Plant Nucleases. II. Properties of Corn Ribonucleases I and II and Corn Nuclease I

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Abstract. A classification system is presented to distinguish 3 corn nucleases—Ribonuclease I, Ribonuclease II, and Nuclease I—which were described in the first paper of this series. The 2 ribonucleases are specific for RNA, are endonucleases, and liberate purine and pyrimidine cyclic nucleotides from dinucleotide monophosphates as well as from RNA. Ribonuclease I and II hydrolyze the purine cyclic nucleotides to 3'-nucleotides, while Ribonuclease II may also act on the pyrimidine cyclic nucleotides. Ribonuclease II is best characterized by its molecular weight of 17,000, by a higher pH optimum than Ribonuclease I, and by its adsorption onto microsomes. Nuclease I is a partially purified endonuclease which produces 5'-nucleotides from RNA, DNA, and dinucleotide monophosphates. The same enzyme may also be a 3'-nucleotidase. The corn nucleases were compared with nucleases found in other plant species.

The preceding paper described the separation and partial purification of 3 nucleases from corn (38). Two enzymes possess RNase activity [Ribonuclease nucleotido-2'-transferase (cyclizing), E. C. 2.7.7.17] while the third has a broader specificity, attacking both RNA and DNA and probably also 3'-nucleotides. Schmidt and Laskowski (24) and Laskowski (13) presented a number of criteria that are useful in the classification of nucleases. The first 4 criteria were used as a start of a classification of plant nucleases, with the enzymes isolated from corn as examples. The terms RNase I and RNase II were selected to correspond with the naming of 2 RNases found in tobacco leaves (22, 23). It is hoped that this classification will be of use to others working on plant nucleases, and will help in comparing enzymes from different species.

Materials and Methods

Enzyme Assays. The assays for RNase and DNase are described in paper I (38). 3'-Nucleotidase was assayed by the method of Shuster and Kaplan (27).

Gel Filtration. Analytical gel filtration of the enzymes on Sephadex G-100 (Pharmacia Fine Chemicals, Inc.)1 is described in paper I (38).

Mode of Action of Nuclease I. The procedure of Clark et al. (2) was followed. S-RNA (obtained from General Biochemicals, 5) was incubated with Nuclease I. Samples were withdrawn at intervals and placed in a boiling water bath to stop enzymatic action. The acid-soluble nucleotides were determined by measuring the A260 before and after precipitation of undigested RNA with uranyl acetate-perchloric acid. The size of the products was determined by gel filtration through Sephadex columns and assay of the fractions for A260. The samples were eluted with 0.2 M NaCl.

Identification of Products of Action. Dinucleotide monophosphates, cyclic nucleotides, nucleotides, and nucleosides were obtained from Sigma Chemical Company. Thin-layer chromatography was performed on cellulose powder with a fluorescent additive (MN 300 F, Macherey, Nagel, and Co.). The solvent systems were: A) ammonium sulfate, 45 g; 1 M sodium acetate, 20 ml; water, 55 ml; and isopropanol, 2 ml; and B) isopropanol, 70 ml; water, 30 ml; ammonium hydroxide (29% NH₃), 5 ml (17). The separations obtained were essentially identical with those obtained earlier with paper chromatography (36). The nucleotides were detected under ultraviolet light. A permanent record was made with a Polaroid camera using a Kodak Wratten K2 filter and 3000 speed film.

The dinucleotides were cleaved by incubating 100 µg of the dinucleotide with the enzyme in 0.1 ml of 0.025 M pH 5.6 acetate buffer, at room temperature. Samples (usually 2 µl) were placed on the thin layer plates before the enzyme was added, immediately after the enzyme was added, and at various intervals after the addition of the enzyme. The spots were dried with hot air.

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1 Trade names are given as part of the exact experimental conditions and not as an endorsement of products over those of other manufacturers.
Results and Discussion

The proposed classification of the known corn nucleases is given in table I. The data to support each criterion will be presented and discussed below. The names RNase I and Nuclease I replace the names RNase A and RNase B previously used for the corn enzymes (35, 36).

