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Purification and Characterization of Endoglucanase from Sorghum (*S. bi-color*) and Millet (*Pennisetum typhoides* and *Digitaria exilis*) Malts

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Authors' contributions

This work was carried out in collaboration among all authors. Author DIU designed the study, wrote the protocol and supervised the work. Authors HCN and CII carried out all laboratories work and performed the statistical analysis. Author ECU managed the analyses of the study. Author CPI wrote the first draft of the manuscript. Author AUE managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Endoglucanase (EC3.2.1.4) from sorghum (*S. bi-color*) and millet (*Pennisetum typhoides* & *Digitaria exilis*) malts were purified to homogeneity through the methods of ammonium sulphate precipitation and gel filtration. Molecular mass of 35 KDa and 41 KDa were determined by SDS-PAGE. The purified enzymes catalyzed the hydrolysis of carboxy-methylcellulose with optimum activity at pH of 4.8, 5.0, 6.0, and temperature of 60ºC, 60ºC and 70ºC for *Digitaria exilis*, *S. bi-color* and *Pennisetum typhoides* respectively. More than 90% activity was retained in *S. bi-color* and *Pennisetum typhoides* and 73% activity in *Digitaria exilis* after 1.0 hour pre-incubation at 60ºC. Km values of 0.11, 0.09, 0.20 mM and Vmax 17.53, 15.0 and 11.10 U/mg/min were obtained for *S. bi-*

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color, *Pennisetum typhoides* and *Digitaria exilis* respectively. Co^{2+} inhibited endoglucanase activity whereas Ca^{2+}, Ba^{2+}, and Zn^{2+} enhanced enzyme activity. The enzyme was inactivated by glucose, a major end product of cellulose hydrolysis. Results indicate that endoglucanase of *S. bi-color* and *Pennisetum typhoides* are more suitable for malting and a blend of the two will produce high quality malt.

Keywords: Endoglucanase; sorghum; millet; malting; enzyme purification.

1. INTRODUCTION

Endoglucanases is one of the cell wall degrading enzymes (cellulase complex- endoglucanases, exoglucanases and glucosidases) activated during the germination period of the malting process [1]. Endoglucanases (EC.3.2.1.4) preferentially cleave the internal glycosidic bonds of cellulosic chains resulting in a rapid decrease in polymer length and gradual increase in the reducing sugar concentration [2,3]. By decomposing the cell wall cellulosic biomass, other hydrolytic enzymes from the aleurone cells are able to have access to the starch reserve in the endosperm. Endoglucanases play key roles in increasing the yield of fruit juices, beer filtration and oil extraction, in improving the nutritive quality of bakery products and animal feed [4].

Endoglucanases have been purified mainly from bacterial and fungal sources by procedures which include ammonium sulphate precipitation, gel filtration, anion-exchange chromatography and SDS-polyacrylamide gel electrophoresis (PAGE) [5-10]. Endoglucanase is generally reported to be thermostable, having profound activity within a broad pH range of 4.0 – 11.0 with considerable stability at pH 4.0 – 7.0. Divalent cations such as Ca^{2+} and Mg^{2+} are noted to stimulate endoglucanase activity [6]. The major end products of cellulose hydrolysis, cellobiose and glucose have been found to inhibit endoglucanases in some cases [5,11,12] while other reports indicate insensitivity of the enzyme to the end-products [13]. Molecular weight in the range of 30 – 60 KDa [13,4,14,10] and as high as 85.1 KDa are recorded for endoglucanases obtained from microorganisms.

Purification and characterization of endoglucanase in plants, even barley is still scanty, in spite its great importance in extract development of these plants. Sorghum and millet malts are used in Africa in the production of local beer and alcohols like Kaffir beer, 'Burukutu' and Opaque beer which contain undegraded starch thereby reducing the product yield and nutritive quality [15]. Study by [16] on the malting and brewing characteristics of some sorghum and millet varieties indicates that some of the varieties have high brewing potentials. Since plant cell wall is a major barrier to the modification of endosperm during processing, a good understanding of the properties of the cell-wall degrading enzymes of sorghum and millet varieties will open doors for improved grain modification. It is against this background that partial purification and characterization of endoglucanase in malted sorghum and millet species were carried out.

2. MATERIALS AND METHODS

2.1 Materials

Dried grains of *Sorghum bicolor (yellow variety* - 'kaura') and *Pennisetum typhoides* ('dauro') were purchased from Samaru market, Zaria while *Digitaria exilis* (fonio-bhull) were purchased from Chori in Kaduna State. [The name 'bhull' is based on the source while the others in brackets are their Nigerian local names]. Materials were identified using breeder's characteristics.

2.2 Preparation of the Raw Grains

About 1.50 kg of each of the grains were weighed and cleaned to remove dirt, stones and broken kernels. About 50 grams of each sample was milled into powdered form using a Thomas Wiley Laboratory mill and then stored safely prior to analysis.

