View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repository of the Academy's Library

Processing and Application of Ceramics 1 [1–2] (2007) 63–67

Entrapment of glucoamylase by sol-gel technique in PhTES/TEOS hybrid matrixes

B. Vlad-Oros[1*], G. Preda[1], Z. Dudas[1], M. Dragomirescu[2], A. Chiriac[1]

[1]*Department of Chemistry, Faculty of Chemistry, Biology, Geography, West University of Timisoara, 300115, Timisoara, Romania,*

[2]*Faculty of Animal Science and Biotechnologies, Banat University of Agricultural Sciences and Veterinary Medicine, 300645, Timisoara, Romania*

Received 31 August 2007; received in revised form 16 November 2007; accepted 23 November 2007

Abstract

Mesoporous silica particles were prepared by the sol-gel method from different alkoxysilane precursors and used as a host matrix for encapsulation of glucoamylase, an enzyme widely used in fermentative industry. The aim was to investigate the physico-chemical properties of the different silica powders and their effect on the enzyme kinetics. The encapsulated enzymes followed Michaelis-Menten kinetics. The Michaelis constant (K_M) and the maximum rate of starch hydrolysis reaction (V_{max}) were calculated according to the Michaelis-Menten and Lineweaver-Burke plots. The values of the Michaelis constant (K_M) of the encapsulated enzymes were higher than those of the free enzyme. The temperature and pH influence on the activity of free and immobilized glucoamylase were also compared. The results of this study show that the enzymes immobilized in organic/inorganic hybrid silica matrixes (obtained by the sol-gel method), allowing the entrapped glucoamylase to retain its biological activity, are suitable for many different applications, (medicinal, clinical, analytical).

Keywords: glucoamylase, phenyltriethoxysilane, sol-gel, kinetics

I. Introduction

Due to the combination of unique properties, mesoporous silica matrixes are very promising materials for biotechnological applications. They possess such excellent basic features as large and accessible surface area, open porosity, narrow and uniform pore size distribution and very convenient pore size [1].

Gels are mostly interesting because an enzyme can be immobilized by building the porous network around it, obtaining efficient and easy recyclable biocatalysts. The preference for silica is because the corresponding gels can easily be tailored to a large range of porous textures, network structures, surfaces functionalities and processing conditions. The pH, gelation time, shaping, transparency or hydrophobicity can be adapted to a particular enzyme or application [2,3].

Biotechnology industries need biocatalysts easy to manipulate and recycle, thus the obtaining of hetero geneous biocatalyst is an important subject for those fields. There are different methods of immobilization (adsorption, covalent binding, cross-linking) among them is entrapment, method that do not affect chemical integrity of enzyme. In the last years, inorganic materials are preferred for entrapment because of their advantages in comparison with the organic ones. Among the inorganic materials, the silica matrixes, obtained by the sol-gel method, are used the most. The sol-gel method is a mild technique that makes compatible the synthesis of inorganic materials with biomolecules. In silica gels, especially the mesoporous ones, both enzyme and cells were successfully immobilized [4,5]. The simultaneous immobilization of enzymes and cells allows one-pot synthesis of different products.

Amyloglucosidase (glucoamylase, AMG) [α–1,4-D-glucan glucohydrolase (E.C. 3.2.1.3)], is one of the most important enzyme for all starch based industries (food, textile, brewing, paper and alcohol industries) and also for the pharmaceutical and fine chemical industries [6–8].

* Corresponding author: tel: +40 256 592 625,
fax: +40 256 592 620, e-mail: *bvladoros@yahoo.com*

The aim of this paper was the immobilization of glucoamylase, as a preliminary step in simultaneous immobilization of enzymes and cells, to obtain ethanol from starch. To prepare the hybrid silica matrixes, by the sol-gel method, different alkoxysilane derivatives ($RSi(OEt)_3$ with R = methyl, ethyl, propyl, phenyl etc.) are used as gel precursors. To entrap glucoamylase we chose three matrixes: PhTES / TEOS (about which there are few literature data) and TEOS (for comparison). The physicochemical properties of the matrixes were studied and then the effects of the temperature and pH on the immobilized glucoamylase activities and the kinetic parameters of these biomaterials were also investigated and compared with those of the native enzyme.

