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Characterization of starch in apple juice and its degradation by amylases

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Abstract

Soluble and insoluble starches from apple juice (Granny Smith variety) at different degree of ripeness were separated and quantified by an iodometric method. Non pasteurised unripe apple juice had as much as 8 g/L starch, 2 weeks before usual harvest date. However, juices obtained from unripe apples stored for 3 weeks at room temperature, had undetectable starch contents. Scanning electron micrographs (SEM) showed apple starch granules are particles of regular shape (mean diameter = 9.21 μm, standard deviation = 2.74). SEM studies also revealed that after pasteurising apple juice (90 °C, 5 min) virtually all the starch granules lost their spherical structure and only gel-like starch fragments dispersed among the other components of haze (pectin, cell wall, etc.) were observed. Röhalase HT (RO) and Tyazyme L300 (TY) amylases normally used in fruit juices clarification, even in doses lower than those industrially recommended, quickly reduced starch contents in pasteurised juices to undetectable values. Microscopy studies also demonstrated that RO enzyme efficiently degraded raw apple starch granules, which showed evidence of centrifugal and centripetal hydrolysis.

Keywords: Apple juice; Starch; Amylases

1. Introduction

The objectives of apple juice concentration are mainly to reduce costs and to increase shelf life. Before concentration, pressed juice must be clarified. Conventional clarification process aim to eliminate insoluble solids and destroy pectic substances by degrading pectin and starch with specific enzymes, flocculating cloudiness with clarifying agents (bentonite, gelatine and/or silicasol) and filtering through plate and frame or vacuum Oliver-type filters, in order to.

Starch is a common problem for apple juice processors. Unripe apples contain as much as 15% starch (Reed, 1975). Polymeric carbohydrates like starch and arabans may make filtration difficult and cause post-process cloudiness. In the presence of starch, the following problems may occur: (i) slow filtration, (ii) membrane fouling, (iii) gelling after concentration, and (iv) post concentration haze.

Apple juice is one of the juices that can contain considerable amounts of starch, particularly at the beginning of the season. The starch content of apples changes from variety to variety and from season to season within a given variety. As an apple ripens on the tree, starch hydrolyses into sugars. Decrease in starch usually begins a few weeks before harvest, but in years when there were relatively low temperatures during the growing season starch content of apple juice may be high.

The standard test for the presence of starch in juice is the iodine test (SI) (IFFJP, 1984). In the presence of starch, iodine will form a characteristic blue–violet colour. A negative test by iodine indicates that all of the starch has been reduced to a chain length of less than nine to twelve glucose units, a size sufficiently reduced to not produce post-bottling hazes.

When a positive iodine test is obtained, starch must be degraded in the production of clear juices and/or

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concentrates. This is achieved by adding starch-degrading enzymes together with the pectinases during depectinization of the juice. Before amylase treatment the juice must be pasteurised to gelatinise the starch and then cooled down 50 °C to avoid enzyme inactivation. Starch in nature is stored in cells as small granules, visible under a microscope. Because of this, starch is insoluble in water at room temperature Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the same starch molecules and with other neighbouring molecules. When an aqueous suspension of starch is heated, the hydrogen bonds weaken, water is absorbed, the granules swell, rupture, and finally gelatinize (Zobel, 1984). When starch granules have not been broken down completely, short-chained dextrins are left. This can lead to a condition known as retrograding. When starch retrogrades, the short-chained dextrin re-crystallise into a form that is no longer susceptible to enzyme attack, regardless of heating.

Besides the generalised application of commercial amylases in the juice industry, there is a lack of information on the apple starch characteristic, the extent of gelatinisation during juice pasteurisation and the action of enzymes on it. The objectives of the present work were: (1) to microscopically characterise and quantify the apple starch and, (2) to study the effect of heat and enzymatic action on it.

2. Material and methods

Unripe apples (UA) were picked two weeks before usual harvest date. These apples had an Effigy firmness >9 kg. Some of these apples were stored at room temperature during one (A_1) and three-weeks (A_3). UA, A_1, and A_3 apples were cut to cubes and pressed in a laboratory hydraulic press (Genovese, Elustondo, & Lozano, 1997). The juice was then filtered through a mesh filter (100 μm). Aliquots of this juice were pasteurized (5 min at 90 ± 1 °C). Hunter *a*, *b*, and *L* parameters were measured with a Hunterlab Ultrascan XE Spectrophotometer (Hunter Assoc. Laboratory, VA, USA) in the transmission mode. Colour was measured on duplicate samples contained in a $5 \times 5 \times 1$ cm cell. Differences in *L*, *a* and *b* determination between replicates were less than 2%.

