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The effect of pore morphology on the catalytic performance of β -glucosidase immobilized into mesoporous silica

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Abstract: β -Glucosidase (BG) was immobilized by adsorption on wrinkled silica nanoparticles (WSNs) and on tannic acid-templated mesoporous silica nanoparticles (TA-MSNPs). The effect induced by a different morphology of the pores of the sorbent on the catalytic performance of β -glucosidase was investigated. A complete textural and morphological characterization of the two samples was performed by Brunauer–Emmett–Teller (BET) method, Fourier Transform Infrared (FT-IR) and transmission electron microscopy (TEM). The results demonstrated that the catalytic performance of the immobilized enzyme depends on the pores size of sorbent but a key factor is the pores morphology. In fact, the BG immobilized on WSNs and TA-MSNPs (BG/WSNs and BG/TA-MSNPs) shows in both cases good catalytic performances in cellobiose hydrolysis, but the catalyst with the best performance is BG/WSNs, in which the support exhibits a central-radial pore structure and a hierarchical trimodal micro-mesoporous pore size. This peculiar morphology allows the enzyme to settle in a place where the interactions with the walls are maximized, increasing its conformational rigidity. Furthermore, the enzyme is prevalently collocated in the interior of pore so that the pores are not completely capped.

Keywords: biomass conversion; enzyme immobilization; Eurasia 2018; β -glucosidase; kinetics; thermal stability.

Introduction

Fossil fuel depletion and environmental degradation, especially evident in the high-impact climate extremes that have been, in part, related to anthropogenic climate change [1], have led researchers towards more sustainable energy sources. In this frame, lignocellulosic biomass, such as forestry and agricultural waste, has been considered as a strategic fuel source [2]. The main component of lignocellulosic biomass is cellulose, a polymer composed by glucose units that can be hydrolyzed to glucose. Glucose is easily fermented into bio-fuels, such as ethanol and butanol [3].

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So far, a great deal of effort has been devoted to the enzymatic degradation of cellulose [4], since it offers many advantages over chemical catalysis, including sustainability, selectivity towards the products and milder operational conditions (lower energy expense and non corrosive environment). The bioconversion of cellulose is achieved with cellulases, a set of enzymes acting sequentially and synergistically on the polymeric chain of cellulose cleaving it until the cellobiose dimmer units are obtained (β glucanases EC 3.2.1.4 and exo-1,4- β -glucanase EC 3.2.1.91) [5]. β -Glucosidase (BG EC3.2.1.21), the third component of cellulases, is considered to be the bottleneck of the whole process, since it hydrolyzes cellobiose to glucose [6] and cellobiose is known to be an inhibitor for both endo and exo-glucanase activities [7].

The cost of enzymes is one of the main obstacles in the large-scale commercialization of cellulose enzymatic hydrolysis. Enzyme recovering and recycling is essential to make the process competitive on an industrial level. Another issue is related to the process operating conditions, which are harsher than those in which the enzyme operates under physiological conditions, which could determine its denaturing. Hence the need to stabilize the enzymes. Both issues can be addressed by enzyme immobilization [8–10]. Among the different technique [11], physical adsorption on insoluble supports is cheap, easily carried out, and tends to be less destructive towards the enzyme than, for example, covalent binding [12].

A variety of organic and inorganic materials are available for enzyme immobilization [13]. Among them, mesoporous silicates are excellent candidates since they offer high porosity and large surface area for high enzyme loading, large pore size for easy enzyme access, easy functionalization and thermal, chemical and biological stability [14–17]. Besides, they can promote enhanced enzyme stability, due to a limited exposure to environmental factors and to the constrains of polypeptide conformational freedom due to the interactions with the pore walls [10].

