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SPECIFIC CLEAVAGE OF BETA-LPH AND ACTH BY TONIN: RELEASE OF AN OPIATE-LIKE PEPTIDE BETA-LPH (61-78)

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SUMMARY. Tonin, a rat enzyme capable of cleaving angiotensinogen, the tetradecapeptide renin substrate and angiotensin I directly to angiotensin II is also shown to cleave beta-lipotropin into beta-LPH 1-50, 1-51, 51-60, 52-60, 61-78 and 79-91, thereby selectively releasing the opiate-like segment beta-LPH 61-78. Its action on ACTH was similar, releasing ACTH 1-8, 1-7, 3-8, 3-7 and 9-39. In both situations the cleavages are of a selective tryptic-chymotryptic type at specific arginine, phenylalanine residues. Comparison of the tonin cleavage with those of trypsin, trypsin in combination with citraconylation of the lysine residues of beta-LPH is made. The data presented show that tonin does not cleave Met-enkephalin and can be used as an enzyme to study the presence of endorphin-like sequences in polypeptides.

INTRODUCTION

Since the discovery of the brain enkephalins by Hughes et al. (1) and the observation of their sequence relationship with the pituitary polypeptide beta-lipotropin (2) comprising within its C-terminal segment the complete sequence of beta-endorphin, a number of investigators turned their attention to the nature of the enzyme(s) involved in the maturation of beta-LPH into beta-endorphin and its fragments. Graf et al. (3) showed that a crude homogenate of porcine anterior pituitary gland contains an endopeptidase capable of cleaving beta-LPH 1-91 specifically between residues Leu_{77} - Phe_{78} yielding beta-LPH 1-77 and beta-LPH 78-91 at an

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optimum pH of 6.5. The pH, however, was found to influence greatly the action of the pituitary homogenate on beta-LPH (4). Thus at pH 8, three other main fragments could be identified, namely beta-LPH 1-46, beta-LPH 1-60 and beta-LPH 1-79 involving cleavages by a trypsin-like enzyme. These authors also showed that at pH 8 the activating enzyme could only be detected in pars distalis and pars intermedia of the pituitary gland and not in pars nervosa and other brain areas (5). In another approach, Austen and Smyth (6) demonstrated that renin, chymotrypsin, Armillaria mellae protease and trypsin under certain mild conditions of digestion could specifically release beta-LPH 61-77, beta-LPH 61-78 and beta-LPH 61-79 from beta-endorphin (beta-LPH 61-91). However, the action of these endopeptidases under similar conditions on beta-LPH 1-91 itself was not investigated.

Recently, we have been working on the primary sequence of a new enzyme, tonin, isolated and purified from the rat submaxillary gland (7,8). This enzyme is capable of cleaving either angiotensinogen, the tetradecapeptide renin substrate or the decapeptide angiotensin I to yield the octapeptide angiotensin II directly. It was thus suggested that tonin may play an important role in the local generation of angiotensin II in tissue. This enzyme was subsequently shown to be a serine protease and showed extensive N-terminal sequence homology with most serine proteases of the trypsin-chymotrypsin family (9). Its selectivity of cleavage, however, was not fully investigated but it seemed to be less wide than these endopeptidases (9). This property of tonin along with its high concentration in the pituitary gland (10) led us to investigate its enzyme properties on pituitary proteins.

In this paper, we report on the preliminary investigation of the cleavage pattern of tonin on biologically important molecules such as beta-LPH, ACTH and methionine-enkephalin. It is shown that although

tonin affords selective cleavages on beta-LPH and ACTH, methionine-enkephalin is resistant to its action. Furthermore, it is demonstrated that tonin can be effectively used in an enzymatic assay for the presence of endorphin-like sequences in polypeptides using a radioreceptor assay on the released fragments of beta-LPH. The comparison of this assay procedure with that using trypsin alone or in combination with citraconylation of the lysine residues of beta-LPH (11) is also evaluated.

MATERIALS AND METHODS

The isolation, purification and partial N-terminal sequencing of rat tonin has already been described (7-9). Ovine beta-LPH and ACTH were purified to homogeneity in our laboratory (2). For digestion of test peptides tonin in 0.1 M K_2HPO_4 pH 6.8 was added to the peptide in the same buffer and digestion continued for 8 hr or 24 hr at 37°C.

