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Identification and localization of an active cutinase in the pollen of *Brassica napus* L.

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Abstract. Polyclonal antiserum and monoclonal antibodies raised to a purified cutinase from *Fusarium solani* f. sp. *pisi* have been used to identify an active cutinase in the pollen of *Brassica napus*. These antibodies recognized a polypeptide with an estimated molecular weight of 22-kDa – a molecular weight indentical to that of the *Fusarium* cutinase – and localized this polypeptide to the intine of the pollen wall. Enzyme assays on the renatured 22-kDa polypeptide after electroelution from a preparative SDS-PAGE gel revealed the polypeptide to be an enzyme capable of catalysing the hydrolysis of tritiated apple cutin and the synthetic substrate *p*-nitrophenyl butyrate. The molecular weight, immunological properties and substrate specificity of the *Brassica* cutinase suggest that this enzyme resembles more closely fungal cutinases than it does the cutinase from the pollen of Nasturtium (*Tropaeolum majus*) – the only angiosperm cutinase so far characterized (Maiti et al., 1979, Arch. Biochem. Biophys. **196**, 412–423). These differences between the pollen cutinases from two members of the Dicotyledoneae are unexpected and predict a diversity of this class of pollen enzyme within the angiosperms.

Key words: *Brassica* (cutinase, pollen) – Cutinase antibodies – *Fusarium* (cutinase) – Pollen (cutinase)

Introduction

Flowering plants with 'dry-stigmas' (Heslop-Harrison and Shivanna 1977) possess stigmatic papillae invested by a continuous cuticle which presents a major barrier to the entry of pollen tubes (Heslop-Harrison 1975; Heslop-Harrison et al. 1975). For a pollination to succeed, this cuticular layer must be penetrated. Although stigmatic cuticles may be thin and easily ruptured mechanically (Elleman et al. 1992) there is much circumstantial evidence from the literature (Christ 1959; Heinen and Linskens 1961; Linskens and Heinen 1962; Heslop-Harrison et al. 1975; Heslop-Harrison and Heslop-Harrison 1975; Knox et al. 1976; Heslop-Harrison 1977; Shivanna et al. 1978) to suggest that a cutinase held in the pollen, or secreted by the pollen tube tip degrades the stigmatic cuticle prior to penetration. Indeed it has been suggested that one of the many non-specific esterases present in the pollen wall (Knox and Heslop-Harrison 1970) fulfils such a role (Heslop-Harrison et al. 1975; Knox et al. 1976).

To date, only one pollen cutinase, from the pollen of Nasturtium (*Tropaeolum majus*), has been identified and characterized (Shaykh et al. 1977a; Maiti et al. 1979). This enzyme proved to be very different from the well-characterized fungal cutinases in molecular weight, substrate specificity, inhibition properties and immunological properties (Kolattukudy et al. 1981). The most striking difference was the non-inducibility of the pollen enzyme (Shaykh et al. 1977a). These authors proposed that the enzyme is synthesized during pollen maturation and stored in the intine layer of the pollen wall. The presence of an active cutinase in *Tropaeolum* pollen is at some variance with a large body of indirect evidence from other species possessing dry cuticularized stigmas (Heslop-Harrison 1975, 1977; Heslop-Harrison and Heslop-Harrison 1975, 1981; Knox et al. 1976; Shivanna et al. 1978) which suggests that the pollen merely contains an inactive cutinase precursor which is activated by a stigmatic component upon pollination. This hypothesis is based on the fact that enzymic removal or detergent disruption of the proteinaceous pellicle (Mattsson et al. 1974), which overlies the stigma cuticle, prevents compatible pollen tubes from penetrating the stigma despite allowing near-normal pollen germination. Knox (Knox et al. 1976) has suggested that esterases from the pollen and pellicle combine to form an 'active cutinase complex' which degrades the cuticle in the re-

Abbreviations: PNB = *p*-nitrophenyl butyrate; PNBase = PNB esterase; PNP = *p*-nitrophenyl palmitate; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate

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gion of penetration, while Heslop-Harrison (Heslop-Harrison and Heslop-Harrison 1975) simply suggests that a yet-unidentified pellicle molecule or molecules is responsible for cutinase activation. Despite this indirect evidence for an inactive cutinase in the pollen of the Brassicaceae (Shivanna et al. 1978), Caryophyllaceae (Heslop-Harrison and Heslop-Harrison 1975), Iridaceae (Heslop-Harrison 1977), Liliaceae (Knox et al. 1976) and Gramineae (Heslop-Harrison and Heslop-Harrison 1981) it remains that the only characterized pollen cutinase, that from *Tropaeolum* (Tropaeolaceae), is present in an active form (Shaykh et al. 1977a).

