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PARTIAL CHARACTERIZATION OF PROTEASE EXTRACTED FROM "YATSIN BIRI" GINGER (*Zingiber officinale*) CULTIVAR OF NORTHWESTERN NIGERIA

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ABSTRACT

The recurrent increase in prices of calf rennet and ethical considerations linked to the production of such enzymes for cheese making and related processes have ignited a flame of scientific enquiries on the possibility and suitability of their substitution by other enzymes of plant sources. In this study, ammonium sulphate fractionation, characterization and milk clotting activity (MCA) of protease extracted from YatsinBiri ginger rhizome cultivar of the family *Zingiberaceae* from northwestern Nigeria were analysed. The protease extracted showed optimum activity at 50 °C and pH value of 5.5. Relative activity of the enzyme was also observed within a broad pH range of 4.5 to 7.0 accordingly. The enzyme was completely denatured at 100 °C and alkaline pH of 11.5. The milk clotting property of the protease indicated 2.83 and 1.81 folds of MCA and MSCA respectively in relation to commercial calf rennet with MCA/PA ratio of 2.18. These properties of YatsinBiri ginger protease, especially its milk clotting activity, broad pH ranges and moderately elevated temperature of 50 °C, may favour its suitability as substitute calf rennet in the food industries, especially in cheese making and related products.

Key words: *Ginger Protease, Milk Clotting Activity, Calf rennet, Characterization,YatsinBiri*

INTRODUCTION

Enzymes are organic catalyst capable of handling biochemical reactions in biological systems. The first use of enzymes occurred more than 5,000 years ago, when people stored milk in animal stomachs, which contains enzymes called "rennet," that turn milk into cheese (Hou-Pin *et al*., 2009; Hashim *et al.,* 2011). There is a wide and considerable use of protease enzymes in food industry nowadays, particularly in meat tenderization, milk curdling and wine and beer turbidity clearance. Ginger protease, gingepain (EC 3.4.22.67) is a plantprotease (Cysteine endopeptidase) from ginger rhizome with a valuable application in food industry. It has 10-folds proteolytic activity in meat tenderization compared to papain from pawpaw and bromelain from pineapple, increasing both the flavour and nutritional value of meat products(Naveena and Mendiratta,2001;Kim *et al*., 2007). It acts as a milk solidifying factor, acting on a milk protein, casein (Zhang *et al.*, 1999; Su *et al*., 2009). The use of ginger proteases as milk coagulants is very interesting since they are natural enzymes that can be used for producing cheeses (Gómez *et al*., 2001; Galán *et al.*,2008).Ginger rhizome (*Zingiber officinale roscoe*), the main source of ginger proteases is grown in Nigeria, particularly in the northwestern region (Job and Philemon, 2013).The plant is widely grown in this region with a massive annual production (FAO, 2010). Southern Kaduna State remains the largest producer of fresh ginger in northwestern Nigeria, majorly around Kachia and Kagarko areas (KADP, 2004;Bernard, 2008). The commonly grown variety produced in those axes is 'Yatsin Biri' gingers cultivar (Kaduna State Ministry of Agriculture, 2007).

Calf rennet has been extensively exploited as a milk coagulating agent, especially in cheese production. However, increasingly higher prices of calf rennet and ethical ground associated with the production of such enzyme for cheese making, especially to the local entrepreneurs have led to scientific enquiries on the suitability of its replacementby other enzymes of plant sources (Sousa and Malcata 1997; Malik *et al*., 2011). However, there is still a paucity of relevant data regarding the milk coagulating ability of ginger proteases and their application in cheese making (Malik *et al*., 2011), particularly proteases from certain ginger cultivars. Research on the potentiality and suitability of proteases from some ginger cultivars is thus required. This research therefore focused on extraction, partial purification and characterization of ginger protease extracted from Yatsin Biri ginger cultivar commonly grown in Kagarko axis in northwestern Nigeria.

MATERIALS AND METHODS

The fresh ginger rhizome was collected from a harvesting site in Kagarko ginger farming area of Kaduna state, north western Nigeria. The sample was identified and authenticated as Yatsin Biri ginger cultivar at the Herbarium Unit of the Department of Plant Biology, Bayero University, Kano. The sample was issued with an accession number (BUKHAN 0299).

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 and tested for the protease characteristics.

