REVISTA ESPAÑOLA DE FISIOLOGIA, 31, 283-288. 1975

Pine Seed Ribonucleases. I. Preparation and Properties

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(Received on 6 May, 1975)

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The enzyme preparation, about 100 fold purified, shows optimal activity at pH 4.8 and 5.9. This activity lessens rapidly at 40-60° C, and retains 20 % at 100° C. These results of heat stability and optimal pH might suggest that the enzyme preparation contains two enzymes. The use of gel filtration clearly shows that pine RNAses are endonuclease.

Crude extracts from many plant sources have been shown to degrade ribonucleic acids (8, 9). Ribonucleases from spinach (14), wheat germ (13), pea leaves (3, 7), barley leaves (5), ryegrass (10, 11), tobacco leaves (2) and corn endosperm (16) have been purified extensively and their modes of action determined. The present communication deals with the isolation and properties of RNAse in Pine seeds. This enzyme is of particular interest to us in connection with our work on ribonucleic acid changes in *Pinus pinea* seeds. By adopting the method described below, a 100-fold purification was achieved.

Materials and Methods

Pinus pinea seeds kindly supplied by the Instituto Forestal of Madrid were used in this investigation. They were utilized within 6 months.

Commercially obtained yeast ribonucleic acid (YRNA) was purified by alcohol precipitation. 10 g of YRNA were dissolved in 100 ml of distilled water and the pH of the solution was adjusted to 6.7 with N NaOH. Any undissolved material was removed by centrifugation. The clear supernatant was brought to pH 5.5 with acetic acid and 3 volumes of absolute alcohol were added to this. After standing overnight at 4° C this was centrifuged. The precipitate was washed successively with 70-95 % absolute alcohol and ether, and then, air-dried. This preparation was used as a substrate for the enzyme.

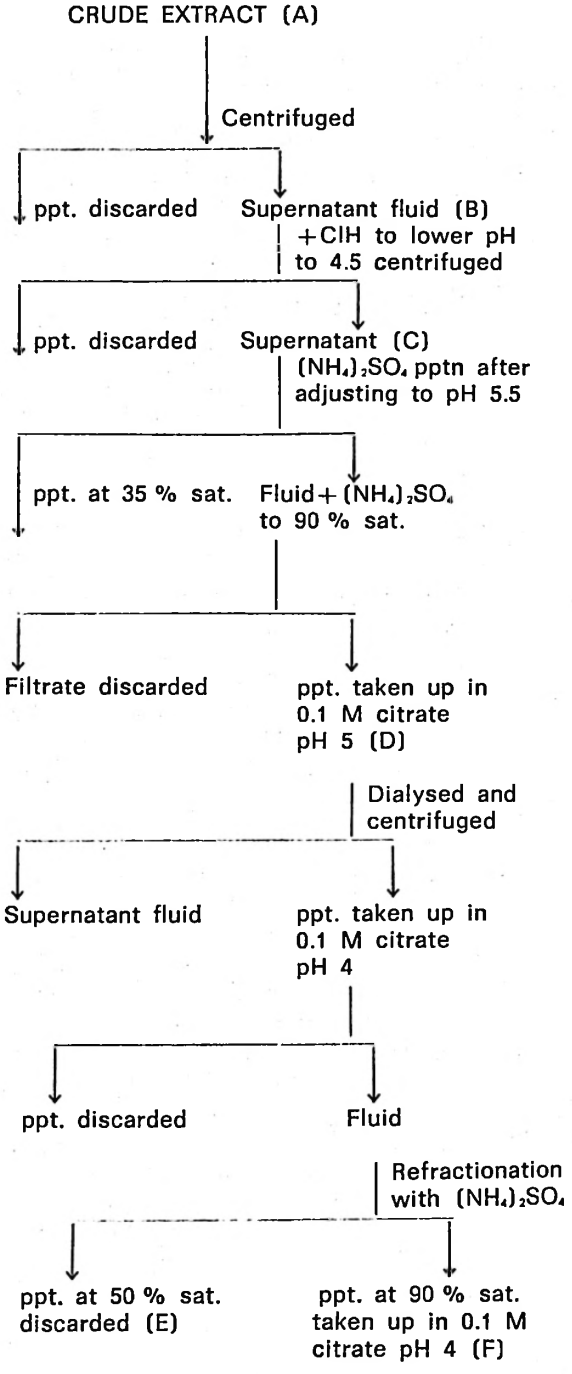
Preparation of crude extract. 10 g of Pine endosperm were deep-frozen

(—20° C) and homogeneized in 40 ml of 0.1 M phosphate buffer, pH 7.6 in a prechilled mortar with quartz sand. The resulting slurry was passed through muslin and centrifuged at 6,000 rpm for 10 minutes at 4° C. The supernatant was used as crude extract.

