Purification and Partial Characterization of an Extracellular Melanoprotein from the Fungus *Venturia inaequalis*

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The fungus *Venturia inaequalis* clone No. 36 isolated from *Malus domestica* cv. Gloster excretes a melanoprotein of 36 kDa in relatively high amounts during growth in liquid culture. The protein was isolated from the culture medium and purified to homogeneity. It was shown to contain melanin. After raising an antiserum against the isolated protein, the protein could be shown to be located in the apoplast fluid of the *V. inaequalis* infected *Malus domestica* cv. Elstar. Partial sequencing of the protein revealed no significant sequence homologies to so far sequenced proteins. The melanoprotein binds ferrous and ferric iron. Moreover, it could be shown that the binding of ferric iron (but not of ferrous iron) leads to a change in the absorbance of the protein suggesting a modification of the protein by ferric, but not by ferrous, iron. In addition to iron, the protein also binds copper, but does not bind manganese or nickel. A possible function of this protein in the recruiting and transport of iron and copper and/or in the protection of the fungus by metal-ion mediated oxidative stress is discussed.

Key words: Apoplast, Melanoprotein, *Venturia inaequalis*

Introduction

The facultative biotrophically growing fungus *Venturia inaequalis* damages apple trees, such as *Malus domestica* cv. Gloster, by causing apple scab. During the infection, the fungus penetrates the cuticle of the host plant and forms a subcuticular mycelium in the apoplast, which eventually leads to new conidia. For prevention or at least reduction of the fungal infection the plant starts its defense machinery which includes an oxidative burst and the synthesis of a number of pathogenesis-related proteins (PR proteins) (Gau *et al.*, 2004). When no successful restriction of the fungus growth could be achieved, the formation of necrotic tissues is initiated to avoid the spreading of the fungus on the plant. To achieve a successful life cycle of the fungus on the plant, a number of protecting reactions are initiated by the fungus.

Although the life cycle of *V. inaequalis* on *M. domestica* is well characterized (MacHardy, 1996),

there is little information available which proteins are excreted by the fungus during infection. Initial work on this aspect provided evidence that *V. inaequalis* (Cke) Wint. excretes a dark-brown protein which was shown to contain melanin (Hignett and Kirkham, 1967; Hignett, 1973; Hignett *et al.*, 1984). This protein causes an increase in the number of lesions on the inoculated plants. Moreover, iron was shown to bind tightly to the protein. When iron salts were applied with the inoculum, the numbers of lesions were greatly reduced. These and additional results were discussed in relation to changes in membrane permeability which occur during infection (Hignett and Kirkham, 1967).

In this paper we present the isolation, a partial characterization, and the localization in the host plant of the melanoprotein isolated from *V. inaequalis* clone No. 36 from *Malus domestica* cv. Gloster.

Materials and Methods

Cultivation of V. inaequalis and preparation of extracellular melanoprotein

A clone of *Venturia inaequalis,* isolated from a leaf of *Malus domestica* (cv. Gloster) in Ruthe

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Abbreviations: ESI Q-ToF, electron spray ionisation quadrupole time of flight; HEPES, 4-(2-hydroxyethyl)-piperazine ethanesulfonic acid; IWF, intercellular washing fluid.

(Sarstedt, Germany) and designated as cultivar No. 36, was cultivated on PDA (potato-dextrose agar) (Parker *et al.*, 1995). For the preparation of the extracelluar melanoprotein a 100 ml Erlenmeyer-flask containing 50 ml PD broth was inoculated with a PDA piece of 1 cm diameter containing mycelium of *V. inaequalis*. The liquid culture was incubated at 19 °C for three weeks under gentle shaking. Afterwards, the liquid culture was harvested and centrifuged for 30 min at $10,000 \times g$. The supernatant was dialysed several times against 10 mM K-phosphate buffer, pH 7.0, at 4 °C and subsequently concentrated by means of a Vivaspin concentrator (cut off 5000 Dalton; Vivascience, Hannover, Germany).

