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Site-directed modification of genetically engineered Proteus sp. lipase K107 variants with a polyethylene glycol derivative

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ABSTRACT

The covalent attachment of PEG to target proteins, known as PEGylation, has broad biomedical and biotechnological applications. Particularly, site-specific PEGylation is widely used owing to its unique properties of preserving bioactivity, improving the stability of conjugated proteins, and achieving a high degree of homogeneity. In this work, linear mPEGs of various sizes (MW = 5, 12, and 20 kDa) were functionalized via dopamine and used for site-specific PEGylation of Proteus sp. lipase K107 derivatives with a single Cys residue introduced by site-directed mutagenesis on the solvent-accessible surface of the protein. The specificity of conjugation was verified by SDS-PAGE and MALDI-TOF mass spectrometry, and the secondary structures of the conjugates were verified by circular dichroism. PEGylated enzymes retained their activity, which was higher in low molecular mass PEG conjugates. Importantly, both pH and thermal stability of enzymes were enhanced by PEGylation, especially at basic pH and above room temperature. Moreover, conjugates with the site of polymer coupling near the catalytic centre were more stable. These results demonstrate a novel, efficient method of site-specific protein modification via catechol-functionalized PEG that could potentially be applied to other enzymes.

1. Introduction

Among the many strategies used to expand the biomedical and biotechnological applications of proteins in recent decades [1,2], the covalent attachment of PEG to target proteins, known as PEGylation, has become one of the popular techniques for modulating physicochemical properties, biocompatibility, and thermal stability [3,4]. In most cases, the sites of PEGylation can be varied, and are usually nucleophilic residues such as Cys, Tyr, and the surface amines of Lys [5]; however, random protein modifications within or near a bioactive site can lead to a loss of structural integrity and hence, protein function [6,7]. Thus, new approaches for generating targeted and specific homogeneous adducts are needed [8].

Site-specific PEGylation can be achieved by covalently attaching PEG to Cys residues, which are relatively rare [9], whereas genetic engineering approaches can be used to introduce surface-exposed, free Cys to proteins [10]. The main requirement for any modification method is to produce a functional, modified protein at a high yield that is soluble in aqueous medium, and active at neutral pH and ambient temperature [11,12].

A simple, biomimetic strategy was developed for site-specific PEGylation of a protein with a catechol-based PEG, in which the catechol unit contributes to adhesion to the thiol group of Cys via a thiol-reactive quinone generated from catechol oxidation under alkalescency [13,14]. Two variants of Proteus sp. lipase (Lip) K107, a 35-kDa monomer lacking free surface Cys [15,16], were prepared. Free Cys residues were introduced in the variants in the lid (T138C) or in the random coil (Q201C) away from the lid domain, both of which are solvent-accessible regions. These Cys served as sites of selective PEGylation with a single catechol-based PEG chains of three different molecular weights (MW = 5, 12, and 20 kDa). The effects of binding site and PEG chain length on the catalytic activity and stability of the modified lipases as well as their structural aspects were evaluated.

2. Materials and methods

2.1. Materials

Primers were purchased from Jie Li Biology (Shanghai, China). The KOD-Plus-Mutagenesis kit was purchased from Toyobo (Osaka,

Abbreviations: PEG, polyethylene glycol; PEGylation, polyethylene glycosylation; MW, molecular weight; mPEG, methoxy PEG; TsT, 2,4,6-trichloro-1,3,5-triazine; DA, dopamine; mPTD, mPEG-TsT-DA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PB, phosphate buffer; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; CD, circular dichroism.

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Japan). Ni^{2+}-nitrilotriacetic acid sepharose (HisTrap HP, 5 mL), Sephadex G-25, and CM Sepharose FF (Hiprep16/10, 1 mL) were obtained from GE Healthcare (Uppsala, Sweden). The 5-, 12- and 20-kDa mPEGs were obtained from Xiamen Sino PEG Biotech (Xiamen, China). DA hydrochloride was purchased from Sigma–Aldrich (St. Louis, MO, USA). TsT was purchased from Adamas Reagent Ltd. (Switzerland, Sweden). All other reagents and solvents used were of analytical or HPLC grade. The plasmid encoding lipase K107 was from a laboratory stock.

2.2. Construction, expression, and purification of LipK107 and Cys variants

Site-directed mutagenesis of LipK107 to generate the Q201C and T138C variants was performed with the plasmid pET-28a containing the LipK107 gene using the KOD-Plus-Mutagenesis kit according to the manufacturer's instructions and the following primers: Q201C-F, 5′-TGT GGA TTA ATT GCT GGG GAA AAG G-3′ and Q201C-R, 5'-AAT ATA GCT ACC AAA ACA GTA GTA ATG AAC C-3′; and T138C-F, 5′-TGT ATT ATC TCA ACA TTT TCT GGC CAT AG-3′ and T138C-R, 5′-ACC AAA TGC ATT TAA TAC TTT TTC GAC AAT ATA T-3′. The constructs were verified by sequencing. LipK107, Q201C, and T138C were expressed in *Escherichia coli* BL21 cultured in 200 mL of Luria Broth medium at 37 °C. When the culture density at 600 nm reached 0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce expression at 25 °C for 12 h. Cells were harvested by centrifugation at 7000 × *g* for 10 min and washed twice with phosphate-buffered saline. Cells were resuspended in loading buffer (40 mL of 20 mM PB and 300 mM NaCl, both pH 7.4) and disrupted by sonication. Soluble crude cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid sepharose column equilibrated with the same loading buffer, and a 0–0.25 M gradient of imidazole was used to elute the proteins. The eluted fractions were dialyzed against 20 mM PB at 4 °C for 12 h and concentrated using Amicon filters (Millipore, Danvers, MA, USA) with a 10-kDa-cutoff membrane. Protein concentration was determined by Bradford's method using bovine serum albumin as the standard. Purified lipases were stored in aliquots at −80 °C.

