SUBTILISIN-CATALYZED HYDROLYSIS OF PEPTIDE METHYL ESTERS

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1. Introduction

It is known that the serine protease subtilisin possesses a specifity for longer peptide amides as opposed to simpler amides [1,2]. Since ester substrates of the serine protease chymotrypsin generally are hydrolyzed much more rapidly (10^5 times) [3] than amides, the subtilisin-catalyzed hydrolyses of specific peptide esters should be much more rapid than those previously studied. Two substrates used here have the highest known reaction rate (k_2/K_s) with subtilisin. These rates, for methyl esters, approximate the rates of the best substrates of α-chymotrypsin [4] which are more highly activated *p*-nitrophenyl esters.

Since in the most specific substrates, the value of k_2 may exceed k_1 (eq. 1), the value of k_{cat}/K_m may reduce to k_1. [4]

$$E + S \overset{k_1}{\rightleftharpoons} ES \underset{P_1}{\overset{k_2}{\rightleftharpoons}} EP_2 \overset{k_3}{\rightarrow} E + P_2 \qquad (1)$$

It is evident that the pH profile of k_1 need not be identical to that of k_2/K_s. [5] Therefore pH profiles of the various hydrolyses were obtained. Since literature data [1,2] concerning the peptide amides similar to the esters is in the form of relative rate data, binding constants of the amides to subtilisin were obtained. These were measured by observing inhibition of the second-order subtilisin-catalyzed hydrolysis of Z-Gly-OnP (see later).

2. Materials and methods

Subtilisin Novo was purchased from Novo Industri, Copenhagen, Denmark. Its molar concentration was determined using a cinnamoylimidazole titration [6]. All substrates and inhibitors were purchased from the Cyclo Chemical Company. *p*-Nitrophenyl *N*-benzyloxycarbonyl glycinate (Z-Gly-OnP) [7] was purified by dissolving it twice in acetone and adding heptane until the onset of precipitation. This was followed by refrigeration. The hydrolysis of this compound was observed at 400 nm, of the tyrosine methyl ester at 237 nm [8], and of the tryptophan methyl ester at 300 nm [9]. Concentrations of Z-Gly-Gly-L-Tyr-OMe used varied from 3 to 10×10^{-5} M, those of Z-Gly-Gly-L-Trp-OMe from 2 to 4×10^{-4} M, and those of Ac-L-Trp-OMe from 3 to 4×10^{-4} M were used in pH dependence studies, and organic solvent concentrations (methanol or DMSO) were 2.5%. Inhibition studies were performed at pH 7.71 I = 0.1 M phosphate buffer [10] with 4.4% *N,N*-dimethylformamide. Inhibitor concentrations ranged from 2 to 20×10^{-3} M. Binding constants were calculated using an expression used previously. [11]

3. Results and discussion

3.1. *Hydrolysis of methyl ester substrates*

Two parameters, the pK_1 of k_2/K_s and k_2/K_s (lim) were measured for Ac-Ty-OMe, Z-Gly-Gly-Try-OMe and Z-Gly-Gly-Tyr-OMe. Figs. 1–3 show the pH profiles and the profile parameters for these substrates. Comparison of the parameters for Ac-Try-OMe and Z-Gly-Gly-Try-OMe shows that the increase in the second-order acylation rate constant k_2/K_s (lim) is not accompanied by any significant change in pK.

The k_2/K_s (lim) value for Z-Gly-Gly-Tyr-OMe (2.6×10^7 M^{-1} sec^{-1}) is the highest yet observed for

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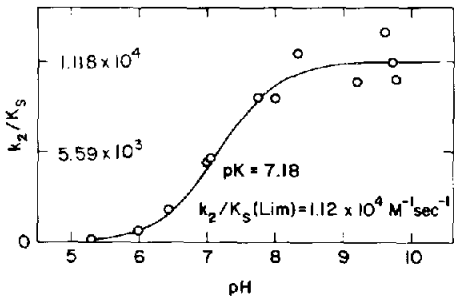

Fig. 1. pH Dependence of the subtilisin-catalyzed hydrolysis of methyl *N*-acetyl-tryptophanate. pK = 7.18, k_2/K_S (lim) = 1.12×10^4 M^{-1} sec^{-1}.

