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Short communication

Application of mesoporous silica materials for the immobilization of polyphenol oxidase

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ABSTRACT

The ability of a number of mesoporous silica materials (SBA-15, SBA-3, and MCM-48) to immobilize polyphenol oxidase (PPO) at different pH has been tested. Pore size and volume are the structural characteristics with higher influence on the PPO immobilization. Mesoropous material SBA-15 adsorbs a larger quantity of PPO at pH 4.00 and offers an inhibition of enzymatic activity close the 50% in apple extracts.

1. Introduction

The control of enzymatic browning is of great economic importance in processed fruits and vegetables. It affects negatively the attributes of colour, taste, flavour and nutritional value of fruits and vegetables. It is estimated that more than 50% of the fruit market losses are a result of enzymatic browning (Whitaker & Lee, 1995).

Several methods have been proven for the inhibition of PPO activity. Sulphites are used as agents to prevent browning, but are also associated with severe allergies in certain vulnerable populations, limiting their presence in food or beverages (Sapers & Miller, 1993). Heat treatments are unsuitable to inhibit such reactions, due to the detriment in nutrient and vitamin content and they can even increase browning reactions (Toribio & Lozano, 1986). Whereas addition of ascorbic acid (Denoya et al., 2012; Gacche, Zore, & Ghole, 2003), anoxic conditions (Rocha & Morais, 2001), refrigeration and non-thermic treatments (Olivas, Mattinson, & Barbosa-Cánovas, 2007; Perez-Gago, Serra, & Del Rio, 2006) have shown good results, their industrial application is not widely developed due to the economic cost or the induction of variations in the organoleptic properties.

Thus, there is a necessity for new methods to inhibit PPO activity in fruits and vegetables. To this effect nanomaterials, in particular high surface area silica based mesoporous materials exhibit properties suitable for irreversible enzyme binding and inhibition. Additionally, they show no apparent toxicity (Fu et al., 2013; Kupferschmidt et al., 2013; Lu, Liong, Li, Zink, & Tamanoi, 2010), their structure includes space for functional groups and a high density of silanol groups able to offer pH dependent electrostatic interactions. This feature provides numerous possibilities for selecting these materials as carriers, adsorbents and supports, or for further reactions with organic functional groups. Consequently, their application has been explored within the food sector in various fields such as catalysis, synthesis of nutritional compounds, as sensors or in the controlled release of bioactive molecules (Bernardos & Kourimska, 2013). However, few studies have focused on the mesoporous material–enzyme interaction with relevance to the food industry (Arroyo, 1998; Datta, Christena, & Rajaram, 2013; Kupferschmidt, Csikasz, Ballell, Bengtsson, & Garcia-Bennett, 2014). Encapsulation of PPO to detect phenolic compounds (Mangrulkar, Yadav, Meshram, Labhsetwar, & Rayalu, 2012) or to produce *o*-diphenols in vitro has been demonstrated (Marín-Zamora, Rojas-Melgarejo, García-Cánovas, & García-Ruiz, 2009), but the inhibitory activity of such materials with respect to PPO activity was not explored. In this context, the aim of the present article is to perform a prospective study of the potential of various silica mesoporous materials as immobilizing or sequestering agents for polyphenol oxidases from fruits or vegetables.

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2. Materials and methods

2.1. Characterization of mesoporous materials

Mesoporous materials SBA-15, SBA-3 and MCM-48 were selected due to their different morphological and textural characteristics. The three materials were prepared according to already well known procedures (Anunziata, Beltramore, Martinez, & Bellon, 2007; Wang, Wu, Sun, & Zhong, 2001; Zhao et al., 1998) and nitrogen adsorption studies, scanning and transmission electron microscopy (SEM, TEM) were performed. The degree of microporosity was calculated from a t-plot analysis and the pore size calculated from Density Functional Theory (DFT). See Electronic supporting information for further details.

2.2. PPO enzyme-silanol surface interation

In order to study the PPO enzyme-silanol surface interaction and aiming to explore a future industrial use of this kind of materials as inhibitors, available commercial PPO was used (PPO lyophilized powder from mushroom, 3130 units mg^{-1} from Sigma-Aldrich). Short contact times (from 15 min) and different pH (pH 1, 3, 4, 5 and 7) were tested. The Bradford method (Bradford, 1976) was used to determine the amount of enzyme.

