Note

Plate Assay for Differentiation of Different Pectinases

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Pectinases which degrade pectic substances in plants are important in plant pathogenesis,¹⁾ in plant physiology such as fruit ripening,²⁾ and in food processing.³⁾ It has been reported that many microorganisms, such as yeast and many plant pathogenic bacteria and fungi, produce combinations of different pectinases which are often common to various genera of microorganisms.^{1~4)} We have developed a simple plate assay for identifying pectinases on the basis of the differences in their substrate specificities, pH optima and cofactor requirements.^{1,5)} Pectate (PA, polymer of galacturonate) and pectin (PN, partially esterified pectate) were added after neutralization to either 50 mM, pH 8.0, Tris-HCl buffer or 50 mM, pH 5.0, sodium acetate buffer, at final concentrations of 1.5% and 1.0%, respectively. In addition, 1 mM calcium chloride or 1 mM

EDTA (0.1 mM for assays of purified enzymes) was added together with 0.2% soluble starch and 1.0% agar to these buffers. Three milliliter aliquots of these solutions were poured into petri-dishes after melting in boiling water. Five μ l aliquots of the enzyme preparations were placed on the plates. After incubation at 28°C for 30 or 180 min, 2 ml of 25 mM periodic acid in 0.7 N sulfuric acid (referred to as the PIo solution) was poured onto each of the plates. The PA plate became turbid due to the acid-insolubility of the long polymer of PA. But, the PA plate remained transparent due to the degradation around the area where the active enzyme preparation was placed. No change was observed on the PN plate at this point. After 10 min, the remaining PIo solution was decanted and 2 ml of 2% sodium arsenite in 0.5 N hydrochloric acid (referred to as the Ars solution) was poured onto each of the plates. Both the PN and PA plates became violet in color due to the reaction between starch and free iodine released from periodic acid, but around the area where the active enzyme preparation was placed remained transparent (Fig. 1).

When an electrophoretically homogeneous preparation of endopolygalacturonase (PG)⁵⁾ from *Aspergillus japonicum* was placed onto these plates, 1.5 units/ml was sufficient for detection on the PA-PH 5.0 plates compared to the 970 units/ml on the pH 8.0 plates (Table I). It was also noted that the addition of EDTA increased the sensitivity of the assay slightly, compared to the addition of calcium chloride. These characteristics coincide well with the reported characteristics of purified endo-PG.⁶⁾ Activity of pectin lyase (PNL), which preferentially cleaves highly methylesterified PA rather than non-esterified PA, was also detected, as indicated by the absence of an iodine-starch reaction on the PN plate around the area where the

Fig. 1. Clear Zones Formed after PIo-treatment (Left) and Ars-treatment (Right).

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Table I. THE ACTIVITIES OF PURIFIED ENDO-PG AND ENDO-PNL REQUIRED FOR DETECTION ON VARIOUS PLATES

Enzyme ^a	Plate	Activities required ^b			
		30 min		180 min	
		Plo	Ars	Plo	Ars
PG	PA (pH 5, EDTA)	7.6	30	7.6	7.6
	PA (pH 5, Ca ²⁺)	15	30	7.6	30
	PA (pH 8, EDTA)	970	970	970	490
	PA (pH 8, Ca ²⁺)	970	970	970	490
PNL	PN (pH 8, Ca ²⁺)	ND ^c	630	ND	40

^a 0.97 units/ml of endo-PG and 0.051 units/ml of endo-PNL were serially diluted in the buffer containing 0.05% of bovine serum albumin. One unit of PG and PNL is defined as the activity required to produce 1 μ mol of galacturonate and unsaturated digalacturonate, respectively.

^b Minimum number of enzyme units required for the reactions after pouring on the Plo and Ars solutions, respectively. The incubation time for the enzyme reaction was either 30 or 180 min.

^c ND, not detectable on treatment with the Plo solution.

Table II. PLATE ASSAY OF A SONICATED EXTRACT OF *Erwinia carotovora* subsp. *carotovora*

Plates	Max. dilution for detection ^a	
	Plo	Ars
PA (pH 5, EDTA)	1/8	1/1
PA (pH 5, Ca ²⁺)	1/8	1/1
PA (pH 8, EDTA)	1/1	1/1
PA (pH 8, Ca ²⁺)	1/32	1/1
PN (pH 6.8)	ND	1/2

^a The extract contained 4.6×10^{-3} units/ml of endo-PL and 1.6×10^{-4} units/ml of PG.

electrophoretically homogeneous PNL solution⁷⁾ was placed (Table I).

The applicability of this plate assay for the detection of individual pectinases in a mixture of different pectinases was examined by using a sonicated crude extract of *Erwinia carotovora* subsp. *carotovora*. This bacterium has been reported to produce endo-PG, endo-pectate lyase (PL) and endo-PNL.^{8,9)} The presence of both PG and PL is suggested by the similarity between the sensitivity on the PA-pH 5 plate and that on the PA-pH 8 plate, and by the stimulation of the activity on the PA-pH 8 plate on the addition of calcium chloride (Table II). Furthermore, the lack of the iodine-starch reaction on the PN plate seems to

Table III. DOUBLE LAYER PLATE ASSAYS OF PECTINASES IN VARIOUS *Erwinia carotovora* subsp. *carotovora* STRAINS

Strains	Overlaid with ^a	
	PA-pH 5-EDTA	PA-pH 8-Ca ²⁺
EC-1	+	++
E7419	+	++
MSR7774	+	++
ASR7778	+	++

^a The bacterial strains were grown on YP+0.5% PA. The next day, a semi-solid agar solution of either PA-EDTA in pH 5 acetate buffer or PA-CaCl₂ in pH 8 Tris-HCl buffer was overlaid, followed by incubation for 180 min. The formation of a transparent zone of less than 5 mm (+) or larger than 5 mm (++) in diameter was recorded.

indicate the presence of PNL in this extract, though a high concentration of the extract was required. The extract was prepared from a culture in the presence of PA which serves as an inducer for the synthesis of PL¹⁰⁾ and possibly of PG, but not of PNL. PNL can only be induced by DNA damaging agents.^{8,9)} The low activity of PNL may be due to the lack of its induction.

The production of PG and PL by this bacterium was also confirmed by that similar results were obtained on overlaying the melted substrate solutions on the basal plates, on which different strains of this bacterial genus were grown to form colonies (Table III). The separation of the assay from the cultivation in this double layer plate method should solve the problems of single layer plate assays^{10,11)} by minimizing the effects of microbial metabolites. Furthermore, the double layer plate method should be useful in the search for an inducer of the synthesis of each pectinase, since the conditions on basal plates for its maximum production can be easily examined.

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