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Ca^{2+}-Dependent Protease I from *Allomyces arbuscula*

Mukti Ojha[1]

Laboratory of General Microbiology, University of Geneva, Switzerland

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A monomeric Ca^{2+}-dependent protease (CDP I) of 39 kDa active at neutral pH has been purified from the aquatic fungus *Allomyces arbuscula.* The enzyme elutes at NaCl molarity of 0.07 M from the DEAE (DE52)-cellulose columns in contrast to the second Ca^{2+}-dependent protease (CDP II) characterized earlier which elutes at 0.18 M NaCl. The enzyme has no basal activity in the absence of Ca^{2+} and requires 1.7 mM Ca^{2+} for half maximum activation of the *in vitro* enzyme activity. The enzyme prefers substrates with Arg in P_1 position but this specificity also depends strongly on the nature of the subsite residues, for example Pro in P_2 position. The enzyme is glycosylated and contains essential cysteine residues in the active site. It appears to be an atypical cysteine protease as it is inactivated to varying degree with some serine protease inhibitors.

The extensively studied Ca^{2+}-activated neutral thiol or cysteine proteases (calpains) were initially reported from mammalian and avian cells (for references see reviews 1–5) but have now been found in invertebrates like *Drosophila* (6) and a parasitic trematode, *Schistosoma mansoni* (7). Two forms of these enzymes are known (8–10). They have the same catalytic function but differ in charge and the concentrations of Ca^{2+} required for the activation of enzyme activity. Because of the charge difference they elute at different ionic concentrations from the ion exchange columns. The enzyme eluting at lower NaCl concentration (calpain I) is activated at μM Ca^{2+} and referred to as μM calpain, the one eluting at higher NaCl concentration (calpain II) requires mM Ca^{2+} for the activation of enzyme activity and is called mM calpain.

Calpains have not been detected in plants (11). We have found a Ca^{2+}-activated neutral thiol protease during the vegetative growth phase of a lower eukaryote, the aquatic fungus *Allomyces arbuscula* (12). This enzyme has an absolute dependence on Ca^{2+} for proteolytic activity and is mainly localized in the apical region of the growing hyphae and is developmentally regulated (13,14). It shares many functional properties with animal calpains, although has different physical organization and substrate specificity (15–19). During the purification of this enzyme, we consistently observed two peaks of Ca^{2+}-dependent proteolytic activities eluting from DEAE (DE52)-cellulose columns. The first peak activity eluted around 0.07 M NaCl and the second one close to 0.2 M NaCl. Because of its relative small amount, the first peak enzyme was not purified and characterized, although its Ca^{2+}-requirements for half maximal activation in a semipurified preparation has been established (16). In this report, we present our results on the purification and characterization of this enzyme.

[1] Correspondence address: M. Ojha, Laboratory of General Microbiology, University of Geneva, Sciences III, 30 Quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland.

Abbreviations: AC, acetyl; 4-APMSF, 4-aminophenylmethane-sulfonyl fluoride, HCl; Boc, 1-butyloxycarbonyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; CHN_2, diazomethane; CHCl TFA, chloro-methyl trifluoro acetate; 5-DMANSF, 5-dimethylaminonapthalene-1-sulfonyl fluoride; DCI, 2-3 dichloroisocoumarin; DTT, dithio-threitol; E64, trans-epoxysuccinyl-L-leucyl amido-(4-guanidino) butane; PMSF, phenylmethanesulfonyl fluoride; pNA, para- nitro-anilide; PTI, pancreatic trypsin inhibitor; STI, soya bean trypsin inhibitor; TCA, trichloroacetic acid; TLCK, N'tosyl-L-lysinechloromethyl ketone; Tos, tosyl (4-toluenesulfonyl); TPCK, N'tosyl-L-phenylalanylchloromethyl ketone.

