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Characterization of an exported monoglyceride lipase from *Mycobacterium tuberculosis* possibly involved in the metabolism of host cell membrane lipids

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Running Title: Exported monoglyceride lipase from *M. tuberculosis*.

Abbreviations used: *Mycobacterium tuberculosis*, *M. tuberculosis*; Terrific Broth, TB; Triacylglycerol, TAG; Diacylglycerol, DAG; Monoacylglycerol, MAG; β -cyclodextrin, β -CD; Phospholipase A and C, PLA and PLC; Tetrahydrolipstatin, THL; diethyl p-nitrophenyl phosphate, E600.

Synopsis

The Rv0183 gene of the *M. tuberculosis* H37Rv strain, which has been annotated as a probable lysophospholipase, was cloned and expressed in *E. coli*. The purified Rv0183 protein did not show any activity when lysophospholipid substrates were used, but preferentially hydrolyzed monoacylglycerol substrates with a specific activity of 290 U.mg⁻¹ at 37°C. Rv0183 hydrolyzes both long chain di- and triacylglycerols, as determined using the monomolecular film technique, although the turnover was lower than with monoacylglycerol. The enzyme shows an optimum activity at pH values ranging from 7.5 to 9.0 using monoolein as substrate and is inactivated by serine esterase inhibitors such as E600, PMSF and tetrahydrolipstatin. The catalytic triad is composed of Ser 110, Asp 226 and His 256, as confirmed by the results of site directed mutagenesis. Rv0183 shows 35% sequence identity with the human and mouse monoglyceride lipases and well below 15% with the other bacterial lipases characterized so far. Homologs of Rv0183 can be identified in other mycobacterial genomes such as *M. bovis*, *M. smegmatis*, and even *M. leprae*, which is known to contain a low number of genes involved in the replication process within the host cells. The results of immunolocalization studies performed with polyclonal antibodies raised against the purified recombinant Rv0183 suggested that the enzyme was present only in the cell wall and the culture medium of *M. tuberculosis*. Our results identify Rv0183 as the first exported lipolytic enzyme to be characterized in *M. tuberculosis* and suggest that Rv0183 may be involved in the degradation of the host cell lipids.

Key words: lipase, Rv0183, expression, monoglyceride, mycobacterium, exported, immunolocalization.

Introduction

Although much research has been focused on Tuberculosis during the last century, it is still the most deadly infectious disease worldwide. It has been estimated that one third of the World's population has been contaminated by, *Mycobacterium tuberculosis* (*M. tuberculosis*), the etiologic agent responsible for this disease. Despite the existence of effective drug treatments, 2.5 to 3 million people die of this disease every year (<http://www.who.int/mediacentre/factsheets/fs104/en/print.html>). The rise of Tuberculosis is directly linked to the spread of AIDS and the development of new multi drug resistance (MDR) strains. *M. tuberculosis* is a highly complex microorganism, and a better understanding of the biochemistry and the physiological role of the enzymes involved in the various metabolic pathways taken by the infectious process could lead to designing new therapeutic agents to fight the bacillus.

Among the various metabolic pathways involved, lipid metabolism is thought to be one of the main pathways taken by mycobacteria, and this pathway therefore needs to be studied in detail. Studies on the lipids present in mycobacteria have been going on for the last 40 years, and considerable attention has focused on the lipids involved in the cell wall. Many unusual lipids, including phthiocerol dimycocerosates, mycolic acid, glycolipids, polyketides, and glycans, have been found to be involved in the virulence and pathogenicity of *M. tuberculosis* [1-4].

An intracellular lipid storage process was recently described in *M. tuberculosis*, forming organites known as lipid bodies. These organites are composed of triacylglycerols (TAG) and other lipids, such as wax esters, polyhydroxyalkanoic acids (PHA) and monomeromycolyldiacylglycerol [4-9]. It has been proposed that these lipids probably provide a carbon source promoting the growth of the bacteria during the chronic infection phase [7, 10]. It might therefore store the energy required during long periods of bacterial,

dormancy [11] and serve as storage form of fatty acids (FA) required for membrane lipid formation [4]. The storage of these lipids *in vivo* may therefore be crucial to the survival of the bacteria and allow them to adapt to the environment provided by the host cells.

It has been suggested by *in vitro* studies that the accumulation or the presence of FA in the culture medium may stimulate the dormancy phase of the bacteria: the growth rate of most mycobacteria can be enhanced by adding oleic or palmitic acid to the culture medium [12-14]. FA can be generated *in vivo* by the hydrolysis of host cell lipids [7, 10]. During the formation of granulomas, mycobacteria are mixed with phospholipids originating from dead cells (host cells and bacteria), which may be hydrolyzed by phospholipase A and C (PLA and PLC, respectively), leading to the release of lysophospholipids, fatty acids and diacylglycerols (DAG) [15]. The resulting DAG may then be hydrolyzed by a secreted or exported lipase and the microorganism will absorb FA released. A synergy of this kind between lipases and phospholipases has been previously described in the case of another pathogen, *Pseudomonas aeruginosa*, which degrades lung surfactants (phospholipids) via the joint action of a PLC and a lipase [16]. Excreted PLA and PLC have been previously described in *M. tuberculosis* [17, 18], but no extracellular lipase has been identified so far in this strain. Two intracellular lipolytic enzymes which may participate in the degradation of intracellular TAG have been identified and purified so far in mycobacteria. The first of these is a native enzyme which was purified from *M. phlei* [19] and characterized. The second lipolytic enzyme was the expression product of the *Rv3097c* gene from the strain *M. tuberculosis* H37Rv in *Escherichia coli* [20].

Using the proteins deduced from the complete genome sequence of *M. tuberculosis* [21], it has now become possible to identify proteins which might be involved in the lipid metabolism by comparing their amino acid sequences with those of other known lipases. Here we report on the cloning and biochemical characterization of a lipase exported by *M. tuberculosis*

H37Rv (accession number Rv0183). This enzyme shows 34% to 36% amino acid sequence identity with monoglyceride lipases from human and mouse adipocytes, respectively [22, 23]. Contrary to what was suggested by genome annotation, which identified Rv0183 as a lysophospholipase, this enzyme does not hydrolyze lysophospholipids but hydrolyzes neutral lipids, showing a clear-cut preference for monoacylglycerols (MAG). Polyclonal antibodies specific to Rv0183 were prepared and used to determine the location of the enzyme in *M. tuberculosis*. Based on all our biochemical data, it seems possible that Rv0183 may contribute to the growth of *M. tuberculosis* bacilli and be involved in the hydrolysis of host cell lipids during the infection process.

Materials and methods

Materials

Pfx DNA polymerase, pDonR221, and pDest14 plasmids were purchased from Invitrogen. Rosetta pLysS *E. coli* cells were purchased from Novagen. Ni²⁺-Agarose gel was obtained from Amersham Biosciences. All vinyl esters, triacylglycerols, cholesterol oleate, phenylmethylsulfonyl fluoride (PMSF), sodium taurodeoxycholate (NaTDC), sodium deoxycholate (DOC), gum Arabic (GA), Triton X-100, diethyl p-nitrophenyl phosphate (E600) and goat anti rabbit immunoglobulins conjugated to peroxidase were obtained from Sigma-Aldrich. Tripropionin was purchased from Acros Organics, and pure mono- and diolein were purified from a commercial low grade DL- α -monoolein from Fluka. Tetrahydrolipstatin (THL) was a gift from Hoffmann la Roche. isopropyl β -D-thiogalactoside (IPTG) was from Sigma. Phthiocerol dimycocerosates, glycerol monomycolates and phosphatidyl-inositol dimannoside were provided by Dr. Martine Gilleron from IPBS-UMRS 5089 (Toulouse, France).

