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## Partial characterization of proteases from *Citrus sinensis* fruit peel

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### ABSTRACT

Proteases are one of the most important enzymes that have various physiological and industrial applications. This study was carried out to purify and partially characterize proteases from *Citrus sinensis* fruit peel. Three active fractions of the proteases (I, II and III) were obtained. The  $V_{max}$  for proteases I, II, III and pooled fraction were 185.19, 192.31, 111.11 and 163.93 U/ml with Michaelis-Menten's constant ( $K_m$ ) 1.01, 0.44, 0.67 and 0.37 mg/ml respectively. The enzymes were optimally active at 40-50 °C. However, they retained activity at 60-70 °C. Protease I was stable up to 60 °C while proteases II and III retained more than 80% activity in the range of 25-70 °C. The optimal pH of proteases II and III was 7 while protease I was optimally active at pH 8. The enzymes were stable at alkaline pH especially between 6 and 9 retaining more than 60% of its activity. The stability of these enzymes at high temperature and different pH may be an indication of its potential applications in food, chemical and laundry industries.

**Keyword:** Proteases, *Citrus sinensis*, kinetic parameters, industries

### 1. INTRODUCTION

Proteases are enzymes that are capable of hydrolyzing peptide bonds and break them down to peptides and amino acids. They can act near the ends of polypeptide chains or within

them (Palma *et al.*, 2002). Proteases have a first place in the world market of enzymes, with estimated sales of approximately US\$3 billion (Leary *et al.*, 2009). This is because they play an important role in biotechnology, considering the fact that proteolytic reaction changes the chemical, physical, biological or immunological properties of proteins.

Proteases constitute the most important group of industrial enzymes currently in use, with an important role in the food and detergent industries, and also in leather processing and as therapeutic agents (Walsh, 2002). Measuring hydrolytic activity on substrates is one of the ways to know the cleavage specificity of these enzymes, which provides important information for biotechnological applications. This is illustrated in the production of bioactive peptides from food proteins (Silva and Malcata, 2005a). Some peptides are hidden and inactive in the original proteins, but when liberated they can have diverse biomedical applications as antihypertensive or antioxidant agents, among others (Perpetuo *et al.*, 2003).

Proteases are also physiologically necessary for living organisms. In plants, the presence of proteolytic activity has been reported in several cell compartments, such as vacuoles, mitochondria, cell wall, chloroplasts and cytosol (Berger and Altmann, 2000; Hamilton *et al.*, 2003). This explains why they are ubiquitous and found in a wide diversity of sources such as plants, animals, and microorganisms (Rao *et al.*, 1998). Proteases have been isolated and characterized from several plant sources but no report is available on the characteristics of protease from *Citrus sinensis* fruit peel. This study is therefore designed to explore possible potentials of using *Citrus sinensis* fruit peel as a source of proteases and provide information on their characteristics.

## **2. MATERIALS AND METHODS**

### ***Citrus sinensis* Fruit Peel**

*Citrus sinensis* were obtained from a plantation at the National Research Institute for Chemical Technology, Zaria. The fruits with the leaves were identified and authenticated at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria. The oranges were properly washed with distilled water and then gently peeled off using clean knife.

#### **2. 1. Extraction and purification of protease**

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 with intermittent switching on and off in order control the heat generated. The blending was also done in an air conditioned environment so as to prevent the denaturation of the enzyme being isolated. 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 ×g 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. The supernatant was then subjected to 75% ammonium sulphate precipitation, dialysis and column chromatography.

## 2. 2. Determination of Protease Activity

Protease activity was determined using the method described by Thangam and Rajkumar (2002) with slight modifications. 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 the 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 were 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 the enzyme required to liberate one microgramme (1 µg) of tyrosine from gelatin per minute at 37 °C under the assay conditions described.

## 2. 3. Substrate Specificity

The ability of the purified protease to degrade various proteins was evaluated with the following substrates (2 mg/ml): casein, bovine serum albumin and gelatin. Protease activity was determined as described earlier.

## 2. 4. Determination of Kinetic Parameters of the Protease

The effect of different substrate concentrations [S], 0.1-1.0mg/ml on the protease activity was investigated. The Michaelis-Menten's constant ( $K_m$ ) and the maximum attainable velocity ( $V_{max}$ ) were determined from the Lineweaver-Burk plot (1/V vs 1/[S]).

## 2. 5. Effect of Temperature on Protease Activity and Stability

The effect of temperature on the enzyme activity and stability was carried out at a temperature range of 25 to 100 °C with an interval of 10 °C.

## 2. 6. Effect of pH on Protease Activity and Stability

The effect of pH of the buffer on the activity and stability of the enzyme was investigated. This was carried out following the method described earlier but the pH was varied for the reaction mixture between a range of 2.0 and 11.0 with an interval of 1.0.

