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Immobilization of Haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26 for Biotechnological Applications

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Abstract

Haloalkane dehalogenases are enzymes capable of converting a broad range of aliphatic halogenated compounds to corresponding alcohols. These dehalogenase-based biotransformations are attractive for various biological processes, e.g. biocatalysis, bioremediation and detoxification, which often require protein immobilization. Different immobilization techniques, including (i) cross-linking using glutaraldehyde, dextran polyaldehyde, disuccinimidyl suberate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, (ii) formation of cross-linked enzyme aggregates, and (iii) entrapment methods using organically modified sol-gel ORMOCER and polyvinyl alcohol particles, were systematically investigated for immobilization of selected haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26. All tested methods and their combinations were critically compared with regard to residual enzyme activity, leaching, and their overall application advantages and limitations. The best results with the 47%, 41% and 33% retention of initial enzymatic activity were obtained by using glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, with adipic acid dihydrazide and cross-linked enzyme aggregates methodology, respectively. Following entrapment of cross-linked enzyme aggregates into polyvinyl alcohol particles, with final 25% retention of the initial enzymatic activity, secures high degree of protection for the enzyme against harsh technological conditions, and provides biocatalyst with superior mechanical properties and easy separation from the reaction media.

Keywords

Immobilization; Enzymes; Enzyme activity; Cross-linking; Entrapment; Biocatalysis

Abbreviations

ADH: Adipic Acid Dihydrazide; APTS: 3-aminopropyltriethoxysilane; BSA: Bovine Serum Albumin; CLEAs: Cross-linked Enzyme Aggregates; DPA: Dextran Polyaldehyde; DSS: Disuccinimidyl Suberate; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; GA: Glutaraldehyde; ORMOCER: Organically modified ceramics

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Introduction

Haloalkane dehalogenases (EC 3.8.1.5) represent an enzyme family, commonly distributed in various bacterial species, which attract attention due to their ability to convert a broad range of halogenated compounds to corresponding alcohols, without requirement of any cofactors. They have been originally isolated from bacterial strains living in a soil contaminated with halogenated compounds [1-7], later also from pathogenic [8], and more recently from extremophilic bacteria [9], potentially possessing unique features for industrial applications. Variety of biotechnological applications have been described for these enzymes, including bioremediation of contaminated sites [10], degradation of warfare chemicals [11,12], recycling of chlorinated by-products from chemical manufacturing [13], synthesis of chiral intermediates for pharmaceutical use [13-16], molecular imaging [17-20], biosensing of halogenated pollutants [21-24], and chemical warfare agents [25]. Several studies focused on expanding their substrate range and enhancing their catalytic properties were reported [15,26,27].

Inspite of the broad biotechnological applicability of haloalkane dehalogenases, only a few examples of single immobilization methods have been reported so far. Dravis et al. [28] described covalent immobilization of haloalkane dehalogenase from *Rhodococcus rhodochrous* on a polyethyleneimine impregnated alumina support, with activity retention of approximately 40%. Johnson et al. [29] attached haloalkane dehalogenase DhlA from *Xanthobacter autotrophicus* GJ10 to iron oxide superparamagnetic nanoparticles, using both affinity and covalent binding, with activity up to 118%. [M. Samorski, Immobilisierung von Haloalkan-Dehalogenasen und Prozessentwicklung der enzymatischen Produktion von optisch aktivem 2,3-Dichlor-1-propanol, Dissertation Thesis, University Stuttgart, Germany, 2008] immobilized haloalkane dehalogenases DhaA on enzyme carrier Eupergit with 22% activity retention.

In this study, various immobilization methods were systematically tested with the haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26. Different immobilization methods were selected and applied with respect to the surface reactive groups of LinB. Cross-linking reagents, e.g. glutaraldehyde (GA), dextran polyaldehyde (DPA), disuccinimidyl suberate (DSS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were tested for immobilization on a glass surface. Cross-linked enzyme aggregates (CLEAs) were prepared to demonstrate the feasibility of carrier-free immobilization for the LinB enzyme. The immobilization methods of entrapment and cross-linking were also used in this study. Activity retention and enzyme leaching were evaluated for all methods; and their advantages and limitations were discussed to facilitate their selection for particular biotechnological application.

