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Purification and use of *E. coli* peptide deformylase for peptide deprotection in chemoenzymatic peptide synthesis

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ABSTRACT

Peptide deformylases (PDFs) catalyze the removal of the formyl group from the N-terminal methionine residue in nascent polypeptide chains in prokaryotes. Its deformylation activity makes PDF an attractive candidate for the biocatalytic deprotection of formylated peptides that are used in chemoenzymatic peptide synthesis. For this application it is essential to use PDF preparations that are free of contamination by peptidases that can cleave internal peptide bonds. Therefore, different purification methods were attempted and an industrially applicable purification procedure was developed based on a single anion-exchange chromatography step of an engineered PDF variant that was equipped with an anionic octaglutamate tag. The deformylation activity and stability of the engineered enzyme were similar to those of the wild-type PDF. This purification method furnished a PDF preparation with a 1500-fold decreased level of contamination by amidases and peptidases as compared to cell-free extract. It was shown that the enzyme could be used for deprotection of a formylated dipeptide that was prepared by thermolysin-mediated coupling.

Introduction

Chemical peptide synthesis, either in solution or by solid phase methods, generally proceeds by elongation at the N-terminal side of the nascent peptide chain. In academic and industrial practice *N*-protecting groups like *tert*-butoxycarbonyl (Boc)[1], benzyloxycarbonyl (Cbz) and especially fluorenylmethoxycarbonyl (Fmoc) are used in combination with advanced coupling reagents such as carbodiimides, phosphonium salts, or uronium/guanidinium salts [1]. Examples of coupling reagents are 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide (EDC/HOSu), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxybenzotriazole (EDC/HOBt) or benzotriazolyloxy-tris-(pyrrolidino)-phosphonium hexafluorophosphate (ByBOP) [2]. Although these coupling methods work well on a laboratory scale and proceed without substantial racemisation of the amino acid building blocks, cheaper protecting groups and coupling reagents are desired for large scale industrial application. In this respect, a particularly interesting N-terminal amino protecting group is the formyl moiety, which can be readily introduced at low cost using formic acid and acetic acid anhydride [3]. The removal of the N-terminal formyl group after the coupling reaction can be performed chemically or enzymatically. Acidic hydrolysis is a conventional method for removing *N*-formyl groups from amino acids and from the amino terminus of peptides [4]. However, the low pH and harsh reaction conditions lead to significant peptide bond hydrolysis when this method is applied in peptide synthesis. A mild and selective enzymatic method would thus be more attractive.

A class of enzymes that can be used for the removal of *N*-formyl-protecting groups from peptides consists of the peptide deformylases. The cellular ribosome-mediated synthesis of proteins starts with a methionine residue. In prokaryotes and eukaryotic organelles (mitochondria and chloroplasts), the methionyl moiety carried by the initiator tRNA. fMet is *N*-formylated prior to its incorporation into a polypeptide [5]. Following initiation of translation, the enzyme peptide deformylase (PDF, EC 3.5.1.88) cleaves the formyl group from the nascent polypeptide chain [6]. Next, methionyl aminopeptidase may remove the N-terminal methionine from the deformylated polypeptide, leading to a mature protein [7]. Because of this deformylase activity, PDF can be an attractive biocatalyst for the deprotection of formylated peptides.

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[1] *Abbreviations used:* PDF, peptide deformylase; EcPDF, *E. coli* peptide deformylase; CFE, cell-free extract; Boc, *tert*-butoxycarbonyl; Cbz, benzyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; EDC/HOSu, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide; EDC/HOBt, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxybenzotriazole; ByBOP, benzotriazolyloxy-tris-(pyrrolidino)-phosphonium hexafluorophosphate; IEC, ion-exchange chromatography; TCEP, tris-(2-carboxyethyl)-phosphine; THF, tetrahydrofuran; DMF, dimethylformamide; DMSO, dimethyl sulfoxide, NMP, N-methyl-2-pyrrolidinone.