## 3. RESULTS

### 3. 1. Substrate Specificity of the Purified Proteases from *Citrus sinensis* Fruit Peel

The ability of the purified proteases from *Citrus sinensis* fruit peel to degrade different proteins is presented in Table I. The relative activity of protease for the hydrolysis of casein was taken as 100%. Results showed gelatin to be the best substrate. Protease I displayed relative activity of 79% with BSA and 112% with gelatin, the relative activities of proteases II and III with BSA are 75% and 91%, but for gelatin 125% and 109% respectively. In all, Protease II exhibited highest substrate affinity and hydrolysis with gelatin (125% relative activity).

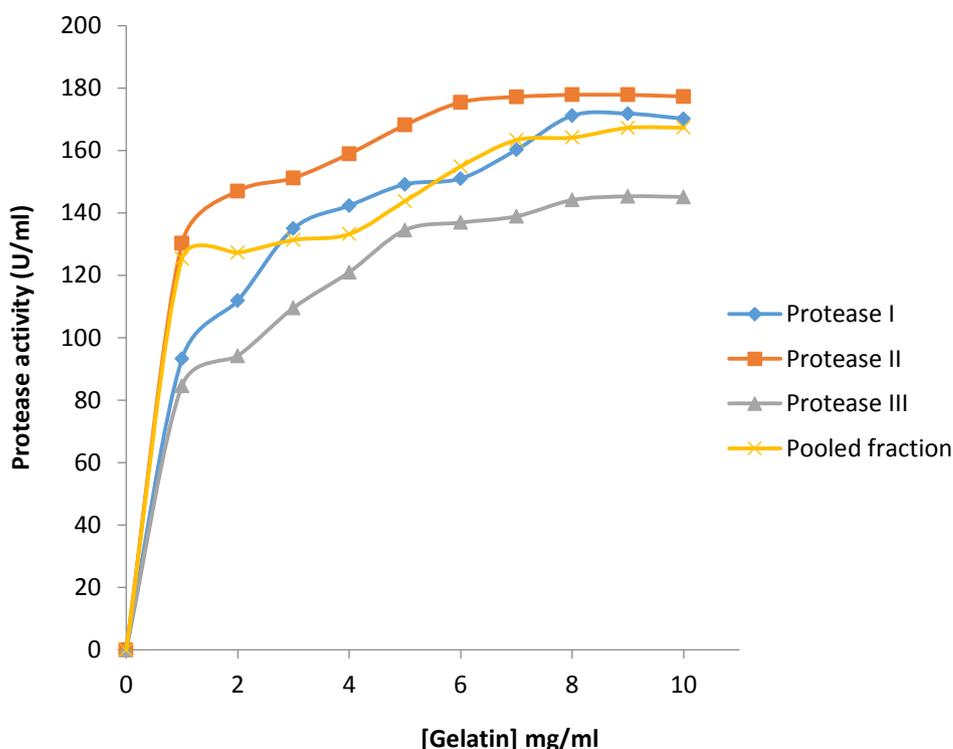
### 3. 2. Kinetic Analyses of the Purified Proteases from *Citrus sinensis* Fruit Peel

The kinetics of the three proteases from *Citrus sinensis* fruit peel on gelatin were determined at various concentrations (1-10 mg/ml). The protease activities of the three active fractions obeyed a typical Michaelis-Menten type kinetics in all the substrates used as shown in Figure 1.

**Table I.** Relative Activity of *Citrus sinensis* Proteases using Different Substrates.

Substrate (2mg/ml)	Relative activity (%)*			
	Protease I	Protease II	Protease III	Pooled fraction
Casein	100	100	100	100
Gelatin	112	125	109	118
Bovine Serum Albumin	79	75	91	80

\*The absorbance readings of the protease using casein was taken as 100% while the relative activities with other substrates was expressed as percentage (%) relative to that of casein.



