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SPECIFICITY AND INHIBITION STUDIES OF *ARMILLARIA MELLEA* PROTEASE

WILLIAM G. LEWIS [a,*], JOHN M. BASFORD [a] and PETER L. WALTON [b]

[a] *Department of Biochemistry, University College, P.O. Box 78, Cardiff, CF1 1XL, and* [b] *Department of Biochemistry, Pharmaceuticals Division, I.C.I. Ltd., Alderley Park, Macclesfield, Cheshire, SK10 4TG (U.K.)*

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Summary

The action of *Armillaria mellea* protease has been evaluated on a number of polypeptide substrates. It has been shown to split the Pro^{7} - Lys^{8} bonds in both native and oxidised lysine-vasopressin and the Ser^{11} - Lys^{12} bond in glucagon. No other splits were detected in these substrates. The enzyme also caused extensive degradation of *S*-carboxymethyl lysozyme, *S*-carcoxymethyl pepsinogen and oxidised ribonuclease A. In each case the only new amino-terminal residue to appear was lysine.

A. mellea protease was inhibited by the chelating agents 1,10-phenanthroline, α,α'-bipyridine and imidazole. The pK_I values (negative $\log_{10}$ of concentration required for 50% inhibition) for these three inhibitors were 3.9, 3.4 and 1.1, respectively.

Lysine, *S*-2-aminoethylcysteine and short chain aliphatic amines also proved to be relatively good inhibitors of *A. mellea* protease while arginine was a poor inhibitor.

Introduction

Armilliaria mellea protease is a proteolytic enzyme isolated from the basidiomycete *Armillaria mellea* [1]. It has been obtained in a highly pure form [2]. Studies using insulin [3,4], aspartate aminotransferase [5,6] and phospholipase A [7] have indicated that the primary specificity of *A. mellea* protease is towards peptide bonds involving the α-amino group of lysine residues. Thus *A. mellea*

* Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada.

protease was found to split only the Pro^{28}-Lys^{29} bond in the B chain of insulin. In aspartate aminotransferase many bonds in which the α-amino groups of lysine residues were involved were found to be susceptible to the enzyme. However, four sequences were found which contained lysine but which were resistant to the enzyme. Three of these sequences had aspartic acid on the COOH-terminal side of the lysine, the fourth having asparagine in this position. In addition, splits were noticed at three positions between arginine and either leucine or isoleucine. One of the purposes of this investigation was to study further the specificity of the enzyme towards polypeptide substrates. In addition, the inhibition of the enzyme by certain amino acids and aliphatic amines was studied to investigate the nature of the substrate binding site.

A. mellea protease is a metalloenzyme containing one atom of zinc per molecule of the native enzyme (Walton, P.L., unpublished observations). We have also studied the inhibition of the enzyme by metal-chelating agents in order to compare its behaviour with that of other zinc-containing metalloenzymes.

Materials and Methods

A. mellea protease. This was isolated according to the method of Walton et al. [1], which is outlined in Fig. 1. This preparation appeared to be homogeneous when subjected to polyacrylamide gel electrophoresis at pH 4.3 [8] and dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis at pH 7.0 in the presence and absence of 2-mercaptoethanol [9]. For the latter method, *A. mellea* protease was incubated at 37°C for 12 h in 8 M urea/2% SDS with and without 0.1% 2-mercaptoethanol.

