THE SIGNIFICANCE OF THE N-ACYL RESIDUE OF L-ALANYL-L-ALANYL-L-ALANINE *p*-NITROANILIDE FOR CLEAVAGE BY PANCREATIC ELASTASE

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1. Introduction

Pancreatic elastase (EC 3.4.4.7) has been lately the subject of increased interest for several reasons. It is of importance in the pathogenesis of acute pancreatitis, especially in damage to the blood vessels [1]. It is not inhibited by clinically used protease inhibitors of the Kunitz type [2]. New specific substrates of various types for the estimation of elastolytic activity have been synthesized [3–5]. We have directed attention to chromogenic substrates, i.e. *p*-nitroanilides of N-acylated tripeptide L-alanyl-L-alanyl-L-alanine. So far *N*-acetyl- [6] and *N-tert*-butyloxycarbonyl-tripeptide-*p*-nitroanilide [7] have been used for this purpose. We have investigated the significance of the hydrophilic acyl residue of the N-terminal amino group $(Ala)_3$–NA_p* for the solubility, rate of enzymic hydrolysis and specificity for pancreatic elastase. For this purpose residues of dicarboxylic acids have been used: maleic acid (Mal), succinic acid (Suc) and glutaric acid (Glt) and the substrates prepared compared with the previously described Ac–$(Ala)_3$–NA_p [6].

Abbreviations: NAp, *p*-nitroanilide; Z, benzyloxycarbonyl.
* Alanine had the L-configuration.

2. Materials and methods

2.1. *Substrates*

The starting substance for the synthesis of N-acylated peptides Ala–Ala–Ala–NAp was Z–$(Ala)_3$–NAp (I) which had been prepared from Z–Ala–Ala–N_2H_3 and Ala–NAp by the azide method. On treatment with HBr in AcOH I afforded the crystalline hydrobromide HBr·$(Ala)_3$–NAp, m.p. 186–8°C; $R_f = 0.22/S_1, 0.69/S_2$. TLC (silica gel G) solvent: S_1: BuOH–AcOH–H_2O (4:1:1), S_2: BuOH–pyridine–AcOH–H_2O (15:10:3:4). *Anal. Calcd.* for $C_{15}H_{21}N_5 \cdot HBr \cdot H_2O$ (450.24): C 40.01, H 5.37, N 15.55, Br 17.75; *found* – C, 40.04, H, 5.17, N, 14.92, Br, 17.17. Treatment of I with aqueous ammonia gave $(Ala)_3$–NAp (II) which yielded by acylation with the appropriate anhydride 2 hr at 80°C in dimethylformamide the corresponding *N*-acyl-tripeptide-*p*-nitroanilides IIIa, IIIb, IIIc and IIId. The synthetic procedure is given in scheme I and the analytical data in table 1.

2.2. *Enzymes*

Porcine elastase was a commercial product of Merck & Co. (batch no. 3676883). The molar concentration of elastase was determined according to Shotton and Hartley [8] using a presumed molecular

Table 1
N-Acylated alanyl-alanyl-alanine *p*-nitroanilides

	M.p. °C $[\alpha]_D^{20}$ a)	Formula Mol. wt.	Calculated/Found %C	%H	%N
I	260–1	$C_{23}H_{27}N_5O_7$	56.91	5.61	14.43
	−49.4° b)	(485.4)	56.64	5.80	14.68
IIIa	348–50	$C_{17}H_{23}N_5O_6$	51.91	5.89	17.80
	−36.3° c)	(393.4)	51.91	6.13	17.39
IIIb	198–201	$C_{19}H_{23}N_5O_8$	50.78	5.16	15.58
	−91.5°	(449.4)	50.91	5.32	15.46
IIIc	247–9	$C_{19}H_{25}N_5O_8$	50.55	5.58	15.52
	−54.8°	(451.4)	50.36	5.66	15.95
IIId	256–8	$C_{20}H_{27}N_5O_8$	51.61	5.85	15.05
	−91.6°	(465.4)	51.46	5.95	15.15

a) Concentration 0.20–0.30 in NeOH;
b) In pyridine;
c) In dimethylformamide.

weight 25 900 [9]. Trypsin and chymotrypsin were commercial products of SPOFA, Czechoslovakia. Elastase was dissolved in 1×10^{-3} M AcOH, trypsin and chymotrypsin in 5×10^{-3} M HCl with 5×10^{-4} M $NaCl_2$. The solutions were kept at 2°C. The enzyme concentrations were 1.27×10^{-7} M.

2.3. *Analytical procedure*

Stock solutions of substrates were prepared in dimethylsulfoxide (Merck). The incubation medium consisted of 2.7 ml 0.1 M Tris buffer, pH 8, 0.1 ml enzyme solution and 0.2 ml substrate solution. The enzymic activity was measured at 25°C monitoring continuously at 410 nm the *p*-nitroaniline liberated using a Unicam SP 800 spectrophotometer.

