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Partial purification and characterization of alkaline proteases from maize (oba super 2)

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Abstract

Proteases are enzymes that degrade proteins into their constituents amino acids and peptide bonds. Despite the availability of plants, their proteases are the least utilized. This study examined the purification and characterization of two different alkaline proteases from Oba Super 2 maize. Standard procedures were used to prepare certified samples, extract and purify enzymes. A substrate casein was used for characterization, and the proteases were identified as alkaline serine proteases (1) and (11) by native polyacrylamide gel electrophoresis, with molecular masses of 62.81 and 63.44 kDa, respectively. The proteases were most active and stable at 50 and 60^{0}C, pH 9 and 8, were activated by Cu^{2+} and dimethyl sulfoxide, while phenylmethylsulphonyl fluoride was the potent inhibitor. In the presence of some surfactants, oxidants, and detergent (ariel), the proteases remained stable. These proteases showed promising qualities for utilization in relevant sectors.

Keywords: Purification; Characterization; Stability; Homogeneity; Formulation; Oba super 2

Introduction

Maize (Zea mays L.) grain is an important staple food crop consumed whole or processed in many countries, including Nigeria (Olaniyan, 2015). Additionally, it plays significant roles in food security and the economy (Wu and Guclu, 2013). It ranks third in global grain production, behind rice and wheat, and first in Nigeria and Sub-Saharan Africa, behind millets and sorghum (Smale *et al*. 2011). Food, animal feed, and industrial processes like brewing, the production of bioethanol, biogas (biofuels), and enzymes are all made from maize (Wallington *et al*. 2012).

Many Nigerian maize varieties, such as Oba Super 2, Oba Super 6, Sammaz, and others, have been shown to exhibit desirable malting properties (NACGRAB, 2009). These include those that have significant malting qualities for brewing, like excellent extract recovery, free alpha amino nitrogen, and strong diastatic power. Oba Super 2 maize, for example, is a locally produced hybrid variety. It is an early-maturing annual herbaceous plant that is resistant to insects and foliar diseases, such as Striga hermonthica (Drought-tolerant maize for Africa, D.T.M.A. 2014). Additionally, it is a high-quality protein maize (QPM), containing about 70% more polyunsaturated essential fatty acids and two essential amino acids, tryptophan and lysine, than normal maize types (Giwa and Ikujeniola, 2009).

Similar to brewing, enzyme manufacture involves malting grains to produce hydrolytic enzymes like amylase and proteases that deplete the endosperm's store of nutrients. Since proteases hydrolyze proteins to peptide bonds and amino acids, their development is essential because complete saccharification and a high extract yield are the final results of effective enzyme development (Mohammed and Addy, 2014).

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The grain endosperm storage proteins can be broken down into peptide bonds and amino acids by extracellular proteolytic enzymes (EC 3-4, 21-24, and 99). These enzymes are also referred to as proteinases, proteases, or peptidases according to Schmitt *et al*. (2013). Depending on how they interact with protein substrates, these proteases are categorized as endo- or exo-enzymes, amino- or carboxypeptidases. Their catalytic mechanism further classified them as glutamic, asparagine, metallo-, threonine, serine, aspartic, cysteine, mixed, or unknown peptidases (Rawlings *et al*. 2018).

Proteases can be found virtually in all living things in nature, including microorganisms, plants, and higher animals (Rao *et al*. 1998). Despite the availability of plants, their byproducts, and wastes, microorganisms are the most frequently utilized (Pant *et al*. 2014), generating almost two-thirds of commercial proteases (Wang *et al*. 2013). Proteases are of great interest to researchers due to their numerous applications and important physiological significance. Food, medicine, pharmaceuticals, silk, diagnostics, detergents, and leather are just a few of the industries that use proteases. However, the purification processes and kinetic properties have a significant impact on their applications (Saranraji *et al*. 2017).

There is a great need for industrial enzymes, particularly those derived from plants, which has not yet been addressed because researchers are concentrating on employing microbes. Since proteases make up one of the three main classes of commercial enzymes and account for around 60% of global production and sales, a paradigm shift is necessary in the field of plant-derived enzymes (Sarrouh *et al*. 2012).

The production and application of enzymes by the food and non-food industries has attracted a lot of attention in recent decades. However, low-efficiency production methods, ethical concerns, environmental concerns, and technical and financial constraints have restricted the manufacture of enzymes from plant and animal sources. Microbial proteases however, have been the most used for the industrial production of extracellular enzymes due to their scientific and economic benefits, their vulnerability to genetic manipulation, and their wide range of biochemical diversity (Jisha *et al*. 2013; Wang *et al*. 2013; Gaur *et al*. 2014; Souza *et al*. 2015; Pant *et al*. 2014).

Specifically, the availability of animals for slaughter, which is controlled by agricultural and political policies, is a prerequisite for the manufacture of proteases from animal sources (Rao *et al*. 1998). Aside other restricting issues, the process of obtaining plants takes a long time because it necessitates a large amount of land for cultivation and a climate that is suitable. However, there have been efforts to produce it both in vitro and in vivo, as well as through cell culture and the creation of recombinant proteases (González-Rábade *et al*. 2011). It's economic advantages over microbiological sources include; ease of purification, low quantities of chemicals that interfere with purification, and good yield (Rao *et al*. 1998).

Numerous microbes have been shown to produce proteases, including bacteria (Gaur *et al*. 2014; Pant *et al*. 2014), viruses and fungi as well as animals (Rao *et al*. 1998). On the other hands, because of their broad substrate specificity, good solubility, high stability, and activity across a wide range of pH and temperature, plant proteases have drawn a lot of interest from the biotechnology and pharmaceutical industries (Asif-Ullah *et al*. 2006; Ademola and Malomo, 2017). Since they exhibit high specific activity, proteases derived from a variety of plant sources, including deteriorated fruit (Ajayi *et al*. 2014), sweet potato (Chen *et al*. 2004), agro waste (Ekpa *et al*. 2010), bulbs (Ndidi and Nzelibe, 2012), and some improved tropical grains, such as rice (Arasaratnam and Kalpana, 2010), sorghum (Ogbonna, 2007), and wheat (Faltermaier *et al*. 2013), have been reported to be essential components of these industries.

According to Arasaratnam and Kalpana's (2010) research on the kinetic examinations of the local rice variety's malt amylase and protease, the ideal pH and temperature for the enzyme activity were 5 and 7, respectively, and 60°C and 50°C. Similarly, De Barros and Larkins' (1990) studies on the purification and characterization of zein-degrading protease from the endosperm of developing maize seeds stated that each of the proteases purified has a fairly comparable apparent molecular weight and net negative charge. According to Ogbonna *et al*. (2004), the sorghum malt SK 5912 was 8.4 times purified and yielded 13.4% in relation to crude activity; the ideal temperature activity was 50°C and pH was 6. Conversely, Ademola and Malomo (2017) used a combination of dialysis, column chromatography, and 95% ammonium sulphate precipitation to partially purify and characterize proteases from Citrus sinensis fruit peel. The enzymes were most active between 40 and 48°C and between pH 7 and 8. The stability of these enzymes at neutral to alkaline pH (6–9) is essential for use in a variety of commercial applications, particularly in the brewing, meat tenderization, fur, and leather industries.

