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Gelatinase Activity in *Mycobacterium bovis* Protein Extract[1]

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Leprosy and tuberculosis are still public health problems in many parts of the world. A number of molecules obtained from mycobacteria have been characterized ([21]). The first proteins identified were intracellular heat shock proteins that could play a role in mycobacterial immunopathology. Another group of proteins comprises secreted antigens, with a major component being complex 85, which presents fibronectin-binding functions and shows extensive homology between *Mycobacterium tuberculosis* and *Mycobacterium leprae* ([11]).

Several molecular components with proteolytic activity have been ignored in mycobacterial studies ([2]). Moreover, there is little information in the literature concerning mycobacteria proteinase detection using classical biochemical techniques.

Proteases have many roles in bacteria, ranging from turnover and modification of cellular proteins, to virulence factors in pathogens. Importantly, in recent years bacterial and viral proteases have been found to interact with the cytokine network ([8]). Consequently, the targeting of proteolytic enzymes is a strategy which shows promise for the control of other bacterial pathogens ([13, 20]).

In order to understand the biology of some mycobacterial proteins, and due to the advantage of being able to cultivate *M. bovis in vitro,* we have continued with the identification of mycobacterial proteins, especially those that present proteinase activities. Therefore, in the present study, gelatinase activity from total proteins (cytosolic and membrane proteins) of *Mycobacterium bovis* (MbSA) was investigated. Our objective was to analyze some biochemical properties of the proteinases present in MbSA, including susceptibility to classical inhibitors and pH dependency of activity.

MATERIALS AND METHODS

Mycobacteria. Danish BCG strain 1331 was cultured in serum free Sauton medium ([16]). The strain was incubated at 37°C for six weeks. The soluble *Mycobacterium bovis* protein extract (MbSA) was obtained by successive passage through a French pressure cell using 10,000 lbs/in^2 ([4]), followed by centrifugation at 27,138 *g* (Sorvall RC-5B, rotor SS-34) at 4°C for 1 hr in PBS pH 7.2 to eliminate bacillary debris, and finally filtered through a 0.22 µm diameter pore size membrane. The protein concentration was determined by the BCA method (Bicinchoninic acid, Pierce-Endogen, Rockford, Illinois, U.S.A.) ([19]).

Zymography (gelatinase activity). Proteinase activity was investigated by SDS-PAGE using gelatin as the proteinase substrate, as previously described ([9]). Activity was studied in 100 µg of protein from a *M. bovis* soluble extract (MbSA). The samples were dissolved 1 hr at room temperature in pH 6.8 Laemmli sample buffer (62 mM

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Abbreviations used are as follows: PMSF = phenylmethylsulfonylfluoride; p-APMSF = p-Amidinophenylmethyl sulfonylfuoride; TLCK = N$^{\alpha}$-Tosyl-Lys chloromethyl Ketone; TPCK = N-tosyl-L-phenylalanine chloromethylcetone; AEBSF = p-Aminoethylbenzenesulfonylfluoride; OPA = ortho-phenantroline; E-64 = trans-epoxysuccinyl-L-leucylamido-[4-guanidino]butane; PCMB = p-chloromercurybenzoate; Pepst A = Pepstatin A; 3,4-DCI = 3,4-dichloroisocoumarin; Lact = Lactacystin; HEPES = N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; CAPS = 3-[cyclohexylamino]-1-propane-sulfonic acid; DTT = dithiothreitol; DMSO = dimethylsulfoxide; 2-ME = 2-mercaptoethanol; SDS = sodium dodecyl sulfate; and SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Tris-HCL, 2% SDS, 50 mM 2-ME, 10% glycerol and 0.5% bromophenol blue). Gelatin (Sigma-Aldrich Co., St. Louis, Missouri, U.S.A.) was co-polymerized at 0.2% in SDS-PAGE gels using a discontinuous gel composed of stacking (4%) and running (8%) resolving gels. After electrophoretic separation, the gels were washed for 30 min at 4°C with Triton-X 100 (Sigma-Aldrich Co.) at 2.5% v/v, followed by three 10 min washes with cold, distilled water, in order to extract the SDS and restore *in situ* proteolytic activities. Finally, the gels were incubated for 18 hr at 35°C in incubation buffer, 50 mM HEPES pH 7.0; 1 mM $CaCl_2$; 0.025% NaN_3. Proteinase activity was revealed as clear zones after gel staining with 0.1% Coomassie Brilliant Blue R-250 (Gibco BRL, Grand Island, New York, U.S.A.). High range protein standards (Gibco BRL), dissolved in a sample buffer containing 2-ME and heated for 3 min at 90°C, were included in all zymograms to estimate the apparent relative mobility (Mr) of the gelatinases under the experimental conditions. The Mr is the mobility of the proteinase with reference to a marker protein, and has been referred to as apparent molecular mass. The position of these protein bands could be identified by partially decolorizing the gels.