Experiment

Materials

All chemicals were of analytical grade and used without further purification. Ammonium ferric sulphate, EDC and potassium dihydrogen phosphate were purchased from Fluka (Switzerland). Ampicillin sodium and isopropyl-β-D-thiogalactopyranoside were

obtained from Duchefa (the Netherlands). Nitric acid was purchased from Lach-Ner (Czech Republic). Solutions of photoinitiator Irgacure, ORMOCER AL657 and ORMOCER KSK1926 were from Fraunhofer Institute (Germany). Polyvinyl alcohol and polyethylene glycol with Mw 1,000 were kindly provided by LentiKat's (Czech Republic). Ethanol for UV spectroscopy, acetone, methanol and toluene of HPLC grade were purchased from Chromservis (Czech Republic). All other chemicals were purchased from Sigma-Aldrich (USA). Aqueous solutions were prepared with MilliQ water obtained using a Water Purificator Simplicity 185 (Millipore, USA).

Preparation of enzyme

Haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26 [3], was overexpressed in *Escherichia coli* BL21(DE3) containing plasmid pAQN::*linB-UT* [30]. The His-tagged LinB was purified using the chromatographic column HR 16/10 (GE Healthcare, Sweden), with Ni-NTA sepharose (Quiagen, Germany) attached to the FPLC system ÄKTA (GE Healthcare, Sweden), as described earlier [31]. The prepared LinB was lyophilized using freeze dryer ALPHA 1-2 LD (Martin Christ, Germany).

Immobilization of the enzyme

Glass slides (26×10×1 mm) were purchased from Merci (Czech Republic), and used as a solid support. They were washed thoroughly with toluene, acetone, ethanol and 1 M sodium hydroxide before immobilization. Then, they were washed with distilled water and dried using paper cloth. Activation of glass slides by silane coupling agent 3-aminopropyltriethoxysilane (APTS) was performed using a modified procedure, previously described [32]. Glass slides were treated with 5% (v/v) hydrochloric acid overnight, and then with piranha solution composed of 95.0-97.5% (v/v) sulphuric acid and 30% (v/v) hydrogen peroxide, in a ratio of 3:1 (v/v) for 30 min. Subsequently, they were washed thoroughly with distilled water, and dried using a paper cloth. The glass slides were immersed in freshly prepared APTS solution (2%, v/v) in acetone for 1 min. Then, they were washed twice with acetone for 1 min and twice with distilled water for 1 min. The glass slides were dried overnight at 37°C.

Cross-linking with GA: The composition of the immobilization mixture was optimized according to Table 1. The mixture (2 µl) was applied on the glass slides, with and without APTS activation, and exposed to GA vapours for 30 min at 21°C. Determination of optimal cross-linking time was performed with the immobilization mixture composed of 10 mg of lyophilized LinB and 20 mg of bovine serum albumin (BSA), dissolved in 120 µl of distilled water, with 40 µl of 99% (v/v) glycerol. This mixture (2 µl) was applied on the glass slides and exposed to GA vapours for 15, 30, 60 or 150 min (Table 1).

Cross-linking with DPA: A solution of the cross-linker DPA was prepared according to a previously described procedure [33]. Lyophilized LinB (3 mg) and BSA (3 mg) were weighed in two Eppendorf tubes and dissolved in 25.5 µl and 27.6 µl of a 10 mM phosphate buffer (pH 7.5), with the addition of 4.5 and 2.4 µl of DPA, respectively. Both immobilization mixtures were applied on glass slides (2 µl), and allowed to polymerize for 90 min at 21°C. Sodium borohydride (2 mg) was dissolved in 2 ml of saturated sodium hydrogen carbonate, and 100 µl of the solution was applied over each immobilized layer. It was allowed to react for 30 min at 21°C. The procedure using 4.5 µl of DPA was used for immobilization of LinB on APTS-activated glass slides.

Cross-linking with DSS: Lyophilized LinB (5 mg) and BSA (1 mg) were dissolved in 445.5 µl of 50 mM triethylamine (pH 8.0). The solution of cross-linker was prepared by dissolution of 5 mg of DSS in 100 µl of DMSO. Proteins were cross-linked by addition of DSS solution to the final concentration of 14.8 mM and 1.48 mM, respectively. Immobilization mixture (4 µl) was applied on the glass slides and allowed to polymerize overnight at 4°C.

Table 1: Composition of immobilization mixture for cross-linking using GA.

Ratio BSA: LinB (w/w)	Lyophilized BSA (mg)	Lyophilized LinB (mg)	Distilled water (µl)	99% (v/v) glycerol (µl)
0:1	0	10	100	0
1:1	10	10	100	0
4:1	40	10	195	0
10:1	100	10	450	0
1:1+glycerol	10	10	60	20
2:1+glycerol	20	10	120	40
4:1+glycerol	40	10	180	60

BSA: Bovine Serum Albumin; GA: Glutaraldehyde.

