New Peptide-pair Screening Strategy and
Peptidylglycine α-Hydroxylating Monooxygenase (PHM) Based Enrichment Method
for the Discovery of Novel α-Amidated Peptides

By

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of the requirements for the degree of
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Dedication

I would like to dedicate this dissertation to my parents Tinghai An and Yinfeng Liu.
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I would like to acknowledge Dr. David Merkler for his steady and encouraging guidance in both science and life. I have been extremely blessed with his support, encouragement and inspiration throughout the past several years. I gave special thanks to Dr. John Koomen who led me into the world of proteomics and trained me in Mass Spectrometry. I would also like to acknowledge other fine scientists on my committee: Dr. Xiao Li and Dr. Kathleen Scott, for their insightful comments and suggestions.

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Abstract

Peptide α-amidation is known as a signature of bioactivity due to the fact that half of the bioactive peptides found in the nervous and endocrine systems are α-amidated and that most known α-amidated peptides are bioactive. α-Amidated peptides are produced by the oxidative cleavage of glycine-extended precursors. Peptidylglycine α-amidating monooxygenase (PAM) is the only known enzyme responsible for catalyzing this reaction and its sole physiological function is to convert glycine extended prohormones to their α-amidated forms. High levels of PAM are found in certain tissues with no corresponding level of amidated products suggesting the presence of undiscovered α-amidated peptide hormones.

Liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) has emerged as a powerful tool for peptide identification due to its advantages of speed, sensitivity and applicability to complex peptide mixtures. Normally, spectra are interpreted using database search engines. However, database searching is inefficient and ineffective for the identification of endogenous peptide with post-translational modifications (PTM) due to its low identification rate and high demand for computing power.
There is a specific mass difference of 58.0055 units between an α-amidated peptide and its corresponding C-terminal glycine-extended precursor. The two peptides will have similar chromatographic retention time and MS/MS fragmentation patterns resulting from the identical amino acids sequences except for relatively the small differences at the C-termini. Based on this, a new LC-MS/MS based strategy for screening for α-amidated peptides was developed. This strategy depends on PAM inhibition and the mass accuracy of mass spectrometry (< 3 ppm). The coexistence of α-amidated peptides and their C-terminal glycine-extended precursors was insured by growing cells in the presence of a PAM inhibitor. After LC-MS/MS, masses and retention times of parent ions were extracted from raw data files and scanned by a script for peptide pairs with similar retention times and a mass difference around 58.0055. Resulting pairs were further validated by comparing their fragmentation patterns in MS/MS spectra. Only peptide pairs that met all three criteria were considered for further interpretation. This reduced the number of MS/MS spectra requiring interpretation by >99% and, thus, enable the manual inspection of MS/MS for the candidate peptide pairs. A total of 13 α-amidated peptides were successfully identified from cultured mouse pituitary AtT-20 cells using this method and a few of these newly identified α-amidated peptides exhibited bioactivity. The adaptability of this strategy to screening for other PTMs is also discussed.
Peptidylglycine α-hydroxylating monooxygenase (PHM) is one of PAM domains which can be expressed separately. It is a copper dependent enzyme that catalyzes the first step of the two-step peptide amidation reaction. Removal of the copper ions results in the loss of enzyme catalytic activity. A PHM based α-amidated peptide enrichment method was developed. This method includes two steps. First, cells grown in culture were treated with a PAM inhibitor to effect the cellular accumulation of glycine-extended peptides. In the second step, copper-depleted PHM (apo-PHM) was used to selectively bind glycine-extended peptides present in the cell extract. All other unbound peptides were removed during wash runs. apo-PHM was then reinstated with copper to convert bound glycine-extended peptides to hydroxylated peptides and release them. Hydroxylated product can be converted to α-amidated peptide under basic conditions. Experiments carried out using model glycine extended peptides showed a 40 – 120-fold enrichment using HPLC-fluorometric assay or MALDI-TOF quantification. This method proved successful when working with complex samples like cell extracts. The relative intensity of a known α-amidated peptide mouse joining peptide (mJP) from an AtT-20 extract was dramatically increased after enrichment experiments.
Chapter 1 Introduction

1.1 Peptide Amidation

1.1.1 Peptide amidation and its biological significance.

Post-translational modification (PTM) is a chemical modification of a polypeptide chain which results from either the addition or removal of chemical groups to amino acid residues, proteolytic processing, or formation of disulfide cross-links. More than 300 different types of PTMs have been discovered and this number is increasing (Witze et al. 2007). As a consequence of PTM, different modified forms of a gene product may be present in vivo each with a different cellular function. Through changing proteins’ mass, charge, structure or hydrophobicity, PTMs can control proteins’ activity and stability, protein subcellular localization and protein-protein interaction (Parekh and Rohlff 1997).

C-terminal amidation is an important PTM. About half of the bioactive peptides found in the nervous and endocrine systems are α-amidated (Eipper, Stoffers and Mains 1992, Eipper and Mains 1988), such as corticotropin-releasing hormone (CRH), thyrotropin releasing hormone (TRH), neuropeptide Y (NPY), substance P, oxytocin, vasopressin and α-melanotropin. For most of these peptides, the presence of the
C-terminal amide structure is essential for their biological activity and stability. Merkler defined a numerical value “potency ratio” to study the contribution of C-terminal amide in α-amidated peptides as compared to the corresponding peptides with free C-terminal acid (Merkler 1994). In this study, the potency ratios of 33 peptides known to be amidated in vivo were summarized. Of these, nine peptides showed a potency ratio greater or equal 1,000 (Table 1). Most peptides (27 out of 33) exhibited at least 10-fold increase in activity with the presence of C-terminal amide. Only 3 of the 33 peptides did not show significant activity change. On the other hand, by replacing free carboxylic acid group with unionizable amide group at C-terminus, α-amidation may change the hydrophobicity of peptide and, thus, increase the affinity of the peptide to the cognate receptor. Studies on cholecystokinin-A receptor (CCK-AR), a G protein-coupled receptor, showed that the amide moiety was a key determinant in the interaction between the receptor and its amidated peptide ligands (Gigoux et al. 1999). This conclusion was further supported by another study on the activities of CCK derivatives without α-amide (Lignon et al. 1987).
Table 1. Potency ratio of endogenous α-amidated peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
<th>Amino acids</th>
<th>C-terminus</th>
<th>Potency ratio&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurokinin A</td>
<td>Mammalian</td>
<td>10</td>
<td>Met-NH₂</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Allatostatin</td>
<td>Cockroach</td>
<td>13</td>
<td>Leu-NH₂</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Lem-KI</td>
<td>Cockroach</td>
<td>8</td>
<td>Gly-NH₂</td>
<td>~10,000</td>
</tr>
<tr>
<td>Thyrotropin releasing hormone</td>
<td>Porcine, ovine</td>
<td>3</td>
<td>Pro-NH₂</td>
<td>4,400</td>
</tr>
<tr>
<td>Red pigment concentration hormone</td>
<td>Shrimp</td>
<td>8</td>
<td>Trp-NH₂</td>
<td>2,500</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Human</td>
<td>32</td>
<td>Pro-NH₂</td>
<td>1,670</td>
</tr>
<tr>
<td>Corticotropin-releasing hormone</td>
<td>Ovine</td>
<td>41</td>
<td>Ala-NH₂</td>
<td>1,000</td>
</tr>
<tr>
<td>Luteinizing hormone-releasing hormone</td>
<td>Porcine</td>
<td>10</td>
<td>Gly-NH₂</td>
<td>1,000</td>
</tr>
<tr>
<td>Leucopyrokinin</td>
<td>Cockroach</td>
<td>8</td>
<td>Leu-NH₂</td>
<td>1,000</td>
</tr>
</tbody>
</table>

<sup>a</sup>The potency ratio is defined as the activity of a peptide amide divided by the activity of its corresponding peptide free acid.

Data adapted from (Merkler 1994).
1.1.2 Biosynthesis of α-amidated peptide and peptidylglycine α-amidating monooxygenase.

Endogenous peptides are derived from prohormone precursors by limited proteolysis within the secretory pathway. Nine mammalian prohormone convertases (PCs) responsible for tissue specific processing of prohormone precursors have been identified. Of these, seven (PC1, PC2, furin, PC4, PC5, PACE4 and PC7) belong to the yeast kexin subfamily of subtilases. These PCs cleave the prohormone precursors on the C-terminal side of two basic amino acid residues separated by 0, 2, 4 or 6 residues (Seidah and Chretien 1999). Another characterized PC is subtilisin kexin isozyme (SKI-1/S1P) which belongs to the pyrolysin subfamily of subtilases. SKI-1 cleaves on the C-terminal side of Leu or Thr while the second residue ahead the cleavage site being hydrophobic and the fourth being basic (Seidah and Chretien 1999) (Table 2). The other PC is neural apoptosis-regulated convertase 1 (NARC-1/PCSK9) which belongs to the proteinase K subfamily of subtilases. This convertase autocatalytically cleaves its prosegment at the motif VFAQ↓SIP (Benjannet et al. 2004).
After the convertase cleavage, one or more residues are usually removed from the C-terminus of peptide-processing intermediate by carboxypeptidases. The peptide may then undergo PTMs before they become active.

### Table 2. Processing sites of mammalian prohormone convertases.

<table>
<thead>
<tr>
<th>Cleavage Site</th>
<th>Prohormone Convertase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K/R)-(X)$_n$-(K/R)$\downarrow$</td>
<td>PC1, PC2, furin, PC4, PC5, PACE4 and PC7</td>
</tr>
<tr>
<td>(K/R)-X-(Hydrophobic)-(L/T)$\downarrow$</td>
<td>SKI-1/S$_1$P</td>
</tr>
<tr>
<td>VFAQ$\downarrow$SIP</td>
<td>NARC-1/PCSK9</td>
</tr>
</tbody>
</table>

X is any amino acid residues. $\downarrow$ denotes the cleavage site.

#### 1.1.2.1 Peptidylglycine $\alpha$-amidating monooxygenase.

A C-terminal glycine is required for amidation in vivo (Eipper and Mains 1988). Peptidylglycine $\alpha$-amidating monooxygenase (PAM; E.C. 1.14.17.3) is the only enzyme known to catalyze the oxidation of inactive C-terminal glycine-extended peptides to their bioactive $\alpha$-amidated products. PAM is a bifunctional enzyme with two distinct catalytic domains: peptidylglycine $\alpha$-hydroxylating monooxygenase (PHM) and peptidylamidoglycolate lyase (PAL). The PHM domain is located at the N-terminal of the PAL domain, separated by a noncatalytic segment exon A. PHM and PAL can also be expressed separately or generated by endoproteolytic cleavage of bifunctional PAM (Prigge et al. 2000).
PAM is a metalloenzyme with two coppers in PHM domain and one zinc atom in PAL domain (Eipper et al. 1995, Bell et al. 1997). The two coppers in PHM are redox-active and cycle between Cu\(^{+}\) and Cu\(^{2+}\) during catalysis (Eipper et al. 1995, Freeman, Villafranca and Merkler 1993). Reduced PHM, with two Cu\(^{+}\) atoms, catalyzes the reduction of molecular oxygen for the hydroxylation of glycine-extended substrates. The PHM-bound Cu\(^{2+}\) atoms can be reduced to Cu\(^{+}\) by a variety of reducing agent, with ascorbate exhibiting the highest V/K for those reductants tested. (Kolhekar, Mains and Eipper 1997, Li, Oldham and May 1994) Ascorbate is, most likely, the reductant in vivo (Eipper and Mains 1991). The role of the PAL-bound Zn(II) remains unclear, as it may serve either a structural or catalytic role (Bell et al. 1997, Takahashi et al. 2009).
Figure 1. Representation of PHM Structure (Prigge et al. 1997).
The active site of PHM is flanked by two coppers represented by brown spheres.
Both copper and zinc atoms bound to PAM can be removed using chelators resulting in the loss of enzymatic activity. Addition of metals restores catalytic activity (Bell et al. 1997).

Figure 2. Representation of PAL structure (Chufan et al. 2009). The zinc atom is represented by a gray sphere.
1.1.2.2 α-Amidation reaction catalyzed by PAM.

The two catalytic domains of PAM work sequentially to catalyze the conversion of glycine-extended peptides to α-amidated peptides (Figure 3). The oxygen, copper, and ascorbate dependent PHM domain removes the pro-S hydrogen for the hydroxylation of the α-carbon of C-terminal glycine. The zinc dependent PAL domain dealkylates the hydroxyglycine intermediate to the α-amidated product and glyoxylate.

**Figure 3. Peptide amidation reaction scheme.**
Bifunctional enzyme PAM consists of two catalytic domains: peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidylamidoglycolate lyase (PAL). PHM domain catalyzes the hydroxylation at the α-carbon of C-terminal glycine. PAL domain converts the peptidyl-α-hydroxyglycine to α-amidated peptide and glyoxylate.
1.1.2.3 The rate limiting role of PAM in α-amidated peptide biosynthesis.

PAM catalyzes the final step in the biosynthesis of α-amidated peptide hormones. For most α-amidated peptide hormones, the amidation is essential to the bioactivity expressed by the peptide (Table 1). The PAM reaction is also the rate limiting step for the in vivo production of the α-amidated peptides. Evidence to support this includes the identification of glycine-extended precursors in tissue extracts. In some cases, the glycine-extended precursors were found in higher concentrations than the mature amidated forms. Examples include the concentration of glycine-extended precursor for thyrotropin releasing hormone (TRH-Gly) being 100-fold higher than the α-amidated form in the rat ventral prostate (Pekary, Knoble and Garcia 1989), and the concentration of glycine-extended form of adrenomedullin being >5-fold higher than that of α-amidated adrenomedullin in healthy human plasma (Kitamura et al. 1998).

Mains et al. (Mains, Bloomquist and Eipper 1991) further studied the rate limiting role of PAM in the biosynthesis of α-amidated peptide hormones by manipulating its expression level in AtT-20 cell line. Model cell lines with significantly increased or decreased level of PAM were generated by transfection with vectors containing PAM cDNA in the sense or antisense orientation. In wild type AtT-20 cells, concentration of
newly synthesized joining peptide in amidated form (JP) is close to that in glycine-extended form (JP-Gly). Cells expressing increased level of PAM produced almost entirely JP while cells with decreased PAM level produced half as much JP as wild type cells (Mains et al. 1991).

1.1.2.4 Accumulation of glycine-extended precursor by PAM inhibition.

Since PAM is the only enzyme catalyzing the amidation reaction and functions as the rate limiting role, accumulation of glycine-extended precursor peptides can be achieved by reducing its activity. In mouse pituitary cancer cell line AtT-20, over half of joining peptide is α-amidated. When incubating with 0.5 µM or 2.0 µM metal chelating reagent disulfiram, percentage of newly synthesized joining peptide in amidated form decreased to 15% and 10%, respectively (Mains, Park and Eipper 1986). Similar experiments have been done \textit{in vivo} with rats in which significant accumulation of glycine-extended precursors was also observed (Marchand et al. 1990, Mains et al. 1986). PAM activity level can also be regulated by modulating its expression level using anti-sense RNA which also results in accumulation of glycine-extended precursors (Mains et al. 1991).
1.2 Current Method to Identify α-Amidated Peptides

Peptide amidation has been termed “signature of bioactivity” because most known α-amidated peptides are bioactive (Cuttitta 1993). Efforts have been made to discover novel peptide hormones through identification of peptides with C-terminal amide.