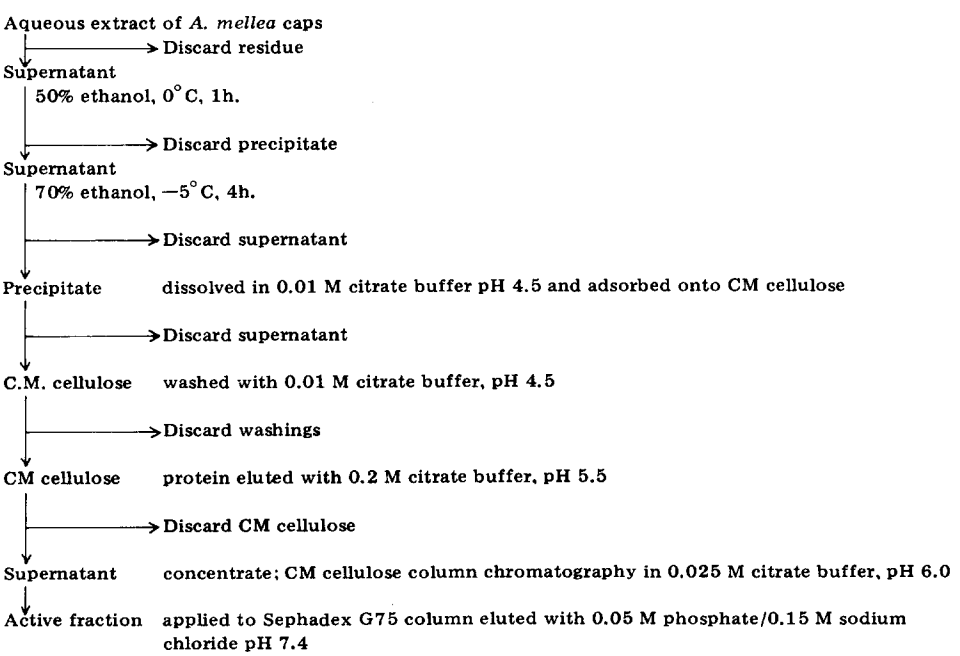

Fig. 1. Scheme for isolation of *A. mellea* protease.

Amino acid analyses. Analyses were performed on oxidised [10] and native samples of protein, according to Moore and Stein [11]. Tryptophan was determined after hydrolysis in 3 M *p*-toluenesulphonic acid containing 2% tryptamine [12]. Analyses were performed on a Locarte amino acid analyser. Calculation of the molecular weight of *A. mellea* protease from its amino acid composition was done by the method of Black and Hogness [13].

NH_2-terminal analysis. The method of Hartley [14] was used.

High-voltage electrophoresis. This was performed at pH 3.5 on a Locarte flat bed apparatus. Electrophoresis was for 1 h at 4000 V in pyridine/acetic acid/water (1 : 10 : 109, by vol.).

(A) Digestion of polypeptide substrates. (a) Lysine vasopression: Lysine vasopressin (grade IV), was obtained from Sigma and was oxidised by the method of Hirs [10]. 0.5-mg quantities of oxidised and native vasopressin were each digested separately with 10 μg of *A. mellea* protease in 100 μl of 0.05% NH_4HCO_3 (pH 8.0) for 6 h. The enzyme was then inactivated by the addition of 2 μl of 200 mM disodium EDTA. The products of both digests were separated by preparative thin-layer chromatography on 0.5-mm silica gel H plates (Merck) using butanol/acetic acid/water/pyridine (15 : 3 : 12 :10, by vol.) as developing solvent. Each of the products was subjected to amino-terminal and amino acid analysis. The products from the digestion of native vasopressin were oxidised before analysis.

(b) Glucagon: Porcine glucagon was obtained from Sigma and 5 mg suspended in 3 ml water. The pH was adjusted to 8.0 and maintained in a Radiometer pH-stat. 40 μg of *A. mellea* protease was added and, after 2 h digestion, a further 40 μg of enzyme were added. When base uptake had ceased (after 5 h), the enzyme was inactivated by addition of 20 μl of 200 mM disodium EDTA.

