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ALKALINE PROTEASE FROM *ASPERGILLUS ORYZAE*: ESTERASE ACTIVITY

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SUMMARY

Kinetic data are presented for the hydrolysis of N-substituted amino acid esters by aspergillopeptidase B, an alkaline protease from *Aspergillus oryzae*. The rate of hydrolysis was followed with a pH-stat at pH 8.5, 25°C and ionic strength 0.1. The overall susceptibility of *N*-acetyl amino acid esters to hydrolysis decreases in the order of Phe, Tyr > Trp > Met > Leu > Lys > His ≫ Val, Gly. The *N*-benzoyl derivatives of His, Lys and Arg methyl esters are approximately thirty times more reactive than the corresponding acetyl derivates. Amino acid esters with free α-amino groups are unable to serve as substrates. These data clearly suggest that before an amino acid ester can serve as a substrate for the alkaline protease of *A. oryzae* (a) the α-amino group must be blocked and (b) an aromatic group is required either in the amino acid side chain or as the N-terminal blocking group. It further suggests that the binding of substrate to the enzyme active site probably takes place through hydrophobic binding.

The hydrolysis of *N*-benzoyl-L-arginine ethyl ester by aspergillopeptidase B showed a sigmoid pH–velocity profile which could be fitted with a calculated titration curve for a single group with a pK' of 6.42 at 20°C and 6.02 at 35°C, giving a ΔH_i of 9.1 kcal per mole at 0°C. These data suggest that the enzyme may require a non-protonated imidazole group for activity.

INTRODUCTION

The presence of proteolytic activity in the culture medium of *Aspergillus oryzae* was first reported in 1922 by Oshima[1]. An apparently homogeneous alkaline protease was isolated by Subramanian and Kalnitsky[2] and was named aspergillopeptidase B.

Abbreviations: Ac, acetyl; Bz, benzoyl; Z, carbobenzoxy; Tos, tosyl. Substituted animo acid substrates are abbreviated according to the Tentative Rules of the IUPAC–IUB Commission on Biochemical Nomenclature *Biochim. Biophys. Acta*, 121 (1966) 1.

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The initial work on the substrate specificity of the crude enzyme indicated that *N*-acetyl derivatives of L-tyrosine, L-tryptophan and L-phenylalanine methyl esters were hydrolyzed at high rates, whereas *N*-carbobenzoxy-L-glycine methyl ester was not hydrolyzed and *N*-benzoyl-L-arginine methyl ester appeared to be most susceptible to hydrolysis by the enzyme[3]. The authors desired to extend the studies of the substrate specificity of this alkaline protease by using a series of structurally related esters. As pointed out by Dixon and Webb[4], a satisfactory investigation of the specificity of an enzyme requires not only the use of pure enzyme and pure substrate, but also requires the determination of K_m and V, so that the two factors, (a) the formation of enzyme–substrate complex and (b) the rate of its breakdown, can be studied separately. Kinetic data were obtained with a series of various N-substituted amino acid esters, RCH(NHR')COOR''. In addition, pH–activity profiles were constructed at both 20 and 35°C.

MATERIALS AND METHODS

Enzyme

The alkaline protease was purified as previously described[2,5]. The purified preparations were stored frozen in aqueous stock solutions of 1.0 mg/ml determined spectrophotometrically, according to the equation, $\varepsilon^{1\%}_{280\,nm} = 9.0$. The molar concentration was computed taking 26 000 as the molecular weight of the enzyme.[5]

Substrates

All the synthetic amino acid esters were purchased from Cyclo Chemical Corporation, Los Angeles, Calif., and were used without further purification. The purity of the esters was checked by paper chromatography. Most of the esters appeared to be homogeneous or contained only trace amounts of impurity in the form of the unblocked amino acid ester. Exceptions are *N*-acetyl derivatives of L-arginine, L-ornithine and L-lysine methyl esters which tend to form some cyclic compounds after being dissolved in water.

