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Silica–enzyme–ionic liquid composites for improved enzymatic activity

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ABSTRACT

Trypsin and pepsin enzyme-catalyzed precipitation of silica, synthesized by sol–gel chemistry in an ionic liquid, produces a composite material that demonstrates high enzymatic activity. This study investigates the structural properties of this silica–enzyme–ionic liquid composite material that allows for the retention of enzyme hydrolysis and condensation activity. The composite was prepared from a mixture of organo-functionalized triethoxysilane and tetraethoxysilane in an ionic liquid *via* enzyme-catalyzed direct hydrolysis and polycondensation reactions. The composite samples have been fully characterized with SEM, TEM, TG–DTA, IR, and N_2 isotherms, and incorporation of the composite's organic function has been demonstrated by ^{29}Si and ^{13}C nuclear magnetic resonance. The presented systematic approach provides valuable information on the influence of the enzyme and ionic liquid on the properties of the silica gel and nature of the silica network as well as on the extent of enzyme and ionic liquid encapsulation. SEM and TEM reveal that the silica–enzyme–ionic liquid composites prepared from trypsin are composed of an aggregate of closely packed spherical structures ~20 nm in diameter; however, the composites from pepsin consist of larger particles of ~1 μm having a smooth surface. In addition, after encapsulation within the silica–ionic liquid composite, enzymes showed extremely high hydrolysis and condensation activities using trimethylethoxysilane as an enzyme substrate, and these activities were better than those of corresponding free enzyme solutions. The simple route employed can yield materials with high enzymatic activities, and this may offer very promising application prospects.

1. Introduction

In recent years, it has become necessary to investigate the feasibility of more environmental friendly and green process routes for the synthesis of inorganic materials that make use of milder processing conditions. This necessity is largely due to increasingly stringent environmental regulations, environmental concerns, and interest in greater efficiency. Because these types of process routes can potentially reduce operating costs (such as handling and waste treatment) and make more efficient use of resources, process efficiency and, hence, economics can be improved [1–3].

A key point in the investigation of possible green process routes is in the application of biomineralization. In this field, systems of inorganic materials using biomimetic and biomineralization approaches have become an important area of research. Biomineralization synthesis is a potential route for the synthesis of inorganic materials under mild conditions, and numerous studies have already demonstrated the use of biotemplates in the synthesis of inorganic materials [4–9]. In the biological world, the organic matrix constituents (proteins, carbohydrates) are important in the synthesis and morphology of inorganic materials. One such extensively studied system is the biomineralization and biomimetic synthesis of highly ordered biosilica from synthetic precursors such as tetraethoxysilane (TEOS) and tetramethoxysilane (TMOS) using biomolecules as templates, which is a system that can offer precise control over the nanostructure of the obtained inorganic material [10–12].

Proteins are important in the synthesis of intricate silica structures in marine organisms. The most widely explored biomolecules are probably silaffins and long-chain polyamines isolated from diatom cell walls and silicateins isolated from marine sponges. These proteins are involved in the formation of the elegant silica structures observed in these organisms. Furthermore, the addition

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of silaffin and silicatein to a silicic acid precursor solution *in vitro* results in the formation of silica under ambient conditions [13–16]. On the other hand, synthetic polyamines and cationic poly($_L$-lysine) were able to catalyze silicic acid deposition [17–23]. For the former case, microphase separation between polyamines and anions was necessary to induce the formation of silica nanospheres. For the latter case, the inherent secondary structure of the polypeptide offered an additional parameter of the resulting structures. Generally, both systems required appropriate counter ions to promote biomimetic silica deposition under ambient conditions. In a previous study [24], we reported on the occurrence of an efficient charge relay effect of peptides on the dehydration reaction of trimethylsilanols (TMSs) when the alternately arranged basic (histidine) and acidic (aspartic acid) amino acid pairs are in the amino acid sequence of the peptide. Also, Wallace et al. [25] reported that an interface consisting of NH^{3+}/COO^{-} promoted silica mineralization more so than that having only NH^{3+} or COO^{-}. These results implied that the charge relay effect of basic and acidic amino acid pairs in silica mineralization is a very important factor for directing the onset of silica nucleation. In particular, we inferred that the secondary structures of synthetic peptides containing basic amino acids could be used to tune silica morphologies by demonstrating that peptides, self-assembled to α-helix and β-sheet conformations, functioned as templates for silica mineralization resulting in wrinkled-paper-like and nanotube silica structures, respectively [26]. Tomczak et al. [27] found that poly($_L$-lysine) of an α-helix conformation precipitated to form hexagonal silica platelets.

The silica structures formed by these means are composite materials in which the inorganic material forms around protein scaffolds that mediate the nucleation and precipitation of the insoluble material. The variability of morphologies derived by biomineralization reactions and the diversity of inorganic oxides that can be synthesized by these techniques has become a versatile tool for nanotechnology. Katagiri et al. [28] reported the development of enzyme-assisted synthesis of titania using a water-soluble titania complex and amorphous phase titania powders were synthesized. Rica and Matsui [29] demonstrated room-temperature synthesis of zinc oxide nanoshell structures using urease as a catalytic template. The enzymatic production of ammonia finely tunes the local pH at the enzyme template surface such that it is adequate for the growth of zinc oxide.

