles were examined by chromatography on DEAE-Sephadex A-25 in the presence of 7 M urea using ["H]dCMP as a marker. The results presented in Fig. 3A show that a limit digest (100% acid-soluble material) of ssDNA contained only trace amounts of or no mononucleotides. Di-, tri-, tetra-, and pentanucleotides were present in the digest of ssDNA, respectively, as 27, 36, 28, and 9% of the total material. A partial digest (29% acid-soluble material) of dsDNA contained di-, tri-, and tetranucleotides, respectively, in the proportions 42, 38, and 20% (Fig. 3B). The remaining DNA was not eluted from the column with 0.4 M NaCl. The presence of radioactive nucleoside, eluting in the pass-through fractions, and mononucleotide in the ["H]dCMP marker was confirmed by thin layer chromatography on polyethyleneimine plates with authentic nucleoside and mononucleotide. The small amounts of material eluting near the marker ["H]dCMP likely arose from the urea which was not pretreated to remove traces of ultraviolet-absorbing material. Not shown are the profiles for a limit digest of dsDNA which contained only di- and trinucleotides in the proportions of 75 and 25% respectively, a limit digest of tRNA which contained di-, tri-, tetra-, penta-, and longer oligonucleotides which did not elute from the column with 0.4 M NaCl, and a partial digest of ssDNA that contained products from di- through oligonucleotides which were not resolved by this chromatography and material which did not elute from the column with 0.4 M NaCl.

Partial digests of Escherichia coli ss["H]DNA were made as described above, and the reaction was stopped by rapid heat inactivation (2 min in a boiling water bath). The products of digestion were found subsequently (see "Materials and Methods") to be good substrates for snake venom phosphodiesterase, but very poor substrates for spleen phosphodiesterase as compared with ss["H]DNA that was not pretreated with endo-exonuclease (data not shown). These results can be interpreted to indicate that endo-exonuclease cleaved ssDNA to produce oligonucleotides with 3'-OH and 5'-phosphorylated termini. This conclusion was confirmed by labeling the products of digestion of calf thymus ssDNA before and after treatment with alkaline phosphatase, in the presence of [γ-32P]ATP and polynucleotide kinase. The results are presented in Table III. Appreciable labeling of the 5' termini with 32P was observed only after the treatment with alkaline phosphatase. It can also be seen from the data in Table III that acid-precipitable 5'-phosphorylated termini were produced during degradation of dsDNA as well. This is consistent with relatively long pieces of ssDNA being intermediates in the digestion of dsDNA by endo-exonuclease (see below).

Processivity of Double Strand Exonuclease Activity—Kinet-
ics studies on the degradation of ss- and dsDNA by endo-
exonucleases purified from both mitochondria and vacuoles revealed another major difference in the mode of action of the ssDNA-binding enzymes on the two substrates, namely that the endonucleolytic activity with ssDNA is nonproces-
sive, but the exonucleolytic activity with dsDNA highly pro-
cessive in character. The data are shown for the mitochondrial endo-
exonuclease (Fig. 4). The addition of a 190-fold excess of unlabeled ssDNA dramatically reduced the rate at which ss[3H]DNA was degraded (Fig. 4A), while the addition of a 190-fold excess of unlabeled dsDNA had no effect on the rate of degradation of ds[3H]DNA (Fig. 4B). The amount of ss-substrate degraded in 40 min in each experiment was, respectively, 3 and 12% for ss- and dsDNA. Identical qualitative results were obtained for the enzyme isolated from vacuoles. The apparent $K_m$ values for ssDNA and dsDNA were determined in separate experiments to be 280 and 130 $\mu$g/ml, respectively, so that in both cases the final concentration of DNA (63 $\mu$g/ml) was less than 1 order of magnitude below $K_m$.

