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Immobilization of β-Glucosidase on carbon nanotubes

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Carbon nanotubes are demonstrated as a good support for the immobilization of β-Glucosidase. This is an enzyme with high molecular weight (ca. 135 kDa). A high enzyme loading of 630 mg per gram of support was achieved in 12 h. The link between the enzyme and the carbon nanotubes surface was by electrostatic interactions due to the different charges of the enzyme and the support at the pH of the immobilization. Immobilized β-Glucosidase showed a catalytic activity above 400 U/g on the hydrolysis of 4-nitrophenyl-β-D-glucopyranoside (*p*-NPG).

KEY WORDS: β-Glucosidase; carbon nanotubes; immobilization; activity.

1. Introduction

Carbon nanotubes (CNTs), which were discovered by Iijima [1] can be considered as the result of folding graphitic layers into carbon cylinders and be composed of a single shell (single-walled nanotubes (SWNTs)), or of several shells (multi-walled nanotubes (MWNTs)). In this time, different practical applications due to their ordered nanostructure have been reported such as biosensors to determine glucose [2], transport and storing of hydrogen [3] and specially, in electrochemistry due to their electronic properties [4–8]. However, their special and steady structural characteristics and morphology are quite suitable for use as catalytic supports [9, 10]. Moreover, it is possible to modify their surface properties through different methods increasing the applications. In this sense, it is possible to employ carbon nanotubes as support to immobilize enzymes. Davis *et al.* [11] immobilized three proteins (Zn_2Cd_5-metalotionein, cytocrome c_1, c_3 and β-Lactamase) on the carbon nanotubes showing that the adsorption was in the internal surface. Azamian *et al.* [12] immobilized ferritine despite the fact that the enzyme was negatively charged being produced strong electrostatic repulsions with the nanotubes. Furthermore, glucose oxidase has been immobilized on aligned carbon nanotubes modified with a gold thin film [13, 14]. Gan *et al.* [15] have studied the immobilization of lactic dehydrogenase of mouse muscle. Recently, Yu *et al.* [16] have shown that the myoglobine and horseradish peroxidase can be immobilized on carbon nanotubes using promoters in the media to favour the formation of amide bond between the terminal carboxylic and the lysine residue.

In this work we report on the immobilization of an enzyme with high molecular weight, such as the β-Glucosidase, on carbon nanotubes and their catalytic activity.

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2. Experimental

2.1. Reagents

β–Glucosidase from almonds (E.C. 3.2.1.21, 12.4 U/mg), 4-nitrophenyl-β-D-glucopyranoside (*p*-NPG) and citric acid were obtained from Fluka. Na_2HPO_4 and *p*-nitrophenol were supplied by Scharlab and Riedel-de Haen respectively. Multi-walled nanotubes (MWNTs) were purchase from SUN-NANOTECH Company.

2.2. Immobilization

Functionalization of the multi-walled nanotubes (MWNTs) was carried out previously to the enzyme immobilization by refluxing with 5 M nitric acid at 120 °C for 3 h. Adsorption of β-Glucosidase was generated by contacting enzyme with a MWNTs suspension in 1.5 ml eppendorf tubes at 25 °C. Adsorption was carried out by adding 0.5 ml of β-Glucosidase from almonds solution (74 μM) to 1 ml of a suspension of MWNTs (2 mg/ml). β-Glucosidase solution and MWNTs suspension were prepared in citrate-phosphate buffer (20 mM) at pH 3.5. The amount of adsorbed enzyme was measured by mass balance from β-Glucosidase concentrations determined before and after adsorption by UV absorption at 280 nm using a UV–Vis spectrometer (UVIKON 930, KONTRON Instruments). Preliminary calibrations with solutions of β-Glucosidase provided the value of the molar extinction coefficient ($\varepsilon_{280} = 59200\ M^{-1}\ cm^{-1}$).

2.3. Enzymatic activity

Catalytic activity of free and immobilized β-Glucosidase were determined by measuring spectrophotometrically

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the *p*-nitrophenol released on the hydrolysis of *p*-NPG. The catalytic assay was carried out at 25 °C for 1 min by adding 0.1 ml of free *β*-Glucosidase solution to 0.9 ml of *p*-NPG 5 mM solution in citrate-phosphate buffer solution (20 mM and pH 3.5). The reaction was stopped by the addition of 2 ml of Na_2CO_3 1 M solution. The same conditions were employed with the *β*-Glucosidase immobilized on MWNTs but using 0.1 ml of suspension. The reaction product, *p*-nitrophenol, was monitored colorimetrically at 400 nm using UV–Vis spectrometer (UVIKON 930, KONTRON Instruments). Preliminary calibrations with solutions of commercial *p*-nitrophenol provided a value of the molar extinction coefficient ($\varepsilon_{400} = 19120\ M^{-1}\ cm^{-1}$). One *β*-Glucosidase unit (U) was defined as the amount of enzyme required to hydrolyze 1 μmol of *p*-NPG· min^{-1} under the above-defined conditions.

