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SEROLOGICAL CHARACTERIZATION OF A RIBONUCLEASE FROM *HORDEUM VULGARE**

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Abstract—Specific rabbit antisera against purified *Hordeum vulgare* seedling RNase I from two winter barley cultivars each formed a single precipitin band when reacted with the homologous crude tissue extract. RNase antigen from either cultivar was equally reactive with both antisera when evaluated by immunodiffusion and immunoelectrophoresis. A small but consistent difference in anti-RNase specificity between cultivars was shown by passive hemagglutination inhibition, suggesting that molecular differences may exist between the two RNase antigens. Immunodiffusion and rocket immunoelectrophoresis were used to qualitatively test the cross-reactivity of protein preparations from various members of the genus *Hordeum* and species from other related grass genera. Neither antiserum showed cross-reactivity with soluble protein preparation from species outside the genus *Hordeum*. A few species within the genus *Hordeum* were cross-reactive. A modification of rocket immunoelectrophoresis was developed to determine the amount of RNase in unpurified tissue extracts. The technique involved a template-reservoir which allowed detection of 250 ng RNase in tissue extract volumes of 50 μl. The amount of RNase in unpurified protein extracts from the two cultivars of barley was similar.

INTRODUCTION

Variation in activity of RNA-degrading enzymes among cultivars of winter barley has been reported [1, 2]. Wilson has described the biochemical properties of a number of plant nucleases [3]. He pointed out the need for more definitive information on these enzymes as an aid in describing specific roles in RNA degradation. In pursuit of this objective, Wong [4] purified an RNase from barley seedlings. The purified enzyme from each of two barley cultivars showed different specificities when assayed with various dinucleoside substrates.

Pitt [5] found serology to be useful in evaluating contaminant proteins in RNase preparations from potato. He also used radial immunodiffusion to quantitate potato RNase in unpurified tissue extracts.

The purpose of this research was to use serological techniques to evaluate the purification method described by Wong [4] for possible contaminants in the RNase fraction. Additionally, it was desirable to learn about the specificity of the antisera prepared against *H. vulgare* RNase toward the RNase component from other cereal crops, including cultivars within *H. vulgare* and several uncultivated species of *Hordeum*. Finally, rocket immunoelectrophoresis was explored as an alternative to radial immunodiffusion as a method to determine RNase content of tissue extracts.

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RESULTS AND DISCUSSION

RNase purification and identification

Pitt reported difficulty in separating potato RNase from acid phosphomonoesterase [5]. A purification schedule for barley RNase involving ion exchange, preparative electrophoresis and Bio-Rad P-100 gel filtration as the final step allowed preparation of enzyme from 4-day old seedlings which was free of enzymes which degrade *p*-nitrophenyl phosphate (*p*NPP) [4]. The yield of purified RNase for both cultivars was *ca* 250 μg from 200 g of fr. shoot tissue. Typically the sp. act. calculated in RNase units per mg protein [2], was 2275 and 2700 for the enzyme from the cultivars 'Dicktoo' and 'Tennessee Winter', respectively.

Identification of the purified RNase was based upon the division of plant nucleases into three categories; nuclease I, RNase I and RNase II [3]. Purified RNase from each barley cultivar corresponded in electrophoretic mobility to RNase I from corn roots [6]. MW for barley RNase, determined by Bio-Rad P-30, for the cultivars 'Tennessee Winter' ($RNase_{TW}$) and 'Dicktoo' ($RNase_{D}$) was shown to be *ca* 22 000 (unpublished data), which falls within the 20 000–25 000 range reported for RNase I [3]. The pH optimum for $RNase_{TW}$ and $RNase_{D}$ was found to be pH 5.4 as

reported by Wong [4]. Wilson [3] reported a range of pH 5–6 for RNase I and pH 6.7 for RNase II. On the basis of the data listed above we have tentatively determined $RNase_{TW}$ and $RNase_{D}$ to be RNase I.

