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PII: S0308-8146(97)00088-5

Food Chemistry, Vol. 61, No. 1/2, pp. 237–241, 1998

0308-8146/98 $19.00 + 0.00

Determination of enzymatic activities of commercial pectinases for the clarification of apple juice

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(Received 6 December 1996; accepted 4 April 1997)

Different methods for testing Polygalacturonase (PG), pectinesterase (PE), and pectinlyase (PL) activities were applied to Röhapect D5S (RHD5) and Pectinol (PA1) commercial enzyme preparations in an apple pectin substrate. The viscometric method for PG activity determination was satisfactory, but foreign proteins could affect the spectrophotometric determination of PL activity in solutions of enzyme preparations. Although 50°C was a well-defined breaking point where enzymes rapidly decrease their activity, rate and range of heat-inactivation were different depending on the activity assayed. While PG activity showed two periods of different thermolability, PL was monophasic and highly sensitive to heat. The pH dependence of the pectic enzyme activities was also studied over the 3–7 range. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Apple production, as far as annual worldwide crop quantity is concerned, ranks second after grapes (Binning and Possmann, 1993). Almost 25% of the apple crop is processed, mainly to juice and concentrate. Clarified apple juice concentrate is one of the most consumed fruit juices in the world. Problems in clarification of apple juice are caused mainly by the presence of pectic substances as suspended insoluble (pulp) particles (Heatherbell, 1981). Commercial pectic enzymes, or 'pectinases', are used in apple juice manufacturing to depectinize pressed juices in order to remove turbidity and prevent cloud-forming (Grampp, 1976). The available commercial pectinase preparations used in apple processing generally contain a mixture of pectinesterase (PE), polygalacturonase (PG) and pectinlyase (PL) enzymes (Dietrich *et al*., 1991). Complete pectin breakdown in apple juices can only be ensured if all three types of enzyme are present in the correct proportions. Apple juice processors generally lack reliable methods for checking the different enzyme activities. Application and success of pectinase products depend on the substrates on which they act. The problems in evaluation of pectinolytic activities are caused by the difficulty of standardizing fruit substrates. Acidity, pH and the presence of inhibitors or promoters of the enzymatic reaction depends on the variety of apple being processed to juice. The objective of this paper was (1) to revise simple methods for the testing of pectinolytic activities and (2) to determine the specific enzymatic activities of two different commercial pectinases in an apple pectin substrate.

MATERIALS AND METHODS

Materials

Röhapect D5S (RHD5) and Pectinol Al (PA1) were purchased from Röhm. GmbH pectinase (from *Aspergillus niger*), apple pectin (AGA: 77%, DE: 7%) and galacturonic acid were from Sigma Chem. Company (St Louis, USA). All the other reagents were of analytical grade and used without further purification.

Determination of polygalacturonase activity

Polygalacturonase (PG) activity was determined by following the viscometric reduction in apple pectin (AP) substrates, according to Lopez *et al*. (1994) with some modification. Viscosity variations were measured with a Brookfield Model RVDV-II viscometer, with fixed shear rate ($g = 122.3\ s^{-1}$) and velocity (100 rpm), at 30°C. The assayed substrate was composed of 13.5 ml of 6.2 mg mL^{-1} AP solution in a 0.1 M citrate–0.2 M phosphate

buffer. A standard curve was constructed by using the Sigma PG enzyme with known activity (11.8 U mg^{-1} protein; pH = 4; T = 25°C). Data were fitted to the following equation:

$$\eta_t = \eta_o e^{-\beta t} \quad (1)$$

where η_t and η_o are the apparent viscosity of substrate (mPa.s) at t = 0 and t = t, respectively, and β = exponential coefficient of viscosity decrease (s^{-1}). Results indicated that the β coefficient increased linearly with the enzyme activity [$\beta = (0.0193 \quad +2.834U) \times 10^{-3}$; $r^2 = 0.954$]. Polygalacturonase activity of commercial enzymes was estimated by measuring the viscosity at selected times after PG addition, and calculating the exponent β from equation 1.

Pectinesterase (PE) activity

Titrimetric method
Assays were performed by stirring 10 ml of AP solution (0.5%) and 0.01 M of NaOH titrating solution. Runs were started by adding 100 μl enzyme solution (RHD5 or PA1). One Unit of PE activity was taken as the amount of NaOH (mEq) consumed per min to keep constant pH value.