(c) *S*-Carboxymethyl pepsinogen: *S*-Carboxymethylated porcine pepsinogen was a gift from Dr. J. Kay of this department. 8 mg were dissolved in 1 ml of 0.5% NH_4HCO_3 and digested with 50 μg of *A. mellea* protease at 37°C for 2 h. A further 50 μg were then added and digestion continued for a further 5 h. The reaction was then stopped by the addition of 200 μl of 200 mM disodium EDTA. The digest was subjected to amino-terminal analysis. The digest was also fractionated by gel filtration on a column of Sephadex G-50 (50 × 1.5 cm, flow rate 19 ml/h, fraction size 2 ml) in 0.5% NH_4HCO_3. Two fractions were obtained and the low molecular weight fraction was peptide mapped by the method of Bates et al. [15].

(d) *S*-Carboxymethyl lysozyme and oxidised ribonuclease A: Chicken lysozyme (Grade I, Sigma) was carboxymethylated [16] and ribonuclease A (Grade XI-A, Sigma) was oxidised [10]. Each preparation was digested for 7 h with *A. mellea* protease in 0.5% NH_4HCO_3 at 37°C using an enzyme/substrate ratio of 1 : 50. The digests were examined by amino-terminal analysis only.

(B) (a) Assay of *A. mellea* protease activity: Azocoll (25 mg; Calbiochem) was suspended in 3 ml sodium cacodylate buffer (pH 7.0, I = 0.1) and 200 μl of enzyme solution were added (containing 0.2—2.0 μg). The mixture was incubated in 20-ml vials in a shaking water bath (Baird and Tatlock, Unitemp) for 30 min at 30°C, with 140 oscillations/min and 4 cm travel. The reaction was stopped by filtration through Whatman No. 1 paper and the absorbance of the filtrate was measured at 520 nm. This assay system gives a linear release of solu-

ble material absorbing at 520 nm over the 30 min incubation time, using an amount of enzyme between 0.2 and 2.0 μg. A control sample (without enzyme) was run each time.

(b) pH dependence of enzyme activity: The effect of pH on enzyme activity was studied using 15 mg Azocoll and 1.2 μg *A. mellea* protease in the assay system previously described. The pH range covered was 4.0—10.05 using the following buffers, all of ionic strength 0.1: Acetic acid/sodium acetate (pH 4.0 - 5.25); cacodylic acid/sodium cacodylate (pH 4.65—7.2); diethylbarbituric acid/sodium diethylbarbiturate (pH 7.6—8.85); $NaHCO_3/Na_2CO_3$ (pH 9.0—10.05).

(C) Inhibition studies with A. mellea protease. (a) The effect of amino acids and aliphatic amines on *A. mellea* protease activity: Enzyme activity was monitored at two different substrate concentrations (15 and 25 mg per 3 ml) in the presence of each of the following compounds: lysine, *S*-2-aminoethylcysteine, arginine, ornithine, methylamine, ethylamine and propylamine. At least four different concentrations of amine were used.

(b) The effect of metal-chelating agents on *A. mellea* protease activity: *A. mellea* protease was assayed in the presence of a range of different concentrations of 1,10-phenanthroline, α,α′-bipyridine and imidazole. For this study, the molecular weight of the protease was assumed to be 16 650 and the calculated concentration of enzyme under the conditions of the assay was $2.1 \cdot 10^{-7}$ M.

Results

Isolation of A. mellea protease. The results of a typical purification of *A. mellea* protease are shown in Table I. The enzyme obtained was homogeneous on polyacrylamide gel electrophoresis at pH 4.3 and on SDS-polyacrylamide gel electrophoresis at pH 7.0 in the presence and absence of 2-mercaptoethanol (Fig. 2). The apparent molecular weight for the enzyme, obtained using the latter method, by comparing its mobility with that of proteins of known molecular weight, was 13 500.

Amino acid analysis of A. mellea protease. The amino acid composition of the enzyme is shown in Table II. Calculation of the most probable molecular weight from the amino acid analysis, gave a value of 16 650.

