

# Endopeptidase-24.11 and aminopeptidase activity in brain synaptic membranes are jointly responsible for the hydrolysis of cholecystinin octapeptide (CCK-8)

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Received 13 July 1984

Endopeptidase-24.11 (EC 3.4.24.11) from pig kidney hydrolysed CCK-8 (sulphated) at two distinct sites: Asp-Tyr(SO<sub>3</sub>H)-Met-Gly<sup>1</sup>-Trp-Met-Asp<sup>6</sup>-PheNH<sub>2</sub>. Under initial conditions, the splitting of the Asp<sup>7</sup>-Phe<sup>8</sup>NH<sub>2</sub> bond proceeded 4-times more rapidly than the Gly<sup>4</sup>-Trp<sup>5</sup> bond. Pig brain striatal synaptic membranes attacked this substrate at the same sites and this activity was inhibited by phosphoramidon. However, other products were detected even in the presence of phosphoramidon. One of these products was identified as free tryptophan. Since their formation was inhibited by bestatin, one or more membrane aminopeptidases is also implicated in the degradation of CCK-8.

*Cholecystinin (CCK-8)    Endopeptidase-24.11    Aminopeptidase    Phosphoramidon    Bestatin    Pig brain synaptic membrane*

## 1. INTRODUCTION

The C-terminal fragment of cholecystinin (CCK-8, Asp-Tyr-Met-Gly-Trp-Met-Asp-PheNH<sub>2</sub>) is present in brain as the Tyr<sup>2</sup> sulphated form [1] and may well play a role as a neurotransmitter [2]. Hence, an understanding of its mode of inactivation by synaptic membranes is important. Only one report of the degradation of CCK-8 by brain peptidases has appeared [3]. These authors solubilized a crude synaptosomal-mitochondrial pellet from rat brains with Triton X-100 and resolved, by DEAE-cellulose chromatography, several peaks of activity capable of degrading CCK-8. Peak I had properties similar to those we have described for endopeptidase-24.11 [4] which is present in the striatum of pig brain [5]. Incubation of Peak I fractions with CCK-8 yielded two peptide products detected by the intrinsic fluorescence of the Trp residue. The main product was identified as Asp-Tyr(SO<sub>3</sub>H)-Met-Gly-Trp, the other was not characterized. This observation implies that the principal site of cleavage by this enzyme preparation was at the Trp<sup>5</sup>-Met<sup>6</sup> bond.

However, in our survey [6] of neuropeptide degradation by endopeptidase-24.11, we did not encounter hydrolysis of an -X-Met- bond even though such a bond was present in two of the substrates examined (Tyr-Gly-Gly-Phe-Met-Arg and Tyr-Gly-Gly-Phe-Met-Arg-Phe). Here we have investigated the degradation of sulphated CCK-8 by purified endopeptidase-24.11 and report that the only bonds split were Gly<sup>4</sup>-Trp<sup>5</sup> and Asp<sup>7</sup>-Phe<sup>8</sup>NH<sub>2</sub>. Pig striatal synaptic membranes hydrolysed the same bonds but additional activity, most probably representing one or more bestatin-sensitive aminopeptidases, was also responsible for the degradation of CCK-8.

## 2. Experimental

### 2.1. Peptides and inhibitors

CCK-8 (sulphated and nonsulphated) and captopril (SQ14225; D-3-mercaptopropanoic-L-proline) were gifts from Squibb Institute for Medical Research, Princeton, NJ. Other CCK-8 fragments and Phe-Met-Arg-PheNH<sub>2</sub> were obtain-

ed from Cambridge Research Biochemicals (Harston, Cambridge). Pentagastrin was supplied by ICI Pharmaceuticals (Macclesfield, Cheshire). Phenylalanyl amide was from Sigma (London). The peptidyl dipeptidase A inhibitor MK422 (*N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-Ala-L-Pro) was provided by Merck, Sharp & Dohme, Hoddesdon, Herts.

### 2.2. Membranes and enzyme preparations

Synaptic membranes from pig striatum were prepared as in [5]. Endopeptidase-24.11 was purified from pig kidneys by immunoaffinity chromatography [7].