Spectrophotometric method
PE activity was also tested by a continuous spectrophotometry method based on the addition of the enzyme to 3.5 ml pectin-indicator solution (100 ml apple pectin (5%) with 10 ml of pH indicator (Bromocresol Green, 0.017%). The pH value was adjusted with 1M NaOH. Absorbance at 617 nm and 25°C (A_{617}) was measured with a Perkin-Elmer Lambda 3 spectrophotometer. Decrease in A_{617} after adding 100 μl of enzyme solution, was registered. A standard curve was made by following the A_{617} change induced by different concentrations of AGA solutions at pH 4.5 and 5.1. A linear response was obtained between ΔA_{617} and AGA content (mEq):

$$\Delta A_{617,\ pH=5.1} = 0.0211 + 0.1312\ \text{AGA} \quad r^2 = 0.995 \quad (2)$$

$$\Delta A_{617,\ pH=4.5} = 0.0130 + 0.0946\ \text{AGA} \quad r^2 = 0.969 \quad (3)$$

One Unit of PE activity was taken as the amount in mEq of AGA released per min.

Pectinlyase (PL) activity

PL activity was determined spectrophotometrically by following the A_{235} value increase due to the double C_4–C_5 bond, forming in the pectin molecule during enzymatic reaction (Spagna *et al.*, 1993). Two ml of 1.0% apple pectin solution were added to 2 ml PL solution in 0.1 M citrate–0.2 M phosphate buffer at 25°C. After 1 min with stirring, 2 ml of 0.5M H_2SO_4 were added to stop the enzymatic reaction. Blank tests were performed by adding the acid previous to the enzyme (no reaction allowed). Samples were read against blank and one Unit (U) of enzyme activity was taken as the amount necessary to increase 0.555 in absorbance in 1 min at 25°C and selected pH.

Activity as a function of temperature and pH

The effect of temperature on the activities of the assayed enzymes was studied by exposing enzyme solutions to different temperatures (50–80°C) and various times. Solutions were then cooled and the residual activity measured by the methods described above. Spectrophotometric determination of PL activity also required centrifugation of samples (10 000×***g*** for 5 min) before absorbance reading, in order to eliminate inactivated enzyme, precipitation due to heat treatment. The effect of pH on enzyme activities was also studied.

Protein content

Protein determination was done according to Gasbarro *et al.* (1972) based on the measurement of the absorbance at 540 nm after reaction between Biuret reagent (EDTA/Cu) and the enzymes, in water. A commercial kit ('PROTI 2'; Wienerlab, Santa Fe, Arg.) including a standard protein solution was used. This standard solution was checked (by supplier) for protein content with the Kjeldahl method. The results given below are the average values of three replicates for each experimental condition.

RESULTS AND DISCUSSION

Activities of commercial enzymatic preparations

Total protein content as well as polygalacturonase (PG), pectinesterase (PE) and pectinlyase (PL) activities of Röhapect D5S (RH5) and Pectinol A1 (PA1) at pH 4 and 5 are listed in Table 1. The amount of foreign proteins was greater in PA1 than in RHD5 pectinase. These proteins and some other unknown compounds (highly coloured polymers) existing in the PA1 solutions were assumed to be responsible for interference which invalidates results during PL activity measurement. On the other hand, lyase activity was low in RH5 while galacturonase activity represented 70–80% of total activity, depending on pH value.

Table 2 compares esterase activities determined by the spectrophotometric and the titrimetric methods. Activities determined by absorbance measurement at pH = 4.5 were lower than those determined titrimetrically. The

Table 1. Enzymatic activities and total protein content

Sample	Proteins (mg mg^{-1})	pH	Activities		
			PG (U mg^{-1})	PE (U mg^{-1})[a]	PL (mU mg^{-1})
Röhapect D5S	0.056 ± 0.003	4.0	0.719 ± 0.017	0.273 ± 0.017	4.39 ± 0.14
		5.0	0.631 ± 0.029	0.145 ± 0.008	8.41 ± 0.99
Pectinol A1	0.138 ± 0.002	4.0	0.092 ± 0.006	0.084 ± 0.002	NE
		5.0	0.076 ± 0.002	0.048 ± 0.004	NE

[a]Titrimetric method.
NE = no evaluated.
Data are average values of three determinations ± sample standard deviation.

difference was attributable to a decrease in bromocresol green sensitivity (Vilariño *et al.*, 1993).

