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Potent cryoprotective activity of cold and CO_2-regulated cherimoya (*Annona cherimola*) endochitinase☆

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ABSTRACT

A cryoprotective chitinase (BChi14) was isolated and purified from the mesocarp of CO_2-treated cherimoya fruit (*Annona cherimola* Mill.) stored at chilling temperature by anion exchange and chromatofocusing chromatography. This hydrolase was characterized as an endochitinase with a M_r of 14.31 kDa and a *pI* of 8.26, belonging to the family 19 of glycosyl hydrolases (GH19). While it was stable over a wide pH range and active in a broad acidic pH range, it had an optimum pH of 7.0. Its optimum temperature was low, 35 °C, and it retained about 30% of its maximum activity at 5 °C. Moreover, BChi14 was relatively heat unstable and its activity was progressively lost at temperatures above 50 °C. Kinetic studies revealed many similarities with other plant endochitinases. However, BChi14 had high k_{cat} (6.93 s^{-1}) value for the fluorogenic substrate 4-MU-$(GlcNAc)_3$, reflecting its great catalytic efficiency. Moreover, a thermodynamic characterization revealed that the purified enzyme displayed a high k_{cat} at 37 and 5 °C, and a low E_a (11.32 kJ mol^{-1}). *In vitro* functional studies indicated that BChi14 had no effect on the inhibition of *Botrytis cinerea* hyphal growth and no antifreeze activity, as shown by the thermal hysteresis analysis using differential scanning calorimetry. However, the purified endochitinase showed very strong cryoprotective activity against freeze–thaw inactivation of lactate dehydrogenase. The PD_{50} was 12.5 times higher than that of the cryoprotective protein BSA, and 2 or 3 orders of magnitude greater than sucrose, comparable with that of most cryoactive plant dehydrins. These results, together with the consolidated microstructure and the integrity of CO_2-treated mesocarp tissue, indicate that BChi14 is functionally implicated in the mechanisms underlying chilling tolerance activated by high CO_2 concentrations.

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1. Introduction

Cold stress is a major environmental stress that limits crop productivity and quality, as well as the postharvest storage life of fruit and vegetables. Many crops and horticultural plants that originate from tropical and subtropical regions are susceptible to damage when exposed to low but positive cold temperatures, causing a set of alterations known as chilling injury. This physiological disorder may occur at temperatures below 15 °C, but by definition, chilling occurs in the absence of ice nucleation in the plant cell (Levitt, 1980). During crop chilling, a complex response to enhance chilling tolerance occurs at the cellular, physiological and developmental levels. Plants have developed an array of genes and biochemical-molecular mechanisms that help them minimize the negative effects of cold stress (Thomashow, 1999). Some of these reactions to cold stress involve the *de novo* synthesis of specific stress-associated peptides and proteins, or the quantitative changes in the content of constitutive proteins. Some such proteins are enzymes involved in carbohydrate metabolism, membrane lipid modifications or in antioxidant defense (Renaut et al., 2006), while others may be antifreeze proteins (AFP: Griffith and Yaish, 2004), cryoprotective proteins like dehydrins (Rorat, 2006) or pathogen-related proteins (PR: Van Loon et al., 2006). Most of these chilling-induced proteins help protect the cell, especially by stabilizing macromolecules and membranes. Alternatively, another specific adaptation to maintain sustainable activity at permanently low temperatures is to produce cold-adapted enzymes with enhanced catalytic potential. This approach is more common in organisms that live at constantly low temperatures and that have therefore had to evolve a higher catalytic efficiency at such temperatures to maintain an adequate metabolism in their environment (Feller and Gerday, 1997).

PR proteins have been identified in numerous plants and they are considered to be ubiquitous in the plant kingdom. These defense-related enzymes accumulate in the apoplastic spaces and

Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; LDH, lactate dehydrogenase; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PD_{50}, protein dosage that renders 50% of cryoprotection; PMF, peptide mass fingerprinting; PR, pathogenesis-related.

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vacuoles of various plant cells in response to both pathogen infection and different kinds of abiotic stress. The best known and most extensively studied examples are members of the PR protein families 2 and 3, which have been functionally identified as 1,3-β-glucanases and chitinases, respectively. There is compelling evidence that 1,3-β-glucanases and chitinases contribute to plant defenses against fungal infection, acting alone or in combination (Neuhaus, 1999). Although the defensive function of chitinases and 1,3-β-glucanases has been the focus of most research, additional functions unrelated to their hydrolytic activity have also been described in plants (Van Loon et al., 2006). In this context, chitinase and 1,3-β-glucanase enzymes that are induced in response to cold and low temperatures exhibit antifreeze (Griffith and Yaish, 2004) or cryoprotective activity *in vitro* (Hincha et al., 1997; Fernandez-Caballero et al., 2009). Generally, hydrolytic PR enzymes can exert a dual function, involving both direct degradation of their substrate and cold protection. Indeed, the presence of these proteins has been strongly correlated with tolerance to cold stress.

Protein chitinases have been studied from a variety of plant sources, although they have not often been purified and characterized from fruit tissues. Most fruit chitinases have been purified from mesocarp tissues of the mature fruit, representing constitutively expressed acidic proteins of between 26 and 34 kDa (Sowka et al., 1998; Sanchez-Monge et al., 1999; Li et al., 2003; Chen et al., 2007). However, little is known about the function of these enzymes during postharvest storage of fruit producing crops. Cherimoya is a highly perishable climacteric fruit that can suffer irreversible chilling injury when the postharvest temperature drops below 8 °C. Nevertheless, short-term treatment with high concentrations of CO_2 enhances the chilling tolerance of this fruit, reducing chilling injury during postharvest storage (Merodio et al., 1998; Maldonado et al., 2002). In addition, cherimoyas were recently shown to contain different acidic and basic chitinases, as well as 1,3-β-glucanase-like isoenzymes. Some of these enzymes are expressed constitutively, while others are induced and highly regulated by both atmospheric storage conditions and the length of exposure to chilling temperatures (Goñi et al., 2009). In particular, short-term CO_2-treated cherimoyas stored for long periods at 6 °C accumulate significant quantities of an induced low molecular mass chitinase (BChi14), thought to be related with the cryoprotective activity in the basic protein extracts.

