Purification and characterization of highly active LasB protease from *Pseudomonas aeruginosa* MCCB 123

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Proteolytic enzymes constitute one of the most important groups of industrial enzymes and have received worldwide attention due to their wide range of biotechnological applications and their sustainability in reducing environmental degradation. In this study, a LasB protease with dual substrate specificities was purified from *Pseudomonas aeruginosa* MCCB 123 with 6.85 fold increase in protease specific activity and 9.05 fold increase in elastase-specific activity. The enzyme revealed the presence of a 33 kDa protein. Optimum temperature and pH of protease activity were 60°C and 9.0, and that of elastase activity were 40°C and 8.0, respectively. Based on the cytotoxicity assay on Hep2 cells, the enzyme has an IC₅₀ (inhibitory concentration) value of 47.28 µg mL⁻¹. According to the deduced amino acid sequence, the MCCB 123 LasB protease is a zinc dependent metalloendopeptidase belonging to M4 neutral protease GluZincin superfamily. Appreciable protease activity of the enzyme at high temperature and alkaline pH proposes its potential application in detergency. MCCB 123 LasB protease is 3.96 fold more active when compared to commercial protease Savinase suggesting its industrial relevance.

**Keywords:** Detergent, Deproteinization, Microbial protease, Savinase

Enzymes are eco-friendly green chemicals that have wide range of applications in industrial sector¹. Microbial protease represents largest group of industrial enzymes which account for 65% of total worldwide enzyme sales². Among microbial proteases, bacterial alkaline proteases secure a prominent position in the enzyme market³, and are commonly used due to their relatively high activity and stability at high pH⁴. They are supplemented as ingredients in detergents, used in food processing, leather industry, waste treatment, dairying, food and feed manufacturing, silver recovery⁵-¹¹ and peptide synthesis⁶,¹². Even though, *Bacillus* spp. has been viewed as a promising group of organisms for protease production towards industrial applications, *Pseudomonas* proteases have also been considered for such uses as they are either similar or more active than those produced by the former. Furthermore, *Pseudomonas aeruginosa* can grow in alkaline conditions and in water-soluble oil and its enzymes are adapted to extreme conditions¹³. But only a few reports exist on the proteolytic enzymes from *Pseudomonas aeruginosa*¹⁴. Predominant proteases secreted by this bacterium are LasA protease, LasB protease, Protease IV and alkaline proteases¹⁵,¹⁶. This organism has been proposed as antagonistic against aquaculture pathogens¹⁷,¹⁸. The *Pseudomonas* proteases do have applications in deproteinization of shrimp and shell waste¹⁹,²⁰, peptide synthesis²¹,²², and also as biocatalysts for enzymatic synthesis in the presence of organic solvents²³,²⁴. In this study, we purified and characterized LasB protease from *Pseudomonas aeruginosa* MCCB 123; and compared its activity with that of commercial protease Savinase® from *Bacillus* sp.

**Materials and Methods**

**Identification of protease producing bacterial isolate**

Protease producing isolate coded as MCCB 123, an organism isolated from a coir retting ground (Chellanum North, Kerala, India), was characterized phenotypically for its identity following Baumann and Schubert²⁵.

**Identification by 16S rRNA gene sequencing**

The identity *Pseudomonas* MCCB 123 was confirmed by 16S rRNA gene sequence analysis. DNA extraction was carried out by Phenol-chloroform method²⁶. Amplification of 16S rRNA
gene was performed according to Reddy et al. using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16 S2 (ACG GCT ACC TTG TTA CGA CTT). The PCR product was separated on 1% agarose gel, purified using QIAEX II gel purification kit (Qiagen), cloned into pGEM-T Easy vector (Promega, USA), and sequenced using the primer walking service of Microsynth AG, Switzerland. The sequences obtained were matched with the database in Genbank using the BLAST algorithm and ribosomal database project (RDP) release 9.

Purification of protease

The protease was purified by ammonium sulphate precipitation followed by DEAE-cellulose chromatography. Ammonium sulphate was added to cell-free culture supernatant up to 30% saturation, precipitates removed by centrifugation at 8260 × g for 15 min at 4°C and discarded, and continued up to 80% saturation, collected the precipitate the same way and resuspended in 20 mM Tris-Cl buffer (pH 8.50). The enzyme was dialyzed against the same buffer using Amicon UF stirred cell (Millipore Corporation USA, Model 8010), with a 10 kDa cutoff membrane and used for further purification. The enzyme was then loaded on AKTA Prime protein purification system equipped with a C10 mm × 20 cm DEAE cellulose column equilibrated with 20 mM Tris-Cl buffer (pH 8.5). After washing the column with the same buffer, the enzyme was eluted by applying a linear gradient of NaCl from 0-1000 mM at a flow rate of 0.5 mL min⁻¹ and fractions of 2 mL were collected. Active fractions were pooled and concentrated by lyophilization.

