CHANGES IN TREHALASE ACTIVITY ARE ASSOCIATED WITH THE HYDRIC STATUS OF *Selaginella Lepidophylla* PLANTS

CAMBIOS EN ACTIVIDAD DE TREHALASA ESTÁN ASOCIADOS A CAMBIOS EN EL ESTATUS HÍDRICO EN *Selaginella Lepidophylla*

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ABSTRACT

Selaginella lepidophylla is a desiccation-tolerant plant known for its ability to desiccate, curl up and survive long dry periods, due its ability to accumulate large amounts of trehalose. Dry *S. lepidophylla* plants were hydrated over 24 hours, and the plants showed fast hydration kinetics. Plants reached a relative water content (RWC) of 48 % after 4 hours of hydration. Fully hydrated plants under dehydration conditions exhibited less accelerated water loss kinetics, with an RWC of 50 % after 8 hours of dehydration. Dry plants exhibited neutral and acid trehalase activity. Maximum neutral trehalase activity was reached at RWC = 48 %, while for acid trehalase activity, the maximum was reached when plants were fully hydrated. Plants subjected to the dehydration treatment for 24 hours exhibited neutral trehalase activity and low acid trehalase activity, with the maximum neutral trehalase activity at RWC = 85 %, and maximum acid trehalase activity at RWC = 50%. The patterns of neutral and acid trehalase activity were closely related to trehalose concentration. It was demonstrated that *S. lepidophylla* plants synthesize acid and neutral trehalase, the activities of which change during water uptake and loss, and this was reflected in the concentration of trehalose.

Keywords: Desiccation tolerance, osmolyte, osmoprotection, resurrection plant, trehalose

RESUMEN

Selaginella lepidophylla es una planta tolerante a la desecación, conocida por su habilidad para sobrevivir largos periodos de sequía, debido a su capacidad para acumular niveles altos de trehalosa. Plantas secas de *S. lepidophylla* hidratadas durante 24 horas mostraron una rápida cinética de hidratación. Las plantas alcanzaron un 48 % de Contenido Relativo de Agua (CRA) a las 4 horas de hidratación. Plantas totalmente hidratadas mostraron una cinética de deshidratación menos acelerada, presentando un CRA del 50 % a las 8 horas de deshidratación. Las plantas secas mostraron actividad de trehalasa neutra y ácida. La actividad máxima de trehalasa neutra se alcanzó a un CRA de 48 %, mientras que el máximo para trehalasa ácida se encontró en plantas totalmente hidratadas. Las plantas sometidas a deshidratación durante 24 horas mostraron actividad de trehalasa neutra y baja actividad de trehalasa ácida. Se encontró un máximo de actividad de trehalasa neutra a un CRA del 85 % y del 50 % para trehalasa ácida. El patrón de actividad enzimática varió en relación con la concentración de trehalosa. Se demostró que la actividad de trehalasa cambia durante la toma y pérdida de agua y qué estos cambios se reflejan en la concentración de trehalosa en plantas de Selaginella.

Palabras clave: Tolerancia a la desecación, osmolito, osmoproteción, planta de resurrección, trehalosa

INTRODUCTION

Selaginella lepidophylla (Hook, and Grev. Spring) is a pteridophyte resurrection plant known by several names, including Rose of Jericho, stone flower, siempre viva and doradilla, among others, owing to its ability to survive while desiccated over long dry periods. *S. lepidophylla* is a desert plant, found from Texas and Arizona southwards to El Salvador. Resurrection plants can lose up to 95 % of their water content, but rehydrate upon watering and are restored to full functionality because their ability to accumulate high concentrations of sucrose and trehalose (Adams *et al*., 1990; Drennan *et al*., 1993).

S. lepidophylla and many anhydrobiotic organisms accumulate trehalose which is thought to protect organisms from severe desiccation stress owing to its capacity for water replacement, glass formation and chemical stability (Crowe *et al*., 1984; Crowe *et al*., 1998). In *S. lepidophylla* trehalose synthesis is catalyzed by trehalose-6-phosphate synthase (TPS) and trehalose-6-phoshate phosphatase (TPP) (Valenzuela-Soto *et al*., 2004).

