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Chromogenic substrate for *endo*-polygalacturonase detection in gels

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ABSTRACT

A simple, sensitive zymogram technique for the detection of *endo*-polygalacturonase (EC 3.2.1.15) in gel slabs after electrophoresis or isoelectrofocusing was developed. This technique employs a new chromogenic substrate prepared by coupling D-galacturonan DP 10 with Ostazin Brilliant Red S-5B dye. The detection of multiple forms of *endo*-polygalacturonase is based on the selective removal of depolymerized dyed substrate from the agar replicas by a solvent system that does not solubilize non-degraded dyed D-galacturonan DP 10 present in agar gel replicas.

INTRODUCTION

Several methods have been described for resolving and detecting pectic enzymes and their multiple forms in electrophoretic gels; of special interest here is the detection of *endo*-polygalacturonase (EC 3.2.1.15).

The first group of these methods uses the incorporation of the substrate (pectic acid or pectin) directly into the separation gel. Following electrophoresis, the gels are incubated in a buffer to allow enzymic digestion of substrate and then stained with ruthenium red, toluidine blue or methylene blue [1–3]. This procedure is not suitable for isoelectric focusing. However, this limitation can be removed by methods using contact of the enzyme with substrate after electrophoretic separation, *e.g.*, by dipping the electrophoretic gel in substrate solution and revealing the enzymes with ruthenium red staining [4]. Even more sensitive and reproducible are methods employing the "zymogram technique". The substrate gel is brought into close contact with the gel in which the pectic enzymes were separated by isoelectrofocusing or sodium dodecyl sulphate electrophoresis. After detachment, the substrate gels are stained with ruthenium red or toluidine blue [5,6]. Pectic enzyme activity can also be revealed by using cetyltrimethylammonium bromide to precipitate the unhydrolysed pectin or pectic acid [7].

This paper describes the preparation of a new soluble chromogenic substrate prepared by coupling the dye Ostazin Brilliant Red S-5B with D-galacturonan and its use for the detection of *endo*-polygalacturonase in electrophoretic gels by the gel-replica technique.

EXPERIMENTAL

Enzyme assays

The *endo*- and *exo*-polygalacturonase activity was determined by measuring the increase in reducing groups [8] with sodium pectate ($M_r \approx 27\,000$), D-galacturonan with a degree of polymerization (DP) of 10 and di-D-galactosiduronate as substrates, using D-galactopyranuronic acid as calibration standard. Activity is expressed in μmoles of reducing groups per minute (1 U).

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Enzymes used

Commercial preparations of pectic enzymes in powder form were used: three samples of pectinase (*Aspergillus niger*) with polygalacturonase activity of 0.7–1.1 U/mg, two samples of pectofoetidin (*Aspergillus foetidus*) with polygalacturonase activity of 0.8–1.3 U/mg (both preparations from the Factory of Microbiological Preparations, Botevgrad, Bulgaria; the samples used differed in date of preparation). One sample of leozyme (*Aspergillus niger* R3) with polygalacturonase activity 1.3 U/mg was the product of Slovak Canning Factory and Distilleries (Leopoldov, Czechoslovakia) and one sample of rohament P (*Aspergillus* sp.) with polygalacturonase activity 3.2 U/mg from Roehm (Darmstadt, Germany).

After dissolution in water and centrifugation, all enzyme preparations were tested with sodium pectate as substrate, reflecting both *endo*- and *exo*-polygalacturonase activities.

exo-Polygalacturonase (EC 3.2.1.67) isolated from carrots [9] as a freeze-dried product had specific activities of 0.52 U/mg with sodium pectate, 0.61 U/mg with D-galacturonan DP 10 and 0.13 U/mg with di-D-galactosiduronate as substrates. A fungal *exo*-polygalacturonase preferring oligomeric substrates was prepared from leozyme (*Aspergillus niger* R3) by chromatography on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephadex A-50. This procedure allowed the separation of the less acidic *exo*-polygalacturonase (pI = 4.8) from the more acidic *endo*-polygalacturonase (pI = 3.0) [10]. This *exo*-polygalacturonase exhibited activities of 1.7 U/mg with sodium pectate, 2.3 U/mg with D-galacturonan DP 10 and 4.4 U/mg with di-D-galactosiduronate as substrates.

