Biochimica et Biophysica Acta, 614 (1980) 102—112

BBA 69005

PURIFICATION AND PROPERTIES OF TWO RIBONUCLEASES AND A NUCLEASE FROM BARLEY SEEDS

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(Received July 17th, 1979)
(Revised manuscript received December 7th, 1979)

Key words: Plant RNAase; Nuclease; Poly(G)-Sepharose; (Barley)

Summary

Three enzymes possessing RNAase activity were isolated from barley seeds. These enzymes were further purified by ammonium sulphate precipitation, DEAE-cellulose chromatography, gel filtration on Sephadex G-75 and DEAE-Sephadex A-50 chromatography.

These enzymes have been characterized and classified as:

1. Plant RNAase I (EC 3.1.27.1). It has a pH optimum at 5.7 and molecular weight of 19 000.
2. Plant RNAase II (EC 3.1.27.1). It has a pH optimum at 6.35 and molecular weight of 19 000.
3. Plant nuclease I (EC 3.1.30.2). It has a pH optimum at 6.8 and molecular weight of 37 000.

Two RNAases were purified to homogeneity by means of affinity chromatography on poly(G)-Sepharose 4B, as shown by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Introduction

Plant RNAases from many species and varieties of plant tissues have been studied for many years (for reviews see Refs. 1—5). These investigations led to the classification of plant ribonucleases proposed by Reddi [1] and later extended by Wilson [2]. According to this classification, plant nucleases fall into three main groups: (a) plant RNAase I (ribonucleate nucleotide-2-transferase (cyclizing) EC 3.1.27.1) (b) plant RNAase II (also EC 3.1.27.1) and (c)

Abbreviation: Mes, morpholinoethanesulfonic acid.

plant nuclease (nucleate 5′-oligonucleotido-hydrolase, EC 3.1.30.2).

A number of purified or semipurified RNAase preparations have been obtained from tissues of various flowering plants. Lantero and Klosterman [6] purified RNAase I from barley leaves and Wilson [2,7,8] isolated all three nucleases from maize. Semipurified RNAase preparations have been obtained from wheat germ [9], oat leaves [10,4], pea cotyledons [11] and tobacco leaves [12]. Few investigations have been made of purified RNAase from dry seeds. The only purified RNAase preparation obtained from dry seeds was RNAase I from corn endosperm [6]. Although the different RNAases may have different functions in vivo, most studies on RNAases in this material have depended upon assays that measure total RNAase activity rather than the activities of the individual RNAase.

It is well known that ribonucleases located in seeds are responsible for hydrolysis of RNA in the reserve material of endosperm [13—15]. It is not clear, however, what types of individual nuclease take part in breaking down the reserve material. It was thought that characterizing the enzymes existing in dry seed might shed some light on the function of these enzymes in vivo. Here we present evidence for the existence of two ribonucleases and one nuclease in dry barley seeds. The methods for obtaining pure RNAase preparations and some of their properties are also presented.

Materials and Methods

Materials. Barley seeds (*Hordeum vulgare* var. Damazy) were obtained from Krakowska Hodowla Roslin. Sephadex G-75, DEAE-Sephadex A-50, Blue Dextran 2000 and CNBr-activated Sepharose 4B were from Pharmacia Fine Chemicals. DEAE-cellulose (DE 22) was from Whatman. Acrylamide, methylene bisacrylamide and sodium dodecyl sulfate (SDS) were from Koch-Light. Membrane UM-10 was from Amicon. Coomassie brillant blue R-250 and G-250, Amido Black, ammonium sulphate, Mes, molecular weight standard proteins and dinitrophenyl-L-alanine were from Serva. Purified agar was from Difco. Aquacide 1 was from Calbiochem. Tris was from Fluka AG. DNA (calf thymus) was from Sigma. Polyribonucleotides were from Miles. Yeast RNA and all other chemicals were from POCh Gliwice.

