Biological Properties of Phospholipase C Purified from a Fleecerot Isolate of *Pseudomonas aeruginosa*

By J. C. CHIN* AND J. E. WATTS†

Immunology, Central Veterinary Laboratory, Department of Agriculture, New South Wales,
Roy Watts Road, Glenfield, NSW 2167, Australia

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A role for one of many exocellular enzymes produced by *Pseudomonas aeruginosa* – phospholipase C (PLC) – as a prime candidate virulence factor in fleecerot dermatitis has been examined. The addition of Tween 80 in tryptose minimal medium effectively perturbed the membrane system of a field isolate of *P. aeruginosa*, resulting in increased production and release of a periplasmic enzyme marker, alkaline phosphatase (AP), and also of PLC. PLC activity levels in the culture supernatant were 10- to 15-fold higher in the presence of Tween than in its absence. Apart from AP, the culture medium contained little or no detectable proteolytic enzyme activity, thereby facilitating the partial purification of a haemolytic form of PLC by anion-exchange chromatography. This enzyme, when injected intradermally into the skin of sheep, elicited histopathological lesions virtually identical to those seen in naturally occurring fleecerot. In addition, serum from each of eight sheep afflicted with fleecerot contained high levels of circulating anti-PLC antibody activity when assayed by ELISA. Since these antibodies did not affect the enzymic function of PLC, it is likely that they do not bind to, or are incapable of conformational modification of, the active site.

INTRODUCTION

Pseudomonas aeruginosa is the predominant bacterium isolated from dermatitic lesions of sheep suffering from fleecerot (Burrell *et al.*, 1982*b*). During the rainy season, high humidity and wet fleece provide ideal conditions for the proliferation of *P. aeruginosa* at the skin surface. Invariably, such rapid bacterial growth exacerbates the mild dermatitis normally associated with transient wetting (Hollis *et al.*, 1982) and this subsequently develops into a superficial inflammatory condition with seropurulent exudation and production of putrefactive odours attractive to the blowfly, *Lucilla cuprina* (Meritt & Watts, 1978). Together the predisposing condition of fleecerot dermatitis and flystrike poses a serious economic problem to wool production.

Although little is known about the role of *P. aeruginosa* as a causative agent of dermatitis in fleecerot, it is likely that the repertoire of extracellular products, such as exotoxin A, haemolysin and proteases, known to be produced by *P. aeruginosa* in other disease syndromes may be involved in the initiation of skin lesions. Another extracellular enzyme, phospholipase C (PLC), may function as a virulence factor since it causes skin lesions in rabbits (Liu, 1976), while a similar enzyme isolated from *Clostridium* species and known as alpha-toxin has been reported to be a major dermonecrotic toxin (Macfarlane & Knight, 1941). However, to establish a possible

† Present address: Animal Science Research, Merck, Sharp and Dohme, PO Box 135, Ingleburn, Sydney, NSW 2565, Australia.

Abbreviations: AP, alkaline phosphatase; PLC, phospholipase C; PNPP, *p*-nitrophenyl phosphate; NPPC, *p*-nitrophenylphosphorylcholine; CTMAB, cetyltrimethylammonium bromide; TDTAB, tetradecyltrimethylammonium bromide; TMM, tryptose minimal medium; Tw, Tween 80.

role for PLC as a dermonecrotizing agent in fleecerot, it was necessary to derive growth conditions favourable for the increased production of PLC in primary cultures of *P. aeruginosa* isolated from fleecerot lesions. Enhanced production of PLC from wild-type cultures would facilitate purification of the enzyme, in contrast to the generation, isolation and use of special PLC-producing mutants (Berka & Vasil, 1982) whose biological properties may have been adversely affected by chemical or ultraviolet mutagenesis. This paper describes the use of Tween for enhanced production of PLC in a field strain of *P. aeruginosa*. The enzyme has been partially purified and some of its biological and immunological effects on sheep skin examined.