2.4. *Kinetic constants*

K_M, k_{cat} and k_{cat}/K_M were determined. The Michaelis constants (K_M) were calculated from Lineweaver–Burk plots obtained with substrate concentrations in the range of $2.5 \times 10^{-3} - 1 \times 10^{-5}$ M (for IIIa the concentrations were $5 \times 10^{-4} - 5 \times 10^{-5}$ M).

3. Results

The maximum solubility in the incubation medium at 25°C was found to be 5×10^{-4} M for IIIa and 2.5×10^{-3} M for IIIc. Substrates IIIb and IIId in the latter concentrations also dissolved completely in the incubation medium. The rate of elastolytic hydrolysis of equimolar solutions of the substrates III(a–d) (3×10^{-4} M) is illustrated in fig. 1. Comparison of kinetic parameters is stated in table 2. At the given experimental conditions the investigated substrates were not cleaved by trypsin and chymotrypsin in an equimolar concentration. The benzyloxycarbonyl derivative I is practically insoluble and entirely unsuitable for enzymic cleavage.

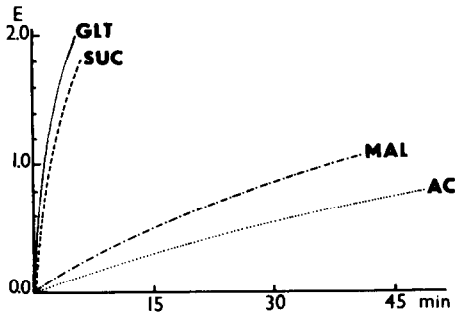
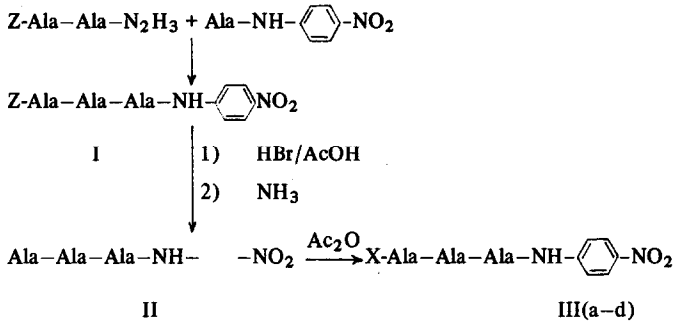


Fig. 1. Rates of elastolytic hydrolysis in equimolar solutions (3×10^{-4} M) of Ac-, Mal-, Suc-, and Glt-(L-Ala)$_3$–NAp at 25°C. Elastase concentration: 1.27×10^{-7} M.

Scheme 1



III(a–d)*

* a = acetyl;
b = 3-carboxy-acryol; (maleyl)
c = 3-carboxy-propionyl; (succinyl)
d = 4-carboxy-butyryl; (glutaryl)

Table 2
Kinetic constants for elastase catalyzed hydrolyses

	K_M(mM)	k_{cat}(sec^{-1})	k_{cat}/K_M($M^{-1} \cdot sec^{-1}$)
IIIa	6.6	3.94	597
IIIb	2.86	6.30	2 202
IIIc	0.625	23.62	37 790
IIId	0.605	22.83	37 730

4. Discussion

The introduction of a dicarboxylic acid residue in the α-amino group of $(Ala)_3$–NAp instead of the acetyl residue brought about a substantially higher solubility of the substrate (at least 5 times higher in comparison with IIIa). According to the kinetic data, the elastolytic hydrolysis of hydrophilic substrates is markedly higher (IIIa < IIIb ≤ IIIc < IIId). In view of these properties and simultaneous high specificity, these substrates should be considered as more suitable for the determination of elastase activity in biological material than the earlier described Ac–$(Ala)_3$–NAp (IIIa).

The adsorption of elastase on substrate (elastin) has according to Gertler [2] two prerequisites: electrostatic charge and nonpolar residue. We presume that the presence of a negatively charged carboxyl anion in N-acylated *p*-nitroanilide peptide makes possible electrostatic bonding with the positively charged enzyme groups. The preference of the nonpolar butyloxycarbonyl group (Boc) over the acetyl group [7] can be considered as an additional reason in favor of Gertler's hypothesis.

The charge of the carboxyl anion alone probably cannot be considered as the decisive factor. The pK_1 values of dicarboxylic acids (maleic 1.8, succinic 4.19, and glutaric 4.42) are in reversed sequence to their K_M or C values. The length of the acyl residue is probably of major importance.

With regard to the mapping of the active enzyme site [10], the position P4–S4 is favoured by an electrostatic rather than a hydrophobic bond. We are of the opinion that this circumstance may explain the more favourable kinetic data of Glt–$(Ala)_3$–NAp (IIId) and Suc–$(Ala)_3$–NAp (IIIc) in comparison with the Boc derivative [7].

Acknowledgements

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