Sampling times were stablished at 0, 15, 30, 60, 120, 360 and 1.440 min in order to follow the kinetics of the immobilization process. The measurement was done five independent times for each mesoporous materials tested.

The immobilization process was modelled by the application of the Power Law (Wise, 1985) and Higuchi equations (Higuchi, 1961). These are standard models to describe the drug release kinetics from a solid porous matrix based on diffusion and derived from by Fick's laws. Here they have been used to explain the kinetics of enzyme adsorption (the opposite to drug release) within the mesoporous matrices. Both Power law and Higuchi model assume that the drug diffuses from inside the porous matrix outwards and as the drug is released, the distance the remaining drug has to travel increases. The amount of drug released is proportional to the square root of time in the case of the Higuchi equation (Higuchi, 1961).

The DFT calculations were developed using the Micromeritics software package TriStar 2030 (Micromeritics Instruments Corp., Atlanta, USA) which provides a method to fit nitrogen isotherms curves from mean-field density functional theory to determine pore size distribution assuming a cylindrical or cage type pore geometries (see i.e. Olivier, Conklin, & Szombathely, 1994)

Additionally, extracts of PPO were obtained from apples and were used to test and confirm the material performance observed in the commercial one. PPO extracts were obtained from Granny Smith cultivar apples according to the method proposed by Guerrero (2009). Then, 15 mg of SBA-15 were added in the presence of 5 mL of extract diluted to 15 mL with phosphate buffer at pH 4. The variation in the activity was monitored at diverse times after contact with the material during one hour.

3. Results and discussion

The three materials are composed of silica and include porous systems with diverse sizes and topology, i.e. while SBA-15 and SBA-3 show 2D hexagonal pore structures with larger and smaller pores respectively, MCM-48 offers a 3D connected cylindrical system of pores. Nitrogen adsorption studies, scanning and transmission electron microscopy (SEM, TEM) confirmed the textural and morphological characteristics of the materials (see Table 1) (Anunziata et al., 2007; Wang et al., 2001; Zhao et al., 1998).

According to the pore size, SBA-15 possesses the largest pore size (133 Å), SBA-3 shows the smallest pore size (29.2 Å) and MCM-48 is lying in between with a pore size of 33.4 Å (Table 1). The only material which has micropores is SBA-15 with two different pore sizes in the microporous range (10.6 Å and 20.0 Å), in addition to its mesoporosity.

A summary of the amount of enzyme immobilized by each material at diverse times and pH can be found in Fig. 1. The standard deviation was never higher than 0.03. For clarity purposes a graph plotting the amount of PPO adsorbed by the different materials at different pH values after 1 h of contact time has been included as Fig. 2.

A general view of the Fig. 1 graphs shows that, in general, the immobilization of the enzyme is not a fast process and increasing the material-enzyme interaction time increases the amount of PPO immobilized on the silica walls. The data also reveals that the immobilization rate is strongly dependent on both, material textural properties as well as pH. On the contrary, the channel configuration (2D-hexagonal channels for SBA-15 and SBA-3, a 3D-channel structure for MCM-48) is not of particular relevance.

SBA-15 at pH 4 offers the highest and fastest loading capacity, being able to load an equivalent amount of enzyme higher than 30% of its mass even at very short times (the first sampling point was stablished at 15 min). Similar results are obtained at pH 3. In contrast, SBA-3 and MCM-48 show slower processes in particular at pH 1 or 7. Since all the materials are composed of silica, a similar behaviour can be expected as a response to pH variations; in fact, Fig. 2 shows that pH 4 offers the maximum capacity of immobilization of PPO for all the materials, followed closely by pH 3; in contrast, the pH value which shows the minimum quantity of enzyme immobilized was in all cases pH 7.

Knowing the structural and morphological properties of the tested materials allows understanding the results of the immobilization assays. Materials having higher pore volume and bigger pore size (SBA-15) can immobilize more PPO than those with low pore volume and size (MCM-48) and the process is faster. Taking into account the similarity of pore volume between the SBA-15 and the SBA-3, the pore size seems to be the key parameter. As the pore size increases, PPO can diffuse more easily and is free to

Table 1
Textural parameters of calcined mesoporous materials calculated from nitrogen adsorption data.