In comparison to animal and microbial proteases, plant proteases have many advantages and economic benefits. These include, but are not limited to, wide substrate specificity, significant stability across a broad range of operating conditions (pH, temperature, salinity, and organic solvents), low

production costs, the lack of need for genetic engineering techniques, and an abundance of protein sources (flowers, latex, node, stem, roots, and fruits) (Antao and Malcata, 2005. The use of in vitro cultures (using cells, tissues, and organs), in vivo methods (using a biological vector to express a specific vegetable protein), and direct production from vegetable biomass (latex, flowers, fruits, and roots) through conventional farming are of benefit economic advantages to industries. While reports have indicated that in vitro and in vivo methods are more expensive than producing protein from crops, these methods have ensured consistent protease production under standard conditions. However, from an economic standpoint, proteases originating from conventional crops are less expensive than those originating from microorganisms.

Proteases from microorganisms, animals, and some plants, including some modified tropical cereal grains, have been extensively studied. However, little is known about indigenous varieties of maize, especially Oba super 2. In light of the aforementioned, the researchers partially isolated and characterized two distinct alkaline serine proteases from the Oba Super 2 (OS2) maize variety. This was accomplished by malting the certified sample at different germination (G) days (0, 1, 2, 3, 4, and 5), different steeping (S) hours (30, 36, and 42), and different kilning (K) temperatures (45, 50, and 55°C). Using ammonium sulphate $(NH_4)_2SO_4$ precipitation or salting out, the crude enzyme (extract) from the malt was partially purified to increase the enzyme activities. It was then chromatographed on carboxyl methyl (CM)-Sepharose ion-exchange chromatography after being dialysis overnight against 5 M sucrose at 4 °C. The elution pattern which showed two major peaks of protease activities between fractions 4 – 11 and 26 – 31 were separately pooled and designated as protease 1 or OS2A and 11 or OS2B. Hydrophobic interaction chromatography (HIC) was then used to further elute the samples on a phenyl Sepharose CL-4B column. The resultant enzymes were characterized using standard procedures to yield two distinct alkaline serine proteases with molecular weights of 62.81 and 63.44 KDa. Analysis of variance (ANOVA) was used to analyze the data at $p<0.05$. Graphs were plotted using Microsoft Office Excel 2016.

Materials and methods

Sample, reagents and chemicals

Premier Seed Nigeria Limited, located in Zaria, Kaduna State, Nigeria, provided the certified Oba Super 2 maize. All the chemicals, salts, reagents, standard protein (colour burst electrophoretic markers) were of analytical grades.

Maize malting

Six hundred (600) grammes of the grain sample were cleaned to remove broken kernels and washed in tap water. To check for microbiological contamination, the sample was surface sterilized by immersing it in a sodium hypochlorite (NaOCl) solution containing 1% (v/v) accessible chlorine for 40 minutes. It was then repeatedly rinsed in tap water (Ogbonna *et al*. 2003). The 200-gram sample was split into three (3) separate portions, malted at distinct steeping (S) hours 30 and germination (G) days 0–5, and then kilned at 45°C. The same malting procedures were used for 36 and 42 hours, as well as at various kilning temperatures of 50 and 55 ˚C and germination (G) days (0, 1, 2, 3, 4, and 5) and the crude enzyme extracted.

Extracting enzymes

With a few adjustments, enzyme extraction was carried out according to Ogbonna *et al*. (2003). 100 g of maize malt was extracted using 250 ml of 0.1 M citrate-phosphate buffer, pH 7.0, which contained 1.075 g of sodium chloride (NaCl) and 0.5% (m/v) cysteine hydrochloride (cyst. HCl). For two hours, the suspension was shaken at 120 rpm at room temperature (±28ºC) in a rotary shaker. Crude protease was recovered from the supernatant after the extract was centrifuged using an Eppendorf centrifuge 5804 R at 4500 x g for 30 minutes at 4°C.

Protease activity assay

A modification of Upton and Fogarty's (1977) methodology was used to measure protease activity. A 0.2 ml reaction mixture comprising 0.1 ml of 1.0% (m/v) casein in 0.1 M citrate-phosphate buffer, pH 7.0, and 0.1 ml of the crude enzyme solution was used to assess protease activity. The mixture was incubated for 30 minutes at 50°C in a water bath with a thermostat (GFL, Burgwedel, Germany). 2.0 ml of 5% (m/v) trichloroacetic acid (TCA) was added to stop the reaction. Whatman No. 1 filter paper was used to filter the reaction mixture, and 1.0 milliliter of the filtrate was obtained. After that, 1 ml of the filtrate was mixed with 5 ml of 0.5 M sodium trioxocarbonate (iv), (Na_2CO_3) solution, and then 0.5 ml of a three-fold diluted Folin-Ciocalteau reagent was then added. Measuring the absorbance at 660 nm using a Jenway 6405 Uv/Vis- Spectrophotometer, the mixture was left to stand at room temperature for half an hour. The above process was used to make a blank tube, with the exception that the TCA solution was introduced before the enzyme.
The quantity of enzyme needed to release 1 mg of tyrosine from the substrate (casein) per minute at 50°C under test conditions is known as one unit (U) of protease activity.

Measurement of proteins

Using bovine serum albumin (BSA) as the standard and measuring the absorbance at 595 nm, Bradford's (1976) method was used to determine the protein level.

Enzyme purification

To purify the enzyme, 250 ml of the extract was saturated with ammonium sulphate overnight at 4°C (Duongly and Gabelli, 2014). The resultant precipitate was dialyzed against 5 M sucrose at 4°C after being re-suspended in 205 ml of 0.1 M citrate-phosphate buffer with a pH of 7.0. On CM-Sepharose that had been equilibrated with 0.1 M citrate phosphate buffer (pH 7.0) (column: 1.8 × 26) and a linear gradient of NaCl (0 – 0.5 M) at a flow rate of 70 ml/h, the concentrated enzyme was chromatographed. Fractions 4 – 11 and 26 – 31 with high protease activities and hereafter designated Oba Super 2 proteases I (OS2A) and II (OS2B), respectively, were separately pooled and dialyzed as previously against 5M sucrose. Hydrophobic interaction chromatography (HIC) on a phenyl Sepharose CL-4B column (2.3 × 8.5 cm) was applied individually to the concentrated enzymes. Proteases 1 and 11 were eluted at a flow rate of 82 ml/h and 80 ml/h, respectively, using a linear gradient of 3, 2, 1, and 0.5 M NaCl in 0.1 M citrate-phosphate buffer (pH 7.0). Fractions with high activities were combined and dialysed as earlier described. The recovered purified proteases were stored frozen for kinetic studies.

