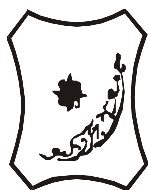

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Milk Clotting Activity of Protease, Extracted from Rhizome of *Taffin Giwa* Ginger (*Zingiber officinale*) Cultivar, from northwestern Nigeria

***[1]Murtala, Y., [1]Babandi, A., [1]Babagana, K., [1]Rajah, M. R., [3]Yakasai, H. M., [1]Ibrahim, A., [2]Shehu, D., and [1]Alhassan, A. J.**

[1]Department of Biochemistry, Bayero University, Kano-Nigeria
[2]Institute of Biological Sciences, University of Malaya, Malaysia
[3]Faculty of Biotechnology and Biomolecular Science, Universiti Putra, Malaysia
Email: murtalakanya@gmail.com

ABSTRACT

The increasing prices of calf rennets, their accessibility and ethical concerns associated with the production of such enzymes for general cheese making have led to systematic investigations on the possibility and suitability of their substitution by other enzymes of plant origin. In this study, ammonium sulphate ($(NH_4)_2SO_4$) fractionation, characterization and milk clotting activity (MCA) of protease extracted from *Taffin Giwa* ginger rhizome cultivar of the family Zingiberaceae from northwestern Nigeria were carried out. The protease extracted showed optimum activity at temperatures near 60 °C and pH value of 6.5 with a relative activity in a broad pH range of 5.0 to 8.0 accordingly. The enzyme was completely denatured at higher temperature of 100 °C and higher pH range of 12.0. The milk clotting property of the protease indicated 3.1 and 2.2 folds of MCA and MSCA respectively in relation to the commercial calf rennet with MCA/PA ratio of 2.52. The properties of *Taffin Giwa* protease shown in this study, especially its milk clotting activity, make it a potential candidate for substituting calf rennet in the food industries, particularly in cheese making processes.

Keywords: Ginger Protease, Milk Clotting Activity, Calf rennet, Characterization, Extraction

INTRODUCTION

Ginger rhizome (*Zingiber officinale* roscoe), the main source of ginger proteases is grown in many parts of Africa, tropical Asia, southeast Asia, India and the West Indies (Hou-Pin *et al.,* 2009). Nigeria is ranked first in terms of the percentage of total hectares of ginger under cultivation, particularly in the northwestern region (Job and Philemon, 2013). Ginger is a popular component of many traditional spices and food additives in northwestern Nigeria. The plant is widely grown in the region with a massive annual production (FAO, 2010). Southern Kaduna State still remains the largest producer of fresh ginger in Nigeria, majorly around Kachia, Jabba, Jama'a and Kagarko axes (KADP, 2000; KADP, 2004; Bernard, 2008). The dominant varieties produced in those axes are '*Taffin Giwa*' and '*Yatsun Biri*' gingers cultivar (Kaduna State Ministry of Agriculture, 2007).

Proteases are a highly demanded and valuable group of enzymes with various industrial applications, covering a market share of 60% of the total enzyme market (Gaur and Wadhwa, 2008; Gaur *et al*., 2010). There is a wide and considerable use of protease enzymes in the food industry nowadays, particularly in meat tenderization, milk curdling and wine and beer turbidity clearance. Plants, animals and microbes are the major natural sources of these proteases. Ginger protease, gingepain (EC 3.4.22.67) is a plant protease (Cysteine endopeptidase) which is obtained from ginger rhizome with a valuable application in food industry. The protease has 10-folds activity in meat tenderization compared to papain from pawpaw and bromelain from pineapple, increasing both the flavour and nutritional value of meat products due to its powerful collagenase activity (Naveena and Mendiratta, 2001; Kim *et al*., 2007). The protease acts as a milk solidifying factor, acting on casein and bean separate proteins (Zhang *et al.,* 1999; Su *et al*., 2009). The ginger proteases are separated into two fractions (GP I and GP II) by a DEAE-cellulose column, with a molecular mass of about 22.5 kDa. The complete amino acid sequence for GP II contains 221 amino acids, while about 98% of the amino acid sequence for GP I have been determined (Ichikawa *et al.,* 1973). GP II is a cysteine protease with two predicted glycosylation sites at Asn 99 and Asn 156 (Choi *et al.,* 1999; Choi and Laursen, 2000). Previous studies indicated that the protease has a strong proteolytic activity and an unusual substrate specificity preference for cleaving peptides with a proline residue at the P2 position. The protease is activated by KCl, $MnCl_2$, $Fecl_2$, mercaptoethanol, EDTA-Na_2, isoascorbate, sucrose and NaCl. The protease

