ENZYMATIC INACTIVATION OF SUBSTANCE P BY A PARTIALLY PURIFIED ENZYME FROM RAT BRAIN

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Summary: Substance P, a unidecapeptide with potent CNS action, is rapidly inactivated by rat brain homogenate with release of all amino acids including Met.NH_2. The inactivating enzyme present in the 100,000 g supernatant was purified on DEAE-cellulose and was eluted in parallel with a neutral endopeptidase degrading hemoglobin and histone. Breakdown was followed by measurement of amino acids released using a method capable of resolving Met.NH_2 and Leu-Met. NH_2. The preferential release of Phe and Leu indicated cleavage at two or more internal sites (-Gln^6-Phe^7- or -Phe^7-Phe^8- and -Gly^9-Leu^{10}-) with release of intermediate peptidyl products. The presence of free Met following inactivation resulted from the deamidation of Met.NH_2 after its liberation from Substance P. A slow release of Arg and Pro indicated that the Arg^1-Pro^2 bond is only slowly cleaved by brain aminopeptidases.

INTRODUCTION

Considerable interest has centered around Substance P in view of its function as a possible neurotransmitter in the spinal cord (1,2). A role for this peptide in neural transmission would necessitate a rapid inactivation at its site of action. Early studies have indicated that brain tissue inactivates Substance P, and an enzyme from brain was partially purified which appeared to possess such activity (3). However, these studies were performed before the purification of Substance P was achieved, and before its structure was established. Thus, the purity of the substrate used and the nature of the enzymes involved could not be defined.

Substance P has been isolated now in pure form from bovine hypothalamic extracts and shown to be a unidecapeptide characterized by a C-terminal methioninamide (4,5). As such it cannot be degraded by classical carboxypeptidases although it might serve as a substrate for novel C-terminal cleaving

peptidases recently found to inactivate other polypeptidyl amides such as oxytocin (6) and lutinizing hormone-releasing hormone (LH-RH) (7). Whole brain extracts were selected for this investigation since they are known to contain a spectrum of proteolytic enzymes some of which specifically inactivate hormonal releasing factors and also other active polypeptides (8).

MATERIAL AND METHODS

Substrates: Substance P (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met.NH_2) was supplied as a gift by Dr. Wilfrid F. White (Abbott Laboratories, North Chicago, Ill.), and also obtained from Peninsula Laboratories, San Carlos, Calif. Met.NH_2 and Leu-Met.NH_2 were obtained from Fox Chemical Co., Los Angeles, Calif. Denatured hemoglobin was obtained from Worthington Biochemical Corp., Freehold, N.J., and histone (Type II) from Sigma Chemical Co., St. Louis, Mo.

Purification: Rat brain from young male adults was homogenized in 10 vol. of cold 0.32 M sucrose containing 0.1 mM Cleland's reagent and then centrifuged at 100,000 g to yield a supernatant fraction. Since preliminary studies indicated that the major inactivating enzyme(s) were labile and required rapid processing, the supernatant fraction equivalent to only one g of brain (10 ml) was passed through a column of DEAE-cellulose, 25 x 2 cm, previously equilibrated with a 40 mM Tris-HCl buffer, pH 7.6, containing 0.1 mM Cleland's reagent. The protein was eluted with a discontinuous salt gradient containing the above buffer and Cleland's reagent, starting with 30 ml of 0.1 M NaCl and continued with 30 ml of 0.2 M NaCl and 0.3 M NaCl. The protein was monitored with a flow cell coupled to the DEAE-cellulose column with a maximum absorption at 254 nm, or protein was measured by the Folin-Lowry procedure (9). Aliquots from all fractions were tested for proteolytic activity using either hemoglobin or histone as a substrate.

Incubations. Appropriate aliquots of crude or purified fractions were incubated for different time periods with Substance P or the protein substrates. In routine experiments, 67 μg (50 nmoles) of Substance P were dissolved in 0.5-1.0 ml containing 0.2-0.4 ml of 40 mM Tris-HCl buffer, pH 7.6, and 0.15-0.5 ml of the different enzyme fractions. Incubations were carried out in duplicate with suitable incubated controls lacking the substrate. The reaction was terminated by the addition of an equal volume of 6% sulfosalicylic acid and centrifuged at 2,000 g for 10 min at room temperature.

For measurement of neutral proteinase activity, denatured hemoglobin or histone was used as a substrate. Hemoglobin, 5 mg in 0.8 ml of 40 mM Tris-HCl buffer, pH 7.6, and a suitable sample of enzyme were incubated for 60 min at 37 C, and then an equal volume of 6% sulfosalicylic acid was added to stop the reaction. When histone was used as a substrate, a solution containing 2 mg/ml in 40 mM Tris-HCl buffer, pH 7.6, was prepared and boiled for 30 min to remove endogenous histonase activity. An aliquot containing 1 mg of histone was then added to a suitable sample of enzyme in a total volume of 1 ml, and incubated for 60 min at 37 C. An equal volume of 6% sulfosalicylic acid was then added to stop the reaction. The mixture was then centrifuged, and the supernatant was assayed for ninhydrin-positive materials using an automated ninhydrin procedure as described previously (10).

