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Purification and properties of a ribonuclease from cowpea cotyledons

E. GOMES-FILHO, C.R.F.M. LIMA, J. ENÉAS-FILHO, F.A.P. CAMPOS, L.A. GONDIM and J.T. PRISCO

Department of Biochemistry and Molecular Biology, Federal University of Ceará, P.O. Box 1065, 60001-970 Fortaleza, Ceará, Brazil

Abstract

The isolation and characterisation of cotyledonary ribonucleases (RNase; EC 3.1.27.1), are basic steps to understand the physiology and biochemistry of RNA turnover and mobilisation during seed germination and seedling establishment, as well as how environmental stresses affect them. RNase was isolated and purified 928-fold, to apparent eletrophoretic homogeneity from 5-d-old seedlings of *Vigna unguiculata*. It is a protein with an apparent molecular mass of 16 kDa having three major isoforms. Its optimum pH is 5.8, which decreases to 5.2 in presence of KCl. It has an apparent K_m of 0.80 mg RNA cm^{-3} and retains 40 % of its activity when heated to 80 °C. It is completely inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+} and is almost insensitive to Mg^{2+}, Ca^{2+} and EDTA. Urea, Fe^{2+}, Co^{2+} and 2-mercaptoethanol partially inhibit its activity. Its amino acid composition shows a resemblance to that of other plant RNases.

Additional key words: enzyme purification and characterization, RNA, *Vigna unguiculata*.

Introduction

The turnover and mobilisation of RNA in cotyledons of legume seeds are considered important steps both for seed germination and seedling establishment (Bewley and Black 1994). It appears that there are several RNases involved in these processes (Bryant *et al.* 1976, Gomes Filho and Sodek 1988) and that the activity of these RNases is affected by mechanical injury (Isola and Franzoni 1986), rust infection (Chakravorty *et al.* 1974), water and salt stresses (Vieira da Silva 1970, Sheoran and

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Fax. (+55) 85 288 9829; e-mail: egomesf@ufc.br

Garg 1978, Gomes Filho and Sodek 1988) and seed ageing (Bewley and Black 1994). In order to understand the physiology and biochemistry of RNA turnover and mobilisation as well as to understand how environmental stresses affect them, it is necessary to isolate and characterise these RNases, and determine their tissue distribution and subcellular localisation. Purification and characterisation of plant RNases have been reported from a wide variety of plants (Wilson 1982, Green 1994, Rojo *et al.* 1994), but our understanding of the properties of RNases from the *Fabaceae* lags far behind those from other families. The purpose of this work was to purify a RNase from cotyledons of 5-d-old seedlings of cowpea (*Vigna unguiculata*) and compare its physical and chemical properties with those isolated from other plants.

Materials and methods

Plants: Pitiuba cowpea [*Vigna unguiculata* (L.) Walp.] seeds were obtained from plants grown under irrigation at the Center of Agrarian Sciences, Federal University of Ceará, Fortaleza, Ceará, Brazil. Seeds were kept at 10 °C in glass flasks containing silica gel. Seeds previously surface-sterilised with sodium hypochlorite were allowed to germinate on filter paper (Prisco and Vieira 1976) at 25 ± 2 °C in darkness.

Enzyme extraction and purification: Cotyledons from 5-d-old seedlings were macerated in 0.1 M potassium phosphate buffer, pH 5.7 (1:3, m/v) at 4 °C and centrifuged at 7 000 *g* for 20 min at 4 °C. The supernatant (crude extract) was brought to 45 - 75 % saturation with ammonium sulfate and the precipitated proteins recovered by centrifugation and dialysed for 24 h against 0.1 M sodium acetate buffer, pH 4.0 and for more than 24 h against distilled water, and then freeze-dried (F_{45-75}). This fraction was applied to a CM-Cellulose column (1.8 × 17.5 cm) equilibrated in 0.1 M sodium acetate buffer, pH 4.0. The proteins bound to the column were eluted with a linear gradient of NaCl (0 to 1.0 M), at a flow rate of 32 cm^3 h^{-1}. The tubes containing RNase activity were pooled and dialysed against distilled water for 24 h (F_{CM}). This fraction was then applied to a *DEAE-Sephadex A-25* column (1.1 × 24.0 cm) equilibrated and eluted with 0.05 M Tris/HCl, pH 7.2. The proteins bound to the column were eluted by applying a linear gradient (0 to 0.4 M) of NaCl in 0.05 M Tris/HCl, pH 7.2. The tubes containing RNase activity were pooled and dialysed against distilled water for 24 h and then freeze-dried (F_{DEAE}). Finally this fraction was applied to a *Sephadex G-50* column, equilibrated and eluted with 0.1 M sodium acetate, pH 5.8. The tubes containing RNase activity were pooled (F_{G50}) and used directly for the assays of enzyme characterisation. Alternatively, the pooled fractions were dialysed against distilled water for 24 h, freeze-dried and stored at -20 °C.