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MATERIALS AND METHODS

Chemicals

Peptide substrates. Bz-Arg-pNA, H-Leu-pNA, Ac-Ala-pNA, Ac-Asp- pNa, Ac -Leu-pNA, Ac-Phe-pNA, Bz-Tyr-pNA, Glu-Phe-pNA, H-Gly-Glu-pNA, H-Gly-Phe-pNA, H-Gly-Arg-pNA, NAC-Pro-Ala-pNA, Bz-Pro-Phe-pNA, Z-Val-Gly-Arg-pNA, P-Glu-Phe-Leu-pNA and Boc-Gly-Gly-Leu-pNA were purchased from Bachem and Z-Arg-Arg-pNA, H-Arg-Pro-pNA, Ac-Pro-Gly-pNA, Bz-Phe-Val-Arg-pNA and Boc-Gly-Gly-Leu-pNA were purchased from Calbiochem. Tos-Gly-Pro-Arg-pNA and Tos-Gly-pro-Lys-pNA were from Boehringer Mannheim.

Inhibitors. Leupeptin, antipain, PMSF, 5-DMANSF and 4-APMSF were from Fluka; benzamidine, aprotinin, STI and ovomucoid trypsin inhibitors were from Sigma; E64 and calpain inhibitor were purchased from Boehringer Mannheim. Dichloroisocoumarin, Z-Tryp-Tyr-CHN_2, Z-Leu-Leu-Tyr CHN_2 and Ac-Gly-Val-Arg-CHCl TFA were generous gift of Dr. Agnali of Biochemistry Department, ETH, Zürich. All other chemicals were of analytical grade from Merck.

Organism and cultural conditions

Sporophytic Allomyces arbuscula, strain Bali was used as experimental material. The maintenance and growth conditions for the production of mycelia have been described elsewhere (15,20). Mycelia after 18 h of growth were harvested by filtration, washed, squeeze-dried and immediately frozen in liquid nitrogen.

Purification of the Enzyme

The enzyme was purified essentially according to the procedures described earlier (15). The only modification was the concentration of leupeptin in the extraction buffer (MOPS 20 mM, pH 7.4, EGTA 2 mM, EDTA 2 mM, $MgCl_2$ 3 mM, leupeptin 5 μM, PMSF 0.5 mM, benzamidine 1 mM, aprotinin 215 μM, pepstatin 0.25 μg/ml, β-mercaptoethanol 10 mM, DTT 1 mM) which was raised from 1 μM used previously to 5 μM. Briefly, the mycelia frozen in liquid N_2 were pulverized and the powder was suspended in the buffer. The homogenate was kept for 15 min in ice and then centrifuged at 48000 *g* for 40 min in a Sorvall SS34 rotor. The supernatant was recovered and passed through a DEAE (DE52)-cellulose column at a flow rate of 1 $ml.min^{-1}$. After washing the adsorbed proteins were eluted in fractions of 2.5 ml against NACl concentration gradient of 0 to 0.6 M, prepared in the extraction buffer. Protein concentration and enzyme activity were estimated by Coomassie brilliant blue (21) and azocoll (described below). The two Ca^{2+}-dependent enzyme activities were pooled separately and purified using sequential Ultrogel and Ca^{2+}-dependent phenyl Sepharose chromatography as described earlier (15).

Enzyme Assay

Unless stated otherwise, the enzyme was assayed with azocoll as substrate in 20 mM Tris-HCl, pH 7.4, containing 4 mM EGTA, 3 mM $MgCl_2$, 1% β-mercaptoethanol and 6 mM $CaCl_2$ (which gives 5 mM free Ca^{2+}) instead of 10 mM used earlier (15) because this concentration had some inhibitory effect on the enzyme activity. When assayed with pNA peptide substrates the reaction was carried out in a total volume of 150 μl containing 1 μg enzyme and 500 μM substrate in the above buffer. After incubation at 37°C for 15 min, the mixture was diluted with 150 μl cold distilled water, centrifuged in Eppendorf and 4-nitro-aniline liberated was read at 405 nm. For calculation, molar absorption coefficient of 4-nitro-aniline at 405 nm was taken as 9500 l/mol × cm (22).

Molecular Mass and Subunit Determination

Molecular mass and subunit determination were determined by gel filtration through calibrated Sepharose S300 column and SDS-PAGE respectively. SDS-PAGE was done according to Laemmli (23).