Electrophoresis and zymography

The lyophilized active fractions of the enzyme were subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 5% stacking gel and 15% resolving gel at a constant current of 12 mA. Zymogram was performed with 4% stacking gel and 10% resolving gel co-polymerized with 0.1% gelatin as substrate under native SDS-PAGE conditions.

Protease assay

Protease assay was carried out following the method of Khembavi et al. An aliquot of 0.5 mL suitably diluted enzyme solution was mixed with 0.5 mL substrate (1% Hammerstein casein in 50 mM Tris-Cl buffer (pH 9) and incubated at 60°C for 30 min. The reaction was stopped by the addition of 0.5 mL 20% TCA; the mixture was centrifuged and the absorbance was measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to liberate 1 µg tyrosine per mL per min. Assays were carried out in triplicates and the mean value was expressed as unit protease activity.

Elastase assay

Elastase activity was determined using Elastin–Congo red (Sigma – Aldrich Co.) as the substrate according to the method of Kessler et al. The reaction mixture consisting of 1 mL enzyme solution, 5 mg elastin-congo red, and 1 mL 50 mM Tris-Cl buffer (pH 8) was incubated at 40°C for 3 h. The reaction was stopped by the addition of 0.1 mL 100 mM EDTA, centrifuged at 8260 × g for 15 min, and the absorbance measured at Abs 495 nm. One unit enzyme activity is the amount of enzyme which causes an optical density increase by 1 unit. Assays were carried out in triplicates and the mean value was expressed as elastase activity.

Protein assay

Quantification of protein was carried out according to the method of Hartree using bovine serum albumin (BSA) as the standard. Based on the molecular weight and elastase activity the enzyme was identified as LasB protease and subjected for further studies.

Effect of pH on LasB protease and elastase activities

Effect of pH on the protease activity was determined over a pH range of 6-12 using the buffers of 50 mM concentrations: sodium phosphate (pH 6, 7), Tris-Cl (pH 8.0, 9.0), glycine-NaOH (10.0, 11.0, 12.0) for 30 min at 60°C. Effect of pH on elastase activity over the same range was determined using the same buffers for 3 h at 40°C. The substrates (casein and elastin – Congo red) were prepared in the respective buffers and assayed under standard conditions.

Effect of pH on LasB protease and elastase stabilities

For the measurement of pH stability, the enzyme was incubated in different buffers for 1 h at room temperature and the residual protease and elastase activities were determined under standard assay conditions. The untreated enzyme was taken as the control (100% activity).

Effect of temperature on LasB protease and elastase activities

Effect of temperature on protease activity was tested by carrying out the assay at temperature ranges
of 30, 40, 50, 60, 70 and 80°C for 30 min in 50 mM Tris-Cl buffer (pH 9.0), and on elastase activity at the same range for 3 h in 50 mM Tris-Cl buffer (pH 8.0).

Effect of temperature on LasB protease and elastase stabilities

Effect of temperature on protease and elastase stabilities was examined by incubating the enzyme at temperatures ranging from 30, 40, 50, 60, 70 and 80°C for 1 h in 50 mM Tris-Cl buffer at pH 9.0 and 8.0, respectively, and the residual protease and elastase activities were measured under standard assay conditions. The non-heated enzyme was considered as the control (100% activity).

Comparison of LasB protease activity with commercial proteases Savinase®

Specific LasB protease activity of the partially purified protease was compared with that of the commercial protease Savinase® from Bacillus sp. (Sigma-Aldrich Co., Product no P3111) under the assay conditions at pH 9.0 and temperature 60°C.

Calculation of specific, relative and residual activities

Specific activity was calculated by dividing the enzyme units with the protein content,

Specific activity (U mg⁻¹) = Total unit activity (U ml⁻¹) ÷ Total protein content (mg mL⁻¹).

Relative activity is the percentage enzyme activity of the sample with respect to the sample, for which the maximum activity was obtained,

Relative activity = [Total activity (U ml⁻¹) × 100] ÷ Maximum activity (U ml⁻¹)

Residual activity is the percentage enzyme activity of the sample with respect to the activity of the control (untreated sample),

Residual activity = [Activity of the sample (U) × 100] ÷ Activity of control (U)

Cytotoxicity analysis of LasB protease

Hep2 cells were seeded in 96-well plates (Greiner Bio-One) with Eagle’s minimal essential medium containing 82 mM glutamine, 1.5 gL⁻¹ sodium bicarbonate and 10% fetal bovine serum. Purified enzyme in concentrations of 0 to 500 µg mL⁻¹ (v/v) was added to the wells in triplicates. Cells in the growth medium (without the enzyme) were kept as control. After 14 h incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed and the percentage of inhibition was determined.

