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Research Paper

Development and characterization of bentonite/wGLP systems

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ABSTRACT

Bentonite (Bent) clay is a component of soil with useful properties for enzyme adsorption. In this work, the previously characterized antioxidant superoxide dismutase (SOD) enzyme named wGLP by wheat germin-like protein was immobilized in raw (Bent) and modified bentonites (Bent-DDA). The physicochemical characterizations of both supports were carried out. Successful adsorption of wGLP onto Bent and Bent-DDA occurred on the surface and within the interlayer spacing as was revealed by X-Ray Diffraction (XRD) and Thermogravimetric Analysis (TGA). The release of wGLP at different times and pHs was tested. While wGLP remained almost totally immobilized into Bent-DDA up to 96 h, it was released from Bent to reach nearly 60% after 72 h at pH 7.5 and preserving its SOD activity. Since tomato cell viability under the presence of Bent-wGLP was maintained, Bent-wGLP complexes are potential carriers of antioxidants in tomato cell suspension cultures.

1. Introduction

Plants produce a diverse collection of valuable molecules such as industrial and pharmaceutical products. Therefore, plant cell culture in liquid media has been accepted as a well-established technology for the synthesis of natural products (Nosov, 2012; Ochoa-Villarreal et al., 2015). However, such experimental approach needs proper cell lines and culture compositions. Thus, for best conditions to growth plant cells, chemical and physical features of media including phytohormones and other components should be thoughtful tested (Ochoa-Villarreal et al., 2016). Antioxidants are protective compounds against plant cell stress conditions. Non enzymatic scavenging systems include bioactives of low molecular weights, such as ascorbic acid, carotenoids and phenolic compounds. However, these low molecular weight antioxidants are easy degraded or volatilized during processing and inside biological fluids (Liu et al., 2017). On the other hand, in spite that are degraded under aggressive processing, high molecular weight antioxidants are usable (Santos et al., 2012). Because of their physical and chemical properties, to immobilize high molecular weight molecules such as polymers or proteins has received great attention in academia and industry. For example, it was proved that incorporating antioxidants into nanoparticles can strikingly produce high stability (Liu et al., 2017). Protein immobilization and release are advantageous for commercial applications due to convenience in handling, reduction of product cost and a possible increase in thermal and pH stabilities (Ansari and Husain, 2012). The aim of our work was to contribute with a novel strategy to immobilize and release the antioxidant enzyme wGLP (Segarra et al., 2003; Mansilla et al., 2012). Especially, linked to a great versatility, wGLP has chemical and thermal stability that favor its use in industrial scales, including plant biotechnology (Bernier and Berna, 2001). Germin-like proteins (GLPs) make up a diverse family of plant glycoproteins belonging to the cupin superfamily (Dunwell et al., 2008). Members of germin and GLPs so far reported to have different activities including, oxalate oxidase (OXO), SOD and ADP glucose pyrophosphatase/phosphodiesterase (AGPPase) (Barman and Banerjee, 2015). GLP with polyphenol oxidase activity has been characterized from *Satsuma mandarine* (Cheng et al., 2014). SOD is an antioxidant enzyme that catalyzes the superoxide (O_2^-) radical dismutation into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2).

An important requirement for enzyme immobilization is that the matrix should provide a biocompatible and inert environment and it should not interfere with the native structure of the protein, which could compromise its biological activity (Mitchell et al., 2002). Because of durability, high mechanical strength and low cost immobilizing antioxidant enzymes by inorganic supports as clays is an area of great interest (de Paiva et al., 2008; An et al., 2015).

Bent is one of the most commonly used clays because of its easy industrial scaling, low toxicity and hydrophilicity (Oztürk et al., 2007;

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Bergaya et al., 2012; Rodrigues et al., 2013). The main advantage of using clays as matrices is their capacity to undergo simple and inexpensive changes because of its chemical reactivity. The importance of Bent is related to its environmental and economic impacts, its natural abundance and its mechanical and chemical resistances that make it useful as reinforcement for polymeric materials among other applications. This type of clay is characterized by a moderate negative charge, known as cation exchange capacity (CEC) (He et al., 2006; Mandalia and Bergaya, 2006; Zampori et al., 2008). It is rather easy to convert these hydrophilic silicates to organophilic ones by ion exchange reactions. The hydrated Na^+ cations present in the interlayer (these cations are not structural) can be replaced by other positively charged surfactants such as alkyl ammonium or phosphonium cations with long alkyl chains (Xie et al., 2002; Picard et al., 2007; Xi et al., 2007). Bent has an interlayer or interlaminar pore usually smaller than 2 nm, so external surface area is mainly accessible to the enzyme molecules (Wang et al., 2014; Mohamad et al., 2015). To change Bent with quaternary salts produces an increase in the interlaminar space, lessens the surface energy and, therefore, became more compatible with hydrophobic materials (Lin et al., 2010).