The ovine beta-LPH digest was purified on a carboxymethyl cellulose column (1 x 10 cm) eluted with a concave gradient previously use to the purification of beta-LPH itself (2,12). The ACTH digest with tonin was mapped on a Whatman 3M paper using chromatography (butanol:aceti acid:H_2O 4:1:5) followed by high voltage electrophoresis (formic acid: acetic acid:H_2O 1:4:45, pH 2.0 at 2 KV for 1.5 hrs) as previously describe (13).

Unless otherwise stated amino acid analysis was performed following 24 hr hydrolysis in 5.7 M HCl at 110°C on a Beckman 120C amino acid analyzer, according to Spackman et al. (14).

For peptide mapping of a portion (200 ug) of the ACTH digest with tonin by high performance liquid chromatography, a Waters u-bondapak C18 column (0.4 x 30 cm) was used. It was eluted with a linear CH_3CN gradient from 5% to 40% in 38 min at a flow rate of 1 ml/min using an original eluant of 0.02 M triethylamine phosphate buffer (TEAP) pH 3.0 (15). The position of ACTH 1-8 is obtained using a synthetic ACTH 1-8 peptide (kindly supplied to us by Dr S. St-Pierre of the University of Sherbrooke, Québec, Canada), and those of ACTH (9-39) and (1-7) were obtained after collection of the peaks followed by hydrolysis and amino acid analysis.

Specific opiate binding assay was performed on membrane fraction from rat brain homogenate prepared as described by Pasternak et al. (16) with slight modifications. Briefly, rat brain homogenate was incubated in 0.4 ml of 0.025 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 7.6, containing 1 x 10^{-9} M ^{3}H-Naloxone (NEN), 10 ug of bacitracin (Sigma) and test peptides at various concentrations for 30 min at 25°C. Incubation was stopped by addition of 3 ml of ice cold HEPES buffer and centrifuged at 1000 x g for 30 min. The supernatant was decanted and the pellets dissolved with 0.2 ml of 2 N KOH and the content of radioactivity measured with a Beckman liquid scintillation counter. Logarithmic dilutions of test peptides were added to the incubation solution and their inhibitory potencies were compared with ovine beta-endorphin purified in this laboratory (17). For each run, samples without peptides were included as control, and the inhibitory

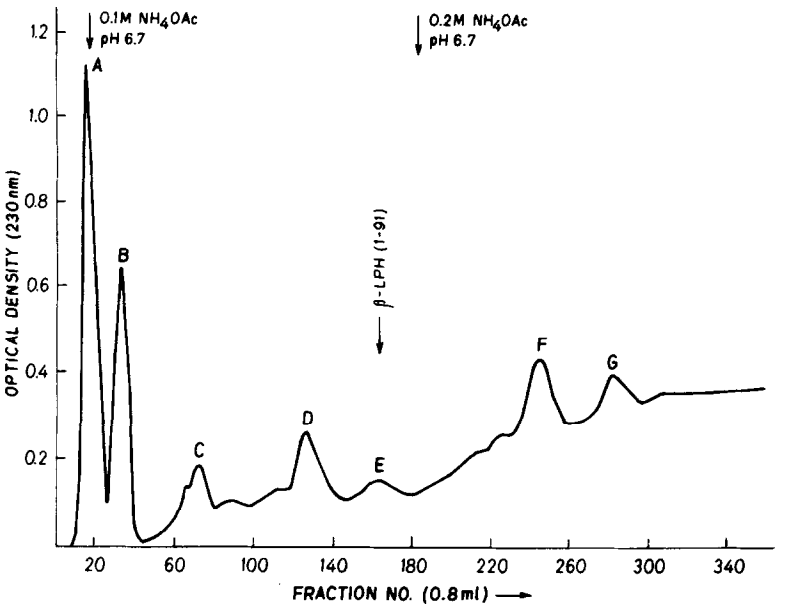


Figure 1. Carboxymethylcellulose (CMC) chromatography of the 8 hr digest of beta-LPH (8 mg) with tonin at an enzyme to substrate ratio of 1:200. The column (1 x 10 cm) was first eluted with 0.01 M NH_4OAc pH 4.6. At tube 20, a concave gradient to 0.1 M NH_4OAc pH 6.7 was initiated and at tube 180 the gradient was changed to 0.2 M NH_4OAc pH 6.7 (2,12).

effect of a peptide displacing the radioactive naloxone was expressed as percent of the control. Each peptide was tested in duplicate at least for three different concentrations.

