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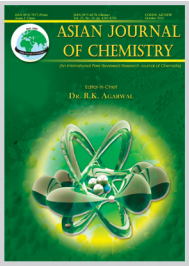

Synthesis and Biocompatible Properties of a New Glycerylsilane

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This paper presents an approach to the preparation of a new glycerylsilane with glycerol and tetraethyl orthosilicate, using Amberlyst 15 (dry), an acidic ion-exchange resin, as catalyst. Trypsin was selected as a model enzyme to study the biocompatible properties of the resultant glycerylsilane by comparing the properties of trypsin in solution, trypsin entrapped in tetraethyl orthosilicate and the resultant glycerylsilane derived sol-gel matrix. The enzyme entrapped into the glycerylsilane derived sol-gel glass exhibits improved properties, which is not found in free enzyme and conventional sol-gel glass and the result indicates the glycerylsilane has good biocompatible properties.

Key Words: Glycerylsilane, Entrapment, Sol-gel, Trypsin, Characterization.

INTRODUCTION

A versatible, mild and biocompatible sol-gel processing method has been used to entrap active biomolecules within a porous silicate matrix[1-4]. Sol-gel which can entrap biomolecules have made use of the silane precursors tetramethyl orthosilicate or tetraethyl orthosilicate in most studies. However, these precursors can liberate a significant amount of alcohol during hydrolysis, which must at least partially be removed to reduce protein denaturation[5-7]. Thus, biocompatible sol-gel precursor needed to solve such problems.

In recent years, a number of biocompatible silane precursors have been reported, which are based on polyhydric alcohol substituted derivatives, such as glycerylsilanes[8-16]. There are two methods to prepare glycerylsilanes. One method is preparing glycerylsilanes by adding acid as catalysts, which was difficult to remove from glycerylsilanes[8]. Furthermore, it will affect the subsequent entrapment process, the properties of gel and even the bioactivity of biomolecules. The other method is preparing glycerylsilanes at a high temperature. However, these precursors cannot be hydrolyzed completely to form a homogeneous solution during hydrolysis. The remained particles needed to be removed before use[11,12]. This disadvantage limited its application in entrapment.

Here we describe a new method of preparing a glycerylsilane with glycerol and tetraethyl orthosilicate, using Amberlyst 15 (dry), an acidic ion-exchange resin, as catalyst under mild conditions. Trypsin was selected as a model enzyme and entrapped in the resulting glycerylsilane derived sol-gel matrix to evaluate the biocompatible properties of the resulting silane precursor.

EXPERIMENTAL

Trypsin was a commercial enzyme available from Amresco. Tetraethyl orthosilicate, glycerol, *N*-α-benzoyl- DL-arginine-4-nitroanilide hydrochloride (BAPNA, 99 %) and Poly(ethylene glycol) 6000 (PEG 6000) were obtained from Acros Organics. Amberlyst 15 (dry) was purchased from Aldrich Chemical Co. All other chemicals were of analytical grade and were used as received.

^{1}H NMR and ^{13}C NMR spectra were recorded in CH_3OD at 500 MHz and 100 MHz respectively on a Bruker Avance III 500 MHz NMR spectrometer. UV-VIS absorbance was carried out on a Shimadzu UV-2450 spectrometer. The morphology of the sol-gel matrix was assessed using a Hitachi S-4700 scanning electron microscopy.

Synthesis of glycerylsilane: To 20 mL of acetonitrile, 1.65 mL of glycerol, 0.2 g of Amberlyst 15 (dry), 10 mL of tetraethyl orthosilicate were added and then the mixtures were stirred at 40 ºC for 96 h. After the completion of reaction, Amberlyst 15 (dry) was filtered off. Subsequently, ethanol and acetonitrile were distilled away under reduced pressure and a white powder was obtained.

Entrapment of enzyme: Entrapment of trypsin was performed according to the following procedures. Briefly, 0.46

mmol of tetraethyl orthosilicate or glycerylsilane, 12 mg of PEG 6000, 0.18 mL of distilled water and 2 μL of H_3PO_4 (1 M) as catalyst were mixed and sonicated to produce a homogeneous solution. The homogeneous solution was then mixed with 0.08 mL of 5 mg/mL buffered trypsin solution (phosphate buffer, 0.2 M, pH 7.0) and allowed to gel at room temperature for 5 min. Then 0.2 mL of PBS was added to the gel. The mixture was then stored at 4 ºC for 72 h for aging.