The aim of the present work was to purify, identify, and biochemically and functionally characterize the activity of the CO_2-low temperature induced BChi14 chitinase in order to assess its role in the tolerance to low temperature in chilling-sensitive fruit. Moreover, the relationships between the induction of this protein and the cellular changes taking place in the structure of CO_2-treated tissues under prolonged cold-storage conditions were studied by low-temperature electron microscopy (LT-SEM).

2. Materials and methods

2.1. Plant material and treatments

Cherimoya (*Annona cherimola* Mill. cv. 'Fino de Jete') fruit of uniform maturity was harvested in Almuñecar (Granada, Spain) and shipped to the laboratory at the "Instituto del Frío" (Madrid) within 20 h. Selected fruits, free from physical and pathological defects, and weighing from 250 to 260 g, were randomly divided into two lots of 20 and stored in the dark at 6 °C. Each lot of fruit was placed in a respiration chamber (20 L) with continuous flow (100 mL min^{-1}) of humidified air (untreated) or a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated). The CO_2 concentration was maintained constant throughout this pre-treatment, and it was measured daily using an automated gas chromatography system equipped with a thermal conductivity detector and a Poraplot Q column (Varian Chrompack CP-20033P). After 3 days of this gaseous treatment, fruits were stored for an additional 6 days in the dark at 6 °C under a continuous flow of humidified air. After the full 9-day storage period, 10 untreated and CO_2-treated cherimoya fruit were collected, peeled, frozen in liquid nitrogen, and they were stored at −80 °C until further study.

2.2. Chitinase purification

All procedures were carried out at 4 °C on ice, unless otherwise stated. Absorbance was measured at 280 nm to monitor the proteins during chromatographic separation, and chitinase activity was assayed colorimetrically at pH 5.0 and 37 °C during chromatographic separation.

(i) *Preparation of crude chitinase from fruit*. Frozen mesocarp tissue (250 g) from CO_2-treated cherimoyas was homogenized for 5 min with a Waring blender in 100 mM sodium acetate buffer (pH 5.0) containing 1.5% (w/v) PVPP, 10 mM EDTA and 20 mM sodium ascorbate (extraction buffer). After stirring for 1 h, the homogenate was centrifuged at 35,000 × *g* for 30 min and the resulting supernatant was filtered by ultrafiltration through an Amicon Diaflo membrane with a 100,000 MW cut-off (Biomax PBHK; Millipore) to remove colloidal carbohydrates. The material retained was washed twice with 50 mM Tricine buffer (pH 8.0) and the filtrates obtained were pooled, concentrated 10 times using an Amicon Diaflo membrane with a 10,000 MW cut-off (YM-10, Millipore), and adjusted to 20% saturation with solid ammonium sulphate. After removal of the precipitated material by centrifugation at 15,000 × *g* for 15 min, the supernatant was brought to 85% saturation with ammonium sulphate and centrifuged at 15,000 × *g* for 20 min. The precipitate was then resuspended in 20 mM Tricine buffer (pH 8.0) containing 10% (v/v) glycerol, and the solution was desalted by ultrafiltration through a YM-10 membrane.

(ii) *Anionic exchange column chromatography*. To separate acidic and basic proteins, the desalted enzyme extract was filtered at 0.22 μm and loaded onto a Mono Q HR 5/5 anion exchange column (GE-Healthcare) attached to a Pharmacia FPLC System (GE-Healthcare) pre-equilibrated with 20 mM Tricine buffer (pH 8.0) containing 10% (v/v) glycerol. Acidic proteins were eluted by increasing the ionic strength of the equilibration buffer to 1 M with NaCl at a flow rate of 1 mL min^{-1}. Basic proteins that passed through the column in the void volume were collected and equilibrated in 25 mM ethanolamine–HCl buffer (pH 9.5) containing 10% (v/v) glycerol, and then concentrated by ultrafiltration in an Amicon Centriplus YM-3 (Millipore).

(iii) *Chromatofocusing*. The non-adsorbed basic protein fraction was further filtered (0.22 μm) and loaded onto a Mono P HR 5/20 chromatofocusing column (GE Healthcare) attached to a Pharmacia FPLC System pre-equilibrated with 25 mM ethanolamine–HCl buffer (pH 9.5) containing 10% (v/v) glycerol. Elution was performed at a flow rate of 0.8 mL min^{-1} by applying a linear pH gradient (9.5–7.0) established with 25 mM ethanolamine–HCl buffer (pH 9.5) and 10% glycerol as the starting buffer, and using a 1:10 dilution of Polybuffer 96-HCl (pH 7.0) and 10% glycerol as the elution buffer (total volume: 34.4 mL). Fractions of 1 mL were collected and chromatofocusing was monitored by measuring the pH of the eluted fractions. In this last chromatographic step, three peaks with chitinase activity were collected, and they were equilibrated in 20 mM sodium acetate buffer (pH 5.0), concentrated by ultrafiltration with a Centricon YM-10 (Millipore) and stored at −20 °C.

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