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ORIGINAL PAPER

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Pectin methylesterase activity in juices from mandarins, oranges and hybrids

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Abstract The pectin methylesterase (PME) activity of fresh and processed juices obtained from Valencia-late oranges, Clementine mandarins (cultivar Nules) and citrus hybrids (Ortanique) was studied. The PME activity was measured using a modification of the traditional method of Rouse and Atkins [1], based on the titration of carboxylic groups generated by PME during the hydrolysis of a commercial pectin solution. Instead of maintaining the pH at a given value by addition of NaOH, the maximum rate of pH decrease is used as measurement of the PME activity. An exponential decay function accurately represents the evolution of pH with time from a starting value of pH = 7.8 (optimum pH of PME activity for all assayed cultivars). This function allows an easy calculation of the PME activity. The total PME activity measured was similar in Clementine and Valencia-late juices, and approximately twice that of Ortanique juices. Ratios between thermostable and total PME activity were 4.0–8.4% for Clementine, 12.6–12.7% for Valencia-late and 24.2–34.2% for Ortanique juices. Soft heat treatments (70 °C, 30 s or 74 °C, 10 s) were enough to reduce the total PME activity of Clementine juices under the maximum residual level (10%) for commercial life of refrigerated juices, according to Irwe and Olson [2].

Keywords Mandarin orange Clementine · Pectin-methylesterase juice

Introduction

The citrus juice cloud is a colloidal suspension of cellular organelles, membranes, cell wall fragments, oil droplets, chromatophores, flavonoid crystals and biopolymers such as pectin, cellulose, hemicellulose and protein [3]. An opaque or cloudy appearance is considered a desirable characteristic in citrus juices. Juice clouds provide not only turbidity and colour but also flavour and aroma. Quantitative analyses of orange juice cloud show approximately 52% protein [4], 4.5% pectin [5], 25% lipid, 2% hemicellulose, 1.5% cellulose, 5.7% nitrogen and 2% ash [6].

Pectin is not the main constituent of citrus cloud but it plays the major role in maintaining colloidal stability through a complex and not well-understood mechanism. Citrus pectin consists of linear α-D-polygalacturonic molecules, with the preponderance of the carboxylic moieties esterified with methanol.

Pectin methylesterase (PME) is a cell wall bound enzyme, which causes the removal of methoxy groups and produces free carboxylic radicals. Once a critical degree of esterification is reached, divalent cations (mainly Ca) can cross-link free carboxylic groups belonging to adjacent pectin chains giving insoluble macropolymers that entrap other components of cloud. This process leads to juice clarification [7]. PME activity also produces gelation in citrus juice concentrates [8].

The inactivation of PME is traditionally carried out in citrus juice plants by heat treatment [9]. Atkins and Rouse [10] studied the PME inactivation at different temperatures and found a break near 70 °C, where residual activity was not destroyed until around 90 °C. The reason is that PME is not an unique enzyme but a mixture of several isoenzymes; 12 forms of PME were detected in citrus fruits [11], with very different heat stabilities. After a treatment of 70 °C during 5 min, some PME activity remains. Versteeg [11] called 'thermostable PME' the fraction responsible for this activity and this denomination has been accepted by other researchers [12, 13]. Snir et al. [12] evaluated the thermostable PME activity in different grapefruits, tangerines and oranges and found that thermostable PME accounts for 0–18.6% of the total PME activity. Thermostable isoenzymes identified by Versteeg [11] and Cameron et al. [14] remain active at 4 °C and at pH ranging from 3.11 to 4.00, causing losses of clouding. Thus the importance of a sufficient inactivation of these isoenzymes.

The classical method of determining PME activity was published by Rouse and Atkins [1], and is based on the

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titration of the carboxylic groups generated by PME during the hydrolysis of a commercial pectin solution. Citrus juice is added to a 1% pectin solution with 0.2 M NaCl and the mixture is quickly adjusted at pH 7.5 with NaOH. The sample is immediately incubated at 30 °C, continuously stirred, and NaOH is added during 30 min periodically to maintain the pH at 7.5. The PME activity is calculated from the consumption of NaOH.

Some modifications have been proposed to this procedure (for example, the use of a pH-stat [12, 15], the adjustment of pH before mixing juice and pectin solution [16], but in any case the accuracy is low and it is usual to carry out several assays of the same sample and give the average value. Sadler et al. [17] assayed in duplicate for each of the two replicates. Lee et al. [18] calculated the average of five replicate samples. King [19] analysed the samples in triplicate, and Nienaber and Shellhammer [20], using a computer-aided titrimeter, analysed each sample in duplicate.

