RNase in *Lasallia hispanica* and *Cornicularia normoerica*: multiplicity of electromorphs and activity changes during a hydration–dehydration cycle

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Abstract

The presence of RNase activity has been detected in the two saxicolous lichen species, *Lasallia hispanica* (Frey) Sancho & Crespo and *Cornicularia normoerica* (Gunn.) DR. Activity was localized in the soluble fraction and had an acid optimum pH in both species. When proteins from the soluble fraction of the two lichens were separated by isoelectric focusing, multiple electromorphs with RNase activity were detected. *L. hispanica* RNase was separated into seven bands, characterized by pIs 7, 6.28, 4.58, 4.45, 4.25, 3.95, and 3.47. In *C. normoerica* four bands were detected, with pIs of 6.28, 3.98, 3.57, and 3.39. The molecular mass of the main RNase of *L. hispanica* estimated by SDS-PAGE was 31.86 kDa, which corresponds to the 33 kDa estimated for the undenatured RNase by gel chromatography. Proteins from *C. normoerica* were resolved by SDS-PAGE in three bands, with estimated molecular mass of 36.07 kDa, 31.86 kDa and 17.13 kDa. In order to improve the detection of RNase activity, gels were incubated after the run (electrophoresis or isoelectric focusing) in a RNA solution, instead of including the substrate in the gel. In both species, RNase activity increased during hydration and decreased during desiccation. This pattern of activity resembles that of other enzyme activities in lichens, which decrease in response to water deficits, and is different from the response of other poikilohydrous organisms such as bryophytes. These results are discussed in relation to the mechanisms that lichens have to withstand dehydration.

Key words: *Cornicularia*, *Lasallia*, lichens, ribonuclease, water stress.

Introduction

The RNA molecules are stable under physiological conditions. However, levels of the different molecular species of RNA, which are needed in tissues at a given moment of their development, are controlled in two ways: by regulation of RNA synthesis at the transcriptional level and by modulation of its hydrolytic breakdown. Therefore, knowledge of the RNA-splitting enzymes is pivotal for the understanding of a number of regulatory and developmental processes in which RNA molecules are involved (Farkas, 1982).

All RNA-splitting enzymes are esterases, but their specific mode of action varies depending on the way, exonucleolytic, of attacking the internal diester bonds of the polynucleotide chain. Moreover, some RNases can attack both RNA as well as DNA (Farkas, 1982).

The RNA-splitting enzymes have been described from a large variety of plant material and four main types have been distinguished in higher plants. The RNase I and RNase II groups include enzymes with an endonucleolytic mode of action, which degrade only RNA; differences between them are related to molecular weights, subcellular localization and optimum pH. The other two groups include enzymes that attack both RNA and DNA (Green, 1994). A RNase, belonging to the RNase I group has been isolated from *Chlorella* (Brown and Marshall, 1977). In plant tissues, the presence of nucleolytic enzyme activity has been detected in the nucleus, chloroplasts, ribosomal preparations, and mitochondria (Farkas, 1982; Green, 1994).

Changes in RNase levels occur at different stages of growth and differentiation in plants: development and germination of seeds, root development and senescence.
Plants also exhibit changes in RNase levels in response to a variety of different endogenous and exogenous stimuli (Green, 1994). Cellular injury, water stress, phosphate starvation, plant disease, and light are some of these factors. An increase in RNase is normally detected when plant cells are subjected to shrinkage of the protoplasm volume caused by a water deficit (Premecz et al., 1977).

It is noteworthy that the increase in RNase activity has been shown, on some occasions, to be partially due to the appearance of new isozymes (Baumgartner and Matile, 1977; Bryant et al., 1976). Moreover, in some cases, increases in RNase observed are not correlated with the decrease in RNA. In these cases, the RNase level can hardly be a controlling factor in the RNA breakdown. So, apart from their function in controlling RNA decay, other roles have been proposed for plant RNases: in the control of self-incompatibility, in P, remobilization during phosphate starvation or in plant defence against pathogen attack (Green, 1994).

So far, RNase activity has not been studied in lichens. As poikilohydrous organisms, lichens are able to withstand quick changes in hydric content, produced by the variable environmental conditions of their natural habitat. The aim of this study was to check the presence of RNase in lichens, to detect the different isozymes in isolectric focused gels and to study the changes in RNase during hydration-dehydration of lichen thalli.

