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Short communication

Sequential conversion by catalytically active MIP and immobilized tyrosinase in a thermistor

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Abstract

To amplify the heat-signal generated by MIP catalyzed solvolysis of phenylacetate the reaction has been combined for the first time in a reactor with the subsequent oxidation by immobilized tyrosinase. The polymer cleaves the substrate and the released phenol is afterwards converted to *o*-benzoquinone by the tyrosinase. The separated and sequentially coupled reactions are characterized by the heat generated in a thermistor. The sequential substrate conversion results in a combined heat generation which results a five times higher signal than compared to the polymer alone.

Keywords: Catalysts; Enzymes; Molecularly imprinted polymers (MIPs); Thermal methods

1. Introduction

High chemical selectivity and catalytic power are properties of biological catalysts based either on proteins or nucleic acids. To complement these recognition elements generated by evolution Wulff, Shea and Mosbach have realized binders and catalysts on the basis of totally synthetic molecularly imprinted polymers (MIPs) (Wulff et al., 1977; Shea et al., 1980; Arshady and Mosbach, 1981) which promise both high chemical and thermal stability and low costs. Despite the potential of MIPs to substitute biological recognition elements in affinity chromatography, biosensors and biochips the widespread applications have yet to come (Piletsky and Turner, 2002; Dickert et al., 2003a).

A barrier for routine application is the signal transduction of the analyte binding to the MIP. Even though all marker types of immunoassays may be applied to the MIP–analyte interactions, direct (label-free) transducers are very promising (Surugiu et al., 2000; Sellergren and Andersson, 2000; Haupt et al., 1998; Ansell and Mosbach, 1998; Lai and Fafara, 1998). In this respect MIP covered quartz crystal micro balances (QCM) as well as surface plasmon resonance sensors have been successfully applied for the recognition of low molecular substances, and so have proteins, viruses and living cells (Kobayashi et al., 2001; Dickert et al., 2003b; Kugimiya and Takeuchi, 2001).

Another possibility for label-free detection is the use of calorimeters. In enzyme technology the enzyme thermistor is widely used for this purpose since enzymatic reactions are generally accompanied by enthalpy changes in the range of 5–100 kJ/mol (Mosbach and Danielsson, 1981; Danielsson et al., 1981; Satoh, 1989). Recently we have shown that binding as well as catalysis of a MIP can be characterized in one run with a thermistor (Lettau et al., 2006).

In this paper we extend this concept to the sequential conversion of phenylacetate by combining a catalytically active MIP with immobilized tyrosinase. This example demonstrates the possibility to couple biomimetic and enzymatic reactions, to establish a reaction sequence.

2. Materials and methods

2.1. Reagents

Phenol, phenylacetate, and methanol were purchased from VWR (Darmstadt, Germany). Glutaraldehyde and tyrosinase from mushrooms were purchased from Sigma–Aldrich

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(Taufkirchen, Germany). All reagents were used as received.

2.2. Polymer preparation

Polymers were prepared with the use of a modified protocol described earlier by Yilmaz et al. (Yilmaz et al., 2000). Therefore the modification of the silica particles was done within a closed filtration device containing pure nitrogen as inert gas.

For the template-modified silica, 1 g of aminopropyl-silica (pores ~9 nm, particle size 35–70 μm, 0.9 mmol of NH_2 groups/g, pore volume 0.9 ml/g) was suspended in 10 ml of toluene, 6 ml (50 mmol) hexamethylene diisocyanate were added and stirred for 6 h at room temperature. The hexamethylene isocyanate modified silica was washed with 250 ml dichloromethane and resuspended in 10 ml toluene, mixed with 0.25 mmol (4-aminobenzyl)-phosphoramidic acid-(4-nitrophenylester) (template) and stirred for 6 h at room temperature for coupling of the template. Two milliliters of methanol was added to block dither remaining isocyanate, stirred over night and finally washed with 50 ml *N*,*N*′-dimethylformamide, dichlormethane and methanol each before drying at 60 °C for 2 h. The control polymer was prepared with the same procedure except of the template addition.