Enzyme assays. RNAse activity was determined by measuring in a spectrophotometer the absorption of the breakdown products which were not precipitated by 0.3 % uranylacetate in 0.2 M perchloric acid (2, 4). The reaction mixture used for the determination was composed of 0.5 ml of 1 % YRNA in 0.1 M acetate buffer, pH 5; 0.2 ml enzyme; and 0.3 ml of 0.1 M acetate buffer, pH 5. This was incubated at 37° C for 30 minutes. At the end of reaction time, the tubes were transferred to an ice bath and 1 ml of 0.3 % uranly acetate in 0.2 M perchloric acid was added to each tube to stop the reaction and to precipitate the unhydrolised material. After standing overnight at 4° C, the tubes were centrifuged in the cold at 6,000 rpm for 10 minutes. 0.5 ml of the supernatant was made up to 5 ml with distilled water and the absorption was measured in a Unicam Spectrophotometer at 260 nm. Suitable blanks were always run side by side.

Gel filtration. Gel filtration was used to distinguish between endonucleolytic and exonucleolytic types of degradation. BIRNBOIM's method (1) was adopted except that Sephadex G-100 was replaced by Sephadex G-50. The incubation mixtures contained 0.5 ml of 1 % highly polymerized yeast RNA, 0.2 ml enzyme, and 0.3 ml of 0.1 acetate buffer, pH 5. In this case 10 μl of dietylpyrocarbonate were used to stop the reaction SOLIMOSY *et al.* (12). 2.0 ml fractions were collected.

Enzyme purification. HOLDEN and PIRIE's procedure was used (3). The course of purification is shown in scheme 1.



Scheme 1. *Purification of pine seed ribonuclease.*

Results and Discussion

Substrate concentration. The measurements of activity were done as described in the experimental part, except that the substrate concentration was varied. At low substrate concentration there was a strict proportionality between enzyme activity and substrate concentration. Afterwards, there was no proportionality. However, there was an increase in the activity (fig. 1).

Enzyme concentration. An investigation of the dependence of ΔA260 on enzyme concentration yielded the results shown in figure 1. There is a proportionality between enzyme activity and enzyme concentration.

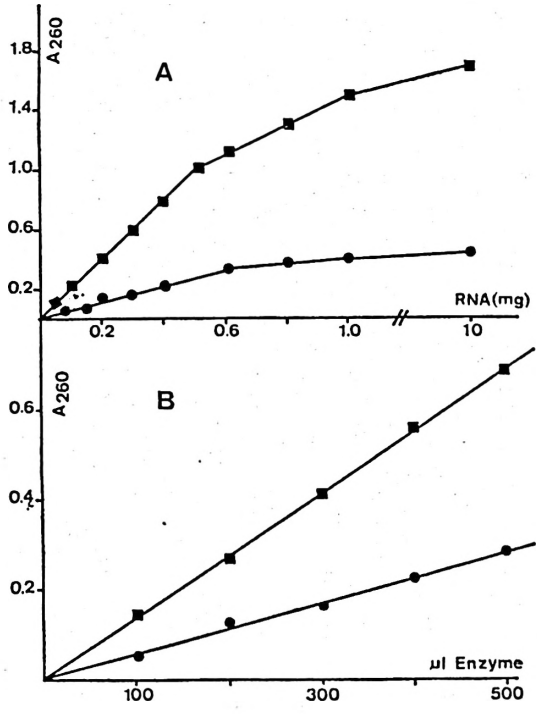


Fig. 1. *Influence of substrate concentration (A) and enzyme concentration (B) on ribonuclease activity.*
Assays were run in 0.1 M acetate-phosphate buffer pH 5 ■---■ and pH 6.2 ●---●.