The protein was precipitated using a modified method of Qiao *et al.,* (2009). The supernatant (80cm^3crude extract) was mixed with acetone which was pre-cooled in refrigerator (1:1) and then, the sediment was collected after centrifuged at 3000rpm 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 20%.The sample solution was incubated at 4ºC for 16 hours. The precipitated protein was then removed by centrifugation at 3000rpm for 20 minutes and 30cm^3 supernatant was collected to which 9.0 g ammonium sulphate was added pinch by pinch for 45 minutes to yield 40% saturation and again incubated at 4ºC for 16 hours. The fraction of precipitated proteins between 20 and 40% 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. The supernatant collected after centrifugation was tested for the protease characteristics.

Total Protein Concentration Determination Total protein was determined using BioAssay Systems' QuantiChromTMprotein assay kit based on an improved Coomassie Blue G method (Bradford, 1976) using Bovine Serum Albumin (BSA) as standard.

The proteases activity (ginger protease and calf rennet) was assayed using casein as substrate. The assay was carried out using a modified method of 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 incubated at 50^{0}C for 30minutes and the reaction was terminated with the addition 100µl of chilled TCA (10%), which were allowed to stand in ice for 15minutes 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. Colorations were observed on both the crude and the precipitated extract upon the addition of 0.lcm^3 Folin-Ciocalteau reagent and the absorbance was measure 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.

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 ranging from 0-100^{0}C at 10^{0}C intervals for 5minutes and the optimum temperature of the enzyme was determined by plotting a graph of enzyme activity against temperature.

The protease activity of the precipitated enzyme was measured at different pH values. The enzyme solution (2cm^3) was mixed with each of the following buffers, 0.1M acetate buffer, 0.1M phosphate buffer, 0.1M Tris-HCl buffer, and 0.1M glycine-NaOH in each labeled test-tube of pH 1.5, 3.5, 5.5, 7.5, 9.5 and 12.0 respectively. The reaction mixtures were incubated with 100µl of casein substrate at 50^{0}C for 30minutes and the optimum pH of the enzyme was determined by plotting a graph of enzyme activity against pH.

Milk-clotting activity (MCA) was measured by a modification of Sousa and Malcata (1998) procedure as modified by Hang *et al*.(2016). The milk substrate was prepared by dissolving 12 g of skim milk powder in 100cm^3 $CaCl_2$solutions (0.01mol L−1). The pH value of the milk was adjusted to 5.5 with 1 M HCl before use. The milk substrate (2 cm^3) was heated at 50^{0}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 skim milk in 1 min at 50^{0}C and pH 5.5 calculated as follows:

$$MCA = 2400/t \times F$$

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

RESULTS AND DISCUSSION

Table 1: Partial Purification of Ginger Protease Extracted from Yatsin Biri 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	740.81	326.8 ± 10	0.44	-	-
Ammonium Sulphate Precipitate b	103.02	77.7 ± 12	0.75	1.70	170%

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

Table 2: Milk Clotting Activities of Protease Extracted Yatsin Biri 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[c]
Calf Rennet	60	0.91	-
Ginger Protease	170	1.65	2.18

a – A unit (U) equals the amount (mg) of enzymes required to coagulate 1cm3 of reconstituted skim milk in 1 min at 500C and pH 5.5.
b – 103.02mg total protein content for ginger protease and 65.7 mg of protein for calf rennet

c -- PA for ginger protease: 77.7 Units

The activity of ammonium sulphate precipitated ginger protease was progressively increased as the temperature rise from the range of 10 to 50^{0}C where the optimum activity of the enzyme was observed near 50^{0}C (figure 1). However, as the temperature progressively elevated above 50^{0}C a decrease in the activity of the protease was observed. An elevated temperature of 100^{0}C denatures the enzyme protein with complete loss of its proteolysis. Considering the optimum temperature (50^{0}C) of YatsinBiri protease, the enzyme may have some applications in food industry, especially dairy and cheese making processes where temperatures near 50^{0}C often relevant (Hashim *et al*., 2011). The optimum temperature of ginger protease extracted from Yatsin Biri ginger cultivar of northwestern Nigeria showed similar temperature trend as previously reported by Hashim *et al.,* 2011; Nafi *et al*., 2013, 2014 in proteases extracted from Chinese and Malaysian ginger cultivars respectively.

Protease extracted from YatsinBiri ginger cultivar of northwestern Nigeria showed proteolytic activity with a broad pH range of 4.5 to 7.5 (figure 2). However, the optimum activity of the protease was observed at pH near 5.5 and at higher pH value of 11 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 has 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) showed a wide range of pH values of 4.5 to 6.0 which was slightly lower relative to the pH

values revealed by this study. Sometime 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 (4.5-7.5) observed inYatsin Biri 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 that favoursthe dissociation of casein from milk micelles (Skelte and Henning, 1997).