II. Experimental Procedure

Glucoamylase from *Aspergillus niger* (300 U/mL) was obtained from Novo. Tetraethoxysilane (TEOS) and phenyltriethoxysilane (PhTES) were purchased from Aldrich (USA) and soluble Zulkowski starch from Merck (Germany). All other chemicals were of analytical grade and were used without further purification.

For entrapping enzyme in silica matrixes, we used the sol-gel method of immobilization in one-step [9], TEOS and PhTES as gel precursors, NaF as catalyst, isopropyl alcohol as solvent and polyvinyl alcohol (PVA) as additive.

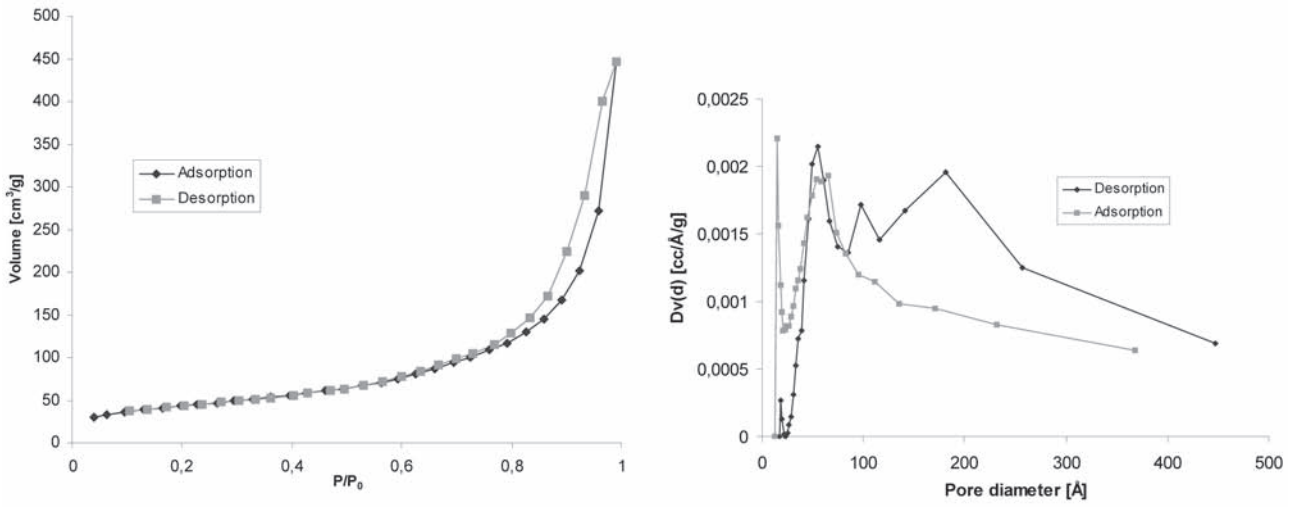
Matrixes were prepared using tetraethoxysilane (TEOS) and a mixture of tetraethoxysilane (TEOS) and phenyltriethoxysilane (PhTES) in different molar ratios (PhTES: TEOS 1: 1 and 2: 1). 1.4 mL mixture of alkoxides and 0.780 mL buffered enzymatic solution (22.5 U AMG in McIlvaine buffer, pH 4.6) were stirred with 200 µL 4% PVA 22.000 (polyvinyl alcohol 22.000), 100 µL NaF 1 M and 200 µL isopropyl alcohol. In a few minutes the mixture became homogenous and, shortly after, the gelation occurred. The gels were left overnight for aging (4°C), washed and dried (4°C).

Specific surface areas were calculated from N_2 adsorption isotherms at 77 K measured on a Quantachrome NOVA 2000 instrument using the multi-point Brunauer-Emmett-Teller (BET) method. The total pore volume (*Vp*) was calculated in the last point of adsorption branch. Pore size distribution curves were calculated from the adsorption (*Dp* [Ad]) and desorption (*Dp* [Ds]) branch of the isotherms using the Barrett–Joyner–Halenda (BJH) method.

Enzyme assay: 0.5 mL soluble starch (1%), 0.4 mL citrate-phosphate buffer (McIlvaine buffer) pH 4.6 and 0.1 mL enzyme solution or 0.05 g immobilized biocatalyst were kept for 5 min at 25°C. 1 mL 3,5-dinitrosalicylic acid (DNS, 1%) was added. The samples were boiled in water 10 min, 10 mL water was added and then absorbance at 540 nm against blank (soluble starch, citrate-phosphate buffer, 3,5-dinitrosalicylic acid and distilled water) was measured. One unit of AMG activity was defined as the amount of enzyme required to produce 1 µmol of glucose in 5 min under the defined assay condition [10].

