Some qualities and fractionation of a nuclease from cotyledons of germinating *Phaseolus vulgaris*

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During gel filtration and subsequent anion exchange chromatography the nuclease activity from cotyledons of germinating *Phaseolus vulgaris* L. was eluted as a single peak, containing high RNase and low inherent DNase activities. The RNase activity was unaffected by EDTA and had no particular requirement for mono- or divalent cations, but was substantially inhibited by Mn^{++} and Zn^{++}. Sodium dodecyl sulphate and the vanadyl ribonucleoside complex were identified as effective inhibitors of the RNase activity. The base preference for homoribonucleic acids was: poly U > poly C > poly A > poly G.

Die nuklease uit saadlobbe van ontkiemende *Phaseolus vulgaris* L. is as enkelfraksies met hoë RNase- en lae inherente DNase-aktiwiteit tydens jelfiltrasie en daaropvolgende anioonuitruilingschromatografie geskei. Die RNase-aktiwiteit word nie deur EDTA beïnvloed nie en het geen besondere behoefte vir mono- of divalente katione gehad nie, maar was aansienlik deur Mn^{++} en Zn^{++} gerem. Natriumdodesielsulfaat en die vanadielribonukleosiedkompleks is as doeltreffende remstowwe van die RNase-aktiwiteit geïdentifiseer. Die basisvoorkeur vir homoribonukleïensure was: poli U > poli C > poli A > poli G.

Keywords: Cotyledons, fractionation, germination, nuclease, *Phaseolus vulgaris*

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Introduction

The hydrolases are the most prominent enzymes in storage tissues of germinating seeds. It is generally accepted that they provide the growing embryonic axis, in the heterotrophic stage, with energy and precursors. Among these hydrolases, the nucleolytic enzymes seem to play a significant role in germination (Gomes Filho & Sodek 1988; Jacobsen 1980; Bryant & Greenway 1976a; Pietrzak *et al.* 1980; Beavers & Splittstoesser 1968; Barker *et al.* 1974; Koshiba *et al.* 1986; Wilson 1975). Our initial studies (unpublished) showed, that as germination proceeds, a slow rate of increase in RNase activity, which could be related to their role in the mobilization of macromolecules, was observed in cotyledons of *Phaseolus vulgaris* L. Earlier work (Walbot 1973), however, showed an accumulation of RNA in cotyledons of beans during germination. This may indicate that RNase, apart from the ordinary hydrolysing of RNA, may have some other functions. It is obvious that the role nucleases play, during germination, is not yet fully understood. For example, the types of nuclease involved are not clear and their individual functions in nucleic acid metabolism are yet to be established. To the best of our knowledge, there seems to be no information available on the characteristics of the nuclease(s) in cotyledons of germinating *Phaseolus vulgaris*. It was thought that some characteristics of this enzyme might contribute to a further understanding of these enzymes *in vivo*. Since the characteristics of plant nucleases are relatively unknown, any additional information would be of aid in the continuing search for new enzymes with interesting features that could serve as tools for genetic engineering.

Materials and Methods

Plant material

Phaseolus vulgaris L. cv. Top Crop seeds were surface sterilized (3 min) in 0.05% (v/v) Panacide (5,5′-2,2′-dihydroxydiphenylmethane) (BDH Chemicals) solution and rinsed with distilled water. The seeds were germinated for 48 h in the dark at 25°C on filter paper soaked with distilled water.

Extraction and fractionation of nuclease

Excised cotyledons were ground in cold 0.3 M sodium acetate (NaAc) buffer (pH 5.5) with the aid of broken glass. After saturation (80%) of the extract with $(NH_4)_2SO_4$ the precipitate was dissolved in a minimum volume 0.3 M NaAc buffer (pH 5.5) and dialysed against the same buffer containing polyethylene glycol (PEG 35000) (Merck) for concentration. Any precipitate formed during dialysis was pelleted (centrifugation) and discarded. The dialysate was treated with dry Sephadex G25 for further concentration before a final solution, of not more than 3 ml, was applied on a Sephadex G75 (40–120 μm) column (48 × 1.5 cm) for gel filtration. The column was developed with 0.3 M NaAc buffer (pH 5.5) at 0.5 ml min^{-1} and fractions (3 ml) collected were assayed for RNase, single- (ss) and double-strand (ds) DNase activities. The pooled active fractions were dialysed extensively against 0.01 M Tris-HCl (pH 7.5) with regular exchanges for fresh buffer. This dialysate was applied to a DEAE Sephacel (Pharmacia) column (8 × 1.5 cm) for anion exchange chromatography. After 10 ml of 0.01 M Tris-HCl (pH 7.5) had been passed through the column, the column was developed (0.5 ml min^{-1}) with a linear gradient (0–0.6 M) NaCl in 0.01 M Tris-

HCl buffer (pH 7.5). Fractions (3 ml) were collected and assayed for RNase, ss and ds DNase activities.