Heat stability

Figure 1 shows the residual polygalacturonase activity of PA1 and RHD5 enzymes after 30 min of heating at different temperatures. They started to become inactivated at temperatures higher than 50°C. Röhapect D5S enzyme was more temperature sensitive and 50°C was a very well-defined breaking point where the enzyme rapidly loses its activity. Thermal inactivation of polygalacturonase activity at optimal pH and 60°C was better followed when log β is plotted against time (Fig. 2). The rate of PG activity decrease could be divided into two periods. The first period was characterized as a thermolabile fraction, with D values equal to 15.3 min and 193 min for RHD5 and PA1, respectively, where D is the decimal reduction time. The second period can be defined as the thermo-resistant fraction of the enzyme, representing 67.3 and 26.5% of initial activity for PA1 and RHD5, respectively. Sakai *et al.* (1993) and Liu and Luh, (1978) reported that the optimal temperature for PG activity was in the range 30°–50°C. Both authors indicated that, for temperatures greater than 50°C, inactivation was notable after a short period of heating. Moreover, the optimal temperature is also a function of the type of substrate to be treated (Ben-Shalom *et al.*, 1986).

Table 2. Comparison between titrimetric and spectrophotometric methods for determination of pectinesterase activities

Sample	pH	Activities (U mg^{-1})	
		Titrimetric method	Spectrophotometric method
Röhapect D5S	4.5	0.316 ± 0.016	0.218 ± 0.004
	5.1	0.113 ± 0.010	0.116 ± 0.024
Pectinol A1	4.5	0.080 ± 0.001	0.059 ± 0.004
	5.1	0.030 ± 0.005	0.027 ± 0.003

Data are average values of three determinations ± sample standard deviation.

The inactivation curve of lyase activity of RHD5 is also shown in Fig. 1. As in the case of PG activities, 50°C can be easily identified as a breaking point where PL rapidly inactivates. However, when representing log activity against time at constant temperature (Fig. 3), a biphasic plot indicating different fractions was not found and the PL activity (RHD5 enzyme) was highly susceptible to heat (D = 6.7 min). Similar results were reported by Alkorta *et al.* (1996) who found that PL from *Penicillum italicum* was active after 1 h at 50°C but was completely inactivated after the same period at 60°C. Another PL (PLY23) produced from *Aspergillus japonicus* showed a half-life of only 5 min at 55°C.

The commercial enzymes assayed in this work were more heat-tolerant than purified fractions (Liu and Luh, 1978). This phenomenon was attributable to the thermoprotective action of impurities.

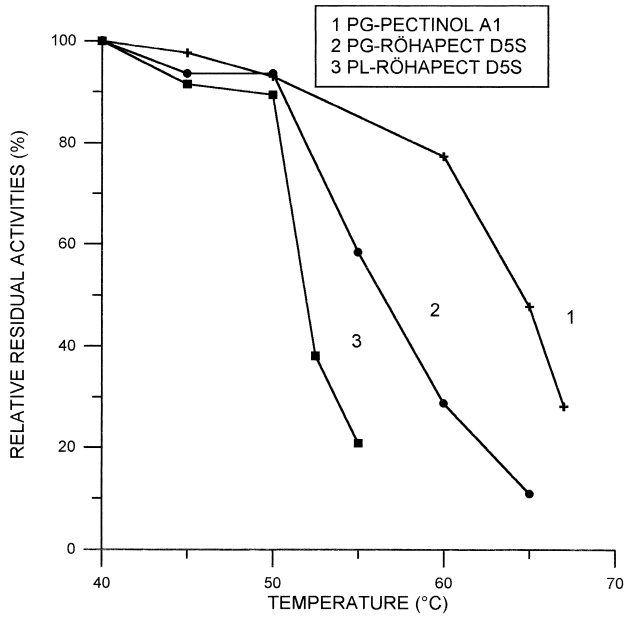

Fig. 1. Enzymatic residual activities after thermal treatment (30 min at different temperatures) of enzyme solutions in 0.1 M citrate–0.2 M phosphate buffers (at optimum pH).

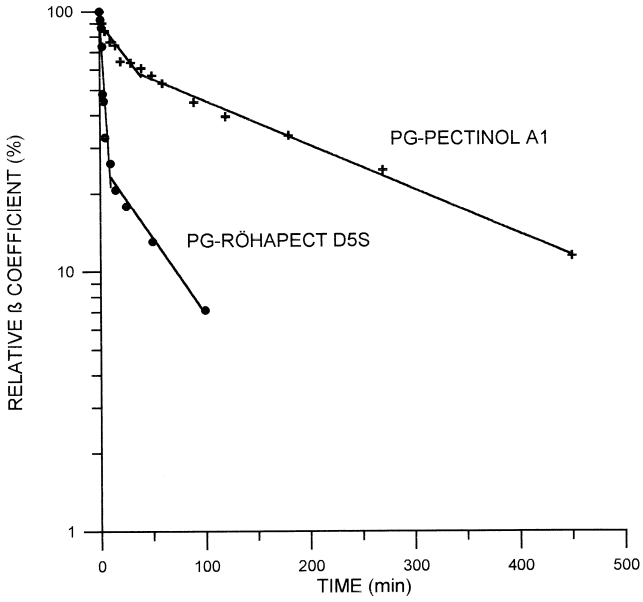

Fig. 2. Thermal inactivation rate of polygalacturonase at 60 °C in Röhapect D5S and Pectinol A1 solutions in 0.1 M citrate–0.2 M phosphate buffers at optimal pHs.

