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Partial Purification and Characterization of α and β-Amylases Isolated from *Sorghum bicolor* cv. (Feterita) Malt

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Abstract: The research aimed to study the diastatic power (PD) of sorghum malt , to purify the α and β-amylase s and to characterize these enzymes. In this study the amylolytic activity initiated during the germination of sorghum was determined. Fourth day of germination of sorghum grains showed the highest of the amount of Diastatic Power (DP) (62.0 IOB), no significant variation ($p \leq 0.05$) between the extraction procedures followed either by distilled water or water with 2% peptone. DEAE-cellulose chromatography was used for the partial purification of α-and β-amylases. The results obtained from the last purification steps is 6.94 fold of α-amylase in fifth day of germination, whereas β-amylase is 35.42 fold in fourth day of germination. These amylases isolated from sorghum malt have interesting characteristics such as, storage stability of purified α and β-amylases at different temperature (-20 and 4°C) for 56 days. No significant loss ($p \leq 0.05$) in the enzymes activity during the storage period for 56 days in-20°C, whereas the loss of enzyme activity at 4°C during the period of 56 days were 19% for α and 34% for β-amylases. The maximum activity of α-amylase was obtained at temperature 70 and 50°C for β-amylase, it was clear that the α-amylase is more stable than β-amylase. The optimum pH for both α and β-amylases were 5.0 and 5.5, respectively. Therefore, these characterizations meet the prerequisites need for food industry.

Key words: Diastatic power, α and β-amylases, sorghum malt, storage stability, enzymes temperature, pH

INTRODUCTION

The cereals are main food and considered as sources of energy, protein, vitamins and minerals for poor people in Sudan and some other African countries. Amylases are useful in abroad range of industrial application which includes food fermentation, textile and paper industries. α-amylases ubiquitous in nature have been isolated, purified and characterized from a number of animal, plant, fungal as well as bacterial sources (Kumar *et al*., 2009).

The diastatic power (DP) refers to the total saccarifying power activity, which is considered as contribution of α and β-amylases as well as α-glucosidases which convert maltose to glucose (Bureng and Worgan,1982). The thermostability of the DP enzymes is critical in determining fermentable sugar yield during mashing, where the mash temperature profile is a balance between the temperature required for starch gelatinization to enable efficient hydrolysis and the rate of thermal inactivation of the DP enzymes (Evan *et al*., 2003). The α and β-amylases of sorghum malt have been purified and their characteristics were studied by Botes *et al*. (1967a,b), Mundy (1982) and Okon and Uwaifo (1984). Zhang *et al*. (2006) showed that the variation in β-amylase activity was mainly attributable to the environment, although the effect of cultivar was also highly significant. Etokakpan and Palmer (1990) calculated activities of α-amylase and the DP from a single extract of sorghum malt using mercuric chloride to inhibit the SH function.

Sudanese sorghum have higher values of amylolytic activity compared to that of Nigerian and Australian sorghum malt as reported by Aniche and Palmer (1990). Etim and Etoakpan (1992) reported that diastatic activity of three sweet potato varieties was principally due to β-amylase. According to Omemu *et al*. (2005), the crude amylase preparation of *A. niger* AMO7 had temperature and pH optima activities at 60°C and 4.0 pH, respectively. Muralikrishna and Nirmala (2005) reported that α-amylases are more stable compared to β-amylases. Egwin and Oloyede (2006) found that the maximum α-amylase yield was attained from 72-120 h of sprouting cereals and the optimum temperature for α-amylase activity for sorghum malt was 70°C. Maize malt characterization showed that α and β-amylase had optimal

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pH between 4-6.5, optimal temperature 50 and 90°C, respectively (Biazus *et al*., 2009). Similarly Nagai *et al.* (2009) reported optimum pH of α-amylase as 6.0-7.0 and optimum temperature 40°C and the enzyme was extremely inactivated at pH higher than 7.0 or lower than 5.0. The heat inactivation occurred at 40°C.

