maintained in the dark until use. DPH is dissolved in tetrahydrofuran and added to the initial lipid solution (1000 : 1 lipid/probe weigh ratio).

The thermotropic behavior of the phospholipid is shown by the thermal variation around the $T_c$ of the fluorescence polarization degree of the probe at 425 nm for excitation at 365 nm. This is analyzed by 1 stepwise increase (10 min equilibration at each temperature) from temperatures below $T_c$. Any change in order and mobility of the phospholipids produced by the protein results in a modification of the thermotropic plot.

Acknowledgments

This work was supported by grant from the MEC (Spain) and NSERC (Canada: R. K., J. D.). A. M.-R. and L.G.-O. are recipients of fellowships from the M.E.C. (Spain).

[22] Secretory Acid Ribonucleases from Tomato. 
_Lycopersicon esculentum_ Mill. 

_By Steffen Abel and Margret Kock_

Introduction

The first secretory ribonuclease from tomato was identified in isolated vacuoles of cultured tomato cells by Abel and Glund. $^1$ It was shown later that cultured tomato cells starved for inorganic phosphate (P_i) induce a limited set of ribonucleases, which comprise extracellular RNase LE, three vacuolar enzymes, RNases LV-1, LV-2, and LV-3, and microsomal RNase LX. Subsequently, these ribonucleases were purified, their primary structures directly determined by protein sequencing, and their enzymatic properties characterized. Isolation of cDNAs coding for RNase LE and RNase LX confirmed the chemically determined primary structures. The deduced secretory targeting signals are in agreement with previous cellular localization studies.

Plant ribonuclease proteins structurally related to RNases LE and LX were purified and characterized from seeds of bitter gourd (Momordica charantia), RNase MC1, and from leaves of Nicotiana glutinosa, RNase NW. $^2,3$ A number of similar plant ribonucleases were identified by gene cloning such as RNS1, RNS2, and

RNS3 from Arabidopsis thaliana or ZRNase I and ZRNase II from Zinnia elegans. This group of related plant enzymes was designated S-like ribonucleases. S-like RNases share conserved sequence motifs that are also found in S-gene products, which play a role in self-incompatibility cell-to-cell interactions, in fungal RNases typified by RNase T2 as well as in animal and bacterial RNases with acidic pH optima. Amino acid residues important to the catalytic activity of S-like RNases, foremost conserved histidine residues, are present in these motifs. All the above-mentioned enzymes are members of the superfamily of acid T2-type ribonucleases, which is also known as the superfamily of T2/S ribonucleases.

It became clear that S-like RNases likely have functions in a number of physiological processes other than in self-incompatibility interactions, which is indicated by differential gene expression in response to a variety of exogenous factors and during plant development. Such physiologic processes include leaf and flower senescence and responses to plant hormones, inorganic phosphate (P_i) starvation, or wounding.

This article focuses on the analysis of secretory acid ribonucleases from tomato, which are synthesized in remarkably high amounts in cultivated tomato cells. We describe enzyme purification from cultured tomato cells and biochemical analysis of several protein parameters of the highly similar RNases. We further discuss structural relationships between the tomato enzymes with regard to their subcellular locations.

Source of Enzymes

All studies are conducted with purified ribonucleases prepared from cell suspension cultures of tomato (Lycopersicon esculentum Mill. cv. Lukullus). Cell suspension cultures provide an advantage in (i) producing and purifying the enzymes of interest in large amounts, and (ii) conveniently carrying out biochemical and immunological analyses in cellular localization studies.

The cell suspension culture is propagated in a modified Murashige–Skoog medium. Suspensions contain mostly small aggregates of 20–50 cells and plasma-rich, single cells to a lesser extent. Cultures are inoculated with 2 × 10^7 cells from 3-day-old exponentially growing cell cultures and are cultivated for 2–6 days in complete medium depending on purpose. The P_i concentration of the...
liquid medium drastically decreases until day 3 of cultivation. Subsequently, cells enter the stationary growth phase during which secretory acid RNases and other P₁-regulated enzymes are induced.¹⁻³,⁹,¹⁰ Alternatively, to induce more efficiently secretory ribonucleases, 3-day-old cells of the logarithmic growth phase can be transferred into P₁-free medium and subcultured for 3–4 days.¹¹

Assay Methods

Spectrophotometric Assay of Ribonucleolytic Activity

*Principle.* Ribonuclease activity is assayed essentially according to the method of Ambellan and Hollander¹² by following the absorbance at 260 nm of the time-dependent release of ethanol-soluble hydrolysis products from high molecular weight RNA substrates.

*Reagents*

- RNA substrate: High molecular weight fraction of yeast RNA (from *Torula* yeast, Type II-S; Sigma), which is prepared by gel filtration on Sephadex G-25, ethanol precipitation and dissolving of the RNA precipitate to a concentration of 5 mg/ml in water
- Assay buffer: 150 mM Sodium acetate (pH 5.6)
- Precipitation reagent: 50% (v/v) ethanol containing 50 mM sodium acetate (pH 5.5), 10 mM magnesium acetate, and 0.8 mM La(NO₃)₃

*Procedure.* Mix 50 µl of assay buffer with 50 µl of appropriate amounts of enzyme in 1.5 ml Eppendorf tubes and preincubate for 5 min at 37°C. Start the assay by adding 50 µl of RNA substrate (250 µg) to the reaction mixture. Continue the incubation for 0–60 min at 37°C and stop the reaction with 1.35 ml of precipitation reagent. Briefly mix and chill for at least 45 min at –20°C. Sediment the precipitating RNA substrate by centrifugation at room temperature (12,000g, 20 min). Carefully remove the supernatant and measure its absorbance at 260 nm against a blank taken at zero time. The ribonuclease assay is linear with respect to time and enzyme concentration for up to an absorbance of A₂₆₀ of 1.0. One ribonuclease unit is defined as the amount of enzyme causing an increase in ΔA₂₆₀ of 1.0 min⁻¹ cm⁻¹ ml⁻¹ according to Wilson.¹³

