Efficient Purification and Characterization of Neem Oil Hydrolysing Lipase from Aspergillus aculeatus for Enrichment of Immunomodulators

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Neem oil hydrolysing lipase from Aspergillus aculeatus was purified to homogeneity with very high yield, i.e., 64.0 per cent with 12.8-fold purification using octyl-sepharose affinity column. The specific activity of enzyme significantly increased from 125 U/mg protein to 1600 U/mg protein. The molecular weight of purified lipase was 42 ± 2 kD as determined by native and SDS-PAGE. This lipase was highly active towards neem oil (350 per cent relative activity) and exhibited 1,3 regiospecificity. In general, this lipase showed high activity towards diglyceride to triglyceride of palmatic series. This novel lipase showed wide temperature tolerance (10-70 °C) with maximum activity at 37°C. Enzyme undergo thermal inactivation at 60°C, whereas it looses its 90 per cent activity after 60 min. The enzyme showed pH tolerance (5.0-10.0) with maximal activity at 8.0. At the concentration of 10μm, this lipase inhibited by some of the serine inhibitors such as diethylparanitrophenyl phosphate and aprotenin, whereas there is no effect of perfibolac and leupeptine. Mg++ and Ca++ are neutral to this lipase, whereas Zn++, Cu++ and Hg++ caused inhibition. Enzyme highly stable in water immiscible organic solvents, whereas water miscible organic solvents generally caused inhibition. As neem oil contains varieties of intact highly hydrophobic immunomodulators the purification and enrichment of these molecules in oil using lipase will be a novel approach.

Introduction

Several microbial lipases have been purified and have received much attention because of their importance in the field of many biotechnological industries. Using three basic catalytic activities, i.e., hydrolysis, esterification, and transesterification many lipase applications have been developed. The lipase used in each of its application is selected based on its substrate reactivity and specificity, temperature, pH, and organic solvent stability dependence. Most of the purification procedures reported involve multisteps method such as precipitation with (NH4)2SO4 or acetone or separation by gel filtration, or ion exchange chromatography. In recent years, affinity chromatographic technique reversed micellar and two-phase system, membrane ultrafiltration and immunopurification have also been used for purification of lipase. Although lipases are being today used commercially, their industrial sources are limited. However, nature still offers a tremendous potential for identifying new source of lipases with novel properties. Neem oil is known to proliferate lymphocyte and exhibited phagocytic activity and expression of MHC class-II antigen and its active principle is highly nonpolar. Enrichments of these molecules by classical chemical routes is very difficult due to non-specific hydrolysis of neem oil which resulted into destruction of active molecules.

Therefore, in present investigation, we describe the successful purification and characterization of neem oil hydrolysing lipase from Aspergillus aculeatus which may be used to enrich the immunomodulatory molecules in neem oil due to specific hydrolysis of neem oil without affecting the active moieties.

Materials and Methods

Enzyme Production

Aspergillus aculeatus was grown in modified minimal medium "Czapekdox" containing corn oil, for 168 h. at 30°C in static condition. The medium contained in g/L distilled water: NaNO3 - 6; KCl - 0.52; MgSO4·7H2O - 0.52; KH2PO4 - 1.52; Cu(NO3)2·3H2O - 0.01; FeSO4·7H2O - 0.001;
ZnSO_4·7H_2O - 0.001; Glucose - 2.0, and corn oil - 1.5 per cent v/v. Initial pH was adjusted to 6.2. The medium was autoclaved at 121°C (15psi) for 15 min. After filtration, 1L of culture filtrate produced under optimized conditions was used as the crude enzyme source for purification.

**Lipase Activity**

Lipase activity was measured by Winkler and Stuckmann method\(^1\). One enzyme unit of lipase defined as that quantity releasing 1nm of free phenol from the substrate (p-nitrophenol palmitate) / min/ mL, substrate under standard assay conditions. Lipase activity in the sample was also determined by NEFA-test (using olive oil as substrate) \(^3\). One unit of enzyme activity is defined as the amount of enzyme required to release 1μM of free fatty acid at 37°C under standard assay conditions (pH 7.0, reaction time 30 min).

**Purification of Lipase**

Culture filtrate 1L, containing lipase was dialyzed in Sigma cellulose tubing (pore size, cut off 12-14kDa) against 0.2M phosphate buffer, pH 7.2 for 24 h, with several changes. The enzyme sample thus obtained was directly applied to to octyl-sepharose affinity column (60 cm x 10 cm) equilibrated with 0.2 M phosphate buffer at pH 7.2. The elute was collected into 5 mL fractions with the help of gradient of methanol ranging from 10 to 30 per cent prepared in 0.2M phosphate buffer at pH 7.2. After dialysis with same buffer the homogeneity of enzyme was confirmed by SDS-PAGE\(^5\).

**Molecular Mass Determination**

The molecular mass of the purified enzyme was determined by SDS-PAGE (10 per cent gel) and activity staining was performed with Victoria blue\(^6\).

