Characterization of a Cholecystokinin 8-generating Endoprotease Purified from Rat Brain Synaptosomes*

Jason C. Viereck† and Margery C. Beinfeld

From the Department of Pharmacological and Physiological Science, St. Louis University Medical School, St. Louis, Missouri 63104

An endoproteolytic activity that specifically cleaves CCK 33, producing CCK 8, has been purified from a rat brain synaptosome preparation. The purification, which included anion exchange, chromatofocusing, hydroxyapatite, and gel filtration chromatography, resulted in a greater than 3000-fold increase in specific activity. This neutral endoprotease (pH optimum 8) exists as a 90-kDa species, which can be dissociated into active 40-kDa species. The enzyme is a non-tryptic serine protease, which is inhibited by diisopropylfluorophosphate and p-aminobezazemidine but not by soybean trypsin inhibitor, phenylmethysulfonfluoride, aprotinin, or a number of thiol or metalloprotease inhibitors. It is highly substrate-specific and cleaves neither trypsin, enteropeptidase, kallikrein substrates, nor analogues of mono- or dibasic cleavage sites of prohormones other than pro-CCK. The endoprotease will not cleave CCK 12 desulfate or CCK (20–29), although these peptides contain common sequences with CCK-33. The protease does cleave [Glu\(\text{2}'\)]CCK (20–29), a peptide in which the glutamate mimics the negative charge normally present on tyrosine sulfate. This suggests that the negative charge at position 27 is important in substrate recognition. The enzyme will also cleave CCK 33 and CCK (1–21) on the carboxyterminal side of a single lysine residue in position 11. The subcellular location and specificity of this endoprotease make it a good candidate for a CCK-processing protease.

Neuropeptides are initially synthesized as relatively large prohormones, which are then processed to the mature form. Modifications of the prohormone during processing may include glycosylation, sulfation, COOH-terminal amidation, or acetylation. In addition, virtually all peptides undergo limited proteolysis, so that the final product is considerably shorter than the original prohormone (1). Processing is often elaborated, so that the final product is considerably shorter than the original prohormone. It is in this context that the information determining where prohormones are cleaved resides within the structure of the prohormones themselves (21). Several investigators have analyzed the structure of prohormone cleavage sites and have found certain features in common. These have involved reverse turns (21), upstream arginine residues (22, 23) and more recently, Q-loops (24).

Endoproteolysis as a modification has attracted special attention, not only because it occurs in virtually all prohormone processing, but also because it is essentially irreversible and represents a definite commitment to cell biological function. It appears that a number of prohormones are cleaved differentially in different tissues (2, 3). Thus, regulation of endoproteolysis is a common factor in differential release of hormones.

Given the evidence for common precursors in various tissues, it is quite possible that tissue-specific processing enzymes are responsible for differences in the structure of neuropeptides in different tissues (4). Other possibilities include differential modification of prohormones by, for example, glycosylation, or regulation of the microenvironment (i.e. availability of co-factors). Identification and characterization of prohormone proteases is in any case essential to an understanding of the tissue-specific reactions of processing.

Classically, basic amino acid doublets have been viewed as sites for proteolytic cleavage. More recently, it has become apparent that many prohormones are cleaved at sites containing a single basic residue, so-called monobasic sites.

One of the problems of prohormone processing is that few processing proteases have been unequivocally identified. The KEX2 gene product of yeast (5) was the first such enzyme described. KEX2 mutants, lacking this gene, cannot process α mating factor. This ability is restored with restoration of the KEX2 gene (6, 7). By searching for mammalian sequences with homology to KEX2, a number of researchers have cloned putative dibasic processing enzymes, which may be involved in processing of POMC1 (8–10). No such model sequence is available yet for monobasic processing enzymes. Currently most of the work being done on prohormone proteases involves the study of putative processing enzymes, so identified because of their specificity and/or subcellular localization (11–20).

