UDC 663.15:577.151.3:661.182
APTEFF, 37, 1-192 (2006) BIBLID: 1450–7188 (2006) 37, 179-186
Original scientific paper

BIOORGANICALLY DOPED SOL-GEL MATERIALS CONTAINING AMYLOGLUCOSIDASE ACTIVITY

Beatrice Vlad-Oros, Monica Dragomirescu, Gabriela Preda, Cecilia Savii and Adrian Chiriac

Amyloglucosidase (AMG) from Aspergillus niger was encapsulated in various matrices derived from tetraethoxysilane, methyltriethoxysilane, phenyltriethoxysilane and vinyltriacetoxysilane by different methods of immobilization. The immobilized enzyme was prepared by entrapment in two steps, in one-step and entrapment/deposition, respectively. The activities of the immobilized AMG were assayed and compared with that of the native enzyme. The effects of the organosilane precursors and their molar ratios, the immobilization method, the inorganic support (white ceramic, red ceramic, purolite, alumina, TiO_2, celite, zeolite) and enzyme loading upon the immobilized enzyme activity were tested. The efficiency of the sol-gel biocomposites can be improved through combination of the fundamental immobilization techniques and selection of the precursors.

KEYWORDS: Amyloglucosidase (AMG); tetraethoxysilane; methyltriethoxysilane; phenyltriethoxysilane; vinyltriacetoxysilane; entrapment; entrapment/deposition; sol-gel

INTRODUCTION

Generally, the sol–gel method has been used in the preparation of inorganic oxidic networks by hydrolysis and polycondensation of alkoxides. Silica host matrices, made by the sol-gel process, have emerged as a promising platform for encapsulation of biomolecules such as enzymes, antibodies and cells. Enzymes find a more stable environment upon encapsulation in a silica host, because the polymeric framework grows around the

Beatrice Vlad-Oros, PhD Student; Dr. Gabriela Preda; Dr. Adrian Chiriac, Professor, Department of Chemistry, Faculty of Chemistry, Biology, Geography, West University of Timişoara, 300115, Dr. Monica Dragomirescu, Faculty of Animal Science and Biotechnologies, Banat University of Agricultural Sciences and Veterinary Medicine, 300645, Dr. Cecilia Savii, Romanian Academy, Institute of Chemistry C. Dragulescu, 300223, Timişoara, Romania

biomolecule, creating a cage and thus protecting the enzyme from aggregation and unfolding. These silica matrices are chemically inert, hydrophilic, and inexpensive to synthesize. They also exhibit higher mechanical strength, enhanced thermal stability, and negligible swelling in organic solvents compared to most organic polymers. Other advantages of silica supports include biocompatibility and resistance to microbial attack. Due to the facilities offered by sol-gel method, this becomes the main way to obtain hybrid materials (1-3). Bioorganically doped sol-gel materials have found increasing application in a wide variety of fields such as biosensing, affinity chromatography and biocatalysis (4,5).

Amyloglucosidase (AMG) (α-1,4-D-glucan glucohydrolase), E.C.3.2.1.3, is an exoenzyme, one of the most economically important enzymes used in many industrial processes. Examples of two industries that have taken advantages of this development are the sweetener and ethanol industries (6,7). To increase the efficiency and profitability of this process, AMG was immobilized by the sol-gel technique on different inorganic supports. Immobilized enzyme preparations were characterized by hydrolysis of starch and compared with the native enzyme.

EXPERIMENTAL

AMG from *Aspergillus niger* was obtained from Novo. The following precursors were used for sols preparation: tetraethoxysilane (TEOS), methyltriethoxysilane (MTES), phenyltriethoxysilane (PhTES) and vinyltriacetoxysilane (VTAS), all from Sigma-Aldrich. Soluble potatoes starch was purchased from Bender & Hobein. All the other chemicals were analytical grade and were used without further purification.

