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Development and analysis of pectic screening media for use in the detection of pectinase mutants

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Summary. Two solid pectic media were devised for mutually exclusive detection of extracellular polygalacturonase and pectin lyase produced by fungi including the vascular parasite of tomato *Verticillium albo-atrum*. These media allowed detection of pectinase-defective mutants. Polygalacturonase detection medium contains non-methylated polygalacturonan (sodium polypectate) is buffered at pH 5.0 (Na citrate, 0.05 M) and is calcium-free. In contrast pectin lyase medium contains polymethylgalacturonan (pectin), is buffered at pH 8.0 (HEPES, 0.05 M) and contains calcium-rich agar. When glucose was added to the media for selection of catabolite-resistant mutants, enzyme synthesis was still evident, whereas in comparable conditions in liquid culture production was almost completely repressed. This apparent discrepancy is discussed in terms of the influence of basal synthesis, colony biomass and accumulation of oligouronides which repress induced synthesis and activity.

Introduction

Different classes of mutant, altered in their ability to synthesise or secrete polysaccharases may be obtained by careful attention to screening procedures and particularly to the design of the plate media that are used (Eveleigh and Montenecourt 1979; Cooper 1986).

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Abbreviations. CR, catabolite repression; CTAB, cetyltrimethyl ammoniumbromide; GALA, galacturonic acid; NAPP, sodium polypectate; PG, polygalacturonase; PL, pectin lyase; TBA, thiobarbituric acid; UGALA, unsaturated galacturonic acid

Use of mutants has led to an improved understanding of regulation of polysaccharase synthesis (Collmer et al. 1982; Cooper 1986). Some regulatory mutants also have industrial applications, e.g. for commercial enzyme production and saccharification of cellulosic waste (Montenecourt et al. 1979; Whaldron and Eveleigh 1986).

Considering the market for pectinases for use in industrial processes (£ 165 million per annum; reviewed by Fogarty and Kelly 1983) it is surprising that there are few examples where production has been optimised by isolation of hyperproductive (often catabolite repression-resistant) fungal or bacterial strains (Fogarty and Kelly 1983).

The use of characterised pectinase mutants of plant pathogens can also provide some insight into the importance of the enzymes in pathogenesis (Cooper 1983; Durrands and Cooper 1987a, b). In common with many fungal plant pathogens *V. albo-atrum* produces an endopolygalacturonase and an endopectin lyase, regulated by dual control involving specific induction by galacturonides and by non-specific catabolite repression (CR) (Cooper and Wood 1975); both enzymes are putative virulence factors. Existing media for screening mutants rely on the different substrate specificities, pH optima and Ca^{2+} requirements of polygalacturonases (PG) and pectin (or pectate) lyases (PL); PG's invariably degrade unmethylated polygalacturonan (polypectate) in acidic conditions, accumulate under acidic conditions and are inhibited by Ca^{2+}; in contrast, most fungal PL's degrade methylated polygalacturonan (pectin) in alkaline conditions, are produced under alkaline conditions and have a Ca^{2+} requirement (Cooper 1983). In devising selection media workers have often ignored the effects of catabolite repression (CR), which results from high levels of energy sources, e.g. yeast extract, peptone and

sorbose, being added to the media (Hankin and Anagnostakis 1975; Howell 1976). Also media were not buffered and were subject to pH drift.

In the present study we have overcome these fundamental deficiencies so that for the first time PG and PL activities can be clearly distinguished in solid media allowing selection of hyper or hypoproducers of PG or PL; adaption of the media should also enable screening for CR^-, and constitutive mutants.

Materials and methods

Culture. The *V. albo-atrum* isolate was provided by Prof. G. F. Pegg of Reading University and was maintained at 4° C on Corn Meal Agar slopes. Liquid media were used for studying PG and PL production and were buffered at pH 5.0 or 8.0 with the non-metabolisable buffers 2-(*N*-morpholino)ethanesulphonic acid (MES) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (0.05 M), respectively. 250 ml Erlenmeyer flasks containing 100 ml basal salts solution (Cooper and Wood 1975) and 1% (w/v) pectin, sodium polypectate (NAPP), glucose or carboxymethyl cellulose (CMC) were inoculated with 1×10^7 conidia removed from 7—14 d Czapek-Dox plates (23° C) and were shaken in a rotary incubator (150 rpm) at 25° C. Selection plates were point inoculated with spores from Czapek-Dox plates.

Buffers and salts solutions were autoclaved at 121° C for 15 min and carbon sources at 115° C for 10 min. Media >pH 7 were autoclaved in two parts; the carbon source was added to the alkaline component after cooling to limit degradation of sugars or pectic polymers.

