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Purification and Biochemical Characterization of Methionine Aminopeptidase (MetAP) from *Mycobacterium smegmatis* mc^{2}155

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Abstract The methionine aminopeptidase (MetAP) catalyzes the removal of amino terminal methionine from newly synthesized polypeptide. MetAP from *Mycobacterium smegmatis* mc^2 155 was purified from the culture lysate in four sequential steps to obtain a final purification fold of 22. The purified enzyme exhibited a molecular weight of ≈ 37 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Activity staining was performed to detect the methionine aminopeptidase activity on native polyacrylamide gel. The enzyme was characterized biochemically, using L-methionine *p*-nitroanilide as substrate. The enzyme was found to have a temperature and pH optimum of 50 °C and 8.5, respectively, and was found to be stable at 50 °C with half-life more than 8 h. The enzyme activity was enhanced by Mg^{2+} and Co^{2+} and was inhibited by Fe^{2+} and Cu^{2+}. The enzyme activity inhibited by EDTA is restored in presence of Mg^{2+} suggesting the possible role of Mg^{2+} as metal cofactor of the enzyme in vitro.

Keywords *Mycobacterium smegmatis* mc^2 155 · Methionine aminopeptidases · L-methionine *p*-nitroanilide · Peptide processing · Metalloenzyme

Introduction

Mycobacterium tuberculosis causes the highest annual global mortality of all known pathogens. Only a few effective drugs are available to treat tuberculosis (TB) infection and rise in drug-resistant strains could give rise to a potentially untreatable form of the disease. Therefore, new drug targets and inhibitors for TB treatment are needed [1]. Among these potential targets, the enzymes responsible for integrating the amino acid methionine into protein biosynthesis, along with enzymes involved in its subsequent posttranslational modification and repair, have emerged as promising candidates for the development of novel antibiotics. Inhibition of these enzymes may have great potential in the treatment of

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such pathologies as cancer and atherosclerosis in humans [2]. Methionine aminopeptidase (MetAP) is one such enzyme, which is primarily involved in the N-terminal methionine excision (NME) from the polypeptides during protein synthesis in both eukaryotes and prokaryotes. Besides their involvement in NME, methionine aminopeptidases are involved in general metabolism of amino acids and proteins, activation and inactivation of biologically active peptides, and in protein degradation and antigen processing to be presented to the major histocompatibility system [3]. The physiological importance of MetAP is apparent in the lethality of its absence in *Saccharomyces cerevisiae*, *Escherichia coli*, *Salmonella Typhimurium*, and *Mycobacterium tuberculosis* [4–7]. MetAP has also been shown to be the probable molecular target of the antiangiogenesis agent fumagillin, a natural product isolated from *Aspergillus fumigatus fresenius*, some of its derivatives, bestatin, amastatin, etc. [8]. The MetAP B from *M. tuberculosis* has less than 48% sequence identity with their human counterpart, HsMetAP1, suggesting that it may be possible to find inhibitors that are selective against this bacterial enzyme [1].

Although *M. smegmatis* and *M. tuberculosis* are relatively distinct species, *M. smegmatis* is often used as a model organism to study *M. tuberculosis* mainly because of its nonpathogenic and fast growing nature [9]. The methionine aminopeptidases from *M. smegmatis* is involved in processing of many of the *M. tuberculosis* proteins heterologously overexpressed in *M. smegmatis*. It is thus important to study the methionine aminopeptidases from *M. smegmatis* in the context of 'Methionine retention' or 'Methionine problem', which happens with many overexpressed proteins in *E. coli* [10]. There have been many reports in recent times on discovery and designing of inhibitors of bacterial methionine aminopeptidase [11, 12]. The characterization of methionine aminopeptidase from *M. smegmatis* can provide many more inputs into screening and designing of inhibitor against *M. tuberculosis* MetAP for treating tuberculosis.