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Although much knowledge is available on PDFs as a target for antibacterial, antiparasitic and chemotherapeutic agents [8–10], data on biocatalytic applications are scarce. Concerning the use of PDF in organic synthesis, the only information available in the literature describes the application of the enzyme in the synthesis of enantiopure amines and amino acid derivatives starting from racemates, based on its ability to hydrolyze *N*-formyl α-aminonitrile and *N*-formyl derivatives of non-functionalized amines and β-amino alcohols in a stereoselective manner [11]. In this paper, the authors also describe an example of the use of PDF for the selective deprotection of *N*-formyl-dipeptides. A prerequisite of the application of PDFs in peptide synthesis is the possibility to isolate the enzyme in a form that is free of contaminating peptidases that may originate from the *E. coli* host strain used for overexpression and could cause peptide bond hydrolysis in the peptide that is under synthesis.

The aim of the work described here is to obtain a method for the rapid, upscalable, and cheap production of purified PDF that is capable of removing N-terminal formyl groups from peptides without concurrent peptide bond hydrolysis. A one-step purification method for PDF based on affinity chromatography with a Met–Lys modified Sepharose matrix was described before [11]. This strategy is in practice only suitable for use on laboratory scale since the matrix is not commercially available and its preparation involves a complicated chemo-enzymatic preparation process making use of expensive materials. In this paper, we explore the development of two simple and industrially applicable purification methods for peptide deformylase from *E. coli* (EcPDF).

Because EcPDF is a monomeric enzyme of 168 aa (19.24 kDa), which is rather small as compared to the average *E. coli* amidases and peptidases, the use of ultrafiltration methods was first examined. Membrane ultrafiltration (UF) is a pressure-modified, convective process that uses semi-permeable membranes to separate species in aqueous solutions by molecular size, shape and/or charge [12]. Alternatively, the selectivity of ion-exchange chromatography (IEC) may be improved by modification of the target protein, e.g. by changing the charge distribution on the surface [13]. The second option that is described makes use of IEC of a variant of PDF that is equipped with a negatively charged octaglutamate tag introduced in a solvent exposed loop on the surface of EcPDF. This allowed efficient purification using IEC.

Experimental

Materials

All standard chemicals were of the highest grade obtainable. H-Phe-NH_2, H-Tyr-OMe, *N*-formyl-Met–Ala-OH, H-Met–Ala-OH, *N*-formyl-Met–Lys-OH, H-Leu–Phe-OH, H-Phe–Leu-OH, H-Asp–Phe-OH, H-Phe–Asp-OH, H-Arg–Phe-OH, H-Phe–Arg-OH, H-Gly–Phe-OH, and H-Phe–Gly-OH were purchased from Bachem (Bubendorf, Switzerland); tris-(2-carboxyethyl)-phosphine (TCEP) was obtained from Fluka (Buchs, Switzerland). If desired, amine groups were formylated in a refluxing mixture containing 1.1 equiv formic acid and 1.1 equiv of acetic acid anhydride [11].

The mono Q HR 5/5 and HiLoad Q Sepharose anion exchange columns were purchased from GE Healthcare Bio-Sciences Ltd (United Kingdom). Centriprep and Centricon centrifugal filter devices were purchased from Millipore Corporation (Billerica, Massachusetts, USA).

E. coli strains CJ236 and JM109 were from Bio-Rad Laboratories GmbH (Munich, Germany). Helper phage M13K07 was from GE Healthcare Bio-Sciences Ltd (United Kingdom).

Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and polynucleotide kinase were obtained from New England Biolabs (Schwalbach, Germany). Adenosine triphosphate and deoxynucleotide triphosphates were purchased from Roche (Mannheim, Germany). Catalase from bovine liver was obtained from Sigma (St. Louis, Missouri, USA). Synthesis of oligonucleotides for site-directed mutagenesis and DNA sequencing were performed by MWG Biotech AG (Ebersberg, Germany).

