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Acid phosphatases and ribonucleases from *Dactylis glomerata* seeds

I. Chromatographic and electrophoretic heterogeneity of the enzymes

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Abstract

Acid phosphatase and ribonuclease extracted with 0.1 M sodium acetate buffer, pH 5.1 from *Dactylis glomerata* seeds, and partially purified by means of 70% ethanol precipitation showed electrophoretic and chromatographic heterogeneity. After chromatography on DEAE-cellulose acid phosphatase and ribonuclease were separated into four peaks. Nonadsorbing acid phosphatase on DEAE-cellulose (peak I) was separated into four peaks on CM-cellulose. The highest activity (11 units/mg) was found in fraction b (acid phosphatase Ib). The enzyme was activated by Mg^{2+}, Ca^{2+}, Li^{+}, Cs^{+}, K^{+} ions and inhibited by Cu^{2+}, Zu^{2+}, F^{-} and Mo^{-6} at optimum pH 5.0. Strong absorbing ribonuclease on DEAE-cellulose (peak IV) was further separated on G-200 Sephadex into two molecular forms: RN-ase$_1$ and RN-ase$_2$. Ribonuclease 1, a thermolabile enzyme with specific activity 807 units/mg, showed an optimal activity at pH 4.8-5.1.

INTRODUCTION

Acid phosphatases and ribonucleases isolated from different vegetative organs of higher plants are characterized by a heterogeneity, determined by plant development and location within cell structure (Dove 1973, Morawiecka et al. 1973, Wieczorek et al. 1975). Molecular heterogeneity of these enzymes has been observed in several grasses during dorment period (Lorenc-Kubis, Wieczorek 1973, Wiśniowska 1977). Crude extracts from *Dactylis glomerata* seeds are characterized by higher activity of acid phosphatase and ribonuclease

compared to extracts from several varieties of rye seeds, as well at other grass seeds (Morawiecka et al. 1976, Wiśniowska 1977).

In the present work attention is paid to heterogeneity of phosphatase and ribonuclease isolated from *Dactylis glomerata* seeds. Preliminary studies were also undertaken on purification and some biochemical properties of these enzymes.

MATERIAL AND METHODS

Studies were carried out on cocksfoot *(Dactylis glomerata)* seeds of the 'Motycka' variety, harvested in 1974. Grass seeds were obtained from the Wrocław Establishment of Cultivation and Seed Production.

Isolation of proteins from seeds was made according to the procedure by Lorenc-Kubis and Morawiecka (1973), extracting ground material with 0.1 M acetate buffer, pH 5.1, at a ratio 1:10 (w/v). Proteins were precipitated from the extract by cool ethanol, to reach final concentration of 70%, and dialysed to water. Dialyser was centrifuged and the supernatant lyophilized.

Proteins were determined with a turbidimetric tannin micromethod according to Mejbaum-Katzenellenbogen (1955), on a photocolorimeter FEK-56, using filter No 8 in 1 cm cells, and on a spectrophotometer Zeiss Jena VSU-2, at 280 nm.

Activity of acid phosphatase was determined measuring p-nitrophenol liberated from sodium p-nitrophenylphosphate. Incubation mixture contained 1 ml of 10 mM sodium p-nitrophenyl phosphate and 1 ml of enzyme (1—30 μg of protein) in a 0.1 m acetate buffer, at pH 5.1. Sample were incubated at 37°C and the reaction was interrupted with 10 ml of 0.1 N NaOH. Extinction was measured on Specol, at 410 nm. A unit of acid phosphatase activity was expressed as an activity of the enzyme which liberates 1 μmol of p-nitrophenol during 1 min, at 37°C; specific activity was defined by a number of units per 1 mg of protein.

Ribonuclease activity was determined according to Anfinsen et al. (1954) in 0.1 M acetate buffer, pH 5.1. A unit of ribonuclease activity corresponded to the activity of the enzyme which resulted in an increase of extinction by 0.1, at 260 nm and layer thickness of 1 cm. Specific activity was expressed in number of units per 1 mg of protein.

