KINETICS OF PEPSIN-CATALYSED TRANSPEPTIDATION: EVIDENCE FOR THE 'AMINO-ENZYME' INTERMEDIATE

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1. Introduction

Swine pepsin (EC 3.4.23.1) catalyses both hydrolysis of the peptide bond between the hydrophobic amino acid residues (A) and transfer of the cleaved C-terminal fragment of a peptide to the acceptor-acylamino acid or peptide with a free carboxy group (B) [1–3].

$$R^1COOH + R^2NH_2 \underset{(A)}{\overset{E,H_2O}{\longleftarrow}} R^1CONHR^2 \xrightarrow[(B)]{E,R^3COOH} R^1COOH + R^3CONHR^2$$

The transpeptidation reaction of the 'amino-transfer' type (B) was the main evidence of formation of an 'amino-enzyme' intermediate ($ECONHR^2$) in pepsin-catalysed hydrolysis [4]. It was therefore desirable to confirm this kinetically. However, up till now no kinetic methods for studying pepsin-catalysed transpeptidation were available. This paper describes a spectrophotometric technique that allows the initial rates of this reaction to be measured by using as acceptor a chromophore-containing compound, *N*-benzyloxycarbonyl-*p*-nitro-L-phenylalanine (I). We compared the transpeptidation rate constants of two different substrates with the same transferable residue, namely *N*-acetyl-L-phenylalanyl-L-tyrosine (II) and *N*-acetyl-L-tyrosyl-L-tyrosine (III). This led to the conclusion that the enzymatic hydrolysis involves the same intermediate, presumably pepsyl-tyrosine for both compounds.

2. Materials and methods

2.1. *Materials*

Swine pepsin, a commercial preparation of the Olaina Plant for Chemical Reagents, was purified by chromatography on DEAE-cellulose according to [5].

N-Benzyloxycarbonyl-*p*-nitro-L-phenylalanine (I), *N*-acetyl-L-phenylalanyl-L-tyrosine (II) and *N*-acetyl-L-tyrosyl-L-tyrosine (III) were synthesized according to [6–8], respectively.

N-Benzyloxycarbonyl-*p*-nitro-L-phenylalanyl-L-tyrosine (IV) was obtained by chymotryptic hydrolysis of the corresponding methyl ester (pH 7.8, 40% CH_3OH) synthesized from (I) and methyl tyrosinate by the hydroxybenztriazole–carbodiimide technique [9]. Compound (IV) was obtained with 36% yield, m.p. 210–211°C, $[\alpha]_D^{20}$ −8°C (DMF).

2.2. *Kinetic studies*

The transpeptidation reactions and the hydrolysis of (IV) were performed on a Cary model 15 double beam recording spectrophotometer at 320 nm. The $\Delta\epsilon$ value was 1000 in all cases. The thermostatted (37°C) spectrophotometric cell of width 0.5–2.0 cm (depending on the acceptor concentration) contained the enzyme, substrate and acceptor in 0.1 M acetate buffer solution (pH 4.61). The reference cell contained the same components, excepting the enzyme. The initial velocity was determined from the linear region of the kinetic curve corresponding to not more than 20% of substrate transformation (fig. 1). It was shown in separate experiments.in which the optical density change at 320 nm was recorded in a system containing the acceptor (I) and pepsin

