

Dehairing, Destaining, and Antibacterial Potential of Protease from Waste Fruit Seeds

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ABSTRACT

Background: Proteolytic enzymes are extensively utilized in medicine, agriculture, and industry. However, limited research exists on the extraction and partial purification of proteolytic enzymes from waste fruit seeds such as Watermelon (*Citrullus lanatus*), melon (*Cucumis melon*), and Cantaloupe melon (*Cucumismelo cantalupensis*). Therefore, the present study focused on extracting and partially purifying proteases from these seeds. **Methods:** Crude enzyme extracts were initially prepared, followed by partial purification through ammonium sulfate precipitation. The proteolytic activity of the enzymes was screened using casein agar medium. In addition, their dehairing and destaining potentials were assessed, while antibacterial activity was evaluated using the agar well diffusion method. **Results:** The findings confirmed the presence of protease enzymes in waste fruit seeds. The extracted proteolytic enzymes effectively removed protein-based stains, demonstrating strong dehairing and destaining potential. Moreover, the partially purified extracts exhibited stronger antibacterial activity than crude extracts against *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. **Conclusions:** Overall, the results indicate that protease extracts from waste fruit seeds can be utilised as promising antibacterial agents with potential applications in dehairing and destaining.

Keywords: Applications of proteases; antibacterial activity; de-hairing assay; de-staining assay

INTRODUCTION

Enzymes are found in every organism and act as biocatalysts that carry out several chemical reactions and are hence used in pharmaceutical, detergents, diagnostic, food and fine chemical industries (Kumar and Takagi, 1999; Gupta et al., 2002). Enzymes are widely used in various applications like the beverage industry, the drug industry, the food industry, the tannery and the textile industry. The main industries that frequently use commercially produced enzymes are pharmaceutical (41%), leather and paper (17%), food and feed (17%), textiles (8%), and

detergents (17%) (Leimkuhler and Iobbi Nivol, 2015). Proteases have extensive applications in various industries (Ullah et al., 2022). The particular applications of mechanical enzymes are in main areas of leather, paper and pulp, detergents, textiles, beverage production, food processing, as well as in animal nutrition (Sawant and Nagendran 2014). Proteolytic enzymes constitute about 60% of the industrial enzyme market and play a key role in the food industry, including meat tendering, production of protein hydrolysate, bread-making and cheese ripening (Poldermans, 1990). With the advancement in

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biotechnology, the use of amylase and protease has been extended into many fields, such as medicine and pharmaceuticals (Negi & Banerjee, 2006).

Protease production can be obtained from various sources such as plants, microbes (Viruses and archaea), and animals. From cells to organs and organisms, the proteases perform important activities and complicated processes like digestion and homeostasis (Rao et al., 1998). During development and germination, seeds accumulate a large amount of proteins serving as a source of nitrogen, sulfur, and carbon, which are necessarily required for growth (Shotwell et al., 1988). These proteins are broken down by the proteases. The main source of protease enzymes is plants (Greenberg and Winnick, 1995). Various physiological processes of proteolytic enzymes determine the potential of proteases that are important in break down of proteins which is present in food, division of cell, protein turnover, series of blood clotting reactions, transduction of signals, programmed cell death (apoptosis) and hormones processing (Frolova et al., 2023; Bouno et al., 2019; Mahajan and Badgajar, 2010). Medicinal plants are used for the production of proteases, which rely upon land which is suitable for agriculture and also has a definite climate (Devaraj et al., 2008). Proteases are ubiquitous in nature and involved in the regulation of all aspects of the life cycles of plants. Plant proteases are involved in germination of seeds, regulation of ageing processes, recycling of damaged plant proteins, and modification of proteins to carry out specific functions in plant cells (Kumar et al., 2011).

Nowadays, various aspects of proteases are extensively studied by scientists because of their demand in various industries and increased hourly consumption throughout the world (Beg and Gupta, 2003). So, the aim of current research was to isolate the proteases from the waste fruit seeds and to evaluate their antibacterial, destaining and dehairing effects (Figure 1). The use of seed proteases could be more effective in the field of medicine to cure the transmission of infectious pathogens.

