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Expression in *E. coli* and In Vitro Amidation of FMRFamide

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ABSTRACT

FMRFamide is a neuropeptide found in molluscs and other invertebrates and is used in vitro to examine molluscan neurophysiology. Our goal was to produce biologically active recombinant FMRFamide as a model for the production of other recombinant neuropeptides. FMRFamide was chosen because of our experience in working with this peptide and its corresponding bioassay. Because FMRFamide is fully biologically active only when α-amidated, amidation was carried out in vitro on a glycine extended precursor, FMRFG, using peptidyl glycine alpha-amidating monooxygenase (PAM). In order to more easily isolate and purify FMRFG, the peptide was generated as a fusion protein with maltose binding protein (MBP). These two regions were separated by a recognition sequence for the coagulation enzyme Factor Xa. This allowed us to generate a convenient amidation strategy, in which the fusion partners could be enzymatically separated following amidation. Recombinant FMRFamide was then purified by HPLC, and analyzed using a clam heart bioassay. This work serves as a model for the generation of other recombinant peptides requiring α-amidation.

INTRODUCTION

α-Amidation is a very important post-translational modification. In vertebrates, half of all biopeptides undergo post-translational alpha-amidation in order to become fully active. In insects, more than 90% of neuropeptides are amidated, and mutations in the gene responsible for amidating peptides is lethal (Jiang et al., 2000). α-Amidated peptides are found widely in vertebrates and invertebrates and include substance P, neuropeptide Y, oxytocin, vasopressin, secretin, calcitonin, gastrin, and pancreastatin (Merkler et al., 1994, Prigge et al., 2000; Tateishi et al., 1994). The amide is formed by hydroxylation of a glycine residue, which produces a peptide amide and glyoxylic acid. The amidation reaction is performed by peptidyl-glycine alpha-amidating monooxygenase (PAM). PAM is a multifunctional enzyme containing two domains which catalyze peptide amidation (Ouafik et al., 1992). The amino terminal domain is peptidylglycine α-hydroxylating monooxygenase (PHM) which catalyzes the stereospecific hydroxylation of the glycine α-carbon of peptidyl-glycine substrates. Near the carboxy terminal end is the peptidyl-α -hydroxyglycine α-amidating lyase (PAL) domain which generates the α-amidated pep-

tide product and glyoxylate (Prigge et al., 1997). It has been shown that PHM and PAL can be expressed independently (Eipper et al., 1991) and in fact exist as independent enzymes in *Drosophila* (Kolhekar et al., 1997).

One of the many neuropeptides that requires α-amidation for full biological activity is FMRFamide. FMRFamide is a four amino acid peptide that is commonly found in molluscs and annelids (Price and Greenberg, 1977). FMRFamide has been shown to have a variety of effects, acting as a neuropeptide, neurotransmitter, and neuromodulator. The biological activity of FMRFamide is mainly the result of the arginine residue and C-terminal amide (Painter et al., 1982). FMRFamide belongs to a class of peptides that integrate signals to and from the nervous system (Krajniak and Price, 1990). Other members of the FMRFamide-Related Peptide (FaRP) family, which consist of N-terminal extensions of the tetrapeptide, are found in *Drosophila* and chordates. The aim of this project was to design a system in which active recombinant FMRFamide could be collected and tested with the possibility of adapting this system to other peptides that require α-amidation. This system makes use of a fusion of recombinant FMRFamide to a secreted version of the maltose binding protein (MBP) located on the plasmid pMal-p2 (New England Biolabs), generating a fusion protein that can be purified from the *Escherichia coli* periplasmic space. At the carboxy terminus of MBP there is a recognition site for the protease factor Xa (Maina et al., 1988). Thus, upon purification of the MBP-FMRFamide fusion protein, a precise cleavage of MBP can occur and the amidated product recovered.

MATERIALS AND METHODS

Expression vector construction

Two single stranded oligonucleotides, (TTTATGCGTTTCGGAT & FMRF AATTATCAGAAACGCATAAA) were synthesized by Sigma-Genosys, kinased, and annealed to create a double stranded oligonucleotide. This double-stranded oligo was ligated to the expression vector pMal-p2 (New England Biolabs), which had been previously digested with Xmn 1 and Eco R1.

