FUNCTIONALITY OF A CLASS I BETA-1,3-GLUCANASE FROM SKIN OF TABLE GRAPES BERRIES

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Abstract

We have analyzed how low temperature storage (0 ºC) and a 3-day treatment of high CO_2 levels (20% CO_2 plus 20% O_2) affect class I β-1,3-glucanase (*Vcgns1*) gene expression and glucanase activity in the red table grape (*Vitis vinifera* L. cv. Cardinal). The results indicate that storage at 0 ºC for 3 days increased *Vcgns1* mRNA levels and β-1,3-glucanase activity in the skin of non-treated grapes. However, the accumulation of the transcripts, and the level of glucanase activity, were lower in the skin of grapes after 3 days of CO_2 treatment, as well as when treated fruit were transferred to air. By using heterologous expression of the *Vcgns1* cDNA in *Escherichia coli*, we showed that it encoded a protein with glucanase activity with an optimum pH and temperature of 6 and 45 ºC, respectively, and a high stability at 0 ºC. Furthermore, the purified VcGNS1 exhibited *in vitro* cryoprotective activity for the freeze labile L-lactate dehydrogenase (LDH) enzyme. In contrast, when the thermal hysteresis activity (THA) of the recombinant VcGNS1 was measured, using differential scanning calorimetry (DSC), the results indicated that it did not show antifreeze activity. The high stability of the recombinant protein at 0 ºC, and its cryoprotective activity shown in this work suggest that VcGNS1 may participate in the response of table grape to combat low temperature conditions.

Keywords: Table grapes; Carbon dioxide; Beta-1,3-glucanase; Gene Expression; Cryoprotective; Antifreeze

1. Introduction

Low temperature storage has always been used as the main method to extend the postharvest life of fruit and vegetables. Table grapes are not susceptible to injury at low (not freezing) temperatures, but their storage life is limited due to their high sensitivity to fungal attack. A number of studies have shown that exposure of plants to a moderate temperature stress not only induces resistance to severe stress of this kind, but can also improve tolerance to other stresses [1,2]. In previous studies we have observed that high CO_2 levels applied at low temperature during 3 days reduced total decay in table grapes, thereby maintaining fruit quality [3,4]. The responses of fruit to high CO_2 levels vary considerably among cultivars, and also depend on other environmental conditions of storage. Non-treated grapes responded to temperature shifts in the first stage of storage at 0 ºC, activating defense responses related to the phenylpropanoid pathway, whereas the application of high CO_2 levels reduced these responses [5].

In recent years, different studies have identified genes and metabolic pathways involved in the perception and signal transduction of plant responses to extreme temperatures (reviewed by [6]). Certain conserved mechanisms may still operate between chilling-sensitive and chilling-tolerant plants, and even between freezing and chilling tolerance [7]; but it is important to note that most of the studies were conducted in *Arabidopsis thaliana* during cold-acclimation to freezing, and little is known about the molecular basis of cold responses in agronomical important plants. Several studies have led to the identification of cold-responsive genes, many of which encode apoplastic antifreeze proteins (AFPs), late embryogenesis abundant proteins or pathogenesis related proteins (PRs), such as β-1,3-glucanases and chitinases [8].

β-1,3-Glucanases (EC 3.2.1.39) hydrolyze the β-1,3-linked glucans found in the cell walls of higher plants, as well as in many fungi [9,10]. Glucanase gene expression may not only be modulated in response to many physiological processes in healthy plants [11,12,13], but also in response to biotic and abiotic stresses [12]. In winter rye, Hon et al. [14] observed that a cold-induced PR protein showed β-1,3-glucanase and cryoprotective activity. Likewise, a class I β-1,3-glucanase purified from tobacco protected thylakoids *in vitro*, isolated from spinach, against freeze-thaw damage [15].

Although isolation of β-1,3-glucanase genes have been reported in grapes [3,16,17], only limited information is presently available on the regulation of gene expression and the function of these proteins. The participation of β-1,3-glucanase in the defense of grapevine leaves against *Botrytis cinerea* has been shown [18,19]. However, in red table grapes we have observed that the efficacy of high CO_2 pretreatment in reducing total fungal decay is not mediated by the induction of class I β-1,3-glucanase and chitinase genes [3], indicating that factors other than fungal infection are involved in the induction of these genes in table grapes.

The aim of the present work was to analyze gene expression of *Vcgns1*, a class I β-1,3-glucanase, in red table grapes as a marker for changes in response to low temperature, and also to assess how high CO_2 levels (20%) modulated its transcript accumulation at 0 ^{o}C. As a part of our study to investigate the possible physiological role of class I β-1,3-glucanase in table grapes as a cryoprotectant, and/or an antifreeze, we also report on recombinant expression of *Vcgns1*.