Homogeneity test

For homogeneities, the native polyacrylamide gel electrophoresis (native-PAGE) was used to assess the purified proteases without the use of denaturing agents (sodium dodecyl sulphate, or SDS) or reducing agents (dithiothreitol, or DTT): The electrophoresis was conducted at room temperature using a buffer of 0.5 M Tris/glycine pH 8.8 with a current of 40 mA and 180 volts until the tracking dye (0.01% w/v bromophenol blue) appeared. To visualize the protein band, Coomassie Brilliant Blue R 250 was used. Unbound protein dye was removed in a de-staining solution.

Relative molecular weight (RMW) estimation

UNSCAN-IT software, version 6.1 (Silk Scientific) (IBM SPSS Statistics for Windows, v. 22, 2013), was used to estimate the RMW. Lysozyme (14.2 KDa), soybean trypsin inhibitor (21 KDa), trypsinogen (bovine) (24 KDa), egg albumin (45 KDa), bovine serum albumin (monomer) (66 KDa), and bovine serum albumin (dimer) (132 KDa) were the reference proteins of the different MWs (Sigma Aldrich color burst electrophoretic markers) that were employed. A calibration curve based on these reference proteins' molecular weights and relative mobility values was used to identify the proteins.

Characterizations of the proteases

Assessment of pH activity and stability

The impact of pH on the activity of the purified protease was investigated using two distinct buffers: 0.1 M NaOH-phosphate buffer, pH 8–11, and 0.1 M citrate-phosphate buffer, pH 3.0-7.0. About 0.1 ml of the purified enzyme and 0.1 ml of 1.0% (m/v) casein in the buffers were both present in the reaction mixture. After 30 minutes of incubation at 50 °C, the enzyme's activity was measured.

An equal volume of the enzyme (0.1 ml) was pre-incubated with each of the buffers (pH 3–11) at room temperature for three hours in order to evaluate the pH stability profile of the protease. After that, the pre-incubated mixture was given an equal amount of 1.0% (w/v) casein in the proper buffers, and it was incubated for an additional half hour at 50°C. After that, the remaining enzyme activity was measured as previously mentioned.

Assessment of temperature activity and stability

Temperature impacts on the activity profile were evaluated between 30 and 90 degrees Celsius. In 0.1 M phosphate-NaOH buffer, pH 9.0, 0.1 ml of the enzyme and 0.1 ml of 1.0% casein were combined, and the mixture was incubated for 30 minutes at the test temperatures. The enzyme (0.1 ml) was pre-incubated individually for 30 minutes at temperatures ranging from 30 to 90°C in order to test for thermostability. The enzyme was then quickly cooled in ice, and the remaining activity was then measured.

Effects of some metal ions on the activity of the enzymes

To check the activity of the purified proteases, a few metal cations (Cu^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+}, Hg^{2+}, Ag^{+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, and Co^{2+}) were employed. At 0.1 M phosphate-NaOH buffer pH 9.0, the enzyme and the individual metal chloride, sulphate, or nitrate at 5 mM were reacted for three hours at 30 °C. After that, a second equal volume of 1.0% casein in buffer was added to the pre-incubated sample before it was re-incubated for 30 minutes at 50 °C and its residual activity was measured. The control tube was considered to have 100% activity since it contained no metal cations.

Effects of some inhibitors on the activity of proteases

At a concentration of 1.0 mM, the effects of several inhibitors on the purified enzyme were investigated. These inhibitors

included ethylene diamine tetraacetic acid (EDTA), phenylmethylsulfonylfluoride (PMSF), iodoacetic acid (IAA), para-chloromercuribenzoate (p-CMB), and ethylene bis (oxyethylene nitrilo) tetra-acetic acid (EGTA).

For ten minutes, the enzyme (0.1 ml) was pre-reacted with 0.1 ml of each inhibitor at 1.0 mM at 30°C. To determine the degree of inhibition, an equal amount of 1.0% casein in 0.1 M phosphate-NaOH buffer, pH 9.0, was then added to the reaction mixture and incubated for 30 minutes at 50°C. The inhibitor-free enzyme was used to pre-incubate the control, and the residual activity was measured.

Effects of detergents and surfactants on enzyme activity

The purified protease's stability and compatibility with detergents and surfactants were investigated. Francois and More's (2015) modified approach was applied. Klin, Ariel, and Omo were the detergents used, and Triton X-100, Tween-80, Tween-20, and sodium dodecyl sulphate (SDS) were the surfactants. 1.0 g per 90 ml of detergents and 1.0 ml per 100 ml of surfactants were dissolved in distilled water to create the detergents and surfactants, respectively. Equal volumes (0.1 ml) were then pre-incubated with the enzyme for 30 minutes at room temperature before an equal volume (0.1 ml) of 1.0% casein was added in 0.1 M phosphate-NaOH buffer, pH 9. The activity was measured after the reaction mixture was re-incubated for 30 minutes at 50°C. The control sample tube, which contained no surfactant or detergent, was considered 100%.

Effect of some oxidizing agents on the activity of proteases

Protease activity was assessed in response to 1–5% (v/v) hydrogen peroxide, H_2O_2, and dimethyl sulfoxide (DMSO). The reaction mixture, which included an equal volume of the oxidizing agents and the enzyme solution (0.1 ml), was incubated for 30 minutes at room temperature (28°C). The enzyme activity was then measured after equal amounts of 1.0% (m/v) casein in 0.1 M phosphate-NaOH were added and incubated for 30 minutes at 50°C.

Impacts of some reducing agents on protease activity

Protease activity was assessed in response to 1–5% (v/v) dithiothreitol (DTT) and 2-mercaptoethanol (2-ME). 0.1 ml of the enzyme and 0.1 ml of the reducing agents made up the reaction mixture, which was then allowed to sit at room temperature for half an hour. Enzyme activity was then measured as previously mentioned.

The relative hydrolysis rates of substrates

Gelatin, bovine serum albumin (BSA), egg albumin (EA), and casein were the substrates utilized. Each substrate, 1.0 percent (m/v) in 0.1 M phosphate-NaOH buffer (pH 9), was incubated separately for 30 minutes at 50°C with an equivalent volume (0.1 ml) of the purified enzyme. After then, assaying for their activities allowed for the determination of their relative hydrolyze rates. The substrate with the highest hydrolytic activity of the enzyme was considered 100%.