RESULTS

A. Beta-LPH - Tonin digestion

(a) Chemical identification of cleavage pattern

Preliminary results on the possible cleavage of beta-LPH by tonin showed that cleavage did occur at enzyme to substrate ratio of 1:10, 1:50, 1:100 and 1:200 at the pH optimum of tonin of 6.8. It was also observed that at the enzyme:substrate ratio of 1:200, beta-LPH was degraded 23%, 90% and 95% after 20 min, 60 min and 480 min of incubation. It was thus decided to carry out an 8 hrs digest for analysis of the released peptides. The peptides were isolated by carboxymethyl (CM)-cellulose chromatography (CMC). In Fig. 1, is presented the CM-cellulose chromatography of the digestion mixture on

TABLE I. AMINO ACID COMPOSITION OF PURIFIED PEPTIDES OF 8 HR DIGEST OF OVINE BETA-LPH WITH TONIN AT TONIN:BETA-LPH RATIO OF 1:200.

CMC PEAK	A	B	C	D	F	G
Beta-LPH Fragment	61-78	1-50	1-51	52-60	51-60	79-91
Amino Acid						
Lys	0.99	2.63	2.91	1.97	1.82	4.1
His		0.75	0.91			1.09
Arg		2.66	4.35	1.22	1.87	
Asp		1.06	1.26	0.84	0.93	2.19
Thr	2.2	0.97	0.94			
Ser	1.53	1.84	1.86	0.79	0.78	
Glu	2.30	12.04*	11.14*			0.77
Pro	1.04	1.92	1.94	2.04		
Gly	1.74	4.06	3.9	0.96	0.93	0.87
Ala		10.84	10.39			2.09
Val	0.87	1.02	1.14			
Met	0.75	0.63	0.79			
Ile						1.05*
Leu	1.85	3.99	3.98			
Tyr	0.79	1.75	1.82			
Phe	1.47	0.74	0.88			

* Hydrolysis for 72 hrs revealed the presence of two residues of Ile and 13 residues of Glu.

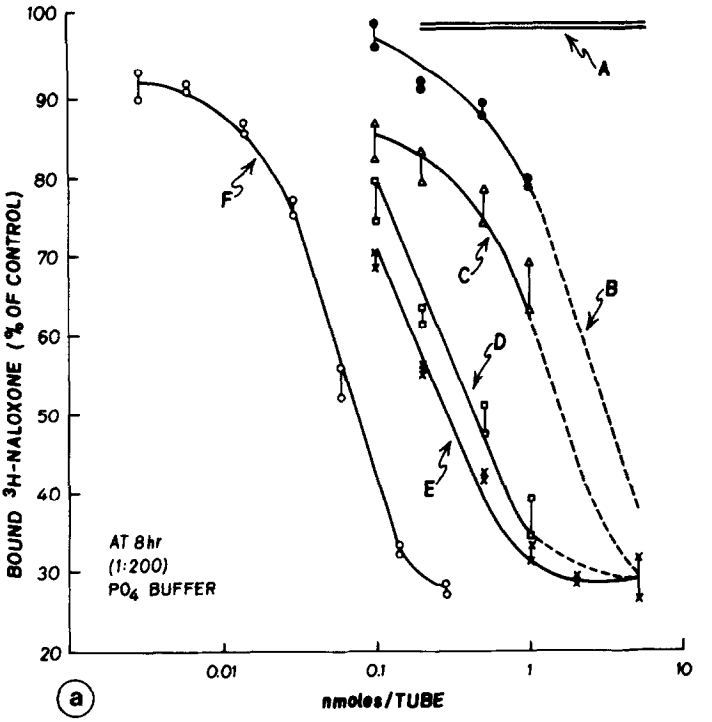
** Peak E on the CMC (See Fig. 1) is $beta_o$-LPH (1-91).

