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Exploitation of the S-layer self-assembly system for site directed immobilization of enzymes demonstrated for an extremophilic laminarinase from *Pyrococcus furiosus*

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Abstract

A fusion protein based on the S-layer protein SbpA from *Bacillus sphaericus* CCM 2177 and the enzyme laminarinase (LamA) from *Pyrococcus furiosus* was designed and overexpressed in *Escherichia coli*. Due to the construction principle, the S-layer fusion protein fully retained the self-assembly capability of the S-layer moiety, while the catalytic domain of LamA remained exposed at the outer surface of the formed protein lattice. The enzyme activity of the S-layer fusion protein monolayer obtained upon recrystallization on silicon wafers, glass slides and different types of polymer membranes was determined colorimetrically and related to the activity of sole LamA that has been immobilized with conventional techniques. LamA aligned within the S-layer fusion protein lattice in a periodic and orientated fashion catalyzed twice the glucose release from the laminarin polysaccharide substrate in comparison to the randomly immobilized enzyme. In combination with the good shelf-life and the high resistance towards temperature and diverse chemicals, these novel composites are regarded a promising approach for site-directed enzyme immobilization.

Keywords: S-layer; Enzyme immobilization; Self-assembly; Extremophilic enzyme; Monolayer; Nanobiotechnology

1. Introduction

Immobilization plays a critical role for the performance, activity and costs of industrially used enzymatic reaction systems. Immobilization prevents loss of enzyme in the process stream and allows re-use and separation of enzyme from the products of the catalytic reaction. On the other hand, transfer of an enzyme from the soluble into the insoluble state through immobilization affects its stability and activity (Chaplin and Bucke, 1990; Aslam and Dent, 1998). Thus, the choice of the right immobilization technique is essential for the application potential of an enzyme.

Currently used immobilization methods are based on the adsorption and covalent binding of enzymes to water-insoluble carriers, the incorporation of enzymes into semi-permeable gels, and the enclosing of enzymes in polymer membranes (Fig. 1a–c) (Angenendt, 2005; Chaplin and Bucke, 1990; Zhu and Snyder, 2003). Self-assembly systems open new possibilities for enzyme immobilization. As a novel approach, we have designed a chimeric protein by fusing an enzyme to a crystalline bacterial cell surface (S-layer) protein (Fig. 1d). The suitability of S-layer protein lattices as matrix for the covalent immobilization of enzymes has already been demonstrated in our laboratory (Neubauer et al., 1993, 1994, 1996). S-layers, in general, are monomolecular proteinaceous arrays that represent the outermost cell envelope component of many prokaryotic organisms (Sára and Sleytr, 2000; Sleytr et al., 1999, 2002, 2005, 2007a; Sleytr and Beveridge, 1999). They are composed of identical (glycol) protein species that are aligned into 2D crys-

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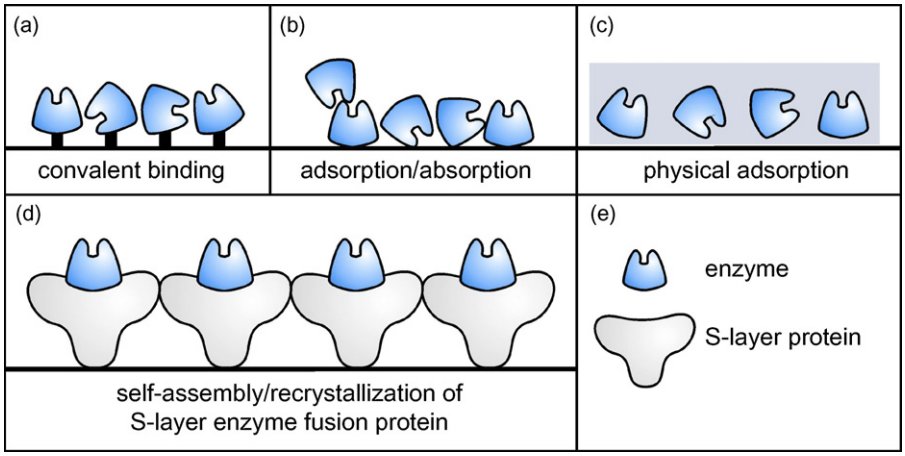