Encapsulation of enzyme by sol-gel process was performed in three different ways:

1. Silica sol was obtained from TEOS, ethanol and water (1.25 : 1.25 : 1, v/v), in acid catalysit (1M HCl). Then the sol was mixed with ethanol and water (1 : 1 : 1.33, v/v), 5 drops NH_3 12% and 1.25 mL buffered enzyme solution, containing 0.1 mL AMG (8).
2. TEOS and enzyme solution, containing 0.075 mL AMG, (1:1.72, v/v) were mixed with PVA 22.000 (polyvinyl alcohol 22.000) 4%, 1M NaF and isopro pylic alcohol (2 :1: 1, v/v) (9).
3. Hybrid matrices were prepared using the following Si-precursors: tetraethoxysilane (TEOS), methyltriethoxysilane (MTES), phenyltriethoxysilane (PhTES) and vinyltriacetoxysilane (VTAS). The mixture containing the alkoxides in different molar ratios, ethanol, water (Table 1) and 11 μL HCl (0.04 M) was stirred for an hour. Then 0.938 mL buffered enzyme solution, containing 0.075 mL AMG and 100 μL 1M NaF were added under stirring (3).

In all cases the gelation occurred in a few minutes. The gels were left overnight for aging (4°C), washed with *n*-hexane and dried (4°C). The gelation was also performed in the presence of 1 g of different supports (celite, white ceramic, red ceramic, purolite, alumina, TiO_2, zeolite).

Table 1. The chemical composition of the hybrid matrices

Alkoxides	Molar ratio	
	Alkoxides	$EtOH/H_2O$
VTAS/TEOS	1:1	0.8:1
PhTES/TEOS	1:1	0.8:1
PhTES/TEOS	2:1	0.8:1
MTES/TEOS	1:1	0.8:1
MTES/TEOS	2:1	0.8:1
MTES/TEOS	3:1	0.8:1

Residual starch concentration assay (I_2/I^-): 0.5 mL soluble starch (0.4%), 0.4 mL phosphate buffer (0.05 M, pH 5.2) and 0.01 g immobilized biocatalyst were incubated for 5 min at 25ºC. 5 mL solution I_2/I^- M/1000 and 15 mL distilled water were added. The samples were filtered. The absorbance was measured at 595 nm against distilled water. One unit of AMG activity was defined as the amount 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.

Reducing sugars assay: 0.5 mL soluble starch (1%), 0.4 mL citrate-phosphate buffer (0.15 M, pH 4.6) and 0.05 g immobilized biocatalyst were incubated for 5 min at 25ºC and then 1 mL 3,5-dinitrosalicylic acid reagent (DNS) was added. The samples were boiled in water for 10 min and 10 mL water was added. The samples were filtered and assayed at 540 nm against blank containing soluble starch, citrate-phosphate buffer, DNS reagent and distilled water. One unit of AMG activity was defined as the amount of enzyme required to produce 1 μmol of glucose in 5 min at 25°C.

Protein concentration was determined by the Lowry method (10). Samples where assayed against the blank prepared in the same way but with water instead sample. A bovine serum albumin calibration curve was used.

RESULTS AND DISCUSSION

The aim of this work was to find the most favorable sol-gel process that enhances the activity of the entrapped AMG. Because of the availability of the precursor and the easiness of the method, a two step sol-gel procedure, based on the typical tetraetoxysilane-water-HCl reaction mixture, was used for the encapsulation of AMG in silica matrices (4). To optimize the immobilization parameters, the enzyme loading (Fig. 1) and the water/TEOS ratio (Fig. 2) were assayed. Our results suggest that the best water/TEOS ratio is 12. By increasing the enzyme loading, in the case of TEOS, the immobilization yields remained relatively small (2 - 15%). The nanoporous nature of these matrices - pore size of approximately 100 Å, total pore volume of 0.408 cm^3/g and BET surface area of 359.8 m^2/g (11), that lead to a low diffusion rate of the substrate and poor accessibility of enzyme, as well as the unfavorable interaction between the matrix and the enzyme may be responsible for the loss of the AMG activity.

