Purification and Characterization of *Digitaria exilis* Protease (White Acha)

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Abstract: The protease of malted *Digitaria exilis* (white acha) species was extracted, purified and characterized. The enzyme was extracted with 200 ml of 0.1M citrate phosphate buffer (pH 7) containing 0.4% (w/v) cysteine and 0.86% NaCl, purified by dialysis against 4M sucrose, Ion exchange chromatography on CM sepharose and gel filtration chromatography on Sepharose 4B gel. On ion exchange chromatography and gel filteration chromatography, the white acha protease was purified 2.21 folds with a specific activity of 362 Umg^{-1} protein. The relative molecular weight of the protease was estimated to be 88,000 daltons by Gel filtration. The white acha protease was optimally active at 50^{o}C and pH 7, but retained about 40% of its activity at 70^{o}C (30 mins) and pH 8. Appreciable stimulation ($P<0.05$) of the white acha protease was only achieved by Mn^{2+}, while the other metal ions (Zn^{2} ,Ba^{2+} , Fe^{2+} ,Cu^{2+} ,Ca^{2+} ,Sr^{2+} & Hg^{2+}) were inhibitory. Guanidine chloride, n-bromosuccinamide and EGTA were inhibitory ($P<0.05$) to the acha protease, while sodium sulphite and 2-mercaptoethanol (2-Me) were stimulatory with striking stimulation obtained with 2-ME. A significant effect ($P<0.05$) of inhibitors on acha protease was recorded. The enzyme exhibited broad specificity (70 – 100%) in the hydrolysis of various proteins (Bovine serum, albumin, casein, egg albumin and gelatin) and showed strongest affinity for casein when its km (0.188 mg/ml) and Vmax (0.208 U/mol) values were obtained, respectively. Therefore, *Digitaria exilis* protease can be useful in food industries if harnessed.

Key word: *Digitaria exilis*, enzymes, extraction, proteases, purification

INTRODUCTION

The world continues to depend and receive sustenance from grain crops (Conklin and Stilwell, 2007). Thus, research and development on acha cereal grains is experiencing renewed interest in Africa and the rest of the world, particularly for its flavour and nutritional qualities (Jideani *et al*., 2000; koreissi *et al*., 2007). Acha proteins have composition similar to that of rice (Temple and Bassa, 1991; Jideani and Akingbala, 1993), but have relatively high sulphur amino acid (Methionine and cysteine) content (De Lumen *et al*., 1993). Sulphur amino acids are crucial for proper heart function and nerve transmission and cereals are an essential source of these amino acids for people with low meat intake (Cirad, 2006). These and other attributes of acha show the uniqueness of the grains and their potential in contributing significantly to whole grain diets. In view of the recent trend in developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment, food, pharmaceutical, detergent industries, and in several bioremediation processes. Acha is potentially an important source of neutraceuticals such as antioxidant phenolics and cholesterol-lowering waxes (Jideani, 1999). The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure-function relationship in the synthesis of peptides and in the sequencing of proteins (Chiplonkar *et al*., 1985). Therefore, this study was aimed at characterizing the white acha protease for industrial application.

MATERIALS AND METHODS

Acha grains used in this study were bought from a neighbourhood market in Onitsha, Anambra state, Nigeria and identified by Botany Department, Nnamdi Azikiwe

University, Awka, Anambra State, Nigeria.

***Enzyme extraction*:** Acha malt grists (20 g) of 4th day of germination was used. The ground samples were extracted with 200 ml of 0.1 M citrate phosphate buffer, pH 7, containing 0.4% (w/v) cysteine and 0.86% sodium chloride for 2 hours using a rotary shaker at 250 rpm.. The obtained extract was centrifuged at 4500 rpm in an Eppendorf refrigerated centrifuge for 30minutes at 4°C and the supernatant was retained for enzyme purification (Ogbonna *et al*., 2003).

Enzyme assay: Protein assay was done by the method described by Upton and Fogarty (Upton and Forgarty, 1977). Casein (1 % w/v) in 0.3 M citrate phosphate buffer was used as the substrate. One milliliter each of casein solution and enzyme in a test tube was incubated in a thermostatically controlled water bath at 40°C (30 mins). The enzyme reaction was stopped by the addition of 6 ml of 5 % (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 10 minutes and then filtered through Whatman No 1 filter paper. To 1 ml of the filtrate were added 3 ml of 7.5 %(w/v) solution of sodium carbonate followed by 1 ml of a 1:3 dilution of Folin-Ciocalteau reagent. A blank was prepared as described in enzyme assay except that 1 ml of water was used in place of the enzyme. After standing the mixture for 30 mins at room temperature, optical density values were read at 660nm in a UV visible spectroscopy (Jenway, 6405). One unit of protease activity is that amount of enzyme releasing 1 mg of tyrosine from casein per minute under the assay condition.