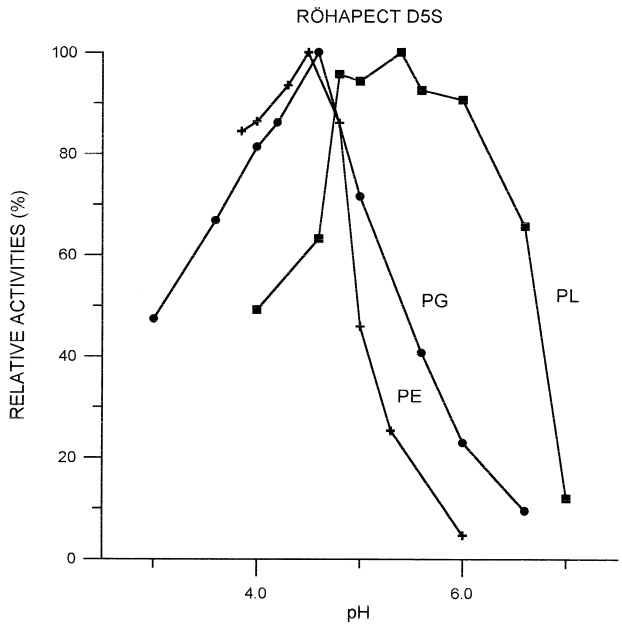

Fig. 4. Effects of pH on the enzymatic activities of Röhapect D5S.

pH-dependence of the pectic enzyme activities

PG and PE activities of RHD5 enzyme vs pH were tested and plotted in Fig. 4. The optimum pH was approximately 4.6. However, the curve for lyase activity as a function of pH was much broader, being difficult to identify as a single optimal value. In this case, an optimal range of pH 5–6 may be defined. Figure 5 also shows results obtained in the case of PA1 enzyme. The optimal activity range for PG was broader in PA1 than in RHD5. Both enzymes (PG and PE) show a rapid decrease in activity at about pH = 5 and become practically inactivated near neutrality. However, this problem becomes irrelevant because pH values of apple juice from Red Delicious and Granny Smith varieties are about 4 and 3.5, respectively (Toribio and Lozano, 1984). It can be deduced from Fig. 4 that, during the enzymatic clarification of the relatively acid Granny Smith juice, as much as 40% of PG and PE inactivation can be expected. It was found that a shift in the optimal pH toward the acid zone (Spagna *et al.*, 1993) or a broadening of the optimal activities range (Ates and Pekyardimci, 1995) could be obtained after enzyme immobilization on appropriate supports. As a

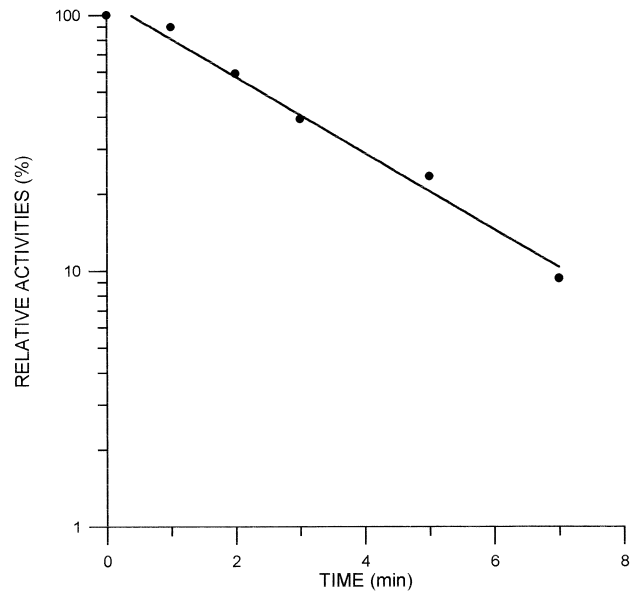

Fig. 3. Thermal inactivation rate of pectinlyase at 60°C in Röhapect D5S solutions at optimum pH.