Antibody production

A single 1 mg RNase injection per rabbit using Freund's complete adjuvant stimulated sufficient antibody production against RNase from both cultivars for standard testing methods. Antisera against both enzymes (anti-$RNase_{D}$ and anti-$RNase_{TW}$) reached a peak passive hemagglutination titer of 4000 in 4–6 weeks. An 85 μg intravenous booster of RNase was administered 8–13 weeks later when the titer fell below 500; however, this did not result in a significant titer increase.

Pitt used 5.75 mg of potato RNase per rabbit to obtain antibody [5] and reported no passive hemagglutination titer values. It appears from our study that a 1 mg RNase injection scheme is adequate when limited quantities of antigen are available. The titer we obtained was sufficient for the serological tests conducted.

Specificity of antisera

Double immunodiffusion and immunoelectrophoresis were used to determine the specificity of both antisera for RNase. All antisera yielded a single precipitin band for both the purified homologous RNase and the homologous anionic protein fraction (APF) obtained in the first purification step. Nonspecific precipitation with either enzyme source using preimmunization sera did not occur. In both antigen–antibody systems the antigenic species in the APF samples corresponded in electrophoretic mobility and precipitin band shape to the purified RNase antigen. Therefore, evidence was obtained that 'pure' RNase was a good immunogen which resulted in antibody formation in rabbits that reacted with only one antigen in the APF samples. Recent results using immunoinhibition, to be reported elswhere, have shown that more than a 50% reduction in RNase activity occurred when desalted tissue extract was assayed in the presence of 4 mM EDTA and a 1/200 dilution of the anti-RNase sera. No activity reduction occurred when using the same dilution of preimmunization serum.

Evaluation of RNase purity was made by determining cross-reactivity of anti-RNase with enzymes active against the substrate *p*-nitrophenyl phosphate (*p*NPP). This compound may be a substrate for more than one enzyme in plants [7]. As experienced by Pitt with potato extracts [5], *p*NPP activity was found after each step leading to barley RNase purification [4]. A fraction of *p*NPP-active enzyme, free from RNase, was available after the final gel filtration step in $RNase_{TW}$ purification. When this enzyme fraction was tested against anti-RNase by immunodiffusion or immunoelectrophoresis, no precipitin bands were observed.

The short-term injection schedule was used to increase specificity for RNase [8], a procedure which may not detect low level contaminants present in the purified RNase sample. However, weekly bleedings up to 1 month after an 85 μg RNase booster resulted in no additional precipitin bands. These data suggest that the purification scheme reported by Wong [4] successfully eliminated detectable non-RNase protein, including rather tenacious enzymes which utilize *p*NPP as substrate.

To determine the specificity of anti-$RNase_{TW}$ and anti-$RNase_{D}$, both antisera were tested by double immunodiffusion against desalted soluble protein samples of spring barley (cv Primus II), winter wheat (cv Winoka), spring wheat (cv ERA), winter rye (cv Von Lockow) and spring oats (cv Wright). Only spring barley (Primus II) antigen formed a precipitin band with either antiserum. The apparent lack of cross-reactivity of RNase in the winter and spring wheat, rye and oat samples was attributed to either low antigen concentration or to antigenic dissimilarity.

Compared to immunodiffusion, rocket immunoelectrophoresis provides greater sensitivity and, therefore, the detection of smaller amounts of antigen. This technique was used for further evaluations of specificity. Soluble protein samples (50 μl) of spring barley, winter and spring wheat, rye and oats were electrophoresed against anti-$RNase_{TW}$ and against anti-$RNase_{D}$. Characteristic precipitin rockets were obtained only from spring barley, indicating that anti-$RNase_{TW}$ and anti-$RNase_{D}$ are specific for only *H. vulgare* RNase (Table 1).