On the other hand, Termote et al. [21] described the competitive inhibition of PME by pectic acid. This mechanism could also affect the measurements of PME activity by the usual methods.

In this paper we studied the PME activity of fresh and processed juices obtained from oranges, mandarins and hybrids by monitoring the pH decrease with incubation time from the pH value corresponding to the maximum PME activity. PME activity is calculated at time zero to avoid the changes on the activity due to pH decrease and pectic acid formation.

Materials and methods

Preparation and processing of juices

Valencia-late and Navelate oranges (Citrus sinensis), Clementine mandarines (Citrus reticulata, cv. Nules and Marisol) and Ortanique fruits (Citrus reticulata × Citrus sinensis) were harvested from a near commercial orchard at Lliria (Valencia) during the period from January to May 2004, and used immediately for juice obtention.

Assays performed to estimate the relationships between deesterification rate and pH (Fig. 1) and to test the proportionality between deesterification rate and enzyme concentration (Fig. 2), were carried out with citrus fruits squeezed by hand using a domestic electric juicer. The juice was then sifted in a kitchen strainer. The experimental points corresponding to the same citrus variety were obtained using the same batch of juice.

Assays corresponding to Figs. 3 and 4 were carried out with citrus juices obtained in our pilot plant. The fruits were washed and processed, using an Exzel in-line industrial squeezer, kindly lent by a local machine manufacturer (Luzzysa, El Puig, Valencia). After the extraction the raw juice was sieved in a screw finisher (holes with 0.5 mm diameter). One part of this fresh juice was packed immediately in 1 l jars and quickly cooled. The rest was pasteurised and cooled at 7 °C in a plate heat exchanger and packed aseptically in 1 l jars previously sterilized by steam. All samples were frozen at −40 °C and stored at −18 °C until the analysis.

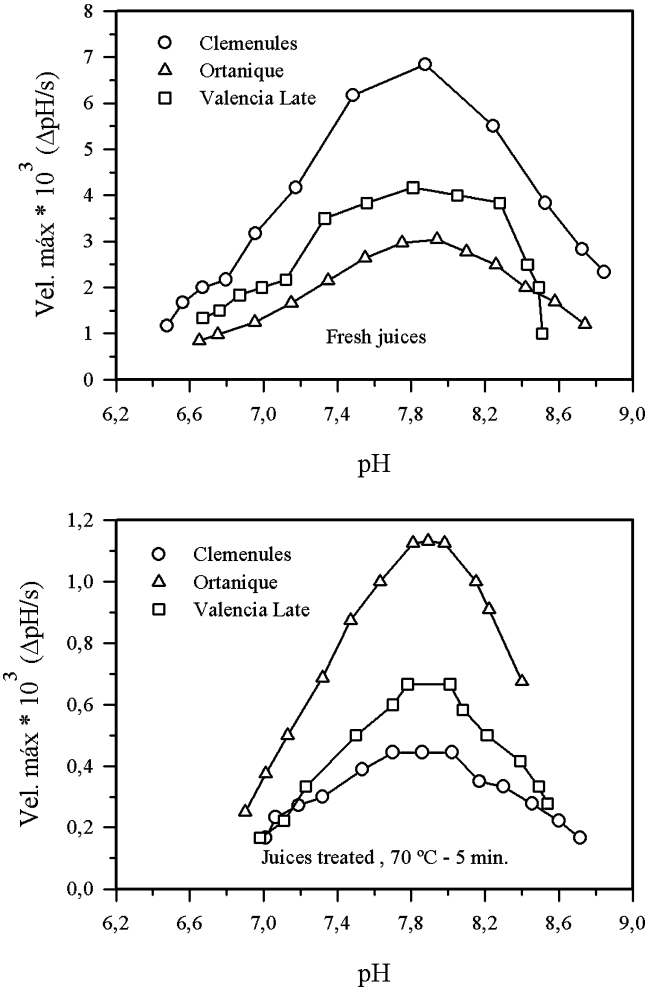

Fig. 1 Relation between the starting pH and the initial rate of the pH decrease

PME activity

PME activity was determined using a modification of the method developed by Rouse and Atkins [1]. Juice samples of 5 ml adjusted to pH 7.8 with NaOH were mixed with 20 ml of a 0.2 M NaCl solution with 0.5% pectin (Grinsted Pectin MRS 351, kind gift from Danisco, Denmark), also previously adjusted with NaOH to the same pH. The decrease of pH caused by the carboxylic groups generated by PME during the deesterification of the pectin solution at pH 7.8 and ambient temperature (22 °C) was periodically recorded for a period of time depending on the enzyme activity. The initial slope of the curve fitted to the evolution of pH against the incubation time was considered as PME activity.