Materials and Methods

Materials

L-methionine *p*-nitroanilide, bestatin, actinonin, amastatin, dialysis tublings, Sephadex G-100 matrix, Q-sepharose, and 1-Napthylamine were obtained from Sigma (USA). Sauton's media, phenylmethanesulfonyl fluoride (PMSF), lysozyme and all other metal ions were procured from Himedia (Mumbai, India). Protein assay kit, molecular weight markers, and chemicals for SDS-PAGE were obtained from Genei (Bangalore, India).

Microorganism and Media

Mycobacterium smegmatis mc^2 155 cells were maintained on Petri plates containing Sauton's agar medium supplemented with 2% glycerol. For all experiments, the culture was grown in Luria Bertani (LB) broth at 37 °C with agitation (180 rpm). Unless otherwise mentioned, the culture was allowed to grow for 48 h until the mid-log phase (OD_{600}=1.0) [13].

Enzyme Preparation and Purification

For enzyme preparation, 200 ml sterile LB broth was inoculated with 2 ml of overnight grown mid-log phase *M. smegmatis* $mc^2$155 cells and was allowed to grow until OD_{600}

reached 1.0. The cells were then harvested by centrifugation at 5,009×*g* at 4 °C. The cell pellet was washed with cold 20 mmol/l phosphate buffer pH 7.4 and stored at −70 °C until it is used. The Mycobacterium cells were suspended in lysis buffer comprising of 20 mmol/l-phosphate buffer and 500 mmol/l NaCl. 0.1 mg/ml lysozyme was added separately and incubated for 15 min at 4 °C. The cells were then subjected to probe sonication (SONICS Ultrasonic processors, USA) for a total time of 9 min at 56% amplitude, in 30-s pulses with cooling interval of 30 s between pulses, on ice. The sonicates were centrifuged at 11,270×*g* for 15 min at 4 °C and the resulting supernatant was further centrifuged at 11,270×*g* for 10 min at 4 °C to remove any suspended particles. The resulting clear supernatant was used for subsequent enzyme purification. The protein concentration was determined with Bradford Protein assay kit (Genei, Bangalore, India).

Ultrafiltration

The clear cell lysate was concentrated using Amicon 10 KDa cut off membrane (Millipore, India). Ultrafiltration was performed as per the manufacturer's instruction. The increase in the specific activity after the concentration was calculated.

Ammonium Sulfate Precipitation

The concentrated filtrate was fractionated using three different ranges of ammonium sulfate concentrations (0–50%, 50–70%, and 70–80%). The protein precipitate obtained at each fraction of ammonium sulfate saturation was dissolved in minimum amount of 20 mmol/l phosphate buffer, pH 7.4, and dialyzed against 20 mmol/l phosphate buffer overnight at 4 °C to remove ammonium sulfate. The fraction with maximum specific activity was used for further purification steps.

Ion Exchange Chromatography

The most active fractions from ammonium sulfate precipitation were pooled and were subjected to ion exchange chromatography in Q-Sepharose column (1.6×20 cm, Amersham Biosciences, UK) that had been preequilibrated with 20 mmol/l phosphate buffer pH 6.5. After washing with the same buffer, the proteins were eluted with 100 ml linear gradient of 0–1 mol/l NaCl in 20-mmol/l phosphate buffer at a flow rate of 10 ml/h. The active fractions were pooled, concentrated by lyophilization and dialyzed against 20-mmol/l phosphate buffer, pH 7.4 at 4 °C.

Gel Filtration Chromatography

The pooled active fraction from the previous step was subjected to gel filtration on Sephadex G-100 column (1.6×80 cm, Amersham Biosciences, UK), previously equilibrated with 20 mmol/l phosphate buffer pH 7.4. Proteins were eluted at 5 ml/h and collected as 0.5 ml fractions. Active fractions were pooled and were concentrated by lyophilization and stored at −70 °C.