The partial purification of Yatsin Biri 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 ginger rhizome crude extract using 20–40% $(NH_4)_2SO_4$ saturation showed 1.70-fold purification and 170% recovery with specific activity of 0.75 Units/mg (Table 1). The one step extraction protocol employed in this study indicates a considerable value of the % yield (170%) with a low value of specific activity of the protease compared with related findings by Nafi *et al,*. (2014). This suggests further purification steps 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).

It is quite interesting using plant proteases as milk coagulants since they are natural enzymes from readily available sources that can be used optimally for producing cheeseand related products (Gómez *et al*., 2001;Galán *et al*., 2008). In the kinetics study of $(NH_4)_2SO_4$ fraction of Yatsin Biri ginger protease carried out in this study, the observed optimal enzyme activity conditions (pH 5.5 at 50 °C) were used in evaluating both the proteolytic and milk clotting activities of the enzyme. The PA of YatsinBiri ginger protease was 77.7Unit/mg (Table 1). This 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 organoleptic attributes. The MCA/PA ratio of Yatsin Biri ginger protease was 2.18 (Table 2), which is relatively a favourable value for cheese processing. Protease MCA/PA ratio is a very important criterion for evaluating protease potential as rennet substitutes (Abel-Fattah and El-Hawwary, 1974;He *et al*., 2012). However, PA and MCA/PA ratio of milk-clotting protease varies significantly with regard to determination and definition methods (Vishwanatha *et al*., 2010; De Castro *et al*., 2014), which often bring about complications in comparisons with related studies. Thus, MCA/PA of different milk-clotting proteases should be evaluated under the similar protocols, and similar to those employed in the cheese-making. Nevertheless, the MCA and MCSA of the Yatsin Biri ginger protease are 2.8 and 1.8 folds respectively compared to that of commercial calf rennet. This finding was in conformity with findings of Su *et al*., (2009), Hashim *et al*. (2011) and Nafi *et al.,* (2013) that ginger protease could be a suitable choice for cheese making compared to some commercial milk curdling agentsand other milk-clotting enzymes of natural origins in improving the bitterness of milk products caused by papain and bromelain. Thus, ginger protease extracted from Yatsin Biri ginger cultivar from northwestern Nigeria possess higher milk clotting activity relative to that of the compared commercial calf rennet.

CONCLUSION

The one-step ammonium sulphate extracted protease from Yatsin Biri ginger rhizome cultivar of northwestern Nigeria showed optimum activity at temperature near 50 °C and a broad range of pH values of 4.5 to 7.5 with an optimum pH at 5.5. The enzyme protein was completely denatured at elevated temperature and alkaline pH. Additional characteristics of the protease obtained in this study, especially its milk clotting activity, broad pH range and moderate temperature make it a suitable candidate for application in the food industries, particularly in cheese making processes. However, further purification of enzyme is required to optimize its application in food industries.

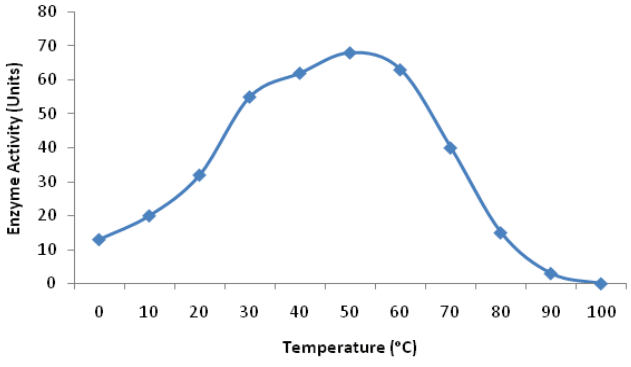

Figure1: Effect of Temperature on Protease from YatsinBiri Ginger

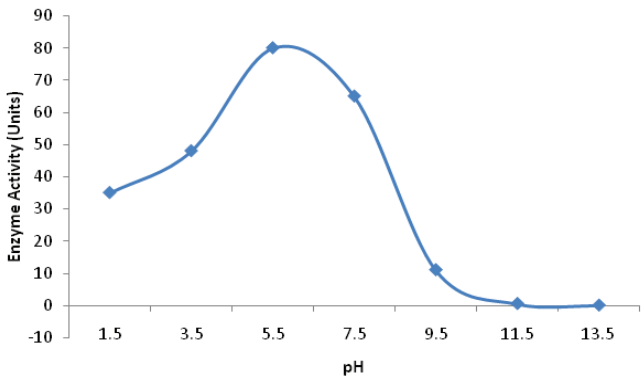

Figure 2: Effect of pH on Protease Extracted from Extracted YatsinBiri Ginger Cultivar

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