Acta Biochimica Polonica

Vol. 44 No. 1/1997

61–68

QUARTERLY

Purification and some properties of a novel dsRNA degrading nuclease bound to rye germ ribosomes*

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Received: 15 December, 1996

Key words: nuclease, double-stranded RNA, germ, ribosome, *Secale cereale* L.

A two-step procedure including affinity chromatography for purification of rye germ ribosomal nuclease that degrades double-stranded RNA from a virus of *Penicillium chrysogenum* and the poly(I)·poly(C) complex was developed. The specific activity towards poly(I)·poly(C) of the obtained nuclease preparations was 30 times as high as that of ribosomes. The recovery of activity was 3.4% when the Octyl-Sepharose column was used, and 2.0% in the case of the Phenyl-Sepharose column. On polyacrylamide/SDS gel electrophoresis the nuclease was resolved into two proteins of molecular mass 62 kDa and 57 kDa, respectively. 2-Mercaptoethanol and Mn^{2+} stimulated the activity of the purified enzyme. Glycerol (20%–50% concentration) stabilized enzyme. In addition to activity towards dsRNA and ssRNA the enzyme cleaves native and denatured DNA. It is suggested that this type of a nuclease takes part in regulation of the mRNA level in cytoplasm.

Ribonucleolytic activity may be associated with plant ribosomes, but the biological significance of this activity *in vivo* has not so far been elucidated [1]. Recently it was found that *Drosophila* ribosomal protein S3 contained DNase activity [2]. We have demonstrated that both ribonucleolytic and deoxyribonucleolytic activity is associated with rye germ ribosomes [3, 4]. These two activities are strongly bound to ribosomes and their subunits, but a part of the activity can be released from ribosomes by washing them with 0.5 M ammonium chloride [4]. It was further noted that this wash is capable of degrading double-stranded RNA from a virus of *Penicillium chrysogenum* and the double-stranded poly(A)•poly(U) complex [5], thus it appears to contain a double-stranded RNA hydrolyzing enzyme. Other nucleolytic enzymes isolated from rye germ did not exhibit such activity. The two ribonucleases isolated previously from rye germ cytosol hydrolyzed only single-stranded RNA [6, 7]. Nuclease type I, isolated from ammonium chloride wash of rye germ ribosomes and purified to a homogeneity, showed no activity towards double-stranded RNA [8].

In view of the previous observations, attempts were made to isolate the enzymes degrading double-stranded RNA from the

*This research was supported by the State Committee for Scientific Research (KBN) grant No. 6 P203 021 06.

Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; ssDNA, single-stranded DNA; poly(I)•poly(C), poly(A)•poly(U) etc., hydrogen-bounded chains of two homopolymers; poly(I), polyinosinic acid; poly(C), polycytidylic acid, poly(A), polyadenylic acid; poly(G), polyguanylic acid.

wash of cytoplasmic ribosomes of rye germs [9] and optimal ionic strength and pH conditions for estimation of their activity were established [10].

Isolation and partial purification of a nuclease from tobacco anthers, that degrades double-stranded RNA was also recently reported [11].

The nucleolytic enzymes of higher plants have classically been grouped into four main enzyme types: RNase I, RNase II, Nuclease I, and Exonuclease I. Nuclease type I enzymes are endonucleases that degrade both single-stranded DNA and RNA, but not double-stranded RNA (for review see: Wilson [12]). Nucleases from rye germ ribosomes and from tobacco anthers which can not be classified into any of the four classical types, seem to form a new type of higher plant nucleases.

This paper presents a two-step procedures for isolation and purification of a novel nuclease capable of degrading double-stranded RNA and describes some properties of this enzyme. The enzyme has been called: rye germ ribosomal nuclease II, to distinguish it from nuclease I, the enzyme purified previously from rye germ ribosomal washes [8].

MATERIALS AND METHODS

Plants. Commercial rye (*Secale cereale* L.) germs were used as the source of ribosomes. Germs were cleaned by sieving from starch contamination. Meshes of 1.2 and 0.6 mm were used. The germs remaining on the 0.6 mm sieve were taken for experiments.

