On the Mechanism of the Action of Thermolysin: Kinetic Study of the Thermolysin-catalysed Condensation Reaction of *N*-Benzyloxycarbonyl-L-aspartic Acid with L-Phenylalanine Methyl Ester

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The mechanism of the thermolysin-catalysed condensation reaction of *N*-benzyloxycarbonyl-L-aspartic acid (*Z*-L-Asp) with L-phenylalanine methyl ester (L-Phe-OMe) giving *N*-benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester, the precursor of the synthetic sweetener L-aspartyl-L-phenylalanine methyl ester (aspartame), was investigated by kinetic measurements. It was found that the reaction is first order in L-Phe-OMe and the Lineweaver–Burk plot of $1/v$ against $1/[Z\text{-L-Asp}]$ yields a straight line, showing that the reaction involves consecutive reactions of *Z*-L-Asp and L-Phe-OMe with the enzyme and with the *Z*-L-Asp–enzyme complex, with the second reaction being the rate-determining step. This fact suggests that an amino-enzyme intermediate is probably not involved in the reaction. It was also found that L-Phe-OMe neither inhibits nor enhances the reaction, whereas *Z*-L-Asp acts as a competitive inhibitor, and moreover the binding constants of *Z*-L-Asp and *Z*-D-Asp with the enzyme are close to each other. From these facts and other evidence, a mechanism which is the reverse of general base catalysed hydrolysis of a peptide bond is proposed for the reaction.

THE elucidation of the mechanism of the action of proteolytic enzymes is an important goal in bio-organic chemistry. In recent years many mechanistic studies have been reported on the reactions of metalloproteinases such as carboxypeptidase A (CPA) [1] and thermolysin; [2-4] whether these reactions take place *via* the acyl-enzyme (anhydride) intermediate or *via* attack of a water molecule at a breakable bond (general base catalysis route) is the subject of active discussion.[3,5]

Thermolysin (EC 3.4.24) is a widely studied neutral metalloendoproteinase which is produced from the thermophilic bacterium *Bacillus thermoproteolyticus*,[6] but its mode of action is not yet well understood. The amino-acid sequence [7] and the three-dimentional structure have been determined.[8] From an *X*-ray crystallographic study of the binding of the enzyme with inhibitors, Kester and Matthews suggested that the active site involves the zinc ion, Glu-143, and His-231, and that hydrolysis of peptide bonds more probably occurs *via* the general base catalysis route than *via* an acyl-enzyme intermediate.[3] They also suggested that CPA and thermolysin may have similar mechanisms because their structures at active sites resemble each other.[9] Recently Morihana and his co-workers studied the thermolysin-catalysed cleavage of L-leucyl-L-leucylamide and *N*-benzyloxycarbonyl-L-leucine, and they postulated pathways involving both ' acyl-enzyme ' and ' amino-enzyme ' intermediates.[4] However there is no unequivocal evidence showing that either of these intermediates involves covalent bond between the active site and the cleaved species.[10]

We have previously found that, in the presence of thermolysin, *N*-benzyloxycarbonyl-L (or DL)-aspartic acid [*Z*-L (or DL)-Asp] and L (or DL)-phenylalanine methyl ester [L (or DL)-Phe-OMe, or L (or DL)-PM] react to give *N*-benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester (*Z*-L-Asp-L-Phe-OMe, or *Z*APM).[11] This enzymatic reaction provides a novel and elegant route to the preparation of L-aspartyl-L-phenylalanine methyl ester (aspartame), the synthetic sweetener which is *ca.* 200 times as sweet as sucrose.[12] It would be expected that a kinetic study of a reverse reaction would produce valuable information which is difficult to obtain from the study of the forward reaction. We performed a kinetic study on this useful reaction and report here some results which are relevant to the elucidation of the mechanism of the action of thermolysin.

