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## Research Article

# Characterization of detergent-stable proteases isolated from *Citrus sinensis* fruit peel

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**ABSTRACT:** Proteases are important group of enzymes with wide industrial applications including laundry. They have been isolated and characterized from different agro wastes but the use of *Citrus sinensis* fruit peel as a source of protease has not been reported. In this study, low molecular weight (10-17 kDa) proteases were purified and characterized from *C. sinensis* fruit peel using a combination of ammonium sulphate precipitation, dialysis and sephadex G150 column chromatography. One unit of protease activity was defined as the amount of the enzyme required to liberate one microgramme (1 µg) of tyrosine from gelatin per minute at 37°C under standard assay conditions. The elution profile obtained from gel filtration chromatography depicted three peaks of protease activity designated as proteases I, II and III. The purification fold obtained for ammonium sulphate precipitation, dialysis and proteases I, II and III fractions were 1.7, 3.85, 9.47, 9.84 and 8.47 respectively. The activities of Proteases I and II were significantly increased ( $p < 0.5$ ) in the presence of 5-10 % w/v, and 1-10 v/v % Tween 80 respectively. However, the activity of Protease III was not significant affected ( $p > 0.5$ ) by Tween 80. Triton-X significantly increased ( $p < 0.5$ ) Protease II activity at 5 to 10% v/v concentrations but did not significantly affect ( $p > 0.5$ ) the activities of Proteases I and III between 1 to 10% v/v concentrations. SDS significantly increased ( $p < 0.5$ ) the activities of proteases I and II at 5-10 % w/v, and 1-10 w/v % respectively but did not significantly affect ( $p > 0.5$ ) Protease III activity. Findings from this study suggest that proteases from *C. sinensis* fruit peel are detergent stable which may be useful in laundry industries.

**KEYWORDS:** *Citrus sinensis*, peel, proteases, detergent stability, laundry.

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## INTRODUCTION

The world production of citrus was 122.09 million tons in 2008 (FAO, 2010) and orange constituted about 60% of the total world citrus production. Several residue materials or wastes like peels, rags and seeds (Chapman *et al.*, 2000) are produced when fresh citrus fruits are processed into juice and canned fruit in developed countries or when they are peeled for direct human consumption as is the case in most developing countries like Nigeria (Oluremi *et al.*, 2010). Citrus peels represent between 50 to 65% of the total weight of the fruits and if not put into use, they can lead to production of bad odour, soil pollution, and harbourage for insects, which can give rise to serious environmental pollution.

In recent years, there is growing interest in efficient use of agro-industrial residues to produce commercial enzymes such as proteases (Ekpa *et al.*, 2010). Proteases (E.C. 3.4) are a group of enzymes that catalyse the cleavage of peptide bonds in proteins breaking them down into polypeptides or free amino acids (Khan *et al.*, 2011). They potentially hydrolyse compounds containing a peptide bond, from a dipeptide up to a large protein containing thousands of amino acids (Khan *et al.*, 2008). They are made up of complex group of enzymes and some of their properties such as substrate specificity, catalytic mechanism, temperature and pH optima and stability profile differ considerably (Sumantha *et al.*, 2005).

Proteases execute large variety of complex physiological functions; they are important in transporting essential metabolites and perform regulatory functions as evident from their occurrence in all forms of living organisms. Some of the functions include activation of zymogens and blood coagulation, cell growth and migration (Liaw and Crawford, 1999), morphogenesis, tissue arrangement (Parde *et al.*, 2014) and inflammation (Holzhausen *et al.*, 2005). They also catalyse important proteolytic steps in tumor invasions or infection cycle of several pathogenic micro-organisms and vessels, a 'quality' that makes proteases a valuable target for new pharmaceuticals (Camerer *et al.*, 2004).

Proteases constitute two thirds of total enzymes used in various industries (Gupta *et al.*, 2002). They have wide application in various industrial processes such as laundry detergents, food, pharmaceuticals, chemical, leather and silk, protein recovery, degradation of gelatin on X-ray films and organic synthesis (Tunga *et al.*, 2003). They are one of the components of standard ingredients used for household laundering and reagents used for cleaning contact lenses or dentures (Raimi *et al.*, 2011).

