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Isolation and Partial Characterisation of α-Amylase from Two Vegetables Consumed in Sokoto Metropolis

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Leaves of cabbage and spinach are edible vegetables eaten all year round in regions of northern Nigeria for nutritional purposes. α-amylase are enzymes that catalyze the breakdown of carbohydrates into soluble absorbable molecules. In this research, α-amylase was isolated and partially characterised from leaves of cabbage and spinach. Conventional methods of protein purification were adopted using successive stepwise purification steps including ammonium sulfate precipitation, dialysis and gel filtration to obtain a corresponding enzyme purification fold of 1, 1.8, 2.2 and 5.7 for *Brassica* (cabbage) and 1, 1.5, 2.3 and 5.1 for *Spinachia* (spinach) respectively. The purified α-amylase from the cabbage and spinach leaves had an optimum pH of 5.5 and 5.1, optimum temperature of 58°C and 60°C, Km of 2.5 mg/mL and 4.2 mg/mL, and V_{max} of 0.24 and 0.20 respectively. The physico-chemical properties and kinetics obtained for the α-amylase from these vegetables may justify that cabbage and spinach can help to improve nutritional availability of foods such as simple absorbable carbohydrates particularly for protein energy malnourished children by facilitating food digestibility and release of macro nutrients.

Keywords: α-Amylase, Enzyme, Kinetics, Cabbage, Spinach.

1. Introduction

Malnutrition in children is a great challenge in many developing nations, accounting for 3.1 million deaths in children annually in 2011 (Black *et al.*, 2013). Severe acute malnutrition (SAM) is particularly problematic because of high fatality rates. According to the joint statement of the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), SAM includes marasmus and kwashiorkor. In developing countries, a common cause of diarrhea is enteric infection, which, when associated with underlying malnutrition, lead to villous blunting and, as a result, impaired carbohydrate absorption. In turn, significant decreases in carbohydrate absorption can lead to severe osmotic diarrhea.

Intestinal microflora population is a complex ecosystem composed of a large variety of bacteria. The metabolic capacity of microflora is extremely diverse and can produce positive and negative effects on gut physiology (Macfarlane and Cummings, 1991). There is, therefore, a great deal of interest in the possibility of altering the intestinal microflora in a beneficial way with the aim of improving the health of the host. Bacterial enzymes are the major microbial glycosidases in the intestinal tract. It has been reported that Enzyme supplementation improves performance and nutrient digestibility of the malnourished children. Therefore, an exogenous supply of amylase might be needed to improve performance early in life. Studies have shown beneficial effects of amylase and protease preparations on growth and efficiency of child development when added to diets.

Alpha amylase (Alpha 1-4 D glucanohydrolase EC.3.2.1.1) catalyzes endohydrolysis of alpha 1-4 glucosidic linkages in starch and any related polysaccharides to produce oligosaccharides and glucose. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolases. Endo-hydrolase acts on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends (Gupta, 2003). Hence, terminal glucose residue and alpha-1, 6-linkages cannot be cleaved by alpha amylase. The substrate that alpha amylase acts upon is starch. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by alpha 1, 4-glycosidic linkage. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units are linked by alpha-1, 4-glycosidic bonds linkage while branching occurs every 15-45

glucose units where alpha-1, 6-glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the condition of hydrolysis and the origin of enzyme (Sharma, 2011). Alpha amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch (Singh, 2011). Alpha amylase catalyses' the first step in this process. Previously, starch was hydrolysed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures (Das, 2011). This limitation is overcome by enzyme hydrolysis of starch to yield high fructose syrup (Gupta, 2003).

Alpha amylase are enzymes with a wide range of substrate specificity, as well as both temperature and pH activity *(*Jens 2001). The functionality of this enzyme has for centuries been exploited in different food processing practices such as malting, brewery, fluidification etc. (Baskaran and Muthukumarasamy 2017). This food processing demand of the enzyme has supported its extraction and purification from different vegetables food, including tuber (Sarker, 2010), leaves (Witt and Sauter 1996) and malted cereals (El Nour and Yagoub 2010). Meanwhile, regarding local endogenous uses of the enzyme. Amylase is extracted from plants through mashing, water extraction and filtration (Glew *et, al.,* 2010). To the best of our knowledge, no scientific work has been undertaken on the characterization of amylases from *Spinachia oleracea* and *Brassica oleracea*. Therefore, this research is aimed at purifying Alpha Amylase from leaf extracts of these plants and investigating its kinetic properties.