Percentage inhibition of cells at each concentration of the enzyme = [100−(Average absorbance (MTT assay) of cells at a particular concentration of the compound ÷ Average absorbance of control cells without the enzyme) × 100)]. Probit analysis for percentage cell inhibition was done with SPSS 17.0 package for Windows.

MTT assay

After replacing the medium, 50 µL of MTT (Sigma Aldrich) solution (5 mg mL⁻¹ in PBS) was added to each well and incubated for 5 hours in the dark. The control consists of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200 µL dimethylsulfoxide. Absorbance was recorded immediately at 570 nm in a microplate reader (TECAN Infinite Tm).

PCR amplification, purification and sequencing of LasB protease gene

Genomic DNA was purified using the phenol-chloroform method as described by Sambrook and Russell. The LasB protease primers were synthesized based on Jellouli et al. Amplification of LasB protease gene was performed using the primers AAGCGTCGGCCGAGTACTTCG (forward) and GACCCGCATTCCTTCTGGAG (reverse). The amplification was performed using DNA Thermal cycler (Eppendorf). Amplified PCR products were analyzed on an agarose gel and purified using gel purification kit of Sigma and sequenced using the primer walking service of Xcelris, Ahmedabad.

Results and Discussion

Identification of the bacterial isolate

The strain MCCB 123 was found to be Gram-negative rods that can denitrify, produce pyocyanin and can grow at 41°C. Further, the molecular characterization based on 16S rRNA gene sequencing confirmed its identity as P. aeruginosa and was deposited in GenBank with the accession no. FJ665510. According to Baumann and Schubert and other researchers, morphological and biochemical characteristics of MCCB 123 agrees with that of P. aeruginosa. 16S rRNA gene sequencing (accession no. FJ665510) further confirmed this identity.

Purification and molecular mass determination of the enzyme

Purified enzyme exhibited dual substrate specificities viz. protease and elastase activities. Purification resulted in 6.85 and 9.05 fold increase in protease and elastase-specific activities respectively (Table 1).
The elution profile of LasB protease on the DEAE-cellulose column is shown in Fig. 1. The enzyme eluted between 0.44 M to 0.68 M NaCl (fractions 44-68). The molecular weight of LasB protease was estimated to be 33 kDa by reducing SDS-PAGE (Fig. 2A). Zymogram of the enzyme showed a clearance zone (Fig. 2B). The purified LasB protease enzyme – with both protease and elastase activities - produced by the strain MCCB 123 showed a 33 kDa band in SDS-PAGE. This molecular mass is in close agreement with the previous reports\textsuperscript{21,33}. The purified enzyme also exhibited an increase in protease (6.85 fold) and elastase (9.05 fold) specific activities.

**Effect of pH on LasB protease and elastase activities and stabilities**

The enzyme showed optimum protease activity at pH 9 and optimum elastase activity at pH 8. The protease activity was stable up to pH 10. The study of the effect of pH on LasB protease stability showed that the enzyme was stable from pH 7.0 to 10 for 1 h and retained more than 90 % of its residual activity from pH 7.0 to 9.0 and 48 % activity at pH 10.0. Elastase activity was stable at pH 7.0, 8.0 and 9.0 with residual activities of 71, 93 and 40%, respectively when compared to the control.

MCCB 123 LasB protease, with its optimum protease activity at pH 9.0, appears to be different from other reported proteases that hydrolyze casein at pH 8\textsuperscript{33}. The enzyme is also stable from pH 7.0 to 10.0. The maximum protease activity at pH 9.0 that entails it to be classified under the category of alkaline protease, also makes it suitable in laundry industry, leather processing, and other industrial processes that are carried out in the alkaline pH range. Alkaline proteases are particularly important because they are active and stable under harsh conditions such as high temperatures (50-60°C) and high pH\textsuperscript{34-37}.

**Effect of temperature on LasB protease and elastase activities and stabilities**

MCCB 123 LasB protease was active over a wide range of temperature from 30 to 80°C with its optimum at 60°C while elastase was active from 30-60°C with its optimum at 40°C. The enzyme retained more than 80 % protease activity and 60% elastase activity from 30 to 50°C.

