Protein Expression and Purification 88 (2013) 7–12

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

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

Expression and purification of chimeric peptide comprising EGFR B-cell epitope and *measles virus* fusion protein T-cell epitope in *Escherichia coli*

Meizhi Wu [a], Lin Zhao [a,b], Lei Zhu [a], Zhange Chen [a], Huangjin Li [a,*]

[a] *Laboratory of Biopharmaceutical, College of Life Science and Biopharmaceutical, Guangdong Pharmaceutical University, Guangzhou Higher Education Mega Center, Guangzhou, Guangdong 510006, PR China*
[b] *Institute of Clinical Pharmacology, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong 510405, PR China*

ARTICLE INFO

Article history:
Received 16 August 2012
and in revised form 2 November 2012
Available online 5 December 2012

Keywords:
EGFR
B-cell epitope
Chimeric peptide
Expression
Purification

ABSTRACT

Chimeric peptide MVF-EGFR$^{237-267}$, comprising a B-cell epitope from the dimerization interface of human epidermal growth factor receptor (EGFR) and a promiscuous T-cell epitope from *measles virus* fusion protein (MVF), is a promising candidate antigen peptide for therapeutic vaccine. To establish a high-efficiency preparation process of this small peptide, the coding sequence was cloned into pET-21b and pET-32a respectively, to be expressed alone or in the form of fusion protein with thioredoxin (Trx) and His_6-tag in *Escherichia coli* BL21 (DE3). The chimeric peptide failed to be expressed alone, but over-expressed in the fusion form, which presented as soluble protein and took up more than 30% of total proteins of host cells. The fusion protein was seriously degraded during the cell disruption, in which endogenous metalloproteinase played a key role. Degradation of target peptide was inhibited by combined application of EDTA in the cell disruption buffer and a step of Source 30Q anion exchange chromatography (AEC) before metal-chelating chromatography (MCAC) for purifying His_6-tagged fusion protein. The chimeric peptide was recovered from the purified fusion protein by enterokinase digestion at a yield of 3.0 mg/L bacteria culture with a purity of more than 95%. Immunogenicity analysis showed that the recombinant chimeric peptide was able to arouse more than 1×10^4 titers of specific antibody in BALB/c mice. Present work laid a solid foundation for the development of therapeutic peptide vaccine targeting EGFR dimerization and provided a convenient and low-cost preparation method for small peptides.

Introduction

B-cell epitope peptide vaccine has an attractive prospect in the treatment of malignant tumors, which is able to evoke continuous and stable humoral immunity response just by limited numbers of injection and achieve similar therapeutic effects of long-term, high-dose and repeated administration of antibody [1–4]. However, B-cell epitope peptide is too small in molecular size to sufficiently elicit high-titer specific antibodies (Abs) usually [5]. Kaumaya reported a novel chimeric peptide consisting of a B-cell epitope from HER2 and a promiscuous T-cell epitope of 18 amino acid residues derived from the *measles virus* fusion protein (MVF), in which the B-cell epitope is fused to the C-terminal of MVF via GPSL linker [6–8]. A series of chimeric peptides based on B-cell epitopes from Her2 have showed good antibody response in Her2 positive breast cancer patients [7]. All reported chimeric peptides like "MVF-GPSL-B-cell epitope" was synthesized by chemical method, But chemosynthesis is high-cost and technically difficult, especially to long peptides more than 50 amino acid residues [9,10]. So it is meaningful to establish a high effective preparation process for the chimeric peptide based on B-cell epitope and MVF by genetic engineering.