Cross-linking with EDC: Lyophilized LinB (5 mg) and BSA (1 mg) were dissolved in 500 µl of 50 mM pyridine (pH 7.0). The cross-linker solution was prepared by dissolution of 7 mg of EDC in 250 µl of 50 mM pyridine (pH 7.0). The EDC solution was slowly added to the proteins to create a final concentration of 13.3 mM and 24.3 mM, respectively. Immobilization mixture (4 µl) was applied on the glass slides, and the mixture was allowed to polymerize overnight at 4°C.

Cross-linking with EDC in presence of adipic acid dihydrazide (ADH): Lyophilized LinB (5 mg) and BSA (1 mg) were dissolved in 500 µl of 50 mM pyridine (pH 7.0). The cross-linker was prepared by dissolution of 7 mg of EDC in 250 µl of 50 mM pyridine (pH 7.0). ADH (8 mg) was dissolved in 500 µl of 50 mM pyridine (pH 7.0). Protein solution was mixed with EDC, to create a final concentration of 13.0 mM and 23.9 mM, respectively. ADH (10 µl) was added to both samples. Immobilization mixture (4 µl) was applied on the glass slides, and the mixture was allowed to polymerize overnight at 4°C.

Preparation of CLEAs: Lyophilized LinB (38.4 mg) and BSA (38.4 mg) were dissolved in 9.6 ml of water, and precipitated by 28.8 ml of saturated ammonium sulfate (pH 8.0) for 45 min under stirring. Precipitated proteins were cross-linked with 3.1 ml of DPA [33], for 45 min. Precipitation and cross-linking steps were performed in an ice bath. After cross-linking, the suspension was centrifuged at 4,000 g for 20 min at 4°C. Supernatant was withdrawn, and resulting CLEAs were resuspended in 30.7 ml of saturated sodium hydrogen carbonate. Sodium borohydride (61.4 mg) was added to the solution, and was allowed to react for 30 min at 4°C under constant stirring. CLEAs were washed three times with 50 mM phosphate buffer (pH 7.5), and separated by centrifugation at 4,000 g for 10 min at 4°C.

Entrapment in ORMOCER: Lyophilized LinB (5 mg) and BSA (5 mg) were dissolved in 50 µl of 20 mM phosphate buffer (pH 7.5). The solution of ORMOCER AL657 was prepared by mixing 1.25 g of ORMOCER AL657, with 0.0125 g of photoinitiator Irgacure. The solution of ORMOCER KSK1926 was prepared by mixing 1.25 g of ORMOCER KSK1926 with 0.0225 g of Irgacure. ORMOCER AL657 or ORMOCER KSK1926 (50 µl) was slowly added to the dissolved proteins. The well-vortexed mixture (2 µl) was applied on glass slides, and kept in the air for 5 min. The immobilized layer was hardened below a high-pressure mercury lamp (100 W, Safibra, Czech Republic) for 60 min. The immobilized enzyme was stored at 4°C.

Entrapment in ORMOCER and cross-linking with GA: Lyophilized LinB (5 mg) and BSA (5 mg) were mixed with 50 µl of 20 mM phosphate buffer (pH 7.5) and 50 µl of solution of ORMOCER AL657. The well-vortexed mixture (2 µl) was applied on glass slides and kept in the air for 5 min. After hardening below a mercury lamp for 60 min, the glass slides were kept in the air for 10 min, and then exposed to 10% or 50% (v/v) GA for 5, 10, 15 and 30 min. The glass slides were then rinsed with distilled water to remove unreacted GA and stored at 4°C.

Entrapment of CLEAs in ORMOCER: CLEAs (50 µl) were vortexed with 50 µl of ORMOCER AL657, or KSK1926. The mixture (4 µl) was applied on glass slides and kept in the air for 5 min. An immobilized layer was hardened below a mercury lamp for 60 min, and then stored at 4°C.

Entrapment of CLEAs in lentiKats: Polyvinyl alcohol (1 g) and polyethylene glycol (0.6 g) were added to 6.6 ml of distilled water. The mixture was heated to 98°C, until polyvinyl alcohol particles were dissolved completely, then slowly cooled to 35°C. A suspension of wet CLEAs (1.8 g) was slowly added to the mixture, and carefully mixed. Small droplets of the resulting mixture were dripped on a smooth plastic plate and dried at 30°C. After drying down to 30% of initial mass, the resulting lenses were incubated in 0.1 M sodium sulfate for 40 min to re-swell. Prepared LentiKats were washed with 400 ml of distilled water and stored at 4°C [34].

