Enzyme and Microbial Technology 105 (2017) 9–17

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Preparation and characterization of carbonic anhydrase-conjugated liposomes for catalytic synthesis of calcium carbonate particles

Keisuke Maeshima, Makoto Yoshimoto*

Department of Applied Molecular Bioscience, Yamaguchi University, Tokiwadai 2-16-1, Ube 755-8611, Japan

ARTICLE INFO

Keywords:
Liposomes
Carbonic anhydrase
Calcium carbonate
Carbon dioxide
Covalent conjugation

ABSTRACT

The biomimetic approach using immobilized enzymes is useful for the synthesis of structurally defined inorganic materials. In this work, carbonic anhydrase (CA) from bovine erythrocytes was covalently conjugated at 25 °C to the liposomes composed of 15 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(glutaryl) (NG-POPE), and the zwitterionic and anionic phospholipids with the same acyl chains as NG-POPE. For the conjugation, the carboxyl groups of liposomal NG-POPE were activated with 11 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4.6 mM *N*-hydroxysulfosuccinimide (sulfo-NHS). The carbonic anhydrase-conjugated liposomes (CALs) with the mean hydrodynamic diameter of 149 nm showed the esterase activity corresponding to on average 5.5×10^2 free CA molecules per liposome. On the other hand, the intrinsic fluorescence and absorbance measurements consistently revealed that on average 1.4×10^3 CA molecules were conjugated to a liposome, suggesting that the molecular orientation of enzyme affected its activity. The formation of calcium carbonate particles was significantly accelerated by the CALs ([lipid] = 50 μ M) in the 0.3 M Tris solution at 10–40 °C with dissolved CO_2 (≈17 mM) and $CaCl_2$ (46 mM). The anionic CALs were adsorbed with calcium as revealed with the ζ-potential measurements. The CAL system offered the calcium-rich colloidal interface where the bicarbonate ions were catalytically produced by the liposome-conjugated CA molecules. The CALs also functioned in the external loop airlift bubble column operated with a model flue gas (10 vol/vo% CO_2), yielding partly agglomerated calcium carbonate particles as observed with the scanning electron microscopy (SEM).

1. Introduction

A metalloenzyme carbonic anhydrase (CA) has been increasingly utilized for the processes involving the capture and sequestration of CO_2 [1,2] because the enzyme can catalyze the transformation of CO_2 into bicarbonate ions [3,4]. The CA molecule includes a cavity at the active site where the cofactor zinc(II) is coordinated by the three histidine residues as well as a water or hydroxide ion, which can realize highly efficient catalytic hydration of CO_2, as exemplary shown for human CA II [4,5]. Compared to the amine-based chemical absorption processes [6,7], the biomimetic CO_2 treatment on the basis of CA-catalyzed reaction would be advantageous in the sense that the latter system can function under relatively mild conditions in an aqueous solution. In this context, study has also been conducted for the preparation of immobilized CA to facilitate practical use of the enzyme [8–14]. The immobilized CA systems, which were fabricated by encapsulation [12], covalent coupling [8,10,11], adsorption [9] and intermolecular cross-linking [14], can improve stability and reusability of the enzyme or allow altering of its apparent catalytic efficiency. The synthesis of calcium carbonate particles on the basis of CA-catalyzed reaction is significant to sequestration CO_2 into functional materials. The calcium ions are consumed for the formation of calcium carbonate, where the rate of reaction should be influenced by the concentrations of both calcium and bicarbonate ions. The immobilized CA systems can potentially induce the solid-water interface with high local concentrations of these reactants. Furthermore, the use of CA-based catalytic systems integrated with gas-liquid contacting reactors would contribute to simplify the process for capturing gaseous CO_2 [15,16] with simultaneous synthesis of calcium carbonate particles [17].

