Process Biochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

Heat treatment increases the bioactivity of C-terminally PEGylated staphylokinase

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ARTICLE INFO

Article history:
Received 2 November 2013
Received in revised form 16 March 2014
Accepted 2 April 2014
Available online xxx

Keywords:
Polyethylene glycol
Staphylokinase
Heat treatment
Bioactivity

ABSTRACT

PEGylation can effectively improve the therapeutic potential of staphylokinase (SAK), a thrombolysis agent for therapy of myocardial infarction. However, polyethylene glycol (PEG) can sterically shield SAK and drastically decrease its bioactivity. In the present study, N-terminally PEGylated SAKs (5 and 20 kDa PEG), C-terminally PEGylated SAKs with phenyl linker and the ones with amyl linker (5 and 20 kDa PEG) were prepared. The effects of the PEG length, the PEGylation site and linker chemistry on the bioactivity of the heat-treated PEGylated SAK were investigated. Heat treatment at 70 °C for 2 h can improve the bioactivity of the C-terminally PEGylated SAKs, where the one with amyl linker and 20 kDa PEG showed the highest increase extent (27%) in the bioactivity. Thus, our study can advance the development of long-acting pharmaceutical protein with high bioactivity.

1. Introduction

PEGylation, covalent attachment of polyethylene glycol (PEG) to a protein, can enhance the serum half-life and reduce the immunogenicity of the protein [1,2]. Thus, dozens of PEGylated proteins have been approved by FDA or in clinical development [3,4]. However, PEGylation is occasionally accompanied by a substantial loss of bioactivity, particularly for most receptor-binding proteins, such as cytokines and chemokines. For example, PEGylation with 40 kDa PEG gives rise to loss of 93% of the IFNα2a activity for treatment of hepatitis C [5]. This is due to a steric shielding effect of PEG on the protein's bioactive domain [6,7].

The steric shielding effect of PEG is related to the PEG length, the PEGylation sites and linker chemistry [8]. Although short PEG chain (e.g., 5 kDa) exhibited a low steric shielding effect, it cannot efficiently improve the serum half-life of proteins. PEG (≥20 kDa) has been widely used to improve the serum half-life of proteins, regardless of its strong steric shielding effect [9]. Site-specific PEGylation at the site far from the bioactive domain of a protein has been widely accepted to minimize the steric shielding effect of PEG [10]. Linker chemistry also altered the steric shielding effect of PEG [1,11]. Recent study suggested that the phenyl linker could result in densely shielding of staphylokinase (SAK) by PEG and thus improve the bioactivity of C-terminally PEGylated SAK [11]. In contrast, the amyl linker could lead to extensively shielding of SAK by PEG [11].

The continual mobility of the PEG domain provides sufficient flexibility to enable high affinity interactions between a protein and its receptor, thereby retaining the bioactivity of a PEGylated protein at a certain extent [12]. In addition, PEGylation can structurally stabilize the protein, due to the hydrated layer of PEG [13]. Thus, enhancement of the PEG mobility by heat treatment can possibly improve the bioactivity of a PEGylated protein.

SAK is an effective thrombolysis agent for therapy of myocardial infarction [14,15]. In the present study, Ald5K and Ald20K were prepared by N-terminal PEGylation of SAK with PEG aldehydes (5 kDa and 20 kDa), respectively (Fig. 1) [16]. MBS5K and MBS20K were prepared by C-terminal PEGylation of SAK with PEG maleimides with phenyl linker (5 kDa and 20 kDa), respectively [11]. EMCS5K and EMCS20K were prepared by C-terminal PEGylation of SAK with PEG maleimides with amyl linker (5 kDa and 20 kDa), respectively (Fig. 1). The six products were used to improve the bioactivity of the PEGylated SAKs during heat treatment process.

