Carbohydrate Polymers 77 (2009) 105–109

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Properties of the isoforms of α-amylase from kilned and unkilned malted sorghum (*Sorghum bicolor*)

Isaac Olusanjo Adewale *, Ayomide Oladejo

Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria

ARTICLE INFO

Article history:
Received 1 July 2008
Received in revised form 3 November 2008
Accepted 8 December 2008
Available online 24 December 2008

Keywords:
α-Amylase
Kilned malted sorghum
Temperature stability
Low K_m

ABSTRACT

We have previously reported the presence of a relatively heat-stable α-amylase with a low K_m for starch in kilned malted sorghum. In order to establish the industrially useful and more efficient isoforms, we have separated this α-amylase into different isoforms using both cation and anion-exchange chromatographies. Unkilned malted α-amylase crude was separated into three different isoforms (a1, a2 and a3) whereas kilned samples were separated into two (a1 and a2). Apparently one isoform (a3) was lost during kilning due to heat lability. a1 isoform which appears to have a neutral p*I* and constitute about 60% of the total α-amylases protein that were induced during germination, have the lowest K_m for starch. They are more generally stable than other isoforms at all the temperatures studied. These isoforms lost only 10% activity at 80 °C for 30 min and still had some residual activity at 100 °C incubation for 30 min. a1 isoform could therefore be adapted for industrial starch conversion processes which are carried out within this range of gelatinizing temperatures because of its properties.

1. Introduction

Amylases are enzymes that catalyze the conversion of starch and other related polysaccharides possessing α-1,4 glycosidic bonds into simpler derivatives such as oligosaccharides or monosaccharides. They are usually of two types – α-amylases (α-1,4-glucan-4-glucanohydrolases; EC. 3.2.1.1) hydrolyses the internal α-1,4 glycosidic links at random to produce less viscous solutions with lower molecular weight products limited by the α-1,6 glycosidic bonds when it attacks starch (Brena, Pazos, Franco-Fraguas, & Batista-Viera, 1996); while β-amylases (α-1,4-glucanmaltohydrolases, EC. 3.2.1.2) are exoamylases that hydrolyze starch beginning at the nonreducing ends producing β-maltose and β-limit dextrins (Brena et al., 1996). Amylases are used for various applications in industries because of these abilities. α-Amylases are produced in large amount in malted grains and we have previously reported this in malted maize, millet and sorghum (Adewale, Agumanu, & Otih-Okoronkwo, 2006).

Generally, grains contain varying amounts of α and β-amylase. For instance, unmalted sorghum has no β-amylase, and very little of it when the grain is malted; barley has significant amount of α and β-amylase which develop during the malting process when the β-amylase is activated from a bound form, which increase the hydrolytic activity substantially (Taylor & Robbins, 1993). Sorghum has also been reported to be a suitable source of α-amylase compared to other grains such as maize or millet because of higher temperature stability profile of its α-amylase (Adewale et al., 2006; Egwim & Oloyede, 2006; Kumar, Singh, & Rao, 2005; Shambe, Voncir, & Gambo, 1989). Grain α and β-amylases could be separated by extraction with buffers having different pH values which would inactivate one species while leaving the activity of the other intact (Muralikrishna & Nirmala, 2005). α-Amylases are inactivated at pH around 4.8–5 while β-amylases are unstable at pH around 6–7 (Muralikrishna & Nirmala, 2005; Ziegler, 1999).

Based on our previous observations (Adewale et al., 2006), and as reported by other investigators (Kumar et al., 2005), malted grains could be a major source of exogenous amylases for various applications such as starch liquefaction and saccharification. Currently, such exogenous enzymes are obtained from bacterial or fungal source. In some brewing and food industries, liquefaction of starch using bacterial enzyme is done at between 85 and 95 °C while saccharification is done at 55–60 °C using fungal enzyme. If a grain amylase should be deployed for this process, it should be able to withstand this temperature treatment. We have therefore decided to investigate the profile of α-amylase isoenzymes in a view to obtaining thermally-stable isoforms which could be recruited in the liquefaction of gelatinized starch either in its native or in a more resistant chemically-modified form. We report here the properties of the unmodified form of purified α-amylase isoforms from both the kilned and unkilned malted sorghum grains.

