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Short communication

Comparative studies of two α-amylases acting on two Sorghum hybrids starches (Montecillos hybrid 2 and 3) and their significant differences in their catalytic activities

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ABSTRACT

Starchy substances constitute the major part of the human diet for most people in the world. α-Amylases are widely distributed enzymes that initiate the hydrolysis of starch into low molecular weight maltodextrins. The effect of pH on the rate of hydrolysis of both sorghum (Montecillo 2 and 3) hybrids starches by industrial bacterial amylase (IBA) and *Aspergillus oryzae* α-amylase(AOA) was studied. This result suggests that both IBA and AOA α-amylases had slightly high affinity for soluble starch of sorghum Montecillo 3 and 1 hybrids, respectively. Using the structural information available, the theoretical pK_a values of its ionizable residues directly involved in the catalytic region were determined. Both our experimental data and prediction studies indicated that the average pK_a values are in good agreement.

1. Introduction

Starch is a reserve chemical form of the energy of the sun found in green leaves, stems, cereal grains, seeds, nuts, and fruits of plants. Many kinds of starches provide the major source of food energy in the diets of humans and are increasingly used industrially as bio-renewable materials for the formation of a diverse array of products, for example, ethanol, high-fructose syrups, cyclomaltodextrins, maltodextrins, adhesives, paper and textile sizing agents, and so forth. Starch consists of two types of polysaccharides, amylose, a linear α-(1 → 4)-glucan, and amylopectin, a branched α-(1 → 4)-glucan with 5% α-(1 → 6)-branch linkages (Robyt, 1998). In general, both polysaccharides are found in 15–30% and 70–85%, respectively.

Sorghum is an important cereal grain resistant to water drought and extreme high temperatures with more than 60 million metric tons harvested from 44 million hectares in 2004 (FAOSTAT, 2004). Sorghum is a staple food in countries (Dendy, 1995). It is estimated that more than 40% of the worldwide sorghum production is used for human food consumption (Rooney & Waniska, 2000).

α-Amylases [EC 3.2.1.1] are widely distributed enzymes formed by bacteria, fungi, plants and animals that hydrolyze the α-(1 → 4)-glucosidic bonds of starch into maltodextrins of varying sizes, depending on the particular enzyme (Robyt, 1998).

This work brings about some experimental and to extend theoretical evidences implying that most probably the amino acid composition of both industrial bacterial (IBA) and *Aspergillus oryzae* α-amylases (AOA) in part those which occur in the vicinity of active site influence the pK_a value of active site titrable groups and subsequently shifts the pH-profile of these enzymes.

2. Experimental

Sorghum starches were freshly prepared from two mature sources (Montecillo 2 and 3) hybrids. These seeds were obtained from Dirección del Instituto de Recursos Genéticos y Productivos del Colegio de Posgraduados in Montecillos, Estado de México, México. Industrial bacterial α-amylase was obtained from Gitss Brocades and *A. oryzae* α-amylase was a crystalline enzyme obtained from Sigma Chemical Co.

Starch extraction was performed using a modified procedure of Schoch & Maywald, 1968. The sorghum seeds are cleaned from impurities. Seeds are milled and sieved through a 3 mm mesh. This material was re-suspended in water with a ratio 1:3 (w/v) and allowed to stay at rest for 24 h. The pH of this mixture should be maintained between 5.9 and 6.3 in order to avoid any acid starch hydrolysis. The starch suspension was filtered through a nylon

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mesh and adding cooled water at 5 °C continuously until the filtered water is clear in order to obtain the maximum amount of starch. After that the water on top of the precipitate is removed very carefully. This precipitate is re-suspended on distilled water with a ratio 1:3 and again filtered using a nylon mesh and refrigerated at 4 °C during 16 h. Then the water is removed and the precipitated is re-suspended in a solution of sodium hydroxide 0.2% in a ratio 1:2 in order to eliminate any protein fine fiber in this precipitate. This procedure is followed twice at pH 6.0. All starch obtained is dried at room temperature and milled in a mortar.

The starch granules were solubilized by suspending 1 g in a 0.02 M phosphate buffer at pH 6.9 and 0.0067 M of NaCl, and maintained this suspension under boiling conditions for 3 or 4 min with continuous and vigorous stirring, followed the dilution with the same buffer solution to reach 100 ml.

