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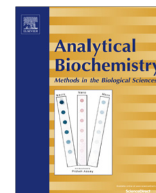

Notes & Tips

Microplate assay for endo-polygalacturonase activity determination based on ruthenium red method

Gastón E. Ortiz [a], María E. Guitart [b], Edgardo Albertó [a], Héctor M. Fernández Lahore [b], Martin Blasco [a,*]

[a] Instituto de Investigaciones Biotecnológicas–Instituto Tecnológico Chascomús (IIB–INTECH, UNSAM–CONICET), Universidad de San Martín, San Martín, Buenos Aires 1650, Argentina
[b] Downstream Processing Laboratory, Jacobs University Bremen, D-28759 Bremen, Germany

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ABSTRACT

Endo-polygalacturonase (endo-PGase) activity determinations generally rely on viscosity changes or reducing sugar ends produced by this activity over polygalacturonic acid. Torres and coworkers [Enzyme Microb. Technol. 48 (2011) 123–128] showed that ruthenium red (RR) is useful for endo-PGase determination. In this article, we present a high-throughput liquid-based endo-PGase assay based on the RR method and compare it with the viscosity determination method. The reduced assay uses a small volume of enzyme solution, 40 μg of polygalacturonic acid, and 45 μg of RR for each sample determination. Furthermore, we obtained an interconversion factor for RR and viscosity activities.

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Pectinases are used in various industrial applications related to the processing of natural products, mainly for extraction purposes [1]. The activity profile within the mixtures (endo- and exo-polygalacturonases and modifying enzymes as rhamnogalacturonases, etc.) generally defines the suitability of an enzymatic product for the different applications [2,3]. Because development and study of enzyme preparations require multiple parallel determinations of different activities in the mixture, suitable methods are demanded in this field [4–7]. To our knowledge, there is no method that accomplishes this requirement for polygalacturonase (PGase,[1] EC 3.2.1.15) activity. This activity is generally determined by using viscosity reduction in pectic substances over time using a viscometer [8] or by increasing reducing sugar ends or metabolites released from pectic substances [1]. As an alternative, Torres and coworkers [9] presented a quantitative method based on the ruthenium red (RR) precipitation of polygalacturonic acid (PGA). Among the drawbacks of the methods currently used, the viscosity determination method is time-consuming and allows few determinations at a time, whereas the reducing sugar-based method could display interference due to other enzymatic activities acting in the same conditions as PGase or compounds such as amino acids [10]. The RR method presented by Torres and coworkers [9] requires a large amount of reagents.

In this context, a reduced scale assay based on RR suitable for high-throughput screening and activity determination is presented and compared with the viscosity method [8].

The scaled down RR assay follows the same general procedures as Torres and coworkers [9] with some modifications that have shown to increase the assay quality. Briefly, it involves one reaction step, followed by precipitation of high-molecular-weight PGA by RR and finally the optical density determination of the remaining RR for quantifying purposes.

The microplate assay was carried out in 96-well polymerase chain reaction (PCR) plates sealed with adhesive film as follows. Mixing was performed after each step involving reagent addition by orbital shaking (5 mm) at 2500 rpm for 20 s, followed by centrifugation in a swinging bucket microplate centrifuge at 4 °C and 3200*g* for 30 s. The calibration curves were prepared from 0.5% PGA sodium salt stock solution in buffer citrate phosphate (BCP: 50 mM, pH 5.0). This solution was obtained by dissolving 0.500 g of PGA sodium salt (Sigma–Aldrich, Germany) in citric acid solution and then adjusting the pH to 5.0 with dibasic sodium phosphate.

The calibration curve of PGA was prepared at final concentrations equivalent to 33.75, 30.00, 22.50, 18.75, 15.00, 11.25, 7.50, 3.75, and 0 μg of PGA per 16 μl of BCP, and 16 μl of the mixture was placed in an ice-cooled microplate by triplicate. The reaction

* Corresponding author. Fax: +54 1140061500x2143.
E-mail address: mblasco@bioimanay.com (M. Blasco).