In this work, raw and modified Bent by cation exchange reaction with a quaternary ammonium salt (Bent-DDA) were selected as matrices to immobilize wGLP. The effectiveness of each modification was discussed throughout characterization assays by XRD, TGA, western blot, optimum pH and release profiles. The cytoprotective action of the immobilized antioxidant enzyme wGLP in tomato-cultured cells is discussed.

2. Experimental Section

2.1. Materials

Sodium Bent was supplied by Minarmco S.A. (Neuquén, Argentina). Its cation exchange capacity (CEC) was 93.9 meq/100 g of clay. The chemical composition (X-Ray Fluorescence) of the Bent (mass %) is summarized in Table 1. wGLP protein was extracted and purified from the extracellular fluid of wheat leaves (*Triticum aestivum*) as described Mansilla et al. (2012).

2.2. Modification of Bent-DDA

Enough quantities of dodecylamine (Sigma-Aldrich, USA), HCl (Biopack, Technical grade, Argentina) and distilled water were measured. The mixture was heated at 80 °C for few minutes to protonate the amine groups. Approximately, 2.5 g of Bent were dispersed in 100 ml of deionized water at 80 °C and 0.435 g of dodecylamine solution (0.435% w/v) and 0.07 ml of HCl (37% w/v) were incorporated. Then, the mixture was stirred vigorously keeping the temperature at 80 °C during 30 min. The suspension was filtered through a Buchner funnel using a grade 50 Whatman filter paper and washed with deionized water until it was free of chloride ions. The organoclay was dried with a freeze dry-system (Karaltay FD-1C-50 Series, China) and stored at room temperature. The procedure was adapted from Ollier et al. (2011).

2.3. Immobilization of wGLP

Approximately, 3 mg of wGLP were dissolved in 10 ml of 0.1 M phosphate buffer (pH 5.8). One gram of Bent and Bent-DDA were dispersed in 10 ml of wGLP solution. The mixtures were incubated at room temperature for 1 h with continuous shaking at 150 rpm. Each immobilized sample was separated by vacuum filtration on a Buchner funnel through grade 50 Whatman filter paper and washed twice with distilled water to remove the unabsorbed soluble protein. Each mixture was dried with a freeze dry-system (Karaltay FD-1C-50 Series, China) and stored at room temperature.

Table 1
Chemical composition of Bent (mass %).

	SiO_2	Al_2O_3	Fe_2O_3	MgO	CaO	K_2O	TiO_2	P	Others
Raw bent	52.3	13.1	22.1	1.9	4.7	1.3	2.0	0	2.4

2.4. Protein assay

The amount of wGLP before and after immobilization was determined by the bicinchoninic acid test (Smith et al., 1985), using bovine serum albumin as standard. The percent of immobilized protein was calculated as described by Ghiaci et al. (2009a):

$$\%\text{immobilization} = [(C_0 - C_f)/C_0] \times 100 \quad (1)$$

Where C_0 is the total amount of protein in supernatant before immobilization and C_f is the total amount of protein after immobilization.

2.5. Characterization of Bent and Bent-DDA

XRD was performed on the clay powder using an X-Pert Pro diffractometer, operating at 40 kV and 40 mA, with CuK_α radiation ($\lambda = 1.54$ Å), at a scanning speed of 1.5°θ/min. The interlayer distance (d_{001}) of clay was determined by the diffraction peak, using the Bragg's equation:

$$n.\,\lambda = 2.\,d.\,\sin\theta \quad (2)$$

where n is a positive integer; λ = wavelength; θ = diffraction angle; d = interlayer distance.

TGA was carried out with a TGA HI-Res™ thermal analyser (TA Instruments, USA) at a heating rate of 10 °C/min from room temperature to 900 °C in airflow. The specimen weight was in the range of 7–15 mg.

