Synthesis and Properties of Different Sol-gel Matrices Containing Bacterial α-Amylase

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Bacterial α-amylase was immobilized in sol-gel matrices using different techniques and precursors (i.e. tetraethoxysilane and methyltriethoxysilane). Sol-gel entrapment procedures were adjusted to enhance the immobilized enzyme activity and the textural properties of the silica matrices. The addition of a template agent, the variation of aging and drying conditions or the gelation performed in the presence of different inorganic supports led to the improvement of the porous biomaterials synthesis and activities.

Keywords: sol-gel, immobilization, á-amylase, templating agent, entrapment/deposition

α-Amylase (1,4-glucan 4-glucanohydrolases, E.C. 3.2.1.1), an endoenzyme that catalyses the breaking of α-1,4-glucosidic bonds in amylose and amylopectin molecules, is present in animals, plants, bacteria and fungi [1]. The conversion of starch to value-added products by enzyme-catalyzed reactions represents the largest industrial use of enzyme molecules [2]. These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries [3].

However, the use of commercial enzymes without appropriate immobilization leads to not only the waste but also the limited reuse of enzymes. In spite of these well-known disadvantages, the immobilization of α-amylase has not been performed frequently because it acts on macromolecular substrate such as starch inside the carriers [4]. The matrix must have pores size sufficiently small to prevent leaching of the enzyme, but large enough to allow the substrate to easily enter the matrix.

The silica materials prepared by the sol–gel technique have drawn attention owing to the propensity to entrap enzymes without affecting their chemical integrity. It is significant that the immobilized biomolecules retain to a great extent their bioactivity. Sol-gel processing entails the hydrolysis of tetraalkoxysilane precursor, under acidic or basic condition. This produces a sol of hydroxylated oligomers, followed by policondesation, which leads to the formation of a porous three-dimensional network around the enzyme. Furthermore, the porous silica matrix provides enzyme accessibility to external reagents and removal of the reaction products through the pore diffusion [5-7].

One of the main advantages of the sol-gel matrices is their tunable porosities and network structures, simply by modifying the process parameters. The reactivity of the sol-gel biocatalysts can be controlled and tailored to meet the requirements of a particular enzyme or application by varying their structural properties [8, 9].

The main purpose of this study was to determine the effects of the immobilization methods on the α-amylase enzymatic activity and textural properties of the sol-gel matrices. The silica matrices were prepared using two precursors: tetraethoxysilane (TEOS) and methyltriethoxysilane (MTES), in two steps or one-step synthesis. The synthesis conditions were modified either by performing the entrapment in the presence of a templating agent, at different aging and drying temperature or by adding various supports (entrapment/deposition). The obtained biomaterials were biochemically and physico-chemically characterized.

Experimental part

Materials and methods

Reagents

Bacterial α-amylase (E.C. 3.2.1.1, 25 IU/mg) and polyvinyl alcohol 22000 (PVA) were purchased from Fluka. The precursors, tetraethoxysilane (TEOS) and methyl-trietoxysilane (MTES), used for sols preparation, were obtained from Aldrich. Soluble potatoes starch was from Bender & Hobein. Folin-Ciocalteus phenol reagent and bovine serum albumine (BSA) were acquired from Merck. All other chemicals were of analytical grade and were used without further purification.

Enzyme immobilization

1. Silica sol was obtained from TEOS, ethanol and water, in acid catalysis, HCl 1N, (5:5:4:0.1, v/v). Then the sol was mixed with ethanol and water (5:5:4, v/v), 5 drops NH_3 12% and 1.25 mL buffered enzymatic solution (25 mg α-amylase in phosphate buffer) [10].

2. The mixture containing 6 mmoles alkoxides (MTES and TEOS) in different molar ratios (MTT 1:1, MTT 2:1 and MTT 3:1), 1 mL ethanol, 0,4 mL water and 1 1μL HCl (0.04 N) was stirred for one hour. Then 0.938 mL buffered enzymatic solution (18.75 mg α-amylase in phosphate buffer) and 100 μL NaF 1M are added under stirring [11].

3. Matrices based on TEOS and a mixture of MTES and TEOS, MTT 1:1, MTT 2:1 and MTT 3:1, (totally 6 mmoles) are obtained by mixing the precursors and 0.780 mL enzymatic solution, containing 18.75 mg α-amylase, with 0.200 mL PVA 22.000 (polyvinyl alcohol 22.000) 4%, 0.100 mL NaF 1 M and 0.200 mL isopropylic alcohol [12].

4. The enzyme was immobilized according to the method 2 but, before the gelation occurred, 1 g of different supports (celite, white ceramic, red ceramic, alumina, TiO_2, zeolite) was added to the mixture.

In all the cases, the gelation occurred in a few minutes. The gels were left overnight for aging, washed and dried. All the experiments were repeated two times and yielded reproducible results.

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Biochemical characterization

Various methods are available for the determination of amylase activity. The reaction is monitored by decrease in the iodine color of the treated substrate or an increase in the reducing sugars levels [3]. The chosen method was residual starch concentration assay (I_2/I^-), specific for the determination of α-amylase activity, because it does not interfere with the templating agent.