Isolation of cytoplasmic ribosomes. The germs (300 g) were grinded in a mortar and then homogenized in a mixer cooled with ice in 10 mM Tris/HCl buffer, pH 8.0, containing 0.25 M sucrose, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. Protein inhibitors were added to the buffer: aprotinin (0.2 mg/ml), leupeptin (0.2 mg/ml), and benzamidine up to the concentration of 0.1 mM. The homogenate was filtered through two layers of nylon cloth and then centrifuged at 12000 ***g*** for 15 min. Triton X-100 or Brij 35 was added to the supernatant up to the final concentration of 0.5%, and the supernatant was centrifuged at 40000 ***g*** for 30 min. The sediment was discarded, and the supernatant was recentrifuged at 160000 ***g*** for 2 h. The resulting crude ribosomal pellet was resuspended in 10 mM Tris/HCl buffer, pH 8.0, containing 10 mM KCl and 10 mM $MgCl_2$. The ribosome suspensions (2 ml) were then applied on top of two-layer discontinuous gradients of sucrose which contained 1 ml of 1.5 M sucrose and 1 ml of 1 M sucrose. The sucrose was suspended in the same buffer as were ribosomes. The samples were centrifuged for 4 h at 140000 ***g*** and the pellet was collected. All operations were carried out at 4°C.

Release of proteins with nucleolytic activity from ribosomes. The ribosomal pellet was washed twice with 30 mM Tris/HCl, pH 8.0, containing 0.6 M KCl, 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 10% glycerol. The suspension was left to standing for 18 h with constant stirring and after that centrifuged at 160000 ***g*** for 2 h at 4°C. The supernatants were used as the material for further purification of the enzyme (wash 1 and 2).

Enzyme purification. All of the purification steps were carried out in a 4°C cold room. The washes were applied to a Sephadex G-100 (60 cm × 1.5 cm) column and eluted with 10 mM Tris/HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 10% glycerol. Fractions showing the activity towards poly(I)•poly(C) were collected and concentrated in dialysing sacks in Ficoll 400 or by centrifugation with Centricon 10. For further purification an Octyl-Sepharose column (3.0 cm × 2.6 cm) or a Phenyl-Sepharose column (3.5 cm × 2.3 cm) was used. Prior to its application to the column the sample was dialysed against 10 mM sodium phosphate buffer, pH 6.8, containing 10 mM 2-mercaptoethanol, and then $(NH_4)_2SO_4$ to saturation of 25% was added. Proteins were eluted from the column with 10 mM phosphate buffer, pH 6.8, containing $(NH_4)_2SO_4$ to 25% saturation and 10 mM 2-mercaptoethanol. The fractions with activity towards poly(I)•poly(C) were pooled and $(NH_4)_2SO_4$ was dialyzed off. The fractions were concentrated and glycerol was added to a concentration of 20%–50%, and they were stored at –70° C until use.

Determination of nucleolytic activity. Nucleolytic activity towards poly(I)•poly(C), RNA or DNA was determined by the modified

Anfinsen's method [13]. The reaction mixture contained 0.1 ml of substrate [poly(I)•poly(C), RNA or DNA 0.5 mg/ml], from 0.01–0.10 ml of the enzyme, and appropriate buffer (total volume 0.5 ml). For estimation of the activity towards poly(I)•poly(C) 10 mM Tris/-HCl buffer, pH 8.5, was used. For estimation of the activity towards RNA 10 mM Tris/HCl buffer, pH 7.8, and towards DNA 50 mM citric acid-sodium phosphate buffer, pH 5.0 were used, respectively.

The samples were incubated at 37°C for 15 min. The reaction was stopped by addition of 20 mM lanthanum acetate (1 ml) in 12% perchloric acid, the sample cooled at 0°C, and precipitate was centrifuged off at 6000 *g* for 15 min. Absorbance of the supernatant was measured spectrophotometrically at 260 nm. One unit (1 u) of nucleolytic activity was defined as the amount of enzyme causing under experimental conditions an increase $\Delta A_{1cm,260} = 0.1$. Specific activity was expressed as units per 1 mg of protein.