(A) Digestion of polypeptide substrates. (a) Lysine vasopressin: Two prod-

TABLE I

PURIFICATION SCHEME FOR *A. MELLEA* PROTEASE (BY THE METHOD OF WALTON et al. [1])

	Units	Protein (mg)	Specific activity (units/mg)	Recovery (%)		Purification	
				Stage	Total	Stage	Total
(i) Aqueous extract (1.5 kg)	17 707	41 179.0	0.43	100	100	—	—
(ii) Ethanol precipitate	14 432	3 608.0	4.00	81.5	81.5	9.3	9.3
(iii) Batch CM-cellulose	13 325	408.5	32.40	91.7	74.5	8.1	75.7
(iv) Column CM-cellulose	6 750	49.5	136.30	51.0	38.1	4.2	378.0
(v) Gel filtration	6 210	30.0	207.00	92.0	35.1	1.5	486.0

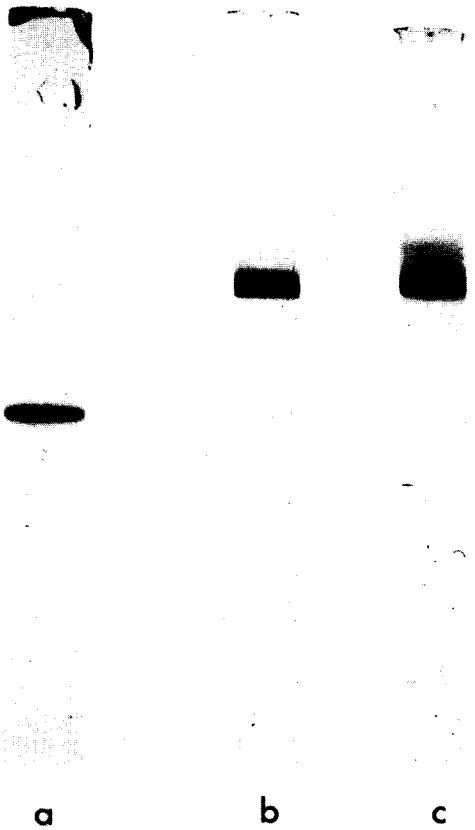

Fig. 2. Electrophoresis of *A. mellea* protease on 15% acrylamide pH 4.3 (a); and 10% acrylamide-dodecyl sulphate gels in the absence (b) and presence (c) of 2-mercaptoethanol.

ucts were isolated by thin-layer chromatography of the native vasopressin digest. They had R_F values of 0.47 and 0.36, respectively. Table III shows the amino acid composition and NH_2-terminal residues for these two products.

Two products were also obtained from the digest of oxidised vasopressin. Their analyses are also shown in Table III.

In each case these were the only two products detected.

(b) Glucagon: Two products were obtained from a digest of glucagon by *A. mellea* protease. One (I) was relatively insoluble and was removed and washed by centrifugation. The second (II) was purified by high voltage electrophoresis at pH 3.5.

The amino acid and NH_2-terminal analyses for the two products are shown in Table IV.

(c) *S*-Carboxymethyl pepsinogen: NH_2-terminal analysis of the digest indicated that the only new NH_2-terminal residue to appear was lysine. The peptide map of the low molecular weight fraction showed nine peptides, three of which were neutral at pH 6.5.

(d) *S*-Carboxymethyl lysozyme and oxidised ribonuclease A: In each case multiple products were obtained. NH_2-terminal analysis indicated that in both

TABLE II

AMINO ACID COMPOSITION OF *A. MELLEA* PROTEASE

Amino acid	Number of residues
Asp/Asn	14.34
The	18.94
Ser	20.97
Glu/Gln	13.87
Pro	3.37
Gly	11.72
Ala	14.84
Val	5.44
Met	0.72
Ile	5.78
Leu	7.19
Tyr	12.97
Phe	5.69
His	5.36
Lys	2.00
Arg	4.03
½-Cys [a]	5.76
Trp [b]	1.29

[a] Determined as cysteic acid.
[b] Determined after hydrolysis with *p*-toluenesulphonic acid.

cases the only new NH_2-terminus to appear was lysine.

