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PEGylation with the thiosuccinimido butylamine linker significantly increases the stability of haloalkane dehalogenase DhaA

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ABSTRACT

Haloalkane dehalogenase (HLD) can catalyze the hydrolytic dehalogenation of halogenated compounds. However, HLD suffers from the poor stability to resist the environmental stress. PEGylation is an effective approach to enhance the stability of enzymes. The linker is an important stabilization factor of PEGylation. Thus, the linkers of the PEGylated HLD were optimized to improve the stability of HLD in the present study. The PEGylated haloalkane dehalogenase DhaAs with methylamine (Ml), carbamate (Cm) and thiosuccinimido butylamine (Tb) linkers were prepared, respectively. The effects of the Ml, Cm and Tb linkers on the stability of the PEGylated DhaAs were investigated under different environmental stresses. Among the three linkers, the Tb linker showed the highest efficacy to improve the stability of the PEGylated DhaA. The Tb linker significantly increased the thermal stability of the PEGylated DhaA by slowing its structural unfolding, and the pH stability of the PEGylated DhaA by slowing the protonation process. In addition, the PEGylated DhaA with the Tb linker showed the maximum resistance to high ionic strength (1 M NaCl) and organic solvent (40% DMSO). PEGylation with the Tb linker is of general interest to effectively improve the stability of proteins, particularly the protein with poor stability.

1. Introduction

Halogenated compounds are widespread throughout the world with high toxicity, long persistence and bioconcentration (Quintero et al., 2005). The halogenated compounds have been the largest sources of environmental pollutants and received serious concerns over their toxicity on the environment and human healthy (Manickam et al., 2006). Haloalkane dehalogenase (HLD, E.C. 3.8.1.5) could catalyze the hydrolytic dehalogenation of haloalkanes by cleavage of a carbon-halogen bond, thus generating the corresponding alcohol, a halide ion, and a proton (Chovancova et al., 2007). HLD belongs to the α,β-hydrolase superfamily and could be used to degrade the halogenated compounds (Janssen, 2004; Camacho-Pérez et al., 2012).

However, HLD suffers from the poor stability to resist the environmental stress (e.g., high temperature, low pH, high ionic strength and organic solvent), which limits its practical application (Chaloupkova et al., 2011). Recently, various methods have been developed to improve the stability of enzymes, including enzyme immobilization (on solid support, sol-gel, or cross-linked enzyme aggregates) and random mutagenesis (Zhao, 2010). For example, the thermal stability of phytase was enhanced by engineering of disulfide bridges (Tan et al., 2016). Chitosan-based peroxidase nanoparticles showed higher thermal stability and the same pH activity profile than those from free enzyme (Alarcón-Payán et al., 2017).

Since the pioneering work of Abuchowski et al., conjugation of polyethylene glycol (PEG) chains with proteins has been one of the most widely used approaches to engineer enzymes with enhanced stability (Abuchowski et al., 1977a, 1977b). For example, PEGylation protects protein from thermal denaturation (Grigoletto et al., 2016; Mero et al., 2016). Because each ethylene glycol unit could bind three molecules of water, PEG forms a hydration layer around the protein and serves as a diffusion barrier against solvent attacks (Veronese and Mero, 2008; Jevsevar et al., 2010). The amphiphilicity of PEG could provide an adequate magnitude of hydrophobic interactions with a protein (Hamed et al., 2015). Thus, the hydration layer and the hydrophobic interactions are the driving forces to underpin the stabilization effect of PEGylation (Veronese and Pasut, 2005; Yang et al., 2011).

The stabilization effect of PEGylation depends upon several factors, including molecular weight (Mw), conjugation site, grafting density of PEG and linker (Li et al., 2008; Xue et al., 2013). Optimization of these factors has been carried out to strengthen the driving forces of PEGylation and thus improve the stability of proteins. For example, the

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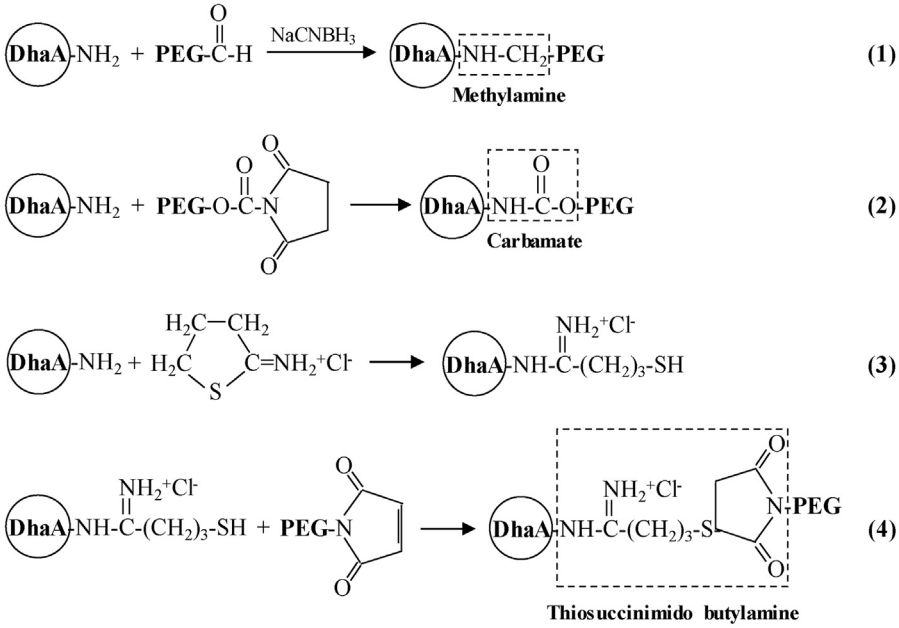