Enzyme assay and protein determination: RNase activity was measured according to Tuve and Anfinsen (1960), with the modification proposed by Gomes Filho *et al.* (1983), using torula yeast type VI RNA (*Sigma Chemical Co.*, St. Louis, USA) as

substrate. Unless otherwise specified, the buffer used was 0.1 M sodium acetate, pH 5.8. Enzyme activity was expressed as the difference in absorbance at 260 nm (ΔA_{260}) or as units of activity (U), one U being defined as a difference in absorbance (ΔA_{260}) of 0.01. Protein was determined by absorbance at 280 nm or according to Bradford (1976).

Enzyme characterisation: The molecular mass of the purified enzyme was determined by PAGE-SDS according to Laemmli (1970) in 12 % slab gels. Discontinuous electrophoresis under non-denaturing conditions was performed according to Robyt and White (1987). The ribonuclease activity associated with the proteins in crude extracts and with purified proteins, was measured according to Gomes Filho and Sodek (1988): after electrophoresis the gels were incubated with RNA at 1 mg cm^{-3} in sodium acetate buffer 0.1 M, pH 5,8 for 20 min, followed by staining for 1 min in toluidine blue, as described by Wilson (1978). The effect of pH on enzyme activity in the pH range of 4.0 to 6.0 was done using 0.05 M sodium acetate and in the pH range of 6.4 to 7.6 with 0.05 M Tris/HCl. The values of apparent K_m and V_{max} were calculated from a Lineweaver-Burk plot, using RNA at a concentration range of 0.1 to 2.9 mg cm^{-3}. For the determination of heat stability, enzyme samples of 0.2 cm^3 in 0.1 M sodium acetate, pH 5.8 were incubated for 10 min at temperatures varying from 30 to 100 °C. After cooling the samples in an ice bath, the enzyme activity was determined. The effects of EDTA and divalent cations were studied by adding each one of them to the reaction mixture up to a final concentration of 3.0 mM. The effects of urea and 2-mercaptoethanol (2-ME) on enzyme activity were studied by pre-incubating the enzyme for 1 h at 25 °C with these substances at 6.0 M and 140 mM, respectively. In the activity assay, the final concentrations of urea and 2-ME were 1.0 M and 140 mM, respectively.

Amino acid composition was determined by *Pico Tag* technology, as described by Uchoa *et al.* (1998). Composition is expressed in numbers of residues per mole of protein based on a molecular mass of 16 kDa. The values presented are an average of five independent analyses.

Results

The enzyme was purified 928-fold, with a yield of 4.4 % (Table 1). In all of the chromatographic steps the enzyme was eluted as a single peak (data not shown). In PAGE-SDS profile of the purified protein a single band corresponding to a molecular mass of 16 kDa was seen (Fig. 1*A*). However, under non-denaturing conditions, after staining with Coomassie blue three major protein bands can be seen (Fig. 1*B*). The position of these bands is correlated with that of three bands which were seen when the same gels were stained for ribonuclease activity (Fig. 1*C*). In these gels a fast-migrating and faint band was also detected.

Table 1. Purification of a cotyledonary RNase from 5-d-old *Vigna unguiculata* seedlings. The values are for one extraction from 1500 cotyledons (110 g fresh mass). For explanation of steps F_{45-75}, F_{CM}, F_{DEAE} and F_{G50}, see Materials and methods.

Step	Volume [cm^3]	Protein [mg]	Total activity [U s^{-1}]	Specific activity [U mg^{-1}(prot.) s^{-1}]	Purification [fold]	Yield [%]
Crude extract	310	1128	971	0.86	1	100
F_{45-75}	75	43.5	644	14.8	17	66.3
F_{CM}	72	2.86	503	176	205	51.8
F_{DEAE}	72	0.13	72	554	644	7.4
F_{G50}	19.2	0.054	43.1	798	928	4.4

