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Easy and efficient protocol for purification of recombinant peptides

Prashant Kumar, Gopala K. Aradhyam *

Department of Biotechnology, IIT Madras, Chennai 600 036, India

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ABSTRACT

Peptide synthesis and purification remains a challenge. Low abundance leads to small yields when peptides are purified from natural sources. On the other hand, synthetic methods are limited by the chemical properties of the amino acids and the concurrent aggregation of peptides. In this paper, we report a versatile, high yielding and general purification method for randomly chosen recombinant peptides of variable sizes (ranging from ~1.7 kDa to ~10 kDa). Expressed as fusion proteins with commonly used tag proteins, these peptides are cleaved by 'PreScission protease' in a volatile buffer that makes concentration and recovery of the peptide easy. Separation of the cleaved peptide is achieved by selective precipitation of the larger tag protein with acetonitrile; leaving the peptide in solution. Our protocol can be used to generate a wide variety of peptides in significant quantities for biochemical, biophysical and physiological studies.

Introduction

Peptides are bioactive molecules that act as ligands (e.g. apelin, angiotensin, insulin, glucagon) [1–4], inhibitors (e.g., cyclic-peptides, peptide analogues) [5], toxins (e.g., conotoxin and snake toxins) [6,7], biomarkers [8], molecules of therapeutic importance (e.g., bacteriocin) [9] and have often been employed for probing the structure–function relationship of proteins [10]. Peptides are obtained either through chemical synthesis, using solid phase chemistry [11], or purified from natural sources [9]. Despite the popularity of solid phase method, peptide synthesis has several limitations e.g., steric hindrance due to bulkier side chains and/or amino acid protecting groups [12–15] and problems with coupling reaction efficiency [16,17]. While recombinant DNA technology helps overcome many of these problems, purification of the peptide remains a major hurdle for large-scale requirements.

Few examples exist of cloning and expression of peptides in the bacterial system, mostly as fusion partners to maltose binding protein (MBP[1]) or glutathione S-transferase (GST) for ease of purification, that either report low yield or have not been applied to purification of peptides of variable sizes and structures [18–23]. Peptides are cleaved from the tag protein either by cyanogen bromide (CNBr) [24] or with enzymes (to cleave at engineered enzyme sites), and are subsequently separated by reversed phase liquid chromatography [25]. Two drawbacks with CNBr cleavage method are (i) its inapplicability to peptides containing methionine and (ii) generation of unwanted homoserine lactone as a by-product [26]. Another problem, emanating from the small size of most peptides, is getting salt-free concentrated peptide.

In order to overcome these limitations we have developed a simple, efficient and versatile strategy to purify peptides of varying sizes (in salt-free condition) expressed as fusion partners to MBP-His_6 or GST in bacterial system. Our procedure involves two steps: (i) cleavage of the peptide from its fusion partner in a volatile buffer (0.15 M NH_4HCO_3) and (ii) selective precipitation of the larger tag protein with acetonitrile. Use of this protocol not only makes desalting redundant but also reduces a purification step. We report the use of this protocol in purification of peptides of variable sizes (from ~1.70 kDa to ~10 kDa) unlike previous attempts [19,21,22,24]. The procedure is quantifiable and amenable to scale up processes. To prove our point we chose peptides from (a) soluble protein (b) an intra-cellular peptide of a trans-membrane protein and (c) a peptide ligand (with varying sizes). We have characterized the secondary structure of these peptides and also demonstrated functional activity of the peptide ligand apelin (~1.70 kDa).

* Corresponding author. Address: Signal Transduction Lab, Department of Biotechnology, IIT Madras, Chennai 600 036, India. Tel.: +91 4422574112; fax: +91 4422574102.

E-mail addresses: candidprashant@gmail.com (P. Kumar), agk@iitm.ac.in (G.K. Aradhyam).

