PURIFICATION AND CHARACTERIZATION OF PECTINASE (PECTIN METHYL ESTERASE) FROM APPLE POMACE

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ABSTRACT

Pectinases have extensive applications in extraction, clarification, and cloud stabilization of fruit juices, in degumming and retting of natural fibers. In the present study, Pectinases/ Pectin Methyl Esterase (PME) was purified from apple pomace by consecutive steps of enzyme extraction and further precipitated by saturated concentration of ammonium sulphate. The results confirm that the enzyme can activated at 45 to 55 °C and work well at a pH of 4.5 to 5.5. The enzyme was further purified and characterized by HPLC. The purified pectinase showed the retention time, 1.812 minutes with reference to standard pectinases which showed the retention time, 1.790 minutes. Purified pectinases were further utilized to observe the degradation of pectin in pectin-agar plate. The zone of degradation of pectin by pectinases was observed by overlaying 1% Iodine solution prepared in 95% ethyl alcohol. Purified pectinases degrade the pectin in pectin-agar plates in comparison to control. The enzyme purified was loaded on agarose gel and further stained with ruthenium red and coomassie brilliant blue separately. The enzyme purified showed molecular weight 43 kDa as compared to standard marker. The enzyme purified was qualitatively assessed for pectin degradation in fruits. It was observed that in case of test, orange slice incubated with pectinases/PME, the pectin degrades and converts the orange slice into juicy lump in comparison to control, in which orange slice was treated with distilled water but not with enzyme. The juice/extract was purified and viscosity was determined. It was found that enzyme treated sample (test) was more viscous in comparison to control. The protein content in pectinases was determined by Bradford method. It was found that total protein content per gram of apple was 1.7 g/apple sample. The antimicrobial activity of purified pectinases was determined by well diffusion method. The
results confirmed the strong antimicrobial potential against all the pathogens studied. The results were found to be more surprising when the purified enzyme showed the maximum activity against MRSA amongst all the pathogens studied.

**KEYWORDS:** Pectin, apple pomace, Pectin Methyl Esterase (PME)/Pectinase, protein content, electrophoresis, HPLC, antimicrobial activity.

**INTRODUCTION**

Pectin is the complex polysaccharide found in fruits and vegetables and helps in ripening. The highest concentration of pectin is found in the middle lamella of cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane. Apple is particularly rich in pectin, the name applied to any one of a group of white, amorphous complex carbohydrates with a high molecular weight. Researchers have found that raw apples are the richest fruit sources in pectin. The characteristics structure of pectin is a linear chain of a \((\beta-1-4)\)-linked D-galacturonic acid that forms the protein-backbone, a homogalacturonan. The highest concentrations of pectin are found in the middle lamella of cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane. \[1\] Pectin esterase catalyzes the deesterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol. \[2\] The resulting pectin is then acted upon by PG and PL. \[3\] Production, biochemical characterization, and applications of PNL have been reviewed extensively. \[4\] Among all pectinases, PNLs are of particular interest because these degrade pectin polymers directly by a b-elimination mechanism that results in the formation of 4,5-unsaturated oligogalacturonides, while other pectinases act sequentially to degrade the pectin molecule completely. Pectin esterases are found in plants, plant pathogenic bacteria, and fungi \[5\] while PGs are widely distributed among fungi, bacteria, and many yeasts. \[6\] Numerous studies on fungal pectolytic enzymes have been carried out and several fungal PNL genes have been isolated and characterized from *Aspergillus niger*, *A. oryzae* and *Glomerella cingulata*. (ramie, hemp, flax, bast), maceration of plant tissues, isolation of protoplasts, and saccharification of biomass.\[7-10]\ Antimicrobial and antioxidant activities of the pectin extracted from apple and pomace were determined. \[11\] In the present study, PME (Pectinases) was purified and characterized from apple pomace. The enzyme was assayed for its biochemical and physical properties. The enzyme was evaluated for its antimicrobial profile against pathogens and drug resistant strains.
MATERIALS AND METHODS

All the chemicals and reagents used in the experiments were procured from C.D.H and Ranchem. Glass wares used were of Borosil. The media and broth used for microbial culture was procured from Hi-Media Ltd., Mumbai.

Collection of Sample

The apple fruit samples were collected in sterilized conditions from local gardens/retail market in sterilized polythene bags and were stored at 4°C in a refrigerator until use.