2.6. Western blot assays

One milligram of Bent, Bent-DDA, Bent-wGLP and Bent-DDA-wGLP were suspended in 50 µl of sample buffer (62.5 mM Tris-HCl pH 6.8; 2.5% (w/v) SDS; 0.002% (w/v) bromophenol blue; 0.71 M β-mercaptoethanol; 10% (v/v) glycerol) and incubated at room temperature during 24 h. Samples were boiled for 5 min and running on 12% SDS-PAGE. Proteins were transferred onto nitrocellulose using a semi-dry blotter (Novex, Invitrogen, USA). Immunodetection was performed using polyclonal antibodies raised against wGLP (Mansilla et al., 2012). The blots were allowed to react with goat antirabbit antibody conjugated with alkaline phosphatase (Sigma-Aldrich, USA) and revealed with the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) according to procedures recommended by the manufacturer (Sigma-Aldrich, USA).

2.7. Measurement of SOD activity

SOD activity was measured according to Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA, and 20 µl of protein sample. Enzymatic reactions were carried out at 37 °C for 15 min in a water-bath fitted with a 22 W Phillips fluorescent lamp. The absorbance was measured at 550 nm. Activity of free wGLP was estimated in units by ml of protein solution, whereas for the immobilized wGLP was calculated in units per mg of clay. One unit of SOD activity was defined as the amount of enzyme that produced a 50% decrease, with respect the control, in the absorbance at 550 nm and it was expressed as:

$$\text{Activity of SOD}\left(\frac{\text{U}}{\text{ml}}\right) = (\text{A control} - \text{A assay/A control}) \times 1/50\% \times (\text{Vtotal/VSOD}) \quad (3)$$

Where $A_{control}$ and A_{assay} are the absorbance units at 550 nm of control and sample, respectively; V_{total} is the total volume; and V_{SOD} is the enzyme volume.

2.8. DPPH antioxidant capacity

The antioxidant capacities of Bent and Bent-wGLP were determined using DPPH (Sigma-Aldrich, USA) as described Molyneux (2004). Briefly, 3 mg of DPPH radical were dissolved in 50 ml of HPLC-grade methanol (Merck, Germany) and adjusted to 1.3 absorbance units at 517 nm. Then, 50 µl of each clay sample (1 mg/ml) were mixed with 180 µl of DPPH in single wells of a 96-well plate. The plate was kept for 30 min in the dark. The absorbance was measured at 517 nm using microplate reader (ELX 800, Biotek, USA). The antioxidant capacity was expressed as percentage of the radical scavenging activity and calculated according to the following equation:

$$\text{DPPH scavenged (\%)} = 1 - [(A_{\text{sample}} - A_{\text{Blank sample}})/A_{Control}] \times 100 \quad (4)$$

Where A_{sample}, $A_{blank\ sample}$ and $A_{control}$ are the absorbance values for the sample, sample without DPPH and control, respectively.

2.9. Viability of tomato cells

Tomato cells (*Solanum lycopersicon* cv. Money Maker; line Msk8) were grown in Murashige and Skoog medium at 125 rpm at 24 °C in the dark (Murashige and Skoog, 1962) supplemented with 5.4 µM 1-naphthalene acetic acid, 1 µM 6-benzyladenine and vitamins (554.94 µM myo-inositol, 8.12 µM nicotinic acid, 4.86 µM pyridoxine-HCl and 29.65 µM thiamine-HCl) as described Felix et al. (1991). All drugs were available from Sigma-Aldrich (USA).

Experiments were conducted with 7-day-old subcultured cells. Suspension cells were individually incubated with 1 mg/ml of Bent-wGLP, Bent or wGLP at 24 °C, overnight. Then, cells were harvested and analyzed. Cell viability was tested by Evans blue staining assay (de Pinto et al., 1999). Evans blue is excluded by living single cells, whereas dead single cells and cell debris are stained a deep blue colour (Huang et al., 1986). Tomato cells were collected by centrifuging at 800 ×*g* for 15 s, washed with phosphate buffer pH 7.4, stained with 1% (w/v) Evans blue for 5 min at room temperature and then, washed three times with buffer to remove unbound dye. Cells were observed by bright field microscopy in an Eclipse E200 (Nikon, Japan) microscope. The percentage of cell death was estimated with some adjustments (Baker and Mock, 1994). Approximately, 500 µl of cells from each treatment were incubated with 10 µl of 1% (w/v) Evans blue for 5 min and washed extensively with distilled water to remove unbound dye. The dye bound to dead cells was extracted with 100% DMSO (Sigma-Aldrich, USA) at 85 °C for 20 min. The optical density of supernatant was determined spectrophotometrically at 565 nm. Cells treated with 1% (v/v) Triton X-100 were used as a positive control.