2. Material and Methods

2.1. Plant Material

Table grapes (*Vitis vinifera* L. cv. 'Cardinal') were harvested at random in Camas (Sevilla, Spain) in July. After harvesting, the field-packaged bunches were transported to the laboratory where fruit were immediately forced-air precooled for 14 hours at –1 ºC. After cooling, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ±0.5 ºC and 95% relative humidity in two sealed neoprene containers of 1 m^3 capacity. One lot was stored under a normal atmosphere for 6 days (non-treated fruit), and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. The grapes were then transferred to air under the same conditions as non-treated fruit until the end of the storage period.

Ten clusters were sampled periodically, and berries from five clusters (approx. 300 g each cluster) were peeled, and the skin and pulp were frozen in liquid nitrogen, ground to a fine powder, and stored at –80 ºC until analysis.

2.2. RNA gel blot hybridization

Total RNA was extracted from the skin of grapes according to the method of Salzman et al. [20]. Samples of denatured total RNA (10 μg) from the skins were fractionated and blotted, as described in Romero et al. [3]. Equal loading was confirmed by membrane staining with methylene blue. The *Vcgns1* DNA probe was a random primer labeled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 ºC in 7% sodium dodecyl sulfate, 0.33 M phosphate buffer, pH 7.2, and 1 mM EDTA, then washed twice in

2 x SSC, 0.1% SDS at room temperature and twice in 0.1 x SSC, 0.1% SDS at 65 ºC, and exposed to Kodak X-Omat SX film at –80 ºC.

2.3. Production of the recombinant VcGNS1 protein in Escherichia coli

The cDNA fragment of VcGNS1 (GenBank accession number DQ267748) without the N-signal peptide and C-terminal extension was amplified using the forward primer (5'-ATT ATC CTC GAG GTG GGT GTA TGC TAT GGA ATG-3') and the reverse primer (5'-GGC GAA TTC AAA GTT GAT AGA GTA CTT CGG -3') containing *Xho*I and *Eco*RI restriction sites, respectively. The PCR conditions were 30 cycles of 40 s at 95 ºC, 40 s at 49 ºC and 40 s at 72 ºC. The amplified DNA fragments were digested with *XhoI* and *EcoRI* and ligated into the multicloning site of the pTrcHisA plasmid (Invitrogen, Carlstad, USA) previously digested with the same enzymes. The vector pTrcHisA-VcGNS1 was transformed into cells of *E. coli* strain TOP10, which were grown at 37 ºC in LB-medium with 50 µg mL-1 ampicillin and 0.8 mM glucose until the optical density at 600 nm reached 0.6, and then induced with 1 mM isopropyl-ß-D-thiogalactoside (IPTG). After 4 h of induction at 37 ºC the cells were harvested by centrifugation at 1760*g* for 20 min at 4 ºC and then frozen. Cells were re-suspended in lysis buffer (50 mM sodium phosphate monobasic, 500 mM sodium chloride, 10 mM imidazole, pH 8.0) and DNase1 RNase-Free (Roche); and then disrupted with 1 g of glass beads (150-252 µm, Sigma) in a FastPrep machine (FP120, Bio101, Savant). The cell extract was centrifuged at 1580*g* for 10 min at 4 ºC. The soluble recombinant His-tag VcGNS1 protein was incubated with Ni-NTA agarose resin (QIAexpress, Qiagen, Germany) for 1 h at 4 ºC with shaking, before the extract-resin mixture was loaded into a column. Contaminating proteins were eluted by washing the column with 8 mL of buffer (50 mM sodium phosphate monobasic, 500 mM sodium

chloride, 40 mM imidazole, pH 8.0). Bound VcGNS1 was eluted from the Ni-NTA column with elution buffer (5 x 1 mL 50 mM sodium phosphate monobasic, 500 mM sodium chloride, 300 mM imidazole, pH 8.0). The purified fusion protein was concentrated with buffer exchange into 50 mM Tris-HCl, pH 7.4 for enzymatic assays, into 20 mM sodium acetate buffer pH 5.0 for *in vitro* antifungal assays or doubled distilled water for *in vitro* cryoprotective and antifreeze assays by ultrafiltration on ultrafree biomax-5K (Millipore), and then stored at -20 ºC. Protein concentration was determined according to the Bradford method [21] using a protein-dye reagent (Bio-Rad) and bovine serum albumin as a standard.

2.4. Gel electrophoresis and protein blot analyses

Protein analysis was performed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [22], using Mini-Protean II Cell (Bio-Rad) equipment. Protein samples were prepared by boiling for 10 min in 2X SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, and 0.05 % bromophenol blue). For reducing conditions, samples were mixed with an equal volume of loading buffer supplemented previously with 10% β-mercaptoethanol. Protein bands were visualized by staining with Coomasie brilliant blue R-250. The molecular mass of the separated polypeptides was estimated by comparison with the mobility of pre-stained standard low molecular mass range proteins (Bio-Rad).

After standard PAGE was performed proteins were electro-transferred to PVDF membranes (Amersham) with a Mini Trans-Blot Cell (Bio-Rad). The Western blot was probed with a 1:5000 dilution of polyclonal anti-PR2 sera from tobacco, kindly provided by

Dr Fritig (Strasbourg, France), which were detected with a 1:5000 dilution of rabbit antiserum against IgG horseradish peroxidase conjugate (Amersham). The immuno-complexes were visualized using the enhanced chemiluminescence (ECL®) detection system (Amersham).