2. Materials and Methods

2.1 Collection and Identification of Cabbage and Spinach

The fresh leaves of spinach and head cabbage were collected between May and June, 2018 along Gagi durbawa road, Sokoto. The plant was transported to Botany unit of the Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto, for identification where the voucher specimen (UDUH/ANS/0003) was deposited.

2.2 Crude α-Amylase Extraction from Cabbage and Spinach

Fifty grams (50 g) of fresh leaves of cabbage and spinach were sliced into small pieces and homogenized in cold phosphate buffer 50 mM, pH 6.0 containing 5 mM of β-mercaptoethanol into a paste using a roaring blender (Kenwood w203). The homogenized sample was then transferred into suitable tubes and centrifuged (Hitachi refrigerated centrifuge) at 3500 rpm for 30 mins at 4^{o}C. The supernatants where syphoned, pooled together into a 250 mL conical flask and then kept on ice chips and taken for the next step of enzyme isolation.

2.3 Determination of α-amylase activity

α- amylase activity was determined by the method of Onu *et al.* (2013) with modifications. The reaction mixture contained 5 mL, 67 mM potassium phosphate buffer, pH 6.8 and 0.2 mL α-amylase of the crude/partially purified enzyme from the vegetables*.* The mixture was equilibrated to 37°C for 5 min. The reaction mixture was activated by the addition of 0.5 mL 10 mM *p*-nitrophenyl-α-glucoside and then incubated for 37°C for 30 mins. Into a test tube containing 8 mL of 100 mM Na_2CO_3 was added 2 mL of the reaction mixture to terminate the reaction. The enzyme activity was monitored by taking the spectrophotometric absorbance of *p*-nitrophenol at 400 nm using optima sp-300 spectrophotometer. One unit of α-amylase was taken as the amount of enzyme liberating 1.0 µmol of *p*-nitrophenyl from *p*-nitrophenyl-α-glucoside per minute at pH 6.8 and 37°C.

2.4 Protein determination

Protein content was estimated by the method of Bradford (1976) using bovine serum albumin as standard.

2.5 Ammonium Sulphate precipitation

The crude extract was treated with different concentration of Ammonium sulphate. 40% and 60% Ammonium sulphate was used for purification. Ammonium sulphate was slowly added to the extract to precipitate out the enzyme. This was done on an ice bath and kept for 1 h under continuous stirring taking care not to produce foaming. The mixture was then centrifuged at 3000 rpm for 20 mins at 4^{o}C. The supernatant and pellet were then separated, and each checked for enzyme activity.

2.6 Dialysis

Exactly 5 mL of the crude extract containing Ammonium Sulphate were transferred onto dialysis bags using a plastic pipette. The bag was sealed with the help of the clip from one end. Excess air was removed from the bag and at least 25% extra space for volume expansion. The other end of the bag was clipped. Dialysis was commenced against 50 mM phosphate buffer, pH 6.0 for 12 h with one change of buffer.

It was ensured that the dialysate was gently stirred throughout the procedure. Upon completion of dialysis. The outside of the bag was dried using tissue and then clips were removed. The volume of sample was measured post dialysis.

2.7 Purification Using Size Exclusion Chromatography

The dialysed ammonium sulfate preparation was applied (50 mL) on to a column (2.6 by 95 cm) of Sephadex pre-equilibrated with 0.025 M Tris-hydrochloride buffer at pH 7.2, containing 0.1 M KCl. Elution of protein was carried out by batch wise addition of 40 mL portions of the equilibrating buffer. A mariotte flask was used to maintain an operating pressure of 25 cm of water. Effluents were collected in 5.0 mL fractions at a flow rate of 20 mL/h. The absorbance at 280 nm as well as the amylase activity in all fractions was tested. The fractions showing a peak activity were pooled together and kept in -20 ^{o}C for further analysis.