2.10. Statistical analysis

One-way ANOVA was performed. Tukey test was performed as post-hoc analysis for one-way ANOVA. Differences between the compared data with *P*-values < .001 were considered statistically significant. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used as statistical software program.

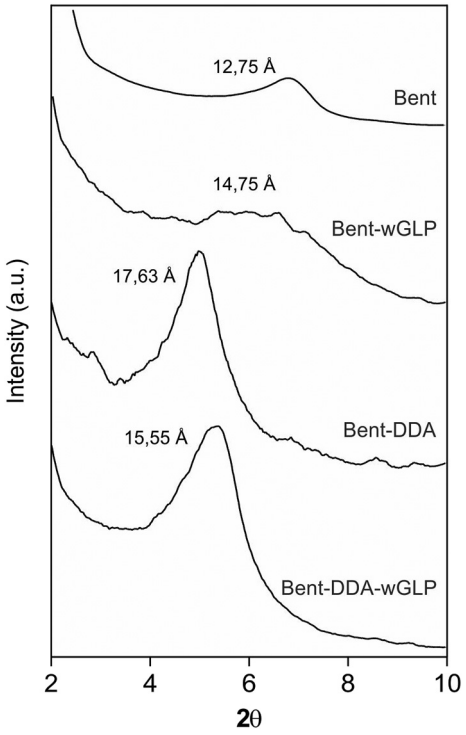

Fig. 1. XRD patterns of non- immobilized and immobilized wGLP onto modified and unmodified Bent.

3. Results and discussion

3.1. Characterization of unmodified and modified Bent

Firstly, based on wGLP amino acid composition its hydrophobicity index by GRAVY calculator (http://www.gravy-calculator.de) was estimated. This index, defined as sum of hydropath values of the total number of amino acids divided by the protein length was 0.357 indicated the hydrophobicity of wGLP. To find an optimum support to immobilize and release wGLP, a comparative study among Bent and Bent-DDA was carried out.

To analyze whether wGLP molecules were mainly adsorbed onto the external surface of Bent or/and it was inserted into the interlayer of Bent a XRD test was performed. It may be noted that the peak position shifts to lower angles as the interlayer distance d001 increases (Günister et al., 2007). The resulting d_{001}-spacing was 12.75 Å and 17.63 Å for Bent and Bent-DDA, respectively (Fig. 1).

After wGLP incorporation into Bent, the diffraction peak widens and shifts to an angle of nearly 6°, giving an interlayer spacing of 14.75 Å (Bragg's equation); thus achieving an increment of nearly 2 Å in Bent interlaminar space. This degree of expansion cannot hold the complete wGLP molecule since its molecular masses correspond to 21 kDa and 66 kDa for monomeric and oligomeric structures, respectively (Mansilla et al., 2012). So, it can contain either the incomplete protein or/and its amino acid residues. This partial intercalation is in agreement with the immobilization of other enzymes in smectite clays (De Cristofaro and Violante, 2001; Serefoglou et al., 2008; Rytwo et al., 2010).

The wGLP incorporation into Bent-DDA modified the interlaminar spacing to 15.55 Å, showing that part of the DDA cations were partially replaced or associated to wGLP, which in turns has a different size and molecular weight than DDA. To examine thermal stability each supports was subjected to TGA (Fig. 2)

The weight loss up to 100 °C for each support is assigned to the absorbed water evaporation; the other two thermal decomposition stages for Bent at 450 °C and 630 °C were associated with dehydroxylation of aluminosilicate (Kakati et al., 2012). In contrast to Bent,