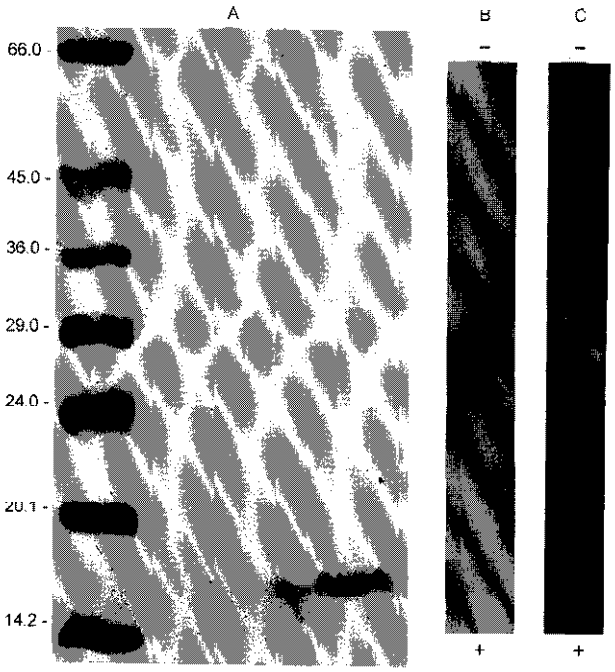

Fig. 1. Assessment of the homogeneity and isoenzyme pattern of the RNase purified from cotyledons of 5-d-old seedlings of *Vigna unguiculata*. *A* - PAGE-SDS of purified RNase (3 μg of purified protein and 25 μg protein of molecular mass kit were applied to the gel; the numbers on the left correspond to molecular mass in kDa. *B* - Electrophoresis of the purified RNase under non-denaturing conditions in which the gel was stained for protein. *C* - Electrophoresis of the purified RNase under non-denaturing conditions in which the gel was stained for RNase activity.

In the absence of 0.16 M KCl the enzyme had an optimum pH between 5.6 and 6.0, while in its presence the optimum pH shifted in range from 4.8 to 5.6 (Fig. 2). In the pH range of 4.0 to 6.0 the enzyme showed a higher activity when KCl was present. However, in the pH range 6.4 to 7.6 the activity was not affected by KCl. The apparent K_m and V_{max} were calculated from the Lineweaver-Burk plot (Fig. 3), and the values were 0.80 mg cm^{-3} and 0.022 ΔA_{260} min^{-1}, respectively. Up to 40 °C, only a small decrease in activity could be noted. However, at temperatures higher

than 40 °C enzyme activity decreased rapidly, reaching 40 % of the control at 60 to 80 °C. Incubating the enzyme at 100 °C abolished 89 % of the enzyme activity (Fig. 4). The activity of the enzyme was completely inhibited by Cu^{2+}, Hg^{2+}, and Zn^{2+}, while urea, Fe^{2+}, Co^{2+} and 2-ME inhibited it only partially. The Mg^{2+} and Ca^{2+} ions caused a slight decrease in enzyme activity, while EDTA slightly stimulated the activity of the purified enzyme (Table 2).

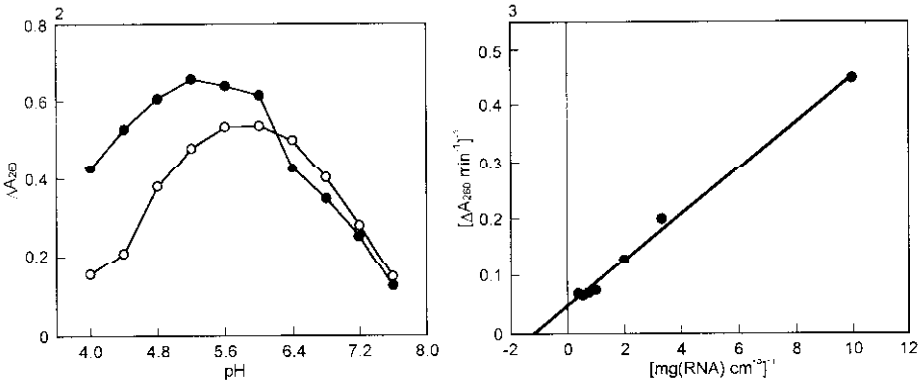

Fig. 2. Effect of pH on the activity of the RNase isolated from cotyledons of 5-d-old seedlings of *Vigna unguiculata*. The activity was measured in the absence (*open circles*) and presence (*closed circles*) of 0.16 M KCl. Means of three independent experiments, each one done in duplicate.

Fig. 3. Lineweaver-Burk plot of the hydrolysis reaction of RNA by the RNase isolated from cotyledons of 5-d-old seedlings of *Vigna unguiculata*. Means of three independent experiments, each one performed in duplicate.