Chromatographic separation of proteins was made on columns with DEAE-cellulose (2.5 × 34 cm) and CM-cellulose (2 × 24 cm), equilibrated with 0.005 M acetate buffer, pH 5.1. Proteins were eluted with increasing concentrations of acetate buffer at pH 5.1 (0.005 M, 0.05 M, 0.2 M), and further on with 0.5 M sodium chloride in 0.2 M acetate buffer. 6 ml fractions were collected at outflow rate of 10 ml for 13 min.

Molecular filtration of proteins was made on Sephadex G-200 column (1.5 × 150 cm). Proteins were eluted with water. 2 ml fractions were collected at outflow rate of 1 ml/10 min.

Electrophoresis on polyacrylamide gel was made according to Davis (1964) and Ornstein (1964) in 7.5% gels in Tris-glycine buffer, at pH 8.4. Proteins in gels were stained with 1% solution of amide black in 7% acetic acid. Excess of stain was removed washing the gels with 7% acetic acid.

Phosphatase activity in gels was located using diazic coupling method in the presence of sodium alfa-naphthylphosphate and Fast Blue B.

Ribonuclease activity in gels was developed according to Wolf (1968).

Substrate specificity of acid phosphatase was determined measuring inorganic phosphorus (Pi) liberated from the substrates under study. Incubation mixture contained 1 ml of 10 mM substrate and 1 ml of the enzyme (5 μg of protein) in 0.1 M acetate buffer, at pH 5.1. After 10 min. incubation in 37°C reaction was interrupted by an addition of 2 ml of 5% trichloroacetic acid. Liberated phosphate was determined with the method of Fiske-Subbarow (1925). Extinction was measured at Specol, at 660 nm.

The effect of temperature on enzyme stability was determined incubating samples (containing about 5 μg of protein in 1 ml of 0.1 M acetate buffer, pH 5.1) for 10 min. in temperatures from 40° to 100°C. Next, samples were cooled for 10 min. in 0°C, and preincubated for 10 min. in 37°C. Enzymatic reaction was carried out with p-nitrophenylphosphate as substrate for phosphatase activity, or 0.8% RNA for ribonuclease activity. Activity of the control sample was treated as 100%.

Optimal pH of enzymatic activity was determined in 0.14 M veronal--acetic buffer, at pH range from 2.6 to 9.8, with sodium p-nitrophenylphosphate as acid phosphatase substrate, or 0.8% RNA as RN-ase substrate.

RESULTS AND DISCUSSION

Extraction and partial purification of acid phosphatase and ribonuclease isolated from *Dactylis glomerata* seeds are presented in Tab. 1. Extract obtained from 100 g of seeds contained 121 mg of proteins with specific activity of acid phosphatase of 2.4 units/mg, and of ribonuclease — 8.0 units/mg. Precipitation of proteins from crude extracts with 70% ethanol removes pigments, balast proteins, and leads to a 3-fold purification of the activity of both enzymes under study. This material, when subject to electrophoresis in 7.5% polyacrylamide gel at pH 8.9

Table 1

Partial purification of acid phosphatase and ribonuclease from *Dactylis glomerata* seeds

Steps	Protein		Phosphatase activity			Ribonuclease activity		
	mg/100 g seeds	%	Specific units/mg	Total units	Yield %	Specific units/mg	Total units	Yield %
Crude extract in 0.1 M acetate buffer, pH 5.1	121.0	100	2.4	290.4	100.0	8.0	968.0	100.8
Ethanol precipitate	13.5	11.1	8.5	114.8	39.5	22.0	297.0	30.7
DEAE-cellulose								
peak I	6.6	5.5	1.7	11.0	3.8	12.5	82.9	8.6
peak II	4.1	3.4	10.1	41.3	14.2	41.0	163.0	17.4
peak III	0.9	0.8	13.5	12.7	4.3	51.2	48.1	5.0
peak IV	1.0	0.8	11.0	10.8	3.7	269.2	269.2	27.3
CM-cellulose (peak I after DEAE)								
peak Ia	0.14	0.12	4.82	0.67	0.23	11.6	1.6	0.2
peak Ib	0.21	0.17	11.10	2.33	0.80	16.6	3.5	0.36
peka Ic	0.18	0.14	2.83	0.51	0.17	23.0	4.1	0.40
peak Id	0.73	0.60	0.50	0.37	0.13	0.4	4.7	0.48