(B) pH vs. activity relationship for A. mellea protease. The pH vs. activity curve for the enzyme is shown in Fig. 3.

(C) (a) Inhibition of *A. mellea* protease by amino acids and amines: The K_I values determined for the various inhibitors are shown in Table V. These were all obtained from Dixon plots of $\frac{1}{v}$ versus $[I]$ as illustrated in Fig. 4 for lysine. All inhibitors gave similar linear plots.

(b) Inhibition of *A. mellea* protease by chelating agents. The results of the

TABLE III

Amino acid composition of peptides isolated from digests of native and oxidised vasopressin by *A. mellea* protease and the compositions of two peptides which would be produced by cleavage between Pro^7 and Lys^8 in vasopressin. Values are in number of residues of each amino acid.

Amino acid	Native vasopressin		Oxidised vasopressin		Vasopressin	
	I	II	I	II	Cys^1-Pro^7	Lys^8-Gly^9
Cys	1.95 *	—	2.06 *	—	2 *	—
Asp	1.15	—	1.15	—	1	—
Glu	0.94	—	0.97	—	1	—
Pro	0.91	—	0.90	—	1	—
Gly	—	1.04	—	0.99	—	1
Tyr	0.90	—	0.87	—	1	—
Phe	1.13	—	1.08	—	1	—
Lys	—	0.95 *	—	1.01 *	—	1 *

* NH_2-terminal residue.

TABLE IV

Amino acid compositions of peptides isolated from a digest of glucagon by *A. mellea* protease and the composition of two peptides which would be produced by cleavage between Ser^{11} and Lys^{12} in glucagon. Values are in number of residues of each amino acid.

Amino acid	Glucagon		Glucagon	
	I	II	His^{1}-Ser^{11}	Lys^{12}-Thr^{27}
Asp	2.81	0.98	1	4
Thr	1.11	1.54	2	1
Ser	1.15	3.46	3	1
Glu	2.07	0.94	1	2
Gly	0.23	1.19	1	—
Ala	0.88	0.19	—	1
Val	1.08	0.04	—	1
Met	0.86	0.09	—	1
Leu	1.83	0.13	—	2
Tyr	1.11	0.94	1	1
Phe	1.09	1.35	1	1
His	0.23	0.72 *	1 *	—
Lys	0.86 *	0.09	—	1 *
Arg	1.94	0.11	—	2

* NH_2-terminal residue.

inhibition by chelating agents as shown in Fig. 5 indicate that the pK_I values for 1,10-phenanthroline, α,α'-bipyridine and imidazole are 3.9, 3.4 and 1.1, respectively.

