Experimental Approach To Optimize the Use of α-Amylases in Breadmaking

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α-Amylases from different origins (wheat, malted barley, fungi, and bacteria) are used extensively to improve breadmaking. However, the enzyme activities, in addition to the differences associated with their origins, are strongly affected by the process conditions and the presence of other compounds in the medium. The activity of different α-amylases was tested under different conditions (pH and temperature), and in the presence of some bread ingredients (salt and sugar), some breadmaking additives (ascorbic acid and sodium propionate), and some metabolites (organic acids and saccharides) generated during the fermentation step, to envisage the behavior of these α-amylases during the breadmaking process. The α-amylase activities were affected to a different extent by the addition of these compounds depending on the enzyme origin. In general, the α-amylases from cereals (wheat and malted barley) were less sensitive to the presence of some ingredients, additives, and metabolites. These results show the great variation of the α-amylase activity with the process conditions and the importance of its knowledge in the selection of the appropriate α-amylase for a specific breadmaking process.

Keywords: *α-Amylases; additives; activity; stability; process conditions; breadmaking*

INTRODUCTION

Nowadays, the use of enzymes has become a common practice in the baking industry, because enzymes can promote effects similar to those of chemical additives but with the advantage of being considered as natural additives by regulators (*1*). The most extensively used enzymes are amylases, lipases, hemicellulases, pentosanases, proteases, and oxidases, etc. (*2*). These enzymes catalyze the hydrolysis of starch, nonstarch carbohydrates, lipids, and proteins, and also catalyze the oxidation of the former compounds. Nevertheless, the α-amylases are the enzymes most frequently utilized in bakeries, because of their increase of bread volume; improvement of crumb grain, crust, and crumb color; and flavor development promoted on the final product (*3*−*6*). Lately, it has been demonstrated that the anti-staling effect of the α-amylases is due to their ability to retard the amylopectin retrogradation during bread storage (*7*−*12*). However, only the bacterial α-amylases of intermediate thermal stability have been confirmed to be useful anti-staling additives by retarding the starch retrogradation (*13*−*15*).

The α-amylase is an endo-enzyme that randomly hydrolyzes the α-1,4 glucosidic linkages in polysaccharides, resulting in short chains further fermented by yeast. However, they can hydrolyze only damaged or gelatinized starch, because they are more susceptible to the enzyme attack. Besides being necessary for fermentation, the polysaccharides obtained from the hydrolytic activity also participate in the Maillard reactions that take place during baking.

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α-Amylases occur naturally in wheat flour but sometimes the endogenous activity is not sufficient to yield fermentable sugars, consequently, flours are frequently supplemented with exogenous α-amylases. Commercial α-amylases can be obtained from fungal, cereal, or microbial sources. They have different thermal stabilities: fungal α-amylase is the most labile, followed by those from cereal, and the most stable are the α-amylases from bacterial sources (*16*, *17*).

Enzymes are proteins characterized by their catalytic activity, high selectivity, and specificity. Nonetheless, their activity is greatly dependent on environmental conditions, i.e., medium conditions (pH, temperature, water activity, and ionic strength) and the presence of different molecules that could modify their catalytic center (*7*, *18*−*20*). Furthermore, some differences are associated with their origin (*21*, *22*). In fact, it is widely known that the activity and thermal stability of fungal, cereal, and microbial α-amylases are pH-dependent. In baking there are enormous variations depending on (i) flour quality, (ii) ingredients used, (iii) presence of additives, (iv) the process followed (sponge, straight dough, or sourdough), and (v) process conditions (pH, temperature, and relative humidity). Therefore, it is necessary to have a thorough knowledge of the enzyme properties for their efficient application; in this case it could be supposed that the α-amylase activity will vary according to both the process conditions and the enzyme origin. In consequence, the enzyme amount supplemented in relation to that of the flour should be optimized depending on the above-cited factors, because not doing so could result in under-dosage or over-dosage in the process.

The objective of this study was to give some information that allows optimized use of α-amylases from

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different origins. A systematic study of the influence of some common ingredients, additives, and process conditions (pH and temperature) on the hydrolytic activity of various α-amylases from different sources will be presented.

In addition, this study was complemented with the analysis of the effect of different metabolites, generated during the fermentation stage, on the α-amylase activity.