2.1. Starch detection and quantification

A solution (126.9 mg/L), containing the same volume of 0.1 M iodine and potassium iodide (5 g/100 mL), was diluted with cold distilled water to obtain a cold iodine solution (around 2 °C). An aliquot of 5 mL of sample was mixed with 2.5 mL of cold iodine solution and after 10 min at 25 °C the absorbance was read at 615 nm with a Perkin–Elmer Lambda 3 Spectrophotometer. Results were compared with a calibration curve made with corn starch (Ind. Qca. Bonaerense, Bs. As.) solutions of different concentrations [$C(\mathrm{g/L}) = 1.227A_{615} - 0.143$, $r^2 = 0.971$] and valid in the absorbance range 0.200–0.660. Starch solutions were made by dissolving starch in 2 M NaOH at room temperature, followed by the addition of an equal volume of 2 M HCl. These solutions were then heated to clear solutions, filtered through Whatman No. 540 paper, and brought to pH 4.6 with 2 M HCl and/or NaOH. Final solutions were made by dilution with 0.1 M acetate buffer (pH 4.6). All the calibration assays and analytical determinations were made in triplicate.

2.2. Insoluble and soluble starch

Unpasteurised juices (UA, A_1, and A_3) were centrifuged at 1000*g* for 20 min, supernatants were collected and pasteurised and sediments were suspended in 2 M NaOH, dissolved as above, and diluted in 0.1 M acetate buffer (pH 4.6). Insoluble starch was determined in these dissolved and diluted sediments by the iodometric method previously described. Soluble starch was determined using the same method as for pasteurised supernatants appropriately diluted with distilled water. Starch determinations were made in triplicate.

2.3. Enzymatic treatment

Pasteurised apple (UA) juice was treated with different concentrations of enzymes at 50 °C several times (up to 30 min). Two commercial amylases, solid Röhalase HT (RO) (AB Enzymes GmbH, Darmstadt, Germany) and liquid, Tyazyme L300 (TY) (Solvay Enzimas, Argentina) were assayed.

Enzyme dilutions in 0.1 M acetate buffer (pH 4.0) were prepared and added to juice in concentrations of 25 and 100 mg/L for RO ENZYME and 10 and 30 μL/L for TY. Enzymatic reaction was stopped by pasteurising the reaction mixture (90 °C, 5 min) and leaving it to cool down in an ice bath. Soluble starch in the reaction mixture was quantified in triplicate by the iodometric method previously described.

2.4. Electron microscopy of the apple starch granules

Particle size, size distribution and shape of apple starch granules were micrographically determined with a JEOL Model 35CF SEM (JEOL LTD, Tokyo, Japan) at 5 kV. Cloudy juice from UA was centrifuged (9000*g*, 1 min) in a Beckman Microfuge centrifuge (Beckman Instruments Inc., USA). Supernatant was removed and replaced with water/ethanol solution (50% v/v). The suspension was treated in an ultrasonic bath and centrifuged. This operation was repeated for three times.

A drop of the insoluble precipitate was put onto a glass slide; vacuum dried at 40 °C and gold covered in a Pelco Model 3 Sputter Coater 91000 metal evaporator. Digital images of starch granules were statistically analysed with the AnalySIS v.2.1 (Soft-imaging Software GmbH) program.

In addition to mean particle diameter, this program also gave information about the lengths of the major axis (L_a) and the minor axis (B_a), valid for estimation of particle sphericity. Each particle diameter was calculated as a projected area. To evaluate maximum and minimum axes the program generates an axis, called evaluation axis, at a defined angle with respect to the particle. Then two lines perpendicular to the evaluation axis are generated, completely including the particle. L_a and B_a maximum diameters can be calculated by moving the evaluation axis angle.