SDS–PAGE and Activity Staining

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [14] was performed in a 5% *w*/*v* stalking gel and 12% *w*/*v* separating gel to analyze all the different

purified fractions. The gel was stained with Coomassie brilliant blue G-250 for visualization of the protein bands.

Activity staining was performed on Native PAGE. The protein was first separated on Native PAGE (4% *w*/*v* staking gel and 10% *w*/*v* separating gel) by electrophoresis at 4 °C. Following this step, activity staining was performed [15]. Briefly, the gel was washed twice with distilled water for 10 min followed by 50 mmol/l Tris–HCl buffer pH 8.5 twice for 5 min. The gel was dipped into 1 mmol/l, L-methionine *p*-nitroanilide and incubated at 50 °C for 20 min. The diazotization of liberated *p*-nitroanilide was done by immersing in 0.1% $NaNO_2$ in 1 N HCl for 2 min. The excess $NaNO_2$ was removed by washing with 1% *w*/*v* urea. The gel was finally immersed in 0.025% *w*/*v* 1-napthylamine solution in 22% ethanol until the pink azo dye was formed.

Methionine Aminopeptidase Assay

The enzyme assay was adopted from Tan and Konings with slight modifications [16]. Methionine aminopeptidase activity was determined spectrophotometrically in a 96-well microtitre plates using a Microplate Reader (Model 680XR, Bio-Rad, USA). The reaction mixture contained 100 μl of 2.5 mmol/l L-methionine *p*-nitroanilide (L-Met-pNA in 100 mmol/l sodium glycine buffer, pH 8.5), 100 μl of 100 mmol/l sodium glycine buffer, (pH 8.5), and 50 μl enzyme sample, making the total reaction volume to 250 μl. The reaction mixture was mixed well and incubated at 50 °C for 10 min. The reaction was stopped by addition of 100-μl glacial acetic acid and the absorbance was measured at 405 nm. Assay was carried out using appropriate substrate and enzyme blanks also. One IU of enzyme activity was defined as the amount of enzyme that hydrolyses 1 μmol/l of L-methionine *p*-nitroanilide per minute. The *p*-nitroaniline released was analyzed according to the standard graph plotted with *p*-nitro aniline.

Biochemical Characterizations

The MetAP was characterized biochemically by studying the effect of temperature, pH, inhibitors, and metal ions. All the experiments were done in triplicates and the average value are taken for representation.

Effect of Temperature and pH on MetAP Activity

Enzyme assay was carried out at six different temperatures ranging from 20 °C to 60 °C and the enzyme activities at different temperatures were compared to find out the optimum temperature. Similarly, the enzyme assay was done at different pH ranging from 4.8 to 11. The buffers used to set different pH were citrate buffer (pH 4.8), sodium acetate buffer (pH 5.8), phosphate buffer (pH 7.4), sodium glycine buffer (pH 8.5), sodium bicarbonate buffer (pH 9.4), and Tris buffer (pH 11).

Thermal Stability

Temperature stability of the enzyme was determined as it was described by Karadzic et al. [17]. The enzyme stability was studied by incubating purified enzyme at pH 7.4 at four different temperatures, 50 °C, 55 °C, 60 °C, and 65 °C. After incubation of the enzyme samples at different temperatures, the activity was estimated as per the standard protocol at 50 °C after every 10 min interval over a period of 1 h. In a separate experiment, thermal

stability of the enzyme was studied by incubating the enzyme at 50 °C over a period of 8 h and the assay was performed at 1 h interval.

Effects of Cations on MetAP Activity

Chlorides of seven different metals namely $MgCl_2$, $CoCl_2$, NaCl, KCl, $CuCl_2$, $FeCl_2$, and $ZnCl_2$ were used in two different concentrations (1 and 5 mmol/l) in the reaction mixture during the assay to study the dependence of enzyme activity on cations [14]. A 100 mmol/l cation stock was prepared and this was accordingly diluted to the above two concentrations in the reaction mixture. Activity was expressed as percentage of activity obtained in the absence of any cations.

