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Considerations on Endopolygalacturonase Activity and Determination of Comparison Ratios with Emphasis on the Influence of the Degree of Substrate Esterification

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A study on the determination and standardization of endopolygalacturonase (EPG) activity is reported, with emphasis on the influence of the degree of substrate esterification using pure yeast EPG. Differences in the results, depending on how the EPG activity unit was defined, are described and discussed. From a theoretical analysis of the expressions established, a general equation for expressing EPG activity in standard international units was obtained, together with the proportional coefficient for each of the substrates studied. It was observed that for a wide range of enzyme concentrations good linear correlations were obtained. Analysis of the comparison ratio (CR) values calculated revealed that these do not differ significantly, except for low-methoxyl apple pectin, confirming the validity of the general expression obtained for pectins with different degrees of esterification. The anomalous CR value found for low-methoxyl (LM) apple pectin is discussed.

KEYWORDS: Endopolygalacturonase; viscosimetry; comparison ratio; pectin

INTRODUCTION

Pectic substances are high molecular weight polysaccharides that are present in the cell walls of higher plants. The pectin molecule consists of a main backbone of α-(1,4)-linked D-galacturonic acid units, which are partially methyl-esterified by the carboxyl group. Pectins are naturally degraded by pectinolytic enzymes, which have been classified in two main groups: pectinesterases, which are able to de-esterify pectin through the removal of methoxyl residues, and depolymerases, which readily split the main chain. The depolymerizing enzymes are divided into polygalacturonases (PG), enzymes that cleave glycosidic bonds by hydrolysis, and pectin-lyases, which break the glycosidic linkages by β-elimination. The latter two enzymes are classified on the basis of whether their mechanism of action is random (endo-) or terminal (exo-).

Pectinolytic enzymes, or pectinases, are used in many industrial food applications, particularly in fruit and vegetable processing. Among such applications are the extraction and clarification of fruit juices, winemaking, cider production, and the maceration of vegetables (*1*−*3*). Commercial pectinases consist of mixtures of several pectinolytic enzymes and other carbohydrases. The macerating and clarification power of these products is related to the level of endopolygalacturonase (EPG) activity. The simultaneous presence of endo- and exo-splitting PG demands consideration of the concept of the comparison ratio (CR), which is the rate between viscosimetric EPG activity and total PG (TPG) activity as estimated by the increase in reducing power when acting on the substrate (*4*). The CR is a measure of the endo character of a pectinolytic enzyme and it is of great technological importance. Commercial enzymes with high CR values are preferred because they are able to afford a considerable degree of depectinization with minimum formation of reducing groups, which may produce alterations in elaborate products such as the Maillard nonenzymatic browning reaction. To date, EPG activity has been defined arbitrarily, and there is no general consensus about what its precise definition should be, thus hindering the comparison of CR values reported so far in the literature.

Additionally,it is generally considered that CR values do represent a measure of the macerating activity of a given pectinolytic enzyme, although little is known about the influence of the degree of esterification (DE) of the substrate on EPG activity. Thus, the aim of the present work was to determine the influence of the substrate DE in the endo character in PG, using two different ways to define the unit of EPG activity measured, and to establish an expression that would allow us to calculate this enzymic activity in standard international units.

MATERIALS AND METHODS

Enzyme Production and Purification. The enzyme was obtained under static culture in YNB (Difco)−glucose medium from the *Kluyveromyces marxianus* CCEBI 2011 yeast strain (Culture Collection of the Industrial Biotechnology Studies Centre, Universidad de Oriente, Santiago de Cuba, Cuba), purified according to the method of Serrat et al. (*5*).

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Substrates. Polygalacturonic acid (sodium salt; PGA) and citrus pectin (60−65% DE), were from Sigma (St. Louis, MO). High-methoxyl (HM) apple pectin (70−75% DE) was from Fluka Chemie AG (Buchs, Switzerland) and low-methoxyl (LM) apple pectin from Ensufarma (Havana, Cuba). All substrates were prepared at 0.5% (w/v) in 50 mM sodium acetate buffer, pH 5.0.