Characterization of immobilized enzyme

Enzymatic activity was determined for the free enzyme, freshly immobilized enzyme, and immobilized enzyme exposed to 10 ml of 100 mM glycine buffer (pH 8.6) for 24 h at 4°C. Activity was measured in 10 ml of 100 mM glycine buffer (pH 8.6) at 37°C, using the Iwasaki method [35]. The substrate 1,2-dibromoethane was added to create a final concentration of 8.7 mM, determined by gas chromatograph (Trace GC, ThermoQuest Scientific, Great Britain). The reaction was started by addition of the enzyme to the reaction mixture, and terminated by mixing with 35% (v/v) nitric acid. Increasing concentrations of reaction products (bromide ions) were measured with mercuric thiocyanate and ferric ammonium sulphate at 460 nm by spectrophotometer Sunrise (Tecan, Switzerland). The amount of reaction products was determined from a calibration curve, prepared using sodium bromide as a standard solution.

Protein leaching was determined for the immobilized enzyme stored in 10 ml of 100 mM glycine buffer (pH 8.6) for 24 h at 4°C. The amount of leached proteins was assessed as the fluorescence of proteins in storage buffer, after removal of the glass slides. Emission spectra were recorded at excitation wavelength 280 nm using spectrofluorometer FluoroMax-4P (HORIBA Jobin Yvon, USA), with a 150-W xenon arc lamp light source, using a quartz cuvette with a 1-cm pathlength. Fluorescence of leached proteins was compared with the fluorescence of the immobilization mixture. Both specific activities and protein leaching were measured in triplicate, and the outlying data were excluded on the basis of Dixon's *Q* test [36]. The results were presented as its mean value.

Results and Discussion

Cross-linking

Cross-linking based on covalent binding of individual molecules is one of the most commonly used immobilization methods, due to its simplicity and rapidity. However, produced cross-linked enzymes are often gelatinous with low mechanical stability and difficulties in handling [37], therefore cross-linking of haloalkane dehalogenase LinB was performed on a glass support. Adapted procedure can be used for dehalogenase immobilization on any modified surface. Several cross-linking agents, with specificity for particular functional groups of LinB, were tested: GA, DPA, DSS and EDC (Table 2).

Cross-linking with GA: GA is a cross-linking agent of choice for enzyme immobilization due to advantages of low costs, simple and fast preparation, and low diffusion limitations. GA was also used for immobilization of LinB, although the content of amino groups available for cross-linking is low (Table 2). Immobilization of pure LinB led to significant loss of enzymatic activity (10.1 ± 0.7%, Figure 1). BSA was added into an immobilization mixture as a proteic feeder, containing more than 50 free amino groups (Table 2), to allow efficient cross-linking of an enzyme [38]. The effect of different BSA:LinB ratios was investigated (Table 1). The highest enzymatic activity was observed for the BSA:LinB ratio of 4:1 (48.1 ± 11.1%, Figure 1). Unfortunately, this immobilized layer suffered from the lack of mechanical stability, which was improved by addition of glycerol to the immobilization mixture. Glycerol is supposed to serve

Table 2: Predicted reactive groups available for immobilization by cross-linking[a] in the catalyst haloalkane dehalogenase LinB, and the scaffold protein bovine serum albumin BSA.A

Reactive group	Specification	Reactive groups on surface of LinB	Reactive groups on surface of BSA	Cross-linking reagent
Primary amine	Lysine N-terminus	4 1	55 1	GA DPA DSS EDC
Carboxylate	Aspartic acid Glutamic acid C-terminus	16 18 1	33 49 1	EDC EDC+ADH
Sulphydryl	Cysteine	0	8	Not tested

ADH: Adipic Acid Dihydrazide; BSA: Bovine Serum Albumin; DPA: Dextran Polyaldehyde; DSS: Disuccinimidyl Suberate; ED: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GA: Glutaraldehyde.

[a]Analysis of available reactive groups was performed using the crystal structure of LinB, RCSB PDB ID: 1MJ5, and the homology model of BSA, MODBASE ID: sp P02769 [48]. Reported numbers of available reactive groups may slightly differ from experimental ones due to protein surface dynamics, salt bridges between the reactive groups, and in BSA also due to lack of information about the first 27 amino acids MKWVTFISLLLLFSSAYSRGVFRRD.

