Department of Food Science and Technology,
University of California,
Davis, Calif. 95616 (U.S.A.)

V. PATEL
A. L. TAPPEL

1 O. Z. SELLINGER, H. BEAUFAY, P. JAQUES, A. DOYEN AND C. DE DUVE, *Biochem. J.*, 74 (1960) 450.
2 J. CONCHIE AND G. A. LEVVY, *Biochem. Soc. Symp.*, 23 (1963) 86.
3 V. PATEL AND A. L. TAPPEL, *Biochim. Biophys. Acta*, 191 (1969) 653.
4 R. G. PRICE AND N. DANCE, *Biochem. J.*, 105 (1967) 877.
5 S. SHIBKO AND A. L. TAPPEL, *Biochem. J.*, 95 (1965) 731.
6 N. G. ASP AND A. DAHLQVIST, *Biochem. J.*, 110 (1968) 143.
7 B. V. PLAPP AND R. D. COLE, *Biochemistry*, 6 (1967) 3676.
8 M. IGARASHI AND V. P. HOLLANDER, *J. Biol. Chem.*, 243 (1968) 6084.

Received July 14th, 1970

Biochim. Biophys. Acta, 220 (1970) 622–624

BBA 63489

Role of histidine in the active site of alkaline protease from *Aspergillus flavus* derived from kinetic data

In the preceding paper[1] we reported the specificity of the alkaline protease from *Aspergillus flavus* which contains an active serine residue. From the results of our experiments with the cleavage of the B-chain of oxidized insulin and of certain synthetic substrates we have been able to conclude that the protease[2] isolated by us belongs to the group of serine proteases with a broad specificity[3]. Another protease which falls into this group is subtilisin which has the same amino acid sequence around the serine active center[4,5] as our protease. The aim of this paper is to report the determination of kinetic constants and the effect of pH and temperature on the catalysis of the hydrolysis of *N*-benzoyl-L-arginine ethyl ester (BAEE) by our protease. From the results of these experiments the role of histidine in the active site can be deduced.

Material. The protease preparation used for the hydrolysis was obtained by the method described elsewhere[2] with the exception that the protease was precipitated first from the fraction obtained by chromatography on DEAE-Sephadex in acetone (−10°; 1:1, v/v) and then, after having been dissolved in water, lyophilized. The preparation thus obtained showed the presence of only one N-terminal group (glycine) and was free from low molecular weight peptide material. Chromatographically pure *N*-benzoyl-L-arginine ethyl ester hydrochloride was purchased from Fluka, A.G. (Buchs, SG; A51073, substrate for trypsin determination).

Determination of esterase activity. The rate of hydrolysis of the substrate was determined in a Model TTT1a pH-stat (Radiometer, Copenhagen) equipped with a titrating device (TTA31) and a temperature-controlled reaction vessel. The temperature of the reaction mixture in the vessel was kept constant with an accuracy of ± 0.1°. The volume of the reaction mixture was 2 ml. All solutions were in 0.1 M KCl

Abbreviations used: BAEE, *N*-benzoyl-L-arginine ethyl ester; ATEE, acetyl-L-tyrosine ethyl ester.

and no buffers were used. A 0.05 M solution of NaOH was used as the titrant. The rates were determined from the slopes of the apparent zero order plots obtained for the initial 5% hydrolysis. At the beginning of the experiment the reaction mixture contained 5–50 mM substrate in 2 ml of 0.1 M KCl and 0.005–0.025 mg of the alkaline protease from *A. flavus* per ml. Within this range the reaction rate was proportional to the enzyme concentration. The kinetic constants were evaluated graphically according to LINEWEAVER AND BURK[6]. For the calculation of K_{cat} (V/E_0), where E_0 is the initial enzyme concentration, a molecular weight of 18 000 was assumed.

