Changes in Barley Leaf Ribonucleases During Early Stages of Infection by *Erysiphe graminis* f. sp. *hordei*

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ABSTRACT

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Ribonuclease fractions from the barley powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei* Marchal, race 3) and a susceptible cultivar of barley (*Hordeum vulgare* 'Prior') have been purified to a high specific activity by Sephadex G-100 gel filtration in the presence of 3.0 M urea. Upon gel filtration the enzymes (termed pH 5-insoluble RNases of molecular weights 24,000 and 10,000 respectively) from healthy and inoculated plants yield two distinct peaks of enzymatic activity. The corresponding enzyme fraction from the powdery mildew fungus yields a single peak of activity of molecular weight about 12,000 daltons. The major peak of RNase activity obtained from inoculated barley leaves 48 hr after inoculation is remarkably different from that obtained from the healthy leaves or from the fungus with respect to its substrate preference as judged by the relative rates of hydrolysis of synthetic ribohomopolymers. These results suggest a dramatic change in the catalytic properties of this RNase of barley leaves at an early stage of host-parasite interactions.

Additional key words: substrate preference, host-parasite interactions, poly (A), poly (U), poly (C), poly (G).

In a number of higher plants, the catalytic properties of RNase change substantially during the early stages of rust infection (4). In genetically susceptible cultivars of flax (5,9), wheat (6,8), *Ribes* spp., and pine (7) inoculated with virulent races of rust fungi, qualitative changes in RNase activity at least in part, are related to the formation of a new class of RNase molecules not found in the healthy (uninoculated) plants or in the rust fungus grown in axenic culture. We have reported similar qualitative changes in two different RNase fractions in barley leaves (*Hordeum vulgare*, 'Prior') inoculated with the race 3 of the powdery mildew fungus, *Erysiphe graminis* f. sp. *hordei* Marchal (2,3). These two RNase fractions, termed pH 5-insoluble RNase and soluble RNase respectively, undergo readily detectable changes in specificity for cleavage sites in synthetic ribohomopolymers (2,3) and in the natural substrates, polysomal messenger and ribosomal RNA (10). Our interest in these enzymes was stimulated because RNases play a central role in the post-transcriptional processing of precursor RNA molecules into mature ribosomal RNA, functional transfer RNA, and translatable messenger RNA and because changes in the catalytic properties of the pH 5-insoluble RNase have been detected in susceptible barley as early as 24 hr after inoculation with the powdery mildew fungus (2,3,10).

The pH 5-insoluble RNase of barley leaves is bound to ribosomes when the subcellular fractions are separated in an aqueous medium and it has a predominantly endonucleolytic mode of action (3). This RNase fraction has been purified to a high specific activity by Sephadex G-100 gel filtration in the presence of 3.0 M urea. We present here a comparison of some important properties of the partially purified enzyme from healthy and inoculated barley leaves as well as those of the corresponding enzyme fraction from the powdery mildew fungus. The results suggest measurable changes in the catalytic properties of this RNase fraction during the initial stages of host-parasite interactions.

MATERIALS AND METHODS

Seeds of barley (*H. vulgare* 'Prior') were obtained from Queensland Wheat Research Institute, Toowoomba, Queensland. This cultivar is highly susceptible to infection by the race 3 of the powdery mildew fungus, *E. graminis* f. sp. *hordei*. The plants were grown in a controlled-environment growth chamber at 20,000 lux for 16-hr days at 23 C and an 8-hr night at 19 C. Seven days after seed sowing, the plants were inoculated with conidia of the powdery mildew fungus and immediately returned to the growth chamber. The uninoculated healthy (control) plants were grown under identical conditions. Exactly 48 hr after inoculation, the leaves from healthy and inoculated plants of the same age were excised, quick-frozen in liquid nitrogen, and stored at −20 C until required.

The fungus spores and hyphae were collected from heavily infected excised barley leaves by gently brushing them into the extraction buffer (50 mM potassium phosphate buffer, pH 6.8) held at 0 C. The material recovered from 50-60 primary leaves 6−8 days after inoculation was adequate for the extraction and Sephadex G-100 gel filtration of the pH 5-insoluble RNase.