2.3 Malting Procedure

The malting procedure of [17] was employed with few modifications. Steeping was at room temperature (28-30ºC). At steep out, the grains were drained and spread on a bench covered with nylon. The grains were shaded by covering slightly with filter paper soaked in water to reduce excessive evaporation. Samples were withdrawn from each variety at 0, 2, 3, 4 and 6 day of the malting and then kilned (between 16-24 hrs) at 50ºC until the rootlets could be removed by

hand. The grains were then ground to powder and packed in airtight containers for analyses.

2.4 Preparation of Extracts

About 0.5 g of malted grain powders per ml of 0.05 M citrate buffer at pH 4.8 was thoroughly shaken (150 oscillations per minutes) at 4ºC for one hour and the slurry centrifuged twice at 3,000 g for 10 mins and then filtered through whatman number one filter paper. The crude extract was dialyzed against 3 volumes of the same buffer for 48 hours with three changes.

2.5 Enzyme Purification

The method [18] was adopted for ammonium sulphate precipitation. Protein extracts were differentially precipitated up to 80%. The precipitates were collected and immersed in 3 mls of citrate buffer then dialyzed against the same buffer with three changes for 2 days. The protein extracts were loaded into a sephadex G-75 column (1.5 × 50 cm) and pre-equilibrated with 50 mM citrate buffer pH 4.8. The column was eluted with the same buffer at a flow rate of 1 ml per 6 minutes. The active fractions were collected and assayed for protein activity.

2.6 Protein Determination

Protein content was measured by the method of [19], with BSA as standard. Protein was also detected by measuring absorbance at 280 nm.

2.7 Enzyme Assay Procedure

Dinitro-salicyclic acid (DNS) method was employed for the enzyme assay in accordance to the procedure of [20]. Exactly 1.0 ml of enzyme solution was added to 1.0 ml of 1% substrate carboxymethyl-cellulose (CMC) and incubated for 30 minutes at 50ºC. DNS reagent (3.0 ml) was then added to stop the reaction. The tubes were placed in boiling water for 5 minutes and cooled thereafter. The reducing sugar was determined spectrophotometrically as glucose at 540 nm. Blanks of buffer enzyme without substrate and substrate without enzyme were used. All assays were in triplicates. One unit of enzyme activity is defined as the amount of the enzyme that liberates one micromole of glucose per minute (1 µmol of glucose = 0.180 mg/ml) from CMC under the specified assay conditions [21]. Specific activity is the number of units per milligram protein.

2.8 Influence of pH and Temperature

The activity of endoglucanase enzyme was measured at different pH values (3.0–9.0) using citrate, phosphate and Tris-HCl buffers at 50ºC by the DNS method.

Enzyme solutions were incubated in citrate buffer, pH 4.8 at different temperatures (30ºC – 90ºC) for 1.0 hour before the addition of 1% CMC.

2.9 Molecular Weight Determination

SDS/PAGE was performed according to the procedure of [22]. Molecular weights were calculated as described by [23].

2.10 Statistical Analysis

The data were expressed as mean ± standard deviation (SD) and statistical analysis were carried out by one-way ANOVA. Data were considered significant for $P < 0.05$ at 95% confidence level. SPSS software was used.

3. RESULTS AND DISCUSSION

3.1 Purification of Endoglucanase

The results of the enzyme purification are summarized in Table 1. The ammonium sulphate precipitation of ‘kaura’, ‘dauro’ and ‘fonio-bhull’ gave purification folds of 2.5, 1.3 and 1.6 and recovery yield of 63, 41 and 38% respectively. The overall recoveries of endoglucanase activities from crude enzyme after gel filtration were 44, 36 and 34% for ‘kaura’, ‘dauro’ and ‘fonio’ respectively. The purification procedure yielded endoglucanase with specific activity of 8.62, 6.09 and 5.09 µmol/min/mg protein and total purification fold of 5.39, 5.25 and 4.07 for ‘kaura’, ‘dauro’ and ‘fonio’ respectively. The elution profiles of endoglucanase from the three samples gave a single peak of enzyme activity as shown in Figs. 1- 3. Fractions 14 - 16 of ‘kaura’ 24 and 25 of ‘dauro’ and 17 - 18 of ‘fonio-bhull’ were the most active while the other fractions showed little or no activity. A single peak of enzyme activity in the elution profiles of endoglucanase from the three samples is an indication that the purification procedure yielded a homogenous endoglucanase. The recovery yield of 63, 41 and 38% for ‘kaura’, ‘dauro’ and fonio-bhull respectively after ammonium sulphate precipitation is consistent with 64, 63 and 44% obtained in cellulase of three strains of *Pseudomonas fluorescens* [24].