METHODS

Source and growth of *P. aeruginosa*. *P. aeruginosa* strain PL42 (identified with a *Pseudomonas aeruginosa* Antisera Set, Serotype 1; Difco) was isolated originally from a sheep with fleecerot. A lyophilized culture was revived and expanded on sheep blood agar. After incubation for 16 h at 30 °C, the bacterium was washed off the plate with sterile saline (0.15 M-NaCl) and adjusted spectrophotometrically to an OD₆₅₀ of 0.4. Portions (2.5 ml) of this suspension were then inoculated into 250 ml tryptose minimal medium (TMM; Stinson & Hayden, 1979) with or without 0.5% (w/v) polyoxyethylene sorbitan monolaurate (Tween 80, or Tw). TMM contained 120 mM-Tris/HCl (pH 7.2), 0.1% (v/v) tryptose (Difco), 20 mM-(NH₄)₂SO₄, 1.6 mM-CaCl₂, 10 mM-KCl and 50 mM-glucose.

Cultures were maintained at 30 °C for 30 h and aerated by stirring with a magnetic flea driven by a stirrer (Wheaton Biostir C) operating at 350 r.p.m. Samples (5 ml) were removed aseptically at specified times for OD₆₅₀ estimations. A portion was plated by limiting dilution on sheep blood agar to assess the number of colony-forming units (c.f.u.) while the remainder was passed through a 0.22 µm Millipore filter and the ultrafiltrate assayed for enzyme activity.

Purification of PLC. PLC was purified by ion-exchange chromatography on DEAE-Sephacel (Pharmacia). An 18 h TMM + Tw broth culture was clarified by centrifugation (2500 g_{av}, 20 min). The PLC-enriched supernatant (250 ml) was concentrated 10- to 20-fold in an Amicon stirred cell equipped with a PM10 (10 kDa cut-off) membrane. The ultraconcentrate (10 ml) was then dialysed against 25 mM-Tris/HCl (pH 7.9) and the volume adjusted to 15 ml prior to loading onto a DEAE-Sephacel column (10 × 2.6 cm) equilibrated in the same buffer. Unbound protein was washed off the column with equilibrating buffer before the application of a salt gradient (0 to 1 M-NaCl) in 25 mM-Tris/HCl (pH 7.9). Finally, a detergent wash [1%, w/v, cetyltrimethylammonium bromide (CTMAB) in 25 mM-Tris/HCl, pH 7.9] was applied to the column immediately after the salt gradient to elute any tenaciously-bound protein. Fractions of 5 ml were collected and protein was monitored by absorbance at 280 nm. Samples from each eluted fraction were assayed for alkaline phosphatase (AP), PLC and haemolytic activity. Fractions with demonstrable PLC and haemolytic activity were pooled and further concentrated by dialysis against Aquacide (BDH). Usually, about 500–1000 µg protein (obtained by scaled-up ion-exchange chromatography of 2.5 litres of TMM + Tw culture supernatant) in a volume of 0.5 ml (40–50 units of PLC activity per µg protein) was loaded and chromatographed on a Sephacryl S200 (37 × 1.6 cm) column equilibrated in 25 mM-Tris/HCl (pH 7.9) containing 0.4 M-NaCl. A single peak which retained both PLC and haemolytic activity was eluted at a flow rate of 0.4 ml min⁻¹. This fraction was used in all subsequent ELISA assays for anti-PLC activity and also for intradermal injections in sheep.

Experimental challenge inoculation of wet fleece with PL42. To simulate natural rain conditions, four sheep were maintained in a closed environment under an artificial sprinkler system. Wetting occurred twice a day for 1 week. These sheep were not challenge-inoculated. A second group of four sheep was challenged by seeding 10 ml volumes of a suspension of *P. aeruginosa* PL42 in saline (2 × 10⁹ c.f.u. ml⁻¹). Post-inoculation humidity was maintained for 1 week by daily wetting with sterile water from a wash bottle along the sheep's back. A control group of another four sheep was kept dry throughout the experiment. Blood was collected from all 12 sheep just before the start of the experiment, and again 2 weeks later. All sera were analysed in an ELISA test for anti-PLC activity.

Skin washings. These were done on the four dry, the four wetted and non-challenged, and the four wetted and challenged sheep, 1 week after the start of the experiment. Wool overlying the skin surface was removed with curved scissors and an open-ended glass cylinder (6 cm diameter by 8 cm long) was affixed end-on by firm pressure onto the skin surface. Then 20 ml saline containing 0.1% (w/v) Triton X-100 was introduced into the glass cylinder and the skin surface was washed rigorously by repeated jetting of the detergent solution with a sterile 10 ml pipette. This process was repeated with fresh detergent solution and pooled washings were passed through a coarse filter (Whatman no. 41), and then successively through a 0.45 µm and a 0.22 µm filter (Millipore). Samples of skin washings were assayed for AP and PLC activity.