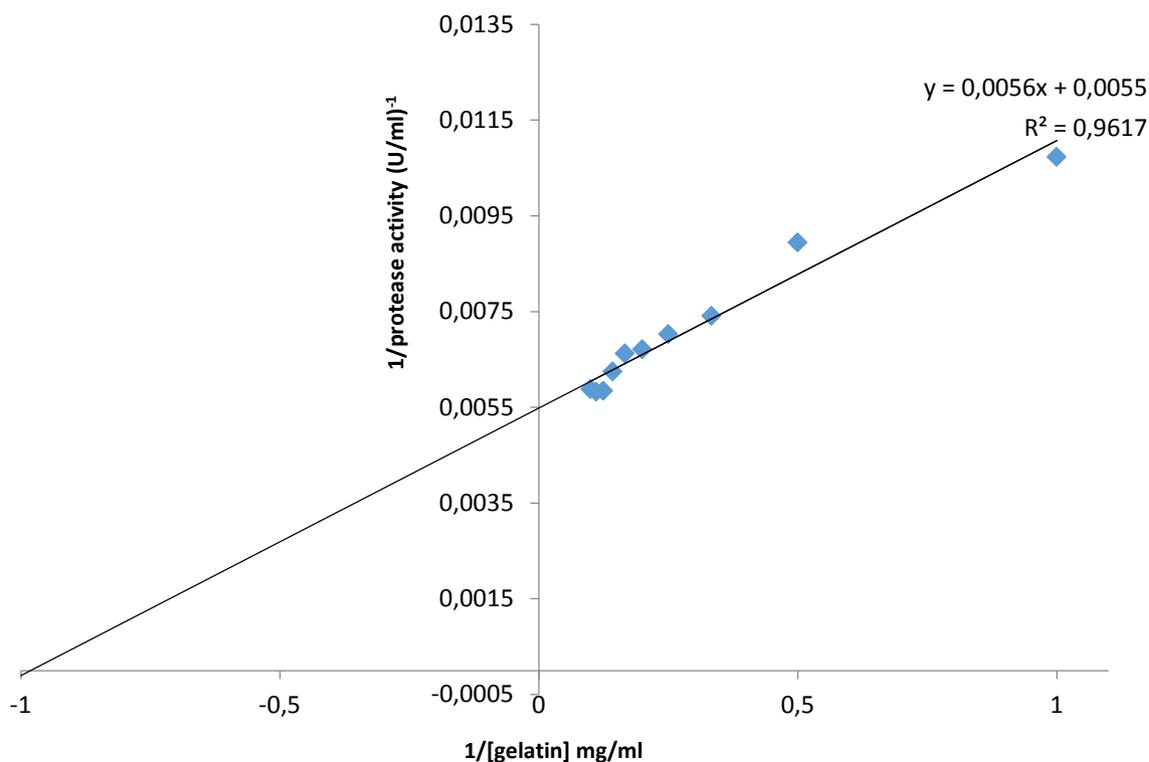
**Figure 1.** Plot of Activities of *Citrus sinensis* Fruit Peel Proteases Versus Gelatin Concentration

The kinetic parameters ( $V_{\max}$  and  $K_m$ ) of proteases I, II, III and pooled fraction for the hydrolysis of the three substrates were obtained (see Table II) from the double reciprocal plot of the velocity versus concentration. The Lineweaver-Burk plots for the proteases are illustrated in Figures 2-5. The  $V_{\max}$  for protease I, II and III were 185.19, 192.31, 111.11 and 163.93 U/ml and the Michaelis-Menten's constant ( $K_m$ ) were 1.01, 0.44, 0.67 and 0.37 mg/ml respectively.

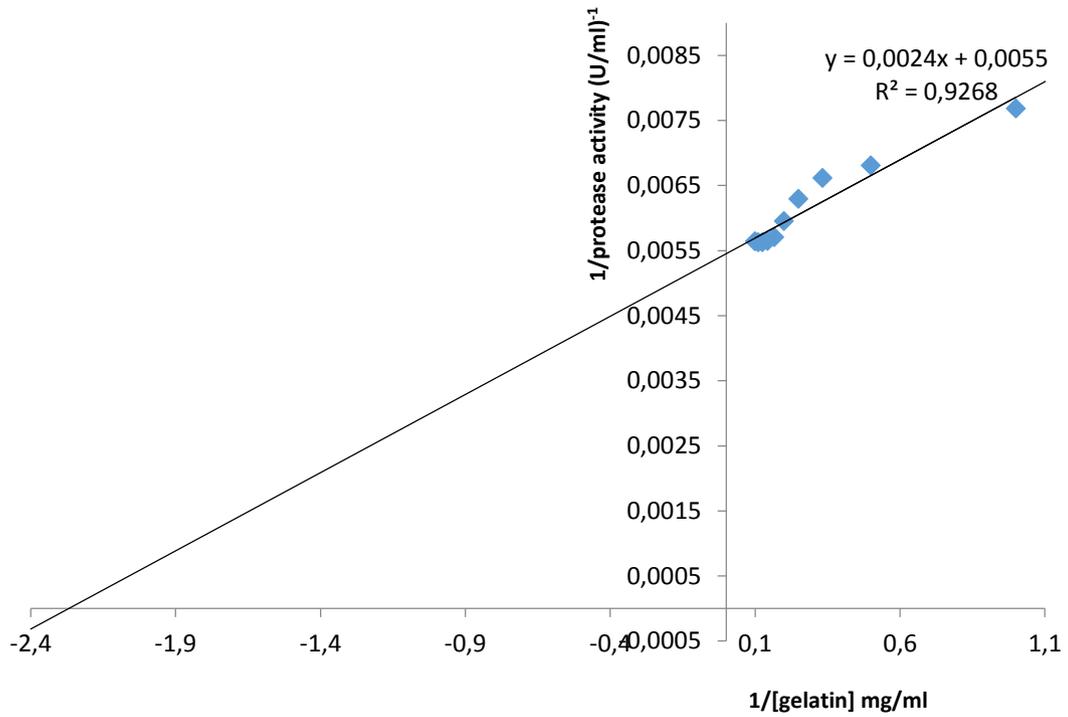
### 3. 3. Effect of Temperature on Activity of Proteases from *Citrus sinensis* Fruit Peel

The optimum temperature for the proteases I, II and pooled fraction was 40 °C while protease III had an optimum temperature of about 48 °C (Figure 6). There was an increase in enzyme activity from 25 °C up to 40 °C in all the proteases before a gradual decrease when the reaction temperature was increased above the optimum of 40-50 °C. Nevertheless, it was observed that the enzymes retained more than 60% of activity at moderately high temperatures of 60-70 °C while the pooled fraction of the enzymes retained more than 70% of activity.