Enzyme Assays. TPG activity was estimated from the increase in reducing power from substrates at 37 °C in 10 min and evaluated by using Somogyi's method (*6*) as modified by Nelson (*7*). A typical reaction mixture contained 400 μL of substrate and 100 μL of the appropriate enzyme dilution in 50 mM sodium acetate buffer, pH 5.0. One unit of TPG was defined as the amount of enzyme producing 1 μmol·min^{-1} of galacturonic acid or equivalent reducing power under these conditions. EPG activity was determined viscosimetrically using a Canon−Fenske capillary viscometer (model 5354/2). The reaction mixtures contained 4.9 mL of substrates and 0.1 mL of a suitable dilution of the enzyme, both preincubated at 37 °C. The enzyme was added to the substrate and mixed rapidly; then, time flows were measured at different time intervals. Time flow at zero time was measured in a reaction mixture containing acetate buffer instead of the enzyme. One unit of EPG activity was defined (i) as the amount of enzyme that reduced the specific viscosity to 50% in 1 min (EPGv) (*8*) or (ii) as the amount of enzyme that increased one unit of specific fluidity in 1 min (EPGf) (*9*). Specific fluidity (F_{sp}) was defined as the inverse of specific viscosity. Specific viscosity (η_{sp}) was calculated as follows:

$$\eta_{sp} = (\text{flow time of sample/flow time of buffer}) - 1 \quad (1)$$

In the evaluation of EPG, the dilution factor (D) during viscosimetric assay (reaction mixture volume/diluted enzyme volume), in addition to the enzyme dilution prior to the assay, was also considered.

EPG Standardization. Assuming that at the beginning of the reaction product formation increases linearly with time, then the average molecular weight (M) of a given product as a function of the reaction time can be expressed as

$$M = C/(At + C/M_0) \quad (2)$$

where C = substrate concentration (μg·mL^{-1}), A = EPG enzymatic activity (μmol·min^{-1}·mL^{-1}), t = reaction time (min), and M_0 = substrate average molecular weight at zero time (g·mol^{-1}).

For diluted polymeric solutions, it can be assumed with a reasonable degree of certainty that

$$\eta_{sp} = K_1(M_v)^a \quad (3)$$

where K_1 and a are constants that depend only on the polymer−solvent system and are completely independent of the molecular weight; for a pectin−water system $a = 1.34$ (*10*). M_v corresponds to the average viscometric molecular weight.

Assuming that the average viscometric molecular weight ratio remains constant during the assay period (which is true at the beginning of reaction because of the random enzymatic attack), one has

$$F_{sp} = K_2/M^a = K_1(At + C/M_0)^a/C^a \quad (4)$$

where

$$K_2 = 1/K_1 \quad (5)$$

because K_2 and C are constants, affording

$$K_3 = K_2/C^a \quad (6)$$

and

$$F_{sp} = K_3(At + C/M_0)^a \quad (7)$$

Because it is assumed that enzymatic activity is proportional to the rate of change of specific fluidity, one has

$$(dF_{sp}/dt) = K_3a(At + C/M_0)^{a-1}A \quad (8)$$

and because enzymatic activity is determined from changes in fluidity at zero time, then

$$(dF_{sp}/dt)_{t=0} = K_3a(C/M_0)^{a-1}A \quad (9)$$

or

$$(dF_{sp}/dt)_{t=0} = K_4A \quad (10)$$

Thus

$$A = K_4^{-1}(dF_{sp}/dt)_{t=0} \quad (11)$$

By combining eqs 5, 6, and 9, we have

$$K_4^{-1} = K_1CM_0^{a-1}/a \quad (12)$$

The K_1 constant was obtained (making TPG = EPG) from the TPG and $(dF_{sp}/dt)_{t=0}$ values for the nonesterified substrate (PGA), and later K_4^{-1} was calculated with this value for the other substrates.

Analytical Determinations. Galacturonic acid was assayed by using the carbazole colorimetric method (*11*), DE was determined from colorimetric estimation of the methanol, according to the procedures of Wood and Saddiqui (*12*), and the molecular weight of the pectic substrates was estimated by viscosimetry (*10*).

Table 1. Substrate Characterization

substrate	mol wt[a] (g·mol^{-1})	galacturonic acid content (%)	DE (%)
PGA	16000	87.2	6.0
LM apple pectin	25700	68.6	28.9
citrus pectin	65600	79.0	63.4
HM apple pectin	48700	70.2	74.8

[a] Viscosimetric average molecular weight.