Effect of substrates concentration on protease activity

Purified proteases activity were measured in a reaction with 0.1 M phosphate-NaOH buffer, pH 9, and varying quantities (0-1.0 mg/ml) of the test protein substrates [casein, egg albumin (EA), gelatin, and bovine serum albumin (BSA)]. The activity was measured after an equal volume of the enzyme (0.1 ml) was incubated with these substrates for 30 minutes at 50°C (Balakireva *et al*. 2019). Using double reciprocal plots of the data in accordance with the kinetic constants (maximum velocity, Vmax, and Michaelis-Menten constant, Km) of the enzyme for each substrate were determined.

Statistical analysis

SPSS software version 22 was used to statistically analyze the collected data. With n = 2, the results were displayed as the mean of duplicates ± standard deviation (± S.D.), with a significance level of $p < 0.05$. One-way ANOVA techniques were used to conduct the analysis of variance. Plotting of the graphs was done with Microsoft Office Excel 2016.

Results and discussion

Protease activity

Table I displays the amount of crude protease that was produced following malting, kilned at 50°C, from day 0 to day 5. Protease development increased steadily until day three (3), when the activity peaked in all steep cycles and then started to drop. But out of all the steeping times, 42 hours produced the best protease development (0.834 U/ml), therefore it was used to purify the enzyme.

Significant differences ($p < 0.05$) were seen in the ANOVA results of the crude protease produced at various steeping and germination times, kilned at 50°C. According to reports, sorghum genotypes showed an increase in protease activity from day zero to day five of germination (Malomo and Alamu, 2013). In the current study, the rise in protease activity levels from the first day of germination to the third day and the subsequent fall was in line with the modifications in the protein content and protease activity of the maize malt.

Table I. Maize malt's crude protease activity (U/ml) at various steeping and germination times, kilned at 50°C

Malting conditions	S_{30}	S_{36}	S_{42}
G_0	0.551±0.000[b]	0.437±0.014[a]	0.482±0.001[a]
G_1	0.562±0.001[b]	0.479±0.001[a]	0.613±0.001[a]
G_2	0.614±0.003[a]	0.496±0.014[a]	0.794±0.000[a]
G_3	0.679±0.001[a]	0.757±0.001[a]	0.834±0.001[a]
G_4	0.635±0.007[b]	0.692±0.001[a]	0.716±0.001[a]
G_5	0.642±0.007[b]	0.656±0.001[a]	0.671±0.001[a]

Key: Go – G5, germination period (0 – 5 days); S30, S36, and S42, steeping at 30, 36 and 42 hours, respectively. Results are presented as means ± SD of two replicates. Using Duncan Multiple Range Test (DMRT) grouping and least significant difference (LSD), values that are followed by the letter "a" are statistically different at $p<0.05$, whereas values that are followed by the letter "b" are not substantially different at $p<0.05$.

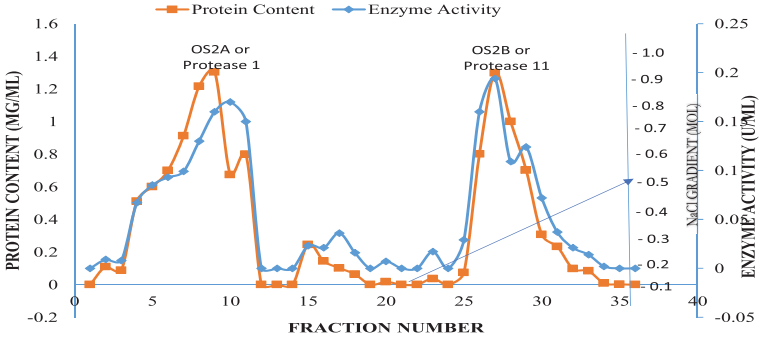

Fig. 1. Protease elution profile using carboxymethyl (CM) sepharose (rapid flow) ion-exchange chromatography from the maize malt variety Oba super 2 (OS2). The linear gradient of the 0.5 M NaCl solution was shown by the arrow, the protein content by the red line, and the protease activity by the blue line.

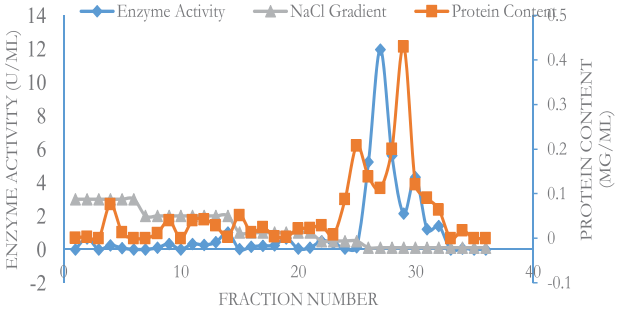

Fig. 2. Elution profile of protease OS2A or 1 on phenyl sepharose CL-4B hydrophobic interaction chromatography. The ash line showed the different 3.0 - 0.5 M concentrations of NaCl solution and elution buffer.

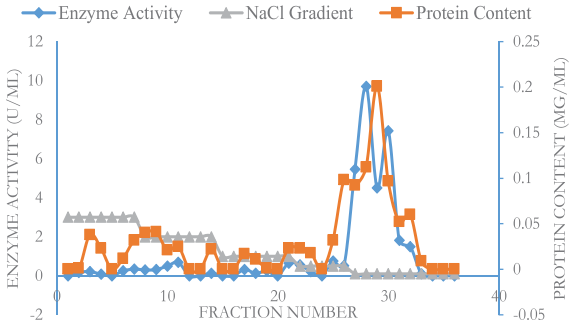

Fig. 3. Elution profile of protease OS2B or 11 on phenyl sepharose CL-4B hydrophobic interaction chromatography The ash line showed the different 3.0 - 0.5 M concentrations of NaCl solution and elution buffer.

The results of the ANOVA of the crude protease development at various germination and steeping periods (days and hours), kilned at 50°C, showed a significant difference ($p < 0.05$) between the parameters [days for germination, hours for steeping, and °C for kilning] on Oba Super 2 (OS2) maize malt.

The protease activity formed in the sample immersed for 30 hours and germinated between zero (G0) to first (G1) and fourth (G4) to fifth (G5) days did not differ significantly ($p > 0.05$), according to the least significant difference (LSD) and Duncan multiple range test (DMRT). However, on the second (G2) and third (G3) days of germination, respectively, a significant difference ($p < 0.05$) was noted.

Between the first (G0) and fifth (G5) days of germination, there was a significant difference (increase) ($p < 0.05$) in the protease activity generated, according to the LSD and DMRT at steeping times of 36 and 42 hours.

Enzyme purification: elution profile of maize malt cultivar

The OS2 protease elution profile on carboxymethyl (CM) Sepharose (rapid flow) ion-exchange chromatography (IEC) is displayed in Figure 1. The elution pattern which showed two major peaks of protease activities between fractions 4 – 11 and 26 - 31 were separately pooled and designated Oba Super 2A (OS2A or protease 1) and Oba Super 2B (OS2B or protease 11). They were subsequently subjected to hydrophobic interaction chromatography

Summary of purification of the proteases

Table II displays the proteases' purification summary. After the proteases were separated from the crude supernatant, the final yields for proteases 1 and 11 were 3.26 percent and 4.24 percent, respectively, with specific activities of 16.21 and 16.75 U/mg and purification factors of 3.805 and 3.932-fold. The purification indices obtained for these proteases were at variance with the KSV8-11, a sorghum malt variety, with 1.4% yield in relation to the total activity after being purified five times (Ogbonna *et al*. 2003).