MATERIALS AND METHODS

A commercial blend of Spanish wheat flour (12.48% protein) from the local market was used to extract the endogenous wheat flour α-amylase. Various α-amylases of different sources, gifts from several companies, were also used. Fungal α-amylase, Fungamyl 1500 BG (Novo Nordisk, Bioindustrial, Spain) hereafter referred to as fungal; microbial α-amylase of intermediate thermostability, Novamyl 1500MG (Novo Nordisk, Bioindustrial, Spain) referred to as bacterial; and α-amylase from malted barley were tested. The cereal α-amylase assay reagent (from Megazyme International Ireland Ltd.) was used to measure the α-amylase activity. Other chemical reagents were analytical grade.

Extraction of α-Amylase from Both Wheat and Malt Flours. Extracts were prepared by homogenizing 10 g of wheat flour or malted flour in 50 mL of water, using a Virtis homogenizer (3 × 10 s strokes at 20000 rpm). The homogenate was centrifuged (12000 rpm, 15 min, 15 °C) and the supernatant was filtered throughout glass wool. The clear extract was kept at 4 °C for further enzyme assays. Previous assays confirmed the stability of the α-amylase at 4 °C during the period of storage. The extract obtained from wheat flour was referred to hereafter as the control, because the activities of α-amylases from different origins were compared to that of the wheat flour endogenous α-amylase.

Determination of α-Amylase Activity. The α-amylase activity was measured by using a blocked *p*-nitrophenyl maltoheptaosido (BPNPG7) as substrate following the method reported by McCleary and Sheenan (*23*) and further adapted to a microplate reader by Sirou et al. (*24*). Briefly, the substrate reagent (30 μL) and then the enzyme extract (30 μL) were pipetted into individual wells of a 96-well microplate, the enzyme reaction proceeded for 15 min at 30 °C, at that time 150 μL of 1% Trizma base solution was added to stop the reaction. In the case of the fungal and microbial α-amylases, the enzyme extracts were easily prepared by dissolving the commercial powder in distilled water.

The pH rise led to the phenoxide colored form of *p*-nitrophenol, which was measured at 405 nm by using a microplate reader (Spectramax 190, Molecular Devices). One unit of α-amylase activity was defined as the amount of enzyme which releases 1 μmole of *p*-nitrophenol per minute under the defined assay conditions. In all cases four replicates were assayed for each experimental point. In the activity assays, the enzyme amount used was varied depending on the source in order to measure comparable activities.

Effect of Some Ingredients and the Breadmaking Process on the α-Amylase Activity. The α-amylase activity was analyzed by measuring the enzyme activity in the range of temperature and pH usually found in the bread-making process. The influence of sugar, salt, and different additives (ascorbic acid, sodium propionate) on α-amylase activity was determined by addition of those compounds to the enzyme reaction medium, testing several final concentrations in the range customarily utilized in bread formulation.

Different carbohydrates (glucose, fructose, and maltose) and organic acids (lactic and acetic), the most common metabolites originated throughout the fermentation stage, were also added to the enzyme reaction medium in order to assess their effect on the α-amylase activity.

RESULTS AND DISCUSSION

Effect of pH and Temperature on the Activity of Different α-Amylases. The enzyme activities of various α-amylases from different sources (cereal, fungal, and bacterial) were analyzed at various pH levels and temperatures usually found through the breadmaking process. In this study a short synthetic substrate was used to measure the enzyme activity, even though it is not the natural substrate, because it is more sensitive to any modification of the enzyme conformation; also, it is a specific substrate of α-amylase, which means that it is not hydrolyzed by β-amylases. The effect of pH on the α-amylase activities is shown in Figure 1. The pH affected the four α-amylases tested in different ways. The greatest activities were observed at pHs higher than 4.5; below that a marked drop of activity was displayed.