Protein determination. Protein content was determined by the method of Bradford [14] or by the modified Lowry's procedure with Bio-Rad assay [15]. Bovine serum albumin was used as a standard.

SDS/polyacrylamide gel electrophoresis. Denaturing polyacrylamide gel electrophoresis of protein was performed as described by Laemmli [16]. Gels were stained with 0.025% Coomassie Brilliant Blue or Silver Kit (BioRad).

Molecular mass determination. This was determined electrophoretically on 10% polyacrylamide gel containing 0.1% SDS. Electrophoresis Calibration Kit (Pharmacia Fine Chemicals, low range) was used for molecular mass standards.

RESULTS

Purification of nuclease

The nuclease studied was released from ribosomes by washing them twice with 0.6 M KCl. Results of the two-step purification procedures are shown in Tables 1 and 2.

The elution profiles obtained from the Octyl-Sepharose column and Phenyl-Sepharose column are shown in Fig. 1A and B. The enzyme activity was eluted with 25% saturated solution of ammonium sulphate in 10 mM sodium phosphate buffer, pH 6.8, as a single peak. The adsorbed proteins, eluted with a gradient of decreasing ammonium sulphate concentration showed no activity towards poly(I)•poly(C).

The enzyme obtained from the Octyl-Sepharose column had about 34-fold higher specific activity towards poly(I)•poly(C) than the corresponding activity of ribosomes, and

Table 1. Purification of rye germ ribosomal nuclease II by Sephadex G-100 and Octyl-Sepharose column chromatography

The procedures used are described in Materials and Methods.

Purification step	Total protein	Total activity towards poly(I)• poly(C)	Specific activity towards poly(I)• poly(C)	Purification factor	Yield
	mg	u	u/mg protein		%
Ribosomes	405.0	11218.5	27.7	1.0	100.0
KCl wash 1	60.5	3309.4	54.7	2.0	29.5
KCl wash 2	14.0	658.0	47.0	1.7	5.9
Sephadex G-100	17.0	2981.8*	175.4	6.3	26.5
Octyl-Sepharose	0.4	378.0	945	34.1	3.4

*KCl wash 1 + 2

the yield of this procedure was 3.4% (Table 1).

After Phenyl-Sepharose column chromatography (Table 2) about 30-fold increase in the enzyme specific activity towards poly(I)•poly(C) was achieved. This was similar as in the previous procedure, but the yield of the latter procedure was a little lower (2%).

Data in Tables 1 and 2 present average values obtained from thirty enzyme purifications experiments.

The two preparations of purified nuclease were compared on 10% polyacrylamide gel electrophoresis in the presence of SDS. Either preparation showed two bands (Fig. 2).

The apparent molecular mass of two enzyme bands, 62 kDa and 57 kDa, respectively, was determined by co-electrophoresis with a number of protein markers.

Table 2. Purification of rye germ ribosomal nuclease II by Sephadex G-100 and Phenyl-Sepharose column chromatography.

The procedures used are described in Materials and Methods.

Purification step	Total protein	Total activity towards poly(I)•poly(C)	Specific activity towards poly(I)•poly(C)	Purification factor	Yield
	mg	u	u/mg protein		%
Ribosomes	440.0	12320.0	28.0	1.0	100.0
KCl wash 1 and 2	56.0	3679.2	65.7	2.3	29.9
Sephadex G-100	21.0	2730.0	130.0	4.6	22.9
Phenyl-Sepharose	0.3	248.7	829.0	29.6	2.0

Properties of the enzyme preparation

Activators and inhibitors

The activity towards poly(I)•poly(C) of washes 1 and 2 or the enzyme preparations obtained after Sephadex G-100 filtration was