Isoelectric Focussing

The IEF gels consisting of 5% acrylamide and 0.2% pH 3.5-10 ampholine were casted and run as described by O'Farrell (24). The gels were charged with the purified protein and focussed for 16 h at 300 volts. The second dimension SDS-PAGE of the IEF gel was done in 12.5 % SDS-PAGE as above.

Staining of the Glycosyl Residues

Purified enzyme was electrophoresed in 12.5% SDS-PAGE as described above, electroblotted on a nitrocellulose membrane for 2 h at 120 volts in Tris-glycine (25 mM Tris-HCl, pH 8.3, and 192 mM glycine-20% methanol) according to Towbin et al. (25). The membrane was stained with 0.05% Ponceau in 3% TCA to check the efficiency of transfer, blocked with 2% BSA in PBS (10 mM phosphate buffer, pH 7.5, 0.136 M NaCl) and processed for glycosyl residues as described by Clegg (26).

pH Profile

pH profile of the enzyme was established in the following buffer system: Na citrate/citric acid 20 mM, pH 2.0 and pH 3.0, Na acetate/acetic acid 20 mM, pH 4.0 and pH 5.0, Imidazole/HCl 20 mM, pH 6.0 and pH 7.0, Tris-HCl 20 mM, pH 8.0 and pH 9.0.

Protease Inhibitors

PMSF, 5-DMANSF, 4-APMSF, E64, diazomethane and calpain inhibitors (Z-Trp-Tyr-CHN_2 and Z-Leu-Leu-Tyr-CHN_2) were solubilized in formamide or methanol, others were dissolved in distilled water to give stock solutions of 5 μM-10 mM. Experiments with reversible inhibitors were done by adding appropriate amounts to the reaction mixture at the time of incubation whereas with irreversible inhibitors like DCI, PMSF, 5-DMANSF, 5-APMSF or E64, TLCK, TPCK and diazomethanes, the protocols described by Dunn (27) and Barrett (28) were followed. The enzyme was preincubated at 12°C in the presence of inhibitors, samples were taken at intervals and the residual activity was measured with Bz-Arg-PNA as substrate. Preliminary experiments on the stability of the enzyme at temperatures of 0, 4, 12, 20, 25 and 37°C showed that, although the enzyme was still not completely stable, 12°C offered a better compromise.

RESULTS AND DISCUSSION

Figure 1 shows the elution profile of peak I and peak II enzyme in the mycelial extract fractionated by ion exchange chromatography. The first peak of enzyme activity eluted at 0.07 M NaCl and the second peak at around 0.2 M NaCl. The proportion of peak I and II enzyme calculated from the elution profile curves from a number of experiments was 20 and 80% respectively. The two extreme values were 8 and 92% and 38 and 62%, respectively.

The purified preparation in SDS-PAGE appeared as a single band of M_r 39 kDa and relatively free of contaminating proteins (Fig. 2a). However, gel filtration through a calibrated Sephacryl S300 column gave a molecular mass of 36 kDa (Fig. 2b). In native gels the molecular mass could not be determined because when electrophoresed in non-denaturating conditions and stained with Coomassie brilliant blue, the protein appeared as a smear indicating that it was probably glycosylated (results not shown). Indeed, specific staining using Concanavalin A - horseradish peroxidase procedure clearly showed the presence of glycosyl residues (Fig. 2c). The two faint glycosylated proteins appearing in Fig. 2c could represent minor copurifying proteins but their relative proportion is very small compared to the purified enzyme. The fact that the protein appeared as a single band of M_r 39 kDa in SDS-PAGE and eluted in a single homogenous peak in gel filtration