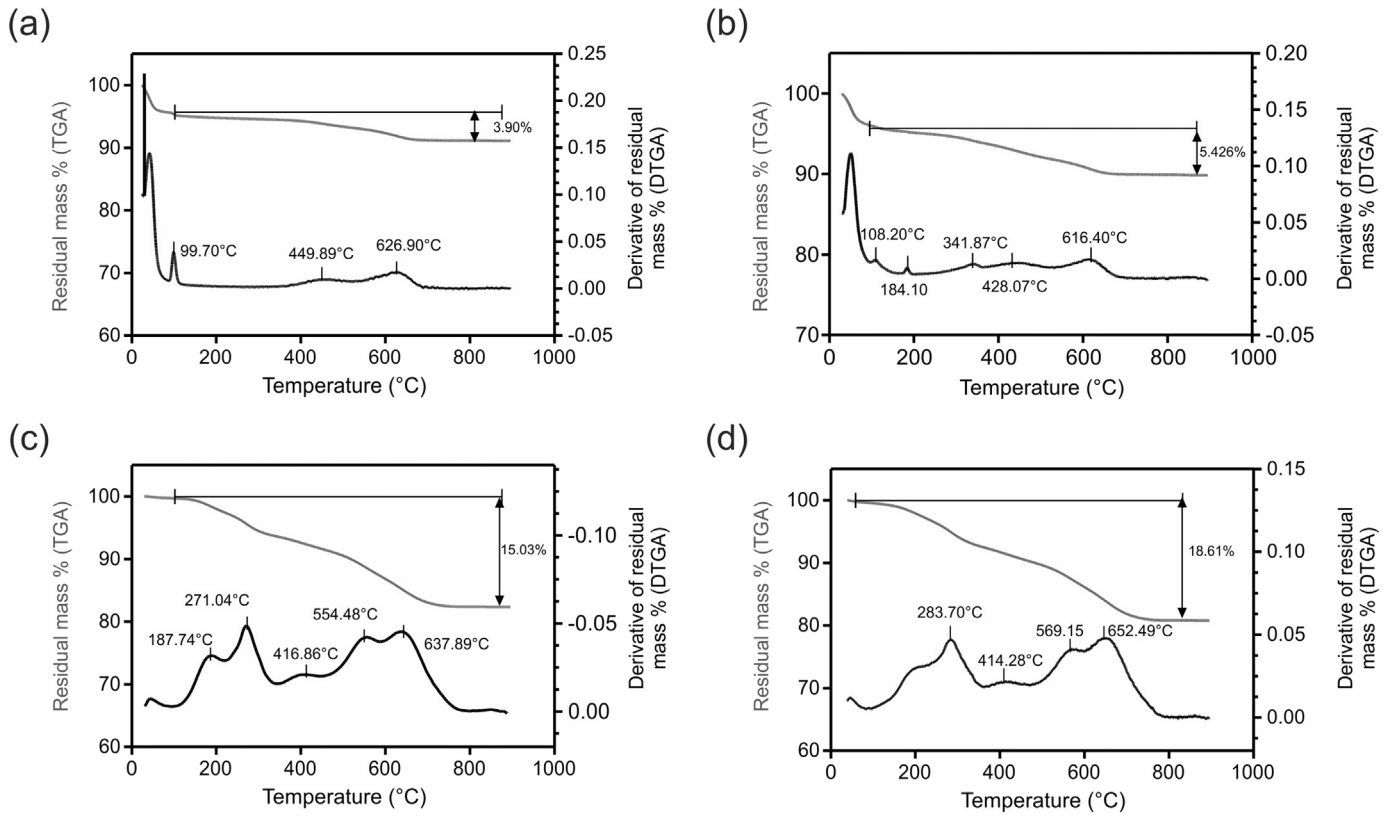

Fig. 2. Residual mass % (TGA) and derivative of residual mass % (DTGA) as a function of temperature for: Bent (a), Bent-wGLP (b), Bent-DDA (c) and Bent-DDA-wGLP (d).

Bent-wGLP display a peak between 184 °C and 341 °C produced by degradation of the intercalated wGLP (Fig. 2b). Interestingly, TGA analysis indicated that the water content (got from residual mass curve until 170 °C) was reduced. It is understandable that all modifications produce a clear decrease on the absorbed water that in turns shows an increased hydrophobicity (Calderon et al., 2008).

Three extra peaks of Bent-DDA, at 187 °C, 271 °C and 554 °C because of DDA decomposition can be detected in TGA curves. For Bent-DDA-wGLP degraded protein peaks overlapped with those of degraded DDA and, therefore not individualized.