Table 1. Purification table of endoglucanase from 'kaura', 'dauro' and 'fonio-bhull'

Stages	Total vol. (ml)	Protein concentration		(µmole /min/ml)	Enzyme activity		Recovery yield (%)	Purification (fold)
		mg/ml	Total protein (mg)		Total activity (µmole/min)	Specific activity (µmole/min/mg)		
Crude								
'Kaura'		77.40	929.20	124.0	1487.20	1.60		
'Dauro'	12	95.14	1141.70	110.0	1324.80	1.16	100	1
fonio		58.13	697.50	72.4	869.20	1.25		
$(NH_4)_2SO_4$ precipitation								
'Kaura'		77.71	233.13	310.5	931.6	4.00	63.00	2.50
'Dauro'	3	120.96	362.90	180.5	541.5	1.49	40.87	1.28
Fonio		55.30	166.00	108.9	326.6	1.97	37.57	1.58
Gel filtration								
'Kaura'		37.66	75.31	324.6	649.2	8.62	43.65	5.39
'Dauro'	2	39.45	78.90	240.5	481.0	6.09	36.30	5.25
Fonio		28.74	57.48	145.3	292.6	5.09	33.66	4.07

3.2 Influence of pH on Enzyme Activity

Enzymatically active fractions dialyzed against 50 mM citrate buffer had optimum activity at pH of 4.8, 5.0 and 6.0 for fonio, 'kaura' and 'dauro' respectively. Fig. 4 shows that enzyme activities were stable over a broad range of pH between 4.0 and 5.5 in kaura and fonio, while in dauro, the enzyme was stable between 5.5 and 7.5. This indicates that endoglucanase of kaura and fonio are highly stable in weak acidic condition while that of dauro tends towards the neutral pH. Enzymes are protein-based substances, thus sensitive to pH. pH can have effect on the ionization of acidic or basic amino acids, shape of the enzyme as well as the shape or charge properties of the substrate, thus affecting the substrate binding to the active site and catalysis. The optimum pH of endoglucanase from fonio, 'kaura' and 'dauro' are similar to that of endoglucanases from *Thermoascus aurantiacus* with optimum pH of 4.4 [4], *Clostridium thermocellum* pH 5.5 [11] and that from *Thermotoga neopolitana* of pH 6.0 [12].

3.3 Influence of Temperature on Enzyme Activity

Fig. 5 shows the result of temperature effect on the initial velocity of CM-cellulose hydrolysis using an incubation period of 30 minutes. Enzyme activity gradually increased up to 60ºC in 'kaura' and fonio and 70ºC in 'dauro'. The study result shows that temperature effect was significant on 'kaura' and 'dauro' than fonio millet. About 78%, 97% and 58% activities remained at 80ºC in 'kaura', 'dauro' and fonio respectively showing that endoglucanases of 'dauro' is more thermostable followed by that of 'kaura'. The decrease in enzyme activity after the optimum temperature is due to denaturation which set in gradually in 'kaura' and fonio but sharply in 'dauro'. The optimum temperatures obtained in this study are similar to optimum temperature of 50-60ºC reported for endoglucanase from *Clostridium thermocellum* [13] and exactly 70ºC for *Thermoascus aurantiascus* [4].

Based on the results of temperature effect on enzyme activity, Arrhenius plots of activation energy (E_{act}) and inactivation energy (E_{inact}) were made. The E_{act} of 11.9, 10.9 and 23.9 KJ/mole (Figure not shown) and E_{inact} of 20.9, 55.4 and 65.4 KJ/mole (Fig. 7) for 'kaura', 'dauro' and 'fonio' respectively were obtained. Activation energy is the minimum energy required to get the chemical reaction started, thus the lower the energy the more the reaction. The results indicate that endoglucanase of 'dauro' reacts faster considering the low activation energy. 'Dauro' also has a broad range of energy for reactive molecules before inactivation at 55.4 KJ/mole. Endoglucanase of 'kaura' had a narrow range of energy (11.9 – 20.9 KJ/mole) therefore requires careful regulation. Higher activation energy in fonio shows that increase in temperature decreases the forward reaction and this might be the reason for its poor enzyme activity under the study conditions.

3.4 Percentage Residual Activity of Endoglucanase

Table 2 shows the result of enzyme solutions incubated in citrate buffer, pH 4.8 at different temperatures (30ºC – 90ºC) for 1.0 hour before the addition of 1% CMC. Endoglucanases of 'kaura' and 'dauro' were stable over a wide range of temperature retaining high activity of 94% and 90% respectively at 60ºC. Fonio had considerable low values. However, at 80ºC reasonable activity remained in the selected cereals. Above 80ºC, the activity rapidly declined indicating denaturation of the enzyme. Exactly 60ºC most stable temperature is reported for endoglucanase of *Bacillus licheniformis* [25], while 83% residual activity after 3.0 hours at 70ºC is reported for *Caldibacillus cellulovarans* [14].

3.5 Effect of Substrate Concentrations

With fixed enzyme concentration, an increase in the concentration of substrate (0.8 – 2.0% CMC) resulted in increase in enzyme activity until a saturation point of 1.6% was reached, beyond which enzyme activity decreased. Based on Lineweaver-Burk plot, the partially purified enzymes gave a K_m value of 0.09, 0.11 and 0.20 mM and V_{max} of 17.53, 15.0 and 11.10 U/mg/min for 'kaura', 'dauro' and fonio respectively (results not shown). This was probably because at high substrate concentration, ineffective complexes were formed between enzyme and substrate. Also, since the substrate molecules were too many around the enzyme molecules, they may be bound to regions on the enzyme which are not the active site or alternatively, may crowd the active site [24].

