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Preliminary studies on ribonucleases from *Poa pratensis* seeds

IRENA LORENC-KUBIS and BRONISŁAWA MORAWIECKA

Department of Molecular Biochemistry, Institute of Biochemistry, University of Wrocław

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Abstract

Ribonuclease was extracted from *Poa pratensis* seeds with 0.1 M acetate buffer, pH 5.1, and then precipitated with alcohol. The enzyme was separated into 5 fractions (I-V) after chromatography on DEAE-cellulose at pH 5.1.

The enzymes were stable at 50°C, at pH 7.1. The activity of ribonucleases I, II, III and V were optimal at pH 7.1-7.3, and that of ribonuclease IV at pH 8.1. All enzymes were inhibited by Ca^{2+} and EDTA. Mg^{2+} inhibited the activity of ribonucleases II, III, IV, and had no influence on that of ribonucleases I and V. Ribonucleases IV and V showed only one activity band in disc electrophoresis, whereas ribonucleases, I, II and III were found to be heterogenous.

INTRODUCTION

Most studies on ribonucleases of higher plants concern isolation and characterization of this group of enzymes occurring in various plant organs (Szarkowski, 1965; Loring et al. 1966; Walter and Loring, 1966; Wyen et al., 1969; Jervis 1972) and their behaviour in the early stages of development (Shuster, 1957; Wilson, 1963). There is little available information on the ribonucleases of seeds in the resting period.

In the present study partial purification and characterization of ribonucleases from *Poa pratensis* seeds was undertaken.

MATERIAL AND METHODS

The investigations concerned seeds of *Poa pratensis* from the 1972 and 1973 harvests at the Experimental Station in Polanowice.

The proteins from ground seeds were extracted with water, 0.9 per cent sodium chloride solution or 0.1 M acetate buffer, pH 5.1 in a 1:10 (w/v) ratio.

After shaking for 1 h the suspension was filtered through cheesecloth and centrifuged at 15 000 g for 20 min. Protein was determined in the supernatant turbidimetrically by the tannin micromethod of Mejbaum-Katzenellenbogen (1955).

Ribonuclease activity was determined toward yeast RNA at pH 7.1 in 0.05 M Tris-HCl buffer. The incubation mixture contained 5—20 μg of enzyme in 1.0 ml buffer and 1 ml of 0.8 per cent. RNA previously dialysed into water for 48 h. The reaction was run at 37°C for 10 and 30 min, and was stoped with 0.5 ml of 0.75 per cent uranyl acetate in 25 per cent $HC10_4$.

The samples were cooled in ice water for 15 min and then centrifuged 0.2 ml aliquot was diluted to 6 ml with water. The absorbance was measured in a VSU 2-P Carl Zeiss Jena spectrophotometer at 260 nm. One unit of ribonuclease activity was defined as the amount of enzyme that under the specified condition caused an absorbance of 0.1 at 260 nm per 10 min. Specific activity of the enzyme was expressed as units per 1 mg of protein.

Ribonuclease from seeds was prepared by the procedure described in a previous paper (Lorenc-Kubis and Morawiecka, 1973).

Partly purified proteins were chromatographed on a 1.8 × 40 cm DEAE cellulose column equilibrated with 0.005 M acetate buffer, pH 5.1.

Protein was eluted from the column by increasing (in a noncontinuous system) acetate buffer concentrations (0.005—0.2 M), pH 5.1. Finally the column was eluted with 1 M sodium chloride in 0.2 M acetate buffer.

Thermostability of the ribonuclease from crude extract and purified ribonuclease was tested at pH 5.1, 7.1 and 8.7. The extracts from seeds, with pH 5.1 were adjusted with 0.5 N NaOH to pH 7.1 and 8.7. The samples were incubated at 40—100°C for 10 min and then cooled in ice water, and enzymatic activity was determined in them at pH 7.1 toward RNA.

Electrophoresis in polyacrylamide gel was performed at pH 8.4 after Ornstein (1964) and Davis (1964) in 7.5 per cent gel subjected to photopolymerization. Protein samples (20 or 100 μg) were dissolved in 20 per cent surcrose and placed on the upper gel. Electrophoresis was run at 4°C for 1.5 h at 2.5 mA/tube for 15 min, and then at 4 mA/tube. Protein was stained for 1 h with a 1 per cent amide black solution in 7 per cent acetic acid. The excess of the dye was washed out with 7 per cent acetic acid.