2.5. Protein extracts preparation

Ground frozen berry skin tissue (3 g FW) was homogenized in 10 mL of 100 mM sodium acetate buffer, pH 5.0, and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 35000*g* for 30 min. After removal of precipitated material the supernatant was adjusted to 95% saturation with ammonium sulphate, and centrifuged at 20000*g* for 20 min. The precipitate was re-suspended in 100 mM sodium acetate buffer, pH 5.0. All steps were carried out at 4 ºC.

2.6. β-1,3-glucanase activity assay

2.6.1. Protein extracts

β-1,3-glucanase activity was quantified measuring the reducing sugar released from the substrate *Laminaria digitata* laminarin (Sigma). 50 µL of extract was added to 300 µL of substrate (0.5 mg) in 100 mM sodium acetate buffer, pH 5.0. The reaction was stopped with 35 µL of NaOH after 8 h of incubation at 37 ºC and cooling on ice for 10 min. The reducing sugar was determined according to Dygert et al. [23]. D-glucose at concentrations from 2.5 to 120 µg mL^{-1} in buffer was used as a standard. Enzyme activity was defined as nmol glucose equivalents h^{-1} g FW^{-1}. Assays were determined in three replicated samples from three independent extractions.

2.6.2. Recombinant VcGNS1 protein

Optimum temperature value was determined by measuring the specific activity of 1 μg of purified VcGNS1 with 2 mg of *L. digitata* laminarin (Sigma) in 100 mM sodium acetate buffer, pH 6.0, at temperatures ranging between 0 ºC and 75 ºC. Optimum pH value was determined at 45 ºC by running the enzymatic assay with 1 μg of purified VcGNS1 in 2 mg of *L. digitata* laminarin (Sigma) in 100 mM phosphoric acid (pH 2.0), sodium acetate (pH 4.0-6.0), sodium phosphate dibasic (pH 7.0), Tris-HCl (pH 9.0) and sodium phosphate monobasic (pH 12) buffers. The mixtures were incubated with shaking for 60 min and the reducing sugars were measured as described above. The activity of VcGNS1 was recorded as the release of μmoles glucose equivalents min^{-1} mg $protein^{-1}$. Assays were determined in three replicated samples from three independent purifications of VcGNS1.

To determine the thermal and pH stability of the recombinant VcGNS1 the purified protein (1 μg) was incubated in 100 mM sodium acetate buffer, pH 6.0, without laminarin**,** for 1 h at temperatures between 0 ºC and 75 ºC and in the buffer at different pHs at 45 ºC, respectively. Subsequently, the residual activity was measured with 2 mg of laminarin as described above. Assays were determined in three replicated samples from three independent purifications of VcGNS1.

2.7. Kinetic parameters

The Michaelis-Menten constants K_m and k_{cat} were determined from the Hanes-Wolf representation of data obtained by measuring the initial rate of substrate hydrolysis. 1 μg of purified VcGNS1 was incubated at 45 ºC with increasing concentrations of laminarin (0-5 mg mL^{-1}) in 100 mM sodium acetate buffer, pH 6.0. After the reaction was stopped the

liberated glucose was measured as described above. The activation energy (E_a) was calculated by the method of Segel et al. [24], using the linear form of Arrhenius equation:

$$\log k = \frac{-E_a}{2.3R}\frac{1}{T} + \log A$$

in which R is the molar gas constant, T is the absolute temperature and k the specific reaction rate constant. For enzyme-catalyzed reactions log V_{max} can be plotted instead of log$k$.

The effect of δ-gluconolactone (Sigma) on the activity of the purified enzyme was measured as described previously Notario et al. [25].

2.8. Assay for cryoprotective activity

The *in vitro* cryoprotective activity was determined following the method described by Lin and Thomashow [26], with slight modifications. Lactate dehydrogenase (LDH, EC 1.1.1.23, Type V-S from rabbit muscle, Sigma, St. Louis, MO, USA) was diluted to 87.6 μg mL^{-1} in 20 mM potassium phosphate buffer, pH 7.5, and used as a freeze-labile stock enzyme solution. Samples, either VcGNS1 protein fractions or bovine serum albumin (BSA, Sigma, St. Louis, MO, USA), were diluted to varying concentrations and mixed with 2.364 μg of LDH from the stock solution. The resulting solution (300 μL) was frozen in liquid nitrogen for 30 s and then thawed at room temperature for 5 min. The freeze-thaw process was carried out three times and the residual LDH activity was then measured. LDH enzymatic activity was determined in aliquots of 30 μL of the mixtures in a final volume of 1.5 mL of the reaction assay buffer (80 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM pyruvate and 30 mM NADH), at room temperature. NAD^+ production was monitored as the

decrease of absorbance at 340 nm for 4 min. The data are showed as the percentage of the activity present in unfrozen controls. Assays were determined in three replicated samples.