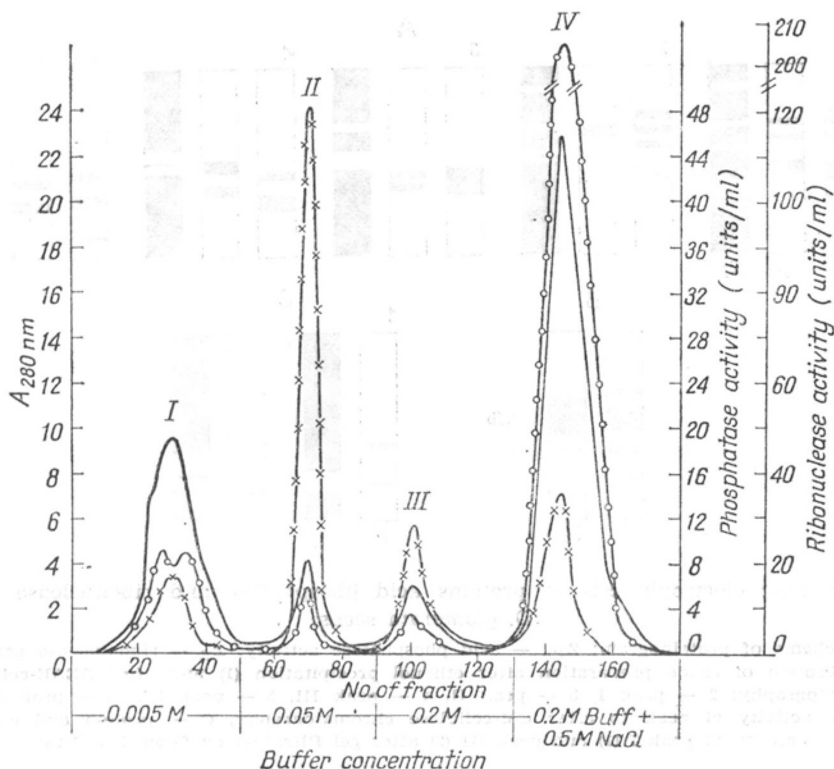

Fig. 1. DEAE-cellulose chromatography of proteins from *Dactylis glomerata* seeds. DEAE-cellulose column (2.5 × 34 cm) was equilibrated with 0.005 M acetate buffer, pH 5.1. Protein was eluted with increasing acetate buffer concentrations and 0.5 M NaCl. 6 ml fractions were collected and analyzed for absorbance at 280 nm (———), acid phosphatase activity (—x—x—) and ribonuclease (—o—o—)

(Fig. 2 — A 1), separates into 3 bands with the activity of acid phosphatase, and 4 bands with ribonuclease activity. Similar electrophoretic heterogeneity was described for partially purified acid phosphatase and ribonuclease from *Poa pratensis* seeds (Lorenc-Kubis, Morawiecka 1973, 1974), rye-grass Wieczorek et al. 1977), and rye (Morawiecka et al. 1976). Chromatographic heterogeneity of partly purified preparation of acid phosphatase and ribonuclease was noted during ion-exchange chromatography on DEAE-cellulose (Fig. 1). Proteins were eluted from the column with increasing concentrations of acetate buffer, pH 5.1, in a non-continous system, and with 0.5 M NaCl. 4 main protein peaks were obtained, possessing both enzymatic activities. Characteristic of particular peaks is presented in Tab. 1. Proteins of peak I, nonadsorbing on DEAE-cellulose, constitute about 50% of the preparation introduced to the column. Compared to other peaks they are characterized by the lowest activity of acid phosphatase and ribonuclease. About 35% of proteins were eluted with