Many mesoporous silica materials have been synthesized since they were first discovered by the Mobil oil company in 1992 [18]. Mesoporous materials with different pore size, pore structure and connectivity have been prepared and the effect of these different parameters on the enzymatic loading and activity has been studied. An important factor influencing enzyme immobilization is the size of mesopores, or more specifically, the pore size relative to the protein molecule size. The pore size of the mesochannels should be sufficiently large to host the biomolecule, and this condition is necessary to adsorb an enzyme [19]. Chen *et al.* [20] prepared two mesoporous silica with different pore size and found that the loading and activity of the enzyme cellulase were correlated with the pore size. Smaller pore size samples showed a lower amount of protein loaded but higher activity with respect to the larger pore size sample. They argued that larger pore sizes may achieve high adsorption amount but the enzyme is easily desorbed and the molecular dense arrangement would inhibit the conformational flexibility causing enzymatic activity loss. Similar results were obtained by Takimoto *et al.* [21] by immobilizing cellulase on SBA-15 with different pore size. Another factor influencing the performance of the immobilized enzyme is the pore structure. Serra *et al.* [22] investigated the immobilization of lipase on different mesoporous materials with different pore size and structure (channel-like and cage-like). They found that pore size was the most important factor limiting the diffusion of the enzyme inside the pore. Restriction to internal diffusion disappeared when the pore size was twice the one of the enzyme. Furthermore, the leaching of the enzyme depended on the pore structure. Cage like pores provided the highest diffusion restriction but the least leaching.

The aim of this work is to investigate the effect induced by a different morphology of the pores of the sorbent on the catalytic performance of β -glucosidase. For this purpose, two different mesoporous silica, wrinkled silica nanoparticles (WSNs) and tannic acid-templated mesoporous silica nanoparticles (TA-MSNPs), with similar average pore size but with different morphology were synthesized. WSNs have a central-radial pore structures in which the radial pore channel size increases going from the interior to the surface [23]. β -glucosidase was already immobilized in WSNs and it was found that the peculiar morphology of this matrix created a favorable microenvironment for catalysis [24]. TA-MSNPs have an interconnected disordered pore structure [25]. These nanoparticles are prepared using a non-toxic and cheap surfactant that can be easily removed without calcination. The obtained nanoparticles have uniform size, shape and large mesopores.

The different pore morphology of the two kind of mesoporous silica are schematized in Fig. 1.

Fig. 1: Pore morphology of TA-MSNPs (a) and WSNs (b).

To best of our knowledge, tannic acid templated mesoporous silica nanoparticles (TA-MSNPs) has been only used in few cases as supports for enzymes immobilization [25–27]. WSNs were only used as a matrix for the lipase immobilization [28]. In our previous study [24], for the first time wrinkled silica nanoparticles (WSNs) have been used to immobilize the enzyme BG. BG was entrapped within the nanoparticles with high activity, reusability and low diffusion limitations, demonstrating that WSNs are an efficient matrix for the immobilization of this. The aim of the present paper is both to immobilize for the first time BG in mesoporous silica nanoparticles using a green porogen agent and to look into the effect of a different morphology of pores on catalytic performance of enzyme.

Experimental

Materials

Tetraethylorthosilicate (TEOS), cetylpyridinium bromide (CPB), urea, cyclohexane, iso-propanol, acetone, hydrochloric acid solution 37%, β -glucosidase from almond (molecular weight 135 000 Da for the dimer), citric acid, sodium citrate hexahydrate, tannic acid (ACS-grade), ammonium hydroxide (28–30 % as NH_3), ethanol (ACS-grade) and glucose oxidase-peroxidase (GOD-POD) assay kit were purchased from Sigma-Aldrich (Milan, Italy). The activity of BG was ≥ 6 U/mg, where 1 U corresponds to the amount of enzyme which liberates 1 μmol glucose per minute at pH 5.0 and 37 °C (salicin as substrate).

