ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 54, nr 3: 241-253
1985

Two acid RNases from *Dactylis glomerata* seeds. Purification, properties and effect of polyamines and lectins on their activity

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(Received: November 19, 1984 Accepted: March 13, 1985)

Abstract

Two glycoproteidic acid RNases (RNase I and RNase II) were obtained and purified from the seeds of *Dactylis glomerata* by extraction with acetate buffer, fractionation with ammonium sulfate, ion-exchange chromatography on DEAE-cellulose, DEAE--Sphadex, affinity chromatography on Con A-Sepharose and gel filtration on Bio-Gel P60. RNase I with a specific activity of 2582 $U \cdot mg^{-1}$ protein and an optimum pH of 4.9 and RNase II with a specific activity of 1928 $U \cdot mg^{-1}$ protein and optimum pH of 4.6, were isolated. They lacked nuclease, phosphodi- and monoesterase activities. Both forms of the enzyme hydrolyzed pyrimidine homopolymers with a preference for poly U and exhibited a low specificity for purine homopolymers (poly G and poly A). RNase I acted with a 3-fold higher hydrolytic activity on poly C homopolymer than RNase II. The hydrolytic activity of both enzymes was inhibited by Zn^{2+}, Fe^{2+}, Cu^{2+} ions when yeast RNA was the substrate. The amines spermine, spermidine and tyramine at a concentration of 0.1 mM increased the enzymatic activity of both RNases by 20 to 60% of the relative activity. The hydrolytic activity of RNases I and II was stimulated by the presence of lentil lectin (LL), soybean lectin (SBA) and potato lectin (STA), and inhibited by the presence of concanavalin A. The 20-200% stimulation and 40-60% inhibition depended on the proportion, on a weight basis, of enzyme to lectin and were reversible in the presence of receptor sugars.

Key words: ribonucleases, seeds, polyamines, lectins

Abbreviations: LL — soybean lectin; STA — potato lectin, Con A — concanavalin A (lectin from jack beans); p-NPP — p-nitrophenyl phosphate; bis-p-NPP — bis-p-nitrophenyl phosphate

INTRODUCTION

Studies on higher plant nucleases conducted over the recent years have concentrated on understanding their structure and biological function.

A role in the regulation of gene expression is attributed to nucleases isolated from the nuclei and chromatin of seeds and leaves (Yupsanis and Georgatos 1983, Chevrier and Sarhan 1980, 1982, Przykorska and Szarkowski 1980). The function of nucleases during seed dormancy is much less understood (Pietrzak et al. 1980, Yokoyama and Hirano 1982).

We have previously given a preliminary molecular characteristic of ribonucleases from *Poa pratensis* and *Dactylis glomerata* seeds (Lorenc-Kubis and Morawiecka 1974, Wieczorek et al. 1978).

As had other authors, we also showed the presence of several forms of ribonucleases in partially purified extracts from grass seeds. The catalytic activities of ribonucleases as well as acid phosphatases from grass seeds were modified by the presence of lectins (Lorenc-Kubis et al. 1981). Natural polyamines also modify the catalytic activity of ribonucleases by inducing structural changes in the enzyme and the high molecular weight substrate (Karpetsky et al. 1977, Kumagai et al. 1977).

In this study we describe the isolation and catalytic properties of ribonucleases from dormant *Dactylis glomerata* seeds. We have tested the reaction of these enzymes to polyamines and lectins as a model of endogenous regulation of enzyme activity.

MATERIAL AND METHODS

This study was carried out on *Dactylis glomerata* L. var. Nakielska seeds from the 1983 harvest obtained from the Wrocław Plant and Seed Concern in Środa Śląska.

Protein was determined by the method of Lowry et al. (1951) with bovine albumin as the standard. Absorption was measured with a VSU-2P spectrophotometer.

Ribonuclease activity was assayed using the method of Anfinsen et al. (1954) with yeast RNA as the substrate. A unit of RNase activity was taken as the activity which gives a 1.0 increase in absorption at 260 nm under the conditions of the Anfinsen method. The specific activity was expressed as the number of activity units (U) per mg protein.

The substrate specificity was determined using 0.8% solutions of homopolyribonucleotides (poly U, poly G, poly A and poly C) and a 0.2% solution of native and denatured DNA. The DNA was denatured in a boiling-water bath for 10 min, after which it was cooled on ice.