The Michealis constant K_m measures inversely the strength of binding between enzyme and substrate. From the results 'kaura' with the

lowest K_m of 0.09 mM has a greater affinity towards carboxymethyl cellulose. High K_m in fonio shows that its endoglucanase is saturated only at very high concentrations of CMC. V_{max} which is the maximum rate of reaction indicates the amount of enzyme that will convert the substrate to product at a given time. Hence fonio endoglucanase is the most effective in the catalysis of CMC. K_m (0.09, 0.11 and 0.20 mM) and V_{max} (17.53, 15.0 and 11.10 U/mg/min) values for 'kaura', 'dauro' and fonio respectively are close to 0.30 mM and 18.4 U/mg/min of thermostable cellulase from *Thermotoga neapolitana* [12].

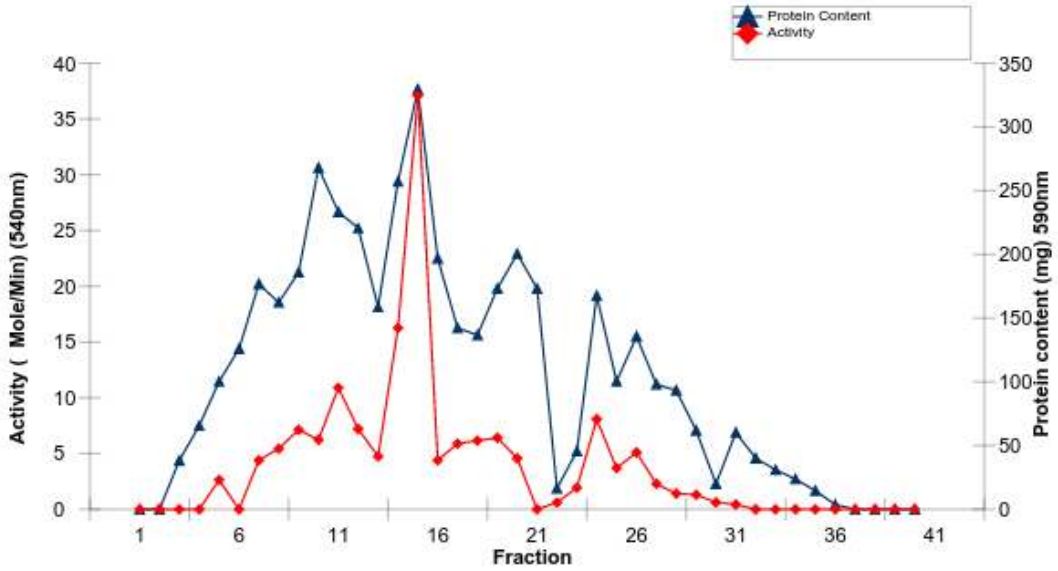

Fig. 1. Elution profile of 'kaura' endoglucanase on sephadex G-75 column (1.5 x 12 cm). The column was eluted at a flow rate of 0.17 cm^3 min^{-1}

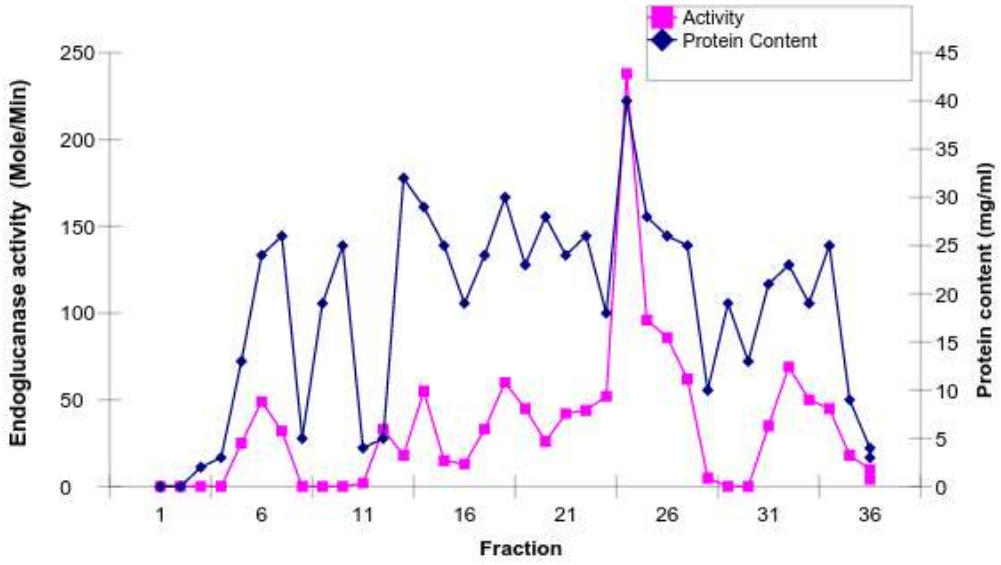

Fig. 2. Elution profile of 'Dauro' endoglucanase on Sephadex G-75 Colum (1.5 x 12 cm). The column was eluted at flow rate of 0.17 cm^3 min^{-1}