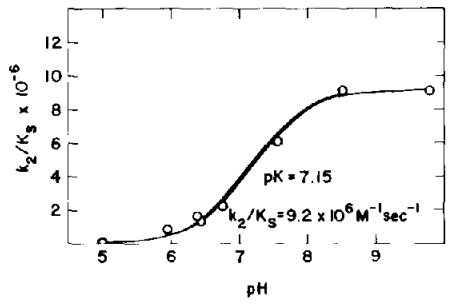

Fig. 2. pH dependence of the subtilisin-catalyzed hydrolysis of methyl *N*-CBZ-glycyl-glycyl-L-tryptophanate. pK = 7.15, k_2/K_S (lim) = 9.2×10^6 M^{-1} sec^{-1}.

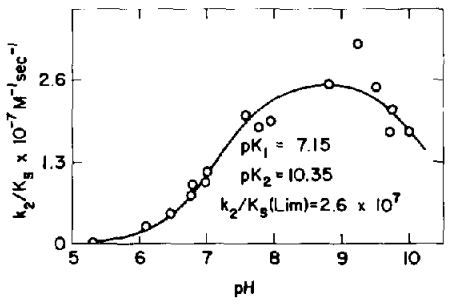

Fig. 3. pH Dependence of the subtilisin-catalyzed hydrolysis of methyl *N*-CBZ-glycyl-glycyl-L-tyrosinate. pK_1 = 7.15, pK_2 = 10.35, k_2/K_S (lim) = 2.6×10^7 M^{-1} sec^{-1}.

a subtilisin substrate. The literature value for Ac-Tyr-OMe is 1.7×10^4 $M^{-1}sec^{-1}$. [12] Hydrolysis of Z-Gly-Gly-Tyr-OMe also shows the pK of 7.15 observed for Ac-Try-OMe.

Very high k_2/K_S values may be associated with rate determining binding, k_1 (eq. 1). Since K_m for subtilisin substrates is independent of pH in the region 6–9, [1,13] the individual constants, k_1 and k_{-1}, are likely to be pH independent in this region. Thus, if k_1 becomes rate determining, the pH profile should be perturbed. [5] This evidently does not happen, and so k_1 for these substrates must be considerably higher than 2.6×10^7 M^{-1} sec^{-1}.

The magnitude of the ester rates observed here may also be compared to the magnitude of k_2/K_S (lim) for the best amide substrate known for subtilisin, Z-Gly Ala-Leu-NH_2, which is 6.3×10^3 M^{-1} sec^{-1}. [1]

Thus, even the most highly specific amide substrates are still hydrolyzed much more slowly (10^3–10^4) than similar ester substrates.

Finally, it is apparent that Z-Gly-Gly-Tyr-OMe shows a k_2/K_S pK near 10.3. Since the subtilisin-catalyzed hydrolysis of Bz-Tyr-OEt also shows this pK [14] while the analogous tryptophan substrates do not, it is reasonable to conclude that the substrate tyrosine phenolic group ionization causes this pK.

3.2. *Inhibition constants of peptide amides*

Table 1 shows binding constants of various peptide amides, measured by observing inhibition of the second-order hydrolysis of Z-Gly-OnP. In each series the binding constant is strongly determined by the presence or absence of the *N*-benzyloxycarbonyl group, which is known to bind as a phenylalanine side-chain [15]. This effect is nearly independent of the peptide chain length. Crystal structure studies [15] indicate that nonproductive binding modes are

Table 1

Inhibitor	K_I (M)
Ac-Tyr-NH_2	1.2×10^{-1}
Z-Tyr-NH_2	7.7×10^{-3}
Z-Gly-Tyr-NH_2	8.6×10^{-3}
Z-Gly-Gly-Tyr-NH_2	5.1×10^{-3}
Z-Gly-Trp-NH_2	7.5×10^{-3}
Z-Gly-Gly-Trp-NH_2	3.6×10^{-3}

present in these complexes which can affect k_{cat}, K_m, and K_I values in a similar way.

If the K_I values are taken to be similar to the K_m value (which includes inhibitory nonproductive components) estimates of k_2 for Z-Gly-Gly-Tyr-OMe and Ac-Tyr-OMe can be made. These are 1×10^4 sec^{-1} for Z-Gly-Gly-Tyr-OMe and 2×10^3 sec^{-1} for Ac-Tyr-OMe. These values are calculated by substituting the value of K_I for K_S in the value of k_2/K_S. These values are quite similar to those of specific chymotrypsin substrates obtained by similar indirect and direct means. k_2 For the chymotrypsin-catalyzed hydrolysis of Ac-Tyr-OEt is 5.33×10^3 sec^{-1} [16] similar to the estimated subtilisin value with the methyl ester.

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