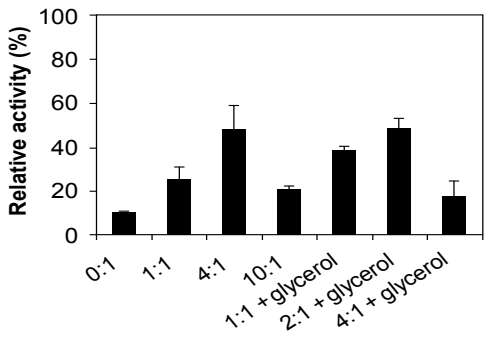

Figure 1: Effect of immobilization mixture composition on activity of haloalkane dehalogenase LinB cross-linked with GA. The composition of the immobilization mixture is expressed as a ratio of BSA and LinB (w/w), in the presence or absence of 25% (v/v) glycerol. The activity was measured with 1,2-dibromoethane, immediately after immobilization.

like a plasticizer to avoid formation of cracks in the enzyme layer, and results in better homogeneity and adhesion of the immobilization layer to the carrier surface [39]. The activity of the BSA:LinB ratio of 1:1, with addition of glycerol almost doubled, in comparison to the same ratio without glycerol. The BSA:LinB ratio of 4:1 exhibited the opposite trend, where the addition of glycerol led to a decrease of enzymatic activity. The best formulation showing maximal enzymatic activity (48.4 ± 4.7%), and good mechanical stability at the same time, was achieved by the co-immobilization of BSA and LinB in the ratio of 2:1, with the presence of 25% (v/v) glycerol. This mixture composition was selected for further optimization (Figure 1).

The effect of different cross-linking times on LinB activity and protein leaching was also investigated. Enzymatic activity and protein leaching decreased proportionally with cross-linking time (Figure 2). The highest enzyme activity, 50.4 ± 3.4%, was obtained using cross-linking time of 15 min. This immobilization time was insufficient, significant protein leaching (16.3 ± 2.9%) was observed. On the contrary, 60 and 150 min of immobilization led to extensive cross-linking, which reduced both the protein leaching and the enzymatic activity. The optimal cross-linking time for immobilization of LinB with BSA, in a ratio of 1:2, was 30 min with an activity retention of 46.5 ± 3.7%, after exposure to buffer. A negligible leaching of 5.6 ± 1.9% was observed for this variant (Figure 2).

APTS-activated glass slides were also tested for immobilization of LinB, to improve mechanical stability of the immobilized layer. APTS provided amino groups for coupling amine-containing ligands, in this case, LinB and BSA, using GA. The immobilization of LinB on APTS-modified surfaces led to a homogeneous layer, without any cracks. The enzyme retained activity of 39.1 ± 2.8% (Figure 3A). However, a further significant activity decrease was observed after 24 hours of exposure to glycine buffer (23.7 ± 0.0%), although the protein leaching was not detected (Figure 3B).

Cross-linking with DPA: Cross-linking agent GA is a small and highly reactive molecule which can penetrate into the interior of the protein, react with catalytic residues, and cause possible deactivation of an enzyme. This effect can be avoided by using bulky polyaldehydes as the cross-linkers, which are obtained by oxidation of dextrans [40]. Enzyme LinB was cross-linked in the presence of BSA, with 2.4 µl and 4.5 µl DPA. Enzymatic activity after cross-linking was 38.0 ± 5.1% and 43.8 ± 3.7%, respectively (Figure 3A). Exposure to glycine buffer for 24 h led to a decrease in activity (18.7 ± 9.0%), for an enzyme cross-linked with 4.5 µl DPA. An immobilized layer prepared using 2.4 µl DPA exhibited limited mechanical stability in the buffer, which resulted in a total loss of enzymatic activity. Leaching of proteins from immobilized layer was confirmed for both variants (Figure 3B). Cross-linking on APTS-activated glass slides provided higher adhesion and cohesiveness of the immobilized layer, even though the observed activity after exposure to buffer was 11.3 ± 1.0% (Figure 3A).

Cross-linking with DSS: Amine-reactive cross-linker, DSS, was further tested for its immobilization capability of LinB and BSA. Two different concentrations of this reagent were tested, 1.48 mM and 14.8 mM. Enzymatic activity for both variants was approximately 40% (Figure 3A), and a higher concentration of cross-linker (14.8 mM), had no effect on enzymatic activity. Exposure of the immobilized enzyme to glycine buffer for 24 h resulted in loss of enzymatic activity, caused by the complete or significant leaching of proteins, which was verified by fluorescence measurements (Figure 3B). Different activity retention, observed after cross-linking with amine-reactive GA, DPA and DSS, reasserts the importance of empirical determination of the best cross-linker.