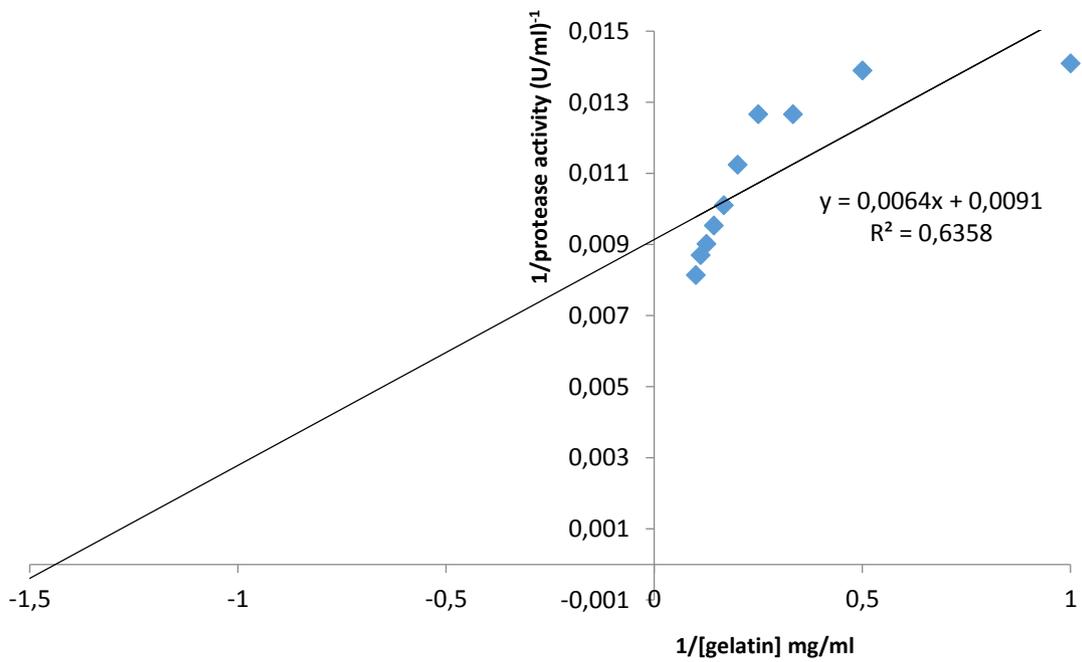
Result of thermostability of the proteases is illustrated in Figure 7. Protease I was stable up to 60 °C with more than 85% activity while proteases II and III retained more than 80% activity in the range of 25-70 °C. However, the residual activity was sharply reduced to less than 40% at temperatures higher than 70 °C.



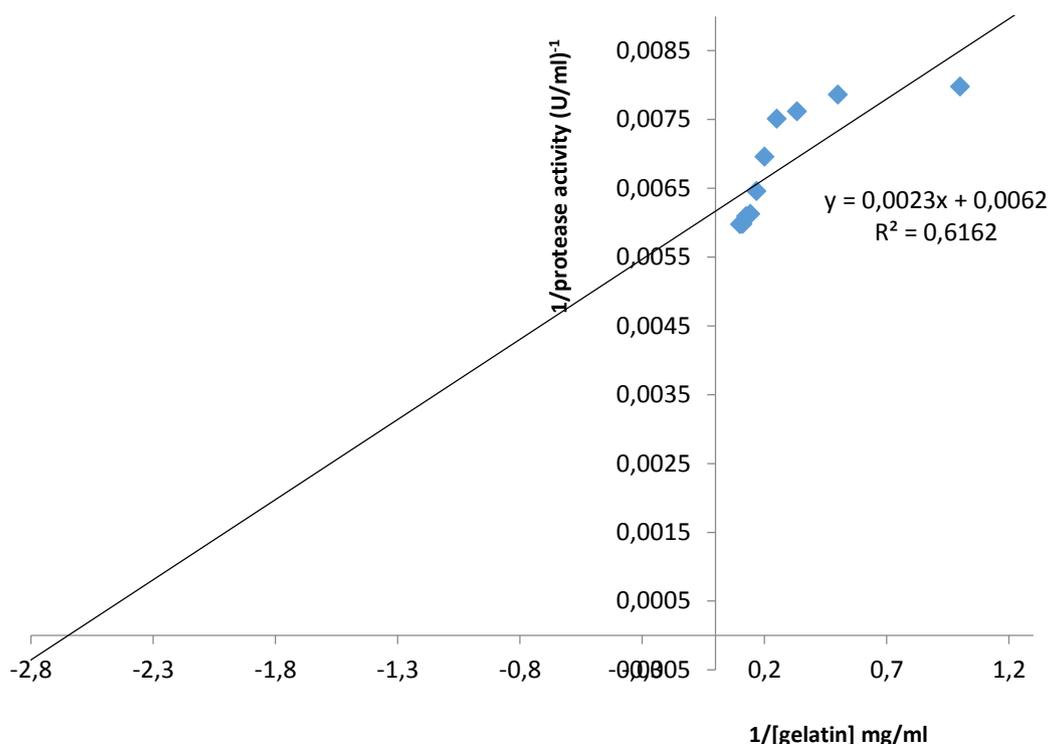
**Figure 2.** Lineweaver-Burk Plot of *Citrus sinensis* Fruit Peel Protease I