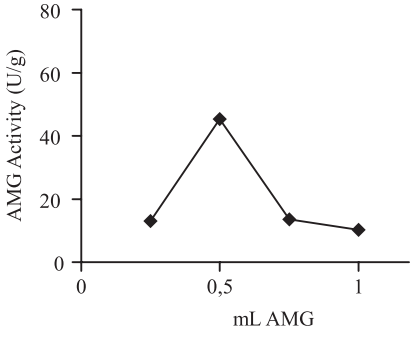

Fig. 1. The influence of enzyme loading on the immobilization efficiency in the case of silica matrices

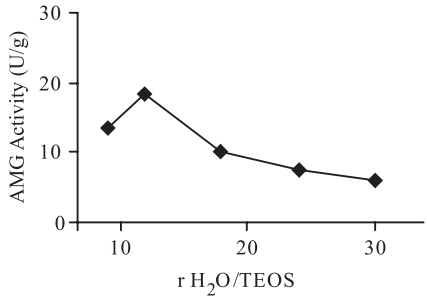

Fig. 2. The influence of H_2O/TEOS ratio on AMG activity

We tried to increase the immobilization efficiency by changing the entrapment technique, using one-step method of immobilization, a combined method – entrapment/deposition and various precursors, respectively. In all the cases the activities were assayed by both methods (I_2/I^-, DNS) and the trends were similar.

Three different methods of AMG immobilization in silica network, using only TEOS as precursor, were compared. The first one is the immobilization technique consisting of two steps; the second method is a one-step method of immobilization; and the third is entrapment/deposition (Fig. 3). Though the second method gave quite good results (the activity was four times higher), the best ones were are obtained by entrapment/deposition, when the activity was increased six times.

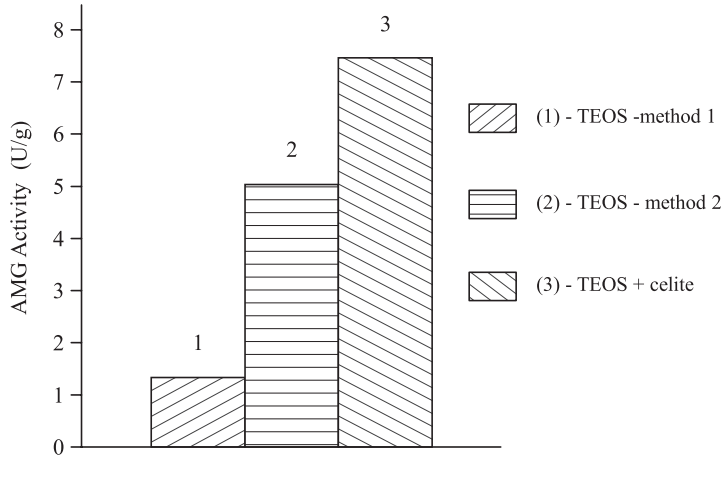

Fig. 3. Comparison of the method of immobilisation of AMG on various supports

Amyloglucosidase was also immobilized in gels derived from TEOS – method 1, and organosilanes (i.e. MTES, PhTES, VTAS) – method 3, through two-step sol-gel processes. To find the best conditions that enhance the activity of the entrapped AMG, the effect of the organosilane precursors and their molar ratios were investigated (Fig. 4). The best results were obtained for MTES/TEOS molar ratio of 1:1.

