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Partial Characterization of Proteases from Culture Filtrate of *Mycobacterium tuberculosis*

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Two proteases were partially characterized from culture filtrate of *Mycobacterium tuberculosis* KIT110. Their molecular weights were approximately 200 and 180 kDa, respectively and they exhibited similar enzymatic characteristics. These enzymes were inhibited significantly by EDTA and to some extent by EGTA. Their activity was enhanced by Ca^{2+} and Mg^{2+} to some degree. However, Cu^{2+} and Ag^{2+} completely inhibited the enzyme activity at the concentration of 2.5 and 5 mM, respectively. The optimal pH was 7.0 and optimal temperature was around 40°C. These enzymes were rapidly inactivated at 80°C. Therefore, they were heat-labile, neutral metalloproteases. These enzymes exhibited antigenicity shown by their reacting with sera from the patients with pulmonary tuberculosis. These enzymes were able to degrade serum proteins including hemoglobin, bovine serum albumin, lysozyme and immunoglobulin G and structural matrix protein such as type I collagen. Therefore, these enzymes may be thought to contribute to tissue necrosis and pathogenesis during infection.

Key words: ***Mycobacterium tuberculosis*, characterization, proteases**

Tuberculosis is a chronic bacterial infection caused by *Mycobacterium tuberculosis*. The disease is spread from person to person by aerosols and remains as one of the most common infectious diseases of mankind (20). The rising incidence of tuberculosis, in large part due to the AIDS epidemic, and the emergence of multidrug-resistant tuberculosis constitute a major public health dilemma that underscores the need for more knowledge about pathogenic mechanisms of *M. tuberculosis*. Live mycobacteria secrete a number of proteins into their surroundings during growth (1, 2, 6, 10, 11, 21, 22, 28, 29) and the isolation and characterization of protein antigens secreted by *M. tuberculosis* have been an active area of investigation for more than 20 years. Such proteins serve important functions in terms of bacterial growth and survival. However, little is known about its exported or secreted proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of *M. tuberculosis* culture filtrate show at least 30 secreted proteins (1, 2, 4, 5, 21, 22, 30, 32). These proteins have been classified according to their molecular weight and their immunological reactivity. Some have been extensively characterized. For instance, secreted proteins of the antigen 85 complex (antigens A, B, and C) are serologically cross reactive proteins of 32 kDa (31). These proteins display affinity to fibronectin and could be involved in the internalization of *M. tuberculosis* into macrophages. Superoxide dismutase (SOD) is an example of an enzyme that was a very distinct function, paralyzing a host defense mechanism by inactivating the toxic superoxide radicals generated by the activated macrophage (9). Other, although immunodominant, antigens of major importance in protective immunity have not been fully identified (3, 23). Obviously, each protein of *M. tuberculosis*, like proteins of any other microorganism, exerts different functions. Regardless of whether a given protein in looked upon as a more or less potent antigen by the immunologist, in every case it serves as a distinct and sometimes indispensable function in the bacterium. Human pathogenic bacteria interact with their hosts in different ways. Each bacterial species has evolved a unique way of either attacking the host or evading host defense mechanisms. Mycobacteria make use of subtle but efficient means of surviving within the host. No true

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exotoxins have ever been described, and much of the tissue damage that occurs in relation to a tuberculosis infection is known to be a result of the host immune response attempting to combat the infection. However, products of the bacteria themselves may also contribute to the pathogenesis including lung damage. Generally, bacterial protease attributes to pathogenesis by various mechanisms (17). *L. pneumophilia* produces a number of extracellular proteases, some of which cause damage when introduced into the guinea pig lung (7). The *P. aeruginosa* elastase has been implicated in lung damage associated with *P. aeruginosa* lung infections in cystic fibrosis patients (8, 12). Recently a gene that has sequence similarity to the *L. monocytogenes* hemolysin (LLO) has been cloned from *M. tuberculosis*. Thus, *M. tuberculosis* may produce a hemolysin or secreted protease which may be associated with its pathogenicity through various mechanisms; however, these were not defined clearly yet. In this study, we characterized the proteases secreted by *M. tuberculosis* and identified them as one potentially important virulence factor of *M. tuberculosis* pathogenesis. To our knowledge, this is the first report about protease of *M. tuberculosis*.