Soluble protein samples obtained from several species of *Hordeum* were also tested for reactivity against anti-$RNase_{TW}$ and anti-$RNase_{D}$ by rocket immunoelectrophoresis. All antigen samples were 50 μl in volume containing a range of 18–25 μg protein per sample. Of the 8 *Hordeum* species tested, 3 showed typical precipitin rockets (Table 2). Three species showed markedly lower rocket heights than the Tennessee Winter and Dicktoo samples, and two species showed no reactivity. Results were the same for either antiserum.

Cross-reactivity of Dicktoo and Tennessee Winter antigens

By the use of double immunodiffusion, purified $RNase_{D}$, purified $RNase_{TW}$, APF_{D} and APF_{TW} samples were all found to be equally cross-reactive with the non-homologous anti-RNase serum. Precipitin bands showed serological identity for the above 4 RNase samples against both anti-$RNase_{D}$ and anti-$RNase_{TW}$ (S. B. Hawthorne, M. S. thesis, 1978). Serological identity indicates that the species possess similar antigenic determinant sites, but does not conclusively demonstrate that the antigens are physio-chemically identical [9].

Passive hemagglutination inhibition

Wong [4] has shown differences in reaction rates between $RNase_{D}$ and $RNase_{TW}$ when tested on dinucleoside substrates. The two enzymes were, however, indistinguishable by double immunodiffusion and immunoelectrophoresis. Passive hemagglutination inhibition was chosen as a more sensitive method to evaluate possible serological differences between the two enzymes. When using anti-$RNase_{TW}$ the minimum inhibiting concentration of $RNase_{D}$ was 2 μg/ml while for $RNase_{TW}$ it was 0.5 μg/ml indicating a very slight, but reproducible preference of the anti-$RNase_{TW}$ for $RNase_{TW}$. Anti-$RNase_{D}$ did not differentiate between

Table 1. Specificity of rabbit antisera obtained from the injection of purified RNase from barley cultivars Tennessee Winter (anti-$RNase_{TW}$) and Dicktoo (anti-$RNase_D$) when reacted against soluble protein samples from 4-day-old shoot tissue of various cereal plants*

Sample source	Growth habit	Anti-$RNase_{TW}$	Anti-$RNase_D$
Hordeum vulgare cv Dicktoo	winter	+	+
Hordeum vulgare cv Tenn. Winter	winter	+	+
Hordeum vulgare cv Primus II	spring	+	+
Triticum aestivum cv Winoka	winter	−	−
Triticum aestivum cv ERA	spring	−	−
Secale cereale cv Von Lockow	winter	−	−
Avena sativa cv Wright	spring	−	−

*The soluble protein sample of each tissue source was prepared by extracting 3 g of 4-day-old fr. shoot tissue in a final vol. of 9 ml. Five milliliters of this sample were desalted on Sephadex G-25 and 6 ml of the eluant showing the highest A_{280} was pooled. Fifty microliters (*ca* 22 μg crude protein) of the pooled eluant were tested using rocket immunoelectrophoresis. Both antisera were diluted 1:10 in the final agarose-gel casting. Reactivity comparable to Tennessee Winter and Dicktoo antigen is indicated by (+). Samples showing no cross-reactivity are indicated with a (−).

Table 2. Specificity of rabbit antisera obtained from the injection of purified RNase from barley cultivars Tennessee Winter (anti-$RNase_{TW}$) and Dicktoo (anti-$RNase_D$) when reacted against soluble protein from 4-day-old shoot tissue of various *Hordeum* species*

Sample source	Anti-$RNase_{TW}$	Anti-$RNase_D$
Hordeum vulgare cv Dicktoo	+ +	+ +
Hordeum vulgare cv Tenn. Winter	+ +	+ +
Hordeum irregulare	+ +	+ +
Hordeum agrocrithon	+ +	+ +
Hordeum stebbinsii	+	+
Hordeum spontaneum	+	+
Hordeum leporinum	+	+
Hordeum glaucum	−	−
Hordeum marimum	−	−

*Fifty microliters of a soluble protein sample (desalted on Sephadex G-25) extracted from 3 g of 4-day-old fr. shoot tissue of each species was used for the rocket immunoelectrophoresis sample (see Table 1). A magnitude of reactivity similar to the *H. vulgare* samples is indicated by (+ +) and no reactivity is indicated by (−). Samples indicated by (+) were cross-reactive, but showed significantly lower rocket heights than the *H. vulgare* samples.

the purified RNases by this test. Even though a relative difference was detected between the RNase from the two cultivars, it was concluded that these results did not absolutely prove that a structural difference exists for the two RNase antigens.