RESULTS AND DISCUSSION

Table 1 shows a characterization of the substrates studied. The values of galacturonic acid contents and DE are consistent with the manufacturer's specifications. Molecular weights increased with DE, except for HM apple pectin, which showed an intermediate molecular weight value between those corresponding to LM apple pectin and citrus pectin. All of the values obtained for these parameters fell within the range reported by the manufacturers. Partial de-esterification of natural pectins is usually carried out through alkaline saponification, which elicits moderate macromolecule degradation. This could explain why the molecules with the lowest DE values also exhibit the lowest molecular weights.

When TPG activity was assayed, it was found to decrease with DE (**Figure 1**). A similar type of behavior for yeast PG was also reported by Barnby et al. (*13*), although in the present study the activity values observed on highly esterified substrates were higher. An interesting observation was that lowering of TPG activity showed a linear match with DE ($R^2 = 0.98$) and also that for the highest DE value almost 50% of TPG activity on PGA was detected. In their study on kinetic constants for the enzyme studied here, Serrat et al. (*5*) found that the cause of the reduced catalytic efficiency of PG on esterified substrates was consistent with the dramatic reduction in enzyme−substrate affinity (as revealed by the K_M value) than with a lower k_{cat}. However, because enzymatic activity determination was carried

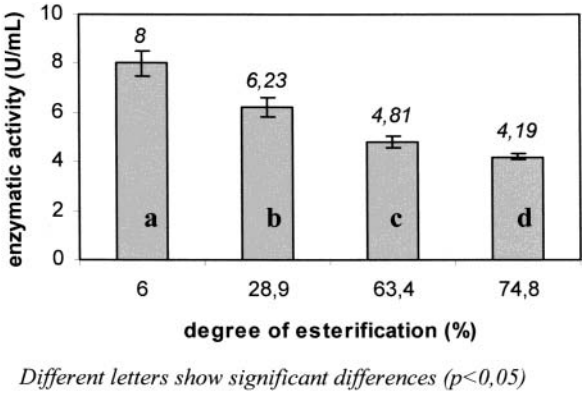

Figure 1. TPG activity according to substrate DE.

out at high substrate concentrations and at the beginning of the reaction, it must be expected that DE would not affect the enzymatic activity values very much. Only if the enzyme is evaluated on a completely esterified substrate can it be concluded that it is a polymethylgalacturonase.

Figure 2 shows the specific viscosity and fluidity curves for each substrate. As can be observed, for all cases—except PGA—a good linear correlation can be established between specific fluidity and reaction time (until a 50% reduction in viscosity has been attained). Thus, the values corresponding to EPGv and EPGf can be calculated from the regression parameters of specific fluidity versus reaction time line. When acting on PGA, a linear behavior for specific fluidity can be considered only up to a 35% reduction in viscosity, and hence in this case EPGv should be calculated directly from specific viscosity measurements. PGA has been usually considered a good substrate for PG activity determination, but the results reported here suggest that the issue should be revised. It is known that the relationship between viscosity and molecular weight varies from polymers with a low degree of polymerization to polymers with a high degree. This in turn could explain the observed differences between PGA and other pectic substrates, which show higher molecular weights.

The results for EPG on the substrates studied are shown in **Figure 3**. It was expected that the EPG values would be proportionally related to the TPG values because pure enzyme was employed. The results, however, did not match such an expectation. The differences between EPGv and EPGf can be explained on the basis that the former would be affected by the initial specific viscosity value (at zero time), whereas the latter would not. Moreover, for those substrates with higher molecular weights it corresponded to a low initial molar concentration and, also, a lower number of bonds must be hydrolyzed to reach any molecular weight fraction. Therefore, it can be concluded that expressing EPG activity as a function of the increase in specific fluidity of the substrate is more appropriate. In an attempt to standardize the determination of another endoglycohydrolase (carboxymethylcellulase; CMCase), Hulme (*14*) proposed a formula that correlates CMCase activity expressed in international units with the rate of change in specific fluidity during the initial minutes of the enzymatic reaction. These differences resulting from the definition used to express EPG activity should be taken into account when EPG values are reported.