MATERIALS AND METHODS

Chemicals, glassware, and equipment

Nutrient Agar, Nutrient Broth, Sodium chloride (NaCl), Sodium Phosphate (Na_2HPO_4

NaH_2PO_4) buffers pH (8.0), 3 Mm DDT (dithiothreitol), 1 Mm Ethylenediaminetetraacetic acid (EDTA), 10 Mm sodium metabisulphite, and 50 Mm Tris buffer (pH 8.0), Muller-Hinton (MH) Agar (4.5g), Casein (0.75g) and Glucose (0.75g), 0.01 M phosphate buffer, ethanol, Laminar flow, Beakers, Petri plates, Mortar and pestle, Centrifuge machine, Autoclave, incubator, shaking incubator, bacterial inoculation loop, spreader, spirit lamp, 250 ml flasks, beakers, testtubes and testube racks.

Seed collection and germination

Fruit seeds of Water Melon (*Citrullus lanatus*), Honeydew Melon (*Cucumis melon*) and Cantaloupe (*Cucumismelo cantalupensis*) were collected from household waste, rinsed with the running tap water to clean the seeds, dried and allowed to germinate in Petri plates on filter paper containing NaCl solution and Na-Phosphate ($\text{Na}_2\text{HPO}_4\text{NaH}_2\text{PO}_4$) buffers (pH 8) in a dark room for 120 h or up to seed germination. The seeds were moistened with distilled water to keep the moisture balance for germination. The germinating seeds were washed with distilled water for further analysis.

Extraction and purification

The germinated seeds were collected, and protease extraction was done using homogenization in a food processor with the solution containing 1 mM EDTA, 10 mM sodium metabisulfite, 50 mM Tris buffer (pH 8.0) and 3 mM dithiothreitol (DTT). After homogenization, sample clarification was done by using centrifugation at 3000 rpm for 30 min. Crude extract (supernatant) was collected, and proteases were purified initially by the ammonium sulphate precipitation method (Nam et al., 2016). After the addition of ammonium sulphate, centrifugation was done at 2000 rpm for 10 min. The pellet was collected and resuspended in phosphate buffer (pH 8.0) (Purwanto, 2016).

Proteolytic assay

Proteolytic activity of both crude and purified protease extract was determined by agar well diffusion method (Rios et al., 1988). The activity was done on medium composed of Muller-Hinton Agar (4.5g), Casein (0.75g) and Glucose (0.75g). The proteolytic activity was checked by making small wells in the medium and filling them with both crude and purified proteases, and then left for 24 h at 37°C. The proteolytic activity was done by the

degradation of casein. Zone of clearance around the wells showed casein hydrolysis and was recorded (Kumar et al., 2005).

Enzymatic dehairing

The dehairing activity was performed using the protocol of Badgujar and Mahajan (2009). The fresh goatskin was cut into 2×2 cm pieces and rinsed with a local detergent. In a container, crude protease enzyme and 0.01 M phosphate buffer (control) in a proportion of 5.0 mL of liquid enzyme per g of skin and 8 to 12 g of the fresh fleshed goat skin were placed. After incubation at room temperature, loose hairs were easily removed by gently scraping the skin gently by using fingers. Dehairing of skin begins with 5.0 U/mg of protein, and it finishes in 18 h at pH 7.0 and temperature 30°C.

Destaining studies

The destaining effect of both crude and purified proteases was determined using the methodology of Banerjee et al. (1999). A small part of the cloth was stained with blood and allowed to dry. A cloth piece, having been stained with blood, was dipped into formaldehyde for 30 min and rinsed with water to get rid of extra formaldehyde. The cloth pieces were incubated at 45-50°C with proteases. After an incubation period of 5-7 h, each piece of cloth was washed with water and then dried. The same process was done for the control.