Phosphatase and phosphodiesterase activities were assayed according to Yoshida and Tamiya (1971) using Na p-nitrophenyl phosphate and Na bis-p-nitrophenyl phosphate as substrates.

Electrophoresis on 10% polyacrylamide gels (PAGE) in Tris-glycine buffer, pH 8.3, was carried out according to Davis (1964); in the presence of SDS (PAGE-SDS), according to Weber and Osborn (1969). The gels were stained for protein with 0.1% Coomassie brilliant blue R-250 according to Chrambach et al. (1966), RNase activity was located using the method of Wilson (1969) with yeast RNA as the substrate. Glycoproteins on the gels were stained using the method of Zacharius and Zell (1969).

The isoelectric point was determined by electrofocusing according to Delince and Radola (1975) in the presence of 1% ampholine in a pH range of 3-10. After electrofocusing, RNase was localized using the method of Anfinsen et al. (1954) to determine the activity in eluates made from successive gel sections (1 cm × 1 cm) with 1 cm^3 distilled water.

The effect of lectins (LL, SBA, STA and Con A) on RNase I or II activity was measured after 20 min incubation of 0.14 µg RNase with increasing concentrations of lectins (0.7, 1.4 and 2.8 µg) in an incubation medium of 50 mM acetate buffer, pH 5.6 and temperature of 37°C.

The reversal of RNase activity after interaction with lectins was done by adding 3.5 µg of the appropriate sugar (mannose and glucose for LL and Con A, N-acetylgalactosamine and N-acetylglucosamine for SBA and STA, respectively), and preincubating them for 20 min with the lectins and then performing the enzymatic reaction with RNase I and II, using yeast RNA as the substrate.

PURIFICATION OF ACID RNases FROM *DACTYLIS GLOMERATA* SEEDS

Ground seeds were extracted for 12 h with 50 mM acetate buffer, pH 5.0 (buffer A) at a proportion of 1:10 (w/v). The homogenate was squeezed through gauze, then centrifuged for 20 min at 5°C and 2700 x g. The proteins in the supernatant were salted-out with ammonium sulfate added to 30% saturation, left for 12 h at 5°C, after which the entirety was centrifuged. The supernatant was saturated to 80% with ammonium sulfate, the precipitate dissolved in buffer A with 50 mM NaCl at a ratio of 1:5 and dialyzed against the same buffer. The proteins salted-out in the 30-80% saturation range of ammonium sulfate were chromatographed on DEAE-cellulose equilibrated with buffer A with 50 mM NaCl. The proteins which were not adsorbed were discarded. Those that had adsorbed and exhibited ribonuclease activity were eluted with 200 mM acetate buffer at

pH 5.0 with 2.0 M NaCl. After dialysis and lyophylization, the preparation was filtered on Bio-gel P60 with buffer A as the eluent. The fractions containing RNase activity were pooled, dialyzed against 50 mM Tris-HCl buffer, pH 7.0 and lyophilized. The lyophilizate was dissolved in 50 mM Tris HCl buffer, pH 7.0 with 50 mM NaCl and the proteins chromatographed on a DEAE-Sephadex A50 column, equilibrated with the same buffer. The adsorbed proteins were eluted from the column with a continuous NaCl concentration gradient of 50-500 mM. The fractions forming two peaks containing RNase activity were pooled separately, and dialyzed against buffer A with the addition of 1 mM Mg^{2+}, 1 mM Ca^{2+}, 1 M NaCl. The collected fractions exhibiting RNase activity were dialyzed against 50 mM acetate buffer, pH 5.0 for RNase I and pH 5.4 for RNase II.

REAGENTS

Synthetic homopolymers, poly U, poly C, poly A, poly G and toluidine blue were obtained from Serva; yeast RNA, riboflavin and NNN'N'-tetramethylethyenediamine — BDH; bovine thymus DNA, methyl α-D-mannoside, cacodylic acid — Sigma; Con A-Sepharose 4B, lentil, soybean lectins, Con A, Sephadex G-75 — Pharmacia Fine Chemicals; fuchsin and Coomassie brilliant blue R-250 — Merck; acrylamide, NN'-methylenebisacrylamide — Fluka AG; Na p-nitrophenyl phosphate, Na bis-p-nitrophenyl phosphate — —Kochlight; ampholine pH range 3.5-10 — LKB; uranyl acetate — Lachema; DEAE-Sephadex A50 — Loba; DEAE-cellulose DE 52 — Whatman. Potato lectin was obtained according to the method of Morawiecka and Arabska (1982). The remaining reagents were from POCh Gliwice.