### 2.3. Hydrolysis of CCK-8

Samples of synaptic membranes (140  $\mu$ g protein) or endopeptidase-24.11 (20–200 ng) in 0.1 M Tris-HCl (pH 7.4) were incubated with 0.5 mM CCK-8 for 30 min at 37°C (final volume 100  $\mu$ l). The reaction was stopped and the products analysed by high-performance liquid chromatography (HPLC) as described for substance P [5], except that each sample was monitored at two wavelengths, 214 and 280 nm.

## 3. RESULTS

### 3.1. Hydrolysis of CCK-8 by endopeptidase-24.11

The peptide products demonstrated as  $A_{214}$  and

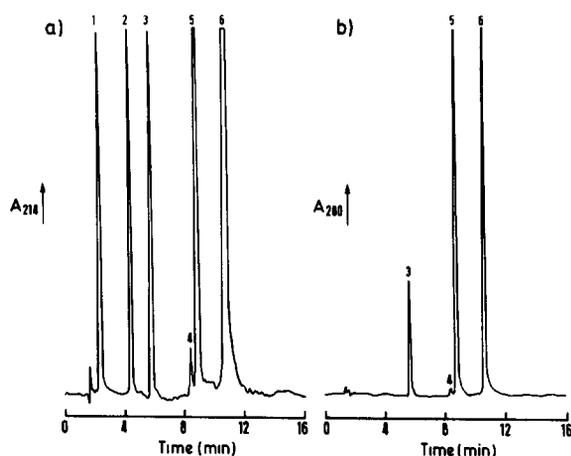


Fig.1. Hydrolysis of CCK-8 by endopeptidase-24.11. The peptide products of CCK-8 hydrolysis were resolved by HPLC and were monitored at 214 nm (a) and 280 nm (b).

Table 1

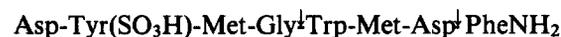
Products released by incubation of CCK-8 (sulphated) with endopeptidase-24.11

Peak no.	Retention time (min)	Peptide identified	Fragment of CCK-8
1	3.52	Fa	8
2	6.28	D-Y-M-G	1-4
3	7.90	W-M-D	5-7
4 <sup>a</sup>	11.06	W-M-D-Fa	5-8
5	11.54	D-Y-M-G-W-M-D	1-7
6	13.80	D-Y-M-G-W-M-D-Fa	CCK-8

<sup>a</sup>Peak 4 was a minor one, inadequate in amount for amino acid analysis; its identity was predicted by co-elution with a marker peptide

See section 2 for details. Data are from incubations achieving 60–90% degradation of CCK-8. The yields calculated from the analysis of each amino acid were  $\pm 20\%$  of those required for the peptides shown. The presence of tryptophan (W) was inferred from the  $A_{280}$  value.

$A_{280}$  peaks are shown in fig.1. Five  $A_{214}$  peaks were identified, 3 of which absorbed at 280 nm and, therefore, contained tryptophan. The identities of these peptides were established by amino acid analyses, shown in table 1. All the products so identified could be attributed to hydrolysis at two sites:



The time course of hydrolysis was investigated. The formation of 3 peptides, Asp-Tyr(SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp (peak 5), Trp-Met-Asp (peak 3) and PheNH<sub>2</sub> (peak 1) is shown in fig.2. The relative rates of hydrolysis at the two sites can be assessed by the initial rates of formation of peak 5 for the Asp<sup>7</sup>-Phe<sup>8</sup>NH<sub>2</sub> bond and peak 3 for the Gly<sup>4</sup>-Trp<sup>5</sup> bond (see fig.2 inset). The former is produced 4-times faster. Peak 4 was not detectable under these conditions.

Since the major site of cleavage of CCK-8 by endopeptidase-24.11 proved to be a rather unusual bond, we further investigated the ability of the enzyme to release a C-terminal PheNH<sub>2</sub> by using two other substrates, Phe-Met-Arg-PheNH<sub>2</sub> and pentagastrin, Boc- $\beta$ Ala-Trp-Met-Asp-PheNH<sub>2</sub>. In both cases, release of the C-terminal PheNH<sub>2</sub> was confirmed (not shown).