Enzyme assay

A modified method of Keys & Zbarsky (1980) was applied. Substrates for nuclease activity were highly polymerized RNA from yeast (250 μg ml^{-1}) (Serva), native (ds) and thermally denatured (ss) calf thymus DNA (400 μg ml^{-1}) (Serva). Known volumes of enzyme samples were incubated at 37°C with 150 μl substrate in 0.04 M NaAc buffer (pH 5.5) for a determined period. After addition of 6% (v/v) $HClO_4$ containing 0.3% (w/v) uranylacetate, to obtain a final relative $HClO_4$ concentration of 2.3% (v/v), and centrifugation, the absorbancy (A) of the acid soluble fractions was determined at 260 nm. Readings were not corrected for dilution with perchloric acid. One unit of enzyme activity (eu) is defined as the amount of enzyme that liberates acid-soluble products with an absorbancy at 260 nm of 1 per min of reaction time under conditions of this assay. Activities are expressed as specific activity [milli-enzyme units (meu) mg^{-1} bovine serum albumin (BSA) protein equivalent] and relative activity (%) where the control A_{260nm} value or the highest A_{260nm} value for a given experiment represents 100% activity. Protein determinations were according to Bradford (1976).

Nuclease recovery after fractional precipitation

Extraction of nuclease was similar to the procedure described. Equal volumes of the same supernatant were saturated with solid $(NH_4)_2SO_4$ and polyethylene glycol 6000 (PEG) to successive percentage values indicated in Table 1. The individual precipitates were dissolved in minimum volumes 0.3 M NaAc buffer (pH 5.5), dialysed and assayed for RNase, ss and ds DNase activities using substrates and assay procedures described.

Effect of ethylenediaminetetra-acetic acid (EDTA), mono- and divalent cations

Extraction and precipitation were according to procedures described. For the effect of cations the precipitate was dissolved in minimum volume distilled (×2) H_2O, dialysed and concentrated (PEG 35000). Aliquots of the concentrated dialysate were assayed for RNase activity in 50 mM MES buffer (pH 5.5) containing the individual chloride salts of monovalent (K^+, Na^+, $NH4^+$) and divalent (Zn^{++}, Ca^{++}, Mg^{++}, Mn^{++}) cations in 1, 10, 50 and 100 mM concentrations respectively. For the effect of EDTA on RNase and DNase activities of cotyledons at different germination stages (1, 12, 24, 48, 72 and 96 h) the protein precipitate was dissolved in a minimum volume 0.3 M NaAc buffer and desalted by ultrafiltration. The standard assay procedure was applied with 5 mM EDTA in the reaction mixture.

Effect of possible effectors

After extraction, precipitation [$(NH_4)_2SO_4$] and ultrafiltration, aliquots of the nuclease-containing solution were assayed for RNase activity in the presence of 8 mM DL-dithiothreitol (DTT) (Sigma), 5% sodium dodecyl sulphate (SDS) (BDH Chemicals) and 13 mM vanadium ribosyl complex (Berger & Birkenmeier 1979) by the standard procedure.

Base preference

Aliquots of the protein dialysate, obtained by the usual procedures were assayed for activity towards polyadenylic acid (poly A), polycytidilic acid (poly C), polyguanylic acid (poly G) and polyuridylic acid (poly U) (Boeringer Mannheim) respectively. NaAc buffer (0.04 M, pH 5.5) containing 250 μg polyhomonucleotide ml^{-1} was used in the assay mixture while the assay procedure remained unchanged.

Results and Discussion

Nuclease recovery after fractional precipitation

Since cotyledons of beans contain large amounts of reserve protein, fractional precipitation was evaluated as an initial purification step of nucleases. For the recovery of RNase from seeds Wiśniowska & Morawiecka (1985) and Pietrzak *et al.* (1980) used the 30–80% fraction after precipitation with $(NH_4)_2SO_4$. Bryant & Greenway (1976b) recovered the 40–60% fraction during the isolation of RNase from cotyledons of the legume, *Pisum sativum*. According to Table 1 the RNase activities were about equally distributed in the 0–40% and 40–80% fractions. Although not widely different, the 0–40% fraction contained more ss and less ds DNase activity than the 40–80% fraction. When precipitation was accomplished with PEG (Table 1) the 0–4% fraction could be discarded without any great loss of nuclease activity. Most of the activity was recovered in the 4–10% fraction. Unfortunately the precipitates obtained from the PEG precipitation procedure were rather difficult to dissolve. It is noteworthy that Mikulski & Laskowski (1970) found that stepwise precipitation up