RESULTS

The successive steps in the preparation of RNase I and RNase II from *Dactylis glomerata* seeds are presented in Table 1.

The proteins which were salted-out from the raw extract in the 30-80% saturation range of ammonium sulfate, were filtered on DEAE-cellulose (Fig. 1). At this stage of purification, RNase activity was found both in the proteins which were and were not adsorbed on DEAE-cellulose. The adsorbed proteins contained 60% of the activity applied to the column and exhibited 7 times the activity of the raw extract. This fraction was filtered on Bio-gel P60. At this stage the preparation was freed from phenolic pigments which were eluted with proteins lacking RNase activity (Fig. 2, second peak). The proteins of the first peak (Fig. 2, first peak) contained the entire RNase activity, which in PAGE was found in two bands having this activity. This preparation was chromatographed on DEAE--Sephadex (Fig. 3). Two well separated peaks having RNase activity were obtained. The proteins in each peak were separated by PAGE, and in

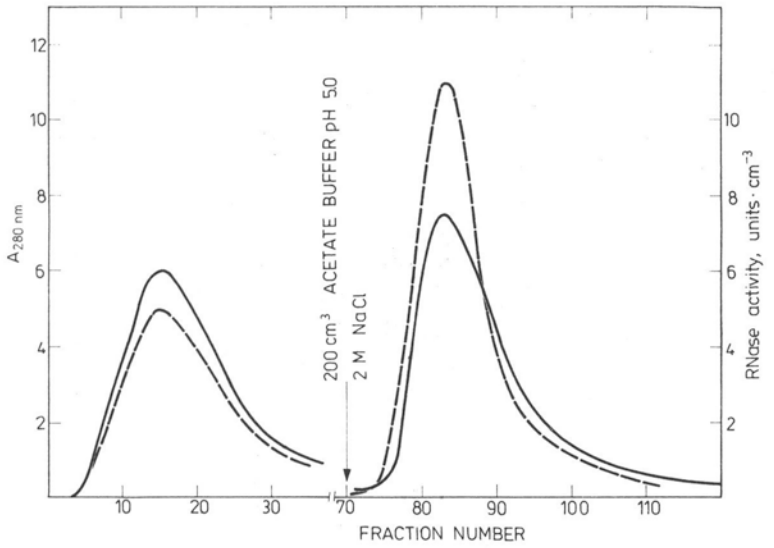

Fig. 1. Chromatography of RNase from *Dactylis glomerata* seeds on DEAE-Cellulose DE52. After washing the column (3 × 35 cm) with 50 mM acetate buffer pH 5.0, elution of proteins was carried out with 200 mM acetate buffer pH 5.0 and 2 M NaCl at a flow rate of 30 $cm^3 \cdot h^-$, 3 cm^3 fractions were collected. Protein $A_{280\ nm}$(——), RNase activity (– – – –)

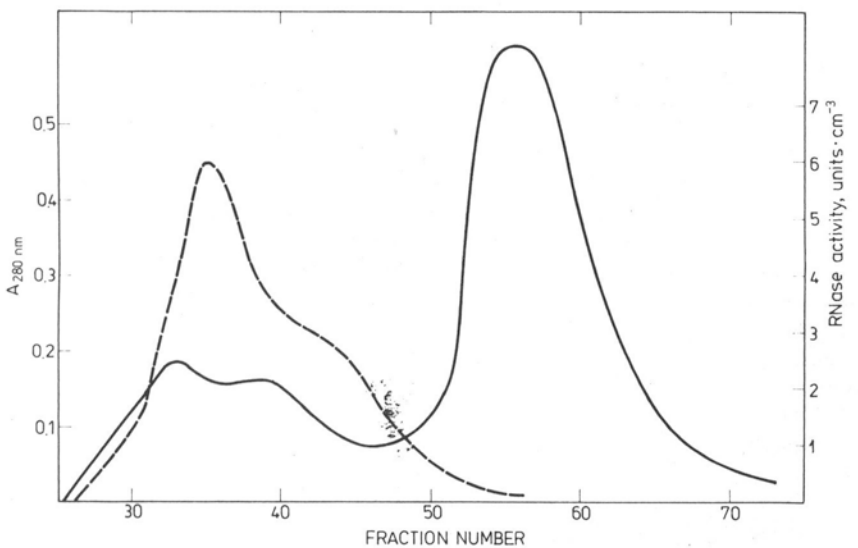

Fig. 2. Gel filtration of RNase from *Dactylis glomerata* seeds on Bio-Gel P60. A column 2 × 135 cm was used and protein eluted with 50 mM acetate buffer pH 5.0, at a flow rate of 12 $cm^3 \cdot h^{-1}$, 2 cm^3 fractions were collected. Protein $A_{280\ nm}$ (——), RNase activity (– – – – – – – –)