Purification of the melanoprotein by DEAE column chromatography

The concentrated melanoprotein was dialysed overnight in the presence of 20 mM Na-acetate pH 4.8, containing 0.05% Tween 20. After a centrifugation step at $10,000 \times g$ for 15 min, the supernatant was loaded onto a DEAE Sepharose column (size 2×10 cm) equilibrated with the above indicated buffer. After washing the column with equilibration buffer the melanoprotein was eluted using a linear salt gradient from 0 to 500 mM NaCl.

Gel electrophoresis, silver staining, antisera production and immunoblotting

SDS-PAGE and sample denaturation were carried out as described by Schägger and von Jagow (1987). Silver staining was done according to Blum *et al.* (1987). A polyclonal antiserum against the melanoprotein was raised in goat. Melanoprotein (500 μg) was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 0.05% SDS. After dilution with an equal volume of Freund's complete adjuvance, the emulsion was divided into two equal parts and injected subcutaneously into a goat and after 3 weeks into the hingleg muscles. The bleeding was 3 weeks after the second immunization. Immunoblotting was performed by using 0.45 μm pore size nitrocelluose filters (Towbin *et al.*, 1979). Blots were blocked with 5% skim milk and incubated with antibodies against melanoproteins (goat, dilution 1:500 in milk), the latter were detected by anti-goat-IgG from rabbit goat labelled with alkaline phosphatase (dilution 1:20,000; Sigma, Munich, Germany).

Protein digestion and sequencing

Proteins were excised after Coomassie brilliant blue staining from SDS-PAGE gels and digested according to Jensen *et al.* (1998). Peptide fragments were extracted from the gels by washing several times with the same volume of extraction solution (50 μl of a 1:1 mixture of 5% formic acid and acetonitrile). The extracted solutions were combined and concentrated with ZipTips C18 (Millipore). *De novo* sequencing was done on a quadrupole/time-of-flight hybrid mass spectrometer (Q-TOF2 Micromass, Waters, Manchester, United Kingdom) in positive-ion mode. Amino acid sequences were identified by homology search using the program PeptideSearch (EMBL, Heidelberg, Germany).

Isolation of thylakoid membranes and measurement of PSII activity

Thylakoid membranes from pea were isolated according to the method described by Berthold *et al.* (1981). PSII activities were determined by O_2 exchange using a Clark type electrode (Specht *et al.*, 1987). The reaction mixture of 2.5 ml contained: 50 mM NaOH, pH 6.5, 1 mM phenyl-*p*-benzoquinone and 25 μg Chl.

Size exclusion chromatography

Size exclusion chromatography was performed on a Superose 12 column equilibrated with 10 mM K-phosphate buffer, pH 7.0 (Amersham Biosciences, Freiburg, Germany), and calibrated with catalase (240 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and cytochrome C (12 kDa). The liquid flow rate was adjusted to 0.5 ml/min.

Isolation of intercellular washing fluid

All leaves of the inoculated plants were selected for the isolation of apoplastic fluid. This was performed according to Gau *et al.* (2004). The leaves were cut off from the petiole, infiltrated for 5 min with PBS buffer under vacuum and fixed between two transparent strips. The leaves were oriented to the cut area to the bottom of the centrifuge tube and centrifuged for 5 min at $50 \times g$ in a Jouan AC50.10 rotor to remove the excess of liquid. The apoplastic fluid was collected by an additional centrifugation step for 15 min at $700 \times g$ and finally stored at −20 °C. Controls were carried out to as-

certain that no leakage of proteins occurred during the centrifugation step as described in the literature (Anderson *et al.*, 1994).

UV/Vis spectroscopy

Absorbance spectra were measured with a Beckman DU7500 absorbance spectra photometer.