Biomolecules such as polysaccharides, phospholipids, amino acids and proteins can modulate the *in vitro* synthesis of calcium carbonate particles [18–21]. Phospholipids forming liposomal structures can offer small aqueous compartment as well as charged lipidic interface. Liposomal systems were applied to control the formation or crystalline stability of calcium carbonate [22–25]. One of the characteristic features of liposomal systems is the electrostatic interaction of lipid

* Corresponding author.
E-mail address: yosimoto@yamaguchi-u.ac.jp (M. Yoshimoto).

http://dx.doi.org/10.1016/j.enzmictec.2017.06.002
Received 18 January 2017; Received in revised form 15 May 2017; Accepted 3 June 2017
Available online 14 June 2017

membranes with calcium ions, which can induce calcium-rich colloidal interface in an aqueous solution [24,26]. Covalent conjugation of the enzyme molecules to the surface of liposomes [27] contributed to stabilize the conformational structure and biological activity of the enzymes, as reported with respect to catalase [28] and D-amino acid oxidase [29]. The liposome-conjugated enzyme molecules can exhibit catalytic activity toward the substrates present in the bulk solution under marginal mass transfer resistance [29]. The liposomal systems are, therefore, potentially applicable to fabricating the catalytic interface where calcium and the CA molecules are present at high local concentrations. The CA molecules were encapsulated inside liposomes for analyzing the permeability of CO_2 or other synthetic substrates through lipid bilayers [30–32] and the kinetics of the liposomal reaction [33]. To the best of our knowledge, relatively little study has been carried out so far concerning the CA-catalyzed mineralization reaction occurring at the outer surface of liposomes.

In this work, we prepared the negatively charged liposomes that were covalently conjugated with the CA molecules. The liposomal catalysts were characterized to clarify the localization of biologically active enzymes and calcium ions on the surface of lipid membranes. Then, the liposomal CA systems were applied to the catalytic hydration of CO_2 with simultaneous formation of calcium carbonate particles in the presence of the liposome-adsorbed calcium. The mineralization reactions were performed using the CO_2-dissolved static liquid system or the external loop airlift bubble column operated with CO_2-containing gas phase.

2. Materials and methods

2.1. Materials

Zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, COATSOME MC-6081, lot 14096811FL, lot 15096811FL), anionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (POPG-Na, COATSOME MG-6081*LS*, lot 14066951L, lot 15066951L) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(glutaryl) (NG-POPE, COATSOME FE-6081GL, lot 10016961A5) were obtained from NOF (Tokyo, Japan). Carbonic anhydrase from bovine erythrocytes (CA, EC 4.2.1.1, Catalog number C3934, lot #SLBM2207V, lot #SLBQ5134V, lot #SLBR0889V), *N*-hydroxysulfosuccinimide (sulfo-NHS) and sepharose 4B gel beads were obtained from Sigma-Aldrich (St. Louis, MO, USA). The above CA is a mixture of isoforms. 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) was obtained from Dojindo Laboratories (Kumamoto, Japan). Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), *p*-nitrophenyl acetate (*p*-NA) and acetonitrile (99.8% <) were obtained from Wako Pure Chemical Industries (Osaka, Japan). The gaseous mixture of CO_2 and N_2 (1:9 vol/vol, lot JTC1415103) was obtained from Sumitomo Seika Chemicals (Osaka, Japan). All chemicals were used as obtained. Water used was deionized and sterilized with an instrument Elix 3UV from Millipore (Billerica, MA, USA).