1.2.1 Chemical assay methods.

1.2.1.1 Tatemoto & Mutt method.

In this method, the target peptide was proteolytically fragmented to a mixture of short peptides, amino acids, and the single C-terminal amino acid amide which could be extracted from the mixture and identified. This approach was pioneered by Tatemoto & Mutt (Tatemoto and Mutt 1978) in the late 1970’s and was used to identify a number of important α-amidated peptide hormones, including galanin (Tatemoto et al. 1983) and pancreastatin (Tatemoto et al. 1986). In the original Tatemoto & Mutt method, the degradation mixture (consisting of peptide fragments, amino acids and an amino acid amide) was dansylated. The hydrophobic dansyl-amino acid amide was extracted into an organic phase followed by two-dimensional TLC identification. With the improvement in separation and detection of C-terminal amino acid amide using HPLC (Simmons and Meisenberg 1983, Schmidt et al. 1987) and capillary electrophoretic chromatography
(Feng and Mitchell 1999), the limit of detection for this method was decreased from approximately 1 nmol to picomole range in model studies using purified α-amidated peptides or amino acid amides. However, when dealing with complex samples, inefficiency in the proteolytic fragmentation, in the labeling chemistries, and in the extraction or separation of the amino acid amide (or its derivative), can dramatically decrease the sensitivity. Moreover, this method can only tell the presence or absence of α-amidated peptides. Further fractionation/purification would be needed to identify peptides of interest. Although being used for identification of a few α-amidated peptide hormones, the Tatemoto & Mutt procedure has not been widely used to discover novel peptide hormones, but to characterize the C-terminal amidated amino acid after the purification of new bioactive peptide (Carlquist, Mutt and Jornvall 1979, Carlquist, Jornvall and Mutt 1981).
Figure 4. Schematic of Tatemoto & Mutt Method.

The target peptide was proteolytically degraded to single amino acids or short peptides. Resulted mixture was dansylated using amine labeling. Under alkaline conditions, the hydrophobic dansyl-amino acid amide was extracted into an organic phase and identified with 2D TLC.
1.2.1.2 Hill method.

Hill et al. (Hill, Flannery and Fraser 1993) presented a different chemical approach for the identification of α-amidated peptides in a complex mixture of peptides from a biological source. Free amines in the peptides were first protected by acetylation. Next the amides were converted to amines via Hofmann rearrangement. The newly formed amines were detected by labeling with ninhydrin. Another reaction for detection of glycine-extended peptides was also developed in which the C-terminal glycine was derivatized to form 2-thiohydantoin. Peptides with C-terminal amide or C-terminal glycine derivatives were detected by HPLC. Hill et al. (1993) argued that the co-elution of the amide and a C-terminal glycine was a strong indicator for the presence of an α-amidated peptide. Overall, the Hill method is very laborious, insensitive (mmol detection limit), and prone to false positives from peptides with Asn and Gln residues. These drawbacks have dramatically hindered the widespread application of this method, as it has never been used for the discovery of novel α-amidated peptide.
Figure 5. Schematic of Hill Method.
Following the protection of peptide free amines by acetylation, the C-terminal amide was converted to amine by Hoffman rearrangement and then colorized by ninhydrin.
1.2.1.3 Carpenter & Merkler method.

Carpenter & Merkler method (Carpenter 2006) was developed based on the detection of the PAM reaction by-product glyoxylate. In this method, PAM inhibitor was used in cell culture to accumulate glycine-extended peptides. Total peptides were then extracted from cells and chromatographically fractionated. The resulting fractions were treated with PAM to convert glycine-extended peptides, if any, to the α-amidated peptides and glyoxylate. The glyoxylate produced in PAM reaction was then quantified using either a spectrophotometric, fluorescent, or chemiluminescent enzyme linked assay (Figure 6). The most sensitive assay for glyoxylate was the chemiluminescent assay which linked the glyoxylate consumption to light emission through hydrogen peroxide. The detection limit was claimed to be 5 nM for hydrogen peroxide and 15 nM for glyoxylate. However, when handling samples from biological sources, this method suffered severe background problem due to the complexity of the samples. The signal to noise ratio was below 25 even for the model α-amidated peptide with high concentration.
Figure 6. Schematic of Carpenter & Merkler method.
A: PAM reaction to convert accumulated glycine-extended peptide to α-amidated peptide and glyoxylate. B: The malate synthase / malate dehydrogenase assay. Tetrazolium was oxidized to yield a formazan dye through an electron shuttling pathway with the concomitant reduction of NAD. Formazan can be detected at 490nm. C: The glyoxylate reductase assay. Glyoxylate was detected by monitoring NADPH loss at 340nm. D: Hydrogen peroxide assay. Hydrogen peroxide was stoichiometrically produced by glycolate oxidase from glyoxylate. Three approaches were presented for the detection of hydrogen peroxide in which hydrogen peroxide consumption was linked to the production of indamine dye (λ<sub>max</sub>=590 nm), fluorophore resorufin (λ<sub>ex</sub>=560 nm, λ<sub>em</sub>=589 nm) or light.
1.2.2 Immunoassay methods.

A possible method to identify $\alpha$-amidated peptides would be the use of antibodies to specifically recognize a C-terminal amino acid amide. To date, no antibody has ever been developed to recognize a single amino acid amide. However, the Grimmelikhuijzen group has produced an antibody against dipeptide amide, Arg-Phe-NH$_2$, and used this antibody for the discovery of a number of novel $\alpha$-amidated peptides (Grimmelikhuijzen and Graff 1985, Grimmelikhuijzen, Leivev and Carstensen 1996, Grimmelikhuijzen, Williamson and Hansen 2002). High quality antibodies against all possible dipeptide amides, a library of 400 antibodies, will be required for a broad application of this approach for the discovery of novel $\alpha$-amidated peptides.

1.2.3 Mass Spectrometry Based Methods.

1.2.3.1 Mass difference based method.

The mass difference between the C-terminal amino acid amide and its cognate free carboxylate form is 0.9840 Da. The combination method of carboxypeptidase Y digestion and fast atom bombardment (FAB) mass spectrometry was developed to exploit this mass difference to identify $\alpha$-amidated peptides (Kim and Kim 1994). In this method, target peptide was digested for various times and the digestion products analyzed using FAB
mass spectrometer. From the mass difference between the parent peptide and its one-amino-acid-short fragment, the identity of the C-terminal residue can be readily determined. Sequence information can also be obtained from mass differences of consecutive digestion products. The limitation of this method is that some amino acids have same or very close mass value and, thus, cannot be distinguished from each other. Examples include the exact same masses for Leu and Ile, and Glu-amide and Gln, and millimass differences between Lys and Gln, Asn-amide and Leu/Ile, and Glu-amide and Lys. Moreover, this method works best with purified peptide.

The small mass difference between peptides with C-terminal amide and free carboxylic acid can be amplified through derivatizations. Kuyama et al. (2009) presented a chemical approach to convert the free C-terminal carboxyl group to methylamide (CONHCH$_3$) via oxazolone chemistry. This method targets only the C-terminal carboxyl group while free carboxyl side chains in Asp and Glu remain intact (Kuyama et al. 2009). Due an incomplete derivatization, peptides with free C-terminal carboxylate exhibit doublet peaks in MS spectra separated by 13 Da (the difference between COOH and CONHCH$_3$), while α-amidated peptides show normal singlet peaks, as such peptides have a protected C-terminus.
1.2.3.2 Fragmentation behavior based method.

It was reported that α-amidated peptides demonstrated specific fragmentation patterns under tandem mass spectrometry (MS/MS). Mouls et al. (Mouls et al. 2006) investigated the behavior of α-amidated and normal peptides upon low energy collision-induced dissociation (CID) and found amidated peptides produced an abundant loss of ammonia from protonated molecules. The side chain amides as in Asp and Gln were found much more stable than C-terminal amide under the CID conditions and, thus, did not affect the detection of α-amidated peptides. Lys and Arg at the C-terminus were stable at the CID conditions. However, ammonia loss from the side chain of Arg residues within peptide chains was observed. This method is useful to identify α-amidated peptides from trypsin digested products, which have all the Arg residues present at C-terminus. Significant false positives from internal Arg residues would show up when handling non-tryptic peptides. CID energy levels required to generate maximal ammonia loss vary from 17% to 80% (normalized energy level), based on the length and amino acid composition of the peptide. This could restrict this method as screening strategy from a complex peptide mixture.
1.2.3.3 Tandem mass spectrometry coupled with database search.

Liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) followed by database search has been emerged as a powerful tool for peptide identification due to its advantages of speed, sensitivity, and applicability to complex peptide mixtures. A few α-amidated peptides have been recently discovered using this method, such as the C-terminal fragments of chromogranin A ER-20amide and AR-28amide (Taylor et al. 2006), as well as the neuroendocrine regulatory peptides NERP-1 and NERP-2 (Yamaguchi et al. 2007).

In a general LC-MS/MS process, the peptide mixture is loaded onto a reverse phase LC column and fractionated based on peptide hydrophobicity. For on-line LC-MS/MS, eluted peptides are usually electrosprayed directly into a mass spectrometer and transferred into ionized form in the gas phase. Masses of peptides are collected and recorded in a MS spectrum. One peptide ion (called parent ion) at a time is selected and isolated for fragmentation to generate the MS/MS spectrum. Shown in Figure 7 are nomenclatures for common peptide fragment ions (Roepstorff and Fohlman 1984). In many mass spectrometry instruments, the major peaks in an MS/MS spectrum are b ions and y ions, where the charges are retained on the N-terminus and C-terminus, respectively. Depending on the fragmentation technique, the mass analyzer, and the
peptide structure, other type of ions could be dominant, as well. An example is electron capture dissociation (ECD) which produces mainly c- and z-type ions. The MS and

![Fragment ions nomenclature](image)

**Figure 7. Nomenclature for fragment ions in mass spectra of peptides.**

a-, b- and c-type ions are N-terminal ions; x-, y- and z-type ions are C-terminal ions. R1, R2, R3 and R4 represent the side chains of different amino acids.

MS/MS spectra are written to a “raw file” in a certain format. One raw file normally contains thousands to hundreds of thousands spectra depending on the complexity of the sample, the LC settings, and the mass spectrometer conditions.

Mapping MS/MS information to peptide sequences is usually done by database search engines such as MASCOT (Perkins *et al.* 1999), SEQUEST (Yates *et al.* 1996) and X!TANDEM (Craig and Beavis 2004). In general, the search engine generates all possible peptide sequences (predicted peptides) from a protein database into an indexed
list. By matching masses of predicted peptides and the observed mass of the experimental peptide, possible candidate peptide sequences are selected. Then, theoretical fragment ions are generated from these sequences and compared with the MS/MS spectrum of the experimental peptide for scoring. The search engine ranks the score of all candidates and reports back the top ranked hit or multiple matches (SEQUEST).

MS/MS spectra can also be interpreted through *de novo* sequencing, *i.e.* sequencing without assistance of a linear sequence database (Seidler *et al.* 2010). *De novo* sequencing can be done manually by comparing mass differences between fragment ions and theoretical masses of amino acid residues. An example of manual *de novo* sequencing of an α-amidated peptide found in AtT-20 cell line is shown in Figure 8. Software have also been developed to automate this approach such as PEAKS (Ma *et al.* 2003), DeNovoX (Thermo, San Jose, CA, USA), PepNovo (Frank and Pevzner 2005) and Vonode (Pan *et al.* 2010). In spite of the continuous growing of protein/genome sequence databases, *de novo* sequencing is still essential in some situations such as identification of protein/peptide from organisms with unsequenced genomes and protein/peptide with non-proteinogenic amino acids or unknown PTMs.
Figure 8. The *de novo* sequencing of a peptide. Mass differences between a set of fragment ions (b ions, marked with red lines) were used to identify the individual amino acids and the peptide sequence. The same sequence was deduced from the other set of fragment ions (y ions, marked with blue lines), which is highly suggestive that the sequence is the actual one.

1.3 Introduction to a Novel Strategy for the Discovery of Novel α-Amidated Peptides

1.3.1 Impetus for the design of an efficient and effective α-amidated peptide screening method.

The only known physiological function of PAM is to convert glycine extended prohormones (including glycine-extended peptides and fatty acyl-glycine) to their amidated forms. No evidences have been found to suggest any other biological role of
PAM or to explain the presence of PAM in tissues not known to produce $\alpha$-amidated products. Moreover, high levels of PAM were found in certain tissues with no corresponding level of known amidated products. One example is the relatively high level of PAM expression in the cardiac atrium (Eipper et al. 1992), yet only minor levels of $\alpha$-amidated peptides have been detected from this tissue. Therefore, it is believed that there are as yet undiscovered $\alpha$-amidated peptide hormones in certain tissues.

To date, there has been no detection method capable to screen for every existing $\alpha$-amidated peptide in every tissue. Known $\alpha$-amidated peptides were discovered by a variety of techniques. Some of them were found by coincidence. The lack of systematic screening method strengthens the hypothesis that there are many undiscovered $\alpha$-amidated peptides.

Reported methods for the identification of $\alpha$-amidated peptides were summarized and discussed in section 1.2 in this chapter. Of these methods, the most promising one to screen for novel $\alpha$-amidated peptides is LC-MS/MS coupled with database search. Advancement in mass spectrometry provides instruments with high resolution and sensitivity. LC or 2D LC systems coupled with mass spectrometers makes it possible to handle complex biological samples. However, several drawbacks need to be overcome in order to apply this method to high throughput screening for novel $\alpha$-amidated peptides.
With automatic spectra interpretation through database search, only as low as around 10% spectra can be successfully mapped (Keller et al. 2002). Moreover, when handling endogenous peptide identification, database searching is extremely time-consuming since all possible cleavage sites must be considered (i.e. “no enzyme” setting has to be chosen) and multiple PTMs must be selected to enhance the successful identification rate (Falth et al. 2006). The expansion of searching space resulted from this settings will in turn generate more false positives.

1.3.2 Novel LC-MS/MS based α-amidated peptide screening strategy.

In biological tissues, α-amidated peptides are surrounded by an ocean of various peptides, modified and unmodified, signal peptides and protein degradation fragments. Modern mass spectrometers coupled to the appropriate separation techniques are capable of identifying parent ion signals of peptides at very low concentration and obtaining their MS/MS spectra. However, high quality MS/MS spectra are not guaranteed, especially for peptides with low abundance and/or PTMs. Screening for α-amidated peptides from large number of MS/MS spectra of all kinds of peptides is akin to finding "needles in a haystack". In this study, we attack this problem from two angles, reducing the haystack and making the needles larger.
1.3.2.1 Peptide pair strategy to reduce the haystack.

As shown in Figure 3, the glycine-extended precursor loses a $[\text{C}_2\text{H}_2\text{O}_2]$ group from C-terminal and gains one proton on the terminal $\alpha$-amide during the PAM catalyzed amidation reaction, resulting in a total difference of 58.0055 Da ($\text{C}_2\text{H}_2\text{O}_2$) between the glycine-extended and amidated forms. Since an $\alpha$-amidated peptide and its glycine-extended precursor have identical amino acids sequences except the relatively small difference at the C-termini, they will have similar hydrophobicities, similar reverse phase retention times, and similar MS/MS fragmentation patterns. By finding peptide pairs which meet these criteria, one can screen for amidated peptides from LC-MS/MS data. A script was developed to scan peptide parent ions in LC-MS/MS data for peptide pairs with 58 mass unit difference and similar retention times. Fragmentation patterns of the two MS/MS spectra in each pair were checked for similarity to validate if these were related $\alpha$-amidated and glycine-extended peptides. Only peptide pairs which met all three criteria were considered for further interpretation. Thus the haystack, namely amount of MS/MS spectra to be interpreted, was greatly reduced.
1.3.2.2 \( \alpha \)-Amidated peptide enrichment to enlarge the needles.

Copper-depleted PHM (apo-PHM) was used to enrich \( \alpha \)-amidated peptides from a complex mixture. When incubating apo-PHM with peptide mixtures, glycine-extended peptide substrates bound to PHM. After the removal of the unbound peptides, PHM was reactivated by addition of copper to release the bound glycine-extended peptides as these were oxidized to the \( \alpha \)-hydroxyglycine peptide products. The hydroxylated intermediates were then converted to the \( \alpha \)-amidated products by adjusting pH > 10 (Jones et al. 1988). As a result, the relative concentrations of \( \alpha \)-amidated peptides were significantly increased.

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Chapter 2 Peptide-pair Screening Strategy for the Discovery of α-Amidated Peptides

2.1 Introduction

A modified peptide and its unmodified counterpart have identical sequences with the only difference being the modified amino acid(s). Thus, modified and unmodified forms of a peptide have a specific mass difference, similar reverse phase retention times, and similar MS/MS fragmentation patterns.