The protease activity of the enzyme at 30°C makes it useful in detergent industry, which is now looking for alkaline proteases that works well under ambient temperature\textsuperscript{18}, to maintain fabric quality and also

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**Table 1—Purification of LasB protease produced by *Pseudomonas aeruginosa* MCCB 123**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>PA (U/mL)</th>
<th>EA (U/mL)</th>
<th>Total Protein (mg)</th>
<th>Specific PA (U/mg)</th>
<th>Specific EA (U/mg)</th>
<th>Purification fold (PA)</th>
<th>Purification fold (EA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>24754.17</td>
<td>654.14</td>
<td>8.55</td>
<td>2895.22</td>
<td>76.50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4) Precipitation</td>
<td>22737.50</td>
<td>574.14</td>
<td>4.09</td>
<td>5559.29</td>
<td>140.37</td>
<td>1.92</td>
<td>1.83</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>13695.83</td>
<td>478.06</td>
<td>0.69</td>
<td>19849.02</td>
<td>692.84</td>
<td>6.85</td>
<td>9.05</td>
</tr>
</tbody>
</table>

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**Fig. 1—Elution profile of LasB protease through DEAE – cellulose C 16/40 column**

**Fig. 2—(A) SDS-PAGE profile of purified LasB protease. Lane 1, molecular weight markers, lane 2, 33 kDa LasB protease; and (B) Zymogram of LasB protease**
for reducing the energy demand\textsuperscript{39}. The temperature stability profile of the LasB protease activity revealed that the enzyme was highly stable from 30 to 50°C retaining greater than 80% of its residual activity. This high-temperature stability of the enzyme can be utilized in the areas where proteases are exposed to high-temperature conditions such as in the recovery of silver from x-ray films, and in detergent industry.

Comparison of LasB protease activity with commercial Protease Savinase\textregistered

Activity of MCCB 123 (LasB) protease was compared with commercial proteases Savinase\textregistered from Bacillus sp. The specific protease activity of purified MCCB 123 LasB protease (14048.91 U mg\textsuperscript{-1}) was 3.96 fold higher when compared to Savinase\textregistered (3546.40 U mg\textsuperscript{-1}). Comparison with the commercial protease Savinase\textregistered from Bacillus sp. indicates that MCCB 123 LasB protease was 3.96 fold more active suggesting its importance in industrial applications and the possible economic viability.

Cytotoxicity analysis of purified LasB protease

The enzyme is nontoxic to Hep2 cells 1-10 µg mL\textsuperscript{-1}. IC\textsubscript{50} value was found to be 47.28 µg mL\textsuperscript{-1} (Fig. 3). Even though 25 µg mL\textsuperscript{-1} concentration of the enzyme inhibited the cells (in terms of MTT assay) morphological changes were absent when compared to the control cells (Fig. 4). Cells exhibited detachment, rounding and shrinkage at 50 µg mL\textsuperscript{-1}. Based on MTT assay values the enzyme was nontoxic to Hep2 cells for up to 10 µg mL\textsuperscript{-1} and inhibited 50% (IC\textsubscript{50}) of the cells at 47.28 µg mL\textsuperscript{-1}. These results are in consensus with the earlier report of Galloway\textsuperscript{39} that elastase (LasB protease) of \textit{P. aeruginosa} is relatively nontoxic with an LD\textsubscript{50} for mice in the range of 60-400 µg depending upon the root of inoculation.

Comparison of deduced amino acid sequence of \textit{P. aeruginosa} MCCB 123 elastase with other elastases

A comparative analysis of deduced amino acid sequence of LasB protease gene of \textit{P. aeruginosa} MCCB 123 with other known proteases is represented in Fig. 5. There are totally 24 amino acids that differ in sequence between MCCB 123 LasB protease and PA7 protease\textsuperscript{41}, 14 amino acids in propeptide region and 10 amino acids in mature protease region. There is difference in one amino acid in propeptide sequence and 4 amino acids in mature protease region from A2 protease\textsuperscript{42} and MN7 protease\textsuperscript{33}. MCCB 123 LasB protease gene sequence (accession number JN118955) is 99% similar to that of \textit{P. aeruginosa}. Classification based on the deduced amino acid sequence demonstrated that the MCCB 123 LasB protease is a zinc dependent metalloendopeptidase belonging to M4 neutral protease GluZincin superfamily. The difference in the deduced amino acid sequence of MCCB 123 LasB protease with the other reported proteases\textsuperscript{33,41,42} indicates its unlikeness among others.

In conclusion, LasB protease was purified from the culture supernatant of \textit{Pseudomonas aeruginosa} MCCB 123 and characterized. The enzyme exhibited good protease and elastase activities. High protease activity at higher temperatures and alkaline pH, and its higher activity in comparison to commercial protease Savinase suggest its industrial relevance.

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**Fig. 3**—Toxicity of LasB protease in Hep2 cells in terms of percentage inhibition determined through MTT assay

**Fig. 4**—Effect of LasB protease on Hep2 cells (40 x) (A) control without the enzyme; (B) 25 µg mL\textsuperscript{-1} enzyme; and (C) 50 µg mL\textsuperscript{-1} enzyme
Acknowledgement

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References