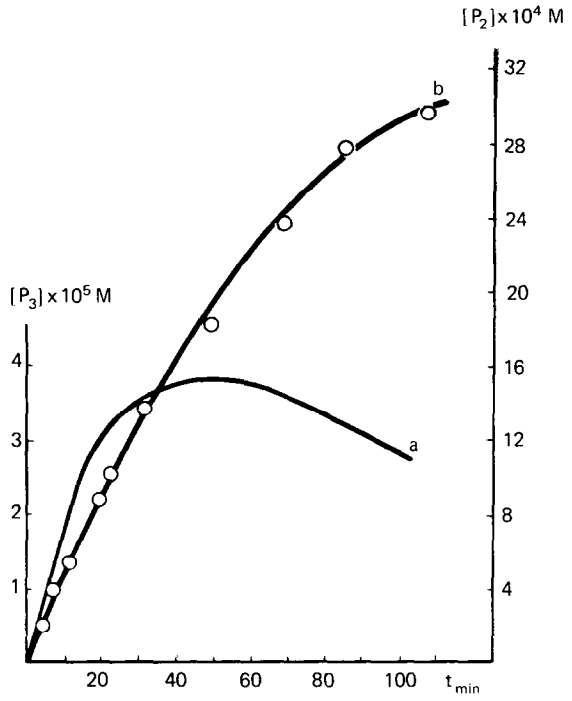

Fig. 1. Kinetic curves for formation of the product of transpeptidation (a) and hydrolysis (b) of *N*-acetyl-L-Phenylalanyl-L-tyrosine (II). $[S]_0$ 8.4 mM; $[A]_0$ 0.5 mM; $[E]_0$ 0.175 mM; pH 4.61 (0.1 M acetate buffer), 37° C.

that 'donor-less' transpeptidation was negligible.

The rates of hydrolysis of (II) and (III) were determined by a modification of Mokrasch's method [10] using 2,4,6-trinitrobenzenesulphonic acid. Rate determinations were made in both the presence (0.5, 0.667 and 1.0 mM) and absence of the acceptor (I).

Values for the kinetic parameters were obtained from double-reciprocal plots (fig. 2) using the least squares method.

3. Results and discussion

The chromophore-containing *N*-benzyloxycarbonyl-*p*-nitro-L-phenylalanine used as acceptor allows one to record the initial rates of formation of the transpeptidation product, (IV), in the case of both substrates: (II) and (III) (table 1). This is possible because formation of this product involves a change in the optical density of the reaction mixture in the region of wavelengths which does not overlap with the absorption of other components of the system ($\Delta\epsilon_{320}$ = 1000). The experimental kinetic curve of fig. 1 contains a sufficiently long linear region to permit highly accurate measurements of the initial rate of formation of the transpeptidation product. Fig. 1

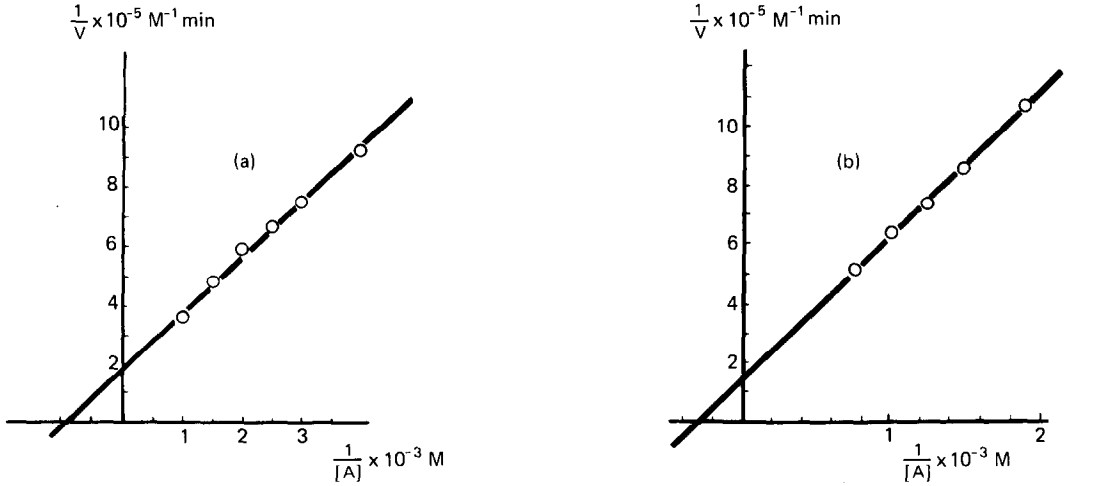

Fig. 2. Double-reciprocal plots for transpeptidation reactions. (a) *N*-Acetyl-L-phenylalanyl-L-tyrosine (II), $[S]_0$ 8.4 mM, $[E]_0$ 0.175 mM. (b) *N*-Acetyl-L-tyrosyl-L-tyrosine (III), $[S]_0$ 16.8 mM, $[E]_0$ 0.293 mM. Other conditions were those as indicated in fig. 1.