Table II. Summary of purification of proteases OS2A (1) and OS2B (11)

Purification steps		Volume (ml)	Total Activity (unit)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme		250	303.50	71.25	4.26	100	1.00
70% sat. of ammonium sulphate		205	210.13	68.06	3.09	69.24	0.725
CM-Sepharose	OS2A	20	13.60	6.06	2.24	4.48	0.526
	OS2B	20	4.16	4.28	0.97	1.37	0.228
Dialysis using 5M sucrose,	OS2A	4.6	5.59	0.62	5.79	1.18	1.359
	OS2B	6.5	4.48	1.37	3.27	1.47	0.768
HIC on phenyl Sepharose	OS2A	10	9.89	0.61	6.21	3.26	3.805
OS2B		12	12.86	0.77	16.75	4.24	3.932

Molecular weight estimation using native polyacrylamide gel electrophoresis

The presence of single protein bands on native polyacrylamide gel electrophoresis (PAGE) verified the homogeneity of the two proteases. According to Figure 4, the calculated relative molecular weights of the enzymes were 62.81 KDa for OS2A (D2) and 63.44 KDa for OS2B (D2*). The enzymes may be similar to the sorghum malt variety

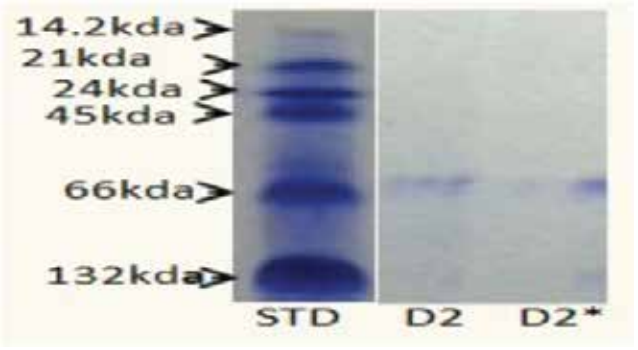

Fig. 4. Native polyacrylamide gel electrophoresis of proteins 1 (D2) and 11 (D2*)
Legend: Native gel electrophoresis of protein samples D2 and D2*. Applied on lane D2, 7 ug and lane D2* 10 ug. Standard proteins on standard lane are; Lysozyme (14.2 KDa), Soy bean trypsin inhibitor (21 KDa), Trypsinogen (bovine) (24 KDa), Egg albumin (45 KDa), Bovine serum albumin (monomer) (66 KDa), and Bovine serum albumin (dimer) (132 KDa)

KSV8-11, which was purified 5.0 times to generate a 1.4% yield in relation to the total activity, based on their low molecular weight (LMW), which indicates that they have short peptide chains (Ogbonna *et al*. 2003). In this investigation, the molecular weights of 62.81 and 63.44 KDa were more than the 54 KDa of MRP (proteinase I) that was extracted from Zea mays L (Goodfellow *et al*. 1993). Using gel filtration, these results were equally comparable to those of 62 KDa serine protease (RSIP), which was likewise isolated from Zea mays L. (James et al., 1996). The 110 KDa serine protease isolated from wheat (Triticum aestivum cv. Pro INTA Isla Verde) contradicted our results (Robert *et al*. 2003).

Physico-chemical properties

Protease stability and activity patterns at different pHs

At different pH levels, the purified proteases displayed stabilities and activity. While protease 11 showed maximum stability at pH 8.0 and optimal activity at pH 9.0, protease 1 showed both optimal activity and stability at pH 9.0 (Figure 5). At neutral to alkaline pH, particularly between 7 and 10, both proteases remained stable. Protease 1 showed a considerable decline in activity and stability with approximately

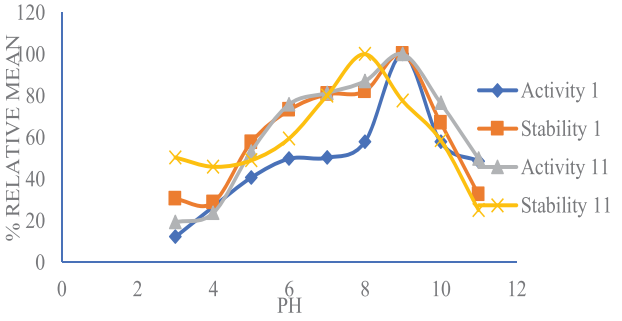

Fig. 5. Effect of pH on the activity and stability of proteases 1 and 11 Enzymatic activity and stability were assayed using casein as a substrate and is expressed as % relative mean

48% and 32%, respectively, at pH 11, and protease 11 showed a matching decline in activity and stability with approximately 49% and 25%, respectively, at pH 11.

The findings of Antao and Malcata (2005), who reported that the majority of plant serine proteases exhibited an optimal pH range of 7–11, are consistent with these findings. According to the current study, the optimal pH of maize proteases indicates that they functioned at neutral to alkaline pH and remained active across these pH ranges. Our results conflict with those of some grain proteases. Contrary to our results, however, the ideal pH for wheat malt protease was found to be 4 (Fahmy and Fahmy, 2005), whereas the ideal pH for sorghum malt was found to be 5 (Ogbonna *et al*. 2003).

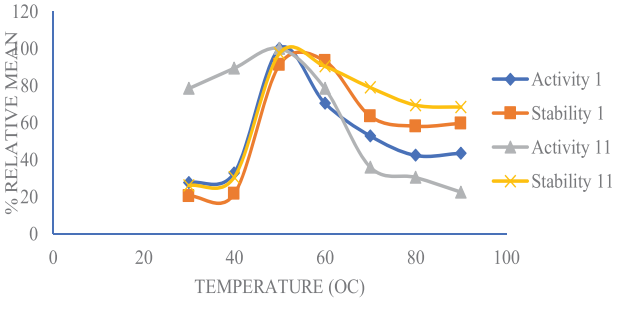

Fig. 6. The impact of temperature on the activity and stability of proteases 1 and 11 Enzymatic activity and stability were assayed using casein as a substrate and is expressed as % relative mean

Additionally, the ideal pH differs somewhat from that found in wheat flour, which has two proteases: a minor protease with an optimal activity at pH 4.4 and a major protease with an optimum activity at pH 3.8 (Mikola and Virtanen, 1980). Furthermore, our results were in contrast to those of the rye grain that germinated, which showed protease activity at pH 3.8 (Wang and Grant, 1969).

