Isolation and Characterization of the Pigment Esters of *Xanthomonas juglandis* (*campestris*)

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Separation of the pigment esters of *Xanthomonas juglandis* (*campestris*) on silica gel columns yielded four bands. Two of the bands, esters 1 and 2, made up over 95% of the mixture. Both esters were phospholipids, with ester 1 containing the alcohol moieties glycerol and sorbitol, and ester 2 only glycerol. Both appeared to be mixtures containing different xanthomonadin pigment molecules but the results suggested that ester 1 consisted of one pigment molecule esterified to glycerophosphoryl sorbitol, whereas ester 2 consisted of 2 pigment molecules esterified to glycerophosphate.

INTRODUCTION

Bacteria in the genus *Xanthomonas* are plant pathogens which produce a non-diffusible yellow pigment, which serves to distinguish them from species of *Pseudomonas* (Starr, 1959; Starr *et al.*, 1977). Pigments in *Xanthomonas juglandis* (*campestris*) are membrane bound and have properties similar to those of carotenoids (Starr, 1944; Starr & Stephens, 1964). However, later work on the structure of xanthomonadin pigments isolated from *X. juglandis* strain XJ103 has shown that the free pigments, isolated as methyl or isobutyl esters, are not carotenoids but are unique brominated aryl octaenes, which are distinguished by either a hydroxy or methoxy group on the ring and the presence of one or two bromine atoms (Andrewes *et al.*, 1973, 1976). In the natural state these pigments are esterified to a lipid-like moiety of unknown composition (Andrewes *et al.*, 1973).

The objective of this investigation was to isolate and characterize the moiety to which these pigments are esterified. We found that the two major esters of these are phospholipids, and that they differ by having glycerol and sorbitol in one ester and glycerol alone in another.

METHODS

Organism and growth conditions *X. juglandis* (*campestris*) ATCC 11329 was obtained from the American Type Culture Collection and maintained on nutrient agar (Difco) slants. Cells for pigment extraction were grown in eight to ten 1 litre batches of double strength nutrient broth (Difco) at 30 °C on a rotary shaker (Eberbach) at 160 r.p.m. for 28 h. Cells were harvested by centrifugation at 7700 g, washed twice with water, resuspended in water and frozen.

Extraction of pigment esters. Frozen cells were thawed and centrifuged, and the pellet was extracted once with methanol and then exhaustively with chloroform/methanol (2:1, v/v) at room temperature. The residue, which still contained some pigment, was refluxed for 10 min at 60 °C in excess solvent as described by Latgé & de Bièvre (1980), cooled to room temperature and filtered. Non-lipid material was removed from the pooled extracts by the method of Folch *et al.* (1959), and the residual water removed with anhydrous Na₂SO₄. The sample was evaporated to dryness in a vacuum evaporator, and then re-dissolved in chloroform and stored under nitrogen at –20 °C.

Thin-layer chromatography (TLC). TLC was done on 0.25 mm layers of silica gel G (Sigma). Mixtures of pigment and lipid in the crude extract were resolved with hexane/diethyl ether/glacial acetic acid (80:20:1, by vol.). Pigments were detected by their colour in visible light, while non-pigmented lipids, as well as pigments, were detected as brown spots on exposure to iodine vapour. Polyols were separated with butanol/water (9:1, v/v), 1-butanol/acetic acid/water (6:3:1, by vol.) and propanol/ethyl acetate/water (3:2:1, by vol.). Polyols were detected

with ammoniacal silver nitrate or ceric ammonium nitrate as described by Stahl (1969). The solvent systems used for the free pigments were 2% (v/v) methanol in benzene and chloroform; extreme care was taken to avoid carcinogenic benzene vapours.