Extraction and Purification of Pectinases (Pectin methyl Esterase)

The enzyme was produced by using the optimized conditions and strain, *Aspergillus niger* was inoculated in apple pomace in solid state fermentation (SSF). For SSF, 800 g of moistened apple pomace was supplemented with optimum concentration of best nitrogen source. The flask was incubated for 48-72 h. After incubation, the whole content of the flask was separately and enzyme was extracted with 0.25 M NaCl at 40 ml/100 ml of medium. The extract was filtered through muslin cloth and was centrifuged at 10,000 rpm for 30 minutes to obtain the clear extract of the enzyme. Pectin esterase in the crude extract was precipitated between 20-80% saturation of ammonium sulphate. The active fractions were pooled out and were stored at 4°C. The partially purified enzyme preparation was used for characterization as well as for evaluation of its activity and other related procedures. Further optimum pH and temperature was determined for proper functioning of the enzyme.

Characterization of Pectinases (Pectin Methyl Esterase) via Chromatographic and Spectroscopic Techniques

Characterization of pectinases (pectin methyl esterase) purified from apple pomace was carried out by spectrophotometric methods as described. Purification and characterization of Pectinases was done by using a combination of different techniques including HPLC and FT-IR.

High-performance Liquid Chromatography (HPLC)

HPLC analysis was performed in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a Shimadzo LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostated flow cell and a selectable two wavelengths of 190 - 370 nm or 371–600nm. The detector signal was recorded on a Shimadzo LC2010 integrator. The column used was a Chiral Column block.
heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 μm. Mobile phase was used containing 50 % acetonitrile along with 50 % Phosphate buffer was used at a flow rate of 3.0 ml/min, column temperature 25°C. Injection volume was 40 μl and detection was carried out at specific wavelength having maximum absorbance as calculated by UV absorption spectra at maximum wavelength.

**Fourier Transform Infrared (FTIR) Studies**
The IR spectrum of purified enzyme was recorded in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a computerized FTIR spectrometer (Perkin Co., Germany) in the range of 4400–400 cm\(^{-1}\) by the KBr pellet technique.

**SDS-PAGE Analysis and Zymography of Pectinases**
The protein profile and the presence of enzyme were confirmed by SDS-PAGE analysis.\(^{[14]}\) The SDS-PAGE was performed in 13 % w/v gels and samples were heated for 10 minutes at 45°C in the sample buffer before loading in the wells. After electrophoresis, gels was soaked in 2.5%w/v Triton-X 100 for 30 minutes, washed in 100 mM Glycine buffer pH, 10.0, 1.5 mM CaCl\(_2\) for 30 minutes and overlaid with 1% agarose gel containing 0.1% polygalacturonic acid in the same buffer as above. After 30 minutes of incubation at 45°C, gel was stained with 0.05% w/v ruthenium red (Sigma) for 10 minutes and was washed with water until PME bands became visible. Further pectinases bands were also observed by staining gel with Coomassie brilliant blue.\(^{[14]}\)

**Qualitative Assays of Pectinases Enzyme**

**(a) For ripening of raw fruits and pectin degradation**
The efficiency of extra cellular activity of pectinases enzyme was determined as per the method prescribed.\(^{[15]}\) Fine orange slices of 20g weight were introduced in two beakers. In one beaker, 5-10 ml of partially purified pectinase was added and in the other beaker, distilled water was introduced in same quantity. This set up (after stirring the contents well enough) was placed inside the boiling water bath at 40°C for 30 minutes. With the help of filter paper, the juicy watery part was filtered from the beakers.

**(b) For Degradation of Pectin in Pectate-Agar Plate**
The enzyme assay of pectinases was performed using Pectate agar plates.\(^{[16]}\) The agar plates were prepared using 0.5% pectin with 1.5% agar. A 10 mm diameter well was punched aseptically with the help of sterile borer. The wells were filled with different concentrations
of the pectinases enzyme sample will be incubated at 37°C for overnight. The substrate utilization zone was observed by overlaying 1% Iodine solution prepared in 95% ethyl alcohol and was kept for 10-15 minutes for the appearance of zones.

3.6 Quantitative Estimation of Protein Content in Pectinases for Enzymatic Activity

Pectinases enzyme was assayed by using TBA (0.01M)/ HCl (1N). One ml of crude enzyme was added to 1.5ml of substrate (1.2% pectin in 0.2M Tris-HCl, pH 8.5) and was incubated at 35°C for 1 hour. The reaction mixture was terminated by adding 1.5ml of 0.01M TBA and 0.7ml of 1N HCl. The contents were boiled in water bath for 20 minutes. Absorbance then after was measured at 540nm in UV-VIS spectrophotometer. The total protein content will be measured using Bradford method. One ml of enzyme sample was mixed with 5 ml of Bradford reagent. BSA (100 µg/ml) was used as standard.

Determination of in Vitro Antimicrobial Activity of Pectinases Enzyme Purified

The pectinases enzyme as such (in dilutions) was used for determination of in vitro antimicrobial activity.