* Corresponding author.
E-mail addresses: olusanjo2002@yahoo.co.uk, iadewale@oauife.edu.ng (I.O. Adewale).

0144-8617/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2008.12.011

2. Materials and methods

2.1. Chemicals and other materials

Sorghum grains were purchased from a local market and the cultivar was identified to be *Sorghum bicolor* in the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. Chemicals and reagents used were of analytical grade and were obtained from Sigma Chemical Company, St. Louis, USA; BDH Chemicals, Poole England or other reputable commercial suppliers.

2.2. Methods

2.2.1. Enzyme source

In a typical extraction process, about 200 g of the sorghum grain which had been screened to remove broken grains and other extraneous materials was steeped in distilled water for 48 h with changes in the steeping water every 6 h interval to prevent microbial growth. After steeping, the grains were removed, blotted to remove excess water and were spread out for germination at 30 °C in a locally constructed malting chamber. In order to maintain sufficient moisture content, the germinating grains were sprayed with water every 12 h during the germination periods. Germination was completed in 72 h. About half of the germinated grains were kilned in hot air oven (Fisher Isotemp Oven Model 175) at 55 °C for 24 h.

About half of the germinated grains were kilned in hot air oven at 55 °C for 24 h. Both the de-rooted kilned and unkilned malted samples were ground into a fine powder or paste and homogenized with 50 mM sodium phosphate buffer pH 6.0 to obtain a 30% homogenate, which was centrifuged at 10,000*g* for 30 min to obtain a clear supernatant.

2.2.2. Purification by cation-exchange chromatography on CM-Trisacryl

In a typical purification, about 75 ml of clear supernatant obtained either from kilned or unkilned malted grains were layered on a 2.5 × 40 cm column of CM-Trisacryl resins which had been equilibrated with 50 mM sodium phosphate buffer pH 6.0. Flow-through fractions were collected and bound proteins were eluted with 0–1.0 M NaCl gradient. Enzyme activity of fractions as well as protein profile were determined.

2.2.3. Purification and separation of isoforms by anion-exchange chromatography on DEAE-Sephacel

Post cation-exchange active fractions were pooled together and applied on a column of DEAE-Sephacel which had been equilibrated with 5 mM Tris–HCl buffer pH 7.0 containing 1 mM $CaCl_2$. A 1.0 M NaCl gradient was used to elute bound proteins. Active fractions were separately pooled for further use.

2.2.4. Protein and enzyme activity determination

Protein concentration was measured using the method of Lowry, Rosebrough, Farr, and Rondall (1951) with bovine serum albumin as the standard proteins. Activity of enzyme was routinely assayed by determination of reducing ends released by the enzyme when it attacks starch. Briefly, an aliquot of the enzyme was incubated with 1% soluble starch in 10 mM phosphate buffer pH 7.0 for 10 min with the appropriate control. The amount of reducing sugar liberated was then determined by Somogyi–Nelson method as modified by Sugita, Kawasaki, Kumazawa, & Deguchi, 1996). One unit of activity was defined as the amount of enzyme which liberated reducing sugar equivalent to 1 μg of D-glucose per minute at 25 °C.

2.2.5. Determination of kinetic characteristics for the isoenzymes

For the determination of kinetic parameters (K_m and V_{max}) for each of the isoenzymes, the experiment was carried out in triplicate, following the method that was described earlier (Adewale et al., 2006). The concentration of soluble starch was varied between 0.1 and 1% while keeping the concentrations of other components constant. The data obtained were analyzed by double-reciprocal plot.