To study the effects of commercial amylases on the apple starch granules, the starch was extracted from cloudy juice (UA) by centrifugating and washing several times with distilled water, as previously indicated. The obtained granules were suspended in 0.1 M acetate buffer (pH 4.0) and treated with enzyme solution at 50 °C during 30 and 150 min. Enzyme concentrations of 100 mg/L and 30 μL/L, for RO and TY enzymes, were used respectively. Enzymatic reaction was inhibited by cooling in ice, the mixture was centrifuged (9000*g*, 1 min), and the precipitate washed with 50% (v/v) ethanol/water three times. Samples for microscopic analysis were made and evaluated as previously indicated.

3. Results and discussion

The main characteristics of unpasteurised juice from UA are summarized in Table 1. Soluble solids content increases as apples ripen. Alone, this is an unreliable ripeness indicator as many factors influence sugar content. Soluble solids content is used in conjunction with days from full bloom, to determine ripeness.

3.1. Starch content

Non-pasteurised UA juice showed a white precipitate attributable to insoluble starch before centrifugation. However, in A_3 juice, precipitate was not observed even after centrifugation. Table 2 lists the starch content in the assayed apple juices. Non-pasteurised UA juice showed, as expected, large quantity of insoluble starch (7.68 g/L). Soluble and insoluble starch content decreased quickly as the apples ripened and undetectable concentrations were observed after the storage of the unripe fruits for three weeks at room temperature.

Table 1
Specifications of the cloudy Granny Smith apple juice[a]

Soluble solids (°Brix)	10
pH	3.4
Color	Greenish white ($L = 79.38$; $a = 1.86$; $b = 17.92$)
Pulp content (%, v/v) (before centrifugation)	<1.0

[a] Elaborated with unripe apples.

Table 2
Soluble and insoluble starch content in apple juices at different degrees of ripeness

Sample	Insoluble starch (g/L)	Soluble starch (g/L)
UA	7.68	0.51
A_1	2.47	0.10
A_3	Nd	Nd

Nd: not detected.

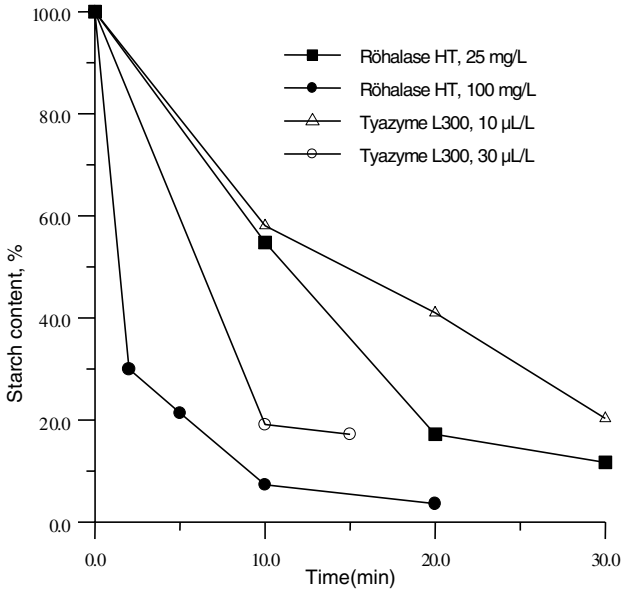

Fig. 1. Kinetic for starch extinction in pasteurised apple juice treated with different amylase doses at 50 °C.

Fig. 1 shows the kinetics of starch extinction in pasteurised juices, treated with RO and TY amylases at 50 °C. Iodine test resulted negative after no more than 30 min of treatment, and depended on enzyme concentration.

3.2. Microscopic observations

The apple starch granules were considered practically spherical (Fig. 2(a)). The results show that the major ($L_a = 9.21$ μm) and minor axes ($B_a = 7.86$ μm) were very similar. A typical frequency histogram obtained through the statistical analysis of apple starch granules is shown in Fig. 3. Apple starch consisted of particles of regular shape with a mean diameter, $D = 9.21$ μm (standard deviation, $\sigma = 2.74$). The results are in agreement with those of Kovács and Eads (1999) who observed rounded granules of 10 μm in size, whose area and perimeter