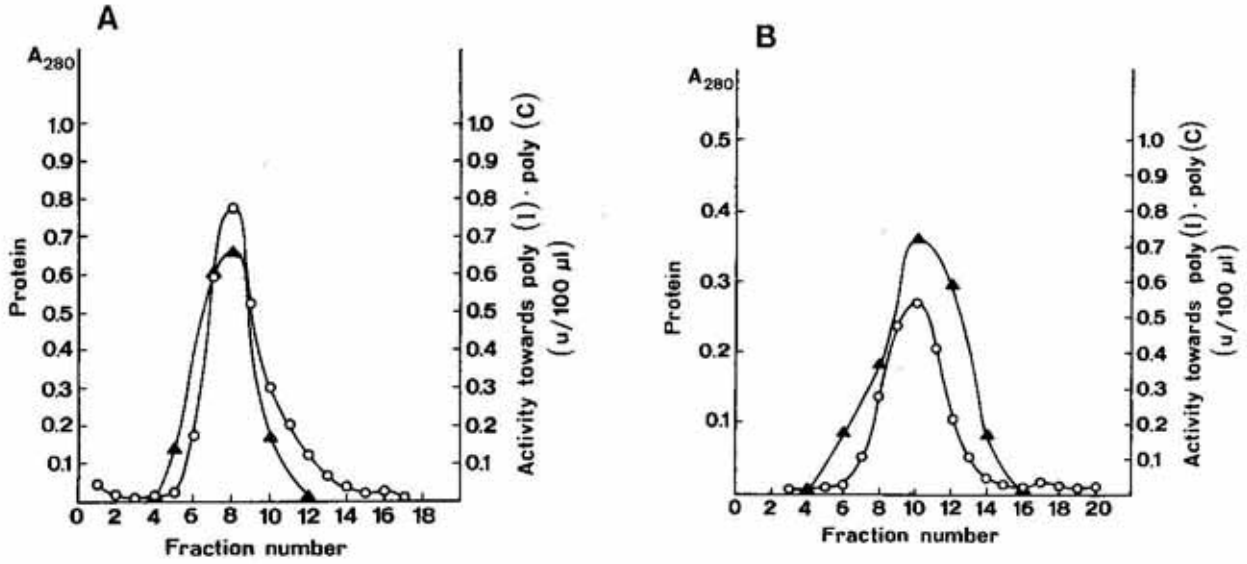

Figure 1. Purification of rye ribosomal nuclease II on Octyl-Sepharose column (A) and Phenyl-Sepharose column (B).

For elution 10 mM sodium phosphate buffer, pH 6.8, containing 10 mM 2-mercaptoethanol and $(NH_4)_2 SO_4$ to 25% saturation was used. ○ , Protein concentration. ▲, Activity towards poly(I)•poly(C).