RNAase assay. RNAase activity was measured by the method of Tuve and Anfinsen [16] using commercial yeast RNA. 1 unit of RNAase activity is defined as the activity that releases 1 A_{260} unit of acid-soluble nucleotide/min at 37 °C [6,32].

Extraction and purification procedure. The ground meal of 2 kg barley seeds (*Hordeum vulgare* L. var. Damazy) was extracted overnight with 6 l 5 mM Tris-HCl (pH 7.2)/0.01% sodium azide. The extract was centrifuged at 2000 rev./min (MSE Mistral 6L) for 30 min to give fraction 1. The supernatant (fraction 1) was adjusted to 30% saturation with solid $(NH_4)_2SO_4$ and allowed to stand at 4 °C overnight. The precipitated material was discarded and supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in 500 ml buffer E (50 mM Tris-HCl, pH 7.2)/0.05 M NaCl and dialysed against two changes of the same buffer (fraction 2). Fraction 2 was applied to a DEAE-cellulose column equilibrated

with 0.05 M NaCl in buffer E. The RNAase activity was eluted with 0.3 M NaCl (fraction 4). Fraction 4 was concentrated by filtration on a UM-10 membrane. It was then applied to a Sephadex G-75 column and eluted with 0.1 M NaCl in buffer E. Fractions containing the two peaks of RNAase activity were pooled to give fraction 5 and fraction 6. Fraction 6 was applied to a DEAE-Sephadex A-50 column and eluted with a 0.1—0.5 M NaCl gradient. The two active fractions obtained (7 and 8) were each dialysed against 10 mM sodium acetate buffer (pH 5.5) and concentrated with Aquacide 1. These fractions were further purified by affinity chromatography on a poly(G)-Sepharose 4B column (1.5 × 14 cm). RNAase was applied on a column washed with acetate buffer and then with one column volume of acetate buffer +0.05 M NaCl. The RNAase activity was eluted from a column with 1 M NaCl in acetate buffer. Fraction 7 gave fraction 9 and fraction 8 gave fraction 10.

Double immunodiffusion technique. The immunodiffusion technique of Ouchterlony [17] was carried out in Petri dishes containing 1% agar in 50 mM Tris-HCl (pH 7.2)/0.15 M NaCl/0.02% sodium azide.

Preparation of poly(G)-Sepharose 4B. CNBr-activated Sepharose 4B was processed according to the makers instructions. 5 g of CNBr-activated Sepharose 4B were reswollen and washed with 1 l of 1 mM HCl. Poly(G) (250 μM P) was dissolved in 10 ml of water and 20 ml of 0.1 M $NaHCO_3$/0.5 M NaCl were added. Gel and poly(G) solution were mixed together and shaken for 3 h at room temperature. The gel was finally washed with 500 ml of bicarbonate buffer.

Polyacrylamide gel electrophoresis. Disc electrophoresis was performed in 10% polyacrylamide gel using the method of Davis [18]. Gels were stained for protein with 0.25% Coomassie brillant blue G-250 in 12.5% trichloroacetic acid [19] and for RNAase activity by the method of Wilson [20], Mes-KOH buffer pH 5.5, was used for RNAase I and Mes-KOH buffer pH 6.45, for RNAase II. Electrophoresis in the presence of SDS was carried out using the method of Laemmli [21].

Protein determination. Protein was estimated by the method of Lowry et al. [22].

Test of endo- or exonuclease activity. Aliquots of purified yeast RNA hydrolysed to different extents were analysed by Sephadex G-75 gel filtration. After hydrolysis in a standard incubation mixture at 37 °C, a 0.2 ml sample was heated in boiling water to inactivate the enzyme, applied to a column (1.6 × 30 cm) and eluted with 50 mM Tris-HCl (pH 7.2)/0.1 M NaCl. Absorption of the fractions was measured at 260 nm.

Identification of hydrolysis products of poly(A) and poly(U). Poly(A) or poly(U) was incubated with RNAase I or RNAase II in 0.1 M Mes-KOH buffer (pH 5.7 and 6.35, respectively). After hydrolysis the enzyme was heat-inactivated at 100 °C. The hydrolysis products were analysed by high-pressure liquid chromatography (HPLC) on pellicular anion exchange resin at 60 °C with a phosphate buffer gradient from 0.01 M—1 M in a Varian apparatus.