Table 1 Nuclease recovery from cotyledons after fractional precipitation with $(NH_4)_2SO_4$ and polyethyeleneglycol 6000 (PEG). (Seeds used for precipitations by $(NH_4)_2SO_4$ and PEG were germinated for 64 and 48 h respectively)

	Total activity (meu)			Specific activity (meu × 10^{-1} mg^{-1} BSA prot.)			Fraction of total activity (%)		
		DNase			DNase			DNase	
Fraction	RNase	ss	ds	RNase	ss	ds	RNase	ss	ds
$(NH_4)_2SO_4$									
0–40%	11616	9041	6500	3728	2901	2086	52	56	42
40–80%	10852	7208	8901	4126	2741	3385	48	44	58
PEG									
0–4%	128	9	–	148	10	–	3	1	–
4–10%	2484	931	–	3516	1319	–	61	75	–
10–15%	1472	292	–	2083	525	–	36	24	–

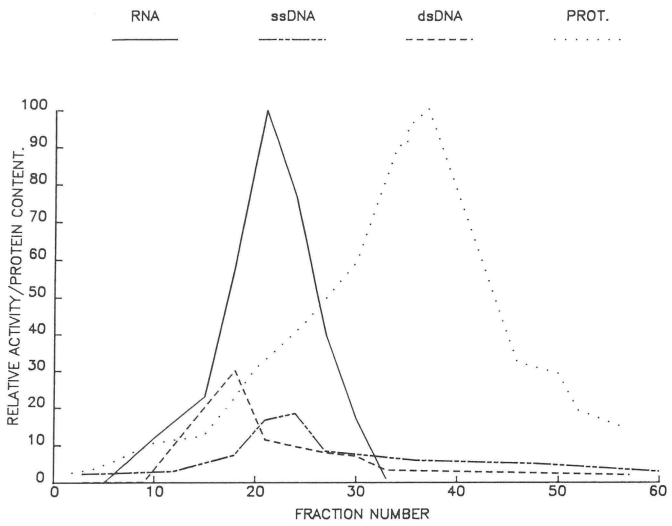

Figure 1 Gel filtration (Sephadex G75) of nuclease from cotyledons. Activity of fractions was assayed towards nucleic acids indicated.

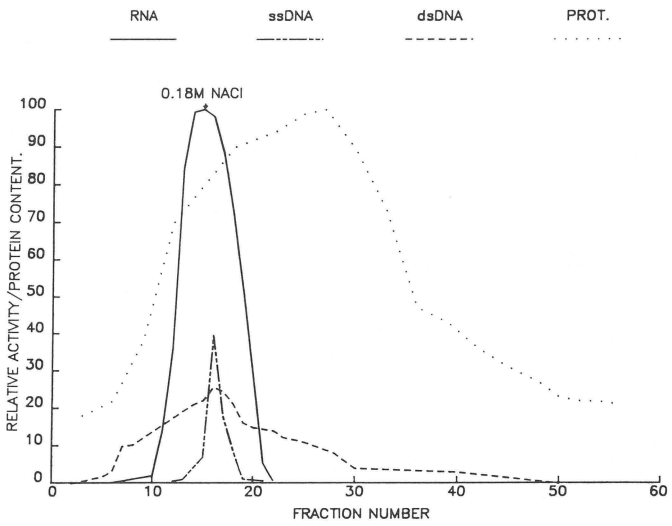

Figure 2 Anion exchange chromatography (DEAE Sephacel) of nuclease-active fraction after gel filtration. Activity of fraction was assayed towards nucleic acids indicated. NaCl molarity at which peak activities eluted is shown.

to 50% saturation with $(NH_4)_2SO_4$ wasted less nuclease activity than a one-step procedure. Since we did not try this procedure and in order to recover most of the nuclease, the precipitate after 80% saturation with $(NH_4)_2SO_4$ was used in follow-up fractionation procedures.