Antibacterial activity

Antibacterial activity of both crude and purified protease extracts of Water Melon (*Citrullus lanatus*), Honeydew Melon (*Cucumis melon*) and Cantaloupe (*Cucumismelo cantalupensis*) was checked by agar well diffusion method (Balouiri et al., 2016). For this purpose, six microbial strains: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* strains were grown in nutrient broth medium at 37°C overnight. Bacterial cultures were mixed with MH agar medium, poured into Petri dishes and placed at room temperature under laminar flow to solidify. After solidification, six wells were made in each plate with the help of sterilised yellow tips (to make 5 mm wells). In three wells, 100 µl crude extract of proteases was added, whereas in the remaining three wells, 100 µl purified protease extract was added. All these plates were placed at 37°C, and the zone

of inhibition was recorded after 24 h of incubation. The growth sensitivity was recorded as (0) for no sensitivity, 1-3 for low sensitivity, >3-7 for moderate sensitivity, and >7 to 15 for highest sensitivity.

Statistical analysis

Each experiment was repeated in triplicate. Mean $\pm$ Standard Deviation from absolute data was calculated using an online calculator (<http://easycalculation.com/statistics/standard-deviation.php>).

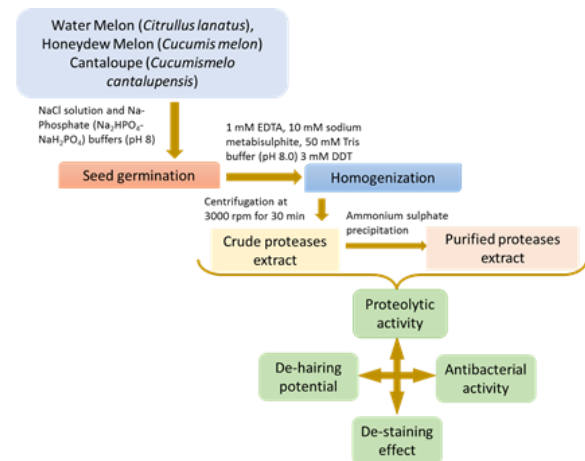


Figure 1: Experimental design to extract and evaluate the biological activities of fruit seed proteases.

RESULTS

Proteolytic activity

The test was conducted to determine the presence of enzyme proteases in seeds of water melon, honeydew melon and cantaloupe melon. During the test, casein was used as a reference, which is favorably to be hydrolysed by enzyme proteases. Clear zone formation was observed at the surrounding of the wells on the agar plates. Figure 2 indicates that proteases that digested the casein are present in both crude and partially purified extracts of seeds of watermelon, honeydew melon and cantaloupe melon.

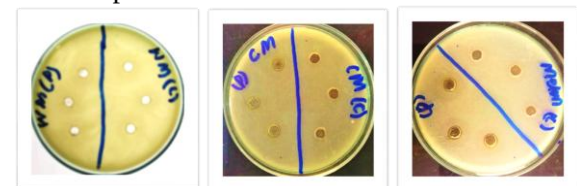


Figure 2: Casein hydrolysis by crude and purified protease extracts of seeds of watermelon, cantaloupe melon and honeydew melon. WM (Water melon); CM (cantaloupe

melon); melon (honeydew melon); P (Purified extract); C (Crude extract).

Dehairing effect

Proteases produced by seeds were used as a dehairing agent by applying them to goat hairy skin to exploit their commercial significance. Results revealed that the skin depilation was completed within 18 h of incubation. The highest dehairing activity was shown by crude and purified extracts of watermelon seeds, while the other two samples showed less dehairing effect. In the case of control, no hair removal was observed (Figure 3).