For a certain PTM, the mass differences between modified peptides and their unmodified forms are specific and constant. Based on this, a tool named Mass Distance Fingerprint (MDF) (Potthast et al. 2007) was developed to detect predominant PTMs. Mass Distance Histogram (MDH) was first established by calculating peptide distances (mass differences) of all peptide pairs. A statistical random background model was then subtracted from the MDH. The Gaussian distributions were fitted to the remaining data to obtain accurate frequent mass distances, which imply modification(s). This method only uses peptide precursor mass and is not database dependent. Therefore, it is rapid and can detect unknown PTMs. However, this method is not capable of detecting less abundant
modifications because both modified and unmodified forms of the peptide must be measured for multiple times in order to yield a reasonable signal above background.

Retention time difference could provide orthogonal supporting evidence for PTM-related peptide pairs. Fu et al. (Fu et al. 2009) presented a sequence database-independent approach to detect abundant PTMs in high-accuracy peptide mass spectra. This approach was based on the assumption that a modified peptide and its unmodified form correlate with each other in masses and retention times. Mass differences and retention time differences between spectra were plotted on a 2D histogram followed by a bivariate Gaussian mixture model to differentiate modification related pairs and random pairs. Frequently occurring peptide pairs with same mass and retention time differences indicated a modification. This method used parent ion masses and retention times to scan the LC-MS/MS data and, hence, was rapid. However, it was designed only to detect abundant PTMs which are found in large number of spectra in order to be discriminated from random spectra pairs.

In addition to mass and retention time differences between parent ions, related fragment ions were also used in a similar approach reported by Savitski et al. (Savitski, Nielsen and Zubarev 2006). Savitski et al. only plotted spectral pairs with similar MS/MS spectra, in contrast to the approach of Fu et al., which plotted all combinations of the
mass retention time differences for all spectral pairs. The similarity was defined as at least four matching fragment pairs in two MS/MS spectra. Matching fragment pairs were either a pair of fragment ions with the same mass, or with a mass difference the same as that of the two parent ions. Both collision induced dissociation (CID) and electron capture dissociation (ECD) were used to get complementary fragmentation. This method is more sensitive for the detection of PTMs relative to the Fu et al. approach, but at a cost of computational efficiency and additional instrumentation. However, spectral pairs with certain PTM still must be identified multiple times for statistical significance. Moreover, multiple fragmentation methods were required, which often cannot be done in many proteomic labs.

When considering the identification/detection of peptides with a known modification, more restrictive screening conditions can be implemented. Instead of statistically analyzing mass differences between all peptide pair combinations, one could just look for peptide pairs with the known mass difference. α-Amidated peptides differ from their corresponding glycine-extended precursors with a mass difference of 58.0055 Da (C₂H₂O₂), as a consequence of the PAM-catalyzed amidation reaction. α-Amidated peptides and the glycine-extended precursors have identical amino acid sequences except for the small difference at their respective C-termini. Therefore, they have similar
hydrophobicities, reverse phase retention times, and MS/MS fragmentation patterns. Moreover, the enzyme responsible for the peptide amidation (PAM) is well studied and inhibitors are available. The application of PAM inhibitors leads to the accumulation of glycine-extended precursors, which can be used not only to insure the coexistence of \( \alpha \)-amidated peptides and their glycine-extended precursors, but also to provide another restriction for screening.

2.2 Material and Methods

2.2.1 Materials.

The mouse pituitary AtT-20 cell line and basal F-12K medium (Kaighn's Modification of Ham's F-12 Medium) were from American Type Culture Collection (www.atcc.org). Fetal bovine serum (FBS) was from Atlanta Biologicals. Donor equine serum was from Thermo Scientific. Mouse pituitaries were from Pel-Freez Biologicals. Disulfiram (1,1',1'',1'''-[disulfanediylbis(carbonothioylnitrilo)]tetraethane) was from Fluka. CHCA (\( \alpha \)-cyano-4-hydroxycinnamic acid) and adenosine 5'-triphosphatase (ATPase) from porcine cerebral cortex were from Sigma-Aldrich. Pen-Strep was purchased from Omega Scientific. Angiotensin-converting enzyme (ACE) was from MP Biomedical. Mouse joining peptide (mJP) and its glycine extended precursor form
(mJP-Gly), the \( \alpha \)-amidated peptide ELEGERPL-NH\(_2\), and a glycine-extended peptide QNEWRIPG were synthesized in-house at the USF Core Peptide Synthesis and Mass Spectrometry Facility. Sep-Pak Plus C\(_{18}\) cartridges were from Waters Associates. ZipTips were from Millipore. All other reagents and solvents were of the highest quality commercially available.

### 2.2.2 Cell growth conditions.

Mouse pituitary AtT-20 cells were cultured in Hams F-12K culture medium supplemented with 15\% (v/v) horse serum, 2.5\% (v/v) fetal bovine serum and 1\% (v/v) of the antimicrobial Pen-Strep (10,000 units Penicillin (Base)/mL and 10,000 units Streptomycin (Base)/mL in 0.85\% NaCl). Cells were grown in 225 cm\(^2\) culture dishes in the presence of 5\% CO\(_2\) at 37 \(^\circ\)C. The AtT-20 cell line was non-adherent. The cells were passed at 1:3 into fresh media after 80\% confluence was reached in each culture flask. A class II sterile laminar flow hood was utilized for all cell culture work. Cells were cultured in a 37\(^\circ\)C incubator with a constant flow of 5\% CO\(_2\). After sufficient growth, cells were collected by centrifugation and evenly split into two 225 cm\(^2\) culture dishes containing 75 mL fresh media. A 75 \( \mu \)L aliquot of 5.0 mM disulfiram (prepared in 70\% ethyl alcohol) was added to one dish to bring the final disulfiram concentration to 5 \( \mu \)M.
An equivalent volume of 70% (v/v) ethanol (vehicle) was added to the other dish as control. The cells were incubated for 20 hours prior to harvest.

2.2.3 Sample preparation.

2.2.3.1 Total peptide extraction.

Cells were collected by centrifugation. The spent media was acidified to 0.1% (v/v) TFA with a 6% TFA stock and centrifuged at 12000g for 30 minutes. The cell pellet was homogenized in a ground glass homogenizer at 4 ºC with 8 M urea solution or acid extract solution containing 0.1M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) TFA (Bennett, Browne and Solomon 1981). The homogenate was centrifuged at 12000g for 15 minutes. The supernatant was collected and added to the acidified media. The mixture was passed through 10 kDa molecular weight cut-off filter (Microcon YM-10, Millipore, Bedford, MA, USA) to remove proteins.

A total of 5 mouse pituitary glands (Pel-Freeze Biologicals, Rogers, AR, USA) were cut into small pieces and homogenized in a ground glass homogenizer at 4 ºC using an acid extract solution containing 0.1M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) TFA. The homogenate was centrifuged at 12000g for 15 minutes. The supernatant
was collected and passed through a 10 kDa molecular weight cut-off filter to remove proteins.

2.2.3.2 Solid phase extraction.

The peptide extract was initially desalted and concentrated by solid phase extraction on a C$_{18}$ cartridge (Sep-Pak Plus, Waters, Milford, MA, USA). A Sep-Pak Plus cartridge was pre-wetted with 10 mL of a solution composed of 0.1%(v/v) TFA/80%(v/v) acetonitrile (ACN) at a flow rate of 2.0 mL/min. The cartridge was then washed with 20 mL aqueous solution of 0.1% (v/v) TFA/2%(v/v) ACN at a flow rate of 2.0 mL/min. The peptide extract was loaded onto the cartridge at a rate of 1.0 mL/min, followed by a wash of 20 mL of 0.1% TFA (v/v) /2%(v/v) ACN at a flow rate of 1.0 mL/min.. Bound peptides were eluted with 4 mL of 0.1% (v/v) TFA/80% ACN at a flow rate of 1 mL/min. The 4 mL eluent was collected and concentrated to 0.5 mL on a Savant Speedvac vacuum centrifuge (Savant Instruments, Farmingdale, New York, USA).

Prior to LC-MS/MS analysis, the extracted peptide mixture was further purified using ZipTip (Millipore, Billerica, MA, USA). A ZipTip was wetted by aspirating 10 µL of methanol and dispensing it to waste 10 times followed by 0.1% (v/v) TFA/80% ACN for 8 times. The ZipTip was then equilibrated with 0.1% (v/v) TFA/2% ACN 6 times by
aspirating and dispensing. Peptides were bound by aspirating 10 µL of the peptide mixture solution and dispensing it back for 15 cycles. After 10 cycles wash using 0.1% (v/v) TFA/2% ACN, bound peptides were eluted by 10 µL of 0.1% (v/v) TFA/80% ACN. Elution was repeated twice with fresh eluting solution. A total of 30 µL of eluent was obtained and concentrated on the Speedvac to 5 µL.

2.2.4 LC-MS/MS assay.

The sample was analyzed by matrix assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometer and linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap). For MALDI-TOF assay, samples from disulfiram treated group and control group were analyzed separately in order to get peptide intensity information so that accumulation filter can be applied when processing data. For LTQ-Orbitrap assay, samples from the two groups were combined and analyzed together.

2.2.4.1 LC-MALDI-TOF/TOF

The sample subject to MALDI-TOF assay was fractionated and spotted onto 192-well plates using a microfraction collector (LC Packings Probot, Dionex, Sunnyvale, CA, USA) interfaced with a capillary liquid chromatograph (Agilent 1200, Agilent, Santa Clara, CA, USA). The sample was loaded on a C$_{18}$ column (100 mm × 150 µm ID, Vydac
MS C₁₈, Grace, Deerfield, IL, USA) and washed for 20 minutes with 95% solvent A (2% ACN + 0.1% TFA) and 5% solvent B (80% ACN + 0.1% TFA). The 65-minute gradient was programmed as: 5% to 15% B in 10 minutes, 15% to 50% B in 40 minutes, 50% to 90% B in 5 minutes, 90% B for 5 minutes and 90% to 5% B in 5 minutes. The flow rate was 1 µL/min. Separated peptides were mixed with α-cyano-4-hydroxycinnamic acid solution (4mg/mL in 50% (v/v) ACN and 5% (v/v) isopropanol) delivered at a flow rate of 1.0 µL/min. Fractions were spotted every 15 s onto blank stainless steel MALDI plates (Applied Biosystems, Foster City, CA, USA).

Spotted peptide fractions were analyzed by MALDI-TOF/TOF mass spectrometry (Applied Biosystems 4700 Proteomics Analyzer, Foster City, CA, USA) using reflective positive mode. MS spectra were obtained with a total of 1500 shots per spot. Up to the 10 most intense precursors per spot were then selected for MS/MS analysis. MS/MS spectra were generated with a total of 5000 shots for each precursor. The data was externally calibrated with 4700 Mass Standards from the manufacture.

2.2.4.2 LC-ESI-LTQ-Orbitrap

A nanoflow liquid chromatograph (U3000, Dionex, Sunnyvale, CA, USA) was coupled to an LTQ-Orbitrap (Thermo, San Jose, CA, USA). The LC system was
equipped with a trapping column (5 mm × 300 µm ID packed with C_{18} reversed-phase resin, 5 µm, 100 Å) and an analytical column (C_{18}, 150 mm × 75 µm ID, Pepmap 100, Dionex, Sunnyvale, CA, USA). The sample was first loaded onto a trapping-column and washed for 8 minutes with aqueous 2% ACN and 0.04% TFA. Trapped peptides were then eluted onto the analytical column. The 120-minute gradient was programmed as: 5% B for 8 minutes, 5-50% B in 90 minutes, 50-90% B in 7 minutes, 90% B for 5 minutes, 90-5% B in 1 minute and 5% B for 10 minutes to re-equilibrate, with solvent A being 2% ACN + 0.1% formic acid and solvent B being 90% ACN + 0.1% formic acid. The flow rate on the analytical column was 300 nL/min. (1/1000 split from 300 µL/min.) Peptides eluted were electrosprayed directly into an LTQ-Orbitrap mass spectrometer. Survey scans were performed in Orbitrap to obtain accurate peptide mass measurement. The resolution was 60,000 at 400 m/z. Mass measurement accuracy was monitored between runs using digested BSA. For each cycle of the survey scan, the five most intense precursor ions were selected for MS/MS analysis. MS/MS spectra were acquired in linear ion trap with normalized CID energy of 30%. Previously selected precursor ions were dynamically excluded for 60 seconds.
2.2.5 LC-MS/MS data processing.

2.2.5.1 LC-MALDI-TOF/TOF data processing.

Mass lists of each MS spectrum (from both disulfiram treated and control group) obtained by MALDI-TOF were exported and combined into one Excel data sheet. The complete list was then imported into Microsoft Visual FoxPro 8.0. Peptides with mass difference smaller than 0.1 \( m/z \) and a retention time difference under 2 minutes were considered the same peptide. The peptide with highest intensity was kept and all others were removed from the list. After removal of redundant data, the list was scanned for peak pairs with 58.01 ± 0.1 \( m/z \) difference and a retention time difference less than 2 minutes. Accumulation filter was then applied. Only peptides showing higher intensity in disulfiram-treated group were kept. The signal-to-noise (S/N) filter was also used to filter out less intensified peptides. Peaks in resulted peak-pairs were subjected to MS/MS analysis. The MS/MS spectra of each pair were inspected manually for similar fragment patterns. Interesting MS/MS spectra were sequenced by database search and/or manual interpretation.
2.2.5.2 LC-ESI-LTQ-Orbitrap data processing.

Information of MS/MS spectra was extracted from RAW data files obtained from LTQ-Orbitrap using RawXtract1.93 (Thermo Corp., San Jose, CA, USA). The list, which contains detailed information of each MS/MS spectrum such as scan number, charge state, monoisotopic $m/z$ and retention time, was imported to Microsoft Visual FoxPro 8.0. Peptides with mass difference smaller than 0.005 $m/z$ and retention time difference under 2 minutes were considered the same peptide and only one was kept for further processing. After removal of redundant data, the list was scanned for peak pairs with mass difference of $58.006 \pm 0.01$ (for singly charged ions), $29.003 \pm 0.01$ (for doubly charged ions) and $19.335 \pm 0.01$ (for triply charged ions), and retention time differences within 2 minutes. The MS/MS spectra of each pair were inspected manually and interesting spectra were sequenced by database search and/or manual interpretation.

2.2.6 ATPase activity assay.

The effects of mouse joining peptide (mJP) and two mJP related peptides on ATPase activity were tested. The standard ATPase activity assay was carried out at 37 °C by the addition of enzyme into 1.5 mL solution containing 20 mM Tris-HCl, pH 7.8, 0.57 mM EDTA, 5 mM MgCl$_2$, 3 mM KCl, 133 mM NaCl and 2.67 mM ATP (tris salt). After 20
minutes incubation, 1.5 mL of 20% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. The mixture was centrifuged at 12000g for 3 minutes to clarify. To measure the phosphate concentration from ATPase hydrolysis, the supernatant (1 mL) was mixed with 2 mL Taussky-Shorr reagent and 1 mL distilled water, the mixture was incubated at 25 °C for 5 minutes, and the absorbance at 660 nm was then measured.

Taussky-Shorr reagent was prepared as follows: 2 g of ammonium molybdate was dissolved in 5 M H$_2$SO$_4$ to a final volume of 20 mL. Distilled water (140 mL) was added followed by the addition of 10 g ferrous sulfate heptahydrate. The solution was brought to a final volume of 200 mL with water.

Standard curve was established by mixing 2 mL Taussky-Shorr reagent, 1 mL of phosphorus standard with a variety of concentration, 0.5 mL of 20% (w/v) TCA and 0.5 mL distilled water, and incubating for 5 minutes followed by absorbance measurement at 660 nm.

2.2.7 Angiotensin converting enzyme activity assay

Angiotensin converting enzyme activity assay was carried out at 37 °C by the addition of 0.01 unit enzyme into a 0.25 mL reaction mix containing 40 mM HEPES, pH 8.3, 240 mM sodium chloride, 0.2% (w/v) hippuryl-L-histidyl-L-Leucine (HHL). The reaction
mixture was incubated for 20 minutes. An aliquot of 1 M HCl (0.25 mL) was then added to quench the reaction. After a 10 minute centrifugation at 12000g, 10 µL of the supernatant was injected to HPLC (Hewlett Packard series 1100 HPLC, Hewlett-Packard, Wilmington, DE, USA) to quantify the amount of enzymatically produced hippuric acid.