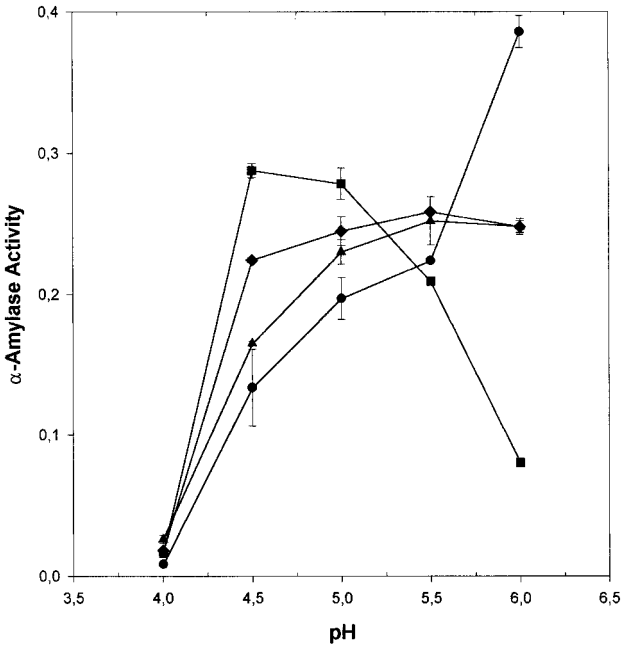

Figure 1. Effect of pH on the α-amylase activities from different sources. Symbols: ♦, control; ■, malted flour; ▲, fungal; ●, bacterial.

The fungal α-amylase and the endogenous one followed a similar trend, keeping their activity constant at pHs higher than 4.5. Different behavior was observed with α-amylases from malted flour and bacteria: the first showed a bell-shaped activity curve reaching a maximum at pH 4.5−5.0, whereas the bacterial type displayed a continuous increase of activity as the pH increased, until the pH reached 6.0.

Several reports described the inactivation temperatures of α-amylases from different sources (*8*, *17*), but there is scarce information about the activity of these enzymes at the temperatures usually employed through the breadmaking process. In Figure 2, the influence of the temperature on the hydrolytic activities of various α-amylases are shown. As expected, the enzyme activity increased with the temperature, but the trend was different depending on the enzyme origin: the bacterial α-amylase showed the highest increase with the temperature. It is known that the bacterial α-amylase of intermediate thermal stability also exhibits the highest thermal stability (*8*), therefore it will be the enzyme with greatest activity during both the fermentation and initial baking steps.

Effect of Some Bakery Ingredients on α-Amylase Activity. There are numerous formulations used in the breadmaking process depending on the type of bread desired. However, there are some ingredients usually

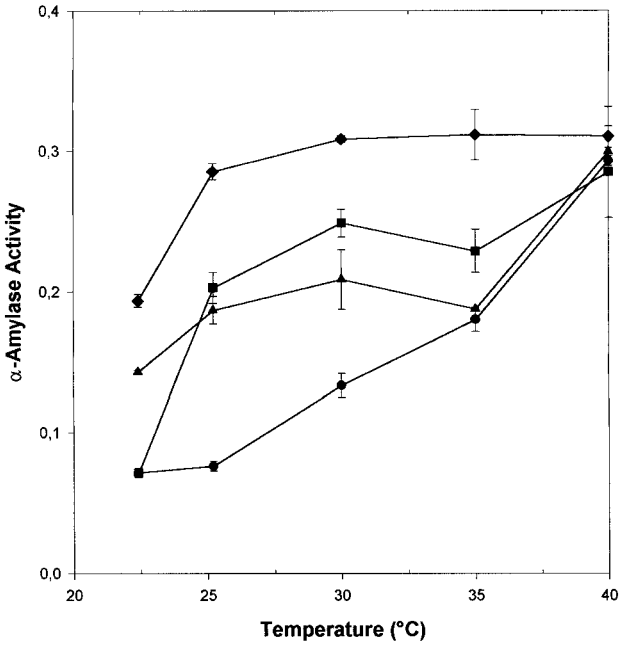

Figure 2. Influence of temperature on various α-amylases from diverse origins. Symbols: ♦, control; ■, malted flour; ▲, fungal; ●, bacterial.

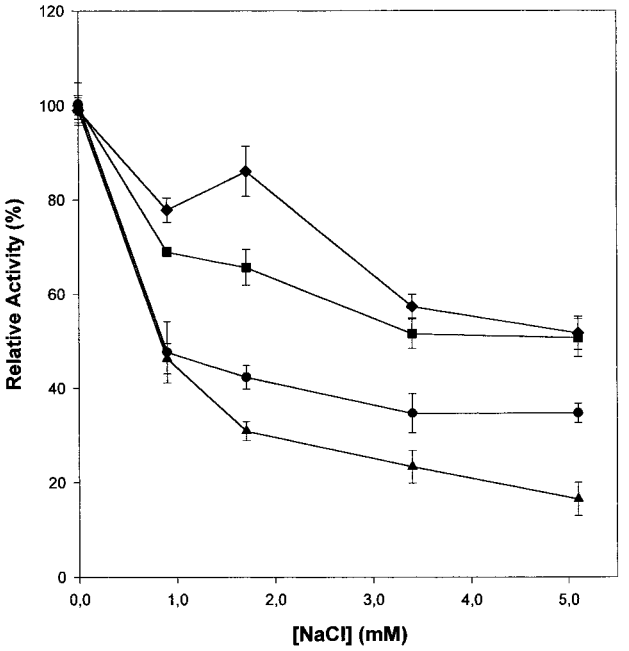

Figure 3. Effect of sodium chloride on the activity of various α-amylases. Symbols: ♦, control; ■, malted flour; ▲, fungal; ●, bacterial.