Fig. 1. Comparison of different enzyme immobilization methods. (a) Random immobilization via covalent binding; (b) random adsorptive binding; (c) random physical adsorption within a 3D gel structure; (d) novel approach for site-directed immobilization of an enzyme via the S-layer self-assembly technique, allowing orientated and dense surface display of the enzyme in its native conformation and ensuring accessibility for the substrate; (e) legend.

talline arrays. Isolated S-layer subunits usually have the intrinsic capability to self-assemble in suspension, on the air–liquid interface, on lipid films or on solid supports (Sleytr et al., 1999, 2004, 2005, 2007b). By genetic engineering, discrete functions could be introduced into S-layer arrays (Breitwieser et al., 2002; Huber et al., 2006; Ilk et al., 2002, 2004; Moll et al., 2002; Pleschberger et al., 2003, 2004; Völlenkle et al., 2004). Based on the high density and regular display of the introduced functions, a broad spectrum of applications of S-layers fusion proteins is envisaged, particularly in the fields of biotechnology, molecular nanotechnology and biomimetics (Sára et al., 2006; Sleytr et al., 2002, 2004, 2007a). Regarding enzyme immobilization, the advantages offered by the S-layer self-assembly system are (i) the requirement of only a simple, one-step incubation process for site-directed immobilization without preceding surface activation of the support, (ii) the provision of a cushion to the enzyme through the S-layer moiety of the fusion protein preventing denaturation, and, consequently, loss of enzyme activity upon immobilization, (iii) the principal applicability of the "S-layer tag" to any enzyme, and (iv) the high flexibility for variation of enzymatic groups within a single S-layer array by co-crystallization of different enzyme/S-layer fusion proteins.

In this study, the feasibility of fusing a hyperthermophilic enzyme to an S-layer protein was investigated. Extremophilic enzymes from microbial resources, in general, attract special interest because of their frequently unusual catalytic capabilities and process-related properties such as stability. For the set-up of a hyperthermophilic biocatalytic system, a 200-amino acid deletion mutant of the 1268 amino acid S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 was used. This approach is based on previous structure–function studies revealing that this C-terminal deletion of SbpA results in significant increase of the spatial accessibility of the C-terminus, while fully retaining the protein's self-assembly capability (Ilk et al., 2002) into a square S-layer lattice with a center-to-center spacing of the tetrameric morphological units of 13.1 nm (Fig. 4a, inset). An advantage of the SbpA system for nanobiotechnological applications is the dependence of its *in vitro* recrystallization on the presence of calcium ions (Györvary et al., 2003), allowing control over lattice formation by varying the calcium ion concentration. As extremophilic enzyme fusion partner for SbpA the laminarinase LamA (LamA, PF0076) derived from *Pyrococcus furiosus* was chosen. LamA is an endoglucanase displaying its main hydrolytic activity on the β-1,3-glucose polymer laminarin. It is extremely thermostable (half life time at 100 °C, 16 h) and thermoactive (temperature optimum, 100–105 °C), and, as a particular property, it is remarkably resistant to denaturation retaining a significant extent of its secondary structure in 8 M guanidinium hydrochloride (GHCl). These properties are paralleled by a notable stability at extremely low pH (∼3) (Chiaraluce et al., 2002; Gueguen et al., 1997; Van Lieshout et al., 2004).

Due to the robustness of the chosen enzyme, the LamA/SbpA construct is well suited for the evaluation of the S-layer self-assembly system for enzyme immobilization. The studies performed with this S-layer fusion protein include (i) determination of enzyme activity when immobilized on different supports, with a focus on membraneous supports, (ii) analysis of the effects of inter- and intramolecular chemical cross-linking of LamA/S-layer subunits, and (iii) overall comparison of the S-layer based immobilization technique with currently used enzyme immobilization techniques.

2. Material and methods

Unless otherwise listed, all solvents and reagents were purchased from Sigma–Aldrich, St. Louis, MO.

2.1. Overexpression, isolation and purification of LamA/SbpA

To construct the S-layer fusion protein (Fig. 2), the PCR product encoding the 263-amino acid enzyme LamA (molecular mass, 33,123 Da) from *P. furiosus* possessing an 11-amino acid C-terminal vsv-g tag, spaced by a flexible linker (Ser-Ala-Ser-Ser-Gly-Gly-Gly-Gly-Ser-Ala) was cloned via a Gly-Gly linker