Reaction mixture was separated on a reverse-phase C<sub>18</sub> column (Hypersil ODS, 4.6 × 100 mm, 5 µm, Keystone Scientific, Inc., Bellefonte, PA). Isocratic elution for 4 minutes with 15% (v/v) ACN aqueous solution was followed by a 4 minute wash run with 65% (v/v) ACN. Hippuric acid eluted at 2.5 min. HHL and L-histidyl-L-leucine eluted during wash run. Eluent was monitored with UV detector at 228 nm.

Standard curve for hippuric acid measurement was established by injecting 10 µL of hippuric acid with a variety of concentration into the HPLC system.

2.3 Results

2.3.1 Proof of concept.

Mouse pituitary corticotropic tumor AtT-20 cells are known to express high levels of PAM and two α-amidated peptides: mouse joining peptide (mJP) and α-melanotropin (α-MSH) (Eipper et al. 1986). For proof of concept, the accumulation of
glycine-extended precursors of these two known peptides was investigated, as well as retention time and mass differences between the two amidated peptides and their precursors, and their fragmentation patterns under CID.

2.3.1.1 Accumulation of glycine-extended precursors.

PAM activity in AtT-20 cells can be inhibited using the copper chelators, disulfiram, resulting in the accumulation of glycine-extended precursors (Mains et al. 1986). The relative levels of these peptides in cells grown with and without PAM inhibitor were investigated using LC-MALDI-TOF. As shown in Table 3, the relative intensity of glycine-extended precursors (in comparison with intensity of amidated form) increased by 2.8-fold for mJP and 9.2-fold for α-MSH when treated with PAM inhibitor.

Table 3. Accumulation of glycine-extended precursors of two known amidated peptides.

<table>
<thead>
<tr>
<th></th>
<th>Control Amidated</th>
<th>Gly-extended</th>
<th>Disulfiram treated Amidated</th>
<th>Gly-extended</th>
<th>Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse joining peptide</td>
<td>2780</td>
<td>5400</td>
<td>1240</td>
<td>6700</td>
<td>2.8</td>
</tr>
<tr>
<td>α-Melanotropin</td>
<td>3750</td>
<td>3100</td>
<td>1300</td>
<td>9940</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Numbers denote the peak intensity of a peptide except in the last column. Numbers in the last columns denote folds of accumulation of corresponding α-amidated peptide in disulfiram treated group.
2.3.1.2 Retention time.

Retention times of mJP and α-MSH were very close to their glycine-extended precursors as shown in Table 4. Under LC conditions for Orbitrap analysis, retention time differences between amidated and glycine-extended peptides were 0.86 minutes and 0.9 minutes for mJP and α-MSH, respectively. Extracted ion chromatograms (XIC) of these 4 peptides are also shown in Figure 9.

Table 4. Retention time differences between the two known α–amidated peptides and their glycine-extended precursors.

<table>
<thead>
<tr>
<th></th>
<th>Average ΔRT(min)</th>
<th>St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse joining peptide</td>
<td>0.86</td>
<td>0.01</td>
</tr>
<tr>
<td>α-Melanotropin</td>
<td>0.9</td>
<td>0.3</td>
</tr>
</tbody>
</table>

RTs are based on the LC conditions as specified in section 2.2.4.2. Values were calculated from 3 readings.
Figure 9. XICs of mJP, α-MSH and their glycine-extended precursors.
A: mJP-Gly; B: mJP; C: α-MSH-Gly; D: α-MSH. The retention time difference between mJP and mJP-Gly was 0.86 minutes. The retention time difference between α-MSH and α-MSH-Gly was 0.64 minutes.
2.3.1.3 Observed mass difference.

Observed masses of two known α-amidated peptides and their glycine-extended precursors were investigated with TOF and Orbitrap mass analyzers. Shown in Table 5 is the distribution of observed masses of the four peptides obtained by TOF mass analyser. Observed mass difference between mJP and mJP-Gly ranged from 57.9228 to 58.1116 while that of α-MSH and α-MSH-Gly ranged from 57.9362 to 58.1187. Based on these data, a window of 58.01 ± 0.1 was selected to screen for peptide pairs in MALDI-TOF data set.

Table 5. Distribution of observed masses of two known α-amidated peptides and their precursors in MALDI-TOF data set.

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>High</th>
<th>Mean</th>
<th>St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mJP</td>
<td>1941.0841</td>
<td>1941.1719</td>
<td>1941.13</td>
<td>0.04</td>
</tr>
<tr>
<td>α-MSH</td>
<td>1622.9843</td>
<td>1623.0680</td>
<td>1623.04</td>
<td>0.03</td>
</tr>
<tr>
<td>α-MSH-Gly</td>
<td>1681.0042</td>
<td>1681.1030</td>
<td>1681.06</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Data were based on at least 6 readings per peptide.
An example MS spectrum of mJP and mJP-Gly in LTQ-Orbitrap data set is shown in Figure 10, in which all peaks were shown in relative m/z to the monoisotopic peak of mJP. The observed mass difference between these two doubly charged peptides was 29.0025, very close to the theoretical value 29.0028. Shown in Table 6 is the distribution of observed masses of the four peptides in doubly charged state obtained by Orbitrap mass analyzer. Observed mass difference between doubly charged mJP and mJP-Gly ranged from 28.9960 to 29.0062 while that of α-MSH and α-MSH-Gly ranged from 28.9987 to 29.0075. Based on these data, a window of 29.003 ± 0.01 was selected to screen for doubly charged peptide pairs in LTQ-Orbitrap data set. Screening windows for triply and singly charged peptide pairs were adjusted accordingly.

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>High</th>
<th>Mean</th>
<th>St. Dev.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mJP</td>
<td>970.9301</td>
<td>970.9342</td>
<td>970.932</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>mJP-Gly</td>
<td>999.9302</td>
<td>999.9363</td>
<td>999.934</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>α-MSH</td>
<td>811.8932</td>
<td>811.8981</td>
<td>811.896</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>α-MSH-Gly</td>
<td>840.8968</td>
<td>840.9007</td>
<td>840.899</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Data were based on 23 readings per peptide.
Figure 10. Mass difference between mJP and mJP-Gly shown in a MS spectrum obtained by Orbitrap.
Monoisotopic peak of mJP was set as zero. All other peaks were shown in relative masses.
2.3.1.4 Fragmentation pattern.

MS/MS spectra of mJP and mJP-Gly are shown in Figure 11. These two peptides demonstrated very similar fragmentation pattern under CID. Corresponding b ions showed same mass value (marked with red arrows) while y ions showed a constant mass difference of 58 (marked with blue lines) resulting from the cleavage of C₂H₂O₂ group from C-terminal of mJP-Gly by PAM.
Figure 11. MS/MS spectra of mouse joining peptide (mJP) and its glycine-extended precursor.
Top: AEEEAHVGDGSPEPSREG; bottom: AEEEAHVGDGSPEPSREG-NH₂.
2.3.2 LC-MALDI-TOF/TOF data set.

2.3.2.1 α-Amidated peptide identified by database search.

Database search yielded one α-amidated peptide as shown in Table 7. This peptide is the C-terminus of chromogranin A (CgA) following a dibasic (Arg-Arg) processing site.

Table 7. An α-amidated peptide identified by database search from LC-MALDI data set.

<table>
<thead>
<tr>
<th>Observed Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Score</th>
<th>Expect</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>3176.24</td>
<td>3175.23</td>
<td>0.57</td>
<td>67</td>
<td>0.005</td>
<td>R.AEDQELESLSAIEAELEKV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AHQLQALRR.G+Amide(C-term)</td>
</tr>
</tbody>
</table>

2.3.2.2 α-Amidated peptides identified by pair finding method.

Surprisingly, two known α-amidated peptides were not reported by database search. The peak list was exported using Data Explorer (version 4.0, ABI, Foster City, CA, USA) and screened for peptide pairs as described previously. Based on the observed mass distribution data of two known α-amidated peptides and their glycine-extended precursors, a window of 58 ± 0.1 was applied to screen for peptide pairs. A list of over three hundred peptide pairs was obtained. An accumulation filter, defined as:
was used to filter out some of false positive pairs. Pairs containing signals with low S/N values were also filtered out since they were unlikely to yield decent MS/MS spectra. After these filters, the number of peptide pairs decreased to ~ 40. Manual inspection of the MS/MS spectra for these peptide pairs found 4 pairs with similar fragmentation patterns. After database search and manual interpretation, 3 of them were successfully interpreted, including the C-terminal fragment of CgA, mJP, and \( \alpha \)-MSH (Table 8).

Table 8. \( \alpha \)-Amidated peptides identified by pair finding method from LC-MALDI data set.

<table>
<thead>
<tr>
<th>Amidated</th>
<th>Gly-extended</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>( m/z )</td>
<td>( \text{intensity} )</td>
<td>( m/z )</td>
</tr>
<tr>
<td>3176.2612</td>
<td>7614.902</td>
<td>3234.247</td>
</tr>
<tr>
<td>1941.0909</td>
<td>1334.641</td>
<td>1999.111</td>
</tr>
<tr>
<td>1622.9706</td>
<td>509.2811</td>
<td>1681.006</td>
</tr>
</tbody>
</table>
2.3.3 LC-LTQ–Orbitrap data set

2.3.3.1 α-Amidated peptides identified by database search.

Over 15,000 MS/MS spectra were obtained from each LC-LTQ-Orbitrap experiment. Nine α-amidated peptides were identified through Sequest and Mascot database search (Table 9). Two sequences received very low probability due to poor spectrum quality.

Table 9. α-Amidated peptides identified by database search from LC-Orbitrap data set.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Probability</th>
<th>Modifications</th>
<th>Observed Mass</th>
<th>Actual Mass</th>
<th>Delta AMU</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)AEEAVWGDGSPEPSPRe(G)</td>
<td>95%</td>
<td>Amide</td>
<td>970.9332</td>
<td>1939.85</td>
<td>-0.004</td>
</tr>
<tr>
<td>(A)EEEAVWGDGSPEPSPRe(G)</td>
<td>95%</td>
<td>Amide</td>
<td>935.4121</td>
<td>1868.81</td>
<td>-0.009</td>
</tr>
<tr>
<td>(E)EEAVWGDGSPEPSPRe(G)</td>
<td>95%</td>
<td>Amide</td>
<td>870.8919</td>
<td>1739.77</td>
<td>-0.007</td>
</tr>
<tr>
<td>(E)EAVWGDGSPEPSPRe(G)</td>
<td>95%</td>
<td>Amide</td>
<td>806.3709</td>
<td>1610.73</td>
<td>-0.007</td>
</tr>
<tr>
<td>(E)AVWGDGSPEPSPRe(G)</td>
<td>3%</td>
<td>Amide</td>
<td>741.8508</td>
<td>1481.69</td>
<td>-0.004</td>
</tr>
<tr>
<td>(A)VWGDGSPEPSPRe(G)</td>
<td>95%</td>
<td>Amide</td>
<td>706.3317</td>
<td>1410.65</td>
<td>-0.005</td>
</tr>
<tr>
<td>(A)WGDGSPEPSPRe(G)</td>
<td>95%</td>
<td>Amide</td>
<td>656.7975</td>
<td>1311.58</td>
<td>-0.000</td>
</tr>
<tr>
<td>(G)DGSGEPSPRe(G)</td>
<td>2%</td>
<td>Amide</td>
<td>535.2485</td>
<td>1068.48</td>
<td>-0.002</td>
</tr>
<tr>
<td>(R)SYSMEHFRWGKPv(G)</td>
<td>95%</td>
<td>Amide</td>
<td>811.8972</td>
<td>1621.78</td>
<td>-0.004</td>
</tr>
</tbody>
</table>
2.3.3.2 α-Amidated peptides identified by pair finding method.

The peptide list was screened for peptide pairs with \(58.006 \pm 0.01\) mass difference and similar retention time (within 2 minutes). A list of 33 pairs was obtained.

**Table 10. Peptide pairs reported by pair finding method from LC-Orbitrap data set.**

<table>
<thead>
<tr>
<th>Obs. (m/z) of amidated</th>
<th>Obs. (m/z) of Gly-ext.</th>
<th>Charge state</th>
<th>Mass difference</th>
<th>R.T. difference</th>
<th>Found by database search</th>
</tr>
</thead>
<tbody>
<tr>
<td>970.9304</td>
<td>999.9359</td>
<td>2</td>
<td>58.0110</td>
<td>-0.01</td>
<td>YES</td>
</tr>
<tr>
<td>935.4109</td>
<td>964.4114</td>
<td>2</td>
<td>58.0010</td>
<td>0.08</td>
<td>YES</td>
</tr>
<tr>
<td>871.8935</td>
<td>899.8951</td>
<td>2</td>
<td>58.0032</td>
<td>0.72</td>
<td>YES</td>
</tr>
<tr>
<td>806.3690</td>
<td>835.3716</td>
<td>2</td>
<td>58.0052</td>
<td>1.22</td>
<td>YES</td>
</tr>
<tr>
<td>741.8502</td>
<td>770.8514</td>
<td>2</td>
<td>58.0024</td>
<td>1.22</td>
<td>YES</td>
</tr>
<tr>
<td>706.3295</td>
<td>735.3329</td>
<td>2</td>
<td>58.0068</td>
<td>1.67</td>
<td>YES</td>
</tr>
<tr>
<td>656.8019</td>
<td>685.8043</td>
<td>2</td>
<td>58.0048</td>
<td>1.10</td>
<td>YES</td>
</tr>
<tr>
<td>563.7588</td>
<td>592.7625</td>
<td>2</td>
<td>58.0074</td>
<td>1.22</td>
<td>NO</td>
</tr>
<tr>
<td>535.2484</td>
<td>564.2518</td>
<td>2</td>
<td>58.0068</td>
<td>1.57</td>
<td>YES</td>
</tr>
<tr>
<td>477.7352</td>
<td>506.7379</td>
<td>2</td>
<td>58.0054</td>
<td>1.09</td>
<td>NO</td>
</tr>
<tr>
<td>811.8932</td>
<td>840.8983</td>
<td>2</td>
<td>58.0102</td>
<td>1.12</td>
<td>YES</td>
</tr>
<tr>
<td>541.5995</td>
<td>560.9345</td>
<td>3</td>
<td>58.0050</td>
<td>0.50</td>
<td>YES</td>
</tr>
<tr>
<td>471.2552</td>
<td>500.2572</td>
<td>2</td>
<td>58.0040</td>
<td>1.42</td>
<td>NO</td>
</tr>
</tbody>
</table>
After manual inspection of these MS/MS spectra for this set of peptides, 13 pairs of peptides were found (Table 10) followed the fragmentation pattern of amidated peptides and precursors, including 9 amidated peptides reported by database search, triply charged $\alpha$-MSH and 3 more interesting pairs (pair No. 8, 10, and 13).

Shown in Figure 12 are MS/MS spectra for peptide pair No. 10. By looking for peaks with mass difference of 58 in two spectra, 4 pairs of y ion with $m/z$ value 458.44 and 400.30, 642.41 and 584.40, 771.46 and 713.23, and 868.66 and 810.51 were identified. These y ions are identical to $y_4$, $y_6$, $y_7$ and $y_8$ ions in the mJP peptide pair (Figure 11), suggesting this pair of peptides are also mJP related peptides. Based on the size of parent ions, the sequences were likely to be GSPEPSPREG and GSPEPSPRE-NH$_2$. Although the complete sequences could not be deduced from the fragments, accurate mass measurements (-0.0013 (2.56 ppm) for GSPEPSPREG and -0.0009 (1.88 ppm) for GSPEPSPRE-NH$_2$) as well as assigned b ions indicated that these were the correct sequences. Similarly, another pair of peptides was identified as GDGSPEPSPREG and GDGSPEPSPRE-NH$_2$ (Figure 13).
Figure 12. Identification of peptide pair No. 10.
Top: MS/MS spectrum of GSPEPSPREG; Bottom: MS/MS spectrum of GSPEPSPRE-NH$_2$. Two spectra have y ions with 58 mass difference marked with blue lines, and b ions with same m/z value marked with red arrows.
Figure 13. Identification of peptide pair No. 8.
Top: MS/MS spectrum of GDGSPESPREG; Bottom: MS/MS spectrum of GDGSPESPREP-NH₂. Two spectra have y ions with 58 mass difference marked with blue lines, and b ions with same \( m/z \) value marked with red arrows.
Spectra of the peptide pair No. 13 were shown in Figure 14. Peaks in two spectra with same m/z value (tentative b ions) were marked with red arrows. Peaks with 58 mass difference (tentative y ions) were marked with blue lines. Through these peaks, partial sequence of this peptide was deduced to be EWR or EGER (Table 11). There was a constant difference of 18 between parent ion mass and the addition of tentative b ions and their corresponding y ions. For example, the addition of a tentative b ion 225.04 and its corresponding y ion 757.53 is 982.57 (with 2 positive charges), 18 less than observed parent ion mass 1000.51 (with 2 positive charges). This indicated water loss in either b ions or y ions. Fragment ions with +18 amu were found for 4 out of 5 tentative b ions while only 1 out 5 were found for tentative y ions, suggesting that water lose was from an amino acid residue at or close to N-terminus and that marked b ions in Figure 14 were actually b-H₂O ions. With that in mind, fragment ions 243.05 and 286.31 were used to deduce the rest sequences at N-terminus and C-terminus, which turned out to be (L/I E) or (Q/K N) for N- and (L/I P )G for C-terminus.

