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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Purification and identification of cutinases from *Colletotrichum kahawae* and *Colletotrichum gloeosporioides*

Zhenjia Chen · Catarina F. Franco · Ricardo P. Baptista · Joaquim M. S. Cabral · Ana V. Coelho · Carlos J. Rodrigues Jr. · Eduardo P. Melo

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Abstract *Colletotrichum kahawae* is the causal agent of the coffee berry disease, infecting leaves and coffee berries at any stage of their development. *Colletotrichum gloeosporioides* is the causal agent of brown blight, infecting ripe berries only. Both fungi secrete the same pattern of carboxylesterases to the fermentation broth when cutin is used as carbon source. By using two different strategies composed of two precipitation steps (ammonium sulphate and acetic acid precipitation) and two chromatographic steps, two proteins displaying carboxylesterase activity were purified to electrophoretic homogeneity. One, with a molecular weight (MW) of 21 kDa, has a blocked N terminus and was identified as cutinase by peptide mass fingerprint and mass spectrometry/mass spectrometry data acquired after peptide derivatization with 4-sulphophenyl isothiocyanate. The second, with a MW of 40 kDa, displays significant carboxylesterase activity on tributyrin but low activity on *p*-nitrophenyl butyrate. N-terminal sequencing for this protein does not reveal any homology to other carboxylesterases. These two enzymes, which were secreted by both fungi, appear homologous.

Keywords *Colletotrichum kahawae* · *Colletotrichum gloeosporioides* · Carboxylesterase · Cutinase · Protein purification · Mass spectrometry

Z. Chen · C. J. Rodrigues Jr.
Centro de Investigação das Ferrugens do Cafeeiro, IICT,
Quinta do Marquês,
2784-505 Oeiras, Portugal

Z. Chen
Environment and Plant Protection Institute,
Chinese Academy of Tropical Agriculture Sciences,
Hainan, People's Republic of China

Z. Chen · C. F. Franco · A. V. Coelho
Instituto Tecnologia Química e Biológica,
Universidade Nova Lisboa,
Oeiras, Portugal

R. P. Baptista · J. M. S. Cabral · E. P. Melo
Centro de Engenharia Biológica e Química,
Instituto Superior Tecnico,
Av. Rovisco Pais,
1049-001 Lisbon, Portugal

A. V. Coelho
Universidade de Évora,
Évora, Portugal

E. P. Melo (✉)
Centro Biomedicina Molecular e Estrutural,
Universidade de Algarve,
Campus de Gambelas,
8005-139 Faro, Portugal
e-mail: emelo@ualg.pt

Plant cuticles are the first defensive barriers that directly penetrating plant pathogens have to overcome (Kolattukudy et al. 1995), and cutin is the main structural component of cuticle. Cutinases catalyse hydrolysis of ester bonds in the cutin polymer and, therefore, have been proposed as being involved in cuticle penetration (Dickman et al. 1989; Degani et al. 2006). They also hydrolyse model substrates such as *p*-nitrophenyl palmitate (NPP), *p*-nitrophenyl butyrate (NPB) and triglycerides (Martinez et al. 1992).

Cutinases are like esterases with an active site composed of the triad serine, histidine and aspartic acid.

Lipases also have a catalytic triad composed of serine, histidine and a carboxyl group, and globally can be defined as carboxylesterases catalysing the hydrolysis (and synthesis) of long-chain acylglycerols (≥10 carbon atoms). However, for lipases, activity on triglycerides is usually greatly enhanced at lipid–water interfaces according to the phenomenon called interfacial activation (Martinez et al. 1992). Cutinases can be classified between esterases and lipases because they can be active on soluble and on emulsified triglycerides (Martinez et al. 1992).

Work on cutinase from the fungus *Fusarium solani* has been carried out during the last years with the aim of evaluating potential biotechnological applications of cutinase (Carvalho et al. 1999; Melo et al. 2003). Indeed, hydrolytic and synthetic reactions catalysed by cutinases have potential uses in many applications such as hydrolysis of milk fat, household detergents, oleochemical industry, synthesis of structured triglycerides, polymers and surfactants, bioscouring and synthesis of chiral compounds (Carvalho et al. 1999; Degani et al. 2006). To include new cutinases from other sources and further explore their catalytic properties, two species of the fungi *Colletotrichum* were tested for their ability to secrete cutinases.