Materials and Methods

Microorganism

Mycobacterium tuberculosis KIT110 was used in this study. The strain was isolated from patient clinically, cultured on Lowenstein-Jensen medium at 37°C and renewed every 2 months.

Preparation of culture filtrate

M. tuberculosis was first adapted to Sauton liquid medium (2 mM $MgSO_4$, 10 mM citric acid, 2.5 mM K_2HPO_4, 30 mM L-asparagine, 0.1 mM ferric ammonium citrate and 830 mM glycerol, in Milli Q water, final pH 7.8) by transferring from Lowenstein-Jensen medium (21). After incubating at 37°C for 3 weeks in Sauton liquid medium, the bacterial pellicle was transferred to a 0.5 liter flask containing 200 ml Sauton liquid medium and incubated at 37°C for 5 weeks. Culture filtrate from 2 liters of *M. tuberculosis* was filtered through 0.22 μm filters (Gelman), concentrated by polyethersulfone membrane (molecular weight cut-off 10,000, Millipore) and further concentrated by lyophilization.

Assay of protease activity

Protease activity was determined by using azocasein as a substrate (19). Azocasein (2%, v/v) was prepared in 50 mM sodium phosphate buffer (pH 7.0). The 75 μl of concentrated culture filtrate (0.5 mg) was added to 125 μl of azocasein solution. Reaction was carried out at 37°C for 24 h. A control without the concentrated culture filtrate was included. The reaction was stopped by addition of 600 μl of 10% trichloroacetic acid. After removal of the precipitated proteins by centrifugation (10,000 rpm for 5 min), an aliquot of 600 μl of the supernatant was added to 700 μl of 1 M NaOH and the absorbance at 440 nm of the resulting mixture was measured. One unit of protease activity was defined as the amount of enzyme which increased the absorbance by 1.0 OD unit under these condition.

SDS-PAGE and substrate gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 10% (w/v) slab gel as described by Laemmli (15). Protein samples were denaturated by boiling for 2 min in 1% SDS, 0.1% 2-mercaptoethanol. A current of 30 mA/gel was applied for 3.5 h. The gels were stained for 2 h with 0.1% coomassie brilliant blue in methanol : acetic acid : H_2O (1 : 2 : 1, v/v) and destained in methanol : acetic acid : H_2O (5 : 1 : 4, v/v) at room temperature. The gelatin SDS-PAGE in slab gels containing SDS and gelatin as copolymerized substrate (13) was used for the detection of the protease activity. Protein samples were mixed with equal volume of sample buffer containing 2.5% SDS, 1% sucrose and incubated at 37°C for 30 min. Electrophoresis was performed at 4°C at a constant current of 30 mA. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h at room temperature to remove the SDS and restore enzyme activity. The gels were then transferred to 50 mM sodium phosphate buffer (pH 7.0) and incubated at 37°C overnight. The gels were then fixed and stained by immersion for 1 h in 0.1% (w/v) amido black and destained in methanol : acetic acid : H_2O (5 : 1 : 4, v/v). The bands of proteolytic activity were revealed as areas depleted of gelatin. SeeBlue prestained standards (NOVEX) were used as molecular weight standard markers.

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.* (16) with bovine serum albumin (Sigma) as the standard.

Effect of protease inhibitors

The effect of inhibitors on the activity of proteases was examined. The concentrated culture filtrate was preincubated at 37°C for 30 min in 50 mM sodium phosphate buffer (pH 7.0) containing each inhibitor. Substrate was then added. The reaction mixtures were incubated at 37°C for 24 h and protease activity was

measured. The inhibitors used in this study were diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), leupeptin, pepstatin A, N_α-tosyl-L-lysine-chloromethyl ketone (TLCK), N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), iodoacetic acid, ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(β-aminoethyl ether) (EGTA). All inhibitors were purchased from Sigma.