2.3. Synthesis of mPTD

The synthesis of 5-kDa mPEG-TsT-DA (mPTD5k) was carried out as follows: 0.55 g TsT (3 mmol) was dissolved in 50 mL anhydrous benzene containing 2 g anhydrous sodium carbonate; 5 g mPEG (1 mmol) was then dissolved in the solution. The mixture was stirred at room temperature overnight and filtered. Ice cold petroleum ether (75 mL) was added to the filtrate while stirring, and the TsT-modified mPEG (mPEG-TsT) was slowly precipitated, collected by filtration, and redissolved in benzene. The procedure was repeated three times to remove unreacted TsT and the precipitated mPEG-TsT was vacuum-dried. Under a stream of N_2, 0.32 g DA hydrochloride (1.7 mmol) was dissolved in 2 mL 1,4-dioxane containing 1.5 g sodium carbonate. A total of 3 g mPEG-TsT (0.6 mmol) was dissolved in 80 mL 1,4-dioxane, which was added dropwise to the above solution with stirring at room temperature over 2 h. After 5 h, the target product, mPTD5k was precipitated with ice-cold petroleum ether (180 mL), collected by vacuum filtration, and the product was dialyzed against ultrapure water with a 1000-Da cutoff membrane at room temperature for 3 days to remove traces of DA hydrochloride. The product was freeze-dried as a white powder and stored at −20 °C. The synthesis of 12- and 20-kDa mPEG-TsT-DA (mPTD12k and mPTD20k, respectively) followed the same procedure.

2.4. Chemical modification of lipase variants

The concentrations of Q201C and T138C were adjusted to approximately 1.5 mg/mL in 20 mM PB (pH 7.4). A 30-fold molar excess of synthesized mPTD of different MWs was dissolved in the solution, and the mixtures were stirred at room temperature for 3–5 h. Unreacted mPTD5k was removed by dialyzing against 20 mM PB (pH 7.4) at 4 °C for 24 h. The conjugation of mPTD5k to lipase variants was analyzed by SDS-PAGE, MALDI-TOF MS, and far-UV CD without further purification. The reaction was incomplete for mPTD12k and mPTD20k despite extending reaction time to 5 h, and these were de-salted on a Sephadex G-25 (5 cm) column equilibrated with 20 mM PB (pH 5.0). To remove free PEG reagent, the solution was loaded onto CM Sepharose FF resin equilibrated with the same buffer, and eluted with a linear 0–1 M NaCl gradient by ÄKTA fast protein LC. Protein fractions were collected and further purified by size exclusion HPLC using a Zorbax GF-250 column (250 × 4.6 mm, 4 μm, Agilent Technologies, Santa Clara, CA, USA) eluted with 0.1 M PB and 0.2 M NaCl (pH 7.2) at a flow rate of 0.3 mL/min. The effluent was monitored by measuring the absorbance at 220 nm. Upon purification and concentration, mono-PEGylated lipase variants were characterized by SDS-PAGE and CD.

2.5. Analytical methods

Mono-PEGylated protein samples were identified by SDS-PAGE with stacking and separation gel of 5% and 12% polyacrylamide, respectively, using a Bio-Rad Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Hercules, CA, USA). Coomassie blue R-250 stain was used to visualize proteins. The MWs before and after PEGylation were determined by MALDI-TOF MS using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) operated in positive linear mode. Far-UV CD measurements were detected in an N_2 atmosphere by Chirascan (Applied Photophysics, Surrey, UK) equipped with a thermostatically controlled unit set at 20 °C. Protein spectra were recorded at wavelengths of 190–250 nm with a 1-nm bandwidth using a quartz cell with a path length of 1 mm. Samples were at a concentration of 0.1 mg/mL in 10 mM PB (pH 7.4). CD data were converted to mean residue molar ellipticity, expressed in deg cm^2/dmol, with Pro-Data Viewer (Applied Photophysics). A mean residual mass of 109 Da was used for calculations.

2.6. Enzyme activity assay

The enzyme activity before and after PEGylation was determined using p-nitrophenyl palmitate as the substrate. The reaction mixture (200 μL) consisted of 50 mM PB (pH 7.4), 0.1% Triton X-100, and 5% isopropanol along with the substrate at a final concentration of 0.5 mM. The production of p-nitrophenyl was monitored for 5–6 min at 30 °C as the increase in absorbance at 405 nm measured using a SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA). The assay was carried out in triplicate. Enzymatic activity was calculated as 1 μmol p-nitrophenol released per min per mg enzyme under the above-described conditions. Specific activity was expressed as U/mg of protein.

2.7. Thermal stability and pH stability assay

The same concentration (0.026 mg/mL in 20 mM PB (pH 7.4)) of native and modified lipases was incubated at temperatures between 20 °C and 50 °C for 2 h, respectively, and cooled at room temperature for 15 min before the enzyme assay was performed. Thermal stability was evaluated as the relative lipase activity (%), with the activity at 4 °C without incubation taken as the control (100%). To assess pH stability, the same concentration (0.026 mg/mL) of native and modified lipases were incubated in