2.2.6. Thermal stability of the isoenzymes

An aliquot of each of the purified isoenzymes was incubated at temperatures ranging between 30 °C and 100 °C for up to 60 min. Aliquots were removed at 10 mins intervals, and placed in ice. The activity of these aliquots were determined and expressed as a percentage of the activity at zero time which was taken to be 100%.

3. Results

3.1. α-Amylases extraction

A large quantity of the enzyme protein was induced in the germination process with a specific activity of 1566 units/mg protein in the unkilned malt which reduced to 615 units/mg protein in the kilned sample. We have previously established that unmalted sorghum has negligible amount of starch-hydrolyzing enzymes (Adewale et al., 2006) Ammonium sulphate or acetone precipitation of the extract led to a reduction in the specific activity, necessitating the use of alternative purification steps.

3.2. Isoenzyme separation and purification

When a crude extract obtained either from unkilned malted sorghum or kilned malted sorghum was applied on cation-exchange column, all the main active peaks came out in the flow-through fractions (Fig. 1A and B).

The active fractions were pooled and were properly separated into different isoforms on DEAE-Sephacel columns (Fig. 1C and D).

For the post CM-Trisacryl fraction of unkilned malted sample, one main peak (a1) was obtained before the gradient. A 0–1.0 M NaCl gradient however eluted two isoenzymes at 0.6–0.7 M (a2) and 0.85–0.95 M (a3) NaCl concentrations respectively. However, only two peaks (Fig. 1D) were obtained when post CM-Trisacryl fraction of kilned sample was purified on DEAE-Sephacel resin. One of the peaks (a1; Fig. 1D) was obtained in the flow-through and the other (a2) appear in the NaCl gradient, between 0.6 and 0.7 M. An attempt was made to separate the isoforms by gel-filtration on Sephadex G-100. There was however non-specific interactions with the resin.

Eluant fractions relating to each individual isoenzyme were pooled for further characterization. The summary of the purification is shown in Table 1.

3.3. Heat stability of the isoenzymes

All the isoenzymes are stable at 30 °C for 1 h (Fig. 2A–D) retaining more than 90% activity. They also retain more than 80% activity at 40 °C. At 100 °C more than 50% activity is retained by a1 isoenzymes from both the unkilned and kilned, malted sorghum. Isoenzyme a2 from kilned and unkilned grains as well as isoenzymes a3 (from the unkilned sorghum) are however unstable at 80 °C or 100 °C, rapidly loosing activity such that residual activity remaining was negligible. Isoenzyme a3 was even more unstable than other heat-unstable isoforms loosing more than half of the residual activity at 50 °C over a period of 1 h. It was not present in the isoenzymes separated from the kilned samples.