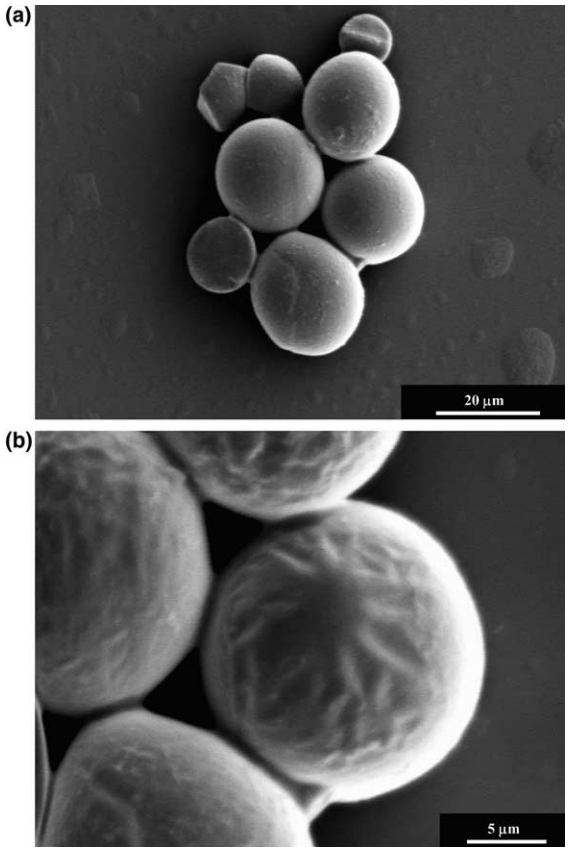

Fig. 2. Scanning electron micrographs of apple starch granules: (a) 5 kV × 2400. (b) 5 kV × 6600.

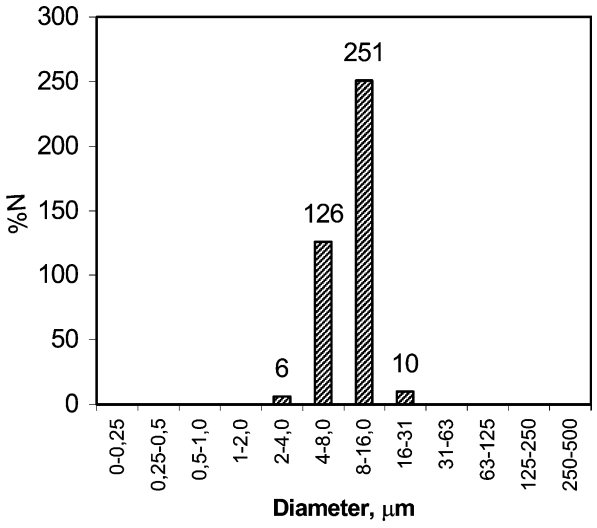

Fig. 3. Particle size distribution histogram (particle relative number, %N vs particle diameter, D) of apple starch granules.

decreased, and whose circularity increased, with the storage time and ripeness of apples.

The surface of untreated starch granules showed some small irregularities (Fig. 2(b)); which would facilitate attacks of the enzyme molecules on starch granules during degradation (Sarikaya, Higasa, Adachi, & Mikami, 2000).

The microscopic study of precipitate from a non-pasteurised cloudy apple juice showed fine particulate, probably containing considerable protein and carbohydrate as principal constituents (Beveridge, 2002) over which the starch granules are easily observed (Fig. 4(a)). Though a few of them presented some degree of collapse, most of granules retained their integrity.

Fig. 4(b) shows a scanning electron micrograph of haze sediment obtained from a pasteurised apple juice sample. This micrograph shows that apple starch granules collapsed after heat treatment and only gel-like starch fragments dispersed among the other components of cloudiness may be observed. Similar behaviour was found when wheat starch was gelatinised by heat in excess water (Lineback & Wongsrikasen, 1980).