Identification of products. Aliquots of deproteinized supernatants

from incubations with Substance P were subjected to quantitative amino acid analysis using the Technicon autoanalyzer and the lithium citrate buffer method of elution (11). In this system, in an eighteen hour run, Leu-Met.NH_2 can be detected as a peak occuring 2 hr after norleucine, with a color value relative to norleucine of 0.16. Met.NH_2 can be detected as a peak occuring 30 min before Arg, with a color value relative to norleucine of 0.67. Since polypeptidyl fragments gave very low color values, and did not interfere with the amino acid peaks, the appearance of free amino acids over background was used as an index for rates of inactivation.

RESULTS AND DISCUSSION

Incubation of 50 nmoles of Substance P with rat brain homogenate prepared in 0.32 M sucrose for one hour led to a release of 70-90% of the constituent amino acids (Table I). A similar rate of breakdown was observed

Table I: Cleavage of Substance P by Crude and Purified Enzyme from Rat Brain.

Fraction	Time (min)	nmoles per cent released								
		H-Arg[1]	Pro[2,4]	Lys[3]	Gln[5,6]	Phe[7,8]	Gly[9]	Leu[10]	Met.NH_2[11]	Met*
Homogenate	60	77	68	95	80	82	80	80	25	50
Supernatant	10	tr	0	0	0	8	0	9	8	0
	30	17	10	12	24	32	17	26	19	tr
	90	47	37	51	59	55	57	58	32	24
	180	70	52	74	69	77	73	81	21	50
DEAE-cellulose (0.2 M NaCl)	180	50	nd	60	55	70	44	72	52	10

*Free Met formed by deamidation of Met.NH_2.

The incubation mixture for brain homogenate (prepared with 10 vol of 0.32 M sucrose containing 0.1 mM Cleland's reagent) and the 100,000 g supernatant contained 50 nmoles of Substance P and 0.15 ml of crude enzyme (0.3 - 1.5 mg protein) in a volume of 0.5 ml containing 8 μmoles of Tris-HCl buffer, pH 7.6. For purified enzyme 0.5 ml of the fraction (0.1 mg protein) eluted from DEAE cellulose by 0.1 - 0.2 M NaCl (Fig. I) was incubated with 50 nmoles of Substance P. Incubations were at 37 C for the time periods indicated. The reaction was terminated by addition of an equal volume of 6% sulfosalicylic acid and the breakdown products determined on an amino acid analyzer by a method capable of resolving Met.NH_2 as described in the Methods section. Values represent the mean of two determinations.

tr = trace quantities nd = not determined.

also in the case of the 100,000 g supernatant. In a study of breakdown with time on incubation with the supernatant fraction, Phe and Leu were detected at 10 min, and Gln and Gly only at 30 min together with traces of Arg, Pro and Lys. In the case of Phe, its rapid release compared to the other residues points to a cleavage at an internal site, either at the -Gln^{6}-Phe^{7}- or -Phe^{7}-Phe^{8}-bond by an endopeptidase acting at pH 7.6; supernatant fractions are known to contain active aminopeptidases capable of liberating Phe from the cleaved products (12). Since brain carboxypeptidases are slower acting, this might explain the delayed appearance of Gln from the remaining fragment. Substance P contains an N-terminal Arg but its slow release indicates that the Arg^{1}-Pro^{2} bond is more slowly hydrolyzed as compared to the N-terminal bond of the cleaved fragments as confirmed also by the slower release of the adjacent amino acids Lys^{3} and Pro^{4}. Evidence for the action of a "neutral endopeptidase" was shown also by a bioassay method using the guinea pig ileum where inactivation preceeded the appearance of free amino acids (Benuck, Marks and Ehrenpreis, unpublished findings).

The release of the C-terminal Met.NH_2 and the penultimate Leu prior to the appearance of Gly indicates a cleavage at a second internal site since Substance P itself is not attacked by carboxypeptidases (2,4). Cleavage of the -Gly^{9}-Leu^{10}-bond would result in the release of Leu-Met.NH_2. The quantity of dipeptide released, however, is too small to detect as a split product owing to its low color value and its rapid hydrolysis by aminopeptidases present in the supernatant (Table II). The mechanism proposed is analogous to that responsible for release of C-terminal Leu-Gly.NH_2 from oxytocin by brain extracts, followed by its subsequent hydrolysis to release Gly.NH_2 (6). A similar mechanism may apply also to other polypeptidyl amides such as vasopressin and LH-RH (7). This was confirmed in separate experiments by incubation of Leu-Met.NH_2 with brain supernatant resulting in the release of Leu, methioninamide and Met. The dipeptide was

Table II: Cleavage of Leucine-methioninamide and Methioninamide by Rat Brain Supernatant.