employed in baking such as salt or sugar. These ingredients could affect the activity of the endogenous α-amylase together with the α-amylase supplemented as a commercial additive. The direct effects of sodium chloride and sucrose on the α-amylase activity was assayed at the different concentrations usually utilized in baking. The addition of salt promoted a decrease of the α-amylase activity in the range of salt assayed, retaining only 50% of its activity at 5 mM NaCl (Figure 3). This inhibition agrees with previous results reported by Harinder and Bains (*25*) showing the increased rate of the falling activity number as the salt concentration increased from 0 to 1.88%; in fact, they proposed the use of salt as a way to control the enzyme activity in high α-amylase flours in order to improve the loaf quality when using sprouted wheat in bakery (*26*, *27*).

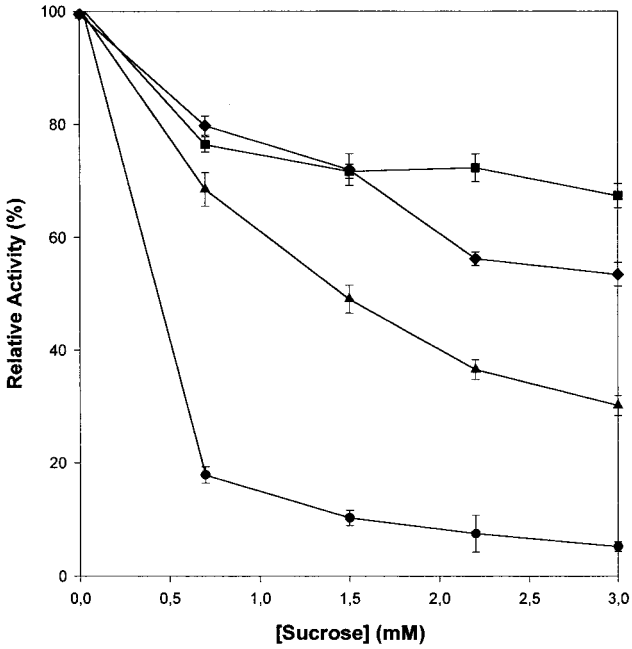

Figure 4. Effect of sucrose on the activity of some α-amylases. Symbols: ♦, control; ■, malted flour; ▲, fungal; ●, bacterial.

However, when the activities of α-amylases from different origins were compared, we observed a diverse grade of inhibition. The α-amylases from cereals were less inhibited by salt than those from fungi or bacteria. Only 40% of the enzyme activity in the case of bacterial α-amylase, or less than 20% in fungal α-amylase, were exhibited at 5 mM salt. In consequence, when doughs were supplemented with fungal or bacterial α-amylases this inhibition should be considered in order to adjust the enzyme dosages.

Sucrose is another ingredient frequently used in baking. Sucrose provides sweet taste, furnishes fermentable sugars to yeast, confers color to bread crust, and improves the texture. The effect of the presence of sucrose in the reaction media on the α-amylase activity has also been analyzed (Figure 4). The α-amylase activity showed a continuous decrease with increasing concentrations of sucrose. However, again, α-amylases from cereals were inhibited in less proportion than α-amylases from fungi or bacteria. The endogenous and malted flour α-amylases exhibited 52% and 69% of their activity at 3.0 mM sucrose, respectively; whereas fungal α-amylase retained 30% of its activity at the same concentration. Bacterial α-amylase was drastically inhibited by sucrose, as less than 15% of its activity remained at 1.0 mM sucrose. This behavior was already envisaged by Adams (*18*), who reported the inhibition promoted by the addition of growing concentrations of sucrose on the α-amylase of *Aspergillus oryzae*.

