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High yield expression and purification of full-length Neurotensin with pyroglutamate modification

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Abstract

Neurotensin (NT) is a 13-residue endogenous peptide found in mammals, with neurotransmission and hormonal roles in the central nervous system and gastrointestinal tract, respectively. The first residue of NT is a pyroglutamate (pGlu) that makes the expression and purification of large amounts of NT with native modification challenging. Here, we describe a simple and efficient procedure for expression and purification of large amounts of NT based on using the small ubiquitin-like modifier (SUMO) as a fusion partner and subsequent enzymatic conversion of the N-terminal glutamine to pGlu. Yields of 13 mg/L and 8 mg/L of pure peptide were obtained from expression in rich and minimal media, respectively. The method is adaptable to expression and purification of proteins and peptides with pGlu modification in a wide range of eukaryotic and prokaryotic expression hosts.

Keywords: neurotensin, pyroglutamate, isotope labeling, SUMO

1. Introduction

Neurotensin is an endogenous tridecapeptide with the sequence: pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu which was isolated from Bovine hypothalamus in 1973 [1, 2]. In mammals NT plays critical roles in the central nervous system (CNS) as a neuromodulator as well as in the gastrointestinal tract to facilitate digestion and absorption of fat [3]. As most of the effects of NT are mediated via neurotensin receptor 1, NTS_1, this interaction is a potential target for the treatment of a wide range of diseases, including schizophrenia and cancer [3]. Structure-activity relationship studies indicate NT8-13 to be the shortest active epitope of NT, which is often used as a model for structural and medicinal chemistry studies. Although these studies help to understand the mechanism of activation of NTS_1, the possible role of the N-terminal region in this process is most often overlooked, even though the N-terminal fragment of NT has been shown to act as an affinity enhancer for the binding of NT to NTS_3 [4].

Being disordered in solution and possibly flexible in the receptor bound state [5], NMR is an important technique for structural characterization of the N-terminal fragment of NT. One of the bottlenecks in NMR studies of full-length NT is the lack of efficient and cost-effective methods for the production of isotope-labelled NT with an N-terminal pGlu. pGlu is a naturally occurring chemical modification that is of structural and functional significance in proteins, formed through spontaneous or enzymatic cyclization of an N-terminal glutamate or glutamine [6]. To date, two systems have been developed for large scale expression and purification of full-length NT in *E. coli* based on expression of NT as a fusion with Glutathione S-transferase or B1 Domain of Streptococcal Protein G (GB1) and cyanogen bromide (CNBr) cleavage of the peptide from the tags [7, 8]. However, both methods suffer from the disadvantage of using CNBr cleavage as well as introducing mutations to the N-terminus of the peptide that might impact the function of the peptide. Moreover, the spontaneous chemical conversion of N-terminal glutamine and glutamate residues to pGlu is typically incomplete causing sample inhomogeneity that hampers NMR characterization [6].

In this context, we developed an efficient protocol for expression and purification of NT with the pGlu at the N-terminus that is adaptable to other peptides and proteins. The method is based on expression

of NT in fusion with the easily cleavable SUMO tag and subsequent enzymatic conversion of N-terminal glutamine to pGlu by human glutaminyl peptide cyclotransferase (hQPCT) in the same reaction. Using the presented method, we could purify up to ~8 and ~13 mg of highly purified wt-NT from minimal and rich media, respectively. The current method is applicable to expression and purification of pGlu-proteins and peptides in eukaryotic and prokaryotic expression hosts without any size limitation, including Aβ-peptides and antibodies [9, 10].

2. Material and methods

2.1. Construct design

SUMO-NT and ulp1 protease genes were synthesized and cloned into pET15b vectors incorporated between Nhe1 and bamH1 restriction sites by GenScript Biotech. The first residue of NT was mutated to glutamine for further enzymatic conversion to pGlu. hQPCT construct was a generous gift from Prof. Turano from the University of Florence, Italy. All the constructs contain the ampicillin resistance gene.