pH profile. The pH proteinase-dependent activity was investigated by zymography using a preparative comb. (It presents only two pockets, a great central pocket and a small lateral pocket. The preparative comb allows separation by electrophoresis of a large number of proteins, which can be analyzed under different conditions.) Five hundred micrograms of total protein was dissolved in the sample buffer and separated in an 8% acrylamide gel containing 0.2% gelatin. After separation and washing, the gel was divided into 7 equal 15 mm wide strips. The gel strips were incubated for 18 hr at 35°C in separate buffer solutions with pH values between 4.0 and 10.0 using 50 mM buffer also containing 1 mM $CaCl_2$ and 0.025% NaN_3. The buffers were as follows: pH 4.0 and pH 5.0, acetate/acetic acid buffer; pH 6, phosphate buffer; pH 7, HEPES buffer (Calbiochem, San Diego, California, U.S.A.); pH 8 and pH 9, Tris-HCL and pH 10, CAPS buffer (Sigma-Aldrich Co.). After the buffer incubation period was completed, the different strips were dyed using Brilliant Blue Coomassie R-250 (Gibco BRL).

Susceptibility to classical proteinase inhibitors. In order to classify the proteinases according to their catalytic mechanisms, their sensitivities to classic inhibitors were analyzed. The inhibitors were diluted in dimethyl sulfoxide (DMSO), whereas p-Aminoethylbenzenesulfonylfluoride (AEBSF) and p-Amidinophenylmethyl sulfonylfuoride (APMSF) were dissolved in water, N-tosyl-L-phenylalanine chloromethylcetone (TPCK) methanol and trans-epoxysuccinyl-L-leucylamido-[4-guanidino]butane (E64) either DMSO or water. The MbSA containing 100 µg of protein was incorporated into a final volume of 20 µl of specific incubation buffer (10 mM HEPES, pH 7.0; 1 mM $CaCl_2$; 0.025% NaN_3) and the samples were pre-incubated with specific proteinase inhibitors for 1–18 hr at 4°C; in other cases the pre-incubation was for 18 hours at 37°C.

After pre-incubation, the samples were solubilized in sample buffer without 2-ME for 1 hr at room temperature and then analyzed by zymography, as described above. The following proteinase inhibitors for serine-type proteinase were studied ([5, 17]): PMSF (Calbiochem), ranging in concentration between 1–10 mM in the presence or absence of 1 mM DTT (Sigma-Aldrich Co.) and AEBSF (Calbiochem) 1–10 mM, APMSF (Carbiochem) 0.1 mM, 3,4-DCI (Calbiochem) in concentrations ranging between 0.125–0.5 mM, TLCK (Sigma-Aldrich Co.) and TPCK (Sigma-Aldrich Co.) 0.1 mM; E-64 (Calbiochem) and PCMB (Sigma-Aldrich Co.) 0.1 mM for cysteine-type proteinase; Pepstatin A (Calbiochem) 10 µM for aspartic-type proteinase; OPA (Sigma-Aldrich Co.) 1 mM for metalloproteinase, and lactacystin (Calbiochem) concentration ranging between 25–100 µm for proteasome.

The volume of the inhibitors did not exceed 5% of the final volume. Controls consisting of 5% DMSO or methanol alone were used to rule out an action of these solvents on the proteinases. The zymograms were analyzed by densitometry, using a densitometer Model GS-690 and Multi-Analyst^R software (Bio-Rad Laboratories, Hercules, California, U.S.A.).