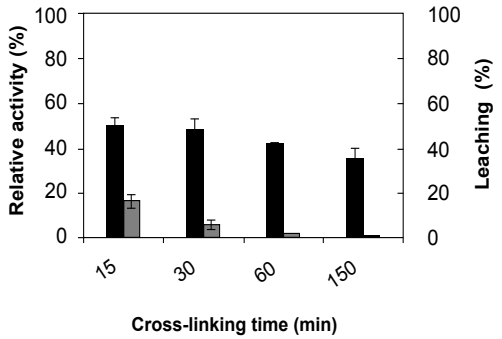

Figure 2: Effect of cross-linking time on activity of haloalkane dehalogenase LinB. Activity was measured with 1,2-dibromoethane, immediately after immobilization with GA (black column). Effect of cross-linking time on protein leaching, after 24 hours of exposure to 100 mM glycine buffer, pH 8.6 (grey column).

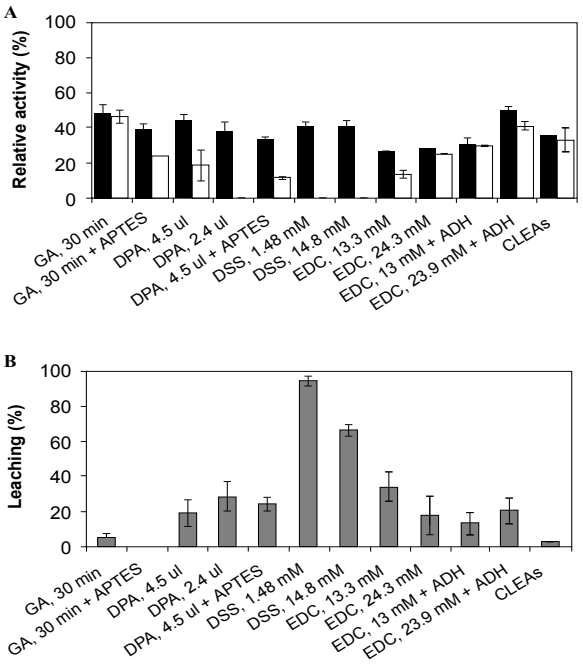

Figure 3: Effect of cross-linking on haloalkane dehalogenase LinB activity. Activity was measured with 1,2-dibromoethane after immobilization (black column), and after 24 hours of exposure to 100 mM glycine buffer, pH 8.6 (white column, A). The effect of cross-linking on protein leaching after 24 hours of exposure to 100 mM glycine buffer, pH 8.6 (B).

Cross-linking with EDC: LinB surface is rich of free aspartic and glutamic acid side chains (Table 2), which can be used for cross-linking with EDC. The reaction provides an active-ester intermediate, and an amide bond is formed in the presence of a primary amine. The interaction of EDC with proteins favours an acidic environment, because a formed intermediate undergoes rapid hydrolysis at higher pH [41]. The requirement of low pH for activation is not compatible with the nature of enzyme LinB, therefore activation of carboxyl groups of LinB in pH 7.0 was investigated. Two concentrations of EDC were tested. Enzymatic activity was 26.3 ± 0.4% for 13.3 mM

EDC and 28.0 ± 0.1% for 24.3 mM EDC (Figure 3A). Activity of immobilized enzyme decreased to 13.6 ± 2.5% and 25.2 ± 0.3%, with respect to cross-linker concentration after exposure to buffer. The lower concentration of the cross-linker in the immobilization mixture was not sufficient for cross-linking of all enzyme molecules, and more than 30% of protein was leached from the immobilized layer during storage in a glycine buffer (Figure 3B).

Cross-linking with EDC in the presence of ADH: EDC can be also used to couple carboxylic groups to hydrazides. If hydrazide is used for coupling, cross-linking requires only carboxylic groups from the protein. The use of hydrazide is advantageous if the protein is poor in free amino groups, but has abundant glutamic or aspartic acids in its side chains. The abundance of reactive groups in side chains in LinB is shown in table 2. Activity of LinB cross-linked with 13 mM EDC, in the presence of ADH was 30.0 ± 4.2%, and the same activity was observed after exposure to buffer for 24 h (Figure 3A). Surprisingly, fluorescence measurement showed that, 13.3 ± 6.3% of proteins were leached from the immobilized layer (Figure 3B). The best results were obtained for LinB cross-linked with 23.9 mM EDC, in the presence of ADH. The initial activity was 50.3 ± 1.5%, and the enzymatic activity decreased to 41.2 ± 2.1%, after exposure to glycine buffer (Figure 3A). The decrease of activity was probably caused by protein leaching (Figure 3B). Cross-linking of LinB with EDC, in the presence of ADH, seems to be a good alternative to cross-linking with GA, providing similar activity retention.

