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Expression, One-Step Purification, and Immobilization of HaloTag™ Fusion Proteins on Chloroalkane-Functionalized Magnetic Beads

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Abstract The presented work introduces a novel method to immobilize enzymes either purified or directly out of a crude extract onto magnetic particles in the micrometer range. This method is based on the creation of a fusion protein consisting of the enzyme of choice and a mutant dehalogenase. The dehalogenase gene is commercially available from the company Promega under the name HaloTag™. When the fusion protein is contacted with magnetic beads having chemically synthesized, chloroalkane ligands on their surface, the dehalogenase and the ligand undergo a covalent coupling leading to stable and spatially defined immobilization. The principle was proved with a lipase fused to the HaloTag™ gene and magnetic poly(methyl)methacrylate beads as carriers. The solubility of the tagged lipase was strongly increased by fusion of the *malE* gene at the N-terminal end of the HaloTag™ lipase gene. This tripartite protein was purified on amylose resin and used for immobilization. About 13 μg protein could be immobilized per 1 mg of beads within a few minutes. Due to the defined binding site, no activity loss was observed in the course of the immobilization. The resulting enzyme carrier was tested with the same beads up to six times for lipase activity over a storage period of 36 days at 8 °C. No loss of activity was found during this time.

Keywords Enzyme immobilization · HaloTag™ · Fusion protein · Magnetic bead · Dehalogenase · Expression

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Introduction

Enzyme immobilization is an important tool in biotechnology because it allows the recovering of valuable enzymes from the reaction solutions for repeated use. Furthermore, it is acknowledged that the immobilization stabilizes many enzymes [1].

Apart from the use of whole cell catalysts, the traditional way of enzyme immobilization requires purified enzyme solutions in order to prevent the immobilization of competing proteins. In many cases, it is necessary to purify an enzyme from crude cell extract usually by precipitation and solid–liquid separation steps, followed by chromatographic steps, if high purities are required. After purification, one or more additional steps for immobilization follow. In many cases, the costs of the purification procedure are the main cost factors in the process of enzyme immobilization [2]. Therefore, new procedures are urgently needed to reduce the costs of the purification and immobilization of enzymes onto carriers.

A major step towards this aim is the production of enzymes by recombinant DNA technology. With modern expression systems, an enzyme or therapeutic protein can be produced by up to about 30% from the total protein in a host strain like *Escherichia coli*. Furthermore, affinity tags can be added to the protein by fusing the respective genes. The resulting tagged fusion proteins show high affinities towards tailored ligands and chromatographic matrices. Together with the overproduction in recombinant cells, this usually allows to purify a fusion protein in one chromatographic step [3]. In many cases, the affinity systems used for protein purification have also been tested as a way to achieve spatially oriented immobilization. The motivation is to achieve an immobilization by which the active sites of the enzyme are not blocked or sterically hindered, resulting in a lower degree of activity loss than in the case of statistically oriented immobilization. For example, Le et al. [4] produced a streptavidin–cellulose binding fusion protein and adsorbed it via the cellulose-binding domain to Avicel, a microcrystalline cellulose.

Linking these two approaches, there have also been attempts to combine the purification and immobilization in a one-step procedure. Ong et al. [5] fused the cellulose-binding domain of an exoglucanase to a β-glucosidase (from *Agrobacterium* sp.). The cellulose-binding domain acts as an affinity tag for simultaneous purification and immobilization on cellulose. Mateo et al. [6] developed a multi-functional chelate epoxy support for purification of poly-his-tagged proteins using the IMAC system and final covalent immobilization by the epoxy groups on the same matrix. Besides more common matrices like agarose or cellulose, also magnetic microspheres have been described as possible carriers for immobilization via affinity ligands. Bilkova et al. [7] used poly(2-hydroxyethyl methacrylate-co-ethylene dimethacrylate) magnetic microspheres modified with hydrazide to bind galactose oxidase from *Dactylium dendroides* through the carbohydrate part. The remaining activity after immobilization was practically 100%.

In summary, it can be said that all combined purification/immobilization procedures the authors are aware of either use adsorption or a bioaffinity mechanism to bind the enzyme onto the carrier surface. However, both of these techniques have their drawbacks. In the case of adsorption, binding generally is too weak to guarantee an attachment of the enzyme over many reaction cycles under harsh conditions. In the case of bioaffinity, the respective ligands are either too weak (e.g., IMAC systems) or the required bioligands are expensive (e.g. biotin).