The effect of the temperature on the activity of native and immobilized enzyme was estimated by measuring reducing sugars, released by action of enzyme at various temperatures. The test tubes were stored in a water bath at specific temperature (25, 30, 37, 45, 60, 70, 80 and 90°C).

The effect of the pH on the activity of native and entrapped enzyme was investigated by reducing sugars assay, but in the presence of citric acid 0.1 M – Na_2HPO_4 0.2 M buffer ranged from pH 2.6 to 8 (McIlvaine buffer, [11,12]) at room temperature.



a) b)

Figure 1. Adsorption/desorption isotherm (a) and pore size distribution curves (b) of TEOS matrix containing glucoamylase

Table 1. Textural properties of glucoamylase – entrapped gels

Matrix	D_p (Ads) [Å]	D_p (Des) [Å]	S_{BET} [m^2/g]	V_p [cm^3/g]
TEOS	33.01–231.36	38.39–256.77	153.1	0.686
PhTES : TEOS 1 : 1	33.19–232.16	22.92–255.57	256.2	0.679
PhTES : TEOS 2 : 1	28.65–229.35	33.03–253.04	214.7	0.595

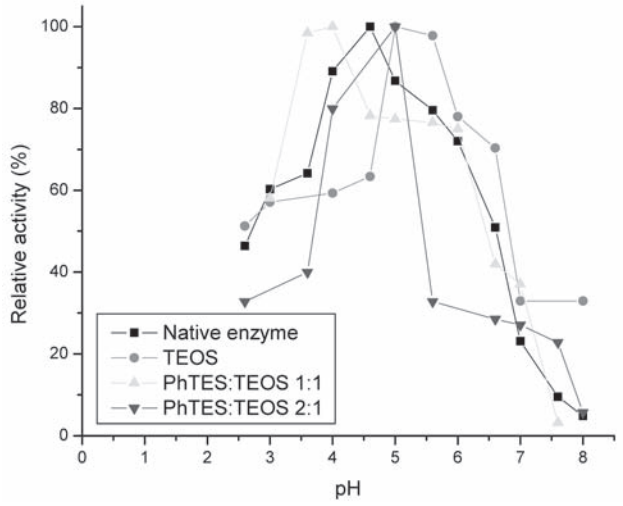


Figure 2. The effect of the pH on activity of immobilized and native glucoamylase

Determination of kinetics parameters of native and immobilized enzyme: K_M and V_{max} were determined by measuring initial rates of Zulkowsky starch hydrolysis. Kinetics studies were conducted in citrate – phosphate buffer 0.15 M, pH 4.6, at 37°C in a 50 mL stirred jacketed batch reactor. The starch concentrations were 1.5–6.25 mg/mL. The reaction was started by addition of the enzyme (4 mL native enzyme and 40 mg immobilized enzyme) and 1 mL samples were collected every 2 minutes, during the first 20 minutes of the reaction. The reducing sugars assay was used for analyze the samples [13].

III. Results and Discussion

The aim of this work was to investigate the physicochemical and biochemical properties of the glucoamylase immobilized in PhTES / TEOS and TEOS matrixes.

Physicochemical characterization

The specific surface area and pore size distribution were determined by N_2 adsorption and desorption method at 77 K. The gels demonstrate the characteristics of mesoporous materials as it can be seen in Fig. 1 – isotherm type IV with H3 hysteresis, according to IUPAC [14,15]. All the gels have presented the same type of isotherm. From their isotherms, the textural properties of the gels were determined as summarized in Table 1. The gels present mesoporous volumes and the introduction of the organic groups into the gels results in a slight decrease in the pore volume. Pores diameters are quite similar, so probably other factors influenced the enzymatic activities (increased hydrophobicity, modified microenvironment).

Biochemical characterization

In biotechnological applications, the main parameters of the immobilized biocatalysts are pH and temperature, inhibitors and activators, substrate concentration. This work presents the immobilization influence on the temperature and pH profile and also on the kinetic parameters of the glucoamylase.

