SUBSTRATE SPECIFICITY OF THE ENZYME TONIN: CLEAVAGE OF SUBSTANCE P

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1. Introduction

Tonin is a novel peptidase isolated and purified from the rat submaxillary gland [1,2]. It is capable of cleaving either angiotensinogen, the tetradecapeptide renin substrate, or angiotensin I to yield directly the octapeptide angiotensin II by the splitting of a phenylalanyl peptide bond. It is believed [2] that tonin plays an important role in regulating peripheral vascular tone by controlling the formation of angiotensin II which is the most active known vasoconstrictor substance. Tonin was also shown to differ from angiotensin I converting enzyme, renin and tissue isorenin [2].

This enzyme has been purified to homogeneity and found to be a polypeptide containing 272 amino acids with a MW of 28 700 [2,3]. N-terminal sequence analysis has permitted the identification of 34 of the first 40 residues [3] and revealed an extensive homology with the sequence of many serine proteases of the trypsin-chymotrypsin family.

We have recently demonstrated that tonin, which is present in the pituitary gland and in the brain [4], is capable of cleaving beta-lipotropin (beta-LPH) and adrenocorticotropin (ACTH) at certain phenylalanyl and arginyl peptide bonds to produce a number of fragments, including an opioid-like material corresponding to portion 61–78 of beta-LPH [5].

The phenylalanyl cleavage sites of tonin with three different substrates (beta-LPH, ACTH and angiotensinogen) [2,5] and the occurrence of two phenylalanine residues at positions 7 and 8 in substance P, a powerful vasodilating agent [6] led us to investigate the activity of tonin on substance P, an unadecapeptide isolated and characterized by Leeman et al. [6].

2. Materials and methods

The isolation, purification and characterization of rat tonin from submaxillary glands has already been described [1–3]. Synthetic substance P was purchased from Peninsula Labs., San Carlos, CA. The peptide was dissolved in 0.1M K_2HPO_4 and digested at 37°C for different lengths of time at a variety of pHs.

The C-terminus directed antiserum [8] showed a complete cross-reactivity with all the biologically active C-terminal fragments of substance P down to fragments 7–11, while the N-terminus directed antiserum [7] cross-reacted only slightly with fragments 2–11 and 3–11 and not at all with the other shorter C-terminal fragments of substance P. Using these two different antisera in conjunction, one may assess the approximate cleavage sites of enzymatic digestion of substance P.

In a first series of experiments, 20 μg of substance P in a total volume of 25 μl (0.6 mM) was digested with 2.5 and 5 μg of tonin (0.0035, 0.007 mM) and the degradation of substance P was followed by radioimmunoassays using two different antisera, one directed against the N-terminus [7] and the other against the C-terminus portion of the molecule [8]. Incubation at pH 7.5 for 8 h were the most appropriate conditions for digestion. These conditions were used for the second series of experiments in which larger amounts of substance P (250 μg) were added, using the same substrate-enzyme ratio. The peptides obtained were separated by high performance liquid chromatography using a Waters Associates instrument, equipped with a Model 450 UV detector. The sample was applied on a Waters u-Bondapak C_{18} (3.9 × 30 cm)

column equilibrated with 5 : 95 (v/v) acetonitrile/10 mM ammonium acetate, pH 4.0 and eluted with a 20 min linear gradient from 5 to 65% acetonitrile in 10 mM ammonium acetate, pH 4.0 at a flow rate of 2 ml/min. The position of substance P was identified with the same synthetic material used for the digestions.

The peaks obtained were collected, dried and analyzed for their amino acid composition following acid hydrolysis in 5.7 N of HCl at 110°C for 24 h.

3. Results

The disappearance of substance P immunoreactivity upon incubation at 37°C was both time- and pH-dependent. As shown in table 1, the more suitable pH being between 7.5 and 8.5. The time course of digestion of substance P at pH 7.5 revealed that an 8 h digestion degraded about 60% of substance P. Incubation of tonin alone under identical conditions did not give rise to any substance P like material.

Since both N-terminal and C-terminal directed substance P radioimmunoassay results were comparable, there was no indication of any accumulation of C-terminal fragments longer than 7–11, and the phenylalanyl bonds were the most likely cleavage sites.

This assumption was verified in the second series of experiments in which 250 μg of substance P was digested for 8 h at pH 7.5 at an enzyme-substrate molar ratio of 1/80. Tonin degraded substance P into 4 major peptide fragments which were separated by HPLC (fig.1A). Peaks 1, 2, 3, 4 were analysed for their amino acid composition. Peak 5 corresponds to standard substance P, while peak 6 co-elutes with the enzyme (fig.1B).