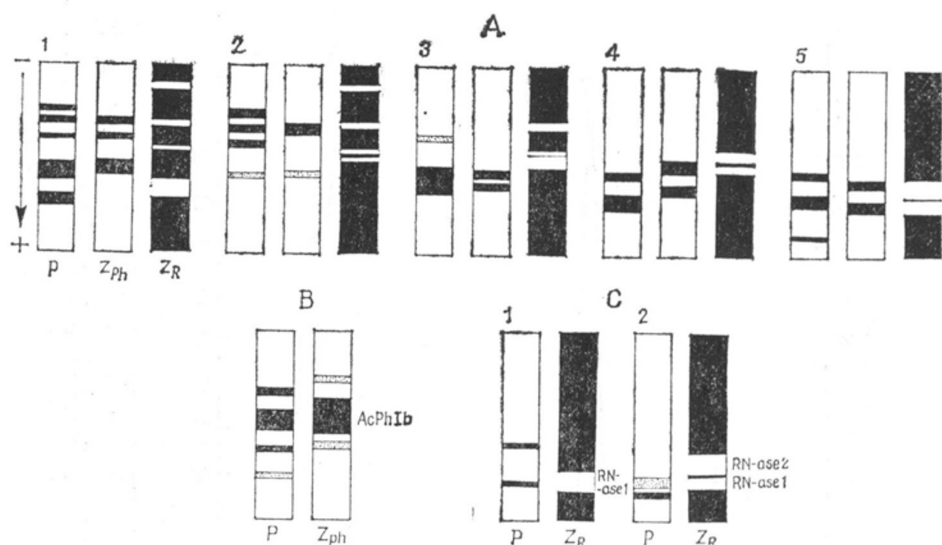

Fig. 2. Disc electrophoresis of proteins acid phosphatase and ribonuclease from *D. glomerata* seeds.

P — scheme of proteinogram; Z_{Ph} — acid phosphatase activity; Z_R — ribonuclease activity. A — Protein of crude preparation after ethanol precipitation (1) and after DEAE-cellulose chromatography: 2 — peak I, 3 — peak II, 4 — peak III, 5 — peak IV. B — protein and enzyme activity of peak Ib after CM-cellulose chromatography, C — protein and enzyme activity of peak I (1) and peak III (2) after gel filtration on Sephadex G-200

0.05 M acetate buffer, pH 5.1, into peak II, whereas peaks III and IV contained respectively 7% and 8% of proteins which underwent ion--exchange chromatography. Phosphatases adsorbing on DEAE-cellulose (peaks II, III, and IV) are characterized by almost similar specific activity, from 10.1 to 13.5 units/mg. The highest acid ribonuclease activity, amounting to 269 units/mg, was noted for proteins most strongly adsorbed on DEAE-cellulose (peak IV). Tsushida and Takeo (1976) obtained four RN-ase fractions from tea leaves on DEAE-cellulose, at pH 6.0, and also in their case the highest activity was noted for proteins eluted with highly concentrated salt. Electrophoretic studies have shown that fractionation of preliminarily purified preparation on DEAE-cellulose leads into partial separation of particular forms of phosphatase and RN-ase. Fig. 2 A presents proteinograms and zymograms of proteins obtained after fractionation on DEAE-cellulose. Proteins of peak I, non-adsorbing on DEAE-cellulose, contain mainly acid phosphatase with the lowest anode mobility, and four weakly staining RN-ases, devoid of the form with the highest anode mobility. Proteins of peak II and III contain two forms of acid phosphatase with average anode mobility, and two or tree forms of RN-ase. Proteins of peak IV contain two bands of acid phosphatase, and two bands of RN-ase, with the highest anode mobility, contaminated by one fraction of balast proteins.

