Journal of Molecular Catalysis B: Enzymatic 110 (2014) 165–170

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Recovery of chymotrypsin using magnetic particles and aqueous micellar two-phase systems: Influence of non-ionic surfactants on enzyme activity

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ARTICLE INFO

Article history:
Received 9 July 2014
Received in revised form 29 August 2014
Accepted 9 October 2014
Available online 18 October 2014

Keywords:
Magnetic bead immobilized enzyme
Biotransformation
Aqueous micellar two-phase systems
Non-ionic surfactant
Enzymatic activity

ABSTRACT

The objective of this work was a proof-of-concept for the use of the immobilized bovine α-chymotrypsin enzyme in a biotransformation process applying an aqueous micellar two-phase system (AMTPS) for separation and recycling of the enzyme immobilisates. Commercially available magnetic polyvinyl alcohol microparticles serve as carriers for the covalent immobilization of the enzyme. For the AMTPS non-ionic surfactants are used, creating a temperature controlled two-phase system which allows for an easy separation of the immobilized enzyme from the reaction medium. We describe the influence of four non-ionic surfactants (Tween 20, Triton X 114, Triton X 100, and Eumulgin ES) on the activity of the immobilized α-chymotrypsin and the optimization of the AMTPS composition via a Design of Experiments approach. Additionally, we demonstrate the reuse of the immobilisates for hemoglobin digestion over eleven cycles in an Eumulgin ES AMTPS. Our findings show that continuous enzymatic conversion processes can be implemented effectively with the help of carrier bound enzymes in surfactant systems.

1. Introduction

Magnetic microparticles have several striking advantages over both non-magnetic particles and particle-free systems. The magnetic property allows for an easy separation, while the large surface to volume ratio offers a high capacity for surface binding of molecules [1]. When magnetic microparticles are used as carriers for an enzyme, the immobilized enzyme can be used many times, because it can be separated easily from the suspension. This also means that the enzymatic conversion can be controlled better [2]. The stability of the enzyme usually is improved by immobilization [3,4], e.g. regarding temperature, pH (organic) solvents or degradation and autolysis over time. Additionally, the product is not contaminated with the enzyme and in many cases does not have to be purified. Finally, continuous processing of magnetic microparticles is possible via Continuous Magnetic Extraction (CME), as will be described below.

CME is a newly developed process which has been applied successfully for protein purification [5,6]. In case of protein purification, use of magnetic (micro-) adsorbents is combined with thermally switchable aqueous two-phase systems. In a first step the microadsorbents are mixed with the feedstock, resulting in a fast binding of the target protein. In a second step a thermoresponsive surfactant is added. If the temperature of the mixture is raised beyond the Lower Critical Solution Temperature (LCST), a two-phase system is formed and the target-loaded microadsorbents partition into the surfactant-rich top phase. In combination with continuously operating, magnetically enhanced phase separation equipment, the fast binding kinetics of microadsorbents allows for a continuous processing of the feedstock.

In order to establish a CME process for use in biotransformation and subsequent recovery of enzyme immobilisates, the effects of the phase-forming surfactant on enzyme activity have to be assessed first. There are many reports about the influence of surfactants on enzyme activity [7–13] in terms of activity increase, inactivation, hyperactivation, and underlying mechanisms. Rubingh et al. [8] describe attachment to the enzyme, alteration of the secondary or tertiary structure, or a change in the reaction environment as possible ways for surfactants to influence enzyme activity. Savelli et al. [9] emphasize the role of charged head groups and alkyl chains for the properties of denaturing agents, while non-ionic surfactants are described to be usually not denaturing. They encountered hyperactivation of α-chymotrypsin in the presence of cationic surfactants [14,15]. Shome et al. [10] report an increase

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http://dx.doi.org/10.1016/j.molcatb.2014.10.005
1381-1177/

Nomenclature

Symbols

A_{free}	activity of free enzyme, U/mg
A_{immo}	activity of immobilisate, U/g
A_{rel}	relative activity of immobilisate, (U mg)/(g l)
c_E	enzyme concentration, mg/ml
c_P	particle concentration, mg/ml
c_{4N}	concentration of 4-nitroaniline, mg/ml
E	extinction, –
L	enzyme loading of immobilisates, mg/g
t	time, s
V_E	volume of enzyme solution, ml
V_P	volume of particle suspension, ml
Y_A	activity yield, %

Abbreviations

CME	Continuous Magnetic Extraction
DOE	Design of Experiments
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
LCST	Lower Critical Solution Temperature

in both activity and stability of lipase in cationic reversed micelles in the presence of non-ionic surfactants (Tween 20). Viparelli and coworkers [11] observed a decrease in α-chymotrypsin activity in the presence of the non-ionic surfactant Triton X 100. A detailed study of the kind of inhibition (competitive–non-competitive, reversible–irreversible) of the activity of α-chymotrypsin by surfactants is given by Schomaecker [13].