The ligation reaction was then transformed into competent JM101 *E. coli* cells. Two ampicillin resistant colonies were selected by blue/white screening. DNA was extracted from both cultures which were then designated rp2.1 and rp2.2. Separate restriction digests were performed on purified DNA from these clones using Eco R1 and Xmn 1, which were then visualized on a 0.7% agarose gel to confirm the presence of the FMRFG insert. PCR analysis was performed and DNA from PCR positive clones was sent to the University of Chicago DNA Sequencing Center for analysis.

Expression, amidation, and purification of recombinant FMRFamide

The fusion protein was then expressed and extracted following the protocol outlined below. Cells containing rp2.1 were grown to a concentration of 2-4 x 10^8 cells/ml in a 1-liter solution of rich broth (Luria containing 3 g/L glucose) supplemented with100 mg/L ampicillin. The cells were then induced using IPTG (0.3 mM) and incubated at 37°C for approximately two hours. The cells were then centrifuged at 4000 x g for 20 minutes. The pellet was resuspended in 30 mM Tris-HCl, 20% sucrose, 1 mM EDTA and shaken for 5-10 minutes at room temperature. The cells were then centrifuged at 8000 x g for 20 minutes at 4°C. The pellet was then resuspended in 100 ml of ice cold 5 mM $MgSO_4$, and

allowed to shake for 10 minutes in an ice bath. The solution was then centrifuged at 8000 x g for 20 minutes at 4°C. The (soluble) protein fraction recovered is termed the cold osmotic shock fraction (COSF). The COSF was concentrated using a 10K Microsep Concentrator cup (Pall Gelman) by centrifuging at 7000 x g (~1ml/hr). This concentrated protein solution (in the retentate) was then analyzed on a 10% SDS-PAGE gel using purified MBP (New England Biolabs) as a standard. The gel was stained with Coomasie Blue R-250 solution for 4 hours, destained, and then visualized on an Eagle Eye Imaging System (Stratagene).

An amidation reaction was prepared using a method similar to Kolhekar et al. (1997). The reaction mixture was prepared in a 10K Nanosep concentrator (Pall Gelman) containing 100 µl of the protein concentrate, 250 µl of 2X amidation buffer [60 mM TES, 0.002% (v/v) Triton X-100, 2% (v/v) EtOH, 10 mM KI, 1 µM $CuSO_4$, 3 mM sodium ascorbate], 135 µl dH_20, 0.85 µl of catalase (10 µg/ml final) and 7 µl of α-AE (PAM); (Unigene Technologies). The reaction was then incubated overnight at 37°C. The buffer was removed by centrifugation through the 10K nanosep filter using until approximately 100 µl remained. To this, 400 µl of wash buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1.0 mM EDTA, 10 mM maltose) was added to dilute out the remaining amidation buffer. The concentration cup was then centrifuged until 200 µl of amidated fusion protein remained in the top of the cup. The amidated fusion protein was then cleaved with 5 µl of 1 µg/µl Factor Xa (New England Biolabs). The Nanosep concentrator was then centrifuged until all of the cleaved peptide solution had gone through the filter.

Analysis of recombinant FMRFamide

The peptide solution (which had gone through the final 10K filter centrifugation) was then collected and loaded onto a reverse phase HPLC column (Supelco Discovery C-18 measuring 25 cm x 4.6 mm) at a flow rate of 1 ml/min using a linear gradient of 16% acetonitrile, 0.1% trifluoroacetic acid (TFA) to 32% acetonitrile, 0.1% TFA over 20 minutes. Fractions were collected every minute and the absorbance of the eluent was monitored at 210 nm. Retention times for authentic FMRFamide and FMRFG were established using commercial peptides (Sigma Chemicals; St. Louis, MO).

HPLC fractions that corresponded to the appropriate retention time for commercial FMRFamide were dried in a speed vac and resuspended in 40 µl ddH_2O. Clam hearts dissected from *Mercenaria mercenaria* (obtained from a local grocery store) were dissected and suspended in a 4 ml chamber filled with Crystal Sea artificial seawater (Price and Greenberg, 1977). One atrium of the heart was connected via a thread to a support rod, and the other atrium connected to a Grass force transducer. A Grass RPS 212 amplifier was used to detect the contractions of the heart. Teaching Lab System software (version 1.2; Sable Systems) was used to record and analyze the frequency and amplitude of the contractions. The heart was first dosed with 40 µl of artificial seawater. Commercial FMRFamide was serially diluted to concentrations of 10^{-8} M to 10^{-4} M in artificial seawater and 40 µl of each was applied to the heart, one at a time, starting with the lowest concentration. Also, 40 µl of the HPLC fraction of in vitro amidated recombinant FMRFamide was administered to the clam heart, flushing with seawater between each dose. Data for all doses were collected and analyzed to determine the percent change in amplitude of contraction caused by each dose. A standard log dose-response curve was created to determine approximately how much FMRFamide was present.

