Optimization of Conditions for Preparation of PEG-Conjugated Phosphotriesterase

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Abstract

New promising group of antidotes against organophosphate poisonings are bioscavengers. Bioscavengers are biomolecules, usually enzymes, able to detoxify toxic organophosphates, such as nerve agents and pesticides. Suitable enzymes for these purposes are cholinesterases (acetylcholinesterase, butyrylcholinesterase), human paraoxonase (HuPON), carboxylesterase and phosphotriesterase (PTE). We prepared polyethylene glycol-conjugated recombinant PTE expressed in *E. coli* with the aim to improve its circulatory stability and immunotolerance. Optimal conditions for modification are discussed.
Keywords: bioscavenger, phosphotriesterase, polyethylene glycol, organophosphate

Introduction

Highly toxic organophosphates, such as nerve agents (NA; tabun, sarin, soman, VX) and some pesticides (oxon form of parathion, malathion, chlorpyrifos) irreversibly inhibit acetylcholinesterase (AChE; EC 3.1.1.7). Subsequent accumulation of acetylcholine, resulting in cholinergic crisis can cause death of the intoxicated organism. Relatively new approach in prophylaxis and therapy of intoxications by these compounds is administration of a bioscavengers capable of neutralizing NA rapidly after their penetration into the organism. Catalytic (paraoxonase, phosphotriesterase, carboxylesterase) and stoichiometric (AChE, butyrylcholinesterase) bioscavengers are intensively investigated for these purposes. The main disadvantage of non-human enzyme bioscavengers is their relatively short half-life in the blood stream and immunogenicity after repeated administration. One way to circumvent these problems is the modification of enzyme bioscavenger by conjugation with polyethylene glycol (PEG) - pegylation. This step can improve its circulatory stability and immunotolerance. A potential catalytic bioscavenger is the phosphotriesterase (PTE; EC 3.1.8.1) from *Pseudomonas diminuta*.

The aim of our work was preparation of PEG-conjugated recombinant PTE (M_w = 36 000) expressed in *E. coli*, optimization of reaction conditions and characterization of modified enzyme. The objective of reaction optimization was to obtain homogeneous and fully PEG-modified PTE.

Experimental part

Lysine (Lys) ε-amino group of were chosen as a target for the modification reaction. There are seven free Lys amino groups on the surface of monomeric PTE and one amino group located at the

N-terminus, which are theoretically capable of reacting with PEG molecules. The enzyme was modified by *O*-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 2 000), *O*-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 5 000), *O*-[(S-succinimidyl)succinyl-aminoethyl]-*O´*-methylpolyethylene glycol (M_w = 2 000) and *O*-[(N-succinimidyl)succinyl]-*O´*-methyl polyethylene glycol (M_w = 5 000) in 0.2 M borate buffer (pH 8.5; 0.1 mM $CoCl_2$) in different ratios (from 1:30 to 1:150 (w/w)) for different time (from 1 to 24 hours) at 25 ºC or 40 ºC. PTE conjugated with *O*-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 2 000) and *O*-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 5 000) was *in situ* reduced using different ratio of sodium cyanoborohydride as reduction agent. The rest of unreacted polymer was removed from modified PTE using ultrafiltration unit (cut-off 10 kDa). Resulting modified PTE was characterized by SDS PAGE and MALDI-TOF methods and by measuring its activity, K_m and V_{max}.

Results and Discussion

We established optimal conditions of the modification such as ratio of enzyme (set as 1), polymer and reduction agent (Table 1). Optimal reaction time for conjugation of recombinant PTE with *O*-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 2 000) and *O*-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 5 000) was 24 hours and for conjugation of PTE with *O*-[(S-succinimidyl)succinyl-aminoethyl]-*O´*-methylpolyethylene glycol (M_w = 2 000) and *O*-[(N-succinimidyl)succinyl]-*O´*-methyl polyethylene glycol (M_w = 5 000) was 3 hours to get fully modified PTE. Optimal reaction temperature was 25 ºC and optimal pH was 8.5. The catalytic activity of prepared modified enzymes was not significantly altered, as well as their K_m values. We obtained fully modified homogeneous population of recombinant PTE with molecular weight around 52 000 and 76 000, respectively.

Our further studies will be focused on investigation of thermal stability of modified enzyme, bioavailability and pharmacokinetics of the conjugate, evaluation of its immunological properties following repeated administration in suitable animal model and estimation of its protective properties.

Table 1: Optimal molar ratios of enzyme : polymer : reduction agent ($NaBH_3CN$)

O-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 2 000)	1	1 200	48 000
O-[2-(6-oxocaproylamino)ethyl]-*O´*-methylpolyethylene glycol (M_w = 5 000)	1	800	40 000
O-[(S-succinimidyl)succinyl-aminoethyl]-*O´*-methylpolyethylene glycol (M_w = 2 000)	1	1 200	-
O-[(N-succinimidyl)succinyl]-*O´*-methyl polyethylene glycol (M_w = 5 000)	1	800	-

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