2.2. Expression and purification of SUMO-NT in rich medium

The ZYP-5052 autoinduction media supplemented with 1% glycerol and 0.3% lactose was used for expression of SUMO-NT in rich media [11]. One liter of culture was inoculated with 10 mL of overnight LB medium pre-culture and supplemented by 100 µg/mL ampicillin. The flasks were incubated at 37 °C until an OD_{600} of ~1 was reached; expression was continued for 48 h at 16 °C. The cells were harvested by centrifugation at 5000×g for 20 min. The cell pellet was dissolved in solubilization buffer, 4 mL per 1 g wet-cell weight, containing 50 mM potassium phosphate pH 7.8, 500 mM NaCl, 10 mM imidazole, 5 mM $MgSO_4$ supplemented with 10 mg DNase, 50 mg lysozyme and one Roche protease inhibitor tablet. The cell suspension was stirred at 4 ºC for 30 min and crushed with 5 cycles using an Avestin Emulsiflex C3 cell crusher. The cell debris was pelleted by centrifugation at 30,000 × g for 45 min and the collected supernatant was incubated with Ni-NTA resin that had been preequilibrated with solubilization buffer, in a gravity flow column for 2 h at 4 ºC. After washing the resin with 50 mL of

solubilization buffer containing 1 mM ATP, the SUMO-NT was eluted by 30 mL of solubilization buffer containing 350 mM imidazole. The presence of ATP and $MgSO_4$ in the washing step removes potential contamination from the bacterial chaperone DnaK [12]. Imidazole was removed by 3 cycles of concentration/dilution by using 3-kDa cut-off amicon centrifugal units, concentrated and then treated with 1 µM of each ubiquitin-like specific protease 1, (ulp1 SUMO protease) and hQPCT in the presence of 1 mM β-mercaptoethanol or dithiothreitol, overnight at 4 ºC. The cleaved peptide was further purified on an Agilent HPLC equipped with Phenomenex C18 column. The peptide was eluted from the column by a gradient of 22-30% TFA-acidified Acetonitrile (ACN) over 20 min.

2.3. ^{15}N labeling of NT in minimal media

10 mL of overnight preculture in LB medium was centrifuged and the cell pellet was added into 1 L of N-5052 autoinduction minimal media supplemented with 1% glycerol, 0.3% lactose and 1 g/L of ^{15}N-NH_4Cl. The flasks were incubated at 37 °C until the OD_{600} reached ~1 and then were transferred to 16 °C for 36 hours. Expression at 20 °C for 24 h resulted in ~20 % reduction of the final yield.

2.4. hQPCT expression and purification

hQPCT was expressed and purified as previously described [13]. Briefly, 1 L of super-broth medium, supplemented with 100 µg/mL ampicillin, was inoculated with 10 mL of overnight preculture and incubated at 37 °C until OD_{600} reached 0.6. Then the expression was induced with 0.2 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG) and culture continued for 48 h at 17 °C. The harvested cells were resolubilized in 50 mM tris buffer pH 8, 150 mM NaCl and 10 mM imidazole. The cells were lysed by cell crusher, cell debris removed by centrifugation at 30000×g and the supernatant was loaded onto gravity flow Ni-NTA resin. After 1 h incubation at 4 °C the unbound proteins were removed, and the resin was washed in lysis buffer supplemented with 5 mM $MgSO_4$ and 1 mM ATP. The hQPCT was eluted with 350 mM imidazole in the same buffer. The imidazole was removed by buffer exchange, and the purified protein was stored in lysis buffer without imidazole at -80 °C for further use. The hQPCT

needs to be reloaded with Zinc ions after purification [13], however, ion reloading was skipped as the purified protein showed enough activity for our experiments.