Measurement of fungal growth. Colony mass produced on solid agar media was estimated by placing excised colonies and attached agar onto dried pre-weighed 9 cm Whatman #1 filter papers. After determination of fresh weight residual agar was steamed from colonies in an autoclave. Filter papers were then dried to constant dry weight at 70° C.

Liquid cultures were harvested on dried pre-weighed Whatman #1 filter paper and dried to constant weight at 70° C.

Analysis of media from solid and shake cultures. Colonies and the surrounding enzyme-cleared zones were homogenised in a chilled mortar with 10 ml 0.1 M K phosphate buffer pH 7.0 and acid washed sand. The extract was filtered through Whatman #1 paper, then 5 ml aliquots were either dialysed overnight for enzyme assay or reserved for assaying uronide and glucose levels.

Samples of culture fluids from each flask were filtered as described above and following centrifugation (1800 *g*, 15 min, 4° C) assayed for galacturonic acid (GALA) and unsaturated GALA (UGALA) with thiobarbituric acid (Ayres et al. 1966); glucose, when employed as a carbon source was assayed with glucose oxidase (Fleming and Pegler 1963). Filtrates were assayed for enzyme activity after dialysis (4° C, 24 h, ca. 200 vol. stirred distilled water, pH 6.5).

A surface electrode was used to measure the pH of solid media.

Enzyme substrates and inducers of synthesis. Sodium polypectate (NAPP) and pectin (Sigma Chemical Co.) were used as inducers of PG and PL. NAPP of higher viscosity (Sunkist Growers Inc., Ontario, Calif.) was used for viscometric assay of PG.

Enzyme assays. The pH optima reported by Cooper et al. (1978) were used in PG and PL assays. PG was assayed viscometrically with NAPP (1% w/v) at pH 5.0 (25° C) and activity is given as Relative Viscometric Units (RVU) (Cooper and Wood 1975). PL was assayed with pectin (0.5% w/v) at pH 9.0 (30° C) by TBA, activity is expressed as μg UGALA released $ml^{-1}\,h^{-1}$ (Ayres et al. 1966).

Selection media. Polygalacturonase detection medium (N5) was prepared in two steps. NAPP (1% w/v) was dissolved in 250 ml distilled water in a high speed Waring blender. 1 g $NaNO_3$, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7\,H_2O$ and 7.5 g low calcium Oxoid #1 agar were added to 250 ml Na citrate buffer (0.1 M, pH 5.0), blended into the NAPP solution and autoclaved (115° C, 10 min). Pectin lyase detection medium (P8) was made in two parts. Pectin (1% w/v) was prepared as described for NAPP and autoclaved (115° C, 10 min) at pH 4.0 (natural pH of pectin which is ca. 75% w/w methylated). 1 g $NaNO_3$, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7\,H_2O$ and 10 g of calcium-rich Oxoid agar #3 were added to 250 ml HEPES buffer (0.1 M, pH 8.0), autoclaved (121° C, 15 min) and mixed with the autoclaved pectin solution. The pH was then readjusted to 8.0 with 1 M NaOH.

PG and PL activity were detected on these media by addition of 1% (w/v) cetyltrimethyl ammonium bromide (CTAB). Enzyme activity was apparent after ca. 5 min at RT as cleared (degraded) zones in the precipitated substrate, which formed a white/grey background.

Selection media for PG (NGluc5) and PL (PGluc8) CR^- mutants contained glucose added to the pectic component of each medium at 2% (w/v) final concentration.

Thin layer chromatography. TLC of oligogalacturonides was performed as described by Cooper et al. (1978).

Results

Mutual exclusivity of PG and PL selection media.

The acidic NAPP medium (N5) excluded PL activity and specifically selected for PG (Table 1). Conversely, alkaline pectin medium (P8) virtually excluded PG (to 11% of the activity around colonies on N5) but induced high levels of PL. Similarly, in liquid culture PG production was highest in acidic media whereas more PL appeared in alkaline media (Table 2). Differences due to pH largely reflect the relative instability of PG in alkaline conditions and in contrast that of PL in acidic conditions (Durrands 1986). PL was not detected on Czapek-Dox medium but PG was detected at basal levels (ca. 1% of induced levels). Similarly, on glucose in liquid culture both PL and PG were only produced at basal levels (Table 2). Low levels were also produced on CM-cellulose confirming that PG and PL are not controlled by derepression alone (Cooper & Wood 1975).

Table 1. Colony growth, PG, PL galacturonide and glucose concentration in solid pectic selection media.