3. Results and discussion

β-Glucosidase is an enzyme that hydrolyses *O*-glucosyl compounds releasing glucose and an aglycon and falls under the category of Glycosidase. *β*-Glucosidase employed in this work was a commercial enzyme from almonds with molecular weight around 135 kDa. Characterization of the enzyme was carried out before the adsorption experiments in order to know the purity. The purity of the enzyme is a parameter important in the immobilization since the impurities could be adsorbed on the support avoiding the adsorption of the enzyme [17]. Figure 1 shows the electrophoresis profiles obtained under denaturing conditions with different concentrations of *β*-Glucosidase from almonds. The predominant form of the enzyme in this analysis was as one monomer with molecular weight around 65 kDa. Analytical Ultracentrifugation (AUC) (XLA Beckman-Coulter) was used to obtain information about the conformation and shape of the enzyme from the sedimentation and diffusion coefficients obtained in sedimentation velocity experiments. Figure 2 shows the species distribution in the sedimentation analysis. Three well-resolved peaks were observed being assigned the main peak at 7.4 S for the dimer (60.1% of the total protein), the peak at 4.9 S for the monomer (30.2% of the total protein) and the peak at 11 S for the tetramer (9.4% of the total protein). The calculated molecular weights were 69 kDa, 128 kDa and 234 kDa for the monomer, the dimer and the tetramer, respectively. The enzyme was symmetric with a calculated frictional and axial ratio of 1.26 indicating that the *β*-Glucosidase presented spherical shape.

Characterization of the carbon nanotubes was carried out by nitrogen adsorption isotherm recorded at 77 K (MICROMERITICS ASAP-2010 apparatus) as is shown in figure 3. The BET method was used to calculate the total surface area. BET surface area was 130 m^2/g. The shape of the isotherm showed the presence of a very slight microporosity in the carbon nanotubes due to the adsorption in the space between the concentric cylinders of the multi-walled nanotubes. According to the literature this separation between this concentric cylinders is inside the microporosity range (around 3–4 Å) [18]. The main adsorption was in the internal and external surface of the MWNTs cylinders.

Figure 4 shows the *β*-Glucosidase adsorption on carbon nanotubes with the time. *β*-Glucosidase was adsorbed on carbon nanotubes reaching in the equilibrium an adsorption of 630 ± 40 mg/g of MWNTs (4.6 ± 0.3 μmol per g of MWNTs). The adsorption yield was around 25% wt. and the equilibrium took ca. 12 h. The amount of immobilized *β*-Glucosidase was relatively high being ca. 40% wt. of the system enzyme/support. The support was washed with buffer solution before the catalytic assay to evaluate the interaction between the enzyme and the MWNTs. In addition, the leaching of the enzyme during the reaction was avoided. Figure 5 shows the spectrometers of the washing solutions. The presence of enzyme in the washing solution

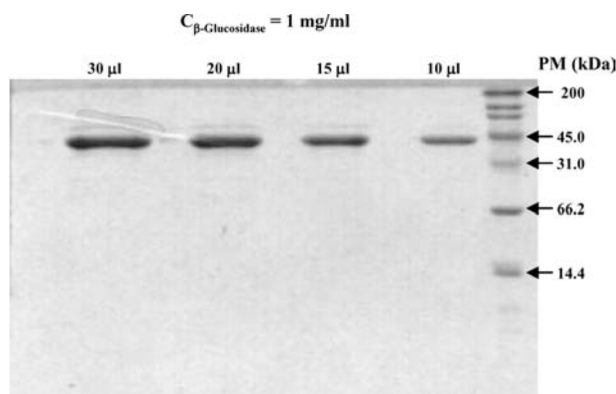

Figure 1. Electrophoresis profiles obtained under denaturing conditions with different concentrations of *β*-Glucosidase from almonds.

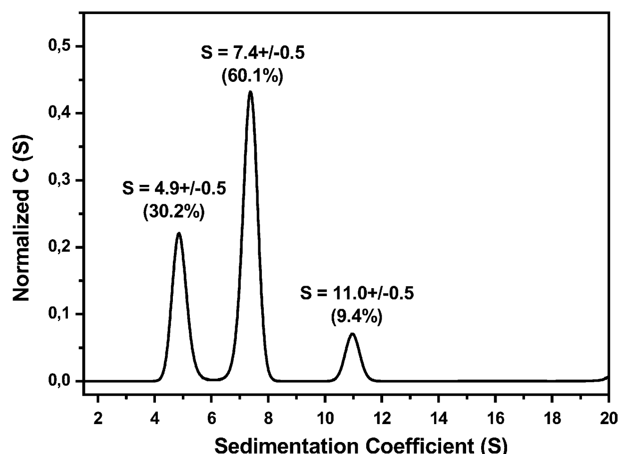

Figure 2. Species distribution in sedimentation velocity experiments. The enzyme was centrifuged at 40,000 rpm for 5 h at a protein concentration in the range 1–4 mg/ml.