EXPERIMENTAL

General.—L-, D-, and DL-aspartic acid and phenylalanine were purchased from Wako Pure Chemical Industries Ltd. Benzyloxycarbonylations of L-, D-, and DL-aspartic acid were performed by the standard Schotten–Baumann method.[13] The products were recrystallized two times from AcOEt–n-hexane. $[\alpha]_D^{25}$ Values of *Z*-L-Asp, *Z*-D-Asp, and *Z*-DL-Asp were +9.4, −9.5, and 0° (*c* 2, AcOH), respectively.† Esterifications of L-, D-, and DL-phenylalanine by methanol were done by the standard Fisher esterification method,[15] and the products (hydrochloride salts) were recrystallized from water. $[\alpha]_D^{25}$ Values of L-Phe-OMe, HCl, D-Phe-OMe, HCl, and DL-Phe-OMe, HCl were −4.7, +4.6, and 0° (*c* 5, H_2O), respectively.‡ Crystalline thermolysin (enzyme activity 8 080 p.u. mg^{-1}) was purchased from Daiwa Kasei Ltd. and was recrystallized according to the literature procedure.[17] Analytical grades of sodium chloride and 1/10M aqueous ethylenediaminetetra-acetic acid (EDTA) were purchased from Wako Pure Chemical Industries Ltd.) and were used without purification. Measurements of pH were made with Horiba M-7 pH meter. Optical rotations were recorded using Union Giken PM-101 automatic polarimeter. Measurements of high performance liquid chromatography (h.p.l.c.) were done with a TSK-HLC-802 instrument (Toyo Soda) equipped with a UV-254 detector. The column packing was TSK-LS-170 (cross-linked polysaccharides gel; particle size 5 μ; Toyo Soda) in a 7.5 mm × 600 mm stainless tube, and the eluant was

† The literature value for the L-isomer is +9.5° (*c* 2, AcOH).[14]
‡ The literature value for the L-isomer is −4.6° (*c* 5, H_2O).[16]

0.5M aqueous AcOH–NaOH buffer solution (pH 6.0) with the flow rate of 9.2 ml min^{-1} (pressure 20 kg cm^{-2}).

Kinetic Measurements.—As has been previously reported, when a concentrated solution of *Z*-L- (or DL)-Asp and L (or DL)-PM is reacted in the presence of thermolysin, the salt of *Z*APM with L (or D)-PM is deposited from the reaction mixture.[11] Therefore kinetic measurements were made with dilute solution of the substrates (0.01—0.07M) in order to avoid precipitation of the product.

The appropriate amounts of each stock solution of *Z*-Asp and PM, HCl, the amounts being chosen to give the desired substrate concentrations when diluted, were taken by a pipette and mixed in a 100 ml beaker, and the volume was made up to *ca.* 80 ml by the addition of distilled water. The ionic strength of the substrates at the desired pH was calculated in the usual manner,[18] and the appropriate amount of NaCl was added to give an ionic strength of 0.364M in the final reaction mixture. The substrate mixture was then adjusted to the desired pH by the addition of N-NaOH. In the present study no specific buffering system was employed, since the condensation reaction does not alter the pH of the mixture significantly, and in addition, the substrate mixture possesses buffering ability by itself in the pH range studied. The contents of the beaker were transferred quantitatively to a 100 ml volumetric flask and made up to the mark with water washings of the beaker. A small portion was withdrawn and subjected to pH measurement. Using a pipette, 50 ml of the contents were transferred to a 100 ml Erlenmyer flask, and

