Carrot ribonucleases

T. GOLASZEWSKI, J. W. SZARKOWSKI

Research on plant ribonucleases (RNAase), especially those of higher plants is far less advanced as compared with that on animal ribonucleases in the first place on bovine pancreatic RNAase. Two RNAase have been obtained in crystalline form from two different bacterial strains (Nishimura 1960; Takahashi 1962). So far, however, only few attempts to extract highly purified RNAase from higher plants have been successful. Among these, RNAase of soya-bean, pea and tobacco leaves, rye-grass, barley sprouts, spinach, and broad bean leaves have been obtained in a relatively pure state (Merola, Davis 1962; Holden, Piri 1955; Friesch-Niggemeyer, Reddi 1957; Shuster 1957; Maciejewska-Potapczyk, Górnjak 1962; Tuve, Anfinsen 1960; Fiers, Vandendriessche 1961).

The present paper deals with RNAase* from the shoots and roots of carrots, its properties and distribution in the cellular subfractions.

MATERIAL AND METHODS

Plants. Carrot (*Daucus carota* L.) roots, Nantes variety, from the previous year were planted in garden soil in boxes at 2-cm intervals, placed in a green-house and grown there for 3—4 weeks at 25° C. One group of plants was kept under normal daylight conditions, the other in darkness (etiolation).

Isolation and purification of the enzyme. The shoots were cut into small pieces and homogenized. The shoots after preliminary cleaning were washed in bidistilled water and with a 0.001 M aqueous solution of ethylenediaminetetraacetic acid sodium salt. After drying the roots were ground and the juice was pressed out from the pulp. The crude extracts were then acidified with diluted acetic acid, to pH 3. After centrifuging off the thus precipitated proteins, the supernatant was alkalized to pH 5, solid ammonium sulphate was added,

* According to the terminology of the International Biochemical Union (mimeographed copy, Brisbane 1964) carrot RNAase is probably identical with ribonucleate nucleotide-2'-transferase (2.7.7.17).

and the proteins salted out in the 0.35—0.80 saturation range were separated by centrifugation at 11,000 g for 30 min. The protein precipitate was suspended in 50 ml of 0.01 M citrate-phosphate buffer, pH 6, and dialysed successively against 0.01, and 0.001 M citrate-phosphate buffer and water for removal of the SO_4^{--} ions. The dialysate was lyophilysed to a small volume. Then a sample was transferred onto a DEAE column and chromatographed.

Purification was carried out in several steps at temperature close to 0°.

Assay of enzymic activity. The RNAase activity was determined spectrophotometrically according to Anfinsen *et al* (1954).

Samples of the enzyme, 0.2—0.6 ml, with 0.8—0.4 ml of buffer solution and 1 ml of 0.4 percent yeast ribonucleic acid (RNA) was incubated at 37° for one hour and then the reaction was stopped by addition of 0.5 ml uranyl acetate in perchloric acid (Anfinsen *et al*. 1954). After 20 min. the excess of nondigested RNA was centrifuged off and a 0.2 ml sample was withdrawn and diluted with water up to 3 ml. The extinction of the sample thus prepared was read in a SP-500 Unicam spectrophotometer at 260 m μ against a blank sample. As unit of RNAase activity (R.u.) was assumed the amount of enzyme producing an increase of extinction by $\Delta E_{260}^{1\text{ cm}} = 0.001$.

The nonspecific phosphodiesterase was assayed by the method of Ostrowski and Walczak (1961) with $\text{Ca}[\text{bis}(\text{p-nitrophenyl})\text{-phosphate}]_2$ as substrate.

Desoxyribonuclease (DNAase) activity was assayed similarly as that of RNAase using herring DNA as substrate, and phosphatase activity according to King (1947).

As substrate for RNAase served a commercial preparation of yeast RNA purified by the Kunitz procedure (1940).

The yeast RNA-core fraction was prepared by exhaustive digestion with RNAase according to Hilmo $\acute{e}$ (1960).

