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Design, characterisation and application of alginate-based encapsulated pig liver esterase

Jan Pauly[a,b], Harald Gröger[b], Anant V. Patel[a,*]

[a] Fermentation and Formulation of Biologicals and Chemicals, Faculty of Engineering and Mathematics, Bielefeld University of Applied Sciences, Interaktion 1, 33619, Bielefeld, Germany

[b] Chair of Organic Chemistry I, Faculty of Chemistry, Bielefeld University, Universitätsstrasse 25, 33615, Bielefeld, Germany

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ABSTRACT

Encapsulation of hydrolases in biopolymer-based hydrogels often suffers from low activities and encapsulation efficiencies along with high leaching and unsatisfactory recycling properties. Exemplified for the encapsulation of pig liver esterase the coating of alginate and chitosan beads have been studied by creating various biopolymer hydrogel beads. Enzyme activity and encapsulation efficiency were notably enhanced by chitosan coating of alginate beads while leaching remained nearly unchanged. This was caused by the enzymatic reaction acidifying the matrix, which increased enzyme retention through enhanced electrostatic enzyme-alginate interaction but decreased activity through enzyme deactivation. A practical and ready-to-use method for visualising pH in beads during reaction by co-encapsulation of a conventional pH indicator was also found. Our method proves that pH control inside the beads can only be realised by buffering. The resulting beads provided a specific activity of 0.267 µmol · min^{-1} · mg^{-1}, effectiveness factor 0.88, encapsulation efficiency of 88%, 5% leaching and good recycling properties. This work will contribute towards better understanding and application of encapsulated hydrolases for enzymatic syntheses.

1. Introduction

In organic syntheses, enzymes play a vital role as regio- and stereoselective catalysts and act as efficient catalysts for a broad range of processes on industrial scale (Davis and Boyer, 2001; Liese et al., 2006; Pollard and Woodley, 2007; Drauz et al., 2012). However, there are organic reactions like olefin metathesis or Pd-catalysed cross-coupling reactions that native enzymes cannot perform. To get access to new fine chemicals or new synthetic routes, the combination of bio- and chemocatalysis in chemoenzymatic one-pot syntheses, either sequentially or in tandem has been gaining attention (Denard et al., 2013; Gröger and Hummel, 2014a,b; Rudroff et al., 2018; Schrittwieser et al., 2018; Gröger and Hummel, 2014a,b; Gröger, 2015; Gröger and Hummel, 2015). Combinations of metal catalysts and enzymes have already been reported since 1980 (Ríos-Lombardía et al., 2015; Fink et al., 2013; Borchert et al., 2012; Boffi et al., 2011; Gauchot et al., 2010; Simons et al., 2006; Allen and Williams, 1996; Makkee et al., 1980; Gomez Baraibar et al., 2016; Denard et al., 2015, 2014), and in the last years the use of organocatalysts along with enzymes has also been reported (Baer et al., 2009; Rulli et al., 2011; Akagawa et al., 2012; Heidlindemann et al., 2014; Simon et al., 2014; Suljic et al., 2015). Despite numerous reports on chemoenzymatic syntheses, there are few studies on such processes utilising immobilised catalysts, which bear several advantages (Gelman et al., 2002; Patterson et al., 2015; Edwards et al., 2016; Brena et al., 2013; DiCosimo et al., 2013; End and Schoning, 2004). Besides stabilisation and recycling of catalysts and simplified downstream processing, encapsulation can contribute to compartmentation of different catalysts, which is an advantage in particular in the presence of compatibility problems (Simons et al., 2006; Gomez Baraibar et al., 2016; Pietruszka et al., 2009; Peters et al., 2014; Sato et al., 2015; Sperl et al., 2016; Pesci et al., 2017). Hence, it is necessary to develop an efficient immobilisate for the enzyme as well as for the chemocatalyst with the ultimate goal to both combine them in a co-immobilised form.