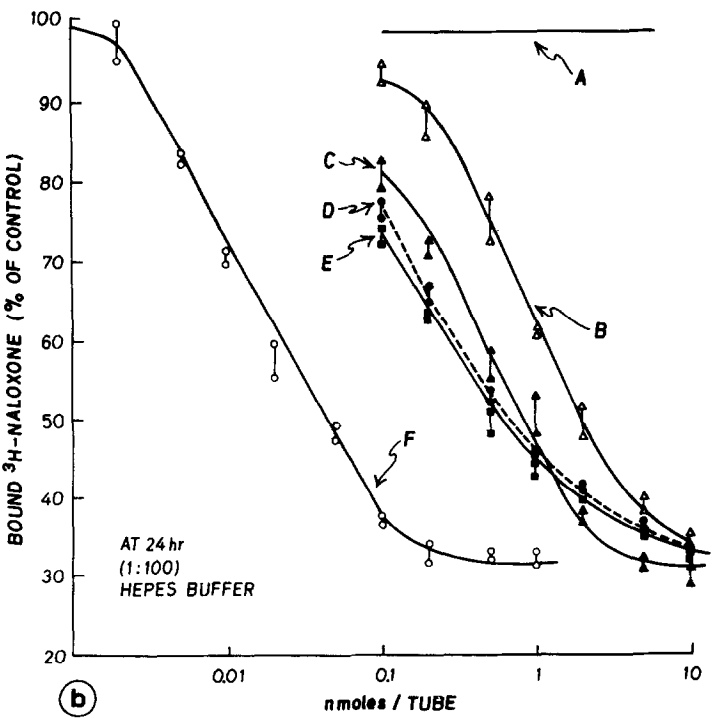
8 mg of beta-LPH after 8 hrs of incubation. The material in these peaks was further purified by a combination of Sephadex G-50 chromatography (1 x 100 cm) and another CM-cellulose column (1 x 10 cm) under the same conditions. Each purified peptide was analyzed on the amino acid analyzer as shown in Table 1. It is seen that the main cleavage occured between $Phe_{50} \downarrow Arg_{51} \downarrow Trp_{52}$, $Arg_{60} \downarrow Tyr_{61}$ and $Phe_{78} \downarrow Lys_{79}$, hence exhibiting selective arginine and phenylalanine cleavages of the

tryptic-chymotryptic type, thereby yielding beta-LPH 1-50, 1-51, 52-60, 51-60, 61-78 and 79-91 peptides. These results could be reproduced at a tonin:beta-LPH ratio as high as 1:25 and show that tonin does not cleave significantly at any of the lysine residues of beta-LPH. It is also seen that only selective arginine and phenylalanine residues are susceptible to tonin attack while others are resistant.

(b) Radioreceptor assay

The release of beta-LPH 61-78 peptide from beta-LPH 1-91, should be accompanied by an increase in opiate-like activity since the intact sequence of Met-enkephalin, necessary for opiate activity (11), is present at its NH_2-terminus. It should thus be possible to monitor such a release by an increase in opiate binding following tonin digestion. Furthermore, the met-enkephalin sequence, beta-LPH 61-65, should be resistant to the action of tonin, otherwise loss of activity will inevitably occur. That this is indeed the case is shown in Figures 2a and 2b. In Fig. 2a, a comparison is made between the increase in opiate-binding as beta-LPH is digested either with tonin, or trypsin, i.e. comparison of the efficiencies of binding of the peptides beta-LPH 61-78 and beta-LPH 61-69 respectively expected to be released by these procedures. It is seen that a displacement of 50% of radioactive naloxone is obtained with about 50 picomoles of native beta-endorphin (range 35-75 picomoles) whereas native beta-LPH, the purported precursor of Met-enkephalin and of the endorphins required at least 5000 picomoles (100 folds more than beta-endorphin). From Fig. 2a, the increase in potency as beta-LPH is digested with tonin is about 7-10 folds, whereas similar digestion with trypsin increases it only two folds. Thus it is apparent from Fig. 2a that the tonin:beta-LPH system is about 5 times as sensitive as the trypsin assay for the detection of the presence of an endorphin-like sequence in polypeptides. Citraconylation of beta-LPH followed by trypsin treatment and decitraconylation (11,12) should


BOUND ^{3}H-NALOXONE (% OF CONTROL)
100
90
80
70
60
50
40
30
20
0.01
0.1
1
10
nmoles/TUBE
A
B
C
D
E
F
AT 8hr
(1:200)
PO$_4$ BUFFER
a


BOUND ^{3}H-NALOXONE (% OF CONTROL)
100
90
80
70
60
50
40
30
20
0.01
0.1
1
10
nmoles / TUBE
A
B
C
D
E
F
AT 24 hr
(1:100)
HEPES BUFFER
b

release beta-endorphin. However, the need of at least one chromatographic step (11) where losses can occur make this procedure only twice as sensitive as the direct tonin assay, especially when low quantities of material are available. The critical test of the stability of the Met-enkephalin sequence is shown in Fig. 2b, where no loss of opiate binding activity is seen upon digestion of Met-enkephalin with tonin for as long a period as 24 hrs at an enzyme:substrate ratio of 1:100. It is also seen that about 500-600 picomoles of Met-enkephalin are required in order to displace 50% of the ^{3}H-naloxone, i.e. a 10 fold higher amount than beta-endorphin.