<table>
<thead>
<tr>
<th>b-18</th>
<th>b</th>
<th>Amino acid residues</th>
<th>y</th>
</tr>
</thead>
<tbody>
<tr>
<td>225.04</td>
<td>243.05</td>
<td>(L/I E) or (Q/K N)</td>
<td></td>
</tr>
<tr>
<td>353.99</td>
<td>372.24</td>
<td>E</td>
<td>757.53</td>
</tr>
<tr>
<td>410.92</td>
<td></td>
<td>G</td>
<td>W</td>
</tr>
<tr>
<td>540.04</td>
<td>558.35</td>
<td>E</td>
<td>571.39</td>
</tr>
<tr>
<td>696.43</td>
<td>714.43</td>
<td>R</td>
<td>442.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(L/I P )G</td>
<td>286.31</td>
</tr>
</tbody>
</table>
Figure 14. Spectra of peptide pair No. 13.
Spectral pair found by the pair finding method. Two spectra have peaks with 58 mass difference (tentative y ions, marked with blue lines) and peaks with same m/z value (tentative b ions, marked with red arrows).
All possible sequences for the tentative glycine-extended peptide in pair No. 13 are shown in Table 12, together with corresponding delta m/z and ppm values. Considering the mass measurement accuracy of Orbitrap mass analyzer, the actual sequence of this peptide is most likely to be (QN)EWR(L/I P)G or (L/I E)EGER(L/I P)G. All 24 permutations for these two combinations were searched against mouse non-redundant protein sequences (NR) database using Basic Local Alignment Search Tool (BLAST) (Parameters: blastp, expect=20000, PAM30, word size 2). Exact match was found for sequence ELEGERPLG. A BLAST search against mouse translated nucleotide database (Parameters: tblastn, expect=100000, no filter, PAM30, word size 2) yielded one exact match for sequence QNEWRI PG.

Table 12. Possible sequences for the tentative glycine-extended peptide in pair No. 13.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>delta m/z</th>
<th>ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(QN)EWR(L/I P)G</td>
<td>0.0032</td>
<td>6.3967</td>
</tr>
<tr>
<td>(L/I E)EWR(L/I P)G</td>
<td>-0.0093</td>
<td>-18.5904</td>
</tr>
<tr>
<td>(KN)EWR(L/I P)G</td>
<td>-0.0150</td>
<td>-29.9846</td>
</tr>
<tr>
<td>(L/I E)EGER(L/I P)G</td>
<td>-0.0020</td>
<td>-3.9979</td>
</tr>
<tr>
<td>(KN)EGER(L/I P)G</td>
<td>-0.0073</td>
<td>-14.5925</td>
</tr>
<tr>
<td>(QN)EGER(L/I P)G</td>
<td>0.0109</td>
<td>21.7888</td>
</tr>
</tbody>
</table>
Peptides QNEWRIPG and ELEG ERPL-NH₂ were synthesized and subjected to LC-ESI-LTQ-Orbitrap analysis. Retention time of the two synthetic peptides and the unknown are shown in XICs in Figure 15. Peptide ELEG ERPL-NH₂ showed a similar retention time to that of the unknown. The MS/MS spectrum of peptide ELEG ERPL-NH₂ as shown in Figure 16 almost exactly matched that of the unknown (the bottom spectrum in Figure 14). Therefore, the peptide sequences in this pair were confirmed as ELEG ERPLG and ELEG ERPL-NH₂.

Figure 15. XICs showing retention time of two synthetic peptides and the unknown.
The unknown refers to the peptide with \( m/z \) value 471.2552 in pair No. 13, a tentative \( \alpha \)-amidated peptide.
Figure 16. MS/MS spectra of synthetic peptides QNEWRIPG and ELEGERPL-NH$_2$.
In summary, a total of 13 peptide pairs were identified using pair finding method as listed in Table 13. Twelve of the 13 α-amidated peptides were identified in AtT-20 cells, including all 9 peptides reported by database search and 3 additional.

### Table 13. α–Amidated peptides identified by pair finding method from LC-Orbitrap data set.

<table>
<thead>
<tr>
<th>Obs. m/z</th>
<th>Charge state</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 970.9304</td>
<td>2</td>
<td>(R)AEEEAVWGDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>2 935.4109</td>
<td>2</td>
<td>(A)EEEAVWGDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>3 871.8935</td>
<td>2</td>
<td>(E)EEEAVWGDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>4 806.3690</td>
<td>2</td>
<td>(E)EAVWGDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>5 741.8502</td>
<td>2</td>
<td>(E)AVWGDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>6 706.3295</td>
<td>2</td>
<td>(A)VWGDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>7 656.8019</td>
<td>2</td>
<td>(V)WGDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>8 563.7588</td>
<td>2</td>
<td>(W)GDGSPEPSPRe(G)</td>
</tr>
<tr>
<td>9 535.2484</td>
<td>2</td>
<td>(G)DGSPSPRe(G)</td>
</tr>
<tr>
<td>10 477.7352</td>
<td>2</td>
<td>(D)DGPEPSPRe(G)</td>
</tr>
<tr>
<td>11 811.8932</td>
<td>2</td>
<td>(R)SYSMEHFRWGKPv(G)</td>
</tr>
<tr>
<td>12 541.5995</td>
<td>3</td>
<td>(R)SYSMEHFRWGKPv(G)</td>
</tr>
<tr>
<td>13 471.2552</td>
<td>2</td>
<td>(R)ELEGERPPl(G)</td>
</tr>
</tbody>
</table>
2.3.4 Complementary peptide extraction methods.

Acidic solvents are commonly used to extract endogenous peptides from biological sources. Properly designed acidic extraction solution can minimize protease activity and increase solubilization of peptides (Bennett et al. 1981). Acidic extraction is often used in combination with heating or boiling to liberate peptides (Conlon 2007, Nylander et al. 1997). However, it has been suggested that the hot acidic extraction conditions may result in protein or peptide degradation, the products of which will obscure endogenous peptide identification (Che et al. 2007). To limit possible degradations, acidic extractions in this study were all carried out at 4 ºC.

Urea (8 M) was also used as a complementary extraction solvent to produce a more comprehensive and less biased total endogenous peptide extract. Urea in aqueous solution will form ammonium cyanate through isomeric transformation over time. The isomerization is spontaneous at high temperature and very slow at low temperature (Dirnhuber and Schutz 1948). Cyanate is highly reactive and will readily, nonspecifically, and irreversibly carbamylate free amine groups. To avoid this unwanted modification of endogenous peptides, the urea solution was prepared freshly just before use and peptide extraction was carried out at 4 ºC.
Table 14. Comparison of identified α-amidated peptides using two extraction solutions.

<table>
<thead>
<tr>
<th>Obs. m/z</th>
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<th>Sequence</th>
<th>Acid ex.</th>
<th>Urea ex.</th>
</tr>
</thead>
<tbody>
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<td>Yes</td>
</tr>
<tr>
<td>935.4109</td>
<td>2</td>
<td>(A)EEEAVWGDGSPEPSPRe(G)</td>
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<td>Yes</td>
</tr>
<tr>
<td>871.8935</td>
<td>2</td>
<td>(E)EEAVWGDGSPEPSPRe(G)</td>
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<td>Yes</td>
</tr>
<tr>
<td>806.3690</td>
<td>2</td>
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<td>Yes</td>
</tr>
<tr>
<td>741.8502</td>
<td>2</td>
<td>(E)AVWGDGSPEPSPRe(G)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>706.3295</td>
<td>2</td>
<td>(A)VWGDGSPEPSPRe(G)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>656.8019</td>
<td>2</td>
<td>(V) WGDGSPEPSPRe(G)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>563.7588</td>
<td>2</td>
<td>(W)GDGSPEPSPRe(G)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>535.2484</td>
<td>2</td>
<td>(G)DGSPEPSPRe(G)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>477.7352</td>
<td>2</td>
<td>(D)GSPEPSPRe(G)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>811.8932</td>
<td>2</td>
<td>(R)SYSMEHFRWGKPv(G)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>541.5995</td>
<td>3</td>
<td>(R)SYSMEHFRWGKPv(G)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>471.2552</td>
<td>2</td>
<td>(R)ELEGERPl(G)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Acid extract solution contains 0.1M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) TFA (Bennett et al. 1981). The 8 M urea solution was prepared fresh before use. All extractions were carried out at 4 °C to minimize degradation and unwanted reactions.

Shown in Table 14 are the α-amidated peptides identified using two solvent systems.

Most of the peptides were found in both extracts. However, the two extraction systems do show complementary results for less abundant peptides: GDGSPEPSPRE-NH₂ was found in urea extract only while DGSPEPSPRE-NH₂ and GSPEPSPRE-NH₂ were only found in the acidic extract.
2.4 Discussion

2.4.1 Identification of endogenous peptides with PTM.

2.4.1.1 Limits of modern database search based endogenous peptide identification.

Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) has emerged as a powerful tool for the identification of endogenous peptides from biological samples. The standard MS/MS data processing procedure is using MASCOT, SEQUEST or other database search engine(s) to compare theoretical spectra generated from database sequences with those obtained experimentally. Despite of the success this approach (as demonstrated in this thesis), problems remain. Endogenous peptides are usually produced from their precursors by a series of chemical modifications catalyzed by different enzymes (Steiner 1998). The specificity for some of the enzymes is currently unknown. Consequently, processing sites of endogenous peptides are variable. Therefore, “no enzyme” has to be used when generating the theoretical spectra from database sequences, which means all possible subsequences of each protein have to be tested. For a protein of N amino acid residues, there are N(N+1)/2 subsequences. Moreover, most endogenous peptides undergo extensive PTMs such as C-terminal amidation, phosphorylation, acetylation and sulfation (Hummon et al. 2006). Operators can specify
some expected PTMs as “variable modifications” in most database search engines before the search. However, database searches take much longer when multiple variable PTMs are chosen because the program has to consider all peptide sequences without PTMs and with PTMs of different combinations. If M variable PTMs are selected, $2^M \frac{N(N+1)}{2}$ subsequences must be tested for a protein of N amino acid residues. Considering the fact that a modern LC-MS/MS instrument generates tens of thousands MS/MS spectra per hour, database searching takes much longer than collecting the spectra and, thus, becomes a bottleneck for endogenous peptide identification. To alleviate this problem, a database specially designed for endogenous peptide identification, SwePep, was constructed (Falth et al. 2006, Falth et al. 2008) which contains known endogenous peptides from different species. To use this database, the masses of experimental peptides will be compared with masses of peptides stored in the database. The identities of matched peptides are then verified by comparing experimental MS/MS spectra with stored spectra. Searching against this database for endogenous peptides can be dramatically increased in contrast to conventional databases. However, this method is good for re-identification of known peptides only and cannot be used for discovery of novel peptides, which are probably not present in the database.
The other problem with database search based peptide identification is the low identification rate. Only 5% to 10% of the MS/MS spectra can be correctly interpreted in a typical LC-MS/MS assay (Keller et al. 2002). A majority of the MS/MS spectra receive a low score due to unknown modifications (Ye et al. 2010, Savitski et al. 2006), poor spectrum quality, (Mujezinovic et al. 2010), and/or mixture spectra (Chen, Drogaris and Bern 2010). On the other hand, a significant number of sequence assignments with high scores are false positives due to misinterpretation of PTMs, incorrect assignment of PTM sites, and incorrect use of isotopic peaks (Chen 2008). False positives due to misuse of isotopic peaks is especially substantial for α-amidated peptide identification. The mass of an α-amidated peptide is 0.984 Da smaller than its unmodified form (note: the one with exact sequence but free C-terminal carboxyl group, not glycine-extended precursor), very close to 1.008 Da which is the mass difference between isotopic peaks. If a higher isotopic peak of an α-amidated peptide was chosen for MS/MS instead of the monoisotopic peak, which is often seen in many cases, the search engine will very likely identify the peptide as a peptide with free C-terminus instead of C-terminal amide.

Shown in Figure 17 is an example of misinterpretation of an α-amidated peptide by database search resulting from the misuse of isotopic peaks. For a small dataset, manual interpretation is likely to identify more peptides of interest. However, manual
interpretation of large data sets containing thousands of spectra is unfeasible for the high throughput identification of potential α-amidated peptides.

Figure 17. A false positive peptide identification by database search due to misuse of isotopic peaks.
A: Assignment of an MS/MS spectrum with a doubly charged peptide VWGDGSPEPSPRE identified by Sequest with a probability of 95%. B: Delta mass errors of fragment ions with high error values for y ions due to misinterpretation of α−amidated C-terminus with free C-terminus. C: Isotopic distribution of the parent peptide. The second isotopic peak m/z 706.83 was used as parent ion instead of the monoisotopic peak m/z 706.33. As a result, the α-amidated peptide VWGDGSPEPSPRE-NH₂ was misinterpreted as an unmodified peptide VWGDGSPEPSPRE with a probability of 95%.
2.4.1.2 Peptide-pair strategy, the solution to the screening of endogenous α-amidated peptides.

Screening a large data set of LC-MS/MS spectra for putative α-amidated peptides can be accomplished using the peptide-pair strategy presented in this study. The essence of our peptide-pair strategy is to greatly reduce the number of spectra requiring interpretation. From the 15,580 MS/MS spectra obtained from the LTQ-Orbitrap experiment, the pair screening method yielded 33 pairs of interesting spectra, a decrease in computing time of 99.6% for the database search. The significant reduction in number of spectra to be interpreted made manual interpretation possible leading to the identification of novel α-amidated peptides. In this study, three more amidated peptides were identified through manual interpretation.

A few tools and methods have been developed to detect general peptide/protein PTMs based on spectral pair finding strategy, such as ModifiComb (Savitski et al. 2006), Mass Distance Fingerprint (Potthast et al. 2007), and more (Bandeira et al. 2006, Fu et al. 2009). These methods were designed for detection of general PTMs without prior assumption of their chemical composition and attachment sites, making them especially useful to detect and characterize novel or unexpected PTMs. However, these methods
only detect predominant and abundant modifications, because spectra of modified and unmodified peptides need to show up repeatedly to be found. Also, these methods work under the assumption that both modified and unmodified peptides are present in the sample, which is not necessarily true in the experimental samples, especially for irreversible PTMs. Different from these approaches, the method presented in this thesis was designed to screen for peptides with known modification, based on a well-defined biosynthetic pathway. By combining the peptides from both a PAM inhibited sample and the control sample, the coexistence of modified peptides and their precursors were guaranteed. Furthermore, since the mass difference was specified when scanning for peptide pairs, this method is sufficiently sensitive to identify peptides that only have one spectrum, like the novel α-amidated peptide ELEGERPL-NH$_2$ identified in LTQ-Orbitrap dataset.

2.4.2 Mass measurement accuracy and false positives.

Mass measurement accuracy plays a key role in this peptide-pair screening method. When MALDI-TOF/TOF and external calibration were used, a wide window of ±0.1 Da was required to screen for peptide pairs due to the relatively low mass measurement accuracy (Table 5). This screening window yielded a list of over 300 peptide pairs, most of which were false positives. To remove these false positives, samples from PAM
inhibited group and control group had to be analyzed separately to obtain peptide intensities in two groups. With this information, an accumulation filter was applied to remove peptides which did not accumulate in PAM inhibitor treated group.