Molecular weight estimation. The approx. molecular weight was determined by gel filtration with a Sephadex G-75 column (1.5 × 87 cm). The column was equilibrated and the sample was eluted with 50 mM Tris-HCl (pH 7.2)/0.1 M NaCl. Cytochrome *c* (12 400 daltons) chymotrypsinogen A (25 000 daltons),

ovalbumin (45 000 daltons), Blue Dextran 2000 and DNP-L-alanine were used for column calibration. The protein standards were determined by the absorbance at 230 nm, while the enzymes were located by enzymatic activity.

Results

Enzyme purification

The purification procedure is outlined in Table I. Approx. 90% of the RNAase activity was precipitated from the crude extract with $(NH_4)_2SO_4$ between 50% and 80% saturation. A sample of the $(NH_4)_2SO_4$ fraction (fraction 2) was subjected to analytical gel filtration on a Sephadex G-75 column. Three peaks of RNAase activity were observed (Fig. 1) in the elution profile. The ribonuclease activity contained in the first peak was not adsorbed on DEAE-cellulose during the preparative purification (fraction 3) and was not studied further, while the activity in the second and third peaks did adsorb on DEAE-cellulose. During the preparative enzyme purification following $(NH_4)_2SO_4$ precipitation, stepwise elution from DEAE-cellulose was chosen rather than gel filtration. In the next step, preparative gel filtration on Sephadex G-75 was used.

Two peaks of activity were observed. A first minor peak of activity contained the enzyme active with RNA and DNA as a substrate and was considered to be nuclease I (fraction 5). A second, larger peak of activity (fraction 6) was further purified by DEAE-Sephadex A-50 chromatography (Fig. 2). Two peaks of RNAase activity were obtained, the first eluting with 0.24 M NaCl (fraction 7) and the second eluting with 0.32 M NaCl (fraction 8). Enzymes contained in these peaks were identified as RNAase I and RNAase II, respectively. Both enzymes were purified to homogeneity using poly(G)-Sepharose 4B affinity chromatography. The final enzyme preparations (frac-

TABLE I

PURIFICATION OF BARLEY SEED NUCLEASES

Procedure	Fraction No.	Vol. (ml)	Total RNAase (units)	Protein (mg/ml)	Units/mg protein
Crude extract	1	4240	13 700	6.0	0.54
Ammonium sulphate	2	600	7 400	11.5	1.1
DEAE-cellulose					
non adsorbed	3		2 600		
adsorbed	4	1200	5 000	1.5	2.8
Sephadex G-75					
1st peak	5	40	430	1.0	10.7
2nd peak	6	230	3 220	0.5	28
DEAE-Sephadex A-50					
1st peak	7	65	702	0.03	350
2nd peak	8	125	1 160	0.02	460
poly(G)-Sepharose 4B					
RNAase I	9	14.5	386	0.004	6430
RNAase II	10	14.5	764	0.007	7640