Colletotrichum kahawae Waller & Bridge and *Colletotrichum gloeosporioides* Penz are two fungi that infect coffee, the former as a pathogen and the latter as a saprophyte or weak pathogen. These two fungi, like many other *Colletotrichum* species, penetrate through the cuticle, but whereas *C. kahawae* is able to infect both green and ripe berries, *C. gloeosporioides* only infects the ripe ones (Chen et al. 2005). In this work we have studied the ability of the coffee fungi *C. kahawae* and *C. gloeosporioides* to secrete cutinases. Cutinases from the two fungi were purified to homogeneity and undoubtedly identified as cutinases by enzyme inhibition, N-terminal sequencing and mass spectrometry.

Materials and methods

Chemicals

Modified trypsin was obtained from Promega; trifluoroacetic acid (TFA) and high-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) were obtained from Riedel; POROS 10 R2 reverse-phase chromatography matrix was obtained from PerSeptive Biosystems; α-Cyano-4-hydroxycinamic acid (α-CHCA) and PepMix1, a mixture of peptide standards, were obtained from LaserBiolabs; tributyrin (TB) was obtained from Fluka; diisopropyl fluorophosphate (DFP) and 4-sulphophenyl isothiocyanate (SPITC) were obtained from Aldrich; NPB, NPP, arabic gum, formic acid, ammonium hydrogen carbonate, dithiothreitol (DTT) and iodoacetamide and other reagents were purchased from Sigma. Cutin was isolated from golden apples by using the method of Kolattukudy et al. (1981).

Fungi

Single-spore isolates of *C. gloeosporioides* from China (Ch27) and *C. kahawae* from Zimbabwe (Z1) were used. The former was isolated from infected ripe coffee berries, and the latter was isolated from infected green ones.

Culture conditions and enzyme production

The isolates were maintained in potato dextrose agar (PDA) plates, in the dark, for about 7–10 days at 22°C. Conidia suspensions with concentration of 2×10^6 conidia per millilitre were obtained. For production of cutinases, 500-ml Erlenmeyer flasks containing 100 ml Czapek–Dox mineral salt medium at pH 7.5 and 0.5 g cutin as sole carbon source were inoculated with 5 ml of conidia suspensions. Controls were identical, but instead of cutin, sucrose at a concentration of 5% (w/v) was used. Flasks were incubated at 22°C without shaking.

Carboxylesterase activity assays

Three substrates were used for determination of carboxylesterase activity. Activity on NPB was determined as described elsewhere (Kolattukudy et al. 1981) and was designated as NPBase activity. Activity on NPB provides a simple measurement of cutinase activity and correlates to hydrolysis of ^{3}H-cutin (Bostock et al. 1999). *p*-Nitrophenyl palmitate (NPPase) was used as longer chain ester substrate. Tributyrin (TBase) was also used to determine carboxylesterase activity by the pH–stat method (Brocklehurst 1993) because this method shows enhanced accuracy at higher pH values (where NPB and NPP are more sensitive to chemical hydrolysis).

Enzyme purification

Ammonium sulphate and acetic acid precipitations

Fermentation broth was first filtrated to remove the mycelium. Granular ammonium sulphate was then slowly added under stirring until 50% (w/v). The precipitated protein was collected by centrifugation at 12,000×*g* for 30 min, dissolved in about 60–100 ml of 50 mM sodium phosphate buffer (pH 8.0) and dialyzed overnight at 4°C (Dickman et al. 1982). Acetic acid precipitation was

performed on dialysed samples by adding small volumes of 30% acetic acid (v/v) until a pH value of 4.7 was reached. Acid precipitation is a very effective step for the purification of cutinase from *F. solani* (Sebastião et al. 1996). The mixture was stirred overnight at 4°C and then centrifuged at 14,000×*g* for 40 min at 4°C. The supernatant was then divided in two fractions: one was dialysed against 20 mM Tris–HCl buffer (pH 8.5), and the other was dialysed against the same buffer at pH 9.5.

Chromatography

The fraction at pH 8.5 was loaded onto a DEAE (diethylaminoethyl) Sephacel column pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.5). Elution was carried out with a gradient from 0 to 1.0 M NaCl. Fractions with NPBase activity were pooled and dialysed overnight against 20 mM Tris–HCl buffer, pH 7.6. The sample was then loaded onto an SP (sulphopropyl)-Sephadex C-25 column, pre-equilibrated with the same buffer at pH 7.6 and eluted with a gradient from 0 to 1.0 M NaCl. The fractions possessing NPBase activity were pooled and assayed for TBase activity. The fraction at pH 9.5 was loaded onto a DEAE column pre-equilibrated with 20 mM Tris–HCl buffer (pH 9.5). The protein eluted in the void volume (no salt) displayed NPBase activity as well as TBase activity and was applied to a Q (quaternary ammonium)-Sepharose fast flow column at pH 9.5. Elution was carried out with a gradient from 0 to 1 M NaCl. Fractions were checked for NPBase activity.