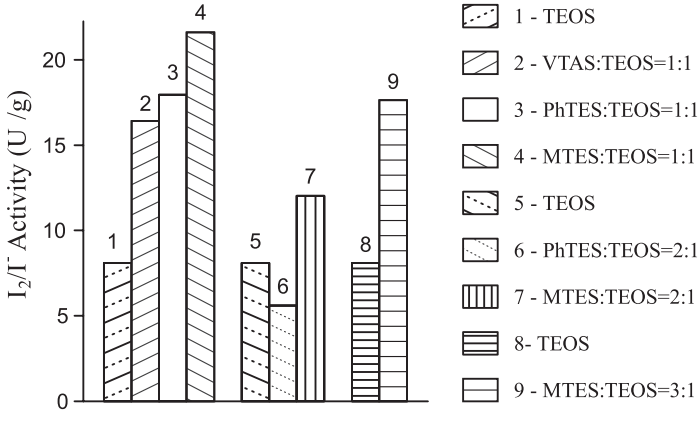

Fig. 4. AMG immobilized on different sol-gel matrices

Glucoamylase was entrapped using a combined method, attempting to reduce diffusional problems present in any network. The AMG was immobilized by sol-gel technique and then an inorganic support, celite, was added. The results obtained using the entrapment and the entrapment/deposition method are compared (Table. 2). By entrapment/deposition the activities increased by 1.75 and 1.5 times, respectively in the case of PhTES/TEOS, and by 5.6, 3.2 and 2.6 times, respectively, in the case of TEOS and MTES/TEOS. The results suggest that the method of choice should be entrapment/deposition.

Table 2. The influence of the immobilization method on AMG activity bound to various supports

Immobilization method	Precursors	AMG activity (U/g)	Protein content (mg/g)
Entrapment	TEOS	1.33	6.59
	PhTES:TEOS=1:1	3.94	13.76
	PhTES:TEOS=2:1	2.83	5.65
	MTES:TEOS=1:1	5.01	8.11
	MTES:TEOS=3:1	2.07	1.37
Entrapment/deposition (Matrices + Celite)	TEOS	7.46	5.23
	PhTES:TEOS=1:1	6.93	12.80
	PhTES:TEOS=2:1	4.45	9.03
	MTES:TEOS=1:1	8.25	7.61
	MTES:TEOS=3:1	5.08	8.78

Using the entrapment/deposition method, the influence of different inorganic supports upon the activity of the enzyme was tested. Some of supports (i.e. white ceramic, red ceramic, purolite, zeolite) are indigenes, from local sources. Some of them are ecological and are used in agriculture, horticulture, etc. The others are well known as supports for enzyme immobilization (alumina, TiO_2, celite). We obtained the best results using zeolite (Table 3).

Table 3. The influence of type of matrices on AMG immobilization by entrapment/deposition

Type of matrices	AMG Activity (U/g)	Protein content (mg/g)
MTES:TEOS=1:1 + Al_2O_3	0.23	15.32
MTES:TEOS=1:1 (Standard)	2.58	8.11
MTES:TEOS=1:1 + purolite	3.54	25.27
MTES:TEOS=1:1 + white ceramic	4.05	15.41
MTES:TEOS=1:1 + red ceramic	4.56	13.63
MTES:TEOS=1:1 + celite	8.25	7.61
MTES:TEOS=1:1 + TiO_2	9.21	12.56
MTES:TEOS=1:1 + zeolite	13.63	22.28

In the case of the silica (MTES : TEOS – 1 : 1)/alumina xerogel the activity was below the standard, so we tried to enhance it by increasing the enzyme loading (Table 4). The enzyme activity increases with enzyme loading to a maximum, then decreases, probably because of steric hindrance and enzyme agglomeration. The best results were obtained at a ratio of 0.75 mL/5 mL sol (26.73 U/mL sol). By increasing the enzyme loading 20 times the activity enhanced approximately 4 times, so the main factor in enhancing the enzyme activity proved to be the support and not the enzyme loading.

Table. 4. Influence of enzyme loading on the immobilization efficiency

MTES:TEOS=1:1 + Al_2O_3 Enzyme loading (mL AMG)	I_2/I^- Activity (U/g)	Protein content (mg/g)
0.05	7.69	7.64
0.1	12.84	15.32
0.25	10.06	19.94
0.5	20.66	33.19
0.75	28.90	28.49
1	28.44	25.11

CONCLUSIONS

The sol-gel immobilization is a promising technique because it allows the obtaining of a solid material, easier to manipulate than native enzyme (liquid in the case of AMG). Also the decrease of catalytic efficiency of the enzyme is partially compensated by the increase of the enzyme stability, as it has to be proved in future work. Even if the most usual method of immobilization uses TMOS and its derivatives (9), this study shows that TEOS, which is less reactive and also less expensive, is suitable too. Further studies should elucidate how the precursors influence the textural characteristics of the matrices.