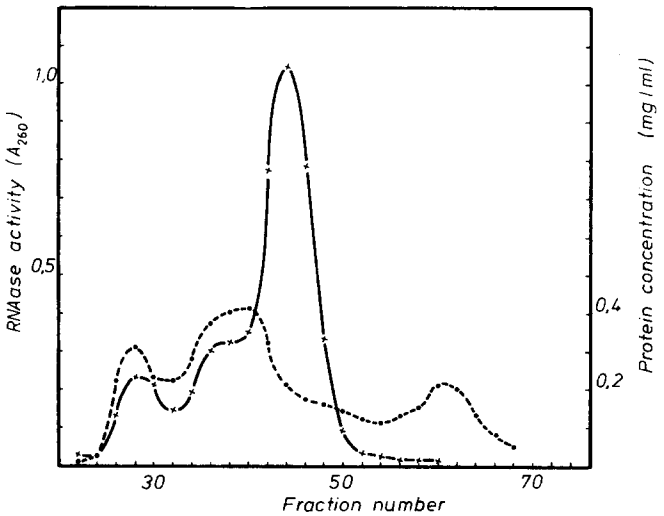


Fig. 1. Analytical gel filtration of nucleases from dry barley seed on Sephadex G-75 column (1.5 × 90 cm). Protein precipitated from crude extract within the range of 50—80% of $(NH_4)_2SO_4$ saturation was applied on the column and eluted with 50 mM Tris-HCl (pH 7.2)/0.1 M NaCl. 2 ml fractions were collected and assayed for protein concentration and RNAase activity (50 µl aliquots). (○- - - - -○), protein concentration; (×———×), RNAase activity.

tions 9 and 10) had specific activities of 6430 and 7640 units/mg of protein, respectively, when assayed under standard conditions.

Polyacrylamide gel electrophoresis

Each enzyme preparation after DEAE-Sephadex A-50 chromatography

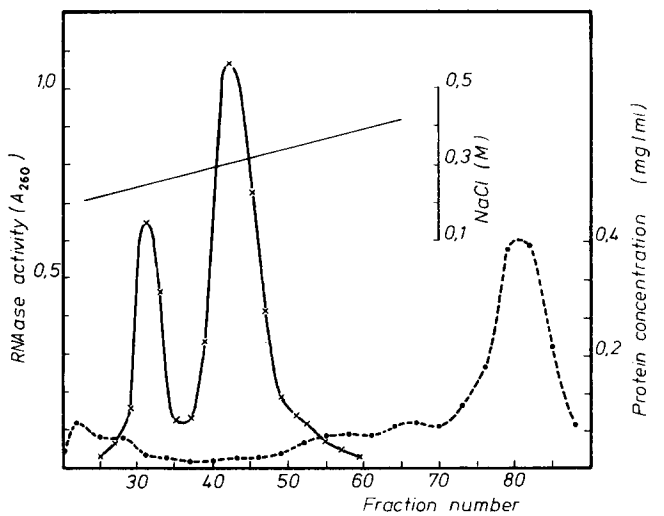


Fig. 2. Chromatography of barley seed RNAase on DEAE-Sephadex A-50 column (2.5 × 30 cm). Protein was adsorbed on a column and washed with two column volumes of 50 mM Tris-HCl (pH 7.2)/0.1 M NaCl. Elution was carried out with a linear gradient of 1 l 0.1—0.5 M NaCl in Tris-HCl buffer. 10 ml fractions were collected and assayed for protein concentration and RNAase activity (10 µl). (○- - - - -○), protein concentration; (×———×), RNAase activity.

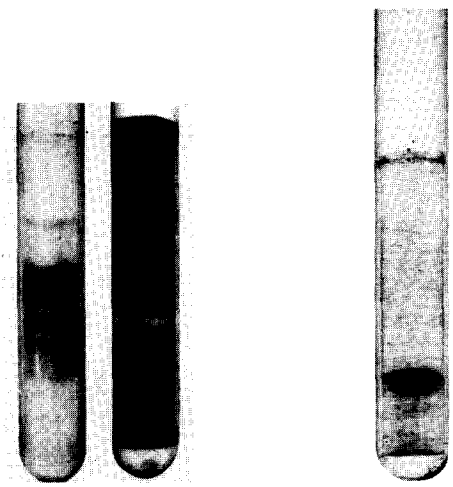
(fractions 7 and 8) was subjected to polyacrylamide gel electrophoresis with and without SDS; each gel revealed a few bands when stained for protein but only one of these bands showed RNAase activity. Electrophoresis of RNAase I without SDS is shown on Fig. 3, a similar result, not illustrated here, was obtained for RNAase II. After affinity chromatography using poly(G)-Sepharose 4B, enzymes subjected to electrophoresis without SDS revealed one, two or three bands of protein with corresponding bands of RNAase activity. When the same enzymes were subjected to electrophoresis in the presence of SDS, only one band of protein was visible. Under this condition the electrophoretic mobility of RNAase I and RNAase II was the same, and these enzymes gave only one band in coelectrophoresis (Fig. 4).

pH optimum

In acetate buffer all three enzymes showed a broad plateau region of highest activity. In contrast, in Mes-KOH buffer the activity curves (Fig. 5) possessed sharply defined peaks at pH 5.7 for RNAase I, ph 6.35 for RNAase II and pH 6.8 for nuclease.