Effect of metal ions

The concentrated culture filtrate (0.5 mg) was incubated in 50 mM sodium phosphate buffer (pH 7.0) containing 1, 2.5 and 5 mM $MgCl_2$, $CaCl_2$, $ZnCl_2$, $AgCl_2$ and $CuSO_4$ at 37°C for 24 h. Then protease activity was measured in comparison with the control which had no metal ions.

Effect of pH

To determine the effect of pH on the activity of proteases, the gelatin SDS-PAGE and azocasein assay were used. Gelatin SDS-PAGE was performed as described previously. The electrophoresed gels were incubated in various pH buffers at 37°C for 24 h. The buffers used were 50 mM sodium acetate (pH 4.0, 5.0), 50 mM sodium phosphate (pH 6.0, 6.5, 7.0, 7.5), 50 mM Tris-HCl (pH 8.0, 8.5) and 50 mM glycine-NaOH (pH 9.0, 10.0). Azocasein assay was performed as described previously using above mentioned buffers.

Effect of temperature and thermal stability

To determine the effect of temperature on the activity of proteases, the concentrated culture filtrate (0.5 mg) and substrate mixtures were incubated at different temperatures from 10°C to 70°C. After 24 h incubation, the activity was measured. To determine the thermal stability of proteases, the concentrated culture filtrate (0.5 mg) was incubated for different time intervals in 50 mM sodium phosphate buffer (pH 7.0) at 60°C and 80°C, respectively. Then remaining activity was measured as described previously.

Substrate specificity

Substrate specificity of proteases was assayed by the method of Kunitz (14). Immunoglobulin G, hemoglobin, bovine serum albumin, fibronectin, lysozyme and collagen (type I) were used as substrates. The 75 μl of concentrated culture filtrate (0.5 mg) was added to 125 μl of substrate solution (1 mg/ml) and incubated at 37°C for 24 h. The reaction was stopped by adding 600 μl of 10% trichloroacetic acid. After removal of the precipitated proteins by centrifugation (10,000 rpm for 5 min), the absorbance at 280 nm of the resulting supernatant was measured. To define further substrate specificity, following fluorogenic synthetic peptide substrates which have a fluorescent leaving group, 7-amino-4-trifluoromethylcoumarin (AFC), were used : CBZ-arg-AFC, CBZ-arg-arg-AFC, CBZ-phe-arg-AFC, CBZ-lys-AFC and CBZ-ala-AFC (Enzyme System Product). The assay mixtures contained 75 μl of concentrated culture filtrate and 10 μl of substrate in 50 mM sodium phosphate buffer (pH 7.0). After incubation at 37°C for 24 h with enzyme solution, the amount of AFC liberated from the fluorogenic substrate was quantified with excitation wavelength at 440 nm and emission wavelength at 505 nm using a fluorometer (Model III, Sequoia-Turner Co.). One unit of the protease activity was defined as nmols of AFC production per min.

Western blotting (Immunoblotting)

Antigens separated by SDS-PAGE were transferred to nitrocellulose membrane and were immunoblotted as described previously (27). In brief, after electrophoretic transfer, the nitrocellulose membrane was incubated in 3% skim milk and 0.5% Tween 20 in phosphate buffered saline (PBS), pH 7.2, to block free protein-binding sites. Human antiserum was applied to nitrocellulose membrane. After washing with PBS, nitrocellulose membrane was probed with peroxidase conjugated anti-human goat IgG (Sigma) diluted 1 : 1,000 in PBS with 0.5% Tween 20. The reaction was visualized by incubating the membrane with substrate solution of 0.05% 3,3'-diaminobenzidine (DAB) (Sigma) and 0.01% H_2O_2. The reaction was terminated by rinsing membrane with distilled water.

Results

Protease activity on gelatin SDS-PAGE

The proteases secreted by *M. tuberculosis* were examined by gelatin SDS-PAGE. Gelatin SDS-PAGE method is highly sensitive and may possibly enable the detection of minor proteases in complex extracts regardless of the class. Gelatin is easily hydrolyzed by numerous proteases and does not tend to easily migrate out of the resolution gel for electrophoretic migration at 4°C, maintaining a uniform distribution of protein substrate in the gel. Thus, using the gelatin as a substrate improves sensitivity and efficiency of the system. Two bands of protease activity were visible at approximate molecular weights of 200 and 180 kDa, respectively (Fig. 1).