Isolation of cell subfractions. Chloroplasts from shoot homogenates were obtained by the differential centrifugation technique of Stäubli (1957) and chromoplasts of roots by the Straus technique (1956). The mitochondrial fraction was prepared from the supernatant after elimination of the heavier cell components by centrifugation at 12,000 g for 20 min. Homogenates of organellae were prepared in the Potter-Elvehjem homogenizer.

RESULTS

In crude extracts of shoot tissue both green and etiolated, and in root juice the RNAase activity was assayed at various pH values.

Figure 1 presents the characteristic curve obtained. It shows two

peaks of optimum activity of the enzyme, both in the acidic pH range. This, in accordance with the results of other investigators (Ostrowski, Walczak 1961), would indicate the presence of two RNAase

Fig. 1. Curve of root juice RNAase activity in dependence on pH.

Ordinate axis — enzyme activity; abscissa axis — pH of incubation mixture. The following buffer mixtures were used: 0.1 M citrate-phosphate buffer. Tris — HCl and glycine buffer in pH range 3—7.0, 7—8.5, 8.6—10, respectively. pH measured with Radiometer-Copenhagen, type 22 pH-metre.

Fig. 2. Chromatographic separation of RNAase preparation isolated from etiolated shoots.

Column (0.8×12 cm) water-cooled to ca 8° , filled with diethylamine-ethyl cellulose (DEAE), equilibrated with 0.1 M citrate-phosphate buffer, pH 6. Sample (3 ml aqueous solution) introduced on column. Eluted successively with: 0.01 M citrate-phosphate buffer, pH 6 (enzyme fraction I — main), 0.09 M sodium chloride solution in 0.2 M citrate-phosphate buffer of the same pH (enzyme fraction II — minor). Protein elution followed by extinction measurement in eluate at 280 m μ in particular collector test tubes of 1.8 ml capacity (ordinate axis, left side). RNAase activity determined in 0.2 ml samples and calculated to total sample volume (ordinate axis, right side). Rate of flow of eluent 2 ml/min.

Fig. 3. RNAase purification.

fractions in the material examined. For isolation and purification of the enzyme a three-step procedure was applied, schematically presented in Fig. 3.

Enzyme activity in the course of purification. At each step, the activity of the enzyme was assayed and the specific activity calculated. The results are listed in Table 1.

Table 1
Specific carrot RNase activity in the course of purification

Purification steps	R.u./mg protein					
	shoots				root	
	green		etiolated			
1 Crude extract (root juice)	240	(1)	390	(1)	800	(1)
2 Extract after centrifugation of proteins, pH 3	495	(2)	1000	(2.6)	1370	(1.8)
3 Proteins salted out with ammonium sulphate 35-80% saturation	2440	(10)	3570	(9)	2220	(3.8)
4 Chromatographic fractions DEAE						
Fraction I	22400	(93)	42400	(110)	46500	(58)
Fraction II		ca 3000				ca 4000

Relative activity of the enzymic preparation as compared with activity of crude extract are given in parentheses. Protein determined by the Lowry et al. method with procedure for insoluble proteins.

Table 2
Total RNase activity at particular steps of purification

Step	Activity, R.u.		
	shoots 120 g fresh tissue		root (100 ml juice from 240 g fresh tissue)
	green	etiolated	
1 Crude extract	225,000	190,000	260,000
2 Supernatant after centrifugation of proteins, pH 3	150,000	100,000	190,000
3 Proteins salted out in 0.35-80 saturation range with ammonium sulphate after dialysis and lyophilisation	20,000	17,000	30,000
4 Introduced on DEAE-cellulose column	15,000	13,000	25,000
5 Recovered	ca 12,000	ca 11,000	ca 22,000
Fraction I	ca 10,000	ca 9,000	ca 20,500
Fraction II	ca 2,000	ca 2,000	ca 1,500

As can be seen the highest increase of specific activity was obtained after column chromatography on the DEAE column. Fig. 2 presents a scheme of the column chromatography.