**Hydrolysis of Neem Oil and Substrate Specificity**

The substrate specificity of *Aspergillus aculeatus* lipase towards various lipids (olive oil, mustard oil, soya oil, corn oil, neem oil and coconut oil) at concentration of 1.5 per cent v/v were studied and relative activity was measured in each case. Extending the experiment specificity towards various glycerides palmitic series towards lipase activity was also investigated by incubating the enzyme 0.1mL (100U) in 0.2M phosphate buffer (pH 7.2) 0.7 mL water and 0.1 mL 10mM glyceride solution (dissolved in N, N-dimethyl formamide). The whole cocktail was incubated at 37°C and after 30 min incubation, reaction was terminated by the addition of Chloroform : Methanol (90:10). Free fatty acid in aqueous phase was estimated by NEFA test and enzyme activity was calculated accordingly\(^14\). The regioselective nature of this novel lipase was determined by incubating 1 mL of triolein and 4mL of 0.2 M sodium phosphate buffer, pH 7.0 with 0.1 mL of enzyme (500U) sample at 37°C for 24 h with shaking at 100 rev/min. The reaction was terminated by using 25 mL of diethyl ether and extract was frozen. It was later analysed by TLC. The solvent system was light petroleum (bp 40-60°C) diethylether : acetic acid (80:30:1). The hydrolyzed spots were detected by using a saturated iodine chamber and were compared with those of standard glyceride\(^7\).

**General Characterization**

The optimum temperature and thermal stability of enzyme were determined by conducting the assay at 10 to 70°C. Thermal activation lipase was performed at 60°C for 60 min. The effect of pH on lipase activity was determined by incubating the purified enzyme at pH value 5.0 to 10.0 pH and stability was checked by incubating the enzyme at various pH 8.0 - 10.0, at various time intervals. At the concentration of 10μM different inhibitors and metals, the modulation of lipase were evaluated. The effect of various water miscible and immiscible solvents were also tested on lipase activity by incubation of 100U enzyme with 50 per cent solvent at 37°C. Relative activity was measured with miscible solvents (Methanol, ethanol, acetone and propanol) and water immiscible solvents (ethylacetate, pyridine, hexane, toluene and tetrahydrofuran) after 30 min.

**Results and Discussion**

Lipase with newer properties still remain at bottlenecks in the enzyme industry and hence screening and purification of lipase from new strain have received a renewed interest in the recent past in order to make lipase novel. Several lipases are purified and used in varieties of lipid transformation with either very poor activity towards the neem oil or no activity\(^3\). *Aspergillus aculeatus* lipase was found to be excellent towards neem oil hydrolysis. The greates advantage of this hydrolysis is to enrich the highly non-polar immunomodulators present in neem oil. Therefore, *Aspergillus aculeatus* lipase was
Table 1 — Purification of lipase produced by *Aspergillus aculeatus* under optimized condition

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total volume (mL)</th>
<th>Total activity (lU)</th>
<th>Fold purification</th>
<th>Specific activity</th>
<th>Yield (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate followed by dialysis</td>
<td>1000</td>
<td>50000</td>
<td>0.00</td>
<td>125</td>
<td>0.00</td>
</tr>
<tr>
<td>Octyl-sepharose affinity column followed by dialysis</td>
<td>100</td>
<td>32000</td>
<td>12.8</td>
<td>1600</td>
<td>64</td>
</tr>
</tbody>
</table>

Figure 1 — SDS-PAGE of *A. aculeatus* lipase showing molecular mass of the lipase. Lane A—purified fraction after extensive dialysis loaded on octylsepharose, lane B—partially purified fraction supernatant direct loaded on octylsepharose, lane C—crude culture filtrate, lane D—protein molecular markers.

purified up to homogeneity (Table 1). Using affinity column octyl sepharose, there is increase in specific activity of lipase from 125 U/mg protein to 1600 U/mg protein. The enzyme was purified approximately 12.8-fold with very high yield (64 per cent) which generally does not occurs in conventional method of purification. *Aspergillus niger* lipase was purified by successive chromatographic ways with maximum yield of 34 per cent by various groups. Recently, we have also purified *Aspergillus terreus* lipase using conventional method only up to 18.04 yield. Lipases from other microbes such as *Mucor mehei*, *Penicillium cyclopium* MI, *Rhizopus delemar*, *Humicola lanuginosa* were also purified only up to 20, 32, 27, 11, 15 per cent yield respectively.

High yield with drastic increase in specific activity of *Aspergillus aculeatus* lipase emphasizes the importance of affinity column in attending homogeneity of the enzyme in few steps. The molecular weight of the purified *Aspergillus aculeatus* lipase has been estimated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and under native conditions to be 42 ± 2 kDa (Figure 1). Other species of lipases which were also purified up to homogeneity with different molecular weight were *Aspergillus niger* (47 ± 1 kDa), *Aspergillus terreus* (42 ± 1 kDa).