Effect of inhibitors and metal ions

The effect of inhibitors on the activity of proteases

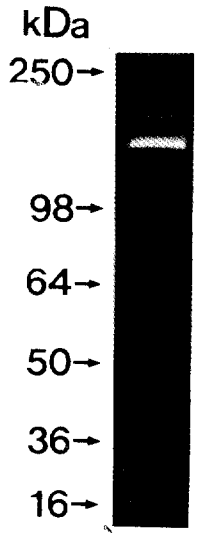

Fig. 1. Identification of proteases secreted by *M. tuberculosis* on gelatin SDS-PAGE. Gel containing 10% polyacrylamide, 0.1% SDS and 0.1% copolymerized gelatin. After electrophoresis, gel was washed in 2.5% Triton X-100 for 1 h at room temperature and then incubated in 50 mM sodium phosphate buffer (pH 7.0) for 24 h at 37°C.

Table 1. Effect of inhibitors on the activity of proteases of *M. tuberculosis*

Inhibitors	Concentration (mM)	Relative activity (%)[b]
Control[a]		100.0
DFP	0.1	82.4
PMSF	0.1	102.7
TPCK	0.01	98.3
TLCK	0.01	88.9
E-64	0.001	104.3
Pepstatin A	0.001	98.7
EDTA	2.5	14.0
	5	5.3
EGTA	2.5	63.7
	5	67.8
2-mer-	1	66.6
captoethanol	2.5	56.1

a : Control represents the activity tested without any inhibitors.
b : % of control.
DFP, di-isopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone ; TLCK, N_{α}-tosyl-L-lysine-chloromethyl ketone ; E-64, epoxysuccinyl-leucylamide-(4-guanidino)-butane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether).

was determined by measuring residual activity following preincubation with inhibitors in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 30 min (Table 1). The activity of enzymes was inhibited significantly by EDTA and some inhibition was observed by EGTA, a calcium-specific chelating agent. However, no significant inhibition was observed with DFP and PMSF (inhibitors of serine proteases), E-64 (inhibitor of cysteine proteases) and pepstatin A (inhibitor of aspartic proteases). 2-mercaptoethanol also showed slight inhibition of the enzyme activity. Fig. 2 revealed the effect of inhibitors on the protease activity on gelatin SDS-PAGE. The two enzymes were completely inhibited by EDTA (Flg. 2, lane

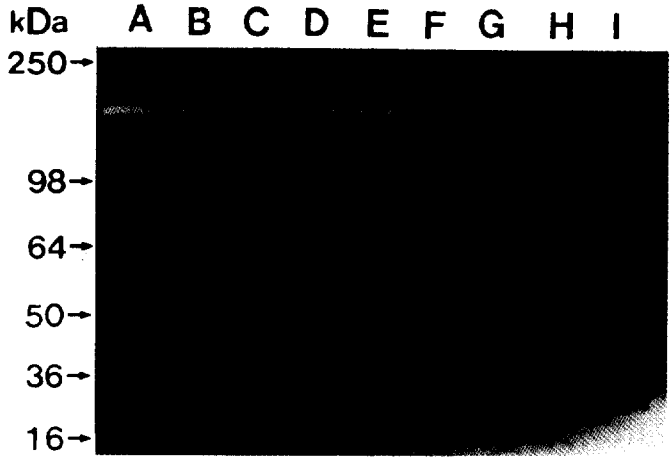

Fig. 2. Effect of inhibitors on the activity of proteases secreted by *M. tuberculosis*. The concentrated culture filtrate of *M. tuberculosis* (0.5 mg) was electrophoresis followed by preincubation with various inhibitors. Lane A, Control without any inhibitor; Lane B, PMSF (0.1 mM); Lane C, DFP (0.1 mM); Lane D, E-64 (1 μM); Lane E, Leupeptin (10 μM); Lane F, Pepstatin A (1 μM); Lane G, Iodoacetic acid (10 μM); Lane H, EDTA (5 mM); Lane I, EGTA (5 mM).

