EFFECT OF PECTINOLYTIC AND AMYLOLYTIC ENZYMES ON APPLE JUICE TURBIDITY

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ABSTRACT

The mechanisms governing the enzymatic clarification of apple juice were studied by electron microscopy techniques. Full ripe and unripe apple juice samples (Granny Smith) were treated with commercial pectinase (Solvay 5XLHA) and amylase (Röhalase HT) enzymes, respectively. Scanning electron microscopy studies revealed that commercial amylolytic enzymes quickly reduced starch content in unripe apple juice to undetectable values. It was also observed that after pasteurization of this juice (90°C, 5 min) all starch granules gelatinized. Using transmission electron microscopy, it was possible to observe pectin bonded to ripe apple juice particles. This protective colloid is known to be responsible for cloudy juice stability. The effect of pectic enzyme to destroy the protective pectin colloid was also detected with this technique. As a result of the enzymatic treatment, average particle size initially increased from 1000 to 1500 nm and decreased thereafter to ~1100 nm, and Z-potential increased in absolute values from −9.6 to −11.4 mV. It was speculated that the destruction of the weak pectin net by the action of the specific enzyme caused particle aggregation, followed by the collapse of aggregates, increasing the number of particles <500 nm.

INTRODUCTION

The main purpose of the clarification treatment employed in industrial apple juice processing is to eliminate constituents responsible for the turbidity and cloudiness in freshly produced juice. Conventional clarification process

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includes hydrolysis of pectin and starch with specific enzymes, flocculation of turbidity with clarifying agents (bentonite, gelatin and/or silica-sol) (Grampp 1977) and filtration through plate and frame or vacuum Oliver-type filters. Application of ultrafiltration (UF) as an alternative to conventional processes for the clarification of fruit juices is gaining acceptance in the industry (Lozano et al. 2000). However, these membrane techniques partially substituted the use of specific enzymes (Mondor and Brodeur 2002), and the adequacy of UF as a technique for preventing haze formation still requires further studies. The other important purpose of clarification is to remove substances that may cause haze and sediment formation during storage, at reconstitution of the concentrate or after bottling of the juice (Tajchakavit et al. 2001).

Apple juice is one of the juices that may contain considerable amounts of starch, particularly at the beginning of the harvest season. Unripe apples contain as much as 15% starch (Reed 1975), and up to 1% starch may be present in juice after milling and pressing (Carrín et al. 2004). Starch is a common problem for apple juice processors, complicating filtration and causing postprocess cloudiness.

The process of depectinization involves the use of commercial enzymes, generally a blend of pectinases (e.g., pectinase, polygalacturonase, cellulase, pectin lyase) to degrade pectic substances. Starch-degrading enzymes such as amylase and amyloglucosidase are also commonly added during the depectinization stage. These enzymes degrade starch into smaller units and may contribute to postbottling haze formation by aggregating among themselves or through the formation of protein–starch complexes. Fining of apple juice also involves the use of gelatin and bentonite as fining agents (Stocké 1998). Differences in the nature of ionic charges of protein, polyphenols and the fining agents induce flocculation and sedimentation and result in the removal of these potential haze precursors from juices.

Turbidity of opalescent apple juice is provided by particulate material that remains in suspension (Genovese et al. 1997). The cell wall of fruit comprises a complex mixture of cellulose, hemicelluloses, pectins and proteins, which incorporate to pressed juice in different ways, providing cloudiness.

Cloud particles have been modeled to consist of a negatively charged pectin wrapped around a positively charged protein–carbohydrate core (Yamasaki et al. 1964; Endo 1965). Pectin slows aggregation by coating the particles, preventing the close-enough approach of one to another needed for the formation of hydrogen bonds or effective dipolar interactions (Van Buren 1989). In addition, they may give particles enough charge such that significant electrostatic repulsion takes place between particles. Particle sizes above 0.5 μm are unstable and may easily settle out (Genovese et al. 1997). Below this range, particles are retained in suspension because of their small size, mutual charge repulsion and protective effect of pectin.
However, the mechanism of particle stabilization in cloudy juices is not completely clear yet. For colloidal stability, the repulsive forces must be dominant. There are different mechanisms that affect dispersion stability in a colloid, including Van der Walls attractive and electrostatic repulsive forces and some others interactions such as steric repulsion, depletion, hydrophobic and hydrophilic interactions (McClements 1999).