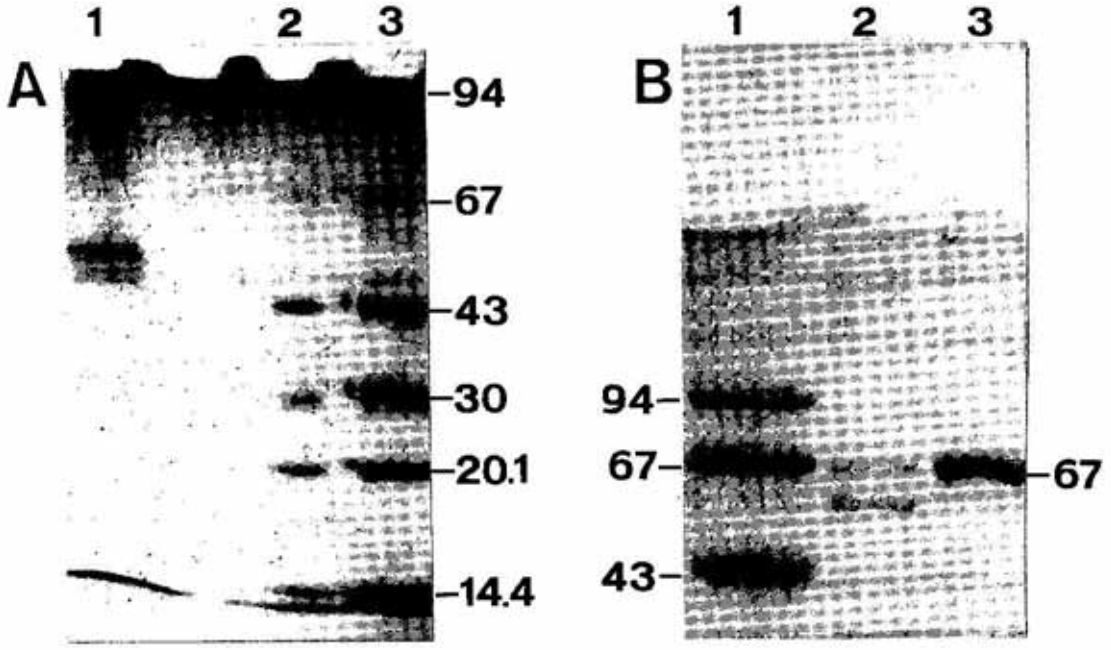

Figure 2. Polyacrylamide gel electrophoresis.

(A) 10% Polyacrylamide gel stained with Coomassie Brilliant Blue. Lane 1, proteins from Phenyl-Sepharose column. Lane 2 and 3, protein standards: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactoalbumin (14.4 kDa). (B) Silver stained 10% polyacrylamide gel. Lane 1, protein standards: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa). Lane 2, proteins from Octyl-Sepharose column. Lane 3, bovine serum albumin (67 kDa).

not affected by the addition of Mg^{2+} (0.5 mM) or Na^+ or K^+ (50 mM) ions. Mn^{2+} ions (5 mM) and 2-mercaptoethanol (10 mM) stimulated the activity at all purification steps (to 125% and 175% of initial activity, respectively).

pH Optimum

As it was found earlier for ribosomal washes [10] the activity towards poly(I)•poly(C) of the enzyme preparations obtained after Sephadex G-100 filtration and Phenyl- or Octyl-Sepharose column chromatography showed a maximum activity in 10 mM Tris/HCl buffer at pH 8.5. The pH optimum for the activity towards RNA is observed in 100 mM Tris/HCl buffer, at pH 7.8, and towards denatured DNA in 50 mM citric acid-sodium phosphate buffer at pH 5.0.

Stability

The preparations of rye germ ribosomal nuclease obtained by either procedures were unstable. Most stable were the ribosomal washes (Table 3). After storage for two

Table 3. Stability of rye germ ribosomal nuclease II preparations after storage in different conditions

Step of purification	Storage conditions	Time of storage	Initial activity	Activity after storage	Loss of activity
		Days	u/mg protein		%
Octyl-Sepharose	solution, 4°C	7	538	0	100
Octyl-Sepharose	frozen and stored at –70°C	30	800	83	90
Octyl-Sepharose	with 50% glycerol and stored at –20°C	30	1200	1040	13
Phenyl-Sepharose	frozen with 20% glycerol and stored at –70°C	30	780	520	33

months at –20°C a 22% loss of their activity towards poly(I)•poly(C) was observed. The enzyme preparations from Sephadex G-100 kept in the same conditions lost half of their activity. Purified enzyme kept at refrigerator temperature (8°C) for a week lost the activity completely.

Table 4. Degradation of nucleic acid substrates by rye germ ribosomal nuclease II.

Experimental conditions as described in Materials and Methods.

Substrate	Specific activity u/mg protein	Relative rate of hydrolysis %
Double-stranded RNA from virus of *Penicillium chrysogenum*	1116	100
Single-stranded RNA from wheat germ	891	80
Native DNA from calf thymus	785	70
Denatured DNA from calf thymus	640	57

Addition of glycerol at a concentration of 20%–50% stabilized the purified enzyme. A similar effect was observed with 30% trehalose. Purified enzyme was stored at –70°C in a small samples (0.5–0.01 ml), with glycerol or trehalose. After one thawing and freezing cycle it retained almost 90% of the initial activity.

Substrate specificity

The relative activity of rye germ ribosomal nuclease II towards double- and single-stranded RNA and double- and single-stranded DNA is shown in Table 4. The highest activity was observed towards dsRNA from a virus of *Penicillium chrysogenum*, but the enzyme degraded also: highly polymerized RNA from wheat germ, and native and denatured DNA from calf thymus. However, it should have be pointed out that the double-stranded RNA and DNA were preferentially degraded.