As for free enzyme which was used to compare the biocompatible properties of the resulting silane precursor, 0.08 mL of 5 mg/mL buffered trypsin solution (phosphate buffer, 0.2 M, pH 7.0) was mixed with 0.2 mL PBS and 0.2 mL of distilled water. The mixture was then stored at 4 ºC for 72 h for the entrapment of enzyme.

Determination of the amount of entrapped enzyme: PBS (1.5 mL) was used to wash entrapped trypsin sample. After washing, the PBS was collected and mixed with 2 mL of coumassie blue dye solution. Then the mixture was determined in absorbance at 595 nm[17]. The protein concentration is linear at the concentration range of 30-140 μg/mL. The amount of the entrapped protein is obtained as follows:

$$W_e = W_t - W_u$$

where, W_e is the amount of entrapped protein; W_t is the amount of total protein introduced in the entrapment process; W_u is the amount of unentrapped protein, which is obtained from the absorption at 595 nm.

Measurement of enzyme activity: The activity was determined by using a chromogenic substrate *p*-benzoyl-DL-arginine-4-nitroanilide (BAPNA). The reaction mixture containing 2.9 mL of *Tris*-HCl buffer solution (13 mM, pH 7.8), 60 μL of water and 30 μg of a trypsin sample was incubated at 37 ºC in a spectrophotometric cuvette. After 75 μL of BAPNA (10 mM stock solution freshly prepared in DMSO) was added, the reaction was monitored by increased absorbance at 408 nm 18. A blank was measured under the same conditions.

Characterization of glycerylsilane derived sol-gel matrix morphology: The glycerylsilane derived sol-gel matrix morphology was assessed using scanning electron microscopy (SEM) for characterization of silica matrix. SEM analysis was done by exposing a fresh surface, which was then coated with a gold film under vacuum to improve conductivity.

Entrapment efficiency and biocompatibility of the sol-gel precursors: The entrapment efficiency of the sol-gel precursor was evaluated in terms of trypsin loading yield (%) as follows:

$$\text{Trypsin loading yield (\%)} = \frac{\text{Amount of entrapped trypsin}}{\text{Amount of trypsin introduced}} \times 100\ \%$$

The biocompatibility of the sol-gel precursor was evaluated in terms of relative activity (%) by comparing the optimum pH and temperature of the free enzyme and entrapped enzyme. Michaelis constant of the free enzyme and entrapped enzyme and stability of the free and entrapped enzyme during long storage time.

Optimum pH of the enzyme: Free trypsin and entrapped enzyme were incubated in different pH *Tris*-HCl buffer solution (50 mM, pH 7.2, 7.4, 7.6 7.8 8.0, 8.2, 8.4, 8.6, 8.8) for 30 min. Measurement of the activity of the free enzyme and entrapped enzyme was performed.

Optimum temperature of the enzyme: Free trypsin and entrapped enzyme were incubated in *Tris*-HCl buffer solution (50 mM, pH 7.8) at different temperatures (30 ºC-86 ºC) for 0.5 h. Measurement of activity of the free enzyme and entrapped enzyme was performed.

Determination of Michaelis constant: The kinetic constant was determined using Lineweaver-Burk plot by initial reaction velocity of the free or entrapped enzyme.

Stability of free and immobilized enzyme during long storage time: The stability of free and entrapped trypsin was measured by calculating the residual activity after a long storage time. Measurement of activity of the free enzyme and entrapped enzyme was performed.