**Figure 3.** Lineweaver-Burk Plot of *Citrus sinensis* Fruit Peel Protease II



**Figure 4.** Lineweaver-Burk Plot of *Citrus sinensis* Fruit Peel Protease III



**Figure 5.** Lineweaver-Burk Plot of Pooled Fraction of *Citrus sinensis* Fruit Peel Protease

**Table II.** Kinetic Parameters of the Purified Proteases Obtained from *Citrus sinensis* Fruit Peel.

Proteases	V <sub>max</sub> (U/ml)	K <sub>m</sub> (mg/ml)
Protease I	185.19	1.01
Protease II	192.31	0.44
Protease III	111.11	0.67
Pooled fraction	163.93	0.37

### 3. 4. Effect of pH on Activity of Protease from *Citrus sinensis* Fruit Peel

In Figure 8, the enzymes were  $\geq 50\%$  active over a range of pH 6-9 with optimum activity at pH 8 for protease I and pH 7 for proteases II, III and pooled fraction. At pH 6, 7, 8 and 9, the protease activity was 46.5, 85, 100 and 92% for protease I, 87, 100, 76 and 23% for protease II, 87, 100, 95 and 92% for protease III and 44.5, 100, 51 and 73% (pooled fraction) respectively using gelatin as substrate. The stability of the proteases at different pH values is illustrated in Figure 9. The enzymes were stable at alkaline pH especially between 6 and 9 retaining more than 60% of its activity. Below pH 5.0 and beyond pH 10 the activity was reduced to approximately 40%.

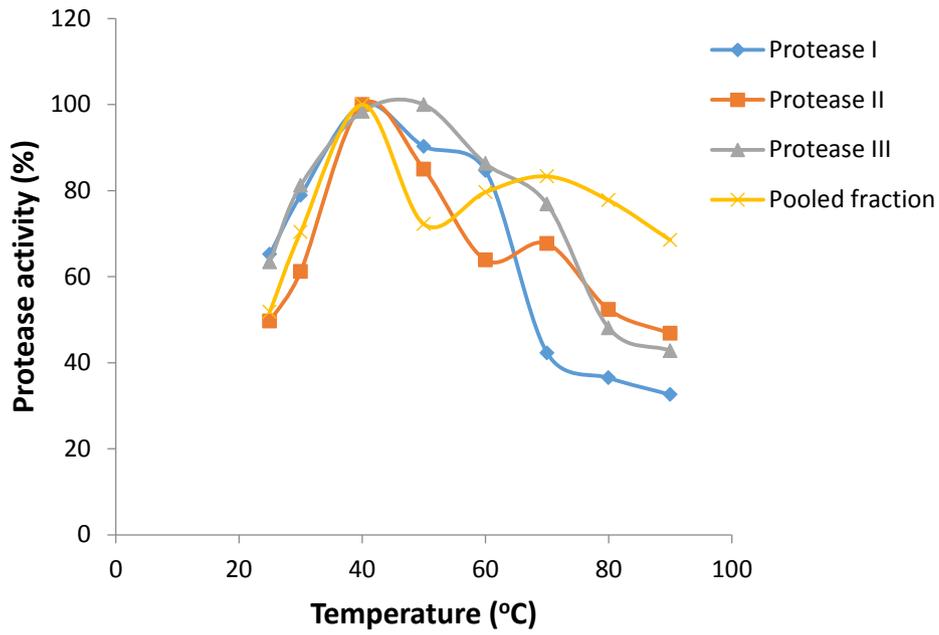


Figure 6. Effect of Temperature on the Activity of *Citrus sinensis* Fruit Peel Proteases