Nuclease II converted completely the closed, double-stranded, supercoiled form of phage ΦX174 DNA into the open (circular) and then linear form (unpublished). This indicates that the enzyme acts endonucleolytically on double-stranded DNA.

The rye germ ribosomal nuclease II showed different rates of activity towards various polynucleotides (Table 5). The highest activity was observed towards double-stranded complexes: poly(I)•poly(C) and poly(A)•poly(U), and then towards homopolynucleotides; poly(U) and poly(A). The lowest activity is observed towards poly(G) and double stranded deoxynucleotide poly d(I)•poly d(C).

DISCUSSION

A novel nuclease bound to ribosomes and degrading double-stranded RNA was isolated from rye germs. Two methods were developed for purification of the enzyme; the first, described previously [9], applies ion-ex-

Table 5. Degradation of homopolynucleotides and double-stranded complexes by rye germ ribosomal nuclease II.

Experimental conditions as described in Materials and Methods.

Substrate	Specific activity u/mg protein	Relative rate of hydrolysis %
Poly(I)•poly(C)	1045	100
Poly(A)• poly(U)	805	77
Poly(U)	794	76
Poly(A)	783	75
Poly(C)	616	59
Poly(I)	606	58
Poly(G)	125	12
Poly(dI)•poly(dC	75	7

change chromatography on CM-cellulose and chromatofocusing on PBE 94 and PBE 118 gels, the second, presented in this paper, applies affinity chromatography with Octyl- or Phenyl-Sepharose. The two methods gave similar results: the obtained nuclease preparations showed specific activity towards poly(I)•poly(C) about 30 times as great as that of rye ribosomes. The previous, 5-step method gave a very unstable product [9]. The newly introduced addition of glycerol stabilizes the enzyme activity towards poly(I)•poly(C). Additionally, the recovery of protein was 5 times as high as in the case of the previous method. The final products obtained by the two methods (data from the first method not shown) gave on SDS-electrophoresis two bands with molecular mass of 62 kDa and 57 kDa. Proteins after elution from native or SDS gels were active towards the poly(I)•poly(C) substrate (not shown).

It should be noted that gel electrophoretic analysis of tobacco anthers revealed four sugar-unspecific nucleases able to digest dsRNA [11]. Bovine seminal ribonuclease which degrades both single- and double-stranded RNAs was isolated as a dimer but can form homo and hetero dimers, and this is important for dsRNA cleavage [17]. The relation between the level of the activity (towards ssRNA and dsRNA) of rye germ ribosomal nuclease II and the structure of this enzyme needs further studies.

In addition to its activity towards dsRNA and ssRNA, the enzyme cleaves native and denatured DNA. These results indicate that it is sugar unspecific nuclease [12]. However, the enzyme differs from the other plant nucleases, classified by Wilson as nucleases of type I [12] by its preference for dsRNA. Thus it appears that rye germ ribosomal nuclease II and nucleases from tobacco anther [11] are examples of a new type of plant nucleases.

Like other plant nucleases the studied enzyme is most active towards poly(U) and poly(A) [10]. Nuclease I, previously isolated from ammonium chloride wash of rye germ ribosomes [7] was most active towards poly(C) and differed from nuclease II.

More detailed studies with 5S rRNA of known sequence confirmed the suggestion concerning base specificity (A, U) of the nuclease II studied (unpublished). It seemed of interest to use this enzyme for studies on the structure of double-stranded RNA fragments and these studies are presently under way. Preliminary results indicate that rye germ ribosomal nuclease II is specific for double-stranded regions in several tRNA and 5S rRNA as well.

The metabolic role of plant nucleases associated with ribosomes has not so far been elucidated [1, 7, 8, 10]. It can only be suspected that this enzyme has regulatory functions in decay of mRNA or in a control of the level of antisense RNA-mRNA transcripts [9]. It has been suggested that ribosomes carry a nuclease capable of degrading mRNA, and that ribosome progression causes changes in mRNA conformation that unmask the nuclease target sites [18]. In fact, in preliminary experiments it was found that rye germ ribosomal nuclease II degraded repeated sequences of certain mRNAs.

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