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Effect of Glycerol on the Activity of α-Chymotrypsin Encapsulated in Sol-Gel Derived Silica Monoliths

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Preparation and properties of a biochemically active sol-gel monolith were investigated by encapsulating the enzyme α-chymotrypsin in a polymerizing tetramethoxysilane in presence of glycerol as an additive and pore forming template. The enzyme encapsulated in the templated mesoporous sol-gels exhibited an improvement in thermal stability and well behaved apparent catalytic activity, compared to those in non-templated microporous host, synthesized in the absence of glycerol. The apparent catalytic activity of encapsulated enzyme was closely associated with the concentration of template in as-synthesized sol-gels. This transparent, mechanically and chemically stable bioactive sol-gel glass may be used for the development of biochemical sensors and for the purpose of chemical catalysis.

Key Words: Sol-gel, α-Chymotrypsin, Glycerol, Additive, Template, Mesoporous.

INTRODUCTION

Encapsulation of biomolecules such as enzyme proteins, cells and microorganisms in porous metal oxides by physical entrapment *via* the sol-gel process has drawn great interest in recent years. The encapsulation of biomolecules by sol-gel methods is commonly carried out through four stages, namely acid catalyzed hydrolysis of alkoxysilane precursors, addition of aqueous buffer solution containing protein, aging and drying to produce optically transparent glass materials with the protein entrapped[1-3].

The structure and properties of doped sol-gels depend not only on the chemical composition of the starting materials, but also on many operational factors involved in the preparation. Several modifications to the immobilization protocol have been explored including substituting tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS) with other precursors, using organic-modified host matrices and stabilizing the protein before encapsulation by co-immobilizing ligands[3].

A variety of additives have been examined as stabilizers of entrapped proteins including ligand-based stabilizers[4], the use of graft copolymers of polyvinyl imidazole and polyvinyl pyridine[5,6], incorporation of charged polymers[7] and the addition of PEG[8]. Such studies clearly show that the interactions between the additive and the protein can be used advantageously to maximize the stability and function of some proteins.

The examination of enzyme stability, reaction kinetics and function all require that the external reagent be able to reach the entrapped protein. The accessibility of reagents to entrapped protein is determined largely by the pore size and electrostatics of the materials. The appropriate porosity of the sol-gel matrix is needed for containing the biomolecules and for mass transport of the reactants and products of the bio-catalyzed reaction. The accessibility can be maximized by several precautions such as incorporation of charged polymers, manipulation of aging conditions to minimize silanol content, manipulation of pH conditions and using molecular templating agents to promote larger pores (mesopores)[9].

Mesoporous silica and organically modified silicate sol-gel materials have been synthesized *via* various surfactant or non-surfactant templating processes. Similar to mesoporous silica gels, the enzyme containing sol-gel materials prepared by the use of pore forming agents or templates have high surface areas, large pore volumes and network pore structure consisting of narrowly distributed mesopores[10,11]. Entrapped enzymes, such as alkaline phosphatase (ALP), acid phosphatase (ACP) and horseradish peroxidase (HRP) in templated mesoporous silica matrices demonstrated much higher remaining activities and thermal stability than in non-templated samples prepared under the same conditions[12,13].

In this work, the modified TMOS-based sol-gel with organic additives and pore forming agents have been applied for the encapsulation of α-chymotrypsin. The effect of glycerol in

the buffered enzymes solution on stability and activity of enzymes encapsulated into TMOS derived silica was examined. The additive was chosen due to its high solubility in aqueous solution and its biocompatibility. α-Chymotrypsin was chosen to demonstrate the utility of having optically transparent materials which allow for spectroscopic studies of the encapsulated enzymes.

EXPERIMENTAL

α-Chymotrypsin (EC 3.4.21.1, type II, from bovine pancreas), *p*-nitrophenyl acetate, glycerol, *tris*-HCl buffer, carbonate buffer, were supplied by Sigma. Tetramethyl orthosilicate (TMOS 98 %) and HCl were purchased from Aldrich.

