INVESTIGATIONS ON NEW TRIPEPTIDYL-*p*-NITROANILIDE SUBSTRATES FOR SUBTILISINS

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1. Introduction

The specificity of subtilisins (EC. 3.2.21.14) in splitting the peptide bonds at different amino acids of polypeptide chains is relatively broad, although X-ray data indicate that the substrate binding site favours non-polar amino acid side chains [1,2]. Several authors reported on the rate of hydrolysis of tripeptides composed of non-polar amino acids, e.g., *Z*–Gly–Gly–Leu–NH_2 [3] and its *p*-nitroanilide derivative [4].

In this paper investigations are described on nine tripeptidyl-*p*-nitroanilide substrates containing polar amino acid residues which increased both the solubility of compounds in aqueous media and the affinity of substrates compared to those studied by other authors [3,4]. The comparison of the Michaelis constants, K_m, indicates that the compounds containing basic amino acid side chain at P_1 binding site are just as good substrates as those with a non-polar side chain at the same site.

2. Materials and methods

Subtilisin Carlsberg (Lot No. 94C-0245) and BPN′ (Lot No. 64C-0261) were purchased from Sigma, St. Louis, Mo. USA, subtilisin Novo was obtained from Serva, Heidelberg, FRG. Kinetic investigations were carried out in 50 mM Tris–HCl buffer, pH 8.1. The relative proteolytic activities of subtilisins were measured on denatured hemoglobin [5] (table 1).

Bz–Phe–Val–Arg–*p*NA (S-2160) and *H*–D–Val–Leu–Lys–*p*NA (S-2251) were obtained from AB Bofors, Nobel Division, Mölndal, Sweden. The other substrates (see table 1) were synthetized in our laboratory [6]. The substrates were dissolved in the buffer except *Bz*–Phe–Val–Arg–*p*NA which was dissolved in distilled water.

Kinetic measurements were carried out at 37°C in a Unicam SP 500 spectrophotometer. The increase in absorption was recorded for 3–5 min at 405 nm where the absorption of substrates is negligible. The enzyme concentration varied between 0.725 nM and 4.35 μM. Initial velocities were calculated from the change in absorption using a molar absorption coefficient of 10 600 for *p*-nitroaniline.

3. Results and discussion

The kinetic measurements performed at least with two different enzyme concentrations showed a linear increase in absorption as a function of time (fig.1). Less than 1% of the substrates was hydrolyzed during the assay, i.e., in 3–5 min under the experimental conditions. The substrates did not release *p*-nitro-

Abbreviation: *p*NA = *p*-nitroanilide

Table 1
K_m Values of tripeptidyl-*p*-nitroanilide substrates for subtilisin

No.	Substrate, with binding site notation [1]	Subtilisin					
		Novo			Carlsberg		
	P_4 P_3 P_2 P_1	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, s^{-1} M^{-1}	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, s^{-1} M^{-1}
1	*Bz*– Phe– Val–Arg–*p*NA	0.04	0.35	88.5×10^2	0.1	0.30	29.7×10^2
2	*Z* –D-Phe– Pro–Arg–*p*NA	0.3	0.08	2.7×10^2	0.3	0.17	5.6×10^2
3	*Z* – Phe– Pro–Arg–*p*NA	0.4	0.14	3.4×10^2	0.5	0.86	17.2×10^2
4	*Z* – Phe– Val–Arg–*p*NA	0.4	0.16	4.0×10^2	1.0	4.32	43.2×10^2
5	*Z* –D-Val– Pro–Arg–*p*NA	0.7	0.03	0.5×10^2	0.7	0.10	1.4×10^2
6	*Z* – Lys–Nleu–Arg–*p*NA	0.7	0.31	4.4×10^2	0.7	4.90	70×10^2
7	*Z* – Lys– Pro–Arg–*p*NA	1.5	0.13	0.8×10^2	4.0	2.75	6.9×10^2
8	*Z* – Asp– Pro–Leu–*p*NA	2.0	0.28	1.4×10^2	0.8	229	2862×10^2
9	D-Val– Leu–Lys–*p*NA	5.0	0.13	0.2×10^2	5.0	26.3	52.6×10^2
	Z – Gly– Gly–Leu–*p*NA				0.8[a]	0.6	7.5×10^2
	Z – Gly– Gly–Leu–NH_2	33.3[b]	14.9	4.5×10^2			
	Relative proteolytic activity with denatured hemoglobin [5]	5			1		

[a] In 7.5% acetonitrile solution (Lyublinskaya et al. [4])
[b] With subtilisin BPN' in 15% dimethylformamide solution (Morihara et al. [3]).

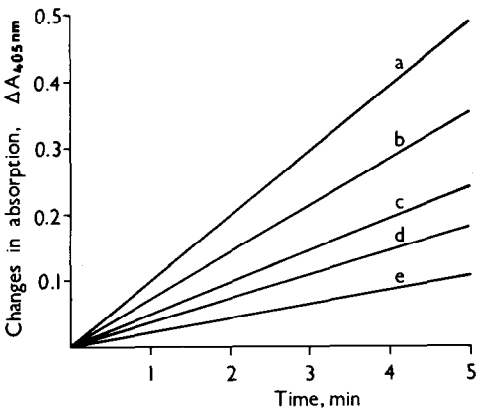

Fig.1. Cleavage of *Z*–Asp–Pro–Leu–*p*NA by subtilisin BPN' in 50 mM Tris–HCl buffer, pH 8.1, at 37°C as recorded at 405 nm. Enzyme concentration 0.2 μM. Substrate concentrations (a) 2 mM, (b) 1 mM, (c) 0.5 mM, (d) 0.33 mM and (e) 0.17 mM.