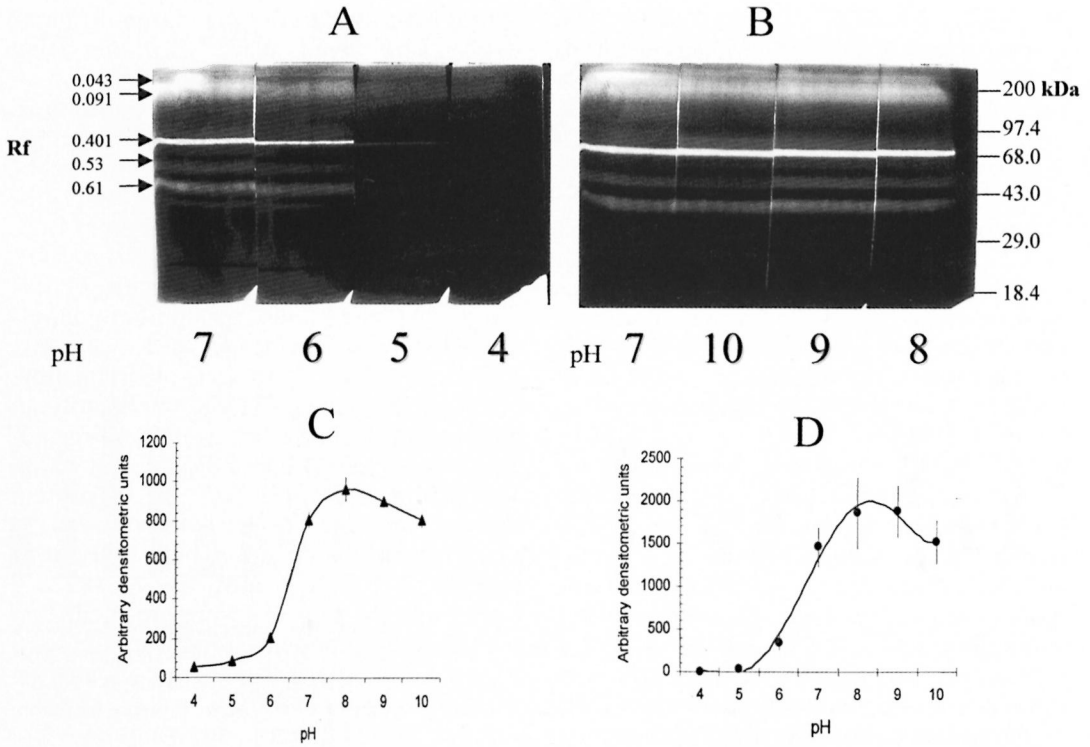

FIG. 1. pH-dependence of MbSA gelatinases. **A** = Zymogram at pH intervals between 4 and 7; **B** = Zymogram at pH interval between 7 and 10; **C** = Mr 200-kDa or Rf 0.091 gelatinase densitometric analysis; **D** = Mr 68-kDa or 0.401 Rf gelatinase densitometric analysis. Protein standards in kilodaltons are indicated on right side and Rf on the left, pH levels on bottom. Values are mean ± S.D., N = 3 experiments.

RESULTS

The zones with proteolytic activity appear as clear bands on a dark background, a consequence of the digestion of the protein substrate (gelatin). Fig. 1 shows the pH-dependency of MbSA gelatinase activity. Five bands of gelatinase activity are appreciated, whose smaller to bigger Rf corresponds to 0.043, 0.091, 0.401, 0.53, 0.61, respectively. Gelatinase activity is minimal at pH 4 and pH 5, increasing progressively from pH 6 and reaching a relative maximum at pH 8, for the 0.091, 0.401, 0.53, and 0.61 Rf bands, except for the Rf 0.043 band, that showed an optimum at pH 7.0. The Rf 0.091 and 0.401 gelatinases were chosen for further analysis since they showed predominant gelatinase activity.

In order to classify the gelatinases according to their catalytic mechanism, we analyzed their susceptibility to classical proteinase inhibitors. Fig. 2 shows a zymogram resulting from pre-incubation of the *M. bovis* protein extracts with different inhibitors at 37°C overnight. In the first place, it is evident that under these conditions the DMSO and methanol solvents produce a decrease of Rf 0.091 gelatinase activity, while they do not affect the Rf 0.40 band. (Compare track number 1 corresponding to the sample in aqueous control with track number 2 (Fig. 2) (5% DMSO) and track number 2 (Fig. 3) (5% methanol), respectively. The reducing agent DTT does not affect the activity of the gelatinases studied (track number 3, Fig. 2).

In Fig. 2, it is evident that PMSF to 10 mM (track 4) or PMSF in the presence of 1 mM DTT produces an inhibition of the gelatinase activities (track 5). Since PMSF was dissolved in DMSO, and, under incubation conditions, solvents may affect gelatinase activity, we investigated the AEBSF inhibition of Rf 0.091 gelatinases under the same incubation conditions, AEBSF was dissolved in water. The inhibition of gelatinases with AEBSF (1–10 mM) was verified (tracks 3 and 4, Fig. 3).