Residual starch concentration assay (I_2/I^-): 0.5 mL soluble starch (0.4%), 0.4 mL phosphate buffer (0.05 M, *p*H 5.2) and 0.01 g immobilized biocatalyst were kept for 5 min at 25°C. 5 mL I_2/I^- M/1000 solution and 15 mL distilled water were added. The samples were filtered. The absorbance was measured at 595 nm against distilled water. One unit of α - amylase activity was defined as the quantity of enzyme required to hydrolyze 1 mg starch in 5 min at 25°C when 2 mg starch was present at the start of the reaction.

Protein content was determined by the Lowry method [13]: 0.01 g immobilized enzyme, 5 mL cupper alkaline reagent and 0.5 mL Folin-Ciocalteu phenol-reagent were kept for 30 min. at 25°C. The samples were filtered and assayed at 660 nm against water. A bovine serum albumin calibration curve was used.

In each experiment, two parallel samples were assayed and the results represent the mean of their analysis.

Physico-chemical characterization

Specific surface areas were calculated from N_2 adsorption isotherms at 77 K measured on Quantachrome instruments, NOVA 2000 Series, high speed gas sorption analyzer version 7.02 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.

Results and discussions

The microporous nature of the conventional sol–gel matrices often leads to a loss of the catalytic activities of the entrapped enzymes due to low diffusion rates of substrate molecules and poor accessibility of enzymes inside the matrices. To circumvent this problem, the templating technique is usually required to increase the pore size of matrix [8]. D-glucose, a template for mesostructure formation, is not only an economical one but also biocompatible with many enzymes [9].

α-Amylase was entrapped in silica matrices according to method 1, in the presence of D-glucose as templating agent. Instead of water, 50% D-glucose solution (wt. %) was added, in an appropriate volume, so the product would contain different amounts of D-glucose. The molar ratios of TEOS:D-glucose were 1:0.3, 1:0.6 and 1:0.9. The specific surface area and pore size distribution were determined by N_2 adsorption and desorption method at 77K. Upon removal of the templating agent by simple but abundant water extraction, the obtained gels present the characteristics of the mesoporous materials. The addition of D-glucose results in an increase of the pore volume and pore diameters. By adding a suitable template agent, the activity of the entrapped enzyme increases by a factor of 1.1 to 1.4 (table 1).

The temperature influences both the enzyme activity and the textural properties of the gels. To study the effect of aging and drying temperature, α-amylase was immobilized in gels using method 1 and 2 and after gelation occurred, the gels were aged and dried in two different conditions: 1) aged and dried at room temperature for 24 h; 2) aged and dried at 4°C for 24 h. As shown in table 2, aging and drying at lower temperature (4°C) enhanced the activity of the immobilized enzyme by 1.5 in the case of TEOS, and by 2.7, 3.1 and 1.7 times respectively in the case of MTT 1:1, MTT 2:1 and MTT 3:1. Although the protein concentration is higher in the case of the matrices aged and dried at room temperature, the activity is lower (table 2). This activity decrease is probably due to a partial activity loss or to the pore formation process, greatly influenced by the temperature. As shown in table 3, aging and drying at low temperature produce gels with large pores size and volume (mesoporous volume) that allow a faster internal diffusion of the substrate and consequently a higher activity of the immobilized enzyme. Therefore a higher efficiency of the enzymatic process was assured even though part of the protein is lost during washing.

Our experimental results suggest that the aging and drying temperature of the sol-gel process should be lower in order to increase the activity of the immobilized enzyme, even after immobilization.

The influence of the immobilization method on the enzymatic activity was studied. Hence, different methods to immobilize α-amylase in silica network, using TEOS

Table 1
INFLUENCE OF GLUCOSE AS TEMPLATING AGENT

Matrix	I_2/I^- Activity (U/g)	D_p [A] (Å)	D_p [D] (Å)	S_{BET} m^3/g	V_p cm^3/g
TEOS (room temperature)	6.24	49.216 – 53.229	41.858	275.7	0.393
TEOS : D-glucose 1:0.3	6.87	52.935	45.133	267.2	0.448
TEOS : D-glucose 1: 0.6	7.06	111	74.328	337.1	0.839
TEOS : D-glucose 1: 0.9	8.53	110.32	74.736	267.5	0.657

Table 2
INFLUENCE OF THE AGING AND DRYING TEMPERATURE ON THE IMMOBILIZATION EFFICIENCY

Matrix	Room temperature		4°C	
	I_2/I^- Activity (U/g)	Protein content (mg BSA/g)	I_2/I^- Activity (U/g)	Protein content (mg BSA/g)
TEOS	6.24	3.92	9.11	1.22
MTT 1:1	5.15	5.02	13.86	4.07
MTT 2:1	5.41	5.94	17.03	4.37
MTT 3:1	4.81	3.66	8.29	3.52