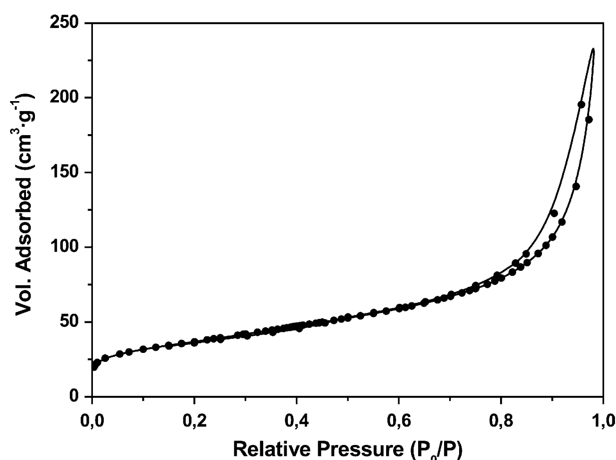

Figure 3. N_2 adsorption isotherms of multi-walled nanotubes (MWNTs).

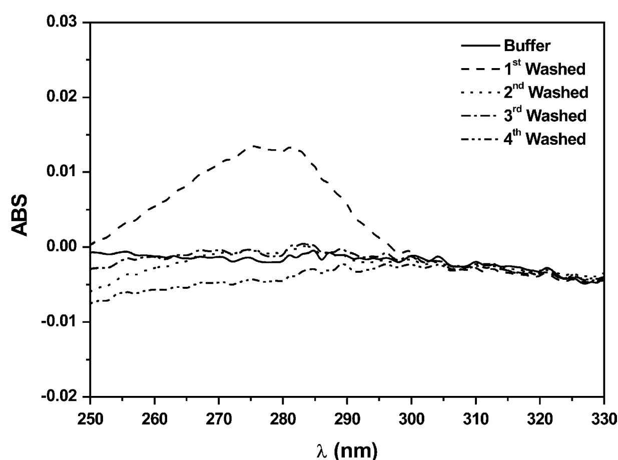

Figure 5. Spectrometers of the washing solutions of the carbon nanotubes after the immobilization of the β-Glucosidase.

was detected by means of the peak at 280 nm. The washing solution after the first washed showed a concentration of 0.03 mg/ml, this means that ca. 15 mg/g were desorbed from the carbon nanotubes. However, since the second washing there was no peak at 280 nm indicating the absence of β-Glucosidase. The loss of enzyme after the first washing could be due to the presence of enzyme solution impregnating the carbon nanotubes and not to the leaching of enzyme. Therefore, the link of the β-Glucosidase with the carbon nanotubes surface was strong.

In order to understand the kind of interactions between the MWNTs surface and the enzyme is important to know what happens during the functionalization process. Carboxylic acid and phenolic hydroxyl groups were formed on the surface of the carbon nanotubes after the functionalization. The isoelectric point (IEP) of the as-grown MWNTs is 5 but after the oxidation with nitric acid the IEP shifts to lower pH values below the pH of the immobilization (pH 3.5) [19]. Therefore, these groups presented negative charges at the pH 3.5 of the immobilization. On the other hand, β-Glucosidase has an isoelectric point around 5.5 [20] being charged positively at the pH of the immobilization (pH 3.5). Consequently, the link between the β-Glucosidase and the surface of the MWNTs should be by electrostatic interactions. In addition, in spite of the oxidation, the MWNTs maintain delocalised π-electrons. These delocalised π-electrons could take part in the link of the enzyme with the support since the enzyme is positively charged and could be formed interactions ion-induced dipole with the carbon nanotube surface. Therefore, the interaction between the carbon nanotubes surface and the β-Glucosidase would be strong.