Materials and methods

Plant material

L. hispanica (Frey) Sancho & Crespo and C. normoerica (Gunn.) DR. were collected in the Sierra de Guadarrama (Madrid, Spain). Thalli were air-dried and maintained in darkness at 10°C before use (one week at maximum).

Subcellular distribution

In order to determine the subcellular localization of RNase activity of L. hispanica, thallus cell-free extracts were fractionated. The lichen was washed in distilled water, pulverized in liquid N₂, homogenized in cold 0.05 M TRIS-HCl, pH 7.3, 0.25 M sucrose (10 ml of buffer per gram of dry thallus) and centrifuged 10 min at 1000 g below 2°C. The supernatant was filtered through a glass fibre filter (Whatman GF/A) and centrifuged 30 min at 26 000 g below 2°C. This supernatant was considered fraction F1. The sediment was washed with distilled water, centrifuged 30 min at 30 000 g below 2°C and the supernatant was considered fraction F2. Ribonuclease activity was measured in fractions F1 and F2 at pH 4.5, 6.5 and 8.0.

Enzyme extraction

To prepare crude enzyme extracts of L. hispanica and C. normoerica, lichen thalli were washed and homogenized as described above, but omitting the centrifugation at 1000 g. The thallus was washed in distilled water, pulverized in liquid N₂, homogenized in cold 0.05 M TRIS-HCl, 0.25 M sucrose and then centrifuged at 26 000 g for 30 min. The supernatant was filtered and used to measure RNase activity.

Crude extracts for isoelectric focusing and electrophoresis were prepared according to the method of Fahselt (1980) with some modifications. Proteins from thalli soaked for 15 min in distilled water were precipitated from the supernatant with (NH₄)₂SO₄ (saturation was 80%) and centrifuged at 23 000 g for 30 min. The pellets were resuspended in 3 ml 1% glycine and transferred to dialysis tubing which had been soaked in 1% glycine. After the dialysis the samples were lyophilized.

Enzyme activity

The reaction mixture contained 100 μl 0.5 M acetate buffer (pH 4.5), 50 μl 1% seroalbumin, 100 μl enzymatic extract and 250 μl 0.2% yeast RNA. RNA hydrolysis was allowed to proceed for 60 min at 37°C. Controls were prepared exactly, but omitting the incubation step. Blank was prepared with distilled water. The reaction was stopped by adding 500 μl perchloric acid/lanthanum solution, left at 0°C for 30 min and the precipitate discarded by centrifugation at 10 000 g below 2°C. The absorbance at 260 nm was measured in the supernatant. Each test was performed in duplicate and final ribonuclease was calculated as a mean of two values. The light absorption of the control mixtures was subtracted from the light absorption of the reaction mixtures. One unit (U) of activity was defined as the amount of RNase that, at 260 nm, releases one absorbance unit at 37°C, under the conditions of the assay.

Optimum pH

The optimum pH was determined by using 0.05 M buffers in the range 4.0–8.0 (pH 4.0–5.5 with sodium acetate buffer and pH 6.5–8.0 with phosphate buffer), under standard conditions.

Gel electrophoresis

Slab-gel electrophoresis on a discontinuous polyacrylamide gel was performed in the presence of SDS according to Laemmli (1970), using Miniprotein II (Biorad). The stacking and running gels were 4% and 12% acrylamide/bisacrylamide, respectively. An equal volume sample-loading buffer (4% [w/v] SDS, 21.4% [w/v] glycerol, and 0.01% [w/v] bromophenol blue in 125 mM TRIS-HCl buffer, pH 6.8) was added to each protein extract before electrophoresis. Mercaptoethanol was omitted because the presence of reducing agents had a negative effect on the detection of RNases in the gel assay (Yen and Green, 1991). Samples were heated at 100°C during 2 min and 4 μg of protein in 10 μl were loaded on to the gel. The experiment was performed with constant 200 V voltage. The gel was stained for total protein (silver stain, Biorad) and for RNase activity. Prior to activity staining, gels were treated as described by Blank et al. (1982) with some modifications: the gel was rinsed with 10 mM TRIS-HCl buffer, pH 8.0, at 20°C (2 × 15 min); isopropanol 25% (v/v) in 10 mM TRIS-HCl buffer, pH 8.0, at 20°C (2 × 15 min) and 10 mM TRIS-HCl buffer, pH 8.0, at 20°C (2 × 15 min). RNase activity in the gels was detected by negative staining with methylene blue: after the gel had been incubated during 60 min with 3 mg ml⁻¹ RNA (Sigma, R-7125) in 0.5 M acetate buffer, pH 4.5, at 37°C, and stained with 0.2% [w/v] methylene blue in 10 mM TRIS-HCl buffer, pH 8.0, the gel was destained with distilled water. The molecular mass standards (Sigma, kit for molecular weights MW-SDS-70) were lysozyme, 14.3 kDa; β-lactoglobulin, 18.4 kDa; trypsinogen, 24 kDa; pepsin, 34.7 kDa; egg albumin, 45 kDa; BSA, 66 kDa.

