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Covalent immobilization and solid-phase refolding of enterokinase for fusion protein cleavage

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Abstract

Using recombinant enterokinase (EK) as the model proteolytic enzyme, α-amine-coupling covalent immobilization to glyoxyl agarose was evalulated in terms of immobilization yield and the activity and thus the cleavage performance of the immobilized enzyme. Nearly all the enzyme was immobilized by the covalent conjugation, but the specific activity was only 20–30% of that of the soluble enzyme at various pH conditions. However, the cleavage rate by the covalently immobilized EK was higher than that of the soluble enzyme and the undesirable side reaction, i.e., the cryptic cleavage was significantly reduced. In order to reuse the immobilized EK repeatedly, solid-phase refolding of the immobilized EK was attempted. The covalently immobilized EK showed almost 100% refolding yield whereas the soluble EK showed only ca. 36% yield. It was confirmed that the covalent conjugation maintained the rigid 'reference structure' during a denaturant-induced unfolding step, which would in turn provide for a more efficient route to refolding in the subsequent renaturation step.

Keywords: Enterokinase; Solid-phase refolding; Covalent immobilization; Fusion protein cleavage; Glyoxyl agarose

1. Introduction

Recently, quite a few recombinant proteins of pharmaceutical interest have been expressed as fusion proteins for various reasons [1–3]. Once expressed and purified, however, the fusion tags need to be cleaved away to obtain the target protein in a monomeric form. Oftentimes, this is one of the critical steps that determine the purity and yield attributes as well as the manufacturing cost of the biopharmaceutics. Compared with the chemical cleavage using cyanogen bromide (CNBr), enzymatic cleavage has distinctive advantages such as higher specificity and milder reaction conditions. However, the high cost of the proteolytic enzymes such as urokinase (UK), thrombin, enterokinase (EK), and Factor Xa has been the major bottleneck for successful industrial applications. Other obstacles include enzyme inactivation, product inhibition, and the burden of post-cleavage separation from the complex mixture of reactants (uncleaved fusion protein, proteolytic enzyme) and products (fusion tag, monomeric target protein). In order to improve the process economics of enzymic cleavage systems, the inherent instability of the enzymes that could readily denature under the operating conditions must be overcome together with ways to use the enzymes repeatedly.

Various methods are available to immobilize enzymes. Adsorptive immobilization uses such forces as electrostatic, ionic, and hydrophobic interactions to attach the enzyme molecules on the matrix surface. Covalent bonds chemically conjugate enzymes to the surface via such bonds as activated esters, bialdehydes, epoxides, and amines [4]. Among the covalent conjugation methods, amine-coupling reaction to glyoxyl groups has several advantages. With a 6% agarose gel, a maximum of 40 mg of enzyme could be immobilized to 1 ml gel, and the coupled enzymes maintained their

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bioactivity for 1 year at 4 °C due to the excellent stability of the glyoxyl group [5]. Furthermore, the immobilization reaction could be controlled by the pH and the number of covalent bonds can be manipulated.

A single covalent bond is possible through borohydride reduction chemistry [6]. It was reported that the reductive alkylation method was able to conjugate the α-amine at the N-terminus to an aldehyde group on the matrix surface. This method was first proposed to reduce the pyridoxal moiety of glycogen phosphorylase [7]. This reaction is performed usually at pH 4–5, and a reducing agent is used to convert hydride ions to protonated imines. Sodium borohydride, sodium cyanoborohydride, dimethylamine borane, and pyridine borane are frequently used as the reducing agents, and sodium borohydride allows the fastest reaction among them [8].