In the following sections, a new way of combining purification and oriented immobilization by generating fusion proteins, including a mutant dehalogenase, shall be described. Dehalogenases are hydrolases which cleave carbon–halogen bonds in halogenated compounds. The enzyme forms a covalent enzyme–substrate complex during the

nucleophilic attack of an aspartic residue on the halogenated substrate. The ester intermediate is resolved by a second nucleophilic attack of a water molecule. This water molecule is activated by a histidine residue which is part of a catalytic triad. The HaloTag™ protein is a mutant dehalogenase in which the histidine residue is replaced by a phenylalanine which impairs the ester hydrolysis step and keeps the enzyme covalently bound to the substrate [8]. This allows covalent binding of ligands to the enzyme. The HaloTag™ interchangeable labeling technology uses the covalent interaction of the HaloTag™ protein and specific fluorescent ligands for imaging eukaryotic cells that express the HaloTag™ protein or fusion proteins between the HaloTag™ and the protein of interest. Besides the binding of small ligands, the enzyme is able to bind to solid surfaces, for example the sepharose-based HaloLink™ resin. This can be used for immobilization of proteins fused to the HaloTag™ protein to study protein–protein interactions for example [9].

For covalent immobilization of enzymes, usually aggressive substances like glutaraldehyde or carbodiimide are used which often partially inactivate enzymes. Fusions with the HaloLink™ technology, by contrast, do not need any aggressive compounds and should therefore be superior to chemical immobilization. The main disadvantages of the HaloTag™ system as offered by the Promega company are (1) that the vectors were mainly developed for expression in mammalian cells which makes production of biotechnologically interesting enzymes expensive and (2) the high amount of inactive protein produced as inclusion bodies during expression of genes fused to the HaloTag™ gene. In the following sections, the improvement of HaloTag™ gene expression by soluble fusion proteins in *E. coli*, the development of a binding matrix based on magnetic beads, and the immobilization of a lipase as a model system for demonstrating the potential of this technology will be presented.

A lipase was chosen since these enzymes represent a biotechnologically important class of highly versatile biocatalysts. The enzyme activity can be determined easily, quickly, and accurately, for example by the hydrolysis of nitrophenyl acyl esters. The lipase from *Bacillus thermocatenulatus* [10] used in our study is thermostable and allowed us to test the immobilized enzymes even under high temperatures. For gene expression, the positively regulated L-rhamnose-inducible promoter was used. This tightly regulated promoter was used for the construction of very stable expression vectors [11, 12] with an excellent performance in high-cell-density fermentations [13].

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

E. coli JM109 [14] was used as host for recombinant plasmids and for gene expression. The strains were cultured in LB liquid media (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) and on LB agar plates supplemented with ampicillin (100 μg/ml) at 37 °C and for induction of the rhamnose promoter at 30 °C. The plasmid pHT2 was purchased from Promega Corp. (Madison, WI, USA). The rhamnose-inducible expression vectors pJOE4056.1 and pJOE3075.1 were published in [15].

Plasmid Constructions

The modified dehalogenase gene in the plasmid pHT2 was PCR-amplified with the primers s4265 (5′-AAA AAA CAT ATG GGA TCC GAA ATC GGT AC) and s4967 (5′-AAA AAA

AGA TCT GAT ATC CAG CCC GGG GAG CCA GC) without stop codon, cleaved with endoR *Nde*I and *Bgl*II, and inserted between the *Nde*I and the *Bam*HI site of the rhamnose-inducible *E. coli* expression vector pJOE4056.1 to give pHM339.1. The lipase gene was PCR-amplified from plasmid pT-BTL-2 [16] without a start codon and signal sequence for export using the primers s4595 (5′-AAG GCG CCG CAT CCC CAC GCG CCA AT) and s4596 (5′-AAA TGT ACA TTA AGG CCG CAA ACT CGC CAA C). The gene was fused to the dehalogenase gene by cleavage of pHM339.1 with endoR *Eco*RV and *Bsr*GI and ligation with the PCR fragment which was cleaved by *Sfo*I and *Bsr*GI (pHM365.21). Finally, the HaloTag™ lipase fusion gene was isolated again from pHM365.21 as *Bam*HI–*Hin*dIII fragment and inserted into pJOE3075.3 cleaved with the same restriction enzymes to give pHM367.4. In pHM367.4 the lipase gene (*lip*) is fused to HaloTag™ as well as to the *malE* gene.