Enzyme immobilisation has been established in the past decades, and therefore many standard encapsulation methods are already known (Brena et al., 2013; DiCosimo et al., 2013; End and Schoning, 2004; Tosa et al., 1966; Nilsson et al., 1972; Hofstee, 1973). Encapsulation in biopolymer hydrogel beads is based on readily available low-cost chemicals and represents a gentle method of high practicability. Nevertheless, for the encapsulation of hydrolases, especially in conventional biopolymer hydrogels like alginate, chitosan and κ-carrageenan,

* Corresponding author.
E-mail address: anant.patel@fh-bielefeld.de (A.V. Patel).

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reported immobilised biocatalysts often have dissatisfying properties with respect to activity, encapsulation efficiency, leaching or recycling (Toscano et al., 2014; Gülay and Şanlı-Mohamed, 2012; Schons et al., 2011; Won et al., 2005; Desai et al., 2004; Betigeri et al., 2002; Tosa et al., 1979; Gülay and Şanlı-Mohamed, 2012). Furthermore, there are no comparative studies on hydrolases encapsulated in different biopolymer beads associated with these properties and whereas the reaction course outside of the capsules can be monitored easily there is often a lack of insight into the reaction conditions inside the capsules.

Thus, an important aim of this study was to understand the influence of different biopolymer hydrogels on crucial parameters in more detail. Towards this end, we chose pig liver esterase (PLE; EC 3.1.1.1), an important and versatile member of the hydrolase enzyme family (Barker and Jencks, 1969; Tamm, 1992; Lam et al., 1986), as a "model enzyme". This biocatalyst has been widely used in organic syntheses, mostly in form of a crude product consisting of six isoenzymes with, at least in part, different catalytic properties and a molecular weight of 168 kDa (Musidlowska et al., 2001; Hummel et al., 2007; Sousa et al., 2000; Schneider et al., 1984; Dominguez et al., 2007). In addition, this biocatalyst has also been applied in a chemoenzymatic one-pot synthesis, in which the formation of diesters, e.g. **1**, by means of a metathesis reaction was combined with their subsequent hydrolysis catalysed by the PLE-type biocatalyst, thus providing the corresponding malonic acid monoester **2** (Scheme 1) (Tenbrink et al., 2011). This PLE-catalysed reaction was also chosen as the test system for evaluation of the enzyme activity. Thus, a further goal of this study was to lay the scientific-technical foundation for a later co-entrapment of the complementary bio- and chemocatalyst. As a chemoenzymatic system with PLE and Grubbs' catalyst in non-immobilised form was developed recently (Tenbrink et al., 2011), we plan to co-immobilise the chemical catalyst as well for the immobilisation of the whole chemoenzymatic system.

This "model enzyme" PLE has already been immobilised in biopolymer hydrogels in various forms before, revealing, however, limited activity, encapsulation efficiency, leaching and recyclability (Desai et al., 2008; Lee et al., 2001). We aim to focus on these attributes, as enhanced thermal and storage stability by encapsulation was, at least in part, already reported for calcium alginate beads (Desai et al., 2008).

As the biopolymer gels are mostly water-based, the catalysed reaction was carried out in a water-based solvent mixture, which along with the other benefits of encapsulation is environmentally benign and meets the standards of green chemistry (Sheldon, 2012; Sheldon and van Pelt, 2013).

As diffusion might act as limiting factor, we were in particular interested in the influence of the different biopolymer hydrogels on this issue. Furthermore, we were interested to gain a deeper understanding of how these factors govern enzyme activity, encapsulation efficiency, leaching and recyclability by investigating interactions between the enzyme and biopolymer hydrogel matrices.

2. Results and discussion

2.1. Design of biopolymer hydrogel beads

In our initial study, we focused on the preparation of various types of biopolymer beads with encapsulated PLE included therein. An overview about the different types of PLE-immobilised hydrogel beads is given in Fig. 1.