Protease stability and activity patterns at different temperatures

The enzymes showed varying stabilities and activity at different temperatures. Protease 1 maintained almost 70% of its initial activity after 30 minutes at 60°C. It was most stable at 60°C and most active at 50°C. Protease 11 exhibited maximum stability and activity at 50°C (Figure 6), and after 30 minutes at 60°C, it maintained more than 70% of its activity. With roughly 68% of the initial enzyme activity at 90°C, protease 11 is marginally more resistant to temperature increases than protease 1, which only retained 60% of its activity after 30 minutes at 90°C.

Our results showed that the proteases were stable at very high temperatures, which implies that they could be helpful in the food industry for processes like baking and brewing that use protease at high temperatures of roughly 50 to 60 degrees Celsius. The sulphydryl or cysteine protease from the sorghum malt variety, KVS8-11, also had an optimal temperature of 50°C, which is comparable to the 50°C found in this investigation (Ogbonna *et al*. 2003). Additionally, according to Arasaratnam and Kalpana (2010), it is consistent with the optimal activity of 50°C, which is derived from a local variety of dehusked, unpolished rice grains known as the "Mottaikaruppan" variety. Senthuran (1997) had previously demonstrated that malted wheat flour's maximum protease activity occurred at 50°C. On the other hand, unstable protease activity at 50°C was reported by Wang and Grant (1969).

Effects of some metal ions on the enzyme activity

Table III displays how certain metallic ions affect the isolated proteases. The proteases' activity were significantly boosted by Cu^{2+}, Ca^{2+}, Mn^{2+}, and Ba^{2+}. There was very little stimulatory activity from mercury (Hg^{2+}). Proteases were slightly inhibited by Ag^{+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, but Co^{2+} gave the highest inhibitory effect. Among metal ions, Cu^{2+} recorded the most stimulatory effect. The stimulatory effect of Cu^{2+} on acid protease of sorghum had been reported (Ogbonna *et al*. 2004). The lack of a -SH group in the proteases' active site is suggested by the fact that Hg^{2+} does not inhibit the proteases. Copper and mercury are heavy metals known for their inhibitory action on enzymes. However, the activation of the proteases by Hg^{2+} and Cu^{2+} at 5 mM concentration corroborate the report of (Liu *et al*. 2015), in which Hg^{2+} and Cu^{2} at low concentration increased the activity of papain but decreased it at higher concentration. In this study, we did not increase the concentration of the metal ions beyond 5 mM. Report had shown that proteases that are stable in the presence of metal cations are are typically used in sewage treatment and leather operations (Anwar and Saleemuddin, 2000).

Effects of some inhibitors on the protease activity

All of the tested inhibitors were inhibiting to the enzymes at different percentages, indicating their impact on the proteases' activity. The PMSF was considered the most potent inhibitor since it had the highest inhibitory action for both proteases, with 60% and 66%, respectively (Table III). Because it is a key control mechanism in biological systems, the inhibition of enzyme activity by particular small molecules and ions is significant. When metal cations are present, they are typically used in sewage treatment and leather operations (Anwar and Saleemuddin, 2000). The type of catalytic site is best demonstrated by the knowledge of protease inhibitors, which serves as the foundation for the categorization of the enzymes (Buller and Townsend, 2013). The two proteases are therefore categorized as alkaline (serine) proteases based on the pH values of the enzymes and their notable inhibition by PMSF

Effect of detergents and surfactants on protease activity

In the presence of the studied detergents (1.0% (m/v) and surfactants (1.0% (v/v), the purified proteases showed different levels of stability and compatibility. The outcome showed that Tween-80, Tween-20, Klin, and Omo were inhibitory, whereas the surfactants (Triton X-100 and SDS) and detergent (Ariel) significantly increased the activities of both proteases. In addition to being stable with a strong anionic surfactant (SDS) at 125.74 percent and a non-ionic surfactant (Triton X-100) at 318.99 percent, protease 1 was also stable with the detergent (Ariel), retaining roughly 136.02% of its activity after 30 minutes of incubation (Table III).

The best indication of catalytic site is provided by the understanding of protease inhibitors. Tween-80 and 20 were inhibiting, whereas, protease 11 remained stable in the presence of 1.0% Triton X-100 at 216.12% and SDS at 116.09%. While Klin and Omo were inhibiting, the enzyme remained equally stable after 30 minutes of treatment with 1.0% Ariel, maintaining roughly 118.77% residual activity (Table III). A good protease should be stable in the presence of commercial detergents because reports have shown that the enzyme's

Table III. Impact of metal cations, inhibitors, detergents and surfactants on protease activity

Metal salts (5 mM)	% Relative activity Protease 1	Protease 11
None	100.00±0.00[a]	100.00±0.00[a]
$CuSO_4$	187.75±0.35[a]	162.95±0.07[a]
$CaCl_2$	180.50±0.14[a]	159.20±0.14[a]
$MnCl_2$	149.20±0.00[a]	150.40±0.00[a]
$BaCl_2$	137.25±0.07[a]	115.50±0.28[a]
$HgCl_2$	117.91±0.01[a]	117.95±0.07[a]
$AgNO_3$	95.95±0.07[a]	95.85±0.07[a]
$ZnSO_4$	72.40±0.14[a]	68.70±0.14[a]
$MgSO_4$	65.85±0.07[a]	70.60±0.71[a]
$FeSO_4$	49.30±0.00[a]	54.10±0.14[a]
$CoCl_2$	29.80±0.28[a]	24.25±0.07[a]
Inhibitors (1 mM)		
EDTA	53.00±0.00[a]	51.30±0.14[a]
EBTA	64.10±0.14[a]	73.70±0.14[a]
IAA	79.30±0.14[a]	76.30±0.14[a]
PMSF	40.20±0.14[a]	34.20±0.14[a]
p-CMB	76.10±0.00[a]	64.50±0.14[a]
Detergents (1.0 %)		
None	100.00±0.00$_a$	100.00±0.00$_a$
Ariel	136.02±0.03[a]	118.79±0.02[a]
Klin	67.89±0.16[a]	49.98±0.01[a]
Omo	36.81±0.28[a]	35.38±0.03[a]
Surfactants (1.0 %)		
Triton X-100	318.99±0.01[a]	216.12±0.01[a]
SDS	127.74±0.40[a]	116.09±0.02[a]
Tween-80	33.90±0.14[a]	49.99±0.02[a]
Tween-20	24.10±0.04[a]	32.20±0.10[a]

Legend: Enzyme activity was regarded as 100% in the absence of metal ions, inhibitors, detergents, and surfactants. The means ± SD of two replicates are used to present the results. Using Duncan multiple range test (DMRT) grouping and least significant difference (LSD), values that are followed by the letter "a" are statistically different at $p<0.05$, whereas values that are followed by the letter "b" are not substantially different at $p<0.05$

stability and activity in the presence of these detergents provide insight into their potential in the detergent business (Devi *et al*. 2008).