The influence of the pH on the activity of the native and immobilized glucoamylases was investigated in the pH range 2.6–8, at room temperature. The optimum pH of the native enzyme was 4.6. After the immobilization, the optimum pH of the entrapped enzymes varies very little. Thus glucoamylase immobilized in matrixes based on TEOS and PhTES : TEOS 2 : 1 has an optimum pH of 5 and for the enzyme immobilized on matrix based on PhTES : TEOS 1 : 1 the optimum pH was 4 (Fig. 2).

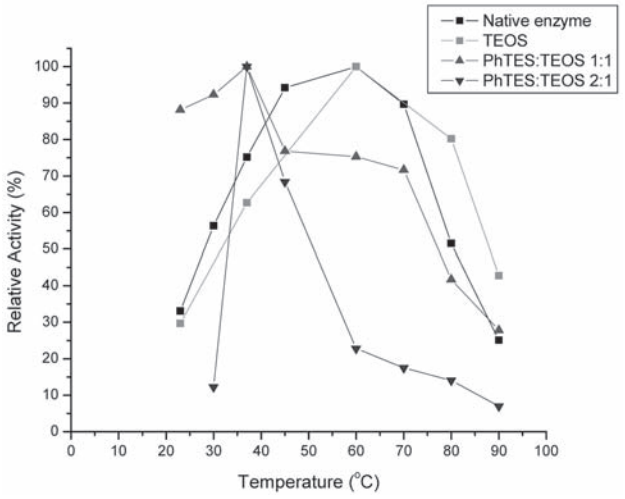


Figure 3. Temperature effect on activity of immobilized and native glucoamylase

The effect of the temperature on the activities of the native and immobilized glucoamylase in the range 20–90°C, was also studied. The optimal temperature for the native glucoamylase was 60°C and remains the same for the enzyme immobilized in matrix obtained from TEOS. In the case of the matrixes based on PhTES: TEOS, the optimal temperature for the entrapped en-

zyme was 37°C, decreased by 23°C in comparison to native glucoamylase (Fig. 3). Therefore, kinetic parameters were determined at this temperature and not in standard conditions.

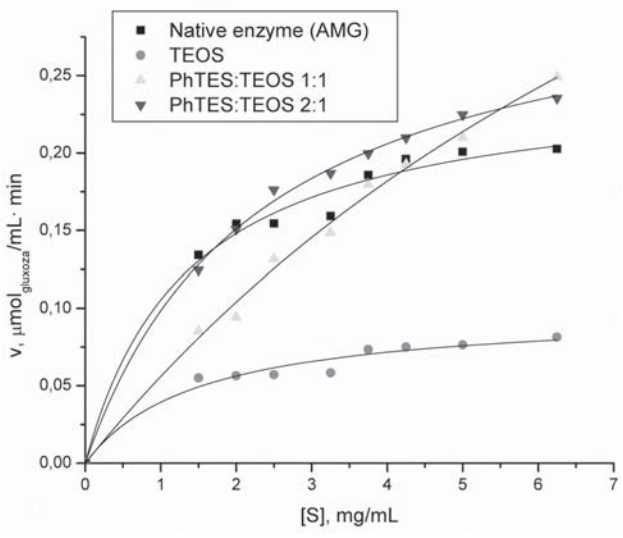


Figure 4. Initial rates (v) vs. substrate concentration ([S]) plots of native and immobilized glucoamylase

A typical profile for Zulkowsky starch hydrolysis using soluble and immobilized glucoamylase is shown in Fig. 4. Table 2 summarizes the kinetic parameters obtained by fitting the experimental data to the Lineweaver-Burke linearization. The Michaelis constant values, for the immobilized glucoamylase, are higher than the K_M of the native one, indicating a lower affinity to the substrate. These results could indicate the presence of partitioning and diffusional effects in the pores of the sol-gel derived matrix or that glucoamylase suffers a conformational change, which affects the active site upon its entrapment into the matrixes (K_M increases). In the case of the PhTES : TEOS matrixes, the V_{max} is increased compared to the maximum velocity of the native enzyme. The immobilization in the TEOS matrix may also lead to a partial protein inactivation or sequestration (V_{max} decreases). However, the V_{max}/K_M ratio shows that the best catalytic efficiency was determined for the native enzyme. Among the immobilized ones, the V_{max}/K_M ratio indicates that glucoamylase entrapped in the most hydrophobic matrix provides the best effectiveness when compared with the native counterpart. Thus, for entrapped enzymes, the best catalytic efficiency was obtained in the case of PhTES : TEOS 2 : 1.