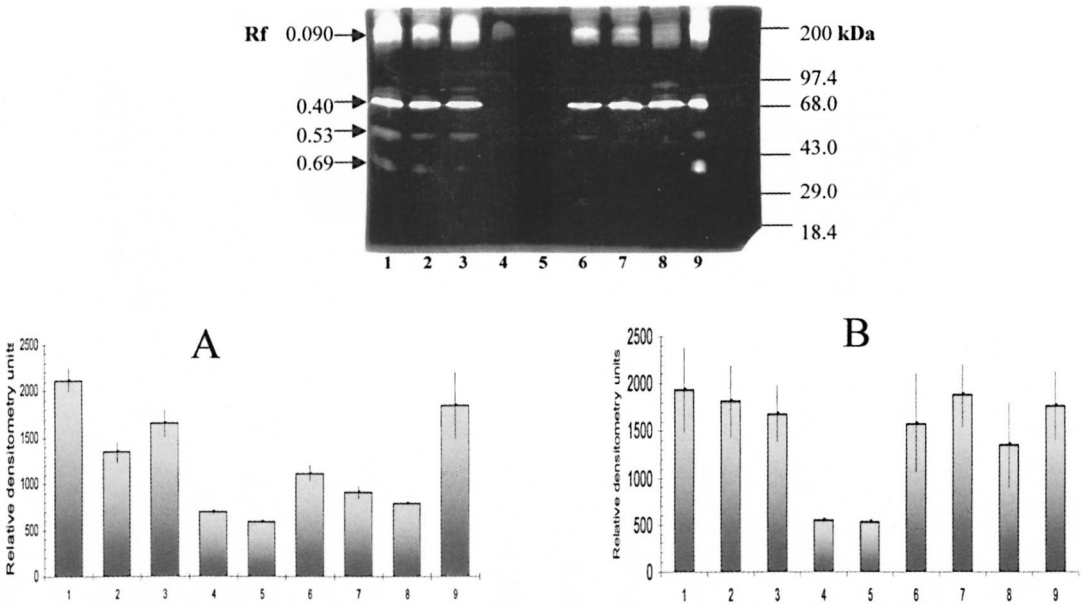

FIG. 2. Susceptibility of MbSA gelatinases to classical proteinase inhibitors. **1**—Aqueous control; **2**—5% v/v DMSO; **3**—1 mM DTT; **4**—10 mM PMSF; **5**—10 mM PMSF/1 mM DTT; **6**—100 μM E64; **7**—100 μM PCMB; **8**—1 mM OPA; **9**—10 μM Pepst A. **A** = Mr 200-kDa or Rf 0.091 gelatinases densitometric analysis; **B** = Mr 68-kDa or Rf 0.40 gelatinases densitometric analysis. Samples were pre-incubated with specific protease inhibitors at 37°C overnight. Values are mean ± S.D., N = 3 experiments.

Although under the pre-incubation conditions mentioned, E64 and PCMB seem to inhibit Rf 0.091 gelatinase (tracks 6 and 7, Fig. 2), it is important to mention that such effects seem to depend on solvent type, temperature, and duration of incubation. In fact, in incubations of one hour at room temperature, or overnight at 4°C, in the presence of 5% DMSO, gelatinase activity is not affected (data not shown). The

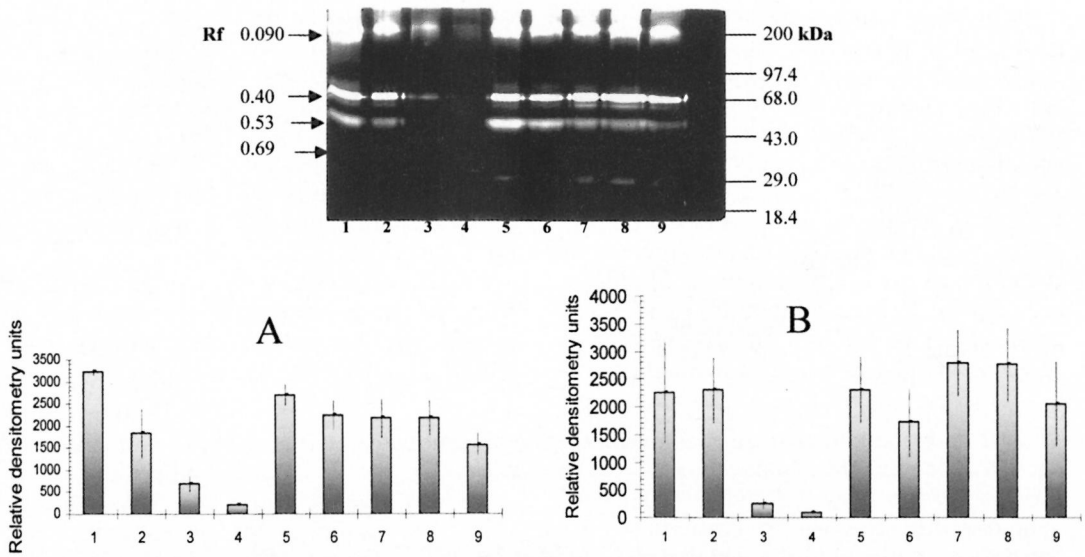

FIG. 3. Susceptibility of MbSA gelatinases to serine-type proteinase inhibitors. **1**—Aqueous control; **2**—5% v/v methanol; **3**—1 mM AEBSF; **4**—10 mM AEBSF; **5**—0.1 mM APMSF; **6**—1 mM APMSF; **7**—0.1 mM TLCK; **8**—1 mM TLCK; **9**—1 mM TPCK. **A** = Mr 200-kDa or Rf 0.091 gelatinases densitometric analysis; **B** = Mr 68-kDa or Rf 0.40 gelatinases densitometric analysis. Samples were pre-incubated with specific protease inhibitors at 37°C overnight. Values are mean ± S.D., N = 3 experiments.