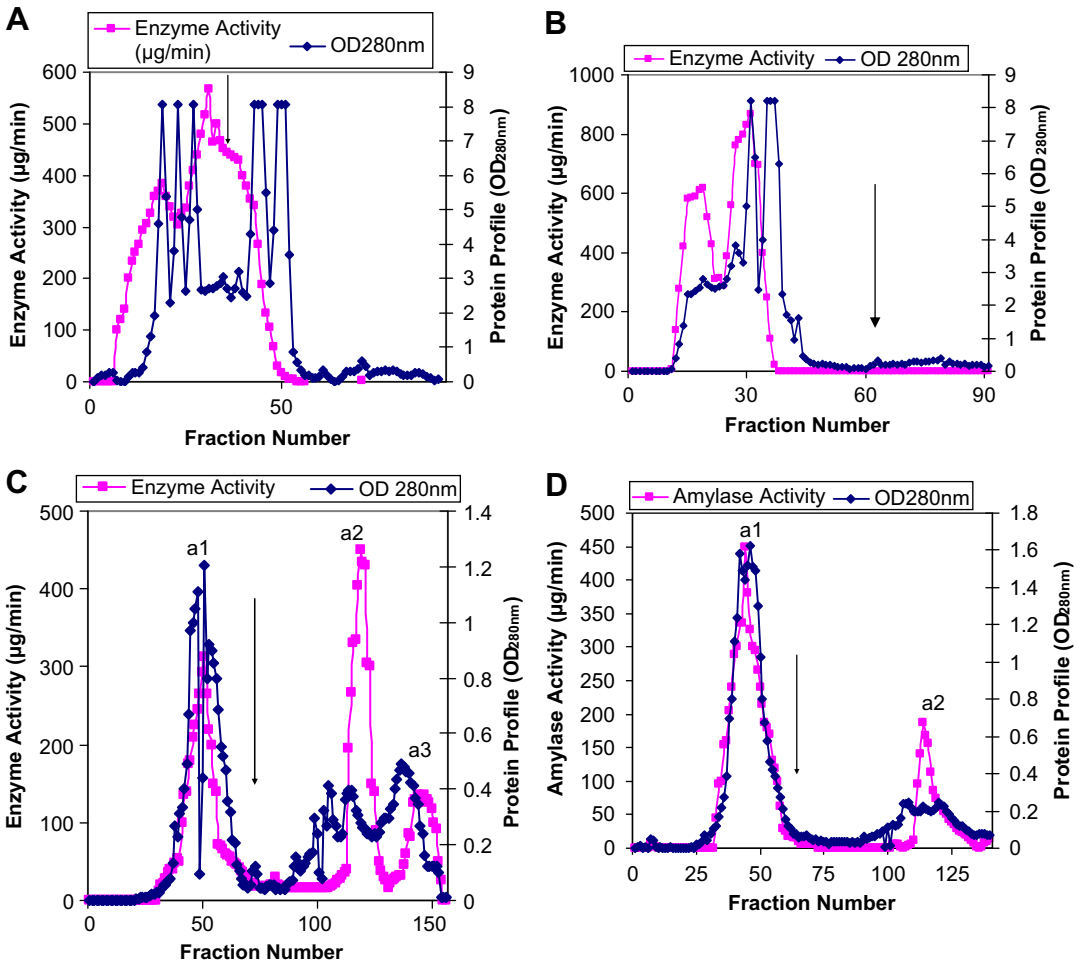

Fig. 1. Ion-exchange chromatography of crude extract of unkilned and kilned malted sorghum (Fig. 1A and B, respectively) on CM-Trisacrylic acid column. After washing the column with phosphate buffer, a linear gradient of 0–1.0 M NaCl dissolved in the buffer, was used to elute bound proteins. All the amylase activities came out in the flow-through fractions. Pooled fractions from the CM-Trisacrylic column of both the unkilned and the kilned partially purified fractions were further purified on DEAE-Sephacel ion-exchange column (Fig. 1C and D, respectively). This column separated the unkilned α-amylase into isoforms a1, a2 and a3 while isoforms a1 and a2 were obtained from kilned malted sorghum. The arrow (↓) indicate the start of NaCl gradient. The activity of the enzyme (■) and the protein (♦) were monitored as described in the text.

Table 1
Summary of purification of α-amylase from kilned and unkilned malted sorghum.

Purification step		Total unit	Total protein (mg)	Specific activity (unit/mg protein)	Purification factor	% yield
Crude (malted, unkilned)		26,765	257	104	1.0	100
CM-Trisacryl	Pool	66,215	357	185	1.8	247
DEAE-Sephacel	a1	12,300	33	374	2	46
	a2	14,337	7.14	2010	10.8	53
	a3	2341	2.95	794	4.3	8.8
Crude (malted and kilned)		11,143	268	41.5	1.0	100
CM-Trisacryl	Pool	60,097	323	186	40.5	540
DEAE-Sephacel	a1	14,166	66	214	1.2	127
	a2	4117	4.14	994	5.4	37

3.4. Kinetic characteristics

The apparent K_m and V_{max} for the isoenzymes are shown in Table 2. Apparent K_ms for the flow-through isoenzymes from the unkilned grains were about twice that of kilned malted grains. The maximal velocities of the isoenzymes from the unkilned grains were however higher than that of isoenzymes obtained from kilned grains. In addition, the maximal velocities of a2 isoenzymes from both the unkilned and kilned grains were higher than their respective a1 isoforms.