Mohamed *et al.* (2009) reported that the activity of α-amylases increased from day 0 to 6 of germination of *Triticum estivum* a local Saudi Arabia wheat variety (Balady). Evans and Monday (2009) found that the rate of α-amylase secretion in sprouting maize, acha, rice and sorghum for 180 h is significantly high ($p \leq 0.05$) with growth period.

Different amylases give rise to oligosaccharides with specific lengths of glucose as major products, for this reasons different types of amylases, with unique properties are isolated and characterized for various application in the starch industry (Egwin and Oloyede, 2006). Kumar *et al.* (2009) found that α-amylase isolated from sorghum is reversible, unfolded by chemical denaturants at pH 7.0 in 150 mM Hepes containing 13.6 mM calcium and mM DTT.

The objectives of this research are to study the effects of malting and peptone addition on development of amylolytic activities in *Sorghum bicolor*, Purification of α and β-amylases isolated from *Sorghum bicolor* malt and characterization of purified α and β-amylases such as storage stability, the optimum temperature and pH.

MATERIALS AND METHODS

This study was applied in Khartoum University, Faculty of Science, Department of Botany, from 2005-2008.

Materials

Sorghum malt: *Sorghum bicolor* cv. (Feterita) grains were purchased from local market. Five hundred grams of sorghum was cleaned and freed from other materials and soaked in water overnight. The grains were spreaded on clean sack made from jute in plastic trays and covered by another jute sack and sprayed with water twice per day at temperature 28±2°C from 1-5 days. Germinated grains were left to dry by the air then ground using an electric grinder, which was operated intermittently so as to avoid heating. The powders were kept in glass bottles for chemical analyses. Three replicates for each experiment were used.

Measurement of diastatic power (DP) of sorghum grains and malt: Five gram of dry, fine powdered sample were taken in a conical flask containing 100 mL distilled water or 100 mL of 2% peptone solution. The diastatic activity during the period of germination of sorghum grains was determined by the method of EBC (1987).

Purification and characterization of α-amylase isolated from sorghum malt

Extraction of α-amylase: The purification and characterization of α-amylase isolated from sorghum malt was done according to the method of Botes *et al.* (1967a). In order to extract the enzyme, eighty gm of sorghum malt were suspended in 300 ml of cooled distilled water using cooled warring blender. The suspension was stirred continuously over night at low temperature (4°C) and then centrifuged at 14,000 g for 20 min.

Purification of sorghum malt α-amylase: Crude α-amylase was prepared by extracting as described above. The clear extract was 40% saturated with solid ammonium sulphate which precipitated the α-amylase. The precipitate was allowed to settle over night, collected by centrifugation, dialyzed to remove the salts and then freeze dried. The supernatant which contained the β-amylase was used for the preparation of β-amylase.

Fifteen grams of the crude α-amylase were dissolved in 1500 mL 0.1M acetate buffer (pH5.9) containing 0.2% calcium acetate and 60 g decolorizing charcoal was added. The mixture was heated at 70°C for 15 min with stirring.

The suspension was cooled to 4°C and the charcoal and coagulated protein removed with sold ammonium sulphate, the precipitate which formed collected by centrifugation, dissolved in 0.1 M tris buffer pH 7.0 +0.2% CaAc and dialyzed overnight against the same buffer.

The dialyzed solution from the previous step was applied to 4×12 cm DEAE cellulose column. The column was first eluted with 0.1 M tris buffer pH 7.0+0.2% CaAc and the eluate discarded. This was followed by elution with 0.1M tris buffer pH7.0 + 0.3 Nacl+0.2 CaAc in which the bulk of the activity appeared.

Enzyme assay (α and β-amylase): Soluble starch (Merck, special for diastatic power determination) was used as substrate for α and β-amylase activity. Apparent saccharifying power was estimated from the amount of reducing sugars, calculated as maltose produced from 2 mL of 2% starch solution in 0.05M acetate buffer of pH 4.6, when treated with 0.1 mL of suitable diluted enzyme solution for 10 min at 30°C. The enzyme reaction was stopped by adding 2 mL of 3,5-dinitrosalicylic acid reagent used for the determination of reducing sugars.