RESULTS AND DISCUSSION

Characterization of glycerylsilane: It is well known that transetherification is hard to proceed completely and it need harsh conditions. Amberlyst 15 (dry) is an acidic ion-exchange resin, which has been used as etherification catalyst[18-21]. In this paper, Amberlyst 15 (dry) was used as catalyst to synthesize glycerylsilane with tetraethyl orthosilicate and glycerol under mild conditions through transetherification. The resulting glycerylsilane is a white solid, which is got after filtering to remove the catalyst and distillating to remove glycerol. ^{1}H NMR (CD_3OD) and ^{13}C NMR (CD_3OD) of the product indicate that the glycerylsilane is a mixture, which included unreacted raw materials, this is due to the reaction which is hard to proceed completely. In addition, glycerol is difficult to be removed by distilling. Since the glycerol is expected to play several important roles in mediating the behaviour of the silica matrix[8,9]. In addition, it was very difficult to obtain pure glycerylsilane by using conventional methods, such as recrystallization, column separation, *etc*. Therefore, the glycerylsilane was used to entrap enzyme in the following experiments without any more purification.

In the entrapment process, the glycerylsilane could be hydrolyzed easily to form a homogeneous solution without particles left, which is different from the glycerylsilane that is prepared under high temperature.

Characterization of of glycerylsilane derived sol-gel matrix morphology: Fig. 1 shows scanning electron microscopy image of the glycerylsilane derived sol-gel matrix, showing the nature of the silica skeleton. As shown in Fig. 2, the three-dimensional network appears to be composed primarily of silica beads that are 0.5-1 μm in diameter. The silica matrix results from the cross-linking of initially formed sol particles. The porous morphology is typical of silica matrix, which appears to be quite similar to that reported by Hodgson *et al.*[11].

Entrapment efficiency of glycerylsilane: The resulting glycerylsilane will be used to entrap enzymes, so its entrapment efficiency is important. The entrapment efficiency was evaluated in terms of trypsin loading yield. The entrapment is more efficient when trypsin loading yield is higher. The trypsin loading yield of tetraethyl orthosilicate and the resulting glycerylsilane was different, 55.3 % for tetraethyl orthosilicate-derived sol-gel glass and 66.9 % for glycerylsilane derived sol-gel glass, which means that the entrapment efficiency of the resulting glycerylsilane is higher than that of tetraethyl orthosilicate.

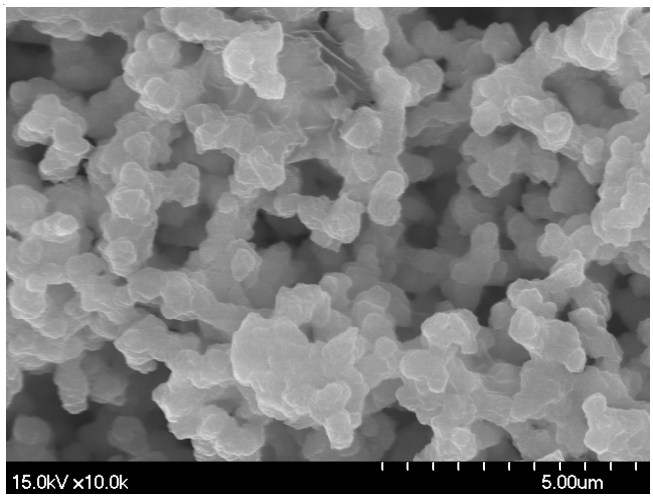

Fig. 1. SEM micrgraph of glycerylsilane derived sol-gel matrix

Biocompatible properties of glycerylsilane: Trypsin, a pancreatic serine protease, can undergo autolysis and lose its activity in free solution. The entrapment of trypsin can avoid those shortcomings. Trypsin was selected as a model enzyme to be entrapped to test the biocompatible properties of the resulting glycerylsilane.

pH is one of the most important parameters altering enzyme biological activity. As shown in Fig. 2, the maximum activity of free enzyme and entrapped enzyme was achieved at pH 7.8-8.0. The optimum pH range of entrapped trypsin was smaller than that of free enzyme, this may be caused by the electrostatic interactions[6]. There are electrostatic interactions between the enzyme and silica matrix because silanols in a silica matrix are negatively charged at pH 7-9, while trypsin has a positive charge (pI = 10.8). The electrostatic interactions will change the microenvironment of the enzyme, which will affect the optimum pH range of entrapped trypsin.