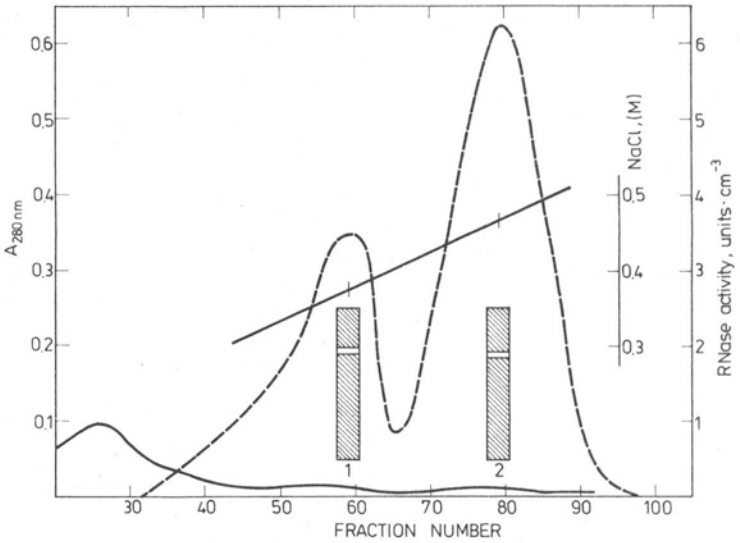

Fig. 3. Chromatography of RNase from *Dactylis glomerata* seeds on DEAE-Sephadex A50. After washing the column (3 × 35 cm) with 50 mM Tris-HCl buffer pH 7.0 and 50 mM NaCl, the proteins were eluted with a linear gradient (50-500 mM) of NaCl in 50 mM Tris-HCl buffer pH 7.0 at a flow rate of 12 $cm^3 \cdot h^{-1}$, 2 cm^3 fractions were collected. Protein $A_{280\,nm}$ (————), RNase activity (– – – – – – – –), 1 and 2 — schemes of zymograms after PAGE of RNase I and RNase II, respectively

Table 1

Purification of RNase I and RNase II from *Dactylis glomerata* seeds

Fraction	Total protein, mg	Specific activity, units[b] per mg protein	Total RNase activity units	Yield, %	Purification factor
Crude extract[a]	11633.0	0.8	2150	100	1
Ammonium sulfate	2304.0	1.4	1355	63	3
DEAE-cellulose DE52					
non-adsorbed		0.3	387	18	
adsorbed	143.0	5.5	796	37	7
Bio-Gel P60	49.0	46.5	516	24	57
DEAE-Sephadex A50					
RNase I	4.0	804.4	129	6	981
RNase II	11.0	688.6	280	13	840
Con A-Sepharose 4B					
RNase I	0.23	2582.0	65	3	3149
RNase II	0.65	1928.0	108	5	2351

[a] Values are given for 500 g of dry seeds.
[b] One unit of RNase activity is defined as 1.0 absorbance unit obtained under standard assay conditions.

each, a single band with RNase activity (Fig. 3) was found in addition to other bands lacking enzymic activity. Affinity chromatography on Con A-Sepharose 4B was employed in order to eliminate these impurities from both preparations (Fig. 4). RNase I and II preparations were completely adsorbed on Con A-Sepharose, from which they were freed with methyl α-D-mannoside. The affinity of RNase I and II to Con A-Sepharose and the positive reaction with alkaline fuchsin used to stain the electrophorograms indicate that both forms of the purified enzymes are glycoproteins. PAGE-SDS gave a similar pattern as that on Fig.b3. The mobilities of RNase I and II bands differed only slightly, which indicates a similar molecular weight of both forms.

SOME OF THE PROPERTIES OF RNase I AND RNase II FROM *DACTYLIS GLOMERATA* SEEDS

The optimum pH for RNase activity, determined in the presence of high molecular weight yeast RNA in acetate buffer, was 5.0 for RNase I and 5.4 for RNase II (Fig. 5).