[1] *Abbreviations used:* PGase, polygalacturonase; RR, ruthenium red; PGA, polygalacturonic acid; PCR, polymerase chain reaction; BCP, buffer citrate phosphate; SSF, solid-state fermentation; SmF, submerged fermentation.

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mixture was performed by placing 8 μl of 0.50% PGA and 8 μl of the enzyme dilution in BCP and then incubated for 20 min at 40.0 °C in a thermocycler or water bath. After the incubation, the plate was placed back in ice, 40 μl of 1.125 mg/ml RR aqueous solution was added to each well, and the plate was sealed and mixed for 30 s. The mixture was diluted by adding 100 μl of 8 mM NaOH solution, mixed for 30 s, and centrifuged at 4 °C and 3200*g* for 10 min. For quantification, a 25-μl aliquot of the supernatant was transferred to the corresponding well of a clear flat-bottom 96-well microplate containing 175 μl of water, and absorbance was read at 535 nm (A_{535nm}) with a microplate reader. Alternatively, for this last step, the measurements could be performed at 492 nm by using 50 μl of supernatant and 150 μl of water.

The calibration curve dependence on the amount of RR was used to adjust the amount of RR for the assay. It was observed that 45 μg of RR (40 μl of 1.125 mg/ml) resulted in good linearity and sensitivity (Fig. 1A), and then this concentration was used in the assays. Furthermore, calibration curves of both methods were analyzed and indicated that dynamic range and sensitivity were equivalent (results not shown).

The enzyme unit was defined as the amount of enzyme required to hydrolyze 1 μg of PGA in smaller fragments unable to precipitate with the dye per minute under the assay conditions. Activities were calculated according to the equation

$$U/mL = \frac{SB - SA}{SLP \times t \times V}, \quad (1)$$

where *SB* is the absorbance of the enzyme sample blank obtained from a mixture of 8 μl enzyme dilution and 8 μl PGA solution without incubation, *SA* is the PGA–enzyme reaction absorbance, and *SLP* is the slope of the least-squares adjusted calibration curve obtained from A_{535nm} versus micrograms (μg) of PGA in 16 μl, *t* is the time (in min) taken for the reaction (typically 20 min), and *V* is the volume (in ml) of enzyme dilution used in the reaction (0.0080 ml).

The activity in the nonincubated reaction (*SB*) was determined to detect the presence of interferences in the sample and used as blank for activity calculations. For screening procedures involving samples from the same medium and strain (i.e., mutant screening), this value could be considered as constant after a short validation step to save space in the plate and then to increase the throughput of the assay.

The microplate assay was compared with the tube assay by analyzing the commercial pectinase mixtures (Fig. 1B): pectinases from *Aspergillus niger* (CAS 9032-75-1, Sigma–Aldrich), P*ectinex 3XL* (*Novozymes*, Denmark), and Fructozyme P (Erbslöh Geisenheim, Germany) at 3200-, 800-, and 400-fold dilutions, respectively. The RR method in tube scale was performed following Torres and coworkers' directions [9] except that absorbance determinations were performed by using 200 μl of the supernatant in 96-well plates. The enzyme activity values obtained by using microplate or tube scale assays presented no significant differences ($P < 0.01$) when analyzed by using a paired *t* test.