Identification of enzymes

Inhibition by DFP

To determine the effect of DFP on the enzyme activity, purified enzyme solutions were mixed with the same volume of 50 μM DFP and incubated at room temperature for 15 min before being tested for NPBase activity.

N-terminal and protein sequence determinations

N-Terminal amino acid sequence analysis was done according to Stone and Williams (1993). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes before being subjected to N-terminal sequencing.

Protein identification by peptide mass fingerprinting

In-gel digestion Excised gel bands from sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) were washed with 50% ACN and dried by centrifugation under vacuum. Digestion was performed as described by Spenglen (1997). For the reduction and carbamidomethylation of cysteines, gel pieces containing the proteins were treated with DTT and iodoacetamide. Modified trypsin was added to the dried gel pieces and incubated at 37°C overnight. Desalting and concentration of supernatant from trypsin digestion were carried out (after acidification with formic acid) with custom-made chromatographic microcolumns containing POROS 10 R2 material filling (20-μm bead size) equilibrated with 2% TFA (Gobom et al. 1999). Peptides were directly eluted to the matrix-assisted laser desorption/ionization (MALDI) plate using α-CHCA in 70% ACN with 0.01% TFA.

MALDI-time of flight MS All mass spectra were obtained using a PerSeptive Biosystems MALDI-time of flight (TOF) MS Voyager-DE STR (Framingham, USA) equipped with delayed extraction, reflectron and with a 337-nm N2 laser. External mass calibration was performed with a mixture of peptide standards, PepMix1. Monoisotopic peptide masses were used to search for protein identification with Mascot software (Matrix Science, UK) (Perkins et al. 1999). Searches were done on a National Center for Biotechnology Information (NCBI) non-redundant protein sequence database.

De novo sequencing of tryptic peptides

The supernatant of the tryptic digestion was derivatized by N-terminal sulphonation as described by Wang et al. (2004) with small modifications. The mixture of tryptic peptides was dried completely by centrifugation under vacuum and resuspended in SPITC (10 μg/μl in 50 mM NH_4HCO_3). After incubation for 30 min at 55°C with gentle shaking, formic acid was added to a final concentration of 5% to stop the reaction. After desalting and concentration as described above, the derivatized tryptic peptides were first analyzed in normal MALDI reflector mode. The *m/z* values resulting from the addition of 215 Da (SPITC addition) were subjected to post-source decay (PSD) analysis to obtain sequence information. PSD fragment ion spectra were acquired for peptides derivatives after isolation of the appropriate precursor ions. Angiotensin was fragmented, and the *m/z* values were used to calibrate the spectra. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflectron. Spectra for each voltage segment were collected using 1,000–1,500 laser shots. The individual spectra were merged together using software developed by Applied Biosystems, Data Explorer version 4.4. The PSD fragment ions measured as isotopically averaged masses were used to search for protein identification with Mascot software (Matrix Science, UK) (Perkins et al. 1999).

Results

Enzyme production

Colletotrichum gloeosporioides from China (Ch27) grew well on sucrose as sole carbon source, reaching 1.2 mg/ml of dry weight of mycelium after 28 days (data not shown). Biomass yields were significantly lower (0.6 mg/ml of dry weight) for the isolate *C. kahawae* Z1 from Zimbabwe. Biomass yield could not be evaluated using cutin as carbon source due to the insolubility of cutin, but isolates Z1 of *C. kahawae* and Ch27 of *C. gloeosporioides* grew well in the cutin-containing medium and hydrolysed roughly 100 mg (20%) of cutin in about 15 days. Cutinases are inducible enzymes secreted to the extracellular medium, and therefore, protein content and carboxylesterase activity were evaluated in the fermentation broth (data not shown). Protein content and NPPase activity increased significantly for the two isolates of *Colletotrichum* after 10 days when cutin was present. In contrast, protein secretion was very low using sucrose as carbon source. No carboxylesterase activity was detected in culture filtrates containing sucrose as the sole carbon source. Activity measurements using NPB as substrate matched those measured using NPP.