Substrate specificity

RNA was hydrolysed to acid-soluble products with all three enzymes. Only nuclease was able to hydrolyse native and denatured DNA at a rate approx. 3 times more slowly than compared to RNA. In addition to hydrolysis of RNA, all three enzymes were able to hydrolyse synthetic polyribonucleotides except for poly(G). This polyribonucleotide was not hydrolysed by RNAase I and



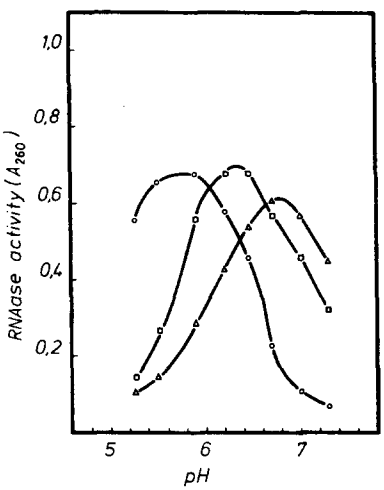


Fig. 3. (Left) polyacrylamide gel electrophoresis of RNAase I after the DEAE-Sephadex A-50 step. Gel concentration was 10%. Left, staining for protein and right, staining for RNAase activity.

Fig. 4. (Centre) coelectrophoresis of RNAase I and RNAase II in 10% SDS-polyacrylamide gel. Equal amounts of RNAase I and RNAase II were mixed together and electrophoresed. Gels were stained for protein.

Fig. 5. (Right) pH optima in 0.1 M Mes-KOH buffer. (○———○), RNAase I; (□———□) RNAase II; (△———△), nuclease.

RNAase II and was only slightly hydrolysed by nuclease (Table II).

For RNA and all the polynucleotides tested, the activity against poly(U) was highest of all three enzymes. The analysis of the poly(A) and poly(U) hydrolysis products showed that both RNAase I and RNAase II produced, after prolonged digestion, 3'- or 2',3'-cyclic nucleotides. In contrast, for nuclease, 5'-nucleotides were the only products formed. In the case of poly(A), both RNAase I and RNAse II produced 3'-nucleotides only, although poly(U) was hydrolysed to 2',3'-cyclic nucleotides by RNAase I and to a mixture of 3'- (21%) and 2',3'-cyclic (79%) nucleotides by RNAase II.

Test of endo- or exonuclease activity

To distinguish between endo- and exonucleolytic modes of action, aliquots of purified yeast RNA hydrolysed to different extents were analysed by Sephadex G-75 gel filtration. The chromatographic profiles showed a continuous distribution of RNA fragments. These results indicate an endonucleotytic mode of action [8—10].

Molecular weight

The apparent molecular weight of each of the three enzymes was determined by gel filtration on Sephadex G-75. The molecular weight values were estimated using the relationship [23] between K_{av} and the log of the molecular weight (Fig. 6). These values were 37 000, 19 000 and 19 000 for nuclease, RNAase I and RNAase II, respectively.