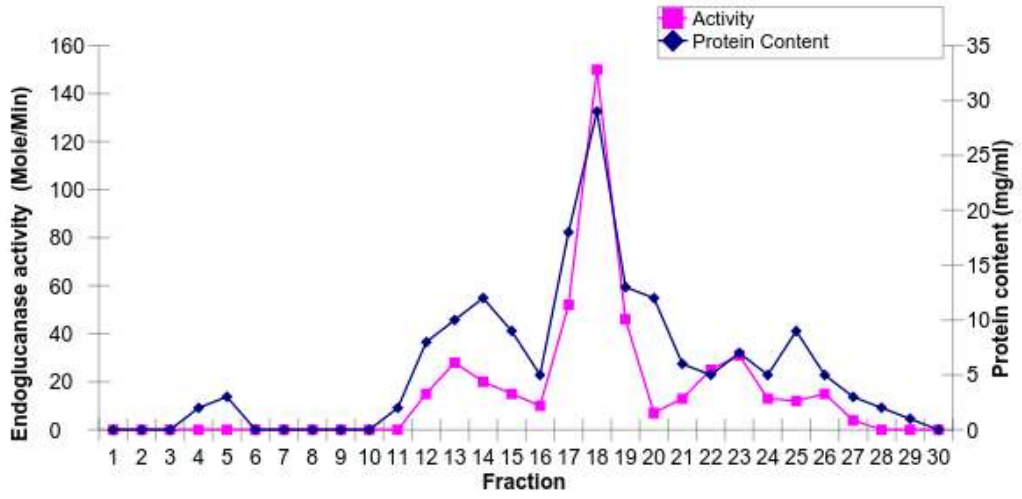

Fig. 3. Elution profile of fonio-bhull endoglucanase on Sephadex G-75 column (1.5 x 12 cm). The column was eluted at flow rate of 0.17cm^3 min^{-1}

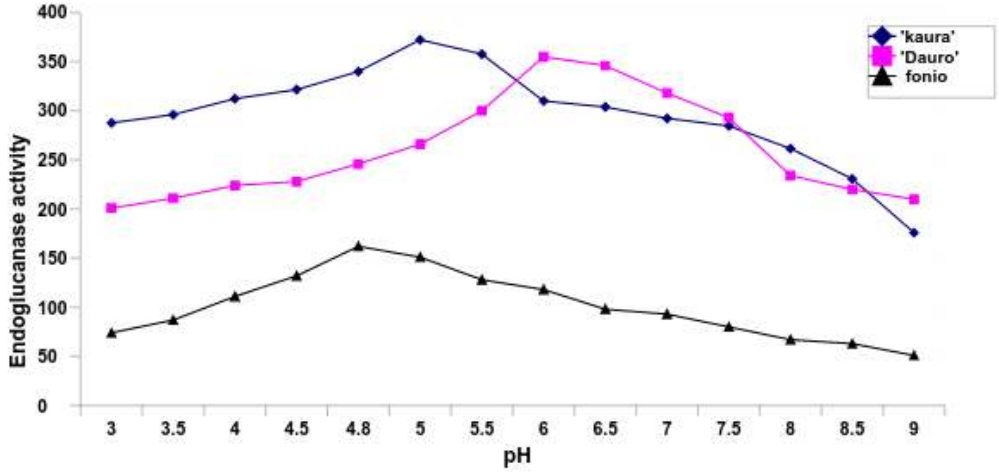

Fig. 4. Effect of pH on endoglucanase activity for 30 min at 50^{o}C with 1% CMC in 50 mM citrate buffer pH 4.8

3.6 Effect of Divalent Cations

The activity of endoglucanase assayed in the presence of ImM Zn^{2+}, Ba^{2+} and Ca^{2+} during the hydrolysis of CMC caused an increase yield in the reducing sugar as shown in Fig. 6. On the other hand, a significant decrease yield of reducing sugar was observed with 1mM Co^{2+} indicating a strong inhibitory effect (Fig. 6). The yield increase in the reducing sugar obtained during the hydrolysis of CMC by the addition of ImM, Zn^{2+}, Ba^{2+} and Ca^{2+} means that the enzyme activity was enhanced by these metal ions, causing more degradation of cellulose. The values of Zn^{2+}, Ba^{2+} and Ca^{2+} for fonio were higher by 37%, 27 % and 11% respectively than 'kaura' which had an increment of 33%, 20% and 8% and 'dauro' with increments of 21%, 18% and 11% for Zn^{2+}, Ba^{2+} and Ca^{2+} respectively. This indicates that endoglucanase of fonio is more sensitive to these metal ions. In the three selected cereals, Zn^{2+} had the highest effect on endoglucanase hydrolysis of CM-cellulose; therefore it is the most stimulatory metal ion. Ca^{2+} activation on endoglucanase of *C. thermocellum* is reported by [6] while Zn^{2+} is reported to have significant inhibition on CMCase from *Caldibacillus cellulovarans* [14]. The increase by

the metal ions would be as a result of proper orientation of substrate within the active site. Divalent cations bind to two or more ligands and induce changes in enzyme-substrate complex conformation. Co^{2+} inhibition may be due to complex-formation with, or catalysis of oxidation of, specific residues by thiol groups or the result of non-specific salt formation [11,6].