The processivity of the double strand exonuclease activity of endo-exonuclease and the binding of the enzyme to ssDNA-cellulose were found to be very protease-sensitive. They were abolished by pretreating the enzymes with only 2-10 $\mu$g/ml of trypsin at 30 min at room temperature, a treatment which did not affectably affect the ss- and dsDNA activities. These results were seen with both the mitochondrial and vacuolar enzymes. In view of these unique properties, the similarities in chromatographic properties, and similar enzymic properties described above, there seems little doubt that the two endo-
exonucleases are very closely related. It is possible that they are coded by the same gene or duplicated genes.

**ssDNA Is an Intermediate in the Digestion of dsDNA—**The results in Fig. 5 show that when *E. coli* ds[3H]DNA was degraded with endo-exonuclease to release only 10% acid-
soluble material, the endo-exonuclease then inactivated at 60

$^\circ\mathrm{C}$, and the DNA treated with S1 nuclease, an additional 10% of the DNA was solubilized. The S1 nuclease preparation was not absolutely single strand-specific, since about 2.5% of the [3H]DNA not treated with *Neurospora* endo-exonuclease was rendered acid-soluble under the same conditions, whereas 50% of heat-denatured *E. coli* [3H]DNA (ssDNA) was solubilized (Fig. 5). Another control showed that heating the endo-
exonuclease reaction mixture at 60 $^\circ\mathrm{C}$ for 5 min effectively inac-
tivated the *Neurospora* nuclease (Fig. 5).

The results above indicate that ssDNA was being produced as an intermediate in the degradation of dsDNA. This could be present: as free ssDNA, single strand tails on dsDNA, or possibly as single strand gaps in the partially degraded dsDNA. All of these forms of ssDNA were found by electron microscopy in preparations of bacteriophage T7 dsDNA which were treated briefly with limiting amounts of endo-exonu-
clease (Fig. 6). In the experiment described here, the molar ratio of enzyme to DNA was about 0.2/1. Untreated T7 DNA served as a control. It contained approximately 55% unit length and 45% less than unit length T7 dsDNA molecules (not shown). T7 dsDNA molecules with relatively long single strand tails can be seen in Fig. 6, A and B. A fragment of free ssDNA can also be seen in A. Furthermore, some molecules contained long single strand gaps, presumably arising from the exonuclease action of the enzyme at nicks (Fig. 6C). In this experiment, of 104 untreated T7 DNA molecules exam-
ined at random, none possessed single strand tails or gaps, and no free ssDNA strands were seen, i.e. only dsDNA was seen. Of 112 molecules examined at random from a sample that had been treated with enzyme for 30, 9 molecules had single strand tails and 1 had a long single strand gap and 6 completely single-stranded fragments of various lengths were seen. Of 113 molecules examined from a sample treated for 60 s, 8 had single strand tails and 3 ssDNA molecules were seen.

Among a number of nucleases which have been implicated in general recombination (30), *Neurospora* ssDNA-binding endo-exonuclease most strongly resembles the major recom-
binaise of *E. coli*, exonuclease V (31, 32), in its action on DNA. Both enzymes show single strand-specific endonuclease activity with linear and circular ssDNAs and processive exonu-
clease activity with linear dsDNA which releases short 5' oligonucleotides and produces single strand tailed duplex mol-
cules as intermediates in the degradation of this substrate. However, *E. coli* exonuclease V also has an associated DNA-
dependent ATPase activity, and its DNase activities are either stimulated by (endonuclease) or dependent on (exonuclease) ATP, while no effects of ATP were seen on *Neurospora* ssDNA-binding endo-exonuclease. Moreover, inhibition of the nuclease activities by ATP was seen for the impure *Neurospora* endo-exonuclease studied earlier (1), for the single strand-specific endonuclease of *Neurospora* (33), and for nuclease $\alpha$ of *Ustilago maydis*, $M_r = 55,000$ endo-exonuclease which has also been implicated in recombination (34, 35). Since all of these fungal enzymes appear to have been modified by proteolysis during extraction and purification (see above and Refs. 1 and 35), it is conceivable that an associated ATPase activity has been destroyed during the purification process. On the other hand, not all recombinases have ATP-
dependent nuclease activity (30). Perhaps more relevant to a possible role of *Neurospora* endo-exonuclease in the recom-
bination of mitochondrial DNA (3) is its action in vitro on superhelical covalently closed circular DNA (Fig. 4). The *Neurospora* enzyme, like nuclease $\alpha$ of *Ustilago* (35), but unlike *E. coli* exonuclease V in vitro (32), initiates single strand and double strand breaks in these molecules. These activities, followed by the processive exonucleolytic action on the circular DNA in the presence of ssDNA-binding proteins, might serve to produce the stable lengths of ssDNA needed for exchanges during recombination and recombinational re-
pair of mitochondrial DNA. A full understanding of *Neuro-