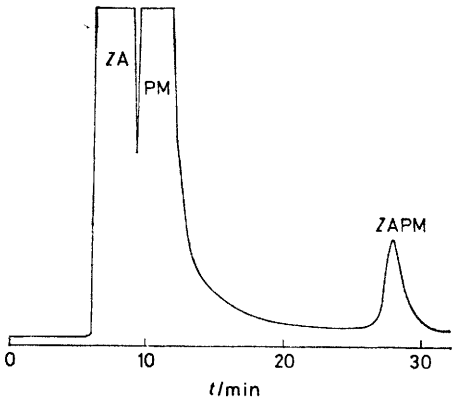


FIGURE 1 H.p.l.c. of the reaction mixture

thermally pre-equilibrated at 40 °C for 5 min. Enzyme stock solution (5 ml), made by dissolving an appropriate amount of thermolysin in distilled water (50 ml) and also thermally pre-equilibrated at 40 °C, was added by means of a syringe. The reaction was started by shaking the mixture vigorously by hand, and then the flask was stoppered and placed in a water-bath shaker thermostatted at 40 °C. At intervals portions of the mixture were withdrawn, poured into an ice-cooled flask containing 0.1M-EDTA solution to quench the reaction, and analysed by h.p.l.c. The *Z*APM peak appeared separately from the others so that the amount of *Z*APM produced could be determined (Figure 1). During the first 10—20 min of the reaction, the amount of *Z*APM produced increased linearly with time (Figure 2); hence the initial rate was determined from this slope.

Attempts to trap the Possible Acyl-enzyme Intermediate.—In a 20 ml round-bottom flask, *Z*-D-Asp (2 mmol) and L-PM, HCl (6.5 mmol) were placed, and N-NaOH (6.5 ml) was added to neutralize the hydrochloride. At this point the pH of the solution was 6.7. After thermolysin (50 mg) was added, the flask was placed in a water-bath shaker thermostatted at 40 °C. The reaction was continued for 17 h, during which the homogeneity of the mixture was maintained and no deposition of the product was observed. The

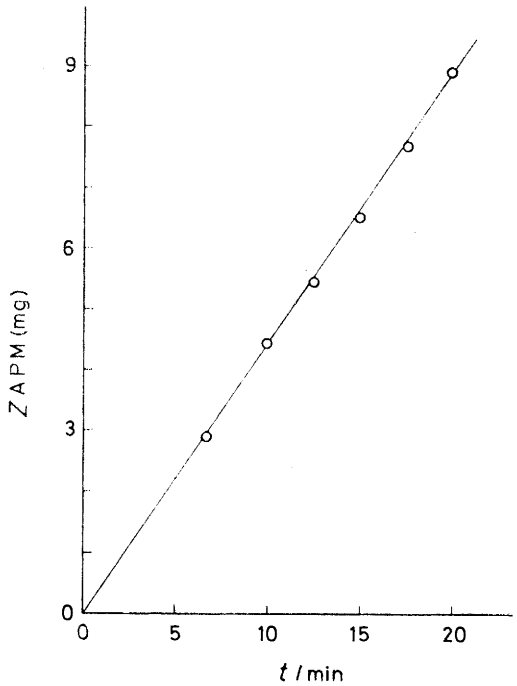


FIGURE 2 Typical time course of the thermolysin-catalysed condensation of *N*-benzyloxycarbonylaspartic acid with phenylalanine methyl ester. [*Z*-L-Asp] 1.82×10^{-2}M; [L-PM] 3.64×10^{-2}M; [E_0] 4.85×10^{-6}M; pH 6.5; μ 0.364M; 40 °C

mixture was thus diluted by the addition of 0.1M-EDTA and water (20 ml), and then analysed by h.p.l.c. as before. No trace of *Z*APM was detected. Under the same reaction conditions, the use of *Z*-L-Asp, instead of *Z*-D-Asp, gave, even after 3 h, an almost quantitative yield of *Z*APM, L-PM, whose deposition made the mixture a stiff, immovable cake.

RESULTS AND DISCUSSION

The rate is dependent on the pH of the medium and at a given pH is first order with respect to the enzyme concentration. In Figure 3, the effects of the concentrations of L-PM and DL-PM on the rate at pH 6.5 are shown. Good linear correlations between the rate and the concentration of the L-isomer as well as of the racemic mixture can be seen with no indication of rate saturation in the concentration range examined. It is of special interest to note that the slope of the rate *versus* [DL-PM] plot is half of that of the rate *versus* [L-PM] plot, and that the rate with L-PM is not affected by the addition of D-PM. These facts indicate that the rate is dependent only on the concentration of L-PM and that the presence of D-PM neither enhances nor inhibits the reaction.