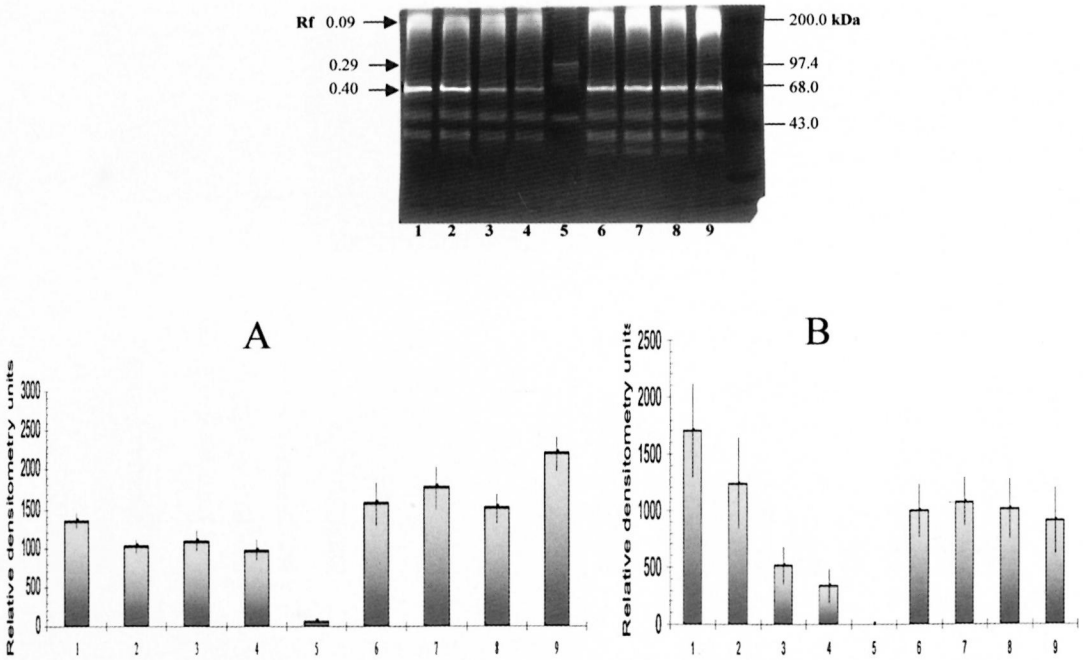

FIG. 4. Susceptibility of MbSA gelatinases to Coumarin, Lactacystin. Orthophenanthroline/Zn^{2+} effect. **1**—Aqueous control; **2**—5% v/v DMSO; **3**—0.125 mM 3,4-DCI; **4**—0.25 mM 3,4-DCI; **5**—0.5 mM 3,4-DCI; **6**—25 μM Lact; **7**—50 μM Lact; **8**—100 μM Lact; **9**—1 mM (OPA/Zn^{2+}). **A** = Mr 200-kDa or Rf 0.091 gelatinases densitometric analysis of **B** = Mr 68-kDa or Rf 0.40 gelatinases densitometric analysis. Samples were pre-incubated with specific protease inhibitors at 4°C overnight. Values are mean ± S.D., N = 3 experiments.

APMSF (tracks 5 and 6), TLCK (tracks 7 and 8), and TPCK (track 9, Fig. 3), and Pepstatin A (track 9, Fig. 2) had no inhibitory effect on these gelatinases.

In order to analyze the partial inhibition of Rf 0.091 gelatinases by incubation with 1 mM OPA track 8 (Fig. 2), we investigated the effect of pre-incubation with equimolar quantities of OPA/Zn^{2+}. In Fig. 4, track 9, we observed the pre-incubation of the homogenates with equivalent quantities of OPA/Zn^{2+} results in an apparent activation of the gelatinases. This result suggests the presence of metalloproteinases. Therefore, we decided to investigate the possible activator effect of the ion Zn^{2+}. Nevertheless, considering the co-migration in this zone with serine proteases, we analyzed the effect of the addition of increasing concentrations of Zn^{2+} at 0–100 μM, in pre-treated samples with 5 mM AEBSF for one hour at room temperature. Fig. 5 shows the results. Surprisingly, in the Rf 0.091 area we did not observe gelatinase activation. However, we did observe activation in two bands of Rf 0.32 with an Mr next to 97-kDa when the Zn^{2+} was increased. Activation by Zn^{2+} was prevented by 1 mM OPA and reverted by treatment with equivalent quantities of OPA/Zn^{2+}.

Finally, in order to investigate whether some of these gelatinases corresponded to proteasome, we studied their inhibition by 3,4-DCI and lactacystin, with pre-incubation

THE TABLE. *Proteinase types and specific proteinase inhibitors used in this study.*

Proteinase type	Specific inhibitor
Threonine-type (proteasome)	Lactacystin
Serine-type	PMSF, AEBSF, APMSF, 3,4-DCI
Metallo-proteinase-type	OPA
Cysteine-type	E64, PCMB
Aspartic-type	Pepstatin A

Note. TPCK and TLCK inhibit serine proteinases and some cysteine proteinases.

with different concentrations at 4°C overnight. In Fig. 4, it can be observed that while 3,4-DCI affects them distinctly, the Rf 0.40 gelatinase, being more susceptible to this inhibitor, is inhibited 0.125 mM, while the Rf 0.09 gelatinase at this concentration is not affected (tracks 3 and 4). With 0.5 mM 3,4-DCI, both gelatinases (Rf 0.091 and 0.40) are completely inhibited, showing the presence of a Rf 0.29 gelatinase (track 5). Lactacystin does not inhibit these gelatinases (tracks 6, 7, and 8, Fig. 4).

DISCUSSION

Proteinases represent a diverse group of enzymes that catalyze the hydrolysis of peptide bonds in proteins. According to the mechanism of hydrolysis of the peptide bonds, proteinases are classified in four classes: serine/threonine, cysteine, aspartic and metallo-proteinases. Based on susceptibility to a group of class-specific inhibitors, it is possible to obtain an initial classification of newly discovered proteinase.

Proteinases carry out a diversity of functions in the physiology of all organisms, ranging from the turnover and modification of cellular proteins, to virulence factors in pathogens ([1, 10]), and the targeting of proteolytic enzymes is a strategy which shows promise for the control of bacterial pathogens. However, our knowledge of proteolysis in prokaryotes is surprisingly quite fragmentary. At the present, only a few proteinases have been examined in mycobacteria.

