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Entrapment of commercially important invertase in silica particles at physiological pH and the effect of pH and temperature on enzyme activity

Akhilesh Rai, Asmita Prabhune, Carole C. Perry *

Biomolecular Materials and Interface Research Team, School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK

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ABSTRACT

We report a simple and economic method to entrap invertase inside silica particles using a biosilicification process at physiological conditions. Larger silica particles (invertase-Si hybrid particles) were formed in the presence of invertase while smaller silica particles were observed in the absence of invertase. The invertase-Si hybrid particles were highly stable and active above the optimum conditions of pH (5) and temperature (50 °C) of the free invertase. The enhanced stability could be attributed to the protective nature and rigidity of silica particles that reduce the freedom of conformational changes of enzymes at higher pH and temperatures. The invertase-Si hybrid particles have an excellent reusability with a significant activity (76%) after 9 cycles of repeated use. This simple route to prepare invertase-Si hybrid particles with enhanced stability might have potential applications in food, beverage and confectionary industries.

1. Introduction

Synthesis of biocompatible materials is an area of considerable interest in bionanotechnology and biosensors with applications in the immobilization of DNA, proteins and enzymes [1–4]. Problems related to the immobilization of enzymes on solid matrices are a major concern to industries due to a significant loss of activity of the enzyme after immobilization. The choice of suitable materials is an important criterion for the immobilization of an enzyme with maintenance of its activity. Sol–gel materials and functionalized silica particles have been used widely for the immobilization of a variety of enzymes; however, the harsh conditions used in these methods cause the reduction in activity of the enzymes [5–7]. In nature, diatoms and sponges form controlled hierarchically complex silica structures in mild conditions using proteins called silaffins and silicatein respectively [8,9]. These proteins are entrapped inside the silica structures during formation under physiological conditions.

Immobilization of enzymes on support material is mostly carried out by covalent binding, adsorption and by encapsulation/entrapment [10]. The main drawback of methods using covalent binding and adsorption is that both processes may affect the active site and impose diffusion restrictions on the enzyme, thus leading to a reduction in activity [11,12]. A very weak interaction during adsorption of an enzyme also results in the leakage of enzyme whilst a very strong interaction causes changes in the conformation of the adsorbed enzyme [13,14]. In contrast, the entrapment/encapsulation process totally entraps enzyme molecules in a one-step procedure during the synthesis of materials without any reduction in the activity of the enzyme. The encapsulated materials also protect the enzyme from adverse conditions of reaction parameters such as pH and temperature. As an example, Stone's group has shown the entrapment of catalase, horseradish peroxidase and butylrycholinesterase enzymes using a silaffin-like polypeptide [15,16]. Zamora et al. have also shown the entrapment of chemically derivatized horseradish peroxidase in silica particles for amperometric sensing applications [17]. Mesoporous silica materials have been used to entrap glucose oxidase and lactose during the synthesis of materials [18,19].

Invertase is a highly efficient enzyme that converts sucrose into glucose and fructose, and has applications in the food and confectionary industries [20]. There are many reports on the immobilization of invertase on solid supports [21,22]; however, to the best of our knowledge, a single attempt has been made to entrap this commercially important enzyme using germania nanoparticles [23]. The entrapment of invertase from baker's yeast into germania nanospheres using a specific peptide sequence (TGHQSPGAYAAH) was demonstrated but this is an expensive process due to the high cost of the peptide [23].

In this study, we show an economical method to entrap invertase inside biocompatible silica particles during *in-situ* synthesis using hydantoin (glycolylurea) at ambient temperature and physiological pH without reducing its activity. Silicic acid species condense around invertase leading to the entrapment of the enzyme inside the synthesized silica particles. The optimum pH and temperature for invertase activity is higher for the entrapped invertase as compared to free invertase due to enhanced stability. This procedure might be useful to entrap other commercially important enzymes for their application

* Corresponding author. Tel.: +44 115 8486695.
E-mail address: carole.perry@ntu.ac.uk (C.C. Perry).

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over a wider range of pH and temperature than normally possible for the enzymes alone.

2. Experimental details

2.1. Materials

Sodium metasilicate nonahydrate, monosodium hydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), Trizma (tris), acetic acid and sodium acetate dihydrate were purchased from Sigma-Aldrich (Minneapolis, MN). Hydantoin and invertase from Baker's yeast (*Saccharomyces cerevisiae*) were purchased from Fluka. Distilled deionized water with a conductivity of ~1 μS was used for all experiments.