Determination of K_m and V for BAEE hydrolysis. The values obtained for the hydrolysis of BAEE at 30° and pH 7.5 at eight different enzyme concentrations are K_m = 7.8 ± 0.5 mM and k_{cat} = 185 sec^{-1}. The found K_m value is in good agreement with K_m = 7.5 mM determined by MORIHARA AND TSUZUKI[3] and K_m = 7.9 mM determined by OTANI AND ISHIKAWA[7], who studied the hydrolysis of BAEE catalyzed by the alkaline protease from *Aspergillus oryzae* under identical experimental conditions. The found values of kinetic constants complement the data obtained in a series of comparison experiments[8] with alkaline proteases from *A. flavus* and *A. oryzae*. The agreement of the values suggests that the enzymes are similar if not identical (*cf.*[5]).

Effect of pH and temperature on hydrolysis of BAEE. The pH profile of the rate of hydrolysis of BAEE at 37° catalyzed by the alkaline protease from *A. flavus* is characterized in the range 4.25–9.5 by a sigmoid curve. All the experimentally determined points lie on the theoretically calculated titration curve of pK' = 5.96 (*cf.*

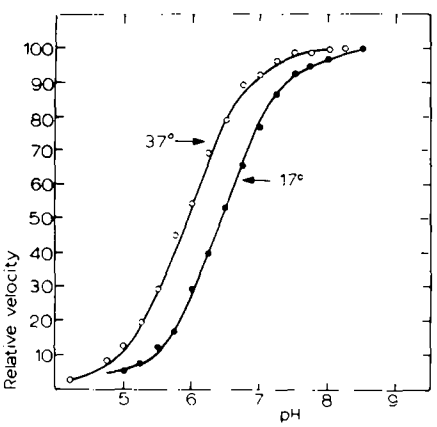

Fig. 1. Relative values of velocity as a function of pH for the hydrolysis of 0.05 M BAEE by alkaline protease from *A. flavus* at 37° and 17°. The curves drawn are theoretical as calculated for a single titratable group of pK' 5.96 at 37° and 6.40 at 17°. The points represent experimental values (see text).

Fig. 1). The K_m values determined at 37° in the pH range 5.0–8.5 are summarized in Table I. All values fall into the K_m range determined for the hydrolysis of BAEE at 30° and pH 7.5 and are therefore independent of pH. The values of relative rates given in Fig. 1 were obtained with substrate concentrations almost 7 times higher than K_m and practically they represent V values. The lack of effect of pH on K_m

indicates that K_m most likely represents the dissociation constant. Our investigation of the pH profile of the reaction rate was carried out under the conditions used by GLAZER[9] in his studies on the cleavage of BAEE by subtilisin. We obtained a very similar pH profile which moreover is analogous to the pH profile of the hydrolysis of

TABLE I

K_m VALUES OF HYDROLYSIS OF BAEE CATALYZED BY ALKALINE PROTEASE FROM *A. flavus* AT DIFFERENT pH-VALUES AT 37°

pH:	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
K_m	8.1	7.3	7.3	8.2	7.6	7.5	7.9	7.9
	7.4	7.7	7.7	7.7	8.0	7.6	7.7	7.6
	7.7	7.9	8.2	7.6	7.7	8.0	7.7	7.8

BAEE by trypsin[10] and of ATEE by chymotrypsin[11]. In agreement with the authors of these studies we also postulate that protonation of a group with a pK' of 5.96 in the active site of the alkaline protease from *A. flavus* results in the inactivation of the enzyme. Fig. 1 also shows the pH profile of the rate of hydrolysis of BAEE catalyzed by the alkaline protease from *A. flavus* at 17°. The experimental data obtained at this temperature are in agreement with the titration curve of pK' 6.40. Clearly, the pK' of the group being titrated varied appreciably with temperature. From van 't Hoff's equation[12]

$$\Delta H_i = -2.303 \cdot RT^2 \frac{dpK}{dT}$$

we calculated the value of ionisation heat $\Delta H_i = -7.5$ kcal per mole at 0°. The ΔH_i values for the imidazole group of histidine in proteins range from 6.9 to 7.5 kcal per mole and the pK of this group is between 5.6 and 7 (ref. 12). Hence, the kinetic data obtained by us in this study are in agreement with the results obtained by GLAZER[9] for the hydrolysis of BAEE by subtilisin. On the basis of his data the author has postulated the presence of an unionized histidine residue in the active site of subtilisin.