Purification and characterization of β-amylase isolated from sorghum malt

Purification steps of sorghum malt β-amylase: The purification of β-amylase as described by Botes *et al.* (1967b) was followed. The supernatant obtained after precipitation of α-amylase at 40% saturation with ammonium sulphate, contained the β-amylase. The

supernatant was precipitated with 70% ammonium sulphate. The precipitate was allowed to settle overnight collected by centrifugation, dialyzed to remove the salts and freeze-dried.

Twenty grams of the crude β-amylase were dissolved at room temperature in 2000 mL 0.1% cysteine (pH 6.0) containing 0.2% EDTA. The pH of the solution was lowered to 3.2 by addition of 5.0 N acetic acid and the solution was kept at this pH for one hour at room temperature. The pH of the solution was then adjusted to 4.6 by the addition of solid sodium acetate.

The solution was cooled to-2°C and fractionated with ethanol at -10 to -12°C. The precipitate obtained at an alcohol concentration between 33.33 and 66.66% v/v contained in a refrigerated centrifuge. The precipitate was dissolved in chilled 0.05 M acetate buffer of pH 5.0 and dialyzed overnight against the same buffer.

The dialyzed solution was applied to a 4×8 cm DEAE-cellulose column. The column was first eluted with 0.05 M acetate buffer of pH 5.0 and the eluate discarded. This was followed by elution with 0.15 M acetate buffer of pH 5.0 which resulted into two partly separated elution peaks. β-amylase activity was associated only with the last peak. Those fractions which contained the enzyme were combined, 90% saturated with solid ammonium sulphate and the precipitate was collected by centrifugation.

Protein determination: Protein was quantified by the method of Bradford (1976) with bovine serum albumin as standard.

Storage stability of α and β-amylases: Twenty fractions of 0.1 mL from purified enzyme preparations were taken in 20 test tubes. Ten of these tubes were stored in a refrigerator at 4°C, the other were placed in a freezer at -20°C. The enzyme activity were assayed with 7 days interval for both the frozen enzyme preparations and the enzyme stored at 4°C. The decrease in the absorbance values was measured and the percent of remaining activity was calculated.

Effect of temperature and pH on the α and β-amylase activity: The optimum temperature of α and β-amylases was determined using different temperatures for enzymes reaction (30.40, 50, 60, 70 and 80°C). The enzymes activity was assayed for each temperature reaction at pH 4.6 and 5.0 for α and β-amylases, respectively for 10 min. The optimum pH values of α and β-amylases for the reaction mixture was determined using acetate buffer at different pH values (2.0, 4.0, 5.0. 6.0, 7.0, 8.0 and 9.0). The enzymes activity was measured at each pH value at 30°C for 10 min.

RESULTS AND DISCUSSION

Diastatic Power (DP): During the process of germination, it was clear that DP was started by the onset of germination from the Ist day and continued until it reached the maximum value at the 4th day of germination as 62.0 IOB. Then it decreased on the 5th day (Fig. 1). These results agree with that obtained by Etim and Etoakpan (1992), who reported that diastatic activity of three potato varieties was principally due to β-amylase. Also the purification of β-amylase in this study revealed that the fourth day of germination of sorghum showed the highest amount of enzyme activity and decreased in the fifth day of germination, these results also confirmed by Egwin and Oloyede (2006). The results showed that the Sudanese sorghum malt have higher values of amylolytic activity compared to that of Nigerian and Australian sorghum malt as reported by Aniche and Palmer (1990). Figure 1 also showed that there was no significant variation ($p \leq 0.05$) between the extraction procedures followed either by distilled water or distilled water with 2% peptone.