4. Discussion

Malted sorghum contains mainly α-amylases; and it has been previously established that grain α-amylases are more stable than β-amylases at pH 6–7, and could be extracted at this pH from grains where they may be present at a concentration as high as 30% of the total protein synthesized during germination (Adewale et al., 2006; Muralikrishna & Nirmala, 2005; Uriyo & Eigel, 1999; Ziegler, 1999). In addition to the fact that they are more heat-sta-

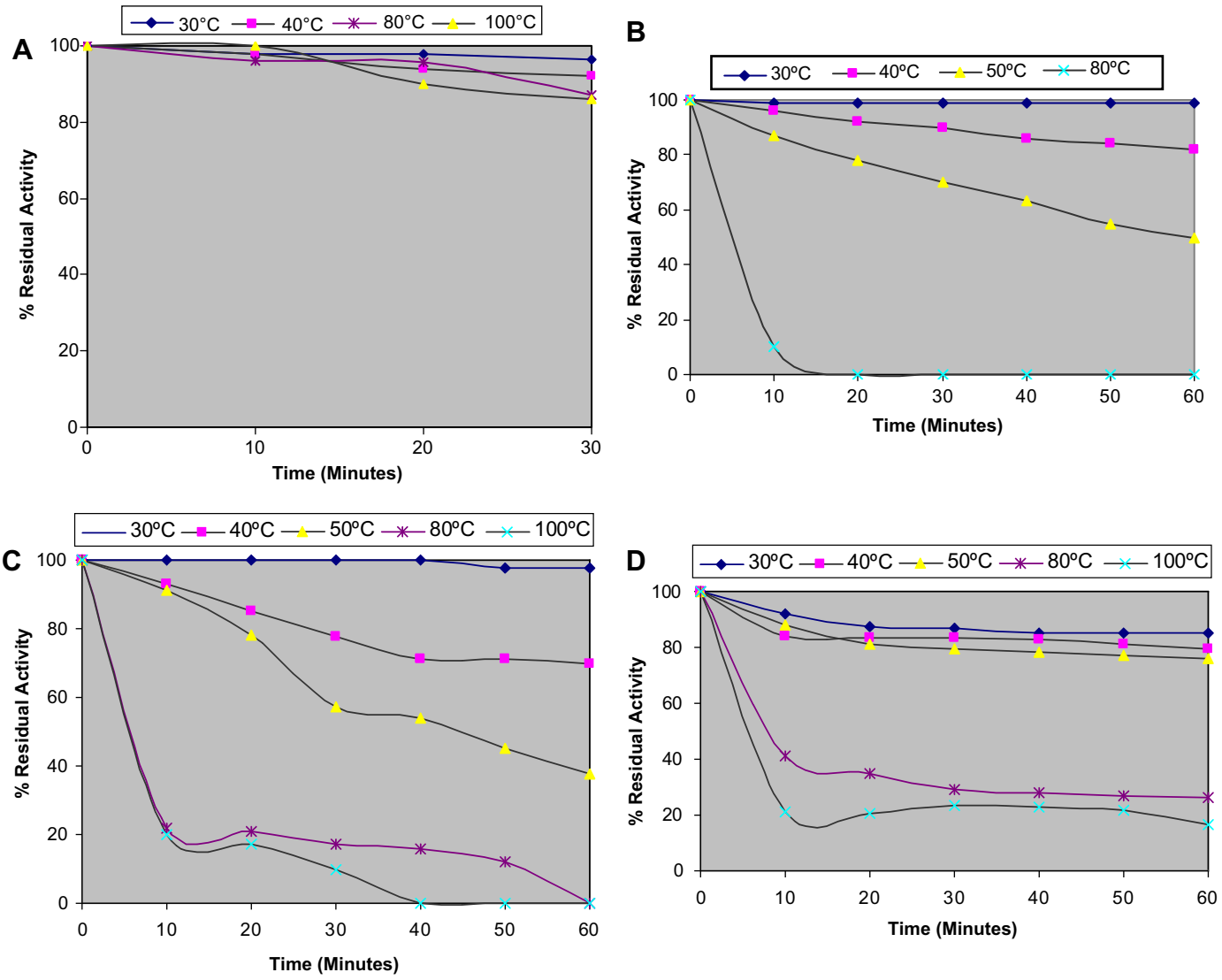

Fig. 2. Effect of heat on the stability of the isoforms of kilned and unkilned malted sorghum α-amylase. (A) An aliquot of purified a1 isoform from unkilned malted sorghum was incubated at temperatures ranging between 30 and 100 °C for a specified time of between 0 and 60 min. An aliquot of the incubated enzyme was withdrawn at specified times and assayed for residual activity. The residual activity was then plotted against the specified time, taking the activity of the enzyme at 0 min incubation, as having 100% activity. A similar pattern of stability was observed for purified a1 isoform from kilned malted sorghum. (B) and (C) are respectively, the heat stability profile of purified a2 and a3 isoforms from unkilned malted sorghum; and (D) is the profile of purified a2 isoform from kilned malted sorghum.

Table 2
Summary of the kinetic parameters of the isoforms of α-amylase from kilned and unklined sorghum with starch as substrate.

Isoforms	K_m (g/ml)	V_{max} (units/min)
a1 from unkilned malted grain	2.535×10^{-4}	250
a2 from unkilned malted grain	5×10^{-3}	2501
a3 from unkilned malted grain	4.16×10^{-3}	348
a1 from Kilned malted grain	1.25×10^{-4}	11
a2 from Kilned malted grain	2.189×10^{-3}	1000

ble, α-amylase could also be distinguished from β-amylase, because of their rapid reduction of viscosity of soluble starch.