Table 2 includes the residual mass of each Bent at 900 °C. As expected, the highest value (96.06%) was obtained for the unmodified Bent, because it is an inorganic material. Decrease of the residual mass correlated with the incorporation of organic fractions degraded at lower temperature. Thus, decline of wGLP was around 8.6%, a quantity associated with the incorporation of wGLP into Bent in the interlaminar space but also in the clay platelets. The organic fraction of Bent-DDA was higher and, depicted a lower residual mass at 900 °C (84.73%) DDA concentration of Bent-DDA estimated from TGA values was nearly 10–14% (Table 2). Finally, the lowest value was displayed by Bent-DDA-wGLP showing that the effective incorporations of DDA and wGLP have taken place. Because Bent-DDA-wGLP had lower interlaminar spacing than Bent-DDA but also a higher organic content, suggest that partial structure of wGLP has been incorporated onto the edges and surface of Bent platelets.

Table 2
Basal spacing and thermal properties of wGLP complexes with modified and unmodified Bent.

Sample	doo1 (A)	Residual mass 900 °C	T_{deg} (°C)
Bent	12.75	96.06	100-450-627
Bent-DDA	17.63	84.73	188-271-417-554-638
Bent-wGLP	14.75	87.49	108-342-617
Bent-DDA-wGLP	15.55	80.07	200-284-566-653

3.2. Protein immobilization efficiency

The amount of wGLP absorbed onto clays was estimated by quantifying protein before and after immobilization. Efficiency of intercalation was approximately 82.7% (± 8.63) and 91.7% (± 3.75) for Bent and Bent-DDA, respectively. Constitution of complexes was confirmed by SDS-PAGE electrophoresis (Fig. 3).

As expected, the immune detected protein of the monomeric wGLP has a molecular mass of approximately 21 kDa (Mansilla et al., 2012). wGLP was specifically detected in both Bent-wGLP and Bent-DDA-wGLP samples showing that it was properly tailored in both clays. These observations agree with the preceding XRD and TGA observations on the interlaminar spacing increase and the final residual mass. wGLP immobilization efficiency was similar for both supports. However, the chemical modification can drastically influence the capacity of Bent for protein loading (Öztürk et al., 2008; Ghiaci et al., 2009b;

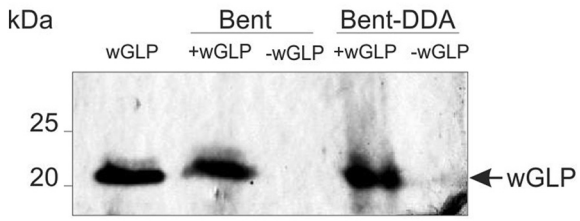

Fig. 3. Western blot of immobilized wGLP. One mg of Bent-wGLP and Bent-DDA-wGLP were run in 12% SDS-PAGE transferred onto nitrocellulose and incubated with polyclonal antibodies against wGLP. Twenty µg of free wGLP were used as control (left lane). The references of molecular mass markers are indicated.

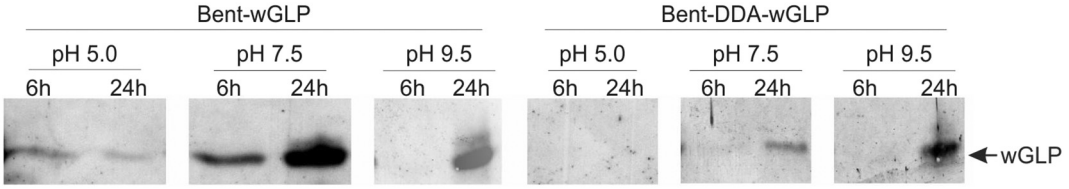

Fig. 4. wGLP released from Bent and Bent-DDA. Ten mg of clay were resuspended in 1 ml of buffer solutions adjusted at pH 5.0, 7.5 and 9.5. At 6 h and 24 h after incubation, the presence of wGLP in each solution was analyzed by western blot and revealed with wGLP antibodies.

Andjelković et al., 2015). Although immobilizing SOD enzymes in chitosan/Fe_3O_4 nanoparticles (Song et al., 2012) or aminopropyl-functionalized KIT-6 mesoporous silica has been done (Falahati et al., 2011), the use of Bent to immobilize a plant SOD enzyme is novel.

3.3. In vitro release of wGLP

The release of wGLP was assayed at different times and pH conditions. A huge amount of protein was released from Bent at pH 7.5 (Fig. 4). However, wGLP was properly associated with Bent-DDA at pH 9.5. Thus, a gradual release from unmodified Bent at neutral pH represented a positive tray for wGLP application in biological fluids.