2.2. Preparation and characterization of silica particles with entrapped enzyme

Thirty millimolar sodium silicate precursor solution was adjusted to pH 7.2 (physiological condition) using HCl. The chosen silica precursor was readily hydrolyzed to monosilicic acid and it was used for the synthesis of silica particles in the presence of 30 mM hydantoin. Hydantoin was added immediately after the hydrolysis of sodium silicate solution, and at this stage the sample contains the highest concentration of silicic acid [24]. Similarly, the invertase enzyme (4 mg mL^{-1}) was added along with 30 mM hydantoin to the sodium silicate precursor solution already adjusted to pH 7.2. Both reactions were kept at room temperature for 12 h to precipitate the silica particles from their respective solutions. The precipitated silica particles were subjected to centrifugation at 3000 rpm for 15 min. The collected pellets were washed three times with water to remove unreacted silica precursors and free enzyme. The synthesized silica particles with and without invertase were frozen in liquid nitrogen and freeze dried at 223 K using a Christ alpha freeze dryer. It should be noted that silica particles synthesized in the presence of invertase (entrapped enzyme in silica particles) are described as "invertase-Si hybrid particles" for simplicity to explain the results throughout the paper. The entrapment of enzyme in silica particles was quantified using the Bradford method [25]. The silica particle solution was centrifuged followed by washing with phosphate buffer. The supernatant and washings were collected and were used for quantification of free invertase. Quantification of protein mass was performed by adding 0.8 mL of enzyme solution to 0.2 mL of Bradford reagent, mixing for 5 min and then monitored using UV–vis spectrophotometry at 595 nm. The entrapped amount was calculated by subtracting the amount of free invertase measured in the supernatants/washings from the original concentration.

The synthesis of silica particles and entrapment of invertase was analyzed by FTIR spectroscopy in transmission mode using a Nicolet Magna IR-750 spectrophotometer at 4 cm^{-1} resolution with 64 scans between 4000 and 400 cm^{-1}. Two milligrams of the dried powder (silica powder or invertase-Si hybrid particles) was mixed with 198 mg KBr and pelletized at 10 t pressure to make discs.

Silica powder and invertase-Si hybrid particles were mounted on aluminum stubs with double-sided adhesive carbon tape and coated with palladium/gold before analyzing using a JEOL JSM 7400F FE scanning electron microscope operated at an accelerating voltage of 20 kV.

Time dependent growth of silica particles in the presence and absence of invertase was measured by dynamic light scattering (DLS) using a Malvern Instrument (model Nano-S).

2.3. Measurement of activity of immobilized invertase

An assay of enzyme activity was performed using the method described by Gascon and Lampen [26]. In general, a defined amount of the free enzyme or invertase-Si hybrid particles was taken and added to a solution of 2% sucrose in 50 mM acetate buffer (pH 4.5) followed by incubation at 40 °C for 10 min in a water bath. The reaction was stopped by adding 1 M phosphate buffer. After centrifugation, the supernatant was removed and used for sugar estimation using the DNSA (dinitro salicylic acid) method. The amount of reduced sugar was estimated by measuring absorbance at 540 nm using an UV–visible spectrophotometer. The same procedure was used to estimate the activity of the free enzyme.

2.4. pH profile

A measured amount of invertase-Si hybrid particles and free invertase was added to 0.5 mL of various buffers (50 mM) of pH from 4.0 to 10.0 and incubated for 10 min at 40 °C in the presence of 2% sucrose. The enzyme assay was performed as described above for each aliquot. Buffers used were acetate buffer (pH 4.0 and pH 5.0), phosphate buffer (pH 6.0 to pH 8.0) and tris buffer (pH 9.0 to 10).

2.5. pH stability

Invertase-Si hybrid particles and free invertase were incubated in various buffers (from pH 4.0 to pH 10.0) as described above followed by incubation for an hour at room temperature. Aliquots were removed after an hour and invertase activity of each aliquot was assayed under standard assay conditions.

2.6. Temperature profile

A defined amount of invertase-Si hybrid particles and an equivalent amount of free invertase were taken in 0.5 mL of acetate buffer (50 mM, pH 4.5) followed by incubation at different temperatures ranging from 30 °C to 80 °C in presence of 2% sucrose. The enzyme assay was performed essentially as described above.

2.7. Temperature stability

To investigate the effect of temperature on stability, the invertase-Si hybrid in acetate buffer (50 mM, pH 4.5) was incubated for an hour at different temperatures ranging from 30 to 80 °C. Aliquots were removed and invertase activity was checked under standard assay conditions.

2.8. Reusability study

A defined amount of invertase-Si hybrid particles were taken in acetate buffer (pH 4.5) in the presence of 2% sucrose at 60 °C for 30 min. After the reaction, particles were recovered from solution using centrifugation and washed twice with buffer prior to re-use.

3. Results and discussion

3.1. Synthesis and characterization of silica particles

The main goal of this study was to entrap invertase enzyme without reduction of its activity inside the silica particles, which were synthesized using an elegant approach under ambient conditions of temperature and pH. Fig. 1A shows the amorphous and aggregated silica particles of size ca. 100 nm formed via a traditional sol–gel mechanism after 12 h of reaction at room temperature. No precipitation of silica particles was observed during the initial period of reaction (0–3 h); however the turbidity of solution started after 1 h of reaction, indicating the formation of very small silica particles in solution (curve 1, Fig. 1C). To entrap invertase inside silica particles, invertase was added during the initial period of reaction between the silica precursor and hydantoin (glycolylurea) at physiological pH