# Proteomic studies of the chromaffin granule demonstrates novel proteolytic processing mechanisms for chromogranin A and proenkephalin by secretory vesicle cathepsin L.

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The chromaffin granule has been utilized by many investigators to understand molecular mechanisms by which neurosecretory vesicles synthesize, store, and secrete neurotransmitters and peptide hormones. Proteomics, in the post-genomic age, provides an ideal approach for elucidating the functional protein and peptide components of this regulated secretory vesicle system. Two proteomic approaches are presented in this study that have (1) defined novel chromogranin A (CgA) cleavage sites for production of the biologically active catestatin peptide[1] that regulates blood pressure, and (2) utilized an activity-based proteomic profiling approach for identification of the secretory vesicle cathepsin L as a proenkephalin and prohormone processing enzyme[2]. Moreover, the novel CgA cleavage sites are compatible with the prediction for CgA processing by secretory vesicle cathepsin L, as well as the prohormone convertases PC1 and PC2. Significantly, these studies demonstrate how selected proteomic studies combined with genetics can lead to novel findings of new regulatory components within neurosecretory vesicles.

**Proteomics of the major proteins of chromaffin granules identifies distinct cleavage sites for chromogranin A (CgA) for biosynthesis of active catestatin.** In proteomic studies of chromaffin granules, proteins were separated by 1-D and 2-D (one- and two-dimensional) SDS-PAGE gels and identified based on peptide sequence characterization via MALDI-TOF mass spectrometry combined with $NH_2$-terminal peptide sequencing by Edman degradation[1]. Multiple proteins of 70 kDa to 17 kDa were identified as catestatin-containing proteolytic fragments derived from chromogranin A (CgA). Catestatin itself, represented by $CgA_{344-364}$ was identified by gel filtration, $NH_2$-terminal sequencing by Edman degradation, and MALDI-TOF mass spectrometry. Results demonstrated that catestatin production requires cleavage of CgA at $NH_2$-terminal side of dibasic and monobasic residues. This cleavage specificity is consistent with the chromaffin granule 'prohormone thiol protease' (PTP) for proenkephalin processing[3-6] However, production of catestatin-containing intermediates appears to

utilize processing at the COOH-terminal sides of dibasic residues, resembling prohormone convertase 1 and 2 processing enzymes[1]. These proteomic studies of the major proteins of chromaffin granules demonstrate CgA-derived catestatin-containing intermediates and biologically active catestatin peptide as major components of chromaffin granules, which secrete catestatin in the regulation of blood pressure[7].

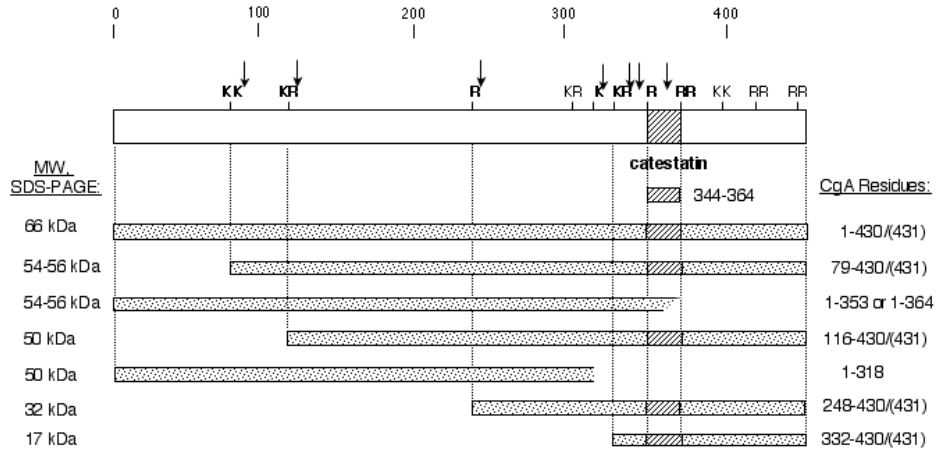


**Figure 1. Proteomic approaches for understanding prohormone processing mechanisms in chromaffin granules.** Catestatin-containing peptides derived from chromogranin A (CgA) were identified by $NH_2$-terminal sequencing and MALDI-TOF mass spectrometry. Chromaffin granules contain active catestatin peptide, $CgA_{344-364}$. Proteomics demonstrated catestatin-related peptides as full-length CgA, $NH_2$-terminally truncated forms of CgA (consisting of residues 79-430(431), 116-430(431), 248-430(431), and 332-430(431)), and COOH-terminal truncated forms of CgA (predicted as 1-353 and 1-318)[1].