The cumulative release of wGLP from Bent and Bent-DDA at a 0–96 h time-course was examined (Fig. 5). Releasing wGLP from Bent gradually increased reaching a maximum of 60% at 72 h. However, wGLP released from Bent-DDA was weak up to 96 h. These results allowed us to suggest that Bent-DDA-wGLP complex is highly stable probably favored by large interactions between hydrophobic dodecyl groups. This feature provided a certain irreversible character to the protein immobilization through hydrophobic interactions.

3.4. Bent-wGLP exerts SOD activity

According to our preceding, we assumed that the unmodified Bent represents a promissory support for immobilizing and releasing of the active wGLP onto plant cell systems. To evaluate if immobilized wGLP preserves its antioxidant properties, SOD activity was measured in release solutions from modified and unmodified clays (Fig. 6). SOD activity from Bent-wGLP was well measured. On the contrary, Bent-DDA displayed a negligible activity that could be because of its low release and/or inactivation. SOD activity was similar between native and released wGLP. Then, this proves that tailored clays were suitable to provide a time-sustainable protein delivery. The structural model for wGLP (Mansilla et al., 2012) predicted an active site composed of three histidine amino acid residues (His81, His83 and His128) and one glutamate residue (Glu88) responsible for manganese ion binding and SOD

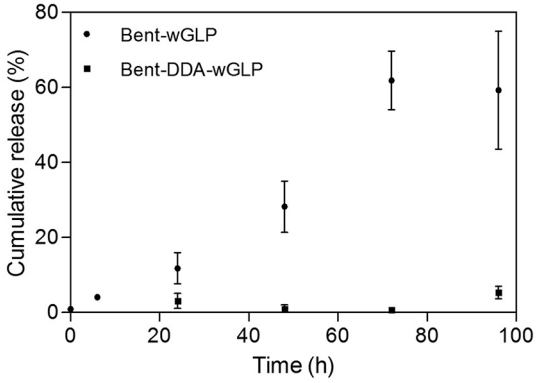

Fig. 5. wGLP released from Bent and Bent-DDA. The protein releasing was carried out by incubating 10 mg/ml of Bent-wGLP (•) and Bent-DDA-wGLP (■) in 100 mM phosphate buffer pH 7.5 or 100 mM borate buffer pH 9.5, respectively. Data are mean values (± SD) of three independent experiments.

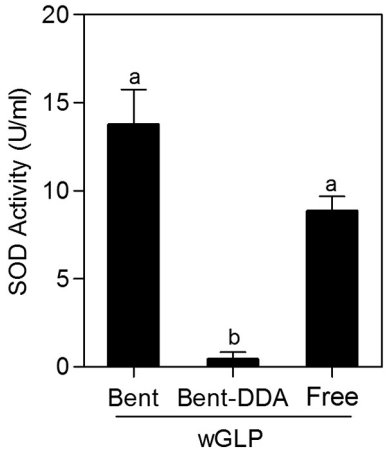

Fig. 6. Measurement of SOD activity. Ten mg of Bent-wGLP and Bent-DDA-wGLP were incubated in 1 ml of 100 mM phosphate buffer pH 7.5 or 100 mM borate buffer pH 9.5, respectively for 24 h. After time, samples were centrifuged and SOD activity was measured in each supernatant. Free wGLP was used as control. Data are mean values (± SD) of three independent experiments. Different letters indicate a significant difference at $P < .001$ (Tukey test).

activity (Woo et al., 2000). Likely, the interaction between wGLP and Bent cannot affect the active site responsible for the antioxidant activity. In this sense, lipase and laccase activities were kept on the immobilization on other types of clay minerals (Tanasković et al., 2017; Olshansky et al., 2018). Thus, Bent results an efficient support to immobilize and release wGLP enzyme in its active form.

3.5. Total antiradical activity measured in Bent-wGLP

Since wGLP could be a promising agent for the management of oxidative stress conditions, the antioxidant capacity was also explored throughout the radical scavenging activity of DPPH compound. The wGLP immobilization into Bent did not affect its antiradical activity (Fig. 7). Both free and released wGLP inhibited nearly 60% the DPPH radical. These results agree with the precedent observation showing that immobilization does not significantly change the SOD active site of wGLP. Therefore, immobilization of wGLP onto Bent is a promising alternative to maintain the enzymatic function and oxidative stability in a proper biological system.