Epidermal growth factor receptor (EGFR) is an attractive target in the treatment of epidermal cancers, which is over-expressed in a variety of epidermal cancers, including head and neck, lung, colon, breast, ovarian, brain and pancreatic cancers [11–15], and its over-expression correlates with poor response to treatment, disease progression, and poor survival [16–20]. A series of anti-EGFR drugs such as monoclonal antibody and tyrosine kinase inhibitor have been developed [21–24], but only 10–25% patients with EGFR over-expression cancer respond to these agents [25,26]. Genetic polymorphism and mutation of EGFR may be the most important reasons for this low response rate. Dimerization plays a key role in the activation of EGFR and the other members of its family, and the highly conserved dimerization interface may be the ideal target of the anti-EGFR [26,27]. In order to develop a B-cell epitope peptide vaccine targeting to the dimerization interface of EGFR, we selected two B-cell epitope peptides EGFR$^{237-267}$ and EGFR$^{237-303}$

* Corresponding author. Address: No. 280 Waihuan East Road, Guangzhou Higher Education Mega Center, 510006, PR China. Fax: +86 020 39352201.
E-mail address: lihuangjin@sohu.com (H. Li).

1046-5928/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2012.11.010

from the dimerization interface of EGFR to construct chimeric peptide like "MVF-GPSL-B-cell epitope". MVF-EGFR$^{237-303}$ has been successfully produced by recombinant *Escherichia coli* [28], but the smaller peptide MVF-EGFR$^{237-267}$ (consisting of 53 amino acid residues) has not been effectively produced because it was highly sensitive to endogenous proteases of host cells.

This work reported a high-effective process for preparing MVF-EGFR$^{237-267}$ by recombinant *E. coli*, which would lay a good foundation for the development of therapeutic vaccine targeting the dimerization of EGFR and provide a novel strategy for the preparation of chimeric peptide and other small peptide.

Materials and methods

Materials and reagents

The plasmid pUC19-MVF-EGFR$^{237-267}$ containing the coding sequence of chimeric peptide MVF-EGFR$^{237-267}$ was constructed and preserved by our laboratory. *E. coli* BL21 (DE3), Expression vector pET21b and pET32a were products of MERK (GERMANY). Taq DNA polymerase, restriction endonucleases, T4 DNA ligase, plasmid extraction kit, DNA ladder and protein marker were purchased from TaKaRa (JAPAN). Enterokinase was purchased from Shanghai Kayon Biological Technology Company (China). BCA kit was purchased from Pierce Chemical Company (USA). HisTrap affinity column, Source 30Q and Sephadex-G25 were the products of GE Healthcare Life Sciences (USA). HPLC BEH C18 column was a product of Waters (USA). 5–8-weeks female BALB/c mice were purchased from Guangdong experimental animals center (China). Freuds complete adjuvant and incomplete adjuvant were the products of Sigma (USA). The peptide EGFR$^{237-267}$ was synthesized at Shanghai Apeptide Company (China).

Construct of expression vectors

The coding sequence of MVF-EGFR$^{237-267}$ was amplified by PCR from pUC19-MVF-EGFR$^{237-267}$ according conventional method, the restriction sites were introduced by PCR primers. The PCR products were inserted into expression vectors pET21b and pET32a after digested with *NdeI* and *HindIII*, *NcoI* and *HindIII*, respectively, and then transformed into *E. coli* DH5α. Recombinants were screened by colony PCR. The recombinant plasmids pET21b-MVF-EGFR$^{237-267}$ and pET32a-MVF-EGFR$^{237-267}$ were extracted and then transformed into *E. coli* BL21 (DE3). Recombinant *E. coli* BL21 (DE3) were shaker-cultured at 37 °C and 170 rpm in LB medium containing 100 μg/ml of ampicillin until the OD_{600} reached 0.4–0.6, then induced by addition of IPTG to a final concentration of 1 mmol/L for 4 h. The cells were harvested by centrifuge at 8000*g* for 5 min at 4 °C, then suspended in 50 mmol/L Tris–HCl (pH7.0) with a ratio of 1% (w/v) and broken by ultrasonication. The lysates were centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant and the precipitate were sampled for SDS–PAGE and gel scanning analysis.

