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Expression and Purification of Recombinant Neurotensin in *Escherichia coli*[1]

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An expression system has been designed for the rapid and economic expression of recombinant neurotensin for biophysical studies. A synthetic gene for neurotensin (Glu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]) was cloned into the pGEX-5X-2 vector to allow expression of neurotensin as a glutathione *S*-transferase (GST) fusion protein. The inclusion of a methionine residue between the glutathione *S*-transferase and the neurotensin has facilitated the rapid cleavage of the neurotensin from its carrier protein. Purification of recombinant neurotensin was performed by reverse-phase HPLC. This method produced a relatively high yield of peptide and offers the potential for economic partial or uniform labeling of small peptides (<15 amino acids) with isotopes for NMR or other biophysical techniques.
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Key Words: **neurotensin; protein expression; *E. coli;* NMR.**

Neurotensin (NT,[2] Glu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]) is a linear tridecapeptide (Fig. 1) responsible for the activation of the G-protein-coupled neurotensin receptor. The neurotensin receptor is found in both the central and peripheral nervous system. In the periphery, the activation of neurotensin receptor by neurotensin stimulates smooth muscle contraction (1,2). In the central nervous system, receptor activation induces a variety of effects including antinociception, hypothermia, and increased locomoter activity (3–5). These effects are probably mediated through the regulation of the nigrostriatal and mesolimbic dopamine pathways. As a result, the pharmacological activity of neurotensin is similar to that of dopamine which functions as an antipsycotic (6,7) and where intervention might provide useful insights into the treatment of conditions such as schizophrenia (8) and Parkinson's disease (9).

Neurotensin belongs to a family of bioactive peptide ligands including xenopsin, bradykinin, and neuromedin N (1), which bind the neurotensin receptor. Notably this family share a common C-terminal motif Arg-Arg-Pro-Tyr-Ile-Leu. This sequence has been shown in the case of the neurotensin to be the minimal sequence showing similar efficacy (K_D = 13 nM) to the native neurotensin (K_D = 2 nM) with the arginines critical for ligand binding (10). Structurally little is known about neurotensin. In free solution, proton NMR studies have revealed that there is no preferred conformation as ascertained by the studies of NOEs and J-couplings (11). These studies were extended to the study of neurotensin in the presence of the membrane mimetic sodium dodecyl d_{25}-sulfate; these indicated that neurotensin adopted no preferred conformation, but charge–charge interactions with the surface of the detergent micelle ordered charged residues within the ligand (11). To date, no crystallographic data have been published for neurotensin as a free ligand or bound to its receptor. In the absence of direct structural data, models for the ligand binding site for the shortest active fragment, neurotensin(8–13), have been proposed on the basis of a heuristic modeling approach supported by site-directed mutagenesis data (13). These propose that the neurotensin adopts a compact conformation upon binding to the neurotensin receptor, with the Arg[9]-Pro[10]-Tyr[11]-Ile[12] adopting a proline type I turn, while the guanidium groups of Arg[8] and Arg[9] interact with the hydroxyl group of Tyr[11] (12); however, this remains to be investigated further.

In the absence of any direct structural information,

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[2] Abbreviations used: NT, neurotensin; GST, glutathione *S*-transferase; pGEX, fusion protein system; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

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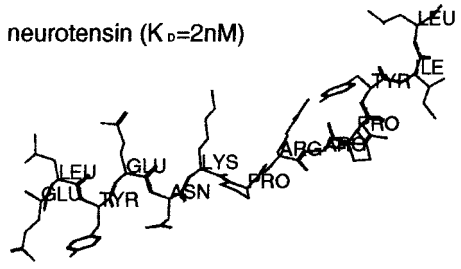

FIG. 1. Diagram showing an energy minimized model of neurotensin (Glu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]), indicating both the sequence and structure of the desired peptide.

our goal was to produce sufficient quantities of peptide labeled with NMR sensitive isotopes (^{13}C, ^{15}N, ^{2}H, ^{1}H) for structural multinuclear NMR studies of neurotensin in both the solid and solution state. To achieve satisfactory expression of small peptides, several criteria were deemed essential, including ease of manipulation and scalability of host, stability and solubility of protein as well as the speed and yield of purification. In an attempt to satisfy these criteria, the glutathione *S*-transferase (GST) fusion protein system (pGEX) was used to express the neurotensin as a GST, neurotensin fusion protein in *Escherichia coli.*

We have used *E. coli* over other hosts due to the ease of scalability and the ability to allow labeling during expression during cell growth. For protein expression *E. coli* strain BL21 was chosen. In this strain, which is protease-deficient, the likelihood of cleavage of the fusion protein is reduced. The choice of the pGEX expression system confers several advantages of other expression systems. The strong inducible *tac* promoter has been shown to give controllable high levels of protein expression. Peptides expressed as fusion proteins have been shown to confer additional protection for the peptide against proteases present in the bacteria (13), while the large GST carrier protein serves to mask the physical properties of the peptide, allowing purification of the recombinant fusion protein with few modifications to existing affinity purification protocols (13). In the vector, we have included a chemical cleavage site at the N-terminus of the neurotensin to allow the precise and rapid cleavage of the peptide from the carrier protein.