---

Determination of Relative Base Specificity by HPLC Analysis of RNA Hydrolysis Products

**Principle.** The enzyme- and time-dependent release of mononucleotides from yeast RNA is followed by isocratic reversed-phase high-performance liquid chromatography (HPLC).\(^{14}\)

**Reagents**

RNA substrate: High molecular weight fraction of yeast RNA (from *Torula* yeast, Type II-S; Sigma), which is prepared as described above
Assay buffer: 50 mM Sodium acetate (pH 5.6)
HPLC system: Merck-Hitachi LiChroGraph (Darmstadt, Germany), pre-packed Octadecyl-Si 100 Polyol column (4.6 x 250 mm, 5 \(\mu\)m, Serva Feinbiochemica, Heidelberg, Germany), 20 mM (NH\(_4\))H\(_2\)PO\(_4\) (pH 6.2) as mobile phase buffer

**Procedure.** Mix 10 mg of RNA and 0.5 units of purified ribonuclease in a total volume of 2 ml of assay buffer and incubate at 37° under moderate shaking. After appropriate intervals (0–60 min), remove 100 \(\mu\)l of the reaction mixture and add to 900 \(\mu\)l of cold ethanol. Briefly mix and chill for at least 2 h at −20°. Centrifuge at room temperature for 20 min at 12,000 g to sediment the precipitating RNA substrate. Carefully remove and evaporate supernatant to dryness. Dissolve residue in 100 \(\mu\)l of mobile phase buffer. Inject sample volumes of 5 \(\mu\)l to 30 \(\mu\)l onto the HPLC column, develop column in the isocratic mode at a flow rate of 1.5 ml \(\text{min}^{-1}\), and monitor eluates at 254 nm. Identify 2',3'-cyclic NMP and 3'(2')-NMP products by comparing their retention times with those of authentic standards. For quantification of the time-dependent release of mononucleotide products, i.e., 2',3'-cyclic NMP and 3'(2')-NMP for each nucleobase, generate calibration curves for each authentic compound. Calibration curves should be linear in the range of 0.1 nmol to at least 4.0 nmol.

Determination of Relative Base Specificity by HPLC Analysis of Diribonucleoside Monophosphate Hydrolysis Products

**Principle.** The enzyme- and time-dependent hydrolysis of the 16 diribonucleoside monophosphate substrates (NpN) is followed by isocratic boronate affinity HPLC.

**Reagents**

Substrates: Diribonucleoside monophosphates (Sigma, St. Louis, MO)
Assay buffer: 50 mM Sodium acetate (pH 5.6)

---

HPLC system: Merck-Hitachi LiChroGraph, prepacked Dihydroxyboronyl-Si 100 Polyol column (4.6 × 250 mm, 5 μm, Serva Feinbiochemica). 10 mM KH₂PO₄ (pH 6.0) as mobile phase buffer

Procedure. Reactions are carried out in a total volume of 100 μl containing assay buffer, 1 mM of the respective NpN substrate, and appropriate amounts of enzyme activity (0.005–0.5 units). Preincubate the enzyme in assay buffer for 5 min at 37°. Start the reaction by adding the substrate to the reaction mixture and continue the incubation for 0–60 min at 37°. Use heat-inactivated enzyme for each substrate in control incubations. Terminate the reactions by injecting a 10-μl aliquot directly onto the HPLC column. Develop the column in the isocratic mode at a flow rate of 1.5 ml min⁻¹ and monitor eluates at 254 nm. Identify NpN substrates and 2',3'-cyclic NMP, 3'(2')-NMP and nucleoside hydrolysis products by comparing their retention times with those of authentic standards. Calculate reaction velocities of enzymatic NpN hydrolysis by quantitating 2',3'-cNMP generation. Use calibration curves as described above.

Assay of Intrinsic Cyclic Nucleotide Phosphodiesterase Activity by HPLC

Principle. The enzyme- and time-dependent hydrolysis of 2',3'-cyclic NMP substrates is followed by isocratic reversed-phase HPLC.

Reagents

Substrates: 2',3'-cyclic NMP (Sigma)
Assay buffer: 50 mM Sodium acetate (pH 5.6)
HPLC system: Merck-Hitachi LiChroGraph, prepacked Octyl-Si 100 Polyol column (4.6 × 250 mm, 5 μm, Serva Feinbiochemica), 20 mM(NH₄)H₂PO₄ (pH 6.2) as mobile phase buffer

Procedure. Reactions are carried out in a total volume of 100 μl containing assay buffer, 1 mM of the respective 2',3'-cyclic NMP substrate, and appropriate amounts of enzyme activity (0.005–0.5 units) essentially as described for the hydrolysis of NpN substrates (see above). Terminate the reactions by injecting a 10-μl aliquot directly onto the HPLC column. Develop the column in the isocratic mode at a flow rate of 1.5 ml min⁻¹ and monitor eluates at 254 nm. Identify 2',3'-cyclic NMP substrates and 3'(2')-NMP hydrolysis products by comparing their retention times with those of authentic standards. Calculate reaction velocities of enzymatic 2',3'-cyclic NMP hydrolysis by quantitating 3'(2')-NMP generation based on calibration curves (see above).