Table 2. Kinetic parameters for the native and immobilized α-amylase, estimated by fitting the experimental data to the Lineweaver-Burke linearization

Sample	V_{max} $\frac{\mu mol_{glucose}}{mL \cdot min}$	K_M $\frac{mg}{mL}$	$V_{max}100/K_M$ $\frac{\mu mol_{glucose}}{mg_{starch} \cdot min}$
Native enzyme	0.24	1.24	19.35
TEOS	0.09	1.12	8.03
PhTES : TEOS 1 : 1	0.66	10.71	6.16
PhTES : TEOS 2 : 1	0.33	2.44	13.52

IV. Conclusions

The studied matrixes present mesoporous volumes, which allow a lower capillary pressure on the gel network and a faster internal diffusion of the substrate than the microporous sol-gel materials. However, the introduction of the organic groups into the gels determines a slight decrease in the pore volume.

The slight variation in the optimum pH and temperature proves that the sol-gel immobilization of glucoamylase in TEOS and PhTES : TEOS matrixes permits their use in the same pH and temperature conditions as the native counterpart.

The encapsulated glucoamylase follows the Michaelis-Menten kinetics. The V_{max}/K_M ratio, that shows the catalytic efficiency, indicates PhTES : TEOS 2 : 1 as the most favorable matrix for glucoamylase immobilization, among those studied.

Our results suggest that the sol-gel entrapment in TEOS and PhTES : TEOS matrixes is biocompatible allowing the entrapped glucoamylase to retain its biological activity.

Acknowledgements: This work was supported by CEEX project, contract number 38/2005, subprogram 9 MATNANTECH.

References

1. D. Fattakhova-Rohlfing, M. Wark, J. Rathousky, "Electrode layers for electrochemical applications based on functionalized mesoporous silica films", *Sensors and Actuators B: Chemical*, 126 [1] (2007) 78–81
2. A.C. Pierre, "The Sol-Gel Encapsulation of Enzymes", *Biocatal. Biotransform*., 22 [3] (2004) 145–170
3. T. Ogoshi, Y. Chujo, "Organic-inorganic polymer hybrids prepared by the sol-gel method", *Composite Interfaces*, 11 [8–9] (2005) 539–566
4. Y. Wei, J. Xu, Q. Feng, H. Dong, M. Lin, "Encapsulation of enzymes in mesoporous host materials via the nonsurfactant-templated sol-gel process", *Mater. Lett.*, 44 (2000) 6–11
5. T. Braniyik, G. Kuncova, "Encapsulation of microbial cells into silica gel", *J. Sol-Gel Sci. Technol.*, 13 (1998) 283–287

6. R. Gupta, P. Gigras, H. Mohapatra, V.K. Goswami, B. Chauhan, "Microbial α-amylases: A biotechnological perspective", *Process Biochem*., 38 (2003) 1599–1616
7. J.M. Sanchez, M.A. Perillo, "α–Amylase kinetic parameters modulation by lecithin vesicles: Binding versus entrapment", *Colloids and Surfaces B: Biointerfaces*, 18 (2000) 31–40
8. B.A. Saville, M. Khavkine, G. Seetharam, B. Marandi, Y.L. Zuo, "Characterization and performance of immobilized amylase and cellulose", *Appl. Biochem. Biotechnol*., 113–16 (2004) 251–259
9. M.T. Reetz, A. Zonta, J. Simpelkamp, "Efficient immobilization of lipases by entrapment in hydrophobic sol-gel materials", *Biotechnol. Bioeng*., 49 [5] (1996) 527–534
10. ***BDH Enzyme Catalog*, Poole, 1981.
11. T.C. McIlvaine, "A buffer solution for colorimetric comparison", *J. Biol. Chem.*, 49 (1921) 183–186.
12. D.D. Perrin, B. Dempsey, *Buffers for pH and Metal Ion Control*, Chapman and Hall, London, 1983.
13. G. Preda, M. Dragomirescu, F. Peter, *Biocatalizatori enzimatici. Obtinere, caracterizare, aplicatii*, Mirton, Timisoara, 2003.
14. IUPAC Recommendations, *Pure Appl. Chem*. 57 (1994) 1739–1758.
15. F. Rosquerol, J. Rosquerol, K. Sing, *Adsorption by Powder & Porous Solids,* Academic Press, San Diego, 1999. 1728.