For LC-MS/MS, Orbitrap is a very accurate mass analyzer; experimental trials using digested BSA between each assay showed errors in the measurement of mass of less than 3 ppm. Consequently, a window significantly smaller was used to screen for peptide pairs. This narrow window successfully decreased false positive rate to an acceptable level and made the accumulation filter unnecessary. Therefore, peptide samples from two groups could be combined and processed together to decrease the effort by half.

With the help of high mass measurement accuracy and accumulation information, false positive rate was confined to a relatively low level using mass difference and retention time as screening conditions. The third restriction, the MS/MS spectra similarity check, eliminated most false positives. However, in some rare cases, two peptides might have very similar sequences and only differ from each other by a few residues. If the mass difference between the distinct parts in two peptides happened to correspond closely to 58.005, the two peptides would meet all three screening conditions and, therefore, would result in a false positive. An extreme situation is two peptides with identical sequences except one having GVT as C-terminus and the other one having KA as
C-terminus. In this case, the two peptides would show similar retention times and would have a mass difference of 58.0055, exactly matches the difference between an α-amidated peptide and its glycine-extended precursor. Moreover, since the N-terminal sequences of the two peptide are the same, they would generate MS/MS spectra that appear like an α-amidated peptide and its glycine-extended precursor, *i.e.* MS/MS spectra with same fragment ions and ions with a 58 mass difference.

Shown in Figure 18 are the MS/MS spectra of a false positive pair found in peptide extract from the AtT-20 cells. The two triply charged peptides demonstrated an observed mass difference of 19.3385 which is in the range of an α-amidated peptide and its glycine-extended precursor. They also showed similar retention times with a difference of only 1.2 minutes. At least 7 related ion pairs (with same mass or with 58 mass difference) in MS/MS spectra were found as marked in Figure 18. However, sequences for these two peptides turned out to be PEPSRSTPAPKKGSKK (Figure 41) and PEPSKSAPAPKKGSKK (Figure 42). The two peptides have very similar sequences except two residues: R, T in one and K, A in the other. Mass difference between the two combinations happens to be 58 amu.

Although this situation happens infrequently, it must be considered when spectra of a peptide pair could not be interpreted with the assumption one being α-amidated peptide
and the other being its glycine-extended precursor. To check if a peptide pair is a false positive, one can compare the relative concentration of the two peptides in samples from PAM inhibited and control group to see if there is any significant difference. The other option is to incubate the sample with PAM to see if the relative amount of the larger peptide decreases. The relative concentrations of two peptides in a false positive pair would not show any relation to PAM activities.
Figure 18. MS/MS spectra for a false positive peptide pair. The two sequences were identified to be PEPSRSTPAPKKGSKK (Figure 41) and PEPSKSAPAPKKGSKK (Figure 42).
2.4.3 α-Amidated peptides identified.

2.4.3.1 Mouse joining peptide related peptides.

Joining peptide is one of the peptides generated from prohormone pro-opiomelanocortin (POMC), which is abundant in pituitary, gut, adrenal gland, and bronchial carcinoids (Bjartell et al. 1990, Cullen and Mains 1987, Fenger 1991). Most peptides derived from POMC are hormones, such as corticotropin, melanotropin, lipotropin, and endorphin (Table 15). Although mouse joining peptide has the typical characteristics of peptide hormones, i.e. C-terminal amidation and flanked with dibasic processing sites, it is the only POMC-derived peptide without a conclusive bioactivity. In UniProtKB/Swiss-Prot database, this peptide is denoted as a “propeptide”.

Efforts have been made to identify the function of joining peptide. In 1992, bovine joining peptide was found to weakly inhibit the sodium pump (IC$_{50}$ = 0.5mM) (Hamakubo et al. 1992). Dose dependent central cardiovascular effects of this peptide were later observed on genetically hypertensive rats (Hamakubo et al. 1993). In 1994, rat joining peptide was also reported with central cardiovascular effects and these effects were mediated by angiotensin II (Yoshida, Hamakubo and Inagami 1994). However,
none of these reported activities for joining peptide have been corroborated by other researchers.

<table>
<thead>
<tr>
<th>Position</th>
<th>Length</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 26</td>
<td>26</td>
<td>Signal peptide</td>
</tr>
<tr>
<td>27 – 100</td>
<td>74</td>
<td>NPP</td>
</tr>
<tr>
<td>77 – 87</td>
<td>11</td>
<td>Melanotropin gamma</td>
</tr>
<tr>
<td>103 – 121</td>
<td>19</td>
<td>Propeptide (Joining peptide)</td>
</tr>
<tr>
<td>124 – 162</td>
<td>39</td>
<td>Corticotropin</td>
</tr>
<tr>
<td>124 – 136</td>
<td>13</td>
<td>Melanotropin alpha</td>
</tr>
<tr>
<td>142 – 162</td>
<td>21</td>
<td>Corticotropin-like intermediary peptide</td>
</tr>
<tr>
<td>165 – 235</td>
<td>71</td>
<td>Lipotropin beta</td>
</tr>
<tr>
<td>165 – 202</td>
<td>38</td>
<td>Lipotropin gamma</td>
</tr>
<tr>
<td>185 – 202</td>
<td>18</td>
<td>Melanotropin beta</td>
</tr>
<tr>
<td>205 – 235</td>
<td>31</td>
<td>Beta-endorphin</td>
</tr>
<tr>
<td>205 – 209</td>
<td>5</td>
<td>Met-enkephalin</td>
</tr>
</tbody>
</table>

As reported in this thesis, a series of mouse joining peptide related peptides with sequential N-terminal deletions were identified in mouse pituitary AtT-20 cells (Table 13). This suggests that mouse joining peptide is a prohormone and, thus, may not, in itself, have significant bioactivity. One or more of the mJP-derived C-terminal fragments could serve as bioactive peptide hormones. To test this hypothesis, mJP, mJP-Gly and one of mJP C-terminal fragments GDGSPEPSPRE-NH$_2$ (mJP-F7) were synthesized and their inhibition activities on porcine Na/K-ATPase were examined. As shown in Figure 19, the glycine extended precursor mJP-Gly showed little inhibition of the ATPase. In
contrast, while mature, $\alpha$-amidated mJP showed about 20% inhibition, which hinted a key role of C-terminal amide for this inhibition activity. Moreover, the fragment peptide mJP-F7 demonstrated stronger inhibition activity than mJP. This suggested N-terminal deletion of mJP may lead to a peptide of greater bioactivity.

**Figure 19. Inhibition effects of three mJP related peptides on Na/K ATPase.** Experiments were performed as indicated in the Material and Method section with the presence of 0.46 mM of the peptide to be investigated. The enzyme activities were converted to relative activities to the control reaction, i.e. reactions with no peptides presented.

Rat joining peptide was reported to be able to increase blood pressure and heart rate by increasing the level of angiotensin (ANG) II (Yoshida *et al.* 1994). ANG II is the most
active form of the angiotensins which is converted from inactive ANG I (DRVYIHPFHL)
by removal of two C-terminal residues, a reaction catalyzed by angiotensin converting
enzyme (ACE). Activation effects of mJP on ACE were tested with two different
concentrations as shown in Figure 20. mJP at 175 µM showed no significant effect on
ACE activity while 350 µM mJP inhibited ACS slightly, 2.9%. These data suggest that the
up-regulation of ANG II by mJP is not through ACE activation. Another possible
mechanism is that the inhibition of sodium pump by mJP decreases the plasma sodium
level which in turn activates renin secretion (Barrett et al. 1989). Renin is the enzyme
which catalyzes the cleavage of angiotensinogen to produce ANG I. Increased renin levels
would produce more ANG I and, thus, more ANG II.

Further screening studies on mJP and its C-terminal fragments are required to validate
the biofunction of this abundant and yet uncharacterized endogenous peptide.
Figure 20. Effects of mJP on ACE activity.
Experiments were performed as indicated in the Material and Method section with the presence of mJP. The enzyme activities were converted to relative activities to the control reaction, i.e. reactions with no mJP presented.

2.4.3.2 β-Lipotropin related peptide.

β-Lipotropin (β-LPH) is another peptide hormone derived from POMC. β-LPH performs lipid-mobilizing functions (Keda Yu and Pankov Yu 1987, Richter, Jacob and Schwandt 1990) and stimulates adrenal steroid production (Oconnell, McKenna and Cunningham 1996, Oconnell, McKenna and Cunningham 1993). It was also found to be correlated with severity of postoperative pain (Matejec et al. 2006a, Matejec et al. 2006b). Shown in Figure 21 is the sequence of mouse POMC 165-235 fragment with β-LPH being marked with the underscore. The N-terminal fragment of β-LPH 165-202,
marked with bold font, is another peptide hormone $\gamma$-lipotropin. An even shorter N-terminal fragment 165-172, marked with red bold font, was discovered in both mouse pituitary tumor cell line and mouse pituitary tissue with C-terminal amidation which is highly suggestive of biological function. This $\alpha$-amidated endogenous peptide has never been reported.

**Figure 21. Sequence of mouse pro-opiomelanocortin.**
Fragment 165-235 (marked with underlined text) is $\beta$-lipotropin. Fragment 165-202 (marked with bold font) $\gamma$-lipotropin. Fragment 165-172 (marked with red bold font) is a novel $\alpha$-amidated peptide discovered in both mouse pituitary tumor cell line and mouse pituitary tissue.

### 2.4.3.3 Chromogranin A related peptide.

Chromogranin A (CgA) is an acidic protein with widespread distribution in secretory cells of the nervous, endocrine and immune system. It has been suggested that one function of this protein is to serve as a prohormone for many bioactive peptides including vasostatins, chromofungin, pancreastatin, catestatin and parastatin (Helle *et al*. 2007, Iacangelo and Eiden 1995). The amidated C-terminal fragment of this protein was first found in 2006 in a rat insulinoma cell line Rin-m5F (Taylor *et al*. 2006). The same
peptide was identified in mouse AtT-20 cells, as reported here (Table 7). Similar α-amidated peptides GR-44 and ER-37 were also found in a human insulinoma tumor extract (Orr et al. 1998, Orr et al. 2002). The C-terminal domain of CgA containing these α-amidated peptides is highly conserved between vertebrate species (Figure 22C). The high degree conservation, basic cleavage sites flanking these peptides, and the presence of C-terminal amidation suggests that these peptides have undiscovered biological functions.
Figure 22. Sequences of chromogranin A of several species.
A: sequence of rat chromogranin A (CgA) with the identified α-amidated peptide marked in red.
B: sequence of human CgA with the identified α-amidated peptide marked in red.
C: High degree conservation of CgA C-terminal region between vertebrate species.
2.5 Concluding remarks

A new strategy for the discovery and identification of α-amidated peptides is described in this chapter. This approach is based on the biosynthesis of peptide C-terminal amide and the high mass measurement accuracy. The coexistence of α-amidated peptides and their glycine-extended precursors was insured by the use of a PAM inhibitor. Peptides were scanned for pairs with mass difference of 58 Da and similar retention times. Interesting pairs were further validated by comparing their fragmentation patterns in MS/MS spectra. This method is able to significantly reduce workload of spectra interpretation and increase the identification rate of amidated peptides. Number of spectra to be interpreted was decreased from 15580 to 66 in LTQ-Orbitrap dataset. Three α-amidated peptides were identified in addition to 9 peptides reported by database search from AtT-20 cell line (Table 13), representing a significant improvement in the detection of these modified sequences.

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modifications and increase spectral identification rate. *Bioinformatics*, 26, i399-i406.


Chapter 3 Enrichment of $\alpha$-Amidated Peptides

3.1 Introduction

3.1.1 PTM specific enrichment.

Traditional PTM identification methods include Edman degradation, amino acid analysis, radioactive isotope labeling, and immunochemistry approaches (Zhao and Jensen 2009). These methods are laborious and incapable of handling complex samples. Within the past two decades, mass spectrometry coupled with liquid chromatography has proven powerful for the study of PTMs due to its high sensitivity and its ability to map PTM sites, to discover novel PTMs, to handle complex samples, and to quantify the changes of PTMs at distinct sites (Larsen et al. 2006). Typically, MS based PTM identification involves four steps: digestion, enrichment, LC-MS/MS assay, and verification (Zhao and Jensen 2009). Digestion can be done before (affinity enrichment) or after (covalent capture enrichment) the enrichment step. For endogenous PTM peptide studies, digestion may or may not be necessary based on the sizes of peptides of interest. PTM-specific protein/peptide enrichment is an essential step because most PTMs are in low abundance. Without enrichment procedures, mass spectrometric analysis of PTM
peptides shows low efficiency despite advances in the sensitivity of mass spectrometers and the development of database searching algorithms (Zhao and Jensen 2009). Current PTM-specific enrichment methods are (a) antibody-based affinity method, (b) ionic interaction-based, (c) enzyme-based, or (d) chemical derivatization-based.

3.1.1.1 Antibody based affinity method.

A few monoclonal antibodies have been developed to target a PTM-of-interest, independent of the specific protein possessing the modification. These antibodies can be used in global PTM analysis. Successful examples include antibodies directed against phosphotyrosine (Mann et al. 2002, Rush et al. 2005), phosphoserine/threonine (Gronborg et al. 2002), acetyllysine antibody (Kim et al. 2006), and nitrotyrosine (Zhan and Desiderio 2006). Antibodies for PTMs which introduce large changes to peptides are relatively easy to develop, especially for those occurring only on certain amino acid residue(s). For PTMs introducing small changes and without specific sites, for example C-terminal amidation, represent a difficult challenge for the development of antibodies for global PTM analysis.

Lectins, sometimes referred to sugar-specific antibodies (Gabius et al. 2002), are carbohydrate binding proteins that recognize specific carbohydrate structures.
Lectin-based enrichment methods have been widely used in glycoprotein and glycopeptide studies (Budnik, Lee and Steen 2006, Dai et al. 2009).

3.1.1.2 Ionic interaction-based affinity method

Negatively charged phosphopeptides bind to immobilized metal ions such as Fe$^{3+}$ and Ga$^{3+}$, and can be eluted after the removal of non-specifically bound peptides. Utilizing this enrichment strategy, Ficarro et al. (Ficarro et al. 2002) identified over 1000 candidate phosphopeptides from *S. cerevisiae* whole cell lysate. Of these, 216 were manually validated and sites of phosphorylation were confirmed. This represents the most extensive list of confirmed phosphorylation sites determined to date. Taking advantage of charge differences between phosphopeptides and non-phosphopeptides, separation technologies such as strong anion exchange chromatography (SAX) and strong cation exchange chromatography (SCX) can be used prior to the enrichment procedure to prefractionate samples and decrease their complexity.

3.1.1.3 Enzyme-based affinity method.

Enzymes are attractive reagents to enrich PTM peptides due to their high specificity. Some enzymes can be deactivated by mutation of one or more amino acid residue(s) in active site, or by removal of metal ions without compromising ligand affinity or global
3-dimensional structure. Such deactivated enzymes can be immobilized and used to enrich their substrate peptides from a complex mixture.

Trypsin is a protease which cleaves peptides at the C-terminal side of lysine (Lys) and arginine (Arg). Conversion of Ser-195 in the trypsin active site to dehydroalanine yields a catalytically inert derivative of trypsin, called anhydrotrypsin, which can still bind Lys/Arg containing peptide substrates (Ishii et al. 1983). As discussed in Chapter 1.1.2, endogenous peptides are synthesized as inactive prohormones and then cleaved by prohormone convertases (Table 2) usually on the C-terminal side of basic amino acid residues (Lys or Arg). Basic amino acid residues at C-termini of the peptides are then removed by carboxypeptidase E (CPE). Che et al. utilized $Cpe^{fat}/Cpe^{fat}$ mice which produce mutated CPE to get accumulation of neuroendocrine peptides with C-terminal basic amino acid residues. These peptides were then enriched and isolated using an anhydrotrypsin affinity column (Che et al. 2001).

3.1.1.4 Chemical derivatization based method.

Chemical approaches have been developed to attach an affinity linker to the PTMs. The affinity linker is used in the subsequent enrichment procedure. A good example is
introducing biotin to PTMs, which can be used to "tag" and isolate PTM peptides from a mixture using avidin conjugated beads.