Rocket immunoelectrophoresis

Precise comparisons of the activity and amount of a particular RNase in different tissue extracts is complicated by the variability of nonspecific nucleases and possible differences in the efficiency of enzyme extraction. Johnson *et al.* [1] have shown RNase I activity in electrophoresed soluble protein samples from 4-day-old shoots to be much higher in the cultivar Tennessee Winter than in Dicktoo. Typically, 1.5–2 units of $RNase_{TW}$ are found per mg soluble protein of tissue extract per minute compared to 1 unit for $RNase_D$, when assayed with 4 mM EDTA, using calculations previously reported [2]. A similar difference in activity has been reported for other divergent phenotypes of winter barley [2], which illustrates a need to determine the actual amount of RNase in the tissue.

Pitt [5] explored the use of radial immunodiffusion to quantitate potato RNase. However, poor reproducibility and the fact that any error in measuring precipitin rings is squared in data interpretation suggested a need to explore another technique as an analytical method. Rocket immunoelectrophoresis [10–12] appeared to be a more suitable technique for quantitating RNase content of tissue. However, the comparatively high antigen concentrations needed and the small sample volumes allowed by existing methods initially prohibited the direct use of this technique

Since the sample is electrophoresed exhaustively it was speculated that the addition of a sample reservoir which would accommodate larger volumes might make this method suitable for dilute samples. The use of a template-reservoir allowed the use of sample volumes of at least 50 μl.

Rocket immunoelectrophoresis was used to determine the quantity of RNase. A standard curve was plotted relating log μg RNase to rocket height for purified samples of $RNase_D$ and $RNase_{TW}$ (S. B. Hawthorne, M.S. thesis, 1978). Linear results of the plotted data were obtained for both cultivars with 5 concentrations of RNase between 0.25 and 6.25 μg ($r^2 = 0.977$).

Using this method, the quantity of RNase I in desalted tissue extracts of each cultivar was shown to be essentially equivalent (Table 3), indicating that the rate of RNA degradation per unit of RNase I from 4-day-old tissue is much higher for $RNase_{TW}$ than for $RNase_D$. At each purification step including the final purified product [4], a higher level of activity is observed for $RNase_{TW}$ per unit of soluble protein. $RNase_{TW}$ activity in desalted extracts is also higher than $RNase_D$ [1], when assayed by the electrophoretic procedure [6]. Therefore, results in Table 3 suggests that regulation of RNase activity in seedlings of the two cultivars appears to be manifested by reaction rate rather than amount of enzyme in the tissue.

EXPERIMENTAL

Protein sample preparation. Ribonuclease was purified from 4-day-old seedling from each of two cvs of *H. vulgare* L. using the method reported in ref. [4]. Following centrifugation (105 000 g for 2.5 hr) and desalting on Sephadex G-25 (pH 7.4) the soluble protein fraction was subjected to Sephadex DEAE-A50 chromatography (0.05 M Tris–HCl, pH 7.4, 0.02 M KCl). The anion bound fraction was washed with eluting buffer equal to 1.5 vol. of the sample. The anion protein fraction (APF) containing RNase was removed from the ion exchanger with 0.6 M KCl. Sephadex DEAE-A50 was used again prior to preparative electrophoresis (Buchler Poly Prep. 200) and once after electrophoresis primarily for vol. reduction. Each of these ion exchange steps was preceded by desalting. Final purification was accomplished by Bio-Gel P-100 gel filtration. In each purification step the RNase fraction saved was determined by activity measurement [13], extent of separation from *p*-nitrophenyl phosphate (*p*NPP) activity and finally molecular size determined by gel filtration [4]. Small-scale soluble-protein samples were extracted as previously described [2]. Details of the RNase and *p*NPP activity assays are outlined in a previous report [2]. Protein was determined by either the method of ref. [14] or the procedure reported by ref. [15].