Trehalose synthesis and its physiological function have been widely studied for several organisms because of the key role as a stress protectant during desiccation, heat stress, freezing, hypoxia, and as a carbon and energy source for bacteria, fungi, plants, and invertebrates (insects, nematodes, etc) (Crowe *et al*., 1984; Drennan *et al*., 1993; Elbein *et al*., 2003; Gadd *et al*., 1987; Goddijn and van Dun, 1999; Hottiger *et al*., 1994; Iordachescu and Imai, 2008; Strom and Kaasen, 1993; Valenzuela-Soto *et al*., 2004; Wiemken, 1990).

Trehalose is hydrolyzed by trehalase (EC 3.2.1.28), which converts the disaccharide into two glucose molecules. This enzyme is widely distributed in microorganisms, inver-

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tebrates and vertebrates (de Aquino *et al*., 2005; Londesborough and Varimo, 1984; Mitsumasu *et al*., 2005; Müller *et al*., 1994; Müller *et al*., 2001; Nakano *et al*., 1977). Different trehalases have been found in bacteria, yeast, fungi, insects and mammals. According to their optimal activity pH, trehalases can be classified as acid or neutral. Acid trehalase has been mainly found at the cell surface (cell wall or plasma membrane), whereas neutral trehalase is a soluble protein located in the cytoplasm (Aesbacher *et al*., 1999; de Aquino *et al*., 2005; Frison *et al*., 2007; Lucio *et al*., 2000; Müller *et al*., 2001).

Although trehalases have been purified and characterized from bacteria, yeast and other fungi (de Aquino, 2005; Inagaki *et al*., 2001; Mittenbühler and Holzer, 1988; Santos Zimmermann *et al*., 1990), invertebrates (Dmitryjuk and Zóltowska, 2003; Lee *et al*., 2001), mammals (Ruf *et al*., 1990), and legume nodules (Müller *et al*., 1992; Müller *et al*., 1994; Aesbacher *et al*., 1999; Tejera *et al*., 2005), few reports describe the purification and characterization of this enzyme from plants (Frison *et al*., 2007).

In resurrection plants, trehalose concentration changes during the hydration/desiccation process, however the regulation of trehalose metabolism has not been studied comprehensively. In this study the aim was 1) to identify the type of trehalase present in *S. lepidophylla*, and 2) determine if the levels of enzyme activity are related to trehalose concentration during hydration-dehydration processes.

MATERIALS AND METHODS

Plant Material

S. lepidophylla plants were collected from arid regions in the state of Morelos in Mexico. Plants were kept at room temperature in darkness.

Hydration and Dehydration Kinetics

Dry plants were hydrated over 24 h in distilled water, with a photoperiod of 10/12 h light-dark at 25°C. Three plants were used for each determination; a sample was collected from each plant at the beginning and 1, 2, 4, 8, 12 and 24 h into the hydration treatment. Fully hydrated plants were dehydrated over 24 h under the conditions described above and a sample from each plant was collected at the beginning and 1, 2, 4, 8, 12 and 24 h into the dehydration treatment.

Measurement of Relative Water Content

Relative water content (RWC) was measured from one gram of stem tissue. Samples were weighed (fresh weight) and then rehydrated over a period of 24 hours in a Petri dish containing strips of wet polyurethane foam; the Petri dish covered with a black sheet of plastic. At the end of the rehydration period, the stems were weighed (saturated weight) and left to dry in an oven at 62 ° C until constant weight was attained (dry weight). To calculate RWC, following formula was used:

RWC = (fresh weight – dry weight)/(saturated weight - dry weight) x 100.

All measurements were performed in triplicate.

Preparation of Cell-free Extracts

Enzyme extracts were obtained from each hydration and dehydration point. Two grams of plant tissue was homogenized with mortar and pestle at 4 °C with one of the following ice-cold buffers in a 2:1 (w/v) ratio: 50 mM HEPES, pH 7 (neutral trehalase) or 50 mM citrate pH 5 (acid trehalase), 10% (w/v) PVP80, and 0.05 mL protease inhibitor mix (AEBSF, PMSF, bestatin, pepstatin A, leupeptin). Extracts were filtered through six layers of Miracloth and centrifuged at 34,354 x *g* at 4°C for 40 min. The supernatant was filtered through a 10 kDa membrane.