Production and Purification of the MalE–HaloTag™–Lipase Fusion Proteins

For production of the MalE–HaloTag™–lipase fusion protein, an overnight culture of *E. coli* JM109 pHM367.4 was diluted (1:100) in 30 ml LB liquid medium with ampicillin, and the culture was grown at 37 °C to an optical density of OD_{600}=0.4. Then, 0.2% L-rhamnose was added for induction of the rhamnose promoter and the culture was incubated at 30 °C. After 7 h of induction, the cells were harvested by centrifugation, washed in 0.05 M sodium phosphate buffer, pH 7.0, and lysed in 3 ml 0.1 M sodium phosphate buffer, pH 7.0, by ultrasonication. The crude extract was cleared by centrifugation at 20,000 rpm, 15 min, 4 °C in a Sorvall SS34 rotor, and after that, the MalE–HaloTag™–lipase fusion protein was purified by affinity chromatography on amylose resin (New England Biolabs). Amylose resin (4 ml) was packed in a 20-ml column and washed 10 times with 2 ml of column buffer (100 mM $NaPO_4$, 0.2 M NaCl, pH 7.4) before use. Crude extract (4 ml) was passed over the resin at 4 °C for 1 h. The column was then washed in a stepwise manner with 40 ml column buffer again. Finally, the protein was eluted in three steps with 1 ml of the same buffer each plus 10 mM maltose. The first two fractions of the eluent were combined and used for enzyme activity determination and immobilization.

Enzyme Activity Assay

The activity of the non-immobilized MalE–HaloTag™–lipase was determined by adding 10 µl crude extract or 10 µl purified enzyme to 990 µl reaction buffer (0.05 mM $NaPO_4$, 5 mM Na-desoxycholate, 0.8 mM *p*-nitrophenylpalmitate, pH 7.2). The lipase activity immobilized on magnetic beads was determined by resuspending the desired volume bead suspension in reaction buffer to a final volume of 1 ml. The reaction mixture was incubated at 30 °C under shaking in a thermomixer (Eppendorf AG, Germany). After 1 min, the beads were separated and the supernatant transferred to a cuvette and measured in a spectrophotometer at 410 nm. One unit lipase corresponds to the release of 1 µM *p*-nitrophenol (molar extinction coefficient=15,200 $mol^{-1} cm^{-1}$) per minute [10]. Protein concentrations were determined according to [17] using bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the method of Laemmli [18]. Proteins in crude cell extracts were separated into soluble and insoluble fractions by centrifugation at 20,000 rpm for 15 min at 4 °C in a

Sorvall SS34 rotor. Gels (12% polyacrylamide) were stained with Coomasie blue. A molecular weight standard (Roth-Mark, Carl Roth, Karlsruhe, Germany) was used as reference.

Synthesis of Chloroalkane-Functionalized Magnetic Beads

All chemicals used were of reaction quality from VWR International except for 2-chloroethanol (analytical quality), poly(vinyl)alcohol (reaction quality), and acrylic acid methyl ester (reaction quality) from Acros Organics and 1,4-divinylbenzene (reaction quality) and methylene blue (reaction quality) from Sigma-Aldrich. The used water was Milli-Q quality (Milli-Q Academic, Millipore, France).

For the synthesis of the magnetite gel, 17.2 g iron(II) chloride and 28.2 g iron(III) chloride were dissolved in 1,200 ml water and heated to 85 °C under an inert nitrogen gas atmosphere. Fifty six milliliters of ammonia (25% w/w) was added quickly to the reaction mixture, and afterwards 30 ml of oleic acid was added in a dropwise manner. The reaction mixture was stirred (700 rpm) until the magnetic gel was formed [19].

Magnetic poly(methyl)methacrylate particles were synthesized by dissolving 25 g poly (vinyl)alcohol in 700 ml water and 25 g sodium chloride in 300 ml water, followed by the combination of the two solutions (water phase). Twenty grams of magnetic gel was dissolved together with 2 g benzoylperoxide in 80 ml hexane (oil phase). The water phase was heated to 60 °C and 1 ml methylene blue was added. Ten milliliters of 1,4-divinylbenzene and 95 ml acrylic acid methyl ester were admixed to the oil phase and combined with the water phase at 60 °C. The reaction mixture was stirred (700 rpm) for 4 h at 85 °C under an inert nitrogen gas atmosphere [20].

For the synthesis of the spacer, 1.5 g polymer particles were brought to reaction with 25.5 ml hexane-1,6-diamine (50 wt.% in water) for 24 h at 60 °C. After this and the following reaction steps, the particles were thoroughly washed five times with deionized water, if not mentioned otherwise. During the decanting of spent wash solutions, the particles were retained easily by means of a strong hand magnet. The particles then reacted with 16.5 ml pentanedial (50 wt.% in water) for 24 h at room temperature. Afterwards, the spacer was enlarged with ethanolamine for 24 h at room temperature. Then, the spacer double bonds were reduced with 60 mg sodium boron hydride in 100 ml water, followed by an activation step with 20 ml sodium hydroxide (10 wt.% in water) for 5 h at room temperature and then reacted with 5.5 ml 2-chloroethanol for 24 h at room temperature. This step was followed by washing of the particles five times with tetrahydrofuran and five times with water. Afterwards, a second activation, the same as above, was conducted. After three times washing with tetrahydrofuran, a final reaction took place with 11 ml 1,6-dichlorohexane and 5 ml triethyl-amine in 100 ml tetrahydrofuran for 24 h at 60 °C. The particles were stored in water at 4 °C after washing five times with tetrahydrofuran and five times with water.