2.5. Ulp1 expression and purification

Ulp1 was expressed in BL21 (DE3) cells in LB media. Cells were incubated at 37 °C to an OD_{600} of 0.6 and expression was induced by 1 mM of IPTG for another 4 h. The cells were harvested, resolubilized in 50 mM phosphate buffer pH 7.8, 300 mM NaCl, 10 mM imidazole and 1 mM β-mercaptoethanol, crushed and centrifuged to remove cell debris. The supernatant was loaded onto Ni-NTA gravity column and incubated for 1 h at 4 ºC. The column was washed with the lysis buffer supplemented with 1 mM ATP and 5 mM $MgSO_4$ and ulp1 was eluted by lysis buffer supplemented with 350 mM imidazole. Imidazole was removed by buffer exchange and the protein was snap frozen in 50 mM phosphate buffe pH 7.8, 300 mM NaCl, and 1 mM β-mercaptoethanol and stored at -80 ºC for further use.

2.6. Expression and purification of NTS_1

NTS_1 was purified as described previously with some minor modifications [14]. The NTS_1 gene in a pDS170 vector was transformed into BL21 (C43) cells and plated onto an agar plate overnight. A single colony was inoculated into overnight LB pre-culture and incubated at 37 ºC shaking at 200 rpm. Next morning, 10 mL of pre-culture was used to inoculate 1 L of ZYP5052 media supplemented with 1% glycerol, 0.3% lactose and 100 µg/ml ampicillin. The culture was incubated at 37 ºC until an OD_{600} of ~1; the flasks were transferred to 20 ºC and expression was continued for another 24-30 h. The cells were harvested, resuspended in 2×lysis buffer, 50 mM HEPES pH 7.8, 400 mM NaCl, 10 mM imidazole, 4 mM $MgSO_4$, 20% glycerol, 10 mg DNase, 50 mg Lysozyme and 1×EDTA-free protease inhibitor tablet. The resuspended cells were sonicated for 10 min at 30% power with 10-sec on/20-sec off cycles. The expressed receptor proteins were extracted from the cell membrane by adjusting the cell lysate to 1% dodecylmaltoside (DDM), 0.12% cholesterol (CHS) and 0.6% CHAPS. The solution was

stirred gently for 2 h at 4 ºC. The cell debris were separated by centrifugation and the detergent-solubilized proteins were loaded onto Talon resin, washed with 25 mM HEPES pH 7.8, 500 mM NaCl, 4 mM $MgSO_4$, 1 mM ATP, 0.05% DDM, 10 mM imidazole and 10% glycerol and eluted with 200 mM imidazole in 25 mM HEPES buffer, 300 mM NaCl, 0.05% DDM and 10% glycerol. The purified receptor was supplemented with 100 mM Na_2SO_4, 1 mM TCEP and 1 μM homemade 3C protease. The cleaved receptor was separated by a cycle of reverse IMAC and polished by applying to a Superdex S200 increase 10/300 GL and eluting in 50 mM potassium phosphate pH 7.4 or pH 6 containing 100 mM NaCl and 0.05% DDM.

2.7. NMR spectroscopy

The purified ^{15}N-labelled peptide was dissolved in 50 mM potassium phosphate buffer pH 6 containing 100 mM NaCl, 0.02% NaN_3, 1×Roche protease inhibitor cocktail, 50 μM EDTA, 50 μM PMSF and 0.5 mM DSS, as a reference, and doped with 10% D_2O. Sensitivity enhanced ^{1}H-^{15}N HSQC spectra of purified peptide were collected on a 700 Avance AVIII HD Bruker spectrometer equipped with a triple resonance TCI cryoprobe. The spectra were collected with spectral widths of 10 ppm on ^{1}H dimension and 22 ppm on ^{15}N dimension, 256 t_1 increments, 2048 t_2 data points and 4 or 32 scans per t_1 point for 0.4 mM solution of NT (where residue 1 is pGlu) and a solution of NT (where residue 1 is Gln) with unknown concentration, respectively. Similar ^{1}H-^{15}N HSQC of 70 μM NT bound to 90 μM NTS_1 receptor was collected with the same parameters with 32 scans per t_1 point. The resonance assignment was performed by collecting 2D ^{15}N-edited-NOESY-HSQC (2048 t_1 and 256 t_2) with a 200 ms mixing time and 2D ^{15}N-edited-TOCSY-HSQC (2048 t_1 and 512 t_2) with 60 ms mixing time on 1 mM peptide solution in the NMR buffer. All spectra were processed using nmrPipe and analyzed by NMRFAM-Sparky [15, 16].