Separation of pigment esters. Pigment esters, along with non-pigmented phospholipids, were separated from other lipids by adding silica gel G to the crude extract in chloroform at a ratio of 20:1 (w/w) (dry weight of extract). The chloroform was evaporated off and non-phospholipids were removed by extraction with hexane/diethyl ether/acetic acid (80:20:1, by vol.). The pigment complexes and other phospholipids were eluted with methanol, and then evaporated to dryness; the residue was dissolved in chloroform. The chloroform solution of this partially purified extract was chromatographed on a column (60 cm $\times$ 5 cm) silica gel G and Celite 545 (1:1, w/w) packed in chloroform. Four pigment fractions were resolved with chloroform/methanol (4:1, v/v). The pigment bands were cut out from the column and extracted with methanol, the solution was dried over Na_2SO_4 and evaporated to dryness, and the pigments were re-dissolved in a minimum amount of chloroform. The two major pigment esters were further purified by chromatography with chloroform/methanol (4:1, v/v) on a column (30 cm $\times$ 2 cm) of dry silica gel G.

Fractionation of pigment esters. The xanthomonadin pigments were released from the esters by mild saponification with 5% (w/v) KOH in methanol at room temperature as described by Andrewes *et al.* (1973). This resulted in the formation of methyl esters of the xanthomonadin pigment but we refer to them as 'free pigment' to distinguish them from the native pigment esters. More vigorous hydrolysis to give the free alcohols was done by heating with aqueous KOH as described by Davies (1976). Saponified samples containing the free alcohols were then acidified with 1 M- H_2SO_4 to pH 2 and partitioned with light petroleum (b.p. 35.8 to 56.5 $^\circ\text{C}$) to remove free fatty acids. The aqueous phase, containing the alcohol moieties of the lipid, was neutralized with 2 M-NaOH and evaporated to dryness *in vacuo*. The residue was extracted with dry pyridine at 100 $^\circ\text{C}$ for 10 min, and then cooled and filtered; the pyridine was removed by evaporation under reduced pressure at 35 $^\circ\text{C}$ (Block *et al.*, 1958). The residue was dissolved in 10% (v/v) 2-propanol and analysed for polyols by TLC.

Infra-red spectra. The IR spectra of pigment esters 1 and 2 were determined on thin films with a NaCl cell and a Perkin-Elmer model 1330 spectrophotometer.

Phosphorus determination. A chloroform solution (50 μl) containing 40 μg of the ester was evaporated under a stream of nitrogen and hydrolysed by the method of Bartlett (1959). Glucose 6-phosphate was used as a positive control. Phosphorus was determined with a Pierce Phosphorus Rapid Stat kit (Pierce, Rockford, Illinois), with KH_2PO_4 as the standard.

Bromine determination. The bromine content of the free pigment was determined by ion chromatography with a Dionex Model 10 Ion Chromatograph with NaBr as the standard. Sample fractions (0.4 μg in methanol) were evaporated to dryness in a glass tube (10 mm $\times$ 150 mm), which was flushed with air and then hermetically sealed, and the fractions combusted at 500 $^\circ\text{C}$ for 12 h. The gas produced by combustion was trapped by freezing to -20 $^\circ\text{C}$. The tube was then broken and the products dissolved in 0.5 ml 1 M-NaOH solution. Bromine was analysed as sodium bromide and recorded as the percentage of bromine.

Pigment determination. Pigment was assayed spectrophotometrically assuming an absorption coefficient ($A_{1\%}^{1\text{cm}}$) of 2200 at 456 nm (Andrewes *et al.*, 1973).

Chemicals. All chemicals used were reagent grade.

RESULTS

General nature of the esters

The pigment esters of *X. juglandis (campestris)* remained at the origin of TLC plates developed with hexane/diethyl ether/glacial acetic acid (80:20:1, by vol.). Non-pigmented lipids, as determined by iodine vapour detection, migrated away from the origin. These results indicate that the pigment ester is either a phospholipid or a very polar lipid since such compounds are reported not to migrate in the solvent system used (Perkins, 1975). This provided a method for separation of the crude pigment esters, along with non-pigmented phospholipids, from other lipids.