Z-potential ($\zeta$) measurements are used to assess the stability of colloidal systems because it is a very good index of the magnitude of electrostatic repulsive forces between colloidal particles. On the one hand, when all the particles have a large negative or positive $\zeta$, they will repel each other and increase dispersion stability. On the other hand, when the particles have low $\zeta$ values, then the net repulsive force to prevent the particles coming together is weak or null. $\zeta$ is a function of the nature of particle’s surface, and the composition of the dispersed phase (pH, ionic strength and concentration of specific ions and polyelectrolyte) (McClements 1999). Genovese and Lozano (2001) worked with the addition of hydrocolloids to cloudy apple juice. They found that $\zeta$ was satisfactory to predict the haze stability in cloudy fruit juices. Scanning electron microscopy (SEM) is a microscopic technique used to visualize the aggregation and flocculation process (Ferworn and Svrcek 1998).

In addition to the generalized application of commercial enzymes in the juice industry, there is also a lack of information about the phenomena that govern the formation of stable colloidal particles on turbid juices and how specific enzymes contribute to make this colloid unstable. The objective of this study was to examine the effect of commercial pectinase and amylase on turbid apple juice using different microscopic techniques, supported by particle size and $\zeta$ determinations.

**MATERIALS AND METHODS**

Figure 1 schematically represents the methodology followed in this study. On the one hand, juice made from unripe apples was used to: (1) isolate starch granules; (2) study the effect of pasteurization on starch; and (3) verify the action of amylolytic enzymes. On the other hand, juice made from ripe apples was used to study the presence of pectin as a protective colloid (nontreated juice, NT) and effect of pectinolytic enzymes on cloud structure and stability (enzyme-treated juice, ET). Particle size and $\zeta$ were also determined in both NT and ET samples.

Cloudy apple juice of 10°Brix was obtained from full ripe and unripe Granny Smith apples at the laboratory. The unripe apples were picked two weeks before normal harvest date and showed a firmness of $>88.3$ N, as determined with an 11-mm-wide probe Effegi FT 327 Penetrometer (Effegi,
Milano, Italy). The generic starch–iodine index chart for apples was used to determine fruit maturity (Blanpied and Silsby 1992). Manufacturing included crushing individual apples in a laboratory mill, steam-heating the mash at 65–70°C for 15–20 s to inactivate native polyphenol oxidase and pressing it in a hydraulic press (100-mesh filter) with the same type of equipment and procedures as previously described (Genovese et al. 1997). Aliquots of juices were pasteurized (90°C, 5 min). Soluble solids were determined with a digital Abbe-type refractometer according to 932.12 AOAC (2000) method. The pectin used was apple pectin from Sigma-Aldrich S.A. (Buenos Aires, Argentina) (Anhydro-galacturonic acid = 77.5). Apple juice turbidity was measured.
as nephelometric turbidity unit (NTU) with an Aqualytic PC-COMPACT (Dortmund, Germany) turbidimeter. Samples of juices (30 mL) were placed in a 30-mL cell, capped and gently inverted twice to ensure even mixing. Presence of starch in juice was determined by the standard iodine test (IFFJP 1984). All the calibration assays and analytical determinations were made in triplicates.

Commercial pectinase and amylase used were a liquid fungal pectinase, Solvay 5XLHA (Genencor Enzymes, Arroyito, Argentina), and a fungal amylloglucosidase, Röhalase HT (RO) (AB Enzymes GmbH, Darmstadt, Germany), respectively. Ceci and Lozano (1998) and Carrín et al. (2002) reported both methods for enzymatic activity determination and optimal conditions for the application of these enzymes to the clarification of apple juice.

Indicated relative polygalacturonase and pectinesterase activities, as percentage of maximum pectinase activity, were 75 and 33%, respectively, at pH = 3.5 and T = 50°C. Amylase (RO) activity was determined (Carrín et al. 2002) to be 1.6 U at pH = 3.6 (1 U of enzymatic activity was taken as the enzyme required to release 1 mg of maltose in 1 min).

The pectic enzyme treatment (80 mg/L) was carried out after the hot technique (Toribio and Lozano 1984). To study the effects of the commercial amylase on the apple starch, pasteurized unripe apple juice was treated with enzyme solution (RO, 100 mg/L) at 50°C for 150 min. Enzymatic reactions were inhibited by cooling in ice; the mixture was centrifuged (9000 × g, 1 min), and the precipitate was washed three times with 50% (v/v) ethanol/water.

Starch granules were isolated by repetitive washing and centrifugation of cloudy juice made from unripe apples. The cloudy juice was centrifuged (9000 × g, 1 min) in a Beckman Microfuge centrifuge (Beckman Instruments, Inc., Irvine, CA). Supernatant was removed and replaced with water/ethanol solution (50% v/v). Suspension was treated in an ultrasonic bath (25°C, 1 min) and centrifuged (9000 × g, 1 min). This operation was repeated three times. The supernatants were collected and pasteurized, and the sediments were suspended in 2 M NaOH, dissolved as previously mentioned 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, as described by Carrín et al. (2004).