B. ACTH - Tonin digestion

When 2 mg of purified pituitary ovine ACTH (18) was digested with 10 ug of tonin (200:1) for 8 hrs at pH 6.8 and the digestion mixture analyzed by peptide mapping the pattern in Fig. 3 was obtained. The material in each spot was eluted individually and analyzed after hydrolysis on the amino acid analyzer as shown in Table 2. The major cleavage fragments were ACTH 1-8, 3-8, 1-7 and 3-7 and 9-39 involving cleavages at Tyr_2 - Ser_3, and Phe_7 - Arg_8 - Trp_9. The last cleavage was expected as ACTH and beta-LPH share a common heptapeptide sequence namely ACTH 4-10 and beta-LPH 47-53. These cleavages are again of a

Figure 2. Radioreceptor assay of enzymatic release of opiate-like fragments from beta-LPH.

Fig. 2a. Tonin digestion was performed in phosphate buffer 0.1 M pH 6.8 and trypsin digestion in NH_4HCO_3 pH 8.2 at an enzyme to substrate ratio of 1:200 for 8 hr at 37°C.

A - digestion buffer (phosphate or bicarbonate)
B - beta-LPH control
C - beta-LPH - trypsin digest
D - beta-LPH - tonin digest
E - citraconylated beta-LPH - trypsin digest (11,12)
F - beta-endorphin standard curve

Fig. 2b. Tonin digestion was performed in HEPES buffer pH 6.8 at an enzyme to substrate ratio of 1:100 for 24 hr at 37°C.

A - digestion buffer
B - beta-LPH
C - beta-LPH - tonin digest
D - Met-enkephalin standard curve
E - Met-enkephalin - tonin digest
F - beta-endorphin standard curve

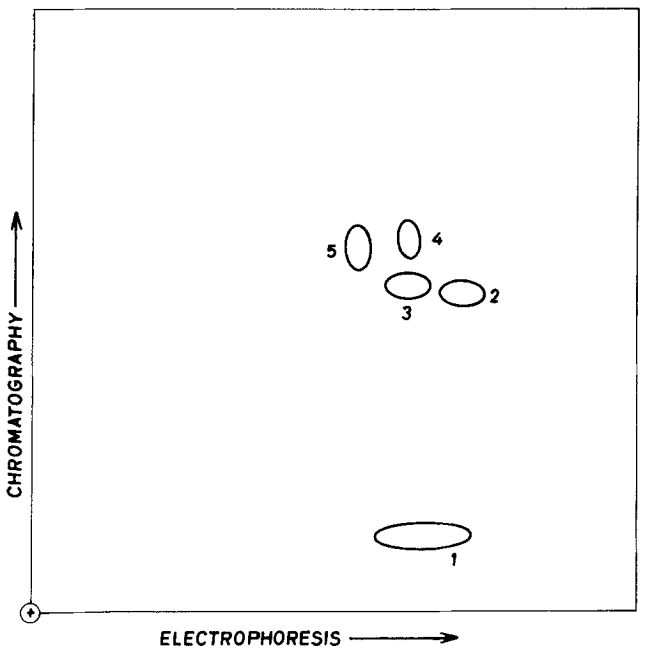


Figure 3. Peptide map of ACTH 24 hr digest with tonin at an enzyme to substrate ratio of 1:200.

selective chymotryptic-tryptic type, and no significant cleavage at any of the 4 lysine residues occured. The monitoring of the release of ACTH 1-8, 9-39 and 1-7 was also made possible by using the high-performance liquid chromatography for separation of these peptides as shown in Fig. 4. It is seen that the ACTH cleavage is complete, since the peak eluting at the ACTH position has disappeared. The resolution in this system is quite comparable if not better than the two dimensional peptide mapping method shown in Fig. 3. Clearly except for the cleavage at Tyr 2 of the ACTH molecule, very similar cleavage patterns are obtained in both the beta-LPH and ACTH systems.

