Antisera Produced to Purified Extracellular Pectolytic Enzymes from Sclerotinia fructigena

By E. C. HISLOP, CLAIRE SHELLIS AND A. H. FIELDING
University of Bristol, Research Station, Long Ashton, BS18 9AF
AND
F. J. BOURNE AND J. W. CHIDLOW
University of Bristol, Department of Veterinary Science, Langford

(Received 21 January 1974; revised 6 March 1974)

SUMMARY

Purified isoenzymes of α-L-arabinofuranosidase (AF I, pI value 3.0) and polygalacturonase (PG III, pI 5.5; PG IV, pI 9.7) separated from culture filtrates of Sclerotinia fructigena by isoelectric focusing and gel chromatography were used to produce antisera in sheep. The serum produced to AF I was specific as judged by immunodiffusion, immunoadsorbent column chromatography and binding of fluorescein-conjugated serum to the antigen separated by polyacrylamide gel electrophoresis. Fluorescein-labelled anti-AF I serum was bound to mycelium producing this isoenzyme in liquid culture, and binding was decreased when AF production was repressed with glucose; no labelling occurred on mycelium infecting apple fruit tissue.

Data for the anti-PG sera were less conclusive. The antigens gave precipitin lines only with homologous sera, but an anti-PG III immunoadsorbent column bound PG III as well as a smaller proportion of PG IV, while an anti-PG IV column bound neither antigen. Fluorescein isothiocyanate-labelled anti-PG III serum was bound to mycelium of S. fructigena grown in liquid culture and to mycelium-infected apple fruit tissue, but labelled anti-PG IV serum was not bound.

INTRODUCTION

Despite the fact that pectolytic enzymes secreted by some fungi have been much studied as potential determinants of pathogenicity in certain plant disease syndromes, they have not, to our knowledge, been used successfully as antigens for the production of specific antisera. We report an attempt to produce antisera to these enzymes since they may be of value in the study of the antigens themselves and may facilitate, by the use of immunological techniques, their direct identification and localization in vivo, which has been impossible hitherto. Furthermore, anti-pectolytic enzyme sera might be used to prepare immunoadsorbent columns for isolation of pure antigens.

METHODS

Preparation of antigens. Sclerotinia fructigena Aderh. & Ruhl. (ATCC26106) was grown in a liquid pectin/ammonium tartrate medium (Byrde & Fielding, 1968) for 10 days. Culture fluid which contained polygalacturonase (EC. 3.2.1.15, poly-(1-4)-α-D-galacturonide glycanohydrolase), PG, and α-L-arabinofuranosidase, AF, was separated from mycelium by filtration, concentrated 20- to 30-fold by lyophilization, dialysed against distilled water.
and submitted to isoelectric focusing in the pH range 3 to 10 (Byrde, Fielding, Archer & Davies, 1973). Individual variations in the subsequent treatments were as follows.

**AFI.** Fractions with pH values 2·5 to 3·5 containing an α-L-arabinofuranosidase isoenzyme (AF I) were re-focused in the pH range 3 to 6, and fractions focusing near to pH 3·0, containing the bulk of the enzyme activity, were collected and dialysed against 0·01 M-sodium acetate buffer pH 4·7, lyophilized and applied to a Biogel 300 P column to remove most of a PG isoenzyme (PG I) which has an isoelectric point similar to AF I.

**PG III.** A PG isoenzyme in pH fractions 5·1 to 5·7 from the broad-range run was re-focused in the pH range 4 to 6 and fractions focusing at pH 5·5, containing the peak of activity, were collected.

**PG IV.** This isoenzyme occurring in pH fractions ranging from 9·0 to 10·5 was re-focused in the pH range 8 to 10 and the peak of activity focusing at pH 9·7 collected.

Fractions of high specific activity containing PG III and PG IV from narrow-range focusing and AF I from Biogel separations were dialysed against phosphate buffered saline (PBS), pH 7·4, and sterilized by passage through a Millipore filter (0·2 μm pore size).