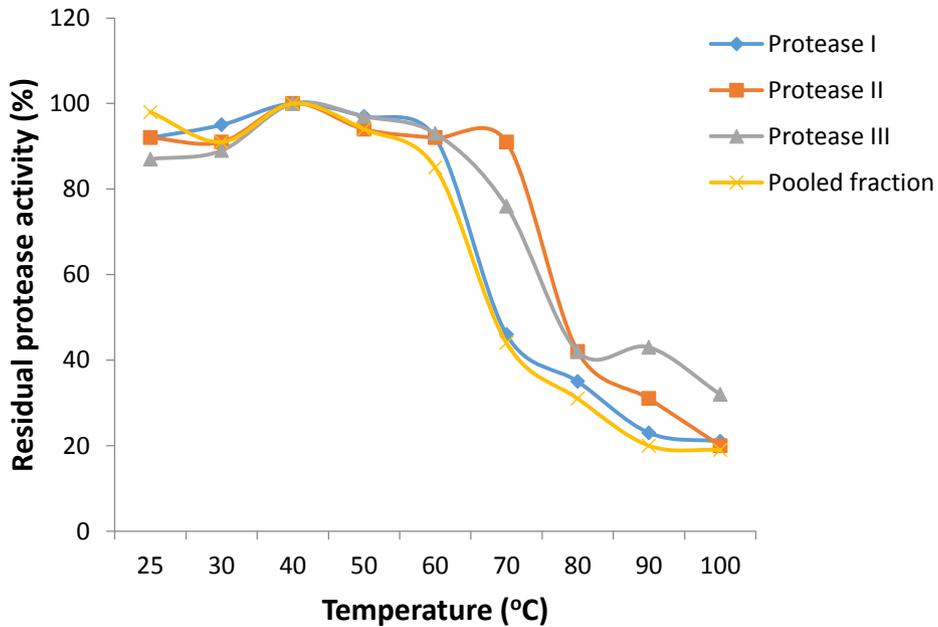


Figure 7. Effect of Temperature on the Stability of *Citrus sinensis* Fruit Peel Proteases