The methodology presented here demonstrates a suitable protocol for the expression and purification of small (<15 amino acids) recombinant peptides, which facilitates their easy and economic labeling for biophysical studies.

MATERIALS AND METHODS

Cloning and Plasmid

A codon optimized synthetic NT gene, designed according to the codon preference in *E. coli,* was cloned into pGEX-5X-2 (Pharmacia) as a *Bam*HI–*Not*I fragment. The sequence of the synthetic oligonucleotides used for cloning is shown in Fig. 2. An ATG codon was included at the 5′ end of the neurotensin-encoding gene, to allow the precise cleavage of the peptide from the GST protein after translation and purification using cyanogen bromide. A *Hin*dIII site was also included in the oligonucleotides at the 3′ end of the NT gene to facilitate the identification of the recombinant plasmids. The sequence of the resulting plasmid pGEX–NT was confirmed by sequencing the NT domain.

Strain and Growth Conditions

E. coli strain BL21 (DE3) (F^-, ompT, hsd, $S(r_B^- m_B^-)$, gal) was used for protein expression. The cells were grown in LB supplemented with 50 μg/ml of ampicillin overnight and used as a 0.1% innoculum for 1 liter. The cells were grown at 37°C to late exponential phase (OD_{600} = 0.6) and induced with 0.1 mM isopropyl β-thiogalactosidase for 4 h (OD_{600} = 1.4).

Purification of GST-NT Fusion Protein

The cells expressing the GST–NT fusion protein were broken in B-Per extraction reagent (Pierce, UK) with mild sonication (six 10-s bursts) and adequate cooling. After clarification, the GST–NT fusion protein was purified by glutathione affinity chromatography. A 2-ml Pharmacia glutathione–Sepharose 4B affinity column was equilibrated in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM Na_2PO_4, pH 7.2). The clarified cell lysate was subsequently loaded onto the column at 0.5 ml min^{-1}. The column was then washed with 10 bed volumes of PBS. The bound protein was subsequently eluted with 10 mM reduced glutathione in 50 mM Tris buffer (pH 8.0).

Cleavage of GST–NT

The neurotensin was cleaved from the fusion protein GST–NT following a cyanogen bromide protocol (17). To a protein solution of 1 to 5 mg ml^{-1} GST–NT was added an equal volume of 0.4 M ammonium bicarbonate solution together with 2-mercaptoethanol to a final concentration of 5% (v/v). The sample was sealed under a nitrogen atmosphere and incubated at room temperature overnight. The sample was then lyophilized with a little heating to remove any remaining ammonium bicarbonate. The sample was then dissolved in trifluoroacetic acid (TFA) at a concentration of between 1 and 5 mg ml^{-1}. Water was then added to make the acid (50% v/v). White crystalline cyanogen bromide was added to the sample in 100-fold excess (typically a mass equal to that of the protein). The reaction was incubated at room temperature for 24 h and then terminated by lyophilization.

