KINETIC STUDIES OF AN AMYLASE USED COMMERCIALLY FOR THE CLARIFICATION OF APPLE JUICE

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ABSTRACT

The effect of substrate and enzyme concentrations on the degradation of soluble corn starch with a commercial amylase (Rohalase HT) recommended for apple juice clarification was studied. The reaction was conducted at 50C at different pH values (3.6-5.6). Starch solutions containing from 2.48 to 14.85 mg/mL were used as substrate in the experiments. Influence of Ca^{2+} on amylase activity was also determined. Obtained results showed that amylase exhibits an optimal activity pH value of 4.6, following a Michaelis-Menten type kinetics (K_m = 8.11 mg/mL and v_{max} = 2.19 U/mg enzyme). The activity of amylase decreased significantly when $CaCl_2$ was added to the amylase solution (final amylase concentration in reaction mixture: 5-10 mM). An empirical equation relating the rate of product of hydrolysis formation (dC_m/dt) with starch (C_{st}) and amylase (C_e) concentrations was obtained:

$$\frac{dC_m}{dt} = 1.18 \times C_e^{0.77} \times C_{st}^{1.03} \times [1-\exp(-0.11 \times t)]$$

INTRODUCTION

Clarified apple juice concentrate is one of the most consumed fruit juices in the world (Binnig and Possmann 1993). Before concentration, pressed juice is generally clarified. Conventional clarification process includes hydrolysis of pectin and starch with specific enzymes, flocculation of turbidity with clarifying agents (bentonite, gelatin and/or silica-sol) and filtration through plate and frame or vacuum filters to eliminate insoluble solids (Grampp 1976). Clarification

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processes may also include ultrafiltration through semipermeable membranes (Heatherbell *et al.* 1977).

Polymeric carbohydrates such as starch result in difficult and slow filtration, membrane fouling, gelling after concentration and/or postprocess cloudiness. Apple juice is one of the fruit juices that contain considerable amounts of starch, particularly at the beginning of the season. Results indicated unripe apple juice made with Granny Smith cultivar had as much as 8 g/L and 0.5 g/L insoluble and soluble starch, respectively, 2 weeks before harvest date. However, total starch content decreased rapidly as the apple matures, becoming undetectable after 3 weeks storage at room temperature (Carrín *et al.* 2001). On the other hand, when unripe apples are processed starch may easily be detected and treated. Treatment may be achieved by adding starch-splitting enzymes together with the pectinases during depectinization of the juice. The use of enzymes in apple juice clarification has been discussed by Heatherbell (1976) and Grampp (1976). Rohalase HT (Röhm Enzyme) is a special product (fungal amylase) for starch degradation in fruit juices at elevated temperatures (up to 50C). Principal activity in this product is an exo-amylase.

Apple juice is rich in Ca^{2+} (Príncipe and Lozano 1991). It is known that these metal ions are often required for the activity of α-amylases (Manelius and Bertoft 1996).

Apple juice processors are in general lacking in both (1) a reliable method for checking the different enzyme activities and (2) information about the kinetics of the amylolysis during clarification. The objective of this paper was to determine the amylolytic enzyme kinetics of a commercial amylase (Rohalase HT) on a soluble starch substrate. Work includes the study of the influence of initial concentration of amylase and starch on the rate of reaction as well as the effect of pH and $CaCl_2$ on amylase activities.

MATERIAL AND METHODS

Rohalase fungic HT-Amylase (from *Aspergillus niger*) was purchased from Röhm GmbH. Amylase solution was prepared by solubilizing the amylase powder in HAcO/NaAcO 0.1 M buffer at different pH values. Soluble corn starch (Química Bonerense S.A., Buenos Aires, Argentina) was dispersed in buffer HAcO/NaAcO 0.1 M at selected pH and boiled for 20 min to attain gelatinization.

All the other reagents were analytical grade and used without further purification. Absorbance measurements were made with a Perkin-Elmer Lambda 3 Spectrophotometer.

Enzyme Activity

Enzymatic reaction was initiated by adding 100 μL amylase solution to 10 mL of starch solution, at 50C. The enzymatic reaction was stopped at selected periods by the addition of 5 mL carbonate-bicarbonate buffer 0.1 M (pH = 10.5) to 50 μL of the reaction mixture. Reaction product content was colorimetrically analyzed with the Nelson-Somogyi method (Somogyi 1951) in 3 mL of this inactivated solution and expressed as maltose content (C_m), through linear relationship $C_m[\mu g/3\ mL] = 207.841 * A_{750} + 2.837$. Blank tests were performed by adding the enzyme to the buffer without starch (no reaction allowed). One Unit of enzymatic activity (U) was taken as the enzyme required to release 1 mg of maltose in 1 min, under the assayed conditions.

Adding of $CaCl_2$

The effect of ion calcium as $CaCl_2$ added either (1) to the substrate or (2) to the amylase solution was also investigated. Amylase (C_e) and starch (C_{st}) concentrations in the reaction mixture at pH = 4.6 were 0.5 and 9.9 mg/mL, respectively. Reaction time was 3 min. Final $CaCl_2$ concentration in the reaction mixture were 5 and 10 mM.

Amylase Concentration (C_e)

The influence of amylase concentration (C_e = 0.01 - 3.46 mg/mL; pH = 4.6) on the amylolytic activity was investigated. Starch solution with C_{st} = 9.9 mg/mL and pH = 4.6 was used. Reaction time was 3 min.

pH and Amylase Reaction

The effect of pH on the amylase reaction was also investigated. HAcO/NaAcO buffer (0.1 M) was used to modify pH value in the range 3.6 - 5.6. The other reaction conditions were: C_e = 0.69 mg/mL; C_{st} = 9.9 mg/mL, and 3 min for the reaction time.

Starch Concentration and Time of Reaction

The effect of starch concentration (C_{st} = 2.48 - 14.85 mg/mL) on amylolytic activity was also determined. Amylase concentration was C_e = 0.69 mg/mL; pH was fixed at 4.6; and time of reaction varied in the range 1-60 min.

Statistical

Experiments were performed in triplicate. Reported values are mean and standard deviations. Data were analyzed with the SYSTAT 5.03 for windows

(SYSTAT Inc.) using nonlinear regression and numerical estimations based in the Quasi-Newton method.

RESULTS AND DISCUSSION

$CaCl_2$ and Amylase Activity

Figure 1 shows the variation of reaction products concentration, as influenced by $CaCl_2$. It can be observed that the influence of $CaCl_2$ on the amylase reaction was different depending at what stage the metal ion was incorporated to the reaction mixture. When $CaCl_2$ was added to the starch solution, reaction rate was practically unaffected. However, a decrease on the amylase reaction rate was observed when $CaCl_2$ was added to the amylase solution. Manelius and Bertoft (1996) reported that Ca^{2+} ion positively affected

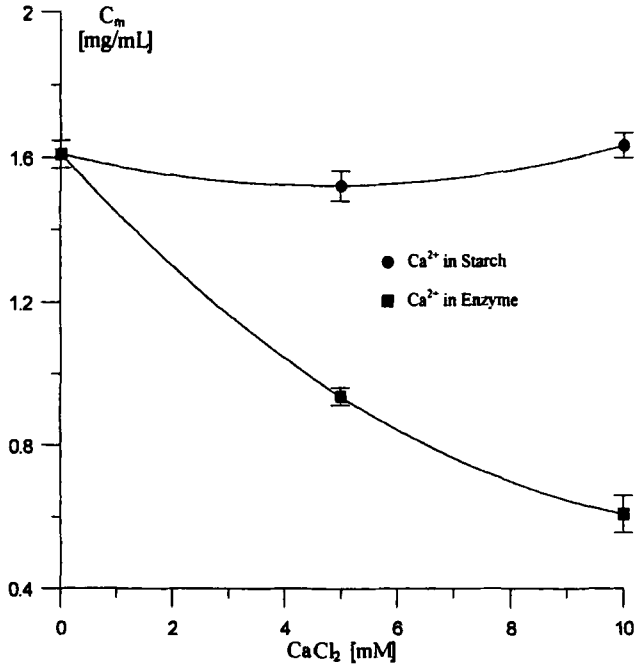

FIG. 1. EFFECT OF $CaCl_2$ ON MALTOSE FORMATION AFTER $CaCl_2$ ADDITION EITHER TO STARCH OR TO AMYLASE SOLUTION

the oats starch solubilization with α-amylase. This effect was irrelevant when corn starch was hydrolyzed with an equivalent amylase. In short, when $CaCl_2$ was added to the amylase solution instead of to the final reaction mixture, amylases were in contact with a relatively high concentration of Ca^{2+}. Witt and Sauter (1996) also found α-amylase activity decreased rapidly when in contact with high Ca^{2+} concentrations. Marchal *et al.* (1999) reported an increase in α-amylase only with small amounts of Ca^{2+}. However, they found certain instability when elevated concentrations of Ca^{2+} were added to the amylase reaction mixture.

Amylase Concentration (C_e)

Figure 2 shows the variation of a starch hydrolysis product, as maltose content (C_m), with amylase concentration. Enzymatic activity for amylase concentrations < 0.1 mg/mL was not detected. Results also indicate that after this induction or lag period maltose production increased linearly up to C_e= 2 mg/mL. Saturation was not reached for the range of C_e studied.

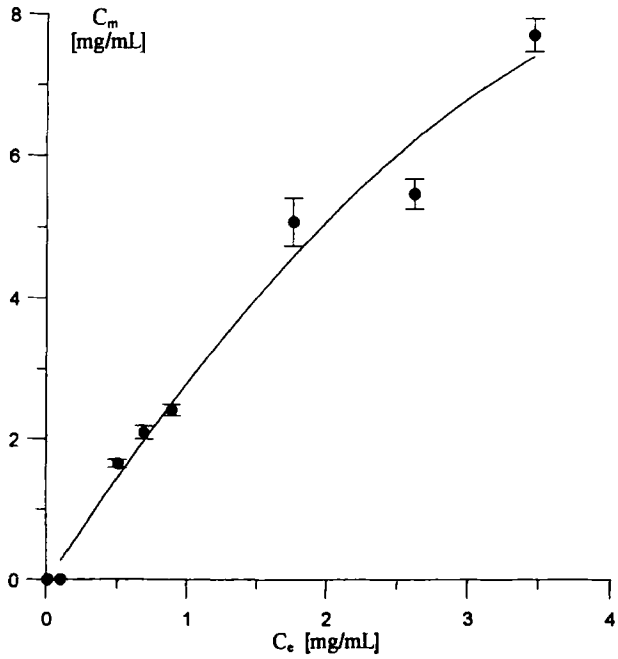

FIG. 2. EFFECT OF ENZYME CONCENTRATION (C_e) ON STARCH HYDROLYSIS AS MALTOSE PRODUCTION

pH and Amylolytic Activities

The behavior of the amylase versus pH of reaction was tested and plotted in Fig. 3. The optimum pH value was approximately 4.6. Assayed amylase shows a rapid decrease in activity at about pH = 5.5. However, this problem becomes irrelevant because pH values of apple juice, depending on cultivar, are in the range 3.5 - 4.0 (Toribio and Lozano 1984).