Test for 3'- and 5'-Nucleotidase Activity by HPLC

Principle. The enzyme- and time-dependent hydrolysis of 3'-NMP and 5'-NMP substrates is followed by isocratic reversed-phase HPLC.
Reagents

Substrates: 3'-NMP, 5'-NMP (Sigma)
Assay buffer: 50 mM Sodium acetate (pH 5.6); 50 mM Tris-HCl (pH 8.8)
HPLC system: Merck-Hitachi LiChroGraph, prepacked Octyl-Si 100 Polyol column (4.6 x 250 mm, 5 μm, Serva Feinbiochemica), 20 mM (NH₄)H₂PO₄ (pH 6.2) as mobile phase buffer

Procedure. Reaction mixtures (total volume of 100 μl) contain assay buffer, 5 mM of the respective 3'-NMP or 5'-NMP substrate, and 4 units of purified enzyme. Incubate for 24 h at 37 °. Terminate the reactions by injecting a 10-μl aliquot directly onto the HPLC column. Develop the column in the isocratic mode at a flow rate of 1.5 ml min⁻¹ and monitor eluates at 254 nm. Identify 3'-NMP and 5'-NMP substrates and nucleoside hydrolysis products by comparing their retention times with those of authentic standards. Purified secretory ribonucleases from tomato should not contain any detectable nucleotidase activity.

Determination of Ribonuclease Activity by in-Gel Assays

Disc gel electrophoresis on native polyacrylamide slab gels (12% or 15% acrylamide) is performed without SDS using the discontinuous buffer system according to Laemmli.¹⁵ Nondenatured proteins in sample buffer lacking SDS and 2-mercaptopethanol are loaded. After electrophoresis, RNases are detected by negative activity staining. The gels are (i) equilibrated with 150 mM sodium acetate buffer (pH 5.6) for 2 x 10 min, (ii) incubated in substrate solution (150 mM sodium acetate buffer [pH 5.6], 2.5 mM EDTA, 0.4% yeast RNA) for 30 min at 37 °, (iii) briefly rinsed in equilibration buffer, (iv) stained in 0.2% (w/v) toluidine blue, 0.5% (v/v) acetic acid for 5 min, and (v) destained in 0.5% (v/v) acetic acid until transparent activity bands appear in the otherwise intensely stained gel. The addition of 2.5 mM EDTA to the substrate solution improves the staining pattern by inhibiting nuclease activities and unspecific reactions. The quality of the RNA used is not critical. We successfully used crude technical preparations of yeast RNA. The substrate and staining solutions can be used several times. Store the substrate solution at −20 °.

Alternatively, SDS–polyacrylamide gel electrophoresis followed by activity staining was described for separating and detecting ribonucleases according to their molecular weight.¹⁶ This assay incorporates the RNA substrate into the gel prior to electrophoresis, which reduces the time required for activity staining. However, since the molecular weights of secretory RNases from tomato are very similar, native gel electrophoresis is the preferred method for in-gel activity assays.

Purification Procedures

Purification of Intracellular RNases (RNase LX, RNases LV-1, LV-2, LV-3)

All steps of the purification protocol are assessed by activity staining in native gels for the presence and enrichment of the individual RNases. The spectrophotometric assay is used to measure total RNase activity.

Cell Extraction. Cells for preparation of protein extracts are grown for 3 to 4 days in Pi-free medium \(^{11}\) and washed extensively with water to remove extracellular RNase LE. The washed cells are rinsed with acetone and dried at room temperature. All the following steps are performed at 4 °C. To prepare cellular extracts, cells (1 kg wet weight) are resuspended in 4 liters of extraction buffer (100 mM citric acid–Na₂HPO₄ buffer [pH 7.0] 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 0.1 % [w/v] Triton X-100) and are disrupted by sonication (200-ml aliquots, 3 × 1 min, 400 W). The homogenate is centrifuged to remove cell debris (4000g, 20 min). The protein fraction of the supernatant precipitating between 60% and 75% acetone is collected (10,000g, 20 min). The proteins are dissolved in water, dialyzed against water, and concentrated using a rotary evaporator under mild conditions.

Gel Filtration. A 1.5 ml aliquot of the concentrated protein extract is applied to a Sephadex G-50 column (1.5 x 80 cm) and eluted at a flow rate of 30 ml h \(^{-1}\) with 75 mM sodium acetate (pH 5.6) containing 0.5 M NaCl. This step removes all acid phosphatase and nuclease activities. Active fractions eluting in the molecular mass range of 18 to 25 kDa are pooled and concentrated by precipitation with 75% acetone. After this step, two approaches can be used to purify cellular ribonucleases to homogeneity. In a first approach, method (A), purification by affinity chromatography is followed by preparative native electrophoresis, which yields highly pure RNase preparations although in low amounts. \(^{11}\) In a second approach, method (B), a series of conventional chromatographic purification procedures are used (Table 1). \(^{17}\)

Method (A)

Affinity Chromatography. Acetone-precipitated proteins of the gel filtration step are dissolved in 5 mM sodium acetate (pH 5.6), and 10-ml aliquots are applied to a UMP-agarose column (0.6 x 5 cm) equilibrated with the same buffer. Elution of the column is performed with 5 mM sodium acetate (pH 5.6) containing 1 M NaCl at a flow rate of 30 ml h \(^{-1}\). The enzyme preparation is free of contaminating phosphodiesterase and phosphomonoesterase activities and is subjected to preparative native polyacrylamide gel electrophoresis on 15% slab gels to separate the different intracellular ribonucleases.

### TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity ($U \times 10^{-3}$)</th>
<th>Specific activity ($U$ mg$^{-1}$)</th>
<th>Purification factor (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell homogenate (pH 7)$^b$</td>
<td>350,000</td>
<td>5920</td>
<td>16.9</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>4000g supernatant</td>
<td>298,000</td>
<td>5660</td>
<td>19.0</td>
<td>1.1</td>
<td>96</td>
</tr>
<tr>
<td>60–75% Acetone</td>
<td>52,000</td>
<td>5356</td>
<td>103.0</td>
<td>6.1</td>
<td>90</td>
</tr>
<tr>
<td>Sephadex G-50</td>
<td>16,300</td>
<td>4194</td>
<td>257.0</td>
<td>15.2</td>
<td>71</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650M$^c$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase LX</td>
<td>68.1</td>
<td>873.5</td>
<td>12,827</td>
<td>759$^e$</td>
<td>14.8$^e$</td>
</tr>
<tr>
<td>RNase LV-1</td>
<td>17.1</td>
<td>109.1</td>
<td>6380</td>
<td>377$^e$</td>
<td>1.8$^e$</td>
</tr>
<tr>
<td>RNase LV-2</td>
<td>31.6</td>
<td>364.9</td>
<td>11,550</td>
<td>683$^e$</td>
<td>6.2$^e$</td>
</tr>
<tr>
<td>RNase LV-3</td>
<td>66.2</td>
<td>1000.2</td>
<td>15,080</td>
<td>892$^e$</td>
<td>16.9$^e$</td>
</tr>
<tr>
<td>UMP-Agarose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase LX</td>
<td>10.8</td>
<td>603.6</td>
<td>55,890</td>
<td>3307$^e$</td>
<td>10.2$^e$</td>
</tr>
<tr>
<td>RNase LV-1</td>
<td>2.2</td>
<td>68.1</td>
<td>30,950</td>
<td>1832$^e$</td>
<td>1.1$^e$</td>
</tr>
<tr>
<td>RNase LV-2</td>
<td>3.6</td>
<td>202.7</td>
<td>56,300</td>
<td>3331$^e$</td>
<td>3.4$^e$</td>
</tr>
<tr>
<td>RNase LV-3</td>
<td>8.1</td>
<td>603.2</td>
<td>74,470</td>
<td>4407$^e$</td>
<td>10.2$^e$</td>
</tr>
</tbody>
</table>

$^a$ From Ref. 17.  
$^b$ The homogenate is prepared from 3 kg cells (fresh weight).  
$^c$ Calculations take into account both chromatographic steps on DEAE-Toyopearl 650M.  
$^d$ One ribonuclease unit is defined as the amount of enzyme causing an increase in $\Delta A_{260}$ of 1.0 min$^{-1}$ cm$^{-1}$ ml$^{-1}$ at 37$^\circ$ according to Wilson.  
$^e$ Data refer to the total cell homogenate and do not consider the percentage of each enzyme in the homogenate.

### Method (B)

**Ion-Exchange Chromatography (I).** Acetone-precipitated proteins of the gel filtration step (approximately 400 mg) are dissolved in 20 ml of 20 mM sodium phosphate buffer (pH 6.7) and are applied to a DEAE-Toyopearl 650M column (1.5 × 70 cm) equilibrated with the same buffer. After washing with 100 ml buffer, the column is developed with a linear gradient of 0–0.1 M NaCl (1200 ml) at a flow rate of 60 ml h$^{-1}$. This step substantially increases the specific activities of RNases LV-1 and LV-2, which elute at 20 mM and 35 mM NaCl, respectively. Both RNase LV-3 and RNase LX elute between 45 mM and 80 mM NaCl.

**Ion-Exchange Chromatography (II).** Rechromatography of the active fractions from the above step on DEAE-Toyopearl 650M under the same conditions and at the same flow rate of 60 ml h$^{-1}$, although at a different pH, further improves purification of the RNase isoenzymes. RNase LV-1: Active fractions are pooled and rechromatographed on the same column using a linear gradient of 0–0.1 M NaCl (1200 ml) in 20 mM sodium phosphate (pH 7.6). The enzyme elutes at 55 mM NaCl. Active fractions are concentrated using a rotary evaporator and desalted by...
gel filtration on a Sephadex G-25 column (1.5 × 50 cm) equilibrated with 50 mM triethylamine/acetate buffer, pH 8.0. **RNase LV-2**: Active fractions are pooled and rechromatographed on the same column using a linear gradient of 0–0.1 M NaCl (1200 ml) in 20 mM sodium phosphate (pH 6.7). The enzyme elutes at 35 mM NaCl. Active fractions are concentrated and desalted as described for RNase LV-1.

**RNase LV-3 and RNase LX**: Active fractions are pooled and rechromatographed on the same column using a linear gradient of 0–0.1 M NaCl (1200 ml) in 20 mM sodium phosphate (pH 5.6). RNase LV-3 elutes at 65 mM NaCl whereas RNase LX elutes at 40 mM NaCl. Active fractions of each enzyme are concentrated and desalted as described above.

**Affinity Chromatography.** After dialysis against 10 mM sodium acetate (pH 5.6), the active RNase fractions of the above step are applied to a UMP-agarose column (1 × 8 cm) equilibrated with the same buffer. After washing with 20 ml of buffer, the column is developed with a salt gradient (60 ml, 0–2.5 M NaCl, 30 ml h⁻¹). RNase isoenzymes elute between 1.0 M and 1.5 M NaCl. After desalting on Sephadex G-25, the purified proteins are lyophilized and stored at −20°C.

