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Immobilization of feruloyl esterases in mesoporous materials leads to improved transesterification yield

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ABSTRACT

Feruloyl esterases are used in biocatalysis for refinement of hydroxycinnamic acids, a group of compounds with antioxidant and antibacterial properties where modification of solubility is necessary for the compounds to be of interest in different commercial products. In industrially feasible and efficient processes, immobilization of enzymes is often required for sufficient enzyme stability and to enable recovery. In recent years, mesoporous materials have become popular as immobilization support due to advantages such as high protein loading capacity and enhanced enzyme activity because of confinement into pores. We used mesoporous silica, for the first time, as immobilization support for feruloyl esterases. The crude enzyme preparation Depol 740L was adsorbed into two SBA-15 mesoporous silica materials of different pore size and the effects of the immobilization on transesterification of methyl ferulate with 1-butanol into butyl ferulate were studied, tested in a reaction system based on 92.5% 1-butanol and 7.5% MOPS buffer (pH 6.0). Immobilization in mesoporous silica with larger pore size (9 nm) showed higher protein loading and higher specific activity compared to immobilization with smaller pore size (5 nm). Importantly, adsorption into mesoporous silica changed the product specificity of the enzymes to favor transesterification and decrease the rate of hydrolysis compared to free enzymes. The immobilized enzyme had a butyl ferulate yield of up to 90%, significantly higher compared to free enzymes. Additionally, the immobilized enzymes showed an excellent operational stability and reusability, retaining ≥70% of the initial activity after 6 sequential runs, each lasting 6 days. Consequently, we show that mesoporous silica is a robust immobilization support for feruloyl esterases to be used in the development of biocatalysts for customization of the antioxidant properties of hydroxycinnamic acids.

1. Introduction

Feruloyl esterases (FAE), is a subclass (E.C. 3.1.1.73) of carboxylic ester hydrolases that catalyze the hydrolysis of ester linkages in plant cell wall materials, releasing ferulic acid (FA) and other hydroxycinnamic acids. Feruloyl esterases show a great diversity in the substrate specificity which lead to a first classification based on substrate specificity on the hydrolysis of four synthetic hydroxycinnamic acid methyl esters [1]. More recently a new classification based on sequence descriptors has been suggested to better describe this diverse group of enzymes [2]. Applications of FAEs have been found within biofuel production, agriculture and pulp and paper industry [3]. Hydroxycinnamic acids have been shown to have tumor suppressing [4], antioxidant [5] and antibacterial properties [6]. To find applications in cosmetics, food products or in therapeutics it is often desirable to change properties of the hydroxycinnamic acids. The carboxylic acid group can be used, through esterification, both to add a functional group or to change the solubility by adding a group that makes the whole molecule more hydrophobic/hydrophilic depending on the application. It is important to have biosynthetic tools available since the esterification reactions with hydroxycinnamic acids are difficult to perform effectively with classical chemical synthesis [3]. Consequently, there is an interest in utilizing FAEs as biosynthetic tools for modification of hydroxycinnamic acids. There are a few examples of FAEs used for esterification (and transesterification) of hydroxycinnamic acids focusing on different model reactions such as: first and secondary alcohols [7,8], glycerol [9,10], saccharides [11,12]. Most of the examples use microemulsions as reaction system both for solubilizing the

Abbreviations: BFA, butyl ferulate; CLEA, cross-linked enzyme aggregate; FA, ferulic acid; FAE, feruloyl esterase; MFA, methyl ferulate; MPS, mesoporous silica.

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substrate and hosting the enzymes. When microemulsions are used, the composition of the solution is restricted to a narrow range that allows formation of the microemulsion. There are also examples of lipases active on hydroxycinnamic acids [13], though the yield when running esterification with ferulic acid is generally low due to specific residues on the benzene ring [14–16].

Low water content prevents unwanted hydrolysis reactions and creates favorable conditions for esterification or transesterification reactions. Therefore, many biosynthetic reactions using esterases or lipases must be run in organic solvents. Substrate solubilization and diffusion properties are also important factors that limit the choices of solvents. However, enzymes will not be as active in organic solvents as in aqueous solutions, since the organic solvent is unnatural to enzymes and can cause denaturation [17]. Therefore, enzyme stability and reusability needs attention and immobilization of enzymes is a way of dealing with this issue. Immobilization can also be a mean of controlling the enzyme activity and selectivity. To the best of our knowledge, FAEs have only been immobilized using cross-linked enzyme aggregates (CLEA) [10,18,19]. However, the reusability of FAE CLEAs (both pure FAEs and crude enzyme preparation with FAE activity) has been limited, since the enzymes lost all their activity after being reused 2–5 times (depending on the reaction conditions).

During the last years, immobilization of enzymes into mesoporous materials and in particular mesoporous silica (MPS) has become increasingly popular. Recent reviews point out that mesoporous materials (mostly commonly mesoporous silica SBA-15 or MCM-41) allow high protein adsorption capacity, enhanced enzyme stability, good diffusion properties and easy recovery in immobilization applications [20–22]. The support material itself has great structural stability in a broad range of pH and temperature. There are extensive possibilities for functionalization of the surface of the material and the pore size is tunable over a range (2–50 nm) that matches the sizes of most enzymes. Additionally, the confining of the enzymes into the mesoporous material can increase the stability of the enzyme, as well as altering the enzymatic activity compared to free enzymes in the bulk solution. Confining of enzymes has also been shown to create protein–surface interactions that alter enzymatic properties and give rise to a specific activity significantly higher than that of free enzyme [23]. Suggested mechanisms include that the active site is stabilized in a more active state or that the packed environment inside the pores resembles that inside a cell. In summary, mesoporous materials as immobilization support have, through enzyme confinement and surface functionalization, the potential to improve control over the enzymatic activity with regards to substrate specificity and fulfill most requirements of a functional biocatalyst. However, the main limitation is still that enzymes immobilized into mesoporous materials through physical adsorption are prone to leaching. The most common strategies to overcome this problem involve covalent binding of the enzyme to the surface or partial closing of the pore entrances which, however, adds complexity and overall cost to the biocatalyst production [24].