Wrinkled silica nanoparticles were synthesized as specified in [24]. Briefly, an aqueous solution was prepared by mixing 9.0 g of cetylpyridinium bromide (0.09 M) and 5.4 g of urea (0.34 M) in 268 mL of water. In order to form a bicontinuous emulsion, 268 mL of cyclohexane and 8.0 mL of iso-propanol were added to the solution. TEOS was added dropwise to the stirred solution for a final volume of 24.0 mL and the reaction was carried out at 70 °C. The nanoparticles were recovered by three centrifugations and the surfactant was removed by chemical extraction with hydrochloric acid 0.80 M in ethanol solution. Several experimental evidences (TEM and FTIR) have shown that the simple washing with ethanol was not enough to remove the surfactant, even increasing the number of centrifugation cycles.

TA-MSNPs were prepared according to Gao and Zharov [25]. $1.6 \cdot 10^{-4}$ mol of tannic acid was dissolved in 50 mL ethanol. Twenty-five milliliters of concentrated ammonium hydroxide (28–30 % as NH_3) was added to the ethanol solution under vigorous stirring, followed by the addition of TEOS (0.3 mL). The onset of turbidity after the addition of TEOS indicated the start of silica sphere formation. After 2 h of stirring, the particles were centrifuged out at 11 500 rpm for 10 min and repeatedly washing with water and ethanol until the supernatant was colorless (ca. 10 times). To ascertain the complete removal of TA, Fourier transform infrared (FT-IR) analysis was performed on recovered nanoparticles.

BG immobilization procedure

In both cases, adsorption was carried out by preparing 17.5 mL a colloidal solution (2.3 mg/mL) for each kind of nanoparticles and adding 2.5 mL of a solution 0.015 mM of β -glucosidase at pH = 5. The resulting mixture was kept under stirring overnight at room temperature. The nanoparticles were then removed by centrifugation at 11 000 rpm for 10 min, washed twice with the buffer solution to remove from the solid support the enzyme that had not been adsorbed, and immediately used in the catalytic assays. The two prepared biocatalysts will be indicated as BG/WSNs and BG/TA-MSNPs in the following.

The Bradford method [29] with serum albumin (BSA) as standard was used for protein concentration determination in the adsorption supernatant. The amount of immobilized enzyme was determined by subtracting the amount of protein in the supernatant after immobilization from the total amount of protein used for immobilization. The immobilization yield (IY) was calculated according to the following equation:

$$\text{IY (\%)} = \frac{C_i - C_s}{C_i} \cdot 100$$

where C_i represents the initial protein concentration (mg/mL) and C_s the protein concentration measured in the supernatant at the end of the immobilization procedure.

Nanoparticle textural analysis

The morphology of WSNs and TA-MSNPs was observed by transmission electron microscopy (TEM) (PHILIPS EM208S microscope equipped with a Mega View camera for digital acquisition of images).

The textural properties of WSNs and TA-MSNPs were determined by N_2 adsorption at -196°C with a Quantachrome Autosorb 1-C, after degassing for 4 h at 150°C . The Brunauer–Emmett–Teller (BET) method was adopted for the calculation of the specific surface area, while pore size distribution was evaluated by means of Barrett–Joyner–Halenda (BJH) adsorption method for mesopores and Dubinin–Astakov (DA) method for micropores.

Biocatalyst characterization

Fourier transform infrared (FT-IR) transmittance spectra were recorded in the $4000\text{--}400\text{ cm}^{-1}$ range, using a Nexus FT-IR spectrometer equipped with a DTGS KBr (deuterated triglycine sulfate with potassium bromide windows) detector. A spectral resolution of 2 cm^{-1} was chosen, and each spectrum represents an average of 32 scans, corrected for the spectrum of the blank KBr pellet. Samples for FT-IR analysis were prepared by mixing KBr and the dried samples powders (0.5 wt %) and pressing into pellets of 13 mm diameter.