Preparation of enzyme containing sol-gel monoliths: Encapsulation of α-chymotrypsin was achieved by hydrolysis and condensation of TMOS in presence of glycerol and α-chymotrypsin at room temperature. In a typical procedure for the preparation of enzyme-encapsulated sol-gel monoliths: TMOS (1 mL) was mixed with H_2O (0.24 mL) and HCl (0.015, 8 μL) in a flask under sonication at room temperature for 0.5 h until a homogeneous mixture was obtained. A volume of 0.75 mL hydrolyzed TMOS was then mixed with 1.25 mL of a *tris*-HCl buffer (40 mM, pH 8.59) containing α-chymotrypsin and between 0 and 40 weight % of glycerol solution. The mixture was placed in a disposable polymethacrylate cuvette which was then sealed with paraffin film and allowed to gel at room temperature. Upon gelation, several small holes were punched in the paraffin film to allow the evaporation of solvents and put into dessicator vacuum for about 5 days. The samples were then placed at room temperature for about 10 days. For fluorescence studies, the monoliths were rehydrated before measurements to avoid cracking. For colorimetric studies, the monoliths were ground into powder and stored in sealed vials in a freezer for subsequent processing, characterization and enzymatic activity assay.

Characterization of enzyme encapsulated sol-gels: Fourier transform infrared (FTIR) spectroscopy was used to characterize the presence of specific chemical groups in the materials and to monitor the template removal. The as-synthesized and water extracted samples were milled and mixed with dried KBr powder. FTIR spectra were obtained within the range 4000 and 400 cm^{-1} during 64 scans.

Fluorescence spectra were recoded on a LS 50 B Perkin-Elmer Luminescence Spectrometer at room temperature. Samples were excited at 290 nm and emission were recorded between 305 and 450 nm with 6 nm band-pass. To obtain fluorescence unfolding profiles, thermal denaturation of free and entrapped enzyme in the absence and presence of additives was assessed by measuring the emission of the tryptophan residues within the proteins as a function of temperature. For solution-based studies, a volume of 1.5 mL of protein in phosphate buffer solution was used. For monolith-based studies, the rehydrated monolith was placed into a cuvette containing 1.5 mL of phosphate buffer solution. In both cases, the proteins were denatured by placing the cuvettes into a water bath. The temperature was raised in 5 ºC increments starting at 20 ºC and increasing to 80 ºC. The samples were allowed to equilibrate for 0.5 h at each temperature. Fluorescence spectra were collected at each point for the sample and blank at an identical temperature. The intensity at each temperature was used to determine the percentage of native enzyme remaining at each temperature following the method of Brennan *et al.*[14] according to the following equation:

$$f_N(T) = 1-\{(F_{max}-F(T))/(F_{max}-F_{min})\} \quad (1)$$

where F_{max} is the maximum fluorescence intensity (at 20 ºC), F_{min} is the minimum fluorescence intensity and F(T) is the fluorescence intensity at given temperature T. The fraction of native enzyme was plotted against temperature to generate intensity based unfolding curves. The unfolding temperature was determined from the unfolding curve which typically corresponded to the temperature where the fluorescence signal had decreased to 50 % of its initial value.

Activity assay for free and encapsulated enzymes: General procedures for enzymatic activity assays of α-chymotrypsin in solution and in immobilized forms were adapted from the literature[12,14,15]. Kinetic parameters for the free and immobilized enzymes were determined using Michaelis-Menten kinetics given by:

$$V = \frac{V_{max}[S]}{K_M + [S]} \quad (2)$$

where V is the reaction velocity, [S] is substrate concentration, V_{max} is the maximal reaction velocity and K_M is the Michaelis constant. The maximal reaction velocity, V_{max} can also be written as:

$$V_{max} = k_p E_o \quad (3)$$

where k_p is the enzyme specific activity and E_o is the enzyme concentration.

The activity of α-chymotrypsin was determined by monitoring the absorbance at 400 nm as a function of reaction time at room temperature. A volume of 1 mL of α-chymotrypsin solution (containing 1 μg) was mixed with 1.89 mL of *tris*-HCl buffer. After 0.5 h equilibration, 22.50 μL of *p*-nitrophenyl acetate (*p*-NPA) 4 mM in acetone solution was added, upon which the absorbance change with time was recorded. For immobilized α-chymotrypsin, all samples were first washed with dilute buffer solution before proceeding with the activity assay. The α-chymotrypsin containing gel was then evaluated for the apparent activity. Activity dependence of the enzymes on the concentration of substrates was conducted at pH 8.59 by varying the concentration of *p*-nitrophenyl acetate from 7.5 to 60 μM. The effect of the pH on the activity of free and immobilized α-chymotrypsin was studied at a fixed concentration of substrate (30 μM). *Tris*-HCl and carbonate buffers were used to obtain pH values required for α-chymotrypsin assays.