Electron micrographs of starch granules isolated from non pasteurised apple juice and treated with amylases are shown in Fig. 5. RO showed a higher ability to degrade or attack raw starch granules, than TY. Apple starch granules showed evidence of attack by RO in spite of short treatment times. α- and β-Amylases bind to and attack starch granules, but generally do not have a large effect on raw starch granules because the granules are very resistant to amylolytic digestion. Electron

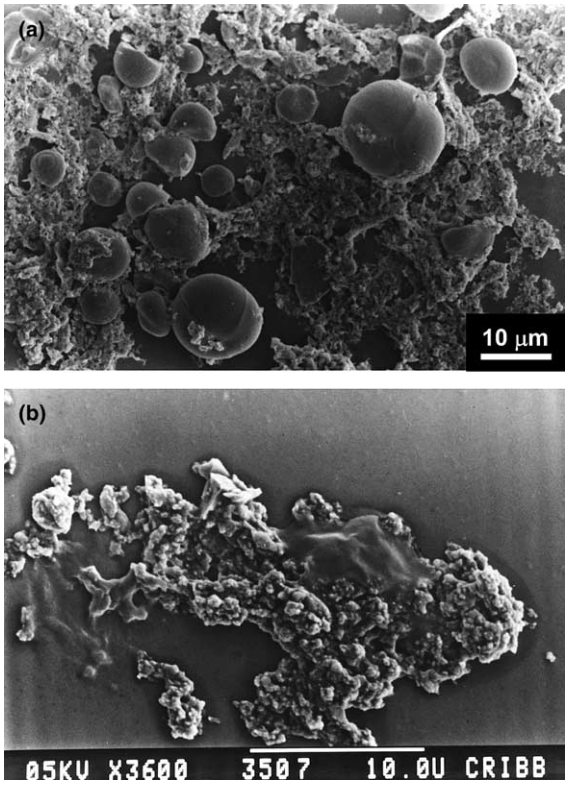

Fig. 4. Scanning electron micrograph of precipitates from: (a) non pasteurised cloudy apple juice (6 kV × 1500), (b) apple juice pasteurised during 5 min at 90 ± 1 °C (5 kV × 3600).

