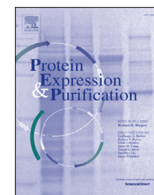
Protein Expression and Purification 129 (2017) 150–157

Contents lists available at ScienceDirect

Protein Expression and Purification

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



Secretory production of designed multipeptides displayed on a thermostable bacterial thioredoxin scaffold in *Pichia pastoris*

Gloria Spagnoli [a], Angelo Bolchi [a], Davide Cavazzini [a], Somayeh Pouyanfard [b], Martin Müller [b], **, Simone Ottonello [a], *

[a] Department of Life Sciences, Biochemistry and Molecular Biology Unit, University of Parma, Italy
[b] German Cancer Research Center, Heidelberg, Germany

A R T I C L E I N F O

Article history:
Received 28 January 2016
Received in revised form
15 April 2016
Accepted 28 April 2016
Available online 29 April 2016

Keywords:
Pyrococcus furiosus thioredoxin
Secretory production
Pichia pastoris
Recombinant peptide antigens
Human papillomavirus minor capsid protein L2
Vaccine

A B S T R A C T

Internal grafting of designed peptides to scaffold proteins is a valuable strategy for a variety of applications including recombinant peptide antigen construction. A peptide epitope from human papillomavirus (HPV) minor capsid protein L2 displayed on thioredoxin (Trx) has been validated preclinically as a broadly protective and low-cost alternative HPV vaccine. Focusing on thioredoxin from the hyperthermophilic archaebacterium *Pyrococcus furiosus* (*Pf*Trx) as a scaffold, we have constructed a modified *Pichia pastoris* expression vector and used a *Pf*Trx fusion derivative containing three tandemly repeated copies of a 19 amino acids peptide epitope from HPV-L2 for expression optimization and biochemical-immunological characterization of the *Pichia*-produced *Pf*Trx-L2 antigen. We show that *Pf*Trx-L2 is produced at high levels (up to 100 mg from a 100 ml starting culture using a multi-cycle induction protocol) and secreted into the culture medium as a highly enriched (>70% pure), non-glycosylated polypeptide that can be purified to homogeneity in a single step. Oxidation and aggregation state, thermal stability and immunogenicity of the endotoxin-free *Pf*Trx-L2 antigen produced in *P. pastoris* were tested and found to be identical to those of the same antigen produced in *Escherichia coli*. Secretory production of endotoxin-free *Pf*Trx-peptides in *P. pastoris* represents a cost- and time-effective alternative to *E. coli* production. Specifically designed for peptide antigens, the *Pf*Trx-expression vector and conditions described herein are easily transferable to a variety of applications centred on the use of structurally constrained bioactive peptides as immune as well as target-specific binder reagents.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Recombinant scaffold proteins (SP) harbouring selected heterologous peptides displayed on their surface are valuable reagents for a number of applications ranging from functional proteomics to high-throughput immune-screenings and peptide-vaccine construction [1–3]. Peptide-SP fusions can be assembled in an end-to-end or an internal grafting configuration. The latter, which mimics the organization of non-terminal (mid-sequence) peptide regions, offers a number of specific advantages such as insertion into a structurally defined site, a superior scaffold-induced constraining and a higher resistance to proteolysis. In an approach named Thioredoxin Displayed Multipeptide Immunogens (TDMI), we have previously exploited internal grafting of selected (multi)peptide epitopes to the disulfide-stabilized, active ("display") site of thioredoxin (Trx) for the construction of recombinant peptide immunogens [4–9]. Thioredoxin is a non-toxic, small-sized (109 amino acids) and highly soluble protein, which has also been successfully employed for peptide aptamer display (i.e., for the construction of artificial, antibody-mimicking

Abbreviations: ABTS, 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); BMMY, Buffered methanol medium; DTNB, 5,5-dithiobis-(2-nitrobenzoic) acid; Endo-H, endoglycosidase H; GST, glutathione S-transferase; HPV, Human papillomavirus; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PBS, phosphate-buffered saline; PSV, pseudovirion; PBNA, pseudovirion-based neutralization assay; *Pf*Trx, *Pyrococcus furiosus* thioredoxin; SP, scaffold protein; Trx, thioredoxin; TDMI, thioredoxin-displayed multipeptide immunogens; VLP, virus-like particle; YPD, yeast extract-peptone-dextrose medium; YPDS, yeast extract-peptone-dextrose medium plus sorbitol.