Iron determination

Protein samples as well as control samples (250 μl) were treated by addition of 1 mM Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+} or Mn^{2+} solution to a final concentration of 160 μM. Subsequently, the samples were dialyzed extensively 3 times against 500 ml water or metal chelator (10 mM Na-EDTA, pH 7.0, for Cu^{2+}, Mn^{2+}, Ni^{2+} or 1 mM *o*-phenanthroline, pH 7.0, for Fe^{3+} or Fe^{2+}) and finally mixed with 500 μl 65% HNO_3 and boiled for 1 h. Afterwards the samples were filled up to 2 ml and after a centrifugation step at $10,000 \times g$ for 10 min, the samples were measured in an inductively coupled plasma at the following wavelengths: 324.8 nm (Cu), 259.9 nm (Fe), 352.4 nm (Ni) and 257.6 nm (Mn) with an inductive coupled plasma atomic absorbance spectrometer (Spectroflame-EOP, Spectro Analytical Instruments, Kleve, Germany).

Results and Discussion

Purification, determination of the molecular mass and partial amino acid sequencing of the melanoprotein from V. inaequalis clone No. 36 isolated from M. domestica cv. Gloster

Preliminary investigations on the interaction of *M. domestica* with the fungus *V. inaequalis* and a comparison of several *V. inaequalis* isolates provided evidence that the clone No. 36 isolated from *M. domestica* cv. Gloster (subsequently called *V. inaequalis*) excreted rather high amounts of a dark-brown protein into liquid culture, the excreted amount being significantly higher than that *e.g.* excreted by *V. inaequalis* clone No. 15 isolated from *M. domestica* cv. Elstar.

Protein purification

The culture medium used for growth of *V. inaequalis* was employed as starting material for the purification of this dark-brown protein. After growth of the fungus for three weeks, the culture was centrifuged and the supernatant containing the dark-brown protein was dialyzed and then concentrated by a Vivaspin concentrator (cut off 5000 Dalton). After concentration, the protein fraction was again dialyzed and then submitted to chromatography on a DEAE Sepharose column (see details under Materials and Methods). At neutral or slightly alkaline pH the protein bound extremely tightly to the DEAE Sepharose column and could not be eluted. For this reason the chromatography was performed at pH 4.8 implying that the protein is a highly acidic one. This chromatography resulted in a homogenous protein of the molecular mass 36 kDa on the basis of SDS-PAGE (see Fig. 1B). The molecular mass of the major fraction (peak No. 2, Fig. 1A) of the native protein determined by size exclusion chromatography was of approximately 30 kDa (26–40 kDa) indicating that the major amount of the protein consisted of monomers. Besides the monomers, a small fraction (approximately 10%; peak No. 1 of Fig. 1A) existed as an oligomer with a molecular mass being larger than the exclusion size of the Superose 12 column (>2000 kDa).
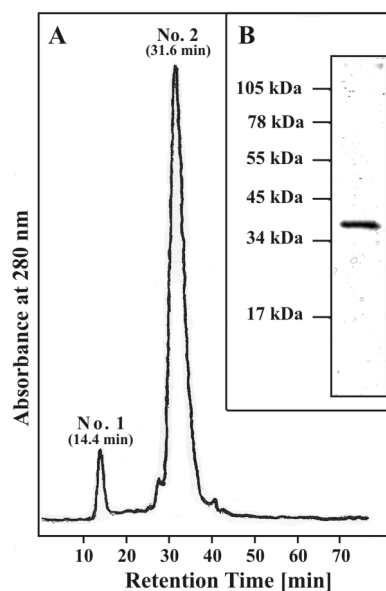

Fig. 1. A: Elution profile of the purified melanoprotein after chromatography on a Superose 12 column. Peak No. 1 represents the high molecular mass complex of the melanoprotein (>2000 kDa) and peak No. 2 the monomer of the protein of 36 kDa. The molecular mass standards were catalase (240 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and cytochrome C (12 kDa). B: SDS-PAGE of the purified melanoprotein (9% acrylamide gel). The lane was loaded with 10 μg protein.