Subsequent fractionation of the partially purified crude pigment esters on a silica gel column resulted in four pigment bands; these were eluted with methanol, and numbered 1 to 4 in order of decreasing polarity. Eluates were concentrated and characterized as to the amount of pigment, dry weight, absorption maxima in the visible spectrum (350 nm–600 nm) and purity as determined by TLC (Table 1). TLC of each fraction yielded only a single spot as detected by iodine vapour. Preliminary experiments showed that the TLC solvent system used would resolve nonpigmented phospholipids from the pigmented phospholipids. Esters 1 and 2 accounted for

Table 1. *Properties of pigment esters of X. juglandis (campestris)*

Pigment complexes 1, 2, 3 and 4 were separated on a column of silica gel G and celite (1:1, w/w) packed in chloroform and developed with chloroform/methanol (4:1 v/v). Esters 1 and 2 were further purified on a silica gel G column as described in Methods. Separated pigments were eluted with methanol, concentrated and analysed. Pigments were chromatographed by TLC using chloroform/methanol (4:1, v/v) as the solvent system, and detected by their colour in visible light and as brown spots on exposure to iodine vapour.

Pigment ester	Absorption maxima in chloroform (nm)	Total free pigment (mg)*	Percentage of total free pigment	R _F value on TLC
1	420, 448, 478	1.28	57.4	0.08
2	432, 456, 483	0.91	40.8	0.15
3	438, 450, 478	0.02	0.9	0.25
4	337, 374	0.02	0.9	0.96

* Values represent the pigment recovered from 10 l of cell culture.

Table 2. *Properties of free pigments of pigment esters 1 and 2*

Pigments 1*a*, 1*b*, 2*a* and 2*b* were released after mild saponification of pigment esters 1 and 2 in 5% KOH in methanol for 1 h at room temperature. The pigments were extracted with chloroform after acidification, concentrated and separated on TLC. Solvent A was 2% (v/v) methanol in benzene; solvent B was chloroform. Bromine was assayed as sodium bromide by ion chromatography after combustion in a sealed tube.

Pigment	R _F values		Absorption maxima in chloroform (nm)	Amount of pigment (mg)*	Percentage of total pigment in ester	Bromine content (%)
	Solvent A	Solvent B				
1 <i>a</i>	0.50	0.43	480 484 414	0.034	54	6
1 <i>b</i>	0.73	0.78	481 448 436	0.029	46	16
2 <i>a</i>	0.50	0.43	480 452 430	0.032	76	6
2 <i>b</i>	0.73	0.78	480 448 436	0.010	24	14

* Values represent the amount of pigment in each spot separated by TLC. The spots were scraped off, eluted with methanol and assayed spectrophotometrically.

over 95% of the *X. juglandis (campestris)* pigments. The absorption maxima of esters 1, 2, and 3 were similar, while those of ester 4 were different. Subsequent studies reported in this paper are confined to esters 1 and 2 (Table 1).

Infra-red analysis

The presence of free hydroxyl groups in the pigment esters 1 and 2 was confirmed by their IR spectra, which showed diagnostic absorption peaks at 3430 cm⁻¹. However, the shape of the peak was indicative of more hydroxyl groups in pigment ester 1 than in 2. The carbonyl peaks at 1750 cm⁻¹ were consistent with ester linkages in both compounds. The relative sizes of the phenyl peaks (750 cm⁻¹) in the two esters indicated more aromatic rings in pigment ester 2 than in 1.

Analysis of free pigments

Mild saponification at room temperature (22–24 °C) of esters 1 and 2 with 5% KOH in methanol for 1 h resulted in the formation of free pigments 1*a*, 1*b*, and 2*a*, 2*b* (Table 2). The pigments in esters 1 and 2 behaved chromatographically as reported by Andrewes *et al.* (1973), and are identical or very similar since pigments 1*a* and 2*a* as well as 1*b* and 2*b* co-chromatographed in two TLC solvent systems. The free pigments had virtually identical visible electronic spectra, but differed in the amounts of bromine that they contained. Pigments 1*b* and 2*b* contained approximately twice as much bromine as pigments 1*a* and 2*a*. The bromine contents of 1*a* and 2*a* corresponded to 0.5 Br atoms per molecule, and those of 1*b* and 2*b* corresponded to approximately one Br atom per molecule.

Table 3. *TLC identification of polyols from pigment esters 1 and 2*

Ester 1 and 2 extracts represent the pyridine extracts of the dried or neutralized aqueous fraction after alkaline hydrolysis. The solvents were: A, 1-butanol/water (9:1, v/v); B, 1-butanol/acetic acid/water (6:3:1, by vol.); C, 1-propanol/ethyl acetate/water (3:2:1, by vol.). ND, Not determined.