The two cations Co^{2+} and Mg^{2+}, which enhanced the enzyme activity, were further used to study their effect on the EDTA-treated enzyme. The enzyme sample was dialyzed against 50 mmol/l EDTA in 20-mmol/l phosphate buffer, pH 7.4 for 2 h. By performing the standard assay described previously, a reduction in enzyme activity was assessed. The enzyme was further dialyzed for another 2 h to remove EDTA against 20 mmol/l phosphate buffer containing 5 mmol/l Mg^{2+} or Co^{2+}. Performing the standard assay at 50 °C assessed the effect of these cations on EDTA-treated enzyme.

Effect of Inhibitors on MetAP Activity

The effects of potential inhibitors of aminopeptidases, such as bestatin, amastatin, actinonin (25 μmol/l and 50 μmol/l), serine protease inhibitor PMSF (1 mmol/l and 5 mmol/l), reducing agent β-mercaptoethanol (1 mmol/l and 10 mmol/l), and metal chelator EDTA (10 mmol/l and 100 mmol/l) were tested. The standard enzyme assay was performed in presence of these inhibitors. Activity was expressed as a percentage of the activity obtained in the absence of any added inhibitor.

Result and Discussion

Enzyme Purification

For enzyme extraction and purification, the selection of culture medium to grow *M. smegmatis* mc^2 155 played an important role. Growth of cells in Sauton's fluid media led to decrease in MetAP yield in comparison with the cells grown in LB broth (results not shown). Among the constituents of Sauton's medium, Tween 80 was found to be responsible for this decrease in yield [18, 19], and this was confirmed by growing the *M. smegmatis* mc^2 155 in LB broth supplemented with different concentrations of Tween 80 ranging from 0.01% to 0.1% and comparing the MetAP activities in the cell lysate. The MetAP activity was reduced up to 53% in presence of 0.1% Tween 80 in the medium (results not shown). Hence, LB medium without any additives was used for growing *M. smegmatis* mc^2 155 cells for purification of MetAP.

Purification was done in four steps, an ultrafiltration, ammonium sulfate precipitation, ion exchange chromatography, and gel exclusion chromatography. These four purification steps led to a final specific activity of 16.2 IU/mg (Table 1). In ammonium sulfate fractionation step, most of the methionine aminopeptidase activity was seen in the 60–80% fraction. In ion exchange chromatography, the active proteins were eluted from Q-sepharose column between 0.5–0.7 mol/l NaCl concentrations. The gel exclusion chromatography

Table 1 Summary of the purification of methionine aminopeptidase from *M. smegmatis* mc^2 155.

Purification Step	Total Protein (mg)	Total Activity (IU)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	51.45	38.08	0.74	1.0	100
Ultrafiltration	29.16	35.0	1.2	1.6	91.9
Ammonium sulfate precipitation	7.48	41.54	5.55	4.67	65.9
Q-Sepharose column	1.94	17.8	9.15	12.4	46.7
Sephadex G-100 column	0.65	10.53	16.2	22	26.3

performed on Sephadex G-100 column removed most of the contaminant proteins and the eluted proteins gave a prominent single band at about 37 kDa on 12% SDS-PAGE (Fig. 1a). The calculated molecular weight from nucleotide-derived amino acid sequence is about 32 kDa. This difference in mobility on SDS PAGE has been reported for many proteins from *M. tuberculosis* [20]. It has been correlated to the number of proline residues present in the protein. Nucleotide-derived amino acid sequence of MetAP from *M. smegmatis* showed a proline content, which contribute to almost 6.5% of proteins molecular mass and which is comparable to other proteins that showed similar mobility. MetAP A from *M. tuberculosis* also exhibits this anomalous mobility on SDS PAGE (unpublished data). The final yields after gel exclusion chromatography was 26.3% and the enzyme was purified 21.9-fold over the crude extract (Table 1).