Until now, immobilization of FAEs has not been tested in mesoporous materials. Therefore, this work aims to explore immobilization of FAE into MPS and study how the activity is affected by confining the enzymes into silica nanopores. A crude enzyme preparation (Depol 740L, Biocatalysts Ltd., UK) was selected, shown to have FAE activity and a high total protein concentration [19]. The immobilization was performed into MPS, of two different pore sizes, through physical adsorption. We chose a model reaction for the transesterification of hydroxycinnamic acid esters: transesterification of methyl ferulate with 1-butanol into butyl ferulate (Fig. 1A), that results in a more hydrophobic compound. The activity and reusability of the immobilized enzymes were studied. Additionally, product selectivity of the immobilized enzymes was compared with that of free enzyme, with evaluation of how the immobilization alters the preference for the side reaction (hydrolysis) that is inherent when working with FAEs (Fig. 1B).

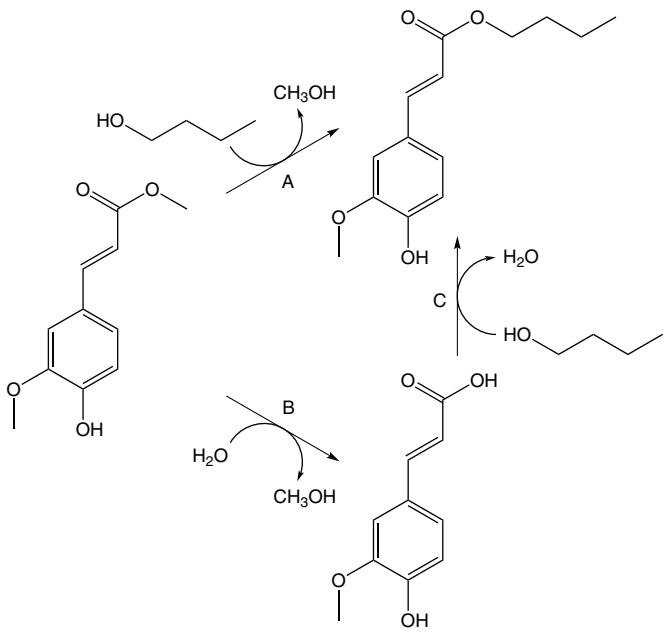

Fig. 1. (A) Transesterification of MFA with 1-butanol generating BFA and methanol. (B) Hydrolysis of MFA generating ferulic acid and methanol (natural reaction at high water contents). (C) Esterification of FA with 1-butanol generating BFA and water.

2. Experimental

2.1. Materials

Ferulic acid (FA), methyl ferulate (MFA) and the other hydroxycinnamic acids and methyl esters were purchased from Apin Chemicals Ltd., UK. MOPS was purchased from Amresco Inc., USA. 1-Butanol (Cat. No. 101990) was purchased from MERCK. Methanol (Cat. No. 34860) was purchased from Sigma–Aldrich. Bradford reagent was purchased from Bio-Rad Laboratories. Butyl ferulate (BFA) was provided as a gift by Evangelos Topakas, National Technical University of Athens, Greece.

2.2. Enzymes

Depol 740L, the crude enzyme preparation used in this study was provided as a free sample from Biocatalysts Ltd., UK. Depol 740L is a multi-component preparation shown to have several other enzymatic activities [19], i.e. out of the total protein concentration measured in this study, only a few percent is likely to be feruloyl esterases. The control experiments with free enzyme was done using Depol 740L, that was washed several times with MOPS buffer using centrifugal filters (Amicon Ultra – 0.5 ml 10 K ultracel membrane). No leakage of active enzymes could be detected using the centrifugal filters. Free enzyme reactions were stopped by rapidly putting the sample at −20 °C.

2.3. Enzyme assay – substrate profile

The substrate profile assay for the hydrolysis of the 4 synthetic hydroxycinnamic acid methyl esters, was adapted from previous

publications [19,25]. In short, substrate solution was prepared by individually dissolving the esters, MFA (15 mM), methyl sinapate (MSA; 15 mM), methyl p-coumarate (MpCA; 5 mM) and methyl caffeate (MCA; 15 mM), in MOPS buffer (100 mM, pH 6.0) and DMSO (10% v/v). The reaction was started by mixing 100 μl enzyme solution with 300 μl substrate-buffer in an eppendorf tube that was put onto a heating block and incubated at 50 °C for 5 min. Reaction was stopped with 400 μl ice-cold 10% acetic acid (FA or MFA stability are not affected by the acetic acid) and the tube stored at −20 °C until analysis.

2.4. Quantification of ferulic acid and its esters

FA, MFA and BFA concentrations were determined by HPLC (Dionex–Ultimate 3000) using a Kinetex 2.6 u C18 100 × 4.6 mm column. Absorbance was measured at 300 nm with a PDA detector. Samples were diluted 2–10 times in methanol:dH_2O (1:1 v/v) prior to analysis. Elution was done with methanol:dH_2O (7:3 v/v) at flow rate of 1 ml/min. Calibration curves was established by running FA, MFA and BFA solutions at known concentrations (0.01–2 mM). Transesterification yield was calculated as the molar amount of generated BFA compared to the initial amount of MFA and expressed as percentage. Unit (U) was defined as amount of enzyme transforming 1 μmol MFA to BFA/min. The product selectivity was defined by the BFA/FA-molar ratio (the concentration of produced BFA divided by the concentration of produced FA). Tests were done to ensure that FA, MFA and BFA were fully stable (data not shown) for at least one week in both the methanol (at 4 °C) and butanol (37 °C) solutions used in the experiments. The sum of the molar amounts of all reactants at the end of the reaction was always within a 10% error margin compared to the starting molar amount of the substrate.

