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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: