

UDC 557.152.321:663.316  
ISSN 1330–9862

*original scientific paper*

(FTB-1100)

## Amylase for Apple Juice Processing: Effects of pH, Heat, and Ca<sup>2+</sup> Ions

*Liliana N. Ceci and Jorge E. Lozano\**

PLAPIQUI (UNS-CONICET) Camino La Carrindanga Km.7, C.C. 717  
8000 Bahía Blanca – Argentina

Received: July 4, 2001

Accepted: November 8, 2001

### Summary

The aim of this paper was to evaluate the effects of pH, heat, and Ca<sup>2+</sup> ions on the  $\alpha$ -amylase activities in a commercial amylolytic enzyme (Tyazyme L300), used for apple juice processing. Kinetics of thermal inactivation was studied in acetate and citrate/phosphate buffers at different temperatures (55–70 °C) and enzyme concentrations (0.276 and 0.552 mL/100 mL). Maximum  $\alpha$ -amylase activity was observed at pH=3.4 in both buffers. Effects of the addition of calcium chloride during and after thermal treatments were also investigated.  $\alpha$ -amylase activities were measured by an iodometric method and thermal inactivation constants and *D* values (time for reducing 90 % of the enzymatic activity) were estimated. The enzyme was more sensible to pH changes and heat when citrate ions were present in the reaction medium. If Ca<sup>2+</sup> in the enzyme structure is bound to citrate then the resistance of the enzyme to pH changes and heat is lowered. Kinetics obtained according to Arrhenius equation and two enzymatic fractions (thermo-labile and thermo-resistant) were observed too. In citrate buffer the following relation was observed for thermo-labile fraction:  $\log(D \text{ value}) = -0.144 t/^\circ\text{C} + 12.992$ . The level of thermal inactivation also depended on the enzyme concentration. Higher thermal inactivation rates were obtained by increasing the enzyme concentration in the case when citrate was present. It was also found that the addition of calcium chloride (1 g/L) after thermal treatment in media containing citrate reactivated the enzyme treated at 60 and 65 °C. The possible implications of these findings in apple juice processing were discussed.

*Key words:*  $\alpha$ -amylase, apple juice, starch hydrolysis

### Introduction

Apple juice may have a high content of starch, when processed from some apple varieties or unripe fruits at the beginning of the season (1). High insoluble starch concentrations (7.68 g/L) were detected in pressed »Granny Smith« apple juice from unripe fruits harvested in »Río Negro« Valley region (Argentina), two weeks before the usual harvest. Soluble starch was also present in these cloudy juices but in significantly lower concentrations (0.51 g/L). The starch contents of pressed

cloudy juices were gradually lower when unripe fruits were stored at room temperature and soluble and insoluble starch were not detected three weeks after the harvest. Insoluble starch was gelatinized when cloudy juices were pasteurized.

Polymeric carbohydrates, as starch, may cause slow filtration, membrane fouling, jellying after concentration or post-process cloudiness. However, starch may be easily detected and treated by adding starch-degrading en-

\* Corresponding author; Phone: +54 291 486 1700; Fax: +54 291 486 1600; E-mail: jlozano@plapiqui.edu.ar

zymes together with pectinase during depectinization of fruit juices (2,3). In a previous work we detected exo- and endo-amylase activities in a commercial enzyme (Tyazyme L300) used for apple juice processing (4).

In recent years, the structure of fungal  $\alpha$ -amylase, a typical starch hydrolyzing endo-enzyme, has been studied. The proposed structure consisted of three domains: A or catalytic, B and C (5). Some microbial  $\alpha$ -amylases have in their carboxyl-terminal regions an extra domain (domain E) that is known to play a role in raw starch adsorption. Domain B is stabilized by  $\text{Ca}^{2+}$  ions (6,7). Moreover,  $\text{Ca}^{2+}$  is typically needed for the activity and stability of these  $\alpha$ -amylases (7). The inactivation of these enzymes in aqueous solutions was assumed to take place in two-stages (8). The first stage involves a reaction between the active enzyme and  $\text{Ca}^{2+}$  ions to produce an inactive apoenzyme. This stage is reversible and the active enzyme can be recovered by the addition of  $\text{Ca}^{2+}$  ions to the reaction medium. During the second irreversible stage, a denaturated enzyme which cannot be readily reactivated by adding  $\text{Ca}^{2+}$  ions, is formed. When a  $\text{Ca}^{2+}$ -chelating ligand (EDTA) is present, the first stage is accelerated. If the ions have the capacity to penetrate in the structure of the enzyme, and the structure has not been largely damaged, the enzyme could be reactivated by addition of  $\text{Ca}^{2+}$  ions (6).

Food and specially fruit juices are complex systems that have  $\text{Ca}^{2+}$  ions and probably  $\text{Ca}^{2+}$  sequestering agents as organic acids. These compounds can affect the action of amylases used for processing and its thermal inactivation.

This work was aimed to evaluate the effects of temperature and pH on the activity of a commercial amylase (Tyazyme L300) used for apple juice processing on soluble starch solution. The influence of citrate ions in the reaction mixture and the addition of  $\text{Ca}^{2+}$  ions to stabilize the enzyme were also analyzed.

## Materials and Methods

Liquid amylolytic Tyazyme L300 enzyme (Solvay, Argentine) and soluble starch from Química Bonaerense (Argentine) were used. Buffers used were 0.1 M sodium acetate/acetic acid (pH=3.4–5.6) and 0.1 M citrate /  $\text{Na}_2\text{HPO}_4$  (pH=2.6–7.0). All reagents were of analytical grade and used without further purification. Enzymatic activities were measured with a Perkin Elmer Lambda 3 spectrophotometer in quartz cells (path 1.0 cm).

### Enzymatic activity determinations

Iodometric method was used to determine the endo-amylase activities (6). The starch solution (1.5 g/L) was made by dissolving starch in 2 M NaOH solution at room temperature, followed by the addition of an equal volume of 2 M HCl. The resulting solution was then heated to a clear solution before its pH was adjusted to desired value by addition of 2 M NaOH. Final solutions were made by dilution with buffer at the desired pH. A solution (126.9 mg/L), containing the same volume of 0.1 M iodine and 5 g/100 mL potassium iodide, was diluted with cold distilled water to obtain a cold iodine solution (around 2 °C).

Activity values were determined at 25 °C by allowing the enzyme solution in buffer (0.5 mL) to react with the starch solution (5 mL) for 2 min. The reaction was then stopped by adding 2.5 mL of cold iodine solution, which converts the non hydrolyzed starch to starch-iodine blue complex. The resulting solution was afterwards placed in a thermostatic bath at 25 °C for 10 min and its absorbance was measured at 615 nm. The amount of the hydrolyzed starch (HS %) was determined as the absorbance value of the hydrolyzed starch, relative to a blank value of non hydrolyzed starch solution, prepared without the addition of the enzyme (6).

### Effect of pH

The enzyme solution was prepared by diluting liquid amylolytic Tyazyme L300 in a buffer. Activity was measured by the above described method. The enzyme concentration in the reaction mixture was 0.025 mL/100 mL. Acetate (pH=3.4–5.6) and citrate/phosphate (pH=2.6–7.0) buffers were used. Enzymatic activities were expressed as relative values (relative HS %), considering 100 % of activity at optimum pH.

### Effects of heat

Thermal treatments were carried out with enzyme solutions placed into thin wall glass containers to ensure a fast heat transfer. The containers were held in an ice bath immediately after the thermal treatment. The samples were then centrifuged (1000 x g, 5 min) to determine residual enzymatic activities in the supernatants.

Enzyme solutions (0.276 mL/100 mL) in both buffers (optimum pH=3.4) were treated at different temperatures (40–85 °C) for 30 min. After thermal treatments, residual enzymatic activities were measured in the supernatants (final enzyme fraction = 0.025 mL/100 mL reaction mixture).

Thermal inactivation kinetics were evaluated using different fraction of enzyme solutions (0.276 and 0.552 mL/100 mL) in citrate/phosphate buffer (pH=3.4) at different temperatures (50–70 °C). Residual enzymatic activities were determined in the supernatants after thermal treatments. Final enzyme fraction in the reaction mixture was 0.025 mL/100 mL. The same enzyme fraction and assay conditions were used to evaluate thermal inactivation kinetics in acetate buffer (pH=3.4).

Arrhenius equation was applied to estimate the thermal inactivation constant ( $k$ ) and the time for reducing 90 % of the enzymatic activity (D value) in all thermal inactivation experiments. Logarithms of relative residual activities vs. time were plotted and  $k$  values were estimated from the slope of the straight lines obtained.

### Effects of calcium chloride addition

Thermal inactivation experiments were carried out under the following conditions: acetate buffer, pH=3.4 (calcium chloride concentrations 0–2 g/L); enzyme fraction 0.276 mL/100 mL and temperature 65 °C. The effects of the addition of calcium chloride solutions in buffer after thermal treatment (final  $\text{CaCl}_2$  concentrations 0–25 g/L) were also studied.

Thermal inactivation kinetics at 60 and 65 °C were evaluated in citrate/phosphate buffer pH=3.4 (enzyme

fraction 0.552 mL/100 mL) with  $\text{CaCl}_2$  solutions in buffer (final  $\text{CaCl}_2$  concentrations 0 and 1 g/L) after thermal treatment. Relative HS (%),  $k$  and  $D$  values were estimated as previously described.

### Statistical analyses

Relative HS (%) values are the average of determinations in triplicate. Grapher 1.09 for Windows software was used to estimate  $k$  and  $D$  values by linear regression.

## Results and Discussion

### pH effects

Maximum enzymatic activity for Tyazyme L300 was observed in both buffers at pH=3.4 (Fig. 1). However, in citrate/phosphate buffer the enzyme was more sensible to pH changes in the reaction medium, showing an optimum pH range 3.4–4.6.

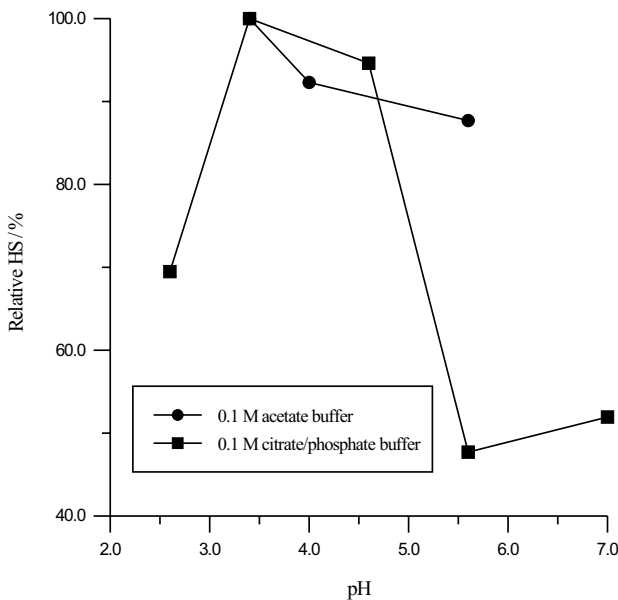


Fig. 1. Effects of pH on the  $\alpha$ -amylase activity

$\text{Ca}^{2+}$  ions could be caught by citrate ions resulting in a higher enzyme sensibility to pH changes. Charges in the catalytic micro-environment of the enzyme may be unbalanced due to the loss of  $\text{Ca}^{2+}$  ions. When the reaction is carried out in acetate buffer, the enzyme could keep its structure undamaged and its resistance to pH changes in the reaction medium. All  $\alpha$ -amylases are calcium-containing enzymes, binding at least one calcium ion per monomeric unit. The calcium ions impart resistance to pH, temperature, proteolysis and denaturation by urea and heat (9).

### Effects of heat

In acetate buffer the enzyme treated at 65 °C during 30 min held back about 80 % of its initial activity. However, the enzyme lost practically 66 % of its maximum

activity when treated in citrate/phosphate buffer (Fig. 2).  $\text{Ca}^{2+}$  ions could be caught by citrate ions shifting forwards the first reversible stage of thermal inactivation.

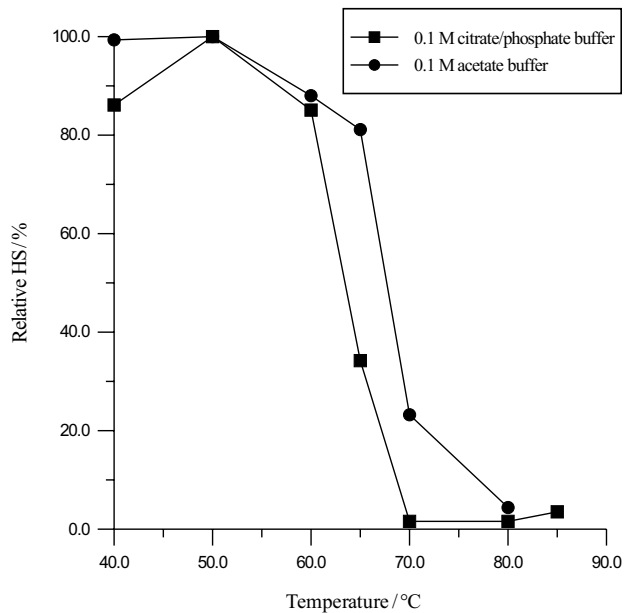


Fig. 2. Residual  $\alpha$ -amylase activities after thermal treatment during 30 min at different temperatures. Enzyme fraction: 0.276 mL/100 mL (thermal treatment) – 0.025 mL/100 mL (residual activity determinations)

Curves in Fig. 2 are typical for fungal  $\alpha$ -amylases whose residual activity decreased from 65 °C and resulted practically in complete inactivation at 75 °C (10).

The obtained results obeyed the Arrhenius equation (Figs. 3–5) but in some experiments two straight lines of different slopes were observed. These results suggested the presence of thermo-labile and thermo-resistant enzymatic fractions in Tyazyme L300. Thermo-labile enzymatic fraction has a major slope in Arrhenius plots. In this case,  $k$  and  $D$  values shown in Fig. 3 correspond to the thermo-labile enzymatic fraction.

$D$  and  $k$  values calculated for thermo-labile fraction at 65 °C, with citrate ions in the reaction medium, were 268 min and 0.0258  $\text{min}^{-1}$  [ $R^2=0.956$ ], respectively. The following relationship applied:  $\log D$  (min) =  $-0.144539 t/^\circ\text{C} + 12.9916$  [ $R^2=0.978$ ] (Fig. 3).

Fungal  $\alpha$ -amylases from some genera as *Aspergillus* and *Thermomyces* were stable after treatment at 50–55 °C during 7–10 h (11,12). Bacterial  $\alpha$ -amylases from microorganisms of *Bacillus* genera, used for industrial processing of starch, are more thermo-resistant than fungal ones.  $\alpha$ -amylase from *Bacillus amyloliquefaciens* has a half-life of denaturation of 17.8 min at 82 °C (13).  $\alpha$ -amylase in Tyazyme L300 has the half-life of denaturation (22 min) similar to the enzyme from *Bacillus amyloliquefaciens*, but at much lower temperature (70 °C). The half-lives of denaturation at 80 °C for  $\alpha$ -amylases from *Bacillus licheniformis* (A and G amylases) were 20 and 25 min respectively (14).

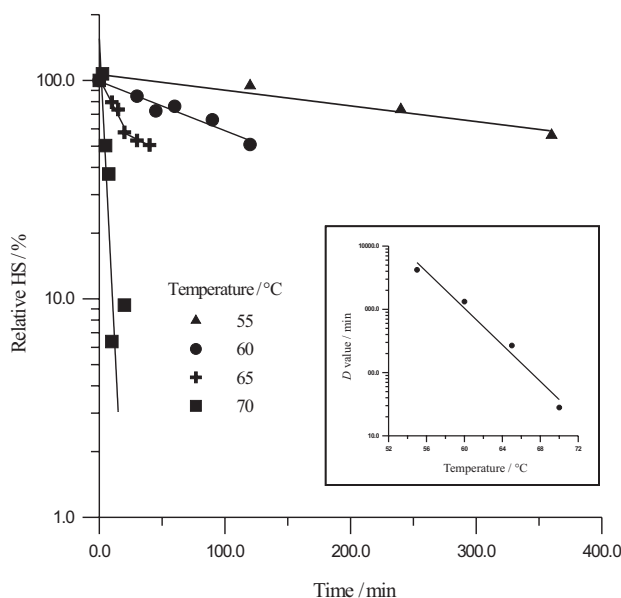


Fig. 3. Thermal inactivation kinetics of Tyazyme L300 in citrate buffer (pH = 3.4). Enzyme fraction: 0.276 mL/100 mL (thermal treatment) – 0.025 mL/100 mL (residual activity determinations)

The inactivation process was also under the influence of the enzyme fraction in the thermally treated solutions (Figs. 4 and 5). In acetate buffer, when the enzyme fraction was increased, the enzyme activity was affected in a lesser degree by thermal treatment.  $D$  value for thermo-labile enzymatic fraction increased from 449 min to 1275 min at 65 °C, when the enzyme fraction in thermally treated solutions was doubled. At 60 °C,  $D$  value increased from 1629 min to 4847 min. These results were similar to those reported by Lecker and Khan

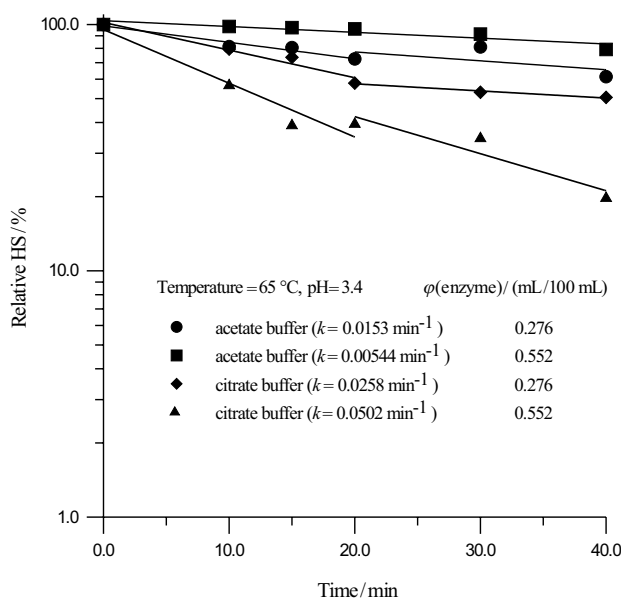


Fig. 4. Effects of enzyme fraction on thermal inactivation of Tyazyme L300 at 65 °C. Enzyme fraction for residual activity determinations: 0.025 mL/100 mL

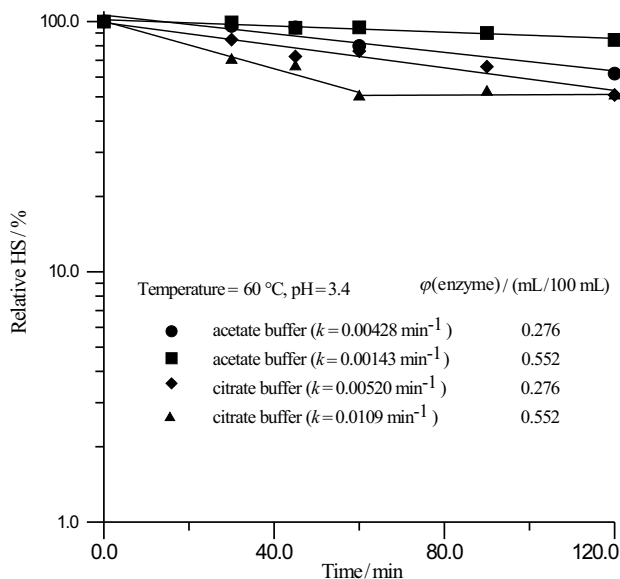


Fig. 5. Effects of enzyme fraction on thermal inactivation of Tyazyme L300 at 60 °C. Enzyme fraction for residual activity determinations: 0.025 mL/100 mL

(6) for  $\alpha$ -amylase from *Bacillus* sp. Diluted enzyme solution was inactivated much more readily than a relatively concentrated solution. A number of enzyme molecules at relatively higher concentrations may form a weak bound cluster, allowing fewer metal ions to be dissociated and hence, lowering the inactivation rate. After dilution, these cluster molecules were gradually separated into component molecules and became more vulnerable to inactivation.

In our experiments with citrate buffer, more concentrated enzyme solutions were more readily inactivated (Figs. 4 and 5). Although  $\text{Ca}^{2+}$  ions were a part of the internal structure of a compact cluster, they could be caught easier by citrate ions making the thermal inactivation. However,  $\alpha$ -amylase from *Bacillus* sp., with EDTA as chelating agent for  $\text{Ca}^{2+}$ , showed a little lower  $k$  value, when it was inactivated in highly concentrated solutions but at the temperature as low as 25 °C (6).

#### Effects of calcium chloride addition

During the thermal process, low concentrations of  $\text{Ca}^{2+}$  ions increased the stability of the studied  $\alpha$ -amylase. When the enzyme solutions in acetate buffer were heated at 65 °C, in presence of 0.05 g/L of  $\text{CaCl}_2$ ,  $D$  value increased for about 30 % and  $k$  values decreased for about 20 % (Table 1). However, the addition of a higher amount of  $\text{CaCl}_2$  induced a decrease in enzymatic thermo-stability, independently of the amount of  $\text{Ca}^{2+}$  added (Table 1).

Addition of  $\text{CaCl}_2$  in any concentration did not practically affect the enzymatic activity (activation <3 %) for non thermally treated enzyme solutions (results not included).

Calcium is known to play an important role in the structure and activity of  $\alpha$ -amylases.  $\text{Ca}^{2+}$  ions are in-

Table 1. Effects of calcium chloride on the thermal inactivation kinetics of  $\alpha$ -amylase in Tyazyme L300

Acetate buffer (pH=3.4), Temperature = 65 °C					
CaCl <sub>2</sub> during thermal treatment <sup>1</sup>			CaCl <sub>2</sub> after thermal treatment <sup>2</sup>		
$\gamma$ (CaCl <sub>2</sub> )	D value	k	$\gamma$ (CaCl <sub>2</sub> )	D value	k
g/L	min	min <sup>-1</sup>	g/L	min	min <sup>-1</sup>
0.00	449	0.0153	0.000	352	0.0197
0.05	579	0.0120	0.025	218	0.0314
1.00	335	0.0207	0.050	300	0.0227
2.00	389	0.0178	1.000	221	0.0314
			25.00	325	0.0212
Citrate/phosphate buffer (pH=3.4) <sup>3</sup>					
Temperature = 60 °C			Temperature = 65 °C		
$\gamma$ (CaCl <sub>2</sub> )	D value	k	$\gamma$ (CaCl <sub>2</sub> )	D value	k
g/L	min	min <sup>-1</sup>	g/L	min	min <sup>-1</sup>
0	633	0.01093	0	137	0.0502
1	1577	0.00437	1	202	0.0342

<sup>1</sup> Enzyme fraction: 0.276 mL/100 mL (thermal treatment) – 0.0250 mL/100 mL reaction mixture (residual activity determination)

<sup>2</sup> Enzyme fraction: 0.276 mL/100 mL (thermal treatment) – 0.0125 mL/100 mL reaction mixture (residual activity determination)

<sup>3</sup> CaCl<sub>2</sub> after thermal treatment. Enzyme fraction: 0.552 mL/100 mL (thermal treatment) – 0.0250 mL/100 mL reaction mixture (residual activity determination)

involved in  $\alpha$ -amylase catalysis and the stability of these enzymes is normally increased by the addition of small amounts of calcium (15–18). The stability arises from the Ca<sup>2+</sup> contribution to the enzyme's tertiary structure, probably by replacing the lack of disulfide bonds. Destabilizing effects of calcium addition at higher concentrations were also reported (8). Some  $\alpha$ -amylases from *Thermophilic Archaea* are activated or non affected by low calcium levels, however, enzymes from other sources were inhibited by high Ca<sup>2+</sup> concentrations (7). As Ca<sup>2+</sup> can bind some catalytic residues,  $\alpha$ -amylase activity may then be inhibited.

The addition of CaCl<sub>2</sub> solutions (final concentrations 0.025–25 g/L) after thermal treatment in acetate buffer did not reactivate the enzyme (Table 1). When Ca<sup>2+</sup> ions were added after thermal treatment,  $\alpha$ -amylase had a tendency towards a lower stability, independently of the amount of Ca<sup>2+</sup> added. It seems that a reactivation process after thermal treatment did not occur by adding Ca<sup>2+</sup>. Results show that the stability demonstrated by the enzyme activity after thermal treatment depended more on how its structure was affected by the treatment than the amount of Ca<sup>2+</sup> added.

When enzyme solutions in citrate buffer were thermally treated, and then CaCl<sub>2</sub> (final concentration 1 g/L) was added,  $\alpha$ -amylase was significantly restabilized (Table 1). Lecker and Khan (6) observed certain restabilization for  $\alpha$ -amylase from *Bacillus* sp. when Ca<sup>2+</sup> ions were added after a thermal treatment in a medium containing EDTA. This re-stabilization was mainly found at temperatures under 40 °C. These findings support the mechanism proposed for  $\alpha$ -amylases thermal inactivation and the reversibility of the first stage of this mechanism.

## Conclusions

Results showed that  $\alpha$ -amylase from Tyazyme L300 was more susceptible to heat and pH changes when Ca<sup>2+</sup> sequestering agents were present in the reaction medium. These agents, such as citrate, are usually present in all fruit juices.

Thermal inactivation level also depended on the enzyme concentration in treated solutions. When Ca<sup>2+</sup> sequestering agents were present in the reaction medium, such as citrate ions in a fruit juice, higher thermal inactivation rates were observed by increasing the enzyme fraction in the treated samples.

A higher  $\alpha$ -amylase thermo-stability would be induced by the calcium content of about 30–120 mg/L, usually present in apple juices, and rarely exceeding 80 mg/L (19). This fact should be considered when the enzymes must be inactivated, after the clarification process.

In recent years many food industries have proposed the use of apple juices fortified with calcium as healthy and non fat alternative especially for children. Concentrations of Ca<sup>2+</sup> ions similar to that observed in fortified apple juice, in a reaction media containing citrate ions, have induced a significant reactivation of the studied  $\alpha$ -amylase. From a practical point of view special attention must be paid to efficiently inactivated amylase activities in clarified juices, if they are planned to be fortified with calcium.

## References

1. G. Reed: Enzyme Inhibition and Activation. In: *Enzymes in Food Processing*, Academic Press, New York (1975).
2. E. A. Grampp, *Fluss. Obst.* 43 (1976) 382–388.
3. D. A. Heatherbell, *Food Tech. in New Zealand*, 5 (1976) 9–11.