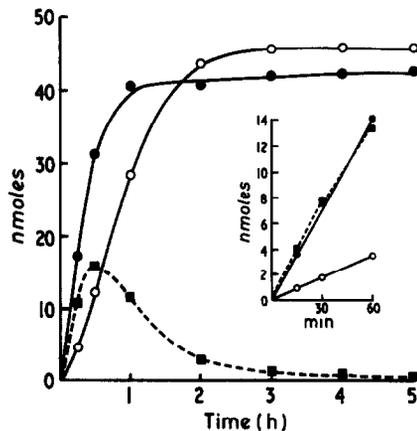


Fig. 2. Time course of CCK-8 hydrolysis by endopeptidase-24.11. Three products were quantified: peak 1 by  $A_{214}$  using a standard (PheNH<sub>2</sub>); Peaks 3 and 5 by  $A_{280}$  using tryptophan as the standard. In the main figure hydrolysis was allowed to proceed to completion (200 ng of enzyme). In the inset figure the conditions were such (20 ng enzyme) that the initial rate of hydrolysis was linear for a 60 min period. ●, Peak 1 (PheNH<sub>2</sub>); ■, peak 5 (D-Y-M-G-W-M-D); ○, peak 3 (W-M-D).

### 3.2. Hydrolysis of CCK-8 by synaptic membranes

The HPLC elution patterns monitored at 214 nm are shown in fig. 3. In the first experiment (fig. 3a), CCK-8 was incubated in the absence of inhibitors. Peaks with identical retention times to peaks 1–3 and 5 generated by endopeptidase-24.11 (fig. 1, table 1) were observed. In addition, two other peaks were seen and one of these was identified as tryptophan (retention time, 6.56 min). Neither peak was seen when bestatin was present. When the incubation was repeated in the presence of 100  $\mu$ M bestatin and 1  $\mu$ M phosphoramidon, to inhibit aminopeptidase and endopeptidase-24.11 activities, virtually no hydrolysis was observed (fig. 3b).

### 3.3. Effect of inhibitors on the hydrolysis of CCK-8 by synaptic membranes

The results of a more detailed study of the effect of inhibitors are shown in table 2. Phosphoramidon, which wholly abolished the hydrolysis of CCK-8 by endopeptidase-24.11 (not shown), was only partially effective with the synaptic membranes. Although the peptide products in peaks

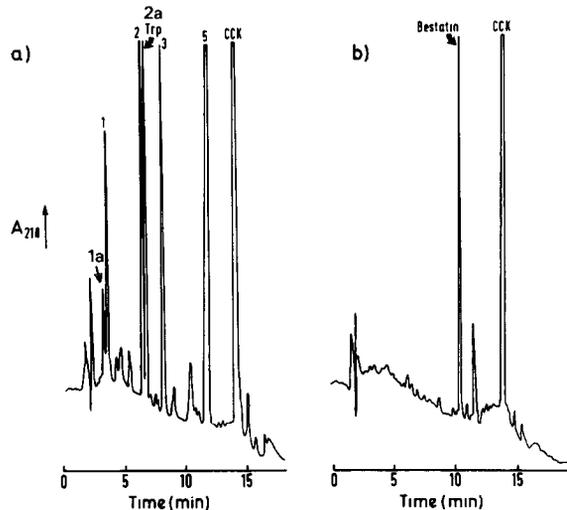


Fig. 3. Hydrolysis of CCK-8 by synaptic membranes. The products were analysed by HPLC, monitored at 214 nm. (a) No inhibitors; (b) incubation in the presence of 100  $\mu$ M bestatin and 1  $\mu$ M phosphoramidon.

1–3 and 5 were very substantially inhibited, phosphoramidon had relatively little effect on product 1a (not defined) and product 2a (tryptophan). Bestatin had no significant effect on peaks 1–3 and 5, but abolished the release of the tryptophan and product 1a. Combination of both inhibitors prevented detectable hydrolysis of CCK-8, as shown in fig. 3b. Specific inhibitors of peptidyl dipeptidase A (EC 3.4.15.1, angiotensin-converting enzyme), namely captopril and MK422, and a general inhibitor of serine peptidases, diisopropyl fluorophosphate, had no appreciable effect.