Material	Particle size[a] µm	Surface area[b] (m^2/g)	Mesoporous		Microporous		
			Pore volume[c] (cm^3/g)	Pore size[d] (Å)	Pore volume[e] (cm^3/g)	Surface area[e] (m^2/g)	Pore size[a] (Å)
SBA-15	1.3-1.5	411.8	1.00	133.0	0.023	66.3	25.2
SBA-3	>5	1238	0.94	29.2	–	–	–
MCM-48	<0.2	311,5	0.36	33.4	–	–	–

[a] Determined from SEM.
[b] Surface area based on the Brunauer-Emmett-Teller (BET) equation.
[c] From nitrogen adsorption isotherm. The pore volume is obtained from the total amount adsorbed at 0.98 in relative pressure.
[d] Pore size derived from Density Functional Theory (DFT) model assuming cylindrical pore geometry.
[e] Microporous parameters calculated from t-plot.

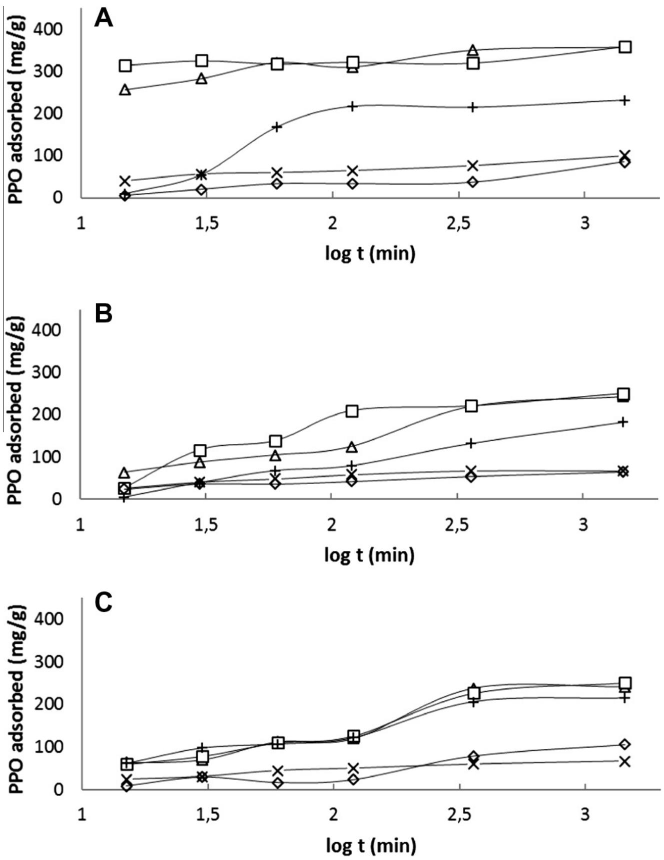

Fig. 1. Immobilization kinetics of PPO (mg of enzyme/g mesoporous material) into the different materials at different pH values (×) pH1, (Δ) pH3, (□) pH4, (+) pH5, (◇) pH7. (A) SBA-15 (B) SBA-3 (C) MCM-48.

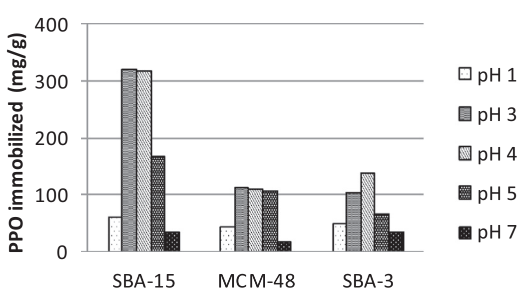

Fig. 2. Amount of PPO adsorbed (mg of enzyme/g of mesoporous material) into the different materials at different pH values after 1 h of immobilization assay.

interact with the silanol groups of the silica walls than in materials such as SBA-3 or MCM-48 which have narrow pore diameters. Similar results have been observed in other studies, being the SBA-15, the material with the higher pore size, the material with higher retention capacity (Vinu, Gokulakrishnan, Mori, & Ariga, 2008). Furthermore, each material tested has a different immobilization behaviour as a function of pH (Fig. 2) but all of them present higher rate at pH 4.00. The silanol groups of mesoporous walls are negatively charged at this pH value (silanols show a pI around 2, see i.e. Cui, Zin, Cho, & Ha, 2005)and the PPO, which has a pI value around 5.0 (Fan & Flurkey, 2004), is positively charged. Favourable electrostatic interactions thus, exist between the enzyme and the silica wall leading to adsorption and immobilization.