Recently, by means of molecular biology techniques, some investigators have identified cloned genes that code putative secreted serine proteinase, present in exoantigens, whose constitutive expression suggests an important role in the biology of *M. tuberculosis* ([18]). Similar studies carried out in *Mycobacterium avium* identified a putative serine protease ([3]). Previously, serine proteases had not been described in *Mycobacteriaceae*. Few papers exist where proteinases in mycobacteria are studied by means of classical biochemical techniques. In this work using gel zymography, and classical proteinase inhibitors, we report the detection and identification of catalytic mechanisms of some proteinases found in *M. bovis* protein extracts. Although zymograms showed five bands of gelatinase activity that differ in their Rf, in this work we studied two: a Rf 0.091 band with a Mr near to the 200-kDa standard and another Rf 0.401 band with a Mr near to the 68-kDa standard. Both gelatinases show the typical curve of many serine proteinases, with an optimum activation around pH 8 ([5]). This behavior is consistent with susceptibility to inhibitors, where classic inhibitors of serine proteases like PMSF, AEBSF and 3,4-DCI, produce a remarkable decrease of gelatinase activity. This suggests the possible existence and participation of a serine amino acid in the active site and the presence of serine type proteinases in *Mycobacterium bovis*. Although both gelatinases show the pH activity profile and inhibition by classic inhibitors of serine proteinases, the behavior of the Rf 0.091 gelatinase in relation to the Rf 0.401 gelatinase in the presence of 3,4-DCI, AEBSF reveals a differential susceptibility. This result provides evidence for differences between inhibition kinetics among these serine gelatinases. Moreover, overnight incubation at 37°C with DMSO and methanol produces a marked reduction

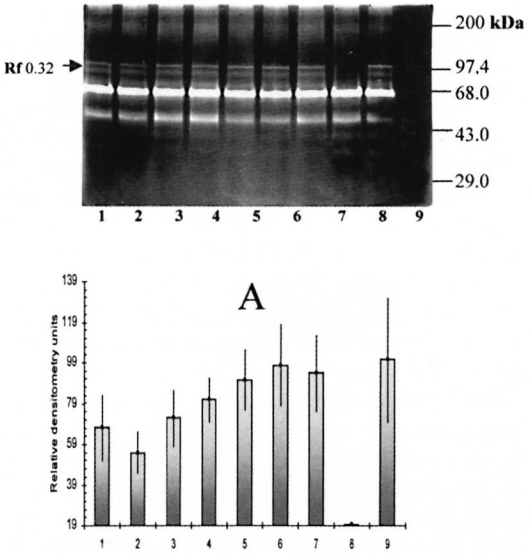

FIG. 5. Effect of increasing Zn^{2+} concentrations on 5 mM AEBSF pre-treated samples. **1**—0 Zn^{2+}; **2**—3 μM Zn^{2+}; **3**—6 μM Zn^{2+}; **4**—12.5 μM Zn^{2+}; **5**—25 μM Zn^{2+}; **6**—50 μM Zn^{2+}; **7**—100 μM Zn^{2+}; **8**—1 mM OPA; **9**—1 mM (OPA/Zn^{2+}). **A** = Mr 97.4-kDa or Rf 0.32 gelatinases densitometric analysis. Samples were pre-incubated with 5 mM AEBSF at room temperature for one hour. Afterward they were incubated for one hr at different Zn^{2+} concentrations. Values are mean ± S.D., N = 3 experiments.

of Rf 0.0901 gelatinases, while it does not affect the Rf 0.401 gelatinases; although we ignore the reasons for this, denaturalization of slow mobility gelatinases could explain the phenomenon. In any case, the behavior of the gelatinase suggests the presence of two serine proteinases in *Mycobacterium bovis.*

The inhibition of Rf 0.091 gelatinases by E64 and PCMB deserves further comments. The E64 is an inhibitor of cysteine type proteases, whose mechanism of action consists in covalent modification of cysteine amino acids that participate directly in peptide bond hydrolysis. In this respect, some experimental evidence suggest these results are due to physical-chemistry factors associated with the temperature, the solvent, and the time of incubation on the activity of the proteinase. First, in support of this proposal, when E64 is dissolved in distilled water and pre-incubation is carried out at room temperature for one hour, or when E64 or PCMB are dissolved in DMSO and pre-incubation is carried out overnight at 4°C, we did not observe, in either case, an effect of these inhibitors on gelatinases. Nevertheless, under the aforementioned conditions, PMSF maintains its inhibitory action on gelatinases. Secondly, cystein proteases are activated by reducing agents like 2-ME and DTT ([5, 17]). The absence of activation of gelatinases by DTT is not favorable evidence for the cysteine-type character of these gelatinases. The absence of an inhibitory effect of Pepstatin A rules out aspartic-type proteinases.

In summary, the experimental evidence is consistent with the serine character of these gelatinases, as follows.