**Purification of RNase LV-3 (Alternative Protocol)**

An alternative protocol for purifying RNase LV-3 is given below, which is based on preferential extraction of the enzyme at low pH.¹⁸

**Cell Extraction.** Cells of a 7-day-old stationary phase culture or of a -Pi culture are used as the source of enzyme. Cells (150 g wet weight) are extensively washed with distilled water to remove RNase LE, resuspended in 1.5 liter extraction buffer (150 mM citric acid/sodium phosphate [pH 3], 0.1 mM PMSF), and disrupted by sonication. After centrifugation of the cell homogenate, the supernatant is adjusted to pH 5 with 2 N NaOH, and the proteins precipitating between 50% and 70% ammonium sulfate saturation are collected. The pellet is dissolved in 100 ml of 100 mM citric acid/sodium phosphate (pH 5.0). The protein solution is incubated for 20 min at 50°C, cooled to 2–4°C, and cleared by centrifugation.

**Gel Filtration.** The supernatant is applied to a Sephadex G-75 column (5.5 × 80 cm) equilibrated with 100 mM sodium acetate (pH 5.0). Proteins are eluted at a flow rate of 70 ml h⁻¹. Active fractions are pooled and adjusted to 10% glycerol.

**Ion-Exchange Chromatography.** Pooled fractions are loaded onto a DEAE-Sephadex A-25 column (1.5 × 10 cm) equilibrated with 50 mM sodium acetate (pH 5.0), 10% glycerol. Proteins are eluted at a flow rate of 30 ml h⁻¹ with a descending discontinuous pH gradient of the same buffer in the following order: 50 ml each of pH 4.6 and pH 4.3, and 100 ml of pH 3.7. RNase LV-3 elutes between pH 4.3 and pH 3.7.

**Hydroxyapatite Chromatography.** The pooled fractions are adjusted to 5 mM sodium acetate (pH 5.5), 10% glycerol and are loaded on a hydroxyapatite column.

(1 × 8 cm) equilibrated with the same buffer. After washing, the column is developed with a 200-ml linear gradient (0–200 mM) of sodium phosphate (pH 5.5) in equilibration buffer (flow rate 80 ml h⁻¹). RNase activity elutes in a sharp peak at 30 mM sodium phosphate.

**Affinity Chromatography.** Active fractions from the previous step are pooled, dialyzed against 20 mM sodium acetate (pH 5.0), 10% glycerol, and loaded on a UMP-agarose column (0.6 × 5 cm) equilibrated with the same buffer. After washing, the proteins are eluted at a flow rate of 30 ml h⁻¹ with a 60-ml linear gradient (0–3.0 M) of NaCl in the same buffer. RNase LV-3 elutes between 1.0 M and 1.5 M NaCl. Active fractions are pooled and dialyzed against 5 mM sodium acetate (pH 5.0), 10% glycerol and are stored at −20°C.

**Purification of Extracellular RNase LE**

**Initial Concentration Step.** Spent culture medium of early stationary cell cultures (4–5 d) is filtered and cleared by centrifugation (10,000g, 15 min, 4°C). Proteins precipitating in the range of 50% to 70% ammonium sulfate saturation are collected (20,000g, 30 min). The pellet is dissolved in 5 mM sodium acetate (pH 5.5) containing 1 mM EDTA, 1 mM PMSF, 1 mM iodoacetic acid, and the protein solution is dialyzed against this buffer.

**Ion-Exchange Chromatography.** The dialyzed sample is applied to a DE 52-cellulose column (1.5 × 15 cm) equilibrated with 50 mM sodium acetate (pH 5.5). The column is eluted at a flow rate of 30 ml h⁻¹ with a discontinuous pH gradient of the same buffer in the following order: 25 ml each of pH 4.5, pH 4.2, and pH 3.8. RNase activity elutes between pH 4.2 and pH 3.8. Active fractions are pooled, concentrated by ultrafiltration, and adjusted to pH 5.5 with 1 N NaOH.

**Gel Filtration.** The protein sample is applied to a Sephadex G-75 column (5.5 × 80 cm) equilibrated with 20 mM sodium acetate (pH 5.5), and the proteins are eluted at a flow rate of 70 ml h⁻¹. RNase LE elutes as a monodisperse activity peak.

**Affinity Chromatography.** Pooled fractions are loaded on a UMP-agarose column (0.5 × 7.5 cm) equilibrated with 20 mM sodium acetate (pH 5.5). The proteins are eluted at a flow rate of 30 ml h⁻¹ with a discontinuous ascending gradient of NaCl (0.5–2.5 M, increments of 0.5 M, 10 ml each) in sodium acetate (pH 5.5). RNase LE elutes between 1 and 2 M NaCl. Active fractions are pooled and desalted. This procedure follows the method described by Nürnberger *et al.*

**Notes on Protein Purification**

The best source of RNase enzymes are 3- to 4-day old -P₁ cell cultures. Stationary phase cells (7 d) of normal cultures are not recommended because of the high content of copurifying secondary metabolites that interfere with biochemical
analyses. To reduce loss of RNase activity, all purification steps should be carried out at 4°C unless indicated otherwise.