AF activity was assayed by the method of Byrde & Fielding (1968), using p-nitrophenyl α-L-arabinofuranoside as substrate. PG activity was measured by the method of Miller (1959), using polygalacturonic acid as substrate. Protein levels were measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

The AF isoenzyme components of eluates from immunoadsorbent columns (see below) were separated by isoelectric focusing in polyacrylamide gels (Wrigley, 1969) using pH 3 to 10 Ampholine (LKB Instruments Ltd, Croydon, Surrey). Samples were polymerized throughout the gel and focused for 16 h at 4 °C (Laborda, Archer, Fielding & Byrde, 1974). Isoenzymes were visualized with 2-naphthyl-α-L-arabinofuranoside (Fielding & Hough, 1971) and Fast Garnet GBC diazonium salt.

PG isoenzyme mixtures from culture filtrates and from immunoadsorbent columns were separated by cellulose acetate strip electrophoresis (Byrde & Fielding, 1968) and visualized by applying strips to sodium polypectate/agar gels (Dingle, Reid & Solomons, 1953). PG activity in different regions of cellulose acetate strips was also measured quantitatively following elution in 0·1 M-sodium acetate buffer, pH 4·7.

**Preparation of antisera.** Antisera were raised in sheep using two animals for each antigen. Injections of antigen with Freund's complete adjuvant were given intradermally, subcutaneously and intramuscularly at weekly intervals for a month using 1 mg AF I or PG IV, or 0·5 mg PG III, for each injection. The sheep were bled at weekly intervals and antibody activity monitored by the agar diffusion method of Ouchterlony (1958).

Gamma globulin fractions were prepared by sodium sulphate precipitation (Handbook of Experimental Immunology, 1967) from serum taken during the fifth week after the commencement of immunization.

**Assays of antisera.** The anti-enzyme activity of sera was measured by incubating antigens and antibodies in various proportions for 1 to 24 h at a range of pH values and then assaying enzyme activity with or without prior centrifugation (25000 g, 6 min). Control tests were done using normal sheep serum in place of immune sera and heat-inactivated enzyme blanks in place of enzyme.

**Preparation of immunoadsorbent columns.** These were prepared from γ-globulin fractions by the method of Avrameas & Ternynck (1969) for glutaraldehyde cross-linked proteins and by a modification (Newby, Bourne, Chidlow & Steel, 1974) of the method of Porath, Axen & Ernbach (1967) for proteins linked to cyanogen bromide-activated Sepharose 4B (Pharmacia). One glutaraldehyde- and one Sepharose-linked column were prepared from
Antisera to pectolytic enzymes of *S. fructigena* Antisera from one of each pair of sheep injected with AF I, PG III or PG IV. Samples (10 ml) of crude concentrated culture filtrate containing known amounts of enzyme activity were applied to each column and allowed to stand for 3 h at room temperature. Unbound enzymes were washed through the columns with PBS until the concentrated washings were free from enzyme activity. Bound enzymes were then eluted with 0.2 M-glycine/HCl buffer at pH 2.8 which was collected in 1.0 M-acetate buffer at pH 5.0 (10 ml eluate; 50 ml acetate buffer) to reduce enzyme inactivation caused by undue exposure to low pH values. The eluate was dialysed overnight against distilled water at 4 °C and then against 0.1 M-acetate buffer at pH 5.0 for 2 h before concentration by dialysis against Carbowax 20 M (G. T. Gurr, Romford).

Fluorescent labelling of antisera. Gamma globulin fractions were conjugated with fluorescein isothiocyanate (FITC, isomer 1; Sigma) by a modification (Goldman, 1968) of the dialysis method of Clark & Shepard (1963), followed by removal of free FITC on a Sephadex G 50 column using PBS to elute the conjugate. This method gave conjugates of fairly low dye/protein molar ratios of 0.6 to 1.0 (1.67 µg FITC/mg protein for anti-AF I, 1.42 µg/mg for anti-PG III; 2.52 µg/mg for anti-PG IV; and 2.16 µg/mg for normal serum). Some conjugates were fractionated by DEAE-cellulose chromatography (see Goldman, 1968) and fractions eluted with increasing concentrations of sodium chloride.