Media	Colony diameter (mm)	Enzyme degradation zone (mm)	Colony dry wt (mg)	PG activity		PL activity		Sugar concentration (μg per g fresh wt colonies and agar)	
				in extract of colonies and agar	per mg mycelium	in extract of colonies and agar	per mg mycelium	GALA/UGALA	Glucose
a	b		c	d	e	f	e	g	h
N5	7±0.3	20±1.0	3.8±0.3	153	7.6	0	0	1477	0
NGluc5	9±0.3	25±1.2	26.8±3.1	460	5.7	0	0	791	1470
P8	14±0.6	16±1.4	5.3±0.4	17	0.26	873	13.4	1105	0
PGluc8	14±0.8	16±0.9	15.9±1.9	4.1	0.06	245	3.3	133	1130
Czapek-Dox	14±0.6	—	20.5±1.8	1.6	0.02	0	0	—	—

Results are representative of 3 replicate experiments

[a] N5, NGluc5, P8 and PGluc8 media are detailed in "Materials and methods", Czapek-Dox contained 3% (w/v) sucrose at pH 6.5

[b] *V. albo-atrum* point inoculated onto agar surface and incubated at 23° C for 7 d; mean value ±SE ($n=20$)

[c] Colony growth was determined by steaming agar from excised colonies onto preweighed filter paper and subsequently dried to constant dry weight at 70° C; mean value ±SE ($n=20$)

[d] PG activity (RVU) in dialysed extract from colony and surrounding agar (see "Materials and methods")

[e] 10 g of colonies and agar were homogenised and after filtration and dialysis the enzyme activities were assessed. Activities are expressed in terms of mg mycelium (dry weight). These dry weights do not relate to those in note (c) which involved separate experiments

[f] PL activity (μg UGALA released $ml^{-1} h^{-1}$), in dialysed extract, determined by TBA (see "Materials and methods")

[g] GALA was released in acidic media (N5 and NGluc5) from the degradation of NAPP by PG; UGALA was released in alkaline media (P8 and PGluc8) by PL; both were determined by TBA. The maximum possible galacturonide concentration in these media was estimated to be 3900 μg per g colonies and ager

[h] Glucose concentration was determined by glucose oxidase assay. Maximum possible glucose concentration in uninoculated media (NGluc5 and PGluc8) was 20 mg per g agar

Table 2. Growth and production of PG and PL on different carbon sources by *V. albo-atrum* in liquid culture; maximum PG and PL activities attained during 13 d incubation at 23° C on a rotary incubator (150 rpm)[a]

Carbon source and initial pH	Final pH	Enzyme activity		Growth dry wt. (mg)	Enzyme activity 100 mg^{-1} final mycelial dry wt.	
		PG (RVU)	PL (μg $ml^{-1} h^{-1}$)		PG	PL
Pectin, pH 5.0	6.0	3965.7±120[b]	60 ± 5.7	408 ±15.3	972.0	14.7
NAPP, pH 8.0	8.2	154.5±14	422.5±22.2	358.3±16.3	43.1	118.0
Pectin/glucose, pH 5.0	6.0	7.3± 0.9	0	464.5±22.1	5.2	—
NAPP/glucose, pH 8.0	8.1	4.7± 0.5	12.5±1.8	1208.3±47.2	0.4	1.0
Glucose, pH 5.0	6.3	19.6± 1.5	0	1507.6±53.2	1.3	—
Glucose, pH 8.0	8.0	1.4± 0.2	0	1587.0±48.1	0.1	—
CM-cellulose, pH 5.0	5.1	10.0± 1.2	0	183.8±16.9	5.7	—
CM-cellulose, pH 8.0	8.0	2.5± 0.2	0	253.15±17.7	1.0	—

[a] Maximum PG activity attained after 11 d incubation and maximum PL activity attained after 5 d on pectin (pH 5.0) and NAPP (pH 8.0), respectively

[b] Mean results of 4 replicate cultures ±SE

PG clearing zones in N5 medium extended much further from colonies than those resulting from PL activity on P8 medium (Table 1). pH drift was minimal in the buffered N5 and P8 media (±0.2 pH units). Citrate (used in N5) did not cause CR in liquid media (unpublished results). MES buffer could not be used as it caused the agar to soften and reduced the clarity of the clearing zones. HEPES (used in P8) failed to support growth when provided as sole carbon source in liquid culture.

Selection media for the detection of derepressed mutants

In liquid culture excess glucose severely repressed pectinase induction (Table 2) and thus comparable conditions in solid media were expected to be repressive. However clearing zones formed around colonies on both NGluc5 and PGluc8 media (Table 1). To determine whether clearing zones were produced as a result of 1) basal synthesis or 2) induced synthesis following the rapid depletion of glucose, PG, PL, glucose, GALA and UGALA levels were estimated in agar around colonies grown on the four media.