2.5. Synthesis of mesoporous silica

SBA-15 of two different pore sizes was synthesized using protocols adapted from Zhao et al. [26,27]. The triblock copolymer Pluronic P123 ($EO_{20}PO_{70}EO_{20}$) was used as a structure directing agent and tetraethyl orthosilicate (TEOS) as a silica source. In a typical synthesis, 4.0 g of P123 was dissolved in 120 g of 2 M HCl and 30 g of deionized water and vigorously stirred at 35 °C for 2 h. 8.5 g of TEOS was added and the solution was stirred at 35 °C for additional 24 h. The gel mixture was transferred to stainless steel pressure autoclaves in Teflon containers and aged for 24 h at 80 or 150 °C, depending on the desired pore diameter. The solid precipitate was recovered by vacuum filtration, washed with deionized water and dried. Finally, to remove the template, the product was calcinated by increasing the temperature from room temperature up to 500 °C during 8 h followed by heating at 500 °C for another 6 h.

2.6. Characterization of mesoporous silica

The pore diameter and surface areas were measured by a Micrometrics Tristar. The pore diameter distributions were determined using the BJH (Barrett–Joyner–Halenda) methods based on the desorption isotherms, and the surface area were determined using the Brunauer–Emmett–Teller (BET) method (material characteristics are summarized in Table 1). The hexagonal structure of SBA-15 was confirmed by TEM imaging on a JEM-1200 EX II TEM operated at 120 kV.

2.7. Immobilization of enzymes

Mesoporous silica and MOPS buffer (20 ml buffer/g MPS) was mixed in a Falcon tube and sonicated (Elmasonic S30H) for 2 × 30 min. After adding the enzyme preparation (7.2 ml Depol 740L/g MPS) the solution was magnetically stirred at 4 °C for 22 h. Thereafter, the MPS was washed (10 min centrifugation at 4000 × g) 3 times with 10 ml MOPS buffer. The amount of immobilized enzyme was estimated by removing the MPS by centrifugation and measuring the residual total protein concentration of the supernatant with a Bradford assay, using BSA as standard [28]. The final pellet was dried in vacuum (CHRIST RCV 2-18) without added heating. The obtained powder was used in the transesterification experiments.

Table 1
Material properties characterized by nitrogen-adsorption.

Sample	Pore diameter (nm)	BET surface area (m^2/g)	Pore volume (cm^3/g)
MPS-5 (SBA-15)	5.01	924	0.73
MPS-9 (SBA-15)	9.32	528	1.17

2.8. Transesterification of MFA with 1-butanol into BFA

In the transesterification of MFA with 1-butanol, MFA (20 mM) was dissolved in 1-butanol:MOPS buffer solutions with solvent ratios of 100/0–85/15% (v/v). The reaction was started by adding MPS particles with immobilized enzyme (10 mg/ml reaction solution) to the reaction solution in an Eppendorf tube which was put onto a shaking incubator (IKA KS 4000, 37 °C, 250 rpm). The reaction was ended by removing the MPS particles by centrifugation (4 min, 18,000 × g) and transferring the supernatant to a new tube, stored at −20 °C until analysis. Control experiments without enzyme were done to ensure that FA and MFA do not adsorb onto the synthesized MPS nor that their stability is affected by the presence of MPS.

2.9. Reusability/stability experiments

The reusability experiments were run as the transesterification experiments described above but after the reaction was stopped, the pellet was dried in vacuum and the reaction restarted with fresh reaction solution (repeated throughout each run). No washing was done in between the runs to minimize potential enzyme leakage. Since it is difficult to separate free enzymes from a solution, the control samples with free enzyme were pre-incubated without substrate (for 3, 6, 12, 19, 26, 33 days) in 750 μl reaction solution buffer at 37 °C and started by adding 250 μl 80 mM MFA 1-butanol:MOPS buffer at the given time points.

3. Results and discussion

3.1. Simple immobilization of Depol 740L onto MPS

During biocatalysis, enzymes are often subject to unnatural conditions and immobilization is thereby a mean of protecting and enhancing the reusability of the enzymes. It is also known that immobilization can alter both stability and activity of enzymes. In this study, FAEs were immobilized into MPS for the transesterification of MFA with 1-butanol. The crude enzyme preparation Depol 740L, was immobilized into SBA-15 mesoporous silica through physical adsorption. In order to evaluate how the pore size affect the protein loading capacity and the activity of the immobilized enzymes MPS of two different pore sizes (5 nm, MPS-5 and 9 nm, MPS-9; Table 1) were selected.

Immobilization into MPS was followed over time to determine the time frame required for adsorption to get close to equilibrium and no more protein being adsorbed. The immobilization was rapid with most protein being immobilized during the first 10 min (Fig. 2).

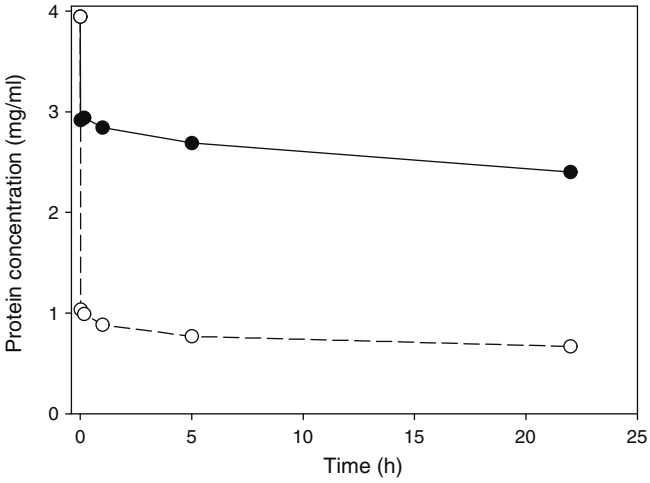

Fig. 2. Residual protein concentration throughout the immobilization. (●) MPS-5D, (○) MPS-9D. The first data point was measured before adding MPS. The last data point is before the washing step.