For the extraction and purification of pH 5-insoluble RNase, healthy or inoculated barley leaves at 48 hr after inoculation were homogenized with 10 times the volume of extraction buffer and, after filtration through two layers of Miracloth®, the homogenate was centrifuged at 10,000 *g* for 30 min. The supernatant fraction was adjusted to pH 5.0 by the dropwise addition of 1 N HCl, allowed to stand at 0 C overnight, and the precipitate was collected by centrifugation as above. The pellets were resuspended in the extraction buffer and clarified by centrifugation. This fraction was called "pH 5-insoluble RNase".

For Sephadex G-100 gel filtration, the pH 5-insoluble RNase fraction was made 3.0 M with respect to urea and was subjected to gel filtration as described previously (5) except that the elution buffer was 50 mM potassium phosphate, pH 6.8, containing 3.0 M urea.

The molecular weights of RNase from different sources were determined by analytical column gel filtration as described by Andrews (1). The standard proteins used and their molecular weights were: cytochrome c (horse heart), 12,000; chymotrypsinogen (bovine pancreas), 25,000; ovalbumin, 45,000; and hexokinase (yeast), 90,000, all from Sigma Chemical Co., St. Louis, MO 63178 USA.

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The standard reaction mixtures for RNase assay contained, in a final volume of 1.0 ml, 40 mM sodium acetate buffer, pH 5.8, 0.6 mg yeast RNA and the enzyme fraction containing either 50–100 μg (pH 5-insoluble RNase) or 10–20 μg (Sephadex G-100 peaks) protein. Duplicate samples also were run as enzyme blanks. All samples were incubated at 33 C for 10 min. The incubation mixtures were chilled quickly and 2.0 ml of a precipitating reagent (1 N HCl in 76% ethanol containing 0.5% $LaCl_3$) were added to each tube. The contents of the tubes were mixed thoroughly, allowed to stand for 10 min in ice, and then centrifuged until the supernatant was clear. The absorbance of the supernatant was read at 260 nm and corrections were made for enzyme blanks (9).

The hydrolysis of polyguanylic acid [poly (G)] and of the ^{3}H-labelled polynucleotides, polyadenylic, polyuridylic, and polycytidylic acids [poly (A), poly (U), and poly (C) (potassium salts) of specific actvity 20 μCi/μmol P, from Schwartz/Mann, Orangeburg, NY 10962] also was estimated by the standard RNase assay except that the appropriate polynucleotide replaced yeast RNA (5-7).

A unit of RNase activity is defined as the amount of enzyme required to catalyze an increase in A_{260} of 1.0 under the standard conditions of assay and with yeast RNA as substrate.

DNase activity was assayed with thermally denatured calf thymus DNA and phosphodiesterase activity with Ca-*bis* (*p*-nitrophenyl) phosphate as substrates as described previously (9).