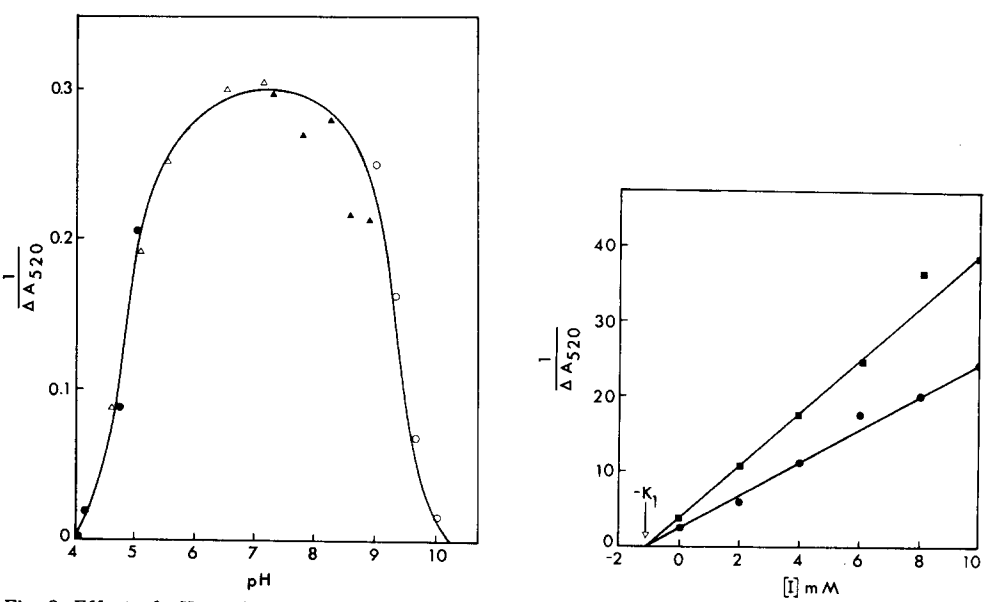

Fig. 3. Effect of pH on *A. mellea* protease activity. Buffers used were: ●———●, acetic acid/sodium acetate (pH 4.0—5.25); △———△, cacodylic acid/sodium cacodylate (pH 4.65—7.2); ▲———▲, diethylbarbituric acid/sodium diethylbarbiturate (pH 7.6—8.85); ○———○, $NaHCO_3$ /Na_2CO_3 (pH 9.0—10.05).

Fig. 4. Plot of $\frac{1}{v}$ vs. [*I*] for the inhibition of *A. mellea* protease by lysine. ●———●, 25 mg Azocoll/3 ml; ■———■, 15 mg Azocoll/3 ml.

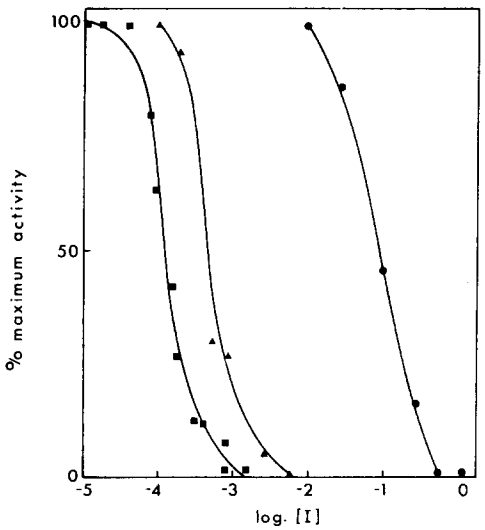

Fig. 5. Inhibition of *A. mellea* protease activity by metal-chelating agents. ●———●, imidazole; ▲———▲, α,α′-bipyridine; ■———■, 1,10-phenanthroline.

TABLE V

K_I VALUES OBTAINED FOR THE INHIBITION OF *A. MELLEA* PROTEASE BY AMINO ACIDS AND ALIPHATIC AMINES

Inhibitor	K_I (mM)
Lysine	1.00
S-2-Aminoethylcysteine	12.70
Ornithine	140.00
Arginine	170.00
Methylamine	23.30
Ethylamine	10.70
n-Propylamine	8.00

Discussion

The preparation of *A. mellea* protease used in this work was highly purified and apparently homogeneous in three electrophoretic systems. It also has a single NH_2-terminal amino acid, isoleucine [1]. The SDS-gel electrophoresis studies also indicate the enzyme to consist of a single polypeptide chain of molecular weight 13 500. Gel filtration studies (Walton, P.L. and Jones, C., unpublished) indicate a molecular weight of 14 000 while the amino acid composition reported here gives a value of 16 650.

The amino acid analysis for the enzyme is quite different from that obtained for Myxobacter AL-1 protease II [17], e.g. there is significantly more aspartic acid/asparagine in the Myxobacter enzyme while significantly less serine. The Myxobacter enzyme has a specificity and molecular weight similar to that for *A. mellea* protease.

Digestion of the polypeptide substrates tested, indicate quite clearly that the

major peptide bond specificity of *A. mellea* protease is towards those bonds where a lysine residue contributes an α-amino group. Thus only the Pro^{7}-Lys^{8} sequence is split in both native and oxidised vasopressin while only the Ser^{11}-Lys^{12} sequence in glucagon is hydrolysed.