Several detergent-stable proteases in the presence of different detergents have been studied (Rao *et al.*, 2009). During industrial applications, the proteases are required to function under diverse environmental conditions where surfactants, oxidants, detergents and solvents are present (Sana *et al.*, 2006). Proteases can be obtained from animal, plant and microbial sources. Some examples include trypsin, chymotrypsin (pancreas), pepsin (stomach), bromelain (pineapple), papain (papaya latex), ficin (ficus latex), subtilisin (*Bacillus subtilis*), collagenase (clostridia), fungal proteases, viral proteases and other bacteria proteases (Evans *et al.*, 2009). They have been isolated and characterized from peels of banana (Ekpa *et al.*, 2010), cocoyam (Raimi *et al.*, 2001) and yams (Raimi *et al.*, 2005) but *Citrus sinensis* peels have not been reported as a source of protease. Therefore, this study was carried out to isolate, purify and characterise protease from *Citrus sinensis* fruit peel with a view to finding possible applications in the industries.

## MATERIALS AND METHODS

### *Citrus sinensis* (Sweet orange) peel

The sweet oranges were obtained from a plantation at the National Research Institute for Chemical Technology, Zaria. The fruits were identified and authenticated at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria with the voucher no 1432. The oranges were washed properly with distilled water and then gently peeled off using clean knife.

### Chemicals

Ammonium sulphate, L-cysteine and sodium azide were products of BDH Chemicals Limited, Poole England while Sephadex G-150 was obtained from Pharmacia Fine Chemicals Uppsala Sweden. The Molecular weight marker for SDS-PAGE was obtained from GE Healthcare UK Limited. All other chemicals are of analytical grade.

### Preparation of crude extract

The crude protease extraction was carried out according to the method described by Raimi *et al.* (2011) with slight modifications. About 250 g of the peel was weighed and blended in 600 ml of ice-cold 150 mM phosphate buffer (pH 7.4) using an electric blender for about ten minutes. The mixture was then filtered using a clean white piece of muslin

cloth. The filtrate was chilled and then left standing for 24 hours before it was centrifuged at 7000 Xg for 10 minutes. The clear supernatant solution obtained was gently decanted and the pellet discarded. The supernatant collected was further filtered to remove any undissolved particles from the solution using Wattman no 10 filter paper.

### Dialysis

The dialysis tube (4 cm x10 cm) containing the crude enzyme solution was suspended in 1 litre-beaker filled with ice-cold 150mM phosphate buffer pH 7.4. The solution was then subjected to dialysis against the buffer for 24 hour with continuous stirring and intermittent replacement of the buffer under temperature-regulated environment using magnetic stirrer.

### Sephadex G-150 chromatography

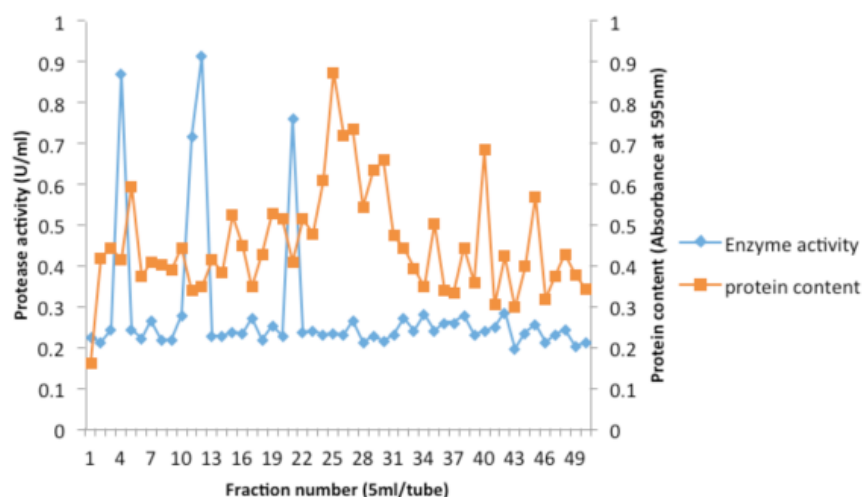
10 ml of dialyzed protein sample was carefully layered on pre-swollen sephadex G-150 that was previously equilibrated. A glass wool was then introduced on top of the protein sample before it was eluted with 50 mM Tris buffer solution (pH 7.8). A flow rate of 0.1 ml/min was maintained and 5 ml fraction was collected into each of the fifty sample bottles. Each fraction was tested for protease activity. The fraction with the highest enzyme activity was then identified and characterized.