Compound	R_F values		
	Solvent A	Solvent B	Solvent C
Arabitol	0.37	ND	0.62
Ribitol	0.40	ND	0.67
Dulcitol	0.25	ND	ND
Erythritol	0.47	ND	ND
Inositol	streak	ND	streak
Mannitol	0.30	ND	ND
Xylitol	0.33	ND	0.62
Glycerol	0.64	0.72	0.73
Sorbitol	0.35	ND	0.60
Ester 1 extract	0.64, 0.35	0.72, 0.54	0.60, 0.73
Ester 2 extract	0.64	0.72	

Analysis of the neutral fraction after harsh saponification

Saponification of the pigment esters by heating with aqueous KOH, followed by acidification and extraction of the free pigments with chloroform, left an aqueous fraction containing the alcohol moieties of the esters; this was neutralized and freeze-dried. TLC analysis of a pyridine extract of this dried neutral fraction showed that pigment ester 1 contained glycerol and sorbitol while pigment ester 2 contained only glycerol (Table 3). These polyols gave colour reactions with ammoniacal silver nitrate and co-chromatographed with pure glycerol and sorbitol. Further proof of identity as a polyol was provided by the characteristic colour with ceric ammonium nitrate. The polyols arabitol, ribitol, dulcitol, erythritol, inositol, mannitol and xylitol could be differentiated from glycerol and sorbitol with the developing solvents used.

Phosphorus analysis

The phosphorus contents of esters 1 and 2 were found to be 3.14% and 4.46%, respectively. The phosphorus content of glucose 6-phosphate was found to be 1.70%, i.e. 93.45% of the theoretical value of 1.82%. If the same recovery as that found for glucose 6-phosphate is assumed, the phosphorus content of esters 1 and 2 would be 3.36% and 4.77%, respectively, corresponding to one atom of P per molecule.

DISCUSSION

The results presented here have characterized for the first time the moiety to which the xanthomonadin pigments of *X. juglandis (campestris)* are esterified. The presence of phosphate, glycerol and sorbitol in pigment ester 1 and phosphate and glycerol in pigment ester 2 indicates that these are phospholipids, with pigment molecules presumably replacing the typical fatty acids normally found in phosphoglycerides. The presence of sorbitol in a phosphoglyceride is also unusual if not unique, inositol being the polyol most often found in phosphoglycerides. To our knowledge this is the first report of bacteria containing phospholipids in which carboxylic acid pigments replace conventional fatty acids. Several glucosides of C_{40} and C_{50} carotenoids have been reported, however (Aasen & Liaaen-Jensen, 1966; Norgard *et al.*, 1970; Weeks & Andrewes, 1970).

Comparison of our experimental results with the theoretical phosphate content for possible pigment esters indicates that pigment ester 1 could be either a monobrominated or dibrominated derivative, while pigment ester 2 could be either two monobrominated derivatives, or one monobrominated and one dibrominated derivative. Although the bromine analysis of the free xanthomonadin pigments was not clear cut, the relative ratios found indicate that the

Fig. 1. Proposed structures for pigment esters 1 (A) and 2 (B). R = H or Br, X = -OH or -OCH₃.

monobromo and dibromo derivatives occur in both complexes. Although the strain of organism used was not XJ103, the free pigments behaved chromatographically as described for that organism by Andrewes *et al.* (1973). The occurrence of both derivatives in ester 1 implies that this complex consists of a mixture which is not resolved by chromatographic separation on silica gel G. The IR spectra show that a greater number of hydroxyl groups is present in ester 1, which is consistent with the analytical data showing both sorbitol and glycerol in this complex whereas only glycerol is found in ester 2. The larger phenyl peak for ester 2 indicates that a greater number of pigment molecules is associated with this ester than ester 1.

Considering the chemical nature of normal phospholipids found in cell membranes, these results suggest the structures for esters 1 and 2 shown in Fig. 1. Rigorous proof of the proposed structures must await development of a method for separating the pigment mixtures, which differ only in the number of bromine atoms or whether a hydroxy or methoxy group is attached to the aromatic ring.

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