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A water-soluble precursor for efficient silica polymerization by silicateins

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ABSTRACT

Silicateins, the spicule-forming proteins from marine demosponges capable to polymerize silica, are popular objects of biomineralization studies due to their ability to form particles varied in shape and composition under physiological conditions. Despite the occurrence of the many approaches to nanomaterial synthesis using silicateins, biochemical properties of this protein family are poorly characterized. The main reason for this is that tetraethyl orthosilicate (TEOS), the commonly used silica acid precursor, is almost insoluble in water and thus is poorly available for the protein. To solve this problem, we synthesized new water-soluble silica precursor, tetra(glycerol)orthosilicate (TGS), and characterized biochemical properties of the silicatein A1 from marine sponge *Latrunculia oparinae*. Compared to TEOS, TGS ensured much greater activity of silicatein and was less toxic for the mammalian cell culture. We evaluated optimum conditions for the enzyme - pH range, temperature and TGS concentration. We concluded that TGS is a useful silica acid precursor that can be used for silica particles synthesis and *in vivo* applications.

1. Introduction

Silicateins are the major spicule-forming proteins from marine demosponges [1]. It is thought that substrates for silicateins in nature are water-soluble salts of silicic acid that present in seawater in small concentrations [2]. These salts are not convenient substance for the experiments *in vitro* due to their influence on pH of the solution and the tendency of precipitate (sol-gel) formation at high concentration at neutral pH. In previous works tetraethyl orthosilicate (TEOS) was used as the silicate ion source [3]. This substance is practically insoluble in water and requires an additional procedure of pre-hydrolyzation. Also, the usage of catechol complexes of silica was proposed [3]. It is more soluble in water, but additional formation of free catechol is the problem due to its high toxicity.

Poor availability of the precursor and weak activity of the protein with TEOS complicate the analysis of the silicatein activity. Since its discovery in 1998 [1] there is a little information about optimal conditions for the silicatein, and its enzymatic activity mechanism remains elusive [4]. Deeper understanding of the enzyme properties would intensify progress in silicatein application for the nanomaterial synthesis [5,6] and potentially in the other fields.

Recently we proposed the water-soluble tetrakis(2-hydroxyethyl) orthosilicate (THEOS), but toxic ethylene glycol is formed upon its hydrolysis [7]. In the present study we introduce a new glycerol-based water-soluble precursor and use it to determine basic biochemical properties of the silicatein A1 from marine sponge *Latrunculia oparinae*.

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2. Materials and methods

2.1. Protein purification and isolation

Coding region of silicatein A1 was PCR-amplified from *L. oparinae* cDNA (Forv-A1 5′-aaatagatctcaatcctatcctgagtccgtg, Rev-A1 5′-aaatctcgagaagagtagggtaggaggcatc, restriction sites are underlined) and cloned into pET40(b+) expression vector with C-terminal His-tag sequence using restriction enzymes BglII and XhoI. For protein expression *E. coli* strain BL21Codon+ was used. Expression was induced by 0.1 mM IPTG at 37 °C for 16 h. Cells were centrifuged, sonicated in 25 mM Tris-HCl pH 7.2, 150 mM NaCl with PMSF protease inhibitor (Thermo Fisher Scientific) and 5 mM DTT. Silicatein A1 was purified from the soluble fraction using Excel metal affinity resin (GE Healthcare Life Sciences), eluted with 25 mM Tris-HCl pH 7.2, 5 mM DTT, 200 mM imidazole, and stored at 4 °C up to 1 week. Prior to the analysis, the protein was transferred to assay buffer. Protein concentration was determined from optical density at 280 nm.

2.2. Substrate synthesis

Compounds THEOS and TGS were synthesized using typical procedure [8]. Tetraethoxysilane (10.4 g, 0.05 mol) was mixed with corresponding alcohol (0.2 mol) and cation exchange resin DOWEX 50 WX 8 (H^+ form, 100 mg). The mixture was stirred at 110 °C for 10–15 h (the liquid phase have to become homogeneous). The mixture was filtered, the residual ethanol was removed using rotary evaporator and the resulting colorless oil was used without further purification.

2.3. Silicatein activity measurement

TEOS was pre-hydrolysed with 50 mM HCl aqueous solution, THEOS and TGS solution were made in Tris-HCl buffer pH 7.2 directly before the experiment.

To evaluate silicatein activity 1 ml of the silica precursor solution was added to the 600 μl of the protein solution (0.06 mg/ml). After 1 h of incubation in the desired conditions samples were centrifuged (16000 g) for 10 min, washed 3 times with 96% ethanol and air dried. The amount of the polymerized silica was determined by the colorimetric molybdate assay.