Methods

Cloning:

The DNA fragment of the *Rv0183* open reading frame (ORF) was amplified by PCR from the bacmid BAC-Rv165 provided by the Pasteur Institute [21, 24]. The full *Rv0183* sequence proposed by Camus *et al.* [25] was analyzed using the software program the SignalP 3.0 Server, which identified a putative signal sequence. A truncated form of *Rv0183* without the signal sequence (starting from Thr 46) was therefore produced. The primers used, which contained the attB1, Shine Dalgarno, kozak, 6 His-Tag and a Tobacco Etch Virus (TEV) Nla (TEV) site sequences at the 5' end and the attB2 recombination site at 3' end, are presented in Table 1. The PCR product was cloned into the expression vector pDest14 in line with the

manufacturer's instructions (Gateway, Invitrogen). The DNA sequence of the *Rv0183* ORF was confirmed by performing DNA sequencing (MilleGen Biotechnologies, France). The recombinant Rv0183 (rRv0183) protein without the 6 His tail and the TEV site is composed of 279 residues and has a calculated molecular mass of 30187 Da, a theoretical isoelectric point of 5.8 and a theoretical extinction molecular coefficient of $0.858 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm.

Mutagenesis:

Three Rv0183 mutants in which the putative catalytic residues Ser 110, Asp 226 and His 256 were mutated into Ala were obtained using the Quickchange[®] site-directed mutagenesis system (Stratagene, La Jolla, CA, USA). A fourth mutant built in with Asp 226 was replaced by Asn. The oligonucleotides used for PCR mutagenesis are specified in Table 1.

Expression and purification of recombinant and Rv0183 mutants:

E. coli Rosetta pLysS cells were transformed with the pDest14-His-Rv0183 plasmid and grown overnight in LB medium with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37 °C. On the following day, the culture medium was diluted 20 times with Terrific Broth (TB) medium containing antibiotics and grown at 37 °C. Protein expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) when the OD₆₀₀ had reached the 0.6-0.8 range. Temperature was then decreased to 25 °C and cells were grown overnight. After 16 hours, cells were harvested at 4 °C by centrifugation at 6,500 g for 20 min. The pellet was resuspended in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.25 mg/mL lysozyme; 30 mL per initial culture liter) and stored overnight at -80 °C. The cell suspension was thawed on ice for 1 hour and 10 µg/mL DNase I and 20 mM MgSO₄ (final concentration) were then added. The mixture was incubated for 30 min. The supernatant containing the recombinant protein was separated from the cell debris by performing a centrifugation step at 17,000 g for 30 min. This supernatant was loaded (3 mL/min) using a FPLC chromatography system (Amersham Biosciences) onto a Ni²⁺-

Agarose column (1 mL resin per 5 mg of recombinant protein) previously equilibrated with buffer A (10 mM Tris-HCl pH 8.5, 150 mM NaCl) containing 10 mM imidazole. The column was then washed using 5 column volumes of 5% and 10% of buffer B (buffer A+500 mM imidazole). rRv0183 was eluted with 100% buffer B. After analysis by SDS-PAGE [26], the eluted fractions containing the enzyme were pooled at a concentration of around 4 mg/mL and loaded onto a size exclusion chromatography (Superdex 200) in 10 mM Tris-HCl pH 8.5 buffer containing 150 mM NaCl. The 6 His-tag in the N-terminal position of rRv0183 was removed by TEV digestion and purified by exclusion from a Ni²⁺-Agarose column. Proteolytic digestion of rRv0183 by TEV was performed overnight at room temperature using a TEV/rRv0183 ratio of 1/10. During the purification process, the amounts of rRv0183 were monitored by measuring the enzyme activity. After the purification process, the protein concentration was calculated from the OD value measured at 280 nm using the extinction molar coefficient (0.858 M⁻¹.cm⁻¹). The enzyme was then concentrated to 12 mg/mL and stored at -80 °C.

pH-stat kinetic assays:

Enzymatic hydrolysis of solutions and emulsions of various esters was monitored potentiometrically for at least 5 min at various temperatures using a pH-stat (TTT 80, Radiometer, Copenhagen, Denmark). Assays were performed in 15 mL of 2.5 mM of Tris-HCl buffer at pH 7.5, containing 150 mM NaCl and 3 mM NaTDC. Fatty acid release was automatically titrated with 0.1 N NaOH. The specific activity of the Rv0183 enzyme was expressed in units per milligram of protein (U.mg⁻¹), where one unit corresponds to the release of one micromole of fatty acid per minute.

Analysis of lipolysis products by thin layer chromatography (TLC):

In order to check whether rRv0183 was able to hydrolyze α -2-monoacylglycerol (2-MAG), this compound was generated from triolein using Human Pancreatic Lipase (HPL). HPL is

known to specifically hydrolyze the *sn*-1 and *sn*-3 ester bonds of triacylglycerol and to specifically release 2-MAG.

Assays were performed at 37 °C using HPL (140 µg) and colipase (molar ratio 1:2) in 15 mL of 1 mM Tris-HCl buffer, pH 7.5 containing 2 mM CaCl₂, 3 mM NaTDC, 150 mM NaCl, and triolein (460 µmol) emulsified with gum arabic (GA) (3.1%, w/v). Five hundred microliters of the reaction mixture were collected at various times: before adding HPL (T0), after 1 hour of incubation with HPL alone and 10, 20 and 120 min after adding rRv0183 (780 µg). The reaction products were then extracted with 5 mL of a chloroform/methanol (2:1, v/v) mixture. The organic phase was separated from the water phase and dried over magnesium sulphate, and the neutral lipids were separated by performing TLC using silica gel plates from Merck and n-heptane/diethyl ether/acetic acid (55:45:1, v/v/v) as the eluent. After spraying the plates with a cupric acetate-phosphoric acid solution, the neutral lipid bands were revealed by heating the plates at 180 °C for 10-15 min.

Monolayer assays:

Lipase activities were determined using a zero order trough, as described by Verger and de Haas [27] using 1,2 dicaprin (DiC10), monoolein, diolein, purified soybean oil and phospholipids (DLPE, DLPG, DLPC and DPPC) as substrates. The aqueous subphase was composed of 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 21 mM CaCl₂ and 1 mM EDTA. In addition, 13 mM β-cyclodextrin (β-CD) were added when long chain glycerides were used as substrates. The long chain FA released upon substrate hydrolysis were solubilised by β-CD to prevent any interference with the surface pressure. The reactions were performed at 25 °C. The subphase in the reaction compartment was continuously agitated with a 2.0 cm magnetic stirrer set at 250 rpm. The enzyme solution (final concentration 5 nM) was injected through the film over the stirrer. The reaction compartment had a surface area of 100 cm² and a volume of 130 mL. The reservoir compartment was 21.9 cm long and 14.8 cm wide.

Effects of pH, temperature and NaTDC on enzyme stability and activity:

The pH stability profile of rRv0183 was determined after incubating the enzyme for 90 min in 1 mL of buffers containing 100 mM NaCl set at various pH values: 100 mM sodium acetate at pH 4.0 and 5.0; 100 mM MES at pH 6.0 and 6.5; 100 mM Hepes at pH 7.0 and 7.5; 100 mM Tris-HCl at pH 8.0 and 100 mM glycine at pH 9.0. The residual activity was determined potentiometrically at 37 °C at pH 7.5 using a monoolein substrate (see pH-stat assays) in the presence of 3 mM NaTDC (final concentration). To draw up the pH and temperature dependence profile, buffer and substrate were pre-equilibrated at selected pH levels. The NaTDC dependence was assessed potentiometrically at 37 °C in 2.5 mM Tris-HCl buffer pH 7.5, 150 mM NaCl containing NaTDC at concentrations ranging from 0 to 6 mM.

Inhibition assay:

Purified rRv0183 (0.7 nmole) in 5 mM Hepes buffer (pH 7.5) containing 500 mM NaCl was pre-incubated at 25 °C with several inhibitors (THL, E600 and PMSF) at a lipase-inhibitor molar ratio of 1/200 in all cases. Each inhibitor was previously solubilized in anhydrous ethanol (10 mM of a stock solution). The reaction was performed in a final volume of 70 μ L in the absence or presence of 3 mM NaTDC. The remaining activity was measured potentiometrically as a function of time using a monoolein substrate.