[1] *Abbreviations used:* MBP, maltose binding protein; GST, glutathione s-transferase; CNBr, cyanogen bromide; APJ C-ter, APJ C-terminal; IPTG, isopropyl β-D-1-thiogalactopyranoside; PMSF, phenylmethanesulfonylfluoride; GdmCl, guanidine hydrochloride; CV, column volumes; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; CCA, alpha-cyanohydroxycinnamic acid; TFE, 1,1,1-trifluoroethanol; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; DMEM, *Dulbecco's modified eagle medium*; FBS, foetal bovine serum.

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Methods

Cloning, expression and purification of rat nesfatin1, APJ C-terminal domain, apelin23 and apelin*

The genes for rat nesfatin1, APJ C-terminal (APJ C-ter), apelin23* and apelin peptides were cloned in bacterial expression vectors. Rat nesfatin1 gene was chemically synthesized (Eurofins-MWG) and inserted into pCR2.1, a TA cloning vector using primers with adaptors for NheI and XhoI restriction sites. It was sub-cloned into a modified pMAL-c5E vector to give MBP-His_6-rat nesfatin1. The vector was modified to have 6× Histidine for purification and PreScission protease cleavage site at the N-terminal of rat nesfatin1. The other peptide gene (APJ C-ter) was amplified by PCR using pcDNA3.0APJ clone as template and primers containing BamHI and EcoRI restriction site adaptors (Supplementary Table 1). It was inserted into pGEM-T vector (a TA cloning vector) and subsequently sub-cloned into pGEX-6P-1 expression vector to generate GST–APJ C-ter. Apelin23* gene was chemically synthesized and inserted into another TA cloning vector (pGEM-T Easy) and subsequently sub-cloned into a single EcoRI site of pGEX-6P-1 vector to generate GST-apelin23*. Extra 8 amino acids at N-terminal domain of apelin23*, generated as a consequence of this protocol, were deleted using site directed mutagenesis kit from Stratagene as per the manufacturer's protocol to generate GST-apelin (Supplementary Table 1). All clones and their orientations were confirmed by nucleotide sequencing.

All the peptides with their respective tags were expressed in bacteria (see below) for purification. MBP-His_6-rat nesfatin1 gene was expressed in *Escherichia coli* BL21 (DE3). Cells were grown at 37 °C until $A_{600} \sim 0.9$ was achieved. Cells were then induced with 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) in *LB* media at 37 °C for 6 h. All other peptides with GST tag (APJ C-ter, apelin23* and apelin) were expressed in *E. coli* ER2566 cells. These cells were grown at 37 °C to achieve $A_{600} \sim 0.9$. Cells were then induced with 200 μM IPTG at 24 °C for 12 h. All cells were harvested by centrifuging them at 18,000×*g* and subsequently stored at −80 °C.

Purification by affinity chromatography: Cells expressing MBP-His_6-rat nesfatin1 were re-suspended in high salt buffer [50 mM Tris–Cl (pH 8.0) and 500 mM NaCl, containing 2 mM β-mercaptoethanol, 1 mM phenylmethanesulfonylfluoride (PMSF) and 2 M guanidine hydrochloride (GdmCl)]. Cells were mechanically lysed in a 'vibrasonic' cell disrupter at ultrahigh (amplitude of 30 db) frequency with 2 s 'on' and 4 s 'off' cycle, for 2 min. Soluble protein fractions were obtained by centrifugation at 18,000×*g* for 45 min at 4 °C and purified by passing through a 4 ml Ni–NTA resin at a flow rate of 0.1 ml/min. The resin was then washed with 8 column volumes (CV) of high salt buffer containing 0.6 M GdmCl (flow rate of 0.4 ml/min) followed by another wash of 6 CV with high salt buffer without GdmCl. The fusion protein was finally eluted with 2 CV of Ni–NTA elution buffer (high salt buffer with 300 mM imidazole).

