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Kinetic investigations of 6-phosphogluconate dehydrogenase confined in mesoporous silica

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ABSTRACT

In this study 6-phospogluconate dehydrogenase (6PGDH) from *Geobacillus stearothermophilus* was adsorbed onto the inner surface of mesoporous cellular siliceous foams (MCF). To initiate different attractive interactions between the surface of 6PGDH and the respective silica hosts, the pore walls of the MCFs have been functionalized with alkyl and aminoalkyl residues consisting of different chain lengths ($-C_3$, $-C_5$, $-C_7$, $-C_{11}$ and $-C_3NH_2$, $-C_5NH_2$, $-C_7NH_2$, $-C_{11}NH_2$). Each modified MCF has been analyzed by nitrogen physisorption, thermal analysis as well as zeta potential titrations. Enzyme uptakes, loading densities, long-time stabilities, leaching as well as enzyme kinetics were investigated. So far kinetic investigations of enzymes immobilized onto mesoporous silica hosts are rare although kinetics give important information about diffusional resistance of substrate or coenzyme. In fact 6PGDH immobilization leads to an increase of the Michaelis-Menten constants (K_m) for the substrate and the coenzyme. The enzyme efficiency for 6PGDH immobilized on the surface of aminoalkyl functionalized MCF is larger than the value gained for the enzyme on alkyl functionalized materials.

1. Introduction

Enzymes are efficient biocatalysts but their application in organic syntheses is often expensive since it is difficult to separate them undenatured from the reaction batch. Therefore it is desirable to stabilize enzymes by immobilization on suitable host materials while retaining most of their activity [1,2]. Since researchers of *Mobil Oil* have fully characterized ordered mesoporous silica phases at first these materials have been established as adequate support materials for enzyme immobilization within the last decade [3,4]. Mesoporous silicas offer a lot of advantages compared to commercial available host materials (e.g. nylon beads, cellulose or agarose) since they are chemically and mechanically stable, easy to produce and reproduce and their tailorable pore sizes match the size of most enzymes [5]. During the immobilization the protein molecules are affected by surface interactions with the pore walls that can result in conformational changes of their tertiary structure [6]. This confinement can lead to higher stability of the biocatalyst even under harsh reaction conditions and protect the enzyme against shear forces [7]. In 1999 Stucky *et al.* published the synthesis of a novel silica foam ("Mesoporous Cellular Siliceous Foams", MCF) consisting of a disordered 3-D pore structure with cage-like pores (20–50 nm) interconnected by smaller windows (9–26 nm) [8]. Due to the pore structure and pore sizes in the upper mesopore range MCF is well suited to host large enzymes. Additionally surface modification using organosilanes can initiate hydrophobic, polar, electrostatic or covalent interactions between the protein surface and the surface of the silica support [4,9–11].

An effective application in biocatalysis requires a detailed investigation of the kinetics of the immobilized as well as the native enzyme, since enzymes offer their highest stability and activity under conditions that correlate with their natural environment [12]. However, the microenvironment of immobilized enzymes differs a lot from their natural environment and thus affects enzyme stability, activity and kinetics. On closer examination the kinetics of immobilized enzymes are more complex and influenced by additional factors, for instance diffusional restrictions of substrate or coenzyme molecules, compared to free enzymes [2]. However, up to now there are only a few reports dealing with Michaelis-Menten kinetics of enzymes immobilized onto mesoporous silica supports [13,14].

Zhao *et al.* investigated the immobilization of penicillin G acylase (PGA) onto functionalized SBA-15 either adsorptively or covalently. They observed a decrease of substrate affinity, indicated by a high K_m value that was assigned to the reduced mobility of the covalently immobilized PGA [13]. Sugunan and coworkers reported

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an enhanced approach of enzyme immobilization combining an adsorptive immobilization followed by cross-linking. The immobilization of β-glucosidase (β-GL) using this approach reduced the maximum velocity (v_{max}) about 10% which was attributed to the hindered flexibility of the cross-linked enzyme. Additionally, the substrate affinity (K_m) of the immobilized and cross-linked β-GL was 58% lower compared to the free enzyme due to diffusional restrictions [14].

In the current paper we present kinetic investigations of an enzyme confined in mesopores of MCF taking 6PGDH from Geobacillus stearothermophilus (*G.s.*) as a model enzyme. 6PGDH is the third enzyme of the oxidative part of the pentose phosphate pathway and catalyzes the oxidation of 6-phosphogluconate (6PG) to ribulose-5-phosphate (Ru5P) with concomitant reduction of β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH by release of one equivalent carbon dioxide (Scheme 1) [15]. Michaelis-Menten kinetics of the immobilized 6PGDH are meaningful since its reaction can be employed in cofactor regeneration [16,17].

In our study 6PGDH was adsorptively immobilized onto aminoalkyl and alkyl modified MCF and the influence of the aminoalkyl as well as the alkyl chain length on the immobilization and the enzyme performance/kinetics was examined.