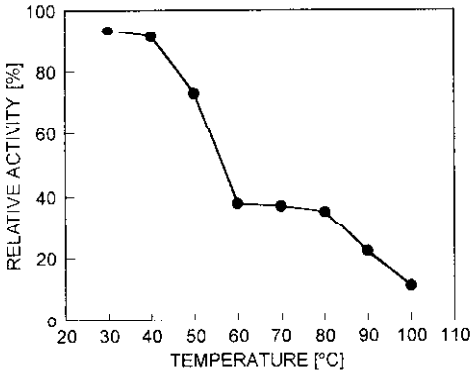

Fig. 4. Effect of temperature on the activity of the RNase isolated from cotyledons of 5-d-old seedlings of *Vigna unguiculata*. The values are expressed as percentages of the control (enzyme not exposed to heat treatment) and represent means of three independent experiments, each one done in duplicate.

The amino acid composition (number of residues) derived from the hydrolysate of the RNase purified from cowpea cotyledons was Lys (7.0), His (4.0), Arg (5.3), Asp/Asn (16.7), Thr (7.3), Ser (20.7), Glu/Gln (22.7), Pro (12.0), Gly (10.7), Ala

(12.3), Val (4.3), Met (2.0), Ile (6.0), Leu (4.0), Tyr (3.0), and Phe (7.7). The levels of Cys and Trp were not determined.

Table 2. Action of some effectors on the activity of the RNase isolated from cotyledons of 5-d-old seedlings of *Vigna unguiculata*. For the effectors 2-ME and urea, the enzyme was pre-incubated with these substances. The values are expressed as percentage of the control (assay without the additon of effectors) and represent the means of three independent experiments, each one performed in duplicate.

Effector	Concentration [mM]	Activity [% of control]
$MgCl_2$	3	94.4
$CaCl_2$	3	92.5
$CoCl_2$	3	68.2
$FeCl_2$	3	50.9
$CuCl_2$	3	0
$HgCl_2$	3	0
$ZnCl_2$	3	0
EDTA	3	107.7
2-ME	140	51.3
Urea	1000	44.9

Discussion

This highly-purified preparation from cotyledons of 5-d-old seedlings of *Vigna unguiculata* is of an RNase, as defined by Wilson (1975, 1982), and Green (1994). It is almost insensitive to EDTA, does not show any DNase activity (Gomes Filho and Sodek 1988), and the products of RNA hydrolysis determined by the latter authors have revealed the formation of all four cyclic (2',3')-nucleotides, which are the precursors of 3'-mononucleotides (Wilson 1975). The purification factor, as well as the yield of recovered activity are in good agreement with values reported by other workers using similar purification protocols (Hirai and Asahi 1975, Beopoulos *et al.* 1978, Yokoyama *et al.* 1982). A molecular mass of 16 kDa for the *Vigna* enzyme is similar to that isolated from barley (Pietrzak *et al.* 1980) and rice (Yokoyama *et al.* 1982). A diversity of isoenzymes seems to be common among plant RNases (Wilson 1982, Yen and Green 1991).

The optimum pH range and its shift due to the presence of KCl in the reaction mixture, as well as the typical Michaelis-Menten kinetics have also been observed in other plant RNases (Frisch-Niggemeyer and Redi 1957, Wilson 1963, 1975, Chevrier and Sarhan 1980). Thermal stability and the effects of divalent cations on the activity are similar to that of most RNases so far isolated from plants (Wilson 1975, Beopoulos *et al.* 1978, Pietrzak *et al.* 1980, Yokoyama *et al.* 1982, El-Shouny and Alexandrescu 1986). The fact that the enzyme is almost insensitive to EDTA, gives further support to the notion that it is a ribonuclease and not a nuclease (El-Shouny and Alexandrescu 1986, Singh *et al.* 1991, Yen and Green 1991), as defined by Wilson (1975). Incubation of the enzyme with urea and 2-ME caused an inhibition of

the purified enzyme of 55 and 49 %, respectively, which contrasts with the results of El-Shouny and Alexandrescu (1986) in respect of an RNase from sunflower roots. The latter authors observed that treatment of the RNase with 2-ME doubled the enzyme activity while urea had no effect. We suggest that the change in activity due to 2-ME might be the result of changes in the tertiary structure of the enzyme dependent on unfolding by reduction of disulphide bridges.

The amino acid composition of the RNase from cowpea cotyledons showed resemblances with RNases from maize endosperm (Wilson 1967), rye germ (Kuligowska *et al.* 1980), bitter gourd seeds (Ide *et al* 1991) and cucumber seeds (Rojo *et al.* 1994). The resemblances were more conspicuous when the high contents of glutamic acid/glutamine, aspartic acid/asparagine, serine, alanine, proline and glycine, and the low levels of histidine, tyrosine and methionine were compared

We are in the process of studying the expression pattern, tissue distribution and subcellular compartmentalisation of the purified RNase, using polyclonal antibodies. We expect that these studies will help to clarify the biological role played by the enzyme.

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