Isoelectric focusing

Isoelectric focusing of ammonium sulphate precipitated proteins from F1 fractions of L. hispanica and C. normoerica, was carried out in polyacrylamide gels (0.3 mm thick) with amphi-
lytes of a final pH range 2–11 (Serva), using LKB Multiphor II. During the run, the temperature was kept at approximately +4 °C with LKB-Multitemp II. Gels were pre-electrofocused for 45 min at a constant 15.0 mA. Samples (8 μg in 10 μl) were applied to the gel near the cathodal end, via a small tab of Whatman No 1 paper. Subsequent electrofocusing was carried out for 180 min at a constant 15 W with a maximum of 1500 V; sample application tabs were removed 30 min after focusing was begun. Broad pl Calibration Kit standards (Pharmacia) were used for pl estimation. The focused gels were stained for total protein (silver stain, Biorad) and RNase activity in the gels was detected by negative staining with methylene blue, as described above. In this study, individual bands were referred to as 'electromorphs' without reference to a particular allelic basis (Hageman and Fahselt, 1990).

**Molecular mass determination by FPLC**

Ammonium sulphate precipitated proteins from crude enzyme extracts of *L. hispanica* were dialysed against 1% glycine, lyophilized, resuspended with 0.05 M phosphate buffer, pH 7.4, 0.15 M NaCl and then loaded (0.15 mg of protein in a loop of 100 μl) on a Superdex 75HR1030 column equilibrated with the same buffer. Elution was achieved at a rate of 0.25 ml min⁻¹, with the same buffer at room temperature and fractions of 0.5 ml were collected. To estimate the molecular mass, the FPLC column was calibrated with standard proteins (Sigma, Molecular weight marker kit, MW-GF-70).

**Hydration–dehydration**

For the hydration process, lichens, after 15 min of soaking in distilled water, were placed for 24 h in a desiccator with an atmosphere of 100% relative humidity, on a thin sheet of sponge fully imbibed in water. The 100% relative humidity was achieved by filling the desiccator with distilled water, until it reached the bottom surface of the sponge. After 24 h of hydration, the water in the desiccator was removed and substituted by silica gel for the dehydration process. The experiment was performed at 10 °C, under a continuous light obtained from two 60 W Philips incandescent lamps (irradiance of 20 W m⁻² at the level of the lichens). At different intervals, lichen samples were taken out, weighed and assayed for RNase activity. To maintain an equal proportion between weight of thalli and extraction buffer volume, an adequate volume of distilled water was added to dehydrated thalli before homogenization. *RWC* (relative water content) of samples were calculated: \( \text{RWC} = 100 \times (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \), where DW = the dry weight of the tissue after constant weight has been reached in a 70 °C drying oven; TW is the maximum fresh weight reached when the tissue is soaked in water. The experiment was done in triplicate. Results of RNase activity were analysed using a Kruskal–Wallis one-way ANOVA by ranks (Colquhoun, 1971) to determine if differences existed between data from hydrated thalli and the dehydrated ones.

**Protein determination**

Protein was determined by Lowry's method (Lowry et al. 1951) or Bradford method (1976), using bovine serum albumin (Sigma) as a standard.

**Results**

**Localization and optimum pH for RNase of *L. hispanica* and *C. normoerica***

The RNase activity detected in *L. hispanica* was mainly present in the soluble fraction referred to as F1, showed an optimum pH at 4.5 (Table 1; Fig. 1A) and was not sensitive to EDTA (data not shown). When the activity was assayed in the F1 fraction from crude enzyme extracts of *C. normoerica*, the same preference for pH acid was exhibited (Fig. 1B). Levels of RNase activity in enzyme extracts of untreated thalli were in the same order of magnitude for both species of lichen (Fig. 2), especially when compared with another species of lichen, *Parmelia omphalodes* (data not shown).