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CCK is a good model neuropeptide system in which to study post-translational processing. It is one of the most abundant peptides in the brain. Aspects of its biosynthesis have been

*This work was supported by National Institutes of Health Grant NS 18667 (to M. C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Pharmacological and Physiological Science, St. Louis University Medical School, 1402 S. Grand Blvd., St. Louis, MO 63104. Tel.: 314-577-8543; Fax: 314-577-8233.

1 The abbreviations used are: POMC, pro-opiomeainocorin; S-28, somatostatin 28; CCK, cholecystokinin; CRF, corticotrophin releasing hormone; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DABITC, 4-(dimethylamino)azobenzene-4'-isothiocyanate; DABSL, 4-(dimethylamino)azobenzene-4'-sulfonyl; DIFP, diisopropylfluorophosphate; FMOC, 9-fluorenylmethoxycarbonyl; PAGE, polyacrylamide electrophoresis; pCMB, p-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; pro-OT/NP, pro-oxytocin/neurophysin; tBOC, t-butoxycarbonyl; HPLC, high performance liquid chromatography; FMRFamide, Phe-Met-Arg-Phe-amide.
investigated (25, 26), although the exact temporal order of cleavages remains a mystery. The prohormone for CCK is relatively simple, containing one copy of the peptide released, which is located in the carboxyl terminus flanked by a carboxyl-terminal extension. The sequence of CCK-33 is given in Table I in the Miniprint Section, along with the sequence of some CCK fragments. The prohormone is differentially cleaved (2). In rat brain, the predominant form is CCK-8 (27), whereas in rat intestine, larger forms (CCK-38, CCK-33, and CCK-22) predominate. In pituitary corticotrophs pro-CCK is processed incompletely (28). Unlike POMC, differential cleavage does not result in the secretion of different peptides, but rather different lengths of the same peptide.

In order to better understand CCK processing in the brain, the enzymatic activity which cleaves CCK-33 to make CCK-8 was examined. A crude preparation of a CCK-producing endoprotease from porcine brain has previously been described (29, 30). This activity was neither purified nor completely characterized. The aim of the present study was to obtain a purified preparation of this endoprotease so that its catalytic properties could be characterized.

MATERIALS AND METHODS

RESULTS

Enzyme Purification

The ability of various extracts to generate CCK-8 from CCK-33 was tested to determine the appropriate starting material for purification of the protease. The rat tissues examined were a crude secretory granule preparation from small intestinal mucosa, brain cytosol (a byproduct of the synaptosome preparation), and both the soluble and membrane-bound fractions of synaptosomes. The intestinal mucosal preparation had no ability to generate CCK-8. Of the other preparations, the soluble fraction of synaptosomes produced by far the greatest amount of CCK-8 (data not shown), and it was thus chosen as the starting material for purification of the enzyme.

The levels of several enzymatic activities measured before and after synaptosomal preparation are outlined in Table II. CCK immunoreactivity and CCK-8-generating activity were both enriched by a factor of about 4.3 in the synaptosomal preparation. The activity of the lysosomal marker enzymes alkaline phosphatase and β-galactosidase activities decreased or did not increase very much. Thus CCK-8, which is present in secretory granules, and CCK-8-generating activity were both enriched relative to markers of other subcellular compartments.

CCK-generating activity was solubilized from synaptosomes following free-thaw lysis in 0.3 M NaCl. The activity is apparently not due to an integral membrane protein, as it did not require detergent for solubilization. Table III summarizes the purification of the enzyme from rat brain. The specific activity of the preparation was increased approximately 3400-fold relative to the brain homogenate. This is, at best, an approximation, however, because of the possible presence of other proteases in the crude sample, which may either produce or degrade the substrate or the product, and because of the difficulty in measuring the protein content of the purified enzyme.

The purified enzyme ran as a single band on SDS-PAGE (Fig. 1). The molecular mass of the protein determined by electrophoresis is 40 kDa.

Some other protease activities were followed throughout the purification. Trypsin, enteropeptidase, and kallikrein activities were assayed using fluorogenic substrates (data not shown). No trypsin activity was found in the synaptosomal preparation. A small amount of activity capable of cleaving the enteropeptidase substrate was detected in the synaptosomal preparation, but this was separated from CCK-8-generating activity by anion exchange chromatography. Significant kallikrein activity was found in the synaptosomal preparation and co-eluted with CCK-cleaving activity on anion exchange. However, this activity could be easily separated by chromatofocusing.

