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An oriented adsorption strategy for efficient solid phase PEGylation of recombinant staphylokinase by immobilized metal-ion affinity chromatography

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ABSTRACT

Conjugation of truncated recombinant staphylokinase (trSak) with polyethylene glycol (PEG) is an effective way to overcome its short plasma half-life and enhance its therapeutic potential. However, conventional amine directed PEGylation chemistry inevitably led to modification at its functionally important N terminus, which resulted in a significantly reduced bioactivity of trSak. In this study, a novel solid phase PEGylation process was developed to shield the N-terminal region of the protein from PEGylation. The process was achieved by oriented adsorption of an N-terminally His-tagged trSak (His-trSak) onto an immobilized metal-ion affinity chromatography (IMAC). His-trSak was efficiently separated and retained on IMAC media before reaction with succinimidyl carbonate mPEG (SC-mPEG, 5, 10 or 20 kDa). The IMAC derived mono-PEGylated His-trSak showed structural and stability properties similar to the liquid phase derived conjugate. However, isoelectric focusing electrophoresis analysis revealed that mono-PEGylated His-trSaks via solid phase PEGylation were more homogeneous than those from liquid phase PEGylation. Moreover, tryptic peptide mapping analysis suggested that a complete N-terminal blockage of IMAC bound His-trSak from PEGylation with 10 kDa- and 20 kDa-SC-mPEG. In contrast, only partial protection of the N-terminal region was obtained for 5 kDa-SC-mPEG. Bioactivities of 10 kDa- and 20 kDa-PEG-His-trSak conjugates without N-terminal PEGylation were significantly higher than those of randomly PEGylated products. This further demonstrated the advantage of our new on-column PEGylation strategy.

1. Introduction

Staphylokinase (Sak), an agent secreted by some strains of *Staphylococcus aureus*, has been known to have profibrinolytic properties [1,2]. Recombinant Sak has been clinically used to treat acute myocardial infarction (AMI) [3,4]. Sak forms a complex with trace amount of plasmin in blood, which is endowed with plasminogen activating properties. The generation of plasminogen activator potential in Sak proceeds via plasmin-mediated removal of the ten N-terminal amino acids with exposure of Lys11 at the new N terminus [5–7]. Lys-11 plays a crucial role in protein function. Deletion or substitution of Lys11 greatly reduced the plasminogen activator properties of Sak. It was believed that Lys11 was important in stabilizing the Sak–plasmin complex through interaction with one of the lysine binding sites within the substrate [5].

In addition to full length Sak, a truncated recombinant Sak (trSak) was shown to have equal bioactivity to that of the full length protein [8]. Besides, deletion of the ten amino acids at the N terminus could potentially reduce the antigenicity of Sak [9]. Polyethylene glycol (PEG) modified proteins have several well-established advantages over their unmodified counterparts, including prolonged residence in body, reduced immunogenicity, and decreased degradation by metabolic enzymes [10–13]. Considering the short blood circulation of trSak, PEGylation of trSak could be an effective approach to further enhance its therapeutic potential.

The most common chemistry for PEGylation targets the ε-amino group of surface lysine residues, which account for approximately 10% of amino acids in a typical protein [14]. Random PEGylation with the ε-amino groups can generate a mixture of positional isomers with PEG attachment at different sites on a protein surface, thereby resulting in a great loss of bioactivity [4,15] and poor reproducibility from batch to batch [16]. Although N-terminus directed