**Activity-based profiling proteomic approach identifies cathepsin L in chromaffin granules as the proenkephalin processing enzyme.** In a focused proteomic study of cysteine protease activity for proenkephalin processing and production of the enkephalin opioid peptide in chromaffin granules, a high degree of enrichment and purification was

required. Moreover, selective activity-based affinity labeling of active cysteine proteases with DCG-04[8] was instrumental for identification of the enzyme responsible for the major proenkephalin cleaving activity in chromaffin granules. The enzyme was analyzed by tandem mass spectrometry of tryptic peptide digests, which indicated cathepsin L as the protease responsible for proenkephalin cleaving activity[2]. Secretory vesicle cathepsin L cleaved enkephalin-containing peptide substrates at prohormone processing sites occurring at the $NH_2$-terminal sides of dibasic or monobasic residues, as well as between the two residues of a dibasic site. These results show that the cleavage specificity of secretory vesicle cathepsin L results in enkephalin peptide intermediates that contain a basic residue extension at their $NH_2$-termini, thus, requiring further processing by an aminopeptidase B-like protease present in chromaffin granules[9]. The cathepsin L and aminopeptidase B proteolytic pathway represents an alternative pathway for prohormone processing, distinct from the prohormone convertase and carboxypeptidase E/H pathway for prohormone processing. The cleavage specificity of secretory vesicle cathepsin L is consistent with processing of CgA to catestatin.

The localization of cathepsin L to secretory vesicles of neuroendocrine chromaffin cells was visualized by immunogold electron microscopy for colocalization of cathepsin L with (Met)enkephalin in these vesicles[2]. Dual immunofluorescence microscopy demonstrated the high degree of colocalization of secretory vesicle cathepsin L with enkephalin-containing secretory vesicles.

Significantly, genetic studies with cathepsin L knockout mice illustrated the *in vivo* role of cathepsin L for enkephalin production. Brain levels of enkephalin were significantly reduced by 50% in cathepsin L knockout mice[2]. These proteomic and genetic findings demonstrate a novel biological function for secretory vesicle cathepsin L in prohormone processing in the regulated secretory pathway.

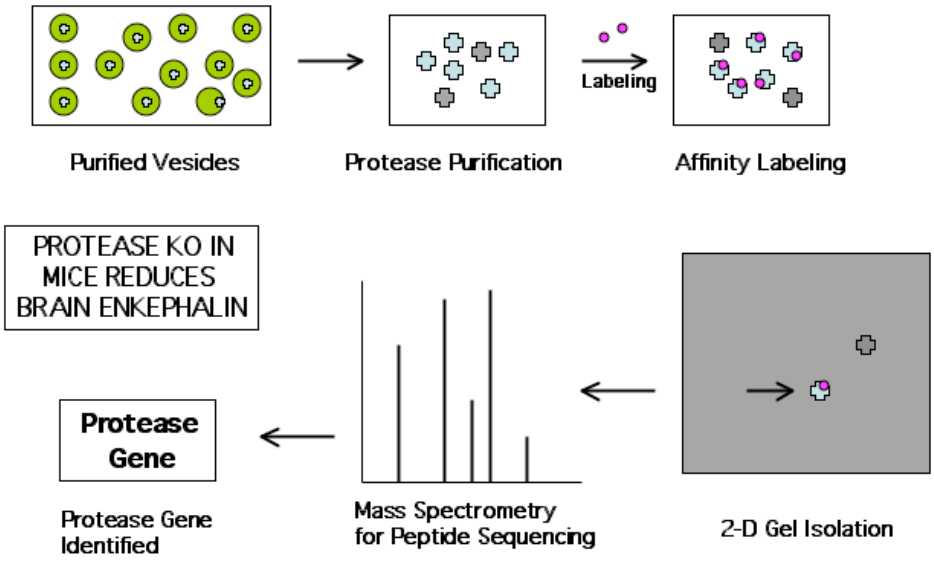


**Figure 2. Activity-based profiling demonstrates cathepsin L in secretory vesicles for the production of the enkephalin opioid peptide.** The cysteine protease activity in chromaffin granules responsible for proenkephalin processing was purified from chromaffin granules (secretory vesicles), identified with the activity-based probe DCG-04 for cysteine proteases, subjected to peptide sequencing by tandem mass spectrometry, and confirmed as the enkephalin-producing protease in genetic studies of cathepsin L knockout mice[2].

**Future analyses of proteins and peptides in chromaffin granules.** Continued proteomic studies of proteins and peptides involved in chromaffin granule function will require a step-wise approach to enrich the sample in proteins/peptides of moderate and low abundance. Resultant proteins can then be analyzed by separation via 1D or 2D gels, or LC (liquid chromatography) approaches, for peptide sequence characterization by mass spectrometry. Successive rounds of protein identification and enrichment of lower abundant proteins, by affinity-based removal of abundant proteins, will provide knowledge of proteins with differing levels of relative abundance. In any cellular system, strategies to examine

subsets of high or low abundant proteins, proteins of different gene families, or proteins localized to particular subcellular organelles are required to begin to obtain knowledge of the protein components of the functional chromaffin granule system that produces, stores, and releases biologically active peptide hormones and neurotransmitters.

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