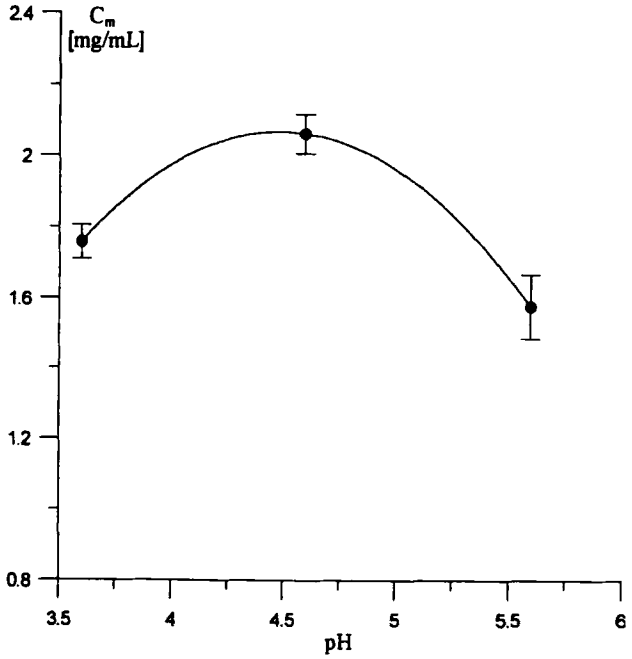

FIG. 3. EFFECT OF pH ON THE ENZYMATIC ACTIVITY OF AMYLASE

Starch Concentration and Time of Reaction

Figure 4 shows the effect of starch concentration on the amylase reaction after different times of reaction. During the initial period of reaction, product concentration (C_m) increased linearly with starch concentration up to approximately 5 mg/mL. For C_{st} > 5 mg/mL a deceleration on the product formation was observed. However, when the amylase reaction was allowed to progress

during relatively extended periods (t = 60 min) product concentration increased linearly as the starch concentration was increased.

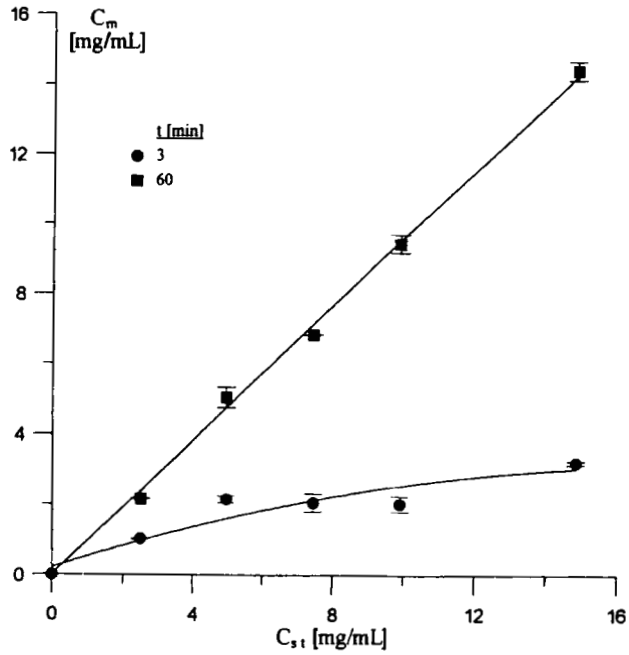

FIG. 4. EFFECT OF STARCH CONCENTRATION (C_s) ON PRODUCT CONCENTRATION (C_m) WITH TIME OF REACTION AS A PARAMETER

Kinetic Parameters

Plot of reciprocal initial reaction rate ($1/r°$) versus reciprocal initial starch concentration ($1/C_{st}$) is shown in Fig. 5. The initial reaction rate ($r°$) represents the rate of change of initial products concentration (ΔC_m) per unit time. The studied amylase showed a typical Michaelis-Menten kinetic behavior. The solid line in Fig. 5 represents Lineweaver-Burk equation:

$$\frac{1}{r^\circ} = \frac{1}{k \times C_e} + \frac{K_m}{k \times C_e \times C_{st}} \quad (1)$$

Where r° is the initial reaction rate; k and K_m are the apparent Michaelis-Menten constants. K_m value resulted in 8.11 mg/mL and maximum velocity ($k \times C_e$) was 2.19 U/mg enzyme.