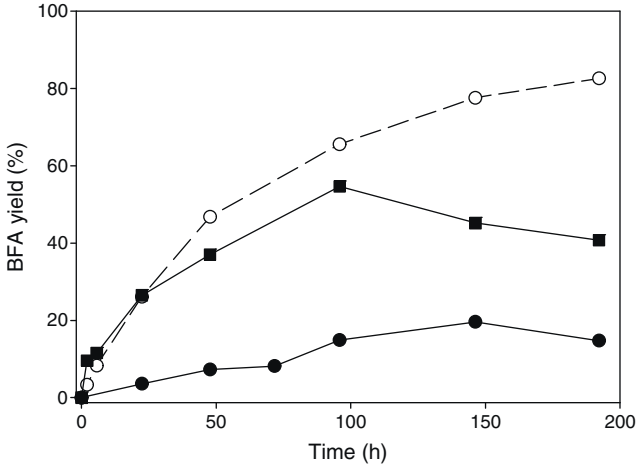

Fig. 3. Transesterification of MFA (20 mM) into BFA over time in 7.5% water for (●) MPS-5D, (○) MPS-9D, (■) free enzyme (amount of free enzyme corresponds to the amount immobilized into MPS-5D). Data shown is the average of 2 replicates.

However, the immobilization was run for 22 h since the time scale of protein rearrangement onto surfaces can be in order of hours [29].

Since Depol 740L contains a mixture of proteins, a substrate profile (for the hydrolysis of four synthetic hydroxycinnamic acid methyl esters) was performed on the supernatant of the immobilization reaction after 22 h. No distinct change in substrate profile was observed in the residual protein solution using MPS-5. This indicates that no selective immobilization of potential FAE isoenzymes occurred (only data for MFA hydrolysis is shown in Table 2). Furthermore, the proportion of immobilized proteins (39% before washing) is reasonably correlated with the proportion of immobilized FAEs (45% MFA activity lost). For MPS-9 no hydrolytic activity could be detected in the supernatant for MSA nor MCA and only a few percent activity was left for MFA and MpCA, whereas the protein measurement showed 83% immobilized before washing. The low residual activity indicates that a majority of the feruloyl esterases in Depol 740L were immobilized into MPS-9. Since the total protein immobilized was lower compared to removed activity, the immobilization was partially selective towards FAEs (assuming free enzymes are not inactivated during the immobilization).

MPS-9 was able to support twice the amount of protein compared to MPS-5 (Table 2). However, if considering the pore volume (Table 1), MPS-9 should only support 60% more protein than MPS-5. Also considering that the surface area is almost a factor 2 larger for MPS-5, it is likely that pore size is the limiting factor for immobilization in MPS-5 and most of the proteins are located on outside the pores. Low protein loading after immobilization, compared to the large available surface area and pore volume, has previously been linked to the fact that MPS with smaller pore sizes are more prone to larger proteins getting stuck in the pore entrance, subsequently blocking the whole pore [30]. It is also believed the density of adsorbed proteins is higher close to the pore entrance, making utilization of the whole pore volume difficult [31]. Nevertheless, using a multi-component preparation and without information regarding size or dimensions of the proteins in Depol 740L, it is difficult to fully interpret the immobilization degree.

3.2. Transesterification yield over time higher with larger pore size

Initially, the transesterification activity of the immobilized enzyme particles was evaluated and measured over time and resulted in a significantly higher transesterification yield for MPS-9D compared to MPS-5D (Fig. 3). The FAEs in MPS-9D are also 3.5 times more active than MPS-5D in terms of specific transesterification activity (Table 2). Assuming that most proteins are located on the surface outside of MPS-5D (as discussed in Section 3.1), one could speculate that it would be less crowded for MPS-9D where a larger proportion is located inside the pores. More space around the enzyme seems to be favorable for the specific enzymatic activity and the transesterification activity detected for MPS-9D is also higher per amount of support material. Enhanced enzymatic activity after immobilization into MPS, has previously been associated with unpredictable conformational changes in the enzyme structure caused by confining the enzyme [32]. The mechanisms behind the changes in activity are not very well understood and considerable amount of effort is put into getting a better understanding on how confining can be used in a rational way to control and direct the enzymatic activity [33].

Comparing the initial activity of MPS-9D with the one of the free enzyme, 40% of the specific BFA activity was lost (Table 2). However, MPS-9D supports a significantly higher overall BFA yield up to 90% and does not have the same tendency of declining yield in the end of the reaction (Fig. 3). Additionally, if the specific transesterification activity would have been calculated at one of the later time points, the advantage of running the transesterification with immobilized enzymes would be even clearer. Previous publications report conversions up to 78–97% for esterification/transesterification of hydroxycinnamic butyl esters [18,19]. In these reports, conversion is defined as the amount of substrate reacted compared to the initial amount of substrate and therefore includes any amount of generated hydrolysis product. The corresponding conversion for MPS-9D is 95%.

Esterification of FA directly to BFA was also tested, but the BFA yield of this reaction was just above the detections limit (data not shown). This result rules out the possibility that MFA would be initially hydrolyzed to FA and then in a second step esterified to BFA (Fig. 1B and C).