2.9. Antifreeze activity measurement

The differential scanning calorimetry (DSC) was used to determine the antifreeze activity by measuring the thermal hysteresis activity (THA). DSC analysis was conducted using a differential scanning calorimeter (DSC822e, Mettler-Toledo Inc., Columbus, OH, USA) equipped with a liquid nitrogen cooling accessory. The instrument was calibrated using Indium and n-octane, and an empty aluminum pan was used as reference. An antifreeze protein (type III) from antarctic fish, kindly provided by Dr. De Vries (Illinois, USA), and BSA (AFP-free solution) were used as standards in order to make a comparison between solutions with and without antifreeze activity. Recombinant VcGNS1 protein (5 $mg \cdot mL^{-1}$) dissolved in double distilled water and 3-4 µl sample was weighed into 40 µL Mettler-Toledo coated aluminum pans. Pans with samples were immediately hermetically sealed and cooled from 25 ºC to -40 ºC at 10 ºC min^{-1}, frozen at this temperature for 5 min, and heated to melt (10 ºC min^{-1}). The samples were cooled at -30°C (10 ºC min^{-1}) for 5 min, and warmed up slowly to various partial-melt temperatures (0.5 ºC min^{-1}). To allow ice-protein interaction, samples were held at this temperature (T_h, hold temperature) for 10 min before being recooled at the same rate of 0.5 ºC min^{-1} to -25 ºC, during which the onset temperature of crystallization (T_c) was recorded using STARe software (Mettler-Toledo). THA was defined as the difference between T_h and T_c, $THA = T_h - T_c$.

2.10. Statistical analysis

The data from at least three replicates per sample were subjected to analysis of variance (One-way ANOVA) using the LHD test to determine the level of significance at $P \leq 0.05$ (Statgraphics Plus version 5.1, STSC, Rockville, Md.).

3. Results

3.1. Effect of low temperature and high CO_2 on Vcgns1 mRNA levels and β-1,3-glucanase activity

In the skin of non-treated grapes low temperature sharply increased the levels of the transcript after 3 and 6 days of storage (Fig. 1A). In contrast, high CO_2 levels applied during 3 days at 0 ºC decreased the accumulation of the *Vcgns1* mRNA, but levels increased slightly when treated grapes were transferred to air for 3 days, although the levels were always lower than in the non-treated. Glucanase activity increased significantly after 3 days at 0 ºC in non-treated grapes, and then decreased after 6 days (Fig. 1B). The increase of glucanase activity after 3 days of CO_2 at 0 ºC treatment was lower than in non-treated grapes, increasing slightly thereafter.

3.2. Expression and purification of VcGNS1

The full-length of the VcGNS1 was previously isolated from the skin of table grape using RT-PCR and 5'-3' RACE strategies [3]. The predicted amino acids of VcGNS1 cDNA shared homology with basic class I β-1,3-glucanases containing a N-terminal signal peptide cleavage site located between residues 22 (Lys) and 23 (Ser), and a C-terminal

extension of 23 amino acids with a *N*-glycosylation site, and required for targeting the protein to the vacuole [27,28].

To determine whether the VcGNS1 cDNA encoded a protein with glucanase activity, and to perform *in vitro* cryoprotective and antifreeze assays, the cDNA fragment corresponding to the mature protein (without the putative N-terminal transit peptide and the C-terminal extension, amino acid residues 23 to 338) was expressed in TOP10 *E. coli* cells as a pTrcHisA-VcGNS1 fusion protein. The VcGNS1 protein was induced in the presence of 1 mM IPTG at 37 ºC for 4 h and purified with a Ni-NTA affinity column. After purification, gel electrophoresis of both reduced and unreduced VcGNS1 yielded a single band of 43 and 40 kDa respectively (Fig. 2A), suggesting that the native protein occurs as a monomer. Western blot analysis with an antiserum against tobacco PR2 showed a positive band with the same molecular mass (Fig. 2B).

3.3. Effects of temperature and pH on enzyme activity and stability

The enzymatic activity of the recombinant β-1,3-glucanase was measured over a pH (2-12) and temperature (0-70 ºC) range by assaying the production of reduced sugars from laminarin (Fig. 3). The pH optimum for recombinant VcGNS1 was 6, decreasing sharply at pH values above 6.5 or below 5.5. The temperature optimum was 45 ºC, but the recombinant VcGNS1 retained some hydrolytic activity at different temperatures assayed. In terms of stability, the recombinant protein was able to catalyze the hydrolysis of laminarin over a wide range of pH, 2 to 9, and temperature, 0 ºC to 45 ºC, being inactive at pH 12 and at temperatures higher than 60 ºC. Interestingly, the recombinant protein showed

the optimum stability at 0 ºC, reaching values significantly higher to those achieved when the activity was measured at 45 ºC.