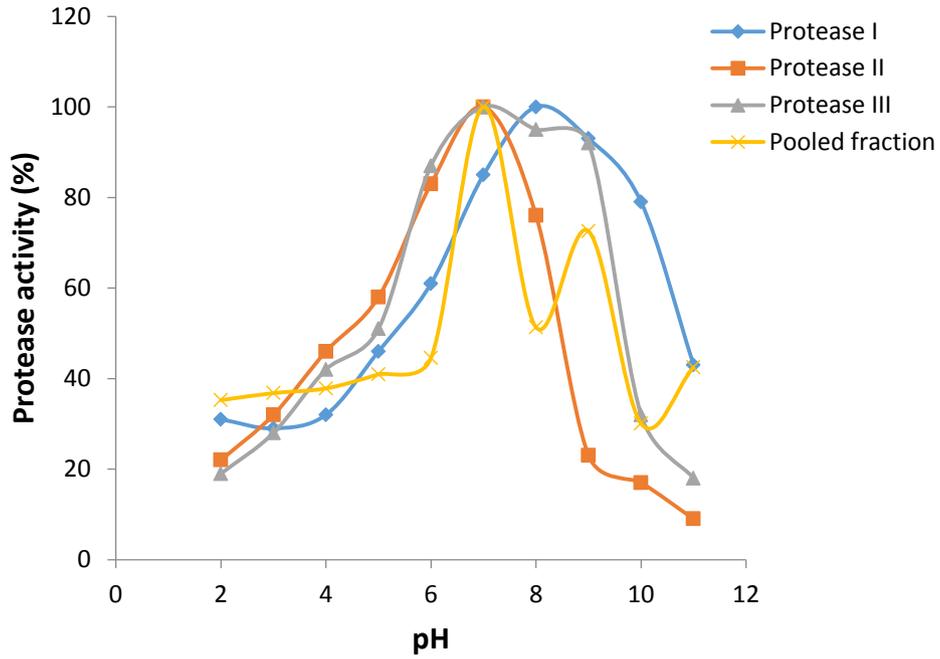


Figure 8. Effect of pH on the Activity of *Citrus sinensis* Fruit Peel Proteases

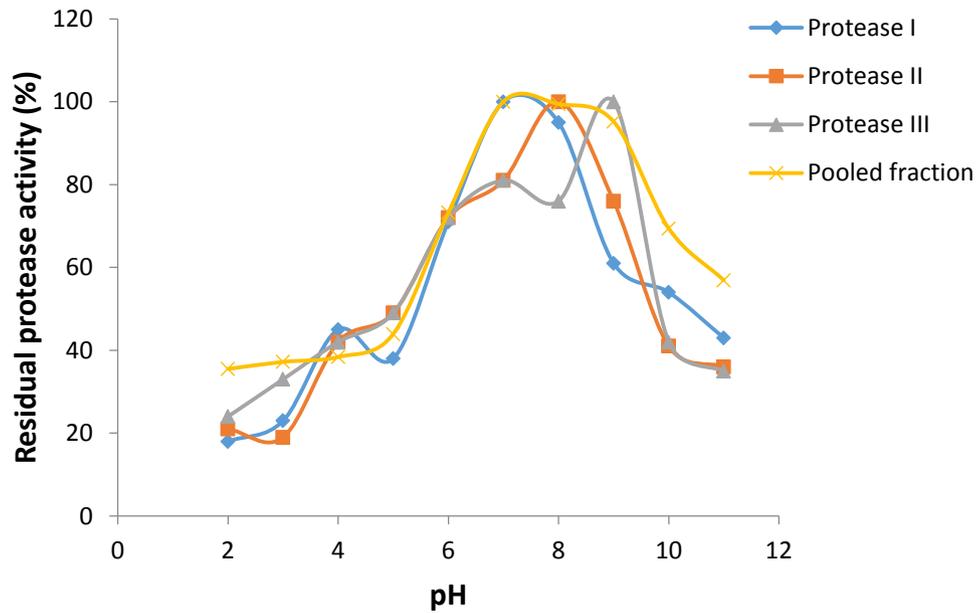


Figure 9. Effect of pH on the Stability of *Citrus sinensis* Fruit Peel Proteases

## 4. DISCUSSION

### 4. 1. Substrate Specificity of the Purified Proteases from *Citrus sinensis* Fruit Peel

The substrate specificity profile of the purified proteases indicates that the enzymes have a wide range of hydrolytic activity on various protein substrates and this quality is a great potential in biotechnological applications. The degradative potential and strong affinity of the proteases for gelatin will make them useful in the decomposition of the gelatinous coating of X-Ray films for the recovery of silver and gelatin hydrolysis in food processing industries (Ire *et al.*, 2011).

Highest hydrolytic activity exhibited in the presence of gelatin in this study was in agreement with the report of Ire *et al.*, (2011) that protease from *Aspergillus carbonarius* showed broad substrate specificity with 100% activity with casein and BSA and 300% with gelatin. However, our finding is at variance with the reports of other workers, e.g. protease from *Beauveria* sp. (Shankar *et al.*, 2011). Two enzymes, PI and PII, from *F. gigantic* displayed higher degradability with azoalbumin and casein compared to gelatin, haemoglobin and albumin (Mohamed, 2005). The following order of affinity was reported for protease of *Bacillus pumilus*: casein (100%) > gelatin (95%) > azocasein > bovine serum albumin (52%). Jaouadi *et al.*, (2008) and Qi *et al.*, (2007) on the other hand, reported the following order: casein > BSA > gelatin for cysteine-like protease from *Stichopus japonicus*. It was also reported that protease from seeds of *Holarrhena antidysentrica* exhibited higher activity towards casein than BSA and gelatin (Khan *et al.*, 2008).

### 4. 2. Kinetic analysis of *Citrus sinensis* Fruit Peel Proteases

The lowest  $K_m$  and highest  $V_{max}$  values recorded for the proteases in the presence of gelatin further suggest that the enzymes possessed high affinity and degradability potential for the substrate than casein and BSA. Ability to degrade various substrates together with other properties is desired for their various industrial applications such as detergent, pharmaceutical, food and biotechnology industry.

Similar observations were also reported by Ire *et al.*, (2011). Fahmy *et al.*, (2004) recorded highest affinity for gelatin compared to haemoglobin and then followed by casein. Our findings were in contrast to the report of other researchers with cysteine protease purified from *Allium cepa*. The least  $K_m$  was reported for casein followed by gelatin and haemoglobin (Ndidi and Nzelibe, 2012). Zhang and Jones (1996) also reported different affinities of the enzymes for different protein substrates. Shankar *et al.* (2011) reported  $K_m$  of 5.1 mg/ml with casein as substrate for protease from *Beauveria* sp. A  $K_m$  value of 0.8% has also been reported for protease from *A. oryzae* MTCC 5341 with haemoglobin as substrate (Vishwanatha *et al.*, 2009) while Devi *et al.* (2008) reported a  $K_m$  of 0.8 mg/ml with casein as substrate for protease from *A. niger*.

### 4. 3. Effect of Temperature on Activity and Stability of Proteases from *Citrus sinensis* Fruit Peel

Our findings indicate that the proteases are stable at considerably high temperature and this suggests that they may be useful in food industry such as brewing and baking that utilize protease at elevated temperature of about 50 to 60 °C. The optimum temperature of 40-45 °C for *Citrus sinensis* fruit peel proteases varies somewhat from those obtained for most alkaline proteases e.g. from senescent leaves of an invasive weed *Lantana camara* which had an

optimum temperature 50-60 °C (Gaur and Wadhwa, 2008), but correlates well with 40-50 °C optimum recorded for banana leaves and peels proteases (Ekpa *et al.*, 2010). An optimum temperature of 45 °C was also reported for protease from *Allium cepa* (Ndidi and Nzelibe, 2012) and 40 °C for fermenting locust beans and melon seed (Evans *et al.*, 2009). Protease of *A. terreus* had optimum temperature of 40 °C (Bushra *et al.*, 2010); 45 °C for *Penicillium sp.* LPB-5 (Germano *et al.*, 2003); 45 °C for *Penicillium crysogenium* Pg 222 (Benito *et al.*, 2002) and *A. oryzae* NRRL 2217 50 °C (Sumantha *et al.*, 2005).