is however, inhibited by some divalent metal ions such as Hg^{2+}, Cu^{2+},$Cd^{2+}$and Zn^{2+} (Ohtsuki *et al.,* 1995; Adulyatham and Owusu-Apenten, 2005; Bhaskar *et al.,* 2006; Kim *et al.,* 2007; Su *et al.,* 2009 Choi and Laursen, 2000; Kong *et al.,* 2012).

The use of these ginger proteases as milk coagulants is very interesting since they are natural enzymes that can be used for producing cheeses aimed at lacto-vegetarian consumers and ecological markets. They can also be used for the manufacture of Kosher and Halal products (Gómez *et al.,* 2001; Galán *et al.,* 2008). Hashim *et al.* (2011), reported the isolation and characterization of milk coagulating cysteine protease from the ginger rhizomes of Asian cultivars which showed higher milk clotting activity in comparison to both calf rennet and papain. The protease also exhibited broad degradation ability for all the three types of caseins (i.e., αs1-, β-, and κ-casein).

Cheese is a product of curdled milk and a popular food in Asia, Europe and Africa (Euromonitor International, 2016). The Euromonitor International's global cheese research in Africa reported that the average unit price of cheese increased moderately in 2014 in Nigeria. In the same year, the cheese had a reported growth value of 7% corresponding to NGN 0.6 billion (US$3011298.0000) (FPA, 2015). The Euromonitor report concerned *wara* cheese which was said to be widely consumed in southwestern Nigeria. In addition, the report indicated that such cheese commodity is becoming a lucrative market which is largely dominated by small scale entrepreneurs.

Calf rennet has been widely employed for a long time as a milk coagulating agent, especially in cheese production. However, increasingly higher prices of calf rennet, its accessibility as well as ethical concerns associated with the production of such enzymes for general cheese making, especially to the local entrepreneurs have led to systematic investigations on the possibility and suitability of their substitution by other enzymes of plant origin (Sousa and Malcata 1997; Malik *et al.,* 2011). This is despite having paucity of information regarding the milk coagulating ability of ginger protease and its application in cheese making (Malik *et al.,* 2011), particularly proteases from certain ginger cultivars. Research on the potentiality of the use of proteases from some ginger cultivars in milk coagulation is thus required. This research was therefore aimed at extracting, purifying, characterizing and evaluating milk curdling activity of ginger protease from *Taffin Giwa* ginger cultivar commonly grown in northwestern Nigeria.

MATERIALS AND METHODS

Collection of Ginger (*Zingiber officinale*) Rhizome

The fresh ginger rhizome was collected from a harvesting site in Jama'a local government area of Kaduna state, northwestern Nigeria. The sample was identified and authenticated as *Taffin Giwa* ginger cultivar at the Herbarium Unit of the Department of Plant Biology, Bayero University, Kano. The sample was issued with an accession number (BUKHAN 0296).

Preparation of Crude Extract Sample

The fresh ginger rhizome was washed and minced. The minced sample (90g) was weighed and homogenized with 180 cm^3 of distilled water. The homogenate was filtered through a piece of cheese cloth and the filtrate was centrifuged at 4000rpm for 30 minutes. The supernatant was collected and filtered through vacuum pump and 80 cm^3 of the filtrate was used for precipitation while the remaining 100cm^3 was used as crude extract which was subsequently tested for the protease characteristics.