2. Experimental

2.1. Chemicals

Pluronic® P123, 6-phosphogluconic acid trisodium salt (≥97%), ammonium fluoride, potassium phosphate monobasic, toluene, acetonitrile, ethanol (96%) palladium on activated charcoal (10% Pd basis) and Platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane complex (2.1–2.4% Pt in xylene, Karstedt's catalyst) were purchased from Sigma–Aldrich. Trimethylbenzene (TMB) and tetraethyl orthosilicate (TEOS) were purchased from Merck. Pentyltriethoxysilane (97%), *n*-propyltriethoxysilane (98%), *n*-propyltrimethoxysilane (98%), pentyltriethoxysilane (97%), (3–aminopropyl)-triethoxysilane (97%), 7-heptene (98%), 1-undecene (96%), triethoxysilane (97%), (11-bromoundecyl)-trimethoxysilane (95%), (7-bromoheptyl)-trimethoxysilane (95%) were produced by ABCR. Sodium azide was delivered from AppliChem. Bradford reagent and BSA standard were purchased from Fisher Scientific. β-Nicotinamide adenine dinucleotide phosphate disodium salt (≥97%) was received from Carl Roth Chemicals. All chemicals were used without further purification.

2.2. Synthesis of MCF

In a typical synthesis 9.6 g Pluronic® P123 was dissolved in 361 mL hydrochloric acid (1.5 mol L^{-1}) at room temperature and 16.7 mL (120 mmol) TMB as well as 111 mg (3.00 mmol) ammonium fluoride were added. The solution was stirred at 40 °C for 60 min. After dropwise addition of 22.7 mL (102 mmol) TEOS the solution was stirred further for 20 h at 40 °C followed by a hydrothermal treatment at 120 °C for 20 h. The obtained precipitate was separated, washed with distilled water and ethanol (96%) and dried at 60 °C. Calcination was carried out in air for 5 h at 550 °C (5 K min^{-1}) [18].

2.3. Alkyltriethoxysilanes and (5-bromopentyl)triethoxysilane

Heptyl-, undecyl- and (5-bromopentyl)-triethoxysilane were prepared as described elsewhere [19]. Under nitrogen atmosphere 30 mmol triethoxysilane, 30 mmol 1-undecene, 1-heptene or 5-brom-1-pentene as well as 35 mg Karstedt's catalyst and 1.2 mL absolute dichloromethane were mixed and stirred for 72 h at room temperature. After removal of the solvents under reduced pressure the crude product was dissolved in absolute ethanol and filtered over neutral aluminium oxide. Ethanol was removed in vacuo to obtain heptyl-, undecyl- and (5-bromopentyl)-triethoxysilane as colorless liquids (S1.).

2.4. Aminoalkyltriethoxysilanes

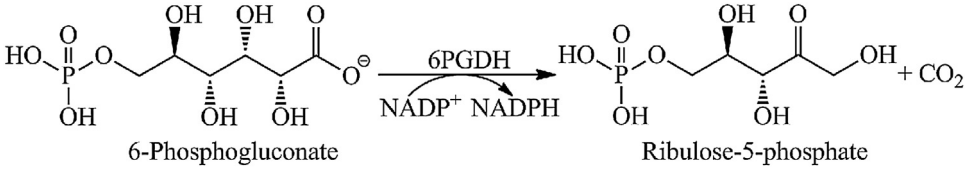
The aminoalkyl silanes (5-Aminopentyl)-, (7-aminoheptyl)- and (11-aminoundecyl)-triethoxysilane were synthesized according to the literature [20]. Under a nitrogen atmosphere 11 mmol of the respective (bromoalkyl)-tri(m)ethoxysilane was dissolved in 60 mL absolute acetonitrile. After addition of 39 mmol sodium azide the mixture was refluxed for 48 h. The solvent was removed under reduced pressure prior to addition of 120–350 mL cyclohexane. The occurring precipitate was separated from the liquid by filtration. 1.0–2.6 g Pd/C was added to the solution that was stirred under hydrogen atmosphere for 24 h at room temperature. After filtration and solvent removal the product was obtained as a colorless liquid (S1.).

2.5. Functionalization of MCF

To organically modify the surface of the host material 200 mg MCF was dispersed in 15 mL dry toluene and 1.5 mmol of the respective organosilane was added. The mixture was heated to reflux for 24 h prior to filtration of the solid. The modified silica was rinsed off with water as well as ethanol and dried at 60 °C [21].

2.6. Immobilization of 6PGDH

The immobilization of 6PGDH was carried out in potassium phosphate buffer (pH 6.5, 50 mmol L^{-1}). 5 mg of the respective modified MCF was dispersed in 1 mL potassium phosphate buffer containing 0.5 mg 6PGDH. The reaction mixture was shaken for 24 h at 25 °C and 350 rpm. After 5, 10, 15, 30 min as well as 1, 2, 3, 4, 5 and 24 h of immobilization 60 μL aliquots of the reaction mixture were removed and centrifuged for 2 min at 4000 rpm. Hereof 50 μL were sampled and kept for the determination of the enzyme concentration in order to follow the progress of immobilization. To remove non-immobilized and loosely attached enzyme molecules the MCF was resuspended in 1 mL potassium phosphate buffer (pH 6.5, 50 mmol L^{-1}) and recovered by centrifugation three times. The supernatants of all purification steps were kept in order to calculate the total amount of immobilized enzyme. All immobilized enzymes were stored at 4 °C in 1 mL potassium phosphate buffer.



6-Phosphogluconate Ribulose-5-phosphate

Scheme 1. Reaction sequence of the pentose phosphate pathway: 6PGDH-catalyzed oxidation of 6PG to Ru5P with concomitant reduction of $NADP^+$ [15].