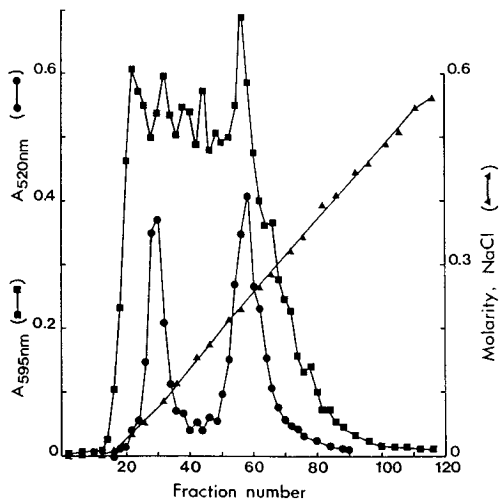

FIG. 1. Elution profile of Ca^{2+}-dependent cysteine protease activity from *Allomyces arbuscula* cell-free extract. Homogenate centrifuged at 48000 *g* and the supernatant chromatographed on DEAE (DE52)-cellulose ion exchange column (flow rate, 1 ml·min^{-1}; 10 ml/fraction). (■) protein concentration; (●) Ca^{2+}-activated proteolytic activity; (▲) NaCl gradient.

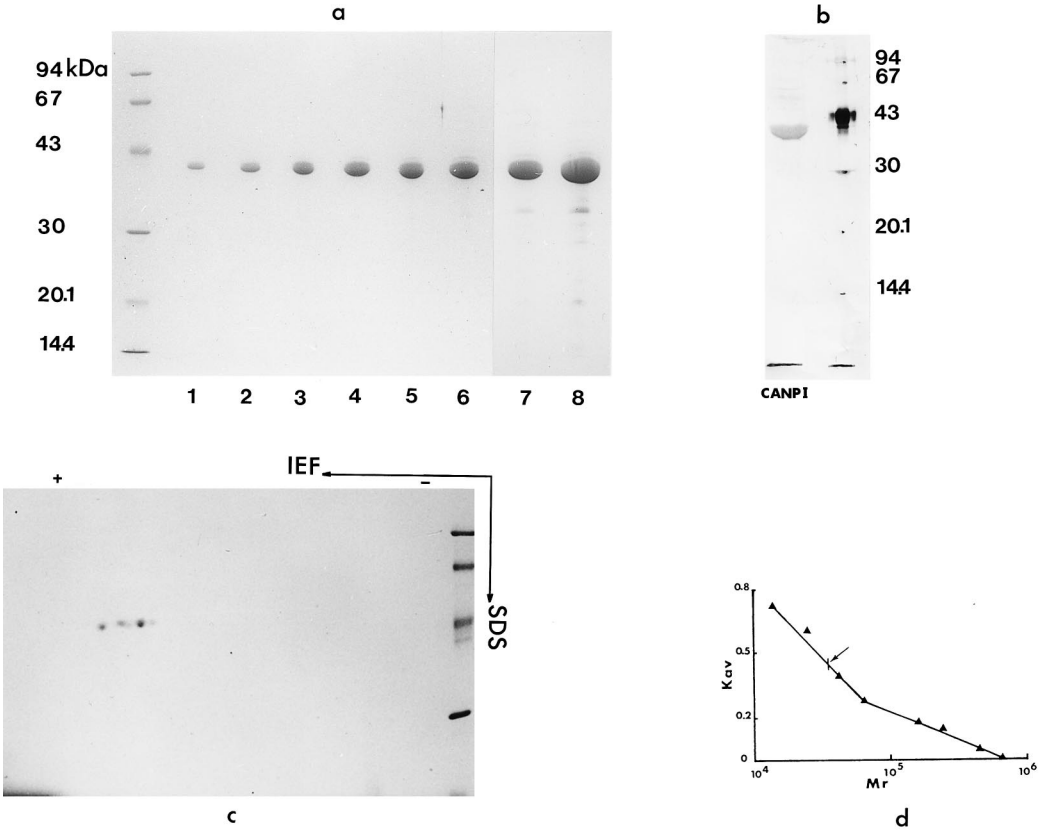

FIG. 2. Characterization of purified Ca^{2+}-dependent neutral cysteine protease from *Allomyces arbuscula.* (a) SDS–PAGE of the purified enzyme; lane 1, molecular mass markers M_r; lanes 2–9 represent, respectively, 0.45, 0.9, 1.8, 2.7, 3.6, 5, 10 and 20 μg purified enzyme electrophoresed and stained with Coomassie brilliant blue. (b) ConA-horseradish peroxidase staining of the glycosyl residues, the enzyme was electrophoresed and transferred to nitrocellulose; the blots were processed according to Clegg (26). (c) Isoelectric focussing of the enzyme in bi-dimensional electrophoresis as described by O'Farrell (24). (d) Analytical gel filtration of the purified enzyme through a calibrated Sephacryl S300 column, molecular mass markers. The Kav is plotted against molecular mass as described in the Pharmacia manual.