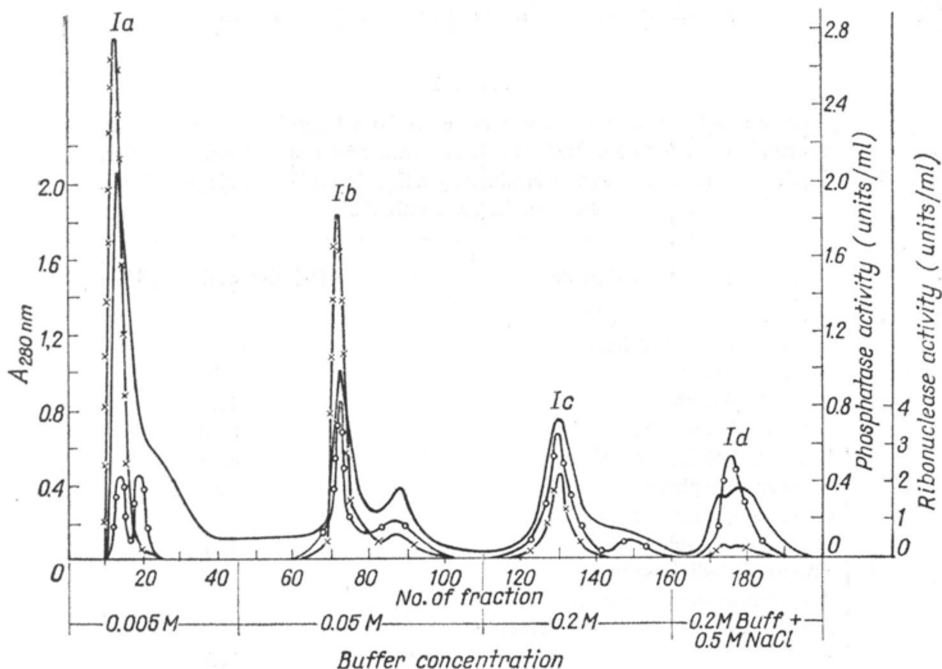

Fig. 3. CM-cellulose chromatography of protein from peak I obtained after DEAE--cellulose.

CM-cellulose column (2 × 24 cm) was equilibrated with 0.005 M acetate buffer, pH 5.1. Protein was eluted with increasing acetate buffer concentrations and 0.5 M NaCl. 6 ml fractions were collected and analyzed for absorbance at 280 nm (———), acid phosphatase activity (—x—x—) and ribonuclease (—o—o—)

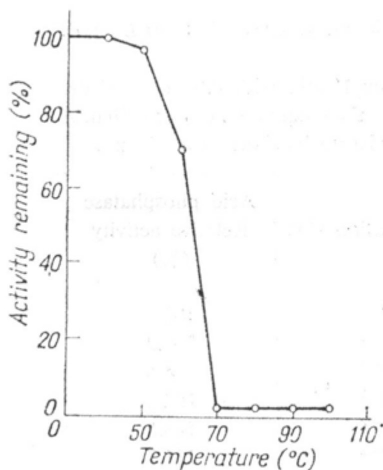

Fig. 4. Heat stability of acid phosphatase Ib from *Dactylis glomerata* seeds.

Aliquots of enzyme were kept in 0.1 M acetate buffer, pH 5.1 for 10 min at various temperatures and then assayed for activity as described in Methods

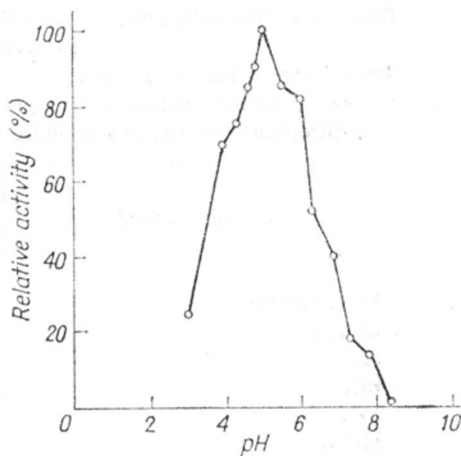

Fig. 5. Effect of pH on the acid phosphatase Ib activity.