Ribonuclease activity in the gel was localized by Wolf's method (1968). The gels were incubated in a 0.4 per cent RNA solution at pH 7.1.

The following special chemical reagents were used: ribonucleic acid, Tris-Fluka A. G. Buchs S. G. Switzerland. Tannin was a French product. DEAE cellulose-D-11--Whatman WR Balston Ltd., England. N-N′-methylenebisacrylamide, 2-mercapto ethanol, sodium 1-naphthylphosphate, acrylamide — Koch — Light, Colnbrook, Buks, England, N, N, N′, N′-tetramethylenediamine — Eastman Organic Co., U. S. A., Fast Blue B — George R. Gurr Ldt., London England.

The remaining reagents were pure for analysis and were products of Polskie Odczynniki Chemiczne, Gliwice.

RESULTS

Table 1 shows the results of protein extraction from seeds and ribonuclease activity. It was demonstrated that the protein extracted with acetate buffer, pH 5.1 constitutes about 14 per cent of the proteins soluble in sodium chloride or water. In all examined extracts ribonuclease activity was detected. The highest enzymatic activity — 45 μ/mg of protein was found in the buffer extracts, pH 5.1.

Table 1

Ribonuclease activity of proteins from *Poa pratensis* seeds extracted with water, sodium chloride and 0,1 M acetate buffer, pH 5.1

Extraction with	Protein g/100 g of seeds	RNase activity	
		specific μ/mg protein	total μ/100 g of seeds
water	0.27	20	5400
sodium chloride 0,9%	0.29	25	7250
acetate buffer 0.1 M pH 5.1	0.04	45	1800

Assays were performed as described in "Material and Methods".

Fig. 1 presents the thermostability of ribonuclease in the crude extract at pH 5.1, 7.1 and 8.7. The proteins extracted with buffer when heated for 10 min at 60°C in pH 7.1 were found to have full enzymatic activity. Above this temperature ribonuclease activity were lost about 20 per cent as compared with that of the unheated extract. Adjustment of pH to 5.1 or to 8.7 caused a decrease of ribonuclease activity at temperature above 40° and 50°C. Proteins with a ribonuclease activity extracted from seeds by means of acetate buffer, pH 5.1 were subjected to preliminary purification by dialysis into water and precipitation with alcohol, according to the procedure

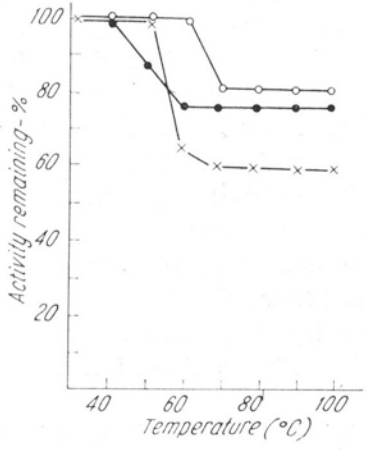

Fig. 1. Thermostability of ribonuclease from a crude extract (0.1M acetate buffer, pH 5.1)
Heat treatment was carried out at pH 5.1 (x—x), pH 7.1 (○——○) and pH 8.7 (●——●), for procedure see Methods.

described in an earlier paper (Lorenc-Kubis and Morawiecka, 1973). The course of preparation is shown in Table 2. Protein precipitation with alcohol of final 64 per cent concentration and then dissolution in water and dialysis into water lead to an about 2.6-fold increase in the specific activity of the enzyme as compared with the crude extract. At this stage of purification the preparations were subjected to lyophilization.

Table 2

Preliminary purification of ribonuclease from *Poa pratensis* seeds

Procedure	Protein		RNase activity		
	g/100 g of seeds	%	specific μ/mg protein	total μ/100 g of seeds	Yield %
Extraction with 0.1 M acetate buffer pH 5.1	0.042	100	45	1890	100
Dialysis of extract into water	0.030	73	60	1800	95.7
Protein precipitation with alcohol and dialysis into water	0.012	30	120	1512	80.4

Protein was determined by the tannin micromethod, RNase assay were carried out at pH 7.1. For reaction conditions see Methods.