Immobilization of MalE–HaloTag™ Fusion Proteins on Chloroalkane-Functionalized Magnetic Beads

The magnetic beads functionalized by the dehalogenase-specific ligands were mixed by gently inverting the vessel to obtain a uniform suspension. Then, 10–80 µl of magnetic bead suspension was dispensed into a 2-ml microcentrifuge tube and a magnetic field was applied for 1 min to pull the beads to the side of the tube (Magnetic Separation Rack from New England Biolabs). The supernatant was discarded carefully and 400 µl of binding

buffer [0.1 M Tris–HCl, 0.15 M NaCl, 0.01% IGEPAL CA-630 (Sigma-Aldrich), pH 7.6] was added. The solution was mixed thoroughly by inverting the tube. Then, a magnetic field was applied for 1 min to remove the supernatant. This step was repeated three times. Finally, the beads were resuspended in 100 µl of binding buffer. The HaloTag™ fusion protein was added to the equilibrated beads. The tube was incubated by mixing on a tube rotator for 60 min at room temperature. After applying a magnetic field for 1 min, the supernatant was removed (and saved for analysis). The beads were washed with 1 ml binding buffer four times. Finally, the beads carrying covalently attached HaloTag™ fusion protein were resuspended in the desired volume of a buffer compatible with downstream applications. The sets of experiments investigating the immobilization efficiency were repeated three times. The data shown in Figs. 4, 5, 6, and 7 represent the calculated mean values, the resulting standard deviation being less than 15% in all cases.

Results

Construction of the HaloTag™–Lipase Expression Plasmids

The plasmid pHT2 contains the modified dehalogenase gene between the CMV promoter and SV40 late poly(A) signal for expression in mammalian cells on a pUC-derived *E. coli* vector. The HaloTag™ gene from pHT2 was amplified by PCR without stop codon and the DNA fragment inserted into the L-rhamnose-inducible *E. coli* expression vector pJOE4056.1. In this way, the eGFP was fused to the C-terminal end of the dehalogenase gene. The eGFP gene was removed from pHM339.1 and replaced by the lipase gene from *B. thermocatenulatus* (pHM365.21). In the original host, the lipase is secreted into the extracellular space via a *sec*-dependant signal sequence. For intracellular production in *E. coli*, the lipase gene was amplified from plasmid pT-BTL-2 [16] without a signal sequence for protein export. The expression of the dehalogenase–lipase fusion gene was induced by growing *E. coli* JM109 pHM365.21 in the presence of L-rhamnose and analyzed for enzyme production by SDS–PAGE analysis and lipase activity assays. The cells produced small amounts of active enzyme and soluble protein. Most of the protein was found in the insoluble fraction of the cell extract (Table 1 and Fig. 1). This inclusion body formation was probably caused by the mutant dehalogenase. Insoluble protein had already been seen before in *E. coli* by the expression of the unfused dehalogenase gene, whereas the lipase was highly soluble (data not shown). There are various ways to improve folding of proteins in *E. coli*. A well-known way is to fuse the genes of problematic proteins with others like *malE* or *nusA* which seem to work like chaperons during protein biosynthesis [21–23]. For this purpose, the HaloTag™–lipase fusion gene was cut out from plasmid pHM365.21 and inserted downstream of the *malE* gene of the rhamnose-inducible expression vector

Table 1 Specific lipase activity (U/mg protein) in crude extracts from *E. coli* JM109-pHM365.21 and JM109-pHM367.4.

	Basal activity (U/mg)	7 h induction by rhamnose (U/mg)
JM109-pHM365.21	0.14	13.7
JM109-pHM367.4	0.28	27.5

The strains were grown in LB liquid medium for 9 h at 30 °C with 0.2% L-rhamnose added after 2 h and without inducer (basal activity)

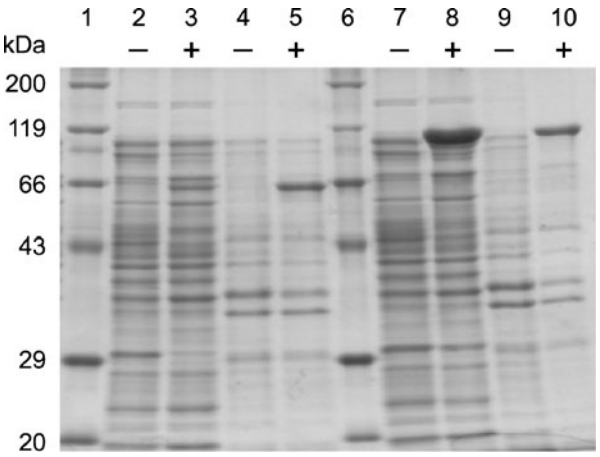

Fig. 1 SDS–PAGE of soluble and insoluble crude extract of *E. coli* JM109 pHM365.21 and JM109 pHM367.4. *Lanes 1*, *6* marker; *2*, *3* soluble crude extract from JM109 pHM365.21; *4*, *5* insoluble fraction of JM109 pHM365.21; *7*, *8* soluble crude extract from JM109 pHM367.4; *9*, *10* insoluble fraction of JM109 pHM367.4; (−) non-induced cells; (+) L-rhamnose-induced cells