2.4. Colorimetric molybdate assay

The assay is based on the method described in Brzezinski et al. [9]. Polymerized silica was dissolved in 200 μl 2M NaOH for 10 min, then 2M H_2SO_4 was added to adjust pH to 1.6–1.8. Sample volume was increased up to 1.46 ml and 135 μl 5% $(NH_4)_2MoO_4$ was added and reaction mixture was incubated for 15 min. If sample color turned to yellow, the sample was dissolved with mixture of the appropriate amount of water, 2M NaOH, 2M H_2SO_4 and 5% $(NH_4)_2MoO_4$ until the color disappeared. Then 135 μl of 10% tartaric acid and 135 μl 1% ascorbic acid were added and samples were incubated at room temperature for 2 h. Silica concentration was determined by the measurement of the optical density at 820 nm using calibration curve. The detection limit of the assay was 1 μM.

2.5. SEM XRF

A drop of sample solution was deposited onto freshly cleaved highly oriented pyrolytic graphite (ZYB quality, NT-MDT, Russia) surface for 1 min. After that, the remaining droplet was removed by a nitrogen flow.

Energy-dispersive X-ray spectroscopy (EDS) microanalysis was performed by SEM (Zeiss MERLIN, emission electron microscope with ESEM capability) using Silicon Drift Detector (SDD) X-MaxN 150 mm^2 (Oxford Instruments, United Kingdom) attached to SEM and AZtecEnergy EDS Software (Version 3.0) to collect backscattered images.

A working distance of 8.5 mm and an acceleration voltage of 15 keV were used to collect all backscattered electron SEM images and EDS data.

High resolution images were acquired using an InLens detector at 1–2 kV and 100–200 pA. Imaging parameters were adjusted so that imaging time did not exceed 1 min.

2.6. Toxicity analysis

Hela cells were grown in the 96-well plate in 100 μl DMEM with 10% FBS up to confluent layer formation. Cultural media were replaced with the precursor solution in DMEM with 10% FBS and 50 mM HEPES. After 24 h of incubation, cell viability assay was performed using alamarBlue™ Cell Viability Reagent (ThermoFisher Scientific, cat# DAL1025) in accordance with the manufacturer's instructions.

3. Results

3.1. Comparison of silica precursors

In this work we present a new glycerol-based silica precursor – tetra(glycerol)orthosilicate (TGS) (Fig. 1A). Synthesis of this substance was based on the well-known transesterification reaction of alkoxysilanes with alcohols in the presence of a strong acid [8]. After the heating of TEOS and glycerol in the presence of cation-exchange resin in H^+ form (Dowex) the homogenous mixture was obtained. This crude product contains the mixture of various glycerol esters (mainly the tetrakis(2,3-dihydroxypropyl) orthosilicate) that cannot be separated by common procedures. Nevertheless, in our work we showed that this crude product can be used without additional purification as a water-soluble substrate for silicateins.

THEOS and TGS were found to be much less stable at physiological conditions than TEOS (Fig. 1B). Both THEOS and TGS do not need pre-hydrolysis and can be directly dissolved in Tris-HCl buffer. However, we found that THEOS is too unstable, especially at high concentrations. At the same time, TGS demonstrated a good balance between the stability level and hydrolysis rate.

Then, we measured silicatein A1 activity with different precursors using reaction conditions similar to that previously described by Cha et al. [3]: 0.1% (w/w) precursor solution (corresponds to 5 mM for TEOS), 0.06 mg of silicatein in Tris-HCl pH 7.2, 1 h at room temperature in 1.5 ml reaction volume. We found that much greater amount of silica particles were formed with water soluble THEOS and TGS compared to TEOS (in fact, under these conditions, no detectable activity of silicatein A1 with TEOS was observed) (Fig. 1C). Parallel control experiments showed that the amount of self-polymerized silica without addition of silicatein was below the colorimetric assay detection limit.

To evaluate the toxicity of the precursors for their potential use in *in vivo* applications we incubated HeLa Kyoto cells for 24 h with the precursors at different concentrations in the cultural media. Compared to TEOS and THEOS, TGS demonstrated 5–10-fold lower toxicity showing no effect on the cell viability at concentrations up to 0.2% (Fig. 1D).

Then we performed scanning electron microscopy (SEM) with elemental X-ray fluorescence (XRF) analysis of the silica particles formed by silicatein A1 with different precursors (a higher protein concentration, 0.2 mg/ml, was used to generate particles with

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