TABLE II

Enzyme activities in homogenate and synaptosome preparation

Activities are expressed per μg of total protein. 1 alkaline phosphatase unit equals 1 nmol of p-nitrophenol produced/30 min. 1 β-galactosidase unit equals 1 pmol of 4-methylumbelliferone produced/30 min. 1 CCK-generating unit equals 1 ng of CCK-8 produced/2 h.

| Activity                  | Crude homogenate | Synaptosomes |
|---------------------------|------------------|-------------|
| Alkaline phosphatase activity | 3.7 units/μg    | 1.0 (27%)  |
| β-Galactosidase activity | 2.8 units/μg    | 4.6 (164%) |
| CCK-generating activity   | 0.4 units/μg    | 1.6 (445%) |
| CCK-like immunoreactivity | 5.7 pg/μg       | 24.4 (428%)|

Enzyme activities in homogenate and synaptosome preparation. The protease migrates as a single band on SDS-PAGE (Fig. 1). The molecular mass of the protein determined by electrophoresis is 40 kDa.

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TABLE III

Purification of the CCK-8-generating endoprotease

Units of specific activity are ng of CCK-8 produced/2 h/μg of protein. After hydroxyapatite chromatography, too little protein was present for accurate concentration determination, and only a lower limit on specific activity could be estimated. Protein totals for crude and synaptosomes refer to total soluble protein.

| Purification step     | Specific activity | Total protein | Total activity | Fold purification |
|-----------------------|-------------------|---------------|---------------|-------------------|
| Crude homogenate      | 0.35              | 245 mg        | 100           | 1.0              |
| Synaptosomes          | 1.56              | 50 mg         | 91            | 4.45             |
| Anion exchange        | 6.2               | 12.6 mg       | 91            | 17.8             |
| Chromatofocusing      | 25                | 580 μg        | 16            | 71.4             |
| Gel chromatography    | 210               | 32 μg         | 8             | 600              |
| Hydroxyapatite chro- |
| matography            | >1000             | <4 μg         | 4             | >3143            |

FIG. 1. SDS-polyacrylamide gel electrophoresis of purified endoprotease visualized with silver stain. The protease migrates as a single band of apparent molecular mass of 40,000 daltons. The gel was prepared in the presence of 2% SDS as described under "Materials and Methods." Bars at the right indicate the positions of molecular weight markers.

*Portions of this work (including "Materials and Methods," Fig. 8, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Characterization

Enzymology—The enzyme was active over a broad range of pH, from 6.5 to 8 with peak activity at pH 8.0. The enzyme was essentially inactive below pH 5 or above pH 9 (data not shown).

Kinetic parameters of the enzyme were estimated by assaying the enzyme in the presence of a range of substrate concentrations. A Lineweaver-Burk plot of the data (Fig. 2) indicates that the $K_m$ value of the enzyme for CCK 33 is 17 $\mu$M.

Enzyme Inhibitors—Assay of the enzyme in the presence of a series of class-specific enzyme inhibitors shows that the enzyme is a non-trypsin serine protease. The enzyme is moderately sensitive to DIPF, but is not inhibited by either soybean trypsin inhibitor or PMSF (Table IV). EDTA does not inhibit the enzyme, nor do divalent cations such as Ca$^{2+}$ or Mg$^{2+}$ affect the activity, indicating that it is not a metalloprotease.

The enzyme is not affected by thiol reagents. Neither pCMB nor o-phenanthroline inhibit the activity, and activity of the enzyme is not enhanced by the thiol regenerating agent cysteine. The enzyme was inhibited by $p$-amino benzamidine, a serine protease inhibitor. Addition of 4 mM Triton X-100 actually increased the activity of the enzyme by a factor of 3.5. It was not possible to tell whether this result was due to an effect on the enzyme or on the substrate.