Influence of Some Bakery Additives on α-Amylase Activities from Different Sources. Ascorbic acid is an oxidant usually added in breadmaking to strengthen dough through the process (for detailed information see Grosch's review; *28*). The presence of growing concentrations of ascorbic acid hardly affected the α-amylase activity, with the exception of the α-amylase from malted flour, which showed a pronounced increase of its activity as the ascorbic acid concentration was augmented (Figure 5). This behavior was explained by

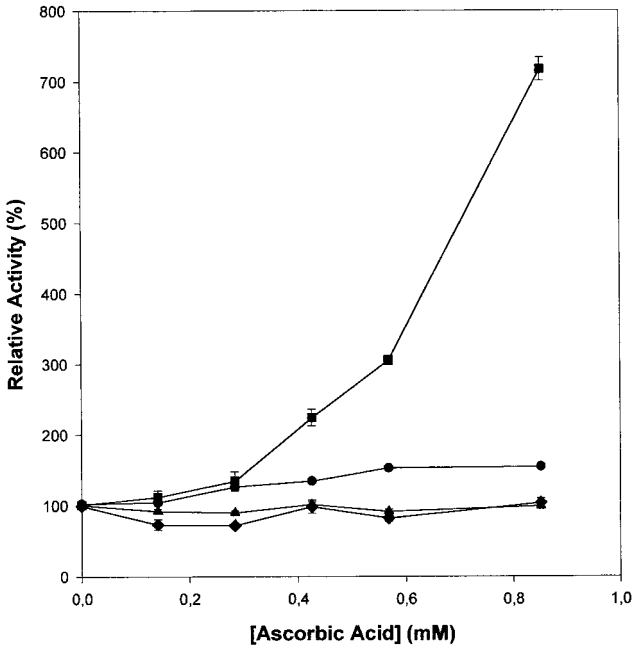

Figure 5. Effect of ascorbic acid on the activity of some α-amylases from various sources. Symbols: ♦, control; ■, malted flour; ▲, fungal; ●, bacterial.

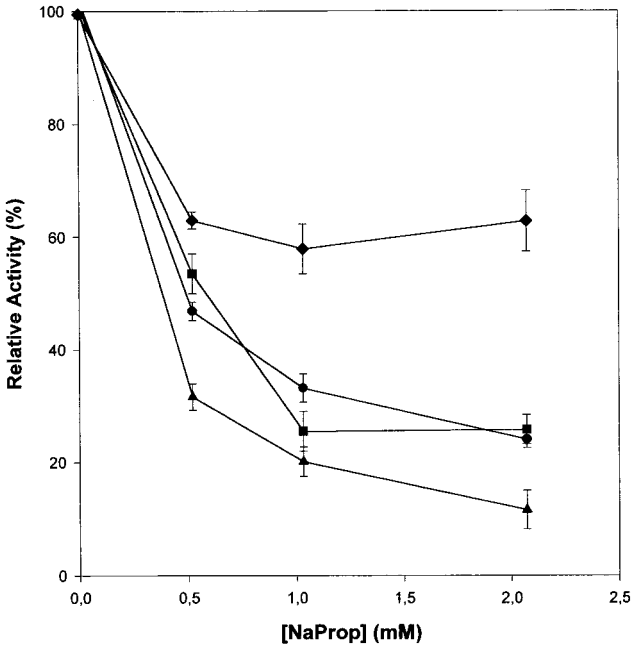

Figure 6. Influence of sodium propionate on the α-amylase activities from different origins. Symbols: ♦, control; ■, malted flour; ▲, fungal; ●, bacterial.

the effect of ascorbic acid on lowering the reaction medium pH, moving that to the malted flour α-amylase pH optimum, because the reaction medium was not buffered (results not shown). These results are in agreement with those previously reported by Purr (*29*), who found that ascorbic acid inhibited *β*-amylases but did not affect the α-amylases of plants.

In addition, other additives commonly used in baking include anti-microbial agents such as different salts of propionic acid. The effect of various concentrations of sodium propionate (NaProp) on α-amylase activity was also investigated (Figure 6). An inhibitory effect of the enzyme activity should be expected because the salts of propionic acid are used as anti-microbial agents, but