Tagging PTMs can be carried out chemically in vitro. In protein phosphorylation studies, the biotin tag can be introduced by β-elimination of phosphoric acid followed by Michael addition (Oda, Nagasu and Chait 2001, Goshe et al. 2001). Also with β-elimination and Michael addition, dithiothreitol (DTT) or biotin pentylamine was attached to sites modified by O-GlcNAc (Wells et al. 2002). A similar approach was applied to the study of S-nitrosylated proteins (Jaffrey et al. 2001), where incorporation of biotin to nitrosylated-cysteine serves both stabilization and enrichment purposes. While useful, in vitro chemical derivatization of PTM suffers from various drawbacks such as sample loss, inefficiencies in the chemical reaction, and unwanted side products. Thus, procedures with multiple reactions should be avoided to minimize sample losses.

PTMs can also be tagged in vivo through metabolic labeling. In studies of S-glutathionylation, glutathione (GSH, the tripeptide, γ-Glu-Cys-Gly) (Huang et al. 2008, Velu et al. 2007), as well as its oxidized form glutathione disulfide (GSSG) (Brennan et al. 2006), and ethyl ester (Sullivan et al. 2000) can be easily biotinylated. Incubation of cell lines with these membrane permeable, biotinylated GSH-based modifiers under
oxidative stress induced their incorporation at S-glutathionylation sites. The biotin moiety of these modifiers was then used to enrich for S-glutathionylated proteins.

3.1.2 α-Amidated peptide enrichment.

No enrichment method specific to α-amidation has been reported thus far. Antibodies that can globally differentiate between peptides with C-terminal amide from peptides with a C-terminal carboxylate have never been successfully developed. Although it has been reported that the C-terminal amide can be converted to amine using the Hoffman rearrangement (Hill et al. 1993), which could be used as chemical derivatization site for tagging and enrichment purposes, preliminary studies were unsuccessful due to many side reactions on peptide side chains (data not shown).

PHM is a copper dependent enzyme that catalyzes the first step of two-step peptide amidation reaction. Removal of the copper ions results in the loss of enzyme catalytic activity (Bell et al. 1997). Therefore, when incubating copper depleted PHM (apo-PHM) with a peptide mixture, glycine-extended peptide substrates should bind and remain enzyme-bound until activity is restored by addition of copper. All peptides that are not bound to apo-PHM could be removed using separation techniques like ultrafiltration or size exclusion filtration. Thus, apo-PHM could emerge as a powerful tool to enrich for
α-amidated peptide precursors. This chapter shows enrichment experiments on several synthetic model peptides. Peptide extracts from cells grown in the presence of PHM inhibitor to accumulated glycine-extended precursors peptides were be used to test the ability of the newly developed enrichment method to handle complex samples.

3.2 Material and Methods

3.2.1 Materials.

Apo-PHM (rat PHMcc residues 42–356 expressed in CHO DG44 cell line) was a generous gift from Dr. N. J. Blackburn (Department of Biochemistry and Molecular Biology, OGI School of Science and Engineering, Oregon Health and Science University, OR, USA). Dansyl-Ala-Arg, Ac-RFMWMK-NH$_2$ and ELPLQNFWLCFR-NH$_2$ were from Bachem (King of Prussia, PA, USA). Dansyl-Tyr-Val-Gly and disulfiram were from Fluka. The mouse pituitary AtT-20 cell line and basal F-12K medium (Kaighn's Modification of Ham's F-12 Medium) were purchased from American Type Culture Collection. Fetal bovine serum was from Atlanta Biologicals. Donor equine serum was from Thermo Scientific. CHCA (α-cyano-4-hydroxycinnamic acid) was from Sigma-Aldrich. Pen-Strep was purchased from Omega Scientific. The glycine-extended precursor of mouse joining peptide (mJP-Gly) was synthesized in-house at the USF Core
Peptide Synthesis and Mass Spectrometry Facility. Sep-Pak Plus C\textsubscript{18} cartridges were from Waters Associates. ZipTips were from Millipore. All other reagents and solvents were of the highest quality commercially available.

3.2.2 \textit{α-Amidated peptide enrichment.}

3.2.2.1 \textit{Preparation of apo-PHM by copper depletion.}

PHM (0.46 mg) was incubated with 200 \(\mu\text{L}\) of 100 mM MES, pH 6.0, 30 mM NaCl, 1\% (v/v) ethanol and 10 mM EDTA at 37 °C for 1 hour. The solution was then transferred to 10 kDa molecular weight cut-off filter (microcon YM-10, Millipore, Bedford, MA, USA) and spun down at 14,000 g to 50 \(\mu\text{L}\).

3.2.2.2 \textit{Incubation with peptide mixture.}

A 150 \(\mu\text{L}\) aliquot of 100 mM MES, pH 6.0, 30 mM NaCl, 1\% (v/v) ethanol and 10 mM EDTA was added to the retentate together with 30 \(\mu\text{L}\) of substrate solution, 15 \(\mu\text{L}\) of 100 mM ascorbate, 3 \(\mu\text{L}\) of 5.75 mg/mL catalase and 2 \(\mu\text{L}\) water to make the final volume 250 \(\mu\text{L}\). The mixture was incubated at 37 °C for 1 hour.
3.2.2.3 Wash.

The volume of the mixture was reduced to 20 µL by ultrafiltration followed by the addition of 180 µL of 100 mM MES, pH 6.0, 30 mM NaCl, 1% (v/v) ethanol. The volume was again reduced to 20 µL by ultrafiltration. For enrichment experiments using the AtT-20 peptide extract, the wash procedure was repeated once.

3.2.2.4 PHM reaction.

PHM reaction buffer (80 µL) containing 100 mM MES, pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 25 µM CuSO4, 100 µg/mL catalase and 10 mM ascorbate was added to 20 µL retentate. After 1 hour incubation at 37 ºC, the mixture was filtered using 10 kDa molecular weight cut-off filter. The pH of the filtrate was adjusted to pH 10 using 2 M KOH solution to convert the α-hydroxyglycine intermediate to the α-amidated product.

3.2.3 Quantitative analysis methods.

3.2.3.1 HPLC-fluorometric assay.

Two dansylated peptides were used for the enrichment experiments: dansyl-Tyr-Val-Gly (dansyl-YVG) and dansyl-Ala-Arg (dansyl-AR). Peptide mixtures were analyzed with reverse-phase HPLC with a C₁₈ column (Hypersil ODS, 4.6 × 100 mm, 5
µm, Keystone Scientific, Inc., Bellefonte, PA) equipped with an in-line fluorometer (Gilson 121, Gilson Instruments, Randolph, MA). The 8-minute gradient was programmed as: 30% B for 3 minutes, 30-40% B in 1 minute and 40% B for 4 minutes, with solvent A being 100 mM sodium acetate and solvent B being 0.1% TFA in ACN. The flow rate was 1.2 mL/min. The column was equilibrated with 30% B for 5 minutes between runs. The fluorometer was equipped with an excitation filter of 352-360 nm and an emission cut-off filter of 482 nm. The chromatograph peak area was calculated using a Hewlett-Packard integrator (HP-3392A, Hewlett-Packard, Wilmington, DE, USA).

3.2.3.2 Relative Quantification by MALDI-TOF/TOF.

Peptide solutions were mixed with α-cyano-4-hydroxycinnamic acid (4 mg/mL in 50% (v/v) ACN and 5% (v/v) isopropanol) and spotted onto a blank stainless MALDI plate. MALDI-TOF MS was performed on an Applied Biosystems (ABI) 4700 Proteomics Analyzer (Foster City, CA, USA) using the reflective positive mode. The data was externally calibrated with a standard peptide mix provided by the manufacturer. The peptides were quantified by their peak areas obtained from MS spectra using Data Explorer (Applied Biosystems, Foster City, CA, USA). The ratios of the peak areas for α-amidated peptides and the reference peptides were calculated manually.
3.2.3.3 Relative Quantification by LC-MS/MS (Extracted Ion Chromatograms).

The sample was separated online by reversed-phase nanoflow liquid chromatograph (U3000, Dionex, Sunnyvale, CA, USA) with mobile phase A being 2% ACN + 0.1% formic acid and solvent B being 90% ACN + 0.1% formic acid. The sample was first loaded onto a trap column (5mm × 300 µm ID packed with C_{18} reversed-phase resin, 5 µm, 100 Å), washed for 8 minutes with aqueous 2% ACN and 0.04% TFA, and then separated with a flow rate of 300 nL/min on an analytical column (C_{18}, 150 mm × 75 µm ID, Pepmap 100, Dionex, Sunnyvale, CA, USA) with a 120-minute gradient programmed as: 5% B for 8 minutes, 5-50% B in 90 minutes, 50-90% in 7 minutes, 90% B for 5 minutes, 90-5% in 1 minute and 5% B for 10 minutes to re-equilibrate. Eluted peptides were electrosprayed directly into a hybrid Linear Ion Trap-Orbitrap mass spectrometer (Thermo, San Jose, CA, USA) operating in data dependent mode. Survey scan MS spectra were acquired in the Orbitrap with a resolution of 60,000 at m/z 400. Following each survey scan, the five most intense ions were selected for CID fragmentation and detection in the linear ion trap with 60 second exclusion. Mass measurement accuracy was monitored between runs using digested BSA.

Relative quantification was done by comparing peak areas of extracted ion chromatograms (XICs) of the amidated peptides and the reference peptides. XICs were
generated from the MS spectra collected over time using the Qual Browser Software (Xcalibur, Thermo, San Jose, CA, USA). The peptide ratios of the α-amidated peptides and the reference peptides were calculated manually from the peak area of the extracted ions.

3.2.4 Cell culture and sample preparation.

3.2.4.1 Cell growth conditions.

Mouse pituitary AtT-20 cells were cultured in Hams F-12K culture medium supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal bovine serum. The cells were passed at a 1:3 ratio after 80% confluence. Cells were cultured in a 37ºC incubator with a constant flow of 5% CO₂. After sufficient growth, cells were collected by centrifugation and evenly split into two 225 cm² culture dishes containing 75 mL fresh media. A 75 µL aliquot of 5.0 mM disulfiram stock solution (prepared in 70% (v/v) ethanol) was added to one dish to get final disulfiram concentration of 5 µM. A 75 µL aliquot of vehicle (70% (v/v) ethanol) was added to the other dish as control group. The cells were incubated for 20 hours prior to harvest.
3.2.4.2 Sample preparation.

Cells were collected by centrifugation. Cell pellet was homogenized in a ground glass homogenizer at 4 °C with 8 M urea solution. The homogenate was centrifuged at 12000g for 15 minutes. The supernatant was collected and passed through a 10 kDa molecular weight cut-off filter to remove proteins.

The peptide extract was desalted and concentrated by solid phase extraction on a Sep-Pak Plus C\textsubscript{18} cartridge. A Sep-Pak Plus cartridge was pre-wetted with 10 mL of 0.1\% (v/v) TFA/80\% (v/v) ACN at a flow rate of 2.0 mL/min. The cartridge was then washed with 20 mL aqueous solution of 0.1\% (v/v) TFA/2\% (v/v) ACN at a flow rate of 2.0 mL/min. The peptide extract was loaded onto the cartridge at a rate of 1.0 mL/min, followed by a wash of 20 mL of 0.1\% TFA (v/v) /2\% (v/v) ACN at a flow rate of 1.0 mL/min. Bound peptides were eluted with 4 mL of 0.1\% (v/v) TFA/80\% ACN at a flow rate of 1 mL/min. The 4 mL eluent was collected and concentrated to 0.5 mL using a Savant Speedvac vacuum centrifuge. The peptide extract from disulfiram treated group was then subjected to α-amidated peptide enrichment experiments.
3.3 Results and discussion.

3.3.1 Enrichment experiments on dansylated short peptides.

3.3.1.1 Separation and standardization of three model peptides.

HPLC-fluorometric assay (Jones et al. 1988) was used to study α-amidated peptide enrichment with two dansylated model peptides: dansyl-Tyr-Val-Gly (dansyl-YVG) and dansyl-Ala-Arg (dansyl-AR). Peptide mixture was analyzed on reverse-phase HPLC equipped with a C$_{18}$ column and a fluorometer. Under the conditions described in methods section, dansyl-YVG, dansyl-AR and dansyl-YV-NH$_2$ were separated with good resolution. The retention times of the three peptides are listed in Table 16.

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dansyl-AR</td>
<td>1.461 min</td>
</tr>
<tr>
<td>Dansyl-YVG</td>
<td>2.326 min</td>
</tr>
<tr>
<td>Dansyl-YV-NH$_2$</td>
<td>6.400 min</td>
</tr>
</tbody>
</table>

Table 16. Retention times of three dansylated peptides.

Standard curves of dansyl-YVG and dansyl-AR (shown in Figure 23 and Figure 24) were established by injection of standard solutions to the HPLC system.
Figure 23. Standard curve for the detection of dansyl-YVG.

Figure 24. Standard curve for the detection of dansyl-AR.
3.3.1.2 Enrichment experiments.

Dansyl-YVG (6.71 nmol) and dansyl-AR (23.71 nmol) were incubated with 0.46 mg apo-PHM as described in methods section. After incubation, 1.04 nmol dansyl-YVG and 18.65 nmol dansyl-AR were found in the filtrate. The relative amount of dansyl-YVG significantly decreased (from 0.28:1 to 0.053:1) which indicated the specific binding to PHM. After washing, loaded apo-PHM was reactivated with copper. Bound dansyl-YVG was converted to dansyl-YV-NH$_2$ and released from PHM. Dansyl-YV-NH$_2$ (4.31 nmol) and 0.32 nmol dansyl-AR (0.32 nmol) were detected in the eluent solution. Relative amount of dansyl-YV-NH$_2$ was dramatically increased to 13.46:1. Compared to the initial ratio 0.28:1 between dansyl-YVG and dansyl-AR, the target peptide was enriched by 48-fold (Figure 25).
Figure 25.  α-Amidated peptide enrichment experiments on dansyl-YV-NH$_2$.
Two dansylated model peptides dansyl-Tyr-Val-Gly (d-YV-G) and dansyl-Ala-Arg (d-AR) were used as model peptides. Peptides were separated and detected using HPLC-fluorometric method as described in methods section. In the enriched sample, the ratio of d-YV-NH$_2$ to d-AR increased to 13.46:1. Compared to the initial ratio (0.28:1) of d-YVG and d-AR, target peptide was enriched by 48 folds.
3.3.2 Enrichment experiments on synthetic mJP.

mJP-Gly was also used as a model peptide in enrichment experiments together with two reference peptides Ac-RFMWMK-NH₂ and ELPLQNFWLCFR-NH₂. A solution containing 100 nmol Ac-RFMWMK-NH₂, 54 nmol ELPLQNFWLCFR-NH₂ and 7.6 nmol mJP-Gly was used as starting material for the enrichment experiments in which 0.46 mg (determined by Bradford assay) apo-PHM was used. mJP-Gly was converted to mJP during the enrichment experiments. The peak areas of the peptides before and after enrichment experiments obtained by MALDI-TOF mass spectrometry (Table 17). The relative amount of mJP to reference peptide Ac-RFMWMK-NH₂ was increased from 0.0076:1 to 0.932:1 with 123-fold enrichment. Using ELPLQNFWLCFR-NH₂ as reference peptide, the enrichment was 38-fold (from 0.0246:1 to 0.934:1). MS spectra are shown in Figure 27.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Obs. MW</th>
<th>Load</th>
<th>Enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-RFMWMK-NH₂</td>
<td>939.2</td>
<td>131103.2</td>
<td>97697.8</td>
</tr>
<tr>
<td>ELPLQNFWLCFR-NH₂</td>
<td>1494.4</td>
<td>40440.6</td>
<td>97432.2</td>
</tr>
<tr>
<td>AEEEAVWGDGSPEPSPREG</td>
<td>1998.3</td>
<td>994.8</td>
<td>3390.8</td>
</tr>
<tr>
<td>AEEEAVWGDGSPEPSPRE-NH₂</td>
<td>1940.3</td>
<td>0</td>
<td>91051.5</td>
</tr>
</tbody>
</table>

Table 17. Peak areas of peptides before and after enrichment experiments.
Figure 26. MS spectra of a peptide mixture before and after enrichment experiments.
A: MS spectra of the starting peptide mixture solution containing 100 nmol Ac-RFMWMK-NH₂ (Rf1), 54 nmol ELPLQNWLCFR-NH₂ (Rf2) and 7.6 nmol mJP-Gly. B: MS spectra of the peptide solution after α-amidated peptide enrichment experiments as described in methods section. The relative intensity of mJP was dramatically increased as compared to that of mJP-Gly in A. Peptides from PHM degradation were also detected.
3.3.3 Enrichment experiments on AtT-20 cell extract.