Our results are consistent with those of Madan *et al*. (2002), who found that activity was retained at 84.5% when commercial detergent (Vim) was present and at over 40% when Nirma Super and Wheel were present. Kiln and Omo's formulations may be the cause of the variations in retention and decrease of activity that they showed. These detergents contain enzymes as additives, although their varied inhibitory effects may also be attributed to other active substances.

Effect of oxidizing agents (O. As) on protease activity

Dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), and O.As. were evaluated at different concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0% (v/v). In the presence of these substances, the purified proteases were stable and displayed varied levels of activity. Protease 1 maintained its activity of 107.8% and 117.5%, respectively, after 30 minutes of treatment with 1.0% H2O2 and DMSO. Enzyme inhibition increased in tandem with increasing O. As concentration from 1.0 to 5.0% (Figure 7a). Protease 11's activity was also marginally increased after 30 minutes of treatment with 1.0% H_2O_2 and DMSO, maintaining 116.50 and 101.80% activities, respectively (Figure 7b).

Protease stability in the presence of DMSO, an oxidant and organic solvent, makes it crucial for synthesis reactions, particularly peptide synthesis that is used to manufacture medicinal drugs. Nowadays, bleach-based detergent formulations often contain proteases that are stable against oxidants, such as DMSO and H2O2. The enzyme in the detergent formulation needs to be active in the presence of oxidants and surfactants, in addition to being stable against temperature, pH, and metal ions (Gupta *et al*. 2002). As a result, the alkaline protease from the maize malt that we examined displayed these characteristics.

Effect of reducing agents on protease activity

The effect of some reducing agents on the proteases were studied at optimal conditions for enzyme activity. The impact

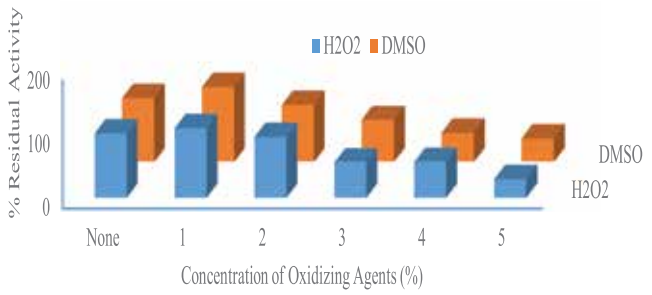

Fig. 7a. Effect of oxidizing agents on the activity of protease 1
The enzyme's activity without oxidizing agent was considered as 100%

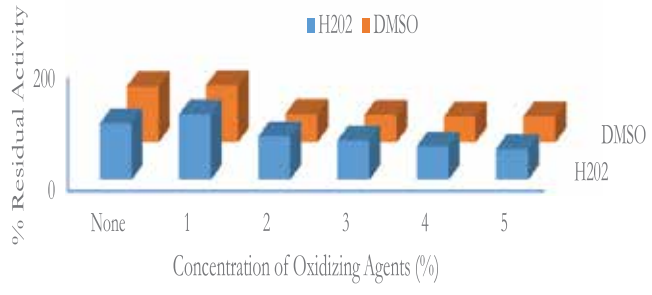

Fig. 7b. Effect of oxidizing agents on the activity of protease 11
The enzyme's activity without oxidizing agent was considered as 100%

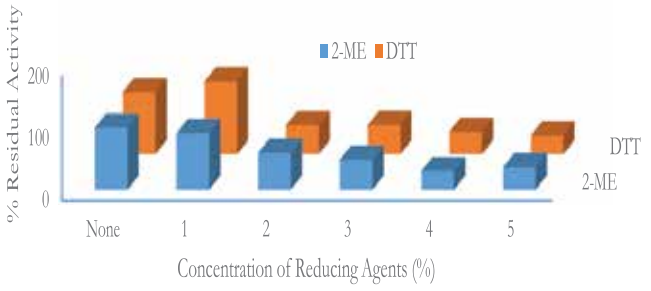

Fig. 8a. Impact of reducing agents on the activity of protease 1
The enzyme's activity without reducing agent was considered as 100%

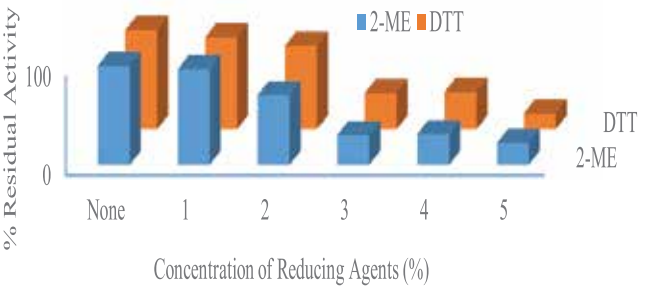

Figure 8b: Impact of reducing agents on the activity of protease 11
The enzyme's activity without reducing agent was considered as 100%

of reducing agents, such as 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) on the proteases were evaluated at different concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0%. In the presence of these agents, the enzymes showed different stabilities and activity. When 1.0% DTT was present, protease 1 was mildly activated at 117.00%; however, when the concentration increased from 2.0% to 5.0%, the activity steadily reduced. Following 30 minutes of treatment with 1.0% of 2-ME, the enzyme displayed reduced activity in the presence of this agent (Figure 8a). When 1.0% concentrations of 2-ME and DTT were present, protease 11 activity was decreased at 96.30 and 92.70 percent, respectively (Figure 8b).

On overall assessment, following a 30-minute incubation period with these compounds, inhibitory activity rose as DTT and 2-ME concentrations increased. According to reports, the reducing agents 2-ME, dithiothreitol, and cysteine HCl enhanced the proteolysis that took place in mash and malt extracts (Jones and Budde, 2003). On the other hand, proteolysis was prevented by the addition of hydrogen peroxide, diamide, or an oxidizing agent. However, a previous study by Kunert (1992) demonstrated that the proteolytic activity of Microsporum gypseum was inhibited in the presence of substrates without a disulphide bond (casein) by sodium sulphate, cysteine, glutathione, 2-ME, and dithioerythreitol at concentrations of 0.1 to 1.0 mmol (L-1), whereas the activity was increased in the presence of substrates with a disulphide bond (BSA and keratin).

Relative rates of hydrolysis of various substrates by the proteases

Ideally, the hydrolytic characteristics of the enzymes were examined on casein, BSA, gelatin, and egg albumin (EA) as substrates. The hydrolytic activity of the protease 1 enzyme was 100% against casein, followed by gelatin (85.10%), BSA (41.50%), and egg albumin (39.50%) (Figure 9a). Protease 11 also exhibited the highest preference for casein at 100%, BSA at 50%, gelatin at 49.40%, and egg albumin at 45.60%. However, it differed from protease 1 in that it preferred BSA over gelatin (Figure 9b).