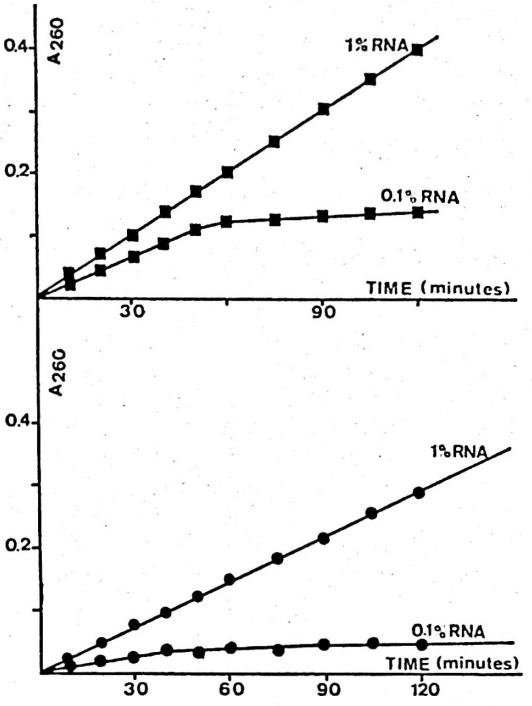


Fig. 2. *Time course of reaction.*
Enzyme activities were assayed as described in the legend to figure 1.

Time course of reaction. The degradation of RNA by the enzyme was seen to follow a practically linear course during the first 2 hours when RNA concentration was 1 %. However, when RNA concentration was lower (0.1 %), this proportionality was only observed during the first 50 minutes (fig. 2). Substrate concentration, enzyme concentration, time course of reaction and heat stability studies was investigated at pH 5.0 and 6.2 values, in 0.1 M acetate-phosphate buffer.

Optimal pH. The activity of enzyme preparations reaches a maximum in 0.1 M buffer at pH 4.8 and 5.9 (fig. 3). A double hump has consistently been observed in the pH optimum curve. This indicates possibly the presence of two RNAses differing slightly in pH optima.

Heat stability. The enzyme was heated during 10 minutes at varying temperature intervals, and samples were cooled in an ice-water bath and assayed by the standard method (fig. 3). The initial rapid loss in activity followed by a slow decrease, might also suggest that the enzyme preparation contains two enzymes, one more thermostable than the other. Similar results were observed by MEROLA and DAVIS (6) in soybean ribonuclease and by WILSON (15) in corn.

Other plant RNAses appear to be similar to pine RNAses. The optimal pH between 5 and 6 compared with the reported values of 5.5 for pea leaves RNAses (3), 5.1 for tobacco leaves RNAses (2) and 4.5 for ryegrass RNAses (10). Tobacco leaves RNAse (2) appears to be heat stable, as the pancreatic enzyme, whereas ryegrass and spinach RNAse seem to have a intermediate heat estability (10,14), and pea leaf RNAse is heathabile (2).

Use of gel filtration to distinguish between endonuclease and exonuclease action. The method of BIRNBOIM (1) de-

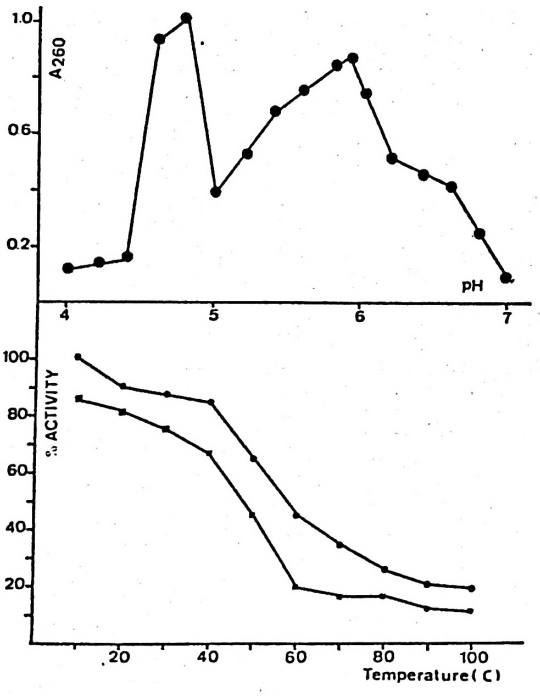


Fig. 3. *Effect of pH and temperature on ribonuclease activity.*
Samples at each temperature were assayed by the standard method in 0.1 M acetate-phospate buffer pH 5 ■---■ and pH 6.2 ●---●.