On the other hand, a plot of [*Z*-L-Asp] against the rate shows saturation of the rate, typical Michaelis–Menten behaviour (Figure 4). It can also be seen that

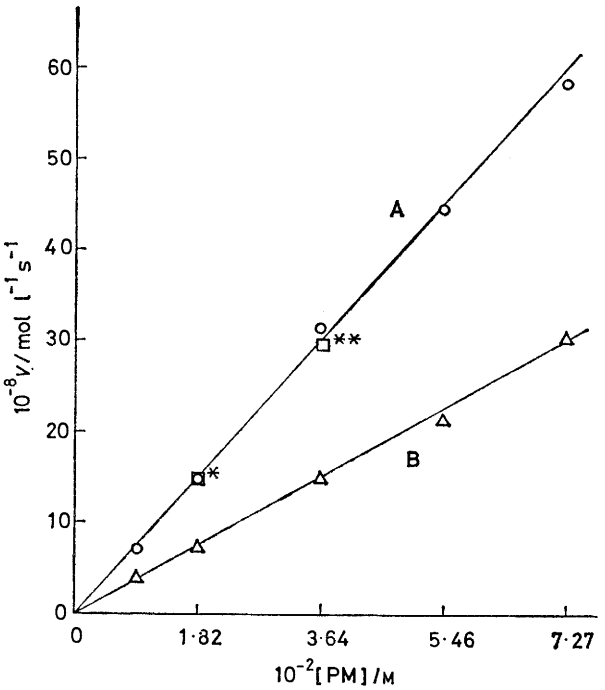


FIGURE 3 The effects of PM concentration on the rate of *Z*APM production. Lines A and B represent the plots of the rate against [L-PM], and [DL-PM], respectively, where □* denotes the reaction rate at [L-PM] 1.82×10^{-2}M in the presence of [D-PM] 9.09×10^{-3}M, and □** denotes the reaction rate at [L-PM] 3.64×10^{-2}M in the presence of [D-PM] 1.82×10^{-2}M: [*Z*-L-Asp] 1.82×10^{-2}M; [E_0] 4.85×10^{-6}M; pH 6.5; μ 0.364M; 40 °C

rate retardation occurs when *Z*-D-Asp is present, indicating that *Z*-D-Asp acts as an inhibitor. The Lineweaver–Burk plots of $1/v$ against $1/$[*Z*-L-Asp], either in the absence or presence of *Z*-D-Asp, show linear correlations, and moreover the two straight lines intersect at the ordinate (Figure 5). This suggests that *Z*-D-Asp acts as a competitive inhibitor.[19]

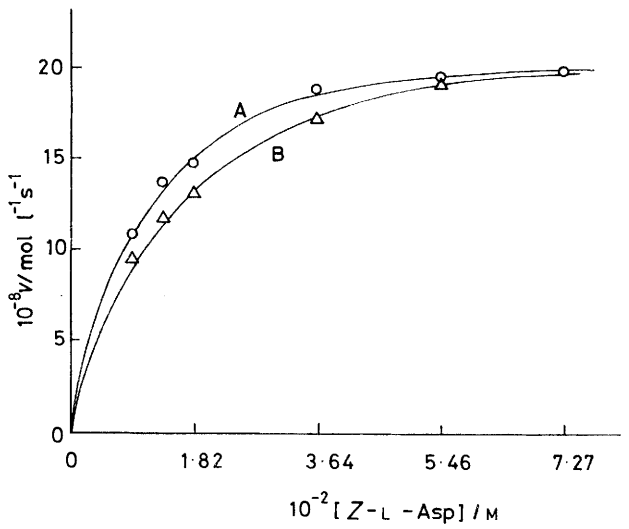


FIGURE 4 The effect of *Z*-L-Asp concentration on the rate of *Z*APM production. Curve A represents the reaction in the absence of *Z*-D-Asp, and B in the presence of *Z*-D-Asp: [*Z*-D-Asp] 9.09×10^{-3}M; [L-PM] 1.82×10^{-2}M; [E_0] 4.85×10^{-6}M; pH 6.5; μ 0.364M, 40 °C