The suitability of microplate assay in screening procedures was evaluated by assessing the activity of crude extracts from four strains of *Aspergillus sojae* grown in solid-state fermentation (SSF) and submerged fermentation (SmF) (Fig. 1C).The former samples were obtained from 6-day-old wheat bran (WB) and sugar beet pellet (SBP) medium as described by Heerd and coworkers [11]. The latter (SmF) samples were obtained from orange peel (OP) and sugar beet syrup (SBS) supplemented with ammonium sulfate

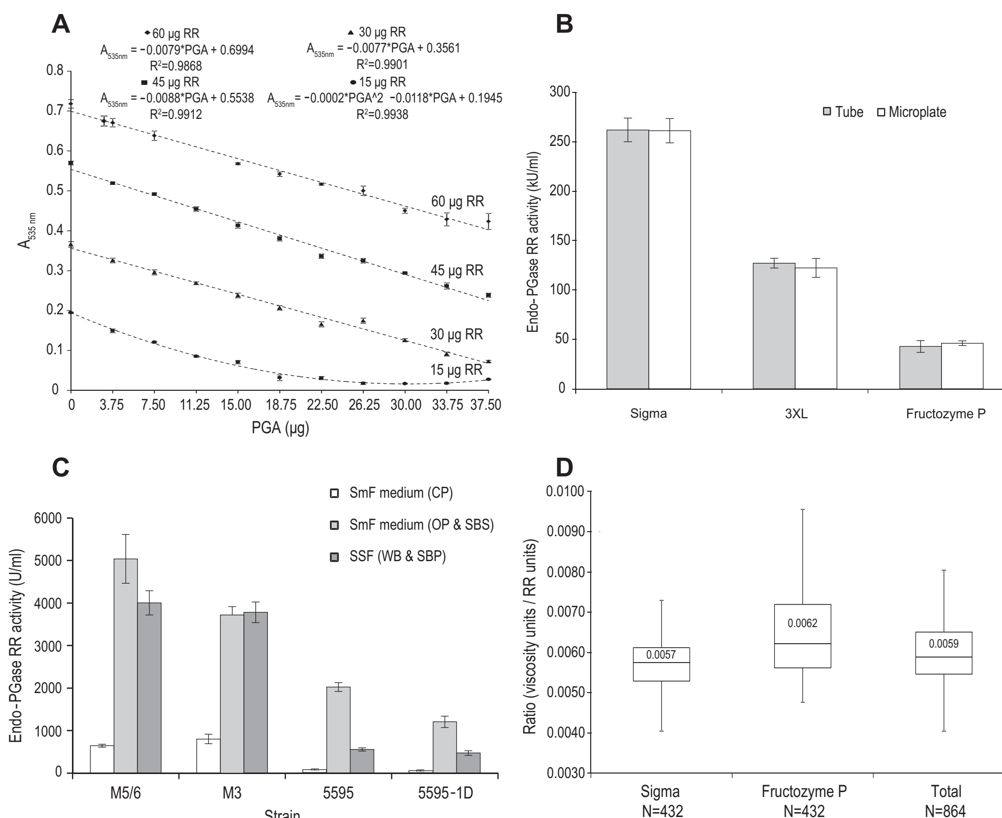

Fig.1. (A) Calibration curves for PGA using microplate assay. Linearity and sensitivity of calibration curves of PGA are shown using different amounts of RR ($n = 3$): ●, 15 μg; ▲, 30 μg; ■, 45 μg; ♦, 60 μg. (B) Commercial samples of PGase activity determination ($n = 6$) using Torres and coworkers' assay (gray bars) and microplate assay (white bars). (C) Activity determination by microplate-based PGase activity of samples from different cultivation methods (SmF and SSF) for four strains of *Aspergillus sojae* using different medium composition (CP, citric pectin; OP, orange peels; SBS, sugar beet pellets; WB, wheat bran; SBS, sugar beet syrup). (D) Box plot for each distribution; the median (within the box) represents the conversion factor between viscosity and RR units, whereas Total is the distribution resulting from the combination of Sigma and Fructozyme P distributions.

Table 1
Comparison between tube and microplate assays.