Preparation of dyed substrate

D-Galacturonan (DP 10 ± 2), prepared according to McCready and Seegmiller [11], was dissolved in water and mixed with an equal amount by weight of Ostazin Brilliant Red S-5B (SODB, Pardubice, Czechoslovakia). To the homogeneous solution, sodium acetate was added to a final concentration of 1% (w/v). The mixture was then made alkaline to pH 11 with sodium carbonate [1.25% (w/v) final concentration], incubated for 2 h at 30°C and neutralized with acetic acid, and the dyed conjugate (OBR–galacturonan DP 10) was then precipitated by adding two volumes of ethanol and two volumes of acetone. The precipitated material was washed on a filter with ethanol–acetone–0.05 *M* acetate buffer, pH 5.0 (2:2:1), then with 96% ethanol and acetone and dried in air [12]. The products obtained contained on average 5% (w/w) of the dye (determined spectrophotometrically in aqueous solution at 540 nm by using Ostazin Brilliant Red S-5B as the calibration standard).

Isoelectrofocusing

Isoelectrofocusing was done on ultra-thin layers of polyacrylamide gel (5% T, 3% C)[a] according to Radola [13], using Servalyte 3–10 (Serva, Heidelberg, Germany) as the carrier ampholyte.

Detection of endo-polygalacturonase activity

OBR–galacturonan DP 10 (300 mg) was dissolved in 10 ml of distilled water by stirring and heating to 70°C. The dissolved substrate was mixed with 20 ml of hot 3% (w/v) agar solution in 0.2 *M* acetate buffer (pH 4.5) and poured between two polyester sheets mounted on glass plates, separated by plastic spacer bars (0.8 mm). After electrophoresis, the polyacrylamide gel was laid on the dyed substrate gel for 5–15 min at room temperature and then the substrate–agar replica was dipped in the solvent ethanol–acetone–0.1 *M* acetate buffer, pH 4.5 (2:1:1). It was possible to detect fungal polygalacturonase multiple forms with isoelectric points below 6 without previous preincubation of electrophoretic gels in an appropriate buffer. The zones of *endo*-polygalacturonase-degraded dyed substrate were continuously destained as a result of solubilization of dyed substrate fragments. The rate of the destaining depended on the degree of substrate digestion.

RESULTS AND DISCUSSION

The detection of multiple forms of *endo*-polygalacturonase after electrophoresis or isoelectrofocusing in polyacrylamide gels was based on the selective removal of depolymerized dyed substrate from the substrate–agar replicas by a solvent which fixed the non-degraded dyed substrate in the gel. The principle of this method is identical with those

[a] C = g N,N'-methylenebisacrylamide (Bis)/%T; T = g acrylamide + g Bis per 100 ml of solution.

described for detection of *endo*-1,4-β-glucanases, *endo*-1,4-β-xylanases and α-amylases [14,15].

The coupling of the dye Ostazin Brilliant Red S-5B with pectic acid ($M_r \approx 27\,000$) and with citrus pectin ($M_r \approx 60\,000$) produced a material containing only 4.5% (w/w) of the dye. This is in contrast to 15–20% of dye which can be attached to neutral polysaccharides such as hydroxyethylcellulose and partially hydrolysed starch [14,15]. Dyed pectic acid, containing *ca.* 2 mol of dye per mole of pectic acid (composed of *ca.* 150 saccharide units), and dyed citrus pectin (containing *ca.* 4 mol of dye per *ca.* 340 saccharide units), were found to be unsuitable for the detection of *endo*-polygalacturonase by the dyed substrate–agar gel overlay technique. OBR–galacturonan DP 10 containing 5.0% of the dye (*i.e.*, on average only each seventh molecule is dye bound) was suitable for the enzyme detection without previous separation of dyed and undyed D-galacturonan DP 10 molecules. An interesting feature of this short polygalacturonase substrate is that the first attack of an *endo*-acting enzyme may liberate fragments soluble in the presence of organic solvents, which would not be the case with dyed pectic acid, which required extensive digestion and overloading of the gel with polygalacturonase to generate short dyed fragments soluble in organic solvents.