The activity staining produced clear pink band on the gel where methionine aminopeptidase was active (Fig. 1b). The staining was very sensitive as it could detect the enzyme band even at higher enzyme dilutions up to 10^{-4} U. However, the renaturation of enzyme from denaturing SDS-PAGE by treating with 2.5% Triton ×-100, as reported for other aminopeptidases [21], was not possible in this case. The protein showed a higher molecular weight on a native gel, which may be attributed to the multimeric nature of protein for its function. However, more studies are required in this regard.

Biochemical analysis

The optimum temperature for MetAP activity against the substrate L-methionine *p*-nitroanilide was found to be 50 °C and activity decreased as temperature increases beyond 55 °C. This temperature optimum is similar to methionine aminopeptidase from *Streptococcus salivarius*

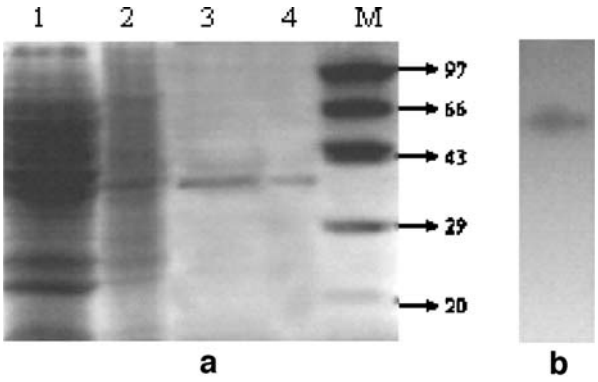

Fig. 1 (a) 12% SDS-PAGE showing the separated proteins stained with Coomassie brilliant blue. *1*: crude extract; *2*: ammonium sulfate fraction (60–80%); *3*: Q-sepharose column fraction. *4*: Sephadex G-100 column fraction. *M* protein marker. (b) Native PAGE showing the signal band corresponding to methionine aminopeptidase activity

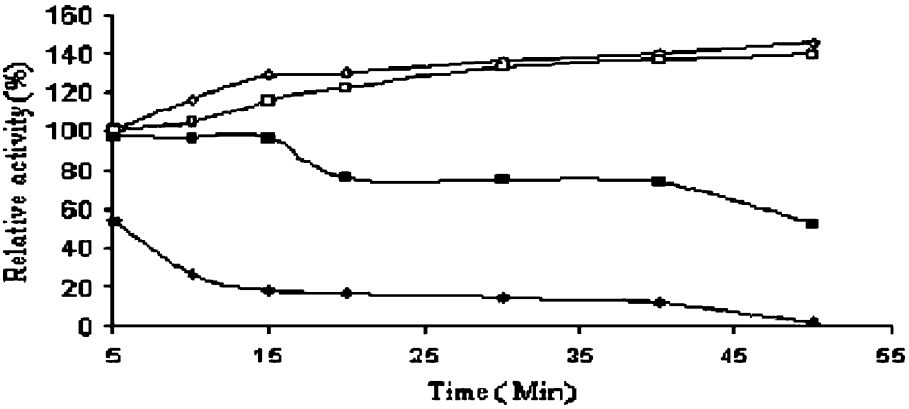

Fig. 2 Thermal stability of methionine aminopeptidase from *M. smegmatis* mc^2 155 at 50 °C (*open diamond*), 55 °C (*open square*), 60 °C (*filled square*), 65 °C (*filled diamond*) over a period of 1 h

and leucine aminopeptidase (LAP) from *Penicillium citrinum* [22, 23]. The optimum pH for MetAP activity was found to be 8.5, which is comparable with the pH optimum of methionine aminopeptidase of *Bacillus stearothermophilus* [24], but much higher than MetAP enzymes from yeast and porcine [25, 26].

