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A THIOL-ACTIVATED AMINOPEPTIDASE OF THE PITUITARY

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Animal tissues appear to contain at least two aminopeptidases which hydrolyze leucineamide. One is activated by Mn^{++} or Mg^{++} , inhibited by EDTA and unaffected by -SH reagents (Smith, 1955). Another aminopeptidase was detected by Fruton (1939) in beef spleen extracts and required activation by cysteine but further characterization of this enzyme has not been pursued. (NASA RP-55)

The Mn^{++} activated aminopeptidase was first observed in pituitary extracts by Adams and Smith (1951). As will be shown below, pituitary extracts also contain an aminopeptidase which is activated by -SH compounds and inhibited by sulfhydryl reagents. Moreover, the -SH activated aminopeptidase, in contrast to the Mn^{++} activated enzyme, hydrolyzes amino acid naphthylamides most rapidly in the following order: Arg > Lys > Phe > Leu > Ala > Tyr.

Pituitary extracts employed in these studies were obtained by centrifugation (30 min. at 6000xg) of a 20% homogenate which had been prepared by homogenization for 10 min. in distilled water and adjusted to pH 7.5. The clear red supernatants gave maximal hydrolysis of $10^{-3}M$ L-lysine-p-nitroanilide (Erlanger et al., 1961; Tuppy et al., 1962) at pH 7.5 in 0.1M Tris-HCl and 0.01M thioglycolate. The reaction rate was determined from the increase in absorbancy at 410 m μ due to the liberation of p-nitroaniline. The kinetics of the reaction were zero order and the rate of hydrolysis was directly proportional to extract concentration (0.01 to 0.08 ml. per 2.5 ml. of assay solution at 37°). The K_m was 3.6×10^{-4} as determined from substrate concentrations ranging from 1×10^{-3} to $10^{-4}M$. Equi-molar concentrations of phosphate, citrate, imidazole or maleate buffers inhibited the rate of hydrolysis by 30 to 40% as compared to Tris buffers. L-phenylalanine-p-nitroanilide was hydrolyzed at 12% of the rate of the lysine derivative while acetyl-Phe- and benzoyl-DL-Arg-p-nitroanilide were not hydrolyzed detectably. The lack of action on the latter two substrates suggests that the enzyme is an aminopeptidase.

The efficacy of different thiols in activating the enzyme at pH 7.5 is shown in Figure 1. It can be seen that a 3- and 5-fold increase in

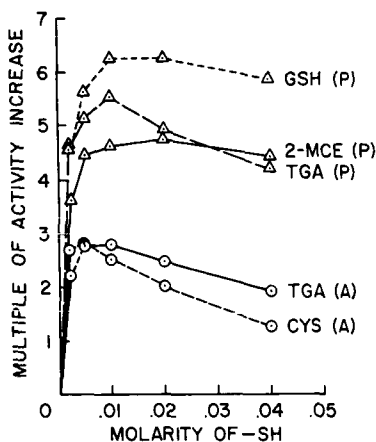


Fig. 1. Augmentation of L-lys-p-nitroanilide activity of bovine pituitary extracts by thiols. GSH, glutathione; 2-MCE, 2-mercaptoethanol; TGA, thioglycolate; Cys, cysteine; P, posterior; and A, anterior pituitary extracts. Assay solution: 1 ml. 2.5×10^{-3} M lys-p-nitroanilide, 1 ml. 0.25M Tris-HCl, 0.1 ml. sulfhydryl solution, and 0.4 ml. diluted extract containing 0.02 ml. of centrifuged extract from a 20% homogenate. Essentially the same results were obtained with porcine and ovine glands.

activity occurs with freshly prepared extracts from anterior and posterior lobes of bovine pituitaries, respectively. The un-activated extract rapidly deteriorates on storage at 5° for 24 hours to about 1/3 of the original activity as determined from assays without added thiol. Thereafter, the activity remains constant for about two weeks and can be fully reactivated with 0.01M thiols to the same levels as with freshly prepared extracts. The activity of the extracts is maximally stable in the range from pH 6.5 to 8 below 45° but can be completely destroyed in 15 min. at 60° . Thiol-activated aminopeptidase was also detected in brain, kidney, liver, spleen, and muscle, but not in thymus extracts.

The activity of freshly prepared extracts without added thiols is inhibited by various disulfides to the extent shown in Table 1. Glucagon at a concentration of 10^{-4} M causes a 50% inhibition, while soy-bean trypsin inhibitor is completely ineffective. N-ethyl maleimide (10^{-3} M) and p-chloromercuriphenyl sulfonate (10^{-5} M) inhibit the activity of crude extracts 79 and 87%, respectively. It is noteworthy that the inactivation of endogenous corticotropin during