2.8. β-arrestin recruitment assay

The potency and efficacy of purified peptide was tested by an arrestin-recruitment nano-BRET assay based on the resonance energy transfer between nano luciferase-fused to the C-terminus of hNTS1 and Venus fluorescent protein fused to the N-terminus of human β-arrestin2. HEK293T cells were transfected with a mixture of 50 ng:200 ng DNA, rat NTS1-nluc: human β-arrestin2-Venus, using lipofectamine. The transfected cells were treated with Furimazine and different concentrations of commercial NT8-13 or the purified NT in duplicates. β-arrestin recruitment was assessed by reading the bioluminescence of nanoluc and fluorescence of Venus at 450 and 535 nm, respectively, over 60 min. The area under the curve for individual ligand concentrations was calculated using GraphPad Prism and plotted against the ligand concentration to obtain dose response curves.

3. Results and discussion

To optimize the expression of NT, chemically competent *E. coli* BL21 (DE3) cells were transformed with pET-15b vector (Genscript) harboring SUMO-NT gene (Fig. 1) and small-scale expression of SUMO-NT in traditional and autoinduction media was performed. The best expression results were achieved by using ZYP5052 containing 0.3% lactose and 1% glycerol for 48 h in 16 °C. The final OD_{600} reached up to ~14-15 and around 30 gram wet-cell weight was harvested per liter of culture. In comparison, IPTG induction in 2YT media reached final OD_{600} of ~5-6. As autoinduction showed a higher cell density it was selected as the method of choice for further experiments. To assess the applicability of this system for expression of isotope-labelled peptide, autoinduction in N5052 media was compared to the method of Marley et al [17]. As expected, significantly higher yields in autoinduction media were obtained suitable for ^{15}N-labelling.

The expressed SUMO-NT protein was purified on Ni-NTA beads and after buffer exchange to remove imidazole, the purified protein was treated simultaneously with Ulp1, to remove the SUMO tag, and hQPCT for cyclization of Gln (Fig. 2). The reaction could be completed in 3 h at room temperature (~22-25 °C), however overnight incubation at 4 °C was preferred to minimize undesired protease activity. The SDS-PAGE analysis of purified SUMO-NT before and after cleavage (Fig. 2a) indicated

complete cleavage of the NT from SUMO, which is much more efficient than reported CNBr cleavage (>70%) [7]. Moreover, the one-pot cleavage and enzymatic treatment minimizes peptide loss that might happen in sequential cleavage and conversion procedures. Further purification of the peptide by HPLC and MS spectra of the purified peptide confirmed the complete conversion of Gln to pGlu (Fig 2b,c).

The lyophilized peptide powder was dissolved in deionized water and the concentration was measured by NMR using DSS as reference. The yield of our method for purification of NT from ZYP5052 and N5052 media is 13 and 8 mg/mL of purified peptide, respectively, with purity of >95%, as was determined by analytical HPLC (Fig. 2b).

The structural and functional characterization of purified NT was assessed by NMR and cell-based assays. The ^{1}H-^{15}N-HSQC spectrum of the free ^{15}N-labelled peptide was collected and assigned. The presence of only one set of peaks arising from the $^{15}NH_2$ side chain of N5, and none from Q1, indicates the conversion of the glutamine to pGlu in purified peptide (Fig. 3a). Interestingly, pGlu conversion affected the resonances from Y3, E4, N5 and K6, indicating the significance of pGlu for proper structural and functional analysis of even small peptides containing this modification. Addition of the receptor to the peptide solution resulted in a very significant chemical shift perturbation and broadening of the peaks from the C-terminal part of the peptide consistent with previous reports on the role of NT8-13 in binding and activation of receptor (Fig. 3b) [18]. In addition, the β-arrestin2 recruitment nano-BRET assays indicated the comparable potency and efficacy of recombinant NT to commercially available NT8-13 (Fig. 3c).