## 4. DISCUSSION

### 4.1. Specificity of endopeptidase-24.11

Only two bonds in sulphated CCK-8, Asp<sup>7</sup>-Phe<sup>8</sup>NH<sub>2</sub> and Gly<sup>4</sup>-Trp<sup>5</sup>, were hydrolysed. The former was cleaved about 4-times faster than the latter. No peptides generated by an attack at the Trp<sup>5</sup>-Met<sup>6</sup> bond were observed at either early or late stages in the degradation of the peptide. There is, therefore, a discrepancy between our results and those of authors in [3] regarding the site of attack. Different species, different regions of the brain and different degrees of purity of the enzyme may possibly have contributed to the discrepancy. But

Table 2

The effect of inhibitors on the hydrolysis of CCK-8 (sulphated) by pig striatal synaptic membranes

Peak	Retention time (min)	Peptide/amino acid	Effect of inhibitors on peak area (control (no inhibitors) = 100 for each peak)					
			Phosphoramidon (P)	Bestatin (B)	P + B	Captopril	MK422	DipF
1a	3.22	n.d.	87	0	0	87	95	81
1	3.52	Fa	0	91	0	90	94	91
2	6.28	D-Y-M-G	0	94	0	93	93	103
2a	6.56	W	43	8	0	95	95	99
3	7.90	W-M-D	8.5	101	0	99	98	101
5	11.54	D-Y-M-G-W-M-D	11	99	5	98	97	95

The inhibitors were added at the start of the incubation period, except for diisopropyl fluorophosphate (DipF) which was preincubated with membranes for 30 min at 20°C. The concentrations were phosphoramidon, 1  $\mu$ M; bestatin, 100  $\mu$ M; captopril, 1  $\mu$ M; MK422, 1  $\mu$ M; DipF, 100  $\mu$ M. The numbered peaks correspond to those shown in fig.1 and table 1. In the absence of any inhibitors, 8% of the substrate was hydrolysed under the conditions used, to a specific activity of 1.0 nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup> protein (n.d., not defined)

a more likely explanation is that the monitoring system used by these workers registered only tryptophan-containing peptides. Hence, the principal product (PheNH<sub>2</sub>) and the N-terminal tetrapeptide (Asp-Tyr(SO<sub>3</sub>H)-Met-Gly) would have escaped detection. Their main product might correspond to peak 5 (Asp-Tyr(SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp) but it is difficult to explain why the sequence analysis stopped short of Met<sup>6</sup>-Asp<sup>7</sup>. Their minor product might be similar to our peak 3 (Trp-Met-Asp). In the 3 methionine-containing peptides that we have used as substrates for pig endopeptidase-24.11 (CCK-8 and the two extended [Met] enkephalins), the -X-Met- bond has resisted hydrolysis. Furthermore, although PheNH<sub>2</sub> was rapidly released from CCK-8, MetNH<sub>2</sub> was not a product from either substance P [5] or [Met<sup>5</sup>] enkephalinamide [6].

#### 4.2. Identity of the synaptic membrane peptidases hydrolysing CCK-8

Phosphoramidon is a specific inhibitor of endopeptidase-24.11 [8]. Our results agree with those of authors in [3] that the release of peptide products was effectively inhibited by this reagent. However, the formation by synaptic membranes of

other products absorbing at 214 nm (peaks 1a, 2a, table 2) was only partially inhibited, because of the successive removal of N-terminal residues by bestatin-sensitive aminopeptidase action. One of these amino acids was identified as tryptophan. The other product which absorbed at 214 nm differed in retention time from tyrosine and probably represents tyrosine sulphate. More tryptophan was produced when the endopeptidase was acting, since the hydrolysis of the Gly<sup>4</sup>-Trp<sup>5</sup> bond exposed that residue to attack by aminopeptidase. The combination of phosphoramidon and bestatin was necessary to block all degradation of CCK-8.

In these experiments, the relative contributions of endopeptidase-24.11 and aminopeptidase activity in the degradation of CCK-8 were assessed by the disappearance of substrate. Endopeptidase activity in the striatal membranes appeared to account for about 80% of the degradation. Since the removal of the C-terminal PheNH<sub>2</sub> residue, which is the main site of the endopeptidase attack, is known to inactivate CCK-8 [9], endopeptidase-24.11 may play a major role in its inactivation in vivo in brain regions such as striatum that are relatively enriched in the enzyme. The release of PheNH<sub>2</sub> in two other natural peptides, gastrin and

Phe-Met-Arg-PheNH<sub>2</sub> may also be an important factor in their biological inactivation at cell surfaces.

#### ACKNOWLEDGEMENTS

We thank the Medical Research Council for support.

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