In samples pre-treated one hour at room temperature with 5 mM AEBSF, a Rf 0.32 gelatinase was stimulated by Zn^{2+}, inhibited by OPA, and reverted with treatment of equivalent quantities of OPA/Zn^{2+}, demonstrating the specific and reversible character of this inhibition. This result is consistent with the existence of Zn^{2+}-dependent metalloprotease in *Mycobacterium bovis.*

In stationary phase culture filtrates of *M. tuberculosis,* a calcium dependent metalloprotease has been identified ([15]). Also another 75-kDa metalloprotease with collagenolytic activity, which shows more activity in the virulent strain H37Rv in comparison with the non-virulent H37Ra, have been identified in *M. tuberculosis* ([12]).

Recently, molecular biology techniques have revealed the presence of genes that code for proteasome in *Rhococcus erytropolis, Mycobacterium tuberculosis* and *Streptomyces coelicolor* ([14]). Taking into consideration the possible presence of a complex proteasomal in our preparation, we examined the effect of a specific inhibitor of proteasome lactacystin on the gelatinases existing in the *M. bovis* soluble extract homogenate. We did not find an inhibitory effect on the gelatinases to concentrations of the inhibitor of 25 μM, 50 μM, and 100 μM. The absence of a lactacystin effect excludes the participation of amino acid threonine in the active site of gelatinases ([14]).

At the present, these protein extracts were used in a cellular activation system and we obtained a good cellular immune response in T cells of lepromatous and tuberculoid leprosy patients. The proteinase identification in such preparations improved our understanding of the biology and immunology of *Mycobacterium bovis.* Given the safety and possibility of *M. bovis* cultures, and its homology with pathogens related to man, such as *M. leprae* and *M. tuberculosis* ([6, 7]), it is possible that the knowledge gained with *M. bovis* could be useful in understanding mycobacterial biology. In this respect, our results are consistent with previous discoveries carried out by other groups that have applied molecular biology techniques to other mycobacteria ([12, 15, 18]).

SUMMARY

Proteases are well-recognized as virulence factors in different pathologies, resulting in tissue damage potential. Despite efforts over the past few years to identify mycobacterial protein antigens, there is little information regarding the role of mycobacterial proteinase activities. In this study, by zymography techniques, we have detected and partially studied some biochemical properties of *Mycobacterium bovis* proteases, such as pH dependency of activity and susceptibility to classical proteinase inhibitors. We observed optimal proteolytic activity at pH 8. Some proteinases were inhibited by classic inhibitors of serine proteases, such as PMSF, AEBSF,

and 3-4 DCI. In some AEBSF pre-treated preparations we observed residual gelatinase activity in Rf 0.32. This gelatinase was stimulated by Zn^{2+} and inhibited by OPA (1 mM). This last effect was reversed by exposure to equimolar quantitative OPA/Zn^{+2} (1 mM/1 mM). These results suggest the existence of serine proteinase and metalloproteinase types in protein extracts of *Mycobacterium bovis.*

RESUMEN

Las proteasas son bien reconocidas como factores de virulencia promotores de daño potencial en diferentes patologías. A pesar de los esfuerzos hechos para identificar antígenos proteicos micobacterianos, todavía hay poca información sobre el papel que juegan las proteasas micobacterianas. En este estudio se utilizaron técnicas zimográficas para detectar y estudiar algunas de las propiedades bioquímicas de las proteasas de *Mycobacterium bovis,* tales como su dependencia del pH y su susceptibilidad a inhibidores clásicos de proteasas. Observamos una óptima actividad proteolítica a pH de 8. Algunas proteasas fueron inhibidas por los inhibidores clásicos de las proteasas con serina, tales como PMSF, AEBSF, y 3-4 DCI. En algunas preparaciones pre-tratadas con AEBSF observamos actividad residual de gelatinasa con un Rf de 0.32. Esta gelatinasa fue estimulada por Zn^{2+} e inhibida por OPA (1 mM). Este último efecto fue revertido por exposición a cantidades equimolares de OPA/Zn^{+2} (1 mM/1 mM). Estos resultados sugieren la existencia de proteasas con serina y metaloproteinasas en los extractos proteicos de *Mycobacterium bovis.*

RÉSUMÉ

Les protéases sont des facteurs de virulence bien connues dans des pathologies variées, dont les conséquences possibles sont des lésions tissulaires importantes. En dépit d'efforts constants pendant ces dernières années pour identifier les antigènes protéiniques des mycobactéries, il y a peu d'informations concernant le rôle des activités des protéinases chez ces dernières. Dans cette étude, par des techniques d'enzymographie, nous avons détecté et partiellement étudié certaines propriétés biochimiques des protéases de *Mycobacterium bovis,* comme la dépendance du pH pour leur activité catalytique et leur susceptibilité aux inhibiteurs classiques des protéinases. Nous avons observé une activité protéolytique optimale à pH 8. Certaines protéinases furent inhibées par des inhibiteurs classiques des protéases de type sérine protéinases, comme le PMSF, AEBSF et 3-4 DCI. Parmi certaines préparation pré-traitées à l'AEBSF, nous avons observé des activités résiduelles de type gélatinase dans la Rf de 0,32. Cette activité gélatinase était stimulée par Zn^{2+} et inhibé par OPA (1 mM). Cet effet pouvait être annulé par l'exposition à une quantité équimolaire de OPA et de Zn^{2+} (1 mM/1 mM). Ces résultats suggèrent l'existence de protéase de type sérine protéinases et de métalloprotéinases dans l'extrait protéique de *Mycobacterium bovis.*