Enzyme purification

The scheme used for enzyme purification is shown in Fig. 1. Firstly, an ammonium sulphate precipitation step up to 50% (w/v) was carried out to concentrate and to clean the enzyme sample from broth components. The resuspended pellets were then submitted to an acetic acid precipitation step (Table 1). The specific activity due to NPBase was distributed between the supernatant and the pellet, but the specific activity due to TBase was close to 100% in the supernatant. In addition, purification factors in the supernatant were larger, especially for TBase activity.

The supernatants of the acetic acid precipitation were dialysed at pH 8.5 and loaded onto the DEAE column (weak anionic exchanger). More than one peak with NPBase activity was detected in the void volume (Fig. 2a). Purification factors were increased 1.5- to 3.0-fold in the major peak eluted in the void volume (Table 1). Elution of the bound protein with a linear gradient of salt displayed a little shoulder (at low NaCl concentration) and two protein peaks, but only the shoulder displayed significant NPBase activity.

The peak eluted from the void volume of DEAE exhibiting high activity was loaded onto an SP-Sephadex C-25 column at pH 7.6 (strong cationic exchanger) (Fig. 2b). One major peak eluted in the void volume of the SP column displayed about a 1.5-fold increase in specific NPBase and about a 2.5-fold increase in specific TBase activity (Table 1). Protein eluted during the salt gradient shows not NPBase activity. The two samples from *C. kahawae* and *C. gloeosporioides* (data not shown) have a very similar chromatographic behaviour in DEAE and SP-Sephadex C-25 columns, and the peak eluted in the void volume of SP-column was analysed by SDS–PAGE (Fig. 3, lanes C and D). One single band around 21 kDa was obtained for both fungi.

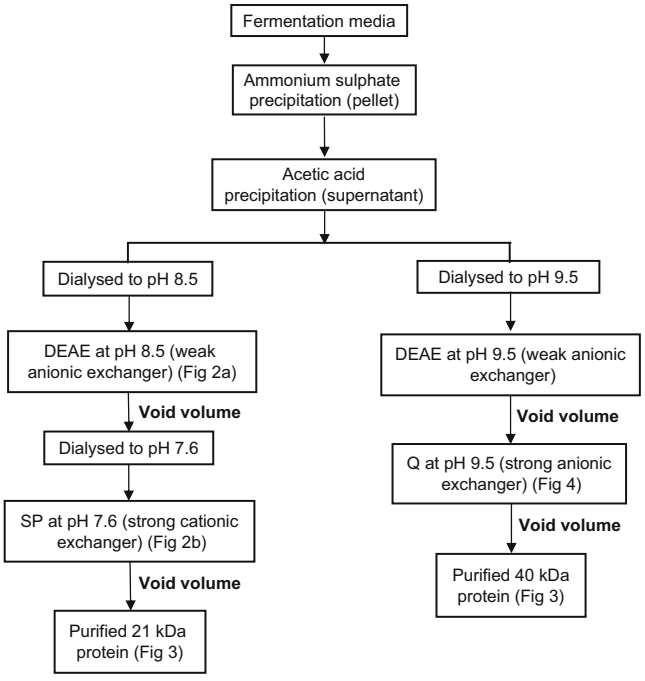

Fig. 1 Flow sheet for the purification of carboxylesterases secreted by *C. kahawae* and *C. gloeosporioides* in the presence of cutin as carbon source

As an alternative strategy, the supernatants of acetic acid precipitation were loaded onto the DEAE column at pH 9.5 (data not shown). Basically, all the NPBase activity was eluted in the void volume. This chromatographic behaviour was unexpected because DEAE at pH 8.5 has fractionated carboxylesterase activity (Fig. 2a). A high pH value such as 9.5 may cause structural changes in some proteins, including aggregation, and thus, change their chromatographic behaviour. The peak eluted in the void volume shows only a small increase in the specific activity when compared with the acetic acid precipitation step (Table 1). This peak was then applied to a Q-Sepharose column at pH 9.5 (Fig. 4), and the protein eluted in the void volume showed a reduction of about sevenfold in NPBase-specific activity and an increase of 1.3-fold in TBase-specific activity (Table 1). When analysed in an SDS–PAGE, gel showed basically one significant band with a molecular weight (MW) of about 40 kDa (Fig. 3, lanes B and E). This enzyme shows a high activity on TB and a low activity on

Table 1 NPBase (micromoles of *p*-nitrophenyl per minute per milligram protein), TBase (micromoles of fatty acid per minute per milligram protein) and PF during the purification of carboxylesterases from *C. kahawae* and *C. gloeosporioides*