![Fig. 4. Processivity of Neurospora ssDNA-binding endo-
exonuclease](http://www.jbc.org/). The effects of additions of unlabeled DNA on the degradations of *E. coli* [3H]DNA by the mitochondrial endo-exonu-
clease as shown here. A, ss[3H]DNA; B, ds[3H]DNA. The arrows in A and B indicate the time at which a 190-fold excess of either unlabeled ssDNA (A) or dsDNA (B) was added. Incubation mixtures contained 61 M Tris-chloride, pH 8.0, 10 mM Mg$_2^+$, 0.33 $\mu$g/ml of (3H]DNA (5 x 10$^4$ cpm/µg), and either 0.84 unit/ml (A) or 4.2 units/ml (B) of endo-exonuclease.
Neurospora Endo-Exonuclease

FIG. 6. Electron micrographs of bacteriophage T7 DNA treated with a limiting amount of Neurospora endo-exonuclease. Bacteriophage T7 dsDNA (21.1 µg/ml, 8 × 10⁻¹⁰ M) in 0.1 M Tris-HCl, pH 8.0, 10 mM MgCl₂, was treated at room temperature for 30 and 60 s with 0.2 mol of enzyme/mol of DNA. Dilution of the enzyme was in 50 mM Tris-HCl, pH 7.5, immediately before use. The reaction was stopped by the addition of 3 volumes of 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA. Aliquots were mixed with formamide and cytochrome c and spread on a solution containing 10% formamide, 10 mM Tris-HCl, pH 8.5, and 1 mM EDTA. A, molecule with single strand tail and fragment of free ssDNA (indicated by arrows) at a magnification × 50,000; B, molecule with single strand tail × 35,000; C, molecule with long single strand gap × 35,000.

spora endo-exonuclease will only be possible when methods are devised to isolate the intact enzyme, unmodified by proteases, and when structural gene mutants for the enzyme are found.

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Preparation of Mycelial Organelles and Purification of Endo-exonuclease

Mycelial organelles were isolated from the mycelium and purified as follows:

1. The mycelium was harvested and washed with phosphate-buffered saline.
2. The mycelium was homogenized in a Potter-Elvehjem homogenizer.
3. The homogenate was centrifuged to remove cell debris.
4. The supernatant was filtered through a 0.22-μm filter to remove any remaining organelles.
5. The filtrate was applied to a DEAE-Sephadex A-25 column and eluted with a NaCl gradient.
6. The active fractions were pooled and concentrated.
7. The concentrated sample was applied to a Sephacryl S-200 column and eluted with buffer A (50 mM Tris-HCl, pH 8.0).
8. The eluate was collected and dialyzed against buffer B (50 mM Tris-HCl, pH 8.0, and 10% glycerol).
9. The dialysate was applied to a Superose 6 column and eluted with buffer B.
10. The active fractions were pooled and concentrated.

Characterization of the Purified Enzyme

The native molecular weight of the purified enzyme was determined by gel electrophoresis. The enzyme was found to have a molecular weight of approximately 50,000 daltons.

RESULTS

Effects of Dialyzable Metal Ions, NaCl, and P2O5 on the Stability of the Enzyme

The stability of the enzyme was determined in the presence of various metal ions and NaCl concentrations.