Table 1. Effect of Different Acids and Sugars on α-amylase Activities from Various Sources

	conc. (*μ*M)	α-amylase activity (%)[a]			
		control	malted flour	fungal	bacterial
acetic acid	0	100.0 ± 0.0	100.0 ± 2.0	100.0 ± 0.2	100.0 ± 12.7
	40	122.8 ± 0.8	161.6 ± 1.6	133.1 ± 2.5	196.1 ± 12.9
	80	122.2 ± 1.6	206.9 ± 9.1	138.7 ± 2.6	214.5 ± 4.9
	125	121.9 ± 2.7	224.1 ± 2.8	134.1 ± 0.6	205.7 ± 13.7
lactic acid	25	105.4 ± 7.6	154.3 ± 6.9	106.4 ± 3.7	103.1 ± 9.9
	50	102.9 ± 2.9	182.2 ± 4.1	111.7 ± 1.9	84.9 ± 2.9
	80	107.5 ± 0.4	230.0 ± 0.8	109.6 ± 0.8	112.7 ± 0.1
glucose	6	105.0 ± 1.6	103.9 ± 1.9	104.4 ± 0.5	92.5 ± 2.9
	10	100.8 ± 0.4	107.4 ± 2.0	90.1 ± 5.3	94.8 ± 5.3
	20	100.0 ± 0.0	123.5 ± 2.5	110 ± 2.6	109.3 ± 3.3
fructose	8	106.4 ± 6.7	142.1 ± 6.1	115.5 ± 3.6	110.6 ± 4.3
	17	105.6 ± 0.8	117.6 ± 5.5	109.0 ± 0.3	103.3 ± 3.1
	25	101.4 ± 5.9	128.5 ± 4.9	111.0 ± 1.4	98.3 ± 5.7
	33	97.8 ± 1.6	96.8 ± 6.0	105.0 ± 2.4	104.2 ± 5.9
maltose	15	104.2 ± 1.2	159.0 ± 4.7	120.7 ± 3.0	111.9 ± 9.8
	30	105.3 ± 1.2	145.2 ± 3.2	114.4 ± 0.1	112.0 ± 2.7
	60	98.1 ± 2.0	158.3 ± 0.3	115.3 ± 4.2	100.3 ± 9.0
	90	101.9 ± 2.3	193.6 ± 5.2	129.5 ± 0.3	108.7 ± 9.0

[a] The α-amylase activity was referenced to the activity of the control (α-amylase activity of the wheat flour). The values are means of four replicates ± SD.

it is not known how these salts affect different α-amylases from various sources. The addition of sodium propionate inhibited the α-amylase activity but to differing extents depending on the enzyme origin. The endogenous α-amylase was the most stable, retaining more than 50% of its activity at 2.0 mM NaProp. A drastic inhibition by this salt was observed with the other α-amylases, with the fungal α-amylase being the most affected (retaining only 9% of its activity at 2.0 mM NaProp).

Effect of Different Metabolites Generated during Breadmaking on the α-Amylase Activity. To determine the possible effect of some compounds generated through the fermentation stage, two acids (lactic and acetic) and various saccharides (maltose, fructose, and glucose) were selected as the most representative metabolites. The effect of these compounds on the activity of the different α-amylases tested can be observed in Table 1. Selection of the range of concentrations to be assayed was based upon the previous results reported by Barber et al. (*30*), who analyzed the acid compositions in several doughs prepared by using sourdough. The presence of acids led to an increase of the α-amylase activity, but large differences were observed regarding both the acid added and the α-amylase origin. Acetic acid yielded an increase of the α-amylase activity, with the α-amylases from malted flour and bacteria showing the highest increase. This effect was mainly attributed to its presence rather than to its concentration, because only slight variations were observed at increasing concentrations of acetic acid. The unique exception to this behavior was the α-amylase from malted barley flour, which showed higher activity at growing acid concentrations; this result was likely related to approaching its optimum pH (as was observed with the addition of ascorbic acid).

The effect of lactic acid was less pronounced than that of the acetic acid. A slight enhancement of the α-amylase activity was observed, except for the α-amylase from malted barley flour, which showed a steady increase of activity with the acid concentration, also explained by the effect of the acid upon the pH.

With respect to the sugars influence, the addition of the sugars tested did not modify the activities of the α-amylases from wheat flour (control) and the bacterial α-amylase, however higher activities resulted when the α-amylases were from malted flour and fungi. Among the sugars studied, maltose produced the highest enzyme activation.

CONCLUSION

From the above results it could be concluded that, although enzymes are really useful in food technology, prior to selecting a specific enzyme it is necessary to have thorough knowledge of its properties and also of the environmental conditions under which it is going to be added. Currently, α-amylases commonly employed in baking are selected as a function of their thermostability, but as has been exposed through this study, all the compounds present and the medium conditions will affect the final enzyme activity. In consequence, thorough knowledge about the system where the enzyme will act is necessary before selecting its source.

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