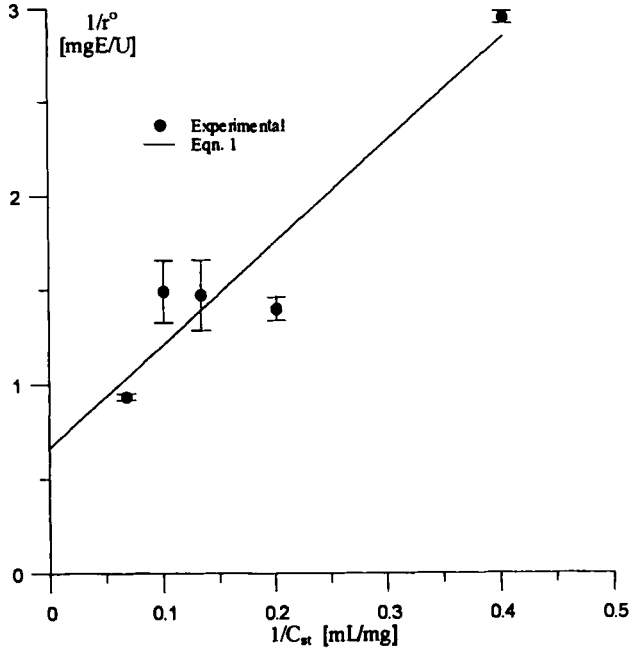

FIG. 5. LINEWEAVER-BURK PLOT OF RECIPROCAL INITIAL REACTION RATE VERSUS RECIPROCAL SUBSTRATE CONCENTRATION (C_e=0.69 mg/mL)
Full line represents Eq. 1.

Empirical Working Equation

The kinetic behavior of assayed enzymes may be modeled with empirical equation representing the formation of maltose as a function of time, with C_{st} and C_e as parameters. Starch hydrolysis rate was fitted to the following Eq:

$$r_{C_m} = \frac{dC_m}{dt} = 1.18 \times C_e^{0.77} \times C_{st}^{1.03} \times [1-\exp(-0.11 \times t)] \quad (2)$$

where C_e, C_{st} and C_m are in mg/mL and t, in minutes. Figure 6 illustrates the experimental data versus fitting results using Eq. 2. It shows that the proposed working equation appropriately fit data obtained under the present experimental conditions. A similar equation but only as a function of reaction time was proposed to describe the potato starch hydrolysis with α-amylase (Marchal *et al.* 1999).

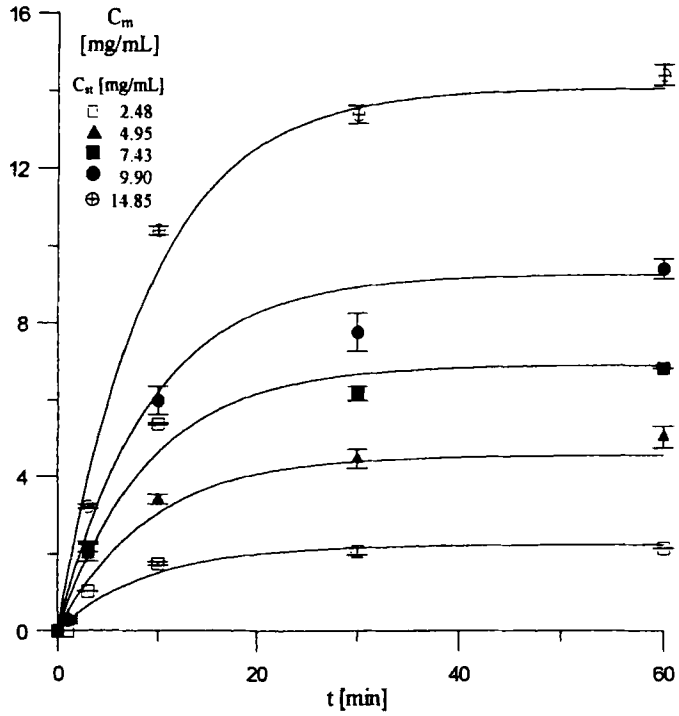

FIG. 6. STARCH HYDROLYSIS AS PRODUCTION OF MALTOSE WITH TIME
(C_e=0.69; mg/mL; pH=4.6)
Full line represents Eq. 2.

It must be emphasized that fruit mash or fruit juices are very complex and variable substrates, making the standardization of enzyme preparation very difficult. In this work a commercial amylolytic enzyme which exhibits amylase activity was used with a starch substrate at concentrations and pH values simulating a real fruit juice. As a hypothetical general model based on purified enzymes failed to describe an industrial enzymatic process, an empirical kinetic equation considering the effect of commercial amylase and starch content is proposed.

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