Although colony diameters were only ca. 30% greater on NGluc5 than on N5, growth (as dry weight) was 7-fold greater with glucose (Table 1); glucose levels remained sufficiently high (> 1400 μg per g colony and agar) to effect CR. PG activity per mg colony (dry weight) was marginally greater on N5 medium and production was apparently unrelated to growth, implying some contribution from induced synthesis.

Glucose-mediated repression in solid media was more pronounced for PL than for PG production (Table 1). *V. albo-atrum* grew 2—3-fold more on PGluc8 than on P8, but total actitivy in the PGluc8 extract was almost 3-fold less (Table 1) and 4-fold less when relative mycelial dry weights are considered. PL production in PGluc8 is attributable to basal synthesis because glucose levels remained repressively high (1100 μg per g colony and agar; Table 1).

On most selection media free galacturonide residues accumulated around colonies at levels sufficiently high to effect CR. Concentrations were especially high on N5 and P8 media (> 1000 μg per g colony and agar; Table 1). The relatively low levels of galacturonides in PGluc8 medium presumably reflect the low PL activity under these conditions.

TLC of extracts revealed that only mono-GALA accumulated around colonies on N5 medium whilst mono- and di-UGALA were found in P8 medium. Free galacturonides were not detected in uninoculated media.

Dialysis of P8 extracts lead to an 11-fold increase in PL activity. In contrast PG activity did not increase following dialysis.

Discussion

The buffered N5 and P8 selection media were mutually exclusive for PG and PL activity, thus, the probability of the absence of one enzyme being masked by the activity of the other was almost entirely eliminated. In contrast we found media used by previous workers (e.g. Howell 1976) were non-specific because of pH drift during growth; a PG selection medium changed from pH 5 to 7.6 and a PL medium from pH 8 to 6.8 thereby allowing concurrent production and activity of both enzymes (unpublished results). A range of PL-deficient mutants have been isolated from P8 medium and PL-deficiency has been verified in liquid culture (Durrands and Cooper 1987a). Furthermore, N5 and P8 media have been used to screen other fungal pathogens, e.g. *Botrytis allii* and *B. fabae* which produced large clearing zones on N5 medium and *Colletotrichum lindemuthianum* which cleared P8 medium, coincident with their major extracellular endopectinases (Cooper 1986).

Specialised media for the selection of catabolite repression-resistant (CR^-) mutants were not succesfully devised because clearing zones were apparent even in the presence of glucose. However, N5 and P8 media per se may also be useful in respect of CR mutants. During growth N5 and P8 media became rich in GALA and UGALA at levels which would cause CR (Cooper & Wood 1975). It is likely that under these conditions of 'self catabolite repression' (Collmer et al. 1982) high enzyme producing colonies would be CR^- mutants.

The behaviour of *V. albo-atrum* on N5 and P8 media in the presence and absence of glucose may be explained as follows. Initial production of PG on N5 medium and PL on P8 medium is clearly by induced synthesis as PG levels were > 100-fold than on Czapek-Dox (Table 1). Further induction of PG and PL on their respective media was repressed by accumulation of GALA or UGALA. This effect is paralleled in liquid cultures containing pectin, where initial pectinase production is accompanied by accumulation of oligo-galacturonides (unpublished results) and significant synthesis does not occur until the oligo-galacturonides have been utilised and CR is relieved. This self CR may explain why clearing zones on N5 were smaller than on NGluc5, because in both cases PG production was repressed to basal levels but with glucose the colonies comprised a greater fungal mass. Differences in PL production on P8 and PGluc8 were more distinct because basal PL levels were extremely low (undetectable in liquid culture).

PL activity was further reduced around the colonies in P8 media because it is vulnerable to end-product inhibition by galacturonides as evi-

denced by the 11-fold increase following dialysis; this inhibition probably explains the comparatively limited zones of PL-induced clearing. Galacturonides in colony extracts on N5 and P8 media corresponded to the final degradation products detected in reaction mixtures of *V. albo-atrum* PG with NAPP and PL with pectin (Cooper et al. 1978). Mutants giving clearing zones larger than that of the wild type on N5 or P8 media could be CR^- or producing pectinases resistant to end-product inhibition.

The regulation of pectinase synthesis in solid media is under dual control as it is in liquid culture. Analysis of the N5 and P8 media has shown that the interpretation of clearing zones is complicated by self-catabolite repression, end-product inhibition and basal activity of the enzymes. Nevertheless, an improved understanding of enzyme production in solid selection media has revealed their potential for selecting enzyme-deficient, CR^- and end-product inhibition resistant mutants.

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