Much recent work has been focused on gene expression during microsporogenesis and pollen development (Reviewed by McCormick 1991) and genes encoding a number of hydrolytic enzymes of potential importance to pollination events have been identified, including pectin esterase from *Brassica* (Albani et al. 1991), pectate lyase from *Lycopersicon* (Wing et al. 1990) and polygalacturonase from *Oenothera* (Brown and Crouch 1990; Allen and Lonsdale 1993) and *Zea* (Niogret et al. 1991). As yet no clones with homology to any known cutinases have been identified.

In this paper we report the identification and the principal properties of an active cutinase from the pollen of *Brassica napus*. While this cutinase resembles that of *Tropaeolum* in that it is readily leached from the pollen in an active form, in other respects it bears greater resemblance to the *Fusarium* cutinase, and to fungal cutinases in general.

Materials and methods

Plant material. Brassica napus L. cv. Topas was grown from seed (supplied by Horticultural Research International, Wellesbourne, Warwicks., UK.) in a growth room (Sanyo Gallenkamp PLC, Loughborough, Leics., UK) at 20° C with a light regime of 16 h light and 8 h darkness. Pollen was collected by gently brushing anthers with a sable-hair brush and stored at −70° C until use. For pollinations, large pre-anthesis buds were used with their petals and sepals removed; after pollination these flowers were maintained in the wells of microtitre plates containing tap water.

Cutinase antibodies, purified Fusarium cutinase and purified cutin. Polyclonal antiserum and specific monoclonal antibodies were raised against a purified cutinase from *Fusarium solani* (Mart.) Appel. & Wr. f. sp. *pisi* (F.R. Jones) Snyd. & Hans. according to Coleman et al. (1994). Pure samples of recombinant *Fusarium* cutinase were kindly provided by Professor P.E. Kolattukudy, Ohio State University, USA. Purified cutin was prepared from Golden Delicious apples according to Coleman et al. (1994).

Chemicals. p-Nitrophenyl butyrate, *p*-nitrophenyl palmitate, thimerosal, tetramethyl benzidine, α-naphthyl acetate, α-naphthyl butyrate and Fast Blue RR salt were obtained from the Sigma Chemical Co., Poole, Dorset, UK. Electrophoresis reagents were obtained from National Diagnostics UK, Aylesbury, Bucks.

Preparation of a crude extract of pollen cutinase. Freshly collected pollen (200 mg) was suspended in 15 ml 50 mM sodium-phosphate buffer (pH 7.0) and the proteins allowed to elute for 1–1$^1/_2$ h at room temperature, with occasional agitation. Following centrifugation at 12000 · *g* for 10 min the supernatant was collected and stored at 0–4° C. For enzyme-linked immunosorbent assays (ELISAs), fractions of the supernatant were diluted 1:4 in sodium-bicarbonate buffer (15 mM $NaCO_3$, 35 mM $NaHCO_3$, pH 9.6). For enzyme assays, Western blotting and preparative SDS-PAGE, proteins contained in the supernatant were precipitated by the addition of ammonium sulphate to saturation; once saturation was reached the solution was allowed to stand at 4° C for 24 h, after which time the precipitated proteins were pelleted by centrifugation at 21000 · *g* for 25 min and resuspended in 300 µl 50 mM sodium-phosphate buffer (pH 7.0).

Enzyme assays. The esterase assays were carried out using *p*-nitrophenyl butyrate (PNB) and *p*-nitrophenyl palmitate (PNP) as substrates (Purdy and Kolattukudy 1973). The reaction mixture contained 0.1 ml of 0.4% Triton X-100, 0.89 ml 50 mM sodium-phosphate buffer (pH 7.0), 0.01 ml 1.76% PNB (v/v in acetonitrile) or 0.01 ml 0.042 M PNP. To this mixture was added 0.6 µg of pure *Fusarium* cutinase in 10 µl 50 mM sodium-phosphate buffer or 10 µl crude pollen protein extract containing 100 µg protein. Activity was measured at 405 nm using a Beckman DU75 Spectrophotometer (Beckman Instruments Inc., Fullerton, Cal., USA); linear rates were observed for 2–15 min. Specific activities of PNB esterase (PNBase) were calculated using the molar extinction coefficient at pH 7.0 = 6830 mol · cm^{-1} (Köller and Parker 1989).

For the cutinase assay, radiolabelled cutin was prepared by the Amersham Corp. (Aylesbury, Bucks., UK) following the method of Köller et a. (1982) by reducing the keto function in apple-fruit cutin with $NaB[^3H]_4$. Tritiated cutin (1.5 mg) was diluted with cold cutin (0.5 g) to give a final specific activity of 16 kBq · (mg cutin)$^{-1}$ (Bonnen and Hammerschmidt 1989). The reaction mixture contained 47 µl 2% Thimerosal, 25 µl 1% Triton X-100, 4.5 mg [^{3}H]cutin in a total volume of 1.0 ml in 0.5 M Tris-HCL buffer (pH 8.9) (Bonnen and Hammerschmidt 1989). To this was added 12 µg purified *Fusarium* cutinase as a positive control or 200 µg protein from the pollen extract, both contained in 20 µl 50 mM sodium-phosphate buffer (pH 7.0). As a negative control, 200 µg of bovine serum albumin contained in 20 µl 50 mM sodium-phosphate buffer was added. The reaction mixture was incubated at 25° C for 18 h and the reaction terminated by the addition of two drops of 6 M HCl. The mixture was twice filtered through Ultrafree-MC Durapore 0.65-µm filter units (Millipore, Bedford, Mass., USA), the filtrate extracted twice in 1.5 ml ethyl acetate, the ethyl acetate evaporated using a centrifugal evaporator (Jouan, Tring, Herts., UK) the extract mixed with 4 ml scintillation cocktail O (BDH, Poole, Dorset, UK) and the radioactivity counted in a Beckman scintillation counter (Beckman Instruments). This was repeated three times and the mean radioactivity calculated (after subtracting the background radioactivity reading of the negative control) and converted into a specific cutinase activity.

