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Stability and catalytic properties of encapsulated subtilisin in xerogels of alkoxisilanes

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ABSTRACT

Subtilisin was encapsulated in glass sol–gel matrices using alkoxysilane precursors of different chain lengths. The entrapment efficiency of the sol–gel glass was about 80%. The resultant glass enzyme had the same optimum pH of 7.0, but the optimum temperature was shifted to a higher temperature of 60 °C. The biocatalyst sol–gel particles retained 50% of the original activity even after 11 cycles of repeat use. The scanning electron micrograph of the immobilized enzyme showed uniform round particles of 5–20 μm. The specific surface area by BET measurement of the immobilized subtilisin in vinyl tri methoxy silane (VTMS) was found to be 38 $m^2 g^{-1}$. This immobilized enzyme was useful for the synthesis of peptides either in a mixture of acetonitrile: dimethyl formamide (DMF) or in 1-butyl 3-methyl imidazolium hexaflurophosphate, an ionic liquid. The formation of dipeptides and tripeptides of L-alanine was confirmed by TLC, HPLC and FT-IR analysis.

1. Introduction

Biocatalysis in non-aqueous media finds broad applications in organic fine chemical synthesis [1–5]. Proteases are becoming increasingly popular as catalysts for peptide bond formation. With respect to the stability of proteolytic enzymes in organic media, the serine proteases, mainly subtilisins are considered to be the most efficient enzymes. To improve the enzyme stability in organic media, various methods such as modification with polyethylene glycol [6,7], site directed mutagenesis [6,8,9], cross-linked enzyme crystals and aggregates [10], immobilization onto various carriers, etc. are there. Enzyme immobilization techniques usually provide, in addition to the desired reuse of the enzyme, unexcelled advantages such as product separation and continuous operation. For successful development and application of an immobilized biocatalyst, the enzyme support is generally considered as the most important component [11]. The incorporation of enzymes in silica matrices has proved to be a good strategy to improve the catalytic efficiency of the enzymes [12]. This can be accomplished by using the sol–gel technology [13,14]. Sol–gels are a new class of materials that have been found to be suitable for the immobilization of enzymes and other biological molecules. Sol–gel is a low temperature process that involves the hydrolysis and polycondensation of suitable precursors to form ceramic materials. They are non-toxic and biologically compatibles and do not swell in aqueous or organic solvents preventing leaching of entrapped biomolecule [15–18]. Compared to the other immobilization matrices, sol–gel have many advantages such as entrapment of large amount of enzymes, thermal and chemical stability, and simplicity of preparation without any covalent modification and flexibility of controlling pore size and geometry. Biomolecules entrapped in sol–gel matrixes typically exhibit improved resistance to thermal and chemical denaturation, and increased storage and operational stability. This technique has been applied to immobilize various biological molecules (proteins, enzymes, antibodies) using the alkoxide route with tetraethyl orthosilicate (TEOS) or tetramethyl orthosilicate (TMOS) as precursors [19]. Their initial hydrolysis in aqueous solution and further polycondensation brings about the formation of sol particles, which on cross-linking leads to the self-organization into a porous three-dimensional network in the bulk. The process allows for high yields of immobilized enzyme [20]. The rigidity of sol–gel polymer framework stabilizes the entrapped enzyme structure and prevents its leaching. The mesoporous structure and high pore volume of these gels allow the diffusion of low to medium molecular weight species into the gel and their free interaction with the enzyme [20,13]. Recently, room temperature ionic liquids have emerged as

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Abbreviations: VTMS, vinyl tri methoxy silane; MTMS, methyl tri methoxy silane; TEOS, tetra ethoxy silane; DMF, dimethyl formamide; IL, ionic liquid; $BIMMPF_6$, 1-butyl-3-methylimidazolium hexafluorophosphate; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; SEM, scanning electron microscope; FT-IR, Fourier transform infrared spectroscopy.

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an exciting alternative while choosing a non-aqueous medium for catalysis [21]. Ionic liquids are organic salts having no vapor pressure and melts below 100 °C and unlike traditional solvents; they are comprised entirely of ions [22]. The interest in ILs systems from their potential as green solvents because of their non-volatile character and thermal stability, which make them potentially attractive alternatives for volatile organic solvents. Here we are reporting the preparation, properties and use of the biocatalytic glass made by sol–gel process.

2. Experimental

2.1. Materials

2.1.1. Enzyme

Subtilisin [Amano, Japan], Vinyl tri methoxy silane (VTMS), methyl tri methoxy silane (MTMS) and tetra ethoxy silane (TEOS), Glutaraldehyde was procured from Sigma, St. Louis, USA. Hexafluro phosphoric acid, 1-methyl imidazole and chloro butane, L-Alanine, poly(vinyl alcohol) were obtained from S.D. Fine Chemicals (Mumbai, India). Acetonitrile, DMF and all the other reagents are of ultra pure analytical quality.

2.2. Extraction and purification of enzymes

5 g enzyme, subtilisin was dissolved in 50 ml phosphate buffer (7.0, 0.1 M) pH and stirred for 1 h at 30 °C. The supernatant was used as such after centrifugation.