Table 2. Effect of metal ions on the activity of proteases of *M. tuberculosis*

Metal ions	Concentration (mM)	Relative activity (%)[b]
Control[a]		100.0
$CuSO_4$	1	9.5
	2.5	0
	5	0
$AgCl_2$	1	12.8
	2.5	3.8
	5	0
$CaCl_2$	1	103.6
	2.5	120.1
	5	125.3
$ZnCl_2$	1	97.9
	2.5	88.1
	5	87.0
$MgCl_2$	1	142.7
	2.5	172.5
	5	192.7

a : Control represents the activity tested without any metal ions.
b : % of control.

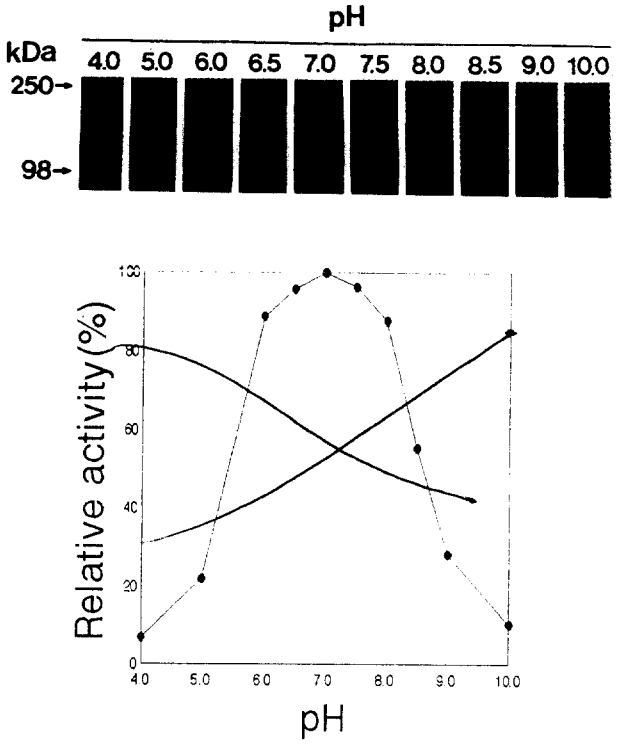

Fig. 3. Effect of pH on the activity of proteases secreted by *M. tuberculosis*. The activity of proteases was assayed in 50 mM sodium acetate buffers (pH 4.0, 5.0), 50 mM sodium phosphate buffers (pH 6.0, 6.5, 7.0, 7.5), 50 mM Tris-HCl buffers (pH 8.0, 8.5) and 50 mM glycine-NaOH buffers (pH 9.0, 10.0).