3.4. Kinetic properties

The recombinant β-1,3-glucanase obeys Michaelis-Menten kinetic. A linear curve was obtained from the Hanes-Wolf plot for the hydrolysis of laminarin at concentrations of 0-5 mg/mL, yielding an apparent K_m value of 0.78 mg/mL which, corresponds to 0.195 mM. The catalytic rate constant, k_{cat}, is 7.96 $s^{-1}$and the specificity or catalytic efficiency factor, k_{cat}/K_m, 40.82 s^{-1} mM^{-1}. The calculated E_a was 19.95 kJ/mol for laminarin hydrolysis (Table 1). Moreover, the VcGNS1 did not show inhibition with δ-gluconolactone (1 to 20 mM), a potent inhibitor of exo-β-glucanases at low concentrations (Notario et al, 1976), indicating an endotype mode of action for the recombinant enzyme (data not shown).

3.5. Cryoprotective activity of recombinant VcGNS1

The ability of recombinant β-1,3-glucanase to preserve LDH activity following freeze-thaw cycles was compared with the level of cryoprotection provided by BSA. The cryoprotective activity of recombinant VcGNS1 and BSA was then assessed by determining whether they could protect LDH against freeze inactivation. Without the addition of a cryoprotectant three freeze-thaw cycles resulted in a reduction of more that 80% in LDH activity (Fig. 4). The addition of VcGNS1 or BSA in protein:enzyme molar ratios from 1 to 4 significantly protected LDH activity. Recombinant VcGNS1 was shown to be less efficient than BSA in protecting LDH at molar ratios from 1 to 3. At a molar ratio of 4, VcGNS1 was as efficient as BSA in protecting LDH activity against freeze

inactivation, maintaining around 70% of initial LDH activity. On the contrary, the addition of sucrose at similar concentrations failed to protect LDH under identical freeze-thawing conditions.

3.6. Thermal hysteresis activity of recombinant VcGNS1

DSC curves of the recrystallization of different partially melted protein-water systems are presented in Fig. 5. A delay of 0.4 ºC in the onset temperature of refreezing was observed in a partially melted AFP system. In recombinant VcGNS1 solution, recrystallization of the melted part started immediately after the temperature dropped, and the exothermic peak appeared without delay. This indicated that the VcGNS1, like AFP-free BSA solution, had no thermal hysteresis effect.

4. Discussion

Grapes are classified as a chilling tolerant fruit, and are normally injured at temperatures below -20 ºC. Previous results indicated that table grapes cv. Cardinal could be sensitive to temperature shifts at 0 ºC, and that high CO_2 levels applied as a pretreatment could regulate these responses [4]. To further identify the mechanisms associated with the response of table grapes at 0 ºC, and to determine whether high CO_2 levels could modulate them, we have analyzed changes in β-1,3-glucanase gene expression in the first stage of storage. There have been several previous reports of PR proteins induced by low temperature. In winter rye, one of the PR proteins induced in response to low temperature showed β-1,3-glucanase and cryoprotective activity [14]. In citrus, Sanchez-Ballesta et al. [29] observed that changes in gene expression of a class III β-1,3-glucanase were linked to postharvest chilling-induced cell damage. In tomato exposed at chilling temperature a class

II β-1,3-glucanase mRNA was induced [30]. In previous work, we have isolated a class I β-1,3-glucanase (*Vcgns1*) cDNA from the skin of table grape [3]. The levels of the *Vcgns1* transcript increased in non-treated grapes after 12 days at 0 ºC, but we did not find any relation between *Vcgns1* gene expression and fungal attack, indicating that factors other than fungal infection are involved in *Vcgns1* gene expression in table grapes. In this work we observed that low temperature by itself has a clear effect on *Vcgns1* gene expression and glucanase activity. Thus, after 3 days at 0 ºC a sharp increase in the level of *Vcgns1* mRNA was observed in the skin of non-treated grapes, maintaining the level after 6 days. In contrast, 3 days CO_2-treatment reduced the accumulation of the transcript observed at 0 ºC, with a slight increase in the levels when treated grapes were transferred to air (Fig. 1A). Likewise, although glucanase activity increased in response to low temperature, the increase was higher after 3 days at 0 ºC in the non-treated grapes. Previously, similar results were observed in non-treated grapes, where phenylpropanoid gene expression, total anthocyanin accumulation and antioxidant activity were activated during the first stage of storage at 0 ºC, whereas the application of high CO_2 treatment reduced these responses [4]. These results reinforce the idea that high CO_2 levels applied at 0 ºC as a pretreatment of 3 days can modulate the responses induced in non-treated grapes in the first stage of storage at 0 ºC.