The activities of industrial bacterial (IBA) and *A. oryzae* α-amylases (AOA) were determined by the measurement of the increase in the reducing value of maltodextrin products of the reaction. The reducing value was determined by the Miller method (Miller, 1959), using maltose as standard. A plot of optical density at 540 nm versus μg of maltose was prepared, and the slope of the linear part of the curve was determined and converted into micrograms of released maltose per min and per mg of protein.

For the assays, a 1% soluble starch was prepared by dissolving 1 g of soluble starch in 0.02 M phosphate buffer at pH 6.9 and 0.0067 M NaCl and then boiled this solution and rigorously stirred during 3 or 4 min, then cooled and calibrated to 100 ml with the same buffer. In the assays, 1.9 ml of the 1% soluble starch solution that were preincubated at 37 °C for 10 min to reach the desirable temperature and then added 0.1 ml of the enzymatic solution. At the end of this time, 4 mL of 3,5-dinitrosalycilic acid was added to stop the reaction, and vigorously stirred to homogenized the mixture. The formation of 3-amino, 5-nitrosalylic acid is carried under boiling water environment during 5 min, then cooled and read at 540 nm. Triplicate assays were made for both starches and enzymes. All buffer solutions used in all experiments at different pH values have an ionic strength of 0.1 M.

For the determination of kinetic parameters, an aliquot of the starch-digesting enzymes was incubated for 10 min with soluble starch, dissolved in 20 mM phosphate buffer and NaCl 6.7 mM, pH 6.9, whose concentration was 1%, while other components were kept constant. The amount of reducing sugar formed was then estimated from where the units of activity were calculated. The data obtained were analyzed by double-reciprocal plot.

3. Results and discussion

Both samples of sorghum varieties for Montecillo 2 and 3 were analyzed for kernel weight and inspected for kernel plumpness. The starch extraction yields for the sorghum varieties are 54% and 43% for Montecillo 2 and 3, respectively.

The effect of pH on the rate of hydrolysis of both sorghum (Montecillo 2 and 3) hybrids starches by IBA and AOA is depicted in Figs. 1 and 2. Both plots of enzyme activity versus pH show a "bell shaped". Both enzymes respond to changes in pH by alterations in their folding and in the ionization of their amino acids side chains. If the pH is either too high or too low, the amino acid side chains in the active site will be titrated one way or the other deviating from the optimal catalytic ionization and thus leading to decreased activity of the enzyme. The curves show the optimum pH's for the fungal and bacterial amylases between 5.5–6.0 and 6.0–7.0, respectively. These values are in agreement with the optimum pH values found for amylase from germinated sorghum starch and amylase from the cover of germinated sorghum (Iturrios & Ma. I., 1987); malt amylase (Bernfelds, 1955); amylase of *Bacillus licheniformes* (Lumdubwong & Seib, 2001); amylase from *Bacillus subtilis*

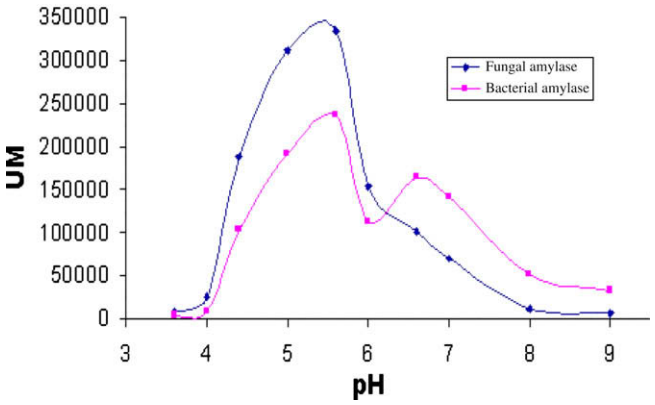

Fig. 1. Effect of pH on the rate of sorghum Montecillo 2 catalyzed by AOA and IBA α-amylases. Experiments were performed at 35 °C according to the procedure described in the text. Initial substrate concentration, 1 mg/ml; enzyme concentrations, 1.5 (fungal) and 3 μg/ml (bacterial); and $t = 5$ min.