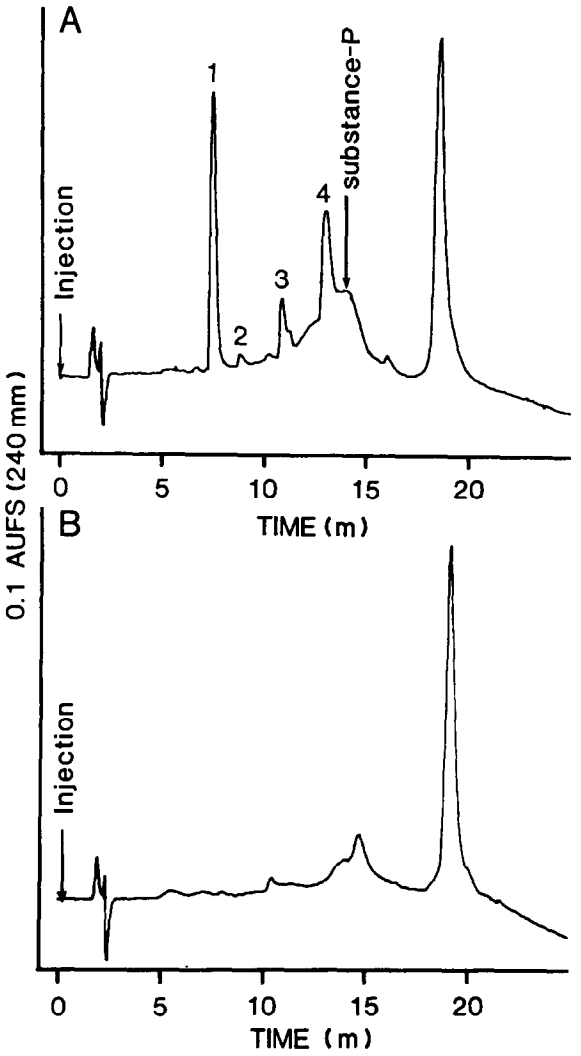


Fig.1(A) High performance liquid chromatographic separation of the peptides released in the digestion of substance P with tonin. The load was 250 μg dissolved in 50 μl of water; (B) Control digestion of tonin without substance P.

Table 1
Percentage of substance P remaining (± SEM)

pH enzyme/substrate ratio	N-terminal antiserum		C-terminal antiserum	
	1/160	1/80	1/160	1/80
4.0	119 ± 13	68 ± 5	129 ± 10	84 ± 4
5.0	141 ± 16	45 ± 1	116 ± 8	58 ± 13
6.8	3 ± 1	0.9 ± 0.0	6 ± 0.7	1 ± 0.0
7.5	0.00 ± 0.0	0.54 ± 0.3	2.24 ± 0.1	0.3 ± 0.0
8.5	0.4 ± 0.4	0.0 ± 0.0	0.19 ± 0.1	0.3 ± 0.1

Degradation of substance P (20 μg, 0.15 mmol) by rat tonin incubated for 24 h at 37°C. Each value is a mean of 4 determinations

Table 2
Amino acid composition of purified peptides isolated by HPLC after an 8 h digest of substance P (250 μg) with tonin at enzyme-substrate ratio of 1 : 80 at 37°C and pH 7.5

HPLC peaks	1	2	3	4
Substance P fragment yield	1–7 (45.5 nmol)	9–11 (10.7 nmol)	1–8 (7.9 nmol)	8–11 (49.8 nmol)
Amino acid				
Glu	2.09		2.18	
Pro	1.97		1.69	
Gly	0.02	1.06	0.23[a]	1.02
Met		0.90		0.97
Leu		1.03		1.02
Phe	0.98		2.0	1.0
Lys	1.01		1.26	
Arg	0.96		0.85	

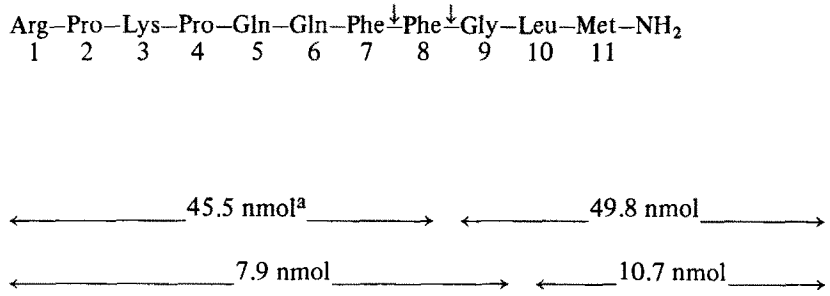
[a] Slightly contaminated by a glycine-containing peptide

The amino acid composition of peaks 1 to 4 is shown in table 2. P_1 is identical to fragment 1–7, P_2 to fragment 9–11, P_3 to fragment 1–8 and P_4 to fragment 8–11 of substance P. The yields of each peptide also reveal that tonin, under this condition, digests substance P at the two phenylalanyl peptide bonds, being 5 times faster on the Phe-Phe bond than on the Phe-Gly bond.

4. Discussion

Tonin was previously found to cleave its substrate preferentially at certain phenylalanyl peptide bonds in angiotensin, beta-LPH and ACTH [1,2,5] while it also cleaves certain arginyl bonds in both beta-LPH and ACTH [5]. There is an arginine residue at the N-terminus of substance P, but it is unlikely to be split by tonin because it is next to a proline residue. There is no indication that the arginine was attacked, since no free arginine could be detected in the digest. This result is comparable to the resistance of other peptide substrates with Arg Pro sequences to tryptic digestion including substance P [6].

However, the preference of tonin for phenylalanyl bond cleavage was also found with substance P, which was mainly degraded at phenylalanine 7 and to a lesser extent at phenylalanine 8. Thus tonin which is present in the blood [2] and in the brain [4], might be involved in the degradation of substance P. This is interesting since the same enzyme (tonin) might act



[a] Yields of peptides isolated from HPLC

Fig.2. Cleavage sites of substance P digested by tonin.

on the vascular tone by simultaneously generating a vasoconstrictor substance (angiotensin II) and degrading a vasodilator agent (substance P). It will be important to test the action of tonin on bradykinin, another circulating vasodilator factor which has phenylalanine at positions 5 and 8 of its amino acid sequence.

Although tonin has a chymotrypsin-like activity, it is not chymotrypsin since it does not split tyrosine, tryptophan and other phenylalanine residues in the substrates already tested, i.e. angiotensinogen, beta-LPH and ACTH [25].

Finally, the activity of tonin on neuropeptides and its presence in the central nervous system [4] makes it worthwhile to study its importance in brain function.

Acknowledgements

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