After lyophilization the preparation was dissolved in 0.005 M acetate buffer, pH 5.1 and fractionated on a DEAE cellulose column equilibrated with the same buffer. Protein was eluted from the column by increasing acetate buffer concentrations (pH 5.1). Fig. 2 presents the profile of protein elution and ribonuclease activity. Five main protein peaks were obtained showing ribonuclease activity.

Specific ribonuclease activity after separation on DEAE cellulose in shown in Table 3. The highest specific activity — 220 μ/mg of protein was exhibited by ribonuclease eluted with the proteins from peak II (ribonuclease II). The proteins from peaks I and V were characterized by a specific activity of about 90 μ/mg of protein. The lowest activity (40 μ./mg of protein) was found in peaks III and IV.

The influence of pH on ribonuclease activity is presented in Fig. 3. It is seen that ribonuclease I and V exhibit the highest enzymatic activity at pH 7.3, ribonucleases II and III at pH 7.1 and the optimum for ribonuclease IV is at pH 8.1.

Fig. 4 shows the thermostability of ribonucleases at pH 7.1. Ribonucleases II and IV presented their full activity up to 60°C, at higher temperatures the activity decreased by more than 65 per cent as compared with that of the unheated enzyme. The remaining ribonucleases showed a decrease of enzymatic activity above 50°C.

The influence of various ions on ribonuclease activity is shown in Table 4. It was demonstrated that EDTA in a 2.5×10^{-2} M concentration completely inhibits the activity of ribonuclease III. The relative activity of the remaining ribonucleases

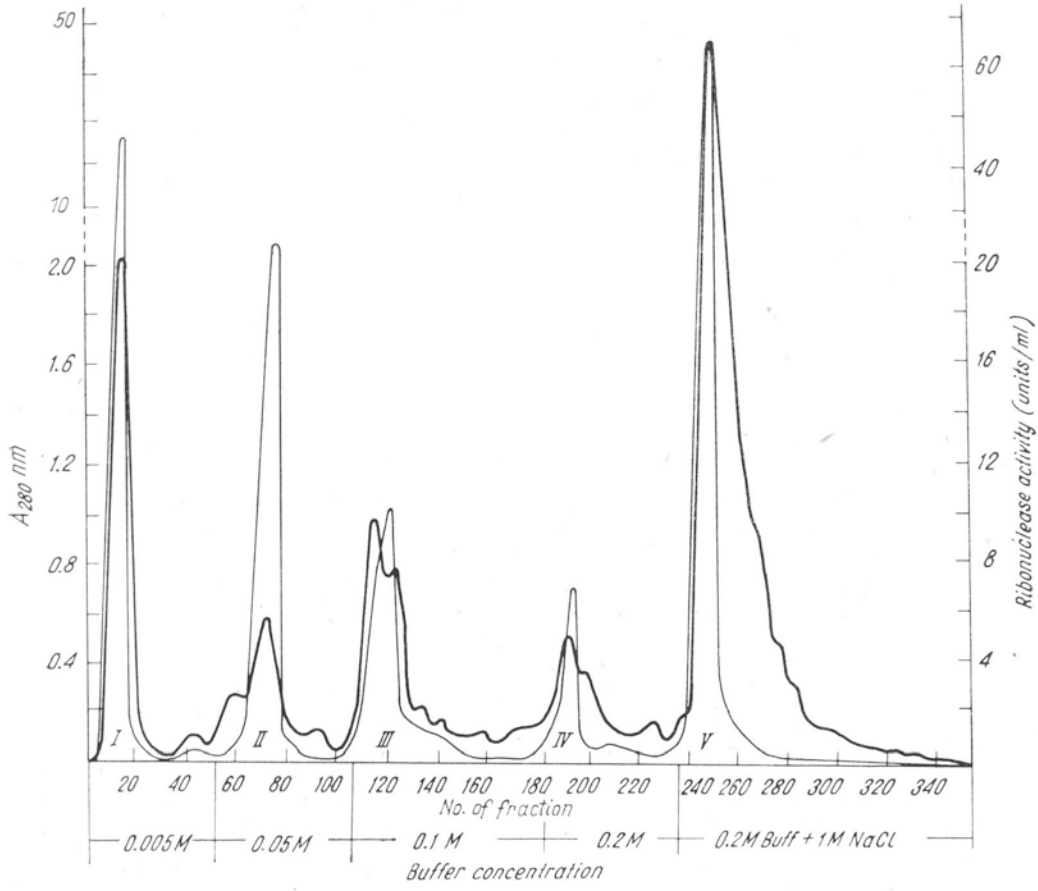

Fig. 2. Chromatography of partialy purified ribonuclease from *Poa pratensis* seeds on DEAE cellulose.