Ammonium Sulphate Protein Precipitation

The protein was precipitated using a modified procedure that was reported by Qiao *et al.,* (2009). The supernatant (80cm^3crude extract) was mixed with acetone which was pre-cooled at -5°C in refrigerator (1:1) and then, the sediment was collected after centrifuged at 3000 rpm for 20 minutes. The sediment was dissolved in 0.05 M phosphate buffer (pH: 6.0) and centrifuged again at 3000 rpm for 20 minutes. At this point the supernatant collected was precipitated using 6.0 g ammonium sulphate to 40 cm^3 of the enzyme extract by gently adding and stirring pinch by pinch for 45 minutes to saturation of 15%.The sample solution was then incubated at 4°C for 16 hours. The precipitated protein was then removed by centrifugation at 3000rpm for 20 minutes, 30cm^3 of supernatant was then collected to which 9.0 g ammonium sulphate was added pinch by pinch for 45 minutes to yield 30% saturation. This was then incubated at 4°C for 16 hours. The fraction of precipitated proteins between 15 and 30% saturation is recovered by centrifugation, the sediment collected at this point was subjected to residual ammonium sulphate removal using 0.05M phosphate buffer (pH 6.0) and then centrifuged at 3000 rpm for 5 minutes. The supernatant collected after centrifugation was tested for the protease characteristic

The total Protein concentration was determined using a BioAssay Systems' QuantiChrom™ protein assay kit based on an improved Coomassie Blue G method (Bradford, 1976). In the procedure, Bovine Serum Albumin (BSA) was used as standard.

Protease Activity Assay

The proteases activity (ginger protease and calf rennet) was assayed using casein as substrate. The assay was carried out using a modified method that was reported by Tsuchida *et al.*(1986). The substrate, 100µl of casein (2mg/cm^3) in 1M

glycine-NaOH buffer (pH 10.5) was added to 100µl of the enzyme solution which were previously incubated at 60 ºC for 30minutes. The reaction was terminated by the addition 100µl of chilled TCA (10%), which were allowed to stand in ice for 15 minutes to precipitate the insoluble proteins. The white soluble precipitate was filtered through Whatman filter paper, 5cm^{3}of Na_2CO_3 (0.5M) solution was added to the soluble product. Changes in the colorations were observed on both the crude and the precipitated fraction upon the addition of 0.1 cm^{3}Folin-Ciocalteau's reagent. Absorbance measurements were conducted on both materials at 660nm.One unit of protease activity is the amount in micromoles of tyrosine equivalents released from casein per minute using 0.2 mg/ cm^3 L-tyrosine as Standard.

Determination of the Effect of Temperature

The precipitated protein (2cm^3) was added to 4cm^3 of tris-HCL buffer. The activity of the enzyme was determined by incubating the reaction mixture with 100µl of casein substrate at different temperatures of 0, 20, 40, 60, 80, and 100 ºC each for 5 minutes and the optimum temperature of the enzyme was determined by plotting a graph of enzyme activity against temperature.

Determination of the Effect of pH

The protease activity of the precipitated enzyme was measured at different pH values. Portions of the enzyme solution (2cm^3) were mixed with the buffers, 0.1M acetate buffer, 0.1M phosphate buffer, 0.1M Tris-HCl buffer, and 0.1M glycine-NaOH, each in a labeled test-tube. The pH values of the buffers were 3.8, 6.5, 7.8 and 10.0 respectively. The reaction mixtures were incubated after the addition of 100µl of casein substrate at 60^{0}C for 30 minutes. The optimum pH of the enzyme was determined by plotting a graph of enzyme activity against pH.