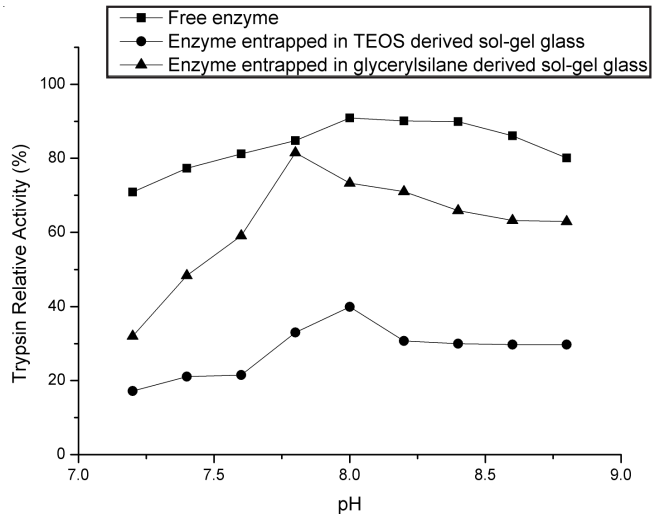

Fig. 2. Effect of pH on the enzyme activity

Temperature stability of entrapped enzyme was determined at different temperatures ranging from 30 to 86 ºC. As shown in Fig. 3, The optimum temperature range of free trypsin was 45-65 ºC, while that of entrapped trypsin was 37-75 ºC, the optimum temperature range of entrapped trypsin was wider than that of free trypsin. This is due to the protective effect of the immobilization matrix against thermal inactivation. The optimum temperature range of trypsin entrapped into glycerylsilane derived sol-gel glass is similar to trypsin entrapped into tetraethyl orthosilicate-derived sol-gel glass. However, the relative activity of trypsin entrapped into glycerylsilane derived sol-gel glass is higher than that of trypsin entrapped into tetraethyl orthosilicate-derived sol-gel glass.

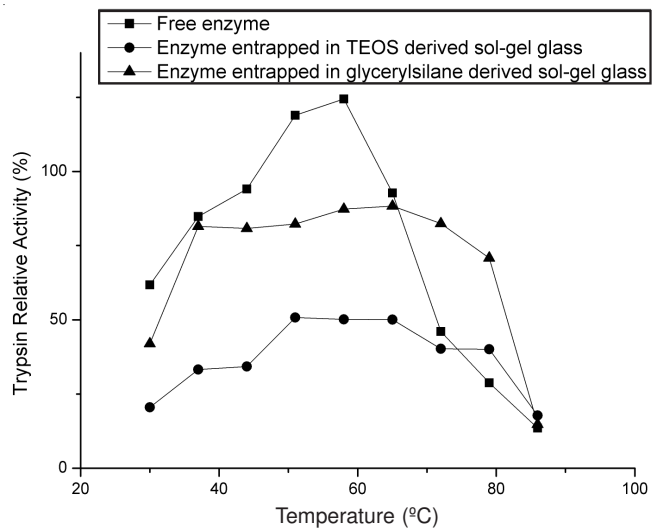

Fig. 3. Effect of temperature on the enzyme activity

The K_m values of free trypsin, entrapped trypsin into tetraethyl orthosilicate and glycerylsilane derived sol-gel glass were found to be 1.06, 6.19, 1.27, respectively. Fig. 4 shows the K_m value of entrapped trypsin was higher than that of free trypsin, which means entrapped trypsin had less affinity to substrate due to the presence of partitioning and diffusional effects in the pores of the sol-gel matrix.