We have extracted α-amylase from kilned and unkilned malted sorghum based on these properties. Sorghum amylase is synthesized in large amount leading to a high specific activity in the crude preparation. In the preliminary experiments, precipitation of the amylase using conventional methods of acetone or ammonium sulphate precipitation led to a decrease in specific activity which could not be ignored. It should however be emphasized that acetone precipitation had previously been used to fractionate α-amylases from malted finger millet, a similar cereal (Nirmala & Muralikrishna, 2003a).

The purification strategy adopted in this work could easily be modified for large scale industrial separation, with good yield to isolate the different isoenzymes constituent of these grains. The enzyme from kilned grains could be recovered in better yield than that from unkilned grains. This may probably be due to in situ modification of the enzyme during the kilning process leading to isoenzymes with different properties. In all, three isoenzymes (a1, a2 and a3) were resolved from unkilned malted sorghum while kilned malted sorghum gave two peaks of isoenzymes namely a1 and a2. The three isoenzymes peaks obtained in this study is similar to the results obtained from malted finger millet, *Eleucine coracana* by Nirmala and Muralikrishna (2003a), who also reported the presence of three isoenzymes during the fractionation of acetone precipitated, crude enzyme extract by DEAE-Sephacel chromatography. Interaction of α-amylase isoenzymes with dextran materials on gel-filtration column suggests

the presence of carbohydrate materials covalently linked to the enzymes amino acid residues.

Except for the in situ modification, which we thought occurred during kilning, isoenzyme a1 from the unkilned and kilned sorghum are identical; so also is isoenzymes a2. We are of the belief that this type of modification with natural constituent of the grains could possibly produce an enzyme with novel catalytic ability which could be deployed for various applications, and this has been demonstrated in this study. The altered characteristics may be due to modification of amino acid side chain groups e.g. by saccharides generated by the enzyme activity.