**Hydration–dehydration test**

Considering that changes in RNase levels do usually occur in response to water stress, the effect of variations

<table>
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<tr>
<th>pH</th>
<th>F1</th>
<th>F2</th>
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<tr>
<td>4.5</td>
<td>99.72</td>
<td>0.27</td>
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<tr>
<td>6.5</td>
<td>99.46</td>
<td>0.53</td>
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<tr>
<td>8</td>
<td>99.72</td>
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**Table 1. Subcellular localization of RNase activity in Lasallia hispanica**

Untreated thalli were fractionated in two soluble fractions, F1 and F2. RNase activity was measured at different pHs and expressed as percentage of the total activity detected.

![Fig. 1](https://example.com/fig1.png)
Two peaks with RNase activity were eluted from the Superdex 75HR1030 column loaded with ammonium sulphate precipitated proteins from crude enzyme extracts of *L. hispanica* (Fig. 3). The main RNase showed a molecular mass of 33 kDa (Fig. 4).

**Isoelectric focusing and gel electrophoresis**

Ammonium sulphate precipitated proteins from crude enzyme extracts of untreated thalli of both lichen species were separated according to their pI by isoelectric focusing and according to their molecular mass by SDS–PAGE, and gels were stained for proteins and for RNase activity. For the detection of RNase activity, it was preferred to incubate the gels after the run in a RNA solution, instead of including the substrate in the gel, because better results were obtained. Moreover, this modification also allowed us to detect the bands of RNase activity in isoelectric focusing and gel electrophoresis.
focusing gels. The crude enzyme of *L. hispanica* was separated into seven bands, characterized by pIs 7, 6.28, 4.58, 4.45, 4.25, 3.95, and 3.47 (Fig. 5C). In *C. normoerica* four bands were detected with pIs of 6.28, 3.98, 3.57 and 3.39 (Fig. 5B). The most intense staining for activity in *L. hispanica* was obtained for the four bands in the pH range 3.95 to 4.58. In *C. normoerica* the main staining corresponded to two bands with pIs 3.57 and 3.98.

The SDS–PAGE of *L. hispanica* proteins showed one band with RNase activity stain whose estimated molecular mass is 31.86 kDa (Fig. 6) which corresponds to that estimated molecular mass by gel chromatography on the Superdex 75HR1030 column of the undenatured RNase (Fig. 3). *C. normoerica* proteins were resolved in two more marked bands, with estimated molecular mass of 36.07 kDa and 31.86 kDa and a minor one with 17.13 kDa.

**Discussion**

RNase activity has been detected in two species of saxicolous lichens with different biotypes, *L. hispanica*, biotype umbilicate, and *C. normoerica*, biotype fruticose. The RNase activity of both lichens was mainly present in the soluble fraction as in other organisms. The major part (up to 80–90%) of nucleolytic activity in plant cell free extracts can usually be recovered in the soluble fraction; this RNase activity is often referred to as 'cytosolic', although enzymes localized in easily disruptible compartments, like vacuoles and chloroplasts, certainly represent major sources of the 'cytosolic' enzyme fractions (Abel and Glund, 1986; Boller and Kende, 1979; Farkas, 1982). So, RNase of the lichen species used in this study, could proceed from the cytosol or from some subcellular compartment because most nucleolytic enzymes can be solubilized even if they are attached to or contained by cell organelles.

The acid optimum pH, together with its localization in the soluble fraction, low molecular mass and insensitivity to EDTA, could suggest that this activity is a RNase belonging to the type I. RNases type I are soluble endoribonucleases with a pH optimum of 5.0–5.5 and a molecular weight of 20–25 kDa; they produce 3'-nucleotide products (EC 3.1.27.1), and are not sensitive to EDTA (Green, 1994). However, due to the lack of substrate specificity assays, these conclusions must be