pJOE3075.3. In this new plasmid pHM367.4, three genes are fused, the *malE* gene at the N-terminal end, the HaloTag™ gene in the middle, and lipase at the C-terminal end.

This construction exhibited twice the lipase activity in the cell crude extract compared to pHM365.21 (Table 1) and by SDS–PAGE; it was shown that most of the recombinant protein was located in the soluble fraction of the cell extract (Fig. 1). The *malE* gene obviously improved the expression of the fusion gene by a factor higher than two to about 20% of the total protein. In addition, it improved the solubility of the fusion protein by about the same extent and finally, the presence of the maltose binding protein as part of the fusion protein enabled the purification of enzyme by affinity chromatography.

Production and Purification of the MalE–HaloTag™–Lipase Fusion Protein

For small-scale production of the MalE–HaloTag™–lipase protein, *E. coli* JM109 pHM367.4 was grown in LB liquid medium and the rhamnose promoter induced by adding 0.2% rhamnose for 7 h. The cells were harvested, lysed, and the fusion protein was purified on amylose resin. The purification was analyzed by SDS–PAGE as shown in Fig. 2. The specific activity was enriched by a factor of about 1.7 by one-step purification. From 30 ml cells (OD_{600}=5), about 0.7 mg protein was obtained (Table 2).

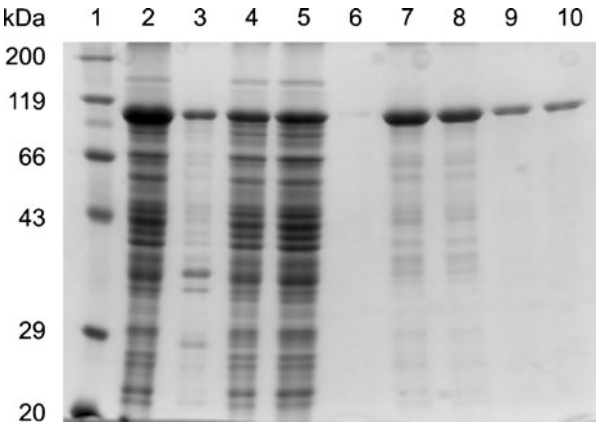

Fig. 2 Purification of the MalE–HaloTag™–lipase by amylose resin. *Lane 1* marker, *2* crude extract from JM109 pHM367.4, *3* insoluble fraction of JM109 pHM367.4, *4* supernatant after pumping through a column with amylase resin, *5* the first wash step, *6* the eighth wash step, *7* the first eluate, *8* the second eluate, *9* the third eluate, *10* the fourth eluate

Table 2 Purification of the MalE–HaloTag™–lipase fusion protein from *E. coli* JM109-pHM367.4 by amylose resin affinity chromatography.

	Volume (ml)	Protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification factor
Crude extract	4	6.42	27.5	176	100	1
Eluate (AC)	2.6	0.71	47.7	34	19	1.74

AC affinity chromatography

Production of the Chloroalkane-Functionalized Magnetic Beads

For the immobilization of the MalE–HaloTag™–lipase fusion protein, magnetic poly (methyl)methacrylate polymer particles were synthesized. Hexane-1,6-diamine was added as a spacer. The spacer was extended by ethanolamine and functionalized by successive reactions with 2-chloroethanol and 1,6-dichlorohexane resulting in the ligand shown in Fig. 3. The produced beads had the following properties: specific surface area 2.2 m^2/g, density 1.21 g/cm^3, magnetic saturation 13.2 A m^2/kg, and non-porous spherical shape with an average bead size of 4.0 μm (size distribution 0.5–40 μm).