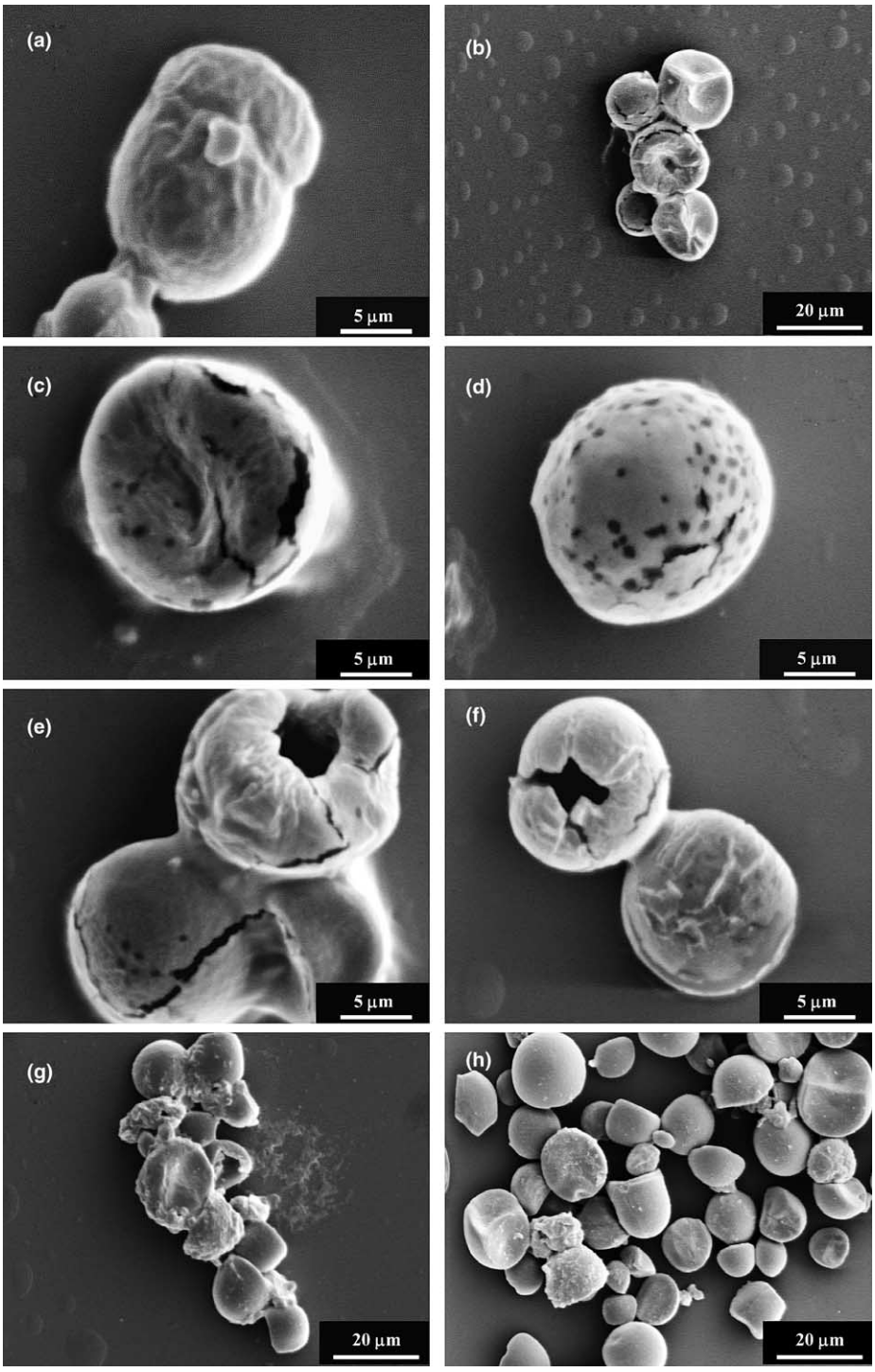

Fig. 5. Scanning electron micrographs of starch granules isolated from non pasteurised apple juice and treated at 50 °C with: Röhalase HT (a–g) and Tyazyme L300(h).

micrographs and kinetic assays confirm that the degree of hydrolysis is related both to types of amylases and to types of starches (Sarikaya et al., 2000).

The mechanism of adsorption of enzymes onto starch granules is still unclear, but probably occurs, through a C-terminal binding domain (Jerpersen, Mac Gregor, Sierks, & Svensson, 1991). Because glucoamylases of some fungi such as *Aspergillus* and *Rhizopus,* with a C-terminal binding domain are very active against raw starch, and the Röhm RO is a ''fungal glucoamylase'' this fact could explain its intense action on raw starch.

Röhalase HT works at a relatively high temperature (50 °C); this also favours it hydrolytic activity. Kim, Namori, and Shninke (1989) have reported that a high

temperature was very effective in raw starch degradation. They found extensive degradation of wheat and potato starch granules at 60 °C.

Helbert, Schülein, and Henrissat (1996) found that α-amylase molecules degrade starch proceeding first from surface toward the center (centripetal hydrolysis), and then the core is completely degraded from within by erosion of its periphery (centrifugal hydrolysis). It was clearly observable that RO produced a centrifugal hydrolysis, which is peripheral in action (Fig. 4(a–d)). Centripetal hydrolysis, also was observed. Centripetal hydrolysis produces very big and deep holes on the granule surface and the interior side appears (Fig. 4(e–g)). This hydrolysis has been described for corn, rice and wheat starch granules treated with BAA (α-amylase from *B. amyloliquefaciens*), while centrifugal hydrolysis was observed in potato and sweet potato starches treated with the same enzyme (Sarikaya et al., 2000). Fissures and pits were notable on the surface of starch granules treated by RO enzyme (Fig. 4(c), (d)), however a laminate internal organisation was not apparent in starch granules enzymatically digested. Rice and wheat granules, treated with BAA, showed this type of laminate internal structure (Sarikaya et al., 2000). Finally, Fig. 5(h) shows that the TY enzyme was generally less effective in hydrolysing apple starch granules.

4. Conclusions

The juices from unripe apples had a high content of soluble and insoluble starch. Three weeks of storage of the fruits at room temperature were sufficient to reduce these contents to undetectable levels in the juices.

Microscopic studies demonstrated that heating of unripe apple juice under conditions similar to those found during industrial pasteurisation, resulted in a practically complete breaking down of the starch granule. In this way, enzymatic starch degradation during apple juice clarification may be easily achieved. The quantitative starch assays demonstrate the amylases used were highly effective, even in doses lower than those industrially recommended, when they act on gelatinised starch.

One of the amylases used, RO enzyme, had relevant degradation ability on raw starch granules isolated from unripe apple juice. The knowledge of the mechanism of how amylases attack starch should lead to improvements in the industrial hydrolysis of starch obtained from different sources.

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