Additional evidence of modification during kilning stems from the kinetic characteristics and heat stability studies. The apparent K_m of the modified isoenzymes is about half of the unmodified enzyme suggesting that the affinity of the isoenzymes for starch was increased after the kilning process. It is also apparent from heat stability data, that the heat-modified isoenzymes are more stable than the unmodified enzymes. A heat-unstable isoenzymes (a3) was however lost during the kilning process. α-Amylases are reputed to be stabilized by calcium and phosphate salts (Taylor & Robbins, 1993). Since Ca^{2+} ions are present at 1 mM concentration in solution with these isoenzymes, they may be contributory to the increased stability of these isoforms. Nirmala and Muralikrishna (2003b) had also reported that the presence of Ca^{2+} at 5 mM concentration has both activating and stabilizing effect on millet α-amylases. Further analysis of the modified isoenzymes to determine how they are modified and the nature of the modifying group(s) would be a guide to produce commercially useful enzyme that can compete with bacterial or fungal enzyme for the same application.

Moreover, a1 isoform obtained from kilned grains are relatively heat-stable, and are present in large proportion; and therefore could be deployed without further modification for commercial uses. This a1 isoform has a higher affinity for starch since its Michaelis constant is about half of the unkilned isoforms. It will however be relatively more effective at low substrate concentration because of its reduced maximal velocity.

References

Adewale, I. O., Agumanu, E. E., & Otih-Okoronkwo, F. I. (2006). Comparative studies on α-amylases from malted maize (*Zea mays*), millet (*Eleusine coracana*) and sorghum (*Sorghum bicolor*). *Carbohydrate Polymers, 66*, 71–74.

Brena, B. M., Pazos, C., Franco-Fraguas, L., & Batista-Viera, F. (1996). Chromatographic methods for amylases –review. *Journal of Chromatography B, 684*, 217–237.

Egwim, E. E., & Oloyede, O. B. (2006). Comparison of α-amylase activity in sprouting Nigerian cereals. *Biokemistri (Publication of the Nigerian Society for Experimental Biology), 18*(1), 15–20.

Kumar, R. S. S., Singh, S. A., & Rao, A. G. A. (2005). Thermal stability of α-amylase from malted jowar (*Sorghum bicolor*). *Journal of Agriculture and Food Chemistry, 53*(17), 6883–6888.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Rondall, R. J. (1951). Protein measurement with folin–phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Muralikrishna, G., & Nirmala, M. (2005). Cereal α-amylases – an overview. *Carbohydrate Polymers, 60*, 163–173.

Nirmala, M., & Muralikrishna, G. (2003a). Properties of three purified α-amylases from malted finger millet (Ragi, *Eleusine coracana*, Indaf-15). *Carbohydrate Polymers, 54*, 43–50.

Nirmala, M., & Muralikrishna, G. (2003b). Three α-amylases from malted finger millet (Ragi, *Eleusine coracana*, Indaf-15)-purification and characterization. *Phytochemistry, 62*, 21–30.

Shambe, T., Voncir, N., & Gambo, E. (1989). Enzyme and acid hydrolysis of malted millet (*Peminsetun tyhoides*) and sorghum (*Sorghum bicolor*). *Journal of the Institute of Brewing, 95*, 13–16.

Sugita, H., Kawasaki, J., Kumazawa, J., & Deguchi, Y. (1996). Production of amylases by the intestinal bacteria of Japanese coastal animals. *Letters in Applied Microbiology, 23*, 174–178.

Taylor, J. R. N., & Robbins, D. J. (1993). Factors influencing beta-amylase activity in sorghum malt. *Journal of the Institute of Brewing, 99*, 413–416.

Uriyo, M., & Eigel, W. E. (1999). Duration of kilning treatment on α-amylase, β-amylase and endo-(1, 3)(1, 4)-β-D-glucanase activity of malted sorghum (*Sorghum bicolor*). *Process Biochemistry, 35*(15), 433–436.

Ziegler, P. (1999). Cereal beta-amylases – a minireview. *Journal of Cereal Science, 29*, 195–204.