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REFERENCES

1. Braun Breton, C. and Pereira da Silva, L. H. Malaria proteases and red blood cell invasion. Parasitol. Today **9** (1993) 92–96.
2. Brown, G. D., Dave, J. A., Gey Van Pittius, N. C., Stevens, L., Ehlers, M. R. W. and Beyers, A. D. The mycosins of *Mycobacterium tuberculosis* H37Rv: a family of subtilisin-like serine proteases. Gene. **254** (2000) 147–155.
3. Cameron, R. M., Stevenson, K., Inglis, N. F., Klausen, J. and Sharp, J. M. Identification and characterization of a putative serine protease expressed *in vivo* by *Mycobacterium avium* subsp. *paratuberculosis.* Microbiology **140** (1994) 1977–1982.
4. Convit, J., Aranzazu, N., Ulrich, M., Pinardi, M. E., Reyes, O. and Alvarado, J. Immunotherapy with a mixture of *Mycobacterium leprae* and BCG in the different forms of leprosy and Mitsuda negative contacts. Int. J. Lepr. **50** (1982) 415–424.
5. Dunn, B. M. Determination of protease mechanisms. In: *Proteolytic Enzymes: A Practical Approach.* 1st edn. Benyon, R. J. and Bond J. S., eds. Oxford: IRL Press at Oxford University Press, 1989, pp. 57–81.
6. Grosskinsky, C. M., Jacobs, W. R. J., Clark-Curtis, J. E. and Bloom, B. R. Genetic relationships among *Mycobacterium leprae, Mycobacterium tuberculosis,* and candidate leprosy vaccine strains determined by DNA hybridization: identification of an *M. leprae*-specific repetitive sequence. Infect. Immun. **57** (1989) 1535–1541.
7. Harboe, M. and Wiker, H. G. Secreted proteins of *Mycobacterium leprae.* Scand J. Immunol. **48** (1998) 577–584.
8. Henderson, B., Poole, S. and Wilson, M. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60** (1996) 316–341.
9. Heussen, C. and Dowdle, E. Electrophoretic analysis of plasminogen activators in poliacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. **102** (1980) 196–202.
10. Johnson, K., Charles, I., Dougan, G., Pickard, D., O'Gaora, P., Gosta, G., Ali, T., Miller, I. and Hormaeche, C. The role of a stress-response protein in *Salmonella thyphymurium* virulence. Mol. Microbiol. **5** (1991) 401–407.

11. Launois, P., Niang, M. N., Drowart, A., van Vooren, J. P., Sarthou, J. L., Lalu, T., Millan, J. and Huygen, K. IgG response to purified 65 and 70 kDa mycobacterial heat shock proteins and to antigen 85 in leprosy. Int. J. Lepr. **62** (1994) 48–54.
12. Masso, F., Paez, A., Varela, E., de Leon, L. D., Centeno, E. and Montano, L. F. Collagen degrading activity associated with *Mycobacterium species*. Thorax **54** (1999) 439–441.
13. Miyagawa, S., Nishino, N., Kamata, R., Okamura, R. and Maeda, H. Effects of protease inhibitors on growth of *Serratia marcescens* and *Pseudomonas aeruginosa*. Microbiol. Pathol. **11** (1991) 137–141.
14. Pouch, M., Cournoyer, B. and Baumeister, W. Characterization of the 20 S proteasome from the actinomycete *Frankia*. Mol. Microbiol. **35** (2000) 368–377.
15. Rowland, S. S., Ruckert, J. L. and Burall, B. N. Jr. Identification of an elastolytic protease in stationary phase culture filtrates of *M. tuberculosis*. FEMS Microbiol. Lett. **151** (1997) 59–64.
16. Sadamu, N., Nagasuga, T., Matsumoto, J. and Coda, K. Isolation of tuberculin skin reactive proteins from heated culture filtrate of *Mycobacterium tuberculosis* H37Rv. Am. Rev. Respir. Dis. **109** (1974) 17–27.
17. Salvensen, G. and Nagame, H. Inhibition of proteolytic enzymes. In: *Proteolytic Enzymes: A Practical Approach*. 1st edn. Beynon R. J. and Bond, J. S., eds. Oxford: IRL Press at Oxford University Press, 1989, pp. 83–104.
18. Skeiky, Y. A. W., Lodes, M. J., Guderian, J. A., Mohamath, R., Bement, T., Alderson, M. R. and Reed, S. G. Cloning, expression, and immunological evaluation of two putative secreted serine protease antigens of *Mycobacterium tuberculosis*. Infect. Immun. **67** (1999) 3998–4007.
19. Smith, P. K. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150** (1985) 76–85.
20. Travis, J., Potempa, J. and Maeda, H. Are bacterial proteinases pathogenic factors? Trends Microbiol. **3** (1995) 405–407.
21. Young, D. B., Kaufmann, S. H. E., Hermans, P. W. M. and Thole, J. E. R. Mycobacterial protein antigens, a compilation. Mol. Microbiol. **6** (1992) 133–145.