Double immunodiffusion of RNAase I and RNAase II

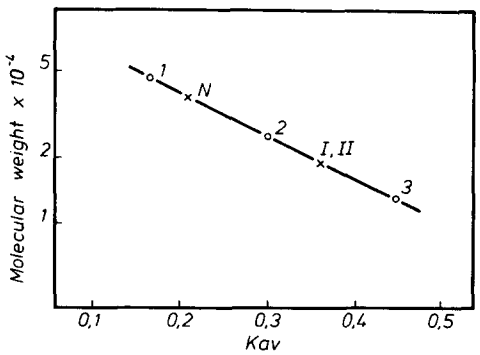
Antibodies against RNAase II after poly(G)-Sepharose 4B purification were produced in rabbits. Double immunodiffusion test's showed only one precipitin arc in the reaction between rabbit antiserum and poly(G)-Sepharose 4B purified RNAase II. This antiserum also reacts with the purified RNAase I preparation. Fig. 7 shows that RNAase I and RNAase II gave a reaction of complete identity with the anti-RNAase II [24].

TABLE II

ACTIVITY OF BARLEY NUCLEASES FOR POLYRIBONUCLEOTIDES

Enzyme activities with different polyribonucleotides as substrates were determined in standard mixture with the appropriate amount of each enzyme, and the activity was related to the activity for RNA, which was taken as 1.

Substrate	Relative activity		
	RNAase I	RNAase II	Nuclease
RNA	1	1	1
poly(A)	1.3	0.77	3.7
poly(C)	0.42	4.1	0.29
poly(G)	0.01	0.01	0.17
poly(I)	2.2	2.1	0.71
poly(U)	3.2	7.0	5.1



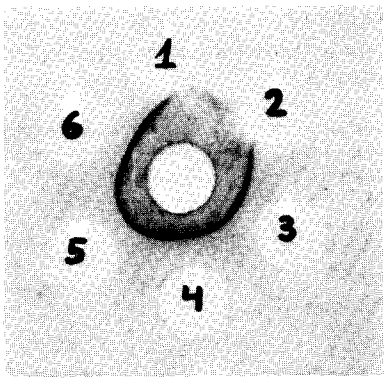


Fig. 6. Molecular weight determination on Sephadex G-75. The relationship between K_{av} and molecular weight is plotted in a semilogarithmic scale. 1, ovalbumin; 2, chymotrypsinogen A; 3, cytochrome *c*; N, nuclease; I and II, RNAase I and RNAase II.

Fig. 7. Double immunodiffusion of RNAase I and RNAase II (Ouchterlony method) in 1% agar against anti-RNAase II. The central well contains 30 μl anti-RNAase II. The peripheral wells (1—4) contain: 5, 10, 20 and 30 μl RNAase II; wells 5 and 6 contain 10 and 20 μl RNAase I, respectively.

Inhibitors

Hg^{2+} and Cu^{2+} are strong inhibitors of the enzyme activity as measured using RNA in a standard incubation mixture. Fe^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} influence the enzyme activity only slightly (Table III).

The effect of the presence of other nucleic acids on the enzyme activity against RNA

Poly(G), which was not hydrolyzed by RNAase I and RNAase II, was found to be a strong inhibitor of the activity of these two ribonucleases (Table IV). As little as 0.4 mg of poly(G)/ml caused inhibition of nearly two-thirds of the activity of RNAase I and more than 90% inhibition of RNAase II. Neither denatured nor native DNA influenced the activity of RNAase I or RNAase II (Table IV). On the other hand, DNA inhibited the activity of nuclease against RNA (Table IV). Denatured and native DNA had almost the same effect.

TABLE III

INHIBITION OF NUCLEASE ACTIVITY WITH DIVALENT METAL IONS

The final salt concentration in the sample was 1 mM.

Salt	Remaining activity (%)		
	RNAase I	RNAase II	Nuclease
$HgCl_2$	8	17	19
$CuSO_2$	7	7	19
$FeSO_2$	83	78	72
$ZnSO_2$	41	32	70
$MnSO_2$	100	88	89
$CoCl_2$	95	80	72

TABLE IV

THE EFFECT OF OTHER NUCLEIC ACIDS ON THE ACTIVITY OF NUCLEASES WITH RNA AS A SUBSTRATE

Nucleic acid added (mg/ml)	Remaining RNAase activity (%)		
	RNAase I	RNAase II	Nuclease
poly(G)			
0.4	37	8.8	—
0.8	21	5.3	—
DNA native			
0.2	—	—	87
0.4	—	—	77
1.0	—	—	64
DNA denatured			
0.2	95	102	70
0.4	94	102	70
1.0	95	100	62