To determine whether the *Vcgns1* cDNA isolated from table grape encoded a protein with glucanase activity, and to analyze its functionality, the protein was produced by the pTrcHis expression system in *E. coli*. The purified recombinant protein showed a single protein band in SDS-PAGE. The molecular mass of the recombinant VcGNS1 was 40 kDa, and this value agreed with the molecular mass calculated from the deduced polypeptide. The purified VcGNS1 showed β-1,3-glucanase activity for laminarin

hydrolysis with a pH optimum higher than other plant β-1,3-glucanase enzymes [31,32], but with similar optimum temperature. The VcGNS1 protein was relatively more thermostable than the β-1,3-glucanases from *Boscia senegalensis* [32] and barley [33], being active over a wide range of temperatures. The recombinant glucanase displayed 27% of relative activity at 0 ºC and, interestedly at this temperature, VcGNS1 showed a higher thermostability after 1 h without substrate. The Ea for VcGNS1 is lower than values obtained for other carbohydrate hydrolases [32] and constant over the studied temperature range, indicating that the enzyme does not undergo major structural changes within this range. In winter rye glucanases presented the maximal activity at temperatures around 30 ºC, and there was clear evidence of activity at zero and subzero temperatures [34]. However, to our knowledge, this is the first report where a β-1,3-glucanase from fruit showed low values of Ea and hydrolytic activity at cold temperatures. Kinetics analyses of the recombinant VcGNS1 during hydrolysis of laminarin allowed the calculation of a K_m value (0.195 μM) which is substantially higher than that reported for β-1,3-glucanases from other plants [32,35]. However, the turnover number (k_{cat} 7.96 s^{-1}) was significantly lower than values reported for plant glucanases [33,35]. Furthermore, VcGNS1 could behave as an endo-splitting hydrolase because of the non inhibitory effect of δ-gluconolactone on the enzymatic activity observed in this work (data not shown). In this respect, this enzyme is comparable to other plant β-1,3-glucanases isolated [32,33,36,37].

It has been reported that proteins identified as PRs that accumulate in winter cereals during cold acclimation function as antifreeze proteins, by inhibiting extracellular ice growth under freezing temperatures [14]. Yaish et al. [34] support the hypothesis that recombinant winter rye glucanases have evolved to inhibit the formation of large ice

crystals, in addition to the capacity to degrade glucans at low temperature. In contrast, the recombinant VcGNS1 did not show antifreeze activity when the THA was determined by DSC. In spinach and cabbage leaves β-1,3-glucanase proteins were accumulated during cold acclimation, and glucanase of class I had potent cryoprotective activity [15]. Likewise, a PR-5 protein from groundnut showed *in vitro* cryoprotective activity against LDH [38]. However, this appears not to be a general effect of stress-induced PR proteins. A tobacco class I chitinase did not demonstrate any cryoprotective effects [15]. In chilling-sensitive mandarins the recombinant class III β-1,3-glucanase protein, likewise, showed glucanase but not cryoprotective activity [29]. In this study we demonstrate that VcGNS1 had cryoprotection activity. Assay for freeze-thawing enzyme inactivation was done with LDH, because of its sensitivity to different stresses. A comparison of the cryoprotection conferred by VcGNS1 revealed that it was similar to that of BSA, which is a known cryoprotectant [39]. Similar results have been observed with WAP18, a PR10 from mulberry [40]; COR15am, a LEA-like protein from *Arabidopsis* [41]; and winter rye apoplastic proteins [42], which in all situations exhibited a similar value to BSA. In contrast the cryoprotective activities of COR85 and PCA60, cold-induced dehydrins, were found to be around four or two-fold higher than that for BSA [43,44]; but CrCOR15, a dehydrin from citrus fruit revealed a cryoprotective activity similar to BSA [45]. The percentage of polar residues in VcGNS1 is around 40% as occur in a PR-10 protein such as WAP18 [40]. These authors indicated that the lower-cryoprotective activity of WAP18, in comparison with other cryoprotectants, may be partially related to the lower-ratio of polar residues in the surface of the protein.

In conclusion, we have identified a class I β-1,3-glucanase from table grapes that displays glucanase activity as well as *in vitro* cryoprotective but not antifreeze activity.

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Figure Captions

Fig. 1. Effect of low temperature and high CO_2 pretreatment on *Vcgns1* mRNA accumulation (A) and β-1,3-glucanase activity (B) in the skin of 'Cardinal' table grapes (A) 10 μg of total RNA from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *Vcgns1* probe. The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. (B) Time courses of β-1,3-glucanase activity in the skin of non-treated and CO_2-treated 'Cardinal' table grapes stored at 0 ºC. Error bars represent S.E (n=9). Values labeled with the same letter are not different at the 5% significance level.

Fig. 2. (A) Recombinant VcGNS1 protein was analysed by SDS-PAGE (12% polyacrylamide) and stained by Coomassie blue. Lanes 2 and 3 were loaded with 2 μg of reduced (with 10 % β-mercaptoethanol) and unreduced protein. Lane 1 was loaded with low molecular mass reference proteins and sizes are indicated on the left. (B) Immunoblot of a similar gel with unreduced VcGNS1. The blot was incubated with polyclonal anti-PR2 sera from tobacco.

Fig. 3. Effect of pH (A) and temperature (B) on the activity (—●—) and stability (—○—) of recombinant VcGNS1. Optimum pH was determined by measuring the activity at 45 ºC in the range of pH 2 to 12. Stability was assessed by measuring the residual activity after 1 h of incubation at different pH and temperatures. Optimum temperature was determined by measuring the specific activity of 1 μg of purified VcGNS1 in a range of temperatures from 0 ºC to 80 ºC at pH 6.0. For all experiments, laminarin degradation was measured under

standard assay conditions. Values presented are expressed in relative activity (percent of the greatest activity detected). Error bars represent S.E (n=9).