The enzyme remained almost stable at 50 °C with a decrease of 32% in relative activity toward the end of the eighth hour. From these data, the enzyme appears to be thermostable at 50 °C, having half-life more than 8 h at this temperature. At 50 °C and 55 °C, the enzyme shows stable activity over a period of 1 h with only a slight decrease of 10% in activity. However, at 60 °C, the relative activity decreases to 50% within 50 min, whereas at 65 °C, the relative activity decreases to 19% and declines to 0 after a period of 50 min (Fig. 2). Methionine aminopeptidase from hyperthermophilic archaeon like *Pyrococcus furiosus* is found to be stable even at 90 °C with half-life period of about 4.5 h at 90 °C [27].

Among a number of metal ions studied, Mg^{2+}, Ca^{2+}, and Co^{2+} ions enhanced the enzyme activity. In the presence of Mg^{2+} the enzyme activity was found to increase twofold. Among the divalent cations, Cu^{2+} and Fe^{2+} abrogated the activity and Zn^{2+} produced only a slight

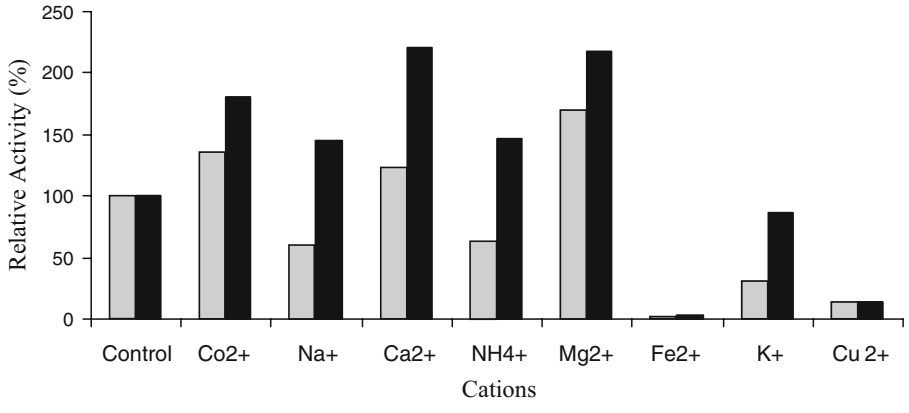

Fig. 3 Effect of selected cations at 1 mmol/l (*open square*) and 5 mmol/l (*filled square*) on the enzyme activity of methionine aminopeptidase from *M. smegmatis* mc^2155

Table 2 Effect of selected metal ions on the EDTA-treated methionine aminopeptidase from *M.smegmatis* mc^2155.

Experiment	Metal ion concentration (mmol/l)	Relative activity of Met AP against the substrate L-Met-pNA (%)
Control	–	100
Enzyme dialyzed against 120 mmol/L EDTA	–	36.25
Mg^{2+} on EDTA-treated enzyme	1	53.75
	5	68.75
Co^{2+} on EDTA-treated enzyme	1	35
	5	37.35

variation in the enzyme activity at different concentrations. Among the monovalent ions, K^+ had an inhibitory effect and Na^+ and NH_4^+ had some positive effect on the enzyme activity at 5 mmol/l concentration (Fig 3). The positive effect of Ca^{2+} has been reported with *Streptomyces griseus* aminopeptidase, which is a Zn^{2+} aminopeptidase, and its activity and stability increases in presence of Ca^{2+}, and also in the case of human methionine aminopeptidase-2 [28, 29]. The present data differ with the previous reports suggesting that Mg^{2+} has either inhibitory or no effect on methionine aminopeptidase activity [24, 26]. The result was further supported by the ability of Mg^{2+} to restore the activity of EDTA-treated enzyme in our experiments. Out of the two metal ions studied, Mg^{2+} was effective in restoring 32% activity of the EDTA-treated enzyme at 5 mmol/l concentration. Co^{2+} was ineffective in restoring any activity of the EDTA treated MetAP (Table 2). Previous studies have indicated that most of the MetAP isoforms have Co^{2+} as cofactor and are inhibited by EDTA [25, 26]. However, as reported herein (Table 2b), Mg^{2+} is a better cofactor than Co^{2+} *in vitro*, and therefore, is likely to be the true cofactor *in vivo* too. This result also supports the results of many previous workers [28, 30] in proving that Co^{2+} may not always be the cofactor involved in the enzyme catalysis of all methionine aminopeptidases. More studies, including atomic absorption spectrum studies and studies at reduced environment, are needed before confirming the role of Mg^{2+} in *M. smegmatis* mc^2155 methionine aminopeptidases. Identifying the actual metal cofactor is very important from the point that many of the