In order to investigate the effect of additives on the enzyme stability, the activity of the two enzymes with and without glycerol was examined spectrophotometrically at various temperatures. Thermal stability of free and entrapped enzymes was estimated from the remaining activity after thermal treatment between 25 and 80 ºC, in comparison with the activity obtained in solution at 25 ºC. The relative activity was plotted against temperature to establish the temperature effect on

activity. The thermal treatment was measured as follows. For an encapsulated enzyme of a few mg, 1 mL of H_2O was added to the sample. For the free enzymes, 1 mL of dilute enzyme solution was added directly. The sample-containing tubes were sealed with paraffin film and incubated at the desired temperature in a water bath for 0.5 h. Immediately after removing them from the water bath, the tubes were cooled to room temperature. The powder samples were then separated from the aqueous phase for the activity assay. Both free and encapsulated samples were assayed for their remaining activities by following the same procedure as described above.

RESULTS AND DISCUSSION

Formation of enzyme-encapsulated monoliths: The preparation of enzyme containing sol-gel materials was performed by adding the enzyme with glycerol additives as templates, into the TMOS derived sol before gelation, followed by aging and drying the gel to yield the transparent monolithic enzyme-containing glasses. The gelation time was shortened by the addition of the buffered enzyme solution which raised the pH of the mixture. The aging and drying processes were accelerated by using a vacuum dessicator to evacuate solvents and by-products of the sol-gel reaction without causing sample cracking which often occurs in a template-free system. All samples, prepared in the presence of 0, 10, 20 and 40 wt % of glycerol were transparent, homogeneous and showed no phase separation. Since the protein and additives are added prior to the gelation, it is possible that a sol-gel structure is formed around the protein with the protein molecule acting as a template. The glycerol molecules may direct the mesophase formation around their aggregates and join the protein molecules in forming the same pores or cages in which the biomolecules exist. The co-existence of the enzyme and the additives in the same cavities would then provide the enzyme molecules with larger space upon template removal, resulting in more freedom for the enzyme to undergo its conformation changes and be more accessible to the external reagents.

Properties of the enzyme-encapsulated sol-gels

Fluorescence studies of encapsulated enzymes: The encapsulation of enzyme into sol-gel derived glass may cause significant conformation changes in the enzyme. In this work, the fluorescence intensity of the enzymes was examined in solution and in TMOS-derived sol-gel in the absence and presence of 20 wt % of glycerol in order to study how encapsulation affected the conformation of these enzymes. Fig. 1 presents fluorescence spectra of α-chymotrypsin in solution and in TMOS sol-gel derived materials without templating. A decrease in intensity (by 80 % for both enzymes) and a red-shift in the emission wavelength for the enzyme (about 23 nm) was observed as the enzymes were heated at 80 ºC (Fig. 1(3)). These observations indicate that there are changes in the tertiary structure of the proteins which result in the exposure of the buried tryptophan residues to the surrounding solvent as the proteins unfold[16]. The similarity of the spectra of these enzymes in solution and in silica matrices confirms that the proteins were encapsulated in the native form.

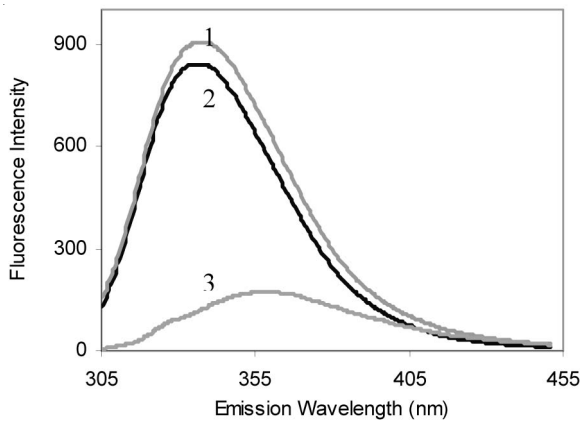

Fig. 1. Fluorescence spectra for α-chymotrypsin. (1) encapsulated α-chymotrypsin (TMOS gel), (2) free α-chymotrypsin (in buffer solution) and (3) denatured α-chymotrypsin

Table-1 gives the emission characteristics of α-chymotrypsin in solution and in silica glasses in the absence and presence of additive. The fluorescence intensity of the enzymes in solution in the absence of additives is taken as 100 % native enzyme. The similar fluorescence characteristics of these enzymes in solution and when encapsulated in the presence of glycerol as shown in Table-1 again indicates that the conformation of these enzymes was not altered upon encapsulation. A very slight red-shift of the emission wavelength for both enzymes relative to the value in solution may be due to changes in polarity of the external solvent that perturb the tryptophan residues. Similar observations have been reported by Brennan and co-workers[14] for a-chymotrypsin in solution and in TEOS-derived materials in the presence of sorbitol or sarcosine.