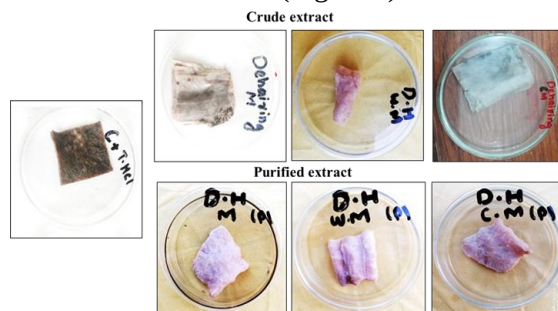


Figure 3: Dehairing from goat skin using crude and purified protease extracts obtained from seeds of watermelon, cantaloupe melon and honeydew melon. WM (Water melon); CM (cantaloupe melon); melon (honeydew melon); P (Purified extract); C (Crude extract).

Removing blood stain

Application of protease as a detergent additive was studied on white cotton cloth pieces stained with human blood. It was observed that the crude extract of all tested seeds showed less destaining power compared to purified extract of seeds (Figure 4).

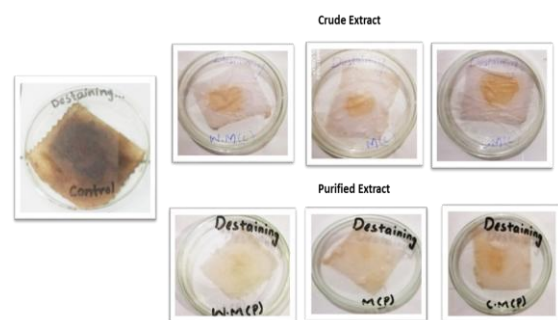


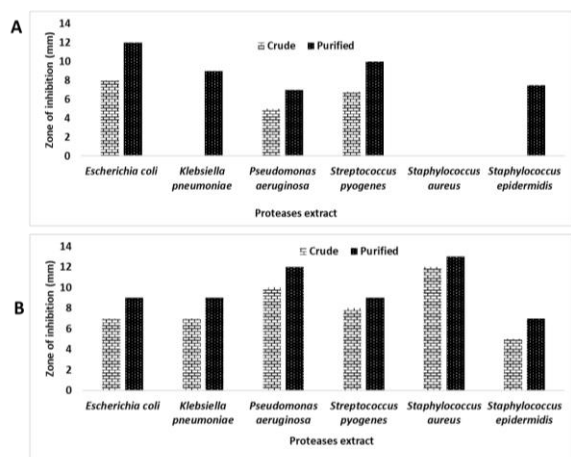
Figure 4: De-staining effect of crude and purified protease extracts obtained from seeds of watermelon, cantaloupe melon and honeydew melon. WM (Watermelon); CM (cantaloupe melon); melon (honeydew melon); P (Purified extract); C (Crude extract).

Antibacterial activity

Antibacterial effect of crude and purified protease extract of all seeds was analysed against clinical bacterial pathogens through the agar well diffusion method. In the present study visualised zone of inhibition ranged from 0-14 mm (Figure 5). All purified protease extracts had a significant antibacterial effect compared to crude protease extracts. It was observed that purified protease extract of watermelon showed maximum inhibition of *E. coli*, *S. pyogenes*, *K. pneumoniae*, and *S. epidermidis* with inhibition zones of 12.0 ± 0.0 mm, 10.0 ± 0.0 mm, 9.0 ± 0.0 mm, and 7.5 ± 0.0 mm, respectively. On the other hand, neither the extract of watermelon nor the extracts of the other species affected the growth of *S. aureus* (Figure 5A).

Crude protease extract of honeydew melon showed maximum inhibition of *P. aeruginosa*, *S. pyogenes*, and *S. aureus* with 10.0 ± 0.0 mm, 8.0 ± 0.0 mm, and 12.0 ± 0.0 mm while purified protease extract of honeydew melon showed maximum inhibition of *P. aeruginosa*, *S. pyogenes*, *S. aureus*, *E. coli*, and *K. pneumoniae* (12.0 ± 0.0 mm, 9.0 ± 0.0 mm, 13.0 ± 0.0 mm, 9.0 ± 0.0 mm, and 9.0 ± 0.0 mm) (Figure 5B).

