COMPETITIVE PEPTIDE-INHIBITORS OF LEUCINE AMINOPEPTIDASE: SPECIFIC INTERACTION OF THREONINE (TERTIARY BUTYL)-PEPTIDES WITH THE ENZYME FROM SWINE KIDNEY AND FROM BOVINE EYE LENS

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1. Introduction

The tertiary butyl derivative H-Thr(But)–Phe–Pro-OH had been shown to be a potent competitive inhibitor of leucine aminopeptidase from swine kidney [1]. It was shown that the voluminous hydrophobic group, protecting the hydroxy function of the threonine side chain was essential for the resistance of this peptide to enzymatic hydrolysis.

To achieve better understanding of the molecular properties of this inhibitor, the compounds

H-Thr(But)-OH, H-Thr(But)-NH_2 and H-Thr(But)–Phe-OH were prepared and their interaction with the enzyme studied.

It is shown in this report that competitive inhibitors derived from *O*-tertiary butyl-threonine can be used to explore relationships between different aminopeptidases from animal tissue.

Abbreviations:

Abbreviations for peptides according to IUB-IUPAC rules: European J. Biochem. 1 (1967) 375.

H-Thr(But)-OH is referred as I in the text,

H-Thr(But)-NH_2 as II,

H-Thr(But)–Phe-OH as III, and

H-Thr(But)–Phe–Pro-OH as IV.

Leucine aminopeptidase, EC 3.4.1.1:LAP.

Leucine aminopeptidase from bovine eye lens: RAP.

Aminopeptidase M, EC 3.4.1.2:APM.

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2. Materials and methods

LAP was obtained from Worthington Biochemicals, Freehold, New Jersey, USA. RAP was a gift of Prof. H. Hanson, Martin Luther Universität, Halle, DDR.

AMP was from Röhm and Haas GmbH, Darmstadt, Germany. The enzymes were used without further purification. Protein concentrations were estimated prior to activation of LAP and RAP from the absorbance of the enzyme solution at 280 and 260 nm. The enzyme catalyzed hydrolysis of leucine *p*-nitroanilide was determined from the increase in absorbance at 405 nm, as measured on an Eppendorf spectrophotometer. The hydrolysis of peptides by LAP and RAP was studied with the aid of an automatic Beckman analyzer, model 121. APM was assayed according to Wachsmuth et al. [2]. LAP and RAP were activated by incubation at 40° in the usual activation system (0.05 M Tris-buffer pH 8.5, 5 mM $MgCl_2$ and 2 mM $MnCl_2$) at a protein conc. of 1 mg/ml. The incubation period to obtain full activity was about 20 min for RAP, whereas nearly 2 hr were required to activate the swine kidney enzyme.

Both aminopeptidases were assayed in 0.05 M Tris-buffer pH 8.5, 5 mM $MgCl_2$, at 30°. The relative

activities with 1 mM leucine *p*-nitroanilide were 1 (LAP):3.8 (RAP):1.8 (APM at 37° and pH 7.0).

The Dixon method [3] was used to calculate inhibition constants (K_i-values).

Peptide synthesis: chemical and physical properties of the threonine(tert.butyl)-compounds will be given elsewhere.

3. Results

Neither the amino acid I, nor its amide II, competed effectively with the substrate, 1 mM leucine *p*-nitroanilide, for the active site of LAP.

Gradually increasing concentrations, ranging from 10^{-6} M to 10^{-3} M, of I and II were added to a 10^{-7} M enzyme solution with substrate (10^{-3} M) present. Even at the highest concentration indicated, no inhibition was observed. In contrast to this, 10^{-5} M of the dipeptide III produced significant inhibition. Kinetic analysis showed that III acted as a pure competitive inhibitor of LAP. The potency of the dipeptide as an inhibitor was distinctly lower, compared with that of the tripeptide IV. A K_i-value of 2.5×10^{-5} M was found. The K_i-value for the tripeptide, as determined earlier [1], is 1.0×10^{-5} M.

With the enzyme from bovine eye lens, the same observations were made in competition experiments. Amino acid and amide were no inhibitors, but dipeptide and tripeptide turned out to be strong, competitive inhibitors of RAP. The clearly competitive inter-

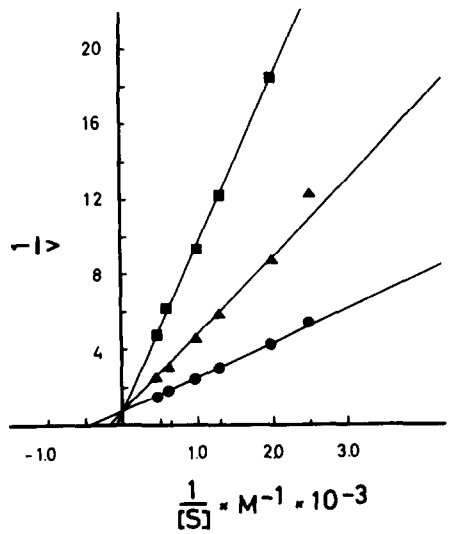

Fig. 1. Lineweaver–Burk plot for the enzyme catalyzed hydrolysis of leucine *p*-nitroanilide by the bovine lens enzyme: 10 μg of protein, 5 min of incubation at 30°. *v* is expressed in μmoles 4-nitroaniline formed. (●—●—●) Uninhibited; (▲—▲—▲) 0.01 mM H-Thr(But)–Phe–Pro-OH; (■—■—■) 0.04 mM H-Thr(But)–Phe–Pro-OH.