3.3. Effect of water content on transesterification activity

The amount of water present in the reaction solution is known to influence the activity of enzymes, especially in organic solvents where a small amount of water usually is required for the enzymes to be active [34]. Therefore, the transesterification reaction was

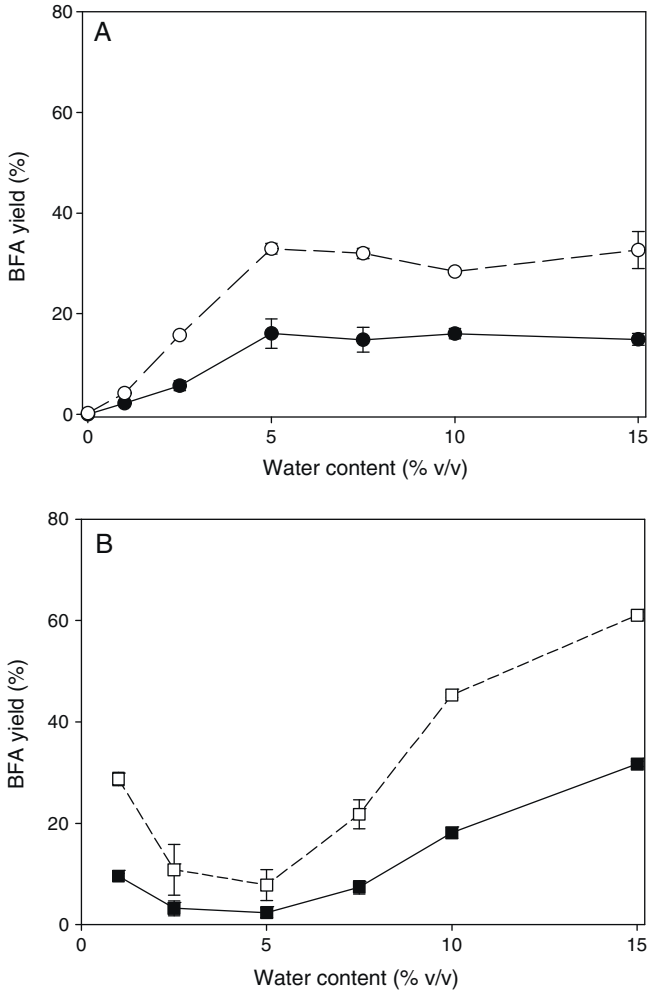

Fig. 4. Transesterification of MFA to BFA tested at varying water content using (A) (●) MPS-5D (136 h) and (○) MPS-9D (22 h). (B) Free enzyme control, after (■) 46 h and (□) 168 h. At water contents >10% the water:1-butanol solution is not fully miscible. The indicated times represents the length of the reaction and a shorter time was selected for MPS-9D to be more comparable with MPS-5D. Data in both graphs are averages of 3 replicates with standard deviation bars.

tested at varying water (MOPS buffer) content. Both the MPS-5D and MPS-9D have a similar BFA yield profile (Fig. 4A) with low yield at low water content (0–2.5%) and relatively stable yield as the water content increases (≥5%). Additionally, MPS-9D consistently has a higher yield compared to MPS-5D at the tested water contents, which further confirms that the larger pore size is indeed favorable for the specific transesterification activity. Previous biosynthetic experiments using FAEs in microemulsions were

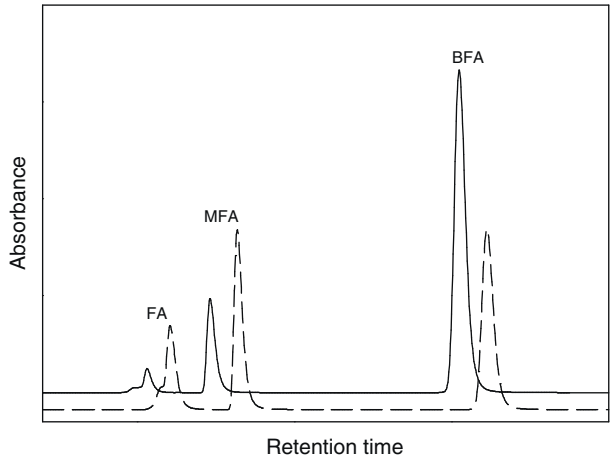

Fig. 5. Typical HPLC chromatogram illustrating the higher BFA/FA molar ratio of immobilized enzyme (solid line) compared to free enzyme (dashed line). Retention times for FA, MFA and BFA were 1.0, 1.5 and 3.0 min, respectively.

generally run at water contents around 2% for optimal esterification activity [25].

In similar transesterification experiments but with free enzyme, the profile turns out noticeably different with a minimum BFA yield at 5% water content (Fig. 4B). Generally, increased water content should lower the transesterification activity and increase the hydrolytic activity [11]. Additionally, decreased transesterification activity with increase water content has been shown for lipases in a broad range of organic solvents [35]. However, we observed that the transesterification yield increased at water contents between 5 and 15%, which might be related to an increase in the hydration of the enzyme. Unexpectedly, as the activity increases, the increase in hydrolytic activity is lower relative to the increase in transesterification activity (further discussed in Section 3.4). Turning the attention to the immobilized enzyme, it is generally believed that hydration of enzymes is closely linked to the enzymatic activity and it has also been shown that proteins inside mesoporous silica are more strongly hydrated [36,37]. Consequently, the immobilized enzymes can obtain the necessary hydration at a lower water content, which would explain why the transesterification activity has no minimum at water content 5%.

3.4. Changed product selectivity

Naturally FAE hydrolyzes the ester bond to yield FA and an alcohol. However, in this study, it was observed that the immobilized enzymes consistently generated less FA (higher BFA/FA ratio) than the free enzymes (Fig. 5). This difference was most evident

Table 2
Degree of immobilization based on residual protein concentration.