Fig. 1. Schematic presentation of the PEGylated DhaAs.

effects of the Mw (5 and 20 kDa PEG), the PEGylation site (N- and C-terminus) and the linker (phenyl and amyl linkers) on the PEGylated staphylokinase (SAK) were investigated at 70 °C (Xue et al., 2014). The C-terminally PEGylated SAK with phenyl linker and 20 kDa PEG showed the highest thermal stability among the PEGylated SAKs.

The linker between PEG and protein has a profound influence on the stability of proteins. The flexibility of the linker was related to the orientation and conformation of PEG in the PEGylated protein, which directly determines the thickness of the hydration layer. However, some linkers did not necessarily improve the stability of the PEGylated proteins. For example, arginine deiminase (ADI) was conjugated with succinimidyl PEG reagents containing succinate, carbonate and propionate linkers, respectively (Zhang et al., 2015). Although PEGylation improved the stability of ADI, the three PEGylated ADIs with these linkers did not show significant difference in the pH and thermal stability. Recently, cases of destabilization upon polymer conjugation have been reported (DeBenedictis et al., 2016; Carmichael and Shell, 2015). Because the studies of the PEGylated HLDs have seldom been reported, it is unknown whether the stabilization effect of PEGylation. Thus, an optimum linker was urgently needed to improve the stability of the PEGylated HLDs.

In the present study, a haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* (Stsiapanava et al., 2008) was used for PEGylation. PEGylation with the methylamine (Ml), carbamate (Cm) and thiosuccinimido butylamine (Tb) linkers was used to improve the stability of DhaA, using 20 kDa PEG as the PEGylation reagent. Three mono-PEGylated DhaAs with Ml linker (mo-Ml-DhaA), Cm linker (mo-Cm-DhaA) and Tb linker (mo-Tb-DhaA) and three multi-PEGylated DhaAs with Ml linker (mu-Ml-DhaA), Cm linker (mu-Cm-DhaA) and Tb linker (mu-Tb-DhaA) were prepared and characterized. As compared with the Ml and Cm linkers, the Tb linker was more effective to improve the stability of the PEGylated DhaA against high temperature, low pH, high ionic strength and organic solvent. Therefore, PEGylation with the Tb linker is of general interest to improve the stability of proteins, particularly the protein with poor stability. Our study is of general significance for long-term storage, transport and practical application of proteins.

2. Materials and methods

2.1. Materials

PEG succinimidyl carbonate (PEG-sc, 20 kDa), PEG propionaldehyde (PEG-ald, 20 kDa) and PEG maleimide (PEG-mal, 20 kDa) were purchased from Jenkem Biotech (Beijing, China). Sodium cyanoborohydride and 2-iminothiolane (IT) were ordered from Sigma (USA). All other reagents were of analytical grade.

2.2. Expression and purification of DhaA

The gene encoding DhaA (GenBank accession no. AAC15838.1) was cloned to the expression vector *p*ET28a. The recombinant vector was transformed into *E.coli* BL21(DE3) as an expression strain. The transformed strain was grown in 100 ml cultures of LB medium at 37 °C. The culture was incubated with vigorous aeration at 37 °C until the density was up to ~0.8 of A_{600}, followed by incubation with 1 mM isopropylthiogalactoside at 16 °C for 16 h. The cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl buffer containing 0.5 M NaCl and 20 mM imidazole (buffer A, pH 8.2). Cells were sonicated in ice bath and the cell debris was removed by centrifugation.

A Ni Sepharose Fast Flow column (5 ml, GE Healthcare, USA) was equilibrated with 5 column volumes (CV) of buffer A. The sample was loaded and the bound protein was eluted with buffer A containing 0.5 M imidazole (pH 8.2). The bound protein was further loaded on a Superdex 75 column (1.6 cm ×60 cm, GE Healthcare, USA). The column was equilibrated and eluted by 20 mM sodium phosphate buffer (PB, pH 7.4) at a flow rate of 3.0 ml/min. The peak corresponding to DhaA was fractionated and concentrated.

2.3. Preparation and purification of the PEGylated DhaAs

2.3.1. Preparation of mo-Cm-DhaA and mu-Cm-DhaA

DhaA (2.2 mg/ml) was allowed to react with PEG-ald and sodium cyanoborohydride at a molar ratio of 1:5:10 in PB buffer (pH 7.4) at 4 °C for overnight (Fig. 1). The reaction mixture was subjected to a Mono Q column (1.0 cm × 10 cm, GE Healthcare, USA), based on anion exchange chromatography. The column was equilibrated with 5 CV of 20 mM Tris-Ac buffer (pH 7.5, buffer A), followed by gradient elution with 10 CV of buffer A containing 0–1.0 M NaCl. The elution peaks corresponding to the mono-PEGylated DhaA (mo-Cm-DhaA) and