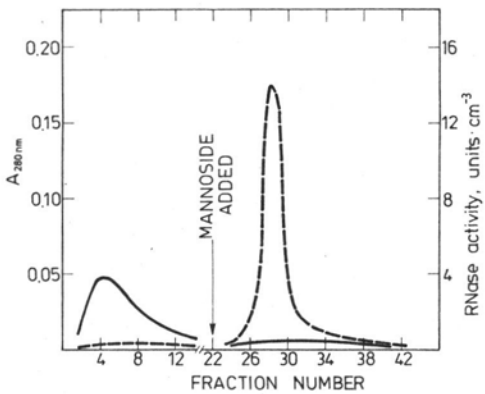

Fig. 4. Affinity chromatography of RNase II from *Dactylis glomerata* seeds on Con A-Sepharose 4B. After washing the column (1.5 × 3 cm) with 50 mM acetate buffer pH 5.0 and 1 mM Mg^{2+}, Ca^{2+} and 1 M NaCl, RNase II was eluted with 5% α-methyl-D-mannoside in the same buffer at a flow rate 12 $cm^3 \cdot h^{-1}$, 3 cm^3 fractions were collected. Protein $A_{280\,nm}$ (————), RNase activity (– – – – – – – –)

When isoelectric focusing was done in ampholine with a pH range of 3.5 to 10, the pI for RNase I was 4.9, for RNase II, 4.6.

The hydrolytic properties of RNase I and II were studied using ribonucleotide homopolymers, DNA, pNPP and bis-pNPP (Table 2). Both RNases preferred poly U from among the homopolymers. RNase I hydrolyzed poly C to the same degree as high molecular weight yeast RNA, RNase II showed a 3 times lower activity towards this substrate. Both forms of this enzyme exhibited poor activity towards purine homopolymers. Native and denatured DNA were not hydrolyzed by the studied RNases. Similarly,

Table 2

Activity of RNase I and RNase II from *Dactylis glomerata* seeds with various substrates

Substrate	RNase I	RNase II
	relative activity*, %	
RNA (yeast)	100	100
Poly U	410	320
Poly C	98	30
Poly G	8	7
Poly A	2	9
DNA native	0	0
DNA denatured	0	0
p-NPP	0	0
Bis p-NPP	0	0

* Enzyme activity was determined under standard conditions (Anfinsen et al. 1954) and expressed as a percentage of the activity towards RNA, which was taken as 100%

Table 3

Effect of inhibitors and activators on RNase I and RNase II from *Dactylis glomerata* seeds

Reagent, mM		RNase I	RNase II
		relative activity, %	
None	0	100	100
K^+	1.0	100	100
$MgCl_2$	1.0	102	104
$MnCl_2$	1.0	98	94
$ZnCl_2$	1.0	62	56
$CuCl_2$	1.0	13	28
$FeCl_2$	1.0	27	37
EDTA	1.0	95	92
Spermine	0.1	115	118
Spermidine	0.1	130	150
Tyramine	0.1	120	160

The enzymes (RNase I — 0.20 μg, RNase II — 0.53 μg) were preincubated at 37°C for 10 min. with the substance to be tested. The activity measured as described in Material and Methods.

no hydrolytic effect on pNPP and bis-pNPP was observed. The purified RNases were typical, acid RNases characterized by their ease in hydrolyzing poly U and did not contain impurities such as nucleases, mono- and diphosphoesterases.

The effect of ions, EDTA and polyamines on RNase I and II activity is presented in Table 3. K^+, Mg^{2+} and EDTA had no effect on

the activity of the studied RNases. Zn^{2+}, Cu^{2+} and Fe^{2+} inhibited the hydrolytic activity of both RNase I and RNase II. Polyamines at a concentration of 0.1 mM stimulated RNase I and RNase II; spermidine and tyramine caused an approximately 50% increase in RNase II activity.

The effect of several lectins on RNase I and II activity is presented on Fig. 6. After 20 min preincubation of the enzyme with a lectin, an increase in the RNase activity dependent on the proportion of enzyme to lectin, on a weight basis, was shown. In the same manner STA, SBA and LL stimulated both RNases (I and II). The presence of LL and SBA almost doubled the relative activity, STA increased it by 62%. Under the same conditions Con A inhibited the RNases. Mannose and glucose in the case of LL and Con A, N-acetylgalactosamine in the case of SBA and N-acetylglucosamine in the case of STA, reversed the stimulatory or inhibitory effect.