A pre-polymerization mixture was prepared by dissolving 94 mg (1 mmol) 4-vinylimidazole and 30 mg (0.18 mmol) α,α′-azoisobutyronitrile in 2 ml (14 mmol) of divinylbenzene and 0.2 ml of methanol. This solution was purged with argon for 10 min and 0.5 ml were added to 1 g of template-modified silica (TSA-silica) or blank-silica (control-silica). By carefully mixing the pre-polymerization mixture with the modified silica, the pre-polymerization mixture was completely absorbed by the silica. These monomer/silica mixtures were again purged with argon for 10 min and then kept at 4 °C for 19 h. After this polymerization took place at 40 °C for 48 h. The composites were treated afterwards at 80 °C for 12 h for final curing of the polymer. To remove the silica the composite materials were suspended in 3 M ammonium hydrogen difluoride over night (Titirici et al., 2003). The resulting imprinted particles were washed on a filter with 2 l ethanol (20% v:v in water) on filter paper until the pH in the effluent was between 6 and 7. Finally the polymer was dried for 24 h at 60 °C.

2.3. Enzyme immobilization

Five milliliters glutaraldehyde (0.5 M glutaraldehyde in 0.1 mM phosphate buffer (Sörensen, pH 6.8)) was coupled to 250 mg aminopropyl-modified porous glass (CPG) (Fluka, Taufkirchen, Germany) at room temperature for 2 h. To remove unreacted glutaraldehyde after incubation the particles were washed six times with 5 ml 0.1 mM phosphate buffer (Sörensen, pH 6.8), alternating with periods of 10 min on a shaker with buffer. Simultaneously 4.6 mg tyrosinase (E.C. 1.14.18.1) is dissolved in 1.5 ml 0.1 mM phosphate buffer (Sörensen, pH 6.8) at 4 °C. After centrifugation the supernatant of the enzyme solution is given to the wet solid support and the coupling is left to proceed for 3 h at room temperature. Finally the residual unbound enzyme is removed by washing with 25 ml 0.1 mM phosphate buffer (Sörensen, pH 6.8). The solid support with the immobilized enzyme is stored in 0.1 mM phosphate buffer (Sörensen, pH 6.8) at 4 °C until usage.

2.4. Thermistor measurements

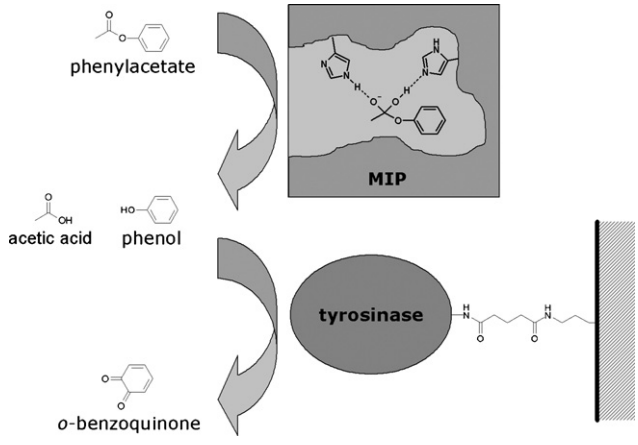
Measurements were made with a flow-through calorimeter developed at the University of Lund—an enzyme thermistor (Mosbach and Danielsson, 1981). For these measurements a 500 μl reactor was filled with 50 mg immobilized tyrosinase and 50 mg of the catalytically active MIP or the control polymer separated by a membrane. Measurements took place at a constant temperature of 30 °C and a flow rate of 1.5 ml/min. Running and sample buffer were 20 mM sodium–phosphate buffer (pH 7.5) containing 10% methanol. Samples were dissolved in this buffer directly before the measurements. As reference for the generated heat a second empty reactor was used.

3. Results and discussion

To establish the sequential substrate conversion, we have chosen the following reaction scheme: first the model substance phenylacetate is hydrolyzed to acetic acid and phenol by the catalytically active polymer (Lettau et al., 2006). The released phenol is afterwards oxidized to *o*-benzoquinone by immobilized tyrosinase under dioxygen consumption. (Scheme 1)

3.1. Phenol conversion

To verify the thermal signal of the subsequent reaction 50 mg tyrosinase was immobilized on porous glass particles via a glutaraldehyde coupling, placed in the reactor of the thermistor and exposed to different phenol concentrations at a flow rate of 1.5 ml/min. The composition of the buffer, i.e. the pH and the additional methanol were optimal for the activity of the polymer but did not coincide with the physiological conditions for the tyrosinase (Lettau et al., 2004). The measurement started with the flow of substrate for 10 min followed by a buffer flow for



Scheme 1. Reaction scheme of the sequential conversion of phenylacetate via phenol to *o*-benzoquinone.