Parameters Affecting the Immobilization of the MalE–HaloTag™–Lipase Fusion Protein

In the following stage, the amount of protein immobilized on the magnetic beads, the binding velocity, and the stability of the fusion protein on the beads were determined. In the first experiment, 1 mg beads was incubated with various amounts of protein ranging from 5 to 55 μg. With lower amounts of enzyme offered (up to about 25 μg), most of the activity was found on the beads (Fig. 4). At this concentration, more than 50% of the activity were immobilized on the beads which corresponds to about 13 μg protein per 1 mg beads. In a second experiment, the protein concentration was kept constant at 25 μg protein (1,300 mU) and various amounts of magnetic beads from 0.1 to 8 mg were added. With 1 mg beads, again about 12 μg protein was bound (Fig. 5). The sum of bound and non-immobilized enzyme activity remained constant under all conditions which showed that there is no loss by inactivation. The immobilization is very rapid as shown in Fig. 6. After 2 min, about 45% of the enzyme are already bound. The amount of bound enzyme increased further from 45% to 55% within the next 20 min and then remained constant. The reaction of the Halo–Tag™ protein with the functional groups on the beads was supposed to be highly specific. Therefore, the binding of the enzyme to the magnetic beads also was expected to be similarly efficient with crude extract instead of purified enzyme. To 1 mg of magnetic beads, various amounts of crude extract (from 16 to 161 μg total protein content) were added and the amount of enzyme bound to the beads was measured by the lipase activity assay. At 32 μg total protein in crude extract corresponding to about 900 mU lipase activity, still about two thirds of the enzyme are bound to the beads (Fig. 7).

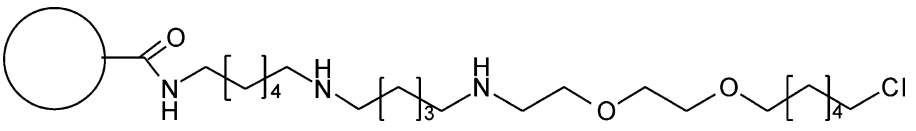

Fig. 3 Schematic representation of the used functionalized beads (the sizes of the bead and the spacer are not true to scale)

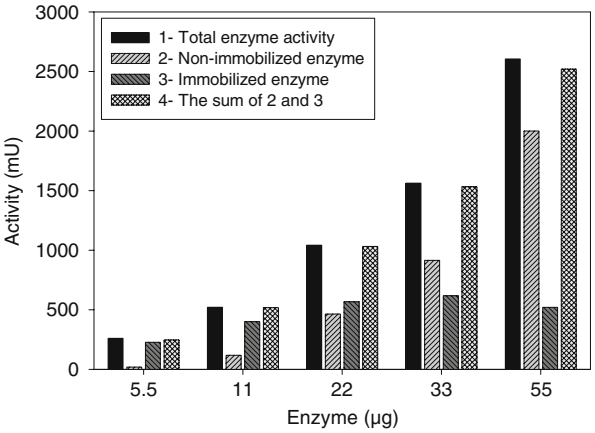

Fig. 4 Immobilization of purified MalE–HaloTag™–lipase fusion protein to chloroalkane-functionalized magnetic beads for different enzyme-to-bead ratios. The used amount of beads was kept constant at 1 mg, while different amounts of enzyme were applied. The activities of non-immobilized and immobilized enzyme were tested independently by an enzyme activity assay with *p*-nitrophenylpalmitate

A direct comparison between the specific activities reached by sequential purification/immobilization and a one-step immobilization from crude extract is shown in Fig. 8. As can be expected when the amount of enzyme per milligram of beads is the same, the use of purified enzyme yields higher specific activities than the use of crude extract. However, it is remarkable that the fast and simple process with crude extract reaches approximately 80% of the results with purified enzyme.

Probably the most important feature of immobilized enzymes is their reusability. Consequently, the enzyme has to be stably bound to the surface and should also be stable against inactivation. To test the storage stability, 1.5 mg beads with immobilized MalE–HaloTag™–lipase protein were assayed for lipase activity, washed, and stored again in reaction buffer without substrate at 8 °C. The activity assays were then repeated up to six times over a period of 36 days with the same beads. No loss of activity was found during that time (Fig. 9).

Discussion

The presented results show that we succeeded in synthesizing magnetic beads that are able to directly capture and immobilize tagged fusion proteins out of crude *E. coli* extract.

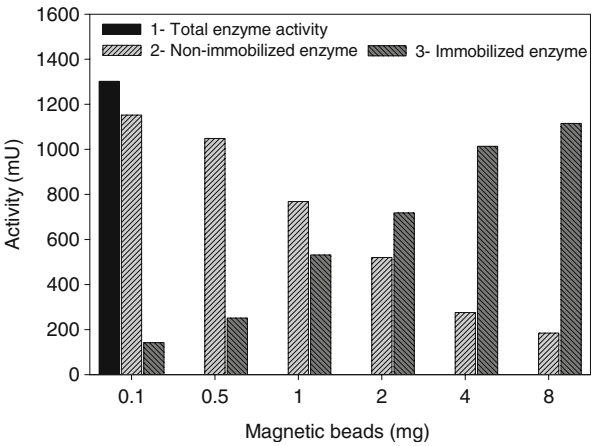

Fig. 5 Immobilization of purified MalE–HaloTag™–lipase fusion protein to chloroalkane-functionalized magnetic beads for different enzyme-to-bead ratios. The used amount of enzyme was kept constant at 25 µg, while different amounts of beads (0.1–8 mg) were applied. The activities of non-immobilized and immobilized enzyme were tested independently by an enzyme activity assay with *p*-nitrophenylpalmitate