The determination of the effect of pH on PME activity was performed by adjusting the pH at different values. In the assays with different enzyme concentration (Fig. 2),

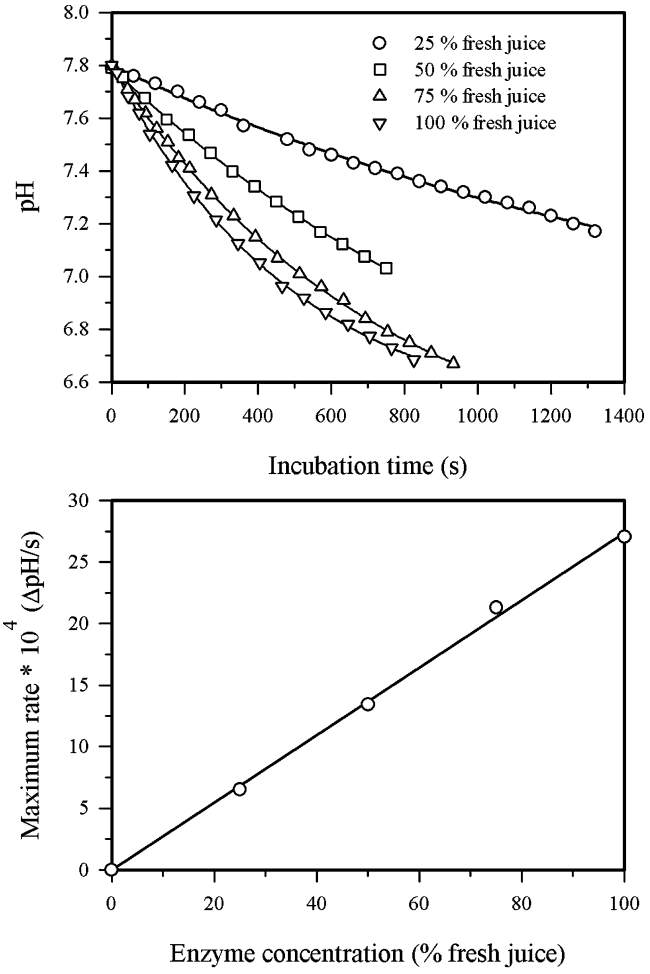

Fig. 2 Relation between the proportion of fresh Ortanique juice and the maximum rate measured by the slope of the fitted curve at time zero

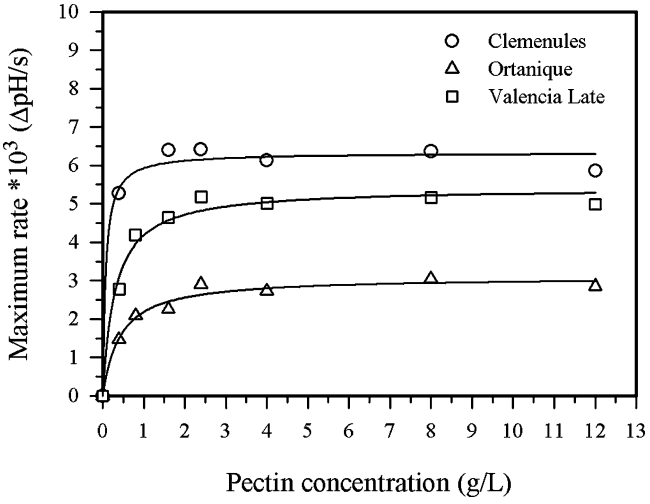

Fig. 3 Relation between the pectin concentration and the PME activity in fresh citrus juices