The observations that the rate increases linearly with the concentration of L-PM and that the Lineweaver–Burk plot for *Z*-L-Asp yields a straight line resemble the kinetic results reported by Chance for the reaction of horseradish peroxidase,[20] suggesting that the thermolysin-catalysed condensation of *Z*-L-Asp with L-PM occur

$$\text{Z-L-Asp} + \text{E} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{Z-L-Asp, E}$$

$$\text{Z-L-Asp} \cdot \text{E} + \text{L-PM} \xrightarrow{k_2} \text{ZAPM} + \text{E}$$

SCHEME 1

via basically the same reaction scheme as in the peroxidase reaction. Thus the reaction mechanism may be as

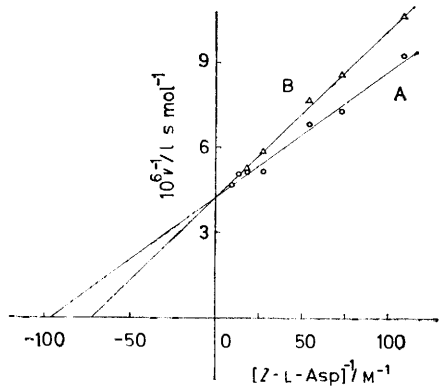


FIGURE 5 Lineweaver–Burk plot of Figure 4

shown in Scheme 1.[20] The rate law for this mechanism can be expressed by equation (1).[20] This equation indicates that, if the second step is rate determining

$$v = \frac{d[\text{ZAPM}]}{dt} = \frac{k_2[\text{E}_0][\text{L-PM}]}{1 + (k_{-1} + k_2[\text{L-PM}])/k_{+1}[\text{Z-L-Asp}]} \quad (1)$$

(*i.e.*, $k_{-1} \gg k_2$[L-PM]), the rate would be first order in [L-PM], whereas saturation of the rate would be seen for [Z-L-Asp] (*i.e.*, linear Lineweaver–Burk plot), since equation (1) can now be expressed by (2) and (3) where $K = k_{-1}/k_{+1}$. The experimental results are in accord

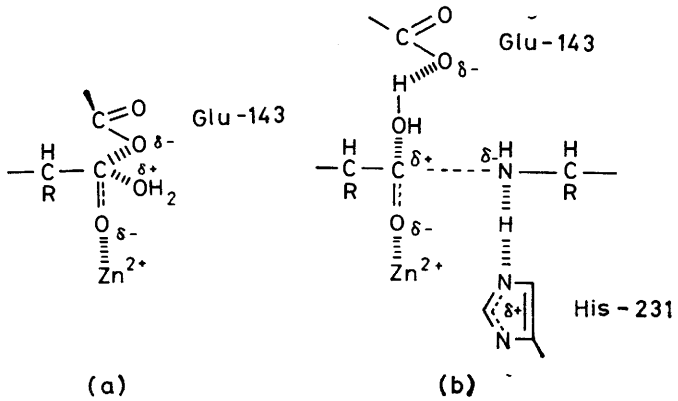
$$v = \frac{k_2[\text{E}_0][\text{Z-L-Asp}][\text{L-PM}]}{K + [\text{Z-L-Asp}]} \quad (2)$$

$$\frac{1}{v} = \frac{K}{k_2[\text{E}_0][\text{L-PM}]} \cdot \frac{1}{[\text{Z-L-Asp}]} + \frac{1}{k_2[\text{E}_0][\text{L-PM}]} \quad (3)$$

with these expectations. From the data in Figures 3 and 5, values of 1.03×10^{-2} mol l^{-1} and 2.65 l mol^{-1} s^{-1} are obtained for K and k_2, respectively. From Figure 5, the inhibition constant of *Z*-D-Asp (K_i) can also be calculated as 2.81×10^{-2} mol l^{-1}. In contrast, the bindings of L-PM and D-PM are extremely sluggish as compared with *Z*-L-Asp and *Z*-D-Asp, and the binding constants could not be determined.