Intradermal injections of PLC. Two sheep were shorn of their fleece cover using electric shears. Care was taken not to nick the skin. Intradermal injections were given using a 28 gauge needle and a 1 ml syringe. PLC at 0.1, 0.01 and 0.001 mg in a final volume of 0.1 ml was administered randomly at five separate sites for each dose per sheep.

ELISA detection of antibodies against AP or PLC. Assays for antibody against AP or PLC were done by ELISA as described by Chin *et al.* (1986). AP or PLC was coupled to microtitre plates at a concentration of $10 \mu\text{g ml}^{-1}$ in 0.035 M-sodium bicarbonate buffer (pH 9.5). Binding of sheep antibodies to the assay antigen was detected with a monoclonal second antibody (ARIMab) which possesses broad-spectrum reactivity against all ruminant immunoglobulins (unpublished results).

Enzyme assays. PLC was assayed in a microtitre plate with *p*-nitrophenylphosphorylcholine (NPPC) as the substrate according to Kurioka & Matsuda (1976). AP activity was determined by hydrolysis of *p*-nitrophenyl phosphate (PNPP) essentially as described by Chin (1983). Enzymic activity is expressed in units where a unit (U) is defined as the amount of enzyme required to release 1 nmol *p*-nitrophenol per min at 21 °C.

Haemolytic assays. Fractions eluted from the DEAE-Sephacel column were diluted (0.1 ml + 0.9 ml) with 0.15 M-NaCl containing 10 mM- CaCl_2 . Sheep erythrocytes (1.0 ml of 0.5% v/v, suspension) in saline were added to each assay and incubated at 37 °C for 1 h. The unlysed erythrocytes were then sedimented by centrifugation (2500 g_{av} , 15 min). The supernatant was removed and the A_{545} determined. Results were expressed as a percentage of the complete lysis of an equivalent amount of sheep erythrocytes (lysed in distilled water).

SDS-PAGE. This was done as described by Laemmli (1970) in a Hoefer SE200 mini-gel electrophoresis system, using 10% (w/v) polyacrylamide. Each track was loaded with 10–50 μg protein. Gels were stained with Coomassie Brilliant Blue 250. The molecular mass of subunit protein was determined under reducing conditions with reference to a set of low molecular mass standards (Bio-Rad).

RESULTS

Growth of P. aeruginosa in TMM with and without Tw

The field isolate of *P. aeruginosa*, strain PL42, was inoculated in TMM with or without Tw at an initial inoculum density of 5×10^5 c.f.u. ml^{-1} . Growth, as determined by an increase in OD_{650} (Fig. 1), in either medium was characterized by a 2–6 h lag phase. In the presence of Tw, the exponential growth phase was restricted to 5–8 h, with growth curves reaching a plateau after 20 h. In contrast, cultures grown in the absence of Tw maintained a 15–20 h exponential growth phase which levelled off after 30 h. Limiting dilution plating was done on culture samples at 25 h to ensure that increases in OD_{650} were not caused by cell lysis. At this time, a cell density of 3.38×10^7 c.f.u. ml^{-1} and 5.2×10^7 c.f.u. ml^{-1} was reached in the presence and absence of Tw respectively. The concentration of Tw used in these experiments was 0.5% (v/v). Increasing Tw to 1% (v/v) further suppressed growth while 2% (v/v) completely inhibited cell proliferation.

Exocellular PLC and AP activities in TMM + Tw

Triplicate 50 μl samples of the clarified culture supernatant were assayed at different times during the culture period for PLC and AP activity. In the presence of Tw, the highest level of PLC detected was at 18 h and this level of enzyme activity was 12 times that in the absence of Tw (Fig. 2). The level of AP activity at 18 h was also higher in the presence of Tw. The production of AP in Tw-free TMM did not appear to parallel the growth characteristics of PL42 (Fig. 2) and decreased while growth was still exponential. In contrast, PLC production in TMM + Tw reached a maximum at the end of exponential growth.