Antibacterial effect of purified protease extract of cantaloupe melon showed the maximum inhibition of *E. coli*, *S. pyogenes*, and *S. epidermidis* (10.0 ± 0.0 mm, 11.0 ± 0.0 mm, and 13.0 ± 0.0 mm). Similarly, crude protease extract of cantaloupe melon showed the maximum inhibition of *S. pyogenes*, and *S. epidermidis* 11.0 ± 0.0 mm, and 8.0 ± 0.0 mm, respectively (Figure 5C). On the other hand, crude protease extract of cantaloupe melon had no antibacterial effect on the growth of *K. pneumoniae* and *S. aureus*.



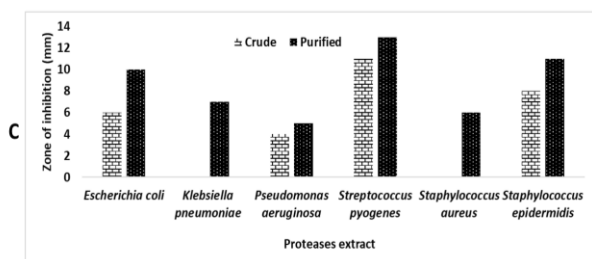


Figure 5: Antibacterial efficacy of crude and purified protease extracts obtained from seeds of watermelon, cantaloupe melon and honeydew melon. A. water melon; B. honeydew melon; C. cantaloupe melon.

DISCUSSION

In fermentation biotechnology synthesis of commercially important enzymes, especially proteases, through an appropriate organism has been a revolutionary achievement. Plant serine proteases are widespread among taxonomic groups, from trees and crops to legumes and herbs. They are also present in almost all plant parts, but seem to be more abundant in fruits (Niemer et al., 2016). Our results are consistent with the findings of Niemer et al., 2016, as in current research, both crude and purified extracts of all types of fruit seeds showed the presence of proteases. Previously, exploration of plant proteases has been widely considered due to their importance in the food and pharmaceutical industries (Kim et al., 2016). Considering the importance of the enzyme, protease enzymes have been widely studied by researchers from various plants and food crops (Sequeiros et al., 2016). Previous literature studies have reported the presence of proteases in various plant sources like rice, tomato plant, cucumber, squash, potato plant, maize, papaya, etc. The protease enzyme can be categorised into four groups: serine, aspartate, metallo and cysteine proteases, respectively. Among these groups, cysteine proteases are predominantly found in plant sources (Domsalla and Melzig, 2008).

Despite its importance in various fields, the extraction of proteases from legumes remains minimal in the current scenario (Foss and Eriksen, 1995). In the current study, seeds of watermelon, melon and cantaloupe melon, belonging to the well-known *Cucurbitaceae*, were considered for germination and the extraction of protease. The seeds were initially germinated, and enzymes were extracted, partially purified, and used for different activities such as destaining and dehairing by

using the existing methods with slight modifications. Determination of protease activity was estimated by using casein agar medium. Casein hydrolysis showed the zone of clearance. These zones also indicate the digestion of protein by proteolytic enzymes present in the seeds of watermelon, melon and cantaloupe melon. Our findings agreed with Marathe et al. (2018). Some earlier workers have also used skim milk agar medium and casein agar medium for the screening of protease (Lakshmi et al., 2014; Gessesse and Gashe, 1997). Our findings agree with earlier reports (Adinarayana et al., 2003). When protease was applied to a white cotton cloth, the blood stain was removed by incubating the cloth with partially purified protease for different time intervals. After an interval of time of incubation, the blood was completely destained by the protease enzyme. It was observed that the partially purified protease from seeds of watermelon, melon and cantaloupe melon had a high capability of removing the blood stain as compared to crude extracts. Results indicated that protease has potential application in the field of detergent industries, and our outcomes are consistent with previous literature (Mahajan et al., 2015). Ramachandran and Arutselvi (2013) reported that the protease from *Bacillus clausii* has the ability to remove blood stains very effectively.