Table 1
Constants of hydrolysis and transpeptidation (0.1 acetate buffer pH 4.61, 37°C)

Substrate	$[S]_0$, mM	$[A]_0$, mM	k_{cat}, min^{-1}	K_m, mM	$k_5 \times 10^2$, min^{-1}	K_T (app.), mM	$\frac{k_{cat}\ (II)}{k_{cat}\ (III)}$	$\frac{K_T\ (app.)\ (III)}{K_T\ (app.)\ (II)}$
(II)	3–20[a]	0	1.62 ± 1.04	43.9 ± 28.6				
	8.4[b]	0.25 – 1.0			3.10 ± 0.4	0.156 ± 0.085		
							2.56	2.72
(III)	8–40[a]	0	0.634 ± 0.274	144 ± 63				
	16.8[c]	0.53 – 1.32			2.93 ± 0.75	0.424 ± 0.18		
(IV)	0.25–1.5[a]	0	0.627 ± 0.11	4.71 ± 0.83	–	–	–	–

a, $[E]_0$ 0.05 mM; b, $[E]_0$ 0.175 mM; c, $[E]_0$ 0.293 mM.

represents also an experimental curve for accumulation of the substrate hydrolysis product, tyrosine, obtained with the aid of trinitrobenzenesulphonic acid as a free amino group reagent.

The following transformations (1–4) should take place in the substrate (S) plus enzyme (E) plus the transpeptidation acceptor (A) system:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} \underset{P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

$$ES' + A \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} ES'\cdot A \xrightarrow{k_5} E + P_3 \qquad (2)$$

$$E + P_3 \underset{k_{-6}}{\overset{k_6}{\rightleftarrows}} EP_3 \xrightarrow{k_7} E + P_2 + A \qquad (3)$$

$$E + A \underset{}{\overset{K_i}{\rightleftarrows}} EA \qquad (4)$$

where ES' is the 'amino-enzyme', P_1 and P_2 are respectively N- and C-terminal amino acid products of the substrate hydrolysis; P_3 is the product of transpeptidation.

When determining the initial rates of formation of the transpeptidation product (P_3), reaction (3) can be neglected. In fact, the data on the rate of hydrolysis of P_3 (table 1) show that decomposition of the transpeptidation product during the time (10–15 min) required for transpeptidation rate determination does not exceed the experimental error. Competitive inhibition of the free enzyme by acceptor can also be neglected, since K_i values (1×10^{-2} M) of acylamino acids under conditions of the experiment (pH > 4) are higher than the acceptor concentration (1×10^{-3} M) [11]. Therefore, the reactions occurring in the system are apparently those given in schemes (1) and (2). On assuming that $[A]_0 > [E]_0$ and $k_2 \ll k_3$ [12] the following equation (5) for the initial rate of formation of the transpeptidation product

$$V_t = \frac{k_5\,[E]_0[S]_0[A]_0}{K_T\frac{k_3}{k_2}(K_m + [S]_0) + [A]_0\frac{k_5}{k_2}(K_m + [S]_0) + [A]_0[S]_0} \qquad (5)$$

where $K_T = \frac{k_{-4} + k_5}{k_4}$ and $K_m = \frac{k_{-1} + k_2}{k_1}$, can be deduced.