indicated that the enzyme is a monomeric protein. Affinity purified enzyme as such or after separation by SDS-PAGE when analyzed by two dimensional electrophoresis, separated into three components having a pI of 5.3, 5.7 and 5.9, the major component being 5.9 (Fig. 2d). There could be three possibilities of this heterogeneity: a) glycosylated nature of the protein, b) limited proteolysis of the enzyme or c) the components represent different isoforms of the enzyme.

The pH profile of enzyme activity, studied in different buffer systems, showed that the enzyme was active in the pH range of 5-9 with optimum activity around neutral pH (results not shown).

The enzyme activity was absolutely Ca^{2+}-dependent, no proteolysis occurred at Ca^{2+} concentrations less than 50 μM. Sr^{2+} could replace Ca^{2+} and in both cases, the half maximal activation of the enzyme required 1.7 mM Ca^{2+} (Fig. 3), but the maximal enzyme activity with Sr^{2+} was lower than with Ca^{2+}. Other divalent cations like Ba^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} had no effect (Fig. 3). The enzyme activity was also absolutely dependent on the presence of reducing agents like β-mercaptoethanol or DTT. Optimal activation required 1% β-mercaptoethanol (results not shown).

The substrate specificity of CDP I was studied using chromogenic peptides containing various amino acids in P_1 position. As shown in Table 1 , the peptides susceptible to cleavage contained arginine in P_1 position. However, this specificity was strongly dependent on the nature of the amino

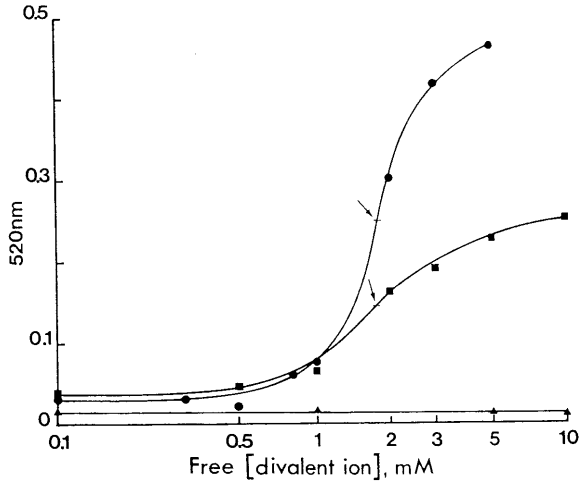

FIG. 3. Effect of divalent cation concentration on the enzyme activity of Ca^{2+}-dependent serine-cysteine protease (CDPI) of *Allomyces arbuscula.* (●) Ca^{2+}; (■) Sr^{2+}; (▲) collectively Ba^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}.

acids in P_2 position. A neutral glycine as in dipeptide H-Gly-Arg-pNA or tripeptide Z-Val-Gly-Arg-pNa was tolerated, but not a second arginine or hydrophobic valine as in dipeptide Z-Arg-Arg-pNA or tripeptide Bz-Phe-Val-Arg pNA. The enzyme preferred proline in P_2 and glycine in P_3 positions as was found with the tripeptide Tos-Gly-Pro-Arg-pNA. These results clearly showed a trypsin-like substrate specificity of the enzyme. The trypsin-like enzymes are also known to prefer lysine in P_1 position but as shown with Tos-Gly-Pro-Lys-pNA which is similar to Tos-Gly-Pro-Arg-pNA, the substitutions of lysine considerably reduced the specificity of the enzyme (Table 1). The kinetic constants of the enzyme, determined with the 3 preferred peptide substrates are shown in Table 2. It is clear that the ideal sequences of amino acid for optimal enzyme activity are Arg, Pro and Gly respectively in P_1, P_2 and P_3 positions.