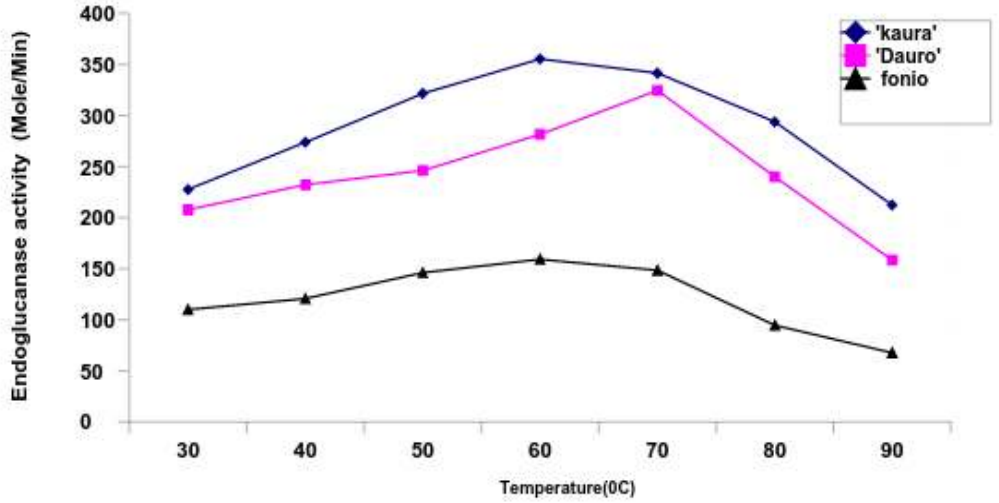

Fig. 5. Effect of temperature on endoglucanase activity for 30 min with 1% CMC in 50 mM citrate buffer pH 4.8

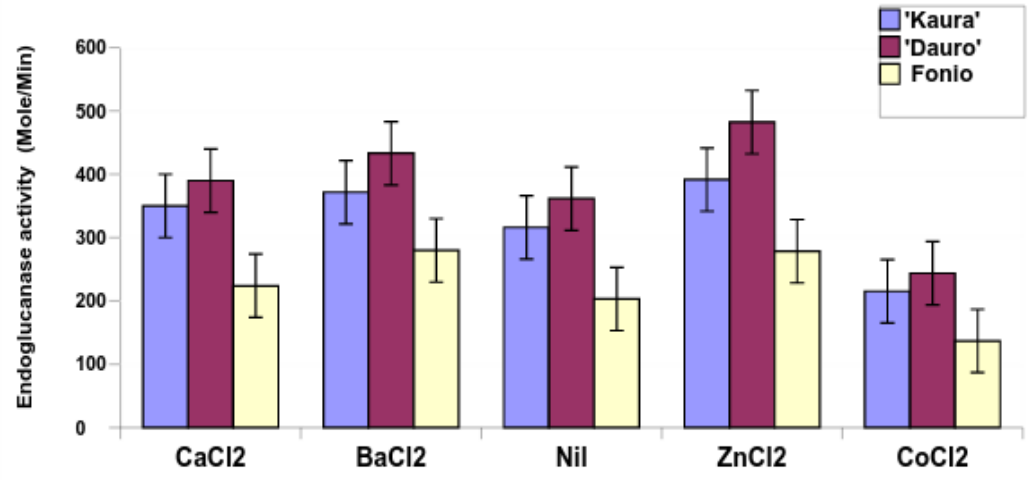

Fig. 6. Effect of divalent cations on endoglucanase activity. The enzyme was assayed for 30 min at 50°C in reaction medium final concentration 1 mM divalent cations with 1% CMC in 50 mM citrate buffer pH 4.8

Table 2. Percentage residual activity of endoglucanase after one hour of pre-incubation at different temperatures

	30°C	40°C	50°C	60°C	70°C	80°C	90°C
Kaura (%)	88	90	93	94	90	88	75
Dauro (%)	73	79	88	90	84	80	64
Fonio (%)	48	54	68	73	65	50	27