TABLE-1
EMISSION CHARACTERISTICS OF α-CHYMOTRYPSIN IN SOLUTION AND IN TMOS-DERIVED GLASSES IN THE ABSENCE AND PRESENCE OF GLYCEROL

Sample	Fluorescence intensity (%)	Emission wavelength (± 2 nm)
Buffer	100	335
Buffer + glycerol	102	336
TMOS	100	336
TMOS-glycerol	100	335

Fig. 2 shows the percentage of native enzyme remaining as a function of temperature for α-chymotrypsin in the presence of varying levels of additives. In each of the fluorescence intensity-based unfolding curves, there was an initial gradual decrease in intensity of up to about 75 % as the temperature was increased to 40 ºC, followed by a rapid decrease corresponding to the main unfolding transition at temperatures above 40 ºC and finally a post-unfolding baseline. The fluorescence intensity changes of the unfolding curves are due to thermally induced effects on the quantum yield and mobility of the tryptophan residue. The unfolding curves indicate that encapsulation of both enzymes in TMOS-derived sol-gel matrices resulted in small increases in the unfolding temperature. This suggests that the stability of protein might be marginally improved on encapsulation. This compared well with avidin[3] and with other enzymes reported in literature[17,18]. The curves clearly show that the presence of increasing levels

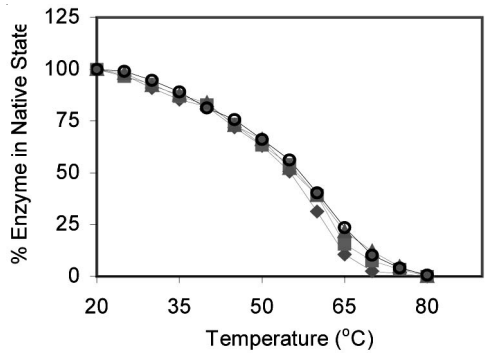

Fig. 2. Fluorescence-based unfolding curves for α-chymotrypsin in the presence of glycerol. (◆) α-chymotrypsin in solution, (■) encapsulated α-chymotrypsin in the absence of additive, (▲) encapsulated α-chymotrypsin in the presence of 10 % additive and (O) encapsulated α-chymotrypsin in the presence of 40 % additive

of additives leads to small but systematic improvements in the unfolding temperature for both encapsulated proteins.

Infrared spectra of encapsulated α-chymotrypsin: The infrared spectra of as-synthesized and extracted samples shown in Fig. 3 exhibited the major bands associated with the network Si-O-Si vibration modes at 460, 790, 1080 and 1220 cm^{-1} along with Si-OH asymmetrical stretching at 960 cm^{-1} and Si-OH stretching at 3400 cm^{-1}. Upon extraction of the samples, the intensity of the band at 2940 cm^{-1} for C-H stretching of the glycerol template component has disappeared, indicating completion of template removal.

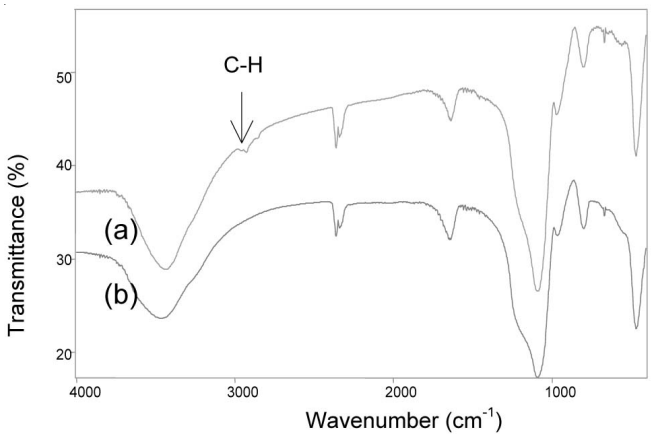

Fig. 3. Infrared spectra of (a) as-synthesized containing TMOS-glycerol sol-gel and (b) after extraction of glycerol