Acid phosphatase activity was determined in 0.14 M veronal buffer. Reaction run at 37° for 10 min. Activity expressed as percentage in reference to highest activity in given experiment

Table 2

Substrate specificity of acid phosphatase Ib from *Dactylis glomerata* seeds
The reaction mixture contained 10 mM substrate and 100 mM acetate buffer, pH 5.1. Enzyme activity was determined by estimation of inorganic phosphate. For details see Methods.

Substrate	Relative activity (%)
p-Nitrophenyl phosphate	100
Phenylphosphate	107.0
α-naphtylphosphate	15.7
β-naphthylphosphate	80.3
Phenolphthaleine phosphate	43.3
β-glycerophosphate	0
Glucose-1-phosphate	0
Glucose-6-phosphate	10.8
Fructose-1.6-diphosphate	0
Bis-p-nitrophenylphosphate	0
5′-IMP, 5′-AMP, 5′-CMP, 5′-GMP, 5′-UMP	0
ATP	68.9
Inorganic pyrophosphate	205.9

Table 3

Effect of various substances on the activity of acid phosphatase Ib from *Dactylis glomerata* seeds
The substances were first preincubated at 37°C for 10 min with 0.05 unit of the enzyme in 100 mM acetate buffer, pH 5.1 (1 ml), then equal volume of 10 mM p-nitrophenyl phosphate was added and the mixture incubated for 10 min.

Substance added	Concentration (M)	Acid phosphatase Relative activity (%)
None (control)	0	100
Li_2SO_4	10^{-2}	160.2
NaF	10^{-2}	8.8
KCl	10^{-2}	152.2
CsCl	10^{-2}	148.5
$MgCl_2$	10^{-2}	211.7
$ZnCl_2$	10^{-2}	11.0
$CaCl_2$	10^{-2}	147.7
$CuCl_2$	10^{-2}	5.1
KH_2PO_4	10^{-2}	73.5
$(NH_4)_6Mo_7O_{24}$	10^{-2}	0
EDTA	10^{-2}	167.6
1.10-Phenanthroline 12.5×10^{-4}		183.8
Urea	2	69.1

Acid proteins nonadsorbing on DEAE-cellulose (peak I) were fractionated on CM-cellulose, pH 5.1. Elution profile of proteins, and phosphatase and RN-ase activities are presented in Fig. 3. Proteins were eluted from the column by a stepwise gradient of ion strength, with acetate buffer at pH 5.1. Characteristic of the peaks with phosphatase activity is presented in Tab. 1. In particular peaks RN-ase activity ranged between 6.4 and 23 units/mg of protein. In proteins of peak Ib almost 7-fold increase of acid phosphatase was noted compared with the activity of material separated on CM-cellulose. Activity of acid phosphatase in the remaining peaks was significantly lower, amouting to 0.5—4.8 units/mg of protein. Further studies were concentrated on a more detailed characteristic of acid phosphatase in peak Ib. Disck electrophoresis (Fig. 2B) showed that proteins of this peak contain mainly acid phosphatase of low anode mobility, accompanied by two trace bands with enzymatic activities.

Substrate specificity of phosphatase Ib was determined against several phosphorus esters; the results are presented in Table 2. This enzyme is most active toward pyrophosphate, similary as acid phosphatases from wheat germs (Verjee 1969), and phosphatases a_2 and a_3 from meadow-grass seeds (Lorenc-Kubis et al. 1975). Lack of activity toward mononucleotides, both purine and pirimidine, and toward some sugar esters points to the similarity to phosphatase from rice bran (Sastry and Rao 1972), and shoots of mung bean (Hagiwara et al., 1969).