	Protein immobilized (%)		Lost MFA activity (%)[a]	Protein load (mg/g_{MPS})	Specific transesterification activity (mU/$mg_{protein}$)[b]	Transesterification activity/support material (mU/g_{MPS})
	Before wash.	After wash.				
MPS-5D (MPS-5)	39	31	45	34	2.2	0.07
MPS-9D (MPS-9)	83	68	94	73	7.8	0.56
Non-porous Si	36	29	32	31	9.3	0.29
Free enzyme	n.r.[c]	n.r.	n.r.	n.r.	13	n.r.

[a] MFA hydrolysis activity on the residual supernatant after immobilization (before washing).
[b] Transesterification activities are derived from the first time points of Fig. 3.
[c] Not relevant.

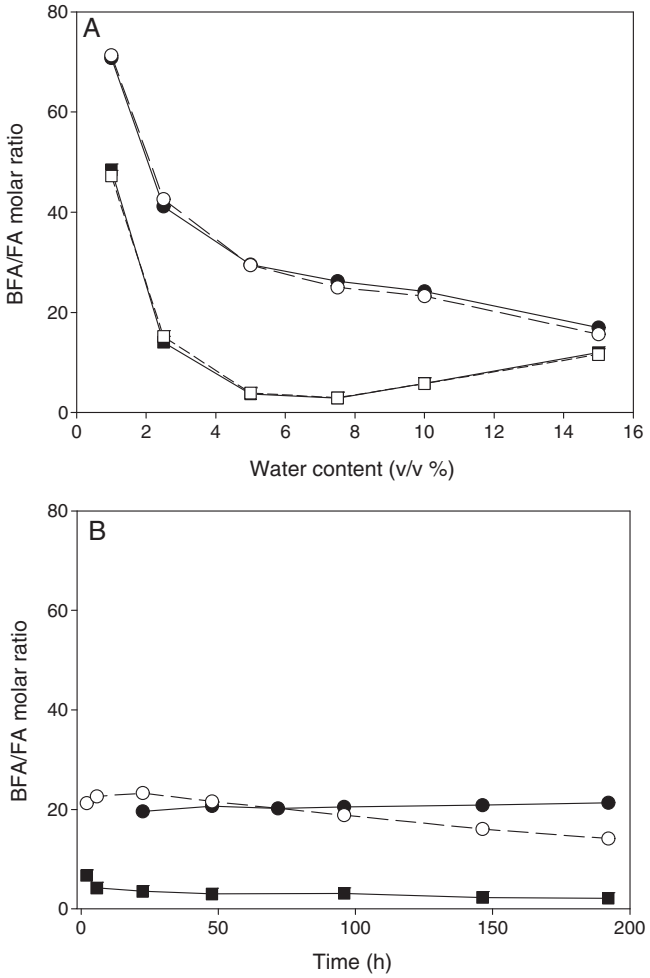

Fig. 6. BFA/FA molar ratio (A) at varying water content for (●) MPS-5D, (○) MPS-9D, free enzyme (■) after 46 h and (□) after 168 h. (B) Different time points for (●) MPS-5D, (○) MPS-9D, free enzyme (■) at 7.5% (v/v) water. The first two data points of MPS-5D were below the detection limit. Data in both graphs are averages of 2–3 replicates.

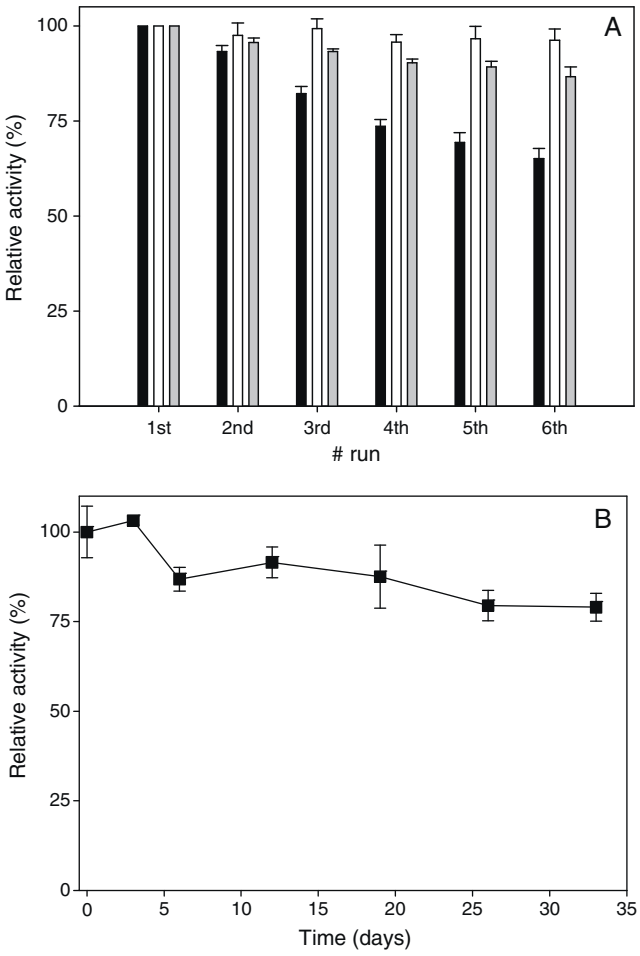

Fig. 7. Reuseability experiments with 7.5% (v/v) water using (A) immobilized enzyme with each run lasting 6 days, (black) MPS-5D, (white) MPS-9D and non-porous silica (grey). (B) Free enzyme. 100% activity is defined as the activity of the first run/first time point. Data shown are averages of 4 replicates with standard deviation bars.

at water contents of 5–10% where the ratio was 4–9 times higher for immobilized enzymes compared to free enzymes (Fig. 6A). The BFA/FA ratios in the reactions with MPS-5D and MPS-9D were closely aligned at the different water contents. Thus the higher BFA/FA ratio is independent of pore size and more likely related to the enzyme being adsorbed to the silica surface. The BFA/FA ratio showed to be relatively stable over time (Fig. 6B). However, MPS-9D showed a slow decrease in ratio at higher yields. A possible explanation for this could be that at higher yields, where the transesterification has slowed down due to substrate limitation, FA can still be generated through hydrolysis of BFA. This could also explain the decrease in yield for the free enzyme (Fig. 3) in the end of the reaction, where selectivity is changed towards hydrolysis.