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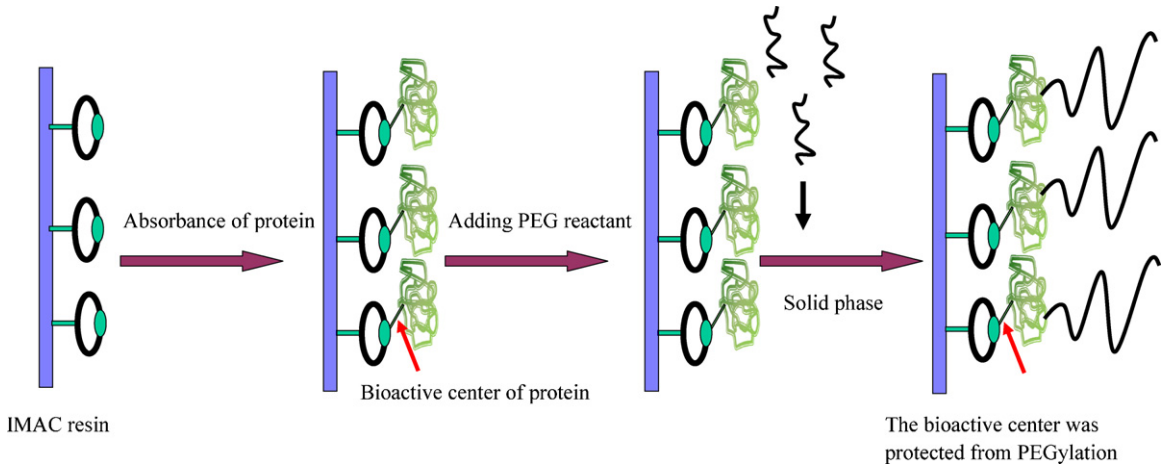

Fig. 1. Solid phase adsorption method for preparation of the PEGylated His-trSak.

aldehyde chemistry could lead to a site-specific protein conjugate [17], PEGylation at this position would significantly affect the plasminogen activation activity of trSak.

Solid phase PEGylation, by which protein is adsorbed onto a solid matrix before PEGylation, has been proved to be an effective method to tackle the heterogeneity problem [18–20]. Diminished multiple site incorporation could be achieved due to the spatial hindrance effect of the solid matrix. A more selected solid phase PEGylation process was reported by oriented enzyme adsorption on affinity chromatography, which resulted in a higher enzymatic activity compared with liquid phase derived conjugates due to the protection of its active sites [21].

In this study, an "oriented adsorption" strategy via immobilized metal-ion affinity chromatography (IMAC) to protect the N terminus of trSak from PEGylation was proposed (Fig. 1). An N-terminally 6× His-tagged trSak (His-trSak) was expressed, efficiently separated and retained on the Ni^{2+}-IDA-Sepharose 4FF media. Succinimidyl carbonate mPEG (SC-mPEG with MW of 5, 10 or 20 kDa) was then introduced into the solid matrix to react with the bound proteins. The mono-PEGylated protein was purified by size exclusion chromatography (SEC). Various characterization methods, such as isoelectric focusing (IEF), tryptic peptide mapping and *in vitro* fibrinolytic assay, were employed to compare the homogeneity and bioactivity between solid and liquid phase derived mono-PEGylated His-trSaks. The results exhibited the advantage of our proposed strategy, as reflected by the absence of N-terminally modified conjugate and a significantly increased bioactivity, in comparison with the randomly mono-PEGylated proteins in liquid phase.

2. Materials and methods

2.1. Materials

Ni^{2+}-IDA-Sepharose 4FF was synthesized as described elsewhere [22]. Superdex 200 10/300 GL and Hiload 16/60 Superdex 200 pg were purchased from GE Healthcare, USA. Succinimidyl carbonate monomethoxy polyethylene glycol (SC-mPEG with MW of 5, 10 and 20 kDa) was purchased from Jenkem Biotech (Beijing, China). Sequencing grade trypsin for peptide mapping was purchased from Promega (Madison, WI, USA). Recombinant *E. coli* (DH5α) strain expressing 6× His-tagged trSak was preserved in our laboratory.

All other chemicals obtained were of analytical reagent grade. All solutions were made using Milli-Q grade water (Millipore, USA).