Total peptide was extracted from both the PAM inhibitor treated group and the control group of AtT-20 cells with 8 M urea solution. Peptide extract from PAM inhibited group was subjected to α-amidated peptide enrichment experiments as described in methods section. All three samples were analyzed by LC-ESI-LTQ-Orbitrap to investigate the enrichment of one known α-amidated peptide, mJP. Shown in Table 18 are XIC peak areas of mJP, mJP-Gly, and the four reference peptides. In the control sample, the intensity of one abundant reference peptide YGGFMTEKSLQPLVTLF was 36-fold higher than that of mJP as measured by XIC peak area. In the enriched sample, intensity of the reference peptide significantly dropped. The relative intensity of mJP was 40-fold higher than the reference peptide which indicated over 1000-fold enrichment (Figure 27). Two less abundant reference peptides SEKSLQPLVTLF and WSRMoxDQLAKELTAE were not detected in the enriched sample. However, it has been noticed that high efficiency enrichment is not always the case with different reference peptides. For example, using peptide EQVLESDAEKDDGPYRVEHF as reference peptide, enrichment of mJP was observed to be only around 4-fold. This indicated some unknown interactions between this peptide and PHM prevented them from being removed during wash runs.
Table 18. XIC peak areas of mJP and reference peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Control</th>
<th>PAM inhibited</th>
<th>Enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEEEAVWGDSPEPSPRE-NH₂ (mJP)</td>
<td>444480</td>
<td>130419</td>
<td>1447282</td>
</tr>
<tr>
<td>AEEEAVWGDSPEPSPREG (mJP-Gly)</td>
<td>961812</td>
<td>947420</td>
<td>54206</td>
</tr>
<tr>
<td>YGGFMTSEKSQTPVLVTBF</td>
<td>16260401</td>
<td>22224358</td>
<td>35157</td>
</tr>
<tr>
<td>SEKSTPVLVTBF</td>
<td>902722</td>
<td>1361254</td>
<td>nd</td>
</tr>
<tr>
<td>WSRMDQLAKELTAE</td>
<td>359606</td>
<td>223177</td>
<td>nd</td>
</tr>
<tr>
<td>EQVLESDAEKKDDGYPYRVEHF</td>
<td>554703</td>
<td>426516</td>
<td>379563</td>
</tr>
</tbody>
</table>

M denotes oxidation. nd denotes not detected.
Figure 27. XICs of mJP and YGGFMTSEKSQTPLVTLF in control (top) and enriched (bottom) samples.
3.4 Conclusion.

An α-amidated peptide enrichment method was described in this chapter. In summary, this method involves two steps. First, cells grown in culture are treated with PAM inhibitor to yield a cellular accumulation of glycine-extended peptides. It is essential that glycine-extended precursors present in the sample because these are the peptides bind to PHM and, thus, be enriched. The mature form of α-amidated peptides are likely to bind to PHM with relatively low affinity and are removed together with other non-binding peptides during the wash steps. In the second step, copper depleted apo-PHM is used to specifically bind glycine-extended peptides in the sample. Without bound copper, PHM is catalytically deficient, but still shows high selectivity. Glycine-extended peptides bind to apo-PHM while all other unbound peptides are removed during the wash steps. Activity is restored in apo-PHM with copper and is, thus, able to convert bound glycine-extended peptides to hydroxylated peptides, releasing them. The hydroxylated intermediate can be converted to α-amidated product under basic conditions.

In enrichment experiments on synthetic model peptides, 40 – 120-fold enrichment was observed using HPLC-fluorometric assay or MALDI-TOF relative quantification. This method has also been proven to work with complex samples like cell extracts. The
relative intensity of mJP in AtT-20 extract was dramatically increased after enrichment experiments.

3.5 References


*Proteomics.* 9, 4632-4641.
Chapter 4 Conclusions and Future Directions

4.1 Conclusions

In this study, a novel LC-MS/MS based strategy was developed for the discovery of \( \alpha \)-amidated peptides. This strategy includes two aspects: an \( \alpha \)-amidated peptide enrichment method to increase the relative concentration of target peptides in samples and a peptide-pair screening method to decrease the amount of MS/MS spectra to be interpreted.

An \( \alpha \)-amidated peptide correlates its glycine-extended precursor with 58.0055 Da mass difference, similar reverse phase retention times, and similar MS/MS fragmentation patterns. The glycine-extended precursor accumulates when PAM is inhibited. By screening for peptide pairs which meet these conditions in LC-MS/MS raw data, the peptide-pair screening method is able to greatly reduce the workload of spectra interpretation. From 15580 MS/MS spectra in an LTQ-Orbitrap dataset, the peptide-pair screening method identified 33 interesting pairs by using mass difference and retention time difference. The great reduction (over 99\%) in the amount of MS/MS spectra requiring interpretation enabled manual interpretation which in turn increased
identification rate and eliminated false positives. As a result, three α-amidated peptides were identified from the LTQ-Orbitrap dataset in addition to the nine reported by database search. The peptide-pair screening method relies on the coexistence of α-amidated peptides and their corresponding glycine-extended precursors which is insured by the use of a PAM inhibitor.

In the newly developed α-amidated peptide enrichment method, cells grown in culture were first treated with a PAM inhibitor to accumulate glycine-extended peptides. The peptide extract was then incubated with inactive, copper-depleted apo-PHM to specifically bind glycine-extended peptides. After the removal of unbound peptides by washing, PHM activity was restored by the addition of Cu$^{2+}$ solution to hydroxylate and release bound glycine-extended peptides. Experiments on synthetic model peptides showed 40 – 120-fold enrichment.

From the model cell line AtT-20, a total of 13 α-amidated peptides were successfully identified as listed in Table 19. Discussions about these peptides can be found in Chapter 2.
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Precursor</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEDQELESLSAIEAELEKVHAQLQALR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>CgA</td>
</tr>
<tr>
<td>AEEAVWGDGSPEPSPRE-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>POMC</td>
</tr>
<tr>
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<td>POMC</td>
</tr>
<tr>
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<td>POMC</td>
</tr>
<tr>
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<td>POMC</td>
</tr>
<tr>
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<td>POMC</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
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<td>POMC</td>
</tr>
<tr>
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<td>POMC</td>
</tr>
<tr>
<td>SYSMEHFRWGKPE-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>POMC</td>
</tr>
<tr>
<td>ELEGERPL-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>POMC</td>
</tr>
</tbody>
</table>
4.2 Future Directions

4.2.1 The application of α-amidated peptide enrichment method to the peptide-pair screening strategy.

The PHM based α-amidated peptide enrichment method can be used in the sample preparation for LC-MS/MS to increase the concentration of peptides of interest and, thus, increase their chances of identification using a database search. The enrichment method can also be used in the peptide-pair screening method together with PAM inhibition to insure the coexistence of α-amidated peptides and their glycine-extended precursors.

In the peptide-pair screening strategy presented in Chapter 2, the coexistence of two parties in a pair was insured by combining the total peptides extracted from cells grown with PAM inhibitor (containing accumulated glycine-extended peptides) and from cells grown without PAM inhibitor (containing α-amidated peptides with natural levels). Instead of the total peptides extracted from the control cells, the enriched sample can be used to provide increased levels of α-amidated peptides. In this way, relative intensities of both parties in samples are increased which would facilitate the detection of less abundant α-amidated peptides.
The other future direction to apply the enrichment method to the peptide-pair screening strategy is to develop elution conditions in which bound glycine-extended peptides can be released from PHM without hydroxylating, and to provide enriched glycine-extended peptides. The combination of a sample containing enriched glycine-extended peptides with a sample containing enriched α-amidated peptides would greatly reduce the sample complexity and render LC-MS/MS more efficient and effective. Possible elution conditions to be considered include high concentration of urea/guanidinium which was used to elute biotin from avidin (Diamandis and Christopoulos 1991, Buckie and Cook 1986), and low pH which was used to elute peptides with C-terminal basic residues (Lys or Arg) from anhydrotrypsin (Yokosawa and Ishii 1979, Ishii et al. 1983, Kumazaki et al. 1986).

4.2.2 Application of peptide-pair strategy to screen for other PTMs.

The peptide-pair strategy can be easily adapted to screen for peptides with other modifications. Mass differences between modified and unmodified form for common PTMs are shown in Table 21. Similar retention time and similar MS/MS fragmentation patterns between modified and unmodified peptides can be expected for most PTMs. The only requirement of the peptide-pair strategy is that modified and unmodified forms of peptides coexist. For most reversible modifications, this condition is usually fulfilled due
to their dynamic nature. Proper treatment of the sample can increase the concentration of the less abundant party and thus make it more detectable. Taking S-glutathionylation as an example, if modified peptide level is low, oxidative and nitrosative stress can be introduced to induce S-glutathionylation (Klatt and Lamas 2000); if unmodified peptide level is low, glutaredoxin can be used for deglutathionylation (Meyal et al. 2008).
<table>
<thead>
<tr>
<th>Modification</th>
<th>Site</th>
<th>Monoisotopic mass change</th>
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<tbody>
<tr>
<td>Acetylation</td>
<td>Lys/N-terminus</td>
<td>42.0373</td>
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<tr>
<td>Biotinylation</td>
<td>Lys/N-terminus</td>
<td>226.2994</td>
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<tr>
<td>Carboxylation</td>
<td>Glu/Asp</td>
<td>44.0098</td>
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<tr>
<td>Cysteinylation</td>
<td>Cys</td>
<td>119.1442</td>
</tr>
<tr>
<td>Deamidation</td>
<td>Gln/Asn</td>
<td>0.9847</td>
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<tr>
<td>Deoxyhexoses</td>
<td>Ser/Thr/Asn</td>
<td>146.143</td>
</tr>
<tr>
<td>Disulphide bond</td>
<td>Cys</td>
<td>-2.0159</td>
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<tr>
<td>Farnesylation</td>
<td>Lys/N-terminus</td>
<td>204.3556</td>
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<tr>
<td>Formylation</td>
<td>Lys/N-terminus</td>
<td>28.0104</td>
</tr>
<tr>
<td>Geranylgeranylation</td>
<td>Lys/N-terminus</td>
<td>272.4741</td>
</tr>
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<td>Glutathionylation</td>
<td>Cys</td>
<td>305.3117</td>
</tr>
<tr>
<td>Hexosamines</td>
<td>Ser/Thr/Asn</td>
<td>161.1577</td>
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<tr>
<td>Hexoses</td>
<td>Ser/Thr/Asn</td>
<td>162.1424</td>
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<tr>
<td>Lipoic acid</td>
<td>Lys/N-terminus</td>
<td>188.3147</td>
</tr>
<tr>
<td>Methylation</td>
<td>Lys/N-terminus</td>
<td>14.0269</td>
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<tr>
<td>Myristoylation</td>
<td>Lys/N-terminus</td>
<td>210.3598</td>
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<td>N-acetylhexosamines</td>
<td>Ser/Thr/Asn</td>
<td>203.195</td>
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<tr>
<td>Oxidation</td>
<td>Cys</td>
<td>15.9994</td>
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<tr>
<td>Palmitoylation</td>
<td>Lys/N-terminus</td>
<td>238.4136</td>
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<tr>
<td>Pentoses</td>
<td>Ser/Thr/Asn</td>
<td>132.1161</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>Ser/Thr/Tyr</td>
<td>79.9799</td>
</tr>
<tr>
<td>Pyroglutamic acid</td>
<td>Gln</td>
<td>-17.0306</td>
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<td>Sialic acid</td>
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<td>Stearoylation</td>
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<td>266.4674</td>
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<tr>
<td>Sulphation</td>
<td>Ser/Thr/Tyr</td>
<td>80.0642</td>
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4.3 References


TRYPTIC DIGESTS OF PROTEINS BY IMMOBILIZED ANHYDROTryptsin APPLICATION TO STRUCTURAL ANALYSES OF THE TAIL SHEATH AND TUBE PROTEINS FROM BACTERIOPHAGE T4.

*Proteins Structure Function and Genetics*, 1, 100-107.


## Appendix A: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-Converting Enzyme</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CCK-AR</td>
<td>Cholecystokinin-A receptor</td>
</tr>
<tr>
<td>CgA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-Cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>Dansyl</td>
<td>5-Dimethylamino-naphthalene-1-sulfonyl chloride</td>
</tr>
<tr>
<td>DMAB</td>
<td>3-(Dimethylamino)benzoic acid</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron capture dissociation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>HHL</td>
<td>Hippuryl-L-histidyl-L-leucine</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>JP</td>
<td>Joining peptide</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography coupled tandem mass spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LPH</td>
<td>Lipotropin</td>
</tr>
<tr>
<td>LTQ</td>
<td>Linear ion trap</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>---------------</td>
<td>----------------------------------------------------------------</td>
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<tr>
<td>Luminol</td>
<td>3-Aminophthalhydrazide</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>MBTH</td>
<td>3-Methyl-2-benzothiazolinone hydrazone</td>
</tr>
<tr>
<td>MDF</td>
<td>Mass Distance Fingerprint</td>
</tr>
<tr>
<td>MDH</td>
<td>Mass Distance Histogram</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>mJP</td>
<td>Mouse joining peptide</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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<td>MSH</td>
<td>Melanotropin</td>
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<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
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<tr>
<td>NAD⁺/NADH</td>
<td>Nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide reduced form</td>
</tr>
<tr>
<td>NADP⁺/NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate/Nicotinamide adenine</td>
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<tr>
<td>H</td>
<td>Neuroendocrine regulatory peptide</td>
</tr>
<tr>
<td>NERP</td>
<td>Neuroendocrine regulatory peptide</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PAL</td>
<td>Peptidylamidoglycolate lyase</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidylglycine a-amidating monooxygenase</td>
</tr>
<tr>
<td>PHM</td>
<td>Peptidylglycine a-hydroxylating monooxygenase</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine methosulfonate</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
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<tr>
<td>S/N</td>
<td>Signal over noise</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
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<td>TRH</td>
<td>Thyrotropin releasing hormone</td>
</tr>
<tr>
<td>XIC</td>
<td>Extracted ion chromatogram</td>
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Appendix B: Figures

Figure 28. Standard curve for the quantification of hippuric acid.

Figure 29. Standard curve for the quantification of phosphorus.
Figure 30. Identification of \( \alpha \)-amidated peptide AEAAVWGDGSPEPSPRE-NH\(_2\).
Figure 31. Identification of α-amidated peptide EEEAVWGDSPEPSRE-NH₂.
Figure 32. Identification of α-amidated peptide EEAVWGDSPEPSPRE-NH₂.
Figure 33. Identification of α-amidated peptide EAVWGDGSPEPSPRE-NH$_2$. 
Figure 34. Identification of α-amidated peptide AVWGDGSPEPSRE-NH₂.
Figure 35. Identification of α-amidated peptide VWGDGSPEPSPRE-NH₂.
Figure 36. Identification of α-amidated peptide WGDGSPEPSPRE-NH$_2$. 
Figure 37. Identification of α-amidated peptide GDGSPEPSPRE-NH₂.
Figure 38. Identification of $\alpha$-amidated peptide DGSPEPSPRE-NH$_2$. 
Figure 39. Identification of α-amidated peptide GSPEPSRE-NH₂.
Figure 40. Identification of α-amidated peptide SYSMEHFRWGWKPV-NH$_2$. 

SYSMEHFRWGWKPV-NH$_2$
Figure 41. Identification of peptide PEPSRSTPAPKKGSKK.
Figure 42. Identification of peptide PEPSKSAPAPKKGSKK.
Top: MS/MS spectrum; Bottom: MS/MS/MS spectrum of $y_{12}^{+2}$ ion.