It has been reported that short alcohols (ethanol and butanol) interact with silanol groups in a combination of hydrogen bonds and van der Waals interactions that allow the alcohol to form chain like structures inside the nanochannels of mesoporous silica (3.1 nm in diameter) [38]. A previous publication also demonstrates that water is adsorbed/arranged along silica surfaces in SBA-15 through hydrogen bonding [39]. Furthermore, the arrangement of water molecules inside the pore changes the stability and movement dynamics of the proteins [40]. Therefore, the environment inside of the pores is significantly different to that of the surrounding bulk. However, since there is no difference in BFA/FA ratio between MPS-5D and MPS-9D, it is more likely that the effect is a general feature of the enzyme being close to a silica surface rather than a confining effect of the pore. This hypothesis was tested by immobilizing Depol 740L onto non-porous silica particles. The BFA/FA ratio using the non-porous particles was the same as for MPS-9D at a water content of 7.5% (data not shown). Still, it remains to determine if the change in BFA/FA ratio is true in other solvents and also if it can be generalized to other transesterification reactions using FAEs. Such studies would aid in deducing whether the change in ratio comes from potential changes in the structure of the enzyme due to interactions with the silica surface or if the effect originates from water and butanol molecules being arranged favorably both inside the pores and on the outer surface of the particles. One should also consider that it can be a challenge to draw conclusions when using a crude enzyme preparation since there can be effects of having other proteins and other active enzymes present. Using pure enzyme, it would also be more relevant to assess parameters such as pH, temperature and surface modifications that could enhance both the immobilization but also potentially allow increased control of the enzymatic activity.

3.5. Reusable and stable biocatalyst

The MPS with immobilized Depol 740L were relatively stable throughout 6 runs (Fig. 7A), retaining 70% and 96% of their activities compared to the first run for MPS-5D and MPS-9D, respectively. The better reusability of MPS-9D can partly be explained by the fact that the reaction had reached a BFA yield (~85%), where a decrease in activity will not be as easily noticeable as for MPS-5D with a significantly lower yield (15–20%). This could also explain the relative high reusability of the non-porous silica (~70% in yield) where the proteins also could be assumed to have adsorbed more strongly due to a larger available surface area per protein, caused by a significantly smaller particles size of the non-porous silica compare to MPS-5D. Nevertheless, another explanation to the observed difference in reusability between the two mesoporous silicas, can be that the enzymes in MPS-9D are conformationally stabilized (assuming a majority of the enzymes are located inside the pores) by protein–surface interactions with the cylindrical shaped pore wall confining the enzyme, as described previously for other enzymes [37,41]. Immobilization of Depol 740L in MPS also considerably improves the reusability compared to CLEAs of Depol 740L where all enzymatic activity was lost after 2 runs (6 days/run in a *n*-hexane/1-butanol/water micro emulsion) [19].

Control experiments were done where the reactions were stopped after 3 days and the supernatant was incubated further 3 days longer. No activity could be detected (data not shown) in the supernatant indicating that the slow decrease in activity of the immobilized enzymes is mainly due to inactivation of the enzyme and not leakage. It also suggests that the interaction to the silica surface through physical adsorption (no covalent linkages) is sufficient for a stable immobilization under the conditions tested in this study. Immobilization in MPS through adsorption are generally prone to leaching and usually require either covalent linkage of the enzyme or partial closure of the pore opening for sufficient enzyme recovery [22]. However, the free enzyme control is also quite stable in the 1-butanol:MOPS solution, retaining 75% of its initial activity after more than 30 days pre-incubation without substrate (Fig. 7B). Nevertheless, MPS should be considered as suitable support material for immobilization of FAEs with a simple immobilization protocol and satisfying reusability.

4. Conclusion

MPS proves to be a robust immobilization support for Depol 740L due to good reusability and stability of the immobilized enzymes and can thereby be considered as a functional biocatalyst for modification of hydroxycinnamic acid esters. The simple immobilization protocol in combination with a crude enzyme preparation shows the potential of the biocatalyst in industrial applications. The immobilization leads to altered enzymatic activity towards transesterification of butyl ferulate while less hydrolysis product is generated, resulting in higher transesterification yield compared to free enzymes. Hopefully, this work can aid in further developing FAE biocatalysts that generally exhibit high transesterification activity also in other solvents and for a broad range of reactions, e.g. hydrophilic modifications with glycerol or saccharification of other hydroxycinnamic acid esters.