Protein determination. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, Ill., USA) with bovine serum albumin as the standard.

Enzyme-linked immunosorbent assay. The ELISA assays ere carried out in 96-well Immulon 2 microtitration plates (Dynatech Labs. Billinghurst, W. Sussex, UK). Wells were coated with antigen in serial dilution from 22 µg · 100 µl^{-1} in sodium-bicarbonate buffer (pH 9.6) overnight at 4° C. All working volumes were 100 µl per well. Wells were washed four times for 2 min with PBST – phosphate-buffered saline (PBS; 13.7 mM NaCl, 2.7 mM KCl, 1.7 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing 0.05% Tween 20 –, dried at room temperature for 30 min then blocked for 1 h with 0.3% (w/v) casein in PBS. Following washing four times for 2 min with PBST, undiluted hybridoma supernatant (monoclonal antibody) or antiserum diluted 1:500 in PBST was added to the antigen-coated wells. After 1 h the plates were washed four times for 2 min with PBST and then incubated for 1 h in horseradish-peroxidase-conjugated goat anti-mouse immunoglobulins (Sigma) diluted 1/1000 in PBST. The wells were washed three times with PBST and substrate solution containing tetramethyl benzidine (100 µg · ml^{-1}) (Dewey et al. 1989) was added. After 30 min the enzyme reaction

was stopped by the addition of 100 µl of 3 M H_2SO_4 and the absorbance read at 450 nm on a Dynatech plate reader (Dynatech Labs.).

Western immunoblotting. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Mini-PROTEAN II electrophoresis system (Bio-Rad, Hemel Hemstead, Herts., UK) employing a discintinuous buffer system (Laemmli 1970) with a 15% polyacrylamide resolving gel, 4% stacking gel and employing low-molecular-weight standards (Bio-Rad.). Proteins were transferred to immobilon-P membranes (Millipore) using a Mini-Trans Blot Electrophoretic Transfer Cell (Bio-Rad) according to Towbin et al. (1979). After blotting, molecular-weight standards were visualized by quickly staining with 0.5% (w/v) Ponceau S (Sigma) in 3% (w/v) trichloroacetic acid, 3% (w/v) sulphosalycylic acid, and destaining with 1% (v/v) acetic acid.

Membranes were then blocked for 1 h in 0.3% (w/v) casein in TBS (Tris-buffered saline: 500 mM NaCl; 20 mM Tris-HCl, pH 7.4), transferred to cutinase polyclonal antibody diluted 1:250 in TBS 0.3% casein or monoclonal antibody (hybridoma supernatant) overnight, washed four times for 10 min in TBS 0.3% casein, transferred to goat anti-mouse gold conjugated secondary antibody (BioCell, Cardiff, UK) diluted 1:100 in TBS 0.3% casein overnight, washed five times for 1 min in distilled water, and the developing bands enhanced using a silver enhancing kit (BioCell).

Indirect immunofluorescence microscopy. Two approaches were adopted: (i) stigmas were pollinated by gently brushing with a newly dehisced anther, the pollen allowed to hydrate and germinate for 1–2 h and then the whole stigma was squashed onto a microscope slide coated with double-sided sticky tape. (ii) Pollen was fixed in 4% (w/v) depolymerized paraformaldehyde, 15% (w/v) sucrose in 50 mM sodium phosphate (pH 7.0) for 2 h, centrifuged at 12000·*g* for 5 min, the supernatant removed, the pollen washed three times in 50 mM sodium phosphate, 15% sucrose and embedded in 15% (v/v) gelatine, 15% (w/v) sucrose in 50 mM sodium-phosphate buffer at 4° C. Blocks of embedded pollen were frozen on solid CO_2 for 5 min. Sections (5 µm) were cut at −20° C using a Fridgocut cryostat (Reichert-Jung, Cambridge Instruments, Cambridge, UK). The sections were collected on gelatin-coated slides, warmed at room temperature for 2 h and then incubated at 37° C in 50 mM sodium-phosphate buffer (pH 7.0) to dissolve away the embedding matrix.