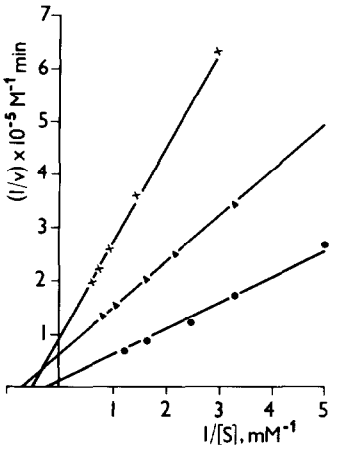

Fig.2. Lineweaver-Burk plots of cleavage of several tripeptidyl-*p*-nitroanilides by subtilisin Novo (×) *Z*–Asp–Pro–Leu–*p*NA, enzyme concentration 0.2 μM, K_m 2.0 mM. (▲) *Z*–Lys–Pro–Arg–*p*NA, enzyme concentration 2.25 μM, K_m 1.5 mM. (●) *H*–D-Val–Leu–Lys–*p*NA, enzyme concentration 2.25 μM, K_m 5.0 mM.

aniline without enzyme, at pH 8.1, for 5 min and their peptide bonds were not cleaved during the measurement.

The Michaelis constants were calculated by plotting the data according to Lineweaver and Burk (fig.2). The k_{cat} values were obtained from the Michaelis-Menten equation.

It follows from the K_m values (table 1) that all compounds tested are reasonably good substrates for subtilisins, since their K_m values fall in the millimolar range. *Bz*–Phe–Val–Arg–*p*NA appears to have the greatest affinity to the enzyme. Although X-ray crystallographic studies [1,2] suggest that subtilisins cleave peptide bonds preferentially at non-polar amino acid side chains, *Z*–Asp–Pro–Leu–*p*NA, which contains a non-polar leucyl residue at the P_1 site, did not prove to be a better substrate when compared to other compounds with basic C-terminal residues.

As far as the P_2 site is concerned, valine can be readily accomodated in the P_2 site (expt. 1) whereas the X-ray data suggested [2] that there was space only for glycine and alanine. This contradiction may be due to the fact that in the affinity labelled subtilisin a covalent bond is formed between the substrate analog and the enzyme. On the other hand, tripeptidyl-*p*NA substrates, due to their rotational freedom, can be accomodated at the active site, even if they contain a side chain greater than alanine at the critical position.

Substrates containing valine or proline at the P_2 binding site (expt. 3 and 4 in table 1) seemed to be equally good, whereas the affinity decreased about twofold when norleucine was replaced by proline (expt. 6 and 7). An inverse phenomenon was observed when lysine was substituted by phenylalanine at the P_3 site (expt. 3 and 7). The binding of substrates was not influenced significantly when L-phenylalanine was substituted by its D isomer (expt. 2 and 3). Thus, in agreement with the conclusion drawn by Kraut et al. [2] from X-ray crystallography, the configuration of the amino acid residue at P_3 site is not crucial. The affinity of the substrates at P_3 site decreases in the following order: D-Phe $\cong$ L-Phe > D-Val > L-Lys (expt. 2, 3, 5 and 7).

Finally, when the benzoyl function was replaced by a benzyloxycarbonyl group at the P_4 site, an about tenfold decrease was observed in the affinity (expt. 1 and 4).

However, when the substrates are compared by comparing the k_{cat} or k_{cat}/K_m values, the situation is different from that discussed above. This is particularly valid for the Carlsberg enzyme, since *Z*–Asp–Pro–Leu–*p*NA was hydrolyzed at the highest rate.

To construct the preferential amino acid sequence the . . .Arg–*p*NA substrates were analyzed according

to structure–activity method of Free and Wilson [8]. Contributions of the different amino acid side chains of the substrates to the biological activity represented by K_m and k_{cat} values were calculated for the P_4, P_3 and P_2 sites. If the K_m values were taken into account *Bz*–D-Phe–Nleu–Arg–*p*NA appeared to be the best substrate for both subtilisin Novo and Carlsberg. The approximate K_m values calculated from this analysis were 0.038 mM and 0.075 mM, respectively. On the other hand, when the k_{cat} values are considered *Bz*–Phe–Nleu–Arg–*p*NA, k_{cat} 0.516 s^{-1} and *Z*–Lys–Val–Arg–*p*NA, k_{cat} 6.206 s^{-1}, seem to be good substrates of subtilisin Novo and Carlsberg, respectively.

It should be mentioned that *Bz*–Phe–Val–Arg–*p*NA is a suitable substrate for proteases that split peptide bonds at basic amino acid residues [7]. However, the affinity of *Bz*–Phe–Val–Arg–*p*NA to trypsin, thrombin and plasmin did not appear to be much greater than that of subtilisin. In addition, *H*–D-Val–Leu–Lys–*p*NA is just as good a substrate for subtilisin as for plasmin, $K_m = 2 \times 10^{-4}$ M (personal communication of Dr E. Berggren).

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