Fig. 4. Cryoprotection of LDH by recombinant VcGNS1. A LDH solution was frozen with different concentrations of VcGNS1, BSA or sucrose. The samples were thawed at room temperature and the LDH activity was measured. The relative activity represents the amount of LDH activity remaining after a freeze-thaw treatment as a percentage of the control enzyme activity. Error bars represent S.E (n=3).

Fig. 5. Refreezing DSC curves (0.5 ºC min^{-1}) of partially melted protein-water systems. A) AFP-III B) BSA C) recombinant VcGNS1.

Table 1. Kinetic properties of recombinant VcGNS1 using *L. digitata* laminarin.

Spact (μmol min^{-1} mg^{-1})[a]	9.70
K_m	
(mg mL^{-1})	0.78
(mM)	0.195
k_{cat} (s^{-1})[b]	7.96
k_{cat}/K_m (s^{-1} mM^{-1})	40.82
E_a (kJ mol^{-1})[c]	19.95

[a] Specific activity.

[b] Catalytic constant rate (k_{cat}) = V_{max}/[E], where [E] = 0.025 nM.

[c] Activation energy determined between 0 and 45 ºC.

Figure 1

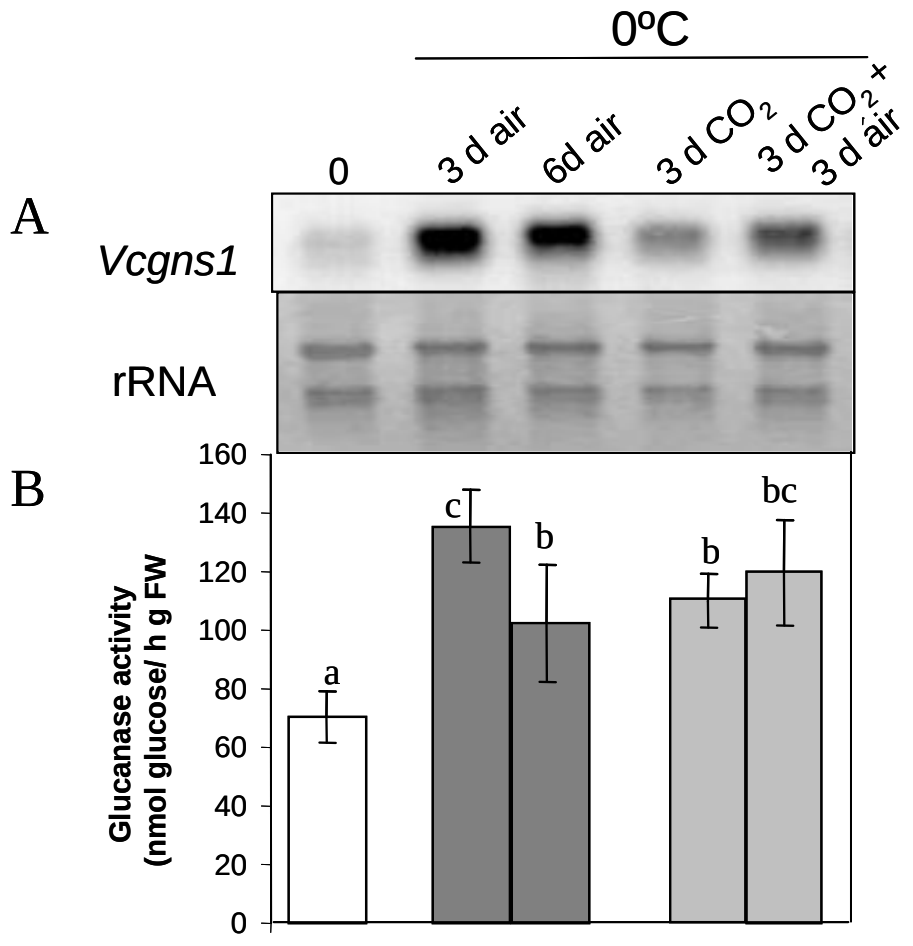
0ºC
0
3 d air
6d air
3 d CO2
3 d CO2 + 3 d air
A
Vcgns1
rRNA
B
Glucanase activity (nmol glucose/ h g FW
160
140
120
100
80
60
40
20
0
a
c
b
b
bc