β -Glucosidase kinetic parameters were determined in buffer (pH 5.0) at 50°C using 8mM cellobiose as a substrate. The amount of BG was 0.15 mg/mL for both BG/WSNs and BG/TA-MSNPs. 2mL of the solution were withdrawn at fixed time (10, 30, 60, 90, 120 and 240 min) and the reaction was stopped by thermal inactivation at 100°C for 10 min. The samples were then centrifuged for 7 min at 11 000 rpm to remove the biocatalysts. Each aliquot of the solution was then analyzed. Glucose (GO) assay kit was used for measuring glucose concentration. The amount of released glucose was determined by incubating an appropriate amount of quenched reaction mixture (1mL), previously diluted, with 2 mL of glucose-measuring reagent at 37°C for 30 min, based on the D-glucose oxidase–peroxidase method [30]. Absorbance (OD) was measured at 540 nm with the help of a spectrometer (Perkin Elmer Instruments, Lambda 25 UV/Vis). The kinetic parameters were evaluated from the time course of the reaction using the integrated rate equation [31].

The effect of temperature was determined for free BG, BG/WSNs and BG/TA-MSNPs. The optimum temperature for soluble and immobilized BGs activity was evaluated using standard assay conditions in buffer at

different temperatures (50, 60, 70 and 80 °C). The biocatalysts were first incubated for 1 h at the experiment temperature, and then cellobiose was added to obtain a solution 8 mM. The reaction was stopped after 1 h by thermal inactivation of the enzyme (10 min at 100 °C) and analyzed for glucose concentration, from which the activity was evaluated.

The operational stability of the immobilized samples was assessed at 50 °C by carrying out the hydrolysis of cellobiose under standard assay conditions. After each cycle, the supernatant was analyzed to determine glucose concentration and the solid was washed with the buffer solution and reused. The yield of hydrolysis (mole of glucose/ $2 \times$ initial mole of cellobiose) after the first cycle was defined as the control and attributed a value of 100 %.

Results and discussion

TEM micrographs displayed in Fig. 2 show the morphology of TA-MSNPs and WSNs and their pore texture. As you can see, both of them have approximately the same size (200–250 nm). TA-MSNPs have almost spherical shape and uniform dimensions. The particles appear highly porous, with higher density in the center of the particles with respect to the periphery, indicating a disordered pore arrangement [25]. On the contrary, TEM images of WSNs disclose spherically shaped particles and a pore texture composed by silica fibers or wrinkles coming out from the center of the particles, spreading uniformly in all directions.

To further characterize the porosity of the two kinds of nanoparticles, N_2 adsorption/desorption analysis was carried out. The results are reported in Fig. 3.

The TA-MSNPs shows a type IV isotherm with two distinct adsorption steps, characteristic of a mesoporous structure. The shapes of the isotherm of WSNs are slightly different from a typical type IV, which has only one step in the middle range of relative pressure (P/P_0). There are additional large N_2 uptakes and H3 hysteresis due to a large amount of macropores with a slit shape [23]. The Brunauer – Emmett – Teller (BET) surface area, total pore volume, and average pore diameter of TA-MSNPs are given in Table 1. The two samples have comparable of specific surface area. The error on specific area is about 5 %. The total pore volume is sensibly higher for WSNs.

Fig. 2: TEM micrographs of TA-MSNPs (left) and WSNs (right) at different magnifications.

Fig. 3: N_2 -adsorption isotherms at 77K (left) and BJH pore size distributions (right) of TA-MSNPs (a) and WSNs (b).

Table 1: Average pore size, BET area and pore volume for TA-MSNPs and WSNs.

Sample	Average pore size (nm)	BET area (m^2/g)	Pore volume (cm^3/g)
TA-MSNPs	8.14	633	0.7
WSNs	12.2	580	1.72

TA-MSNPs show a monomodal pore size distribution in the range 2–10 nm, with a maximum centered at ca 5 nm and an average pore size of 8 nm; additionally, some microporosity is inferred in the range 1–2 nm. WSNs show a more complex distribution characterized by: (i) mesopores in the range 5–50 nm, having a mean value of 12.2 nm which corresponds to inter-wrinkled distances, (ii) a second mode in the range of 2–4 nm, suggesting the presence of a mesoporous structure in addition to wrinkles, (iii) additional microporosity in the range of 1–2 nm. The presence of this additional micro/mesoporosity indicates that the wrinkles are themselves porous and hence the pores are interconnected.

