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Thermoswitched immobilization—A novel approach in reversible immobilization

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Abstract

The present work is based on the finding that the mesophilic carbohydrate-binding domain from *Clostridium cellulovorans* fused with thermophilic enzymes from *Pyrococcus furiosus* can be reversibly denaturated and renaturated by a simple switch of temperature. Modular recombinant enzymes are active and free in the reaction mixture at 80–90 °C and deactivated and immobilized by affinity adsorption on cellulose at 40–30 °C. The temperature transition between both modes is rather sharp and occurs within the range of 40–50 °C. Due to the elevated temperature, there is no limitation by a diffusion step, and contamination does not occur during the reaction. After the reaction, the enzymes are quickly deactivated, adsorbed on the affinity matrix, removed from the reaction mixture, and ready for use in another reaction cycle.

Keywords: Enzyme engineering; Immobilization; Biocatalysis; Phosphomanno-mutase; GDP-mannose phosphorylase; Glycerol kinase

1. Introduction

The use of the enzymes as biocatalysts able to assist in the industrial manufacture of fine chemicals and pharmaceuticals has enormous potential. However, to fully realize the potential of enzymes in industrial applications, it would be necessary to tailor the catalyst properties so that they were optimal not only for a given reaction, but also in the context of the industrial process in which the enzyme is applied (Burton et al., 2002). For the majority of enzyme-catalyzed industrial syntheses, the cost of biocatalyst plays a key role, and therefore, the procedure of enzyme isolation and purification should be reduced to the minimum number of steps. Since the catalyst must be easily recovered for a new synthesis, the enzyme immobilization is employed for this purpose. A packed bed of the immobilized enzyme has a clear advantage with respect to space and time requirements, which can lead to reduced costs of product manufacturing. However, the hydrodynamics of a packed bed reactor, as characterized by plug flow, is associated with a number of complications, such as

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generation of concentration gradients of substrate and product, the impossibility of pH adjustment during a single pass through a packed bed, and, which is the most critical, multiphasic mixtures cannot be handled in a packed bed (Burton et al., 2002). Moreover, the industry is equipped preferentially with the stirred-tank reactors, therefore it is convenient to adapt biocatalysts to be used with them. As follows from the above observations, due to limitation by a diffusion step resulting in appearance of the concentration gradients, it seems that the enzyme encapsulation could be excluded from the search of the optimum biocatalysis conditions, and moreover, enzyme adsorption should be ideally used only for biocatalyst recovery and not for a reaction.

The potential contamination and operational stability are other issues that should be taken into consideration in a search for an ideal biocatalyst. Fortunately, evolution has provided a relatively vast repertory of natural biocatalysts and enzymes from hyperthermophilic microorganisms, which are active in a rather broad range of temperatures and can retain high level of activity and/or stability up to 100 °C. The risk of contamination, leading to undesired complications, is reduced at high temperatures. The thermal stability is also accompanied with an enhanced resistance to proteolysis and the action of detergents and chaotropic agents (Sellek and Chaudhuri, 1999). Moreover, at the elevated temperatures the solubility of many reaction components, in particular polymeric substrates, is significantly improved. Thermophilic proteases, lipases, and polymer-degrading enzymes have already found their way into industrial applications (Burg, 2003).

For the above-mentioned reasons, we have tried to design new thermoswitched biocatalysts, which would be free and active at high temperatures, and adsorbed by affinity only after the completion of the desired reactions. *Pyrococcus furiosus* GDP-mannose phosphorylase (GMP, EC: 2.7.7.22), phosphomanno-mutase (PMM, EC: 2.7.7.22), and glycerol kinase (GK, EC: 2.7.1.30) were used as the model enzymes in the present paper. The thermophilic modules were fused to mesophilic cellulose-binding domain from *Clostridium cellulovorans* cellulosome (CBD_{clos}). CBD_{clos} was chosen as the fusion domain because it is well characterized (Goldstein et al., 1993) and is used commercially with cellulose in many applications (Levy and Shoseyov, 2002).

2. Materials and methods

2.1. Cloning, expression and isolation of the recombinant enzymes

Novagen ligation-independent cloning system (pET-34 Ek/LIC Kit, Fig. 1) was used for cloning and expression of *P. furiosus* genes in *Escherichia coli* expression system. *P. furiosus* genomic DNA was obtained from American Type Culture Collection (ATCC Catalog No. 43587D). The primers were designed according to Ek/LIC system (Table 1).

Freshly transformed *E. coli* BL21(DE3) harboring the recombinant plasmid was grown in 50 ml of LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) with kanamycin (30 μg ml^{-1}) overnight (30 °C, 225 rpm), then transferred into fresh LB (1 l) containing kanamycin for another 3 h at 37 °C. When A_{600} value reached 0.8–1.0, the culture was induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h at 37 °C. The cells were harvested by centrifugation (4500 × *g*, 10 min, 4 °C) and lysed with a non-ionic detergent (50 ml, CelLytic B, Sigma, Catalog No. B3553). After centrifugation of the lysate (20,000 × *g*, 10 min, 4 °C), the debris were washed in 100 ml buffer (50 mM Tris–HCl, pH 7.5), harvested by centrifugation and dissolved in 50 ml urea solution (4.5 M urea, 40 mM Tris–HCl, pH 11.3). Refolding was performed by a dilution method. The urea solution was introduced dropwise into a hot renaturation solution

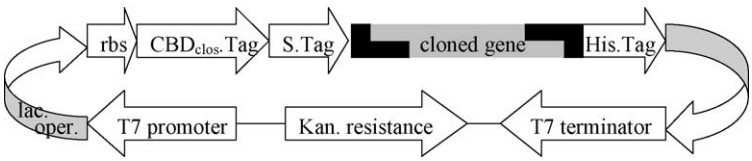

Fig. 1. Schematic construction of pET34b plasmids.