Enzyme activity: Activity assays were carried out for the enzymes in order to further explore the stabilizing effects of the glycerol. The activity of free and encapsulated α-chymotrypsin was measured *via* the hydrolysis of *p*-nitrophenyl acetate as follows:

$$O_2NC_6H_4CH_3COO + H_2O \longrightarrow O_2NC_6H_4OH + CH_3COOH \quad (4)$$

Effect of temperature on the activity of free and enacapsulated α-chymotrypsin: Fig. 4 illustrates the changes in relative activity as a function of temperature for α-chymotrypsin in solution and in TMOS-derived glasses in the presence of additives. All results are normalized to the activity obtained for protein in solution in the absence of additive at room temperature. For α-chymotrypsin in solution, the activity of the enzyme increases as the temperature increases to 30 ºC and reaches its maximum activity at about 35-40 ºC. The activity then decreases dramatically at 60 ºC and the enzyme becomes inactive above 65 ºC. This result is in agreement with that observed from fluorescence measurements (Fig. 2) where the unfolding temperature for this enzyme is about 55 ºC. A similar trend is observed for encapsulated α-chymotrypsin, though the enzyme activity is lower. The addition of glycerol results in a slight increase in activity relative to the encapsulated enzyme in the absence of additive. These results are again in agreement with those obtained from fluorescence studies (Fig. 2) and confirm that glycerol can stabilize the encapsulated enzyme, but only a small extent, against denaturation. As compared with the enzyme in solution, the apparent activity of the encapsulated enzyme is much lower.

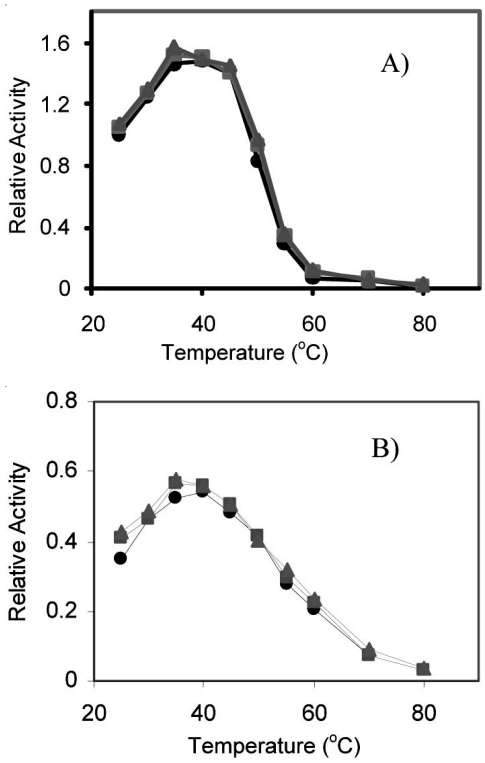

Fig. 4. Changes in α-chymotrypsin activity at pH 8.59 as a function of temperature. (A) α-chymotrypsin in solution and (B) encapsulated α-chymotrypsin. (●) no additive, (■) 20 % glycerol and (▲) 20 % D-glucose. Activities are relative to those in solution at 25 ºC

Effect of substrate concentration on the enzyme activity: Fig. 5 displays the reaction kinetics of free and encapsulated α-chymotrypsin at varying concentration of *p*-nitrophenyl acetate. The experimental data were fitted by using eqn. 2 and the kinetic parameters are presented in Table-2. The excellent fits indicate that the reaction again obeys Michaelis-Menten kinetics. By comparing the specific activity of the free and encapsulated α-chymotrypsin, the activity of α-chymotrypsin decreases upon encapsulation in silica sol-gel matrices this time to about 40-57 % of the free enzyme. The hydrophobic properties of the active sites of α-chymotrypsin could result in weak protein-silica interactions and more accessibility to substrate. It looks like the kinetics for α-chymotrypsin is less affected by the gel.