Discussion

RNAases have often been isolated and purified from leaves or roots but rarely from dry seeds. However, because of the important role of these enzymes in the early stages of germination, we wanted to characterize the nucleolytic enzymes of dry seed in detail. RNAase activity and its changes have been measured in dry seeds or during germination to clarify its role during plant development [11,13,14,25,26]. Most of these studies used crude or only partially purified preparations and thus did not provide accurate information on metabolic functions of the individual enzymes involved.

Results presented in this paper showed the existence of different enzymes possessing RNAase activity in dry barley seeds. Two of the enzymes studied were specific for RNA and the third was able to hydrolyse both RNA and DNA. All three enzymes showed the mode of action characteristic for endonucleases. These results suggest that the enzyme hydrolyzing RNA and DNA could be nuclease I and that the two other enzymes specific for RNA only could be RNAase I and RNAase II. To confirm this, an analysis of reaction products was made which distinguished between RNAase I and RNAase II [1]. The RNAase that produced 2′,3′-cyclic pyrimidine nucleotide as a final product might be classified as RNAase I; the second RNAase which is able to hydrolyse 2′,3′-cyclic pyrimidine nucleotide might be classified as RNAase II [1,32]. Barley seed RNAase I and RNAase II showed sharply defined pH optima in Mes-KOH buffer at 5.7 and 6.35, respectively. These pH optima were characteristic for RNAase I and RNAase II [32]. The order of hydrolysis of synthetic polyribonucleotides was similar for both ribonuclease, except that RNAase II showed a high rate of hydrolysis of poly(C) as compared to barley RNAase I and RNAases from other sources. Poly(C) was the slowest hydrolysed polynucleotide with RNAase from wheat germ [9] or oat leaf [10]. Hg^{2+} and Cu^{2+} were strong inhibitors of activity for both RNAases (Table III), this has also been reported for other plant RNAases [6,9,12].

Denatured DNA was not hydrolyzed by either of the ribonucleases and was not an inhibitor of their activity (Table IV). Poly(G), which was not hydrolysed, appeared to be a strong inhibitor of these enzymes. This property was used to purify plant ribonucleases by means of affinity chromatography on poly(G)-Sepharose 4B. This method produced homogeneous preparations of both plant RNAases, and was previously applied for purification of RNAase from Citrobacter [27] and RNAase C from human plasma [28].

Although RNAase I and RNAase II differed in their action and pH optimum, they shared such properties as molecular weight (19 000) and identical reaction in double immunodiffusion against anti-RNAase II (Fig. 7). This suggests that structural similarities between these two enzymes exist. RNAase I and RNAase II described in other plant species are usually similar with respect to molecular weight and mode of action [2,8,12]. The molecular weight of the enzymes isolated from barley seeds was different, however, from the molecular weight (25 000) of the RNAase I isolated from barley leaves [6]. The third nucleolytic enzyme found in barley seeds was nuclease. Properties of several plant nucleases have been described previously [1,4,29,32]. The molecular weight of the enzyme studied in this paper was determined by gel filtration as approx. 37 000. This value was slightly higher than the molecular weights determined for oat leaf nuclease (33 000) [4], corn nuclease (31 000) [1] and tobacco nuclease (35 000) [30].

The pH optimum of barley seeds nuclease was 6.8; this is in the range usually found for this enzyme in other plants [4,30—32] and was higher than the optimum pH of RNAase I. Barley seed nuclease showed higher activity towards RNA than native or denatured DNA. DNA was an inhibitor of nuclease activity toward RNA and denatured and native DNA showed almost the same effect (Table IV).

Poly(A) and poly(U) were hydrolysed faster than other polynucleotides with the barley seed nuclease (Table II). These results agree with results obtained by Wyen et al. [4] for oat leaf nuclease.

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