Table 1. Inhibition of Lysine-p-nitroanilide Hydrolysis by Disulfides

| Extract | Molarity of Disulfide | Hydrolysis Rate Δ O.D. ₄₁₀ /min./ml. | % Inhibition |
|----------------------|------------------------|---|-----------------|
| <hr/> | | | |
| XXII-19 ¹ | | | |
| 0.05 ml. | None | 0.22 | 0 |
| | GSSG | | |
| | 1 x 10 ⁻³ | 0.11 | 50 |
| | GSSG | | |
| | 5 x 10 ⁻³ | 0.12 | 45 |
| | Oxytocin | | |
| | 1.2 x 10 ⁻⁵ | 0.06 | 73 |
| | Oxytocin | | |
| | 1.2 x 10 ⁻⁶ | 0.084 | 61 |
| <hr/> | | | |
| XXII-3 ² | | | |
| 0.02 ml. | None | 0.14 | |
| | Vasopressin | 0.085 | 39 |
| | 3 x 10 ⁻⁶ | | |
| 0.06 ml. | None | 0.14 | |
| | Vasopressin | 0.10 | 29 |
| | 3 x 10 ⁻⁶ | | |
| 0.10 ml. | None | 0.12 | |
| | Vasopressin | 0.095 | 21 |
| | 3 x 10 ⁻⁶ | | |
| <hr/> | | | |
| XXII-62 ³ | | | |
| 0.1 ml. | None | 0.16 | |
| | Formamidine | | |
| | disulfide | | |
| | 1 x 10 ⁻⁵ | 0.14 | 13 |
| | 1 x 10 ⁻⁴ | 0.04 | 75 |
| <hr/> | | | |

1, 2 Posterior and 3 anterior pituitary extract. The assay solutions were as indicated in Fig. 1 except for 0.1 ml. disulfide solution and 0.4 ml. of diluted extract containing the indicated amount of undiluted extract. GSSG: oxidized glutathione.

incubation of pituitary homogenates is also inhibited by the octapeptide disulfides and glucagon (Barrett and Sayers, 1958).

In the absence of added thiols, all metals tested, with the exception of Mg, Ca and Fe⁺⁺ showed a strong inhibition with both undialyzed extracts and with extracts dialyzed against EDTA (10⁻⁴M)

followed by water. The range of inhibition by divalent metals at $10^{-3}M$ was as follows: Mn, 50%; Co, 40%; Hg, 80%; Cu, 90%; Zn, 95%. Fe^{+++} ($10^{-3}M$) caused a 70% inhibition whereas Fe^{++} ($10^{-4}M$) increased the activity to 115%. EDTA (10^{-3} and $10^{-4}M$) when pre-incubated with the buffered extract prior to the addition of the substrate caused a logarithmic loss of activity amounting to 60% after 30 min., while pre-incubation with both EDTA and 0.01M 2-mercaptoethanol resulted in a 95% loss of activity. The addition of Fe^{++} or Co^{++} ($10^{-3}M$) to the extracts which had been inhibited with EDTA alone produced an 80 to 100% regeneration of activity.

As with crude extracts, the partially purified enzyme was strongly activated by thiols, but the inhibitory effect of EDTA, either alone or with 2-mercaptoethanol, was no longer evident. The purified enzyme was neither inhibited or activated by Fe^{++} , Co^{++} , Mn^{++} or Mg^{++} . The partially purified enzymes employed in these studies were essentially concentrates of follicle-stimulating hormone, or derived sub-fractions, which had been obtained by chromatography on DEAE-cellulose according

Table 2. Hydrolysis of β -Naphthylamides by Bovine Anterior Pituitary Extract

| β -Naphthylamide Substrate | Hydrolysis Rate | |
|--|-------------------------------|----------------------------|
| | no addition mmoles/min/ml. | SH added mmoles/min/ml. |
| Arg | .11 | .42 |
| Lys | .044 | .38 |
| Phe | .016 | .31 |
| Ala | .010 | .28 |
| Leu | .036 | .26 |
| Tyr | <.01 | .18 |
| Pro | <.01 | .016 |
| Ser | <.01 | .011 |
| Val, α -Glu, γ -Glu His, Thr, Gly | <.01 | <.01 |
| Cys-di- β -NA ($1 \times 10^{-5}M$) | .032 | .070 |

Assay solution: $1 \times 10^{-4}M$ β -naphthylamide, 0.0625M Tris-HCl, 0.01 M 2-mercaptoethanol, 0.0125cc centrifuged extract. Total volume 4.0 cc, pH 7.5, 37°. Hydrolysis rate calculated on the basis of 1 ml. of centrifuged extract from a 20% homogenate.

to methods outlined elsewhere (Ellis, 1960, 1961).

For the purpose of establishing the rates of hydrolysis of different L-amino acid derivatives relative to lysine, the β -naphthylamides (Goldstein et al., 1962; Nachlas et al., 1962) were employed at a concentration of 10^{-4} M. The rates of hydrolysis were determined fluorimetrically (Greenberg, 1962) employing 0.1M Tris-HCl, pH 7.5, 0.01 M 2-mercaptoethanol and 0.015 ml. of extract. At this concentration of substrate, the reaction was zero order and the hydrolysis rate was directly proportional to the concentration of extract. Table 2 shows the rates of hydrolysis of various amino acid β -naphthylamides observed with anterior pituitary extracts. As with the p-nitroanilides, the addition of -SH to the naphthylamides also produced marked activation. With the purified enzyme, the relative rates of hydrolysis were the same with the exception of Phe whose rate equaled that of Arg. Purified leucine aminopeptidase from hog kidney (Worthington) hydrolyzed the arginine derivative at only 3% of the rate of leucine- β -naphthylamide.

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