From the values for TPG and EPGf for PGA obtained in the present study it is possible to propose a general expression that allows one to express EPG activity in international units:

$$\mathrm{EPG} = 0.456[\mathrm{d}(1/\eta_{sp})/\mathrm{d}t]_{t=0}D$$

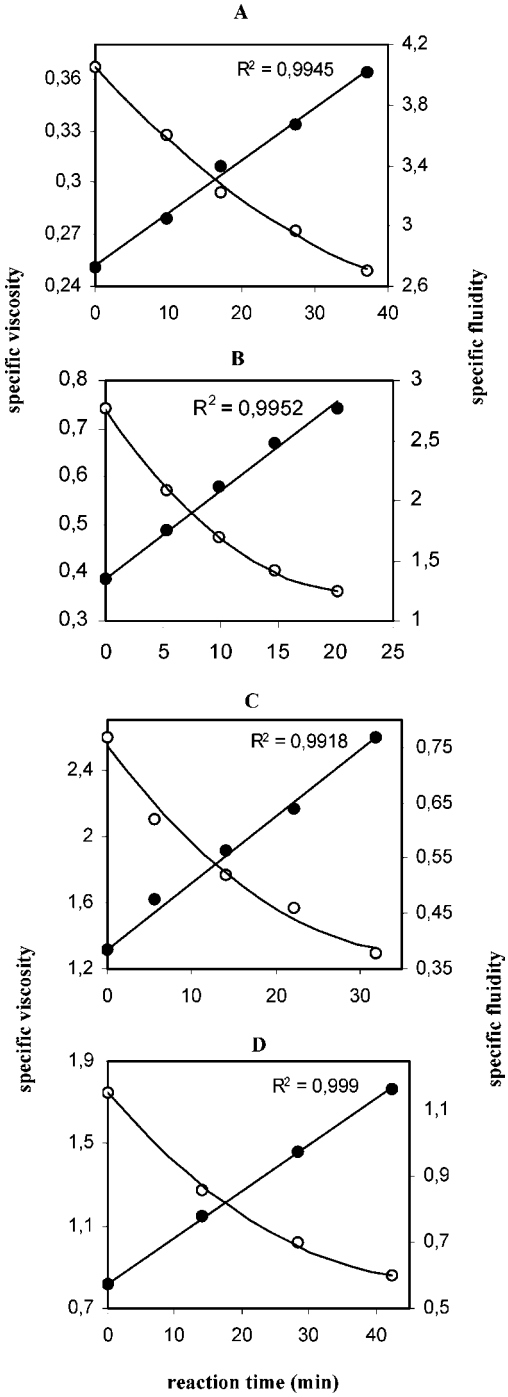

Figure 2. Changes in specific viscosity and fluidity during the course of the enzymatic reaction: ○, specific viscosity; ●, specific fluidity; (**A**) PGA; (**B**) LM apple pectin; (**C**) citrus pectin; (**D**) HM apple pectin.

Specific fluidity in relation to the reaction time derivative is calculated from the regression line at the very beginning of the enzymatic reaction (as much as up to a 50% decrease in the specific viscosity). Thus, CR calculated using EPG values does express the true *endo*-acting activity and allows reliable comparisons between different CR values.

Table 2 shows K_4^{-1} and the standardized EPG and CR values. For highly esterified substrates, the CR values do not differ significantly ($p < 0.05$) from 1.0, in agreement with what would be expected, because pure enzyme was used in the assays. An anomalous CR value was obtained from LM apple pectin (28.9% DE), and this differed significantly from unity. Assuming that the methoxylated galacturonic acid residues are randomly

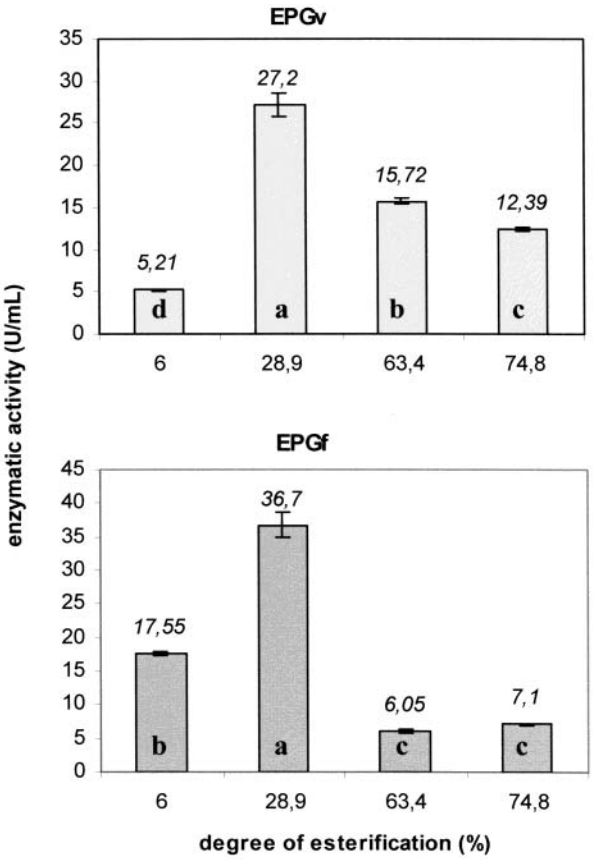

Figure 3. EPG activity according to the definition used and substrate DE.