Fluorescence tracing was carried out by the routine ‘direct’ method following incubation of mycelium or infected tissue slices in labelled sera diluted with PBS, or by the ‘indirect’ method using FITC-labelled rabbit anti-sheep serum (Wellcome Reagents Ltd, Beckenham, Kent).

RESULTS

Antisera decreased enzyme activities to varying extents (Table 1). Except for sheep No. 89 there were no indications of cross-reactions between heterologous mixtures. Similar results were obtained with whole serum fractions, except that rather higher levels of enzyme inactivation were achieved. Mixtures of antigens and antibodies incubated at pH values between 5.5 and 7.5 for 1 to 24 h failed to precipitate, thus these assays only measure enzyme inactivation due to the formation of soluble complexes which are stable under the conditions of the enzyme assays. Precipitation of antigen/antibody complexes with an anti-sheep serum was not examined in the present tests.

Most further work was carried out with anti-AF I serum from sheep No. 87, with anti-

<table>
<thead>
<tr>
<th>Table 1. Decrease of enzyme activity by antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme antigens</td>
</tr>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Anti-AF I</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Anti-PG III</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Anti-PG IV</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. (a) Immunodiffusion patterns in agar of reactions between an AF isoenzyme (AF I, pl value 3.0) and PG isoenzymes (PG III, pl 5.5, and PG IV, pl 9.7) with their respective antisera from sheep numbered 87, 88 and 90. (b) Cross-reaction patterns of AF (AF I, pl 4.5) and PG (PG I, pl 2.8; PG II, pl 4.6) isoenzymes, separated from Sclerotinia fructigena culture filtrate by isoelectric focusing and purified by chromatography, with anti-AF I (87), anti-PG III (88) and anti-PG IV (90) sera.

PG III serum from sheep No. 88, and with anti-PG IV serum from sheep No. 90. The immunodiffusion patterns of these three sera against the three antigens after 48 h incubation (Fig. 1 a) indicate that while the AF I antigen produced at least one precipitin line with each serum, by far the strongest reaction occurred with the homologous serum. Likewise, the strongest reactions with the PG antigens occurred with their homologous sera, and the low levels of cross-reactivity seen in these tests generally parallel the enzyme inactivation data in Table I. The precipitin pattern obtained from sheep No. 89 (not shown) also corresponded with the results in Table I, in that a precipitin line was seen in the interaction of PG IV and the anti-PG III serum, but the reason for this anomaly is not known.

The antiserum specificity to a variety of PG and AF isoenzymes (Fig. 1 b) indicates some cross-reactivity between PG I and PG III, but none between PG III and PG IV or between AF I and AF isoenzymes II and III. AF II, unlike AF I and AF III, is not secreted by mycelium grown in liquid culture (Laborda, Fielding & Byrde, 1973).

When concentrated dialysed culture filtrate was applied to Sepharose and glutaraldehyde cross-linked anti-AF I immunoadsorbent columns, almost all of the original AF activity was recovered from the Sepharose column but some 25% was lost on the glutaraldehyde column. Subsequent studies with the Sepharose–serum 87 immunoadsorbent column indi-
Antisera to pectolytic enzymes of S. fructigena

Fig. 2. Localization of AF isoenzymes I and III separated by isoelectric focusing in polyacrylamide gels by a histochemical reaction (A) and localization of AF I by adsorption of fluorescein isothiocyanate-conjugated anti-AF serum (B). Sclerotinia fructigena culture filtrate, purified AF I and AF III were applied to pairs of gels 1, 2 and 3 respectively, and fluorescence was only seen in gels 1 and 2 in the area where AF I was present.

Table 2. Fluorescence staining of mycelium grown in vitro and in infected apple fruit tissue

Mycelium was washed in phosphate buffered saline (pH 7.4) for 10 min and stained with FITC-labelled antisera for 30 min (direct method) or incubated with undiluted unlabelled antisera (30 min) followed by washing and treatment with diluted FITC-labelled rabbit anti-sheep serum (indirect method). Note that slight non-specific staining occurred even when normal serum was used with the indirect method.