Pollen/stigma squashes and pollen cryosections were incubated with cutinase antiserum 1:25 in PBS for 1 h, washed four times for 5 min in PBS, incubated in goat anti-mouse-fluorescein isothiocyanate (FITC) conjugate (Sigma) 1:40 in PBS for 1 h, washed twice for 5 min in PBS and once in distilled H_2O, dried and mounted in a drop of 'Citifluor' (Citifluor, City University, London, UK). Fluorescence microscopy was carried out using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany.) (excitation 485 nm, dichroic beam splitting 510 nm and long-pass emission 420 nm).

Esterase detection. Following SDS-PAGE, gels were washed for 1 h in 2.5% (v/v) Triton X-100 to remove the SDS (Lockwood et al. 1987). Non-specific esterase activity was detected using α-naphthyl acetate as substrate in a coupling reaction with Fast Blue RR salt (Pearse 1972). Esterases capable of hydrolysing butyrate esters were detected using 5 ml 1% (v/v) α-naphthyl butyrate as substrate instead of α-naphthyl acetate (modified from Pearce 1972).

Partial purification of 22-kDa polypeptide. Preparative SDS-PAGE of 240 µl crude pollen extract containing 4200 µg protein was carried out on two 15% polyacrylamide mini gels. Following electrophoresis, the ends of each gel were removed and quickly stained for esterase activity. The gel pieces were then realigned with the gels and the regions containing the 22-kDa and 42-kDa esterases excised from the gels. The proteins contained in the excised gel pieces were then electroeluted for 4 h at 3 W in electrode buffer (0.005 M Tris, 0.036 M glycine, 0.001% SDS, pH 8.3) using an ISCO sample concentrator (ISCO, Lincoln, Neb., USA). The eluted protein samples were resuspended in 200 µl of electrode buffer and the SDS removed by incubating with 100 µl 2.5% (v/v) Triton X-100 for 30 min. The sample was then spun through a Sephadex G-25 column washed with 0.05 M sodium-phosphate buffer (pH 7.0) to remove salt and detergent. This was carried out twice. The sample was then concentrated to a final volume of 100 µl using a centrifugal evaporator (Jouan). As a positive control, 15 µg of purified *Fusarium* cutinase contained in excised gel fragments was subjected to the same electroelution and renaturation. The pollen protein samples and *Fusarium* cutinase samples were then assayed for cutinase activity.

Results

Hydrolysis of [^{3}H]cutin and PNB by a crude extract of pollen proteins. A crude extract of pollen proteins catalysed the release of labelled cutin monomers from [^{3}H]cutin over a period of 18 h with a specific activity of 363.3 Bq (mg protein)$^{-1}$·h^{-1}. This extract also catalysed the hydrolysis of *p*-nitrophenyl butyrate (PNB) in a linear fashion for 15 min with a specific activity of 168 nmol·(mg protein)$^{-1}$·min^{-1}, but did not catalyze the hydrolysis of *p*-nitrophenyl palmitate (PNP).

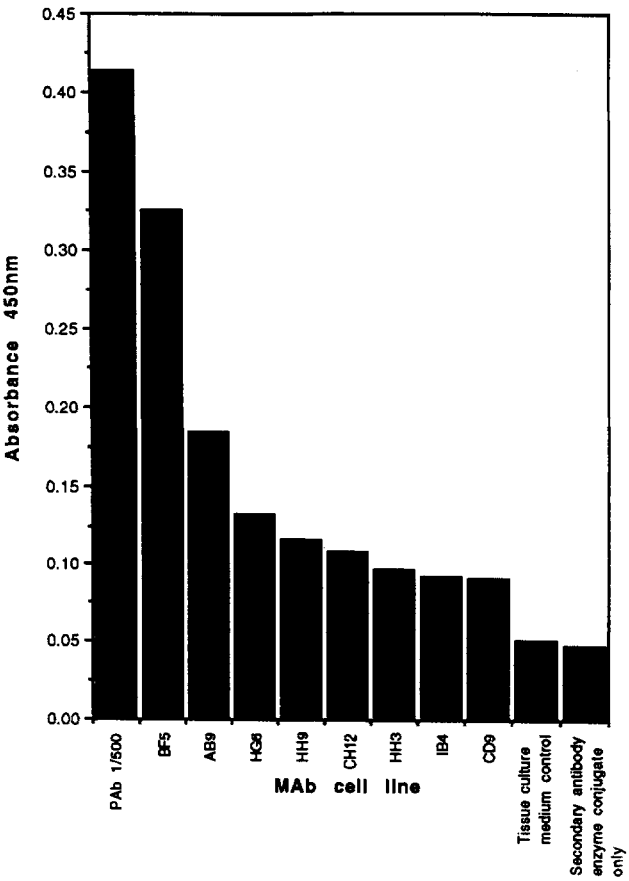

Fig. 1. Polyclonal antiserum and monoclonal antibodies to *Fusarium* cutinase tested by ELISA against a crude protein extract from the pollen of *Brassica napus*. *PAb*, polyclonal antiserum; *BF5*, *AB9*, *HG6*, *CH12*, *HH3*, *IB4*, *CD9*, monoclonal-antibody (*MAb*) cell lines