Enzyme Activity Assay

Assay mixtures contained 100 mM trehalose and 0.1 mL enzyme extract in a total volume of 0.12 mL. Assay mixtures were incubated at 30 °C for 30 min, and the reaction was stopped by heating at 100 °C for 3 min. Afterwards, samples were kept in ice for 10 min and centrifuged at 34,354 x *g*. Glucose release was measured by the glucose oxidase-peroxidase method using a commercial kit (Sigma-Aldrich) according to the manufacturer's instructions. Each determination was done in triplicate. One unit of trehalase was defined as the amount of enzyme producing 2 µmol of glucose per minute at 30 °C and pH 7.0 or pH 5.0.

Trehalose Extraction

Aqueous or ethanol extracts were obtained from each hydration and dehydration point. *S. lepidophylla* stems (2.5 g) were homogenized in 15 mL H_2O or ethanol (80% v/v), and incubated for 8 h at 50°C. The suspension was centrifuged at 10,000 x *g* at 25 °C for 30 min, and the supernatant used to determine trehalose concentration by HPLC with the method described by Vázquez-Ortiz and Valenzuela-Soto (2004).

RESULTS AND DISCUSSION

Hydration and Dehydration Kinetics

The RWC of dry *S. lepidophylla* plants was 5 %, and after adding water for 6 h plants showed fast hydration kinetics, reaching 48 %, after 2 more hours, and achieving their maximum RWC of 96 % at 24 hours (Fig. 1A). *S. lepidophylla* plants are fully extended and green at 48 % RWC; therefore it is assumed that photosynthesis is operating.

S. lepidophylla plants in the dehydration treatment exhibited slow water loss kinetics: after 8 h of treatment RWC was 50 % (Fig. 1B) and at 24 h it was 5 % (Fig. 1B).

The relative water content (RWC) of tissues has been used as an indirect measure of cell volume and as a parameter of water status (Merchant *et al*., 2007). In a previous study of *S. lepidophylla*, we demonstrated that RWC was correlated with hydric potential measured with a Scholander pump; both methodologies showing fast hydration kinetics (Figueroa-Soto *et al*., 2004).

Trehalase Activity During Plant Hydration

Dry plants (RWC = 5 %) showed acid and neutral trehalase activity. Neutral activity was 0.054 µmoles/min/gDW,

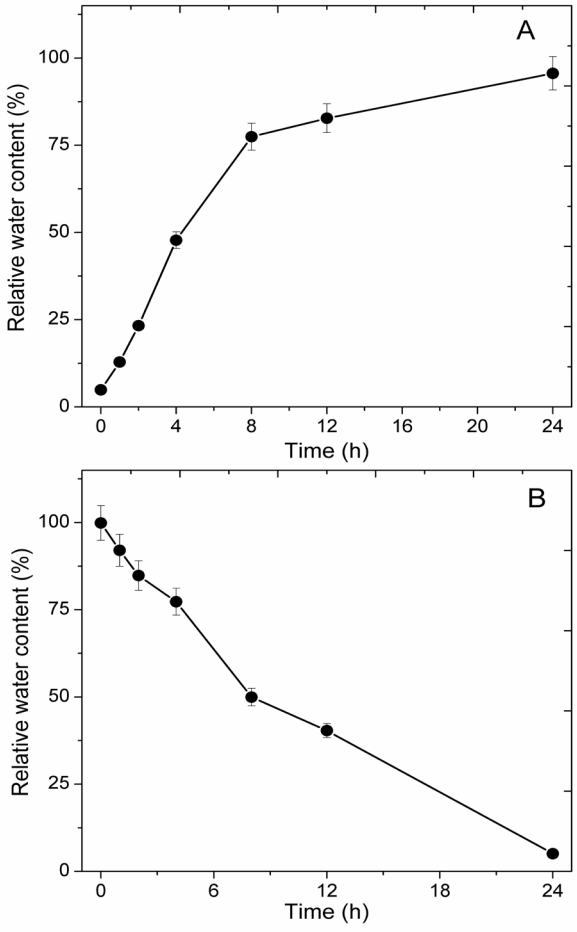

Figure 1. Effect of water uptake on the relative water content of *S. lepidophylla* plants. A) Hydration treatment; B) Dehydration treatment. Each point is the mean ± S.D. for three independent experiments.
Figura 1. Efecto de la toma de agua en el contenido relative de agua en plantas de *S. lepidopylla*. A) Tratamiento de hidratación; B) Tratamiento de deshidratación. Cada punto es la media ± D.S. para tres experimentos independientes.

whereas acid activity was 0.057 µmoles/min/ gDW (Fig. 2). In desiccated plants, ten enzymes involved in carbohydrate metabolism, as well as RUBISCO, were active in *S. lepidophylla* (Eickmeier, 1982; Harten and Eickmeier, 1986).