1. Substrate. Corn RNase I has no activity upon DNA (36), and the slight DNase activity of corn RNase II preparations comes from small amounts of contaminating Nuclease I (38). The initial reaction (see below) of both enzymes is the production of 2', 3'-cyclic nucleotides, which cannot be formed from DNA. Corn Nuclease I acts on both RNA and DNA, though somewhat faster on RNA under the conditions used in this laboratory. A careful study of DNA digestion conditions has not been made. Passage of corn Nuclease I through a column of Sephadex G-100 gives a single peak for RNase and DNase activity. Walters and Loring (33) reported that a similar enzyme preparation from mung beans lost DNase activity without the loss of RNase or 3'-nucleotidase activity, but this has not occurred with any corn Nuclease I preparations. Preliminary studies on the 3'-nucleotidase activity of corn Nuclease I revealed that it is eluted from the Sephadex G-100 column in the same region as the RNase and DNase activities.

2. The Mode of Action. This criterion divides the enzymes into the endo- and the exo-nucleases. When partial RNA digests produced by RNase I and RNase II were examined by paper chromatography, slow moving compounds were detected which disappeared upon longer digestion. It is assumed that these were oligonucleotides, and that RNase I and RNase II are endonucleases. Further study is obviously needed. Nuclease I attacks S-RNA (36), a molecule with a molecular weight near 30,000 (5). The digestion products of a partial digest of S-RNA by Nuclease I were examined by gel filtration (fig 1). The untreated S-RNA gave a sharp peak with Sephadex G-25, G-50, and G-75. The first digest produced 10% acid-soluble nucleotides. If the method of attack was from one end, it would be expected that the products would be a mixture of mononucleotides plus RNA of almost the same size as originally present (2). Random cleavage would produce few mononucleotides, but would rapidly reduce the number of molecules of the original size while liberating oligonucleotides of intermediate size. The bulk of the RNA in the first digest appeared in the initial peak region on G-25, while the smaller molecules did not form a discrete peak in the region of the mononucleotides. On G-50 and G-75 the first peak was shifted to a position intermediate between the original sharp peak and the final mononucleotide peak. The second digest, with 83% acid-soluble nucleotides, yielded nothing but small molecules, presumably mononucleotides. The broad peak from the G-25 column also occurs with a mixture of standard nucleotides. The conclusion is that Nuclease I cleaves RNA at random, and thus is an endonuclease.

3. Products Formed. In earlier work it was found that the products of RNA digestion by RNase I (36) and RNase II were the 4 cyclic nucleotides, identified by paper chromatography. A more elegant procedure involves the use of dinucleotide monophosphates as substrates, for the number of possible end products is limited. Figure 2 shows the products of the 3 corn enzymes. In all cases, at least some breakdown occurred in the short time needed to place the sample on the thin-layer plate and dry it. Figure 2a shows the action of RNase I.