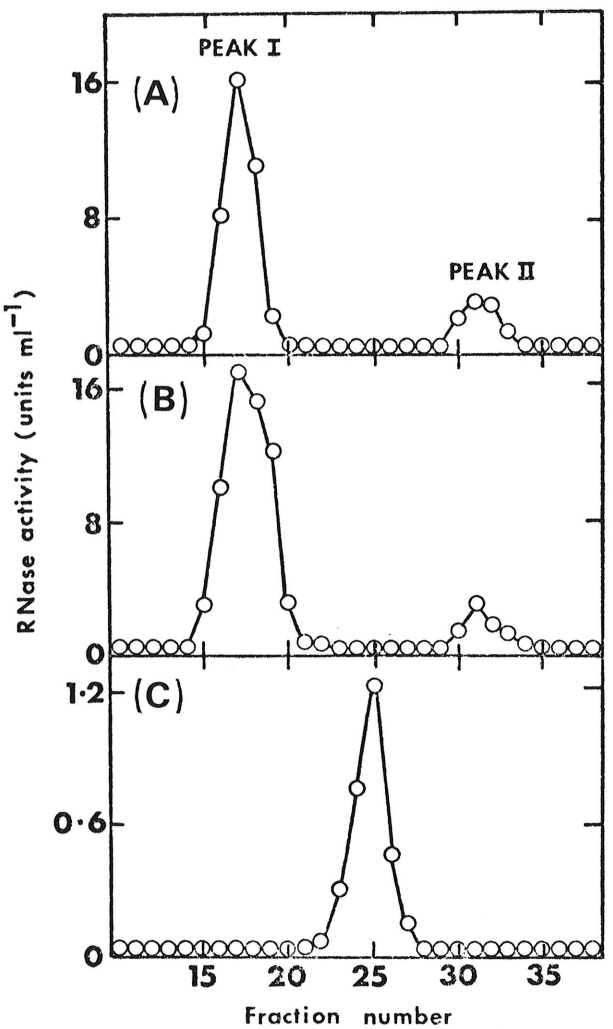

Fig. 1. Sephadex G-100 gel filtration profiles of the pH 5-insoluble RNase fraction from **A)** healthy barley leaves; **B)** barley leaves at 48 hr after inoculation; and **C)** powdery mildew spores and mycelium. Approximately 5.0-ml fractions were collected at 20 ml/hr and 0.2 ml of each fraction was assayed for RNase activity.

RESULTS

A barley leaf RNase fraction, termed pH 5-insoluble RNase, undergoes changes in substrate preference during the early stages of mildew infection (2,3). To purify this enzyme further, the fractions containing pH 5-insoluble RNase from healthy and inoculated barley leaves as well as from the mildew fungus were subjected to Sephadex G-100 gel filtration. The activity profiles are presented in Fig. 1. The enzyme from healthy and inoculated barley leaves yielded two peaks of enzymatic activity: a major one, peak I, and a relatively minor one, peak II (Fig. 1 A, B). The pH 5-insoluble fraction from the powdery mildew fungus yielded a single peak of activity (Fig. 1C). Data from the Sephadex G-100 gel filtration profiles of the enzyme from healthy and inoculated plants revealed no obvious difference. The single peak of RNase activity of the fungal enzyme occupied an intermediate position between peaks I and II of the enzyme from healthy and inoculated leaves.

The pH 5-insoluble RNase fraction from healthy and inoculated barley leaves was subjected to Sephadex G-100 gel filtration in the absence of urea, but the results were unsatisfactory: the resolution was poor and the two peaks (peaks I and II in Fig. 1A,B) were incompletely separated (Chakravorty and Scott, unpublished).

The enzyme peaks isolated by Sephadex G-100 gel filtration from all three sources are extremely unstable: 90% of the activity is lost during storage for 24 hr at 0–4 C or overnight dialysis against 50 mM potassium phosphate buffer, pH 6.8. Therefore, measurement of their properties, were made immediately after isolation and without storage.

The effect of EDTA on the activity of the enzymes before and after Sephadex G-100 gel filtration is shown by the data in Table 1. The activity of the relatively crude pH 5-insoluble RNase fraction as well as that of the peak I enzyme from healthy and inoculated leaves is stimulated by EDTA in varying degrees. The peak II enzyme is slightly inhibited by EDTA. The fungal RNase is severely inhibited by EDTA both before and after Sephadex G-100 gel filtration. Several plant RNases described in the literature (11) are either insensitive to EDTA or inhibited by the reagent. The highly reproducible finding that both relatively crude preparations of the pH 5 insoluble RNase as well as the partially purified enzyme from barley leaves are stimulated by EDTA suggests that EDTA stimulation is caused by a direct effect of the reagent on the activity of the enzyme rather than by interactions of EDTA with impurities.

A summary of some important properties of the protein peaks from Sephadex G-100 gel filtration of extracts from healthy and inoculated barley leaves as well as from the powdery mildew fungal spores and hyphae is presented in Table 2. The relative rates of hydrolysis of the radioactive substrates by the peak I and peak II enzymes from healthy and inoculated barley leaves are quite different. The substrate preference of peak I enzyme from

TABLE 1. Effect of EDTA on the RNase activities before and after Sephadex G-100 gel filtration

Source and enzyme	RNase specific activity[a]	
	Control	EDTA (15 mM)
Healthy leaves:		
pH 5-insoluble fraction	10.5	26.0
Sephadex peak I	700.0	1,250.0
Sephadex peak II	20.0	15.0
Inoculated leaves:		
pH 5-insoluble fraction	18.9	38.9
Sephadex peak I	700.0	1,365.0
Sephadex peak II	15.0	12.0
Powdery mildew fungus:		
pH 5-insoluble fraction	16.0	3.7
Sephadex peak	400.0	32.0

[a]Specific activity is: units of RNase per mg protein.