Figure 2

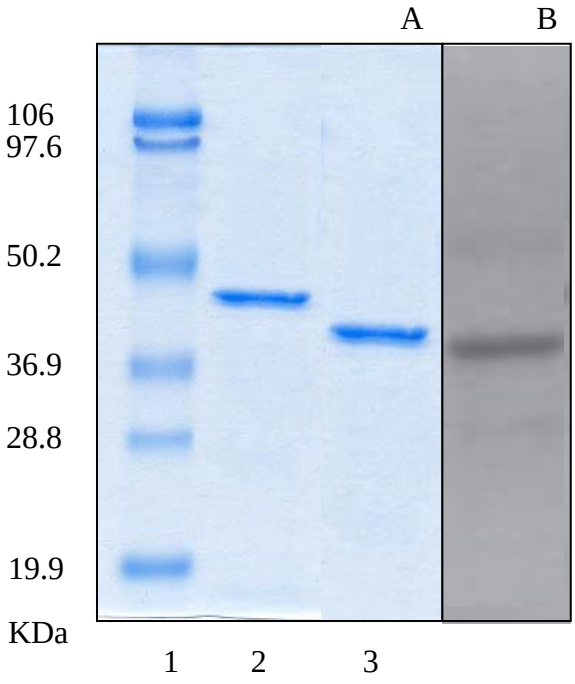

Figure 3

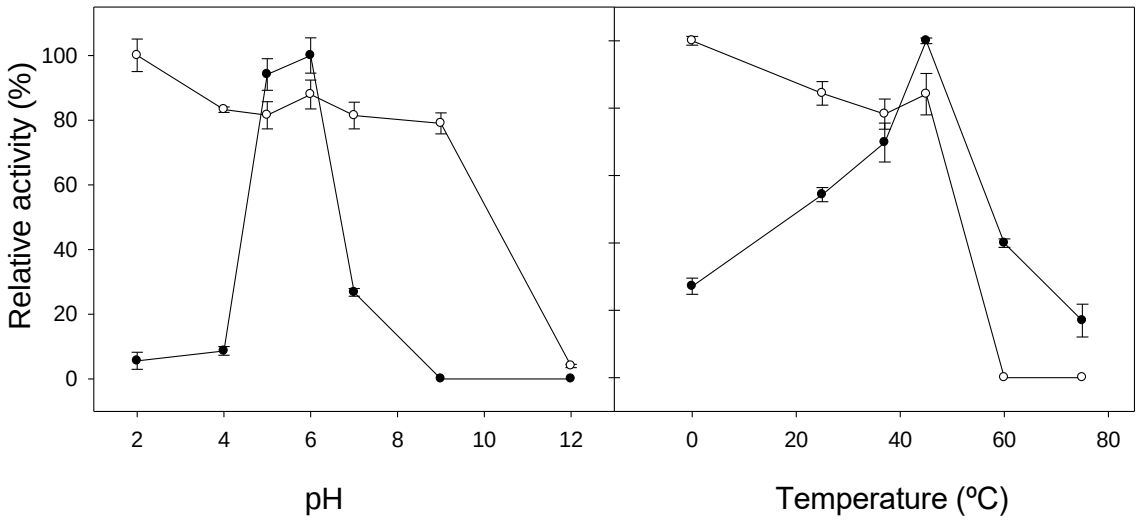
Relative activity (%)
100
80
60
40
20
0
2
4
6
8
10
12
pH
0
20
40
60
80
Temperature (ºC)

Figure 4

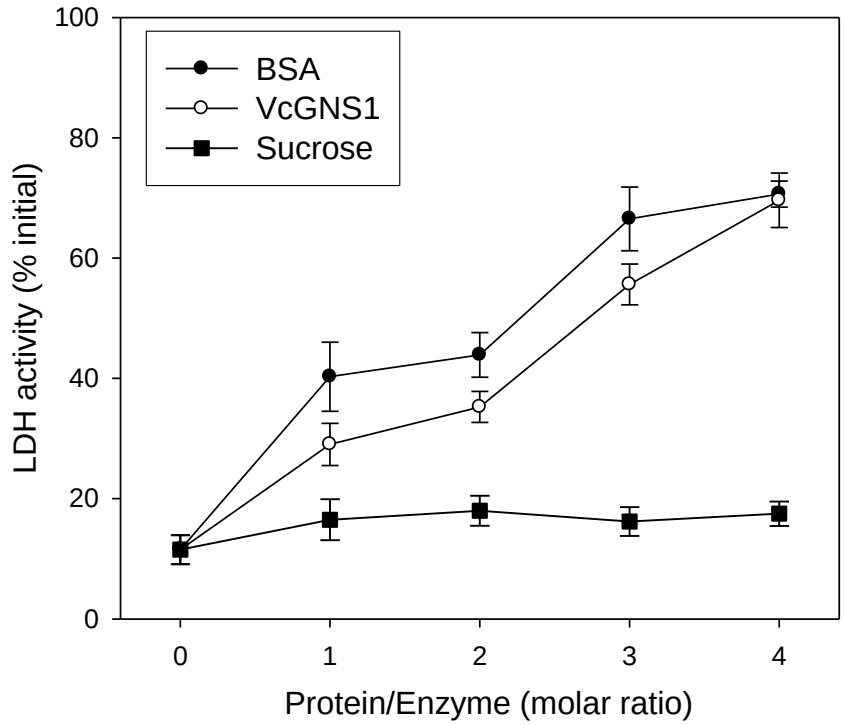
BSA
VcGNS1
Sucrose
LDH activity (% initial)
Protein/Enzyme (molar ratio)
0
20
40
60
80
100
0
1
2
3
4

Figure 5