Estimation of initial velocity

Rates of hydrolysis were determined with the aid of a Radiometer Type TTT 1a pH-stat equipped with a syringe drive, recorder and a thermostatically controlled reaction vessel which was maintained to within 0.1°C. The reaction mixture (3.0 ml) containing substrate and 0.1 M KCl was adjusted to the desired pH by addition of minute volumes of 0.5 M NaOH. To minimize the absorption of CO_2, a stream of N_2 was passed over the surface of the reaction fluid which was mixed continuously with a magnetic stirrer. Concentrations of NaOH between 0.002 and 0.01 M were used as the titrant. Blanks were established by monitoring base uptake for 10 min prior to the addition of enzyme solution; the reaction was then initiated by adding 10 μl of enzyme solution to the reaction fluid by means of a microsyringe. The base uptakes per unit time after subtracting the blanks are initial rates and considered to be accurate to approx. 2%, except in the case of substrate concentration much lower than the K_m values where marked curvature of reaction plot was encountered.

Determination of the point of cleavage in N-blocked amino acid esters

Synthetic esters were first subjected to alkaline protease hydrolysis for 24 h under conditions similar to those described for the rate measurements. The reaction mixture was then developed on Whatman No. 1 chromatography paper, along with authentic samples of unblocked amino acid ester and N-blocked amino acid. The developing solvent was either 1-butanol:acetic acid:water (4:1:5, by vol.) or 1-butanol:pyridine:water (1:1:1, by vol.). The resultant spots were detected by specific spray reagents[7].

RESULTS

Determination of products of the reaction

From the paper chromatogram of the enzymatic hydrolysate of *N*-acetyl-L-tryptophan ethyl ester, only spots corresponding to the N-blocked amino acid (*N*-acetyl-L-tryptophan) and the ester of the N-blocked amino acid (*N*-acetyl-L-tryptophan ethyl ester) could be detected. The same was also true for the hydrolysate of *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-histidine methyl ester. This evidence demonstrates that the ester bond, not the peptide bond between the blocking group and amino group, is the site of cleavage.

Optimum pH

The alkaline protease of *Aspergillus oryzae* readily hydrolyses *N*-benzoyl-L-arginine ethyl ester in the pH range 7–9, with the optimum at pH 8.50–8.75 (Fig. 1). This was determined by plotting initial velocities from the pH-stat against pH at a

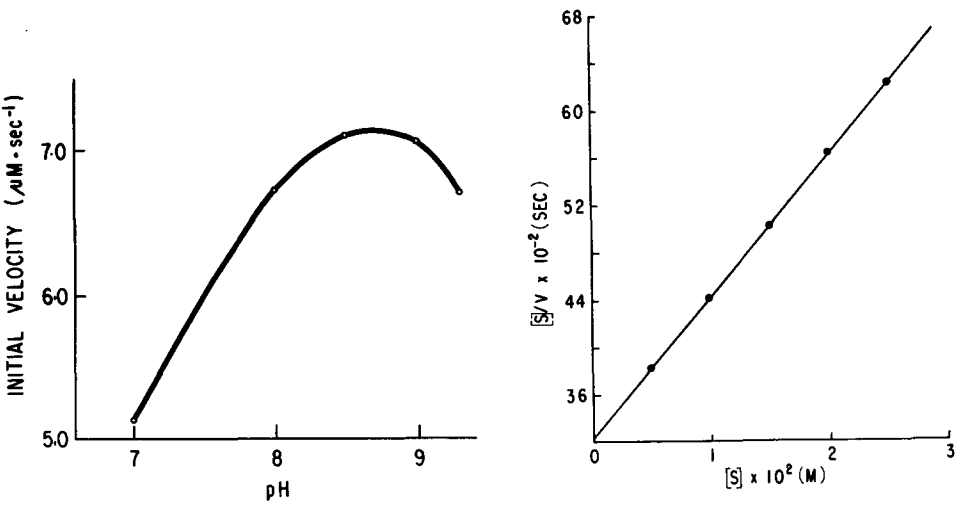

Fig. 1. Effect of pH on the hydrolysis of 0.06 M *N*-benzoyl-L-arginine ethyl ester by the alkaline protease from *A. oryzae*. All measurements were performed at 25 °C in the presence of 0.1 M KCl and 0.128 μM enzyme.