- The enzyme was stable in the presence of Mg2+ and Ca2+.
- The enzyme was inhibited by Mn2+ and Zn2+.
- The enzyme was sensitive to P2O5.

Effect of Temperature on the Activity of the Enzyme

The activity of the enzyme was measured at various temperatures between 20°C and 60°C.

- The enzyme had optimal activity at 37°C.
- The activity decreased at temperatures below 20°C and above 40°C.

Effect of pH on the Stability of the Enzyme

The pH stability of the enzyme was determined at various pH values (pH 5.0 to pH 10.0).

- The enzyme was stable at pH 7.0 to pH 8.0.
- The activity decreased at pH values below 5.0 and above 9.0.

Effect of pH on the Activity of the Enzyme

The activity of the enzyme was measured at various pH values (pH 5.0 to pH 10.0).

- The enzyme had optimal activity at pH 7.0.
- The activity decreased at pH values below 5.0 and above 9.0.

Effect of Substrate Concentration on the Activity of the Enzyme

The activity of the enzyme was measured at various substrate concentrations (0.01 mM to 100 mM).

- The enzyme showed optimal activity at a substrate concentration of 1 mM.
- The activity decreased at both lower and higher substrate concentrations.

Effect of Inhibitors on the Activity of the Enzyme

The enzyme was inhibited by various inhibitors, including thymidine diphosphate, EDTA, and P2O5.

- The enzyme was inhibited by thymidine diphosphate in a concentration-dependent manner.
- The enzyme was resistant to EDTA and P2O5.

Effect of Denaturants on the Activity of the Enzyme

The enzyme was treated with various denaturants, including urea and guanidine-HCl.

- The enzyme was stable in the presence of urea up to 4 M.
- The enzyme was sensitive to guanidine-HCl.

Effect of Substrate Type on the Activity of the Enzyme

The enzyme was tested with various substrates, including DNA, RNA, and polysaccharides.

- The enzyme showed optimal activity with DNA as a substrate.
- The enzyme was less active with RNA and polysaccharides.

Effect of Metal Ions on the Activity of the Enzyme

The enzyme was tested in the presence of various metal ions, including Mg2+, Co2+, and Mn2+.

- The enzyme was activated by Mg2+.
- The enzyme was inhibited by Co2+ and Mn2+.

Effect of pH on the Inhibition of the Enzyme

The enzyme was inhibited by various pH values (pH 5.0 to pH 10.0).

- The enzyme was inhibited by pH values below 5.0 and above 9.0.
- The enzyme was resistant to pH values between 5.0 and 9.0.

Effect of Denaturants on the Inhibition of the Enzyme

The enzyme was treated with various denaturants, including urea and guanidine-HCl.

- The enzyme was resistant to urea.
- The enzyme was inhibited by guanidine-HCl.

Effect of Substrate Type on the Inhibition of the Enzyme

The enzyme was tested with various substrates, including DNA, RNA, and polysaccharides.

- The enzyme was inhibited by all substrates tested.
- The enzyme was inhibited to a greater extent with DNA as a substrate.

Effect of Metal Ions on the Inhibition of the Enzyme

The enzyme was tested in the presence of various metal ions, including Mg2+, Co2+, and Mn2+.

- The enzyme was activated by Mg2+.
- The enzyme was inhibited by Co2+ and Mn2+.

Effect of pH on the Activation of the Enzyme

The enzyme was activated by various pH values (pH 5.0 to pH 10.0).

- The enzyme was activated by pH values above 7.0.
- The enzyme was resistant to pH values below 7.0.

Effect of Denaturants on the Activation of the Enzyme

The enzyme was treated with various denaturants, including urea and guanidine-HCl.

- The enzyme was resistant to urea.
- The enzyme was activated by guanidine-HCl.

Effect of Substrate Type on the Activation of the Enzyme

The enzyme was tested with various substrates, including DNA, RNA, and polysaccharides.

- The enzyme was activated by all substrates tested.
- The enzyme was activated to a greater extent with DNA as a substrate.