* Corresponding author. Department of Life Sciences, University of Parma, Parco Area delle Scienze 23/A, 43124, Parma, Italy.
** Corresponding author. DKFZ ATV F035, Im Neuenheimer Feld 242, 69120, Heidelberg, Germany.
E-mail addresses: martin.mueller@dkfz.de (M. Müller), s.ottonello@unipr.it (S. Ottonello).

http://dx.doi.org/10.1016/j.pep.2016.04.012
1046-5928/© 2016 Elsevier Inc. All rights reserved.

"binders") [10–15]. Some thioredoxins, e.g., Trx from the hyperthermophilic archaebacterium *Pyrococcus furiosus* (*Pf*Trx) also have a remarkably high thermal stability (>12 h at 100 °C, apparent Tm > 95 °C [7]).

An extensively characterized TDMI construct, named *Pf*Trx-L2(20-38)$_3$, comprises a fusion between *Pf*Trx and three tandem repeats of a 19 amino acids peptide derived from the N-terminal region (amino acid positions 20–38) of human papillomavirus (HPV) minor capsid protein L2 [7,8]. *Pf*Trx-L2(20-38)$_3$ is one of variously assembled L2-based recombinant immunogens, all centred on the same immunodominant, linear cross-neutralizing epitope region of L2 [16–20], that are being developed as broadly protective and lower cost alternatives to the current HPV vaccines [21]. The latter (Gardasil® and Cervarix®) are produced in yeast and insect cells, respectively, in the form of higher-order assemblies of major capsid protein L1, named virus-like particles (VLP) [21]. Despite a proven safety and efficacy profile, both L1-VLP vaccines suffer from a few important limitations, including the requirement for a stringent cold-chain distribution and a high cost of production.

With the aim of further streamlining *Pf*Trx-L2 production, especially with regard to protein yield, ease of purification and elimination of the endotoxin removal step, which is time-consuming and requires downstream testing of residual endotoxin levels, we transferred production of our Trx-HPV16-L2 immunogen to the methylotrophic yeast *Pichia pastoris*. We show here that *Pf*Trx-HPV16-L2(20-38)$_3$ is produced in *P. pastoris* as a secreted and highly enriched non-glycosylated protein, which can be used as such or further purified in a single step and employed for immunization studies without any additional processing. Importantly, *P. pastoris*-produced *Pf*Trx-HPV16-L2(20-38)$_3$ displays an immunogenicity as well as a thermal stability identical to those of the bacterially produced antigen. Using a multi-cycle induction protocol (i.e., multiple additions of the methanol inducer to the same primary culture), we could produce up to 100 mg of the *Pf*Trx-HPV16-L2 antigen after four methanol-induction cycle**s** applied to a single (100 ml) starter culture. Given the wide applicability of the thioredoxin scaffold [4–6,8,9,12,22,23], this system represents a general tool for the high-yield production of secreted, endotoxin-free and properly folded Trx-peptide fusion proteins to be used for immunization as well as diagnostic, functional, and structural analysis purposes.

2. Materials and methods

2.1. *Pf*Trx-HPV16-L2(20-38)$_3$ construction and *P. pastoris* transformation

An engineered, chemically synthesized *Pf*Trx sequence, previously utilized for bacterial expression of the *Pf*Trx-L2 antigen (pET26b-*Pf*Trx-HPV16-L2(20-38)$_3$; [7]), was modified by PCR using the following oligonucleotide primers (*Pf*Trx-XhoI-Pp):

Fwd: TAAATATAAA**CTCGAG**AAAAGAGAGGCTGAAGCTATTATCGAGTATGACGGCGAAAT
Rev: AATTATTTTA**CTCGAG**TTACTCCTGCAGCTCTTTCAG

These primers insert two *XhoI* restriction sites (*bold*) at both ends and a sequence coding for the last six amino acids of the α-factor secretion signal peptide (*underlined*) to the 5′-end of the amplicon.

The latter served to reconstitute a full-length signal peptide sequence, part of which was lost due to the presence of an XhoI site both in the polycloning site of the pPICZαA vector (Invitrogen) and in the 3′-end region of the α-factor-coding sequence (see Fig. 1A). The reason for choosing XhoI as a cloning site is that it allows to avoid the addition of any extra-amino acid to the *Pf*Trx polypeptide. The amplification product thus generated was eluted with the Zymoclean DNA gel recovery Kit (Zymo Research), digested with *Xho*I (Takara) and cloned into the *Xho*I site of pPICZαA, using the DNA ligation Mighty Mix kit (Takara). In this way, we obtained the intermediate plasmid pPICZα-*Pf*Trx, suitable for directional (*Cpo*I-mediated) insertion of any peptide of interest grafted to a *Pichia* expressible and secretable form of *P. furiosus* thioredoxin. A synthetic DNA fragment coding for the HPV16-L2(20-38)$_3$ tripeptide [7], consisting of three tandemly repeated sequences coding for the L2(20-38) peptide (KTCKQAGTCPPDIIPKVEG) with a GGP spacer interposed between each peptide unit, was then digested with *Cpo*I (a type IIP restriction enzyme with a 7 bp recognition site) and directionally inserted into the unique *Cpo*I site of pPICZα-*Pf*Trx to generate pPICZα-*Pf*Trx-HPV16-L2(20-38)$_3$. Following transfer into *Escherichia coli* DH10 cells and sequence verification, the latter construct was used for *P. pastoris* transformation. To this end, *P. pastoris* KM71H cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) to an OD_{600} of 2.0, sedimented (3000 × g, 5 min) and resuspended in one-fifth of the initial culture volume of ice-cold 100 mM Tris–HCl (pH 8.0) containing 10 mM dithiothreitol (DTT), 100 mM lithium acetate and 0.6 M sorbitol. This was followed by electroporation-mediated transformation with the *SacI*-digested pPICZα-*Pf*Trx-HPV16-L2(20-38)$_3$ plasmid and selection on YPDS agar (YPD + 18.22% sorbitol) containing different amounts (0.1–2.0 mg/ml) of zeocin (Invivogen) as reported previously [24,25] and described in detail in the EasySelected Pichia Expression Kit User Manual (Invitrogen).

2.2. *Pf*Trx-HPV16-L2(20-38)$_3$ expression and purification

A single colony of a *P. pastoris* transformant resistant to the highest zeocin concentration was used for the initial testing of *Pf*Trx-HPV16-L2(20-38)$_3$ expression, which was carried out under standard BMMY medium (1% yeast extract, 2% peptone, 0.1 M K-phosphate buffer, 1.34% YNB, 0.5% methanol and 4 mg/ml biotin) conditions, i.e., incubation at 30 °C with continuous shaking at 200 rpm and 0.5% methanol added every 24 h, as specified in the EasySelect Pichia Expression Kit version G.2005 Instruction Manual (Invitrogen). The growth medium of the methanol-induced culture was used for the initial assessment of *Pf*Trx-HPV16-L2(20-38)$_3$ expression by SDS-PAGE analysis. This was followed by a systematic examination of different experimental parameters known to affect heterologous protein production in *P. pastoris* [24], namely culture medium pH (varied from 5.0 to 8.0 in 0.5 pH units increments), growth temperature (varied from 20 °C to 32 °C in 3° increments), cell suspension:culture flask volume ratio (1:5, 1:10, 1:20), and methanol inducer concentration (varied from 0.5% to 2.5% v/v in 0.5% increments). Growth at 23 °C, pH 6.5, with a 1:10 cell suspension:flask volume ratio and a 2% concentration of the methanol inducer were identified as the best conditions for *Pf*Trx-HPV16-L2(20-38)$_3$ expression. These conditions were transferred to a multi-cycle induction protocol, which allows to increase the protein yield that can be obtained from a single small-scale (100 ml) batch culture and is based on an initial 5-days induction cycle with daily 0.5% methanol additions, followed by cell harvesting and three additional induction/culture cycles. In each of these cycles, yeast cells were sedimented by centrifugation (3000 × g, 5 min), followed by conditioned medium recovery, resuspension of the cell pellet in 100 ml of fresh, 2% methanol-containing BMMY, and culturing for five days without any further methanol addition. The combined growth media, containing the *Pf*Trx-HPV16-L2(20-38)$_3$ polypeptide in a >70% pure form as verified by SDS-PAGE, were exchanged into 25 mM MES (pH 5.8) and loaded onto a 1 ml HiTrap Capto-S column (GE Healthcare), which was eluted with a 40 ml

Download English Version:

https://daneshyari.com/en/article/5516178

Download Persian Version:

https://daneshyari.com/article/5516178

Daneshyari.com