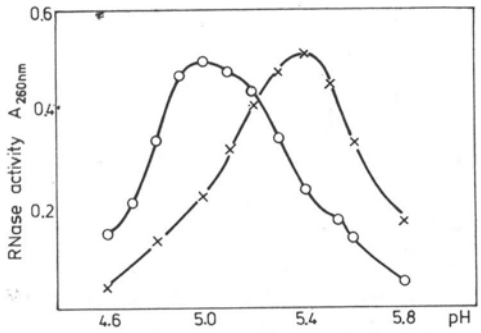

Fig. 5. Effect of pH on the RNase activity. 50 mM acetate buffer was used, RNase I (— o — o —), RNase II (— x — x —)

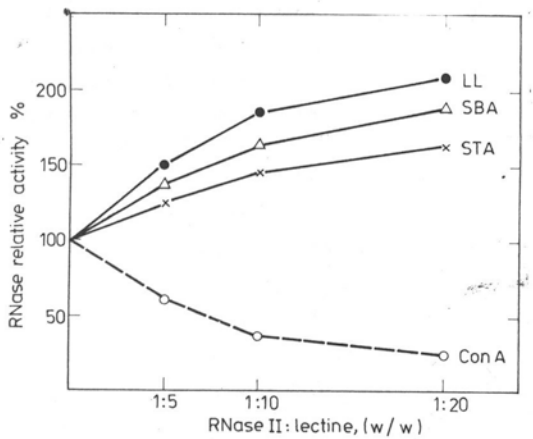

Fig. 6. Effect of lectins (LL, SBA, STA, Con A) on RNase II activity. RNase II (0.14 μg) was preincubated in increasing concentrations of lectins (0.7 μg, 1.4 μg, 2.8 μg) in 50mM acetate buffer pH 5.4 at 25°C for 20 min, then activity was assayed under standard conditions

DISCUSSION

Dormant seeds are a rich source of hydrolytic enzymes, among others, of ribonucleases (Pietrzak et al. 1980, Kuligowska et al. 1980, Torti et al. 1973). In addition to acid phosphatases, grass seeds also contain ribonucleolytic activity in forms having different electrophoretic mobility (Lorenc-Kubis and Morawiecka 1974, Wieczorek et al. 1978). The dominant RNases in seeds are acid RNases, presumably glycoproteins. In this study, two highly purified (3150 and 2351 times) RNases were obtained from *Dactylis glomerata* seeds. These enzymes were free of DNase, phosphomono- and phosphodiesterase activities, and their glycoprotein nature was confirmed.

The pH optima found for RNase I (5.0) and RNase II (5.4) are similar to values found for RNases from pine seeds (Martinez-Honduvilla and Martinez-Honduvilla 1975), rye germ (Kuligowska et al. 1980), as well as rice bran (Yokoyama and Hirano 1982). A trait which differs them from most known plant RNases (Wilnon 1975) is their high specificity for pyrimidine homopolymers with a preference for poly U and low specificity for purine polymers. A similar preference for poly U was shown for two ribonucleases isolated from barley seeds (Pietrzak et al. 1980), and two ribonucleases from wheat leaves (Chevrier and Sarhan 1980) as well as from the nucleoplasm of rye germ (Przykorska and Szarkowski 1980). Ribonucleases from barley seeds also exibited the lowest ability to hydrolyze poly G (Pietrzak et al. 1980) as did cytosol nucleases (Kuligowska et al. 1980) and those from rye germ nucleoplasm (Przykorska and Szarkowski 1980).

The inhibitory effect of Fe^{2+} or Cu^{2+}ions on the activity of acid ribonucleases found in this study was also observed earlier by other authors in rye germ (Kuligowska et al. 1980), wheat leaves (Lantero and Klosterman 1973, Chevrier and Sarhan 1980) and barley grains (Pietrzak et al. 1980). Mg^{2+}, Mn^{2+} and EDTA do not stimulate *Dactylis glomerata* seed RNase I and II. Similarly insensitive to EDTA are RNases from rye germ (Kuligowska et al. 1980), barley leaves (Pietrzak et al. 1980) and wheat germ (Torti et al. 1973).