RESULTS AND DISCUSSION

Expression vector design
Restriction digests and PCR analysis indicated that the oligos had been integrated into clone rp2.1. DNA sequencing (data not shown) indicated that the rp2.1 sample consisted of a double stranded recombinant plasmid containing the FMRFG sequence and this clone was chosen for the expression and amidation steps.

Expression, amidation, and purification of recombinant FMRFamide
A 10% SDS-PAGE gel was run to evaluate the proteins in the COSF. The visible band in the COSF had a similar migration rate to that of the maltose binding protein standard, suggesting that this was the MBP/FMRFG fusion protein (results not shown). These results indicate that the fusion protein was appropriately expressed and transported to the periplasmic space of the *E. coli* cells.

The results of the in vitro amidation and subsequent cleavage with Factor Xa were analyzed using HPLC. Commercial FMRFamide showed a peak at 13.08 minutes, and commercial FMRFG showed an elution time averaging 14.43 minutes. These times were used as a standard for comparison of the recombinant product. The peak for the recombinant peptide eluted at 13.29 minutes, indicating the presence of FMRFamide (Figure 1). The amount present was estimated to be approximately 200 pmol.

Analysis of rFMRFamide activity
HPLC purified recombinant FMRFamide was then tested in a clam heart bioassay. The change in amplitude of contraction was calculated by measuring the parameters for five beats before and after administering each dose. The control dose containing only seawater showed no bioactivity. The bioassays for the experimental samples showed substantial activity, with a 93% increase in amplitude (Figure 2). The data from dilutions of commercial FMRFamide was used to create a standard curve allowing for the calculation of the concentration of recombinant FMRFamide (Figure 3). This was determined to be approximately 200 pmol, which is the same concentration as determined by HPLC analysis.

CONCLUSIONS

The results obtained from the clam heart bioassay and HPLC indicate the presence of active recombinant FMRFamide. This system has the potential to be used for the in vitro production of other products that require α-amidation. Also, this system can be used in further experiments to assess the bioactivity of other peptides that require amidation. In vitro amidation of other peptides such as salmon calcitonin have also been completed in vitro (Ray et al., 1993 & Hong et al., 2000). The techniques used in the production of calcitonin also require the initial production of a fusion protein to a glycine extended substrate followed by amidation. However, additional steps are included that are not required with the methods outlined within this paper. After production of the recombinant fusion protein, it was cleaved by cyanogen bromide and then recovered through affinity chromatography. Calcitonin was then amidated by recombinant α-amidating enzyme, and then purified again using CEX-HPLC (Ray et al., 1993). However, the techniques out-

lined within this paper are more streamlined. After the production of the recombinant peptide and transfer to the periplasmic space, the COSF protocol offers an easy way to recover the fusion protein through breaking down the outer cell wall. The use of spin filters to concentrate the recombinant protein allows for very little loss of protein due to the large size of the MBP-fusion protein. The protein can then also be amidated within these same spin filters through the addition of the amidating enzyme, catalase, and necessary cofactors for each enzyme. After washing the amidation co-factors through the filter, the enzyme Factor Xa cleaves MBP, allowing the recombinant protein to now flow through the filter to be recovered. It is hoped that the techniques outlined within this paper will lead to the high efficiency production of any peptide requiring amidation for full activity.