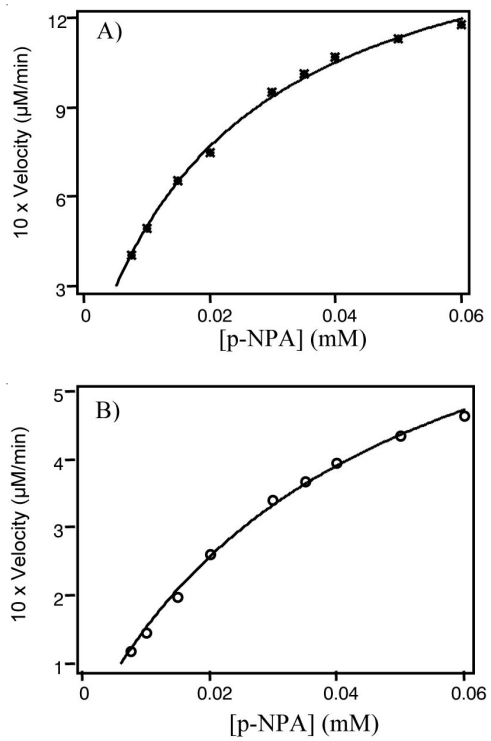

Fig. 5. Reaction kinetics for α-chymotrypsin at varying concentration of *p*-nitrophenyl acetate (A) in buffer solution and (B) in TMOS gel; lines show fits to eqn. 2

TABLE-2
KINETIC PARAMETERS FOR ALKALINE PHOSPHATASE AND α-CHYMOTRYPSIN IN SOLUTION AND IN TMOS-DERIVED GLASSES

Sample	K_M (mM)	Specific activity (U mg enzyme^{-1})
Solution of α-chymotrypsin	0.023	0.309
TMOS	0.042	0.106
TMOS-glycerol 10 wt %	0.040	0.113
TMOS-glycerol 20 wt %	0.033	0.121
TMOS-glycerol 40 wt %	0.029	0.132

Effect of additives on enzyme activity: Fig. 6 shows the substrate dependence plot for encapsulated enzymes prepared in the presence of glycerol template. For all samples, the apparent activity increased with the concentration of substrate until reaching a maximum and then tended to level off as the substrates concentration increases. The kinetic data displays the dependence of the enzyme activity on glycerol concentration, in agreement with report by Wei *et al.*[12] who investigated the activity of alkaline phosphate encapsulated in TMOS and TEOS prepared by using D-glucose as template.

The addition of template to the glasses led to significant alterations in the pore morphology, including a larger pore diameter (*ca.* 14 Å for TMOS-derived materials prepared in the absence of template to *ca.* 32 Å for glycerol-doped samples) and larger pore volume[19]. An increase in pore size would result in reducing interaction between the enzyme and surrounding matrix pore walls. The enhanced pore size is perhaps more important in achieving the improved enzyme activity as this requires good mobility of substrates through the host silica matrices. However, the higher the additives concentration incorporated, the higher is the possibility of enzymes leaching

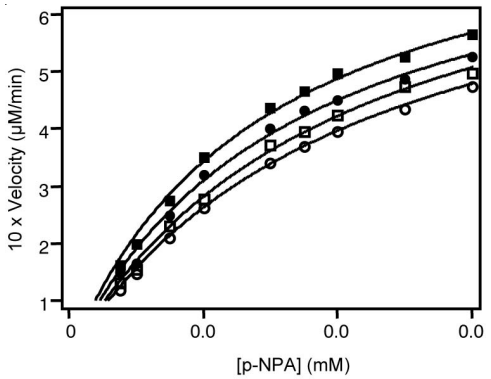

Fig. 6. Activity of α-chymotrypsin as a function of substrate concentration. (○) TMOS, (□) TMOS-glycerol 10 wt %, (●) TMOS-glycerol 20 wt % and (■) TMOS-glycerol 40 wt %; lines show fits to eqn. 2

out from their cavity during additive extraction. However, no leaching was found in the samples investigated here.

Conclusion

α-Chymotrypsin were successfully encapsulated in silica matrices *via* the hydrolysis and condensation of tetramethyl orthosilicate (TMOS) in presence of glycerol as pore forming agents. Based on a comparison of the fluorescence properties of free and encapsulated α-chymotrypsin, the native structure of these enzymes is largely unperturbed by encapsulation in sol-gel glass matrices. The enzyme-containing sol-gels had slightly improved thermal stability and well behaved apparent catalytic activity. The apparent activity of encapsulated enzymes systematically increased with the concentration of glycerol in as-synthesized sol-gels. The mesoporous silica matrix, with pore parameters tunable by adding varied amounts of glycerol, not only effectively retained the enzyme without leaching, but also reduced mass transfer of the substitute into and product out of the matrix. In the sol-gel system investigated here, α-chymotrypsin showed retention of its activity.

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