TABLE 2. Substrate preference and molecular weights of the enzymes separated by Sephadex G-100 gel filtration

Source and enzyme	Relative rates of hydrolysis: Radioactive substrates (dpm solubilized mg protein × 10^{-3}) Poly (A)	Poly (U)	Poly (C)	Nonradioactive substrates ($\Delta A_{260\ nm}$/mg protein) Poly (G)	DNA	Approximate molecular weight
Healthy leaves						
Peak I	1,880.0	2,564.0	1,590.0	6.8	No detectable activity	24,000
Peak II	93.0	18.8	3.2	7.0	2.3	10,000
Inoculated leaves						
Peak I	4,890.0	16,700.0	16,470.0	12.0	No detectable activity	24,000
Peak II	60.6	5.8	0	6.0	5.2	10,000
Fungal mycelium and spores	1,012.5	33.3	0	7.5	No detectable activity	12,000

inoculated leaves, in descending order, is poly (U) ≡ poly (C) > poly (A). This is strikingly different from the substrate preference of the corresponding enzyme peak from healthy leaves [poly (U) > poly (A) > poly (C)] and from that of the only peak of fungal enzyme [poly (A) > poly (U)].

The data in Table 2 also show that peak II enzyme from healthy barley leaves exhibits relatively low depolymerization of poly (C) but the corresponding enzyme peak from inoculated leaves and the only peak of the fungal enzyme are unable to hydrolyze poly (C) under standard assay conditions. The enzyme peaks from all sources hydrolyze poly (G). The major peak of activity from healthy and inoculated leaves and the fungal enzyme have no detectable DNase activity. The peak II enzyme from healthy and inoculated barley leaves hydrolyzes thermally denatured DNA. The approximate molecular weights of the peak I and peak II enzymes from both healthy and inoculated leaves are 24,000 and 10,000, respectively. The molecular weight of the fungal enzyme is 12,000. None of the enzyme peaks shows phosphodiesterase activity with Ca-*bis* (*p*-nitrophenyl) phosphate as substrate (Chakravorty and Scott, unpublished).

DISCUSSION

The results presented indicate that the major component of the pH 5-insoluble RNase fraction from barley leaves at 48 hr after inoculation with the powdery mildew fungus (Fig. 1, peak I) has remarkably different catalytic properties than those of the corresponding enzyme from healthy leaves (Table 2). A somewhat similar, but much less dramatic, difference in the substrate preference of the relatively crude preparations of the pH 5-insoluble RNase from healthy and inoculated barley leaves and from the powdery mildew fungus was reported previously (2,3). Because the amount of fungal material associated with the host tissue is negligible at 48 hr after inoculation, the observed changes in the catalytic properties of this enzyme are unlikely to be due to a contribution of fungal component to the enzyme isolated from inoculated leaves. This conclusion is corroborated by the finding that Sephadex G-100 gel filtration profile of the pH 5-insoluble RNase from inoculated leaves (Fig. 1B) does not reveal a peak of activity corresponding in position to that of the fungal enzyme (Fig. 1C). The substrate preferences of the major peak of activity from inoculated barley leaves and that of the only peak of activity from the powdery mildew fungus (Table 2) also are significantly different. These results provide direct evidence that the changes in the substrate preference of this enzyme at 48 hr after inoculation are due to parasitically induced alterations in the host enzyme.

It has been reported previously that there is an increase in the RNase activity of plants following mechanical injury (7,9). However, this change is only quantitative because the catalytic properties of RNase from mechanically injured tissues essentially is identical to that from healthy plants.

Readily detectable changes in host enzymes involved in transcription and post-transcriptional processing of RNA have been observed in a number of rust-diseased higher plants (4). It has been suggested that these changes are brought about either by the modification of preexisting enzymes or the synthesis of new enzyme molecules with novel catalytic properties even though they have the same molecular weight as the preexisting host enzymes (4,5). Work now in progress in this laboratory has been designed to ascertain whether a similar mechanism operates during the early stages of barley-powdery mildew interactions.

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