Fig. 2. Linear plot of $[S]/v$ *versus* $[S]$ for the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by the alkaline protease from *A. oryzae*. All measurements were performed at 25 °C and pH 8.5 in the presence of 0.1 M KCl and 0.128 μM enzyme. Slope = $1/V$; Intercept = K_m/V; therefore, V = 8.43 $\mu M \cdot s^{-1}$ and K_m = 27.4 mM.

single substrate concentration ([S] = 0.06 M = 8.73 K_m). The maximum difference in the K_m value over the pH range 5.5–8.5 was 2-fold and was not considered significant. The optimum pH for the hydrolysis of *N*-acetyl-L-methionine methyl ester was also at pH 8.50–8.75 ([S] = 0.15 M = 2.18 K_m). At pH values above 9.3, measurements were not carried out because the rate of nonenzymatic hydrolysis of the substrate reached a level comparable to that of the enzymatic catalysis.

Estimation of V, K_m and k_3

The Michaelis–Menten equation may be plotted in several different ways[8] for the determination of V and K_m. Statistical considerations[9] indicated that the linear

TABLE I

KINETIC PARAMETERS OF THE HYDROLYSIS OF *N*-ACETYL AMINO ACID ESTERS CATALYZED BY THE ALKALINE PROTEASE FROM *A. oryzae*

All measurements were performed at 25 °C and pH 8.5 with 0.01 M NaOH as titrant. The reaction mixture (3.0 ml) contained 0.128 μM alkaline protease and 0.1 M KCl.

Substrate	K_m *(mM)*	k_3 (s^{-1})	k_3/K_m $(mM^{-1} \cdot s^{-1})$	*Relative reactivity*
Ac-Phe-OEt	14.0	35.0	2.50	33.3
Ac-Tyr-OEt	27.4	66.4	2.42	32.2
Ac-Tyr-OMe	24.4	60.8	2.49	33.2
Ac-Trp-OEt	3.6	5.8	1.59	21.2
Ac-Met-OMe	68.8	68.3	0.99	13.2
Ac-Leu-OMe	34.8	20.2	0.58	7.7
Ac-Lys-OMe	249.0	77.2	0.31	4.1
Ac-Ala-OMe	144.0	31.5	0.22	2.9
Ac-His-OMe	224.0	16.7	0.08	1.0

plot $[S]/v$ *versus* $[S]$ was preferable. An $[S]/v$ *versus* $[S]$ plot for a typical experiment on the hydrolysis of *N*-acetyl-L-tyrosine methyl ester by the alkaline protease at 25°C, pH 8.50 is shown in Fig. 2.

The k_3 values were obtained from the equation $V = k_3 E_0$ where the enzyme concentration was 0.128 μM. Kinetic parameters derived from these equations are summarized in Tables I and II.

TABLE II

THE EFFECT OF N-SUBSTITUTION ON THE KINETIC PARAMETERS OF THE HYDROLYSIS OF AMINO ACID ESTERS CATALYZED BY THE ALKALINE PROTEASE FROM *A. oryzae*

All measurements were performed at 25 °C and pH 8.5 with 0.01 M NaOH as titrant. The reaction mixture (3.0 ml) contained 0.128 μM alkaline protease and 0.1 M KCl.

Substrate	K_m *(mM)*	k_3 (s^{-1})	k_3/K_m $(mM^{-1} \cdot s^{-1})$	*Relative reactivity*
Ac-Lys-OMe	249.0	77.2	0.31	1.0
Bz-Lys-OMe	29.4	257.0	8.74	28.2
Ac-His-OMe	224.0	16.7	0.08	1.0
Bz-His-OMe	16.9	40.4	2.39	31.9
Tos-Arg-OMe	38.3	16.3	0.43	1.0
Z-Arg-OMe	12.0	20.2	1.68	3.9
Bz-Arg-OEt	6.9	30.1	4.38	10.2

Effect of pH and temperature on hydrolysis of N-benzoyl-L-arginine ethyl ester

The rate of hydrolysis of *N*-benzoyl-L-arginine ethyl ester by alkaline protease was measured as a function of pH at 20°C and at 35°C with an enzyme concentration of 0.0128 μM. The concentration of Bz-Arg-OEt (0.1 M) exceeded the K_m value at

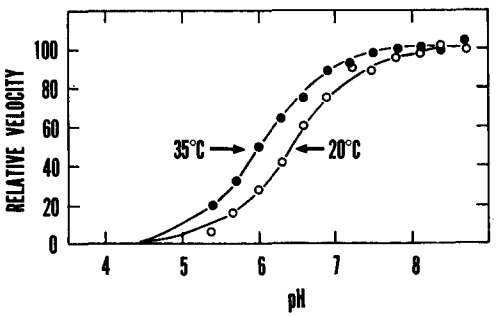

Fig. 3. Relative velocity as a function of pH and temperature for the hydrolysis of 0.1 M *N*-benzoyl-L-arginine ethyl ester by the alkaline protease from *A. oryzae*. The open and closed circles are data collected at 20 and 35 °C, respectively. The curves are theoretical as calculated for a single titratable group of pK' 6.42 at 20 °C and 6.02 at 35 °C. The initial reaction volume, 3.0 ml, contained 0.0128 μM alkaline protease and 0.1 M KCl.