Purification of α and β-amylase: Table 1 represented the results of the purification steps of α-amylase. Heating the crude enzyme for 15 min at 70°C increased the specific activity of sorghum malt from one to five days by 1.64, 2.1, 2.56, 3.16 and 3.51 folds. At the same time the total protein decreased by 43, 57, 71, 74 and 76% in the same order as compared by the initial value for sorghum malt. The values of enzyme yield were 93, 90, 74, 82 and 82% for sorghum malt. It is clear that heat step of purification is successful step, this result supported the findings of Kumar *et al.* (2005) and Egwin and Oloyede (2006).The

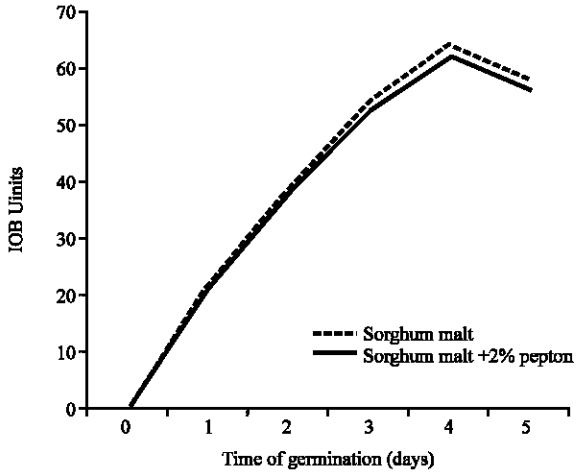

Fig. 1: Comparative development of Diastatic Power (DP) in *Sorghum bicolor* malt

Table 1: Purification steps of α-amylase isolated from *Sorghum bicolor* (Feterita) germinated for five days

Days	Step of purification	Total Vol. (mL)	Protein (mg mL^{-1})	Total protein (mg)	Enz. spec. Act. (U mg^{-1}) prot	Total enz. activity units	Yields (%)	Purification fold
1	Crude Jα amylase	0.70	4.90	329.0	82.60±0.09	27205.7	100	_
	40% $(NH_4)_2$ $2SO_4$ fractionation	52.0	4.20	218.4	120.10±0.23	26241.5	96	1.45
	Heat at 70°C for 15 min	08.4	3.90	187.2	135.60±0.72	25391.4	93	1.64
2	Crude Jα amylase	72.0	4.92	354.2	96.12±0.42	34050.5	100	_
	40% $(NH_4)_2$ $2SO_4$ fractionation	49.0	4.00	196.0+	165.20±2.2	32388.3	95	1.75
	Heat at 70°C for 15 min	43.0	3.57	153.5	201.80±.60	30979.3	90	2.1
3	Crude Jα amylase	76.0	5.07	385.7	121.70±1.2	46979.6	100	_
	40% $(NH_4)_2$ SO_4 fractionation	40.8	4.60	187.6	211.00±0.76	39603.9	84	1.73
	Heat at 70°C for 15 min	32.0	3.48	111.3	312.70±0.34	34822.9	74	2.56
4	Crude Jα amylase	74.0	5.40	399.6	157.90±1.30	63132.1	100	_
	40% $(NH_4)_2SO_4$ fractionation	36.0	4.21	151.6	356.90±0.62	54124.9	85	2.25
	Heat at 70°C for 15 min	29.0	3.56	103.2	500.50±0.43	51669.2	82	3.16
	DEA-cellulo chromatography	25.0	1.92	48.0	1046.80±0.3	50250.1	79	6.63
5	Crude Jα amylase	77.0	5.18	398.9	169.70±1.35	67677.9	100	_
	40% $(NH_4)_2SO_4$ fractionation	40.0	4.10	172.0	367.60±1.35	63223.6	93	2.16
	Heat at 70° C for 15 min	27.0	3.44	92.88	596.20±3.5	55373.5	82	3.51
	DEA-cellulo chromatography	23.0	1.91	43.93	1178.00±2.02	51757.4	76	6.94