TABLE 1
Substrate Specificity of Ca^{2+}-Dependent Protease I from *Allomyces arbuscula*

Substrate	Enzyme activity	
	Specific	**Normalized(%)
Bz-Arg-pNA	5.0×10^{-3}	100
Z-Arg-Arg-pNA	0.45×10^{-3}	9
H-Gly-Arg-pNA	3.02×10^{-3}	60
Bz-Phe-Val-Arg-pNA	0.93×10^{-3}	18
Z-Val-Gly-Arg-pNA	3.2×10^{-3}	60
Tos-Gly-Pro-Arg-pNA	48.2×10^{-3}	964
Tos-Gly-Pro-Lys-pNA	2.9×10^{-3}	58

The reaction mixture, in a total volume of 150 μl, contained 500 μM substrate, 1 μg enzyme and appropriate amount of tris-Ca^{2+} buffer (Tris–HCl 20 mM pH 7.4, EGTA 4mM, $MgCl_2$ 3mM and $CaCl_2$ 6mM). The mixture was incubated for 15 min at 37°C and the reaction interrupted by the addition of 150μl chilled distilled water. The mixture was centrifuged and the absorbance of the supernatant was read at 405nm. For calculation of specific activity the molar coefficient of 4-nitroaniline at 405nm was taken as 9500 1/mol × cm. All peptides listed in the "Materials" were tested but only those showing detectable activity are shown in the table.

* M pNA released.min^{-1} mg $protein^{-1}$.

** Normalized activity with respect to BZ-Arg-pNA as 100%.

TABLE 2
Kinetic Constants of Ca^{2+}-Dependent Protease I with P1-Arginine Containing Peptides

Peptide Substrate	Kinetic constants		
	Km mM	Kcat $mM^{-1}S.^{-1}$	Kcat/Km
Bz-Arg-pNA	0.3	6000	2000
Z-Val-Gly-Arg-pNA	0.3	4700	1500
Tos-Gly-Pro-Arg-pNA	0.22	85600	40000

The reactions were carried out at substrate concentrations ranging from 66.7 μM to 667 μM. The K_m and V_{max} values were determined from the linear regression calculation of K_{cat} values the M_r of CDP I was taken as 36 kD.

Experiments done to classify the enzyme according to its susceptibility to class specific inhibitors showed (Table 3) that the enzyme was inhibited by the natural serine protease inhibitors with trypsine-like substrate specificity for example, PTI and STI (requiring Arg or Lys in P_1 position) and benzamidine. Specific serine protease inhibitors like DCI, PMSF, 5-DMANSF and 4- APMSF which react directly with active serine residue either did not effect (DCI) or inhibited the enzyme activity only partially (maximum inhibition: PMSF 15%, 5-DMANSF 25% and 4-APMSF 32%) in time dependent reactions (Fig. 4). Much of the inhibitable reaction was achieved in 2 h preincubation period. Similar but more pronounced inhibition profile was obtained with TLCK and Ac-Gly-Val-Arg-CHCl TFA (Fig. 4). Another chloromethyl ketone, TPCK and diazomethyl peptides Z-Trp-Tyr-CHN_2 and Z-Leu-Leu-Tyr-CHN_2, the last two, specific inhibitors of mammalian calpains, had no effect (results not shown). This is not surprising since the substrate specificity requirement of *Allomyces* CDP I is basic arginine residue in P_1 position but these inhibitors have

TABLE 3
Effect of Protease Inhibitors on Ca^{2+}–Dependent Neutral Protease I Activity

Class of protease inhibitor	Inhibitor	Inhibitor conc.	Inhibition %
Serine	PMSF	1 mM	20
	2,3-DCI	50 μM	3.7
	Benzamidine	1 mM	71.2
		10 mM	91.4
	Soya bean Trypsin	40 μM	0
		100 μM	47
	Pancreatic Trypsin	50 μM	37.2
		100 μM	78
	Ovomucoid Trypsin	1 mM	8
Cysteine	E64	100 μM	0
	Leupeptine	50 μM	92.6
		100 μM	92
	Antipain	50 μM	94.1
		100 μM	96.4
	HgCl2	500 μM	90.5
	Iodoacetate	1 mM	33
Aspartic	Pepstatin	1 mM	0
Metallo	EDTA	1 mM	98.4
	EGTA	1 mM	98

The reaction in a total volume of 500 μl contained 2.5 mg azocoll, tris–Ca^{2+} buffer and 1 μg enzyme. Appropriate amount of inhibitors were added at the time of incubation which was carried out at 37°C for 20 min. After the incubation the mixture was centrifuged and the absorbance of the supernatant was read to 520 nm.