Titration of the sulfhydryl group:

Reaction mixtures were prepared as follows in the presence and absence of 2% SDS: 190 μ L of protein solution (0.4 mg/mL) were mixed with 810 μ L of 5 mM Hepes pH 7.5, 150 mM NaCl containing 0.35 mM 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) at 25 °C. A 5 mM stock solution of DTNB was initially prepared in methanol. The reactions were carried out in 1 cm pathlength cells with stirring. The changes in absorbance occurring at 412 nm, corresponding to the formation of the TNB product ($\epsilon_{412} = 13.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [28]), were monitored on a Kontron spectrophotometer (model SFM 25).

Production and purification of anti-rRv0183 polyclonal antibodies:

Antiserum against rRv0183 was prepared in rabbits (Bourgogne Fawn) using 4 mg of purified antigen. Rabbits were injected subcutaneously every 2 weeks with 0.5 mg antigen emulsified with complete Freund's adjuvant. Rabbits were bled by ear vein puncture before the beginning of the immunization procedure (pre-immune sera), then 1 week after the second booster injection and weekly injections were administered thereafter. The antibody response was assessed by performing a direct binding ELISA using rRv0183 antigen. This immunization procedure yielded an anti-Rv0183 serum with a titer of 1/40,000. Polyclonal antibodies were purified from the serum by performing affinity chromatography using a column of immobilized rRv0183 prepared as follows: Eleven milligrams of immobilized rRv0183 were coupled to 3 mL of AffiGel-10 (Bio-Rad) with a high efficiency of up to 90% and packed into an IBF column (diameter 1 cm). Serum was loaded onto the AffiGel-10-rRv0183 column and incubated overnight at 4 °C. After washing the column with 10 mM Tris-HCl pH 7.0 and eluting pure immunoglobulins at an acidic pH level (2.4), the solution was immediately neutralized to pH 7.0 with a 2 M Tris-HCl pH 9.0. Purified antibodies (20 mg) were concentrated at 2 mg/mL and stored at -20 °C.

Western blotting analysis of Rv0183 in cellular fractions from *M. tuberculosis*:

Samples of *M. tuberculosis* were kindly provided by the "TB Vaccine Testing and Research Materials" Contract funded by the NIH and NIAID (National Institutes of Health). Proteins were separated on 12% SDS-PAGE before being transferred onto a nitrocellulose membrane [29]. The purified polyclonal antibodies directed against rRv0183 (final dilution 1:1,000) were incubated for 1 hour with the nitrocellulose membrane previously saturated with 3% dry milk powder (Régilait®). After 3 washes, the membrane was incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit IgG (at a dilution of 1:4,000). The immunoreactive proteins were revealed after covering the membrane with enhanced

chemiluminescence (ECL) reagent (Amersham Biosciences, ref. RPN 2109), after exposing Kodak Biomax MS film to the membrane.

Results

Selection of Rv0183:

BLAST searches against all available microbial genomes showed that homologs of *Rv0183* gene (without its putative signal peptide sequence) are to be found in many mycobacterial species and other microorganisms such as *Burkholderia* and *Streptomyces* sp. Sequence identity between the mycobacteria is 100% in the case of *M. bovis*, 79% in that of *M. leprae* and 68% in that of *M. smegmatis*, corresponding to e-values ranging from 0 to 10^{-111} (Figure 1).

To our knowledge, none of the homologs identified among the microbial genomes have been purified and characterized so far. Despite their low sequence identity with lysophospholipases (14 to 18%), these proteins have been annotated as putative lysophospholipases. However, a blast search against human and mouse genomes identified two hits with e-values ranging from 10^{-35} to 10^{-37} corresponding to a sequence identity ranging from 34% to 36%. Both hits correspond to monoglyceride lipases (MGL) involved in fat mobilization processes in adipose tissues [22, 30]. In *Rv0183* and all the mycobacterial homologs, two lipase motifs including the GX SXG pentapeptide containing the catalytic Ser and a His-Gly dipeptide are conserved. In addition, the His and Met residues present in the pentapeptide containing the active serine (G-H-S-M-G) are strictly conserved in *Rv0183* and in human and mouse MGL. Secondary structure predictions suggested that *Rv0183* may belong to the α/β hydrolase fold family and that it is composed of 8 β -strands and 9 α -helices (Figure 1), but no significant hits have been obtained from the PDB data base with which to build a dependable model.

Cloning, expression and purification of rRv0183:

Under the conditions described in the Methods section, 14 to 15 mg of pure rRv0183 was purified from the 23 mg of protein produced per culture liter (giving a yield of between 60 and 65%). The migration of purified rRv0183 observed upon performing SDS-PAGE

corresponds to a molecular mass (of approximately 30 kDa) which is in agreement with the theoretical mass deduced from the DNA sequence (Figure 2, lane 2). The 6 His-Tag in the N-terminal position was removed by performing TEV cleavage with a yield of 70%. The difference between the molecular masses of rRv0183 with and without the 6 His-Tag can be seen in Figure 2. Masses determined by MALDI-TOF mass spectrometry were 31940 Da and 30223 Da, respectively. About 10 mg of purified rRv0183 without the His tail were obtained from 1 liter of culture medium. All biochemical data (see below) were obtained with both forms of the rRv0183 and it was noted that the presence of 6 His-Tag did not affect the biochemical behavior of the enzyme. Further characterization using circular dichroism and dynamic light scattering methods showed that rRv0183 is a monodisperse α/β protein in solution (data not shown).

Biochemical properties:

Substrate specificity

To investigate the potential role of Rv0183, activity measurements using various substrates were performed using the pH-stat, TLC and monomolecular film techniques (Table 2 and Figures 3 and 4).

The rRv0183 activity measured using the pH-stat technique showed that this enzyme was not able to hydrolyze emulsified triacylglycerols, whatever the chain length of the substrates tested (Table 2). Only diacylglycerols with short and medium fatty acid chains were hydrolyzed, and specific activities of 27 and 46 U.mg⁻¹ were measured with DiC6 and DiC10 emulsified with gum Arabic. No activity was recorded with the diacylglycerols having the longest chain fatty acids, even in the presence of gum Arabic and detergents (NaTDC, CHAPS or Tween). rRv0183 was found to be active, however, on synthetic monoacylglycerols with short, medium and long chain fatty acids (MC4, 1-monobutyryl rac glycerol; MC8, 1-monooctanoyl rac glycerol; MC10, 1-monodecanoyl rac glycerol; MC12, 1-

monolauroyl rac glycerol; MC14, 1-monomyristoyl rac glycerol; MC18:1, 1-monooleyl rac glycerol) (Table 2). The maximum specific activity recorded using monoolein as substrate was obtained at 37 °C and reached 290 U.mg⁻¹. 1-monopalmitoyl rac glycerol (MC16) was also tested, but under our assay conditions, MC16 was found to be insoluble due to the absence of double bonds, and no lipase activity was recorded.

Activity of rRv0183 was also checked on 2-MAG, the physiological MAG usually produced by 1, 3-regioselective lipase. Triolein was first converted into 2-MAG and free oleic acid by adding HPL prior to rRv0183 (Figure 3, lane 2). Ten minutes after rRv0183 was added to the reaction mixture, 2-MAG decreased rapidly, and it was completely hydrolyzed within 20 min (Figure 3, lane 3 and 4). These data shown that rRv0183 is able to hydrolyze both 2-MAG and 1-rac MAG. Since we also established that rRv0183 is not able to hydrolyze diolelylglycerols (Table 2), the disappearance of 1,2-dioleyl rac glycerol observed after adding rRv0183 (Figure 3, lanes 3-5) can be attributed to the HPL activity also present in the reaction mixture. Overall, the concomitant action of HPL and rR0183 results in the complete conversion of triolein into free oleic acid (Figure 3, lane 5). These data support the idea that rRv0183 is a monoglyceride lipase, and that this enzyme is responsible for completing the process of TAG lipolysis initiated by other lipases.