Analysis of enzymatic stability

Host cells were induced and harvested as above, then broken by ultrasonication in 50 mmol/L Tris–HCl (pH7.0) buffer containing no inhibitor, 1 mmol/L EDTA, 1 mmol/L PMSF, and EDTA + PMSF, respectively, then centrifuged at 12,000*g* for 10 min at 4 °C, and the supernatants were sampled for SDS–PAGE and gel scanning analysis.

Purification and recovery

Method 1

Recombinant *E. coli* BL21 (DE3) was induced as above in 1L LB culture medium. The paste of cells was suspended in 200 ml of 500 mmol/L NaCl, 20 mmol/L imidazole, mmol/L Tris–HCl, pH7.0, then broken by ultrasonication and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was filtrated through 0.45 μm membrane, then loaded on HisTrap affinity column according to the manufacturer's instructions. The fusion protein was eluted by 200 mmol/L imidazole, 500 mmol/L NaCl, mmol/L Tris–HCl, pH7.0. The sample was loaded on Sephadex G25 column to replace the buffer, then cleaved by enterokinase at 16 °C overnight in 50 mmol/L NaCl, 20 mmol/L Tris–HCl, pH8.0. The digestion product was loaded on HisTrap affinity column again and collected the flow-through peak as the recovered MVF-EGFR237–267 chimeric peptide.

Method 2

Recombinant *E. coli* BL21 (DE3) was induced as above in 1L LB culture medium. The paste of cells was suspended in 200 ml of 1 mmol/L PMSF, 1 mmol/L EDTA, 50 mmol/L Tris–HCl, pH7.0, then broken by ultrasonication and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was filtrated through 0.45 μm membrane, then used as the sample for Source 30Q AEC. Source 30Q column (16 mm × 200 mm) was equilibrated with 4 column volume (CV) of 50 mmol/L Tris–HCl, pH7.0, and the sample was loaded at a linear flow rate of 300 cm/h, then the column was stepwise eluted with 50, 100, 250, 1000 mmol/L NaCl in the 50 mmol/L Tris–HCl buffer (pH7.0). The elution peak with 250 mmol/L NaCl were pooled as the fusion protein sample, then polished by MCAC, digested by enterokinase, and recovered by a second MCAC as above.

SDS–PAGE and gel scanning analysis

SDS–PAGE was carried out according to conventional method, the expression level; purity and content of target protein were assayed by gel scanning analysis with Tanon gel scanning system (China) according to the manufacturer's instructions.

Purity assay for chimeric peptide

The purity of final chimeric peptide was measured by high performance liquid chromatography (HPLC) on Waters HPLC BEH C18 column. Recovered chimeric peptide was desalted by Sephadex G25 chromatography, then 20 μg desalted peptide was loaded on C18 column. The elution was carried out using a linear gradient of 30–70% acetonitrile at a flow rate of 0.8 ml/min in the presence of 0.1% trifluoroacetic acid (TFA).

Assay of protein concentration and yield

Protein concentration was determined by BCA method with BCA kit according to the manufacturer's instructions. The yield of target protein was calculated according to the following formula: $m = c \times p \times v$, where m, c, p, v represent mass (mg), concentration (mg/ml), purity (%) and volume (ml), respectively.

Immunization of animals and ELISA

18 BALB/c mice were divided into three groups, and immunized with chimeric peptide prepared by method 1, chimeric peptide prepared by method 2 and adjuvant alone (as control), respectively. Animals were immunized with 0.05 mg of peptide dissolved in double-distilled water (ddH_2O) emulsified (1:1) in Freuds complete adjuvant, then were boosted with 0.05 mg of peptide dissolved in ddH_2O emulsified (1:1) in Freuds incomplete adjuvant at 3 weeks intervals. Blood was collected via retro-orbital sinus and sera tested for Abs titers. ELISA was performed in 96-well plates according to the method of Voller et al. [29]. Briefly, the 96-well plates were coated with 100 μl of synthetic peptide