Parameter	Torres et al. [9]	This work	Scaling down fold
Amount of RR per reaction	200 μg	45 μg	4.44
Amount of PGA per reaction	187 μg	40 μg	4.67
Reaction volume	100 μl	16 μl	6.25
Water amount for measurement	6 ml	175 μl	34.28
Enzyme dilution volume	Variable	8 μl	–
Ratio RR/PGA	1.07	1.12	–
Enzyme reaction at 40 °C for 20 min	Test tubes	96-well PCR microplate	–
Read at 535 nm	1 ml in 10-mm spectrophotometer cuvette	200 μl in standard optical 96-well plate	–

medium (SmF–OP–SBS: 10 g/L OP, 61.9 g/L SBS, and 8.5 g/L ammonium sulfate) and from Czapek containing 1% citric pectin (CP) medium named SmF (CP). Shorter incubation times were assayed, and it was observed that PGase activity quantification could be achieved by 4-day SmF and SSF without processing (i.e., purification, concentration) of the sample.

During the scaling down, it was observed that the addition of RR directly to the enzyme reaction increases the lineal range of the method by reducing the amount of soluble RR obtained at high concentrations of PGA, thereby increasing the dynamic range and confidence at low enzyme activities. Furthermore, the use of 8 mM NaOH instead of water during the dilution of RR–PGA complex results in a better performance of the assay, reducing background at high PGA concentrations. It is recommended not to increase the initial PGA concentration due to the presence of soluble RR products. This situation was observed previously and could be related to the presence of pectin esters in the PGA solution [12].

Due to the importance of unit conversion for product comparison and yield calculations, the RR method was compared with the viscosity method. The viscosity units for commercial samples were obtained following Gusakov and coworkers' directions [8]. Measurements were performed at 40.0 °C using a capillary viscometer of 0.60 mm internal diameter, 0.03 K constant, 1.2 to 18 $mm^2\ s^{-1}$ measuring range (kinematic viscosity) (516 13, SI Analytics, Germany).

The conversion factor between the viscosity method and the RR method was calculated as the ratio between viscosity units and RR units (Fig. 1D). For this purpose, several dilutions of the enzyme Fructozyme P and Sigma pectinases were assayed in the linear range for both methods. To obtain all possible combinations of ratios, the activity values from Sigma and Fructozyme P were arranged in a Punnett square, where the first row and first column contain the activity values from the RR method and the viscosity method, respectively. The resulting distribution was analyzed statistically using the software G-Stat (version 2.01), and the median of this distribution (0.0059 RR units/viscosity units) was considered as the conversion factor. To establish the contribution of each formulation to the final value, the Sigma and Fructozyme P distributions were calculated separately as described earlier.

The viscosity activity determination method indicated that the Fructozyme P preparation resulted in 350 ± 40 U/ml activity (n = 10, mean ± standard deviation, 1:5 to 1:40 dilutions for determination), whereas the Sigma pectinases resulted in 1800 ± 200 U/ml (1:10 to 1:100 dilutions for determination). The dynamic range, calculated over the basis of enzyme dilutions, was approximately one order of magnitude (5^{-1} to 40^{-1} and 10^{-1} to 100^{-1} for Fructozyme P and Sigma pectinases, respectively). On the other hand, the RR method displayed a lower range of dilutions, resulting in linear correlation compared with the viscosity determination (440^{-1} to 830^{-1} and 2400^{-1} to 3840^{-1} for Fructozyme P and Sigma pectinases, respectively). Besides, it is noteworthy that the amount of enzyme required for the RR method is 10 to 20 times less than that required for the viscosity-based method.

Considering that most of industrial processes involving pectinases are related to viscosity reduction, the conversion factor obtained for RR to viscosity units could be used as reference for activity determination using a viscometer, although validation for particular enzyme mixtures should be accomplished.

The scaled down assay presented here reduces the reagent volume, space usage, and pipetting effort and increases the throughput of the assay (Table 1).

The assay reduction resulted in a lower cost per test, making it the preferred option to carry out high-throughput screening of PGase activity. Considering the difference in the limit of detection for viscometer-based determination and the time required for each determination, this method should be considered as a first choice in PGase activity screening.

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