### Molecular weight determination

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was carried out using the method described by Singh *et al.*, (2012) on vertical slab-gel unit Mini Protean II electrophoretic cell (Bio Rad Laboratories). The gel was run for about 30 minute at 75 V and later increased to 150 V for another one hour.

### Protease assay

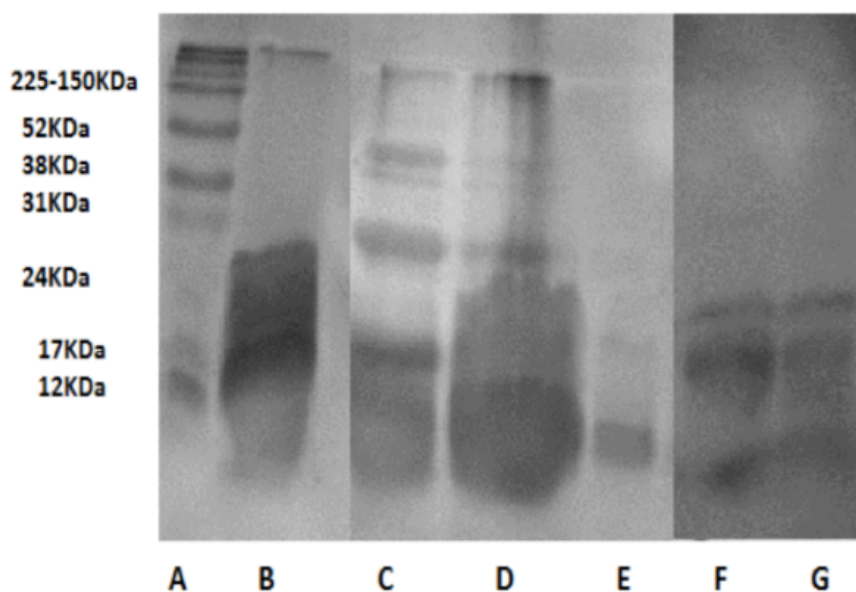
Protease activity was determined using the method described by Thangam and Rajkumar (2002) with slight modification. 0.1 ml of the crude enzyme source was added to 0.2 ml of 0.5% casein in 50 mM phosphate buffer (pH 7.4). The reaction mixture was incubated at 37 °C for 30 minutes and terminated by adding 1.5 ml of 5% TCA. The mixture was allowed to stand for 10 minutes and filtered through Whatman No.1 filter paper. To 1 ml of filtrate, 3 ml of 0.5 M Na<sub>2</sub>CO<sub>3</sub> solution and 1 ml of 3-fold-diluted Folin-Ciocalteu reagent was added and mixed thoroughly. A blank was prepared as described above except that the TCA solution was added before the enzyme. The colour developed after 30 minutes of incubation at 30 °C was measured in SpectrumLab 725s UV-Vis spectrophotometer at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate one microgramme (1 µg) of tyrosine from casein per minute at 37 °C under the assay conditions described.



**Figure 1: Elution profile of *Citrus sinensis* peel protease on Sephadex G-150 chromatography**

**Table 1: Summary of purification steps of protease from *Citrus sinensis* peel**

Purification step	Total activity (U/ml)	Total protein (mg)	Specific activity (U/ml/mg)	Purification fold	Yield (%)
Crude	100.07	94.98	1.05	1	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	37.74	21.17	1.78	1.7	37.72
Dialysis	63.64	19.05	3.34	3.85	63.61
Gel filtration					
Peak 1	23.46	2.36	9.94	9.47	23.44
Peak 2	24.37	2.36	10.33	9.84	24.35
Peak 3	20.98	2.36	8.89	8.46	20.97



**Figure 2: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Different Stages in the Purification of Proteases from *Citrus sinensis* Fruit Peel.** Lane A is the molecular marker; B is the dialysed protein; C is the ammonium sulphate fraction; D is the crude enzyme; E is the protease III (peak III); F is protease I (peak I); and G is protease II (peak II). Lanes from different SDS-PAGE images were spliced together in this figure to allow side by side comparisons.

## Protein determination

Protein concentration was quantitatively determined using Lowry's method and Bovine Serum Albumin used as standard (Lee *et al.*, 2010). The protein concentration was then estimated from a standard curve.

## Effect of surfactants (detergents) on the stability of the proteases

Triton X-100, Tween 80 (each in v/v) and sodium dodecyl sulphate, were used to assess the effects of surfactants on protease stability. 0.1 ml of varying concentration (0.1 - 10%) of each of the surfactants was incubated with equal volume of the enzyme preparation in 2 ml of 100 mM phosphate buffer at 37 °C for 30 minutes. Thereafter, 0.2 ml of the substrate was then introduced and the reaction carried at standard condition described earlier. Residual protease activity was then measured.