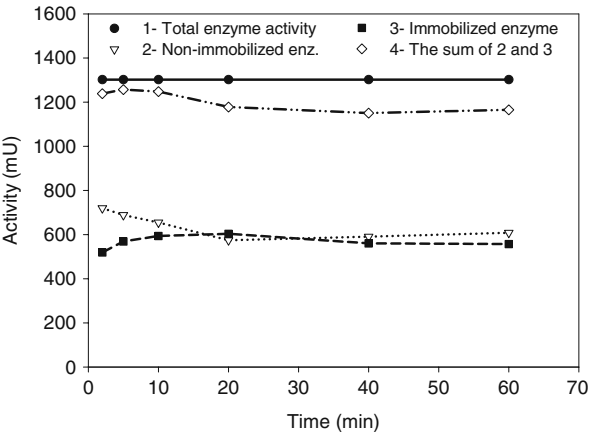

Fig. 6 Time dependence of the immobilization of purified MalE–HaloTag™–lipase fusion protein (25 μg) to chloroalkane-functionalized magnetic beads (1 mg). After different reaction times, the beads were separated magnetically from the supernatant and the activity left in the supernatant and immobilized onto the beads was tested independently

Suspension polymerization is an easy and cheap way for bead production with a small average bead size. The beads can be produced on the technical scale (kg) and aliquots are used for one-step immobilization of the suited fusion proteins as described above. Bead sizes smaller than 5 μm are hardly subject to diffusion limitation [24] which is a disadvantage of beads of greater size. Beads with an average size of more than 100 μm can be separated easily by sieving, while beads with small sizes are difficult to separate selectively from crude feedstocks, and filtration will quickly result in high pressure drops. This problem does not occur in the case of magnetic beads of micrometer size because they can be easily separated by magnets [25]. Commonly used beads, such as Eupergit® (Röhm, Darmstadt, Germany) or Sepabeads® (Resindion S.r.1., Mitsubishi Chem. Corp., Milan, Italy), are usually of greater size (100–300 μm) and porous (25–50% with pore size of 30–60 nm). Porous beads have the advantage that they are able to immobilize greater amounts of proteins compared to non-porous beads of the same size. However, they are prone to severe fouling in the case of solutions containing suspended solids or colloids, something which happens to a much smaller extent for non-porous magnetic enzyme carriers [26]. In addition, the proposed magnetic microbeads can be produced in large quantities and derivatized easily with chemical ligands.

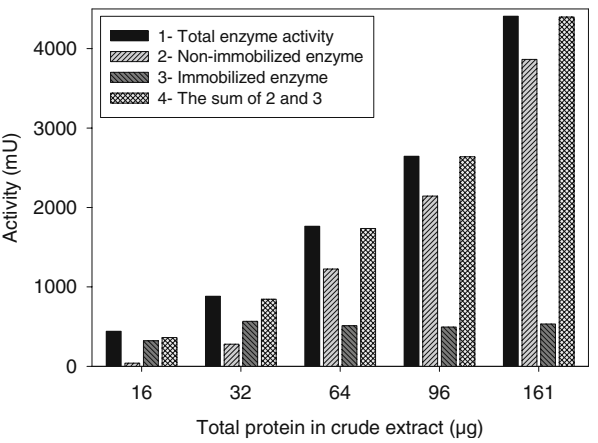

Fig. 7 Immobilization of MalE–HaloTag™–lipase fusion protein from crude extract to chloroalkane-functionalized magnetic beads for different enzyme-to-bead ratios. The used amount of beads was kept constant at 1 mg, while different amounts of crude extract were applied. The activities of non-immobilized and immobilized enzyme were tested independently by an enzyme activity assay with *p*-nitrophenylpalmitate

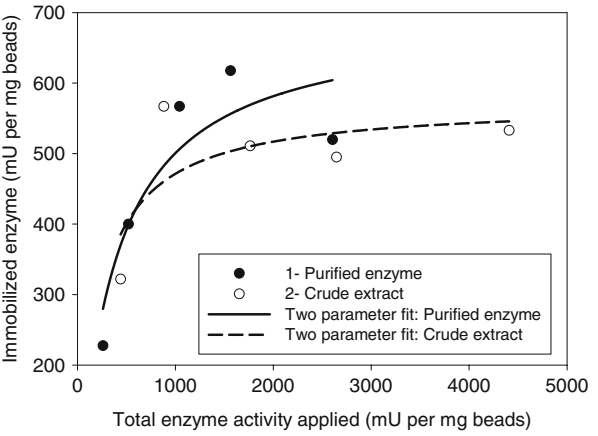

Fig. 8 Comparison of the resulting specific activities in the case of immobilization using purified enzyme or crude extract. In the figure, the total amount of enzyme offered (expressed in milliunits per milligram bead) is plotted versus the amount of active enzyme immobilized (also expressed in milliunits per milligram bead). The fitted lines were obtained using the 'Hyperbola, 2 Parameter' equation $y = ax/(b + x)$ of the Software Sigma Plot 8.0 distributed by SPSS Inc.