Table 2. K_4^{-1}, Viscosimetric and Standardized EPG Activities, and Comparison Ratio Values for the Substrates Studied

substrate	DE (%)	K_4^{-1}	EPGf[a] (units·mL^{-1})	EPG[a,b] (units·mL^{-1})	CR[a,c]
PGA	6.0	0.456	17.55 ± 0.32	8.00 ± 0.15	1.00 ± 0.08 b
LM apple pectin	28.9	0.536	36.70 ± 1.94	19.67 ± 1.04	3.16 ± 0.38 a
citrus pectin	63.4	0.737	6.05 ± 0.19	4.46 ± 0.14	0.93 ± 0.07 b
HM apple pectin	74.8	0.666	7.10 ± 0.21	4.72 ± 0.14	1.13 ± 0.07 b

[a] Values are given as means ± standard deviation. [b] Standardized EPG. [c] Different letters show significant differences ($p < 0.05$).

distributed as blocks on the backbone (*15*) and that the catalytic efficiency of the enzyme is higher on glycosidic bonds between non-methyl-esterified galacturonic units, then enzymatic splitting will be limited to the fraction of the galacturonan backbone that is not esterified. This could explain the apparent reinforcement in the endo character observed for LM apple pectin, which affords large fragments in the course of enzymatic hydrolysis. For higher DE (>60%) the probability of formation of small fragments must be higher, and similar TPG and EPG values will be obtained. It must also be taken into account that TPG activity determination is carried out during longer (2.5 times) reaction times than EPG, which means that it is also affected by other factors such as a reduction in the segment size of nonesterified regions (*5*). Other structural aspects due to the complexity of pectins (i.e., branching and neutral sugars) must also be considered.

The validity of the general expression for determining EPG activity (eq 11) at different enzyme dilutions was evaluated,

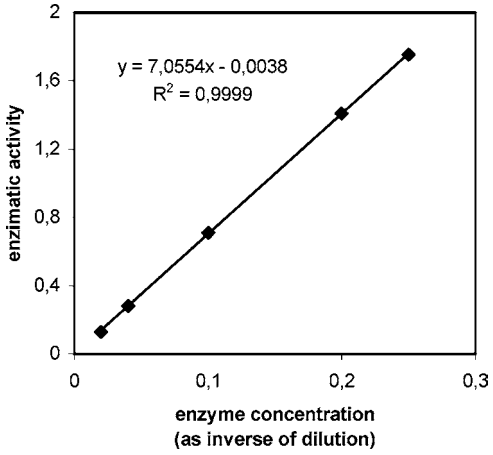

Figure 4. Effect of dilution on EPG viscosimetric determination, using HM apple pectin as substrate.

and the results using HM apple pectin are shown in **Figure 4**. A good linear correlation for EPG activity was obtained for a broad range of enzyme concentrations (up to 2 units/mL).

The results reported here should allow us to unify the expression of both EPG enzymatic activity and CR values on the basis of standard international units and should also help to correlate the values of different authors.

ABBREVIATIONS USED

CMCase, carboxymethylcellulase; CR, comparison ratio; DE, degree of esterification; *D*, dilution factor; EPG, endopolygalacturonase; *A*, EPG enzymatic activity (μmol·min^{-1}·mL^{-1}); HM, high methoxyl; LM, low methoxyl; *M*, average molecular weight; PG, polygalacturonase(s); PGA, polygalacturonic acid; *t*, reaction time (minutes); F_{sp}, specific fluidity; η_{sp}, specific viscosity; M_0, substrate average molecular weight at zero time (g·mol^{-1}); *C*, substrate concentration (μg·mL^{-1}); TPG, total polygalacturonase; M_v, average viscosimetric molecular weight; YNB, yeast nitrogen base.

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