The effect of some metal ions, urine, EDTA, and phenanthroline on the activity of phosphatase Ib is presented in Tab. 3. Ions of Mg^{2+}, Ca^{2+}, Li^{+}, Cs^{+}, K^{+}, as well as EDTA and phenanthroline activates this phosphatase. Activating effect of EDTA was also noted for acid phosphatases from rice bran (Igaue et al., 1975), seeds of meadow-grass (Lorenc-Kubis et al., 1975), and seed of French rye-grass (Wieczorek et al., 1977). Activity of phosphatase Ib from *Dactylis glomerata* seeds was, on the other hand strongly inhibited by ions of Cu^{2+}, Zn^{2+}, and F^{-}. Ammonium molybdate totally inhibited enzymatic activity. Inhibiting effect of molybdate ion was observed for acid phosphatase from

Table 4

Activity of ribonucleases after gel filtration on Sephadex G-200

Material	Total protein mg	Total activity units	Specific activity units/mg	Purification factor
Protein of peak IV (peak IV after DEAE)	18.2	4799.4	269.2	—
Sephadex G-200				
peak I	1.8	1623.6	807.6	3
peak III	2.5	2692.0	1076.8	4

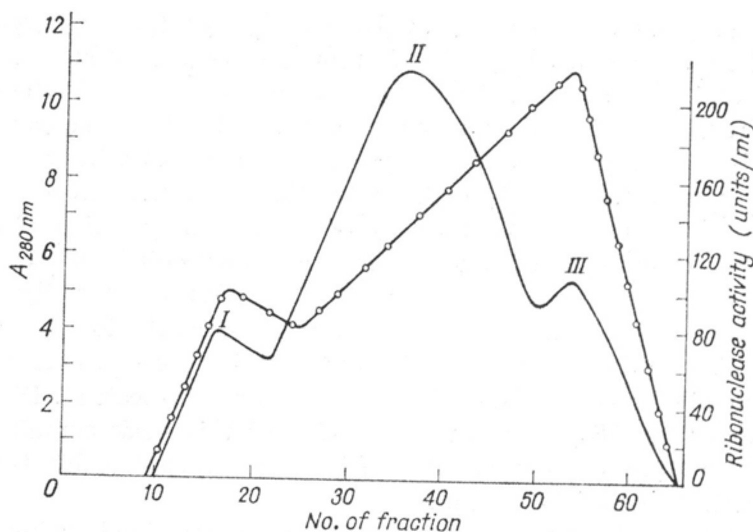

Fig. 6. Sephadex G-200 gel filtration of partialy purified ribonuclease from *Dactylis glomerata* seeds.

18.2 mg of protein was applied to a column (1.5×150 cm) of Sephadex G-200. Elution was carried out with water. Fractions (2 ml) were collected and analysed for absorbance at 280 nm (———) and ribonuclease activity (—o—o—)

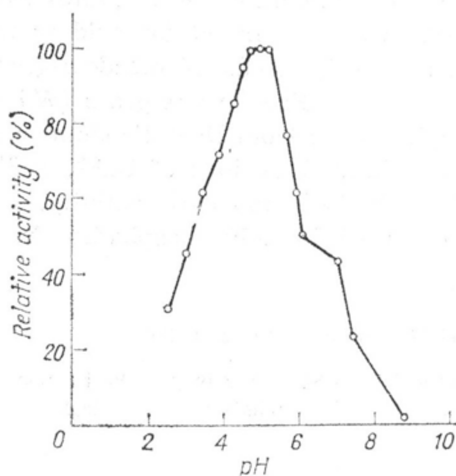

Fig. 7. Effect of pH on RN-ase 1.

Acid phosphatase activity was determined in 0.14 M veronal buffer. Reaction run at 37° for 10 min. Activity expressed as percentage in reference to highest activity in given experiment

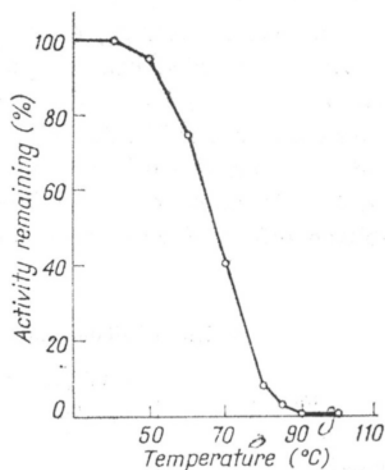

Fig. 8. Heat stability of RN-ase 1.