Fraction	*Colletotrichum kahawae* Z1				*Colletotrichum gloeosporioides* Ch27			
	NPBase ($\times 10^{-3}$)	PF[a]	TBase	PF	NPBase ($\times 10^{-3}$)	PF	TBase	PF
Ammonium sulphate precipitate	2.78±0.39	1	2.8±0.3	1	3.38±0.90	1	1.6±0.2	1
Supernatant of acetic acid precipitate	14.5±2.9	5.2	24.6±3.8	8.8	9.9±2.1	2.9	10.5± 2.2	6.6
DEAE (pH 8.5, major peak of void volume)	43	15.4	34.2	12.2	24	7.1	26.3	16.4
SP (pH 7.6, void volume)	59	21.2	86.7	30.9	42	12.4	50.2	31.4
DEAE (pH 9.5, void volume)	17	6.1	33.6	12.0	16	4.7	12.3	7.7
Q (pH 9.5, void volume)	2.44	0.9	43.4	15.5	2.08	0.8	26.4	16.5

[a] Purification factors calculated by the ratio between specific activities. NPBase activity was determined by using NPB as substrate at pH 7.6, and TBase activity was measured using tributyrin as substrate at pH 10.5.

NPB. The elution of the bound proteins from the Q column with the NaCl gradient led to two protein peaks. The first peak displayed high NPBase activity and includes the 21-kDa protein purified by using the former strategy (data not shown).

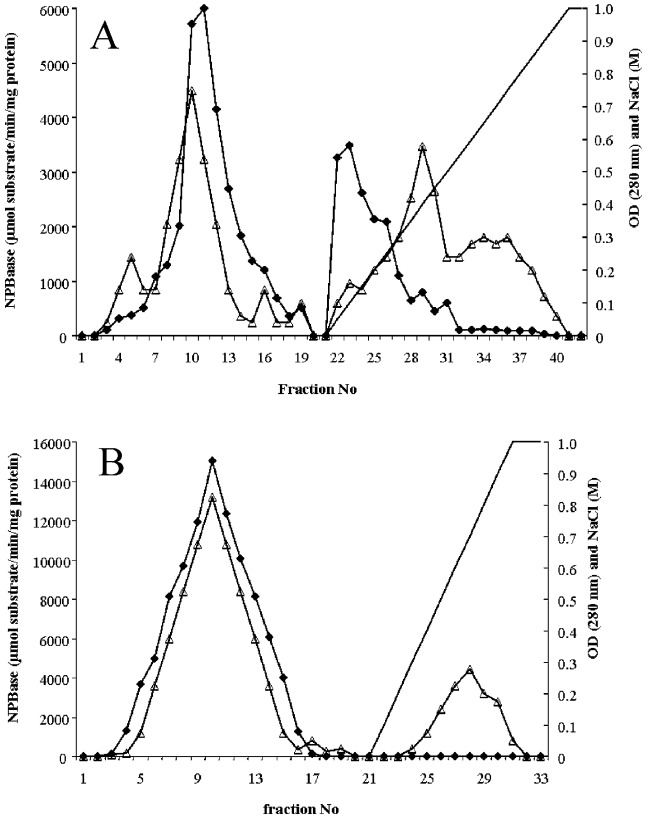

Fig. 2 **a** Chromatogram of *C. kahawae* Z1 sample in a DEAE Sephacel column at pH 8.5 for the supernatant of acetic acid precipitation. **b** Chromatogram of *C. kahawae* Z1 sample (the major peak eluted in the void volume of DEAE column) in an SP-Sephadex C-25 at pH 7.6. The protein was eluted from the columns using a gradient of 0–1.0 M NaCl (*solid line*). Both optical density at 280 nm (Δ) and NPBase (♦) activity were monitored. A similar chromatogram was obtained for *C. gloeosporioides* Ch27 sample

The presence of cutin has induced a complex response, and two enzymes displaying carboxylesterase activity were secreted. Using two different strategies composed of two precipitation steps and two chromatographic steps, it was possible to purify to electrophoretic homogeneity two enzymes secreted to the extracellular medium that display carboxylesterase activity. The MW of these enzymes was determined by a linear plot of electrophoretic mobility vs log MW of standard proteins (Fig. 3). For *C. kahawae* Z1 MWs of 21.7± 0.5 and 40.5±0.3 kDa were determined for these two enzymes (lanes c and b, respectively). For *C. gloeosporioides* Ch27 similar values were obtained (20.8± 0.7 and 40.2±1.0 kDa for lanes d and e, respectively). For simplicity, these enzymes will be designated 21 and 40 kDa enzymes.