In the absence of Tw, the pH of the culture medium remained relatively stable over 25 h, decreasing from 7.2 to 6.4. In contrast, cultures grown in the presence of Tw showed a rapid decrease in pH from 7.2 to 4.7 over the same time period. This probably accounted for the earlier arrested growth rate observed in cultures containing Tw.

Purification of PLC

Clarified supernatants from cultures grown for 18 h in TMM + Tw (containing maximum detectable levels of PLC) were used for enzyme purification. Each ion-exchange column was usually loaded with 15 ml of culture supernatant concentrated from 250 ml of original culture medium. A typical elution profile is shown in Fig. 3. An unbound peak (A) enriched in AP was eluted with 25 mM-Tris/HCl, pH 7.9. Application of a 0 to 1 M-NaCl gradient eluted two additional peaks (B and C) which contained only PLC activity. At the end of the salt gradient, a detergent elution was done with CTMAB, since Berka & Vasil (1982) reported that PLC bound

Fig. 1

Fig. 2

Fig. 1. Growth of *P. aeruginosa* PL42 in the presence (●) or absence (○) of Tw (0.5%, w/v) in TMM. Samples were removed at specific times from a 250 ml culture and their OD_{650} determined. The experiment was repeated twice with essentially similar results.

Fig. 2. Effect of Tw on the production of PLC and AP in the culture supernatant. AP (---) and PLC (—) assays were done on the supernatants of cultures with (●, ▲) and without (○, △) Tw, activity being measured as the release of *p*-nitrophenol in a 30 min assay. Essentially similar results were obtained in a repeat experiment.

Fig. 3. Enzymic and haemolytic activities of PLC and AP present in the fractions collected during ion-exchange chromatography, on a DEAE-Sephacel column, of concentrated Tw-containing supernatants. Upper panel: ▲, haemolytic activity expressed as a percentage of complete haemolysis of sheep erythrocytes by water. Lower panel: △, A_{280} ; ■, AP activity; ●, PLC activity. Essentially similar results were obtained in at least four other separate experiments.

Fig. 4. SDS-PAGE of PM10-concentrated broth culture (lane A) and PLC after Sephacryl S-200 chromatography (lane B) run under reducing conditions in a 10% polyacrylamide gel.

Table 1. *Purification of PLC from TMM + Tw broth*

Purification step	Volume (ml)	Protein (μ g)	Specific activity [U (μ g protein) $^{-1}$]	Recovery of activity (%)	Purification (-fold)
Culture supernatant	250	21 250	0.5	100	1
PM10-concentrated supernatant	15	8 600	0.78	62	1.5
Fraction B after ion-exchange chromatography	5	44	48.7	48	97

tenaciously to ion-exchange resins so that desorption could be achieved only with tetradecyltrimethylammonium bromide (TDTAB). A small protein peak (D) was obtained which contained neither AP nor PLC activity.

The ability of individual fractions separated by ion-exchange chromatography to lyse sheep erythrocytes was examined semi-quantitatively (Fig. 3, upper panel). The AP-containing peak A possessed no haemolytic activity; haemolysis was confined essentially to peak B (PLC). Fractions constituting this PLC peak were pooled, concentrated by dialysis against Aquacide and applied to a Sephacryl S-200 column. A single major peak with an estimated molecular mass of 80 kDa was eluted and this contained all the PLC activity (data not shown). SDS-PAGE analysis of the Sephacryl S-200 peak under reducing conditions (Laemmli, 1970) revealed a major band with an apparent molecular mass of 80 kDa (Fig. 4). Peak C fractions contained detectable but low levels of PLC activity. None of these fractions lysed sheep erythrocytes.

The purification and percentage recovery of PLC which retained both enzymic and haemolytic activity are summarized in Table 1. Pooled peak B fractions contained 48% of the total PLC activity detected in TMM + Tw, and the enzyme had been purified 97-fold. This enzyme was relatively unstable; it could be stabilized by the addition of sorbitol to 60% (w/w) in 25 mM-Tris/HCl, pH 7.2 (unpublished). Further chromatography on a molecular sieve column failed to increase purification and was associated with a further loss in activity (20–30% recovery).