Preparation of CLEAs: Carrier-free immobilization of dehalogenase LinB, in a form of CLEAs, was also tested. The CLEAs were successfully prepared by co-aggregation of LinB and BSA, to obtain an efficiently cross-linked enzyme, despite of low content of the surface amine residues (Table 2). DPA was used as a mild cross-linker for preparation of CLEAs, owing to data reported by Mateo et al. [40]. They observed higher activity retention for CLEAs of several enzymes, using DPA, compared with commonly used GA. Freshly prepared dehalogenase CLEAs exhibited activity of 35.5 ± 3.6% (Figure 3A). These CLEAs retained similar activity (33.0 ± 6.7%), after a 24-hour incubation in the glycine buffer, followed by subsequent separation by centrifugation. Minimal protein leaching (2.8 ± 0.5%) was observed (Figure 3B). This approach resulted in higher mechanical stability of the immobilized enzyme, compared to cross-linking on a glass slide, and facile separation from the reaction media by centrifugation or decantation. This carrier-free immobilization is also of interest for industrial applications due to low production costs [42].

Entrapment

The entrapment of haloalkane dehalogenase LinB in sol-gel was tested, since the inclusion of an enzyme in a polymer network should avoid any negative influence on the protein structure. Sol-gels prepared from inorganic precursors belong to widely used materials for entrapment. LinB was entrapped in organically modified sol-gel ORMOCER [43]. This hybrid material combines desirable properties of inorganic solids–hardness and chemical resistance–with those of organic polymers–toughness and elasticity. These attractive features and biocompatibility make organically modified sol-gels promising materials for enzyme immobilization in optical and electrochemical biosensors [44-46].

Entrapment in ORMOCER: Two types of ORMOCER were compared for immobilization of LinB. Activity of entrapped LinB was similar in ORMOCER AL657 and KSK1926, 38.2 ± 5.4% and 42.7 ± 2.3%, respectively (Figure 4A). Total loss of enzymatic activity was observed after exposure to glycine buffer, for both matrices. ORMOCER, similarly to other sol-gels, is a highly porous material enabling fast diffusion of a substrate, but with high tendency to cause enzyme leaching. Fluorescence measurements confirmed significant leaching, approximately 80%, during storage in glycine buffer (Figure 4B). Additional covalent attachment or formation of bigger enzyme complexes was required to prevent enzyme leaching.

Entrapment in ORMOCER and cross-linking with GA: Cross-linking of LinB entrapped in ORMOCER AL657 by using GA, was tested to prevent enzyme leaching. Different cross-linker concentrations and different cross-linking time were tested. Firstly, the entrapped enzyme was exposed to 50% (v/v) GA for 5, 10, 15 and 30 min. Then, the immobilized layer was carefully rinsed with distilled water to remove any un-reacted cross-linker, and prevent non-reproducible and too extensive cross-linking. Measured enzymatic activity and protein leaching correlated with the cross-linking time. The highest activity (22.6 ± 2.7%, Figure 4A) was determined for LinB entrapped in ORMOCER, and cross-linked with GA for 5 min. Activity was less than 10% after exposure to glycine buffer (which was associated with protein leaching of 10.5 ± 4.0%) for 24 h (Figure 4B). Similar retention of enzymatic activity after exposure to buffer was observed for the cross-linking time of 10 min. Cross-linking of entrapped enzyme for 15 and 30 min led to minimal activity (less than 5%), and negligible leaching. The low protein leaching observed in all tested variants indicated that the loss of activity was mainly caused by enzyme inactivation. The concentration of GA was reduced to 10% (v/v), to prevent extensive cross-linking. This milder cross-linking for 5, 10 and 15 min led to higher initial enzymatic activity. However, the activity was reduced to less than 12% after exposure to glycine

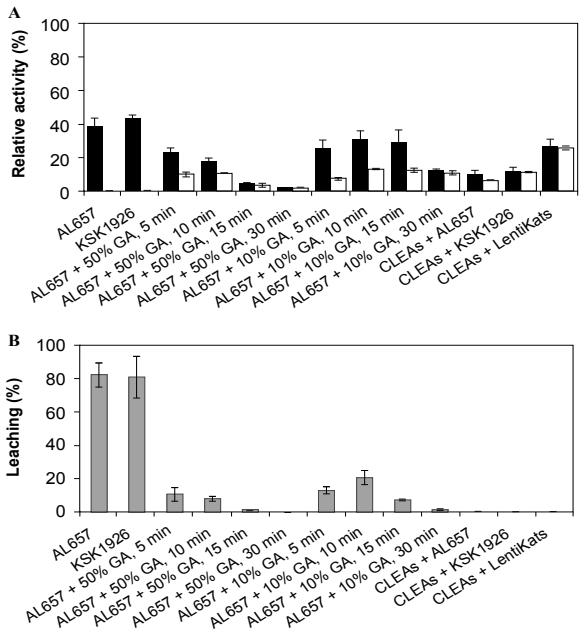

Figure 4: Effect of entrapment on activity of haloalkane dehalogenase LinB. The activity was measured with 1,2-dibromoethane, after immobilization (black column), and after 24 hours of exposure to 100 mM glycine buffer, pH 8.6 (white column, A). The effect of entrapment on protein leaching after 24 hours of exposure to 100 mM glycine buffer, pH 8.6 (B).