After enzyme adsorption in TA-MSNPs and WSNs and successive nanoparticles separation, the supernatants were analyzed for residual enzyme concentration. The results indicate that both TA-MSNPs and WSNs absorb 30 % of the initial enzyme mass, obtaining 150 ± 1 mg/g of support. The amount of adsorbed enzyme in the two kinds of nanoparticles is about the same, despite WSNs have the same surface area but higher pore volume (more than double). This shows that the pores of WSNs are not completely filled, consistently with what was previously observed from the TEM images of the WSNs after BG adsorption [24]. The peculiar morphology of this kind of pores allows the enzyme to be placed in the inner cavity of the pore, where the interactions with the walls are maximized, preventing pore blocking.

After BG adsorption, the two obtained bioconjugates were analyzed by FT-IR. In fact, the activity of an enzyme is strongly influenced by its secondary structure [32]. The secondary structure of the protein during adsorption can undergo modifications due to the interactions with the support. This will affect the resulting enzymatic activity. Infrared spectroscopy is a well established technique for conformational analysis of proteins and has recently been used to study the secondary structure of adsorbed BG confined in porous materials [24]. FT-IR spectra of the two bio-conjugate compounds and lyophilized BG are displayed in Fig. 4 (whole spectra on the left, a zoom on the right). The presence of the BG is testified by the appearance of the bands between 1500 and 1700 cm^{-1} . Amide I band, corresponding to (CO) carbonyl stretching mode is placed between 1700 and 1600 cm^{-1} [33]. Its position is very sensitive to the secondary structure of the polypeptide, since it consists of a group of overlapped signals, which depend on the environment of the carbonyl groups [34]. The band centered at around 1547 cm^{-1} is assignable to the amide II band, which is an out-of-phase combination mode of the NH in plane bend and the CN stretching vibration with smaller contributions from the CO in plane bend and the CC and NC stretching vibrations [33].

Fig. 4: FT-IR spectra of lyophilized BG (a), BG/WSNs (b) and BG/TA-MSNPs (c) (left), a zoom on amide I and II region (right).

Figure 4 shows that BG immobilized in WSNs exhibits amide bands similar to free (lyophilized) BG, indicating little or no modifications of the protein native structure. In contrast, BG immobilized in TA-MSNPs exhibits a slight but neat shift of the amide I band to lower wavenumber and a decrease of amide II absorbance, implying a different conformation of BG in this material [35].

BG/WSNs and BG/TA-MSNPs were tested in the hydrolysis of cellobiose. The time course of the reaction, reported in Fig. 5, was followed by measuring glucose concentration vs. time. Both samples followed a Michaelis-Menten kinetic and the conversion cellobiose to glucose is complete after 4 h. From the linearization of the kinetic curves, K_M and V_{max} were extrapolated. Their values are reported in Table 2 and compared with those of free BG [24].

The kinetic results can be explained by considering both the structure of the protein immobilized within the supports and the porous texture of the support. The decrease of V_{max} depends on the enzyme structure with respect to its native one [20, 36]. Its value is almost unchanged for BG/WSNs, whereas it is about half for BG/TA-MSNPs. These results are consistent with FT-IR analysis of the two samples. For BG/WSNs, which show no modification of the enzyme secondary structure, V_{max} is unchanged with respect to free BG, while for BG/TA-MSNPs, in which the shift of amide I band is indicative of conformational changes, there is a decrease in V_{max} . This detrimental modification in the enzyme conformation, and/or the loss of the conformational flexibility, can be caused by interactions between the polypeptide molecules and the matrix.

K_M can be influenced by several factors, such as diffusion limitations, the affinity of the enzyme for its substrate, or the substrate concentration near the active sites [37, 38]. K_M is lower for BG/WSNs and higher for BG/TA-MSNPs. The decrease in K_M for BG/WSNs suggests no diffusion limitation and that the local concentration of the substrate near the active site is higher than is the bulk solution [24]. This can be due to

Fig. 5: Glucose concentration vs. time for cellobiose hydrolysis catalyzed by BG/WSNs (squares) and BG/TA-MSNPs (circles).