120 mg of protein in 10 ml was added to a column of DEAE cellulose equilibrated with 0.005 M acetate buffer, pH 5.1. Protein was eluted from the column with increasing acetate buffer concentrations. 5 ml fractions were collected and analysed as described in Methods. Adsorbance at 280 nm (———) ribonuclease activity (— — —)

Table 3

Ribonuclease activity in peaks I, II, III, IV and V after chromatography on DEAE-cellulose

Peak	RNase activity μ/mg of protein
I	95
II	220
III	40
IV	38
V	80

RNase activity was measured in tris-HCl buffer, pH 7,1. For reaction conditions see Methods.

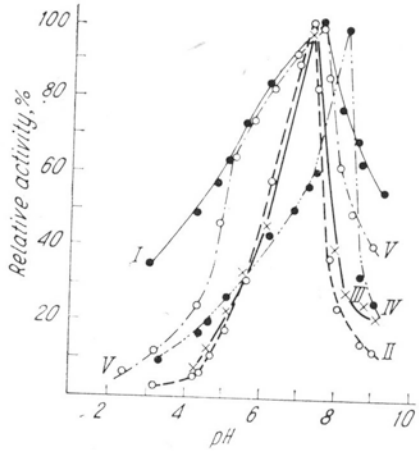

Fig. 3. Effect of pH, on the activity of *Poa pratensis* ribonucleases.

RN-ase activity was determined in 0.14 M veronal buffer. Enzyme concentration was 5-20 μg. Reaction run at 37° for 30 min. Activity expressed as percent in reference to highest activity in given experiment. RN-ase I (●——●), II (○— — —○), III (x——x), IV (●—..—●), V (○—.—.—.○)

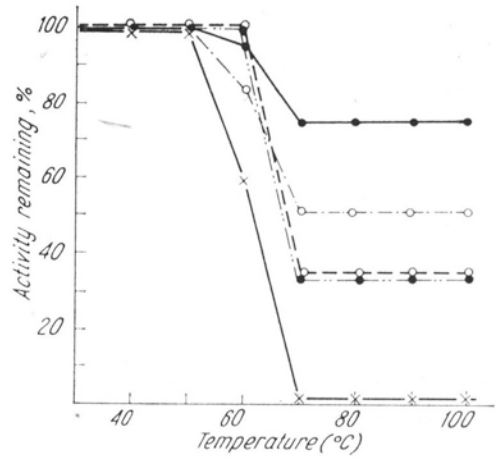

Fig. 4. Heat inactivation of *Poa pratensis* RN-ases at pH 7,1.

RN-ases (5-20 μg/ml) were heated for 10 min at indicated temperatures an then assayed for activity as described in Methods. RN-ase: I (●——●), II (○— — —○), III (x——x) IV (●—..—●), V (○—.—.○)

in the presence of this salt was on the average about 30 per cent. Ca^{++} and Mg^{++} ions exert a weaker inhibitory effect.

Proteinograms (P) and zymograms (Z) are schematically presented in Fig. 5. In polyacrylamide gel the proteins of the particular peaks were separated into 3—6 fractions. The largest number of fractions was obtained from peak I. The proteino-

Table 4

Effect of various compounds on the activity of ribonuclease from *Poa pratensis* seeds

Substance added	relative activity (%) of RN-ases: I	II	III	IV	V
None	100	100	100	100	100
KCl	100	100	100	100	100
$CaCl_2$	98	82	80	66	—
$MgCl_2$	100	79	80	70	100
NaF	100	115	100	120	100
EDTA	36	23	0	40	38

Substance concentration in incubation mixture — $2{,}5 \times 10^{-2}$ M. Enzymes concentration 5-20 μg. Reaction run at 37° for 10 and 30 min. Activities were expressed as percent of that without added substance.

gram of peak II showed the presence of two of the main fractions and 3 trace ones. Four fractions were noted in the case of proteins of peaks IV and V. The smallest number fractions was occurred in proteins of peak III. The fraction dominating among the three protein fractions showed highest anodic mobility. In the simultaneously performed zymograms the presence of 1—3 bands of ribonuclease activity