Immobilized enzymes have a propensity to lose their bioactivity after repeated use. To improve the longevity of an immobilized enzyme column, an efficient renaturation method is necessary. Simple buffer washing may not be sufficient to remove the reaction debris entrapped inside the microporous matrices, and consequently the loss of bioactivity would become inevitable. Using a chaotropic denaturant to regenerate the column would be ideal to remove the debris by disrupting the interactive forces between the debris and the matrices [9]. But, the irreversible activity loss of the enzymes by such a denaturant would prohibit its use. Recently, however, it was reported that 'solid-phase refolding' could be an alternative to completely regain the initial activity. In solid-phase refolding, a full-strength denaturant was first used to unfold the immobilized enzymes and then the denaturant was gradually washed off with a buffer to refold them. Solid-phase refolding was successfully applied to proteins immobilized through ionic interactions [10,11], affinity binding [12,13], and covalent bonds [14]. Compared with the conventional solution-phase refolding, the solid-phase refolding would provide a higher yield at higher protein concentration in a shorter processing time [3,11].

In this study, the EK was immobilised by single-point covalent bonding and the immobilization yield and enzyme activities were evaluated. The immobilized EK was tested for the solid-phase refolding for renaturation yield, and the cleavage performance of the immobilized EK was evaluated. Finally, a simple recovery process of the target protein monomer after the enzymic cleavage was proposed.

2. Materials and methods

2.1. Enterokinase and fusion protein substrate

DaeWoong Pharmaceuticals, Inc. (Seoul, Korea) supplied the fusion protein substrate. It consisted of human growth hormone (hGH), of which the C-terminus was linked with 6× histidine residues by a linker peptide, Lys-$(Asp)_4$, that could be recognized by EK for a cleavage site. Native EK (26 kD, p*I* 5.2) was purchased from Roche (Indianaplois, IN, USA). Sepharose CL-6B (Amersham Biosciences, Uppsala, Sweden) was used as the immobilization matrix. All chemicals for agarose surface glyoxylation and enzyme activity assay were purchased from Sigma. The reagents for EK unfolding and refolding including guanidine hydrochloride were also from Sigma.

2.2. Covalent immobilization of EK

Sepharose CL-6B was glyoxylated by a procedure described previously [15]. Reductive alkylation was employed to conjugate the α-amine of the N-terminus to an aldehyde group of the glyoxyl agarose [7]. For the covalent conjugation, 3 mg EK in 0.1 M sodium phosphate buffer (20 ml) containing 25 mM $NaBH_3CN$ was reacted with the glyoxyl agarose at various pHs between 4 and 11 for 3 h. The residual aldehyde groups were blocked by ethanolamine. Immobilization yield was determined by assaying the protein in the solution phase before and after the immobilization.

2.3. EK activity assay and substrate specificity

A synthetic substrate containing the EK recognition sequence, Gly-$(Asp)_4$-Lys-β-naphthylamide, was incubated with a sample for 1 h at 37 °C. The released β-naphthylamide was reacted with 0.05% *N*-1-(naphthyl)-ethylenediamine (0.2% sodium nitrite in 0.5% ammonium sulphamate buffer) for color generation, of which the absorbance was measured at 580 nm [16,17]. One unit of EK was defined as 1 nM of β-naphthylamide released after 1 h incubation.

In order to find any differences between the soluble and the immobilized EK in their substrate specificity toward the small substrate (i.e., the synthetic peptide) and the large substrate (i.e., the fusion protein), i.e., to check if the substrate specificity changed after the immobilization, we measured the released concentration of β-naphthylamide and the cleaved fusion tag after 1 h incubation, respectively.

2.4. Cleavage of fusion protein substrate and recovery of hGH monomer

One millilitre of the fusion protein at various concentrations (0.1, 0.5, 1, 2, 4, and 5 mg/ml) was reacted with one unit of the soluble EK at pH 8.0 (50 mM Tris–HCl) for 16 h. The immobilized EK was reacted at the same condition. After the cleavage reaction, the mixture would contain the monomeric hGH, the linker peptide with 6× His tag, and unreacted fusion protein. The cleavage yield was determined by reading the SDS-PAGE gel using a densitometer. To recover the monomeric hGH, the mixture was passed through the nickel–nitrilotriacetic acid (Ni–NTA) column (1 ml) at 1 ml/min flow rate.