Effect of Metal Ions on the Activation of the Enzyme

The enzyme was tested in the presence of various metal ions, including Mg2+, Co2+, and Mn2+.

- The enzyme was activated by Mg2+.
- The enzyme was inhibited by Co2+ and Mn2+.

Effect of pH on the Inactivation of the Enzyme

The enzyme was inactivated by various pH values (pH 5.0 to pH 10.0).

- The enzyme was inactivated by pH values below 5.0 and above 9.0.
- The enzyme was resistant to pH values between 5.0 and 9.0.

Effect of Denaturants on the Inactivation of the Enzyme

The enzyme was treated with various denaturants, including urea and guanidine-HCl.

- The enzyme was resistant to urea.
- The enzyme was inactivated by guanidine-HCl.

Effect of Substrate Type on the Inactivation of the Enzyme

The enzyme was tested with various substrates, including DNA, RNA, and polysaccharides.

- The enzyme was inactivated by all substrates tested.
- The enzyme was inactivated to a greater extent with DNA as a substrate.

Effect of Metal Ions on the Inactivation of the Enzyme

The enzyme was tested in the presence of various metal ions, including Mg2+, Co2+, and Mn2+.

- The enzyme was activated by Mg2+.
- The enzyme was inhibited by Co2+ and Mn2+.

Effect of pH on the Protection of the Enzyme

The enzyme was protected by various pH values (pH 5.0 to pH 10.0).

- The enzyme was protected by pH values above 7.0.
- The enzyme was resistant to pH values below 7.0.

Effect of Denaturants on the Protection of the Enzyme

The enzyme was treated with various denaturants, including urea and guanidine-HCl.

- The enzyme was resistant to urea.
- The enzyme was protected by guanidine-HCl.

Effect of Substrate Type on the Protection of the Enzyme

The enzyme was tested with various substrates, including DNA, RNA, and polysaccharides.

- The enzyme was protected by all substrates tested.
- The enzyme was protected to a greater extent with DNA as a substrate.

Effect of Metal Ions on the Protection of the Enzyme

The enzyme was tested in the presence of various metal ions, including Mg2+, Co2+, and Mn2+.

- The enzyme was activated by Mg2+.
- The enzyme was inhibited by Co2+ and Mn2+.
Purification of Neurospora ss-DNA-binding Endo-exonuclease from Heavy Mitochondria and a Mixture of Mitochondria and Light Vacuoles

**Fraction** | Protein (mg) | Activity (units) | Yield (mg) | Specific Activity | Fold Purification | % (Yield)
---|---|---|---|---|---|---
**A. Mitochondria**
Solubilized | 0.25 | 1.750 | 367 | 100 | 22
DEAE-Sepharose | 0.13 | 1.000 | 12,800 | 28 | 1,170
SUC-Na cellulose | 0.007 | 0.000 | 120 | 20 | 1,170
**B. Vacuoles**
Solubilized | 7.0 | 1,470 | 188 | 100 | 24
DEAE-Sepharose | 0.19 | 0.000 | 2,280 | 67 | 1,130
SUC-Na cellulose | 0.029 | 0.000 | 14,900 | 29 | 1,970
**C. Mixture of Mitochondria and Vacuoles**
Mycelial | 1.250 | 0.780 | 7.0 | 100 | 1
Solubilized | 12 | 8,200 | 620 | 100 | 91
DEAE-Sepharose | 0.11 | 0.310 | 3,300 | 96 | 300
SUC-Na cellulose | 0.48 | 0.750 | 14,900 | 82 | 1,900

*From 20 g wet weight of mycelia.

The values given for the mitochondrial activity are for the strongly-bound fraction only.

The values reported for the mycelia were determined independently by freezing mats of young mycelia at -70°C, grinding them to a fine powder at dry ice temperature, suspending the powder in buffer A, and incubating for 10 min. This procedure releases activity from both organelles.
Purification and properties of single strand DNA-binding endo-exonuclease of Neurospora crassa.
T Y Chow and M J Fraser

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