<table>
<thead>
<tr>
<th>‘Stain’ method</th>
<th>Anti-AF I</th>
<th>Anti-PG III</th>
<th>Anti-PG IV</th>
<th>Normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dilution</td>
<td>Stain</td>
<td>Dilution</td>
<td>Stain</td>
</tr>
<tr>
<td>Direct</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>in vitro</em></td>
<td>10</td>
<td>++</td>
<td>16</td>
<td>+++</td>
</tr>
<tr>
<td><em>in vivo</em></td>
<td>0</td>
<td>–</td>
<td>16</td>
<td>++ (+)</td>
</tr>
<tr>
<td>Indirect</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>in vitro</em></td>
<td>100</td>
<td>++</td>
<td>100</td>
<td>+++</td>
</tr>
<tr>
<td><em>in vivo</em></td>
<td>40</td>
<td>+ (+)</td>
<td>40</td>
<td>+</td>
</tr>
</tbody>
</table>

-, No fluorescence; +, slight fluorescence; ++, moderate fluorescence; ++++, intense fluorescence.

It is indicated that 13% of the applied AF activity could not be eluted with PBS, but that when this was eluted with glycine/HCl buffer and applied to a polyacrylamide gel for electrofocusing, activity was only seen in the region where AF I isoenzyme is focused. The other major isoenzyme (pl 6.5) present in culture filtrate was absent from the glycine/HCl eluate.

When culture filtrate and authentic samples of AF I and III isoenzymes were separated by electrofocusing on polyacrylamide gels, the typical pattern (Laborda et al. 1974) was obtained (Fig. 2). However, if gels containing the eluate from the anti-AF I column or containing other samples of AF I, e.g. from culture filtrates, were incubated in FITC-labelled anti-AF I serum, fluorescence was seen in the gels only in the region where this isoenzyme was focused. Unfortunately, attempts to use this technique for the characterization of the
Fig. 3. Fluorescent mycelium of *Sclerotinia fructigena* grown in a liquid pectin/ammonium tartrate medium and 'stained' with fluorescein-conjugated anti-AF I serum. Moderate staining (+ +) is more or less evenly distributed over the mycelium and is not concentrated over hyphal tips.

labelled anti-PG III and PG IV sera were negative, as no fluorescence could be seen on the gels containing these isoenzymes.

The Sepharose-serum 88 immunoadsorbent column bound 27 % of the applied PG activity, and almost all of this was eluted with glycine/HCl. When this eluate was subjected to cellulose acetate electrophoresis it was seen to contain both PG III and PG IV isoenzymes, although quantitative assays indicated that the ratio of the PG III to PG IV isoenzymes had doubled compared with the original culture filtrate.

The glutaraldehyde-serum 88 column bound rather less total PG activity than the Sepharose column, and electrophoresis on cellulose acetate again indicated that it removed a similar mixture of isoenzymes and in similar proportions to the Sepharose column.

Results of applications of culture filtrate to the two columns containing cross-linked anti-PG IV serum showed no binding of PG to the column.

Fluorescence 'staining' reactions using both the direct and indirect methods were performed (Table 2). In general, unfractionated labelled sera, and sera fractionated by DEAE-cellulose chromatography, behaved similarly. 'Staining' could be considerably reduced or eliminated by prior incubation of mycelium or infected tissue slices with unlabelled antisera. Repression of AF production by mycelium in vitro following supplementation of the normal growing medium with 1 % glucose (Hislop, Barnaby, Shellis & Laborda, 1974) reduced binding of labelled anti-AF I serum but had no such effect on the binding of labelled anti-PG III serum; glucose does not repress the production of total PG by mycelium grown in the pectin/tartrate medium. AF I localization on mycelium grown in liquid culture (Fig. 3) was not reproduced on mycelium infecting apple fruit tissue. PG III
Antisera to pectolytic enzymes of *S. fructigena*

Fig. 4. Tungsten light (a) and u.v. light (b) photomicrographs of apple fruit tissue parasitized by *Sclerotinia fructigena* and 'stained' with fluorescein-conjugated anti-PG III serum. The highly stained areas (arrowed) are the cut ends of mycelium where the cytoplasm has adsorbed antiserum particularly strongly.
localization on mycelium in vitro was apparently identical to that of AF I, but in this case the reaction was reproduced in vivo (Fig. 4b). Fig. 4(a) is a bright field photograph of the area in Fig. 4(b). PG IV could not be localized by fluorescent staining of mycelium growing in vitro or in vivo.