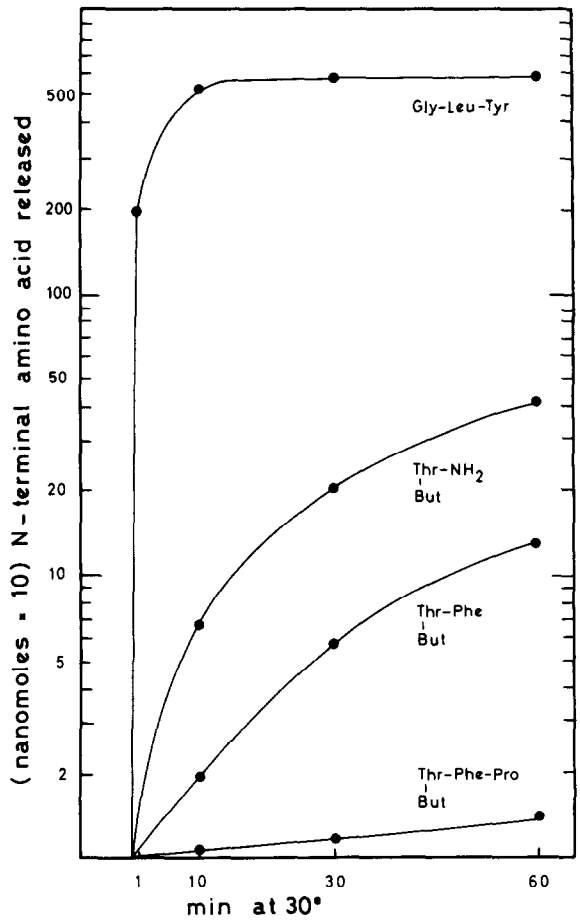

Fig. 2. Digestion of threonine(tert.butyl)-derivatives by leucine aminopeptidase from bovine lens. 1.6×10^{-6} M enzyme was incubated with 25 mM substrate solutions at 30°. The reaction was terminated at various intervals of time by adding 0.38 N sodium citrate buffer, pH 2.2. The amount of N-terminal amino acid liberated was determined by amino acid analysis.

action of the inhibitors with RAP is illustrated for IV in fig. 1.

The K_I-values found for III and IV were identical with those that had been determined for the swine kidney LAP (table 1).

None of the threonine(tert.butyl)-derivatives had any detectable effect on APM, the enzyme from the microsomal fraction of swine kidney.

Added in various concentrations up to 10^{-3} M, none of the compounds I–IV was able to inhibit the reaction of about 10^{-7} M APM with its substrate, 10^{-3} M leucine *p*-nitroanilide.

A qualitative digestion experiment carried out with 25 mM peptide solutions and an enzyme concentration of 10^{-6} M, indicated that neither III or IV was degraded by APM at a significant rate.

Similar results were also obtained with a bacterial aminopeptidase of broad specificity, AP I from *Bacillus stearothermophilus* [4].

This enzyme liberated only minor quantities of

$$\overset{\text{But}}{\underset{|}{}}$$

N-terminal H-Thr-OH from III and IV after prolonged incubation near its optimum temperature for substrate binding and catalysis, 55°. As APM, it was not inhibited by I, II, III or IV at 10^{-3} M.

A quantitative digestion study with LAP and RAP indicated that the threonine(tert.butyl)-derivatives II–IV, represented different stages of resistance to hydrolysis by leucine aminopeptidase (fig. 2).

It must be noted that even the rate of hydrolysis for the amide (5–9% of a 25 mM substrate solution was hydrolyzed by $1-2 \times 10^{-6}$ M enzyme in 1 hr at 30°) is very low compared with that of a normal peptide-substrate.

The rate of hydrolysis of the dipeptide III is 3–5 times lower than that of the amide. About 1 molecule in 1000 of III is cleaved by leucine aminopeptidase per hr at 30°, to yield I and H-Phe–Pro-OH (fig. 2).

4. Discussion

The interaction of the threonine(tert.butyl)-compounds I–IV with LAP and RAP, as summarized in table 1, is compatible with the idea that leucine aminopeptidase from swine kidney and from bovine eye lens are two forms of the same enzyme. This is consistent with the conclusions of Hanson et al. [5], who suggested a close relationship between the "classical" leucine aminopeptidase and the bovine lens enzyme on the basis of immunological evidence and specificity.

According to Glässer et al. [6], RAP is not a lens specific protein, as immunologically related enzymes occur in various tissues of vertebrates.

The competitive inhibitors III and IV might serve here to explore the similarity of the active site region of various forms of leucine aminopeptidase which may show considerable variations in their physico-chemical properties, depending on tissue and species of origin.

The failure of APM and AP I to cleave II, III and IV at a significant rate, and the inefficiency of the peptides as inhibitors of these aminopeptidases, is best interpreted by a very low or lacking affinity of the threonine(tert.butyl)-derivatives for these enzymes. From this inhibition study, a close relationship between the swine kidney enzymes LAP and APM must be denied.

The high affinity of IV to leucine aminopeptidase, and in addition the potentially high resistance of the peptide to the catalytic action of other proteolytic enzymes, make it promising to use the inhibitor in studies on the biological role of leucine aminopeptidase in various tissues.

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