2.3. Protease assay

Proteolytic activity of native and immobilized subtlisin was determined at 60 °C in 50 mM phosphate buffer, pH 7.0, using casein as substrate [23]. One unit of protease was equivalent to the amount of enzyme required to release 1 μmol of tyrosine/ml/min. Protein concentration was estimated as described by Lowry's et al. [24] using bovine serum albumin as standard.

2.4. Entrapment of subtilisin in sol–gel materials

The subtilisin was encapsulated in sol–gel matrices by modification of the method of Katsuya [25]. The silanes (1.14 g, 6 mmol) was added to a mixture of 400 μl Subtilisin, (103.26 mg protein/ml, Sp. activity 0.611 units/mg), 100 μl 1N aqueous sodium fluoride, 200 μl 4% (w/w) aqueous poly vinyl alcohol, (Mw 22,000D) and 164 μl distilled water. The mixture was vigorously stirred in a vortex mixer for 10 s, and then was shaken by hand for another 5 min. The sealed tube was kept at room temperature for 16 h. The material was air-dried at room temperature for 1–3 days and the xerogel was ground in a mortar. The powder was washed with distilled water, and was used as the biocatalyst.

2.5. Determination of optimum temperature, pH and kinetic parameters

Optimum temperature, pH, K_m and V_{max} were determined by changing each of the parameters by keeping all the other conditions constant and the protease activity was assayed. The activity of native and immobilized enzyme was studied in a temperature range of 20–90 °C at pH 7.0. The activity profile was studied in different pH range of 6.0–10.5 at 60 °C using 50 mM buffer (pH 6–8, phosphate buffer; pH 9–10.5, NaOH/glycine buffer). The kinetic studies were done by changing the substrate concentration from 0.25 to 2.5%. The thermo stability profile was studied by incubating the enzyme at various temperatures (50, 60 and 70 °C) for different durations and then the residual enzyme activity was determined.

2.6. Ionic liquid synthesis [$BIMMPF_6$]

1-Butyl 3-methylimidazolium chloride was prepared by reacting equal molar amounts of 1-methyl imidazole and chloro butane in a round bottomed flask, fitted with a reflux condenser, and heating at 70 °C under stirring for 48–72 h. The resulting viscous liquid was allowed to cool to room temperature and then was washed three times with 200 ml ethyl acetate and the traces of ethyl acetate were removed under vacuum at 70 °C. To prepare the ionic liquid, hexaflurophosphoric acid (1.3 M) was added slowly to a mixture of 1-butyl 3-methyl imidazolium chloride (1 M) in 500 ml of water. After stirring for 12 h, the upper acidic aqueous layer was decanted and the lower ionic liquid portion was washed with water to remove acidity. The ionic liquid was then heated under vacuum at 70 °C to remove any traces of water.

2.7. Use of entrapped enzyme for the synthesis of peptides

The enzymatic synthesis was carried out either by using ionic liquid or acetonitrile/DMF mixture. A solution of L-alanine (0.75 g) in ionic liquid (1.5 g) or Acetonitrile: DMF (760 μl) was cooled to 0 °C. It was vigorously stirred for 5 min for making it into slurry and then added sol gel immobilized enzyme (500 mg). The reaction mixture was allowed come to room temperature and then stirred for 24 h. The product was isolated from the reaction medium by first extracting with diethyl ether and evaporating the diethyl ether off. The product was then precipitated with methanol, filtered, and the filtrate was again extracted in ether. The ether was evaporated to get the product.

2.8. TLC analysis

TLC analyses were carried out on 0.25 mm thick silica gel GF 254 plates (Merck). The plates were air dried and activated at 120 °C for 1 h before use. Methanol was used as the developing solvent. The chromatograms were directly visualized by the spraying with 3% ninhydrin in *n*-butanol.

2.9. HPLC analyses of the peptides

The di and tri peptides were analyzed by reverse phase HPLC on a Lichrocart 250–4 mm ODS C18 column (Merck, Darmstadt, Germany) using a HPLC [model LC 10AD, Shimadzu, Japan] at room temperature (25 °C) equipped with dual pumps and a SPD-10A UV/vis detector. The mobile phase was methanol:water (70:30) with a flow rate of 1 ml/min. The samples were filtered through a 0.22 μm PTFE membrane (Millipore) before analysis and 20 μl was injected to the column. The eluting compounds were identified by a UV detector at 210 nm. The peaks were identified by the CSL Data processing software and the quantification was performed using external standards. The standard deviations of the analyses were less than 5%.

2.10. FT-IR

The chemical structures of the standard amino acid and newly formed peptides were characterized by FT-IR. The IR spectra were recorded on a PerkinElmer System 2000 FT-IR spectrophotometer (PerkinElmer Cetus Instruments, Norwalk, CT) with KBr pellets.

2.11. Microstructure studies by SEM

The morphological structures of the sol–gel immobilized enzymes were studied by scanning electron microscope (JSM-