4. Conclusion

A robust tag is required for overexpression of small peptides to facilitate tracking of the purification process and to prevent peptide degradation by cell proteases [19]. SUMO is one of these fusion tags that allows high yield expression of recombinant proteins in eukaryotic and prokaryotic expression hosts [19]. The clean cleavage of the SUMO tag from the peptide N-terminal is another advantage of

SUMO compared to other expression systems [20, 21]. The latter feature is essential for expression and purification of small peptides where additional residues might significantly affect peptide activity [19].

Here we showed that the SUMO tag is an efficient fusion protein for the high yield of expression of NT in *E. coli*. To achieve modification of the peptide N-terminus to pGlu, SUMO cleavage using Ulp1 protease was followed by conversion of the introduced N-terminal Gln to pGlu using hQPCT. We obtained ~13 and ~8 mg/L of highly pure NT, > ~95%, in rich and minimal media, respectively. The purified NT was successfully assessed in NMR experiments in solution and in the presence of NTS1 as well as functional assays, which showed similar properties as commercially available NT8-13. Given the importance of N-terminus of NT in binding to NTS_3 [4], the current method provides the opportunity to investigate the structural basis of subtype selectivity of full-length wt-NT toward different neurotensin receptors. Moreover, the current protocol is adaptable to expression and purification of pGlu modified peptides and proteins including Aβ and monoclonal antibodies as well as therapeutic peptides in different expression hosts [6, 22-24].

Author statement

K.A. developed expression, purification schemes and with K.H. and F.Y. purified all proteins. D.J.S. and L.A.Z performed receptor assays, K.A., K.H. and P.R.G collected and analysed NMR data. K.A. and P.R.G. wrote the manuscript and all authors edited. P.R.G. and D.J.S. supervised the project.

Declaration of competing interest

The authors declare no conflict of interest.

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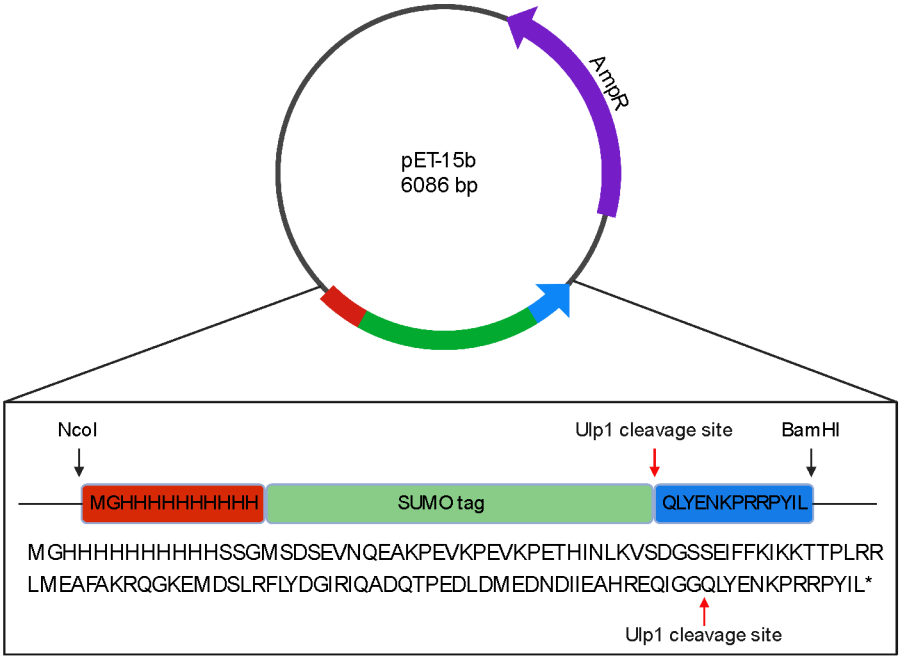

Figure 1. Schematic of SUMO-NT construct used to express the full-length NT. The construct was cloned into pET-15b vector between NcoI and BamHI restriction sites (arrows). The sequence of SUMO-NT with the Ulp1 cleavage site with a red arrow is shown.