25°C more than 10-fold and therefore the initial rates should approximate V values. When relative velocities were plotted as a function of pH, sigmoidal profiles were obtained (Fig. 3).

DISCUSSION

The term "Proteolytic coefficient" is defined as the first-order reaction constant per mg of enzyme nitrogen per ml (ref. 10). This is calculated from decimal logarithms and is usually determined at 0.05 M substrate concentration. This term has been used to express enzymatic activity and to compare the activity of the same enzyme toward several structurally related substrates. The limited validity of such interpretations is evident from the fact that the first-order reaction constant is dependent on both K_m and on the initial substrate concentration[11,12]. The use of k_3/K_m ratios for the description of the relative specificity of proteolytic enzymes has been thoroughly discussed by Knowles[13] and by Bender and Kézdy[14]. They concluded that for the purpose of correlating structure and specificity, k_3 and the complex constant k_3/K_m appear to be the most useful.

According to the values of k_3/K_m for the alkaline protease catalyzed hydrolysis of synthetic esters listed in Table I, the rate of cleavage of the ester bond decreases in the order of Ac-Phe-OEt; Ac-Tyr-OEt > Ac-Trp-OEt > Ac-Met-OMe > Ac-Leu-OMe > Ac-Ala-OMe. Thus amino acid residues containing an aromatic structure are more susceptible to hydrolysis than those composed of aliphatic chains. Another related and striking feature which can be seen in the data summarized in Table I is the great difference in the K_m values between substrates containing aromatic side chains and those with basic side chains. The kinetic parameter K_m is observed to increase from a low value of 3.63 mM for a good substrate such as *N*-acetyl-L-tryptophan ethyl ester to a high value of 249 mM for *N*-acetyl-L-lysine methyl ester, which

is a poor substrate. Therefore, it appears that the poor ability to form the enzyme-substrate complex could account for the relatively low susceptibility of *N*-acetyl-L-histidine methyl ester to hydrolysis by the alkaline protease of *A. oryzae*. The possible explanation for the ability of methionine to replace an aromatic amino acid in these substrates may be found in its well-known chemical similarity to benzyl and allyl compounds as pointed out by Kaufman and Neurath[15].

The effect of various N-substituted groups on the hydrolysis of the amino acid ester bond can be evaluated from Table II. Substitution of the benzoyl group for the acetyl group promotes hydrolysis about 30-fold,

$$\frac{k_3/K_m \text{ Bz-Lys-OMe}}{k_3/K_m \text{ Ac-Lys-OMe}} = 28.2, \quad \frac{k_3/K_m \text{ Bz-His-OMe}}{k_3/K_m \text{ Ac-His-OMe}} = 31.9$$

and the resulting decrease in K_m values is much greater than the increase in the k_3 values. It further suggests that the greater activity of the *N*-benzoyl amino acid ester, as compared to their *N*-acetyl analogs, depends mainly upon the greater affinity of the former for the enzyme surface. The same property has been observed in the case of the α-chymotrypsin-catalyzed hydrolysis of amides of *N*-benzoyl-L-tyrosine and *N*-acetyl-L-tyrosine[16]. The alkaline protease of *A. oryzae* does not hydrolyze substrates with a tosyl or carbobenzoxy group as effectively as substrates containing a benzoyl group, the rates being in the proportion of about 1:4:10 for Tos-Arg-OMe:Z-Arg-OMe:Bz-Arg-OMe. This effect may be a consequence of the position of the aromatic ring of the N-substituted group in relation to the sensitive ester bond. Indeed, from a study of atomic models the aromatic ring of the benzoyl group can be placed in a closer proximity to the bond which is hydrolyzed by the enzyme. When acetyl is the N-blocking group of arginine methyl ester, the ester is not hydrolyzed.