Table I. Classification of Corn Nucleases

<table>
<thead>
<tr>
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<th>RNase I</th>
<th>RNase II</th>
<th>Nuclease I</th>
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<tr>
<td>1</td>
<td>Substrate</td>
<td>RNA</td>
<td>RNA</td>
</tr>
<tr>
<td>2</td>
<td>Mode of action</td>
<td>2',3'-Cyclic</td>
<td>2',3'-Cyclic</td>
</tr>
<tr>
<td>3</td>
<td>Products formed</td>
<td>nucleotides</td>
<td>nucleotides</td>
</tr>
<tr>
<td>3a</td>
<td>Secondary products</td>
<td>purine nucleotides</td>
<td>3'-nucleotides</td>
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<tr>
<td>4</td>
<td>Base specificity</td>
<td>Purine&lt;pyrimidine</td>
<td>Purine&lt;pyrimidine</td>
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<tr>
<td>5</td>
<td>Mol wt</td>
<td>23,000</td>
<td>17,000</td>
</tr>
<tr>
<td>6</td>
<td>pH optimum</td>
<td>5.2</td>
<td>6.7</td>
</tr>
<tr>
<td>7</td>
<td>Location in cell</td>
<td>Soluble</td>
<td>Microsomes</td>
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<tr>
<td>8</td>
<td>Enzyme commission classification</td>
<td>2.7.7.17</td>
<td>(Ribosomes?)</td>
</tr>
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Abbreviations: ApA = adenyl-(3',5')-adenosine; ApC = adenyl-(3',5')-cytidine; CpA = cytidylyl-(3',5')-adenosine; GpA = guanylyl-(3',5')-adenosine; UpA = uridylyl-(3',5')-adenosine; A-cyclic-p = adenosine 2',3'-cyclic monophosphate; C-cyclic-p = cytidine 2',3'-cyclic monophosphate.
separated from all of the products on the thin layer plates. RNase I (fig 2b) initially produced A-cyclic-p and adenosine (spot 2), then hydrolyzed the cyclic nucleotide to 3'-AMP (spots 3 and 5). In contrast, after the production of A-cyclic-p and adenosine (fig 2c, spot 2), RNase II hydrolyzed the cyclic nucleotide to adenosine. No traces of 3'-nucleotides were seen as intermediates during RNase II action on any of the dinucleotides tested, and A-cyclic-p and C-cyclic-p were both hydrolyzed. However, the RNase II preparations were contaminated with a small amount of Nuclease I, which possesses sufficient 3'-nucleotidase activity to hydrolyze the 3'-nucleotides as fast as they are formed. The action of RNase II presumably stops after the hydrolysis of the cyclic nucleotide.

Nuclease I hydrolyzed CpA to 5'-AMP and cytidine (fig 2d, spots 1–5) and ApC to 5'-CMP and adenosine (spots 9–13). No other products were detected. Note that spots 6 and 8 are the standards for the products of each dinucleotide.

None of the 3 enzymes caused any detectable hydrolysis of adenylyl-(2',5')-cytidine. They are specific for the 3',5' linkage. Similar specificities have been found for spinach (32) and soybean (20) RNases.

4. Base Specificities. The experiments reported here were not quantitative, and the conditions of the assay were not selected to give optimum rates of enzymatic activity. Further, only 5 of the 16 possible dinucleotides were tested. Both RNase I and RNase II split ApA, ApC, and GpA much more rapidly than CpA or UpA. Both enzymes rapidly hydrolyzed the purine cyclic nucleotides to the 3'-nucleotide in the case of RNase I and to the nucleoside in the case of RNase II. RNase I preparations definitely hydrolyzed the pyrimidine cyclic nucleotides, but RNase II acted very slowly, if at all, on the pyrimidine compounds. The RNase I preparations were essentially free of contaminating protein, but the RNase II was only partially purified, and its secondary activity on pyrimidine compounds might well be due to other enzymes. Stockx et al. (28) found a plant phosphodiesterase which hydrolyzed cyclic nucleotides.

No distinct base specificities were detected for Nuclease I using dinucleotides as substrates. It did release deoxy-AMP more rapidly from DNA and AMP from RNA than the other nucleotides, in similar fashion to the mung bean nuclease (31, 34).

5. Molecular Weight. The apparent molecular weights of the 3 enzymes were determined by gel filtration on Sephadex G-100 (38), which can be used for impure enzymes. Nuclease I has a molecular weight of 31,000, RNase I has a molecular weight of 23,000, and RNase II has a molecular weight of 17,000. This procedure, along with the differing adsorption onto CM-cellulose (38), most clearly distinguishes RNase I from RNase II.