Removal of blood stains with minimum use of commercial detergent within a short time can be done through proteases (Badgujar and Mahajan, 2012). Destaining results of crude and purified extracts of seeds are in good accordance with the earlier explanation, investigated in washing presentation of protease of *Bacillus subtilis* (Mahajan et al., 2015) and *Bacillus* sp. JB-99 (Shivasharana and Naik, 2012).

Dehairing of the skin was completed after 18 h, while the skin which is treated with only Tris HCl remained unaffected. Therefore, seed-based proteases could potentially be applied for dehairing purposes in the leather industry, and our results agreed with Akhtar et al. (2024). They produced, purified and optimised the production of oilseed-based protease that showed efficient destaining and dehairing. To improve leather quality plant plant-derived protease can be the best source over chemically produced proteases, and this trend is getting more attention nowadays

(Sohag et al., 2013; Sivasubramanian et al., 2008). Recently plant protease are widely used because they are eco-friendly which are helpful in reducing environmental pollution which is caused by leather processing.

Plant derived proteases are also used in bioremediation processes. Seventy percent pretanning process of leather are directly involved in environmental pollution (Ramasami and Prasad, 1991). Skin and hair composition is mostly proteinaceous. Sodium sulfide and lime are hazardous chemicals that are added to the environment during leather processing. Most of the proteolytic enzymes are used for the hydrolysis of proteinaceous of the skin (Rao et al., 1998). Result suggested that plant derived enzyme can be a used as alternative in dehairing applications. Reduction of environmental pollution and improvement in leather quality can also be achieved by using plant proteases.

Antibacterial affect of crude and purified extract of seeds of watermelon, melon and cantaloupe melon was analyzed against clinical pathogens (*E. coli*, *P. aeruginosa*, *S. pyogenes*, *K. pneumoniae*, *S. aureus* and *S. epidermidis*) through agar well diffusion method. Results revealed that Purified extracts of all seeds of fruits had bactericidal effect as compared to crude extracts. Purified extract of water melon showed highest inhibition of *E.coli*, melon showed maximum inhibition of *S. aureus* and cantaloupe melon showed highest inhibition of *S. pyogenes*. On the other hand crude extract had low or even no bactericidal effect. Our results are consistence with the findings of Osman et al. (2021). Our results are agreed with the findings of Ngai and Ng (2004). They reported that Napin line protein from cabbage seed had antibacterial effect against *P. aeruginosa*, *B. subtilis*, *B. cereus* and *B. megaterim*. Mohajeri et al. (2010) concluded that actinidin from kiwi fruit extract has antibacterial effect. Hafezi et al. (2010) illustrated the strong proteolytic effects of actinidin. Basile (1997) demonstrated the significant antibacterial activity against bacterial strains via using Actinidia chinensis extract. In current research protease extracts of all types of fruit seeds showed the antibacterial effect against both Gram-negative bacteria (*E. coli*, and *P. aeruginosa*) and gram-positive bacteria (*S. aureus* and *S. pyogenes*). The antibacterial effect differences were due to the differences of both Gram negative and Gram-

positive bacterial cell wall structure and enzyme concentration. Our results agreed with the Farouk (1982). He concluded the bactericidal effect of proteolytic enzymes was enzyme concentration dependent. In current research proteases showed antibacterial effect against both Gram⁺ and Gram⁻ bacteria. Antimicrobial proteases are broadly present in agricultural plant species and are helpful in medicinal field. Walsh (2002) reported that proteolytic enzymes play important role in food and detergent industries as well as therapeutic agents. The presence of antimicrobial peptides indicates their role against clinical pathogens. These peptides suggest that they play a key role in pharmaceutical industries (Glazebrook, 1999).

CONCLUSION

Based on the present findings, these seed sources can be considered a promising resource for the production of plant proteases with significant applications in both the detergent and pharmaceutical industries. The extracted proteases demonstrate potential for use as effective dehairing and destaining agents, as well as for therapeutic purposes.

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Statement of conflict of interest

No conflict of interest.

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