Table 3 Effect of inhibitors, chelators and reducing agents on the activity of methionine aminopeptidase from *M. smegmatis* mc^2 155.

Inhibitors	Concentration μmol /l	Relative Activity (%)
Control		100
Bestatin	25	57.5
	50	44.9
Amastatin	25	40.4
	50	29.7
Actinonin	25	27.7
	50	12.6
EDTA	10,000	53.7
	100,000	33.7
PMSF	1000	67.9
	5,000	29.4
β-mercaptoethanol	1000	12.8
	10,000	0

methionine aminopeptidase inhibitors identified recently are specific to the divalent metal occupying the active site [12].

In the absence of any known specific inhibitors against methionine aminopeptidase, some of the known inhibitors of other aminopeptidases were studied and were found to show inhibition of the MetAP activity, with actinonin being the best inhibitor followed by amastatin and bestatin. (Table 3). Actinonin is a known hydroxamate-containing inhibitor of several metallohydrolases including peptide deformylase. The hydroxamate-containing compounds are very potent inhibitors of metalloenzyme as the chelating groups bind to the metal ion of the enzyme [31]. In this case too actinonin must have inhibited the enzyme activity by binding to the metal ion. Bestatin is a known leucine aminopeptidase inhibitor, and to work as a good methionine aminopeptidase inhibitor, it needs slight modification in the N-terminal side chain [8]. In this case, both amastatin and bestatin showed inhibition of the enzyme activity by reducing the activity to less than 50% at 50 μmol/l concentration. This high inhibition could be because of the relatively high concentration of the inhibitors used in the experiments than the previous reports. β- mercaptoethanol was found to completely inhibit the enzyme at 10 mmol/l concentration. This inhibition is thought to be by reduction of the disulphide bonds. Many aminopeptidases have been reported to be unaffected by β- mercaptoethanol and some even show enhanced activity in the presence of reducing agents like DTT [32]. However, leucine aminopeptidases from *Bacillus* sp. was completely inhibited by DTT [33]. Partial inhibition of MetAP enzyme was seen with PMSF. Methionine aminopeptidase and leucine aminopeptidase from *Bacillus stearothermophilus* and other *Bacillus* sp. were not inhibited by PMSF at 0.1–0.5 mmol/l concentrations [24, 33]. In our studies too, a lower concentration of PMSF did not prove effective for inhibiting the MetAP, which points to the fact that serine residue may not be directly involved in the catalysis.

Conclusion

Methionine aminopeptidase from *M. smegmatis* mc^2 155 is a metalloenzyme with specificity toward N-terminal methionine. Mg^{2+} ion is thought to influence the methionine aminopeptidase activity positively and some aminopeptidase inhibitors like actinonin and bestatin are found to inhibit this enzyme. Identifying the true metal cofactor of methionine aminopeptidases *in vivo* will be useful to specifically screen some inhibitors against the enzyme. Studies with overexpressed recombinant protein and the elucidation of the 3D structure of the protein will be of great use to understand more about the enzyme and for possible exploration of importance of this enzyme in pathogenic strains like *Mycobacterium tuberculosis*.

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