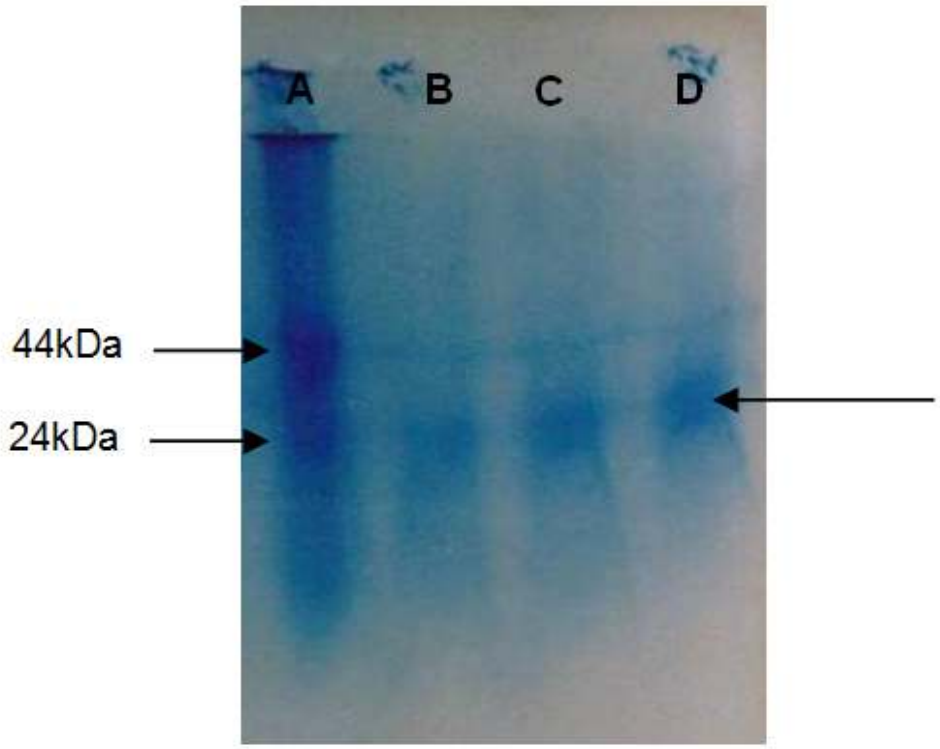

Fig. 7. SDS – PAGE of the partially purified endoglucanase on 12% acrylamide gel.
Lane A: Molecular weight markers, Lane B: 'Kaura' Lane C: 'Dauro', Lane D: Fonio

3.7 Effect of Glucose on Enzyme Activity

10 mM glucose added to different concentrations (0.8 – 2.0%) of the substrate inactivated enzyme activity of the three samples investigated. Lineweaver-Burk plot of the data obtained from the enzyme assay gave a competitive inhibition pattern for 'kaura' while non-competitive pattern was obtained for 'dauro' and fonio (Results not shown). The inhibitory constant (K_i) values of 10.25 mM, 12.54 mM and 12.72 mM were obtained for 'kaura', 'dauro' and fonio respectively.

The inhibitory effect suggests that endoglucanases of the samples are sensitive to the end-product (glucose) as reported by [12], but in contrast to the result of [6] which shows that 200 mM glucose had no effect on endoglucanase from *C. thermocellum*. Competitive inhibition pattern obtained in 'kaura' is similar to that of *Thermotoga neapolitana* [12]. The inhibitory constant (K_i) measures the extent of binding between the enzyme and the inhibitor at given concentration of the inhibitor. 'kaura' with the lowest K_i value at 10 mM glucose shows that glucose has greater degree of inhibition on its endoglucanase than 'dauro' and 'fonio'. Higher K_i compared to K_m of the three samples is a clear indication that the enzyme is more inclined to the substrate CMC than glucose.

3.8 SDS-PAGE Result and Molecular Weight

The enzyme preparations migrated as a single band on SDS PAGE (plate 1). Molecular weight of 35 KDa for 'kaura' and 'dauro' and 41 KDa for 'fonio' were obtained based on [23] formula. The molecular weight of 35 KDa for 'kaura' and 'dauro' and 41 KDa for 'fonio' are close to molecular weight 34 KDa of CMCase from *T. aurantiascus* and 50 KDa of *Populus elba* determined by SDS PAGE [4,16]. They are within the range 20 to 60 kDa estimated for cellulase isolated from *Amitermes evuncifer* bacteria [24] but lower than 94 KDa of CMCase from *Sinorhizobium fredii* [26] and 76 KDa and 83 KDa of endoglucanase from *Clostridium thermocellum* [11,6]. This result indicates that endoglucanase of 'kaura', 'dauro' and 'fonio' are low molecular weight endoglucanase.