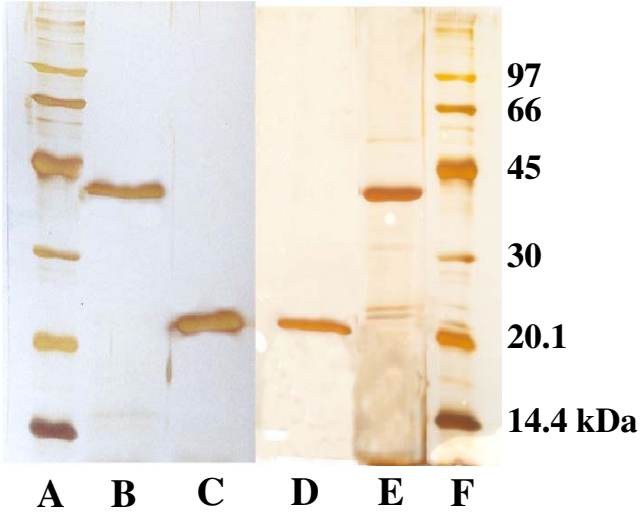

Fig. 3 SDS–PAGE gel under reducing conditions for the samples from *C. kahawae* Z1 and *C. gloeosporioides* Ch27 showing purified 21- and 40-kDa proteins. *C* and *D* are the sample eluted in the void volume of SP Sephadex C-25 column for *C. kahawae* Z1 and *C. gloeosporioides* Ch27, respectively. These samples display a single band of about 21 kDa; *B* and *E* are the samples eluted from the Q-Sepharose column for Z1 and Ch27, respectively, and they display basically a single band of about 40 kDa. *A* and *F* show the molecular weight standards

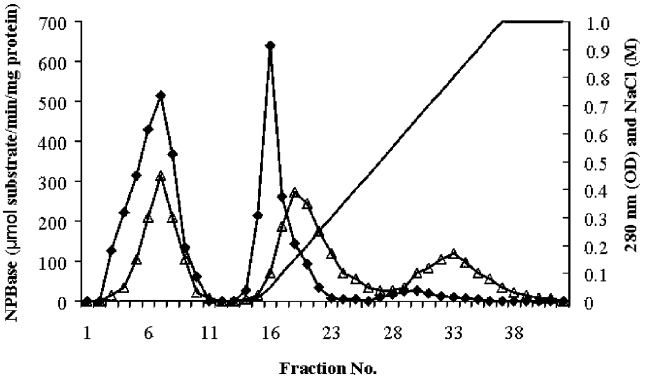

Fig. 4 Chromatogram of *C. kahawae* Z1 sample (eluted in the void volume of DEAE column) in a Q-Sepharose column equilibrated at pH 9.5. The protein bound to the column was eluted with a gradient of NaCl (*solid line*). Both optical density at 280 nm (Δ) and NPBase (♦) activity were monitored. A similar chromatogram was obtained for *C. gloeosporioides* Ch27 sample eluted in the void volume of DEAE column

Enzyme identification

The four purified enzymes displaying carboxylesterase activity, two with MWs of about 21 and two with 40 kDa were totally inhibited by the lowest concentration (25 µmol) of DFP tested (data not shown). DFP is a potent serine inhibitor (Dickman et al. 1982), which has been used as chemical probe for serine residues that are part of serine hydrolase active sites. DFP acts by phosphorylation of the active site serine residue and leads to full inhibition of the enzyme activity Köller and Parker (1989).

To identify clearly which of the four proteins was in fact cutinase, attempts to sequence the N terminus were done. The four proteins were transferred to PVDF membrane, and the N-terminal sequence was analysed. This was successful for the two 40-kDa proteins. For *C. gloeosporioides* Ch27, the N-terminal sequencing was DINGGGATLPQKLYQT (S)GVLT, with some uncertainly for the serine between brackets. Protein–protein BLAST (basic local alignment search tool) was done at the Web site of the NCBI, and the following significant identities were found: 95% identity to the N terminus (residues 3–23) of the protein p27(sj) of plant *Hypericum perforatum* and 85% identity to the N