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References

[1] V.F. Crepin, C.B. Faulds, I.F. Connerton, Appl. Microbiol. Biotechnol. 63 (2004) 647–652.
[2] D.B.R.K.G. Udatha, I. Kouskoumvekaki, L. Olsson, G. Panagiotou, Biotechnol. Adv. (2010), doi:10.1016/j.biotechadv.2010.09.003.
[3] E. Topakas, C. Vafiadi, P. Christakopoulos, Process Biochem. 42 (2007) 497–509.
[4] A. Murakami, Y. Nakamura, K. Koshimizu, D. Takahashi, K. Matsumoto, K. Hagihara, H. Taniguchi, E. Nomura, A. Hosoda, T. Tsuno, Y. Maruta, H.W. Kim, K. Kawabata, H. Ohigashi, Cancer Lett. 180 (2002) 121–129.
[5] H. Kikuzaki, M. Hisamoto, K. Hirose, K. Akiyama, H. Taniguchi, J. Agric. Food Chem. 50 (2002) 2161–2168.
[6] S. Naz, S. Ahmad, S.A. Rasool, S.A. Sayeed, R. Siddiqi, Microbiol. Res. 161 (2006) 43–48.
[7] S. Giuliani, C. Piana, L. Setti, A. Hochkoeppler, P.G. Pifferi, G. Williamson, C.B. Faulds, Biotechnol. Lett. 23 (2001) 325–330.
[8] N.S. Hatzakis, I. Smonou, Bioorg. Chem. 33 (2005) 325–337.
[9] M. Tsuchiyama, T. Sakamoto, T. Fujita, S. Murata, H. Kawasaki, Biochim. Biophys. Acta 1760 (2006) 1071–1079.
[10] C. Vafiadi, E. Topakas, V.R. Nahmias, C.B. Faulds, P. Christakopoulos, J. Biotechnol. 139 (2009) 124–129.
[11] E. Topakas, C. Vafiadi, H. Stamatis, P. Christakopoulos, Enzyme Microb. Technol. 36 (2005) 729–736.
[12] C. Vafiadi, E. Topakas, P. Christakopoulos, Carbohydr. Res. 341 (2006) 1992–1997.
[13] N.T.L. Chigorimbo-Murefu, S. Riva, S.G. Burton, J. Mol. Catal. B: Enzym. 56 (2009) 277–282.
[14] B. Guyot, B. Bosquette, M. Pina, J. Graille, Biotechnol. Lett. 19 (1997) 529–532.
[15] H. Stamatis, V. Sereti, F.N. Kolisis, J. Mol. Catal. B: Enzym. 11 (2001) 323–328.
[16] M.H. Katsoura, A.C. Polydera, L.D. Tsironis, M.P. Petraki, S.K. Rajacic, A.D. Tselepis, H. Stamatis, New Biotechnol. 26 (2009) 83–91.
[17] A.M. Klibanov, Nature 409 (2001) 241–246.
[18] C. Vafiadi, E. Topakas, A. Alissandratos, C.B. Faulds, P. Christakopoulos, J. Biotechnol. 133 (2008) 497–504.
[19] C. Vafiadi, E. Topakas, P. Christakopoulos, J. Mol. Catal. B: Enzym. 54 (2008) 35–41.
[20] S. Hudson, J. Cooney, E. Magner, Angew. Chem. Int. Ed. 47 (2008) 8582–8594.
[21] C. Ispas, I. Sokolov, S. Andreescu, Anal. Bioanal. Chem. 393 (2009) 543–554.
[22] C.H. Lee, T.S. Lin, C.Y. Mou, Nano Today 4 (2009) 165–179.
[23] C.H. Lei, Y. Shin, J.K. Magnuson, G. Fryxell, L.L. Lasure, D.C. Elliott, J. Liu, E.J. Ackerman, Nanotechnology 17 (2006) 5531–5538.
[24] M. Hartmann, D. Jung, J. Mater. Chem. 20 (2010) 844–857.
[25] E. Topakas, H. Stamatis, M. Mastihobova, P. Biely, D. Kekos, B.J. Macris, P. Christakopoulos, Enzyme Microb. Technol. 33 (2003) 729–737.
[26] D.Y. Zhao, J.L. Feng, Q.S. Huo, N. Melosh, G.H. Fredrickson, B.F. Chmelka, G.D. Stucky, Science 279 (1998) 548–552.
[27] D.Y. Zhao, Q.S. Huo, J.L. Feng, B.F. Chmelka, G.D. Stucky, J. Am. Chem. Soc. 120 (1998) 6024–6036.
[28] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[29] A.M. Moulin, S.J. O'Shea, R.A. Badley, P. Doyle, M.E. Welland, Langmuir 15 (1999) 8776–8779.
[30] H. Takahashi, B. Li, T. Sasaki, C. Miyazaki, T. Kajino, S. Inagaki, Chem. Mater. 12 (2000) 3301–3305.
[31] M. Hartmann, Chem. Mater. 17 (2005) 4577–4593.
[32] C.H. Lei, Y. Shin, J. Liu, E.J. Ackerman, Nano Lett. 7 (2007) 1050–1053.
[33] A.K. Dunker, A. Fernandez, Trends Biotechnol. 25 (2007) 189–190.
[34] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 8017–8021.
[35] L.F. Garcia-Alles, V. Gotor, Biotechnol. Bioeng. 59 (1998) 684–694.
[36] V. Kurkal, R.M. Daniel, J.L. Finney, M. Tehei, R.V. Dunn, J.C. Smith, Biophys. J. 89 (2005) 1282–1287.
[37] R. Ravindra, Z. Shuang, H. Gies, R. Winter, J. Am. Chem. Soc. 126 (2004) 12224–12225.
[38] T. Kamijo, A. Yamaguchi, S. Suzuki, N. Teramae, T. Itoh, T. Ikeda, J. Phys. Chem. A 112 (2008) 11535–11542.
[39] B. Grunberg, T. Emmler, E. Gedat, J. Shenderovich, G.H. Findenegg, H.H. Limbach, G. Buntkowsky, Chem. Eur. J. 10 (2004) 5689–5696.
[40] G. Schiro, M. Sclafani, F. Natali, A. Cupane, Eur. Biophys. J. Biophys. 37 (2008) 543–549.
[41] Y. Urabe, T. Shiomi, T. Itoh, A. Kawai, T. Tsunoda, F. Mizukami, K. Sakaguchi, Chembiochem 8 (2007) 668–674.

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