By exploring or combining available methods or knowledge, or by exploiting the positive attributes of each immobilization method, the performance of immobilized enzymes can be improved the levels that were previously unattainable by using single immobilization methods. A rational combination of various immobilization methods is a valuable approach to obtain robust immobilized enzymes, which cannot be obtained by the straightforward immobilization.

ACKNOWLEDGEMENT

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

REFERENCES

1. Jitianu, A., A. Britchi, C. Deleanu, V. Badescu and M. Zaharescu: Comparative study of the sol–gel processes starting with different substituted Si-alkoxides. J. Non-Cryst. Solids **319** (2003) 263–279.
2. Bhatia, R. B., C. J. Brinker, A. K. Gupta and A. K. Singh: Aqueous sol-gel process for protein encapsulation. Chem. Mater. **12** (2000) 2434-2441.
4. Peter, F., L. Poppe, C. Kiss, E. Szocs, G. Preda, C. Zarcula and A. Olteanu: Influence of precursors and additives on microbial lipases stabilized by sol-gel entrapment. Biocatal. Biotransform. **23**, 3/4 (2005) 251-260.
5. Brinker, J. and G.W. Scherer, Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing, Academic Press, Boston (1990) p. 839.
6. Cao, L.: Immobilised enzymes: science or art? Curr. Opin. Chem. Biol. **9** (2005) 217–226.
7. Rani, A.S., M.L.M. Das and S. Satyanarayana: Preparation and characterization of AMG adsorbed on activated charcoal. J. Mol. Catal. B: Enzym. **10**, 5 (2000) 471-476.
8. Vera-Avila, L.E., E. Morales-Zamudio and M.P. Garcia-Camacho: Activity and reusability of sol-gel encapsulated α-amylase and catalase. Performance in flow-through systems. J. Sol-Gel Sci. Technol. **30** (2004) 197–204.
9. Preda, G., C. Savii, A. Chiriac, F. Peter, M. Dragomirescu, J. Halász, I. Stan and Z. Dudás: Properties of protease immobilised in/on innorganic supports. Annals West University (Timişoara). Ser. Chemistry **12**, 2 (2003) 205-212.

10. Reetz, M.T., A. Zonta and J. Simpelkamp: Efficient immobilization of lipases by entrapment in hydrophobic sol-gel materials. Biotechnol. Bioeng. **49**, 5 (1996) 527 – 534.
11. Lowry, O.H., N.I. Rosebrough, A.L. Farr, R.J. Randall: Protein measurement with the Folin-Phenol reagents. J. Biol. Chem. **193** (1951) 265 – 275.
11. Preda, G., C. Savii, A. Chiriac, F. Peter, M. Dragomirescu, J. Halász, Z. Dudás and I. Stan: Amylases entrapped in sol-gel derived silica matrixes. Annals West University (Timişoara). Ser. Chemistry **12**, 2 (2003) 199-204.

БИООРГАНСКИ ДОПИРАНИ СОЛ-ГЕЛ МАТЕРИЈАЛИ СА МИКРОБИОЛОШКИМ АМИЛАЗАМА

Beatrice Vlad-Oros, Monica Dragomirescu, Gabriela Preda, Cecilia Savii and Adrian Chiriac

Амилоглукозидаза (АМГ) из гљиве *Aspergillus niger*, је инкапсулирана у капсулу са различитим матриксима који су добијени од тетраетоксисилана, метилтриетоксисилана, фенилтриетоксисилана и винилтриацетоксилана, различитим методама имобилизације.

Имобилизација ензима је постигнута у два корака, у једној фази и инкапсулирањем/депозицијом, редом. Активности имобилисане АМГ су испитане и поређене са невезаним ензимом. Испитивани су утицај прекурсора на бази органосилана и њихов моларни однос, метода имобилизације, неорганski носач (бела керамика, црвена керамика, алумина, TiO_2, зеолити, целит, пуролит) и активност имобилисаног ензима. Ефикасност сол-гел биокомпозита може се побољшати комбинацијом основних техника имобилизације и избором прекурсора.

Received 22 May 2006
Accepted 10 October 2006