The results from the digestions of pepsinogen, lysozyme and ribonuclease, while giving no direct evidence of the bonds split, indicated that the primary points of attack were again bonds where lysine contributed an α-amino group. While the evidence for this statement is based only on NH_2-terminal analysis by the 'Dansyl' method (for the digests of lysozyme and ribonuclease), the sensitivity of this method (sub-nanomol) would have allowed detection of other NH_2-terminal residues had they been present at a concentration less than 1% that of lysine.

Doonan and co-workers [5,6] have shown that certain peptide sequences in which lysine residues are followed by either aspartic acid or asparagine are resistant to *A. mellea* protease. Our results with *S*-carboxymethyl pepsinogen indicate it is likely that sequences where aspartic acid follows lysine are susceptible in this protein. Only three neutral peptides can be produced from pepsinogen [18,19] by cleavage on the amino side of lysine residues, and three are indeed found in the digest. For these peptides to be produced the enzyme would have to hydrolyse the sequences Ile-Lys^{18}-Asp and Leu-Lys^{23}-Asp. However, due to lack of material, we do not yet have any direct evidence for the structures of the neutral peptides.

A. mellea protease can apparently work close to the termini of polypeptide chains. It releases the NH_2-terminal isoleucine from a bee venom peptide [7] where lysine is the penultimate residue and dipeptides from the COOH-termini of both insulin and vasopressin. Further studies in this laboratory have also indicated that the terminal degradation products of poly-lysine are lysine and lysyllysine (Williams, M.P., personal communication).

Although other substrates have been employed (e.g. synthetic peptides, proteins), the most suitable assay system for the enzyme has been found to be that using Azocoll® [20]. This is a general protease substrate and has certain disadvantages despite its relatively high sensitivity. Thus the assay is discontinuous and can only be used within certain limits. To date no low molecular weight substrate has been found for the enzyme although several potential candidates have been investigated.

The pH vs. activity curve (Fig. 3) indicates a dependence of activity on the ionization of groups having p*K* values of 4.9 and 9.4. These may be in the enzyme, substrate or both. No further study has yet been made on the nature of these groups. Probable candidates would appear to be side-chain carboxyl and ε-amino groups, respectively. Also, no investigation was made of the reversibility of these pH effects although the work of Gregory [4] would seem to indicate that the effect of low pH is reversible to some extent.

Inhibition of *A. mellea* protease by various amino acids and amines is of interest. Although there is no direct evidence, it is assumed that they inhibit by binding in a hypothetical pocket on the enzyme which binds the lysine side chains of susceptible polypeptide substrates. If such a pocket exists and the inhibitors bind there then certain features are apparent: (a) Lysine binds quite tightly to this pocket (K_I 1 mM). (b) Arginine (K_I 170 mM) is a very poor

inhibitor compared to lysine indicating that the hypothetical pocket may be too narrow to accommodate the bulky guanidino group. (c) Ornithine (K_I 140 mM) is also a poor inhibitor compared to lysine, possibly indicating the depth of the pocket. Decreasing K_I values for the increasing chain length of the aliphatic amines tested also support the idea of a pocket able to accommodate at least three methylene groups in addition to the amino group. The fact that *S*-2-aminoethylcysteine is a relatively good inhibitor (K_I 12.7 mM) also correlates with the observation that such residues are susceptible to the enzyme in intact polypeptides [6,21].

The conditions for the determination of the pK_I values for the chelating agents studied, were chosen to allow a direct comparison with the similar inhibition of another zinc-containing enzyme, thermolysin [21]. The affinity of *A. mellea* protease for its zinc atom appears to be similar to that of thermolysin. The pK_I values for inhibition of thermolysin by 1,10-phenanthroline, α,α'-bipyridine and imidazole are 4.4, 3.4 and 1.3, respectively. It will be interesting to see if the zinc ligands in *A. mellea* protease are the same as in thermolysin and carboxypeptidase A.

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