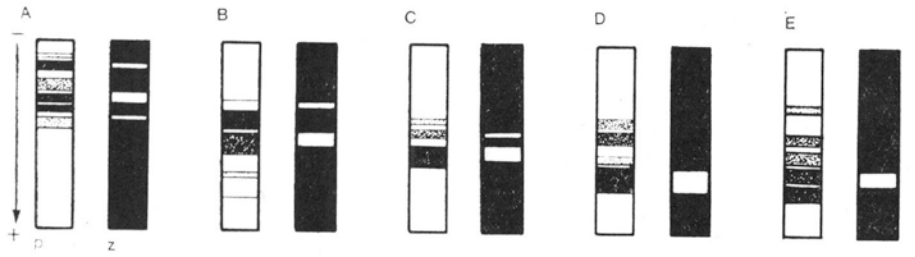

Fig. 5. Separation of proteins from *Poa pratensis* seeds on polyacrylamide gel.

P — scheme of proteinogram, Z — scheme of ribonuclease activity.
Proteins after chromatography on DEAE cellulose. A — peak I, B — peak II, C — peak III, D — peak IV, E — peak V. Electrophoresis was performed in tris-glycine buffer, pH 8,4 for 1,5 h at 4°. Staining for protein and RN-ase activity see Methods.

was demonstrated. Three bands of enzymatic activity occurred in zymograms of peak I and two in those of peaks II and III. In the zymograms of peaks IV and V one band with ribonuclease activity was localized with a mobility corresponding to the dominating protein fraction.

DISCUSSION

Wilson (1963) isolated and characterized two ribonucleases present in maize seedlings and Szarkowski (1965) in rye blades. The enzymes differed in substrate specificity and optimum pH. In the present study the presence of 5 ribonucleases was demonstrated in the seeds of *Poa pratensis*. Ribonuclease IV showed optimum enzymatic activity at pH 8.1 and the remaining ones at pH 7.1—7.3. A similar pH optimum was noted in the case of ribonuclease from *Streptomyces aureofaciens* (Zelinkova et al., 1971) and *Rhizopus oligosporus* (Woodroof and Glitz, 1971). Calcium ions are inhibitors of all the ribonucleases investigated in *Poa pratensis* seeds as well as of ribonuclease I occurring in rye blades (Szarkowski, 1965) and ribonuclease isolated from *Acrocylindrium* sp. (Suhara et al., 1972). The chromatographic heterogeneity of ribonucleases from *Poa pratensis* demonstrated in the present work is confirmed by electrophoretic investigations in polyacrylamide gel. The presence of several zones of ribonuclease activity has also been demonstrated by Kubicz, Wieczorek and Morawiecka (1972) in extracts from tubers of various potato varieties subjected to electrophoretic separation in polyacrylamide gel. In spite of extensive investigations on ribonucleases of higher plants as well as of microorganisms, to data the principles of their molecular heterogeneity remain obscure.

The biological significance of the presence of several ribonuclease groups in seeds is probably connected with a protection function against bacterial or viral infection (Barnard, 1969).

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Author's address:
doc. dr Bronisława Morawiecka
dr. Irena Lorenc-Kubis
Institute of Biochemistry
University of Wrocław
ul. Tamka 2, 50-137 Wrocław
Poland

Streszczenie

W wyciągach wodnych, chlorkowych oraz buforowych z nasion wiechliny łąkowej stwierdzono obecność białek o aktywności rybonukleazowej. Rozdział białek rozpuszczalnych w 0,1 M buforze octanowym o pH 5,1 na kolumnie z DEAE celulozy wykazał obecność 5 grup rybonukleaz. Optimum działania tych enzymów wynosi: dla rybonukleazy I i V — 7,3 dla rybonukleazy II i III — w pH 7,1, natomiast dla rybonukleazy IV — 8,1. Rybonukleazy zachowują pełną aktywność enzymatyczną podczas ogrzewania do temperatury 50°. Badane rybonukleazy hamowane są przez jony Ca^{2+} oraz wersenian dwusodowy. Jony Mg^{2+} hamują aktywność rybonukleazy II, III i IV, pozostają bez wpływu na aktywność rybonukleazy I i V.