**SEM of Apple Starch and Unripe Juice**

Drops of both cloudy juice of unripe apples and isolated starch granules were put on glass slides, dried in oven under vacuum at 40°C (30 min), gold-covered in a Pelco model 3 Sputter Coater 91000 metal evaporator (Ted Pella, Inc., Redding, CA) and observed with a Japan Electron Optics Laboratory (JEOL) model 35CF SEM (JEOL Ltd, Tokyo, Japan) at 5 kV.
Transmission Electron Microscopy (TEM) of Pectin

To verify the role of pectin and pectic enzymes in turbidity, cloudy juice of ripe apples was centrifuged (9000 × g, 1 min). The supernatant was discarded, and the sediment was diluted (1:100) with distilled water. One drop of each dispersion, made with both ET and NT juices, was fixed with 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH: 7.4–7.6) with 0.3% ruthenium red, dehydrated with 25, 50, 70, 90% and three times 100% acetone (30 min each step), post-fixed with 2% osmium tetroxide with 0.3% ruthenium red for 30 min at room temperature, embedded with Spurr’s resin and cured at 70°C overnight. Thin sections were cut with a diamond knife in an LKB Ultramicrotome, mounted in 200-mesh grids and examined and photographed in a JEOL 100 CXII transmission electronic microscope (JEOL Ltd). Selected cuts were stained with 1% aqueous uranyl acetate for 30 min, washed with water, stained with lead citrate for 3 min, washed with a 0.01 N NaOH solution and placed on a filter paper in a Petri dish to dry. ET and NT apple juice samples were examined with a JEOL 100 CXII transmission electronic microscope at 80 kV.

Particle Size and $\xi$ Determinations

Ripe apple juice particle size was determined by photon correlation spectroscopy. The $\xi$ was determined by laser Doppler electrophoresis applying the Smoluchowski equation. Both particle size and electrophoretic mobility measurements were conducted with a Malvern Zetasizer 3000 (Malvern Instrument, Inc., London, England) as in a previous work (Genovese and Lozano 2000). The juice samples were centrifuged (4200 g × 5 min) in advance, and no dilution was required. The results given are the average values of five samples for each treatment.

RESULTS AND DISCUSSION

Starch

The juice from unripe apples showed a large quantity of insoluble starch granules. Apple starch granules could be considered practically spherical (Fig. 2). Mean diameter ($\phi$) was determined to be $\phi = 9 \pm 3$ μm, which is similar to what is reported in previous work (Carrín et al. 2004). Nonpasteurized juice showed fine particulates, supposed to contain considerable protein and carbohydrate as basic constituents (Endo 1965; Beveridge 1997) over which the starch granules are easily observed (Fig. 3a). Although a few of them collapsed to some degree, most of the granules retained their integrity.
Figure 3b shows an SEM micrograph of the pasteurized sample (90°C, 5 min). After heat treatment, mainly gelatinized starch was detected. This micrograph (Fig. 3b) shows that apple starch granules collapsed after heat treatment and only gel-like starch fragments dispersed among the other components of cloud may be observed. Similar behavior was found when wheat starch was gelatinized by heat in an excess of water (Lineback and Wongsrikasem 1980).

SEM studies revealed that amylase normally used in fruit juices clarification quickly reduced starch contents in pasteurized juices to an undetectable level (micrographs not shown). Starch was not detected by iodometry either.

**Pectin**

Ruthenium red binding to free carboxyl groups of pectin with different degrees of esterification was investigated by Hou et al. (1999). Ruthenium red with glutaraldehyde for staining mucopolysaccharides, and with osmium tetroxide for enhancing contrast, is a well-known staining reagent in histology. In Fig. 4, TEM micrographs of NT apple juice at different magnifications are presented. Typical particle shapes, including vesicles and agglomerations...
already described in the literature (McKenzie and Beveridge 1988), were identified (Fig. 4a). Presence of pectin fibers in a network structure, specifically stained with ruthenium red, was observed at a higher magnification (Fig. 4b). Presence of fibrous material in apple haze, attributable to pectin, was also observed by Beveridge et al. (1996). Ruthenium red is a dye, which selectively binds to the intramolecular spaces between carboxyl groups of pectin. The dye group links the carboxyl oxygen of one galacturonide moiety to a hydroxyl oxygen of an adjacent neighbor galacturonide in the pectate chain. The number of staining sites for ruthenium red depends on the number

FIG. 3. SCANNING ELECTRON MICROGRAPHS OF PRECIPITATES FROM UNRIPE APPLE JUICE
(a) Nonpasteurized; (b) Pasteurized (5 min, 90°C).
of monomers of anhydrogalacturonide units in the pectin polysaccharide (Sterling 1970).