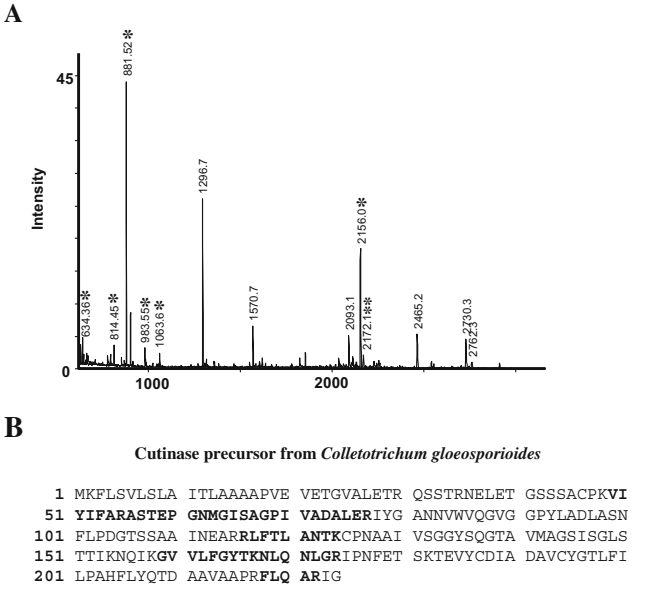

Fig. 5 Peptide mass fingerprinting of the 21-kDa protein from *C. gloeosporioides*. **a** Peptide mass map obtained after digestion with trypsin of the 21-kDa protein from *C. gloeosporioides* (*mass values matched with tryptic peptides of cutinase precursor from *C. gloeosporioides*; **peptide from residues 56–77 with methionine oxidised). The database search program MASCOT (http://www.matrixscience.com) revealed a top score of 82 for cutinase precursor from *C. gloeosporioides*, which means a positive (not random) identification ($p<0.05$ for scores >76). The sequence coverage for this cutinase precursor is shown in **b**. The matched peptides are highlighted in *bold*

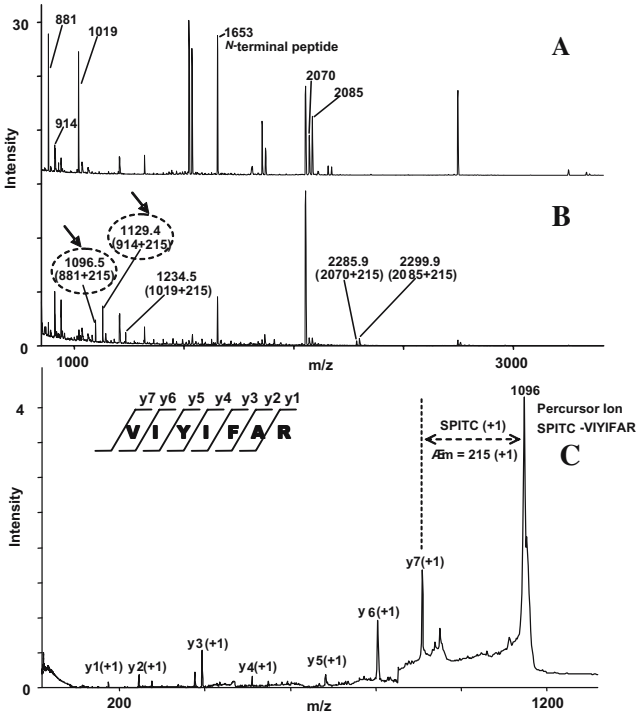

Fig. 6 Mass spectra from *C. kahawae* Z1 after trypsin cleavage. **a** MALDI-TOF MS spectra of the tryptic peptides before derivatization with SPITC (peptides that were derivatized are labelled). **b** MALDI-TOF MS spectra of the tryptic peptides after derivatization with SPITC. Peptides derivatized with SPITC (increase of 215 Da in *m/z* value) were subjected to PSD analysis to obtain sequence information. *Arrowheads* indicate the peptides that were subjected to further sequencing. **c** Peptide sequence from peptide *m/z* 1,096. The resulting ions from *m/z* 1,096 fragmentation were identified in the database as the sequence VIYIFAR (MASCOT, MS/MS ions search). This peptide was found in cutinase precursor form *C. capsici*. The score of 63 for this identification is higher than the minimum of 38, which indicates identity or extensive homology ($p<0.05$)