By fractionation on the DEAE column, two protein fractions ex-

Table 3

Comparison of enzymatic degradation products of RNA and RNA-core

RNase fraction	Extinction (E_{260}) of enzymatic hydrolyse products non sedimented by uranyl acetate	
	yeast RNA	RNA-core
Main fraction from green shoots	0.41	0.36
Main fraction from etiolated shoots	0.26	0.27
Main fraction from roots	0.65	0.60
Minor fraction from green shoots	0.14	0.17

hibiting RNAase activity were obtained. The first (I) — the main fraction — exhibited 80—90 percent of the over-all activity of the material introduced on the column, the second (II) was a minor fraction (see Table 2).

As already stated, the main fraction showed optimal activity within the pH range 6.2—6.5, thus at the pH value of the cell sap of carrot tissues. The optimum activity of the minor fraction lay within pH 5—5.4.

The main fraction isolated from green and etiolated shoots contained no DNAase, nonspecific phosphodiesterase nor phosphomonoesterase contaminations, in contrast to the fraction from roots in which some traces of these enzymes were detected.

The main fraction, hydrolysed completely yeast RNA, so that no trace of what is known as the RNA-core remained, as it did in the case of pancreatic RNAase (Kunitz 1940). The same enzyme from carrot digested the RNA core fraction from yeast at the same rate as it did undigested RNA. The results are summarized in Table 3.

The chromatographic fraction I of shoot RNAase was strongly

Table 4

Influence of metal ions on RNase activity of shoots and roots main fraction

Metal ion, Me ⁺⁺ concentration $2 \times 10^{-2} M$, pH 6	Enzyme activity %
Cu	10
Co	40-30
Mg	110
without Me ⁺⁺ ions added	100

Table 5

Total RNase activity in the particular subcellular fractions
and in crude extract of green shoots ca 100 g fresh tissue

Cellular fraction	Total RNase activity	
	R.u.	%
Crude extract	140,000	100
Chloroplasts	2,000*	1
Mitochondria	8,000	5
Postmitochondrial supernatant	125,000	90

*Activity measured at pH 5

inhibited by the ions of bivalent metals such as copper and cobalt, whereas magnesium ion at the same concentration and pH value gave only an insignificant effect (see Table 4).

The most interesting property of carrot shoot RNase is its thermostability. After 5 min. heating at 100° at pH 5 both the fractions from shoots still retained about 95 percent of their initial activity as compared with the nonheated sample. However, when heated in the same conditions for 15 min. they lost about 60 percent of their activity. Root RNAase-activity decreased by 40 percent after 5 min. of heating at 100°.

Distribution of RNAase in subcellular fractions. The distribution of RNAase in subcellular fractions was examined in carrot tissue homogenates. In the supernatant obtained after centrifugation of the mitochondrial fraction two RNAase fractions seem to be present. The curve for enzyme activity as the function of pH value is similar to that obtained for the crude extract. The chloroplasts seem

Table 6

Specific RNase activities in subcellular fractions

Subcellular fraction	Specific RNase activity in R.u. mg of protein after pigments extraction
Chloroplasts of green shoots	200
Chloroplasts of roots	trace activity
Mitochondria of green shoots	150
Mitochondria of roots	120
Postmitochondrial supernatant:	
green shoots	410
roots	570

to contain only the fraction exhibiting optimum activity at pH 5, whereas in the mitochondria the fraction with optimum activity at pH about 6.5 was present.

It was found that carrot RNAase is chiefly concentrated in the post-mitochondrial supernatant, may be in the ribosome fraction. To the chloroplasts and mitochondria falls only a relatively small part of the total RNAase activity (Table 5).

Similarly, postmitochondrial RNAase of shoots showed the highest specific activity as compared to that found in other subcellular fractions. As seen from Table 6, the specific activities of chloroplast and mitochondrial RNAase are of the same order. RNAase activity in the postmitochondrial supernatant of shoots is 2—3 times higher than in plastids and mitochondria.

Chromoplasts of the roots, on the contrary, show at most only traces of RNAase activity.

Thus, the results obtained would indicate a nonuniform distribution of RNAase in carrot cells. Nevertheless, notwithstanding the heterogenous nature of carrot cell RNAase, both those of chloroplasts and mitochondria seemed to be homogenous enzymes.