A TEM micrograph of a commercial pectin gel (0.017 g pectin/mL) is also presented for comparison (Fig. 4c). As the figure shows, size and charac-
teristics of commercial and native pectins are very similar, taking into consideration the difference in concentration. High methoxyl apple pectin is composed of open networks, with the strands arranged in bundles or loose aggregates. Lofgren and Hermansson (2002) also found pectin gels that showed aggregated networks with large pores around 500 nm.

Apple juice particles after the treatment with Solvay 5XLHA enzyme may be observed in Fig. 5a. The effect was a reduction in the size of vesicles and an increase in the number of particles smaller than 0.5 μm. In Fig. 5b, ET particles can be observed at a higher magnification. It was determined, after analyzing nearly 40 micrographs, that fiber-like pectin practically disappears after the action of pectinase, as expected.

FIG. 5. TRANSMISSION ELECTRON MICROGRAPHS OF RIPE APPLE JUICE PARTICLES AFTER PECTINOLYTIC ENZYME TREATMENT AT DIFFERENT MAGNIFICATIONS
(a) 12,000×; (b) 40,000×.
g; particle agglomerates; v; vesicles.
In Fig. 6a, pectin fibers connecting particles in an NT apple juice, was identified at a higher magnification (40,000×). Figure 6b is a micrograph of an ET sample (40,000×) to have better view on the effect of pectin hydrolysis on particle structure. It can be also verified (Fig. 6b) that pectin fibers disappeared in apple juice particles after the enzymatic treatment.

### Turbidity, ξ and Particle Size Determinations

Figure 7 shows the variation in turbidity of an apple juice sample treated with pectic enzyme. After reduction in the first 10 min, the juice turbidity
recovered back to the initial range (1300–1400 NTU). On the contrary, average particle size increased right after the enzyme addition, reached a maximum in about 10 min, decreased sharply afterwards and finally remained stable around 1100 nm. This behavior may be explained by considering that some particles aggregated immediately after addition of the pectolytic enzyme. During aggregation, particles increased in size and decreased in amount, while their total mass remained constant. When the total aggregate mass remains constant, above a critical φ of 0.3 μm for an aggregate, the

FIG. 7. CHANGE IN TURBIDITY (NEPHELOMETRIC TURBIDITY UNIT, NTU) AND MEAN PARTICLE SIZE (nm) OF RIPE APPLE JUICE DURING PECTINASE TREATMENT
Vertical bars represent SDs.
scattered light intensity declines in proportion to $1/\phi$ because the particle count decreases in proportion to the cube root, although the $\phi$ increases in relation to the square (Anon 2004). This may explain the observed decrease in the measured turbidity during initial aggregation.

Moreover, as a result of the enzymatic treatment, $\xi$ increased in absolute values from $-9.6$ to $-11.4$ mV, after 2 h.

We propose that enzyme depectinization had two effects:

1. It destroyed the weak soluble pectin net, and reduced juice viscosity. The role of pectin in increasing cloudy apple juice viscosity was discussed previously by Beveridge (2002);
2. It caused the aggregation of cloud particles. Insoluble pectin forms a protective coat around proteins in suspension (Lozano 2003). In acidic environment (apple juice typically has a pH of 3.5), pectin molecules carry a negative charge. This causes them to repel one another. Pectinase degrades the pectin molecule and exposes part of the positively charged protein beneath. The electrostatic repulsion between cloud particles is thereby reduced so that they clump together.

However, it seems that after most of the native pectin was destroyed, aggregation structures collapsed, increasing the number of small particles, resulting in the decrease in size and increase in turbidity as observed in Fig. 7 after the first 10 min. This fact, which is associated to the changes observed in $\xi$, may explain the need of other clarifying agents (bentonite and gelatin) for flocculation.

**CONCLUSIONS**

This study has used electron microscopy to show the distribution of starch and pectin in cloudy apple juice. SEM studies and specific starch test revealed that amylases normally used in fruit juices clarification quickly reduced starch contents to undetectable levels. By using TEM, it was possible to identify: (1) a weak net of soluble pectin suspended in the NT cloudy juice; and (2) insoluble pectin bonded to apple juice particles. The effect of pectinolytic enzyme, destroying both the pectin protective colloid and the pectin net, came also to light with this technique. It is known from industrial practice that viscosity reduction is one of the controlling steps during apple juice clarification. This study revealed that the role of pectinase during clarification was more associated with an increase in particle mobility (because of the pectin net disintegration) than a reduction of electrostatic repulsion between cloud particles.
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