This can be represented in the double-reciprocal form (Eqn. 6):

$$\frac{1}{v_t} = \frac{K_T\frac{k_3}{k_2}}{k_5\,[E]_0}\left(\frac{K_m + [S]_0}{[S]_0}\right)\frac{1}{[A]_0} + \left(\frac{1}{k_5\,[E]_0} + \frac{K_m + [S]_0}{k_2\,[E]_0\,[S]_0}\right) \qquad (6)$$

Thus, by determining the initial rates of transpeptidation at constant substrate and variable acceptor concentrations one can obtain from the vertical intercept of the double-reciprocal plot (fig. 2a, b) the value

$$a = \frac{1}{k_5\,[E]_0} + \frac{K_m + [S]_0}{k_2\,[E]_0[S]_0} \qquad (7)$$

and from the horizontal intercept, the value

$$b = \frac{1}{K_{T\ (app.)}} \left(\frac{[S]_0}{K_m + [S]_0} + \frac{k_5}{k_2} \right) \qquad (8)$$

where $K_{T\ (app.)} = K_T \frac{k_3}{k_2}$

In Eq. (7) the second member is the reciprocal of the substrate hydrolysis rate in the absence of acceptor, and so can be determined by an independent experiment (table 1). This allows calculation of the transpeptidation rate constant (k_5). Using this constant and assuming $k_2 \sim k_{cat}$, as is generally accepted for pepsin hydrolysis [12], we can find the value of $K_{T\ (app.)}$.

Some interesting conclusions can be drawn by analysing Eq. (5). First, the transpeptidation rate constants for substrates with the same C-terminal residue must be equal, the substrates therefore forming an identical 'amino-enzyme' intermediate. Second, the apparent Michaelis constants for the transpeptidation reaction ($K_{T\ (app.)}$) must be inversely proportional to the hydrolysis rate constants of these substrates. The rate of formation of the hydrolysis product (P_2) in the presence of the acceptor is determined by Eq. (9):

$$v_h = \frac{k_3 K_T [S]_0 [E]_0}{K_T \frac{k_3}{k_2} (K_m + [S]_0) + [A]_0 \frac{k_5}{k_2} (K_m + [S]_0) + [A]_0 [S]_0} \qquad (9)$$

and the ratio between the hydrolysis and the transpeptidation rates is given by Eq. (10):

$$\frac{v_h}{v_t} = \frac{k_3 K_T}{k_5} \cdot \frac{1}{[A]_0} \qquad (10)$$

Thus we arrive at the third conclusion that the v_h/v_t ratio is a linear function of the reciprocal of the acceptor concentration and is equal for all substrates with the same transferable C-terminal amino acid.

The data presented in table 1 and in fig. 3 show that all three aforementioned conclusions are satisfied. Therefore, hydrolysis of the substrates does involve formation of the intermediate complex between the enzyme and C-terminal fragment of the substrate. This does not seem to be a non-covalent complex of pepsin with tyrosine, since the enzyme affinity for free amino acids is very low ($K_i \sim 1 \times 10^{-1}$ M [11]).

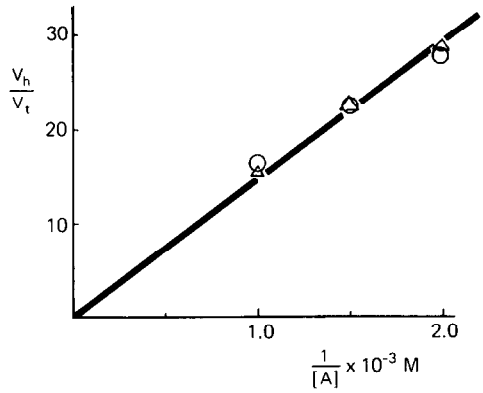

Fig. 3. Ratio between hydrolysis and transpeptidation rates vs. acceptor concentration. (○——○) *N*-Acetyl-L-phenyl-alanyl-L-tyrosine, (△——△) *N*-acetyl-L-tyrosyl-L-tyrosine. Conditions were those as indicated in figs. 1 and 2.

It is quite plausible that the complex is of the 'amino-enzyme' type.

Recently Silver et al. [13] have shown that transpeptidation does not proceed in the case of substrates with a protected C-terminal carboxy group. We believe, however, that the difficulties encountered in detection of the transpeptidation product in this case are caused by an exceedingly large $k_3 K_T / k_5$ ratio (Eq. (10)) so that $v_h \gg v_t$. Perhaps the reaction would be detectable with acceptors having high affinities (low K_T) and sufficient solubilities. We are at present in the process of checking this assumption.

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