Aliquots of enzyme were kept in 0.05 M acetate buffer, pH 5.1 for 10 min. at various temperatures and then asseyed for activity as described in Methods

Fusarium moniliformae (Yoshida and Tamiya, 1971), sweet potato (Uehara et al., 1974), and seeds of *Avena elatior* (Wieczorek et al., 1977).

Studies on thermal stability of phosphatase Ib (Fig. 4) have shown that 10 min. heating in 50°C, at pH 5.1, does not affect the activity of the enzyme, while in 70°C the enzyme becomes totally inactive.

Acid phosphatase Ib is characterized by enzymatic activity within a broad pH range, from 3 to 8 (Fig. 5). The highest enzymatic activity was noted at pH 5.0.

RN-ase of peak IV, after DEAE-cellulose, was subject to molecular filtration on Sephadex G-200 (Fig. 6). Three poorly separated protein peaks were obtained. In I and III peak activity of acid RN-ase was localized. Enzymatic activity of these two forms of acid RN-ase is 3 to 4 times higher than the activity of material obtained after purification on DEAE-cellulose (Tab. 4), and 100 to 130 times higher compared with the activity of raw seed extracts. In disc electrophoresis (Fig. 2C) proteins of peak I, after Sephadex G-200, contained a single band with the activity of acid RN-ase, while proteins of peak III — two active bands. Optimum of enzymatic activity of RN-ase I (after Sephadex G-200) is noted between pH 4.8 and 5.1 (Fig. 7).

10 min. incubation in 70° resulted in a 60% decrease of the enzymatic activity, while incubation in 87° resulted in total loss of the activity (Fig. 8). RN-ases from some strains of *Penicillum,* as also from rye leaves, are characterized by similar thermal stability (Fujimoto et al., 1977, Gołeszewski et al., 1967).

Ion-exchange chromatography applied on DEAE-cellulose for the separation of partly purified extract from *Dactylis glomerata* seeds showed the presence of several molecular forms of acid phosphatase and RN-ase. Molecular heterogeneity of these enzymes is probably caused by their function and localization in particular anatomic parts of seeds.

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Fosfatazy kwaśne i rybonukleazy nasion Dactylis glomerata I. Heterogenność chromatograficzna i elektroforetyczna enzymów

Streszczenie

Fosfatazę kwaśną i rybonukleazę z nasion *Dactylis glomerata* izolowano za pomocą ekstrakcji do 0,1 M buforu octanowego o pH 5,1, frakcjonowania 70% etanolem, chromatografii jonowymiennej na DEAE-celulozie, CM-celulozie oraz sączenia molekularnego na Sephadex G-200. Po rozdziale białek na DEAE-celulozie i elektroforezie dyskowej wykazano 3 do 4 formy fosfatazy kwaśnej i 4 formy rybonukleazy. Fosfataza kwaśna nieadsorbująca się na DEAE-celulozie ulega rozdziałowi na 4 do 5 form molekularnych na CM-celulozie. Fosfataza kwaśna Ib

o aktywności właściwej 11 jedn./mg jest termolabilna i posiada optimum aktywności enzymatycznej w pH 5,0. Aktywatorami tego enzymu są jony Mg^{2+}, Ca^{2+}, Li^{+}, Cs^{+}, K^{+} oraz EDTA i fenantrolina. Silnymi inhibitorami są jony molibdenianowe, fluorkowe, miedzi i cynku.

Rybonukleaza silnie adsorbująca się na DEAE-celulozie (IV szczyt) po oczyszczeniu na Sephadex G-200 dzieli się na dwie formy molekularne — RN-aza$_1$ i RN-aza$_2$. RN-aza$_1$ o aktywności właściwej 807 jedn./mg wykazuje optimum aktywności w pH 4,8—5,1 i jest enzymem termolabilnym.