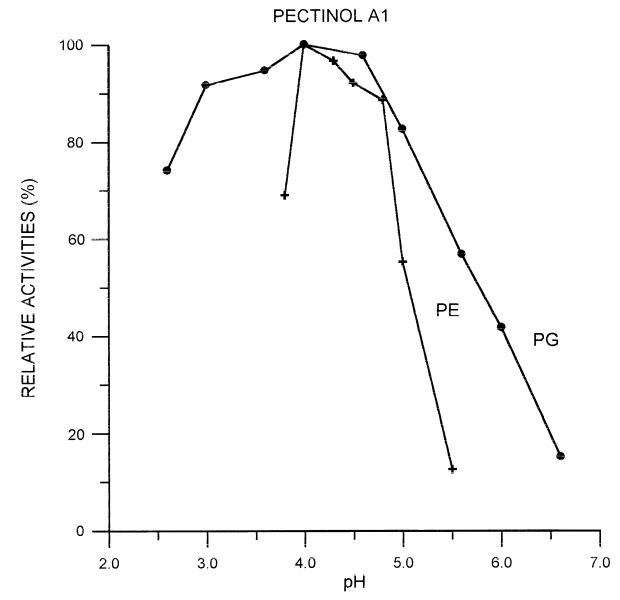

Fig. 5. Effects of pH on the enzymatic activities of Pectinol A1.

matter of fact, the author of this work is involved in the immobilization of commercial pectinase and evaluating its possible application in apple juice clarification.

Effect of enzyme concentration on pectin hydrolysis

The exponential coefficient of viscosity reduction (β) defined in equation 1 increased linearly with the amount of enzyme added to the apple pectin substrate:

$$\beta = a\varepsilon + b \tag{4}$$

where ε = concentration of enzyme (mg ml^{-1}), and a and b are fitting parameters. Results and corresponding regression coefficient are listed in Table 3. Similar results were obtained by Lopez *et al.* (1994) for commercial Borozym M5 (Endo-polygalacturonase from *Rhizopus oryzae*) using sodium polygalacturonate as substrate. The rate of absorbance decrease during the PE activity test also showed a linear dependence on enzyme concentration added to the apple pectin (plus pH indicator) solution:

$$Abs_r = \alpha\varepsilon + \delta \tag{5}$$

where Abs_r = rate of Absorbance decrease (min^{-1}); ε = enzyme concentration (mg ml^{-1}), and α and δ are fitting parameters (Table 4). Vilariño *et al.* (1993) found similar results for fungic pectinesterase at the same pH as the indicator. Finally, the increase in A_{235} when measuring PL activity at pH = 6 also followed a linear relationship with enzyme concentration. Fitting parameters are also listed in Table 4.

In conclusion, we have determined the different pectinase activities of two commercial enzymes usually utilized in the apple juice industry for clarification purposes.

Table 3. Parameters of equation (4), valid for apple pectin substrate hydrolysis at pH = 5 and PG activity

Enzyme	a 10^3 (ml s^{-1} mg^{-1})	b 10^3 (s^{-1})	r^2
Röhapect D5S[a]	24.5	0.0569	0.994
Pectinol A1[b]	2.91	0.0589	0.985

[a]Valid up to $\varepsilon = 0.2$ mg ml^{-1}.
[b]Valid up to $\varepsilon = 0.4$ mg ml^{-1}.

Table 4. Parameters of equation (5), for PE and PL hydrolysis of apple pectin substrate

Enzyme	Activity	Concentration (mg ml^{-1})	α 10^3 (ΔA ml min^{-1} mg^{-1})	δ 10^3 (ΔA min^{-1})	r^2
Röhapect D5S	PE[a]	0– 1.4	86.6	−6.33	0.948
	PL[b]	0– 5	84.8	74.2	0.908
Pectinol A1	PE[a]	0– 5.5	17.1	−0.666	0.987

[a]pH = 5.1.
[b]pH = 6.

The viscometric and spectrophotometric assay here proposed are reasonably good for commercial enzyme characterization. However, some interference attributable to foreign proteins can invalidate results when measuring the lyase activity of pectinase solutions. As apple juice clarification is usually done at 45°–50°C, special care must be taken to avoid excessive inactivation when lyase activity is considered important.

It must be noted that a more specific evaluation of commercial pectinases should be done with the natural substrate (apple juice), because some differences may be observed when checking pectinolytic activities with apple pectin solutions. Moreover, it is known (Dietrich *et al.*, 1991) that commercial enzyme preparations cause a certain degree of side activities other than those studied in this work. However, the simple tests evaluated and modified here are useful tools for pectinase evaluation and comparison.

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