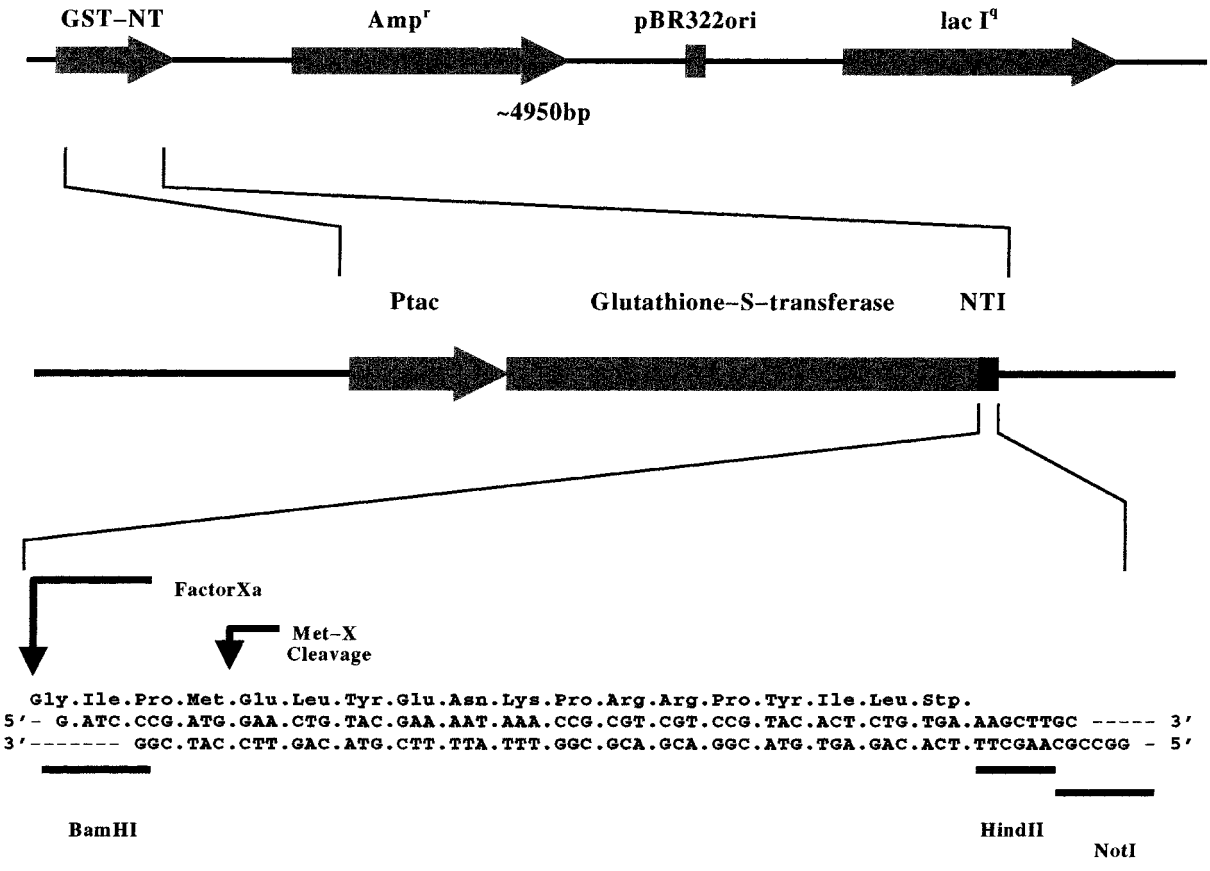

FIG. 2. Structure of the synthetic gene for the expression of the glutathione *S*-transferase, showing the oligonucleotides synthesized, and the relevant protease/chemical cleavage sites.

Purification of NT

Separation of the cleavage products following the cyanogen bromide cleavage was performed by reverse-phase HPLC, using a C18 Dynamax 83-213-C 20-ml preparative column with a flow rate of 3 ml min^{-1}. The column was equilibrated with 0.1% TFA in water until a stable baseline was attained; 100 μl of 1 mg ml^{-1} protein was subsequently loaded onto the column. The products were eluted with a gradient of acetonitrile from 0 to 40% over 25 min.

RESULTS AND DISCUSSION

Neurotensin was expressed in *E. coli* as a GST C-terminal fusion. The fusion of small peptides to carrier proteins such as GST has been shown to improve stability and enhance solubility (13). The fusion of neurotensin peptide with GST allows rapid purification using a single affinity column. The purification steps are shown in Fig. 3. Lanes 4 and 5 show that one major protein species with an apparent molecular weight of 30 kDa was purified. This is likely to correspond to the GST–NT. This was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF) which gives a higher resolution analysis of molecular weight than conventional SDS–PAGE. Early mass spectroscopic studies indicated a range of molecular species separated by 317 Da. This is consistent with the presence of multiple glutathione adducts. Treatment of the GST–NT with 100 mM dithiothreitol gave rise to a single molecular species of 28395 ± 20 Da, which is consistent with the predicted molecular weight. This confirmed that the purified species corresponds to the full-length GST–NT and that no C-terminal truncations were present. From 1 liter of culture, 35 mg of GST–NT protein was typically purified by affinity chromatography.