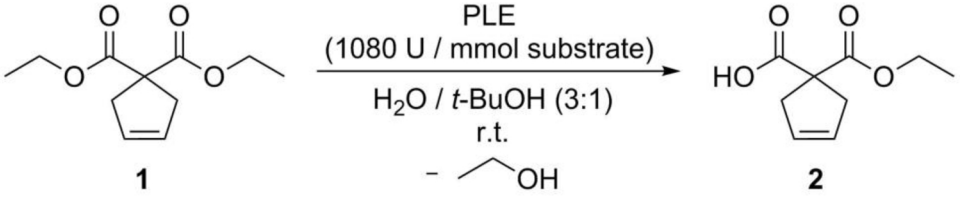
The benchmark of our study represented the automatically titrated hydrolytic reaction of diester **1** under formation of the acid **2** with a non-encapsulated (free) enzyme, which showed a specific enzyme activity of 0.305 ± 0.043 μmol · min^{-1} · mg^{-1} (Fig. 2a).

The first investigated hydrogel matrix in our study was calcium alginate, which has been used for enzyme immobilisation since the 1970 s (Kierstan and Bucke, 1977; Martinsen et al., 1989). Reported diffusion tests with different proteins at pH 7 showed that even large molecules with a molecular weight of 341 kDa diffuse out of the matrix, although relatively slowly (Tanaka et al., 1984). In accordance with this study, encapsulated PLE (168 kDa) in calcium alginate (A) showed a dissatisfying encapsulation efficiency of 70.7 ± 7.0% (Fig. 2b). The encapsulation efficiency represents the amount of enzyme being immobilised in relation to the total amount of enzyme used in the encapsulation process. To compare the activities of the immobilisates more easily, the effectiveness factor η was introduced which was calculated by dividing the specific enzyme activity of an immobilised enzyme by the specific enzyme activity of the free enzyme. For calcium alginate (A), specific enzyme activity (Fig. 2a) of 0.042 ± 0.009 μmol · min^{-1} · mg^{-1} and thus, an effectiveness factor of 0.14 was observed.

The leaching during the reaction was low with 5.2 ± 1.8% (Fig. 2c). To enhance encapsulation efficiency, we introduced a cationic chitosan coating (A-C) in a one-step method, increasing it to 98.1 ± 0.2% (Fig. 2b). Leaching decreased slightly but not significantly (Fig. 2c). Increased encapsulation efficiency and low leaching can be explained by the reduction of hydrogel pore size (Sezer and Akbuga, 1999). The specific enzyme activity (Fig. 2a) and thus, the effectiveness factor doubled to 0.30. The increasing reaction rate may be explained by neutralisation of the anionic matrix by cationic chitosan, which led to better diffusion of non-ionic substrate.

To rationalise the positive influence of the chitosan coating, we tested non-ionic silicate-coated alginate beads (A-S) providing a dissatisfying encapsulation efficiency of 76.1 ± 1.7% (Fig. 2b). This resulted from the two-step coating procedure based on alginate. The significantly lowered leaching of 1.2 ± 0.2% (Fig. 2c) as well as the noticeably low specific enzyme activity (Fig. 2a) and thus, effectiveness factor of 0.01 indicated that the diffusion limitation was intensified by a tight coating, which is in accordance with a study of silicate-coated alginate beads (Gülay and Şanlı-Mohamed, 2012). In addition to the characterisation of the prepared beads in terms of specific enzyme activity, encapsulation efficiency and leaching, we also compared their macroscopic properties (bead size) prior to and after the reaction (Table 1). The mechanical stability of A-S-beads was enhanced, leading to the lowest reduction of diameter after reaction.

Encouraged by the promising results with the chitosan coating, we decided to produce alginate-free chitosan beads. With alginate hollow beads containing dissolved chitosan, mechanical stability problems occurred. To stabilise the chitosan, it was cross-linked with glutaraldehyde. However, cross-linked chitosan without alginate layer (C) resulted in brittle beads that fragmented during the reaction into smaller particles, leading to strongly reduced bead diameter after reaction (Table 1). In contrast, all other beads showed only low deviation in diameter after reaction. Considering the margin of error, these beads basically remained unchanged after reaction unlike chitosan beads. By creating alginate-coated cross-linked chitosan beads (C-A), stable beads were obtained, which provided an effectiveness factor of 0.17, an



Scheme 1. Studied reaction to evaluate enzyme performance in immobilisates.