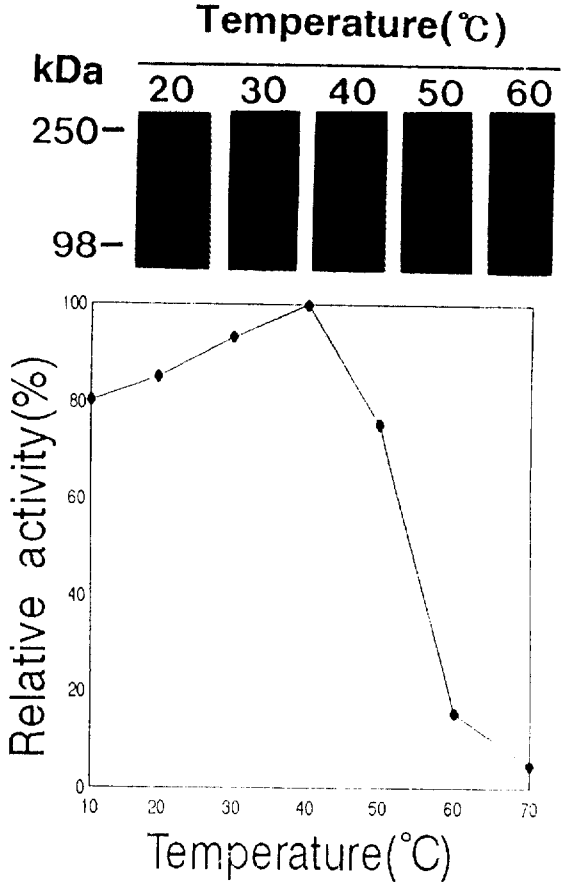

Fig. 4. Effect of temperature on the activity of proteases secreted by *M. tuberculosis*. The concentrated culture filtrate of *M. tuberculosis* (0.5 mg) was incubated at different temperatures from 10℃ to 70℃. After 24 h incubation, the remaining activity was measured. Maximal activity was shown as 100%.

H) and some inhibition were exhibited by DFP and EGTA (Fig. 2, lane C and I). Interestingly, the protease bands were detected in higher molecular weights when treated with EGTA. This probably was due to EGTA affecting the mobility of proteins in some mechanism. The protease bands were also detected in slightly higher molecular weight region when treated with 2-mercaptoethanol (data not shown). This indicated that the proteases were reduced by 2-mercaptoethanol and had a relaxed tertiary structure. However, the smaller protease bands were not detected. This indicated that these proteases had monomeric structures. The effect of various divalent metal ions on the activity of proteases are shown in Table 2. The activity of proteases was inactivated completely by 2.5 mM Cu^{2+} and 5 mM Ag^{2+}. Zn^{2+} had no significant effect on the activity of proteases. Addition of Ca^{2+} and Mg^{2+} enhanced the enzyme activity to a degree. These results indicated that the proteases secreted by *M. tuberculosis* could be classified as metalloproteases which were activated by Ca^{2+} and Mg^{2+}.

Effect of pH and temperature

The effect of pH on the activity of proteases was examined by using various pH buffers (Fig. 3). The proteases were active over a narrow pH range, from 6.0 to 8.0 and the optimum pH was 7.0. The activity decreased gradually below pH 6.0 and above pH 8.0. It suggested that the optimum pH of these enzymes was neutral. These enzymes showed a broad optimum temperature with a maximum activity around 40℃ (Fig. 4) and very unstable at 80℃ (Fig. 5).

Substrate specificity

The proteases secreted by *M. tuberculosis* degraded immunoglobulin G, hemoglobin, bovine serum albumin, lysozyme and collagen (type I). However, incubation with fibronectin, generated little or no proteolysis (Table 3). To determine further their substrate specificity, the concentrated culture filtrate of *M. tuberculosis* was incubated with the following synthetic peptide substrates : CBZ-arg-AFC, CBZ-arg-arg-AFC, CBZ-phe-arg-AFC, CBZ-lys-AFC and CBZ-ala-AFC. These enzymes hydrolyzed CBZ-arg-AFC, CBZ-arg-arg-AFC and CBZ-phe-arg-AFC and exhibi-

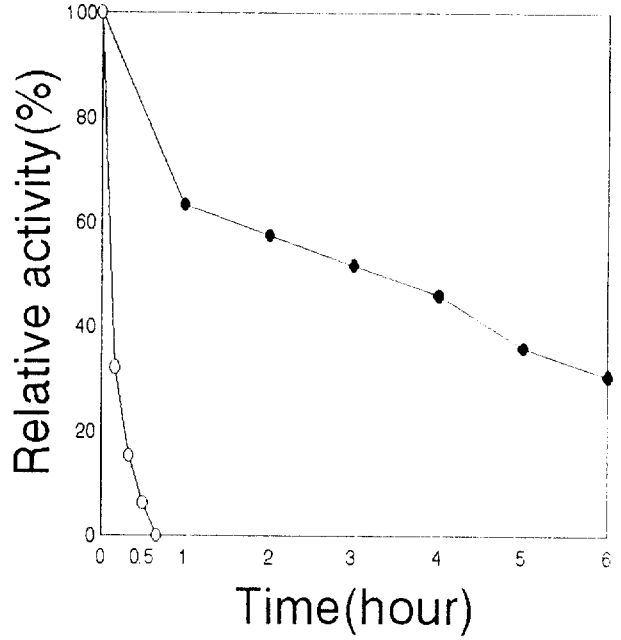

Fig. 5. Thermal stability of proteases secreted by *M. tuberculosis*. The concentrated culture filtrate (0.5 mg) was incubated at 60℃ (●) and 80℃ (○) for various time intervals and remaining activity was measured.