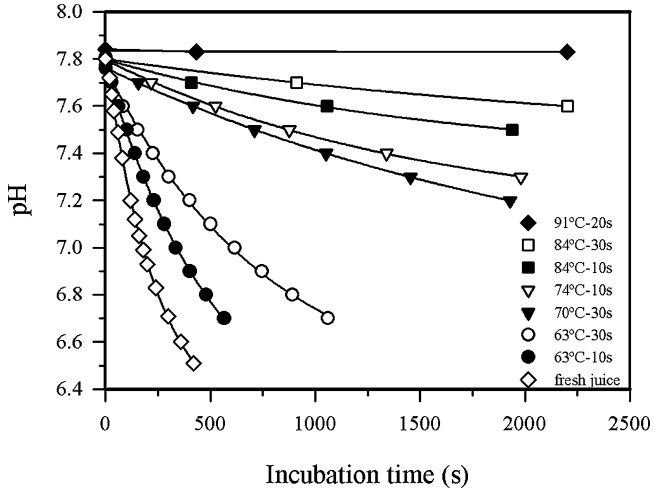

Fig. 4 Evolution of pH with the incubation time of a fresh Clemenules juice and the same juice treated at different heating conditions

Ortanique fresh juice was previously diluted with different amounts of the same juice after being boiled for 5 min.

In the evaluation of thermostable PME activity, fresh juice samples were subjected to a heat treatment at 70 °C for 5 min [11].

Dilution of the juice with NaOH solutions for the pH adjustment was taken in consideration for the calculation of the PME activity. The dilution of the pectin solution was neglected since only a very small amount of NaOH solutions were added and, according to Michaelis kinetics, this small dilution of substrate cannot affect the PME activity.

Results and discussion

The optimum pH for the PME activity was checked in juices obtained from Clemenules mandarin, Valencia-late orange and Ortanique hybrid. In each experience, the starting pH of the juice–pectin solution mixture was adjusted at a different value, and the change of the pH with the incubation time was recorded. The slope at time zero, calculated in these experiences by fitting of points recorded during the first 3 min to a straight line, was taken as measurement of the PME activity at the starting pH. Figure 1 represents the curves showing the response of total PME activity (upper plot) and thermostable PME activity (lower plot) to the pH. As it can be observed, the optimum pH ranged in all varieties from 7.8 to 7.9, either in fresh juices or in juices treated at 70 °C for 5 min. The PME activity estimates were not corrected by the effect of alkaline deesterification, since it had no influence on the determination of optimum pH. Its share to the pH decrease was 0.15×10^{-3} ΔpH/s at pH 8.5, but only 0.04×10^{-3} at pH 8, and not detected at pH 7.8.

The evolution of pH with time due to PME activity followed a monotonically decreasing curve for any starting pH (Fig. 2). When the initial pH was 7.8, experimental points could be accurately fitted ($R^2>0.999$) to

a single exponential decay curve reaching an asymptote [pH=(7.8−a)exp(−kt) +a], and the value of the slope at zero time [slope = (7.8−a)k] can be considered the PME activity. This is an easy way to calculate relative PME activities, being the units of PME activity reported as ΔpH/s.

These units (ΔpH/Δt) can be converted to the International System of Units for enzyme activity as follows: $\Delta pH = pH_0 - pH_t = -\log [H^+]_0 - (-\log [H^+]_t) = \log ([H^+]_t/[H^+]_0)$, where subscripts 0 and t indicate the initial state and t seconds after, respectively.

$$\frac{[H^+]_t}{[H^+]_0} = 10^{\Delta pH}$$

$$[H^+]_t = [H^+]_0 \times 10^{\Delta pH}$$

$$[H^+]_t - [H^+]_0 = [H^+]_0 \times (10^{\Delta pH} - 1)$$

The International Unit of PME activity is:

$$\frac{[H^+]_t - [H^+]_0}{\Delta t} = \frac{[H^+]_0}{\Delta t}(10^{\Delta pH} - 1),$$

When t tends to zero, ΔpH also tends to zero and ΔpH/Δt is the slope of the fitting curve at t=0. Then, the expression ($10^{\Delta pH}$−1) tends to (ln 10)×ΔpH=2.3ΔpH. The International System of Units for enzyme activity is then 2.3(ΔpH/Δt)$[H^+]_0$. In our assays, since pH_0=7.8 and the dilution factor of juice = 5, PME activity (katal) = ($10^{-7.8}$×2.3×5)×(ΔpH/Δt) = 182.26 × 10^{-9} ×(ΔpH/Δt). The SI unit habitually used to express the enzyme activity is the nanokatal (nanomol per second), thus the conversion factor is 182.26.

An accurate adjustment of pH of both solutions to a given pH is not a easy duty. Small amounts of the added dilute solutions of NaOH strongly modified the pH of the pectin solution. On the other hand, in citrus juices adjusted at pH 7.9 with NaOH, the pH does not remain stable but decreases due to the PME acts on native pectin, giving free carboxylic groups. When adding 5 ml of citrus juice to 20 ml of pectin solution (each solution with the pH adjusted but with unavoidable differences between them), the pH of the mixture tends to that of the citrus juice (more buffered than the pectin solution) but quickly decreased, and it is necessary to work very fast. The solution we adopted was to reach a starting pH of the mixture around 7.9 and began to record time (t) and pH from the moment that a pH = 7.8 was reached.