![Fig. 5. Isoelectric focusing of proteins from *L. hispanica* and *C. normoerica*. Ultrathin layers (0.3 mm) of polyacrylamide with ampholytes pH 2–11 were used. Proteins from thalli soaked for 15 min in distilled water (8 μg in 10 μl) were applied to the gel near the cathodal end and temperature was kept at approximately +4°C. The gel was stained for total protein (silver stain, Biorad) and for RNase activity (negative staining with methylene blue). Lane A: silver staining of proteins from Broad pl Calibration Kit (Pharmacia). Samples of 3 μg of protein in 3 μl of 1% glycine were applied. Lane B: ammonium sulphate precipitated proteins (8 μg in 10 μl) from F1 fraction of *C. normoerica*, stained for RNase activity. Lane C: ammonium sulphate precipitated proteins (8 μg in 10 μl) from F1 fraction of *L. hispanica*, stained for RNase activity.](https://academic.oup.com/jxb/article-abstract/47/12/1927/545648)
interpreted with caution. Moreover, many plant RNA-degrading enzymes cannot be easily classified into one of the four main classes as the distinction between the classes is not always clear.

The electromorphs pattern suggests that in lichens there exists multiple RNases differing one from another in isoelectric point and electrophoretic behaviour, as occurs in other organisms (Kazmierezak and Knypl, 1994). An acid RNase is broadly present in plant tissues (Blank and McKeon, 1991a, b). Both species have a RNase pattern with acid and neutral electromorphs. The two lichens present different electromorph patterns; only one band with an isoelectric point of 6.28 and one band with 31.8 kDa are common. The great diversity in isoelectric points observed, especially in \textit{L. hispanica}, may be due to the dual nature of lichens. The same enzyme may have a different isoelectric point in the algae and the fungus; moreover a certain strain of lichen may contain different algal species/strains and the electromorphs can belong to the different systems (Skult et al., 1986). Moreover electromorph patterns can be modified in different physiological situations. In lichens, as in higher plants, seasonal variation has often been shown to modify the isozyme banding pattern and enzymatic activity (Skult et al., 1986, 1990).

One would expect a close correlation between the mode of action of a nucleolytic enzyme and its physiological function. Strangely enough, the large amount of work devoted to the isolation and characterization of plant nucleases is not very illuminating in this respect (Farkas, 1982). The knowledge of which RNases participate in mRNA decay in plants and other higher eukaryotes is extremely limited. The half-lives of different transcripts vary over a wide range in plants, so the decay process must be highly regulated and RNases probably do not play a primary role in it (Green, 1994). Several RNases, differing in molecular size or isoelectric point, are usually present in a single organism and they increase or decrease from the normal, vegetative status to a stress situation. However, at present no correlation has been shown between changes in the level of a particular RNase and a decay process, which could assign to the enzyme a primary role in its control. In \textit{L. hispanica} and \textit{C. normoerica}, RNase activity does not increase during desiccation, as it has been shown to increase in bryophytes and other organisms that can withstand dehydration (Bewley and Krochko, 1982; Gniazdowska-Skoczek and Bandurska, 1994). The changes in RNase activity in these two lichen species follow the same pattern as other enzyme activities in lichens: they increase during hydration, as lichens have to survive drought and re-establish metabolic activity after each rehydration, and decrease during dehydration because metabolic activity comes to a standstill during dry periods (Yagüe and Estévez, 1988). The same behaviour has been observed in the saxicolous lichen \textit{Parmelia omphalodes} (data not shown). The fact that a significant RNase activity is present in extracts from dry lichens implies that, at least some forms of the enzymes are
stable. If a particular RNase plays a role in desiccation in these lichens, its increase is probably obscured by the decrease in general RNase activity.

It is also possible that drought tolerance is achieved in lichens by mechanisms that do not include RNase intervention. The drought responses of the whole lichen thallus are, of course, a combination of algal (photobiont) and fungal (mycobiont) components. Water deficit stress induces changes in photosynthesis that reflect the metabolic state of the algal component (Bewley and Krochko, 1982). Changes in respiration in relation to drought stress in lichens are well documented as well as the importance that polyols have during wetting and drying cycles (Gupta, 1991). In the bryophyte Tortula ruralis, after rewetting, an increase in superoxide dismutase activity has been detected, which is related with the increased oxygen uptake observed (Krochko et al., 1979). However, this mechanism seems not to operate in the lichen Peltigera polydactyla (Brown et al., 1983).

At present, the proteins from hydrated and dehydrated thalli are being separated by isoelectric focusing in order to detect some differences in bands of RNase activity. If physiological adaptation to water deficit involves the participation of a particular RNase, the knowledge of the variations in the electromorphs pattern will help in the understanding of the process.

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References