4. M. Poncetta, L. N. Ceci, J. E. Lozano, *Abstracts of the 10<sup>th</sup> Latinoamer. & Caribbean Seminar of Food Sci. & Technol.* Buenos Aires, Argentina (1997) pp. 16–11.
5. K. Ohdan, T. Kuriki, H. Kaneko, J. Shimada, T. Takada, Z. Fujimoto, H. Mizuno, S. Okada, *Appl. Environ. Microbiol.* 65 (1999) 4652–4658.
6. Douglas N. Lecker, Arshad Khan, *Biotechnol. Progr.* 12 (1996) 713–717.
7. Emmanuel Lévêque, Stefan Hanecek, Bernard Haye, Abdel Belarbi, *Enzyme Microb. Technol.* 26 (2000) 3–14.
8. L. M. Marchal, J. Jonkers, G. Th. Franke, C. D. de Gooijer, J. Tramper, *Biotechnol. Bioeng.* 62 (1999) 348–357.
9. H. Tümtürk, S. Aksoy, N. Hasirci, *Food Chem.* 68 (2000) 259–266.
10. R. E. Kirk, D. F. Othmer: Enzimas Industriales. In: *Enciclopedia de Tecnología Química*, UTEHA, Mexico (1961).
11. F. Guillen Moreira, F. Arias de Lima, S. R. Fazzano Pedrinho, V. Lenartovicz, C. G. Márquez de Souza, R. M. Peralta, *Revista de Microbiología*, 30 (1999) 157–162.
12. R. S. Mishra, R. Maheshwari, *J. Biosci.* 21 (1996) 653–672.
13. Van Loey, A. Arthawan, M. Hendrickx, T. Haentjens, P. Tobback, *Lebensm. Wiss. Technol.* 30 (1997) 94–100.
14. V. Ivanova, E. Dobрева, M. D. Legoy, *Acta Biotechnol.* 4 (1998) 339–351.
15. K. Igarashi, Y. Hatada, H. Hagihara, K. Saeki, M. Takaiwa, T. Uemura, K. Ara, K. Ozaki, S. Kawai, T. Kobayashi, S. Ito, *Appl. Environ. Microbiol.* 64 (1998) 3282–3289.
16. K. Ikawa, H. Araki, Y. Tsujino, Y. Hayashi, K. Igarashi, Y. Hatada, H. Hagihara, T. Ozawa, K. Ozaki, T. Kobayashi, S. Ito, *Biosci. Biotechnol. Biochem.* 62 (1998) 1720–1725.
17. W. Witt, J. J. Sauter, *J. Exp. Bot.* 47 (1996) 1789–1795.
18. J. R. Wykes, P. Dunnill, M. D. Lilly, *Biochim. Biophys. Acta*, 250 (1971) 522–529.
19. R. Binning, P. Possmann: Apple Juice. In: *Fruit Juice Processing Technology*, S. Nagy, C. S. Chen, P. E. Shaw (Eds.), Agscience Inc., Auburndale, Florida (1993).

## Amilaza za preradbu jabučnog soka: utjecaj pH, topline i kalcijevih iona

### Sažetak

Svrha je rada utvrditi utjecaj pH, topline i kalcijevih iona na aktivnost  $\alpha$ -amilaze u komercijalnom amilolitičkom enzimu (Tyazyme L300) koji se koristi u preradbi jabučnog soka. Proučavana je kinetika toplinske inaktivacije u acetatnom i citratnom/fosfatnom puferu pri raznim temperaturama (55–70 °C) i udjelima enzima (0,276 i 0,552 mL/100 mL). U oba pufera opažena je maksimalna  $\alpha$ -amilazna aktivnost pri pH=3,4. Također je ispitivan utjecaj dodatka kalcijeva klorida tijekom i nakon toplinske obradbe. Aktivnost  $\alpha$ -amilaze mjerena je jodometrijskim postupkom, a određene su i toplinske inaktivacijske konstante te  $D$ -vrijednosti (vrijeme za snizivanje 90 % enzimске aktivnosti). Enzim je bio puno osjetljiviji na promjene pH i na toplinu u prisutnosti citratnih iona u udjelu mediju. Kalcijev ion u strukturi enzima može biti vezan na citrat, a pritom se smanjuje otpornost enzima na promjene pH i utjecaj topline. Razmatrana je kinetika dobivena prema Arrheniusovoj jednadžbi, a opažene su i dvije enzimске frakcije (termolabilna i termootporna). Za termolabilnu frakciju u citratnom puferu dobivena je ova vrijednost:  $\log D = -0,144 t/^\circ\text{C} + 12,992$ . Razina toplinske inaktivacije ovisi i o udjelu enzima. Povećavajući udjel enzima u prisutnosti citrata, povećava se brzina toplinske inaktivacije. Ujedno je nađeno da dodatak kalcijeva klorida (1 g/L), nakon toplinske obradbe u mediju sa citratom, omogućava reaktivaciju enzima obrađenog pri 60 i 65 °C. U raspravi su razmotrene moguće primjene tih rezultata u obradbi jabučnog soka.