Protein estimation: Total protein was estimated by the dye-binding method of Bradford (1976) using BSA as standard at wavelength of 595 nm.

Purification of enzyme: Prior to enzyme purification process, the enzyme solution was concentrated through an overnight dialysis at 4°C using 6 M sucrose solution. The enzyme concentrate was centrifuged at 4500 rpm (4°C) and the supernatant applied to the column (1.7x35 cm) containing the CM-Sepharose previously equilibrated with phosphate buffer pH 7. After a wash with the buffer, proteins were eluted with 250 ml of phosphate buffer (pH 7.0) and equal volume of 0.5 M NaCl solution in buffer. Elution was done at 10 ml per 15 mins. A total of 30 fractions were collected and protein assayed for enzyme activity and protein. The protein absorbance values were read using an Eppendorf Biophotometer at 280 nm and fractions 6-9 which showed enzyme activity were pooled. The enzyme concentrate was applied to a column (2x55 cm) packed with Sepharose 4B which was equilibrated with about 200 ml of 0.1 M citrate phosphate buffer and elution continued with 0.1 M of citrate phosphate buffer at a flow rate of 1 ml/4.5 mins. The fractions (10ml per tube) with protease activity were pooled and their enzyme activities determined.

Estimation of molecular weight: The molecular weight of the protease was determined by the method of Andrews (1964) using Sepharose 4B column (2 cm by 55 cm) equilibrated with 0.1 M citrate phosphate buffer (pH 7.0). Marker proteins (0.2 mg/ml of each protein) consisted of bovine serum albumin (mol wt. 66,500 daltons), *Aspergillus niger* amyloglucosidases (mol. wt. 97,000 daltons) and *Bacillus licheniformis* amylase (mol.wt. 55,200 daltons) (Roche diagnostic).

Temperature activity profiles and stabilities: The temperature activity profiles of white acha protease was determined by reacting 0.2 ml of the enzyme with 0.2 ml of 1% (w/v) casein in buffer at various temperatures (40°C, 50°C, 60°C, 70°C) for 30 mins. For the temperature stabilities, 0.2 ml of the enzyme contained in thin-walled test-tubes was incubated at different temperatures for 30 mins. The enzymes were promptly chilled in ice-chips and the remaining activities assayed.

pH activity profiles and stabilities: The pH activity profiles of acha protease were determined by incubating separately 0.2 ml of the enzyme with 0.2 ml of 1%(w/v) casein in various buffers (0.1M acetate buffer pH 3 – pH 5 and 0.1M citrate phosphate buffer pH 6 – pH 8) at 50°C for

30mins. The enzyme activity was assayed. For pH stabilities, 0.2 ml of the enzyme was incubated with 0.2 ml of the various buffers of different pH values for 24 hours at room temperature. Thereafter, 0.2 ml of casein solution was added and the enzyme assay carried out against a blank at 50°C.

Effect of metal ions and inhibitors: The effects of various cations ($FeSO_4$, $CaCl_2$, $BaCl_2$, $MnSO_4$, $CuSO_4$, $SrNO_4$, $ZnSO_4$, $HgSO_4$) and some inhibitors such as 2-mercaptoethanol (2-ME), ethylene glycoltetraaceticacid (EGTA). n-bromosuccinamide (NBS), guanidine hydrochloride (Guanidine) and sodium sulphite (NaS) on the activities of the proteases were examined. The reaction mixtures contained 0.2 ml of 1mM concentration of the reagents solutions, 0.2 ml of the enzymes and 0.2 ml of substrate (casein). The mixtures were incubated at 50°C for 30 mins to assay for enzyme activity. A control was set up without the salts and inhibitors.

Effect of substrate concentration on enzyme activity: The various concentrations (0 – 1 mg) of the protein substrates (BSA, egg albumin, gelatin and casein) in 0.1M phosphate buffer, pH 6 for the white acha purified enzyme were prepared. To 0.2 ml of the substrate was added 0.2 ml of the enzyme which was incubated at 50°C for 30 mins. Enzyme activity was determined as described earlier. The km values of the enzymes were determined by the Lineweaver-Burk linear transformation of the Michalis-Menten equation.

Statistical analysis: Analysis of variance (ANOVA) was used to analyze the effects of various cations and inhibitors on the properties of the purified enzymes. The significance level used was $P \leq 0.05$.