Milk-clotting activity assay

Milk-clotting activity (MCA) was measured using a modified version (Hou-Pin *et al.* 2009 and Hang *et al.* 2016) of the procedure that was initially reported by Sousa and Malcata (1998). The milk substrate was prepared by dissolving 12 g of skimmed milk powder in 100cm^3 $CaCl_2$ solutions (0.01mol L−1). The pH value of the milk was adjusted to 6.0 with 1 M HCl before use. The milk substrate (2 cm^3) was heated at 60 ◦C, and then thoroughly mixed with 0.2 cm^3 of the enzyme solution. The time for the formation of fragments was measured with a stopwatch. One unit of milk clotting activity (MCA) is equal to the amount (mg) of enzymes required to coagulate 1cm^3 of reconstituted skimmed milk in 1 min at 60^{0}C and pH 6.5. The MCA was calculated using Equation 1:

$$MCA = 2400/t \times F \qquad \text{(Equation 1)}$$

Where t is the time for the formation of fragments (s), and F is the dilution coefficient.

RESULTS AND DISCUSSION

Effect of Temperature on the Activity of *Taffin Giwa* Ginger Protease

The activity of the ammonium sulphate precipitated ginger protease was increased as the temperature was increased from 20 to 60^{0}C. The optimum activity of the enzyme was observed near 60^{0}C (figure 1). As can be seen in figure 1, further increase in the temperature above 60^{0}C led to the decline in the activity of the protease. A higher temperature of 100^{0}C denatures the enzyme protein and led to the complete loss of its function. Considering the optimum temperature (60^{0}C), ginger protease may have some applications in food industry, especially in meat tenderization, dairy and cheese making processes where high temperatures are often required (Hashim *et al.*, 2011). The optimum temperature of ginger protease extracted from *Taffin Giwa* ginger cultivar of northwestern Nigeria showed similar temperature trend as previously reported by Hashim *et al.*, (2011); Nafi *et al.,* (2013); Nafi *et al.,* (2014) in proteases extracted from Chinese and Malaysian ginger cultivars respectively.

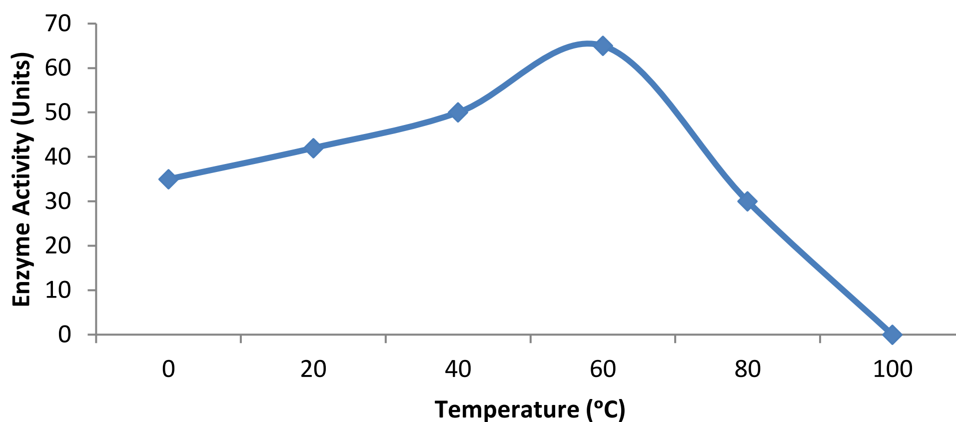

Figure 1: Effect of Temperature on Protease Extracted from *Taffin Giwa* Ginger Cultivar