## Statistical Analysis

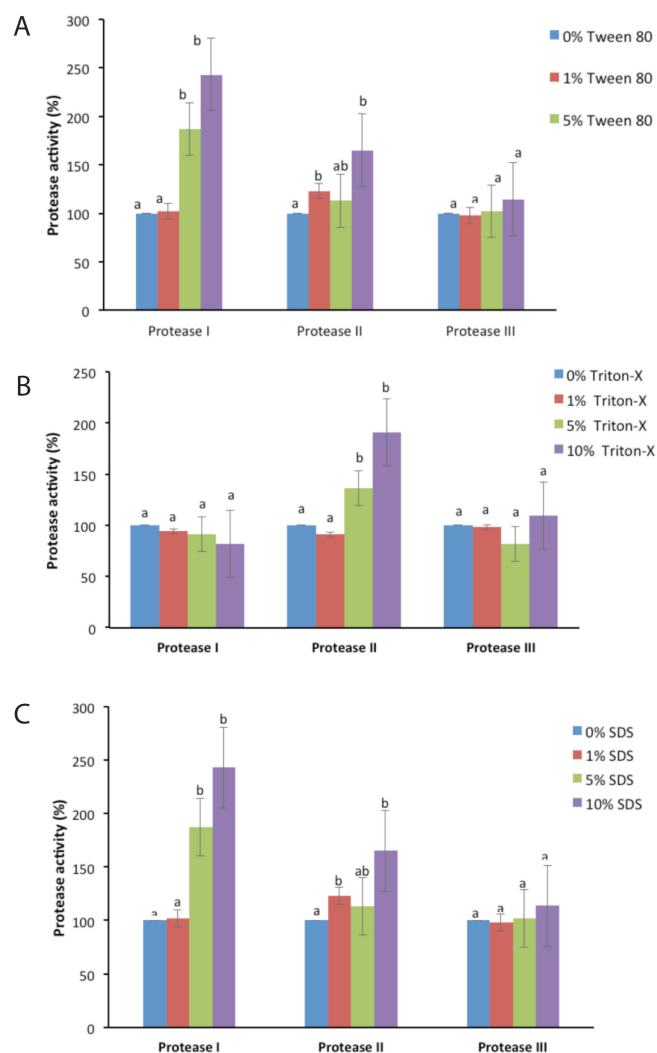
Results are expressed as mean of 3 determinations  $\pm$  standard error of the mean (S. E.M.). One-way ANOVA followed by post hoc Duncan's multiple range tests were used for analysis of data; differences were considered significant at  $p < 0.05$  when compared with control. The procedures were performed using SPSS software (20.0).

# RESULTS AND DISCUSSION

## Isolation of proteases from *Citrus sinensis* peel

Isolation of proteases in *Citrus sinensis* fruit peel has not been reported previously. In this study, proteases were isolated and characterized from *C. sinensis* fruit peel using a combination of ammonium sulphate precipitation, dialysis and sephadex G150 column chromatography. The elution profile obtained from gel filtration chromatography depicted three peaks of protease activity designated as proteases I, II and III (Figure 1). The purification fold obtained for ammonium sulphate precipitation, dialysis and proteases I, II and III fractions were 1.7, 3.85, 9.47, 9.84 and 8.47 respectively (Table 1). The overall yields of proteases I, II and III were 23.44, 24.35 and 20.97% respectively (Table 1).

The enzyme yields obtained in this study are like those reported in other plant proteases such as 12% yield of acidic protease from Indian beans, 8.0% yield of vicillin peptidohydrolase from mung bean seedlings and 3.38% yield for GA3-induced protease (Nzelibe and Wasike, 1995).



**Figure 3: Effects of tween 80 (a), triton-X (b) and sodium dodecyl sulfate (c) on the activity of *Citrus sinensis* fruit peel proteases.** Values are mean  $\pm$  S.E.M. of 3 determinations. Bars for each protease with the same alphabet are not significantly different ( $p > 0.05$ )

## Estimation of molecular weight of *Citrus sinensis* Fruit Peel Proteases

Figure 2 shows the SDS PAGE analysis of the proteases from *Citrus sinensis* fruit peel together with the molecular weight markers. The estimated molecular weight was 17, 17 and 10 kDa for proteases I, II and III respectively. This indicated that the proteases from *Citrus sinensis* fruit peel are of low molecular weight. The single band observed for protease III indicated the homogeneity and monomeric nature of the fraction. However, the multiple bands in the



lanes for proteases I and II suggest that these proteases may either likely have subunits or contaminated with another unwanted protein(s).

Proteases with low molecular weight have been previously isolated from different sources: 22 kDa from *Allium cepa* (Ndidi and Nzelibe, 2012), 22 kDa *Bacillus cereus* (Singh *et al.*, 2012), 23 kDa from *Aspergillus parasiticus* (Tunga *et al.*, 2003), 25 kDa from seeds of *Holarrhena antidysenterica* (Khan *et al.*, 2008), 25 kDa from *Aspergillus oryzae* (Lee, 2010) and 28 kDa from *Bacillus sp.* Though these enzymes have higher size than our finding, they are all classified as low molecular weight proteases. Proteases with relatively higher molecular weight have also been isolated from different sources: 31 kDa from germinated barley (Zhang and Jones, 1996), 39.5 kDa from *Bacillus circulans* (Rao *et al.*, 2009), 40 kDa from potato leaves (Guevara *et al.*, 2001), 46 kDa from capsules of *Capparis spinosa* (Demir *et al.*, 2008), 59 kDa from *Serratia sp.* (Li *et al.*, 2011), 61-63 kDa from wheat germ (Yang *et al.*, 2011), 64kDa from *Raphanus sativus* leaves (El-Sayed, 2001) and 67 kDa from *Cucumis trigonus* (Asif-Ullah *et al.*, 2006). Findings from this study and previous studies revealed the diversity of proteases from different sources.

#### Effect of surfactants (detergents) on the activity of *Citrus sinensis* fruit peel proteases

Surfactants are extensively used for solubilizing protein from lipid membranes and other biological materials, and for maintaining the solubility of certain proteins in the solution. The effects of surfactants (Tween 80, Triton-X and sodium dodecyl sulphate-SDS) on the activity of proteases from *Citrus sinensis* fruit peel at different concentrations (1-10 % w/v) were investigated in this study. The activity of the enzyme without any surfactant or detergent (control) was taken as 100%. The activities of Proteases I and II were significantly increased ( $p < 0.5$ ) in the presence of 5-10 % w/v, and 1-10 v/v % Tween 80 respectively (Figure 3a). However, the activity of Protease III was not significant affected ( $p > 0.5$ ) by Tween 80 (Figure 3a). Triton-X significantly increased ( $p < 0.5$ ) Protease II activity at 5 to 10% v/v concentrations but did not significantly affect ( $p > 0.5$ ) the activities of Proteases I and III between 1 to 10% v/v concentrations (Figure 3b). SDS significantly increased ( $p < 0.5$ ) the activities of proteases I and II at 5-10 % w/v, and 1-10 w/v % respectively but did not significantly affect ( $p > 0.5$ ) Protease III activity (Figure 3c). These findings indicated that optimal concentrations of Tween 80 and SDS enhanced the activities of Proteases I and II and sustained the stability of Proteases I, II and III. Triton-X on the other hand enhanced the activity of Protease II and sustained the stability of Proteases I, II and III.

Commercially available proteases, such as Subtilisin Carlsberg, Subtilisin BPN', Alcalase, Esparsase and Savinase, have been shown to exhibit great stability in the presence of detergents or surfactants (Gupta *et al.*, 2002). This property makes them suitable as detergent additive (Joo *et al.*, 2003; Sana *et al.*, 2006). Therefore, the stability of proteases I, II and III in the presence of optimal concentrations of the surfactants as observed in this study suggest that the enzymes can be used as detergent additive.

#### Conclusion

Proteases have been isolated and characterized from different agro wastes but the use of *Citrus sinensis* fruit peel as a source of protease has not been reported. In this study, low molecular weight (10-17 kDa) proteases were isolated and characterized from *C. sinensis* fruit peel using a combination of ammonium sulphate precipitation, dialysis and sephadex G150 column chromatography. Our results indicated the presence of three proteases designated as proteases I, II and III. These proteases were stable in the presence of optimal concentrations of surfactants (Tween 80, Triton X and SDS). This property (detergent stability) of the proteases is a desirable characteristic that can be exploited for their applications in laundry industry.

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