As shown above, it is now evident that the condensation reaction involves initial binding of *Z*-L-Asp to the

enzyme to form the *Z*-L-Asp–enzyme complex and then attack by L-PM on the complex as the rate-determining step to form the condensation product. From the ordered binding of the substrates and the sluggishness of the binding of L-PM as compared with *Z*-L-Asp, it is reasonable to assume that an 'amino-enzyme intermediate' (covalently bonded or its non-covalent equivalent), which was suggested for thermolysin-catalysed transpeptidation reactions,[4] is not involved in the present reaction. Although the exact nature of the *Z*-L-Asp–enzyme complex cannot be described at this moment because of the limited information, some speculation is possible from the present results and other available information. The most important aspect of the reaction mechanism is whether the reaction proceeds *via* attack of the amino-substituent of L-PM at the α-carboxy-group of *Z*-L-Asp to produce the peptide bond directly, or *via* nucleophilic attack of Glu-**143** of the enzyme to form the acyl-enzyme intermediate. This is the reverse of the postulated mechanism for the hydrolysis of a peptide bond, and correspond to the general base catalysis route and the acyl-enzyme route, respectively. The transition states of these routes are shown in Scheme 2.



SCHEME 2 Possible mechanisms for the synthesis of the peptide: (*a*) acyl-enzyme route; (*b*) general base catalysis route.

In general, it can be assumed that the binding of the L- and D-isomers to an enzyme occur with about the same ease, whereas the rate of deacylation of an acyl-enzyme intermediate, and presumably the acylation rate, too, is greatly dependent on the stereochemical configuration of a substrate.[21,22,*] Consequently, if the first step in the reaction involves initial binding of Z-L-Asp followed by formation of the covalently bonded acyl-anzyme intermediate, the values of *K* for *Z*-L-Asp and *Z*-D-Asp would be significantly different. The fact that the *K* values of *Z*-L-Asp and *Z*-D-Asp are similar therefore suggests that these values are only a function of the binding step, and accordingly covalent bond formation to give the acyl-enzyme intermediate is probably not involved in this reaction.

One frequently employed technique to show the existence of an acyl-enzyme intermediate is trapping by potential nucleophiles.[24] We have thus carried out the enzymatic reaction using L-PM and *Z*-D-Asp, instead of *Z*-L-Asp, with the intention of trapping a possible acyl-enzyme intermediate from *Z*-D-Asp–enzyme complex, if it were in fact formed (see Experimental section). However, no trace of *Z*-D-Asp-L-Phe-OMe was detected by h.p.l.c. even for reactions performed under conditions which give an almost quantitative yield of the product if *Z*-L-Asp is used. This result is again evidence against the acyl-enzyme intermediate route.

In summary, our results are most consistent with a mechanism involving the reverse of general base catalysed hydrolysis of a peptide bond. This mechanism has also been suggested by Kester and Matthews from their crystallographic study of the binding of inhibitors to thermolysin.[3]

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* For instance, the binding constants of the L- and D-isomers of *N*-acetyltryptophan ethyl ester with chymotrypsin are 2.5×10^{-3} and 0.8×10^{-3} mol l^{-1}, respectively.[21] In contrast, relative rates for the deacylation, k_3^L/k_3^D, in the chymotrypsin-catalysed hydrolyses of $AcNHCHRCO_2C_6H_4NO_2$ were found to be 18 000 (R = CH_2-indole), 6 400 (R = $CH_2C_6H_5$), and 148 (R = Pr^i).[23] Although the stereochemical requirement for the carboxymethyl group of *Z*-Asp may not be as big as those for the above substituents, electronic interaction with Glu-143 would bring about similar or even greater effects.

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