Table 2: Purification steps of β-amylase isolated from Sorghum bicolor (Feterita) germinated for five days

Days	Step of purification	Total Vol. (mL)	Protein (mg mL^{-1})	Total protein (mg)	Enz. spec. Act. (U mg^{-1}) prot	Total enz. activity units	Yields (%)	Purification fold
1	Crude β amylase	68.0	4.70	319.0	0.327±0.07	104.59	100	_
	70% $(NH_4)_2SO_4$ fractionation	14.0	3.80	53.2	1.472±0.05	78.349	75	4.59
	Acid treatmentand alcohol fractionation	10.0	2.20	22.0	3.163±0.13	69.586	66	9.67
2	Crude β amylase	64.0	5.12	327.6	0.440±0.01	146.562	100	_
	70% $(NH_4)_2$ SO_4 fractionation	14.0	4.30	60.0	2.072±0.08	124.778	85	4.63
	Acid treatment and alcohol fractionations.	12.0	2.10	25.2	4.527±0.07	114.085	78	10.1
3	Crude β amylase	60.0	5.21	312.0	0.981± 0.09	306.9	100	_
	70%$(NH_4)_2$ SO_4 fractionation	17.0	3.21	54.4	4.909±0.05	267.1	87	5.0
	Acid treatment andalcohol fractionation.	12.0	1.42	17.0	14.840±0.40	252.8	82	15.1
4	Crude β amylase	70.0	5.40	378.0	1.418±0.37	536.1	100	_
	70% $(NH_4)_2$ SO_4 fractionation	11.0	4.92	54.1	9.436±0.79	510.0	95	6.65
	Acid treatment andalcohol fractionation	8.0	1.73	13.8	32.730±0.52	452.5	84	23.07
	DEAE-cellulose chromatogr.	5.5	1.50	8.58	50.240±0.92	431.0	80	35.42
5	Crude β amylase	75.0	5.30	397.5	1.580±0.00	628.8	100	_
	70% $(NH_4)_2$ SO_4 fractionation n	13.0	5.00	65.0	8.626±0.10	560.2	89	5.44
	Acid treatment and alcohol fractionation	9.0	1.90	17.1	30.700±0.40	524.2	83	19.37

results using DEAE-cellulose chromatography indicated that the purification folds were 6.63 and 6.94 for 4th and 5th day of germination, respectively and the yields of enzyme activity were 79 and 76%, respectively. The reduction of protein was 87 and 88%, respectively, compared to that of initial value.

The specific activity was increased by 9.67, 10.12, 15.10, 23.17 fold at day 1,2,3,4 respectively and then decreased to 19.37 at the 5th day of germination compared to initial value. At the same time the total protein were decreased by 93, 92, 94, 96 and 95%, respectively. The enzyme yields were 66, 78,82, 84and 83%, respectively (Table 2). Botes *et al.* (1967b) reported that the acid treatment reduced α-amylase while the β-amylase activity remained constant then alcohol fractionation removed the last traces of α-amylase and gave considerable purification of β-amylase. In this study it was clear that high purification fold (35.42) was obtained during the 4th day of germination, where DEAE-cellulose chromatography was used, these might confirm the Botes *et al.* (1967b) results. The total protein was reduced by 98% and the yield of enzyme activity was 80% compared to initial value. At the same time during the 5th day of germination the specific activity was reduced in all steps of purification treatment.

Storage stability of α and β-amylases: The storage stability of purified α and β-amylases at -20 and 4°C for 56 days was determined. It was observed that there was no significant loss in the enzymes activity during the storage period for 56 days in-20°C for both enzymes isolated from sorghum malt. The loss of activity at 4°C during the period of 56 days was 19% for α, whereas the loss of β-amylase activity during storage period at 4°C at days 35, 42, 49 and 56 were 20, 25, 30 and 34% (Fig. 2). Theses results confirm the findings of Muralikrishna and Nirmala (2005),who observed that α-amylases are more stable compared to β-amylase.