Antibody production. New Zealand white rabbits were each immunized once by injecting a total of 1 mg purified RNase protein dissolved in 1.5 ml 0.05 M Tris, pH 7.8, emulsified with an equal vol. of Freund's complete adjuvant. Equal injections (0.6 ml) were made in 5 different sites; intramuscularly in each hind flank, subcutaneously in each axillary region and a subcutaneous injection in the nape of the neck. The rabbits were bled by cardiac puncture starting 4-weeks after injection and following 18-hr starvation.

Immunological characterization. Passive hemagglutination, used to determine antibody titer, and passive hemagglutination inhibition using 25 μl of diluted antiserum in microtiter plates, was performed by the methods described in refs. [16] and [17]. For these tests formalized sheep red-blood cells (srbc) were sensitized with the RNase antigen by a standard method [18] using 30 μg protein per ml of 2.5% srbc. Micro adaptations of standard methods for immunodiffusion (ID), immunoelectrophoresis (IE), and rocket immunoelectrophoresis (RIE), [8–10] were employed. Agarose was used as the support medium in concns of 0.75% for ID and IE and 1% (final concn) for RIE. It was dissolved in either a Tris–barbitol, pH 8.6 buffer ($\frac{1}{2}$ strength Gelman buffer) for ID or 0.1 M Na_2HPO_4–KH_2PO_4, pH 7.6, for IE and RIE. Uniform agarose thickness was obtained by placing warm agar between two slides separated by Scotch No. 88 electrical tape fastened to the parallel edges of one slide. Two layers of tape were used for ID and 3 for IE and RIE. Pi buffer (0.1 M Na_2HPO_4–KH_2PO_4, pH 7.6) was used in the electrophoretic chamber. Sample spacing was achieved by using 6 mm thick Plexiglass templates for ID and RIE and a LKB cutter for IE. The use of a template having funnel-shaped wells (6 mm top and 1.5 mm bottom dia) for RIE provided the additional

Table 3. The quantity of RNase in 4-day-old shoot tissue of 2 barley cultivars, determined by rocket immunoelectrophoresis,* compared with the relative RNase activity†

Cultivar	μg Protein per g tissue	Rocket height (mm) Set 1	Rocket height (mm) Set 2	μg RNase per g tissue Set 1	μg RNase per g tissue Set 2	Units RNase per mg protein
Dicktoo	1530	17.5	17.6	49	49	1.4
	1550	18.2	17.8	52	50	1.4
Tenn. Winter	1550	18.0	17.5	51	49	2.7
	1670	18.0	18.2	51	52	3.0

*RNase was quantitated using antisera against 5 samples (0.25–6.25 μg) of purified $RNase_{TW}$. A standard curve was obtained by plotting log μg RNase against rocket height, which yielded a straight line having a $r^2 = 0.977$. Quantitation of RNase using antisera against purified Dicktoo RNase also showed nearly equivalent quantities in both cultivars. Fifty microliters of a soluble protein sample (desalted with Sephadex G-25) extracted from 3 g of 4-day-old shoot tissue of each cultivar was used for the rocket immunoelectrophoresis sample (see Table 1). The two sets of data presented represent two RNase determinations using duplicate protein samples from each cultivar.

†Activity determined in the presence of 4 mM EDTA and calculated per min on the basis of extracted soluble protein [2].

advantage of increasing sample sizes from 0.4 to 50 μl. Unprecipitated protein was removed from the slides by soaking in physiological saline prior to staining 10 min with 0.2% naphthol blue black (dissolved in 5:5:1 MeOH–H_2O–HOAc). The slide was then exhaustively destained in 4% HOAc.

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