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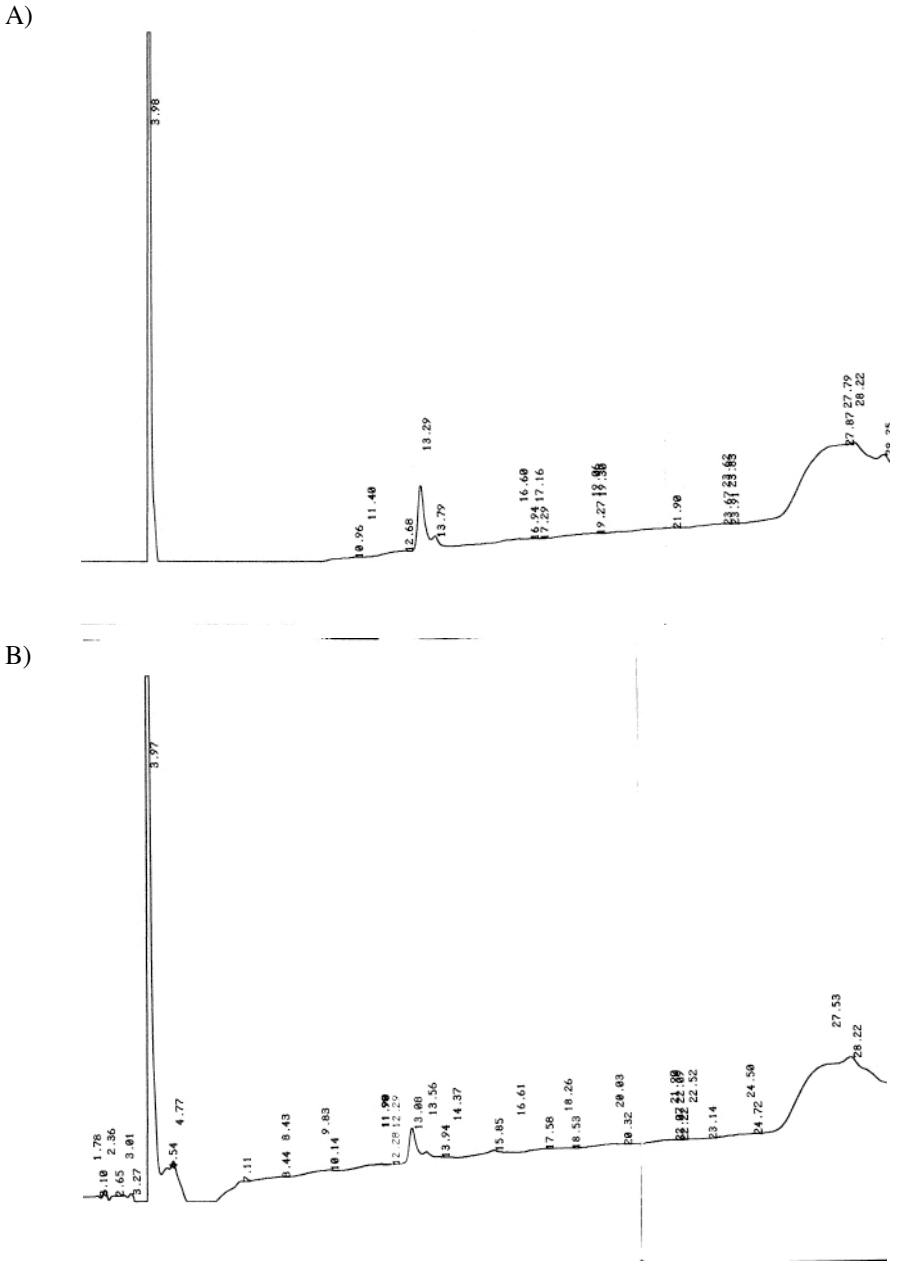

Figure 1. HPLC tracings comparing the retention times of commercial and in vitro amidated recombinant FMRFamide. (A) Commercial FMRFamide with a peak at 13.29 minutes. (B) In vitro amidated recombinant FMRFamide with a peak at 13.08 minutes. Similar retention times were noted over several trials.