Fig. 1 presents the results of isoelectric focusing and subsequent detection of *endo*-polygalacturonase in four commercial pectic enzyme preparations. The sensitivity of the method is in the range 10^{-2}–10^{-3} U of endopolygalacturonase. The gel replicas can be preserved by fixing on a chromatographic paper or by drying under vacuum.

OBR–galacturonan DP 10 was not a substrate for *exo*-polygalacturonases. Neither carrot nor *Aspergillus niger exo*-polygalacturonases produced a positive response even after incubation for 10 h of the separation gels with OBR–galacturonan DP 10–agar replicas (in a wet chamber). With high-molecular-mass substrates, *exo*-acting glycanases, for obvious reasons, do not liberate short dyed fragments from the corresponding dyed polysaccharides. For instance, amyloglucosidase does not attack Cibacron Blue–amylose [16] and Cibacron Blue–pachyman does not serve as a substrate for *exo*-1,3-β-glucanase [17]. The pectin covalently dyed with N-(1-{4[(3,6-disulpho-1-naphthyl)azo]naphthyl})ethylenediamine is considered to be a substrate for endopectinase only [18].

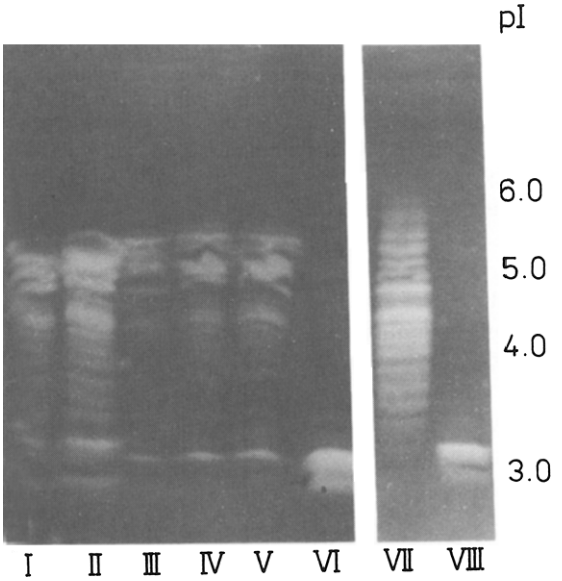

Fig. 1. Isoelectric focusing of commercial samples of pectic enzymes in ultra-thin layers of polyacrylamide gel with a pH 3–10 gradient. A 1-μl volume of enzyme solution was applied. *endo*-Polygalacturonase was detected with OBR–galacturonan DP 10–agar detection gel kept in contact with the separation gel for 10 min. Lanes: I = pectofoetidin, sample 1 (10 μg); II = pectofoetidin, sample 2 (10 μg); III = pectinase, sample 1 (8 μg); IV = pectinase, sample 2 (10 μg); V = pectinase, sample 3 (10 μg); VI = leozyme (12 μg); VII = rohament (4 μg); VIII = leozyme (7 μg).

However, OBR–galacturonan DP 10 could theoretically serve also as a substrate for *exo*-polygalacturonase, as two or three subsequent cleavages from the non-reducing terminal could produce dyed fragments soluble in the presence of organic solvents, as fragments formed by the action of *endo*-polygalacturonase. Why this does not occur remains unclear, but the following possibilities are suggested: the dye is attached exclusively to D-galacturonic acid units present on the non-reducing terminus of the D-galacturonate DP 10, thus preventing the action of exopolygalacturonase; and/or the bulky dye (M_r = 615) present on a relatively short substrate prevents the formation of the enzyme–substrate productive complex. The latter alternative finds some support in our previous observations that *exo*-polygalacturonase did not interact with partially acetylated D-galacturonic acid units in the pectic acid molecule [19].

OBR–galacturonan DP 10 was successfully used to follow the separation and purification of *Aspergillus endo-* and *exo*-polygalacturonases having different p*I* values; parallel detection of both *exo-* and *endo*-polygalacturonases was performed by using non-dyed D-galacturonan DP 10–agar gel overlay technique with subsequent ruthenium red staining [5].

OBR–galacturonan DP 10 could theoretically serve also as a substrate for *endo*-pectate lyase detection, but during these experiments a specific *endo*-pectate lyase was not available.

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