terminus (residues 1–21) of a human synovial protein stimulatory for T cells (Darbinian-Sarkissian et al. 2006; Hain et al. 1996). For *C. kahawae* a shorter very similar sequence was obtained (DINGGA) showing again no identity at all to other cutinases. Attempts to sequence the N terminus of the two 21-kDa proteins did not succeed, most probably because the N terminus was blocked. Cutinases from *F. solani* f. sp. *pisi*, *Fusarium roseum culmorum*, *F. roseum sambucinum*, *Ulocladium consortiale*, *Colletotrichum capsici* and *C. gloeosporioides* (from papaya) are known to have the N-terminal amino group containing a covalently attached glucuronic acid molecule (Lin and Kolattukudy 1980; Kolattukudy 1984; Hodeland et al. 2002). This supports the possibility that the two 21-kDa proteins are indeed cutinases. These two 21-kDa proteins were then digested by trypsin (which cleaves proteins at the carboxyl end of lysine and arginine residues) for identification by peptide mass fingerprint (PMF). As shown in Fig. 5, the peptide mass map of the 21-kDa protein from *C. gloeosporioides* allowed a positive identification based on seven tryptic peptides mass values that matched to cutinase precursor protein from *C. gloeosporioides*, also known as *Glomerella cingulata* (Swissprot database accession no. p11373). This identifies the enzyme produced by *C. gloeosporioides* isolated from ripe coffee berries as a cutinase.

Although having four tryptic peptides common to *C. gloeosporioides* cutinase (data not shown), the 21-kDa protein from *C. kahawae* could not be identified by PMF with a significant MASCOT score. Therefore, sequence information of tryptic peptides was obtained by PSD MALDI-MS (Spenglen 1997). To overcome the complexity of spectra generated by the appearance of different types of fragmentation ions (*y*-type, *b*-type, *a*-type etc.), several derivatization methods have been developed to produce predictable sequence specific fragment ions. To enhance fragmentation towards the peptide bond, as described by Wang et al. (2004), a sulphonic acid group was introduced at the N terminus of the tryptic peptides using SPITC reagent (Fig. 6). Two of the peptides derivatized with SPITC were sequenced. After a search in the database for identification of the MS/MS ions generated, one of the peptides revealed 100% homology to a highly conserved peptide from a cutinase precursor from *C. capsici* (Swissprot database accession no. p10951) and from *C. gloeosporioides*.

Another evidence of the N-terminal blockage in this 21-kDa protein from *C. kahawae* is also shown in Fig. 6. When the signal peptide is removed from cutinase sequence of *C. gloeosporioides* (Swissprot database accession no. b27451), it originates an N-terminal peptide after tryptic digestion with a monoisotopic mass of 1,470 Da ($[M+H]^+$). The N terminus of cutinases is usually blocked with glucuronamide (Lin and Kolattukudy 1980), and in terms of masses, this originates a N-terminal peptide with 1,653 Da [1,470+183 (glucuronamide)=1,653]. In the peptide mass map of the 21-kDa protein from *C. kahawae* (Fig. 6a), a peptide with a monoisotopic mass of 1,653 ($[M+H]^+$) is present, suggesting the blockage of the N terminus with a glucuronamide residue. This is one more evidence that the 21-kDa protein from *C. kahawae* is indeed a cutinase, confirming the other data collected (activity and inhibition by DFP).

Concluding remarks

Cutinases secreted by two *Colletotrichum* species were purified using a DEAE matrix (weak anionic exchanger) at pH 8.5 as the first chromatographic step. This served basically to remove proteins with no NPBase activity and also to separate cutinase from the other 40-kDa carboxylesterase because the latter binds to the column and elutes at low salt concentration. An additional chromatographic step using an SP matrix (strong cationic exchanger) bound additional proteins with no activity to the matrix and allowed purification of cutinase to electrophoretic homogeneity. Cutinases purified in this work should have a pI within the range 7.5–8.5 because they neither bound to DEAE at pH 8.5 nor to SP at pH 7.6. Cutinase from *F. solanipisi* has a pI of 7.6, for instance (Koops et al. 1999). By using an alternative strategy, cutinase at pH 9.5 bound to the Q matrix (strong anionic exchanger) and the 40-kDa protein can be purified from the void volume of this matrix.

Cutinases from *C. kahawae* and *C. gloeosporioides* could not be identified by N-terminal sequencing due to the blockage of the N-terminal as described for other cutinases (Lin and Kolattukudy 1980; Kolattukudy 1984; Hodeland et al. 2002). Peptide mass fingerprint has allowed the identification of cutinase from *C. gloeosporioides* because the gene coding for it was previously sequenced (Ettinger et al. 1987). For *C. kahawae*, identification of the 21-kDa as cutinase was only possible based on the sequence of an internal peptide that shows homology to other cutinases.

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