Using the monolayer technique, we also observed that rRv0183 shows considerable rates of activity on 1-rac monoolein and 1,2 dicaprin, as well as being able to hydrolyse diolein and purified soybean oil monomolecular films (Figure 4). The activities measured using a 1,2 dicaprin substrate were comparable to those recorded with dog gastric lipase (DGL) under similar conditions [31]. When 1-rac monoolein was used as the substrate, the lipase activity increased with the pressure up to the collapse value (30 mN.m⁻¹) without reaching a maximum. With 1,2 dicaprin, diolein and purified soybean oil substrates, the activities reached a maximum of 11, 8, 3 mmole.cm⁻².min⁻¹.M⁻¹ at 15, 20 and 7 mN.m⁻¹, respectively,

and decreased at higher surface pressure values. The activity recorded with diolein and purified soybean oil was found to be almost zero before the collapse of the monomolecular film. When 1,2-dicaprin was used as the substrate, the hydrolytic activity decreased more slowly as a function of the surface pressure without reaching zero, even near the collapse value. This result might explain why the rRv0183 activity was recorded with emulsified dicaprin using the pH-stat technique ($46 \text{ U} \cdot \text{mg}^{-1}$), whereas no activity was found to occur on diolein, triolein or purified soybean oil under these assay conditions. Although the monomolecular film technique does not make it possible to determine specific activities with each of substrates, these data clearly indicate that rRv0183 is able to hydrolyze TAG, DAG and MAG and that these compounds can therefore be accommodated in the active site of the enzyme. However, the absence of activity observed on long chain DAG and TAG emulsions under bulk conditions may possibly be due to the fact that the physical state of the substrate induces denaturation of the enzyme at the oil/water interface, or else it may show that rRv0183 has a poor affinity for oil-water interfaces.

The fact that rRv0183 showed high rates of activity on monoglycerides under bulk conditions probably results from rRv0183 having a better affinity for micellar substrates rather than emulsified substrates. This is an important finding for understanding the role of Rv0183 *in vivo*.

Since Rv0183 has been annotated in the genome as a lysophospholipase, we checked its activity using lyso-PC as substrate (data not shown). Despite many attempts to induce activity in this case, we did not observe any hydrolysis of this substrate. Actually, this result was not very surprising, since the homologous human and mouse monoglyceride lipases do not hydrolyze lysophospholipids [22, 23, 30]. In order to investigate another potential substrate, trials were performed with several phospholipids using the monomolecular film technique. rRv0183 was not found to hydrolyze cationic (dilauroylphosphatidylethanolamine, DiC₁₂PE),

anionic (dilauroylphosphatidylglycerol, DiC₁₂PG) or zwitterionic (dipalmitoylphosphatidylcholine, DPPC; dilauroylphosphatidylcholine, DiC₁₂PC) phospholipids (Table 2). We also checked the activity of rRv0183 on lipids constituting the cell wall such as phthiocerol dimycocerosates, glycerol monomycolates and phosphatidyl-inositol dimannoside. No activity was detected on these various substrates (data not shown).

Chain length dependence

rRv0183 was also found to hydrolyse synthetic vinyl esters with various acyl chain lengths. To determine the acyl chain length specificity, the specific activity of rRv0183 was measured on these vinyl esters and monoacylglycerols having 4 to 18 carbon atoms, and the highest values were those recorded with monoacylglycerol substrates having between 8 and 18 carbon atoms (data not shown). Similar specific activities were recorded with vinyl laurate (VC₁₂), vinyl butyrate (VC₄) and vinyl propionate (VC₃) (64, 45 and 57 U.mg⁻¹, respectively; Table 2). These data show that the enzyme activity does not depend significantly on the chain length, and that the active site can therefore accommodate substrates with short, medium and long acyl chains.

pH stability and pH dependent activity

The pH stability and the pH dependent activity of rRv0183 were investigated using a monoolein substrate (Figure 5A and 5B). The results obtained show that rRv0183 is stable at alkaline pH values ranging from 7.5 to 9 and that 100% residual activity occurs at pH 9 after 1 hour of incubation at room temperature (Figure 5A). At lower pH values, the residual activity dropped rapidly to 50% at pH 6.5, and it was completely abolished after 1 hour of incubation at pH levels of 6.0 and below. The curve describing the activity of rRv0183 as a function of the pH (Figure 5B) is superimposable on the pH stability curve (Figure 5A). Since the purified rRv0183 was found to be unstable at pH values below pH 6.5, it was impossible to determine the real effects of the pH on the rate of enzyme turnover at lower pH levels.

Temperature dependence

The temperature dependence profile obtained using the monoolein substrate (Figure 5C) shows that the specific activity of rRv0183 increased linearly 3-fold from 25 °C to 50 °C before decreasing slowly at higher temperatures. Although rRv0183 showed a high rate of activity at 55 °C, it cannot be classified as a thermostable enzyme, since no residual activity was detected after 1 h of incubation at 55 °C, which suggests that rRv0183 is completely denatured at this temperature. Based on these data, 37 °C was used in all the present biochemical studies. At this temperature, it was possible to test volatile vinyl substrates as well as triglycerides and the activity of the enzyme remained linear during several minutes on all the substrates tested.

NaTDC dependence

Lipase activity on MAG and DAG was enhanced by adding various detergents, such as tetradecyltrimethyl- ammonium bromide (a cationic detergent), Triton X-100 (a non-ionic detergent), CHAPS (a zwitterionic detergent), DOC (an anionic detergent) (data not shown) and NaTDC (an anionic detergent) (Figure 5D). In the absence of detergent, no activity was recorded with monoolein, DiC₆, DiC₁₀ and VC₁₂ substrates. When monoolein was used as the substrate, the lipolytic activity increased with the NaTDC concentration, peaked at 1 mM NaTDC, and then remained stable up to 5 mM NaTDC (Figure 5D).

The fact that the activity profiles recorded with other detergents under the same assay conditions were similar to those obtained with NaTDC, shows that the charge of the detergent does not affect the activity of rRv0183. These data suggest that the binding of rRv0183 to substrate micelles, which is a prerequisite for lipolytic activity to occur, is probably not mediated by electric charges but only by hydrophobic interactions. NaTDC, which is commonly used in lipase assays, was used here to measure the activity of Rv0183. The standard activity assay developed for use with rRv0183 was performed in 2.5 mM Tris-HCl,

150 mM NaCl, 3 mM NaTDC at pH 7.5 at 37 °C using 9.3 mM of monoolein (50 µl) as substrate.

Absence of the disulfide bridge

The amino acid sequence of rRv0183 possesses two cystein residues. Under native conditions, no free sulfhydryl groups were detected during the incubation of rRv0183 with the DTNB reagent. However, after adding 2% SDS to the rRv0183 protein solution in the absence of reducing agent, two moles of sulfhydryl group were titrated per mole of enzyme. These results show that two free cysteines are present but that they are not involved in a disulfide bridge and not accessible to the solvent in the native Rv0183.

Identification of the catalytic triad

The high level of sequence identity observed between Rv0183 and the human and mouse monoglyceride lipases, in which a catalytic triad has been identified [22, 30], led us to study the possible involvement of Ser 110, Asp 226 and His 256 in the catalytic triad of Rv0183 (Figure 1). The corresponding Ala mutants were expressed and purified and correct folding was checked using the circular dichroism technique. The enzymatic activity on vinyl esters and monoglycerides was found to be completely abolished when the following residues were mutated: Ser 110 into Ala, Asp 226 into Ala or Asn, and His 256 into Ala.

Effects of serine esterase inhibitors on rRv0183 activity

After preincubating pure rRv0183 with E600, PMSF or THL at a molar excess of 200 in the absence of NaTDC, the lipolytic activity observed on monoolein was inhibited (Figure 6A): after a 180-min incubation step, the inhibition rate of rRv0183 was 100% with PMSF, 80% with E600 and only 22% with THL. In the presence of 3 mM NaTDC, the inhibitory process was accelerated. The activity was completely inhibited after a 25-min incubation step with PMSF and a 103-min incubation step with E600. After a 150-min incubation step with THL, the rate of rRv0183 inhibition was 62% (Figure 6B).

These results differ from those obtained with classical lipases such as HPL, DGL and TLL, the inhibition of which requires the presence of a detergent such as NaTDC: these enzymes are known to have a lid domain covering the active site, and only in the presence of NaTDC the lid adopt an open conformation giving inhibitors access to the active site [32, 33]. The kinetic behavior of rRv0183 in the presence of an inhibitor supports the idea that the catalytic site of this enzyme is probably not covered by a lid.