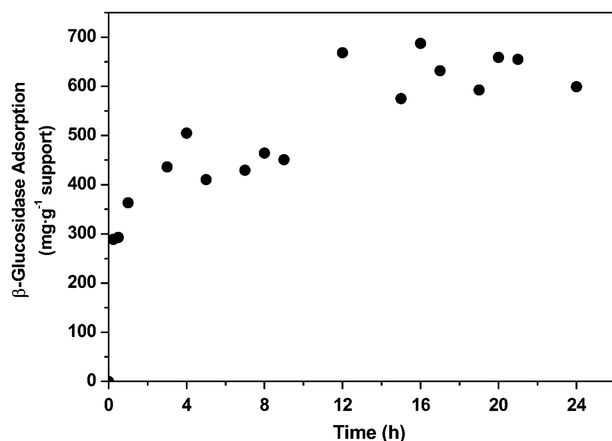

Figure 4. β-Glucosidase adsorption on carbon nanotubes at 25 °C, buffer solution (20 mM citrate-phosphate, pH: 3.5).

The amount of enzyme adsorbed on the carbon nanotubes during the immobilization process is important to design a bioreactor that can be used in industrial processes. However, the adsorbed enzyme must have catalytic activity. The catalytic activity of the β-Glucosidase immobilized on MWNTs is showed in figure 6. Catalytic activity was increased as the amount of immobilized β-Glucosidase was increased. The immobilized activity in the equilibrium was above 400 β-Glucosidase units per gram of MWNTs. Figure 7 shows the *p*-nitrophenol formation during the catalytic assay with free and immobilized β-Glucosidase. The activity of the immobilized enzyme was lower than the catalytic activity for the same amount of free enzyme. Immobilized enzyme after the immobilization process maintained around 20% of the catalytic activity corresponding to the free enzyme. The loss of the catalytic activity with the immobilization is relatively common and could be due to changes in the conformation of the enzyme. The enzyme could change their active conformation as a result of the strong interactions with the MWNTs surface. On the other hand, the β-Glucosidase could be immobilized inside the cylinders being only accessible to the substrate molecules the enzyme placed near the mouth of the cylinders.

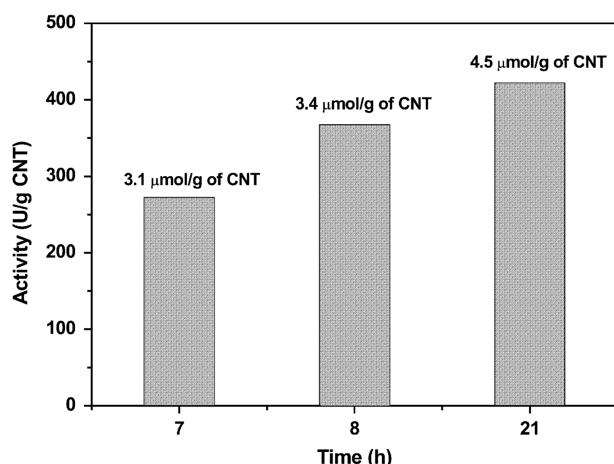

Figure 6. Catalytic activity of the immobilized β-Glucosidase on the hydrolysis of the 4-nitrophenyl-β-D-glucopyranoside (p-NPG).

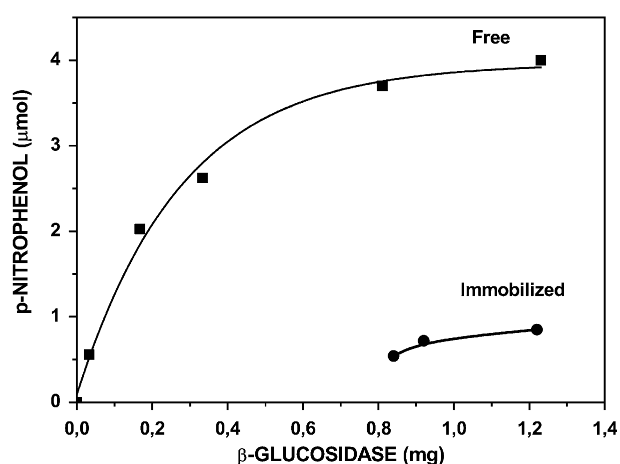

Figure 7. Variation of the produced p-nitrophenol during the hydrolysis of p-NPG with free and immobilized β-Glucosidase.

4. Conclusions

The main feature of this work is the excellent adsorption of the β-Glucosidase on the carbon nanotubes. This support is relatively new and there are few literature reports about their use in the immobilization of enzyme. Another remarkable aspect of this research is the use of an enzyme with high molecular weight (ca. 130 kDa) for the main conformation in solution (dimer). The immobilized β-Glucosidase showed a strong interaction with the carbon nanotubes surface due to the electrostatic interactions. These strong interactions may significantly change the conformation of the enzyme resulting in the loss of immobilized enzyme activity with respect to the free enzyme. In spite of this, the immobilized β-Glucosidase showed an acceptable catalytic activity above 400 U/g of MWNTs with the advantages of the immobilization. This feature of our results is the subject of further investigation.

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