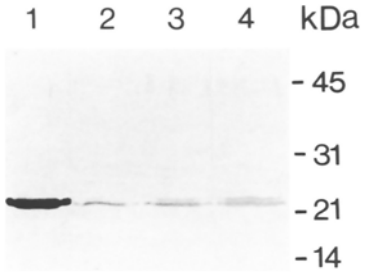

Fig. 2. Western blot of *B. napus* pollen protein extract probed with polyclonal antiserum to *Fusarium* cutinase. Bound antibodies were detected using gold-conjugated secondary antibody. *Lane 1, Fusarium* cutinase (1.8 μg protein). *Lanes 2–4*, pollen protein extract: *lane 2*, 50 μg protein; *lane 3*, 75 μg protein; *lane 4*, 100 μg protein

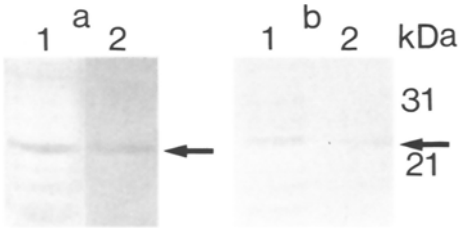

Fig. 3a, b. Western blot of *B. napus* pollen protein extracts probed with monoclonal antibodies to *Fusarium* cutinase. **a** MAb BF5; **b** MAb CH12. *Lanes 1*, pollen protein extract (75 μg protein); *lanes 2, Fusarium* cutinase (1.8 μg protein). Bound antibodies detected as in Fig. 2

Enzyme-linked immunosorbent assays of crude pollen extract using cutinase polyclonal antiserum and monoclonal antibodies. Antigens present in the crude pollen extract of *B. napus* gave high absorbance values in ELISA tests with antisera (polyclonal antibodies) raised against a purified cutinase from *Fusarium solani* f. sp. *pisi* (Fig. 1). A high absorbance value was also obtained with a monoclonal antibody, BF5, raised against the same cutinase while lower, but appreciable absorbance values were obtained with seven other cutinase monoclonal antibodies: AB9, HG6, HH9, CH12, HH3, IB4 and CD9. Of these, AB9 gave the highest absorbance value.

Identification of the major pollen antigen recognized by the cutinase antibodies by Western blotting. Because the antiserum to *Fusarium* cutinase gave the highest absorbance values in ELISA tests with the pollen antigens, it was used to probe Western blots of the crude pollen extract (Fig. 2). The polyclonal antiserum recognized a protein doublet present in the pollen extract which had the same R_f as purified *Fusarium* cutinase, giving an identical estimated molecular weight of 22 kDa.

Western blots of the crude pollen extract were also probed with the monoclonal antibodies BF5 and CH12 which also recognized the 22-kDa protein (Fig. 3). This recognition was weaker than with the antiserum, such that a protein doublet was not discernible.

Indirect immunofluorescence localization of the pollen antigen. When cryosections of pollen were treated with *Fusarium* cutinase polyclonal antiserum followed by FITC-conjugated secondary antibody, strong fluorescence was visible in all pollen grains. This fluorescence was confined predominantly to the intine region of the pollen wall (Fig. 4a, b), although faint fluorescence was also detectable within discrete regions of the cytoplasm (Fig. 4b). Control sections treated with mouse pre-immune serum and FITC-conjugated secondary antibody showed no fluorescence (Fig. 4c, d).

When squash preparations of pollinated stigmas were treated as above, fluorescence was detected in very few pollen grains. In preparations made 1.5 h after pollination a small number of pollen grains showed localized fluorescence confined to an area of the pollen wall which was in contact with stigmatic papillae (Fig. 4e). Some pollen grains which had become detached from the stigma also showed fluroescence in regions of their walls usually near to a colpus (Fig. 4f).

Esterase and cutinase assays of the 22-kDa pollen polypeptide. Three proteins present in the crude pollen extract catalysed the hydrolysis of α-naphthyl acetate in polyacrylamide gels after removal of the SDS with Triton (Fig. 5a). One of these proteins had an estimated molecular weight of 22 kDa identical to that of the pure *Fusarium* cutinase and the pollen polypeptide recognized by the *Fusarium* cutinase antibodies on Western blots; the two other esterases had estimated molecular weights of 31 kDa and 42 kDa – neither of these esterases was recognized by the cutinase antibodies. Both the 22-kDa esterase and the 42-kDa esterase also catalysed the hydrolysis of α-naphthyl butyrate (Fig. 5b), but the 31-kDa esterase did not.