Immunolocalization of rRv0183 in cell fractions:

Anti-Rv0183 polyclonal antibodies (pAbs) were used to determine the cellular and subcellular distribution of Rv0183 in *M. tuberculosis*. As shown in Figure 7, pAbs recognized the pure rRv0183 (lane 1) as well as the native Rv0183 present in the *M. tuberculosis* culture medium (lane 3) and the cell wall (lane 4). As can be seen from Figure 7, Rv0183 was not detected in the culture medium filtrate passed through a 0.22 μ m filter (lane 2), which suggests that Rv0183 bound to large particles. No trace of enzyme was observed in either the membrane or the cytosol fractions (lane 5, 6, 7). The fact that native Rv0183 was detected in the culture medium is in agreement with data obtained in studies in which the *M. tuberculosis* proteome was analyzed [34, 35].

Discussion

Understanding the molecular mechanisms involved in lipid metabolism in *M. tuberculosis* is likely to be of both fundamental and clinical importance. Although many studies have been performed on the synthesis of lipids in *M. tuberculosis* [11], little is known so far about their degradation [20]. No secreted or exported lipases have been identified up to now in *M. tuberculosis*, although lipids play an important role in this microorganism: (phospho)-lipids in the host cells may be hydrolyzed into fatty acids and glycerol and absorbed by the mycobacteria. These fatty acids are therefore used to synthesize the lipids (TAG) occurring in the *M. tuberculosis* lipidic inclusion bodies, which are thought to be required for the survival of the bacteria during the dormant and reactivation phases [7]. During these phases, intra- and extracellular lipases may play an important role in lipid metabolism processes. In the *M. tuberculosis* genome, which was completed in a recent study, 24 genes have been annotated as putative lipases (Lip C to Z except K and S) [20, 21]. However, among these 24 targets, 9 do not possess the G-X-S-X-G pentapeptide, which is the minimum moiety included in the larger lipase consensus pattern [LIV]-{KG}-[LIVFY]-[LIVMST]-G-[HYWV]-S-{YAG}-G-[GSTAC], according to the PROSITE database. Among the 15 remaining targets, only Lip Y (Rv3097c) has been found so far to be a lipolytic enzyme [20], while Lip H (Rv1399c) and Lip F (Rv3487c) were not classified as lipolytic enzymes after being biochemically characterized [36, 37]. However, more specific research on the *M. tuberculosis* genome based on comparisons with genes coding for biochemically characterized enzymes found to be lipolytic has shown that other genes may be lipases (data not shown). One of these potential lipases, the *Rv0183* gene, which codes for a putative lysophospholipase [21], was selected because of its high amino acid sequence identity (~34%) with known mammalian monoglyceride lipases. After having cloned, expressed and biochemically characterized the recombinant Rv0183, this protein was found to be a lipolytic enzyme hydrolyzing long chain

monoacylglycerols, which does not show any lysophospholipase activity. This enzyme is the first exported lipase to be identified in *M. tuberculosis*. The optimum conditions of expression obtained in Rosetta pLysS *E. coli* cells at 37 °C using Terrific Broth as growth medium yielded around 10.5 mg of purified soluble recombinant protein per liter of culture medium. The optimum assay conditions were then established using the pH-stat technique, which is a useful means of performing kinetic studies. The biochemical properties of rRv0183 are very similar to these of human and mouse MGL [22, 30, 38, 39]: amino acid sequence alignment (Figure 1) and mutagenesis experiments showed that the catalytic triad is composed of residues Ser 110, Asp 226 and His 256. The results of inhibition studies performed in the absence and presence of NaTDC support the idea that unlike many other lipases, rRv0183 does not possess a lid covering its active site. This is actually not very surprising, since many bacterial lipases have been found to be devoid of a lid domain [40].

The biochemical characteristics of rRv0183 determined using the monomolecular film technique clearly show that this enzyme is also potentially active on long chain TAG and DAG. Therefore, rRv0183 possesses an active site accommodating various glycerides. The absence of rRv0183 activity on TAG and DAG in the pH-stat assay probably resulted from the occurrence of poor interactions between the enzyme and the lipid-water interface, whereas the monomolecular film technique made it possible to screen a larger range of interfacial conditions.

The high levels of expression of rRv0183 obtained here will hopefully lead to further crystallization studies. Information about the rRv0183 3-D structure will help to explain the substrate specificity of the enzyme, which it is difficult to interpret both at the molecular level and in terms of the putative physiological role of Rv0183.

The cellular location of Rv0183 means that native enzyme can be exported through the cell wall and may play a role both in the cell wall and in the culture medium: Rv0183 may act

outside the bacilli, and be directly involved in the host lipid degradation process initiated by another partner. Many studies have shown that protein fractions originating from the culture medium are potential candidates for the production of vaccines, and Rv0183 might therefore be one of them. Since no Rv0183 can be detected inside the cytoplasm, Rv0183 obviously does not play the same physiological role *in vivo* as the first intracellular lipase identified [20]. Several hypotheses can be put forward, as follows: i) Rv0183 may be able to hydrolyze extracellular TAG, DAG and MAG with different rates, leading to the uptake of free fatty acids and their further storage in lipid inclusion bodies when *M. tuberculosis* infects adipocytes [10], ii) Rv0183 may hydrolyze diglycerides released by PLC and then the resulting monoglycerides, thus triggering the release of glycerol and absorbable fatty acids, iii) Rv0183 may mainly hydrolyse the monoglycerides resulting from the action of another, as yet unknown exported lipase. The latter scheme is known to occur in human and mouse adipocytes, where the homologous MGL catalyzes the hydrolysis of monoglycerides after the action of adipose triglyceride lipase (ATGL) and hormono-sensitive lipase (HSL) on the TAG and DAG, respectively [41, 42].

The absence of Rv0183 in the culture medium passed through a 0.22 μ m filter suggests that Rv0183 may aggregate in large vesicles after being exported, or that Rv0183 may be exported from the membrane by these extracellular vesicles. Vesicular trafficking from phagosomes containing bacteria has been found to occur in previous studies [43, 44]. Mycobacterial lipids were found to be present in extracellular vesicles isolated from the culture medium. Via the release of these vesicles, the effects of the bacteria may be exerted on more distal cells or tissues rather than being restricted to the nearest cells. In this context, the possibility can not be ruled out that Rv0183 may be involved in trafficking processes of this kind during the infection stage. Although Rv0183 was detected in the culture medium, the processes underlying its up-regulation are not yet known. Its physiological role will now have to be

investigated using specific antibodies. This powerful tool combined with electronic microscopy will make it possible to detect the presence of Rv0183 during the process of phagocytosis of mycobacteria by macrophages.

Although Rv0183 is not thought to be one of the main targets involved in the infection process [45, 46], this does not rule out the possibility that this enzyme may contribute to the reactivation of the bacteria and help the mycobacteria to survive in the mature granulomas during the dormant phase. This hypothesis is in agreement with previous studies showing that bacteria grown *in vitro* had a preference for carbohydrates, whereas bacteria grown *in vivo* preferred fatty acids as their carbon source [47, 48].

Likewise, the presence of a homologous enzyme in *M. leprae* (ML2603), which is known to have only the enzymes necessary for its replication in the host cells, suggests that this enzyme may play an important role in the lifecycle of the microorganism. Studies on the behavior of Rv0183 might also provide indirect information about the lipid metabolism in *M. leprae*.

In conclusion, the results obtained in the present study show the existence of an exported lipolytic enzyme, which preferentially hydrolyses monoacylglycerols and is closely related to human and mouse monoglyceride lipases. Based on the present biochemical findings, this exported enzyme may be involved in the hydrolysis of host cell lipids during the infection process, but its exact physiological contribution to the process of lipid metabolism still remains to be elucidated.