Amide added	nmoles per cent released		
	Met	Leu	Met.NH_2
Leu-Met.NH_2	36	100	68
Met.NH_2	37	0	62

The incubation mixture contained 250 nmoles of each amide and 0.15 ml of an 100,000 g supernatant prepared from rat brain in a volume of 0.5 ml containing 8 μmoles of Tris-HCl buffer, pH 7.6. Incubations were carried out for 3 h at 37 C and terminated by the addition of sulfosalicylic acid to a final concentration of 3%. Cleavage products were determined by an autoanalyzer capable of detecting Met.NH_2 and Leu-Met.NH_2 as described in the Methods section.

cleaved within 3 hr with 100% release of Leu implying also a complete release of Met.NH_2 (Table II). Met.NH_2 is deamidated only slowly, 33% within 3 hr, providing evidence that release of Met occurred after liberation of the methioninamide. For Substance P itself a similar mechanism is evident since significant quantities of Met were found only at the later time periods (Table I). The enzyme(s) responsible for deamidation are probably aminopeptidases similar in specificity to leucine aminopeptidase for which monoacyl amides are used as substrates (13). Amides such as Phe.NH_2, Leu.NH_2 and Tyr.NH_2 are deamidated by crude brain extracts (14); Leu.NH_2 is deamidated by a purified brain aminopeptidase (15).

The studies reported above imply the action of a neutral proteinase as the major inactivating enzyme. Previously it was shown that brain neutral proteinases are highly labile and require rapid processing (16). Therefore, brain extracts were partially purified in small batches using a simple two step procedure as described in the Methods section. Fractions were monitored for neutral proteinase activity using histone or hemoglobin as the substrate,

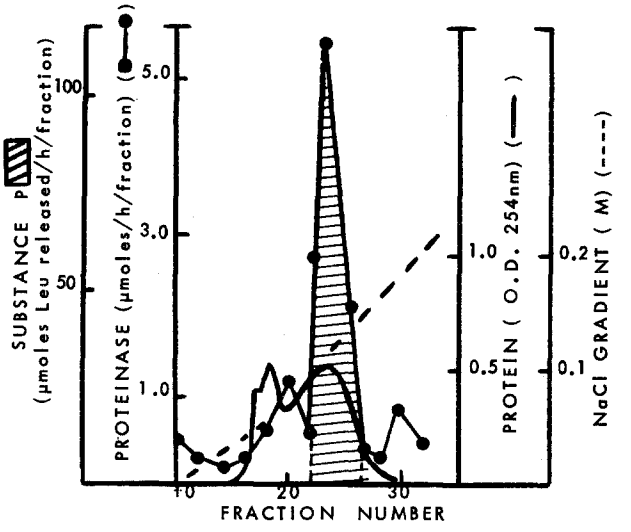

Figure 1. Separation of neutral proteinases (●—●) and enzyme inactivating Substance P (▨) from 10 ml of a 100,000 g supernatant fraction applied to a column of DEAE cellulose. Protein (——) was monitored in a flow cell with a maximum absorption of 254 nm. The column was equilibrated with a 40 mM Tris-HCl buffer, pH 7.6, containing 0.1 mM Cleland's reagent and 5 ml fractions were collected on elution with a NaCl gradient (- — —). The rate of Substance P inactivation was based on the release of Leu. Proteinase active at neutral pH was measured with histone as the substrate as described in the Methods section.

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and the active fractions were then tested with Substance P.

Chromatography of the supernatant fraction on DEAE-cellulose gave two protein peaks (Fig. I). The protein eluted with 0.1 - 0.2 M NaCl contained the enzyme inactivating Substance P which coincided with a neutral proteinase degrading hemoglobin or histone. The specificity of the purified enzyme, based on the appearance of free amino acids, was similar to that of the brain supernatant. After a three hour incubation with the purified fraction, the amino acids released in greatest amount were Phe and Leu, implying as with the brain supernatant two internal cleavage sites.

Purification using DEAE cellulose leads to a mixture of endo- and exopeptidases capable of acting sequentially on Substance P and its cleaved products with release of internal amino acids. The release of Leu was used,

Table III: Partial Purification of Substance P Inactivating Enzyme

Fraction	Protein	Substance P Inactivation	Specific Activity	Yield
	(mg)	(nmoles Leu/hr)	(nmoles Leu/mg pr/hr)	(per cent)
Homogenate	105	2660	25	100
Supernatant	25	1300	52	50
DEAE-cellulose 0.1 - 0.2 M NaCl	5	800	160	33

Enzyme was purified rapidly for reasons cited in the text using 10 ml of a 10% rat brain homogenate prepared in 0.32 M sucrose containing 0.1 mM Cleland's reagent as described in the Methods section. Substance P inactivation was determined from the quantity of Leu released as measured by amino acid analysis.

therefore, as a measure of inactivation. On this basis, the enrichment obtained after purification through DEAE-cellulose was 6 fold, compared to the whole homogenate, with a recovery of 33% (Table III).

This study represents the first to define the points of cleavage of the unidecapeptide Substance P by brain enzymes. The results with crude and partially purified enzyme show cleavage of two internal peptide bonds by a brain neutral endopeptidase; as such, this enzyme has similarities to that recently reported to inactivate LH-RH and bradykinin (7,17).

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