In this work α-chymotrypsin was used as a model enzyme for biotransformation purposes which was immobilized successfully on magnetic supports in the past [16,17]. We studied the influence of phase-forming surfactants on enzyme activity and demonstrated multiple reuses of the enzyme immobilisates using a permanent magnet setup for their recovery. The surfactants were found to have an influence on the applied analysis, an effect which must be taken into account in order to correctly interpret the experimental findings.

2. Experimental

2.1. Enzyme

As a model enzyme, α-chymotrypsin from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA) was used. α-chymotrypsin is a proteolytic enzyme with an optimum activity at about 37 °C [18] and pH 8 [19]. The enzyme was dissolved at a concentration of 10 g/l in 1 mM HCl solution (pH 3) in order to minimize auto-digestion during handling. This enzyme stock solution was diluted with substrate or surfactant solution as needed prior to use.

2.2. Particles

The particles used in all trials were M-PVA C22, highly-carboxylated superparamagnetic particles, of about 2 μm in size (PerkinElmer chemagen Technologie GmbH, Baesweiler, Germany; batch No. C22-0111071). They are easily separable by simple permanent magnets and redisperse readily when the magnetic field is removed.

2.3. Immobilization procedure

Covalent immobilization of alpha-chymotrypsin on M-PVA C22 beads was accomplished via the widely used method [20] of coupling with EDC (1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide hydrochloride, Merck, Germany). A short description of the procedure is given in the following sections, detailed information can be found in [21]. The particles were first activated with a 10 g/l EDC solution in 0.1 M MES buffer (pH 5.3) for 35 min at 11 °C on an Eppendorf shaker. After two times washing of the particles with MES buffer, the enzyme solution (pH 7.1) was added to the particles and the mixture was again incubated on a shaker for 150 min at 25 °C. Finally, the particles were washed five times with 0.1 M Tris buffer (pH 9) containing 0.02 M $CaCl_2$ and stored in 1 mM HCl buffer at 4 °C until use. The immobilisates reached a loading of approx. 25 mg/g (used in scale-up trial) and approx. 40 mg enzyme/g particle (applied in DOE, validation, and storage trials).

2.4. Surfactants

When using thermoresponsive surfactants, a temperature increase of an aqueous surfactant mixture will result in the formation of a two-phase system. This process is reversible and can be repeated indefinitely. Four thermoresponsive surfactants exhibiting a LCST were used: Eumulgin ES (LCST 25 °C), Triton X-100 (LCST 65 °C), Triton X-114 (LCST 23 °C), and Tween 20 (LCST 95 °C). While LCSTs higher than approx. 45 °C are not suitable for the used model enzyme, they might be interesting in combination with enzymes from thermophilic bacteria, which is why they are included in the screening. All surfactants applied are non-ionic, which means that they are least denaturing and most suitable for use with proteins [8].

2.5. Enzyme activity determination

If not stated otherwise, samples were analyzed in triplicate. A total volume of 200 μl was used for activity determination. The activity determination was done in 96 well microplates using an automated liquid handling station with an integrated monochromatic plate reader (PerkinElmer, USA). The substrate n-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide was used, since the conversion product p-nitroaniline is easily detectable with UV-/Vis-spectroscopy. The specific activity A_{free} of the free enzyme (based on the mass of enzyme) was determined under standard conditions (0.1 M Tris buffer at pH 8 and 38 °C, without surfactant) using the following equation:

$$A_{free} = \frac{\Delta E * V_E}{\Delta t * m_E * \varepsilon_{410\,nm} * d} \quad (1)$$

with the total volume V, the extinction difference ΔE over the time interval Δt, the path length d, the extinction coefficient $\varepsilon_{410\,nm}$, and the mass of enzyme m_E. Furthermore, the activity of the immobilized enzyme A_{immo} was determined (based on the mass of particles):

$$A_{immo} = \frac{\Delta E * V_P}{\Delta t * m_P * \varepsilon_{410\,nm} * d} \quad (2)$$

Here, m_P is the particle mass in the trial. Samples of the incubation solution containing the enzyme (free or immobilized) and substrate were taken 1, 2, 3, and 5 min after the start of incubation. The conversion was terminated by a 1:4 dilution of the sample with 11.5% acetic acid, with the decrease in pH minimizing the enzymes' catalytic activity. Prior to absorption measurements, the magnetic particles suspended in the samples with immobilized enzyme were removed using a 96 well magnetic plate (Alpaqua, USA).