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Table 1: Oligonucleotides used in this study

	Forward	Reverse
Cloning		
	5' tcgaaggagatagaaccat <u>g</u> catcaccatcaccatcacgaaa acctgtacttcagggtactaccacccggactgaacgg 3'	5' cctacaaccgctcggtgagccag 3'
Mutagenesis		
Ser 110 Ala	5' gtgctcgggacg <u>cc</u> atgggcggcggc 3'	5' gccgcgcgccatggcgtgcccgagcac 3'
Asp 226 Ala	5' cacggcaccgat <u>ccc</u> ggctgatcccc 3'	5' ggggatcagccgggcacggtgccgtg 3'
Asp 226 Asn	5' cacggcaccgata <u>acc</u> ggctgatcccc 3'	5' ggggatcagccggttatcggtgccgtg 3'
His 256 Ala	5' cccgggctgtac <u>cc</u> gaggtgttcaac 3'	5' gttgaacacctcggcgtacagcccggg 3'

The underlined bases indicate the site of the mutation.

Table 2: Specific activities in the hydrolysis of lipids

Experiments were performed with tri, di and monoacylglycerides at 37 °C in 2.5 mM Tris-HCl pH 7.5 buffer, 150 mM NaCl under stirring. Assays with vinyl esters were performed at 25 °C in the same buffer. The procedure used in the experiments on cholesterol oleate was strictly the same as that described by Ben Ali *et al.* [49]. Activities on phospholipids were determined on monomolecular film at 25 °C (see Materials and Methods section).

Substrates	Specific activity (U.mg ⁻¹)
Triacylglycerides (at 37 °C)	
Triacetin (TC2)	0
Tripalmitin (TC3)	0
Tributyrin (TC4)	0
Triolein (TC8)*	0
Triolein (TC18)*	0
Diacylglycerides (at 37 °C)	
DiC ₆ *	27± SD
DiC ₁₀ *	46± SD
DiC ₁₄ *	0
Diolein (DiC18)*	0
Monoacylglycerides (at 37 °C)	
MC4	31± SD
MC8	307± SD
MC10	334± SD
MC12*	328± SD
MC14*	95± SD
Monoolein (MC18)*	290± SD
Vinyl esters (at 25 °C)	
Vinyl acetate	0
Vinyl propionate	57± SD
Vinyl butyrate	45± SD
Vinyl laurate*	64± SD
Cholesterol oleate	0
Phospholipids (at 25 °C)	
DLPE	0
DLPG	0
DLPC	0
DPPC	0

1 unit corresponds to 1 μmole of fatty acid released per min. Values are means ± SD (*n* = 4).

*(GA (3%)±3mM NaTDC).

Figure Legends:

Figure 1: Amino-acid sequence alignment between Rv0183, without its putative signal peptide identified from the bioinformatic data, and 3 homologous proteins from *Mycobacterium: bovis* (Mb0189, 100%), *leprae* (ML2603, 79%) and *smegmatis* (68%) and 2 human (34%) and mouse (36%) monoglyceride lipases. This figure was generated by the ESPrit program (available from the expasy web site) using the alignment function performed by Clustal W (from EBI web site) and the secondary element structure obtained from the PredictProtein website [50]. Conserved residues are shown with a black background. Residues potentially involved in the catalytic triad are indicated by a heavy triangle (▲). The two lipase motifs are indicated by heavy circles (●). The secondary structure elements (β -strands and α -helices) are indicated at the top.

Figure 2: SDS-PAGE for purification and TEV cleavage of rRv0183. Protein samples were loaded onto a 12% SDS-PAGE under reducing conditions. The gel was stained with Coomassie brilliant blue R250. *Lane 1* Molecular weight markers from Amersham Biosciences; *lane 2*, purified rRv0183 (10 μ g); *lane 3*, purified rRv0183 after TEV cleavage and after exclusion from Ni^{2+} -NTA agarose (10 μ g).

Figure 3: TLC analysis of triolein degradation by HPL and rRv0183. Reaction was performed on triolein emulsion with HPL (140 μ g) for 1 h and rRv0183 (780 μ g) was then added to the mixture containing the HPL degradation products. Lipids were extracted with a chloroform/methanol solution and separated on silica gel by TLC (see Materials and Methods section). *Lane 1*, Reaction mixture before incubation with enzymes; *lane 2*, products released after 1 h of incubation with HPL *lanes 3, 4 and 5*: products released 10, 20 and 120 min after adding rRv0183, respectively; *lane 6*, lipid markers.

Figure 4: Lipolytic activity of rRv0183 on 1,2 dicaprin, 1 rac monoolein, diolein and purified soybean oil monolayers as a function of the surface pressure. Lipolytic activity was determined after injecting 15 μ g of rRv0183 into the reaction compartment. Collapse point of each substrate was reached at 41, 32, 28 and 13.8 mN.m^{-1} in the case of 1,2 dicaprin, monoolein, diolein and purified soybean oil, respectively. Each experiment was performed in triplicate (error \pm 5%).

Figure 5: Kinetic assays on rRv0183 using a monoolein substrate. (A) pH stability, (B) pH effects on rRv0183 activity, (C) Temperature dependence and (D) NaTDC dependence (for details of the experimental procedure, see Materials and Methods section).

Figure 6: Inhibitory effects of E600 (\circ), THL (\diamond) and PMSF (\square) on rRv0183 activity as a function of time. (A) Without NaTDC and (B) With 3 mM NaTDC. The protein/inhibitor ratio was 1/200 in all experiments. Enzyme activity was determined as described in the Methods section.

Figure 7: Location of Rv0183 in *M. tuberculosis* cellular compartments. Aliquots (15 μ g of proteins per well) of *M. tuberculosis* cellular compartments were subjected to immunoblotting analysis using purified polyclonal antibodies directed against rRv0183. *Lane 1*, rRv0183 purified protein (400 ng); *lane 2*, protein from culture medium obtained using a 0.22 μ m filter; *lane 3*, protein from culture medium; *lane 4*, cell wall; *lane 5*, membrane; *lane 6*, Triton X-114 extracted proteins; *lane 7*, cytosol. *M. tuberculosis* cellular samples were kindly provided by the TB Vaccine Testing and Research Materials funded by NIH and NIAD (Colorado State University).