3.6. Bent-wGLP exerted protective action in tomato cell suspension cultures

Plant cell cultures represent effective experimental models to study the effect of metabolic regulators involved in cell viability (Woltering et al., 2007). It is well-known that there is a considerable positive association between antioxidant agents and viability in cell suspensions cultures. In recent years, the antioxidant activity of pure compounds, foods, and dietary supplements were extensively studied throughout developing antioxidant activity assays that include human and animal cell cultures (Liu and Finley, 2005). Immobilizing enzymes and cells has received increasing attention since it can reduce some of the physical problems associated with the cell cultures such as, forming aggregates,

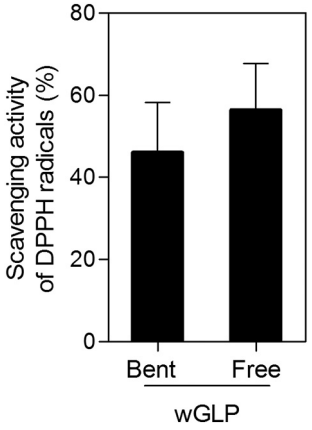

Fig. 7. Scavenging effect of wGLP against DPPH radical. Ten mg of Bent-wGLP were incubated in 1 ml of 100 mM phosphate buffer pH 7.5 for 24 h. Ten μg of free wGLP was used as control. The scavenging capacity on DPPH radical was determined spectrophotometrically at 517 nm. Data are mean values (± SD) of three independent experiments.

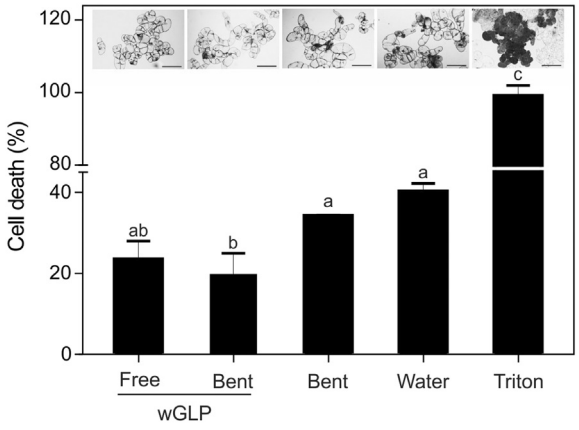

Fig. 8. Tomato cell viability. Suspension-cultured tomato cells were treated with 1 mg/ml Bent, 1 mg/ml Bent-wGLP and 1 mg/ml wGLP for 24 h. Water and 1% Triton X-100 were used as the negative and positive controls, respectively. The relative increase in cell death was estimated by monitoring Evans blue retention. Data are mean values (± SD) of three independent experiments. Different letters indicate a significant difference at $P < .05$ (Tukey test). Inset shows the cells from each treatment observed under bright field microscopy. Bars = 20 μm.

and susceptibility to mechanical stress (Datta et al., 2013).

Next, wGLP was tested as a potential source of a natural antioxidant to prevent oxidative stress-related disorders in tomato-cultured cells. Tomato cells cultures containing Bent-wGLP were incubated for 24 h. After time, the effect of wGLP on cell viability was evaluated by Evans blue exclusion (Fig. 8). To determine the protective action of wGLP on tomato cells we worked with weakly stressed tomato cell cultures. As expected, a cytoprotective effect of free wGLP was detected. Preservation of tomato cell viability under the presence of Bent-wGLP was also measured. The protective effect of Bent-wGLP on tomato cells could be related to the redox functionality of wGLP corroborated by total antiradical and SOD activities. However, we cannot discard that Bent-wGLP interacts either directly or indirectly with other compounds whose activities are vital for cell viability. Cytoprotection by a plant antioxidant enzyme was demonstrated in *Morinda citrifolia* cells (Deshmukh and Wadegaonkar, 2012). However, the action of immobilized antioxidant enzymes in tomato cells has not been reported yet. Indeed, future research will be welcome to deepen on considerations of bioavailability and the antioxidant mechanism exerted by wGLP in tomato cells.

4. Conclusions

In this work, wGLP was immobilized in unmodified and DDA-modified Bent by a simple and efficient laboratory method that is, in turn, scalable. XRD and TGA allowed us to assume that wGLP was incorporated to these two studied bentonites. The amount of protein incorporated was improved by modification with DDA that is a common approach to change clay physicochemical properties. Raw Bent is a successful matrix for immobilization, release and preservation of the antioxidant activity of wGLP. Bent-wGLP has also a protective effect on cell viability and, therefore, conferring it useful and potential properties for different applications in plant biotechnology.

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