Culture Media

The media used for antibacterial and antifungal test was Nutrient agar/broth and Sabouraud’s dextrose agar/broth respectively procured from Hi media Pvt. Bombay, India.

Inoculum

The pure pathogenic bacteria cultures were separately inoculated into nutrient broth and incubated at 37°C for 4 h and the suspension will be checked to provide approximately 10^5 CFU/ml. Similar procedure was done for fungal strains by inoculating in Sabouraud’s dextrose broth for 6 h.

Microorganisms Used

Pure cultures of various pathogenic bacterial and fungal strains, E. coli NCIM 2065, Lactobacillus plantarum NCIM 2083, Micrococcus luteus ATCC 9341, Salmonella abony NCIM 2257, Candida albicans NCIM 3471, Aspergillus niger NCIM 1196 and Multidrug resistant (MDR) strains of Staphylococcus aureus isolated from clinical specimens viz. pus and blood of infected patients were procured with authentication for the study.
Agar Well Diffusion Method
The agar well diffusion method was modified. [17] Nutrient Agar medium (NAM) was used for bacterial cultures. The culture medium was inoculated with the bacterial strains separately suspended in nutrient broth. Sabouraud’s dextrose agar (SDA) was used for fungal cultures. The culture medium was inoculated with the fungal strains separately suspended in Sabouraud’s dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with pectinases (100 µl) and solvent blank (N-saline) separately. Standard antibiotic (Azithromycin/Erythromycin, concentration 1mg/ml) were used as positive control. The bacterial plates were then incubated at 37°C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The same procedure was used for determining antifungal activity but in this case standard antibiotic (Fucanazole, concentration 1 mg/ml) was used as positive control and fungal plates were incubated at 37°C for 72 h.

RESULTS
Purification and Characterization of PME (Pectinases)
Pectinases/ Pectin Methyl Esterase (PME) was purified from apple pomace by consecutive steps of enzyme extraction and further precipitated by saturated concentration of ammonium sulphate. The extraction process is shown in Image 1. The results also confirm that the enzyme can activated at 45 to 55 °C and work well at a pH of 4.5 to 5.5. The enzyme was further purified and characterized by HPLC. The purified pectinase showed the retention time, 1.812 minutes with reference to standard pectinases which showed the retention time, 1.790 minutes. The HPLC chromatograms of purified pectinase and standard are recorded in Figure 1 (a &b). Further FT-IR spectra were recorded of the purified pectinases. The FT-IR spectrum of PME is shown in Figure 2.

SDS PAGE Analysis and Zymography of PME (Pectinases)
The enzyme purified was loaded on agarose gel and further stained with ruthenium red and coomassie brilliant blue separately. The enzyme purified showed molecular weight 43 kDa as compared to standard marker. The results are shown in Figure 3 and 4.

Qualitative Assays of Pectinases Enzyme
The enzyme purified was qualitatively assessed for pectin degradation in fruits. It was observed that in case of Test, orange slice incubated with pectinases/PME, the pectin degrades and converts the orange slice into juicy lump in comparison to Control, in which orange slice was treated with distilled water but not with enzyme. The juice/extract was
purified and viscosity was determined. It was found that enzyme treated sample (test) was more viscous in comparison to control. The results are shown in Figure 5.

Purified pectinases were further utilized to observe the degradation of pectin in pectin-agar plate. The zone of degradation of pectin by pectinases was observed by overlaying 1% Iodine solution prepared in 95% ethyl alcohol. Purified pectinases degrade the pectin in pectin-agar plates in comparison to control. The results are shown in Figure 6.

**Quantitative Estimation of Protein Content in Pectinases**

The protein content in pectinases was determined by Bradford method. It was found that total protein content per gram of apple was 1.7 g/apple sample. The results are shown in Table 1 and Figure 7.

**Antimicrobial Activity of Pectinases**

The antimicrobial activity of purified pectinases was determined by well diffusion method. The results confirmed the strong antimicrobial potential against all the pathogens studied. The results were found to be more surprising when the purified enzyme showed the maximum activity against MRSA amongst all the pathogens studied. The results are shown in Table 2 and Figure 8 (a & b).

![Image 1: Extraction of pectinase from apple pomace.](image-url)
Figure 1 (a): HPLC chromatogram of standard pectinases (PME)

Figure 1 (b): HPLC chromatogram of purified pectinases (PME)
Figure 2: FT-IR spectra of purified pectinases (PME)

![FT-IR spectra](image)

Figure 3: Electrophoretogram showing bands of PME stained with ruthenium red showing molecular weight 43 kDa (MM, Marker; CE, Enzyme fraction before ammonium sulphate precipitation; CM, Enzyme Fraction after ammonium sulphate precipitation; Sdex 75, Final Gel chromatography fraction)

![Electrophoretogram](image)
Figure 4: Electrophoretogram showing single band of PME stained with Coomassie brilliant blue showing molecular weight 43 kDa.