![Figure 2](image-url)

**Table IV**

Effect of enzyme inhibitors on CCK 8-generating activity

| Inhibitor                | Concentration | % control activity |
|-------------------------|---------------|--------------------|
| DIPF                    | 5 mM          | 58                 |
| PMSF                    | 5 mM          | 97                 |
| Soybean trypsin inhibitor | 100 $\mu$g/ml | 100                |
| EDTA                    | 5 mM          | 103                |
| Ca$^{2+}$               | 10 mM         | 90                 |
| Mg$^{2+}$               | 10 mM         | 85                 |
| 1,10 o-Phenanthroline   | 5 mM          | 100                |
| pCMB                    | 5 mM          | 103                |
| Cysteine                | 10 mM         | 99                 |
| Aprotinin               | 25 $\mu$g/ml  | 98                 |
| Leupeptin               | 100 $\mu$M    | 100                |
| Bestatin                | 500 $\mu$M    | 100                |
| $p$-Aminobenzamidine    | 1 mM          | 54                 |
| Triton X-100            | 4 mM          | 350                |

Molecular Size of the Enzyme—When the synaptosomal preparation was subjected immediately to gel filtration, CCK 8-generating activity eluted in a peak corresponding to a molecular mass of 90 kDa (Fig. 3). If the synaptosomal preparation was treated with either detergent or high ionic strength, the activity eluted at 40 kDa, as described above. Activity also eluted near the void volume of the column (fraction 20) corresponding to a molecular mass well over 100 kDa. This peak may represent high molecular mass protein aggregates.

The molecular size of the activity was further examined by correlating with gel electrophoretic mobility. A partially purified preparation of the endoprotease was electrophoresed into two separate lanes. One lane was silver-stained for protein, while the other was cut into segments which were assayed for CCK 8-generating activity. The bulk of this activity can be seen to co-elute with the protein band at 40 kDa in the stained lane (Fig. 4).

![Figure 3](image-url)

**Figure 3** Gel filtration of CCK 8-generating activity before and after treatment with 0.5% CHAPS detergent. A crude synaptosomal extract was subjected to Sephadex G-150 filtration either before (solid line) or after (dashed line) 48-h treatment with 0.5% CHAPS at 4 °C. One-ml fractions of each sample were collected and assayed for CCK 8-generating activity. Molecular weight markers are indicated at the top of the plot (BSA, bovine serum albumin; OB, ovalbumin; CA, carbonic anhydrase).

![Figure 4](image-url)

**Figure 4** Correlation of CCK 8-generating activity with electrophoretic mobility. A crude preparation of the endoprotease (purified by anion exchange and hydroxyapatite) was subjected to polyacrylamide gel electrophoresis in the absence of SDS and stained for protein. In an adjacent lane, an identical sample was electrophoresed but not stained. This lane was cut into 10 equal segments, which were assayed for CCK 8-generating activity. Assay results are shown in the histogram to the left. Activity was concentrated in a segment corresponding to a protein band of molecular mass = 40 kDa. Some activity could also be detected at a much higher molecular mass (>90 kDa). Bars at the right indicate the positions of molecular mass markers.
Specificity of the Endoprotease

Analog of CCK—Fig. 5 illustrates the ability of the enzyme to generate CCK 8 from CCK 33. Surprisingly, the enzyme did not cleave CCK 12 (CCK 22-33), although its sequence is very similar to that which is cleaved in CCK 33. Possible explanations are either that CCK 12 is too short for proper recognition by the protease or that a sulfated tyrosine residue (as occurs in CCK 33) is required for recognition. In order to examine the latter possibility, two other peptides were tested: CCK (20-29) and [Glu22]CCK (20-29). The sequence of these analogues is given in Table IV.

CCK (20-29) was also not cleaved. In order to determine if the negative charge of the sulfate group on Tyr22 in CCK 33 is an important factor in substrate binding, the analogue [Glu22]CCK (20-29) was tried. In this peptide, the negatively charged glutamate replaces the tyrosine. The peptide was indeed cleaved by the enzyme (Fig. 6), although not as readily as CCK 33. Using similar enzyme and substrate concentrations, only about 50 pmol of [Glu22]CCK (20-29) is cleaved after 10 h of incubation, as compared with 150–200 pmol of CCK 33 after only 3–4 h.