Effect of PLC on sheep skin

In the natural environment of wet fleece, *P. aeruginosa* colonizes the skin surface and is non-invasive during fleecerot formation. As shown in Fig. 5(a), skin sections from natural fleecerot

Fig. 5. Vertical skin sections from sheep (magnification $\times 312$). (a) Lower half of wool follicle and dermal tissue from a natural fleecerot lesion. A microabscess (mabsc) at the outer margin of a wool follicle has formed by infiltration of polymorphonucleocytes (PMN). Note that the margins of the wool follicle (WF) are completely lysed. (b) An intact wool follicle from normal sheep skin. (c) Extensive necrosis of the follicular and epidermal margins of a wool follicle following intradermal injection with PLC. As in (a), inflammatory cells comprising predominantly polymorphonuclear leucocytes have infiltrated the necrotic cell zones.

Table 2. AP and PLC activity from skin washings of fleecerot-affected sheep and their anti-PLC antibody levels compared to animals that had been injected intradermally with PLC

Treatment	Number of sheep	Activity in skin washings*		Anti-PLC antibody (ELISA A_{492})†	
		AP	PLC	Before wetting	After wetting
Dry/not challenged	4	<1	<1	0.18 ± 0.08	NA
Wetted/not challenged	4	137 ± 15	26 ± 8	0.16 ± 0.09	0.95 ± 0.18
Wetted/challenged	4	248 ± 11	87 ± 9	0.20 ± 0.1	1.43 ± 0.25
Intradermal injection of PLC	2	NA	NA	0.17 ± 0.05	0.64 ± 0.13

NA, Not applicable.

* Results represent the mean ± SEM of six washings from six sites per animal one week after wetting, and are expressed as U (mg protein)⁻¹.

† Blood was collected prior to wetting (with or without challenge) or before intradermal injection with PLC. All animals were bled again 2 weeks later. Serum was diluted 1 in 200 before being assayed in triplicate for anti-PLC activity by ELISA. The results are means ± SEM.

dermatitic lesions are characterized by dense infiltration of leucocytes with ensuing exocytosis of predominantly polymorphonucleocytes as a microabscess (arrowed) in the epidermis. Commonly, these microabscesses develop at the outer margins of wool follicles and eventually discharge their seropurulent contents onto the skin surface. Follicle bulb cells are characteristically lysed *in situ* (Fig. 5a) by presumptive PLC activity; this contrasts sharply with the morphology of a normal wool follicle (Fig. 5b).

A role for PLC of *P. aeruginosa* strain PL42 as a dermonecrotizing agent was investigated by intradermal injection of the enzyme followed by histological analysis. Sections of skin biopsied 24 h after exposure to PLC at a dose of 0.1 mg per injection site revealed the presence of a central abscess with extensive liquefactive necrosis of the wool follicles (Fig. 5c). With 0.01 mg PLC, a less severe dermatitic response was noted (similar to Fig. 5a) and this was characterized by dense infiltration of predominantly polymorphonuclear leucocytes into the dermis. Intradermal injections with varying doses (0.1–0.001 mg) of partially purified AP (peak A), failed to elicit the inflammatory responses observed with PLC.

Immunological response to PLC in sheep affected with fleecerot

In a limited study, fleecerot dermatitis was initiated in sheep under different conditions. In the first case, four sheep were wetted in an artificial water sprinkler system (as described in Methods) to simulate natural rainfall, but were not challenged with *P. aeruginosa*. They developed characteristic greening lesions after about 3–4 d. A second group of four animals was similarly wetted except that the sheep were also challenged with *P. aeruginosa*. These challenged animals developed greening lesions 12–14 h later.

As shown in Table 2, proliferating fleece bacteria identified microbiologically as *P. aeruginosa* and also by virtue of the characteristic greening lesions of the fleece (unpublished observations), produced assayable quantities of AP and PLC in skin washings. Similar washings from unwetted, unchallenged sheep showed no detectable enzyme activity. The activities of AP and PLC in skin washings from wetted and challenged sheep 1 week after treatment were much higher than those in wetted and unchallenged animals. Accordingly, higher levels of anti-PLC activity were detected in the sera of wetted and challenged sheep compared to their unchallenged counterparts. Intradermal injections of PLC did not elicit as high a level of anti-PLC activity as found in the sera of sheep experiencing fleecerot (either wetted, or wetted and challenged). Pre-incubation of anti-PLC serum (from sheep that had been wetted, wetted and challenged, or injected intradermally) with PLC for 1 h at 37 °C did not inhibit the enzymic activity of PLC when compared to similar incubations using control sera collected from the same sheep before the experiment. All control sera possessed only background levels of anti-PLC activity (<0.2 ELISA A_{492} units).

