



Chitosan/clay nanocomposite films as supports for enzyme immobilization: An innovative green approach for winemaking applications



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ABSTRACT

Protein haze formation in white wines during storage is considered the most important instability phenomenon of non-microbial origin. The use of proteolytic enzymes, covalently immobilized on solid supports, has recently proved to be a promising approach for reducing the haze potential of white wines. In this study, supports based on chitosan and nanoclays were produced by solvent casting technique, and investigated for use as a covalently linked bromelain carrier in wine-like medium. Various kinds of nanoclays were tested, including montmorillonite, sepiolite and bentonite, in different amounts (1–5% w/w with respect to chitosan). More specifically, unmodified and organically modified clays and an activated bentonite authorized for contact with food (i.e. OPTIGEL CK) were considered, and their effect on the final microstructural, thermal and mechanical properties of the obtained composite systems was investigated, on the basis of their different chemical composition, morphology, hydrophilicity/hydrophobicity and surface charge, resulting in different interactions with the polymeric matrix and a different enzyme loading.

The nanocomposite films were used as innovative supports for the covalent immobilization of pineapple stem bromelain, selected as reference enzyme, and the kinetic parameters of the immobilized bromelain were analyzed.

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1. Introduction

Protein haze formation is the most important instability phenomenon of non-microbial origin, which occurs in white wines during storage (Batista, Monteiro, Loureiro, Teixeira, & Ferreira, 2009; Waters et al., 2005). Bentonite is the most commonly used fining agent for stabilizing white wines, despite its unspecificity toward haze forming proteins and its detrimental effect on wine quality (Ferreira, Picarra-Pereira, Monteiro, Loureiro, & Teixeira, 2002; Sauvage, Bach, Moutounet, & Vernhet, 2010). For this reason, various authors have evaluated the effectiveness of proteolytic enzymes, either in free form (Marangon et al., 2012; Younes, Cilindre, Jeandet, & Vasserot, 2013) or covalently immobilized on

solid supports, for reducing the haze potential of white wines (Benucci, Esti, & Liburdi, 2014; Liburdi, Benucci, & Esti, 2010), as a promising alternative to bentonite fining.

In recent years, many inorganic (i.e. glass, mineral kissiris, γ -alumina, polygorskite, montmorillonite, hydromica, porous porcelain, pumice stone) and organic materials (i.e. alginate, cellulose, carrageenan, agar, pectic acid and chitosan) commonly found in nature, have been proposed as immobilization supports to be used in winemaking (Colagrande, Silva, & Fumi, 1994; Divies, Cachon, Cavin, & Prevost, 1994; Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). Among the organic materials, thanks to its hydrophilicity, biocompatibility and biodegradability, chitosan (CS), a natural polyaminosaccharide obtained by *N*-deacetylation of chitin, is deemed to be ideal for producing supports in various forms (particles, gels, spheres, fibres and membranes) for the immobilization of food enzymes (Altun & Cetinus, 2007; Benucci et al., 2016; Boadi & Neufeld, 2001; Dwevedi & Kayastha, 2009; Krajewska,

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2004; Malmiri, Ghaz Jahanian, & Berenjian, 2012; Noda, Furuta, & Suda, 2001; Spagna, Barbagallo, Casarini, & Pifferi, 2001; Zappino et al., 2015). In a previous study, membranes based on CS from animal (shell fish) and microbial (*Aspergillus niger*) sources have been obtained by solvent casting method, using glycerol as plasticizer (Zappino et al., 2015), for applications in acidic medium mimicking wine. However, several authors have affirmed that CS films have low mechanical properties and thermal stability (Cengiz, Çavaş, & Yurdakoç, 2012; Tang et al., 2009; Wang, Shen, Zhang, & Tong, 2005; Chang & Juang, 2007a, b), and that adding proper nanofillers (i.e. carbon nanotubes, nanoclays and silica) to the polymeric matrix may be an effective way of improving the mechanical and thermal properties (Panzavolta et al., 2014).

In particular, nanoclays are widely used for the preparation of CS based composites, due to their low cost, high availability, high cation exchange capacity, strong adsorption ability and high surface area (Chang & Juang, 2007b; An, Zhou, Zhuang, Tong, & Yu, 2015; Bertolino et al., 2016; Daraei et al., 2013). Among the clays, the most widely studied ones are montmorillonite (MMT, a hydrated 2-1 layered alumina-silicate, composed of two silica tetrahedral sheets attached to a central Al octahedral sheet), and sepiolite (SP, a microfibrillar hydrated magnesium silicate, composed of two tetrahedral silica sheets enclosing a central magnesium sheet), employed in numerous industrial and environmental applications (i.e. oil refining, wastewater treatment, odour removal, pharmaceuticals, and pesticide carriers, paper and detergent industries) (Hsu, Wang, & Lin, 2012; Lewandowska, Sionkowska, Furtos, Grabska, & Michalska, 2015; Sedaghat, Ghiaci, Aghaei, & Soleimani-Zad, 2009). Moreover, several studies have been devoted to the use of organomontmorillonite (oMMT), produced by exchanging inorganic cations of MMT with organic ammonium ions, in order to make the MMT more hydrophobic and suitable for several applications, such as clay-based nanocomposites (Ray & Okamoto, 2003) and absorbents of organic pollutants (Adebajo, Frost, Klopogge, Carmody, & Kokot, 2003). Indeed, the surface is characterized by a stable negative charge and by hydrophilicity, due to the presence of inorganic cations (e.g. Na^+ and Ca^{2+}). Therefore, attempts have been made to modify the clay surface with cationic surfactants, such as alkyl ammonium salts (Gupta & Suhas, 2009), since replacing exchangeable cations with cationic surfactants changes the clay surface from hydrophilic to hydrophobic, originating organoclays (Gupta & Suhas, 2009; He, Duchet, Galy, & Gérard, 2006).

In particular, CS films loaded with MMT/oMMT and SP have also been used to produce supports for enzyme immobilization (Azeredo, 2009; Gopinath & Sugunan, 2007) and as green plastics (Darder, Aranda, & Ruiz-Hitzky, 2012; Wang, Chen, & Tong, 2006). In fact, in the absence of other organic modifiers, this biopolymer is able to intercalate into the pristine clay via the cationic exchange process, originating intercalated and exfoliated nanostructures, which can provide strong interactions between the clay and CS (Tang et al., 2009). To the best of our knowledge, in literature there is a lack of systematic studies about the influence of several clays on the final properties of composite CS based supports as enzymatic carriers for application in winemaking. Therefore, the aim of this study was to produce biopolymeric nanocomposite films as carriers for the covalent immobilization, via glutaraldehyde (GDH) cross-linking, of a proteolytic enzyme (bromelain from pineapple stem) to be applied in winemaking process. In order to achieve this goal, on the basis of a previous study by the same authors (Zappino et al., 2015), low molecular weight CS blended with 25% glycerol was selected as biopolymeric matrix and different kinds of nanoclays were added as fillers to the polymeric matrix. More specifically, organically modified montmorillonites, bentonites (unmodified and modified) and sepiolite were tested in different amounts (1–5%

w/w with respect to CS). We decided to use, as immobilization procedure, the covalent coupling of the protease to glutaraldehyde-activated supports, being the most commonly applied (also for food applications), quite simple and efficient (Garavand, Rouhi, Razavi, Cacciotti, & Mohammadi, 2017). The chemical, thermal, microstructural and mechanical properties of the obtained systems were analysed by infrared spectroscopy (FTIR/ATR), differential scanning calorimetry (DSC), scanning electron microscopy (SEM) and uniaxial tensile tests.

2. Materials and methods

2.1. Materials

Films, to be used as supports for enzyme immobilization, were produced using CS powder obtained from an animal source, composed of shellfish powder with low molecular weight (Lot#MKBG3334V; 50–190 kDa; percentage of deacetylation: 75%; Sigma Aldrich). Five different nanoclays, kindly provided by various suppliers (Table 1), were added to the CS-based films as fillers: i) high purity organically modified montmorillonites (DK1N and DK2); ii) selected and activated bentonite authorized for contact with food (OPT); iii) high purity modified bentonite (SMP); iv) high purity unmodified sepiolite (PAN). The characteristics of the used clays are summarized in Table 1.

The organoclays DK1N and DK2 were modified by different levels of cation exchange reaction with octadecylammonium salt in order to broaden their interlayer spacings, according to the method described in literature (Kornmann, Lindberg, & Berglund, 2001), and had an average thickness of approximately 25 nm (aspect ratio 100–1000).

The tripeptide chromogenic substrate, Bz-Phe-Val-Arg-*p*-nitroaniline (pNA), used for evaluating protease activity, was purchased from Bachem (Bubendorf, Switzerland). Stem bromelain (EC 3.4.22.32), which was selected as referred enzyme, glycerol ($\geq 99.5\%$), glutaraldehyde (GDH) (25%), and all of the other reagents were obtained from Sigma-Aldrich (Milan, Italy).

2.2. Preparation of chitosan/clay nanocomposite films

Films were prepared by solvent casting technique, using low molecular weight CS (1% w/v) blended with glycerol in the weight ratio CS:glycerol 75:25, selected on the basis of previous results by Zappino et al. (2015). The five different nanoclays were added in various w/w percentages with respect to CS (i.e. 1, 3, 5% w/w), thus obtaining three different compositions for every clay (DK1N-x, DK2-x, OPT-x, SMP-x, PAN-x, where x is the clay w/w percentage).

Firstly, the nanoclays were ultrasonicated in aqueous solution (Ultrasonicator Sonics Vibracell CV33, 750 W, 20 kHz, Amplitude 30%, time 30'), subsequently acetic acid (2% v/v), low molecular weight CS (1 %w/v) and glycerol were added. The obtained suspensions were magnetically stirred overnight. The CS solutions were poured onto plastic Petri dishes, which were placed in a fume hood at room temperature for 48 h to allow for solvent evaporation and film formation.

Moreover, as a reference, the clay-free film, composed of CS and glycerol, was produced following the same procedure.

2.3. Chemical, thermal, mechanical and morphological characterization of chitosan/clay nanocomposite films

The typical functional groups of the produced films were investigated by means of FT-IR spectrometer (Jasco, FT/-6600), equipped with an attenuated total reflectance (ATR). Spectra were acquired by pressing the samples into contact with the ATR cell, in

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