In terms of stability to high temperature, the enzymes retained more than 80% activity in the temperature range of 25-70 °C. The broad, optimal temperature range for maximum protease activity and stability reveals the thermostable nature of the proteases from *Citrus sinensis* fruit peel. Thermal stability is desirable for most biotechnological applications of proteins. Thermal stability increases the efficiency of proteins and is one of the essential features for their commercial exploitation (Pandhare *et al.*, 2002).

The activity of alkaline proteases at broad temperature ranges is a desirable characteristic for their application in detergent formulations. Manachini *et al.* (1988) reported an alkaline protease from *Bacillus thermoruber* that is active at temperature range of 10-80 °C, with an optimum of 45 °C while alkaline protease from *B. clausii* I-52 was observed to be stable in the temperature range of 30-80 °C, with almost 100% activity in the temperature range of 30-50 °C (Joo *et al.*, 2003). Sareen and Mishra (2008) also reported a thermoalkaline protease from *B. licheniformis* which was active at a temperature range of 30-90 °C and had the maximum activity at 50 °C. Abusham *et al.*, (2009) also reported an alkaline protease from *B. subtilis* strain Rand with 100% stability in the temperature range of 35-55 °C.

#### 4. 4. Effect of pH on Activity and stability of Protease from *Citrus sinensis* Fruit Peel

The optimum pH of *Citrus sinensis* fruit peel proteases in the present study suggests that they act at near neutral pH and are still very active at alkaline pH condition. Most plant proteases are known to be active over a wide range of pH and temperature values. Our finding is in agreement with most plant proteases. The optimum pH for the leaf and peel of banana proteases were found to be 8 and 9 respectively (Ekpa *et al.*, 2010), while Cucumisin-like protease had an optimum pH range of 8-10 (Patel *et al.*, 2007). Optimum pH reported for latex protease from *Euphorbia* species was between 6 and 8 (Khan *et al.*, 2008) and that of a protease from *Thaumatococcus daniellii* waste was 7 (Raimi *et al.*, 2011).

Our findings contrast with some reports on the optimal pH of protease of plant origin. The optimal pH of 4 has been reported for wheat (Fahmy and Fahmy, 2003), pH 5 for sorghum malt (Ogbonna *et al.*, 2003), pH 5.5 for horse gram (Rajeswari *et al.*, 2009), pH 7.5 for mushroom (Zhang *et al.*, 2010), pH 7.9 for *Euphorbia milii* (Yadav *et al.*, 2006) and 8 for sweet potato root (Chen *et al.*, 2004). Most of the plant serine proteases showed optimum pH in the range of 7-11 (Antao and Malcata, 2005). In barley and germinated barley, the majority of aspartic and cysteine proteases were found to be active under acidic conditions, while serine proteases and metalloproteinases were more active at pH  $\approx$  7 and pH > 7 respectively (Jones, 2005).

The remarkable activity and stability over such pH range reveals the alkaline nature of the proteases, which makes them suitable for applications in alkaline environments and with detergents.

Several other researchers have also described proteases with broad pH activities and stabilities. An alkaline protease from *B. subtilis* PE-11 was observed to be stable in the pH

range of 8.0-11.0, with the highest activity at pH 10.0 (Adinarayana *et al.*, 2003). Joo *et al.* (2003) reported an alkaline protease from *B. clausii* I-52 that was stable in the pH range of 4.0-12.0, with maximum activity at pH 12.0. An alkaline protease from *B. licheniformis* RSP-09-37 was observed to be active in a broad pH range of 4.0-12. It was 100% active at pH 10.0 and exhibited 14, 28 and 40% residual protease activities at pH 4.0, 5.0 and 12.0, respectively (Sareen and Mishra, 2008).

The largest share of the enzyme market has been held by detergent alkaline proteases active and stable in the alkaline pH range (Gupta *et al.*, 2002). An industrially important alkaline protease, isolated from a selective strain of *Bacillus*, has been shown to be useful as a catalyst for the resolution of N-protected amino acids having unusual side chains. The major component of this enzyme is subtilisin, which is a serine protease and is widely used as a detergent additive (Chen *et al.*, 1991). In the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients (Raimi *et al.*, 2011).

Stability of these enzymes at neutral and alkaline pH is essential for their use in a range of commercial applications especially in removal of protein hazes in brewing industry, meat tenderization, fur and leather industries.

## 5. CONCLUSION

Proteases are essential enzymes in almost of all aspects of human life. In this work, proteases were purified and partially characterized from *Citrus sinensis* fruit peel. The  $V_{max}$  for proteases was 111.11-192.31 U/ml with Michaelis-Menten's constant ( $K_m$ ) 0.37-1.01 mg/ml. The enzymes were optimally active at 40-48 °C but retained activity at 60-70 °C. The optimal pH of proteases was 7-8. The information obtained from this study indicates that *Citrus sinensis* fruit peel can serve as a source of proteases and provides opportunity to further explore the suitability of these enzymes from citrus wastes in relevant industries.

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