The GST–NT was cleaved with cyanogen bromide and the resulting fragments were purified by reverse-phase HPLC. Native neurotensin (Sigma, UK) was used as a standard and eluted with an acetonitrile concentration of 27%. Separation of the cyanogen bromide cleavage reaction by reverse-phase HPLC gave rise to the chromatogram shown in Fig. 4A. The peak eluting at an acetonitrile concentration of 27% was collected, lyopholized, and resuspended in 200 μl of water. The HPLC chromatogram of recombinant neu-

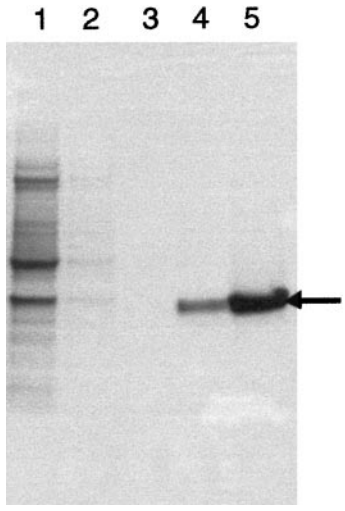

FIG. 3. 12% Tris SDS–PAGE gel of a typical purification of GST–NT. Lane 1, 10 μg of total cell extract. Lanes 2 and 3, first and second washes of affinity column prior to elution. Lanes 4 and 5, 5 and 20 μg, respectively, of the eluted GST–NT (indicated with arrow).

rotensin shown in Fig. 4B corresponded to that of native neurotensin. This was confirmed by loading an equimolar solution of native and recombinant material. MALDI-TOF MS of the recombinant NT showed a single molecular ion peak in addition to the monosodium, disodium, and monopotassium salts. The recombinant peptide was further characterized by N-terminal sequencing and MALDI-TOF fragmentation analysis. These experiments confirmed both the sequence of the neurotensin peptide and its purity. The final yield of peptide from 35 mg of pure GST–NT was 1.8 mg as determined by UV spectroscopy ($E^{280}_{1\text{mg ml}} = 1.37$) giving an 83% recovery with respect to the theoretical yield. The results of this purification are summarized in Table 1.

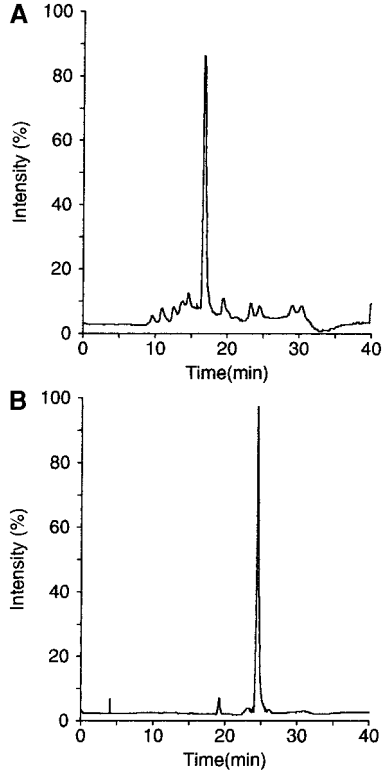

FIG. 4. Reverse-phase HPLC chromatogram (254 nm) showing the separation of the cyanogen bromide cleavage mix (A) and of the purified material obtained upon collection of the appropriate fraction (B).

TABLE 1

Purification of Neurotensin from *E. coli*

Fraction	Yield	Purity (%)
Mass of wet cells	4.97 g	
Pooled fractions of GST–NT	35.0 mg[a]	>95[b]
Pooled fractions of NT	1.8 mg[c]	>95[d]

[a] Determined using a modified Lowry assay.
[b] Determined by densitometry of gel (data not shown).
[c] Determined as described in text.
[d] Determined by HPLC chromatogram of pure NT.

CONCLUSIONS

In this work we have developed an expression system that permits the production of sufficient quantities of neurotensin (~mg's) from 1 to 2 liters of culture for solid-state NMR studies. The levels of recombinant fusion protein are consistent with those previously described in the literature, whereas the purification of the recombinant peptide from the cyanogen bromide cleavage mix occurred with high (~80%) recovery. It has become apparent that the limiting factor associated with the expression system employed here is neither expression nor purification. The major consideration is the quantity of carrier protein (glutathione *S*-transferase) produced in contrast to the desired product (only 6% of the mass of all protein expressed is the required neurotensin). Alternative strategies have been proposed for the expression of small recombinant peptides (17) which may circumvent some of these problems. However, the properties inferred by the carrier protein which aid both the expression and purification of the recombinant peptide outweighs the potential disadvantages that may occur from using smaller carrier proteins. Thus, we propose this system as an efficient and economic method for uniform labeling of small (<15 amino acids) peptides with isotopes for NMR and other biophysical studies.

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