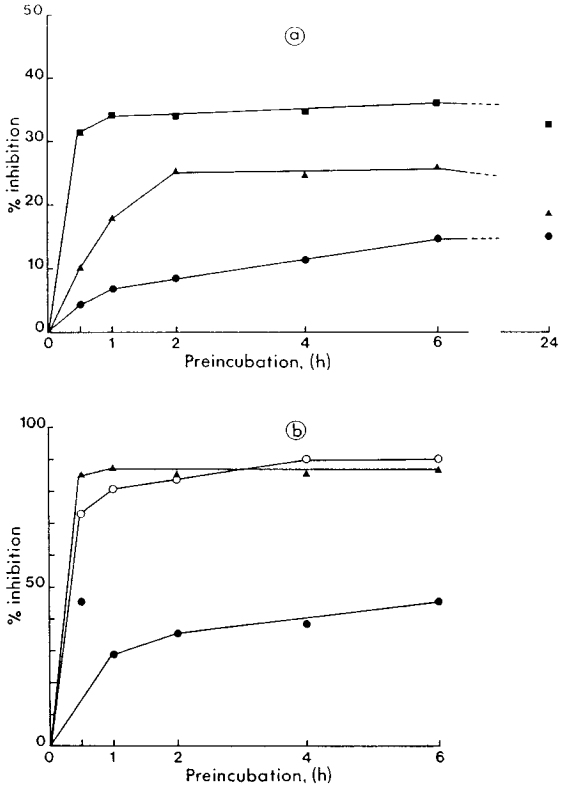

FIG. 4. Inhibition of Ca^{2+}-dependent neutral protease activity by active site serine reacting sulfonyl fluorides (a) and substrate related inhibitors TLCK and Ac-Gly-Val-Arg-CHCl TFA (b). 3 μg of dialyzed enzyme in a total volume of 32 μl in tris buffer containing a final concentration of 10 mM Ca^{2+} and 2 mM, respective, sulfonyl fluoride inhibitors or 50 μM - 1 mM TLCK and 5 μM Ac-Gly-Val Arg-CHCl TFA were incubated at 12°C. 4 μl of the reaction mixture was taken at intervals and the residual enzyme activity measured with Bz-Arg-pNA as substrate as described in the methods. Each point in the curve represents a mean of 4 experiments. (a) ● PMSF, ▲ 5-DMANSF, ■ 4-APMSF. (b) ▲ 5 μM Ac-Gly-Val Arg-CHCl TFA, ●; 50 μM TLCK, ○ 1 mM TLCK.

hydrophobic amino acid residues Phe, Trp or Tyr in P_1 position. The most potent inhibitors were peptide aldehydes like leupeptin and antipain, but these inhibitors are known to inhibit both serine and cysteine proteases.

The presence of essential cysteine residue at the active site, first deduced from the absolute dependence of the enzyme activity on the presence of reducing agents like β-mercaptoethanol or DTT, was confirmed by a strong inhibition of the enzyme activity by $HgCl_2$ (Table 3). However, iodoacetate also a thiol binding reagent was only partially inhibitory and E64, a natural peptide inhibitor of some cysteine protease, had no effect when added in the reaction mixture or in time dependent preincubation experiments. The strong inhibition of the enzyme activity with metal chelators reflected the absolute Ca^{2+}-dependence of the enzyme.

Therefore, in contrast to the Ca^{2+}- dependent enzyme II purified and characterized earlier (13–18) which was a typical cysteine-protease, the enzyme I is probably an atypical-cysteine protease containing in addition to the essential cysteine possibly a serine residue as well and requires Ca^{2+} for the activation of proteolytic activity.

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