2.8 Specific activity of enzyme

α-amylase activity was assayed according to the procedure of Benfield (1951). The amount of protein content in the culture filtrate was assayed by Lowry method. The enzyme was incubated along with buffer, with 5 mL of freshly prepared alkaline solution and incubated for 10 min at room temperature. 0.6 mL of FC reagent was added and incubated for 30 mins. The absorbance of the solution was read at 660 nm. The protein content was estimated using calibration curve of standard BSA protein. Then the specificity of enzyme was calculated by the amount of protein in mg present per mL of sample. The specific activity was expressed as activity unit/mg protein.

2.9 Effect of temperature and pH on activity and stability

The optimal pH for amylase activity was determined using 0.1 M acetate buffer (pH 2.5-5.5), 0.1 M phosphate buffer (pH 6.0-7.5), 0.1 M Tris-HCl buffer (pH 8.0-9.0) and glycine-NaOH (pH 10). The tested pH values were 2, 3, 4, 5, 6, 7, 8, 9 and 10. The enzymatic assay was done as described in the earlier section above. The effect of pH on enzyme stability was evaluated by measuring the residual activity after a pre-incubation time of 4 h at 4 °C and at various pH (2.0 to 10.0). Residual activity was expressed in percent and was determined under standard assay conditions.

The effect of temperature on amylolytic activity was determined by incubating the reaction mixture for 5 min at the following temperatures: 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90 and 100 °C. Also, the thermo-stability of the enzyme at the different temperatures was determined after a pre-incubation time of 1 h followed by determination of residual activity.

2.10 Determination of Kinetic constants

The Michaelis constant (K_m) and the maximum velocity (V_{max}) for α-amylase, were determined from Lineweaver-Burk plots. 0.5 mL of *p*-nitrophenyl-α-glucoside concentrations of 0.2, 0.4, 0.8 and 1.6 mM were used and activity was assessed at 37°C and at pH 6.0. Obtained data were inserted in Graph 4.4.2 (downloaded from www.padowan.dk) and line regression was drawn by using concentrations of substrate and observed enzymatic velocity.

3. Results

3.1 Purification scheme of Amylase Activity of Brassica and Spinachia

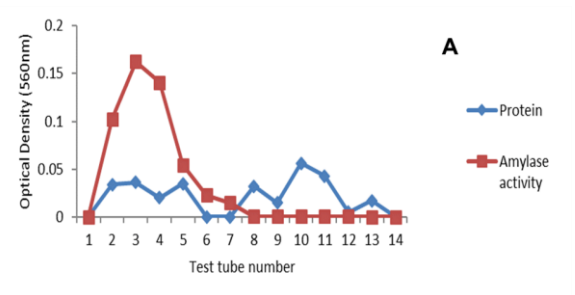
The results of the purification scheme of amylase enzyme from Brassica and Spinachia using ammonium sulphate precipitation, dialysis and size exclusion chromatography are presented in Table 1. The results showed that ammonium sulphate precipitation gave 99 % yield with a corresponding 1.72 purification fold for brassica while 98 % yield and corresponding 1.34 purification fold was recorded for spinachia. Dialysis and gel sephadex size exclusion chromatography gave 69 and 58 % yield for brassica and 90 and 60 % yield for spinachia respectively. Size exclusion chromatography gave a higher purification fold for spinachia (47.69) as compared to brassica (5.66).

Table 1. Purification scheme of Amylase activity of Brassica and Spinachia

	Purification Steps	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Yield %
Brassica	Crude Extract	879	4601	5.23	1	100
	Ammonium Sulphate	506	4553	9.00	1.72	99
	Dialysis	289	3196	11.06	2.11	69
	Gel Sephadex	91	2693	29.59	5.66	58
Spinachia	Crude Extract	878	3783	4.31	1	100
	Ammonium Sulphate	637	3693	5.79	1.34	98
	Dialysis	380	3388	8.92	2.07	90
	Gel Sephadex	11	2261	205.55	47.69	60

3.2 Elution profile of size exclusion chromatography of Amylase from Brassica and Spinachia

Figures 2 A and B give the elution profile of amylase from Brassica and Spinachia respectively using size exclusion chromatography. From the results, it can be seen that amylases from brassica eluted in the first 6 test tube corresponding to approximately 30 mL washes of the equilibrated buffer while amylases from spinachia eluted in the first 4 test tubes corresponding to 20 mL washes of the equilibrated buffer. Presence of protein in brassica was detected in test tube 8 to 13 but there was no amylase activity. Similarly, protein was detected in only test tube 8 but had no amylase activity as well