4. CONCLUSION

The results of purification and characterization procedures obtained in this study indicate that endoglucanase of 'kaura', 'dauro' and 'fonio' are active within a wide range of pH and temperature but that of "kaura' and 'dauro' were found to be more thermostable. Endoglucanase from these cereals were also found to be metallo-proteins of low molecular weight. Useful properties of endoglucanase from 'kaura', 'dauro' and 'fonio' indicate that 'kaura', and 'dauro' are more suitable for malting process at optimum pH of 5.0 and 6.0 and temperature of 60ºC and 70ºC respectively. A blend of sorghum and pearl millet malt will produce malt with high profile.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Jadhav AR, Girde AV, More SM, More SB, Saiqua K. Cellulase production by utilizing agricultural wastes. Research Journal of Agriculture and Forestry Science. 2013; 1(7):6-9.
2. Begiun P, Aubert JP. The biological degradation of cellulose FEMS. Microbiol Rev. 1994;13.
3. Wood PJ, Weisz J, Blackwell BA. Structural Studies of (1→ 3) (1→4) – β-D-glucans by 13-nuclear magnetic resonance spectroscopy and by rapid analysis of cellulose-like regions using high performance anion-exchange chromate-graphy of oligosaccharides released by lichenase. Cereal Chem. 1994;71:301-307.
4. Parry NJ, Beever DE, Owen E, Nerinckx W, Clacyssens M, Beeumen, and Bhat, MK. Biochemical characterization and mode of action of a thermostable endoglucanase purified from *Thermoascus aurantiacus.* Archives of Biochemistry and Biophysics. 2002;404(15):243–253.
5. Chang WT, Thayer DW. The cellulose system of a cytophaga species. Can J Microbiol. 1977;23:1285.
6. Romaniec MPM, Fauth U, Kobayashi T, Huskisson NS, Barker PJ, Demain AL. Purification and characterization of a new endoglucanase from *Clostridium themocellum* Biochem J. 1992;283:69-73.
7. Nakamura S, Hayashi T. Purification and properties of an extracellular Endo-1, 4-β-Glucanase from Suspension –Cultured Poplar Cells Plant and Cell Physio.1993; 34:1009-1013.
8. Dawar V, Jain V. Cell wall degrading enzymes and permeability changes in sunflower (*Helianthus annuus)* infected with *Alternaria helianthin.* Int J Agric Env Biotech. 2010;3(3):321-325.
9. Hubballi M, Sornakili A, Nakkeeran S, Anand T, Raguchander T. Virulence of *Alternaria alternate* inflicting Nori associated with production of cell wall degrading enzymes. J Plant Prot Res. 2011;51(1):87–92.
10. Swati D, Pratibha S, Niranya B. Optimization of extracellular cellulose enzyme production from *Alternaria brassicicola.* Int J curr Microbiol App Sci. 2014;3(9):127-139.
11. Fauth U, Romaniec MP, Kobayashi T, Demain AL. Purification and Characterization of endoglucanase Ss from Clostridium thermocellum Biochem J. 1991;279:67-73.
12. Bok J, Yernool DA, Eveleigh DE. Purification, characterization and molecular Analysis of thermostable cellulases Cel A and Cel B from *Thermotoga neopolitana.* Applied and Environmental Microbiology. 1998;64:4774–4781.
13. Golovchenko RN, Singh GA, Velikodvorskay A, Akimenko VK. Isolation and characterization of a Lichenin – degrading hydrophobic endoglucanase of clostridium thermocellum Applied microbiology and Biotechnology. 1993;39: 74-78.
14. Xiao PH, Monk C. Purification and characterization of cellulase (CMCase) from a newly isolated thermophilic aerobic bacterium *Caldibacllus cellulovorans.* World J of Microbiol and Biotech. 2004; 20:85-92.
15. Singh T, Hannder K, Bains GS. Malting of Finger Millet: Factors influencing α – amylase activity and wort characteristics. J Amer Society of Brewing Chemists. 1988; 46:48-51.
16. Nzelibe HC, Obaleye S, Onyenekwe PC. Malting characteristics of different varieties of fonio millet (*Digitaria exilis*). Food Res Technol. 2000;211:126-129.
17. Hough JE, Briggs DE, Steven R. The Chemistry and Biochemistry of Mashing,

Malting and Brewing Science Chapman and hall, London. 1971;95-105.
18. Wiser MF. Lecture Notes for Methods in Cell Biology. 2003;53-62.
19. Ryan MT, Chopra RK. Protein determination by biuret method. Biochem Biophys Acta. 1976;427:337-349.
20. Miller GL. Use of Dinitrosalicyclic acid reagent for determination of reducing sugar. Analytical chemistry. 1959;31: 426-428.
21. Dahot UM, Noomrio HM. Microbial production of cellulases by *Aspergillus fumigatus* using wheat straw as carbon source. Journal of Islamic Academy of Science. 1996;9(4):119-124.
22. Laemmli UK. Cleavage of Structural proteins during the assembly of the head of bacteriophage T4 nature. 1970;277: 680-685.
23. Weber KJ, Pringle JR, Osborn M. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel methods Enzymol. 1972;26:3.
24. Bakare MK, Adewale IO, Ajayi A, Oshonuka OO. Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas flourescens.* African Journal of Biotechnology. 2005;4:894-904.
25. Bischoff K, Rooney A, Li X, Liu S, Hughes S. Purification and characterization of a family 5 endoglucanase from a moderately thermophilic Strain of *Bacillus licheniformis*. Biotechnology Letters. 2006; 28:1761-1765.
26. Chen P, Wei T, Lin L. Purification and characterization of carboxymethyl cellulase from *Senortizobium fredii*. Bot, bull Acid Siri. 2004;45:111-118.

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