Subsequent studies have taken advantage of *in vitro* reactions for facile enzyme immobilization and encapsulation within silica matrices for numerous biotechnological applications. The extension of biomineralization to enzyme immobilization was derived from the observation that silica-precipitating species become entrapped during silica formation [30–34]. We described that urease-templated precipitation of silica synthesized by sol–gel chemistry produces a composite material allowing high urease activity [35]. The composite has a mesoporous structure composed of closely packed spherical structures ~20–50 nm in diameter. In addition, we have recently demonstrated the encapsulation of the enzymes trypsin and pepsin in a silica matrix during the direct hydrolysis and polycondensation of TEOS in the ionic liquid 1-butyl-3-methyl-imidazolium hexafluorophosphate ([bmim][PF_6]) [36]. The enzymes encapsulated in the silica–ionic liquid composites retained enzymatic activity higher than that of free solution enzymes. As an extension of this work, we simultaneously explored silica precipitation by direct enzyme-catalyzed reaction of silicon alkoxide having organo-substitution. In this report, we present the details of our investigation on the encapsulation of the hydrolytic enzymes trypsin and pepsin in a sol–gel silica matrix using enzyme-assisted direct condensation reactions of silicon oxide with organo-substitution of ethyltriethoxysilane (Et-TEOS) and phenyltriethoxysilane (Ph-TEOS) in the ionic liquid ([bmim][PF_6]). The resulting silica–enzyme–ionic liquid composites were thoroughly characterized in terms of morphology, size, and pore structure. In particular, incorporation of the organic components into the silica matrix was evaluated by solid-state (^{29}Si and ^{13}C) nuclear magnetic resonance (NMR). The activity and stability of the encapsulated enzymes were also assessed.

Room temperature ionic liquids are organic salts with a melting point below room temperature. Because of their unique properties, they have emerged recently as potential replacements for organic solvents in biocatalytic transformations [37–40]. Recently, ionic liquids have been used as solvents, reactants, and templates for the fabrication of inorganic materials [41–43]. To the best of our knowledge, the formation and characterization of silica–enzyme composites in an ionic liquid by direct enzyme-assisted reaction have not yet been reported.

2. Experimental

2.1. Chemicals

Trypsin (from bovine pancreas, Cat. No. T9201) and pepsin (from porcine gastric mucosa, Cat. No. P7000) were purchased from Sigma–Aldrich, St. Louis, MO. TEOS, Ph-TEOS, and Et-TEOS were obtained from Shin-Etsu Chemical Co., Ltd., Tokyo, Japan. The ionic liquid 1-butyl-3-methyl-imidazolium hexafluorophosphate ([bmim][PF_6]) was purchased from Kanto Chemical Co., Tokyo, Japan. All materials were of analytical grade and used as received without further purification.

2.2. Preparation of silica–enzyme–ionic liquid composites

Silica–enzyme–ionic liquid composite synthesis was carried out using trypsin or pepsin in [bmim][PF_6] containing a phosphate buffer as a co-solvent according to our recent report [36]. Trypsin or pepsin enzyme (30 mg) was added to a mixture of [bmim][PF_6] (1.5 mL) and 1 mM phosphate buffer of pH 6.0 (0.15 mL). The mixture of TEOS (1.8 mL) and organo-substituted silane (Et-TEOS or Ph-TEOS, 0.2 mL) was added, and the mixture was stirred for 3 days at 25 °C. On gel formation, the solid material was crushed in distilled water and the suspension was centrifuged to collect the silica–enzyme–ionic liquid composites. The silica gel was freeze-dried, and the obtained solid was refrigerated before use in activity measurements and material characterization.

2.3. Characterization of the composites

Particle morphology of silica–enzyme–ionic liquid composite materials was determined by scanning electron microscopy (SEM) using a Hitachi S-3000 (Hitachi, Ltd., Tokyo, Japan) field emission (FE)-SEM system with a 10 kV accelerating voltage for imaging. For transmission electron microscopy (TEM) studies, a small aliquot was taken from a suspension of methanol and placed in a lacey carbon-coated TEM grid that was pulled through the suspension and allowed to dry in air. The resulting sample was examined with a JEOL JEM 2010 microscope (JOEL Ltd., Tokyo, Japan) operated at 200 kV. The surface area, pore diameter, and pore volume were measured using nitrogen adsorption/desorption measurements in a Shimadzu TriStar 3000 system (Shimadzu Co., Kyoto, Japan). Pore diameter distributions were calculated from desorption branches by the Barrett–Joyner–Halenda (BJH) method. Specific surface area was calculated by the Brunauer–Emmett–Teller (BET) method based on desorption isotherms. Solid-state NMR was recorded on a Varian Unity Inova 300 NMR spectrometer (Varian Inc., Santa Clara, CA) equipped with Doty 7 mm (^{13}C nuclei) and Varian 7 mm (^{29}Si