The effect of temperature and pH on α and β-amylases: The influence of temperature on α and β-amylases activity showed that enzyme activity increased

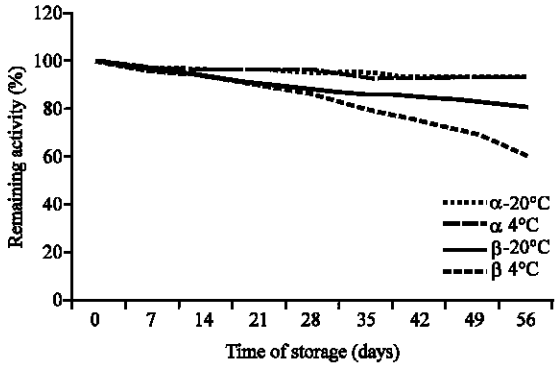

Fig. 2: *Sorghum bicolor* α-and β-amylases stability at two different storage conditions

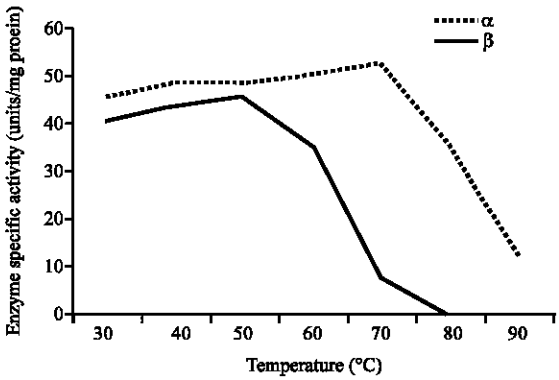

Fig. 3: Temperature dependent activity profile of *Sorghum bicolor* malt α-and β-amylase

progressively with increase in temperature from 30°C reaching a maximum at 70°C for α-amylase and 50°C for β-amylase as shown in (Fig. 3). Above 70 and 50°C there was a reduction in α and β-amylases activity, respectively. These results agree with that reported by Egwin and Oloyede (2006), they found that the optimum temperature for α-amylase activity isolated from sorghum malt was 70°C.The results showed that the activity of α-amylase decreased markedly after being heated for 15 min at temperature up to 70°C this agree with the findings of Bureng and Worgan (1982). On the other hand, the β-amylase enzyme was stable at 50°C for 10 min; otherwise it showed markedly destroyed at temperature above 50°C.

The effect of pH on α and β-amylases activity isolated from sorghum malt was showed that the enzymes specific activity was reached a maximum value at pH 5.0 and 5.5, respectively (Fig. 4). These results conversely with that obtained by (Egwin and Oloyede, 2006). As the same time the obtained data agree with the finding of Nirmala and Muralikrishna (2003) who found that the optimum of pH α-amylases for finger, millet was a range of

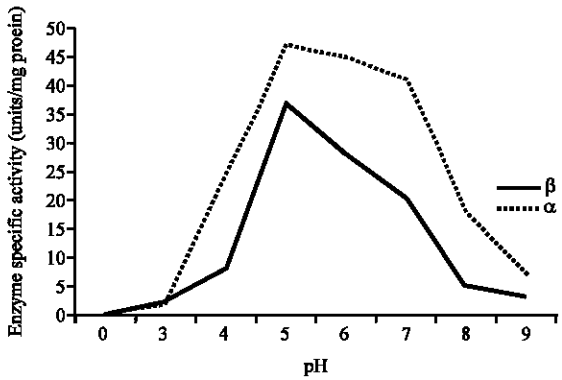

Fig. 4: *Sorghum bicolor* malt α-and β-amylases activity as a function of pH at 30°C

5.0-5.5. Mohamed *et al.* (2009) reported that the optima of different α-amylases of wheat have broad pH optima range from 5.0 to 7.0. This pH finding of β-amylase agrees with that of Okon and Uwaifo (1984) who reported that the optimum pH of β-amylase was 5.5.

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