The cell extraction at pH 3 provides a powerful enrichment step that removes most of the cellular protein (approximately 99%) but recovers most of the vacuolar RNase LV-3 activity. However, this procedure is not advised for purification of RNases LV-1, LV-2, or LX because of severe isoenzyme activity loss. The most convenient analytical method to reliably distinguish between the different secretory tomato RNases is native polyacrylamide gel electrophoresis followed by in-gel activity assays. Therefore, all steps of the purification protocols are assessed by activity staining in native gels for the presence and enrichment of the individual RNases. When purifying intracellular RNases, care has to be taken not to contaminate the preparations with extracellular RNase LE that accounts for about 80% of the total RNase activity of cultivated cells. Excessive washing of the cell cake with at least 20 volumes of distilled water is recommended. Intracellular RNases are more stable in citric acid/Na₂HPO₄ (pH 7.0) or in sodium acetate (pH 5.5) than in other buffers. All final enzyme preparations are essentially homogeneous as judged by SDS–PAGE and protein silver staining.

Subcellular Localization of Intracellular Ribonucleases

Step 1: Protoplast Isolation

Reagents

Cell protoplast washing solution (CPW): 10 mM CaCl₂, 1 mM KI, 1 mM KNO₃, 1 mM MgSO₄, 0.2 mM KH₂PO₄, 0.1 mM CuSO₄
Solution A (CPW, 0.5 M sorbitol); Solution B (CPW, 0.4 M sorbitol); Solution C (CPW, 0.4 M sucrose, 5% [w/v] Ficoll 400).

Procedure. Incubate 4.8 × 10⁷ cells in 12 ml solution A containing 1% (w/v) Driselase (Sigma) and 0.01% (w/v) HUPc-cellulase on a gyratory shaker at 75 rpm for 10 to 12 h at 23°C in the dark. Filter the digested material through a nylon net (45 μm mesh size). Centrifuge the filtered crude protoplast suspension at 100 g for 10 min. Resuspend the pellet in solution B and wash the protoplasts three times in this solution by repeated centrifugation. Further purify protoplasts by an additional flotation step. After the final sedimentation step, resuspend the pellet (containing about 10⁷ protoplasts) in 5 ml solution C and overlay with 2 ml of a 4:1 (v/v) mixture of solution C and B, followed by 2 ml of a 4:1.5 (v/v) mixture of solution C and B and by 1 ml of solution B only. After centrifugation at 100g for 10 min, collect purified protoplasts from the upper interphase. Dilute

collected protoplasts into solution B and sediment protoplasts (100g, 10 min) to remove Ficoll 400.

**Step 2: Isolation of Vacuoles**

*Reagents*

Buffer I (5 mM MES-Tris [pH 6.0], 0.4 M sorbitol); Buffer II (15 mM KH$_2$PO$_4$/K$_2$HPO$_4$ [pH 8.0], 0.4 M sorbitol); Buffer III (15 mM KH$_2$PO$_4$/K$_2$HPO$_4$ [pH 8.0], 0.4 M sucrose, 5% [w/v] Ficoll 400)

*Procedure.* Resuspend the sedimented protoplasts in buffer II supplemented with 5 mM EDTA to accelerate protoplast lysis. Vigorously shake protoplast suspension at 200 rpm for about 10 min at 30°. Centrifuge the suspension for 30 s at 500g to remove protoplast debris. Transfer supernatant to a new tube, resuspend the lysed protoplast pellet in the above buffer, and repeat the centrifugation step. Combine the supernatants, which contain intact vacuoles but are largely free of protoplasts. Mix 3 volumes of the crude vacuole suspension with 2 volumes buffer III. Overlay the diluted vacuole suspension sequentially with 2 ml each of the following mixtures of buffer II and buffer III: first, 3 volumes buffer II/1.5 volumes buffer III and second, 3 volumes buffer II/0.5 volume buffer III. Finally, overlay the step gradient with 0.5 ml of buffer II. After centrifugation at 2500g for 10 min, collect the purified vacuoles from the upper interphase and directly analyze for RNase activity and vacuolar and extravacuolar marker enzymes.20

**Step 3: Isolation of Microsomal Fractions**

*Reagents*

Lysis buffer (10 mM HEPES [pH 7.5], 1 mM 2-mercaptoethanol)
Gradient buffer (50 mM HEPES [pH 7.5], 10 mM MgCl$_2$, 2.5 mM EDTA, 5 mM 2-mercaptoethanol, 10% [w/v] glycerol)

*Procedure.* Incubate cultured tomato cells from -Pi cell cultures with cell-wall degrading enzymes supplemented with 1% (w/v) cellulase Onozuka R-10 in solution A for 2 h as described in Step 1 (see above). Wash protoplasts three times in solution B and resuspend in 0.2 ml of 50% sucrose. Carefully mix protoplast suspension with 1.6 ml of lysis buffer on ice. Protoplast lysis can be assisted mechanically by repeatedly pressing the dense suspension through a thin needle. After centrifugation at 1500g for 5 min at 4°, load 3 ml of the pooled supernatants on

---

Fig. 1. Isoelectric focusing of purified intracellular ribonucleases. Lanes (1) RNase LV-3, (2) RNase LV-2, (3) RNase LV-1, (4) RNase LX, and (5) IEF marker proteins. Aliquots of purified enzymes (10 μg each) and marker proteins (100 μg Protein Test Mixture, Serva) were separated on SERVALYT PRECOTES 3–10 (Serva) using the buffer system recommended by the manufacturer (anode buffer: 25 mM L-aspartic acid, 25 mM L-glutamic acid; cathode buffer: 25 mM L-arginine, 2.5 mM L-lysine, 1.5 M ethylenediamine) for 4 h (starting voltage at 200 V, 1000 V max.). Proteins were stained with Coomassie blue (B. J. Davis, Ann. N. Y. Acad. Sci. 121, 407, 1964).

a discontinuous sucrose gradient consisting of 7 ml 13% and 1.5 ml 50% sucrose in gradient buffer. After centrifugation of the sucrose gradient (100,000 g, 2 h at 4 °C) in a SW 41 Ti rotor (Beckman), collect the turbid fraction at the interphase (total microsomal fraction) according to the method of Sticher et al.21 Directly analyze fractions for total RNase activity and microsomal marker enzymes [antimycin A-insensitive NADH cytochrome-c reductase (ER), inosine diphosphatase (Golgi apparatus)]22 in addition to above-mentioned marker enzymes of cell organelles.