Alternative Cleavage of CCK—When the products from an assay of the purified enzyme were analyzed by gel filtration chromatography over Sephadex G-50, a CCK-immunoreactive peak was found with a molecular mass intermediate between that of CCK 8 and CCK 33 (data not shown). This peak may have been due to cleavage of CCK 33 by the enzyme at a site other than that which generates CCK 8. In order to investigate whether the enzyme cleaves the NH2-terminal region of CCK 33, the substrate CCK 1-21 was used.

When this peptide was incubated with the CCK 8-generating enzyme, it was cleaved at a single site (Fig. 7). Amino-terminal analysis of the liberated fragment indicated that it had an NH2-terminal asparagine. Since there is only 1 asparagine in the peptide, the cleavage site could be unequivocally identified. Cleavage of CCK 33 at this site would liberate CCK 22.

Other Peptide Substrates—The purified enzyme was incubated with a variety of peptide analogues of mono- and dibasic cleavage sites to investigate whether it would recognize such sites from other prohormones. The sequence of these analogues is shown in Table I (see Miniprint Section). The purified enzyme would not cleave analogs of cleavage sites from pro-somatostatin (neither the monobasic or dibasic site), the carboxyl terminus of pro-CRF, pro-oxytocin/neurophysin (pro-OT/Np) or dynorphin A. A small peak is generated corresponding to the fragment Asp-Gly-Met-Gly (DEMG), indicating cleavage at the Arg-Asp bond. There was no cleavage at the Arg-Ile bond, as shown by the absence of the expected fragment His-Arg.

DISCUSSION

This study reports the purification of a CCK 8-generating endoprotease from rat brain synaptosomes. The product was monitored by a RIA specific for the sulfated, amidated CCK peptides as well as by chromatographic methods. A synaptosomal preparation was chosen as starting material for the purification because the endoprotease was expected to colocalize with CCK in vesicular fractions of rat brain. When the CCK 8-generating activity was compared to left-over cytosol, this was found to be the case. Furthermore, the activity was found to reside in the soluble fraction of synaptosomes, rather than the detergent-soluble fraction.

In the preparation of synaptosomes, a 4-fold enrichment of the endoprotease, as well as CCK, was achieved. This is
comparable with enrichments of 3-4-fold previously reported in several studies (31, 32). Though this result does not prove that the endoprotease is localized with CCK in synaptic vesicles, it does suggest that both are present in the nerve terminal.

The four-step purification resulted in at least a 3400-fold increase in the apparent specific activity of the endoprotease. The purified enzyme is a serine protease with a neutral pH optimum. It is not kallikrein-like nor trypsin-like based on its insensitivity to inhibitors and its inability to cleave substrates for these proteases. Although tissue kallikrein is present in the brain (33), the results of this study indicate that the CCK 8-producing endoprotease is distinct from it. The CCK 8-producing endoprotease does not have critical thiol groups, nor is it divalent cation-dependent.

The molecular mass of the purified enzyme was determined to be 40 kDa. Before purification, the activity had a molecular mass of 50 kDa. Thus, the enzyme may exist in vivo as a dimer of non-covalently bound subunits. It is also possible, however, that the active enzyme may bind to inert proteins in the synaptosomal preparation, showing a larger apparent molecular mass.

This enzyme is very specific, cleaving the Arg-Asp bond in CCK 33, yet not cleaving the same bond in the context of similar sequences. The enzyme also does not cleave a series of mono- or dibasic cleavage sites from other prohormones. These features are characteristic of a processing, rather than degradative enzyme.

Straus and colleagues (34) first described a CCK 8-producing activity in crude porcine brain extracts, although this activity was neither purified nor extensively characterized. The activity was found to cleave CCK 33 but not gastrin 17 and was not inhibited by lima bean trypsin inhibitor. This suggested that it was a specific rather than nonspecific protease. They reported an apparent molecular mass of less than 70 kDa, consistent with the present study. The CCK 8-generating endoprotease described here may be a purer preparation of this previously reported activity.