**DISCUSSION**

Our results show that some pectolytic enzymes secreted by Sclerotinia fructigena can act as antigens when injected into sheep, and record the first successful immunological study of these enzymes.

Antiserum to the α-L-arabinofuranosidase antigen was apparently highly specific to one isoenzyme (AF I) as judged by its adsorption on the immunoadsorbent column and the binding of labelled serum to the isoenzyme on polyacrylamide gels. The molecular weights of the isoenzymes are very different (AF I 200,000, AF II 400,000, and AF III about 40,000); we have no evidence for their interconversion, and their immunological specificity indicated appreciable differences in the proteins. The reason why the anti-PG III and anti-PG IV sera gave precipitin lines to the AF I antigen is not known; it was probably due to a common contaminant, although the only contaminant that we know of – an extracellular polysaccharide – was not the common antigen.

FITC-labelled anti-AF I serum bound more or less uniformly to mycelium of Sclerotinia fructigena grown in liquid culture, confirming previous histochemical studies on the distribution of total α-L-arabinofuranosidase which showed that activity occurred throughout mycelium and was not particularly concentrated at hyphal tips (Hislop et al. 1974). Likewise, the absence of binding of a fluorescent anti-AF I conjugate to the pathogen in apple fruit tissue also parallels inability to demonstrate the enzyme in mycelium in vivo, even though an identical histochemical technique gave good localization of acid phosphatase (Hislop et al. 1974). AF I has been identified (Laborda et al. 1974) in homogenates of S. fructigena-infected apples, but the amounts recovered were low and apparently below the limits of detection by the histochemical or immunochemical methods examined. The availability of an antiserum specific to only one isoenzyme may be of considerable value in the immunohistochemical localization of the homologous isoenzyme in the presence of the two other isoenzymes which occur in mycelium. Conventional histochemical methods applied to fixed mycelium visualize only a small proportion of the total enzyme activity, and it is not possible to ascribe activity to particular isoenzymes.

Data relating to the PG antigens are less easy to interpret than those for AF, and indicate a need for caution in their interpretation and for further study. On the basis of agar diffusion and enzyme inhibition tests, PG III and PG IV would probably be judged to be different proteins. However, the anti-PG III immunoadsorbent column bound a (small) quantity of PG IV as well as the homologous antigen. In contrast, the anti-PG IV immunoadsorbent column bound neither of the PG antigens, and this observation is presumably related to our inability to bind FITC-labelled serum to mycelium of S. fructigena grown in liquid culture (from which the antigen was originally obtained) or in infected apple fruits where the antigen also occurs (Byrde et al. 1973).

Since there is doubt about the specificity of the anti-PG III serum, and as we were unable to demonstrate binding of FITC-labelled anti-PG III serum to polyacrylamide gels containing the homologous antigen, we cannot conclude that the fluorescent ‘staining’ of mycelium from liquid culture and in infected apples is definitely due to the localization of PG III on the mycelium. In fact, PG III has not yet been identified in extracts of infected apple tissue, but neither have pectin trans-eliminase and other cell-killing factors which occur
Antisera to pectolytic enzymes of *S. fructigena* in culture filtrates and which are thought to be produced *in vivo*. For the present, therefore, it is only possible to suggest that the PG III antiserum contains antibodies to PG III or to other extracellular fungal products which have a very similar PI value to PG III and which occur in mycelium grown *in vitro* and *in vivo*. These antibodies have facilitated dramatic fluorescent visualization of mycelium in infected tissues, where the red autofluorescence of the host contrasts strongly with the yellow/green fluorescence of the antibody-labelled pathogen.

We acknowledge the advice and assistance of Dr Verrier-Jones during earlier abortive attempts to produce anti-PG IV serum in rabbits.

REFERENCES