Effect of pH on the Activity of *Taffin Giwa* Ginger Protease

Protease extracted from *Taffin Giwa* ginger cultivar of northwestern Nigeria showed proteolytic activity with a broad pH range of 5.0 to 8.0 (Figure 2). However, the optimum activity of the protease was observed at pH values around 6.5. At higher pH value of 12, the enzyme completely lost its activity due to denaturation of the enzyme protein. This broad range of effective pH (slightly acidic and mildly alkaline) observed, may suggest a possibility of the presence of multiple proteases in the ammonium sulphate precipitate. Nafi *et al.* (2014) reported similar findings that protease from Malaysian ginger crude extract had a broad range of effective pH which could be advantageous in food processing. In some studies on the properties of the pure ginger protease by Thompson *et al.* (1973) and Hashim *et al.* (2011), the enzymes showed activity in wide range of pH values between 4.5 and 6.0. These were slightly lower relative to the pH values revealed by this study. Often, the difference in optimum pH could be attributable to factors such as plant variety, types and nature of proteolytic enzymes present in the source, extraction protocols and degree of purity of the enzyme. The effective pH range (5.0-8.0) observed in *Taffin Giwa* ginger protease may be an advantage to the enzyme on its proteolytic action on skim milk that has a pH that is often slightly acidic. This acidic pH favours the dissociation of casein from milk micelles (Skelte and Henning, 1997).

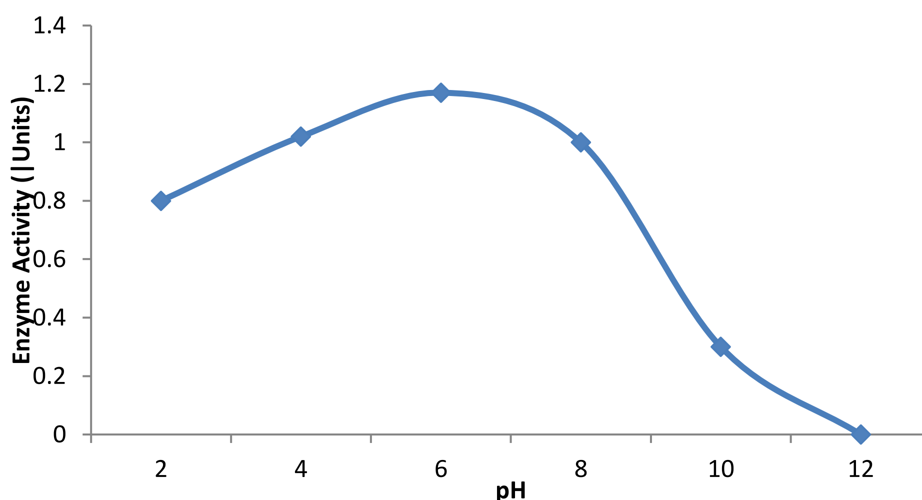

Figure 2: Effect of pH on Protease Extracted from *Taffin Giwa* Ginger Cultivar

Partial Purification of the Ginger Protease

The partial purification of *Taffin Giwa* ginger protease using one step ammonium sulphate ($(NH_4)_2SO_4$) fractionation, an extensively used technique in enzyme purification (Qiao *et al.,* 2009; Malik *et al.,* 2011) was performed on the crude extract. The enzyme obtained from the ginger rhizome crude extract using 15–30% $(NH_4)_2SO_4$ saturation showed 0.57-fold purification and 57% recovery with specific activity of 1.29 Units/mg (Table 1). The one step extraction protocol employed in this study indicates a considerable value of the % yield (57%) with a low value of specific activity of the protease compared with related findings by Nafi *et al.* (2014). This suggests further purification steps such as ion-exchange chromatography, size exclusion chromatography or electrophoresis are required to optimize the purity of the enzyme protein as indicated in the previous findings by Qiao *et al.* (2009); Nafi *et al.* (2014).