These authors also reported that the CCK 8-generating preparation would cleave the dipeptide Arg-Asp and suggested that the activity is not hormone-specific but bond-specific. This result is not in agreement with the results reported here: that the CCK 8-generating endoprotease is quite specific and will only cleave the Arg-Asp bond in the context of the correct surrounding sequence. It seems likely that contaminating proteolytic activities complicated the earlier study.

Monobasic enzymes are widely varied, including thiol proteases, metallo-proteases, and serine proteases, although almost all are optimally active at neutral or slightly alkaline conditions. Many of the prohormone proteases described are membrane bound (11, 12, 19). The CCK 8-generating endoprotease is not an integral membrane protein, as it can be extracted without detergent. It is true that its activity is enhanced in a membrane-like environment (4 mM Triton X-100). However, this may be due to an effect on the substrate. It is likely that the secondary structure of CCK, as well as other peptides, changes upon introduction into a membrane-like environment. It is not known to what extent the endoproteolysis taking place during processing is associated with the membrane during packaging and sorting into secretory granules.

The variety of enzymes which have been described supports the idea that there are many prohormone-specific proteases, rather than a few general processing enzymes. Certainly, the enzyme described here is distinct from previously described proteases.

The Arg-Asp bond cleavage is not common. Two other prohormones are cleaved at such a site: FMRFamide of Drosophila (35) and porcine brain natriuretic peptide (36). In the case of FMRFamide, there are a series of similar cleavage sites of which the Arg-Asp cleavage is one member. The processing endoproteases associated with FMRF prohormones have not yet been characterized.

The enzyme described here cleaves CCK 33 at Lys11-Asn12 and at Arg25-Asp26. This is surprising as these two sites are very different, and one involves the kallikrein-like Arg and the other a single Lys. The Arg-Asp site is surrounded by charged residues, 2 aspartic acids and the sulfated tyrosine, while the Lys-Asn is surround by non-charged residues. How the conformation of the peptide as a whole may contribute to the observed cleavages is not known. Certainly substrate conformation seems to determine not just whether a substrate is cleaved, but also where (37).

The enzyme cleaves a synthetic CCK substrate [Glu2] CCK(20-29) in which a glutamic acid was substituted for the sulfated tyrosine found in CCK 33. This suggests that the negative charge is part of the recognition site for the enzyme, at least for the Arg-Asp bond. Sulfation of CCK in vivo may play a role in substrate recognition.

CCK 22, the peptide resulting from cleavage of pro-CCK at the Lys11-Asn12 bond, is not a major form of CCK found in the rat brain (27), although small amounts of CCK 22 have been detected in porcine cortex (38). This may be due to the fact that in the brain, CCK 33 is efficiently and rapidly processed to CCK 8. This is supported by the isolation from porcine brain of significant amounts of the amino terminus of CCK 33 minus CCK 8 (39). The peptide produced by cleavage of CCK 33 at both Lys11-Asn12 and Arg25-Asp26 also appears to be secreted from CCK-producing cell lines in culture.3

For some prohormones, cleavage has been shown to be an ordered process, with particular bonds being cleaved first. Whether the cleavage at Lys11 occurs before or after that at Arg25 deserves additional study, as does the more basic question of the relative affinity of the enzyme for that site. The ability of the enzyme to cleave other mono and dibasic sites in pro-CCK is under investigation.

Based on the subcellular localization and specificity of the CCK 8-generating endoprotease, it is a good candidate for a CCK-processing endoprotease. Given the diversity of putative prohormone-processing proteases, it is apparent that individ-

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3 M. C. Beinfeld, unpublished observations.
ual proteases are involved in regulating the processing of specific peptides. Thus better understanding of these proteases in general is crucial to unraveling the molecular mechanisms of peptide expression. In particular, further study of this CCK 8-generating enzyme is a cornerstone of studies of the tissue-specific processing of CCK.