When the regions of preparative SDS-PAGE gels containing the 22-kDa esterase and the 42-kDa esterase were excised and their protein contents electroeluted and renatured by removal of the SDS with Triton, 15.2 μg of protein was recovered from the 22-kDa band and 12.3 μg from the 42-kDa band. When these protein extracts were assayed for PNB and cutinase activities (Table 1) both the 22-kDa and the 42-kDa proteins catalysed the hydrolysis of PNB but only the 22-kDa proteins catalysed the release of labelled cutin monomers from [^{3}H]cutin (Table 1).

In a control experiment to assess the efficiency of the electroelution and enzyme renaturation technique, 4.8 μg of protein was recovered from a starting total of 15 μg of pure *Fusarium* cutinase. This renatured cutinase was still able to catalyse the hydrolysis of tritiated cutin (Table 1), although its activity was reduced by nearly 10 times its activity before the treatment, showing that the enzyme renaturation, although successful, was only partial.

Discussion

We have shown that the pollen of *Brassica napus* contains an active cutinase capable of catalysing the hydro-

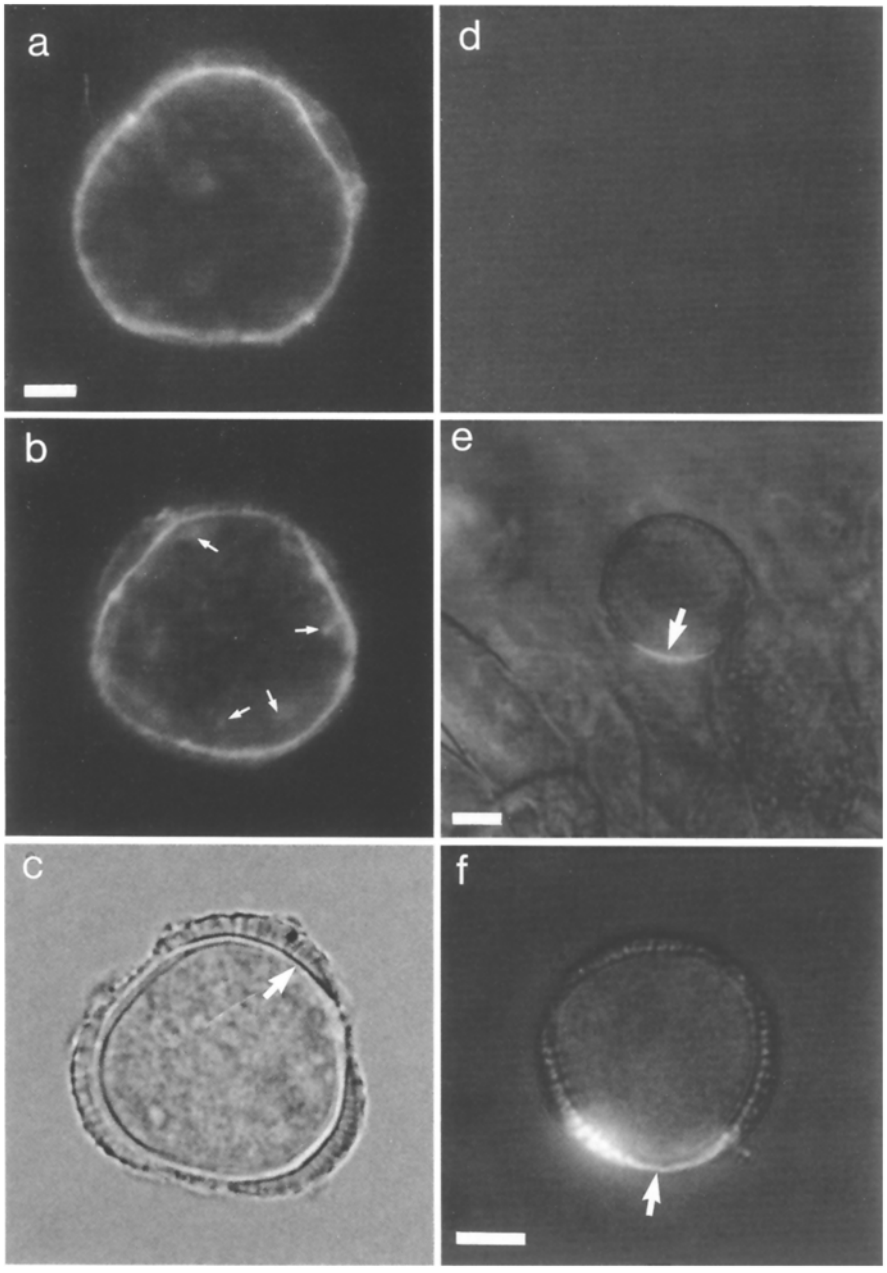

Fig. 4a–f. Immunolocalization of the 22-kDa pollen antigen from *B. napus* recognized by polyclonal antiserum to *Fusarium* cutinase. **a–d** Cryosections of pollen (×1500; bar = 5 μm). **a, b** Fluorescence micrographs of sections treated with cutinase polyclonal antiserum followed by FITC-conjugated secondary antibody. Note that fluorescence is associated primarily with the intine region of the pollen wall together with discrete regions of the cytoplasm (*arrows* in **b**). **c, d** Light micrograph (**c**) and fluorescence micrograph (**d**) of a pollen-grain section treated with mouse pre-immune serum followed by FITC-conjugated secondary antibody as a control (*arrow* in **c** indicates the intine). **e, f** Fluorescence micrographs of a stigma squashed 1.5 h post-pollination then treated with cutinase polyclonal antiserum followed by FITC-conjugated secondary antibody. In **e** note that fluorescence is confined to a region of the pollen grain in contact with the stigma (×750; bar = 10 μm). **f** A pollen grain which has become detatched from the stigma. Note the fluorescence in the pollen wall either side of a colpus (*arrow*) (×1150; bar = 8 μm)

lysis of radiolabelled cutin. The differential catalytic behaviour of the crude pollen extract towards the short-acyl-chain ester PNB (C4) and the long-acyl-chain ester PNP (C16) indicates a surprising resemblence to that exhibited by fungal cutinases (Kolattukudy et al. 1981) and suggests fundamental differences between this pollen cutinase and the enzyme from the pollen of *Tropaeolum* (Shaykh et al. 1977a) which catalyses the hydrolysis of *p*-nitrophenyl esters of both short- and long-chain fatty acids with almost equal ease (Maiti et al. 1979). This apparent similarity between the crude extract of *Brassica* pollen cutinase and fungal cutinases was unexpected.