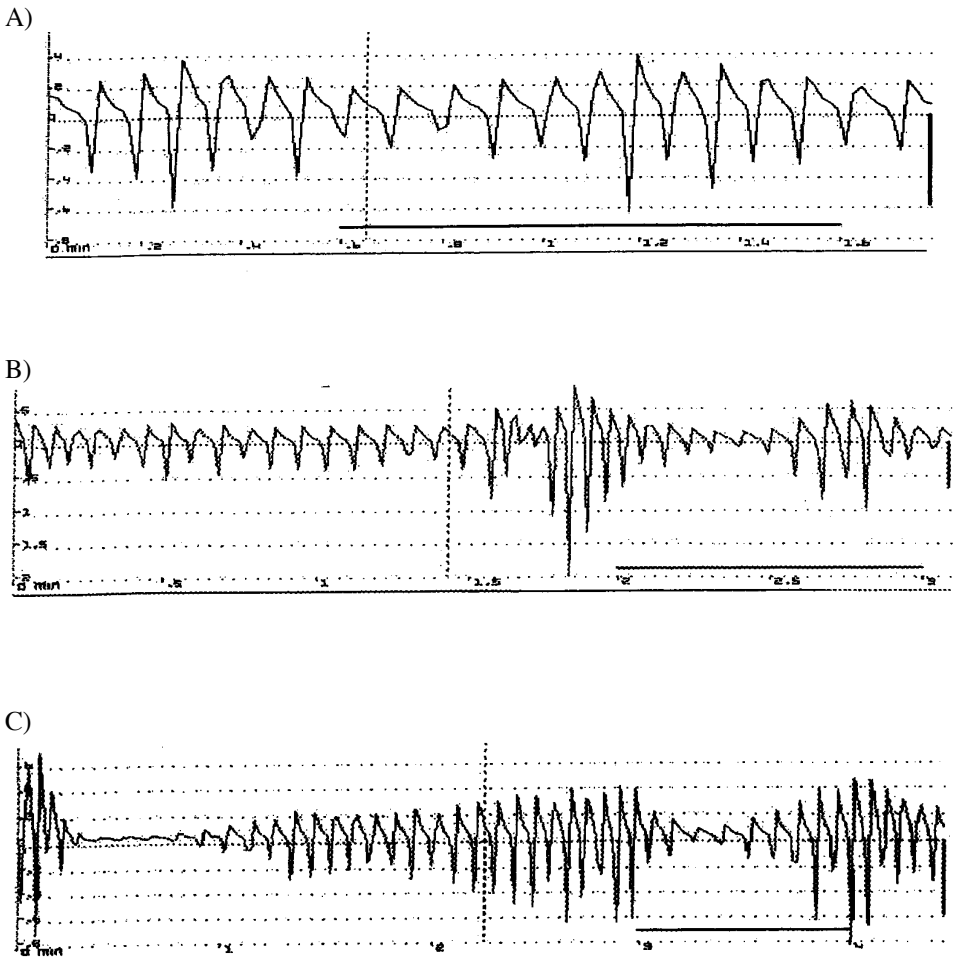

Figure 2. Clam heart bioassay, comparing the effects of (A) artificial seawater, (B) commercial FMRFamide (10^{-6} M), and HPLC purified in vitro amidated recombinant FMRFamide (see Figure 1). Vertical axis indicates amplitude of contractions, while the horizontal axis indicates frequency of contraction over time. Horizontal dashed line indicates point in time at which the sample was added. Concentration of recombinant FMRFamide was determined using a standard curve (Figure 3).

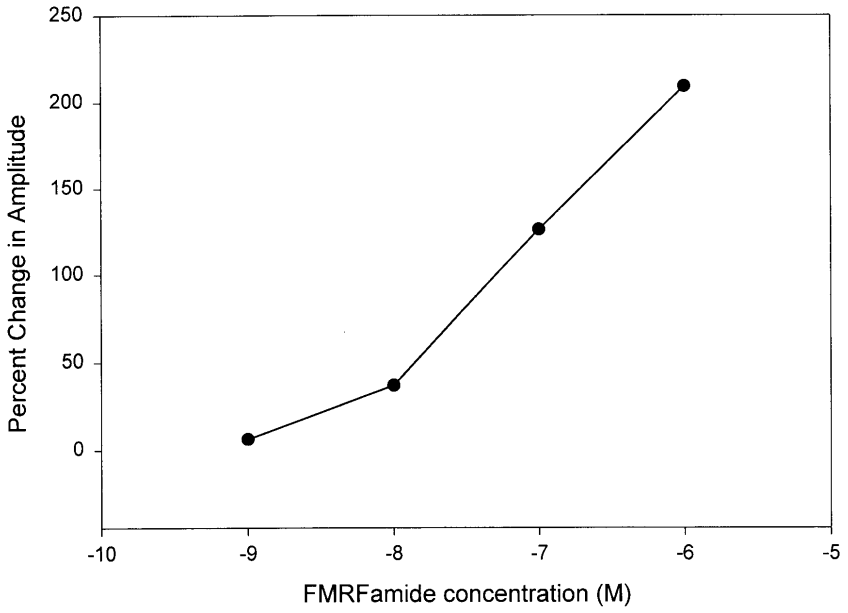

Figure 3. Standard curve based on concentration of commercial FMRFamide at various concentrations (as in Figure 2) versus the amplitude of contraction caused by that treatment. This was used to determine the concentration of recombinant FMRFamide.