6. pH Optimum. The pH activity curves presented in paper I (38) showed that RNase I pos-

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Fig. 1. Sephadex column elution patterns of S-RNA before and after treatment with Nuclease I. Experimental conditions given in Materials and Methods section. □□□□, untreated S-RNA ○○○○, S-RNA after enzymatic hydrolysis released 10% acid-soluble nucleotides; ▲▲▲▲, S-RNA after 83% release of acid-soluble nucleotides. Columns 1.6 × 30 cm, fractions 2.8 ml, sample size 1.0 ml.

on CpA² and ApC. CpA (spots 1–5) was split to adenosine and C-cyclic-p. After long incubation and with a large amount of sample on the thin-layer plate, it was possible to detect a small amount of 3'-CMP (spot 5). ApC was split to cytidine and A-cyclic-p. The latter was then hydrolyzed to 3'-AMP (spots 13 and 14). The rapid hydrolysis of A-cyclic-p must be enzymatically catalyzed, but the small amount of hydrolysis of C-cyclic-p might be due either to slow enzymatic activity or to spontaneous breakdown (30).

ApA is a good substrate for the demonstration of the secondary enzymatic action, for ApA is
sessed optimum activity at pH 5.2 and Nuclease I at pH 6.2. RNase II had the highest activity at pH 7.0 in that experiment, but in repeated experiments it appeared to be characterized by a plateau region between pH 6 and pH 7. A Crestfield type RNA (Sigma Chemical Co.) was used in these assays, but similar results were obtained with commercial yeast RNA from P-L Biochemicals. Yeast RNA obtained from Schwarz BioResearch produced a pH optimum of 5.8 for RNase I and gave a different response to salt concentration. The above experiments were done with 0.16 M KCl in the assay medium, for the pH activity curves are known to be affected by ionic concentration (35). The type of RNA, the buffer, the assay technique, or the state of purification might all have some effect on the pH curve. Thus it is sometimes difficult to compare results from different laboratories, especially when only one enzyme is studied. However, RNase I does have a definitely lower pH optimum than either RNase II or Nuclease I under all conditions examined in this laboratory.

7. Location in Cell. This must be a tentative criterion. RNase I from corn is soluble in water and does not tend to be adsorbed onto cell particles or membranes. RNase II from corn was isolated from a microsome preparation (38), and may possibly be found on partially purified ribosomes (unpublished work in this laboratory). If the tissue is extracted with a high ionic strength buffer, RNase II will be found in solution. Nuclease I was isolated from particles precipitated from a sucrose homogenate of corn roots (38). It might be located in the nucleus, mitochondria, spherosomes (25), endoplasmic reticulum, or vacuoles (18).

8. Classification. The assignment of RNase I and RNase II to the Enzyme Commission Classification of 2.7.7.17, ribonuclease nucleotido-2'-trans-
erase (cyclizing) presents no problems. The classification system has not been developed to the state where these 2 enzymes of such similar activity could be assigned different numbers. Nuclease I might be placed in class 3.1.4.x as a phosphodiesterase with a specificity toward polynucleotides, while the class 3.1.3.6 might also apply if the 3'-nucleotidase activity is located on the same enzyme as the RNase and DNase.

A survey of the literature on plant nucleases revealed a small number of enzymes that can be fairly well identified with the proposed classes, a larger number that might fit one of the classifications, and only a few enzymes which apparently did not fit.

RNase I type enzymes are widespread in plants, and are generally characterized by the production of all 4 cyclic nucleotides and the hydrolysis of the purine cyclic nucleotides (22). Corn RNase I is a soluble enzyme and is relatively stable; thus it presents fewer problems during purification than other nucleases and is more likely to be retained during purification (38).

An RNase II type enzyme was isolated from tobacco, and can be identified on the basis of its location on the microsomes and its pH optimum (23). The identification of impure plant RNase II by the hydrolysis of pyrimidine cyclic nucleotides (22) may not be reliable when other enzymes are present. Microsomes from pea (19) and wheat (9) contain nucleases, the latter perhaps having a mixture of a Nuclease I type enzyme and an RNase. Tests for DNase activity and molecular weight determinations might help to resolve some of the questions about microsomal nucleases.