Recently, molecular studies have revealed considerable sequence homology to exist between pollen cDNA clones for pectate lyase (Wing et al. 1990) and pectin esterase (Albani et al. 1991) and equivalent genes in the plant pathogen *Erwinia chrysanthemis*. Our finding that cross-reactivity occurred between polyclonal and monoclonal antibodies raised to a cutinase from the pathogenic fungus *Fusarium solani* f. sp. *pisi*. (Coleman et al. 1993) and a cutinase from the pollen of *Brassica* should there-

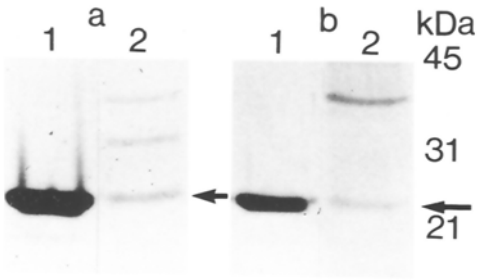

Fig. 5a, b. Detection of esterases in extracts of *B. napus* pollen protein. Following SDS-PAGE the gels were washed with 2.5% Triton X-100 to remove SDS. **a** Gel stained for non-specific esterase activity using α-naphthyl acetate and Fast Blue RR salt; **b** gel stained for butyrate-esterase activity using α-naphthyl butyrate and Fast Blue RR salt. *Lanes 1*, *Fusarium* cutinase (1 μg protein); *lanes 2*, pollen protein extract (100 μg protein)

Table 1. *p*-Nitrophenyl-butyrate esterase and cutinase assays of the 22-kDa and 42-kDa *B. napus* pollen esterases after electroelution from a preparative SDS-PAGE gel and removal of SDS with 2.5% Triton X-100. Also cutinase assays of pure *Fusarium* cutinase (as a positive control) before and after the same treatment. The PNBase activity was assayed spectrophotometrically at 405 nm according to Purdy and Kolattukudy (1973) and cutinase activity was assayed according to Bonnen and Hammerschmidt (1989) by measuring the disintegration per minute (60 dpm = 1 Bq) of cutin monomers released from tritiated cutin. Protein extracts were incubated with tritiated cutin for 18 h

	PNBase activity[a]	Cutinase activity[b]
22-kDa esterase fraction	86.7	280
42-kDa esterase fraction	99.5	0.00
Fusarium cutinase		
Before SDS-PAGE, electroelution and renaturation	n.t.[c]	$1.58 \cdot 10^4$
After SDS-PAGE electroelution and renaturation	n.t.[c]	$1.66 \cdot 10^3$

[a] nmol·(mg protein)$^{-1}$·min^{-1}
[b] Bq·(mg protein)$^{-1}$·h^{-1}
[c] not tested

fore not be totally unexpected. Remarkably, these antibodies recognized a pollen antigen of 22 kDa, a molecular weight identical to that of the *Fusarium* cutinase. Furthermore, studies using indirect immunofluorescence microscopy showed that the antigen is localized to a region of the pollen, the intine, which is not only known to be a reservoir for readily elutable hydrolytic enzymes (Knox and Heslop-Harrison 1970) but has been predicted as a location for the pollen cutinase of *Tropaeolum* (Shaykh et al. 1977a). The antigen was also localized to an area of contact between pollen and stigmatic papillae at a time (1.5 h post pollination) just prior to pollen-tube penetration when erosion of the papillar cell cuticle is apparent (Dickinson and Lewis 1973; C.J. Elleman, Department of Plant Sciences, University of Oxford, personal communication) and when non-specific esterase activity is at its highest (Heslop-Harrison and Heslop-Harrison 1981). Interestingly, in pollen grains which had become detached from papillae, fluorescence was usually associated with one of the colpi – the pores through which germinating pollen tubes emerge and from which intine-held hydrolases readily elute (Knox 1973).