Properties of Tomato Ribonucleases LE, LX, LV-1, LV-2, and LV-3

The secretory RNases from tomato share very similar enzymic properties, but differ slightly in protein chemical parameters such as molecular mass and isoelectric point (Fig. 1). The different cellular locations are the most conspicuous differences between the tomato enzymes.9,11,17,18

Enzymatic Properties

The secretory tomato ribonucleases studied, RNase LE, LX, and LV-1 to LV-3, are endoribonucleases that are specific for single-stranded RNA substrates. During RNA hydrolysis, the tomato RNases generate by a phosphotransferase reaction 2',3'-cyclic NMP as obligate monomeric products. Further hydrolysis of 2',3'-cyclic NMP to 3'-NMP is a side reaction of the tomato enzymes, which proceeds significantly more slowly than the generation of 2',3'-cyclic NMP from synthetic diribonucleoside monophosphate or natural RNA substrates. Based on these mechanistic properties, the secretory tomato ribonucleases are classified as of the RNase l-type (EC 3.1.27.1). According to Wilson, RNase I enzymes are endoribonucleases that hydrolyze RNA substrates via 2',3'-cyclic NMP intermediates.

All secretory tomato ribonucleases studied have an acidic pH optimum (between pH 5 and pH 6) with yeast RNA as the substrate and preferentially release purine, in particular guanine, mononucleotides from diribonucleoside monophosphate or RNA substrates (relative nucleobase specificity: G>>A>U>C). As determined for RNase LE, RNase LX, and RNase LV-3, hydrolysis of RNA does not require divalent metal ions (Mg²⁺, Mn²⁺, Ca²⁺, Co²⁺) and is not affected by the presence of 2.5 mM EDTA. However, RNase activities are slightly stimulated by the presence of 25 mM sodium citrate (170%) or 25 mM sodium phosphate (153%), when compared to 25 mM sodium acetate (100%). Secretory tomato RNase activities are completely inhibited by incubation in 10 mM Ag⁺, Zn²⁺, Hg²⁺, Cu²⁺, or Al³⁺ ions, and 1 mM p-chloromercuribenzoic acid causes 80% inhibition of RNase LE activity. Possible end products of RNA hydrolysis and derivatives at concentrations of 1 mM do not affect the enzyme activities.

Stability

Secretory tomato RNases can be stored without measurable loss of activity for at least 2 months at −20°C. The enzymes are less thermostable in comparison to other RNases. Total inactivation is observed for all secretory tomato RNases within 5 min by incubating the enzymes at 100°C in buffers of different pH and ionic strength. Five disulfide bridges are present in RNase LE, one of which is unique to the subfamily of acid ribonucleases (Cys²⁵−Cys⁸¹). This disulfide bridge appears to stabilize the structure of RNase LE and to protect the enzyme against proteolytic cleavage.

Primary and Secondary Structures

The primary structures of RNase LE and RNase LX were directly determined by sequencing of the proteins, which allowed determination of the signal peptidase cleavage site. Both enzymes consist of a single polypeptide chain. Mature RNase LE contains 205 amino acid residue, whereas RNase LX is composed of 213 amino acids. Although other members of the superfamily of T2/S ribonucleases are glycoproteins, conserved glycosylation sites do not occur in the primary structures of both enzymes. Likewise, experimental tests for glycosylation such as adsorption of RNases LE and RNase LX to concanavalin A or digestion with endoglycosidase H suggested that the tomato enzymes are not modified by glycosylation.

The primary structures of RNase LE and RNase LX share 62% amino acid sequence identity. Amino acid residues are particularly conserved in the active site cassettes important for catalysis. Amino acid sequence identity between tomato RNases and other members of the T2/S superfamily ranges from 25% (fungus RNases) to 30% (S-RNases). RNase LE shares the highest amino acid sequence identity with RNase NE and RNase NW from Nicotiana species (about 85%), with ZRNase II from Zinnia elegans, and with RNS1 from Arabidopsis thaliana (about 70%). RNase LX is closely related to ZRNase I from Zinnia elegans and to RNase NGR3 (Nicotiana glutinosa) (EMBL AB032257), and the sequence similarity extends to the C terminus.

Partial amino acid sequences, including N- and C-terminal sequences, were determined for RNases LV-1, LV-2, and LV-3. Enzyme purification and characterization studies by Abel and Glund and Nümberger et al. as well as direct sequencing of the proteins strongly suggest that RNase LE and RNase LV-3 are identical enzymes. The two proteins (i) comigrate in native and denaturing polyacrylamide gels, (ii) have identical protein chemical (CD spectra, IEP) and enzymic properties, (iii) give similar peptide fingerprints after tryptic digestion, and (iv) share identical amino acid sequences in N-terminal and internal tryptic peptides. About 50% of the RNase LV-2 and 20% of the RNase LV-1 protein were sequenced, including the N and C terminus of RNase LV-2. Except for the C-terminal peptides, tryptic maps of both RNases were identical. Comparison of the primary structure of RNase LX with the vacuolar isoenzymes reveals differences only at the truncated C termini of RNases LV-2 and LV-1.