Neutral trehalase activity increased linearly with RWC during the first four hours of hydration (Fig. 2). Acid trehalase activity had a more variable pattern, increasing and decreasing throughout the hydration process (Fig. 2). From plant 48% RWC, acid trehalase activity increased until it reached its maximum (RWC = 97%) (Fig. 2). Maximum neutral trehalase activity was 0.21 µmoles/min/gDW at RWC = 48 % (Fig. 2), while the acid trehalase activity maximum was 0.62 µmoles/min/gDW when plants reached RWC = 96% (full hydration) (Fig. 2).

Changes in Trehalose Concentration During Plant Hydration.

Trehalose concentration in dry plants (RWC = 5 %) was 12 mg/gDW. This increased to 14 mg/gDW, coinciding with a decrease in acid trehalase activity and with an increased neutral trehalase activity at RWC = 13 % (Fig. 2). Trehalose concentration increased (43 mg/gDW) at the same time there was a decrease in acid trehalase, but neutral trehalase activity increased slightly. As acid trehalase activity increased, trehalose concentration decreased to 34% (Fig. 2).

Trehalose has been proposed as source of carbon skeletons for anabolism and as a source of glucose for energy (Elbein *et al*., 2003). Harten and Eickmeier (1986) demonstrated that several respiratory enzymes are active in *S. lepidophylla*, in both desiccated and fully hydrated fronds, and these enzymes require glucose during the hydration process, most likely, we think, from trehalose hydrolysis carried out by trehalase. Interestingly, trehalose was not fully hydrolyzed when plants reached 97% RWC. Adams *et al*. (1990) suggested that fully hydrated *S. lepidophylla* plants synthesize and accumulate trehalose to prepare for drought stress. Our data supports this idea since fully hydrated plants contain 34 mg/gDW trehalose (Fig. 2).

Trehalase Activity During Plant Dehydration

The dehydration treatment was carried out with plants that had been hydrated for over 30 h. Fully hydrated plants (RWC = 99%) had a neutral trehalase activity of 0.62 µmoles/min/gDW; this value increased and decreased during the dehydration treatment (Fig. 3). Neutral trehalase maximum activity occurred at RWC = 85 % (1.05 µmoles/min/ gDW). Later it peaked again at RWC = 50 % to finally decrease at RWC = 5 % to its minimum activity (0.06 µmoles/min/gDW) (Fig. 3).

For acid trehalase, fully hydrated plants exhibited low enzyme activity (0.24 µmoles/min/ gDW) compared to neutral trehalase. Acid trehalase maximum activity was 0.5 µmoles/min/gDW at RWC = 50 %, and decreased again to no more than 0.14 µmoles/min/gDW in plants with 5% RWC (Fig. 3).

During the desiccation process of *S. lepidophylla* plants, neutral and acid trehalase seem to complement each other before reaching RWC = 50%, when the activity of both enzymes peaked (Fig. 3). Neutral trehalase activity was higher than that of acid trehalase, but dropped to a lower level at RWC = 5 %.

Changes in Trehalose Concentration During Plant Dehydration

Fully hydrated plants had 34 mg/gDW trehalose, and trehalose concentration decreased (28 mg/gDW at RWC = 85%) during the first two hours of dehydration process (Fig. 3). As RWC was descending trehalose concentration increased until it reached 50 mg/gDW at RWC = 50% (Fig. 3).