Table 1: Partial Purification of Ginger Protease Extracted from *Taffin Giwa* Ginger Cultivar from Northwestern Nigeria

Purification Steps	Total Protein (mg)	Proteolytic Activity (PA) (Units) [a]	Proteolytic Specific Activity (PSA) (Units/mg)	Purification Fold	% Yield
Crude Enzyme Extract	674.81	300.0 ± 07	2.25	-	-
Ammonium Sulphate Precipitate [b]	92.02	71.1 ± 13	1.29	0.57	57%

a –One Unit of enzyme activity is the amount in micromoles of tyrosine equivalents released from casein per minute
b – Precipitation under 15% to 30% Saturation

Proteolytic and Milk Clotting Activities of Precipitated *Taffin Giwa* Ginger Protease

In some countries, the use of calf rennet substitutes for cheese-making, such as porcine pepsin A, porcine pepsin C, *Mucor miehei* protease and *Endothia parasitica* protease is common practice (Macedo *et al.,* 1993). Ginger protease from Asian ginger extract is one of the powerful milk clotting enzymes (Hashim *et al.,* 2011) that could be employed for cheese making.

The optimal enzyme activity conditions (pH 6.5 at 60 ^oC) for the $(NH4)_2SO_4$ precipitated *Taffin Giwa* ginger protease observed in this study were used in evaluating both the proteolytic and milk clotting activities of the protease. In the evaluation procedure, the protease MCA/PA ratio has been a very important criterion for evaluating protease potential as rennet substitutes (Abel-Fattah and El-Hawwary, 1974; He *et al.,* 2012). The PA of *Taffin Giwa* ginger protease was found to be 71.1 Unit/mg (Table 1). This lower value suggests a possibility of good yield in cheese processing. Milk-clotting protease with strong PA would excessively hydrolyze casein substrate, and thus led to reduction in cheese yield and any required organoleptic attributes. The MCA/PA ratio of *Taffin Giwa* ginger protease was found to be 2.52 (Table 2). This is relatively a favourable value for cheese processing. PA and MCA/PA ratio of milk-clotting protease varies greatly in terms of determination and definition methods (Vishwanatha *et al.,* 2010; De Castro *et al.,* 2014), which complicates the comparisons among different studies. Thus, MCA/PA of different milk-clotting proteases should be assessed under the same conditions, and similar to those employed in the cheese-making. Nevertheless, the MCA and MCSA of the *Taffin Giwa* ginger protease are 3.1 and 2.2 folds respectively compared to that of commercial calf rennet. This finding agrees with Su *et al.* (2009), Hashim *et al.* (2011) and Nafi *et al.* (2013) that ginger proteases could be a suitable choice for cheese making compared to some commercial milk coagulating agents as well as other natural milk-clotting enzyme source to improve on the bitterness of milk products caused by papain, ficin and bromelain. Thus, ginger protease extracted from *Taffin Giwa* ginger cultivar from northwestern Nigeria possesses higher milk clotting activity relative to that of commercial calf rennet.

Table 2: Milk Clotting Activities of Protease Extracted *Taffin Giwa* Ginger Cultivar and Calf Rennet

	Milk Clotting Activity (MCA) (Units/cm^3) [a]	Milk Clotting Specific Activity (MCSA) (Units/mg of Protein) [b]	MCA/PA Ratio
Calf Rennet	58	0.87	-
Ginger Protease	179	1.95	2.52

a – A unit (U) equals the amount (mg) of enzymes required to coagulate 1cm^3 of reconstituted skimmed milk in 1 min at 60 ^{0}C and pH 6.5.
b – 92.02 mg total protein content for ginger protease and 66.67 mg of protein for calf rennet
c- 71.1 (Unit/) PA for ginger protease

CONCLUSION:

The protease extracted from the *Taffin Giwa* ginger rhizome cultivar that was obtained from northwestern Nigeria showed optimum activity at temperatures near 60 °C and at a broad range of pH values of 5.0 to 8.0. The enzyme protein was completely denatured at 100 °C and pH value of 12.0. The properties of the protease as shown in this study, especially its milk clotting activity make it a potential candidate for application in the food industries, particularly in cheese making processes.

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