Table 3. Substrate specificity of proteases of *M. tuberculosis*

Substrates	ABS[b] (280 nm)	Relative activity[b](%)	Activity[c] (Unit)	Relative activity[d](%)
Immunoglobulin G	0.36	85.7		
Hemoglobin	0.35	83.3		
Collagen(type I)	0.25	59.5		
Lysozyme	0.34	80.1		
Fibronectin	0	0		
Bovine serum albumin	0.42	100.0		
CBZ-arg-AFC			679.7	93.3
CBZ-arg-arg-AFC			728.6	100.0
CBZ-phe-arg-AFC			362.8	49.8
CBZ-lys-AFC			7.4	1.0
CBZ-ala-AFC			4.8	0.7

a : absorbance at 280 nm.
b : relative activity compared to maximum activity of bovine serum albumin regarded as 100%.
c : nmoles of AFC production per min.
d : relative activity compared to maximum activity of CBZ-arg-arg-AFC regarded as 100%.

ted maximum activity against CBZ-arg-arg-AFC. However, they did not hydrolyze CBZ-lys-AFC and CBZ-ala-AFC. This indicates that these enzymes cleave preferentially at the carboxylic sides of arg, but does not cleave at the carboxylic sides of lys or ala. The cleavage was significantly inhibited when the N-terminal amino acid of arg was phe (phe-arg-X).

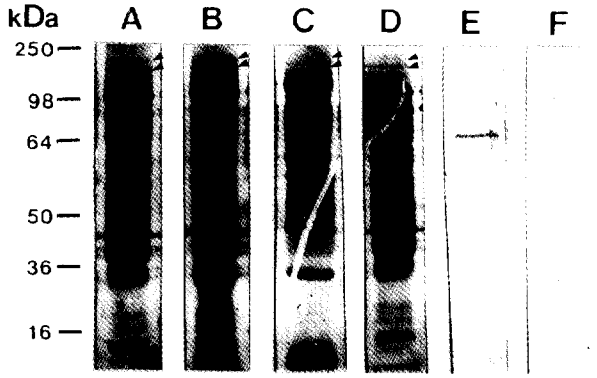

Fig. 6. Western blot analysis of culture filtrate of *M. tuberculosis*. SDS-PAGE and electrophoretic transfer of proteins onto nitrocellulose membrane were performed as described in materials and methods. Lane A, B, C and D, patient's sera; Lane E and F, normal human sera.

Antigenic property

Fig. 6 shows a immunoblotting pattern after SDS-PAGE of culture filtrate of *M. tuberculosis*. After being blotted onto nitrocellulose membrane, the membrane was exposed to human antiserum. The bound antibody was visualized by reacting with peroxidase conjugated anti-human goat immunoglobulin G. Two bands corresponding to the protease bands on SDS-PAGE were recognized. Therefore, the proteases secreted by *M. tuberculosis* have an antigenic property.

Discussion

In this study, we have partially characterized two proteases from culture filtrate of *Mycobacterium tuberculosis*. Protease activity was detected using generalized substrate, azocasein, and gelatin SDS-PAGE. Two proteases were detected on gelatin SDS-PAGE, whose molecular weights were approximately 200 and 180 kDa, respectively and had highly similar enzymatic characteristics. They were inhibited significantly by EDTA and some inhibitions were observed by DFP, EGTA and 2-mercaptoethanol (Fig. 1, Table 1). However, the concentration of DFP used by us is sufficient to rapidly inactivate most (but not all) extracellular serine proteases and these proteases were not inhibited by PMSF. Accordingly, these enzymes are not thought to be serine proteases. The fact that these enzymes were not inhibited by E-64, a cysteine protease-specific inhibitor, although 2-mercaptoethanol inhibited somewhat the activity of proteases, indicating that these enzymes are not cysteine proteases. Interestingly, when accomplished gelatin SDS-PAGE followed by treatment of 2-mercaptoethanol, the protease bands observed in somewhat