Peptides with GST tag were purified by GSH resin. GST–APJ C-ter failed to bind to GSH resin efficiently in cleavage buffer alone [20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM PMSF and 1 mM dithiothreitol (DTT)]. Hence, a variety of conditions were attempted with different concentrations of urea (e.g., 0.5 M, 1.0 M and 2 M) in the cleavage buffer. The presence of 2 M urea yielded the best binding conditions. The entire cellular protein (12 ml) was incubated with 4 ml GSH resin for 12 h at 4 °C with end-to-end mixing (batch method). The resin was washed thrice with 4 CV of the cleavage buffer in 2 M urea. The bound protein was eluted with 4 CV of elution buffer (10 mM glutathione in 50 mM Tris–Cl, pH 8.0). Bacterial cells over-expressing the other tagged proteins (GST-apelin23*, GST-apelin and Pre-Scission protease) were lysed in the cleavage buffer alone and the resultant soluble cellular protein of GST-apelin23*, GST-apelin and PreScission protease were passed through GSH resin at a flow rate of 0.1 ml/min, subsequently washed with 10 CV of cleavage buffer at a flow rate of 1 ml/min. The protein was then eluted with 3 CV of elution buffer.

The eluted samples obtained of all the purified proteins were pooled and divided into two parts and buffer exchanged into either cleavage buffer or volatile buffer. The purity of all the tagged proteins was checked on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) [27]. The fusion proteins yields were estimated by Bradford method [28].

Peptide cleavage

Peptides were cleaved by PreScission protease to release them from their affinity tags. PreScission protease (with a unique octamer cleavage sequence, LEVLFQ/GP) tagged to GST used throughout this study, was also purified to ~99.0% by GSH affinity chromatography [29]. The purified fusion proteins were digested either in cleavage buffer or volatile buffer with PreScission protease. The concentration of the fusion proteins was kept at 0.5 mg/ml for GST–APJ C-ter, and 1 mg/ml for MBP-His_6 rat nesfatin1, GST-apelin23* and GST-apelin respectively. The cleavage of the fusion proteins was done at 8 °C for 16 h with protein-to-protease ratio at 25:1 (w/w) in both the buffers.

Peptide purification

All the peptides released from tags by protease cleavage were purified in the following manner. The cleaved tags, GST of APJ C-ter peptide and MBP-His_6 of rat nesfatin1 peptide were precipitated with acetonitrile in ratio of either 1:1 or 2:1 (acetonitrile: buffer; v/v) in cleavage or volatile buffers at 25 °C for 20 min. The solution was mixed by inverting it 3–4 times and kept standing for 20 min to complete the precipitation. The soluble fractions containing the peptides were separated by centrifugation at 19,000×*g* for 20 min at 4 °C. The supernatants were freeze-dried and stored for further analysis. The purity of the peptides was checked by analyzing the samples on Tricine–SDS–PAGE [30]. In fact, acetonitrile precipitation in the ratio of 2:1 (acetonitrile: volatile buffer; v/v) was also tested for its efficiency on purification of rat nesfatin1 at different fusion protein concentrations 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml.

For large-scale purification of the peptides, 50 mg each of GST-apelin23* and GST-apelin fusion proteins were digested in volatile buffer and precipitated with acetonitrile in the ratio 2:1 (acetonitrile: buffer; v/v) in buffer as mentioned above. The soluble fractions containing the peptides were freeze-dried and reconstituted in 1 ml of 30% acetonitrile and 0.1% trifluoroacetic acid (TFA) to check purity. Peptide fractions were loaded on size-exclusion "peptide column" (Superdex peptide 10/300 GL column, GE Life Sciences). The column was run at 0.5 ml/min and fractions of 1-ml size were collected (monitored by absorbance at 280 nm). The fractions corresponding to peaks were analyzed by MALDI-MS. The purified peptides rat nesfatin1, apelin23* and apelin were estimated by fluorescamine method [31].

MALDI-MS analysis of apelin23 and apelin*

The purity of apelin23* and apelin peptides, and their sizes were analyzed on MALDI-MS for which the samples were initially mixed in 1:1 ratio of CCA resin (alpha-cyanohydroxycinnamic acid). Subsequently, the resin (10 mg/ml) was prepared by re-suspending it in 1:1:1 ratio of water/acetonitrile/0.1% TFA. Spectra were recorded