Table 2: Kinetic parameters of free BG and BG immobilized on TA-MSNPs and on WSNs.

Kinetic parameters	Free-BG [24]	BG/TA-MSNPs	BG/WSNs [24]
K_M (mM)	5.4	7.1	4.3
V_{max} ($\mu\text{mol}/\text{min} \cdot \text{mg}$)	43	24	41

(i) the peculiar geometry of the pores that hinders pore blocking, (ii) the presence of a hierarchical porous structure that improves substrate diffusion and (iii) a high affinity of the substrate for the support, due to hydrogen bonds. In BG/TA-MSNPs there is an increase in the apparent K_M . This can be attributed to the pore morphology: pore size is slightly bigger than the protein diameter (about 6 nm) and pore blocking is highly possible.

The effect of temperature on the catalytic activity was evaluated for BG/TA-MSNPs, BG/WSNs and free BG. In Fig. 6, the percentage residual activities at different temperatures are displayed. Both immobilized samples showed increased thermal stability with respect to the free form. An increase of thermal stability, often observed upon immobilization [36, 38, 39], can be attributed to the multipoint attachment to the support, increasing enzyme rigidity and preventing denaturation through constrains of the conformational freedom and thermal vibration [40, 41]. Circular dichroism (CD) studies have demonstrated that free β -glucosidase continuously lose its secondary structure with increasing temperature, which may be due to a loss of higher order secondary structure (α -helix) [42]. Furthermore, by CD it is demonstrated that enzymes anchored by more than one bond is stabilized against thermal unfolding [43]. It is also demonstrated by fluorescence anisotropy that immobilized enzymes can maintain the thermal stability against the unfolding temperature comparing to that of the enzyme free in solution [44].

The sample showing the best thermal stability is BG/WSNs, preserving 100 % of its activity at 60 and 70 °C. Once again this performance can be explained with the different morphology of the pores in the two kinds of materials. As already outlined, the radial morphology the pores in WSNs, different from the channel-like pores in TA-MSNPs, allows the enzyme to settle in a place (inside the pores) where the interactions with the walls are maximized, increasing its conformational rigidity.

The immobilized BGs were used repeatedly in 5 consecutive hydrolysis cycles. The results are displayed in Fig. 7. In both cases the enzyme demonstrated operational stability, since it can be reused three times

Fig. 6: Residual Activity (%) at different at different temperatures for free BG (empty squares), BG/TA-MSNPs (circles) and BG/WSNs (plain squares).

Fig. 7: Operation stability of immobilized BG during cellobiose conversion for BG/WSNs (full bars) and BG/TA-MSNPs (empty bars).

without loss of activity. In the fourth reuse, about 80 % of activity is preserved, while in the fifth 40–50 % is preserved. This decrease could be due to the physical visually detected loss of small amounts of biocatalyst during the transfer procedures, rather than enzyme leaching or denaturation.

Conclusions

β -Glucosidase was immobilized in two kinds of mesoporous silica nanoparticles, WSNs, with central radial pores and TA-MSNPs, with disordered channel-like pores. The average pore size of both nanoparticles was suited to physically adsorb the enzyme. Both materials showed high adsorption ability towards β -Glucosidase and proved to be suitable supports for the immobilization of this protein, improving its thermal and operational stability. The performance of β -Glucosidase was found to be strongly dependent on the pore

morphology, which plays a very important role in determining the catalytic performance of an immobilized enzyme. In particular, BG immobilized in WSNs showed improved kinetics and strongly ameliorated thermal stability. In this case the enzyme is prevalently collocated in the interior of pore so that the pores are not completely capped. Furthermore, the peculiar morphology of the pores allows the enzyme to settle in a place where the interactions with the walls are maximized, increasing its conformational rigidity.

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