2. Materials and methods

2.1. Materials

Sodium cyanoborohydride, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), and 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (EMCS) were purchased from Sigma (USA). Methoxyl PEG amine with M_w of 5 kDa (PEG5K-NH_2) and 20 kDa (PEG20K-NH_2), methoxyl PEG propionaldehyde with M_w

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http://dx.doi.org/10.1016/j.procbio.2014.04.001
1359-5113/

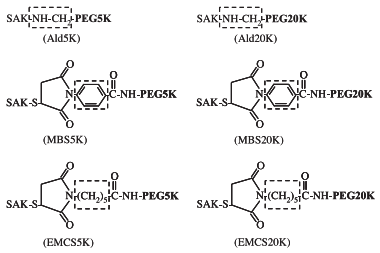

Fig. 1. Schematic presentation of the PEGylated SAKs.

of 5 kDa (PEG5K-ald) and 20 kDa (PEG20K-ald) were ordered from Jenkem Biotech (China).

2.2. Preparation and purification of the PEGylated SAKs

The recombinant SAK consisted of native SAK with a peptide of Gly-Gly-Cys fused at its C-terminus. SAK was prepared and purified as Liu et al. [17]. N-terminally PEGylated SAKs (Ald5K and Ald20K) were prepared as Mu et al. [18]. PEG5K-NH_2 (2 mM) was allowed to react with 2-fold molar excess of MBS or EMCS in 20 mM phosphate buffer (pH 7.2) at room temperature for 3 h, respectively. The reaction mixtures were subjected to extensive dialysis against 20 mM phosphate buffer (pH 7.2) to obtain PEG5K-MBS and PEG5K-EMCS. C-terminally PEGylated SAKs with phenyl linker (MBS5K) and amyl linker (EMCS5K) were prepared by incubation of SAK (0.1 mM) with 0.4 mM PEG5K-MBS and 0.4 mM PEG5K-EMCS at 4 °C overnight, respectively. Similarly, MBS20K and EMCS20K were prepared essentially as MBS5K and EMCS5K, respectively. The six PEGylated SAKs were purified as Mu et al. [18].

2.3. Analytical methods

Size exclusion chromatography (SEC) analysis of the SAK samples was carried out as Liu et al. [17]. The concentrations of the SAK samples were measured by a bicinchoninic acid assay kit. SDS-PAGE analysis was conducted using a 15% polyacrylamide gel. The gel was stained with Coomassie blue R-250. The *in vitro* bioactivities of the SAK samples were measured by radial fibrinolytic assay [19]. Circular dichroism (CD) spectra of the SAK samples were recorded on a Jasco J-810 spectropolarimeter (Jasco, Japan) as Xue et al. [11]. Fluorescence measurement was carried out on a Hitachi F-4500 Fluorescence spectropolarimeter (Hitachi, Japan) as Suo et al. [15].

2.4. Heat treatment of the PEGylated SAKs

The SAK samples were at a protein concentration of 0.6 mg/ml in 20 mM phosphate buffer (pH 7.2) and incubated for different time period at 70 °C. Aliquots of the heat-treated SAK samples (10 μl) were withdrawn after 0.5, 1.0, 2.0, 3.5 and 5.0 h, immediately followed by storage at −20 °C. In addition, the SAK samples (0.6 mg/ml) in 20 mM phosphate buffer (pH 7.2) were incubated at 25–80 °C for 2 h, immediately followed by storage at −20 °C. The relative bioactivities of the heat-treated SAK samples were measured as described above. The bioactivity of the corresponding untreated SAK sample was set as 100%.

2.5. Sedimentation velocity

Sedimentation velocity measurements were conducted by analytical ultracentrifugation on a ProteomeLab XL-1 (Beckman, USA) equipped with an An-60Ti rotor. The SAK samples at the nominal concentration (A_{280} = 0.6) were centrifuged and analyzed as Xue et al. [11].

2.6. Molecular dynamics simulation

The structure of SAK was obtained from the Protein Data Bank (1SSN). For N-terminal PEGylation, PEG chain was linked to Met^1 of SAK via a propyl moiety. For C-terminal PEGylation, PEG chain was linked to Cys^{130} (i.e., C-terminus) of SAK through succimidyl moiety with phenyl and amyl, respectively. Molecular dynamics simulations were performed in GROMACS version 4.5.4 [20] with GROMOS96 53a6 force field [21]. The simulated annealing was carried out as Mu et al. [18].