RESULTS AND DISCUSSION

Enzyme purification

The elution profile of white acha protease on CM Sepharose ion exchange chromatography showed major peaks of protease activity between fractions 6-9 (Figure 1) with 10% retention of the overall activity, a 1.6 and 1.5-fold purification and final specific activity of 311 and 340 Umg^{-1} protein, respectively (Table 1). Similarly, on gel filtration chromatography, major peaks were recorded between fractions 7-10 (Figure 2) with 6% retention of the overall e Umg^{-1} protein, respectively (Table 1)

The relative molecular weight of the acha protease was estimated to be 88,000 daltons. Thus, the acha protease could be said to be a high molecular weight protein.

A summary of purification of the proteases shows that about 6% of the original activity was recovered after gel filteration chromatography, but its specific activity increased (362 Umg $protein^{-1}$) along the purification steps. This specific activity value was lower than that obtained (402.1 Umg $protein^{-1}$ from a sorghum protease (KSV8-11 variety) on Q- Sepharose ion exchange chromatography and gel filtration chromatography on sephadex G-100 (Ogbonna *et al*, 2003).

The relative molecular weight (88,000 daltons) is comparable with that of an acid proteinase (80, 000) from germinated sorghum (Garg and Virupaksha, 1970), but higher than that of a protease (62,000 daltons) from a sorgum malt variety (Ogbonna *et al*., 2003) and protease (35,000-40,000 daltons) from two millet varieties (Taylor, 2004) .

The protease enzyme from white acha species demonstrated optimal activity and stability at 50^0C; such feature places the enzyme at a good advantage for industrial purposes**.** The white acha protease demonstrated pH activities from pH 3.0 to 7.0 with an optimum at pH 6 but was optimally stable at pH 4. Most malt enzymes are known to be metal ion dependent for their activity (Barett, 1995).

Inhibitors give the clearest evidence to the type of catalytic site which forms the basis for the classification of the enzymes (Odibo, 1987). The purified white acha protease was significantly stimulated by 2-ME which is generally known as an activator of enzymes and also contains an –SH group. Another stimulatory effect was obtained with sodium

sulphite which contains sulpur and this stimulatory effect supports the presence of sulphur amino acids (methionine and cysteine) in white acha grain. The white acha protease was inhibited by guanidine hydrochloride; an inhibitor of serine proteases and other enzymes. Ethylene glycol tetraacetic acid (EGTA); a chelating agent, inhibited the protease and must have removed the Mn^{2+} from the active site thereby inhibiting it. The inhibition of the proteases by n-bromosuccinamide indicates the presence of tryptophan group in the active site of the enzyme. The white acha protease could be classified as a metalloprotease. The white acha protease obeyed the rule modeled by Michaelis-menten by producing linear plots, thus permitting the determination of Km and Vmax showing that white acha protease enzyme had strong affinity for proteins and the strongest affinity for these enzymes was demonstrated by gelatin and casein. The relative rate of hydrolysis of proteins by the enzyme occurred at varying rates (63-100%) which indicated broad substrate specificity.

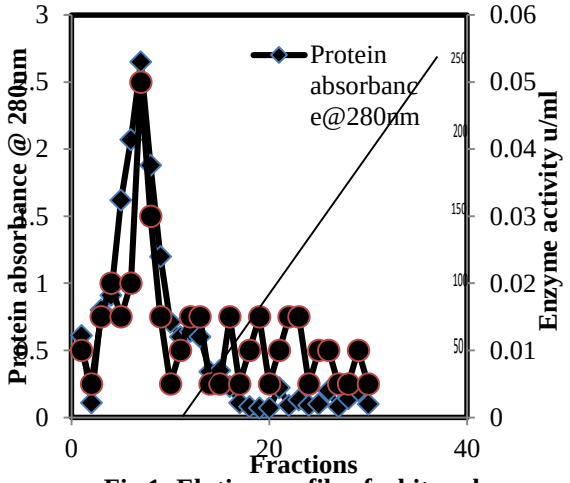

Fig.1: Elution profile of white acha malt protease on CM Sepharose Ion exchange chromatography

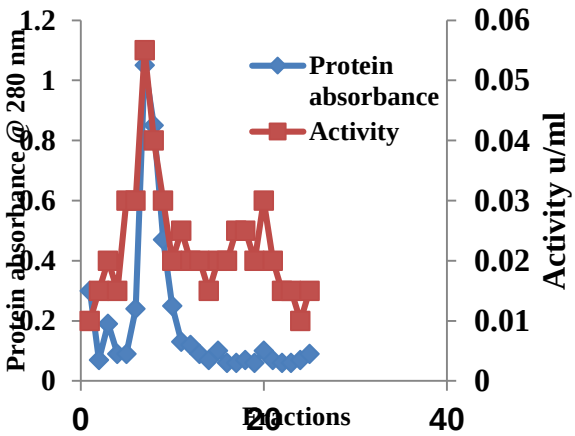

Fig.2: Elution profile of white acha malt protease on 4B Gel filtration Chromatography