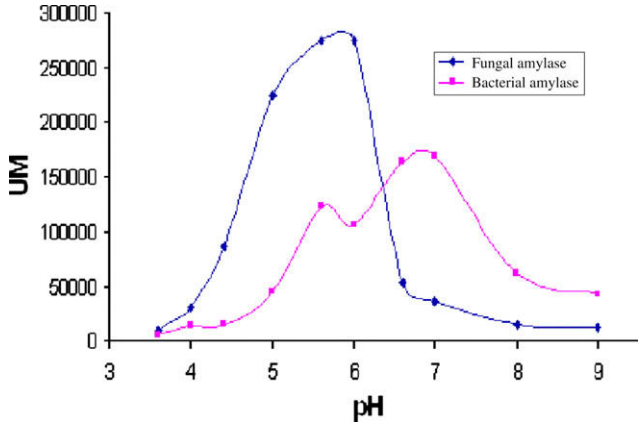

Fig. 2. Effect of pH on the rate of sorghum Montecillo 3 catalyzed by AOA and IBA α-amylases. Experiments were performed at 35 °C according to the procedure described in the text. Initial substrate concentration, 1 mg/ml; enzyme concentrations, 1.5 (fungal) and 3 μg/ml (bacterial); and $t = 5$ min.

(Whitaker, 1994) with values of 6.5–7.0, 6.5–7.5, 5.0–6.0, 5.0–7.0 and 5.8–6.0, respectively.

The values of the kinetic parameters obtained for each of the IBA and AOA α-amylases acting on sorghum (Montecillo 2 and 3) hybrids starches are shown in Table 1. A typical double-reciprocal plot for the determination of kinetic parameters is shown in Figs. 3 and 4. IBA α-amylase had the lowest K_m for sorghum Montecillo 3 hybrid, whereas AOA α-amylases had the lowest K_m for sorghum Montecillo 2 hybrid. This result suggests that among the two amylases compared, both IBA and AOA α-amylases had slightly high affinity for soluble starch of sorghum Montecillo 3 and 1 hybrids, respectively.

α-Amylase has been studied extensively from various aspects: structure and function, secretion, and industrial application. The three-dimensional X-ray structures have been reported for α-amylases from *A. oryzae*, (AOA) (Swift et al., 1991), *Aspergillus niger* (Brady et al.,1991), and *B. licheniformis* (Machius, Vértesy, Huber,

Table 1
Kinetic parameters of IBA and AOA α-amylases acting on sorghum (Montecillo 2 and 3) hybrids starches

Grain sample	Bacterial amylase, IBA		Fungal amylase, AOA	
	K_m (mg/ml)	V_{max} (U/min)	K_m (mg/ml)	V_{max} (U/min)
Montecillo 2	1.5	5	0.12	2
Montecillo 3	1.0	5	0.14	2

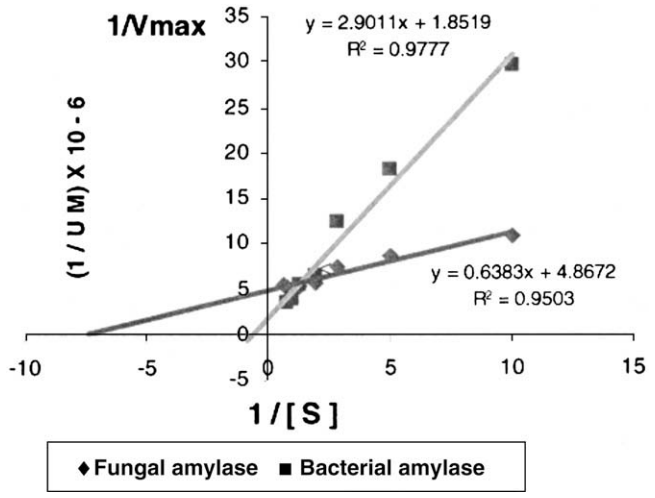

Fig. 3. A typical Lineweaver–Burk plot of the activity of the activity of IBA and AOA α-amylases acting on sorghum Montecillo 2 hybrid starch.