buffer, and significant protein leaching was observed in all variants (Figure 4B). The time of cross-linking, 30 min, was probably too long, and resulted in a significant activity decrease (11.3 ± 1.1%). Protein leaching for this variant was negligible, with no significant change of activity observed after exposure of the immobilized enzyme to glycine buffer. To summarize, the enzyme preserved higher activity in milder conditions (shorter cross-linking time, or lower concentration of cross-linker), but exhibited more intensive leaching. More intensive cross-linking (longer exposition or higher concentration of cross-linker) produced a strongly immobilized enzyme with a low level of leaching, but with extensively reduced activity. Combination of these reciprocal effects resulted in similar activity of the final immobilization material.

Entrapment of CLEAs in ORMOCER: CLEAs were prepared by co-precipitation of LinB and BSA, and entrapped in both types of ORMOCER. ORMOCER AL657 is not suitable for the immobilization of CLEAs due to low miscibility of ORMOCER solution, with CLEA suspension. The resulting enzymatic activity after exposure to buffer was only 6.3 ± 0.2% (Figure 4A). Better miscibility was achieved with ORMOCER KSK1926, reaching the activity of immobilized LinB 10.9 ± 2.7% (Figure 4A). This activity was also retained after exposure to glycine buffer, and no protein leaching was observed (Figure 4B).

Preparation of LentiKats: The second matrix tested for entrapment of LinB was polyvinyl alcohol hydrogel. A mild immobilization method, based on partial drying of the hydrogel at room temperature, provided lens-shaped particles (LentiKats), exhibiting good stability and easy separation [34]. However, free enzymes, owing to their smaller size, can diffuse out of the hydrogel and consequently, leach into the buffer [47]. In order to efficiently entrap LinB in LentiKats, the size of the enzyme had to be increased by formation of CLEAs. While the activity of freshly prepared CLEAs was 35.5 ± 3.6%, a slight decrease of activity was observed after entrapment of CLEAs in LentiKats (26.2 ± 4.8%, Figure 4A). This activity was fully retained after 24-hour exposure of LentiKats to a glycine buffer, which was in good agreement with a negligible degree of protein leaching (0.2 ± 0.0%, Figure 4B). High retention of activity after immobilization, and excellent mechanical stability, enables utilization of this immobilization strategy in biocatalysis.

Conclusions

Haloalkane dehalogenase LinB was immobilized by using different methods of cross-linking and entrapment, and by combination of both methods. The activity and stability of the immobilized enzyme strongly depended on the applied method. The best results were obtained by using cross-linking by GA, providing high activity retention with negligible leaching. Co-immobilization of LinB with BSA, acting as a proteic feeder, was highly favourable due to a significant increase of immobilization efficiency, and retention of activity. This method has recently been successfully applied for immobilization of LinB on a fiber tip of the optical biosensor [21]. Two other tested amine-reactive cross-linkers were less efficient due to weaker cross-linking activity. These results indicated that five primary amines available on the protein surface may not be sufficient for cross-linking by using mild cross-linkers, even in the presence of BSA. Cross-linking using carboxylic acid residues could be a solution for the immobilization of LinB, possessing a low amount of amino groups. EDC and ADH, utilising the carboxylic groups and mild amine-reactive cross-linkers, produced an active and mechanically more stable immobilized layer.

Immobilization of LinB by the entrapment procedures was also tested. Enzyme entrapment into ORMOCER produced mechanically stable immobilized layer. However, high porosity of the matrix led to extensive protein leaching. Simple cross-linking and preparation of CLEAs was tested for improved immobilization of LinB in ORMOCER. Low retention of enzymatic activity was observed due to leaching or enzyme inactivation. Prepared dehalogenase CLEAs were also entrapped in polyvinyl alcohol particles, LentiKats. Although the enzymatic activity was lowered in comparison to simple cross-linking, LentiKats provide a good potential for broad application in biocatalysis and bioremediation. Robustness of immobilized biocatalyst, facile separation from the reaction media, and repeated use are valuable features, increasing the industrial potential of haloalkane dehalogenases.

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