Construction of *E. coli* PDF wild-type expression vector

Wild-type peptide deformylase from *E. coli* ($EcPDF_{wt}$) was produced with the expression vector pBAD/*Myc*-His-DEST using Gateway cloning technology (Invitrogen). The PDF gene was first amplified by PCR using forward primer 5′-GGG*GACAAGTTTGTACAAAAAAGCAGGCT*AGG-AGGAATTAACCA**ATG**TCCGTGCTTCAAGTGT TACATATTCC-3′ as (*attB1* site in italics, Shine–Dalgarno sequence underlined, start codon in boldface), and reverse primer 5′-GGG *GACCACTTTGTACAAGAAAGCTGGGT***TTA**AGCCCGGGCTTTCAGACGATC CAGTTTTTC-3′ as (*attB2* site in italics, stop codon in boldface) and plasmid pTL7-1. The PCR reaction was performed using AccuPrime Taq DNA polymerase (1U) and accompanying buffer (Invitrogen). The amplification reaction was started with an initial denaturation of 2 min at 95 °C, followed by 30 cycles of 15 s at 95 °C, 30 s at 58 °C and 1 min at 68 °C, with an additional cycle of 5 min at 68 °C. The amplification product was purified using the QIAquick PCR purification kit (Qiagen), after which this product was introduced into the pDONR201 vector via a Gateway BP recombination reaction in a vector:insert molar ratio of 1:2. After transformation of *E. coli* TOP10, recombinant cells were selected by plating on LB plates containing kanamycin (50 μg/mL), followed by overnight incubation at 28 °C. The PDF-encoding gene in pDONR201 was subsequently recombined to expression vector pBAD/*Myc*-His-DEST in a standard Gateway recombination reaction using LR clonase (Invitrogen) and a molar ratio of destination vector vs. entry vector of 1:2.4 (150:300 ng). After transformation of *E. coli* TOP10, recombinant cells were selected by plating on LB plates containing carbenicillin (100 μg/mL) followed by overnight incubation at 28 °C. Finally, a correct clone, as established by plasmid DNA isolation and restriction enzyme analysis was designated pBAD/Myc-His-DEST PDF_{wt}, or pBAD-PDF_{wt}.

Construction of EcPDF variant expression vector

The Kunkel method [14] that we used for site-directed mutagenesis of the *def* gene was performed according to the instruction manual of the Muta-Gene phagemid kit (Bio-Rad Laboratories GmbH, Munich, Germany). The *def* gene from *E. coli* K12 (EMBL accession number U00096, nucleotides 3,431,712–3,432,221) was cloned between the EcoRI and HindIII sites of phagemid pTZ18U [14], placing the gene under the control of the *lac* promoter [15]. The resulting phagemid was used for mutagenesis. A DNA segment encoding an octaglutamate tag between codons for Glu64 and Asn65 was obtained using the primer prPDF-Etag (5′-CGTCAC GGTCCTCTTCTTCTT CCTCCTCTTCCTCTTCCGAAACATC-3′, AvaII restriction site underlined). The oligoglutamate tag is encoded by a mixture of CCT and CTT codons (in bold) to prevent hairpin formation. For the construction of PDF_{short} the primer was prPDFstop (5′-GTTGTTTCA**CTTAAG** ACAGATAATCC-3′, AflII site underlined) which changes the CCG codon for Pro148 into a stop codon (TAA, in bold). After synthesis of dsDNA, this was used to transform *E. coli* JM109 (*ung*+) which contains uracil *N*-glycosylase for inactivating the uracil-containing template. The resulting PDF_{Etag}- and the PDF_{short}-encoding plasmids were respectively called pTL7-1 and pTL7-2. For expression, the mutated genes were recloned into the pBAD/*Myc*-His-DEST vector as described above, yielding pBAD-PDF_{Etag} and pBAD-PDF_{short}.