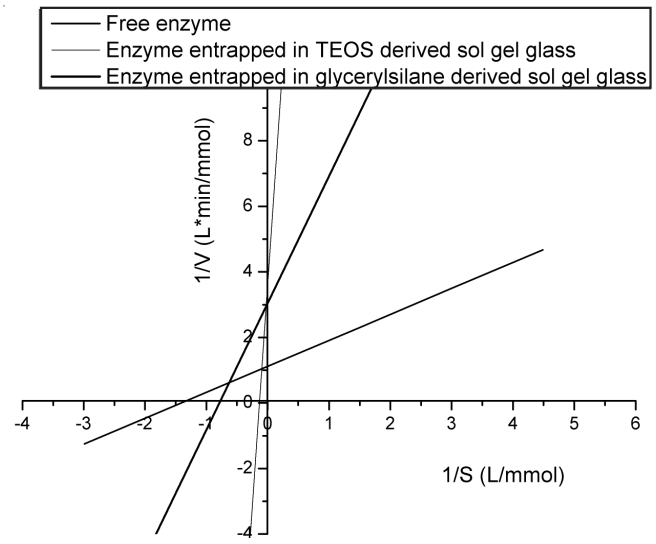

Fig. 4. Line-weaver Burk plots of free and entrapped enzyme

Fig. 5 shows the stability of free and entrapped trypsin at 4 ºC for long time. The activity of entrapped trypsin in glycerylsilane derived sol-gel glass remained more than 80 % after 11 days. However, the activity of free trypsin and

entrapped trypsin in tetraethyl orthosilicate-derived sol-gel glass decreased dramatically, the activity of free trypsin dropped from 84.8 % to 46.7 % and the entrapped trypsin in tetraethyl orthosilicate-derived sol-gel decreased from 33.1 % to 21.4 %. The dramatic decrease of free trypsin activity can be attributed to the fact that the free trypsin can undergo autolysis and lose its activity in free solution. The dramatic decrease of entrapped trypsin in tetraethyl orthosilicate-derived sol-gel activity may be due to the liberation of alcohol during hydrolysis, which is harmful to the enzyme activity. The results indicated that the resulting glycerylsilane was capable of maintaining entrapped trypsin in an active state for a significant amount of time.

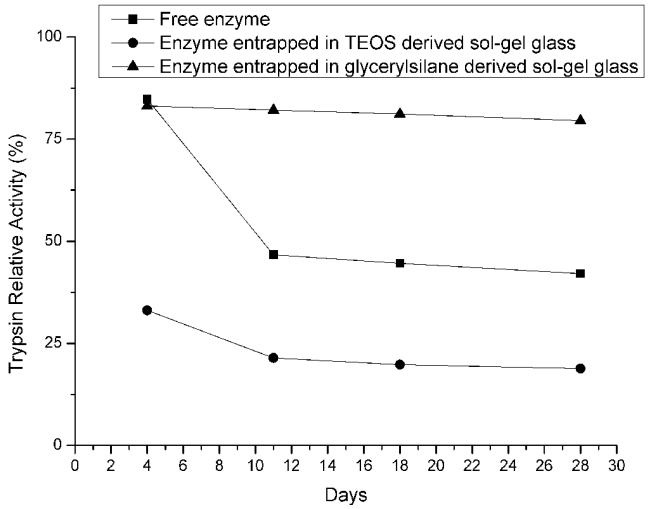

Fig. 5. Long-term stability of free and entrapped enzyme

The entrapped trypsin in glycerylsilane derived sol-gel matrix showed higher activity and better long-term stability than that of trypsin which entrapped in conventional sol-gel glass. This result indicated the glycerated silane had better biocompatible properties than that of conventional sol-gel glass. This may result from the biocompatible reagent, glycerol and glyceryl in the glycerylsilane, which is expected to play several important roles in mediating the behaviour of the silica monoliths, especially when they contain proteins. Glycerol has protein-friendly nature, as it is unstable to leave the gel, the glycerol/water syrup also plasticizes the gel to the benefit of entrapped proteins. Glycerol also can act as a drying control additive, which will reduce the shrinkage and cracking of silica monolith. These factors and differences in precursor hydrolysis, sol-gel glass structure and biomolecule-matrix interactions probably all contribute to the biocompatible properties of the glycerylsilane[8-10].

Conclusion

A new glycerylsilane was prepared with amberlyst 15 (dry) as catalyst. The reaction is performed under mild conditions. The product is not contaminated by catalyst, which is good to the following sol-gel process and activity of enzyme. The entrapment efficiency of the resulting glycerylsilane is higher than that of tetraethyl orthosilicate. The enzyme entrapped in glycerylsilane derived sol-gel glass showed better long-term stability than that of conventional sol-gel glass. The glycerylsilane has entrapment efficiency and good biocompatible properties, which provides a promising tool for development of biosensors, affinity supports and immobilized enzyme reactors.

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