Nuclease I presents a real problem in determining whether it might represent a general class of plant nucleases or if it is a mixture of several enzymes. Sung and Laskowski (31) purified a similar enzyme from mung bean, but Walters and Loring (33) reported that the DNase activity of mung bean could be lost independently of the RNase activity. Carlsson and Frick (1) found 2 RNase-DNase enzymes and 1 RNase in garlic, with the RNase having the smallest size. Freeman (4) found that his semi-purified ryegrass RNase would liberate cyclic nucleotides at low pH and 5'-nucleotides at high pH. The widespread occurrence of at least 1 plant enzyme which liberates 5'-nucleotides from RNA or DNA is shown by the use of crude extracts from several species to produce 5'-nucleotides on a large scale (12, 14, 15). The type of hydrolysis was controlled by suitable inhibitors, activators, or pH. Holbrook et al. (8) purified a barley nuclease which differed from Nuclease I in being an exonuclease and in having a molecular weight near 122,000. Harvey et al. (7) found a phosphodiesterase in carrot which was an exonuclease and had a pH optimum at 9.0 to 9.5, and also had a high molecular weight. Evidence for a second enzyme possessing DNase activity was found in corn root tips (38).

Further confusion enters when we consider enzymes determined as phosphodiesterases, often with bis-p-nitrophenyl-phosphate as the substrate. They may have little (28) or no (6) activity on polynucleotides. Corn and pea seedlings possess 2 different phosphodiesterases, determined with p-nitrophenyl thymidine 5'-phosphate as the substrate (21), but activity against polynucleotides was not determined.

A third enzymatic activity involved in the corn Nuclease I is that of 3'-nucleotidase. Associations of partially purified nuclease activity with 3'-nucleotidase activity have also been reported for mung bean (16), ryegrass (4), and barley (8). However, mung beans (29) and peas (3) contain a phosphatase which acts on 3'-nucleotides but not on RNA. Wheat contains a nonspecific phosphatase which possesses a puzzling specificity toward certain oligo- and polynucleotides (11). Relative changes in nuclease activity and 3'-nucleotidase activity during the germination of ryegrass (26) and corn (10) suggest that not all of the 3'-nucleotidase activity is found in a single enzyme. The evidence is fairly
FIG. 2. Thin-layer chromatography of the digestion of dinucleotides by corn nucleases. Solvent system (a) developed for about 90 min. A line marks 10 cm from points of application. Standards for figure 2a, b, and c (listed from slowest to fastest): spot 6: A-cyclic-p, G-cyclic-p, C-cyclic-p; spot 7: adenosine, guanosine, cytidine; spot 8: 3'-AMP, 2'-AMP, 3'-GMP, 2'-GMP, 2' + 3'-CMP; spot 9: 5'-AMP, 5'-GMP, 5'-CMP. Standards for figure 2d: spot 6: 5'-AMP, cytidine; spot 7: 3'-AMP, 2'-AMP, guanosine, 3'-GMP, 2'-GMP; spot 8: adenosine, 5'-CMP; spot 14: A-cyclic-p, 2' + 3'-CMP. Treatments. Figures 2a and b, RNase I: Spots 1 and 10 - no enzyme; spots 2 and 11 - immediately after adding 200 units of enzyme; spots 3 and 12 - 15 min incubation; spots 4 and 13 - 3 hr incubation; spots 5 and 14 - 3 hr incubation, but 5 times as much sample applied. Figure 2c: RNase II: spot 1 - no enzyme; spot 2 - immediately after adding 44 units of enzyme; spots 3 and 12 - 15 min incubation; spots 4 and 1 hr incubation; spots 5 and 13 - 3 and one-half hr incubation. Figures 2d. Nuclease I: spots 1 and 9 - no enzyme; spots 2 and 10 - immediately after adding 12 units of enzyme; spots 3 and 11 - 15 min incubation; spots 4 and 12 - 1 hr incubation; spots 5 and 13 - 3 and one-fourth hr incubation.
strong, but not conclusive, that there is a plant enzyme which is capable of hydrolyzing the 3'-phosphate bond of either a mononucleotide or a polynucleotide.

These studies and those reported in the previous paper (38) on corn Nuclease I, have not carried the purification far enough, nor have they critically examined all of the possible enzymatic activities in sufficient detail to answer the questions raised.

Literature Cited