3. Results

3.1. Characterization of the PEGylated SAKs

SEC analysis suggested that the PEGylated SAKs were all eluted as a single peak that was left-shifted as compared with SAK (Fig. S1a). In addition, the PEGylated SAKs with 20 kDa PEG were all eluted earlier than the ones with 5 kDa PEG. This indicated that the hydrodynamic volume of SAK increased upon PEGylation and 20 kDa PEG can induce a larger hydrodynamic volume of SAK than 5 kDa PEG.

SDS-PAGE analysis showed that the PEGylated SAKs all exhibited a single band with aberrant migration, indicating high purity of the PEGylated SAKs (Fig. S1b). The aberrant band migration was due to that PEG can bind water and form a hydrated layer surrounding SAK. Moreover, the PEGylated SAKs with 20 kDa PEG (Lanes 6–8) all displayed slower band migration than the ones with 5 kDa PEG (Lanes 3–5).

3.2. Bioactivity of the heat-treated SAK samples

Aliquots of the SAK samples (10 μl) were incubated for different time period at 70 °C. As shown in Fig. 2a and b, the relative bioactivity of SAK progressively decreased as a function of incubation time. In contrast, PEGylation can retard the decrease in the relative bioactivity of SAK. EMCS5K displayed higher relative bioactivity than Ald5K and MBS5K. Particularly, the relative bioactivity of EMCS5K even slightly increased to 102.5% upon heat treatment for 2 h (Fig. 2a).

The PEGylated SAKs with 20 kDa PEG (Fig. 2b) showed higher relative bioactivity than the ones with 5 kDa PEG (Fig. 2a). The relative bioactivity of Ald20K was essentially unchanged upon heat treatment for 3.5 h, followed by a progressive decrease (Fig. 2b). MBS20K exhibited a slightly progressive increase in the relative bioactivity during heat treatment for 2 h with a peak value of 113.0%. In contrast, EMCS20K exhibited a pronounced increase in the relative bioactivity with a peak value of 127.0% during heat treatment for 2 h.

Aliquots of the SAK samples (10 μl) were incubated at different temperature for 2 h. As shown in Fig. 2c and d, the relative bioactivity of SAK progressively decreased as a function of temperature. EMCS5K showed higher ability than Ald5K and MBS5K to retain the relative bioactivity upon heat treatment at 25–70 °C. The bioactivity of EMCS20K progressively increased upon heat treatment at 25–70 °C and was higher than those of Ald20K and MBS20K, followed by a decrease when the temperature was higher than 70 °C (Fig. 2d). Thus, heat treatment at 70 °C for 2 h was used for the next experiments.

The bioactivities of the six PEGylated SAKs were compared with each other, where the bioactivity of SAK was set as 100%. EMCS5K showed a bioactivity higher than Ald5K and lower than MBS5K (Fig. 3). Particularly, heat treatment can decrease the bioactivities of Ald5K and MBS5K and slightly increase the bioactivity of EMCS5K. In contrast, the PEGylated SAKs with 20 kDa PEG showed lower bioactivity than the ones with 5 kDa PEG. However, heat treatment increased the bioactivities of Ald20K, MBS20K and EMCS20K. Although the bioactivity of the heat-treated EMCS20K was lower than that of the heat-treated MBS20K, EMCS20K showed the highest increase in the bioactivity upon heat treatment (Fig. 3).

3.3. Circular dichroism spectroscopy

As measured by CD spectroscopy, the secondary structure of SAK did not essentially change upon PEGylation (Table S1). After heat treatment, α-helix and β-turn of SAK significantly decreased, whereas β-sheet content significantly increased. The decrease extent in secondary structure of Ald5K was lower than the one of SAK and higher than the one of Ald20K upon heat treatment. In contrast, the secondary structures of the four C-terminally PEGylated SAKs were essentially not altered upon heat treatment. Thus, the structural loss of the four C-terminally PEGylated SAKs was essentially not observed upon heat treatment.

Please cite this article in press as: Xue X, et al. Heat treatment increases the bioactivity of C-terminally PEGylated staphylokinase. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.04.001