Polyamines are known to be factors regulating transcription (Karpetsky et al. 1977). This is accomplished by the interaction of polyamines either with RNA or active sites, among others, of ribonucleases. RNase I and RNase II from *Dactylis glomerata* seeds, like RNases from *Citrobacter* (Levy et al. 1973), *Aspergillus saitoi, Aspergillus oryzae* (Igarashi et al. 1975) and *E. coli* (Kumagai et al. 1977), are activated in the presence of spermine, spermidine and tyramine. Putrescine and cadaverine stimulate rye germ acid RNase and spermidine inhibits this enzyme (Kuligowska et al. 1980).

The interaction of lectins with plant acid phosphatases evokes changes in the catalytic properties of these enzymes. Lorenc-Kubis and Bøg-Hansen (1980) have demonstrated an increwse in *Poa pratensis* sèed acid phosphatase activity in the presence of several lectins. LL, SBA, STA stimulate both partially purified RNase (Wiśniowska and Strzyż 1981) as well as highly purified RNases I and II from *Dactylis glomerata* seeds; Con A causes inhibition of these enzymes' activity. The stimulatory and inhibitory effects, at a constant incubation time, depend on the proportion of enzyme to lectin. As does ATPase (Riordan et al. 1977), RNases from *Dactylis glomerata* seeds and glycoproteinic phosphatases from the seeds of other grasses, change their pH optimum in the presence of Con A in the acidic direction (Lorenc-Kubis et al. 1981, Wiśniowska and Strzyż 1981). Con A also causes a slight increase in the thermostability of glycoproteinic RNases from *Dactylis glomerata* seeds (Wiśniowska and Strzyż 1981). The stimulation and inhibition of both RNases is reversed by the sugars specific for a given lectin. It can be deduced from this that the interaction of the studied lectins with RNases I and II takes place through mannose, glucose or N-acetyglucosamine and N-acetylgalactosamine, which are elements of the sugar component of the enzyme. A similar interaction takes place with Con A, whose receptor sugars are mannose and glucose, however, the result of this interaction is the inhibition of the purified RNases I and II. Both Con A and LL interact with RNases through the same sugars, and the effect they cause is expressed by either activation or inhibition of the same enzymes. This can be explained by the stereospecificity of the interaction of the Con A polypeptide chain with the active sites of the RNases. After the reaction, the enzyme's active site is either blocked or uncovered for the substrate. The proven wide distribution of lectins may point to their role in the regulation of various metabolic processes. It seems that the ability of lectins to stimulate or inhibit ribonucleases, among others, hides in itself the mechanism of *in vivo* regulation of this enzymic activity.

Acknowledgment

This study was supported through the MNSzWiT Program R. 1. 9. 02. 04.

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Dwie kwaśne RNazy z nasion Dactylis glomerata. Oczyszczanie, właściwości i wpływ poliamin oraz lektyn na ich aktywność

Streszczenie

Dwie glikoproteidowe RNazy kwaśne (RNaza I, RNaza II) oczyszczono z nasion *Dactylis glomerata* stosując ekstrakcję do buforu octanowego, frakcjonowanie siarczanem amonowym, chromatografię jonowymienną na DEAE-cellulose, DEAE-Sephadex i powinowactwa na Con A-Sepharose oraz sączenie molekularne na Bio-Gel P60. Wydzielono RNazę I o aktywności właściwej 2582 U na mg białka o optimum pH 4,9 i RNazę II o aktywności właściwej 1928 U na mg białka o optimum pH 4,6, pozbawione aktywności nukeazowej, fosfodi- i monoesterazowych. Obie formy enzymu hydrolizują homopolimery pirymidynowe z preferencją wobec poly U i wykazują słabą aktywność wobec homopolimerów purynowych (poly G i poly A). RNaza I wykazuje trzykrotnie wyższą aktywność hydrolityczną niż RNaza II wobec homopolimeru poly C.

Jony Zn^{2+}, Fe^{2+} i Cu^{2+} hamują hydrolityczne działanie obu RNaz wobec RNA drożdżowego jako substratu. Aminy biogenne: spermina, spermidyna i tyramina w stężeniu 0,1 mM wywołują wzrost aktywności enzymatycznej obu RNaz w zakresie 20-60% aktywności względnej. Aktywność hydrolityczna RNazy I i II wzrasta w obecności lektyny soczewicy (LL), soi (SBA) i lektyny ziemniaczanej (STA) a obniża się w obecności konkanawaliny A. Wzrost aktywności od 20 do 200% i hamowanie od 40 do 60% zależą od stosunku wagowego enzymu do lektyny i ulega rewersji w obecności cukrów receptorowych.