2.2. Analytical method

2.2.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were heated to 100 °C for 5 min in sample buffer containing 2% 2-mercaptoethanol, and applied to 15% homogeneous polyacrylamide gels containing SDS as described by Laemmli [23]. Afterwards, the gels were subjected to silver staining [24].

2.2.2. Reverse phase HPLC

Reverse phase HPLC analysis was performed using a Proteonavi C4 column (4.6 mm × 250 mm, SHISEIDO, Tokyo, Japan) equilibrated with 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) on an analytical HPLC system (Agilent Technologies 1100 series, Agilent, USA). The column was eluted with 5% acetonitrile containing 0.1% TFA for 15 min, followed by elution with a linear gradient of 5–50% acetonitrile containing 0.1% TFA for 100 min. The flow rate was 0.5 ml/min and the effluent was monitored at 214 nm.

2.2.3. High performance size exclusion chromatography (HPSEC)

The Superdex 200 10/300 GL column (10 mm × 300 mm, GE Healthcare, USA) was mounted on an analytical HPLC system (Agilent Technologies 1100 series, Agilent, USA). The column was equilibrated and eluted with a buffer containing 20 mM sodium phosphate and 100 mM Na_2SO_4 (pH 7.0) at a flow rate of 0.5 ml/min. The effluent was monitored at 280 nm.

2.2.4. Far-UV circular dichroism spectroscopy

Far-UV circular dichroism (CD) spectra of the mono-PEGylated and unmodified His-trSak were recorded on a JASCO J-810 spectropolarimeter (Tokyo, Japan) at 25 °C using a 0.1 cm light path cuvette. For the 250–200 nm absorbance spectra, the concentration of the His-trSak samples was 0.15 mg/ml. All samples were in 20 mM sodium phosphate buffer, pH 7.0.

2.2.5. Fluorescence spectroscopy

Intrinsic fluorescence measurements of the samples were performed using a Hitachi F-4500 fluorescence spectrometer (Hitachi, Japan) at room temperature. The emission spectra were recorded from 300 to 400 nm using an excitation wavelength of 280 nm. Excitation and emission slit widths were 5 nm and 2.5 nm, respectively. All the samples used were at the protein concentration of 0.15 mg/ml in sodium phosphate buffer at pH 7.0. A cuvette with 1 cm path length was used.

2.3. Preparation of PEGylated His-trSak in liquid phase

The cell pellets were lysed by sonication at 150 kHz, using VC-600-2 sonicator (Sonics & Materials Inc.) with a 13-mm probe. This cycle was repeated three times for a total sonication time of 20 min with an interval of 2 min for cooling. The cell extracts were then cleared by centrifugation (10,000 × *g* for 30 min) and applied to an IMAC column (XK 200 mm × 26 mm ID, GE Healthcare, USA) packed with 50 ml Ni^{2+}-IDA-Sepharose 4FF. The unadsorbed protein was removed with 40 mM sodium phosphate buffer (pH 7.5) containing 0.04 M imidazole and 0.1 M NaCl. Bound proteins were eluted with 40 mM sodium phosphate buffer (pH 7.5) containing 0.25 M imidazole and 0.1 M NaCl.

Purified His-trSak (1 mg/ml) in 40 mM sodium phosphate buffer (pH 7.5) was reacted with 5, 10 and 20 kDa SC-mPEG at 8-fold molar excess over His-trSak, respectively. The reaction mixture was incubated at room temperature for 2 h. The mono-PEGylated protein was purified by a Hiload 16/60 Superdex 200 pg (16 mm × 600 mm, GE Healthcare, USA) for further characterization.

2.4. PEGylation of His-trSak on solid phase

After sonication and centrifugation of the *E. coli* cells, 100 ml supernatant was loaded onto a IMAC column (XK 200 mm × 16 mm ID, GE Healthcare) packed with 15 ml Ni^{2+}-IDA-Sepharose 4FF, followed by elution with 40 mM sodium phosphate