According to reports, the key characteristic of proteases is their capacity to distinguish between competing substrates; the usefulness of these enzymes is frequently determined by their substrate specificity (Shankar *et al*. 2011). The proteases' substrate specificity profiles show that they have a broad range of hydrolytic activity on different protein substrates, which is a highly promising property for biotechnological applications. In this study, the maximum hydrolytic activity in the presence of casein is consistent with other researchers' findings. Protease from *Holarrhena antidysentrica* seeds was found to have more action against casein than BSA and gelatin (Khan *et al*. 2008). According to Ademola and Malomo (2017), proteases from the fruit peel of *Citrus sinensis* had the highest substrate affinity and hydrolysis with gelatin (125 % relative activity).

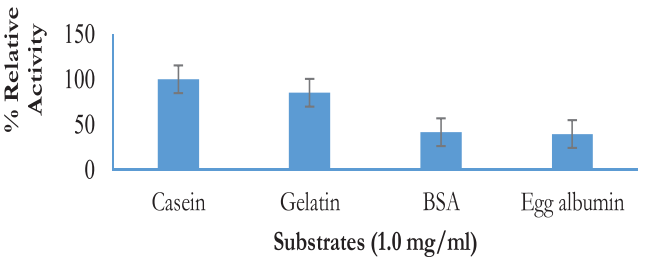

Fig. 9a. Relative rates at which protease 1 hydrolyzes different substrates.
The error bars display the duplicates' standard deviation (±SD).

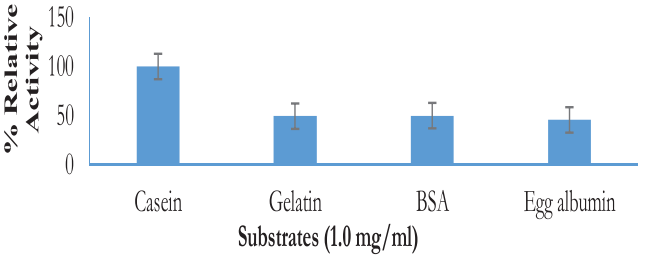

Fig. 9b. Relative rates at which protease 11 hydrolyzes different substrates.
The error bars display the duplicates' standard deviation (±SD).

Effect of substrates concentration on protease activity

Figures 10a and 10b illustrated how the activity of the purified enzymes is affected by different amounts of the substrates (casein, BSA, gelatin, and egg albumin). The proteases were hyperbolic in all substrates examined and had typical Michaelis-Menten's type kinetics.

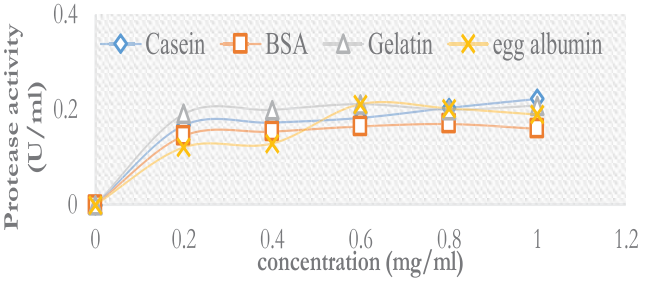

Fig. 10a. Impact of substrate concentrations on protease 1 activity

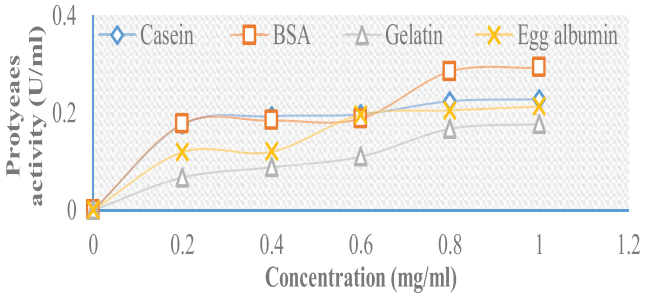

Fig. 10b. Impact of substrate concentrations on protease 11 activity

yield that can be utilized for enzyme production. The molecular masses of 63.44 and 62.81 kDa, the enzymes optimal activities at 50°C, pH 9, highly stimulatory effects of Cu^{2+}, Ca^{2+}, hydrogen peroxide and dimethyl sulfoxide, as well as their decreased activities by Fe^{2+}, Co^{2+}, dithiothreitol and 2-mercaptoethanol, while phenylmethylsulphonyl fluoride (PMSF), however, was the potent inhibitor, are all indications that the partially purified proteases were alkaline (serine). Additionally, the enzymes remained thermally stable in the presence of surfactants, oxidants, and detergent at alkaline pH and

Table IV. Proteases kinetic parameters

	Protease 1		Protease 11	
Substrates	Vmax (mg/ml/min)	Km (mg/ml)	Vmax (mg/ml/min)	Km (mg/ml)
Casein	0.217	0.069	0.234	0.071
Gelatin	0.213	0.022	0.240	0.547
BSA	0.172	0.038	0.284	0.142
EA	0.241	0.215	0.249	0.247

Table IV shows the kinetic parameters (*Km and* V_{max}) of the proteases for the hydrolysis of the substrates obtained from the double reciprocal plot of the data according to The enzymes' affinities for the various substrates varied. The maximum affinity for gelatin was shown by protease 1 (Km = 0.022 mg/ml, Vmax = 0.213 mg/ml/min), whereas the highest affinity for casein was shown by protease 11 (Km = 0.077 mg/ml, Vmax = 0.234 mg/ml/min). The results of this investigation are consistent with the reports of Ademola and Malomo (2017) on gelatin. Nonetheless, casein was shown to have the lowest Km value, followed by hemoglobin and gelatin (Ndidi and Nzelibe, 2012). Our results differ equally from those of other researchers using *Beauveria sp*. protease, who reported a Km of 5.1 mg/ml using casein as the substrate (Shankar *et al*. 2011). Using casein as the substrate, sorghum malt cultivars KSV8-11 have been reported with Km values of 18 mg/ml and Vmax of 11.1 µmol/ml/min (Ogbonna *et al*. 2003). Additionally, Ogbonna and Okolo (2005) used casein as a substrate and obtained a Km of 21 mg/ml and a Vmax of 8.2 µmol/ml/min for the sorghum malt variety, KSV8-1.

Conclusion

This study had shown that the Nigerian maize variety Oba Super 2 had good malting properties and high extract temperature of 60 and 50°C, respectively. The information obtained in this research provides opportunity to further explore the suitability of these enzymes in relevant sectors.

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Recommendations

Due to availability and cost factors, we advise using locally grown grains as a compelling substitute for imported grains in the production of protease. The usefulness of this enzyme in other pertinent industries, particularly in brewing, pharmaceuticals, sewage treatment, etc., should be investigated further. The findings should be implemented as soon as possible. Researchers, the enzyme industry, and the brewing sector should synergize to ensure that more insights can further be explored.

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