Figure 1

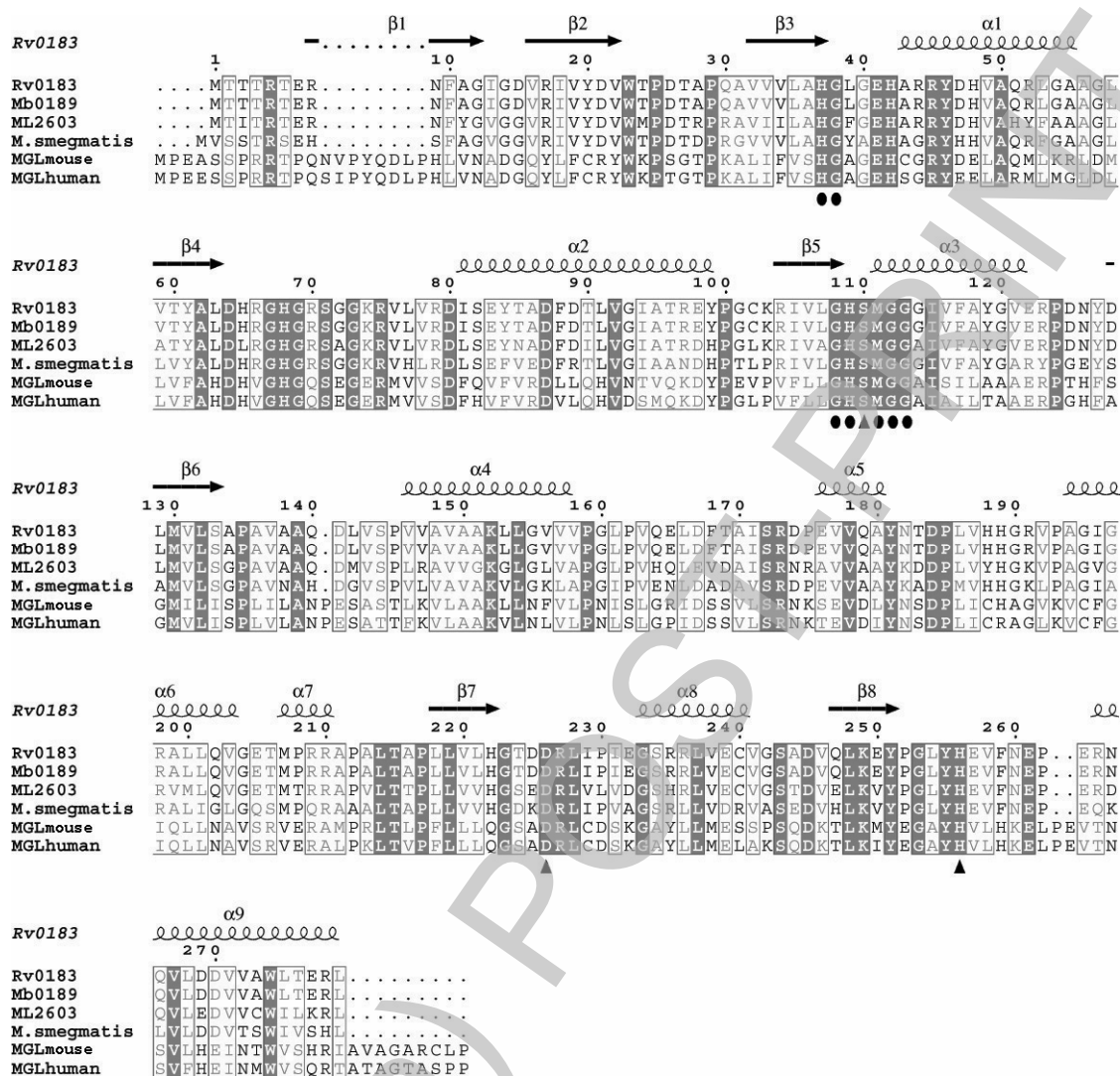


Figure 2

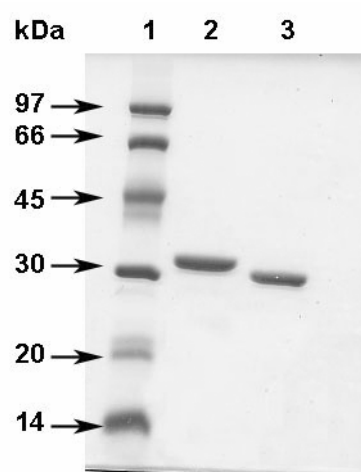


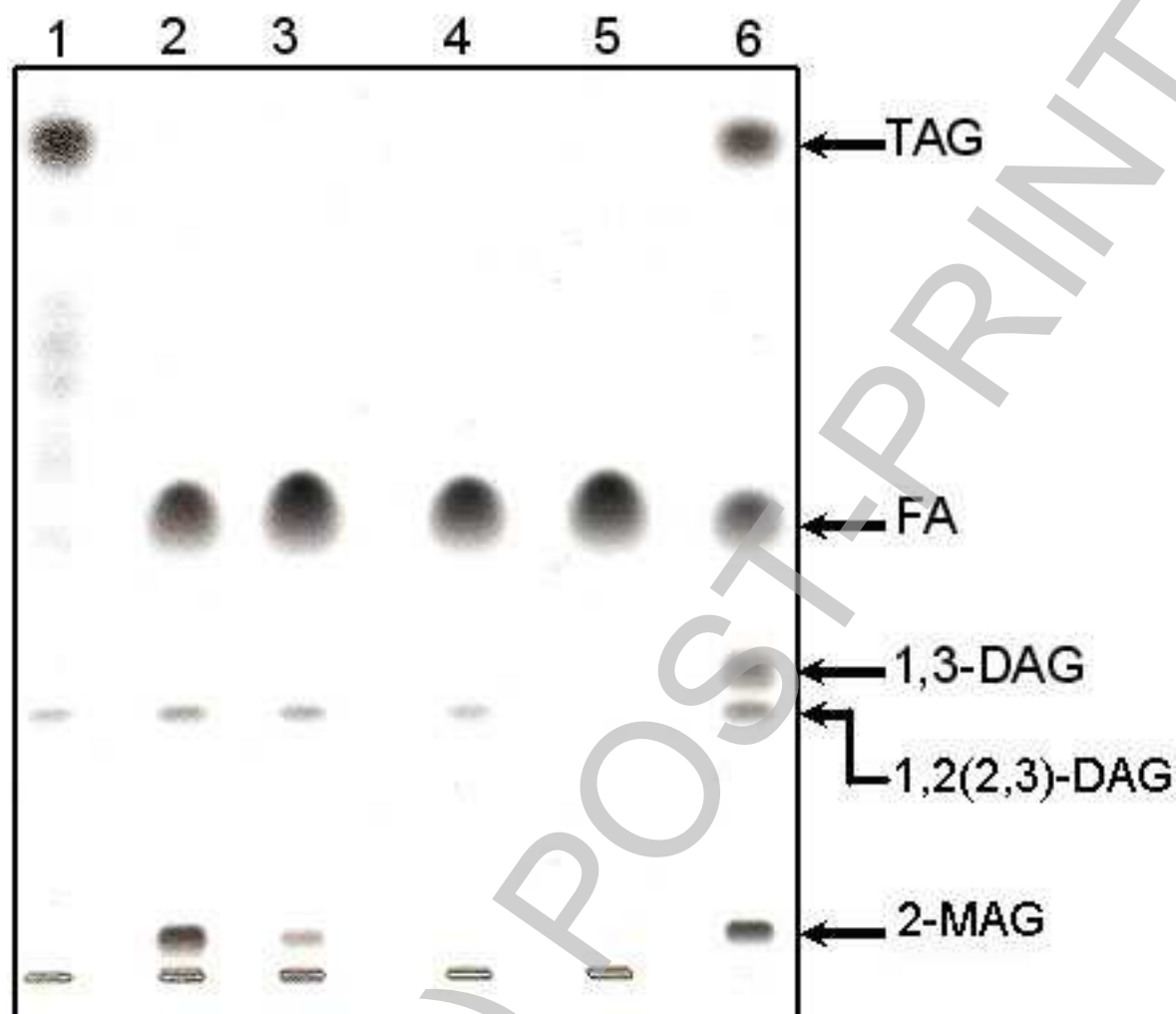
Figure 3