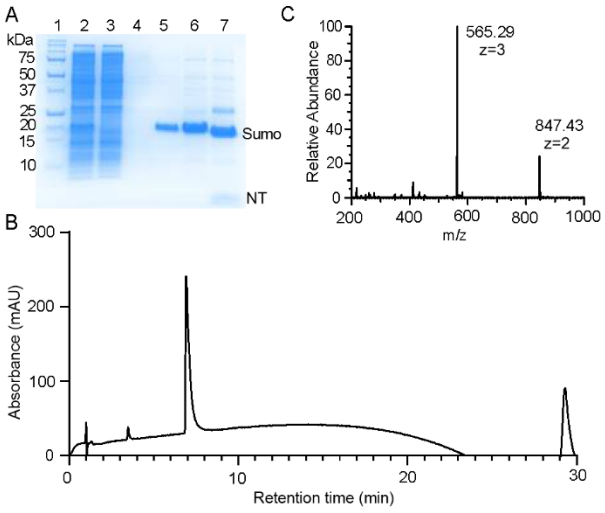

Figure 2. Purification of uniformly labelled ^{15}N-NT. a) the SDS-PAGE analysis of NT purification steps on a 12% tris-tricine gel. (Lane 1 protein markers, 2 soluble fraction of cell lysate, 3 IMAC flow through, 4 wash, 5 eluate, 6 concentrated protein, 7 after cleavage and modification. b) The analytical HPLC chromatogram of purified NT on analytical C18 column over a gradient of acetonitrile for 25 min indicates the high purity of the purified peptide. c) the MS spectra of ^{15}N-NT.

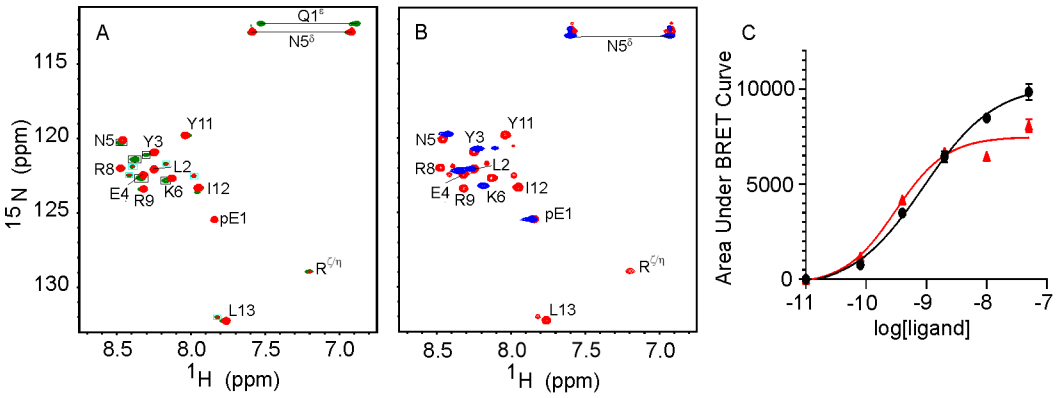

Figure 3. Structural and functional characterization of NT. a) ^{1}H-^{15}N HSQC spectra of free NT (where residue 1 is Gln) (green) compared to NT (where residue 1 is pGlu) (red). The spontaneous partial chemical conversion of Gln to pGlu causes spectral heterogeneity (black boxes). The lower intensity set of peaks originates from cis-proline isomers (cyan boxes). The only signal from the side chain $N^{\delta}H_2$ of Asn5 confirms the conversion of Gln to pGlu. The NMR spectra are presented in different contour levels. b) ^{1}H-^{15}N HSQC spectra of NT (where residue 1 is pGlu) free (red) and in complex with neurotensin receptor 1 (blue). c) Recombinant NT (red) shows comparable potency and efficacy to commercially supplied NT8-13 (black) in β-arrestin2 recruitment assays. The error bars indicate the standard error of mean in duplicate experiments (SEM).