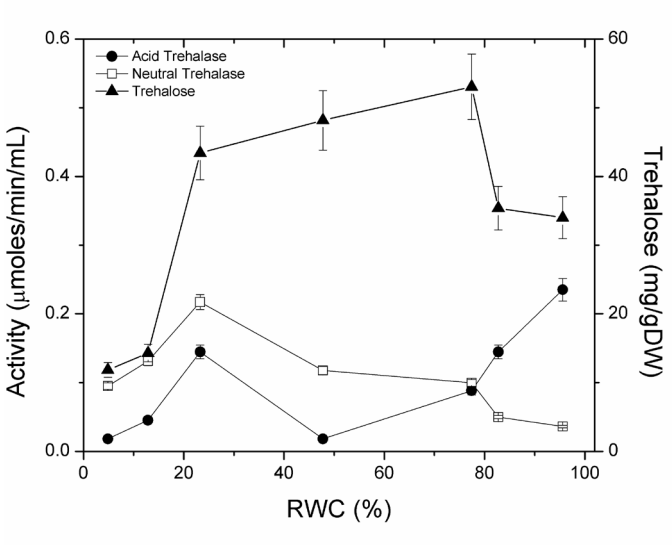

Figure 2. Hydration effect on trehalase activity and trehalose content of *S. lepidophylla* plants. Each point is the mean ± S.D. for three independent experiments.
Figura 2. Efecto de la hidratación en la actividad de trehalasa y el contenido de trehalosa en plantas de *S. lepidophylla*. Cada punto es la media ± D.S. para tres experimentos independientes.

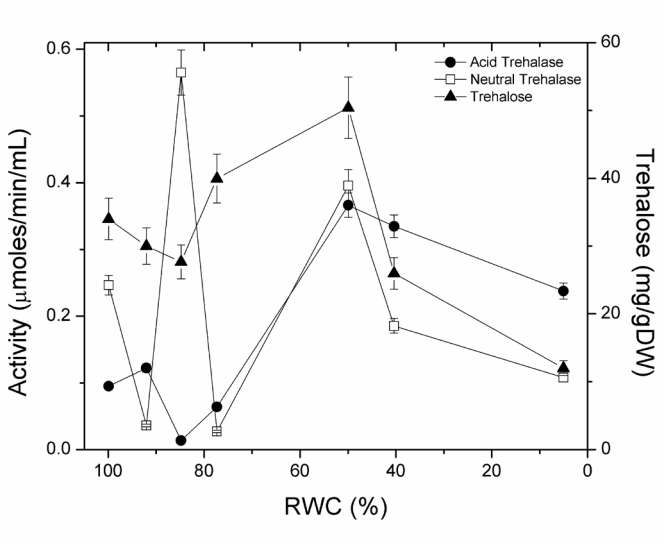

Figure 3. Effect of dehydration on trehalase activity and trehalose content of *S. lepidophylla* plants. Each point is the mean ± S.D. for three independent experiments.
Figura 3. Efecto de la deshidratación en la actividad de trehalasa y el contenido de trehalosa en plantas de *S. lepidophylla*. Cada punto es la media ± D.S. para tres experimentos independientes.

After reaching its maximum value, trehalose concentration decreased to 12 mg/gDW at RWC = 5% (Fig. 3). Trehalose concentration increased even though the plant showed activity of both of the trehalases, however the activity of both was low compared with that determined during hydration process. Previous studies showed that trehalose 6-phosphate synthase activity increased with desiccation in *S. lepidophylla* plants (Figueroa-Soto *et al*., 2004), which can explain the increase in trehalose concentration.

There are some reports on the biochemical changes that occur in *S. lepidophylla* during desiccation and rehydration; however they were measured at only one point in time (Eickmeier, 1982; Harten and Eickmeier, 1986; Pandey *et al*., 2010). In our study, the hydration and desiccation processes were recorded over a 24 h period. Changes in enzyme activity coincided with changes in trehalose concentration. It was interesting to find two trehalase activities, each with a different activity pattern, which suggests that as water availability changes in the plant there are changes in pH in the cell compartments where trehalose is metabolized.

In a previous study we found that trehalose concentration increased when TPS activity increased (Figueroa-Soto *et al*., 2004), and in the present study trehalose concentration decreased when trehalase activity increased. The changes in TPS, and in neutral and acid trehalase were closely related to the hydric status of the *S. lepidophylla* plants, suggesting that plant hydric status is being sensed by the plant in some way. However, we found no information related to how *S. lepidophylla* senses changes in its water status.

CONCLUSIONS

S. lepidophylla plants exhibited neutral and acid trehalase activities, and these activities changed as consequence of the hydric status of the plant. In *Selaginella lepidophylla* the dehydration process appears to follow a different route than that used for hydration, and this requires further investigation. The pattern of enzyme activity was closely related to trehalose concentration.

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