DISCUSSION

The preliminary study of the selectivity of cleavage by tonin, on beta-LPH, ACTH and met-enkephalin has been presented. The obvious

TABLE 2. AMINO ACID COMPOSITION OF 8 HR DIGEST OF OVINE ACTH WITH TONIN, AT TONIN:ACTH RATIO OF 1:200.

SPOT NUMBER	1	2	3	4	5
ACTH fragment	9-39	3-8	1-8	3-7	1-7
Amino Acid					
Lys	3.13				
His		1.01	0.97	0.90	1.01
Arg	2.02	1.08	1.01		
Asp	2.04				
Thr	1.11				
Ser	4.13	0.74	1.79	0.74	1.92
Glu	4.16	1.29	1.19	1.17	1.23
Pro	3.07				
Gly	3.02				
Ala					
Val	2.9				
Met		0.79	0.83	0.44	0.64
Ile					
Leu	0.81				
Tyr	0.92		1.16		1.03
Phe	1.72	1.24	1.19	0.99	1.07
Trp	1*	-	-	-	-
Yields**	15%	11%	13%	1%	5%

* This spot is the only one which gave a positive reaction with the Erlich reagent (20).
** The yields are calculated on the basis of 2 mg ACTH digested with tonin. They are not corrected for losses during peptide extraction with 1M acetic acid from the Whatman 3M paper.

potential use of such a selective enzyme in the study of molecules susceptible to contain the active endorphin sequences within their primary structure has been investigated using a sensitive radioreceptor assay. The importance of this work lies in the fact that beta-LPH is selectively cleaved into beta-LPH 61-78. A similar peptide was found to be released

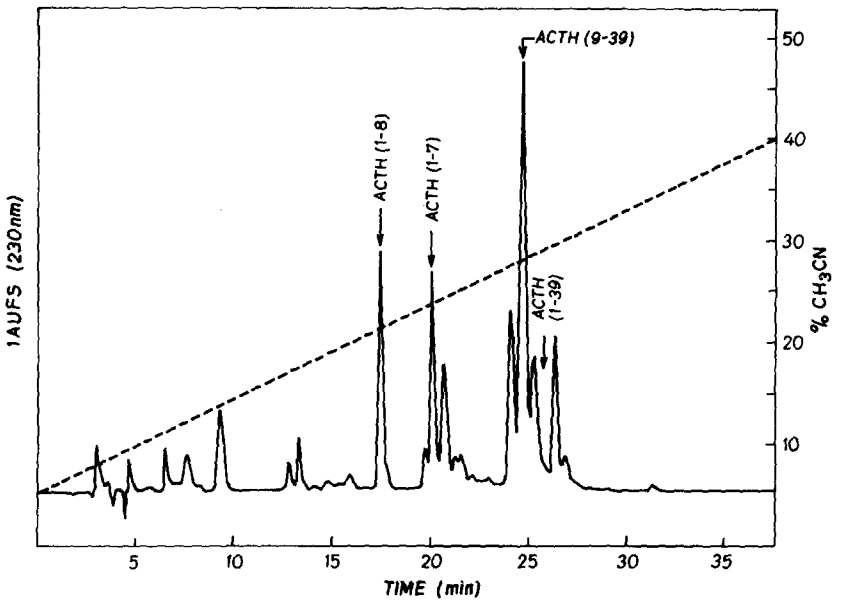


Figure 4. High performance liquid chromatographic separation of the peptides released in the digestion of ACTH with tonin. The load was 200 ug of the digest.

by Austin & Smyth (6) using chymotrypsin only at very low 1:500 enzyme:substrate ratio on beta-endorphin, but no such study was undertaken on beta-LPH and furthermore at high concentration chymotrypsin cleaves Met-enkephalin. In our search for the endogenous enzyme involved in the maturation of beta-LPH into its segments we looked into the tonin effect on this molecule. This work was all the more valuable since preliminary studies using both radioimmunoassay on tonin (19) and the fluorimetric detection of cleavage of angiotensin I to angiotensin II (8) showed that tonin is indeed present in large amounts in rat pituitary extracts in more than 1000 fold concentration than in whole brain (10).

From the data presented above, it thus appears that tonin, an enzyme which is able to split angiotensinogen into angiotensin II directly is also able to cleave beta-LPH into a fragment with opiate-like activity. As important is the resistance of Met-enkephalin to tonin digestion. These results raise the question whether tonin might

be one of the maturation or catabolic enzymes of beta-LPH and ACTH. We are now investigating this possibility and measuring the relative presence of this enzyme in various tissues.

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