2.2. Preparation of carbonic anhydrase-conjugated liposomes (CALs)

The carboxyl group-bearing liposomes for coupling with CA were prepared by the extrusion method as follows. The lipid composition of liposomes was POPC/POPG/NG-POPE (molar ratio 65:20:15). Each lipid was weighed and solubilized in a 100-mL round-bottom flask with 4.0 mL of chloroform, followed by removal of the solvent by a rotary evaporator. The total amount of lipids was 0.1 mmol. The lipids were dissolved in 4.0 mL of diethylether and the solvent was removed. This procedure was performed twice. The dry lipid film was vacuumed for 2 h in the dark with a freeze-dryer instrument for removing the residual organic solvent molecules. Then, the formation of multilamellar vesicles (MLVs) was induced by adding 2.0 mL of 5.0 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer solution (pH 5.5) followed by the freezing for 7 min in dry ice/ethanol and thawing for 7 min at 37 °C. This freezing-thawing cycle was repeated 7 times. The MLVs were repetitively passed through a polycarbonate membrane with nominal pore diameter of 200 nm using an instrument Liposofast with a stabilizer from Avestin (Ottawa, Canada) [34]. The concentration of POPC in a liposome suspension was determined with a series of enzymatic reactions catalyzed by phospholipase D, choline oxidase and horse radish peroxidase using an enzyme kit Phospholipid C Test Wako from Wako Pure Chemical Industries. The total concentration of lipids $C_{L,tot}$ was calculated with the known lipid composition based on the concentration of POPC measured. The water solutions containing 100 mM sulfo-NHS (90 μ L) and 250 mM EDC (90 μ L) were added to a liposome suspension (900 μ L, $C_{L,tot}$ = 10 mM) prepared as above, and the mixture was incubated for 10 min at 25 °C to activate the carboxyl groups of liposomes. Then, the 50 mM borate buffer solution (pH 7.4, 900 μ L) containing 4.0 mg/mL CA was mixed with the suspension of activated liposomes to give the total volume of 2.0 mL. Note that the CA solution with the above concentration was prepared on the basis of the commercially available CA powder. The purity of CA was determined spectrophotometrically on the basis of absorbance at 280 nm with the molar extinction coefficient ε_{280} of 56,000 $M^{-1}{\cdot}cm^{-1}$ [35]. The molecular mass of CA was taken as 29,000 [5]. The mixture was incubated for 3 h at 25 °C to induce the formation of covalent bonds between the primary amino groups within the CA molecules and the activated lipids in lipid membranes. The reaction mixture was subjected to the gel permeation chromatography (GPC) with a sepharose 4 B column (1 × 20 cm) in order to remove the free (unbound) CA molecules from the CALs. As eluent, 50 mM Tris-HCl buffer solution (pH 9.0) was used. Each 1.0-mL fraction collected was analyzed in terms of the concentration of POPC as well as the esterase activity of CA (see section 2.3). For the fraction containing CALs, the amount of CA was determined spectrophotometrically in the presence of 40 mM sodium cholate for solubilization of lipid membranes ($C_{L,tot}$ = 1.47 mM). The GPC was also performed with respect to the mixture of liposomes and CA without EDC/sulfo-NHS to clarify the physical adsorption of the enzyme molecules to the liposomes. The free CA solution with or without EDC/sulfo-NHS was also subjected to the GPC for assignment of the peak corresponding to the elution of the free CA molecules.

2.3. Measurements of esterase activity of CALs

The esterase activity of CALs was measured at 25 °C with *p*-NA as substrate [36] at $C_{L,tot}$ = 50 or 200 μ M. The CAL suspension was diluted with the 50 mM Tris buffer solution (pH 7.5) to give the total volume of 1485 μ L. To initiate the enzymatic reaction, the acetonitrile solution of *p*-NA (15 μ L, 100 mM) was added to the above CAL suspension to give the total volume of 1.5 mL and the initial concentration of *p*-NA of 1.0 mM. The reaction mixtures contained 1 vol/vol% acetonitrile. Note that the reaction pH of 7.5 was employed in order to decelerate the decomposition of *p*-NA in the absence of enzyme. The CAL suspension purified with the 50 mM Tris-HCl buffer solution of pH 9.0 was diluted 20–50 times with the 50 mM Tris-HCl buffer solution of pH 7.5. When the buffer solution of pH 9.0 was diluted more than 20 times with the buffer solution of pH 7.5, the change in the pH value of the latter solution was less than 0.1. The CAL-catalyzed formation of *p*-nitrophenol was continuously followed for up to 300 s on the basis of absorbance at 348 nm (ε_{348} = 5400 $M^{-1}{\cdot}cm^{-1}$ [37]) corresponding to the isosbestic point of *p*-nitrophenol and *p*-nitrophenolate ion [36,37] using a spectrophotometer UV-550 from JASCO (Tokyo, Japan) equipped with a Peltier-type temperature controller (EHC-477T, JASCO). The reaction was performed in a quartz cuvette with an optical path length of 1.0 cm. The enzyme activity was determined as the initial formation rate of *p*-nitrophenol subtracted by the rate observed in the absence of enzyme. The intrinsic activity of enzyme in the CALs was measured in the presence of 2.0 mM non-ionic detergent Triton X-100 which could completely solubilize the lipid membranes into the lipids/Triton X-100 mixed micelles. Triton X-100 showed an inhibitory effect