Acknowledgments—We thank Paul Cohen and Viktor Mutt for providing some of the peptide substrates. We gratefully acknowledge the technical assistance of Lloyd R. Allard.

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Materials and Methods

1. Enzyme Purification

A. Assay:

Sprague Dawley rat weighing 200-300g were obtained from Harlan (Indianapolis, IN). The animals were fasted before use in the assay. Homogenate was centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant was collected, chilled to 6°C and a 1:4 dilution of the supernatant was prepared.

B. Substrate and Assay Buffer:

The enzyme assay was performed in a 20 mM phosphate buffer, pH 7.2, in a final volume of 0.1 ml. The assay mixture contained 100 ng of CCK-8 tracer and 1.25 pmol of CCK-8 substrate (20-28). The reaction was stopped by addition of 2 ml glycine-EDTA buffer pH 10.5. Fluorescent assays were performed using a Fluoroscan microplate reader (Microplate Reader #4000).

C. Prefixation:

The HPLC system consisted of a Waters 600 multisolvent delivery system, Waters 990B column, an Applied Biosystems 577 absorbance monitor and Waters 780 data module. All solvents were degassed with He gas prior to and during analysis. DABITC and DABITC-chloride were obtained from Pierce. Specific HPLC conditions used are described in figure legends.

2. Polyacrylamide Gel Electrophoresis and Protein Assay:

Proteins (1% w/v) for electrophoresis were prepared by modification of Laemmli (43,44), and were stained with Coomassie blue stain. Protein determination was done using a Bio-Rad protein assay kit.

3. Tryptophan and Tyrosine Assay:

Protein concentration was modified to (31-41)NH2. Protryptophan was obtained from Sigma (St. Louis, MO). Tryptophan (Trp) was determined using a Bio-Rad Protein Assay kit.

D. Western Blotting:

The reaction mixture was transferred to a gel and electrophoresed. The gel was stained with Coomassie blue and destained with 2% acetic acid.

4. HPLC Chromatography:

The HPLC system consisted of a Waters 600 multisolvent delivery system, Waters 990B column, an Applied Biosystems 577 absorbance monitor, and Waters 780 data module. The solvents were degassed with He gas prior to and during analysis. DABITC and DABITC-chloride were obtained from Pierce. Specific HPLC conditions used are described in figure legends.

IV. Materials:

DEAE Sephadex was purchased from Sigma. Disposable columns for anion exchange, PBE and Polybuffer were obtained from Perkin.

Table 1: Sequence of Peptide Substrates used with the CCK 8 Generating Enzymes

| Peptide | Sequence |
|---------|----------|
| CCK 8   | Lys-Asp-Pro-Arg-Asp | |
| CCK 11-21| Lys-Asp-Pro-Arg-Asp | |
| CCK 28-29| Lys-Asp-Pro-Arg-Asp | |

V. Enzyme Assays:

A. CCK Generating Activity:

CCK generating activity was routinely assayed by incubating 10-30 minutes of sample with 1 mg of CCK 33 or 30 minutes of CCK 38 in 10 mM phosphate buffer, pH 8, at a final concentration of 50 ng/ml. After 2 hours at room temperature, the reaction was stopped by the addition of 2 ml glycine-EDTA buffer pH 10.5. The reaction mixture was transferred to a gel and electrophoresed. The gel was stained with Coomassie blue and destained with 2% acetic acid.

II. Characterization of a Cholera Toxin 8 Generating Endoprotease from Rat Brain Synaptosomes

J. C. Vriesek and M. C. Bertrand

Supplementary Material:

Characterization of a Cholera Toxin 8 Generating Endoprotease from Rat Brain Synaptosomes

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Figure 8: Purification of CCK 8 Generating Activity. Steps in the purification included A) DEAE Sephadex anion exchange chromatography, B) Cibacron blue, C) Sephadex G-15 gel filtration, and D) Hydroxypatite chromatography. Bars in each case indicate CCK 8 generating activity. Details are provided in the text.