Figure 4

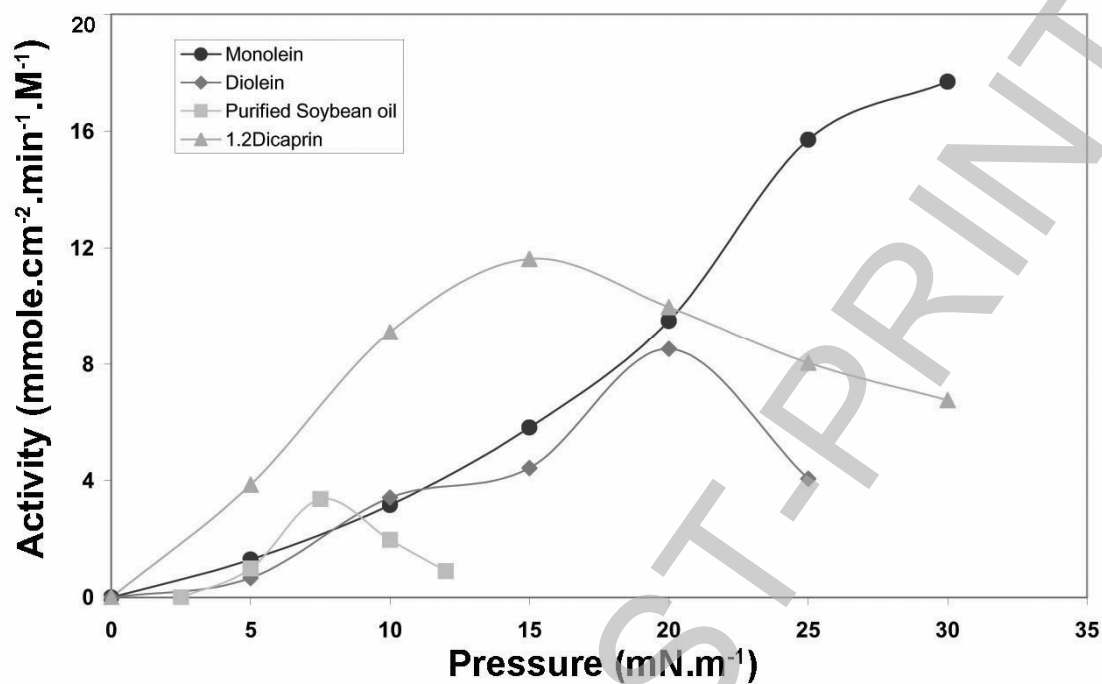


Figure 5

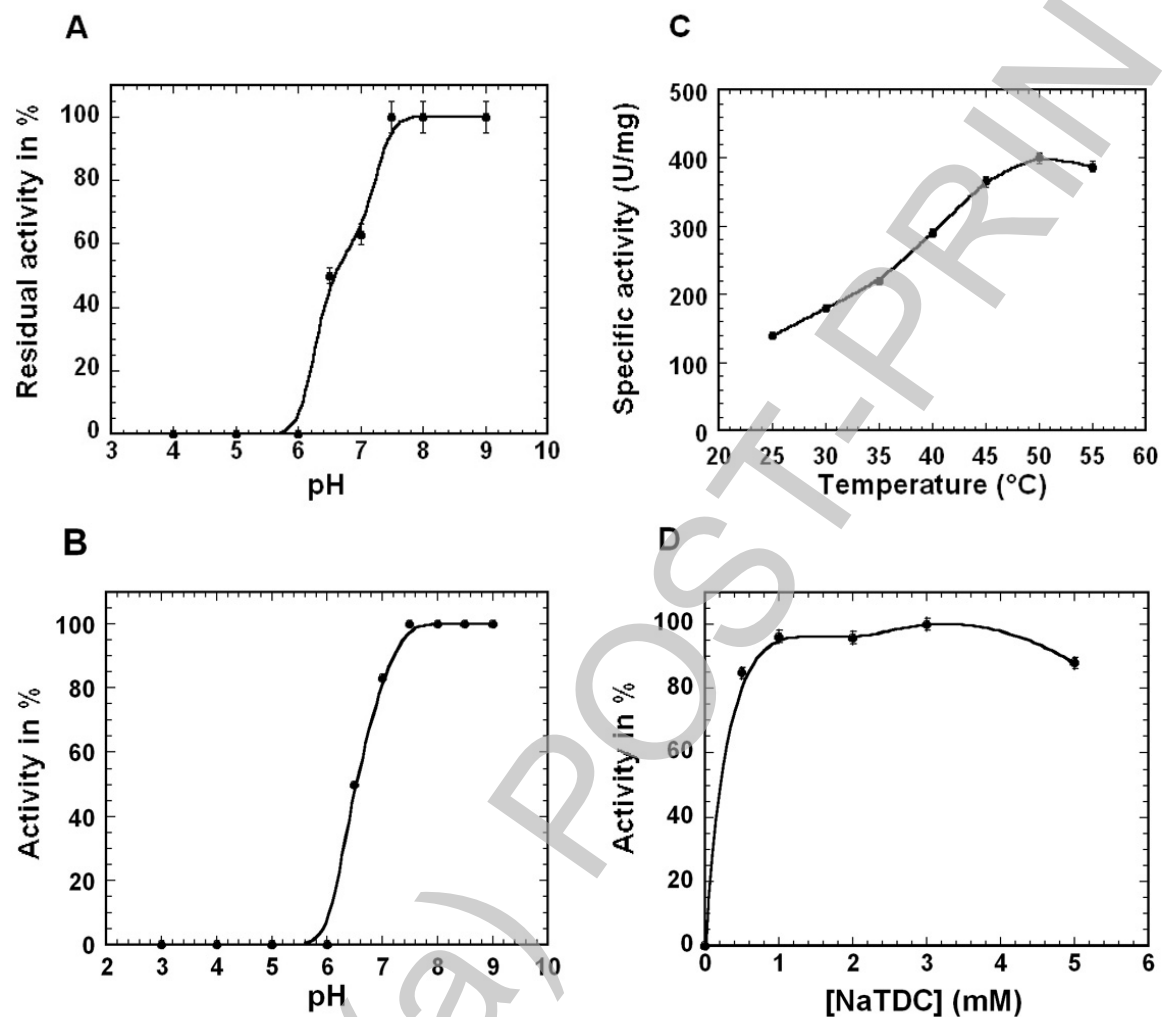


Figure 6

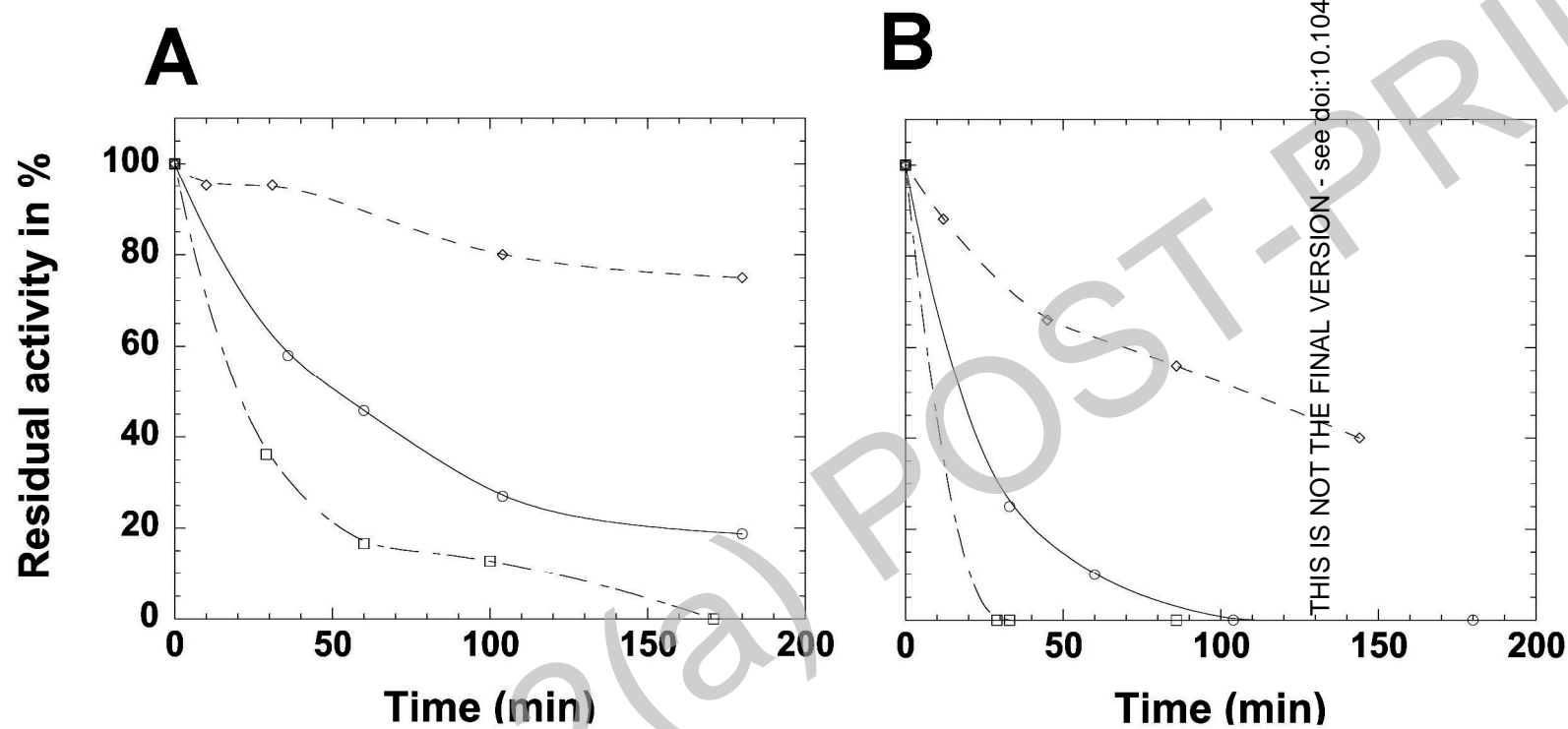


Figure 7