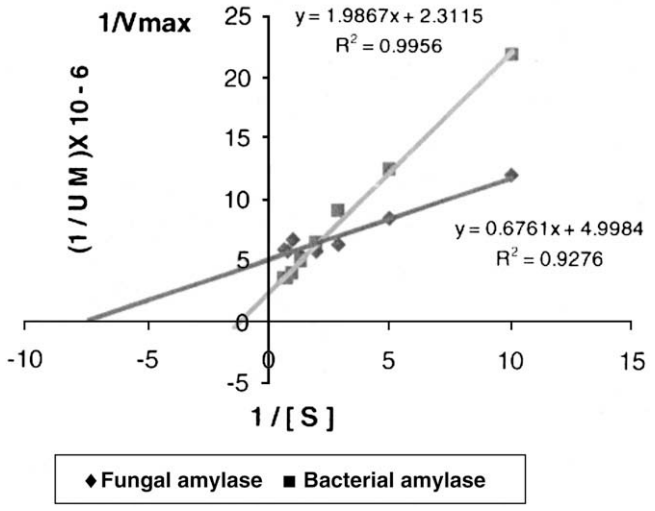

Fig. 4. A typical Lineweaver–Burk plot of the activity of the activity of IBA and AOA α-amylases acting on sorghum Montecillo 3 hybrid starch.

& Wiegand, 1995). These studies have provided the overall fold of α-amylases. Despite differences in their amino acid sequences, they have similar three-dimensional structures with three domains: domain A consisting of a central $(\beta\alpha)_8$-barrel flanking the active site, domain B overlaying the active site from one side and domain C consisting of a β-structure with a Greek–Key motif or forming a "beta sandwich". However, the identification and roles of the catalytic residues of α-amylase have been under long debate. α-Amylases have conserved two aspartic acids and one glutamic acid, which are now considered the catalytic residues. Site-directed mutagenesis experiments on the presumed catalytic residues showed that the substitution of any of these residues caused almost complete loss of activity and suggested that all the three residues were important for activity (Søgaard, Kadziola, Haser, & Svensson, 1993).

Once the three-dimensional structure of an enzyme is known, then the dependence between measurable properties of an enzyme can be exploited, namely the pK_a values of its ionizable residues directly involved in the catalytic region. The pK_a values are a measure of the chemical reactivity of ionizable residues and determine the pH-dependence of the rate for many enzymes. The pK_a value of a given residue depends on the local protein geometry surrounding it (Li, Robertson, & Jensen, 2004). Thus, the measurement of pK_a values can be predicted based on the protein structure in a matter of seconds using the program PROPKA (Li, Robertson, & Jensen, 2005). The pK_a values predicted for the conserved three active site residues: two aspartic acids and one glutamic acid for AOA α-amylase (TAKA AMYLASE, Protein Data Bank Code 6TAA): Asp206, Glu230, Asp297 and for bacterial α-amylase (*Alteromonas haloplanctis bacterium* α-amylase, Protein Data Bank Code 1BLI): Asp231, Glu261, Asp328 are 1.79, 9.71, 4.82: 6.05, 3.71, 10.01, respectively. The AOA α-amylase (TAKA AMYLASE) contains a Ca^{2+} cation. The presence of this cation modifies ionization environment at the catalytic site. The average pK_a values involving the triad active sites residues for AOA and *A. haloplanctis bacterium* α-amylases are 5.44 and 6.59. From the pH-activity profile of AOA and bacterial α-amylases (IBA) using sorghum (Montecillo 2 and 3) hybrids starches as substrates, the pK_a's values were determined using the Dixon–Webb method (Whitaker, 1994). The pK_a values determined for AOA and IBA α-amylases using sorghum (Montecillo 2 and 3) hybrids starches as substrates are 4.75, 6.4; 4.95, 6.3 with average pK_a values of 5.58 and 5.63; and 4.8, 7.4; 5.0, 7.6 with average pK_a values of 6.1 and 6.3, respectively. These average pK_a values are in good agreement with the predicted values of 5.44 and 6.59 based on the protein structure using the program PROPKA (Li et al., 2005). The above pK_a average values are in good agreement with the predicted values.

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