The tag used is a mutant dehalogenase commercialized under the name HaloTag™ by the company Promega. The problems with wrongly folded and, hence, inactive dehalogenase protein were solved to a large extent by adding a second fusion partner, which acts as a solubility fusion tag, presumably by promoting the proper folding of the mutant dehalogenase. A disadvantage might be the large size of the fusion proteins (118.8 kDa). The molecular weight of MalE alone already is 42.5 kDa. The HaloTag™ protein adds another 33 kDa to the 43.3 kDa lipase. Assuming a maximal protein synthesis capacity of the cells, the synthesis of MalE certainly reduces the amount of target protein. The bulkier size of the fusion proteins might also reduce the number of proteins which can bind to the magnetic beads. A factor Xa cleavage site exists between the maltose binding protein and mutant dehalogenase, but the removal of the 42.5 kDa MalE protein by protease would add another costly step to the immobilization procedure. In the long run, it would be useful to improve the folding kinetics of the dehalogenase by directed evolution approaches. During the preparation of this manuscript, we became aware of HaloTag7, a genetically engineered version of HaloTag™ with improved solubility [27]. By now, with our version of the HaloTag protein, the higher solubility by far compensates this disadvantage. The maltose binding protein might also have this positive effect on the

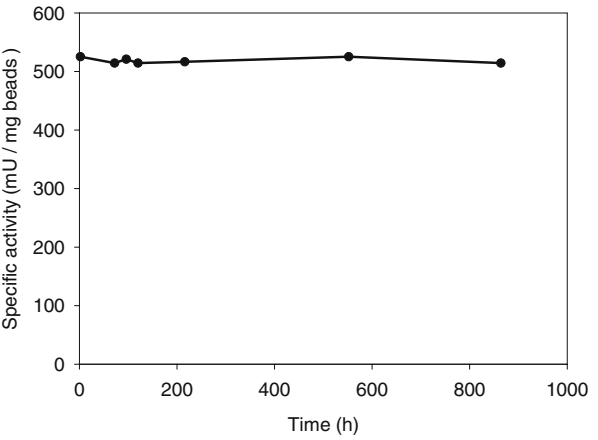

Fig. 9 Storage stability of MalE–HaloTag™–lipase measured as enzyme activity of immobilized enzyme at different storage periods at 8 °C

folding of the second protein fused to the dehalogenase. However, this cannot be seen with the lipase used in our experiments since this protein is highly soluble and produced in active form and high amounts.

In combination with a specially designed small chemical ligand, the dehalogenase forms a stable covalent bond permanently immobilizing the fusion protein. While we expected enzyme immobilization using the HaloTag™ to work in principle because of the reported applications of this system in other biological fields, there were many open questions regarding the yield and loss of enzyme activity during the procedure. As can be seen from Figs. 4 and 5, enzyme immobilization using the HaloTag™ resulted in practically no activity loss because the sum of the activity left in solution and the activity bound to the beads always equaled the original activity supplied. The probable reason is the fact that the HaloTag™ results in a directed immobilization with a well-defined spatial location of the binding site. In contrast to more conventional immobilization techniques resulting in a more or less statistical spatial distribution of the binding sites, the active site of the fusion enzyme is protected.

Another question was whether immobilization will work in the presence of a vast number of other proteins in crude extract. As shown in Fig. 8, the immobilization of purified enzyme results in slightly higher specific loadings only. The difference of up to 20% must be seen in relation to the additional purification effort and cost needed to purify the crude extract. Besides the cost savings, even the yield of direct immobilization from crude extract becomes superior, if the enzyme loss during purification is taken into account. Only about one third of the enzyme originally present in the crude extract is recovered in purified form. It is without doubt that the used purification scheme can be optimized. However, the enzyme loss during purification will stay in the range of the excess that is needed to match the same enzyme loading by immobilization from crude extract instead of using pure enzyme. This observation, in combination with the measured high stability of the covalent immobilization using a modified HaloTag™, should make the approach quite interesting for industrial use. Future investigations with industrially relevant enzymes on a larger scale and over a longer time period will show whether these expectations are met.

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