DISCUSSION

The great difficulties encountered in preparation of highly purified RNAase preparations from plant material, particularly those from higher plants are the main obstacle in the study of the properties, distribution and role of this enzyme. Carrot shoots seem, therefore, a convenient source of plant RNAase, particularly the etiolated ones.

Carrot RNAases seem to be very similar to those of other higher plants as regard to their optimum activity in the acidic pH range, complete digestion of yeast RNA and sensitivity to metal ions. The relatively high resistance of carrot shoot RNAase to elevated temperature indicates its similarity to broad-bean (Maciejewska-Potapczyk, Górniak 1962) and spinach RNAase (Tuve, Anfinsen 1960). The RNAase fractions revealed in carrot thus point to the probability of the presence of multiple RNAase systems in other plant organisms such as bacteria (Nishimura 1960; Ostrowski, Walczak 1961; Szarkowski, Gołaszewski msc.). In higher plants: wheat, apple leaves and maize, heterogenous RNAase has recently been detected. Some preliminary results obtained by the present authors (not reported here) seem to suggest the possibility of existence of multiple RNAase systems in e.g. the thallus of *Cladonia silvatica*, in rye and in the leaves of *Ginkgo biloba*. Thus the phenomenon of heterogeneity of plant RNAases seems to be quite common. The high concentration in carrot of RNAase in the postmitochondrial fractions is in agreement with the findings

in tobacco leaf extracts (Sissakian 1958). These results are interesting in view of the reported presence of RNAase in *Escherichia coli* cells exclusively in the ribosome fraction (Spahr, Hollingworth 1961). This would indicate that the enzyme plays a certain role in protein biosynthesis (Tal, Elson 1963).

SUMMARY

It was found that carrot shoots and roots contain active ribonuclease (RNAase). The enzyme can be easily isolated and purified to a relatively high degree. In carrot tissues, beside the main RNAase fraction, exhibiting optimum activity in the pH range 6.2—6.5, a second minor component is present. The distribution of RNAase activity in the subcellular fraction was nonuniform. In the chloroplasts and mitochondrial fractions there seem to exist single but different enzyme fractions.

Some properties of the main enzyme fraction were studied.

*Institute of Biochemistry and Biophysics
Polish Academy of Sciences
Warszawa, Poland*

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*Rybonukleazy marchwi (Daucus carota L.)**

Streszczenie

W ekstraktach z pędów i korzeni marchwi (*Daucus carota* L.) wykryto rybonukleazę. W pędach i korzeniu marchwi, obok frakcji głównej o optymalnej aktywności przy pH = 6—6,5, stwierdzono frakcję poboczną enzymu rybonukleolitycznego wykazującą optymalną aktywność przy pH = 5—5,4 (ryc. 2, 3). Frakcja główna jest wrażliwa na obecność jonów miedzi i kobaltu (tab. 4), przeprowadza hydrolizę granicznego oligonukleotydu (tab. 3) i jest enzymem cieplotrwałym. Podgrzewanie wyizolowanej i oczyszczonej, głównej i pobocznej rybonukleazy pędów, przez 5 min. w temperaturze 100° i pH 5, zmniejsza jedynie nieznacznie aktywność preparatu.

Aktywność rybonukleazy stwierdzono we frakcji mitochondrialnej, w chloroplastach i supernatancie uzyskiwanym po odwirowaniu mitochondriów (tab. 6). Z chloroplastami związana jest frakcja poboczna enzymu, w mitochondriach przeważa frakcja główna rybonukleazy. Chromoplasty korzeni wykazują jedynie słabą aktywność rybonukleazy.

Stwierdzono nierównomierne rozmieszczenie aktywności rybonukleazy we frakcjach podkomórkowych. Przeważającą część aktywności, stwierdzoną w surowych ekstraktach, odnajdowano w supernatancie po odwirowaniu mitochondriów (tab. 5).

Marchew, a zwłaszcza jej pędy etiolowane wydają się być dogodnym źródłem rybonukleazy dającej się oczyścić w znacznym stopniu.

*

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