Table 3
PHYSICOCHEMICAL PROPERTIES OF THE MATRICES OBTAINED BY AGING AND DRYING AT 4°C

Matrix	D_p [A] (Å)	D_p [D] (Å)	S_{BET} m^3/g	V_p cm^3/g
TEOS	33.187 – 110.137	35.777 - 84.202	182.3	0.546
MTT 1:1	72.413	54.373	619.9	1.000
MTT 2:1	35.194 – 64.407	35.7832 – 74.862	439.3	0.760
MTT 3:1	38.503	35.446	327.3	0.494

(method 1 and 3) and a mixture of TEOS and MTES (method 2 and 3) were compared (fig. 1). In all the cases, the one-step method of immobilization gives the best results. The α-amylase activities increased by 1.55 times in the case of the matrix based on TEOS and by 1.5, 1.35 and 2.1 times for the MTT 1:1, MTT 2:1 and MTT 3:1 matrices, respectively. The enhanced activities for the α-amylase immobilized by one-step method are presumably due to the macromolecular additive, PVA, which protect the enzyme from denaturing.

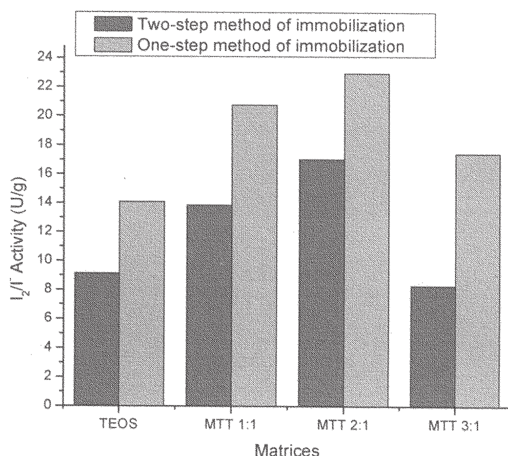

Fig. 1. Influence of the immobilization method on the enzyme activity

Among the studied hybrid matrices, MTT 3:1, obtained by method 2, presents the lowest activity. In an attempt to increase its activity, by reducing diffusional problems, α-amylase was entrapped using a combined method - entrapment/deposition, thus exploiting the positive characteristics of each technique. α-Amylase was immobilized by sol-gel technique (method 2) and before the gelation was complete, different inorganic supports were added (white ceramic, red ceramic, alumina, TiO_2, celite, zeolite). Hence the enzyme entrapment in a hybrid gel formed on the outer surface of the inorganic support particles was achieved. Some of these supports are well known for enzyme immobilization. The others (white and red ceramic, zeolite) are indigenes and used in agriculture, horticulture etc, being ecological. The influence of these supports on the enzymatic activity was tested. The results, obtained using the entrapment (method 2) and the entrapment/deposition technique (method 4), were compared. The enzymatic activity enhances by using either ones of these supports. The gels containing the enzyme are dispersed on the surface of the supports, which, probably, served as dispersion medium and prevented the aggregation of the gels fine particles. Perhaps there are optimum locations where the enzyme molecules are neither entirely embedded in nor completely deposited on the matrix. Apparently the mechanical properties of the gels, e.g. rigidity - elasticity, are also important factors for the catalytic activity and stability of the immobilized enzyme. Thus, the protein could be prevented from unfolding when the enzyme molecules are placed in the close proximity of the gel surface which is sufficiently but not too rigid [14]. The best results were obtained in the case of MTT 3:1 and celite, when enzymatic activity is increased by 2.05 times and also using zeolite and red ceramic, when activity is enhanced by 2.03 and 1.98 times, respectively (fig. 2).

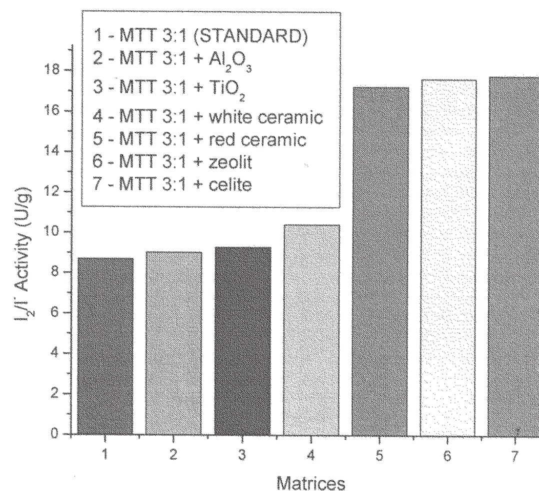

Fig. 2. α-Amylase immobilization by entrapment/deposition on different supports

Conclusions

The obtained matrices present mesoporous volumes, which allow a faster internal diffusion of the substrate to the entrapped enzyme. These results suggest that the physicochemical properties of the biomaterials are determinant parameters affecting their enzymatic activity.

These structural features can be modified by using a template agent, by lowering the aging and drying temperature or by using a combined method, entrapment/deposition, and an appropriate inorganic support.

The efficiency of the sol-gel biocomposites can be improved through combination of the fundamental immobilization techniques, control of the synthesis parameters and selection of the precursors and templating agents. Consequently, the sol-gel method of immobilization can be applied also for enzymes with macromolecular substrates.

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