The reported binding of neutral tosyl-L-phenylalanine chloromethyl ketone to the active histidine of chymotrypsin[13] and of basic tosyl-L-lysine chloromethyl ketone to the active site of trypsin[14] led us to examine the effect of these inhibitors on our enzyme. As in the case of subtilisin[15] and of the alkaline protease from *Aspergillus sojae*[16], we have not been able to show the inhibition of proteolytic activity of our protease by these inhibitors, either. The reason for this phenomenon may be the difference in character of the binding sites.

From recent work on the mechanism of action of serine proteases it is obvious that we are likely to meet with the same arrangement of the catalytic site not merely in pancreatic proteases, *i.e.* chymotrypsin[17], trypsin[18], and elastase[19], but also in the microbial proteinase subtilisin[20], whose primary and tertiary structure is entirely different. Even though the amino acid composition[2] and the amino acid sequences which have been determined so far of peptides isolated from digests of the mold alkaline protease from *A. flavus* (unpublished results) indicate that the primary structure of the protein is different, we assume that our data permit us to classify our enzyme as belonging to the above mentioned group of serine proteases.

We wish to acknowledge the skillful technical assistance of Mrs. J. Lukešová in the experimental part of this study.

Institute of Organic Chemistry and Biochemistry, J. TURKOVÁ
Czechoslovak Academy of Sciences, Prague (Czechoslovakia)

1 J. TURKOVÁ AND O. MIKEŠ, *Biochim. Biophys. Acta*, 198 (1970) 386.
2 J. TURKOVÁ, O. MIKEŠ, K. GANČEV AND M. BOUBLIK, *Biochim. Biophys. Acta*, 178 (1969) 100.
3 K. MORIHARA AND H. TSUZUKI, *Arch. Biochem. Biophys.*, 129 (1969) 620.
4 H. F. NOLLER AND S. A. BERNHARD, *Biochemistry*, 4 (1965) 1118.
5 O. MIKEŠ, J. TURKOVÁ, NGUYEN BAO TOAN AND F. ŠORM, *Biochim. Biophys. Acta*, 178 (1969) 112.
6 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
7 Y. OTANI AND Y. ISHIKAWA, *J. Ferment. Technol.*, 47 (1969) 424.
8 O. MIKEŠ AND J. TURKOVÁ, *Abstr. Meeting Czech. Biochem. Soc., Martin, 1970*, IV-6, p. 88 (in Czech).
9 A. N. GLAZER, *J. Biol. Chem.*, 242 (1967) 433.
10 H. GUTFREUND, *Trans. Faraday Soc.*, 51 (1955) 441.
11 B. R. HAMMOND AND H. GUTFREUND, *Biochem. J.*, 61 (1955) 187.
12 M. DIXON AND E. C. WEBB, *Enzymes*, Longmans, Green and Co. Ltd., 1958, pp. 149 and 170.
13 G. SCHOELLMANN AND E. SHAW, *Biochemistry*, 2 (1963) 252.
14 E. SHAW, M. MARES-GUIA AND W. COHEN, *Biochemistry*, 4 (1965) 2219.
15 E. L. SMITH, F. S. MARKLAND, C. B. KASPER, R. J. DE LANGE, M. LANDON AND W. H. EVANS, *J. Biol. Chem.*, 241 (1966) 5974.
16 K. HAYASHI, M. TERADA AND K. MOGI, *Agr. Biol. Chem. Tokyo*, 34 (1970) 627.
17 D. M. BLOW, J. J. BIRKTOFT AND B. S. HARTLEY, *Nature*, 221 (1969) 337.
18 T. A. STEITZ, R. HENDERSON AND D. M. BLOW, *J. Mol. Biol.*, 46 (1969) 337.
19 D. M. SHOTTON AND H. C. WATSON, *Nature*, 225 (1970) 811.
20 C. S. WRIGHT, R. A. ALDEN AND J. KRAUT, *Nature*, 221 (1969) 235.

Received August 3rd, 1970