Enzyme fractionation

The nuclease enzyme activity was separated from most of the major contaminating proteins during gel filtration on a Sephadex G75 column (Figure 1). The activities towards RNA, ss and ds DNA were represented by single peaks and were mainly eluted together. Maximum ds DNase activity eluted somewhat earlier than maximum RNase and ss DNase activities which raises the possibility of separate ss and ds specific iso-enzymes with somewhat different molecular masses. The unequal distribution of ss and ds DNase activities during fractional precipitation with $(NH_4)_2SO_4$ (Table 1) may also be an indication of this. Prentice & Heisel (1985) were able to separate the ds DNase activity of barley roots almost completely from the RNase activity during gel filtration on a Bio Gel P-30 column. However, they did not measure ss DNase activity which could have given an indication as to whether ss and ds specific activities are separated. The RNase character of the enzyme and low DNase activity are clear (Figure 1). After dialysis of this nuclease-active fraction, a recovery of 24% and purification factor of 28 relative to the crude extract was obtained (Table 2).

During subsequent anion exchange chromatography a single fraction containing relative high RNase and low DNase activities was eluted (Figure 2). The enzyme was not totally separated from contaminating proteins and further purification steps will be needed. Three RNase iso-enzymes could be distinguished after anion exchange chromatography of the nucleolytic enzyme from bean embryonic axes of the same germination period (48 h)

Table 2 Purification of nuclease activity

	Total protein (μg BSA protein equivalents)	Total activity (milliunits) RNase	Specific activity (milliunits μg^{-1} protein)	Yield (%)	Purification (fold)
Crude supernatant	463335	42690	0.09	100	1
Precipitation (80% ammonium sulphate) and concentration (PEG and Sephadex G25)	32407	27340	0.84	64	9.3
Sephadex G75 and dialysis (fractions 15–30)	3941	10245	2.6	24	28
DEAE Sephacel	1226	3678	3.0	8.6	33

Table 3 Effect of EDTA (5 mM) on activity of nuclease from cotyledons of different germination periods

	Hours of germination					
	1	12	24	48	72	96
Nature of activity	Relative activity (100 without EDTA)					
RNase	101	87	95	104	102	105
ss DNase	67	117	174	215	176	209
ds DNase	143	150	158	144	119	74

(van der Westhuizen *et al.* 1989). This, to some extent, illustrates the difference in nucleic acid catabolism of embryonic axes and cotyledons during germination. Bryant & Greenway (1976b) have isolated two RNase iso-enzymes from the cotyledons of 5-day old seedlings of another legume, *Pisum sativum* L., by ion exchange chromatography (CM-cellulose). They also found that the iso-enzyme composition changed with the age of the seedlings. It is interesting that the ds DNase activity eluted over a wide range of salt concentrations while the ss activities (RNase and ss DNase) were confined to a smaller number of fractions (Figure 2).

Effect of EDTA

The RNase activity of the nuclease from cotyledons of different germination periods was not affected to a great extent by EDTA (Table 3). RNase activities of different plants were found to be insensitive towards EDTA (Gomes Filho & Sodek 1988; Isola & Franzoni 1981; Meza-Basso *et al.* 1986; Torti *et al.* 1973; Wilson 1975; Wiśniowska & Morawiecka 1985). The ss DNase activity was initially inhibited and increasingly stimulated thereafter by EDTA as germination proceeded to 48 h (Table 3). The stimulatory effect on the ds DNase activity maximized after 24 h of germination, thereafter it decreased to an ultimate inhibitory effect at the 96-h germination period. This may be indicative of changing requirements of the DNase activity as germination proceeds. However, the stimulatory effect on the DNase activity is rather unusual and requires further attention.

Table 5 Effect of divalent cations on RNase activity

Concentration (mM)	Relative activity				
	Mn^{++}	Mg^{++}	Ca^{++}	Zn^{++}	Cu^{++}
0	100	100	100	100	100
1	94	93	100	72	–
2	–	–	–	–	78
4	–	–	–	–	83
10	52	101	102	38	–
50	64	101	102	31	–
100	64	101	93	60	–

Effect of cations

Monovalent cations (Na^+, NH_4^+) had virtually no, or very little, effect on the RNase activity of cotyledons (Table 4). K^+ had a stimulatory effect (+10%) at concentrations of 10 mM and more. The insensitivity of the RNase activity towards Na^+ is in agreement with the observations of Meza-Basso *et al.* (1986) and Wyen *et al.* (1971) for the RNases from leaves of maize and oats respectively. Hanson & Fairley (1969) found that the DNase and RNase activities of wheat seedlings were only slightly stimulated by K^+, Na^+ and NH_4^+. In contrast, ss DNase activity of *Petunia* was inhibited (*ca.* 12%) by Na^+ and NH_4^+ (Plischke & Hess 1980). Wilson (1963) also reported the stimulation of two RNase activities from maize seedlings by KCl.