DISCUSSION

Of several exocellular products excreted by *P. aeruginosa*, PLC and an associated haemolysin have been described as dermonecrotizing agents (Kurioka & Liu, 1967). *P. aeruginosa*, under conditions of phosphate limitation, synthesizes and releases into the culture medium an inducible AP and PLC (Poole & Hancock, 1983). Although the mechanism for AP and PLC release has not been clearly defined, it may occur after membrane perturbation or destabilization with Tris-based medium (Bhatti & Ingram, 1982; Irvine *et al.*, 1981) or by cold shock treatment (Hoshino & Kageyama, 1980). In preliminary experiments, we attempted to elicit PLC production by membrane perturbation using detergents such as Triton X-100, Nonidet P40 and Tween 80 in cultures grown under phosphate-limited conditions. Growth of PL42 was inhibited to varying degrees by all three detergents, depending upon the concentration. Only with Tween 80 was growth considered to be satisfactory (60–80% of that in the absence of detergent) and this was accompanied by a 12-fold increase in PLC production. Under similar conditions, the level of detectable AP in TMM + Tw was only 2 to 2.5 times that in the absence of Tw.

To investigate the role of PLC as a virulence component in fleecerot, it was necessary to purify the enzyme from its major contaminant – AP. This was accomplished by ion-exchange chromatography, where AP was not retained on a range of anion resins tested including DEAE-Affi-gel Blue (Bio-Rad), DEAE-Sephacel (Pharmacia) and Trisacryl DEAE (Linbrook, LKB). PLC bound to and could be eluted under low salt conditions from all three anion-exchange resins. This property differs significantly from that described by Berka & Vasil (1982), who found that under their conditions PLC bound tenaciously to anion resins and could be eluted only with a quaternary ammonium detergent (TDTAB). It is likely that the PLC produced by the PLC-hyperproducing strain of the latter authors, which was isolated after chemical mutagenesis of *P. aeruginosa* PAO1, may differ in charge from the PLC produced by the natural fleecerot isolate.

The ion-exchange conditions used for the purification of PLC from supernatants of cultures grown in TMM + Tw permitted the identification of two peaks (B and C) with PLC activity. Only peak B possessed haemolytic activity in addition to enzymic activity against NPPC. It is conceivable that peak C is either one of two different isoenzymic forms of PLC (Berk *et al.*, 1987) or it is a denatured form of PLC which has lost its haemolytic activity.

Although the biological activity of peak B PLC from strain PL42 was confirmed by haemolysis of sheep erythrocytes, its role in eliciting dermatitis was further tested by intradermal injection of varying doses of the enzyme into the skin on the back of shorn sheep. The ensuing dermatitis and folliculitis associated with such treatment, although deep-seated, was nevertheless remarkably similar to that seen in skin sections from natural fleecerot lesions. Unpublished results using intradermal injections of crude culture supernatants devoid of PLC but containing high levels of proteolytic elastase and collagenase activity failed to elicit equivalent pathology. AP was neither haemolytic nor dermatitic.

Sheep after experiencing several fleecerot episodes should become sensitized to one or several exoenzymes produced by *P. aeruginosa*. When analysed by ELISA, serum from sheep with fleecerot contained high levels of circulating antibody against PLC. However, these antibodies were incapable of neutralizing PLC activity *in situ*, and it is likely that the enzyme molecule may contain immunodominant epitopes at non-catalytic site(s). It is interesting that although proliferating *P. aeruginosa* produces AP as well as proteolytic enzymes on the wet sheep skin, the humoral immune system of the sheep recognizes PLC as the dominant immunogen. It therefore appears unlikely that protection against fleecerot by immunization with crude bacterins or concentrated culture supernatants enriched in exoenzymes (Burrell *et al.*, 1982a; Burrell, 1985) may be effective as a result of antibody neutralization of dermatitic enzymes of *P. aeruginosa*, unless such antibodies can be targeted to the active site and shown to inactivate enzyme function.

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