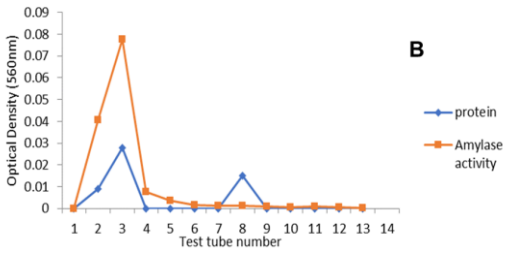


Figure 1A. Elution profile of size exclusion chromatography of Amylase from Brassica B. Elution profile of size exclusion chromatography of Amylase from Spinachia

3.3 Effect of temperature and pH on activity and stability

Figure 1 A and B show the effect of temperature on the activity and stability of amylase isolated from brassica and spinachia respectively while Figure 1 C and D show the effect of pH on activity and stability of amylase isolated from brassica and spinachia respectively. The results show that amylase isolated from brassita was stable with an optimal temperature at 58^{o}C (Figure 1A) while amylase isolated from spinachia was stable with an optimal temperature at 60^{o}C (Figure 1B). Similarly, the amylase from both brassica and spinachia had optimum pH of 5.5 and 5.1 respectively.

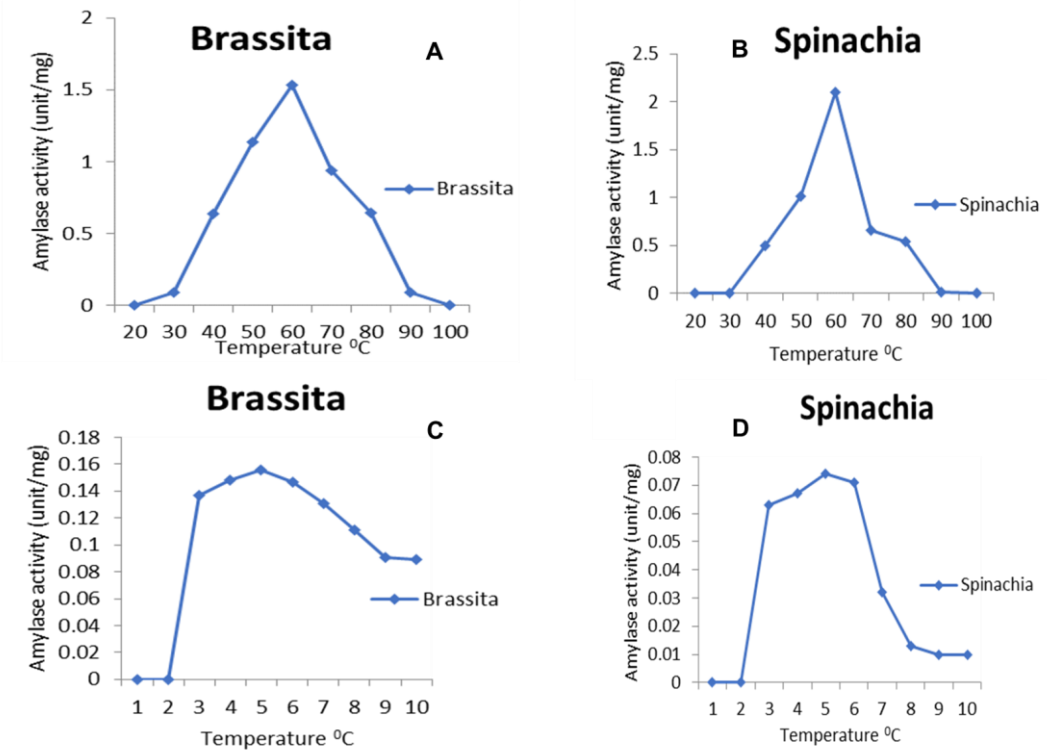


Figure 1. A - Plots of effect of temperature on activity and stability of Amylase isolated from Brassica; B - Plots of effect of temperature on activity and stability of Amylase isolated from Spinachia; C - Plots of effect of pH on activity and stability of Amylase isolated from Brassica and D - Plots of effect of pH on activity and stability of Amylase isolated from Spinachia.