These immunological data therefore strongly suggest that the pollen antigen recognized by the *Fusarium* cutinase antibodies is itself a cutinase. This conclusion was confirmed when the 22-kDa polypeptide was electroeluted from a gel, renatured and shown to hydrolyse tritiated cutin and PNB. Further confirmation that the *Fusarium* cutinase antibodies are recognizing a cutinase in the pollen of *Brassica* has come from our preliminary attempts to immunoprecipitate the pollen cutinase using IgGs from the polyclonal antiserum conjugated to protein-G-coated Sepharose beads (data not shown). The immunoprecipitation complex has been shown to catalyse the hydrolysis of PNB and tritiated cutin and work is now in progress to use this technique to purify the enzyme by affinity chromatography.

The immunological and molecular-weight similarities of the *Brassica* cutinase and the *Fusarium* cutinase are all the more important when it is considered that the *Tropaeolum* pollen cutinase was found to have a molecular weight of 40 kDa and was not recognized by antibodies to *Fusarium* cutinase (Maiti et al. 1979). Interestingly, a second esterase with PNBase activity was also detected in the crude *Brassica* pollen extract. The estimated molecular weight of this esterase, 42 kDa, was very similar to that of the *Tropaeolum* cutinase. However, when this esterase was assayed for cutinase activity it was found to be incapable of catalysing the hydrolysis of tritiated apple cutin, suggesting that it is unlikely to be a functional cutinase.

It is perhaps not surprising that the spores of pathogenic fungi and the pollen of flowering plants with 'dry' stigmas should possess cutinases, because to achieve successful infection and pollination, respectively, a cuticular barrier must be breached. Despite almost overwhelming evidence for the importance of cutinases in the infection of host tissues by certain pathogenic fungi (Shaykh et al. 1977b; Maiti and Kolattukudy 1979; Köller et al. 1982, 1990; Dickman et al. 1989) a recent report (Stahl and Schäfer 1992) has questioned the central role of cutinase in this process. Parallel studies of the role played by pollen cutinases in penetration of the stigmatic cuticle by pollen tubes will no doubt add further fuel to this debate.

The presence of pre-formed active cutinases in the pollen of *Brassica* and of *Tropaeolum* is in sharp contrast to the situation in the spores of necrotrophic fungi such as *Fusarium* where cutinase synthesis occurs as a result of transcripitional induction by cutin monomers (Woloshuck and Kolattukudy 1986; Podila et al. 1988; Bajar et al. 1991). Interestingly, in *Crocus* cutinase activity could only be detected in the pollen after contact with a stigma (Heslop-Harrison 1977); such a finding suggests that there may be yet another type of pollen cutinase

which is either induced or activated by stigmatic components. Indeed, the pollen cutinases of a number of other species have been proposed to be active only after stigmatic contact (Heslop-Harrison and Heslop-Harrison 1975, 1981; Knox et al. 1976; Shivanna et al. 1978) but direct proof of this cutinase activation has never been forthcoming. Instead the *Brassica* cutinase, which was proposed by Shivanna et al. (1978) to be activated by stigmatic contact has now been shown to be present in the pollen in an active form. So while the *Brassica* and *Tropaeolum* cutinases differ in their molecular weights, immunological properties and substrate specificities they are similar in the fact that their synthesis is not induced by contact with the stigma. Such findings, together with those of Heslop-Harrison (1977), predict a diversity of angiosperm pollen cutinases paralleling that found in fungal cutinases (Trail and Köller 1990). Spores of the biotrophic fungi *Erysiphe graminis* (Kunoh et al. 1990) and *Puccinia* sp. (our observations, not shown), unlike those of necrotrophic fungi, such as *Fusarium* and *Colletotrichum* (Kolattukudy 1985) have been shown to contain a pre-formed active cutinase which is released when the spore alights on a solid surface (Kunoh et al. 1990).

It will be interesting to discover how widespread and diverse pollen cutinases are within the Angiosperms and whether the possession of cutinase by the pollen is confined to those species with "dry" cuticularized stigmas. If cutinase is important in pollen-tube penetration it follows that the pollen of species possessing non-cuticularized "wet" stigmas (Heslop-Harrison and Shivanna 1977) may lack such an enzyme. The stigmas, or the equivalent receptive surfaces of the carpels, of species from Angiosperm families considered to be primitive, such as the Magnoliaceae and Hamamelidaceae (Takhtajan 1969), are dry (Heslop-Harrison and Shivanna 1977), which indicates that their pollen may contain a cutinase, and raises the possibility that this class of enzymes may have arisen early in the evolution of the Angiosperms.

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