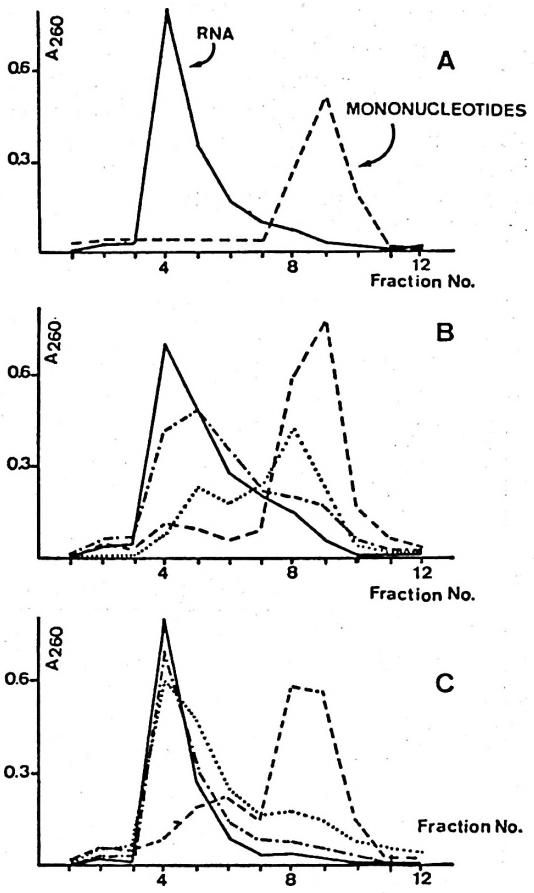


Fig. 4. *Characterization of endonuclease activity.*
Gel filtration on Sephadex G-50 of A) a mixture of RNA and mononucleotides. B) breakdown products of RNA at various stages of digestion with RNase A. C) products obtained by hidrolysis of RNA with Pine Seed Ribonucleases. Incubation times at 37° C. — 5 min; ------ 10 min; ··· 30 min; --- 60 min.

Table I. *RNAse purification.*
Details of the different fractions are give in scheme I. One RNAse unit is defined as the amount of enzyme which causes a change in absorbance of 0.01 under the given conditions (Material and Methods).

Vol (ml)	Fraction	RNAse units/ml	Total units	Yield (%)	Specific activity (units/mg protein)	Degree of purification
A	600	22.2×10^3	$13{,}320\times10^3$	100.0	716	1.00
B	500	20.6×10^3	$10{,}323\times10^3$	77.5	1,660	2.32
C	450	10.8×10^3	$4{,}874\times10^3$	36.6	1,880	2.63
D	300	13.4×10^3	$4{,}039\times10^3$	30.3	4,372	6.16
E	226	0.04×10^3	1.04×10^3	0.007	380	0.53
F	40	13.6×10^3	554×10^3	4.16	69,320	96.80

veloped for deoxyribonucleases was adapted for testing the endo- or exonuclease activity of RNAse. Sephadex G-100 was replaced by G-50 and 0.1 M acetate buffer pH 5.5 used for the elution of nucleotides and undigested RNA from 0.9 × 15 cm column. Before the method was applying to pine ribonuclease its reliability was ascertained by using pancreatic ribonuclease A, an enzyme well known as being an endonuclease.

High-molecular-weight yeast RNA, was digested with enzymatic extract for various intervals, and the digestion products were chromatographed on Sephadex G-50. The chromatographic profiles are shown in figure 4. At zero time, a single peak representing the undigested RNA was obtained. At short incubation times, a small amount of digested material appeared as a shoulder of the main-peak. After a longer incubation a peak, corresponding to the position of the first peak, was shifted towards higher elution volumes corresponding to a decrease in average molecular weight. After extensive digestion, the hydrolisis products yielded upon chromatography a single peak corresponding to mononucleotides. The shoulder probably represents low-molecular-weight oligonucleotides. The results clearly show that the RNAses investigated are endonuclease. WYEN *et al.* (17) observed a similar property of avena leaf RNAse. A general property of higher plant RNAses seem to be that they are endoenzymes.

Enzyme purification. The results of the pine seed RNAse purification are summarized in table I, and about 100 fold purification was achieved.

Acknowledgement

The authors wich to express their gratitude to Prof. A. Santos-Ruiz for his help and criticism during the preparation and realization of this work.

Resumen

La preparación enzimática de *P. pinea*, purificada unas 100 veces, muestra un óptimo de actividad a pH de 4,8 y 5,9. Entre 40-60° C existe una pérdida rápida de actividad del enzima reteniéndose un 20 % a 100° C. Los resultados anteriormente expuestos podrían sugerir la existencia de dos enzimas en la preparación enzimática. El uso de gel filtración muestra que las ribonucleasas de pino son endonucleasas.

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