3.4 Kinetic constants

The results of the line weever burk plot showing the kinetic constant of amylase activity isolated from brassica and spinachia are presented in Figure 2 A and B respectively. The results show that amylase isolated from brassica had a K_m of

2.5 mg/mL and V_{max} of 0.24 unit/mg (Figure 2A). Furthermore, it was observed that amylase isolated from spinachia had a K_m of 4.2 mg/mL and V_{max} of 0.2 unit/mg (Figure 2B).

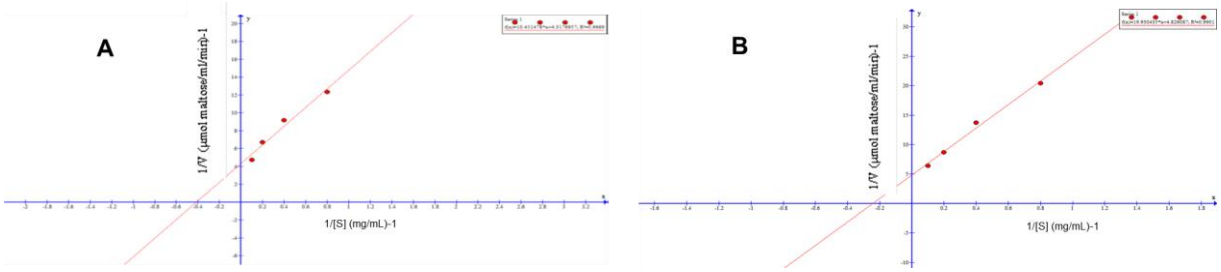


Figure 2. A - Lineweaver-Burk plot of Brassica amylase for the determination of kinetic parameters.
B - Lineweaver-Burk plot of Spinachia amylase for the determination of kinetic parameters.

4. Discussion

4.1 Purification of Amylase

From the elution profile, it is observed that the purified enzymes are eluted as a well resolved peak of amylase activity [Figure 1]. Each step of the purification increases the specific activity of the amylase enzymes and, at the end of chromatography on Gel Sephadex, purification folds of 6 and 51 are achieved respectively for *Brassica* and *Spinachia* with corresponding specific activity of 30 and 206 Units/mg. It is therefore envisaged that the amylolytic properties of *Spinachia* leaves and *Brassica* head cabbage were exclusively the result of alpha amylases contained in these plants. The protein recoveries of the two enzymes in these conditions are respectively 58 and 60%. The purification data of α-amylase from the two plants are summarized in (Table 1). Chromatography on Gel Sephadex appears as the most determining step for purification of the two enzymes. The protocol of purification used in this study allows a better purification fold for Spinachia and a better yield for Brassica oleracea.

4.2 Optimum pH and Temperature

The purified enzymes display an optimum pH value of 5.1 for amylase from Brassica, and 5.5 for amylase from Spinachia [Figure 2]. Regarding the temperature effect, the amylase activity increases and is stable with increasing temperature, up to an optimum activity at 58°C for enzyme from Spinachia, while Brassica amylase activity has its optimum temperature at 60°C [Figure 3]. The enzyme activity then declines above these optimum temperature values, indicating loss in the active conformation of the protein.

4.3 Kinetic Constants

Kinetic studies, carried out under standard conditions using soluble starch, display for amylase from Brassica, a K_m value lower than that of amylases from Spinachia. This indicates high affinity of Brassica amylase for soluble starch, compared to the amylase from Spinachia. V_{max} values vary between 0.24 µmoles/min/mg for Brassica and 0.2 µmoles/min/mg for Spinachia.

5. Conclusion

The study confirms the presence of α-amylase in both *Brassica* (cabbage) and *Spinachia* (spinach) which are common vegetables consumed in Sokoto metropolis. Conventional methods of protein purification were able to isolate α-amylase to significant yield. The amylases in both vegetables were however different in their optimal pH, temperature and kinetics constants. It can therefore be suggested that if these vegetables were prepared at their optimal physiological conditions they may serve as better alternative of exogenous supply of amylase to improve nutrient release when added to diets.

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Conflict of interest

The authors declare no conflict of interest.

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