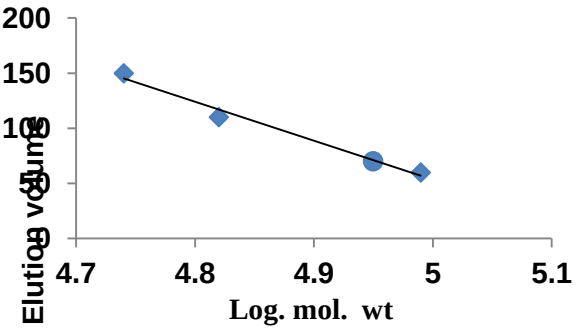

Fig.3 Estimation of relative molecular weight of protease from acha species using Sepharose 4B.

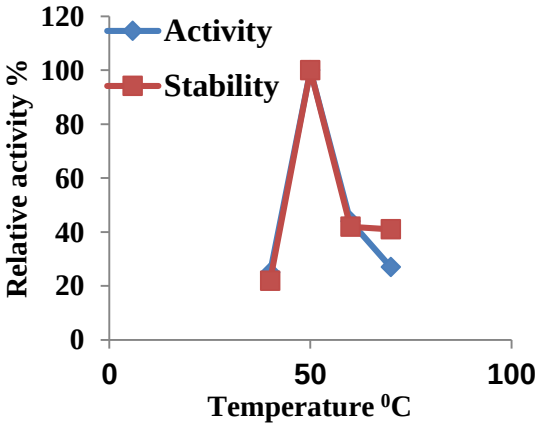

Fig.4: Temperature effect on activity and stability of white acha malt protease

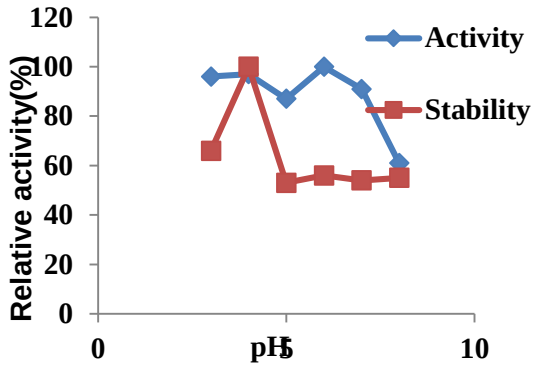

Fig. 5: pH effect on the activity and stability of white acha malt protease

Table 1: Purification summary of Protease from white Acha malt

Purification Steps	Vol (ml)	Total enzyme activity (U/ml)	Total protein (mg)	Specific activity (Umg protein^{-1})	Yield (%)	Fold
Crude enzyme	100	2520	15.4	164	100	1
Dialysis in 6M Sucrose solution	40	1172	4.56	257	46.5	1.56
Ion exchange Chromatography	10	258	0.83	311	10.23	1.89
Gel filtration Chromatography	8	152	0.42	362	6.03	2.207

Table 2: Effect of metal ions on the white and acha proteases

Metal ions (1×10^{-3}M)	White acha P < 0.05 protease % remaining activity
None	100
$SrNO_3^-$	95±1
$ZnSO_4$	56±2
$MnSO_4$	111±2
$BaCl_2$	63±1
$FeSO_4$	59±2
$CuSO_4$	65±1
$CaCl_2$	64±1
$HgSO_4$	53±2

Key: Values represent mean ± standard deviation of duplicate determinations

Table 3: Effect of some inhibitors on acha malt proteases (% relative activity)

Inhibitors (1×10^{-3})	White acha P < 0.05 protease
None	100
EGTA	85±1
NBS	97±2
NaS	125±2
Guanidine	57±1
2-ME	1137±3

Key: Values represent mean ± standard deviation of duplicate determinations, EGTA – Ethylene glycol tetraacetic acid, NBS - N-Bromosuccinamide, NaS - Sodium sulphate, Guanidine – Guanidine hydrochloride, 2-ME – 2-Mercaptoethanol

CONCLUSION

White acha malt protease (enzyme) qualities recorded in this study are such that can be useful, for example, its thermal activities and stabilities, pH broad range and specificity of the enzyme reflects its potential as a good protein source.

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