The following amino acid esters are not hydrolyzed at a significant rate by this alkaline protease under the conditions of these experiments: Ac-D-Phe-OEt, Ac-D-Tyr-OEt, Ac-diiodo-L-Tyr-OMe, Ac-Val-OMe, Ac-Gly-OMe, Ac-Pro-OMe, Ac-Orn-OMe, Ac-Arg-OMe, Ac-β-Ala-OMe, Bz-Gly-OMe, Tyr-OEt, Arg-OMe, Gly-OMe, His-OMe, Lys-OMe, Met-OMe, Met-OEt and sarcosine-OMe. These results give us some further insight concerning the specificity requirements of the enzyme. The enzymatic activity of the alkaline protease is restricted to those esters in which the amino acid residue is of the L-isomeric form. This stereospecificity is adequately demonstrated by the findings that the *N*-acetyl derivatives of D-phenylalanine and D-tyrosine ethyl esters are resistant to hydrolysis, whereas the corresponding L-isomeric esters are good substrates (Table I). *N*-acetyl-3',5'-diiodo-L-tyrosine methyl ester is also not hydrolyzed, indicating that the addition of two bulky groups on the benzene ring causes a steric shielding effect. The extreme difficulty in hydrolyzing the ester of *N*-acetyl-L-valine may be interpreted as a steric inhibition due to the branching of the side chain β to the potentially reactive carbonyl[17,18]. The failure to find evidence of hydrolysis of N-blocked glycine methyl esters suggests that the alkaline protease of *A. oryzae* contains a binding site for the amino acid side chains. The esters of *N*-acetyl-L-ornithine and *N*-acetyl-L-arginine are not only resistant to hydrolysis, but were also unable to inhibit the hydrolysis of *N*-benzoyl-L-arginine ethyl ester even when present in greater than equal molar amounts. The lack of ability to inhibit is probably due to the failure to form an enzyme–substrate complex.

The inability of amino acid esters with free α-amino groups to be hydrolyzed suggests that this alkaline protease also contains a binding site for the N-substituted group. Thus it appears that for substrates of the type RCH(NHR')COOR'', at least two binding sites are involved in the formation of the alkaline protease–substrate complex: one site is capable of effective combination with the amino acid side chain R while the other site is capable of effective combination with the N-substituted group R'. Both interactions are probably through hydrophobic binding. In order to characterize more fully the specificity of this enzyme, the hydrolysis experiments with the A and B chains of oxidized insulin and with reduced and alkylated ribonuclease A as substrates are under investigation.

The hydrolysis of Bz-Arg-OEt by alkaline protease at 35°C showed a sigmoid pH–velocity profile over the range from 5.4 to 8.7 (Fig. 3) and the experimental points could be fitted with a calculated titration curve for a group with a pK' of 6.02. The pH–velocity profile is very similar to those obtained for the trypsin–Bz-Arg-OEt[19], chymotrypsin–Ac-Tyr-OEt[20], Carlberg substilisin–Bz-Arg-OEt[21] and *Aspergillus flavus* alkaline protease–Bz-Arg-OEt[22] systems and suggests that, protonation of a group with a pK' of 6.04 ± 0.04 in the active site of alkaline protease, results in the inactivation of this enzyme. The pH–velocity profile obtained at 20°C is also shown in Fig. 4 and could be fitted with a calculated titration curve for a group with a pK' of 6.42 ± 0.04. This clearly shows the pK' of the group being titrated varied appreciably with temperature. The most likely group responsible for the pK' value is the imidazole group of a histidine residue. The heat of ionization (ΔH_i), calculated from the Van 't Hoff equation, is 9.1 kcal/mole at 0°C. This value is in good agreement with the ΔH_i value of 9.3 kcal/mole at 0°C obtained with chymotrypsin–Ac-Tyr-OEt (calculated from the data in ref. 20), and is only slightly higher than the ΔH_i values (6.9–7.5 kcal/mole) for an imidazolium group in peptides[23]. The value of 9.1 kcal/mole is also within experimental error the same as that found for other alkaline protease–amino acid ester systems[19,21,22]. Therefore, it is believed that a histidine residue is present at the catalytic site of the alkaline protease from *Aspergillus oryzae*.

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