Figure 5: Conversion of orange juice by purified pectinases.
Figure 6: Degradation of pectin by purified pectinases on pectin-agar plate.

Table 1: Estimation of protein content in pectinases by Bradford Method.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>BSA aliquots/Sample (ml)</th>
<th>Phosphate buffer (ml)</th>
<th>Bradford’s reagent (ml)</th>
<th>Absorbance (540 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Blank</td>
<td>1.0</td>
<td>5</td>
<td>0.00</td>
</tr>
<tr>
<td>2.</td>
<td>0.2</td>
<td>0.8</td>
<td>5</td>
<td>1.27</td>
</tr>
<tr>
<td>3.</td>
<td>0.4</td>
<td>0.6</td>
<td>5</td>
<td>1.32</td>
</tr>
<tr>
<td>4.</td>
<td>0.6</td>
<td>0.4</td>
<td>5</td>
<td>1.42</td>
</tr>
<tr>
<td>5.</td>
<td>0.8</td>
<td>0.2</td>
<td>5</td>
<td>1.43</td>
</tr>
<tr>
<td>6.</td>
<td>1.0</td>
<td>----</td>
<td>5</td>
<td>1.47</td>
</tr>
<tr>
<td>7.</td>
<td>Test; Purified enzyme (1.0)</td>
<td>----</td>
<td>5</td>
<td>1.43</td>
</tr>
</tbody>
</table>

Figure 7: Protein content in purified pectinases.
Table 2: Antimicrobial activities of purified pectinases.

<table>
<thead>
<tr>
<th>Sample</th>
<th>E. coli</th>
<th>S. abony</th>
<th>M. luteus</th>
<th>L. plantarum</th>
<th>MRSA 35</th>
<th>MRSA 8</th>
<th>niger</th>
<th>C. albicans</th>
</tr>
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<tbody>
<tr>
<td>Purified Pectinases/ PME</td>
<td>22</td>
<td>11</td>
<td>11</td>
<td>13</td>
<td>30</td>
<td>35</td>
<td>38</td>
<td>21</td>
</tr>
<tr>
<td>Erythromycin (1 mg/ml)</td>
<td>27</td>
<td>28</td>
<td>32</td>
<td>33</td>
<td>30</td>
<td>NA</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Fucanazole (1 mg/ml)</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>24</td>
<td>12</td>
</tr>
</tbody>
</table>

*NA, No Activity; NT, Not Tested

Figure 8 (a): Graphical representation of antimicrobial spectrum of purified pectinases.
DISCUSSION

Previous studies already have already documented the enormous industrial significance of enzyme pectinases/PME. Apple pomace is more susceptible to microbial decomposition and thus acts as an ideal substrate for the production of pectinases. Pectinases constitute a unique group of enzymes which catalyze the degradation of pectic polymers present in the plant cell
walls. \cite{18} Pectinases are produced by many organisms such as bacteria, \cite{19} fungi \cite{20} and yeasts. \cite{21} In the industrial sector, acidic pectinases are used in the extraction and clarification of fruit juices, whereas alkalophilic pectinases are in immense use in the degumming of ramie fibers, \cite{22} retting of wax, \cite{23} plant protoplast formation and treatment of effluents discharged from fruit processing units. Pectinase enzyme which includes pectin methyl esterase and depolymerizing enzymes find extensive application in fruit processing industries for clarification of fruit juices and wines, in the manufacturing of pectin free starch, curing of coffees, cocoa and tobacco, refinement of vegetable fibres and is used as an analytical tool for the estimation of plant products. \cite{24-26} Pectinase is produced by several fungi but \textit{Aspergillus} is found to be the most prominent source. \cite{19} Therefore the study was undertaken to reveal the various pharmacological properties of pectinase. Although the present studies also adds a new chapter in the pharmacological importance and significance of enzyme, pectinases/Pectin methyl esterase. The present investigation emphasized the role of apple and other fruits for intake of pectinases which can be utilized as an effective strategy in treatment of severe and non treatable microbial infections. Apart from this the purified pectinases has molecular weight of 43 kDa which can be utilized as a standard protein marker in determination of other unknown proteins and enzymes. The results also confirm that the enzyme can activated at 45 to 55 °C and work well at a pH of 4.5 to 5.5. The enzyme can be utilized in extraction of fruit juices without adding synthetic extractants in fruits. The work thus can create a stepping stone in area of enzyme research. Further studies can be done in order to investigate other pharmacological properties of the enzyme.

**REFERENCES**