The RNase activity of cotyledons was practically unaffected by the divalent cations Mg^{++} and Ca^{++} (Table 5). Van der Westhuizen *et al.* (1989) showed that the RNase from embryonic axes of beans of similar germination stages also had no Mg^{++} or Ca^{++} requirement for activity. Mn^{++} and to a greater extent Zn^{++} inhibited RNase activity to a substantial degree (Table 5). The inhibitory effect of Cu^{++} however, was less effective. According to Torti *et al.* (1973) Cu^{++} or Zn^{++} at much lower concentrations (0.5 mM) caused a 90% inhibition of the RNase activity from wheat germ. Reports on the effect of divalent cations on the activity of nucleolytic enzymes from plants are divergent. The impression is that different divalent cations were mostly inhibitory to RNase and DNase activities (Meza-Baso *et al.* 1986; Prentice 1987; Prentice & Heisel 1986; Torti *et*

Table 4 Effect of monovalent cations on RNase activity

Concentration (mM)	Relative activity		
	K^+	Na^+	NH_4^+
0	100	100	100
1	105	101	95
10	111	99	93
50	111	103	95
100	113	105	97

Table 6 Effect of possible effectors on RNase activity

Effector	Relative activity
–	100
DTT (8 mM)	94
SDS (5%)	3
VRC (13 mM)	0

DTT = DL-Dithiothreitol; SDS = sodium dodecyl sulphate; VRC = Vanadyl-ribonucleoside complex

al. 1973; Wiśniowska & Morawiecka 1985; Zilberstein *et al.* 1987). Stimulatory effects have been reported especially for Zn^{++}, Mg^{++} and Ca^{++} (Hanson & Fairley 1969; Johnson & Lawskowski 1970; Nakamura *et al.* 1987; Wilson 1963). These results (Table 4) confirm the results of the effect of the metal scavenger, EDTA, on the RNase activity (Table 3).

Possible effectors of RNase activity

The vanadyl-ribonucleoside complex (VRC), a widely used inhibitor of RNase (Berger & Birkenmeier 1979), totally inhibited the RNase activity of cotyledons (Table 6). SDS was about equally effective. Dithiothreitol (DTT) caused an inhibition of only 6%. The effect of DTT on the RNase activity from plant material varies from stimulatory at low concentration (1 mM) (Frost & Small 1984) to inhibitory (17% and 50%) at concentrations of 2.5 mM (Prentice 1987) and 50 mM (Prentice & Heisel 1986), respectively.

Base preference

The nuclease from cotyledons seems to prefer the pyrimidine bases of the homopolyribonucleotides poly U and poly C (Table 7). The order of preference would be poly U > poly C > poly A > poly G. The high preference for poly U could be connected with the RNase nature of the enzyme also depicted in Figures 1 and 2. Van der Westhuizen *et al.* (1989) showed a similar sequence of preference for the embryonic axes from *Phaseolus vulgaris* of the same germination period. The low affinity for poly G (Table 7) probably results from the highly ordered structure of poly G which is resistant to hydrolysis (Brown & Ho 1987).

Conclusions

We have shown that the nuclease activity from cotyledons of germinating *Phaseolus vulgaris* is essentially an RNase with an inherent but low DNase activity which remains part of the enzyme during successive separations by gel filtration and anion exchange chromatography. According to definition (Wilson 1975) therefore, the enzyme can be classified as a nuclease. Although the unique base specificity is an obstacle to a detailed classification of the enzyme, we realize that a more detailed study will be needed to categorize the enzyme according to the table of Wilson (1975). The separation procedures applied could only demonstrate a single peak of RNase activity compared with the three RNase active molecular species found for the nuclease of embryonic axes of a similar stage of germination in earlier studies (van der Westhuizen *at al.* 1989), thus illustrating the difference in nucleic acid catabolism between these two organs. The RNase activity was virtually unaffected by EDTA and therefore had no particular requirement for metal cations. In contrast Mn^{++} and Zn^{++} were inhibitory. Furthermore substances such as sodium dodecyl sulphate and vanadyl ribonucleoside complex were identified as effective inhibitors of the RNase activity which could be useful for quantitative recovery of RNA from cotyledons. The enzyme has a pronounced preference for poly U and would thus probably, preferably, cleave uridine-rich regions of RNA.

Table 7 Activity of nuclease from cotyledons towards homopolyribonucleotides

Substrate	Relative activity (%)
Poly U	100
Poly C	60
Poly A	47
Poly G	0.3

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