higher molecular weight region compared with 2-mercaptoethanol untreated control. These indicate that some cysteine residues, which formed disulfide bonds influencing on enzyme activity, may be exist in internal amino acid sequences of these enzymes and slight molecular weight change and some inhibition of enzyme activity may be due to these enzymes are more relaxed or structurally changed by cleavage of disulfide bonds. The activity of enzymes was enhanced by Ca^{2+} and Mg^{2+}. However, the activity of enzymes was inactivated completely by 2 mM Cu^{2+} and 5 mM Ag^{2+} (Table 2). Sensitivity to the metal chelating agent EDTA is typical of metalloproteases, many of which are stabilized or activated by Ca^{2+} and Mg^{2+} (18, 26). Therefore, these enzymes seem to be metalloproteases activated by Ca^{2+} and Mg^{2+}. These enzymes were active over a narrow pH range, from 6.0 to 8.0 and pH optimum was 7.0 (Fig. 3). Optimal temperature was about 40°C (Fig. 4) and the activity of enzymes were inactivated rapidly at 80°C (Fig. 5). These results suggest that these enzymes are heatlabile, neutral proteases. The proteases secreted by *M. tuberculosis* degraded immunoglobulin G, hemoglobin, bovine serum albumin, collagen (type I) and lysozyme, but not fibronectin (Table 3).

The inability of *M. tuberculosis* proteases to degrade the fibronectin may be explain in two respects. These enzymes have a limited substrate specificity and are not able to degrade fibronectin naturally or the antigen 85 complex of *M. tuberculosis*, which binds human fibronectin with specific affinity (4), bind specifically to fibronectin prior to enzyme's attack on fibronectin and form the steric hindrance which disturb attack of enzymes and inhibit the proteolysis of fibronectin. The resistance of fibronectin against the proteases secreted by *M. tuberculosis* is thought to be meaningful for internalization of *M. tuberculosis* into macrophages closely related to fibronectin (4, 24, 25). Therefore, to define the relationships of fibronectin and proteases of *M. tuberculosis* more clearly, further studies with purified antigen 85 complex and proteases of *M. tuberculosis* against fibronectin must be performed. The proteases secreted by *M. tuberculosis* were able to degrade serum components such as hemoglobin and bovine serum albumin. Therefore, *M. tuberculosis* could use these serum proteins as nutrient sources during growth and survival. Moreover, these enzymes were able to degrade immunoglobulin and collagen type I. Immunoglobulin G is the major immunoglobulin in normal human serum accounting for 70~75% of the total immunoglobulin pool. Therefore, degradation of immunoglobulin G by the proteases secreted by *M. tuberculosis* may induce negative affect of the host immune system against infected *M. tuberculosis*. Collagen normally functions as structural protein to maintain tissue integrity. Therefore, degradation of collagen results in tissue necrosis (7, 12). Thus, degradation of collagen by the proteases secreted by *M. tuberculosis* implicate the possibility that *M. tuberculosis* proteases may contribute tissue necrosis and other pathological symptoms during pulmonary infection. These proteases exhibited antigenicity against patient's sera. One of two normal sera reacted weakly to some antigens, especially to the 70 kDa antigen. This may be due to antibodies produced by BCG vaccination. However, the enzymes did not react with the normal sera. This may be due to low antibody levels detected them in western blot or it may be because the antibodies which react to the proteases are not produced by BCG vaccination. These suggest the possibility of proteases as useful diagnostic antigens. Furthermore, if the definite roles of proteases as virulence factors in pathology of tuberculosis are revealed more clearly, it is possible to use the proteases for vaccine, diagnostic or treatmental purpose. In order to more specifically characterize the proteases of *M. tuberculosis*, further purification must be done and to support the hypothesis that *M. tuberculosis* proteases may contribute to the pathology observed in tuberculosis, *in vivo* studies with inhibitors are required.

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