Ten repetitions of the measurement of the PME activity in the same sample (fresh juice from Clemenules), working in the operating conditions described above, and fitting the experimental points to the exponential decay function, gave values with a variation coefficient of 7.8%.

The proportionality between PME concentration and activity was tested by adding different mixtures (25, 50, 75 and 100%) of fresh juice with juice boiled (100 °C during 5 min) to the pectin solution. Figure 2 shows the results obtained with Ortanique juice. As it can be observed, experimental points were accurately fitted to the exponential function (upper plot), and the calculated maximum rate is proportional to the fresh juice concentration (lower plot).

Figure 3 shows the relation between PME activity and pectin concentration. Experimental points were fitted to the Michaelis function. The curves show that substrate (pectin) concentrations above 2.5 g/l does not practically affect the measurement of the PME activity of citrus juices. This result is helpful for the routine work in the laboratory, since the preparation of pectin solutions is a time-consuming operation due to its low solubility. For measuring PME activity most researchers [12, 13, 16, 17, 22] use pectin solutions of 10 g/l but more diluted solutions could be used. In our work we chose a pectin concentration of 5 g/l.

This procedure for the determination of PME activity presents as main advantage its easiness and its accuracy, and needs only the use of a pH-meter and a chronometer.

Pectin modifying enzymes (polygalacturonase and PME mainly) play an important role in the loss of firmness of fruit during its maturation [23]. Thus, these activities can change along the ripening processes. Table 1 shows the PME activity in four cultivars of citrus fruits at different ripening stages.

In all theses cultivars, PME activity in vitro decreased during the early ripening stages assayed. Clementine cultivars (Clemenules and Marisol) and Valencia-late oranges showed similar levels of total PME activity, and similar

Table 1 Total and thermostable PME activity for different cultivars and several maturity indices

Cultivar	Maturity index	PME activity (nanokatal/ml)		Thermostable/total PME (%)
		Total	Thermostable	
Clemenules 1	8.6	1.1527	0.0813	7.1
Clemenules 2	12.4	0.9861	0.0826	8.4
Marisol 1	8.5	1.2833	0.0588	4.6
Marisol 2	8.7	1.0157	0.0637	6.3
Marisol 3	11.1	0.9976	0.0521	5.2
Marisol 4	13.3	0.9680	0.0391	4.0
Valencia-Late 1	9.6	1.1904	0.1514	12.7
Valencia-Late 2	11.6	0.9773	0.1229	12.6
Ortanique 1	10.2	0.6511	0.2227	34.2
Ortanique 2	13.5	0.5093	0.1233	24.2

Table 2 Residual PME activity of Clemenules juices corresponding to curves of Fig. 4

Heat treatment (T (°C)−t (s))	PME activity (nanokatal/ml)	Residual activity (%)
63 °C−10 s	0.5581	46.9
63 °C−30 s	0.3344	28.1
70 °C−30 s	0.0812	6.8
74 °C−10 s	0.0774	6.5
84 °C−10 s	0.0469	3.9
84 °C−30 s	0.0231	1.9
91 °C−20 s	0.0006	0.05
Fresh juice	1.1904	100

evolutions with fruit ripening, but a different thermostable PME activity (2–3 times higher in Valencia-late).

PME activity of juice from the Ortanique showed a very different behaviour. Its total PME activity was lower (approximately half) than those from the other cultivars assayed but the thermostable PME was higher. The ratios between thermostable and total PME activities were 4.0–8.4% for Clementine mandarins, 12.6–12.7% for Valencia-late oranges and 24.2–34.2% for Ortanique fruits. This result may justify the different behaviour of juices from Clementine and Ortanique in heat treatments.

Figure 4 shows the curves corresponding to the PME activities of fresh and heat-treated juices from Clemenules, and Table 2 the residual activities calculated by fitting each curve to the decay exponential function. Soft heat treatments (70 °C, 30 s or 74 °C, 10 s) were enough to reduce the PME activity under the maximum residual level (10%) suggested by Irwe and Olson [2]. To reach a similar residual level of PME activity, the Ortanique juices required heat treatments at 85 °C for 10 s, such as we reported in a previous paper [24].

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