The other two pH values tested near from this value (pH 3.00 and 5.00) also present high immobilization rate. By contrast, the solutions far from pH 4 (pH 1.00 and 7.00) show almost no PPO immobilized – less than 100 mg of PPO per g of mesoporous material. This behaviour can be explained because at these pHs both enzyme and material have the same charge (Fan & Flurkey, 2004; Cui et al., 2005). At pH 1.00, both are charged positively; whereas at pH 7.00 both have a predominately negative charge.

In order to gain deeper insight about the immobilization process, all the kinetic curves were fitted by power law and Higuchi equations (Higuchi, 1961; Wise, 1985). These parameters are shown in Table 2. Drug release data are frequently plotted as percent (or fractional) drug released versus $t_{1/2}$ using the Higuchi model. Here, an attempt to do the same, but with loading amount instead of release was done. A linear plot indicates a diffusional controlled drug loading (Higuchi, 1961). The same curves were calculated fitting a power law equation and have a similar shape. Hence, we can assume that the PPO immobilization into mesoporous materials is a diffusion controlled mechanism. The coefficients of correlation of each model are considerably high. Table 2 shows that the time needed to incorporate 50% of PPO depends directly on the pore size and volume of the material. Materials with higher pore volume and pore size, such as SBA-15, need less than 1 min to incorporate the same quantity of enzyme as MCM-48 does in 2 h.

The reported results show that the tested materials retain significant amounts of PPO. The good response observed, together with the idea to develop a new and useful strategy for the food industry encouraged us to further study the ability of SBA-15, the most active material, to immobilize PPO directly from an apple extract. Apples (*Malus domestica*) were selected as they are one of the most important sources of polyphenols (phenolic compounds) in the human diet (Hertog, Hollman, & Katan, 1992; Kammerer, Kammerer, Valet, & Carle, 2014) and a classic example of fruit susceptible to enzymatic browning, which is a major problem for the fruit processing industry (Coseteng & Lee, 1987; Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). Several studies have shown that enzymatic browning in apple pulp is associated with its polyphenol content and/or polyphenol oxidase activity (Holdenbaum, Kon, Kudo, & Guerra, 2010; Murata, Noda, & Homma, 1995; Walker, 1964).

As expected, in only 15 min the enzyme activity decreased to 51% of its original value, however further time of contact does not increases significantly the inhibition for the concentrations and conditions of the assay.

Table 2
Parameters of power law and Higuchi equations for the loading curves of PPO loading into mesoporous materials at pH 4.00.

	Power law: $\ln F = \ln k_p + n \ln t$			Higuchi: $F = k_H t^{1/2}$		
	k_p	n	R	k_H	R	$T_{50\%}$ (min)
SBA-15	0.87	0.01	1.00	0.02	0.97	0.10
SBA-3	0.28	0.32	0.99	0.27	0.80	18.04
MCM-48	0.76	0.37	0.99	0.74	0.91	131.32

k_p: kinetic constant; n: loading exponent; R: coefficient of correlation; $T_{50\%}$: the time loaded 50% of PPO; k_H kinetic constant for the Higuchi model; k_p for the power law model; F is the equivalent to the rate of release in case of being applied to release.

4. Conclusions

In conclusion, results suggest that silica based mesoporous materials offer a suitable approach for the development of new procedures of inhibition of the PPO in fruits and vegetables, in particular in apples. The enzyme immobilization is a diffusion controlled process in which the two main factors are pH and pore size. However, pore volume can be also relevant in particular with respect to the loading capacity. The highest immobilization rate was observed at a pH value of 4.00 probably because the electrostatic interactions are favourable between the enzyme and the silanol groups of the mesoporous walls. Wide pore size and volume materials such as SBA-15 offer the fastest and higher loading capacity with a loading of a 50% in only 15 min. These results suggest that SBA-15 can be an interesting material for the inactivation of PPO in juices and fresh cut fruits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.08.027.

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