Although lipases from different species of microorganisms resemble each other, and in many instance their specificities overlap, it is well known that they attack various synthetic and natural triglycerides at different rates. The substrate specificity of *Aspergillus aculeatus* lipase towards various natural lipids was tested. It is evident that this lipase is highly active on the substrate neem oil (350 per cent relative activity) and least observed in mustard oil (60 per cent relative activity) (Table 2). This difference might be caused by the dependence of lipolytic activity on the structure of both the fatty acid and alcohol moities of the substrate. This has also been suggested by Lawrence and Macrae and Hammond. The above properties can be useful to enrich the immunomodulatory molecules in oil by specific cleavage of triglycerides and its derivative.
into fatty acids without affecting the target molecule which is generally responsible for phagocytotic activity and expression of MHC-class II antigen\(^1\). Amplification of these activity in crude oil will reduce the dosage of neem oil during design of contraceptive.

The *Aspergillus aculeatus* lipase showed pronounced regiospecificity and hydrolysed triolein into its intermediate product diolein and monolein (Figure 2). As the lipase showed 1,3-regiospecificity towards the outer chain of triglyceride, this result is in accordance with our previous report of *Aspergillus terreus* lipase other lipases such as *Aspergillus niger* and *Rhizopus delemar* lipases\(^2\). On the other hand, lipases from *Geotrichum candidum* inn. and *Penicillium cyclopium* lipases attacked the fatty acid chains of triglycerides regardless of their position (non-specific). Both these type of lipases have been reviewed by Macrae and Hammond\(^3\). In the present investigation, this lipase interestingly showed high activity towards diglyceride in comparison to triglycerides and monoglycerides (Table 2), hence it can be used for purification of oil.

With few exceptions microbial lipases are more active within \(30-40^\circ C\)\(^4\). The novel lipase also showed maximal activity at \(37^\circ C\) and it undergoes thermal inactivation at \(60^\circ C\) and it looses its 90 per cent activity after 60 min. (Figure 3 and 4). It is clear from Figure 5 that *Aspergillus aculeatus* lipase could tolerate a broad range of pH 5-10 with maximum activity at pH 8.0. It undergoes drastic inactivation at pH 10.0 (Figure 6). Our finding of optimum pH 8.0 is accordance with work of Liu et al\(^23\) where they obtained pH optima at 8.0 in *Humicola lanuginosa*. Higher pH optima for activity have been also reported by Aiasaka and Terada\(^24\) (*Rhizopus japonicus* pH 8.3) and Ruiz et al\(^25\) (*Penicillium candidum*). The purified lipase of *Aspergillus aculeatus* was incubated with serine inhibitors, perfobolac, leupeptine, apotinin and diethyl p-nitro phenyl phosphate. The results presented in Table 3 show that PNPP and aprotanin caused inhibition whereas perfobolac and leupeptine failed to do so. Higher degree of inhibition with PNPP suggested that this novel lipase belongs to class of serine hydrolase (Gilbert et al\(^26\); Brozowskii et al\(^27\). It is known that activation and inactivation of lipase are controlled by various ions, hence the effect of different ions on novel lipase was tested. Divalent cations at the concentration of \(10 \text{ mM}\) and Mg\(^{++}\) neutral to lipase activity, whereas Zn\(^{++}\), Cu\(^{++}\), Ca\(^{++}\), Hg\(^{++}\), Fe\(^{++}\) ions caused inhibition (Table 3). This result is in sharp contrast to our previous findings where we have reported the
stimulatory effect of Mg$^{++}$ and Ca$^{++}$ in *Aspergillus terreus* lipase$^1$.

Extending the experiment the stability of *Aspergillus aculeatus* lipase in various organic solvents was tested in order to understand the protein stability. Enzyme is highly sensitive to water miscible solvents such as ethanol, acetone, and propanol, whereas it loses 100 per cent activity with 50 per cent solvent. Interestingly methanol caused less inactivation (20 per cent). Water immiscible solvent such as butanol and tetrahydrofuran significantly retain the enzyme action 94 per cent and 84 per cent suggesting that their regioselectivity may be ideal reaction system of organic solvents (Table 4). The above properties of enzyme make it an ideal candidate for bio-catalyst in organic solvents particularly in water immiscible solvents.
Table 4 — Stability of Aspergillus aculeatus lipase in various organic solvents.

<table>
<thead>
<tr>
<th>Organic solvents</th>
<th>Relative activity (per cent)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Methanol</td>
<td>80</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.00</td>
</tr>
<tr>
<td>Propanol</td>
<td>0.00</td>
</tr>
<tr>
<td>Butanol</td>
<td>95.73</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>76.43</td>
</tr>
<tr>
<td>Pyridine</td>
<td>64.13</td>
</tr>
<tr>
<td>Hexane</td>
<td>74.95</td>
</tr>
<tr>
<td>Toluene</td>
<td>74.02</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>84.97</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Conclusion

Aspergillus aculeatus lipase is excellent fungal lipase with the ability to hydrolyse neem oil and can be used in enrichment of hydrophobic immunomodulators present in oil. This lipase can be easily purified up to homogeneity and the properties of enzyme make it an ideal candidate for neem oil transformation both in aqueous and organic solvents.

Acknowledgements

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References

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