Tertiary Structures

The crystal structure of RNase LE has been determined at 1.65 Å resolution. The enzyme is composed of seven α helices and seven β strands. Five disulfide

bridges have been identified but only two are common to all acid T2-type RNases. Another two disulfide bridges are common to the animal/plant subfamily (including S-RNases), but their position differs in the fungal RNase Rh. One disulfide bridge (Cys\textsuperscript{25}–Cys\textsuperscript{81}) and an adjacent cis-peptide bond (Pro-82) are unique to RNase LE. Since the related tomato enzyme, RNase LX, and other members of the plant S-like RNase subfamily have conserved amino acid residues at identical positions, it is very likely that they share similar secondary structures, which may explain similar biochemical properties of these enzymes. It is expected that the three-dimensional structure of RNase LE will provide a basic framework for the animal/plant subfamily of RNase T2 enzymes, including S-RNases. Based on the sequence alignment with RNase Rh, the catalytically important residues of RNase LE were predicted to be His-39, Trp-42, His-92, Glu-93, Lys-96, and His-97, which has been confirmed by crystal structure analysis of RNase LE.

**Gene Structures**

By screening a cDNA library prepared from P\textsubscript{T}-starved cells, we isolated cDNA clones for RNase LE and RNase LX. The deduced amino acid sequences for RNase LE and RNase LX contain N-terminal signal sequences of 25 and 24 amino acids, respectively. The hydrophobic core and other properties of the predicted signal peptides are consistent with a function in cotranslational transport into the endoplasmic reticulum. Southern blotting analysis revealed that both RNases are encoded by single-copy genes in tomato. cDNA library screening and Southern analysis under low stringency conditions did not indicate the presence of additional, related genes in the tomato genome. Therefore, it is likely that the extracellular and vacuolar RNases are the products of posttranscriptional processing and/or posttranslational modification reactions.

**Localization**

When cultured tomato cells and the spent liquid medium were analyzed for ribonuclease activity, four intracellular RNases and one extracellular RNase were identified by native gel electrophoresis. The most mobile intracellular enzyme, RNase LV-3, and the enzyme secreted into the cell culture medium, RNase LE, share identical electrophoretic properties and likely share the same primary structure (see above). Nonetheless, both proteins have different destinations in the secretory pathway. The absence of a vacuolar targeting signal in RNase LE and its high levels in the cell culture medium, which severalfold exceed the amount

Fig. 2. Electrophoretic separation of ribonucleases from different cellular fractions using native PAGE. RNases are detected by negative activity staining with RNA as substrate. Positions of the different RNases in the gel are marked on the right. Medium corresponding to $5 \times 10^5$ cells grown for 48 h under $+P_i$ (lane 1) or $-P_i$ (lane 2), and cell extracts of $5 \times 10^5$ cells grown for 48 h under $+P_i$ (lane 3) or $-P_i$ (lane 4), isolated vacuoles corresponding to $5 \times 10^5$ cells (lane 5), and the isolated microsomal fraction (lane 6). $[+/- P_i]$ indicates whether cells were grown with or without phosphate in the cultivation medium. Gradient fractions of isolation procedures for vacuoles and microsomes were characterized by calculation of contaminating cellular enzymes and by cofractionation with marker enzymes according to Glund et al., Shore and MacLachlan, and Kaletta et al.

of RNase LV-3 in the vacuoles, strongly suggest that the extracellular space is the location of RNase LE function. This is supported by studies in transgenic tomato plants that constitutively overexpress RNase LE. As predicted, RNase LE is almost exclusively detectable in the extracellular fluid collected from the intercellular spaces of tomato leaves.

Using isolated vacuoles prepared from protoplasts of cultured tomato cells, it was shown by in-gel activity assays under native and denaturing conditions that three of the four intracellular RNase activities copurify with the vacuolar organelles. These enzymes were designated RNase LV-1, LV-2, and LV-3. The fourth RNase activity is of extravacuolar origin and was named RNase LX (see Fig. 2). Further localization studies demonstrated that RNase LX is retained in the endoplasmic reticulum (ER) of tomato cells by virtue of the C-terminal amino acids, HDEE. The C terminus of RNase LX was shown to function as a novel ER retention signal. Interestingly, as revealed by C-terminal sequencing

with carboxypeptidase Y, RNase LV-2 lacks the C-terminal ER retention signal. \(^{28}\) Although the C-terminal amino acids of RNase LV-1 could not be identified, it is likely that the enzyme lacks more amino acid residues than the HDEF motif at the C terminus. This assumption is based on electrophoretic properties of RNase LV-1, which has a lower molecular weight but migrates more slowly native gels than RNase LV-2. The electrophoretic behavior of RNase LV-1 is possibly caused by the absence of two aspartic acid residues, which are present at the very C terminus of RNase LV-2. We suspect that a minor fraction of the RNase LX population that can escape the endoplasmic reticulum is transported into vacuoles. In this lytic environment, RNase LX is proteolytically cleaved, which results in loss of the C-terminal ER retention signal peptide, and further degradation may occur.

Acknowledgment

We are grateful to Dr. A. Löffler for making available Fig. 1. Parts of this work was supported by grants of the Deutsche Forschungsgemeinschaft, SFB 363, to M.K.

[23] Leczyme

By KAZUO NITTA

Introduction

We have isolated two sialic acid-binding lectins (SBLs) from frog eggs (SBL-C, from *Rana catesbeiana*; SBL-J, from *R. japonica*) that not only agglutinate a large variety of tumor cells\(^{1-5}\) but also hydrolyze RNA,\(^{6,7}\) and have named them leczyme.\(^{8}\) Leczyme from *R. catesbeiana* (SBL-C) was found to inhibit

References: