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Full inhibition of enzymatic browning in the presence of thiol-functionalised silica nanomaterial

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ABSTRACT

Darkening processed fruits and vegetables is caused mainly by enzymatic browning through polyphenol oxidase (PPO) action. Accordingly, we explored the potential of four silica-based materials (MCM-41 nanometric size, MCM-41 micrometric size, UVM-7 and aerosil), non-functionalised and functionalised with thiol groups, to inhibit PPO activity in the model system and apple juice. All materials showed relevant performance when immobilising and inhibiting PPO in model systems, and support topology is a main factor for enzyme immobilisation and inhibition. Thiol-containing silica UVM7-SH showed the greatest inactivation, and similar browning values to those obtained by acidification. The enzyme's kinetic parameters in the presence of UVM-7-SH suggested non-competitive inhibition, which indicated that the material interacted with the enzyme, but beyond the active centre. In real systems, UVM-7-SH completely inhibited enzymatic browning in apple juice (cv. Granny Smith and cv. Golden Delicious) up to 9 days after 5 min of contact.

1. Introduction

Consumer acceptance of new food products depends on their organoleptic properties, with appearance and colour being the most important factors when making buying decisions, especially about fruits and vegetables. Therefore, maintaining food colour during shelf life is a main objective in the food industry given its possible economic impact. The main reason for colour change with fruits and vegetables is known as enzymatic browning, and it has been estimated that this process is responsible for more than 50% of waste (Whitaker & Lee, 1995).

Enzymatic browning is a complex chemical reaction that is divided into several phases, enzymatic hydroxylation, enzymatic oxidation and non-enzymatic polymerisation. The two first steps are catalysed by polyphenol oxidase (PPO). This process transforms phenolic compounds into polymeric structures, which produce the characteristic brown colour (Bello Gutierrez, 2000).

The polyphenol oxidase (PPO) enzyme (EC 1.14.18.1 o EC 1.10.3.1), also known as tyrosinase, catechol oxidase, monophenol oxidase and creolase, has two copper (II) ions, and each is linked to three histidines (type 3 copper enzyme). In nature, the PPO enzyme can be found in two different forms to catalyse two distinct reactions. In the first reaction, the hydroxylation of mono-phenols generates *ortho*-phenols, while the enzyme oxidises these *ortho*-phenols into quinones in the second one. These forms are met-tyrosinase and oxi-tyrosinase, but only oxi-tyrosinase is able to hydroxylate mono-phenols (Rolff, Schottenheim, Decker, & Tuczek, 2011; Sánchez-Ferrer, Neptuno Rodríguez-López, García-Cánovas, & García-Carmona, 1995). The last enzymatic browning process step consists in the non-enzymatic polymerisation of quinones, which gives rise to melanoides (Rouet-Mayer, Ralambosoa, & Philippon, 1990) that are responsible for colour changes.

The main factors that affect food enzymatic browning are: pH, temperature, enzyme activity, quantity of polyphenols, and presence of oxygen (Martinez & Whitaker, 1995). For this reason, processing vegetal foods with large amounts of active PPO and polyphenols involves the risk of enzymatic browning, which causes colour changes and reduces consumer acceptability.

It is important to note that in Europe, and according to FAOSTAT, apple (*Malus Pumila*) is the second fruit in consumption and production terms, and is also one of the most important polyphenol sources in diet.

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Given their high polyphenols content, apples are one of the fruits most affected by enzymatic browning (Hertog, Hollman, & Katan, 1992). Apples contain several forms of polyphenols, of which chlorogenic acid is the most important given its high concentration in this fruit (Picinelli, Suárez, & Mangas, 1997). Another is L-tyrosine (Rocha & Morais, 2001), which is also found in other foods such as mushrooms and crustaceans, and it provokes the same browning problems as in apples.

Over the years, the industry has adopted different chemical and physical strategies to lessen enzymatic browning and to, therefore, reduce fruit and vegetable losses. Traditionally, heat treatment has been used as an enzymatic inactivation method (Williams, Lim, Chen, Pangborn, & Whitaker, 1986). However, this process has many problems since fruits and vegetables have a considerable amount of thermosensitive compounds, such as vitamins (Bomben, Dietrich, Hudson, Hamilton, & Farkas, 1975), carotenoids and anthocyanins (Buckow, Kastell, Terefe, & Versteeg, 2010), which may be affected during treatment. Besides, temperature applications must be fast and enzymatic inhibition must be complete or the browning process accelerates (Toribio & Lozano, 1986).

When chemical treatments are applied, acidulants that lower pH, or chelating agents that interact on the active centre of the enzyme, have been used to inactivate PPO (Sapers et al., 1989). Sulphites have also been employed to prevent colour change, but the potential induction of allergenic reactions in consumers has limited their use in food and beverages (Sapers, 1993).

Other non-thermal treatments, such as ultrasounds (Abid et al., 2013), CO_2 supercritical (Gui et al., 2007), electrical pulses (Ho & Mittal, 1996), high hydrostatic pressure (HHP) (Juarez-Enriquez, Salmeron-Ochoa, Gutierrez-Mendez, Ramaswamy, & Ortega-Rivas, 2015) and ultraviolet light (Müller, Noack, Greiner, Stahl, & Posten, 2014) having shown good results to inactivate PPO, they have their limitations, such as high cost and large machinery requirements.

From another point of view, nanotechnology is opening up new research areas in several fields, such as medicine and pharmacology (Vallet-Regí, Balas, & Arcos, 2007), and also in the food industry (Pérez-Esteve, Bernardos, Martínez-Máñez, & Barat, 2013). However in the food industry, the use of nanoparticles has focused on developing encapsulated bioactive compounds and designing active packaging, but applications in industrial processes are still scarce. Silica mesoporous materials are nanomaterials that are synthesised by combining surfactant micellar aggregates with reactive silica precursors (Beck et al. 1992). Depending on the surfactant being used, the resultant materials have different structures and pore sizes, which vary between 2 nm and 50 nm. These materials can also be functionalised easily with diverse chemicals groups. Many silica mesoporous materials have been developed in the last 25 years when the M41S family was discovered by a scientist at Mobil Oil (Beck et al., 1992); e.g., MCM-41 is one of the most investigated materials and has a 2D hexagonal structure. UVM-7 is also a mesoporous material that was synthesised at the University of Valencia in 2002 based on the "atrane route" (El Haskouri et al., 2002). This material is characterised by having both intra-particle and inter-particle pores, which provide the material with a large surface area and a stable pore distribution. These features make these supports ideal for hosting and interacting with enzymes (Ispas, Sokolov, & Andreescu, 2009). Nevertheless, the interactions between silica mesoporous materials and polyphenol oxidase have barely been studied as a strategy to avoid enzymatic browning in food systems (Corell Escuin, García-Bennett, Ros-Lis, Argüelles Foix, & Andrés, 2017), with only a partial inhibition in model systems and no tests available in real samples.

The aim of this work is to study interactions between four silica mesoporous materials (MCM-41 nanometric size, MCM-41 micrometric size, UVM-7 and Aerosil 200), and their parent materials functionalised with thiol groups, with the PPO enzyme, to evaluate their ability to inhibit enzymatic browning in both model systems and apple juice.

2. Materials and methods

2.1. Chemicals

Aerosil 200 was purchased from Evonic industries. Mushroom tyrosinase, Dopamine hydrochloride, L-tyrosine, chlorogenic acid, sodium dihydrogen phosphate and disodium hydrogen phosphate were acquired from Sigma-Aldrich, and were used without further purification. Finally, two different varieties of apples (cv. Granny Smith & cv. Golden Delicious), obtained from a local retailer, were used to prepare juice.

2.2. Synthesis and characterisation of silica materials

Materials were prepared following known procedures. A detailed description of the synthesis of the three mesoporous nanomaterials, the functionalisation with the thiol groups and their characterisation can be found in the Supplementary Material.

Silica materials characterisation was done by low-angle X-ray powder diffraction (XRD) in a Bruker D8 Advance using CuKα radiation. A JEOL-jem-1010 was employed for the transmission electron microscopy (TEM) characterisation. The amount of thiol groups in the four materials was measured by a TGA/SDTA 851e Mettler Toledo (TGA).

The nitrogen adsorption/desorption isotherms were measured in a volumetric adsorption analyser (Micromeritics ASAP 2020) at a liquid nitrogen temperature (−196 °C). The Barret-Joyner-Halenda (BJH) model (Barrett, Joyner, & Halenda, 1951) was fitted to estimate pore size distribution and pore volume, while the specific surface area was calculated by the BET model (Brunauer, Emmett, & Teller, 1938) within the low-pressure range. Wall thickness and a_0 cell were calculated from the porosity and XRD data (Neimark, Ravikovitch, Grün, Schüth, & Unger, 1998).

2.3. Enzyme kinetics in model systems

Mushroom tyrosinase was used to prepare the model systems. Dopamine, L-tyrosine and chlorogenic acid were tested as substrates. In a typical experiment, 1.25 mL of a solution that contained 0.005–2.5 mM of substrate in the presence of 10 mM phosphate buffer at pH 5.5 is mixed with 0.25 mL of the enzyme solution (0.14 mg/mL–375 U/mL). Absorbance is measured every 20 seconds at 420 nm. Enzyme kinetic studies were performed at 20 °C in duplicate. Solutions at pH 3.5 and 4.5 were also prepared for dopamine.

The initial reaction rate was calculated from the slope of the linear part of the absorbance-time curves. The saturation curve was obtained by plotting the reaction rate values *versus* the different substrate concentrations. Since tyrosinase enzymatic reaction follows the Michaelis-Menten equation (Espín et al., 2000), the corresponding kinetic parameters, K_m and V_{max}, were obtained from the Lineweaver-Burk plot (Doran, 1998). Afterwards, the catalytic constant (K_{cat}) and the specific constant were calculated from the kinetic parameters for each substrate and pH. A one-way analysis of variance (ANOVA) was applied to determine the influence of the different substrates and the influence of pH in the case of dopamine.

2.4. Study of enzyme-material interactions in model systems

In order to determine the nature of the enzyme-material interaction, two types of studies were conducted: enzyme kinetics in the presence of the material and quantification of enzyme immobilisation. In the kinetic studies, 0.25 mL of the enzyme solution (375 U/mL) were added to 1 mL of phosphate buffer 10 mM (pH = 4) that contained 1 mg of material. The resulting suspension was stirred for 2 h to ensure that the interactions between the enzyme and the material were as high as possible. Afterwards, 0.25 mL of dopamine 0.12 mM were added and colour enhancement was monitored for 60 min by measuring