Protein digestion and sequencing

For obtaining partial amino acid sequences, the isolated protein was digested with trypsin and the polypeptide fragments were identified by ESI Q-ToF mass spectrometry. This resulted in 11 tryptic peptide fragments with a contiguous amino acid sequence from 6 to 23 amino acids, corresponding to 14,610 Da which represents about 40% of the protein. The derived amino acid sequences are listed in Table I. Homology search in different databases (NCBI, Swiss Prot, EMBL) only resulted in weak similarities to known proteins, expressed sequence tags or unknown ORFs in sequenced genomes. The peptides No. 1 and No. 4 have some similarity to a hypothetical protein of *Aspergillus nidulans* that shows in part homology to β-1,3-glucosidase from *Fusarium sporotrichioides* which might be an indication that the protein has a hydrolytic activity, although this is not supported by the other obtained sequences.

UV/Vis spectroscopy

The logarithmic absorbance spectrum of the isolated protein shows an increase in the region from 350 to 500 nm. The calculation of the logarithmic absorbance slope of −0.0038 indicates the presence of melanin in the purified dark-brown protein, since the logarithmic absorbance slope of the synthetic melanin obtained after oxidation by persulfate as well as the isolated melanin extracted by alkaline treatment followed by acid precipitation from the intact *V. inaequalis* strain 3B3-1 (El Bassam *et al.*, 2002) have a value of −0.0040. This result implies that the dark-brown chromophore of the protein isolated from *V. inaequalis* clone No. 36 isolated from *M. domestica* cv. Gloster also contains melanin and thus represents a melanoprotein.

Interaction of the melanoprotein with metal ions

Previous work has provided evidence that the melanoprotein from *V. inaequalis* isolate E_1 interacts with ferrous sulfate and also with ferric citrate, while neither calcium nor magnesium ions were retained by the melanoprotein after prolonged dialysis. Our investigations confirm the binding of ferrous and ferric ions to the melanoprotein, and also confirm that iron in part is tightly bound, since a small fraction of iron remains bound to the protein, even after dialysis against the chelator *o*-phenanthroline or EDTA. In addi-
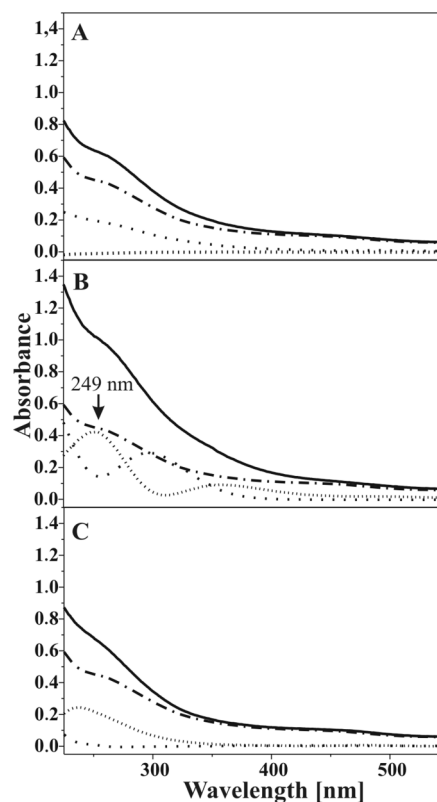

Fig. 2. Influence of selected metal salts on the isolated melanoprotein from *V. inaequalis* (50 μg/ml). Curves represent the absorbance of (-·-·-) melanoprotein from *V. inaequalis* without addition, (——) melanoprotein after addition of metal salt, (· · · ·) metal salt, (····) melanoprotein after addition of metal salt and subtraction of the absorbance spectra of melanoprotein and metal salt. A: Effect of 160 μM $FeSO_4$; B: effect of 160 μM $FeCl_3$; C: effect of 160 μM $CuCl_2$.

tion to iron, the protein binds also Cu(II) but does not bind Mn(II) and Ni(II) (see Table II). Moreover, it could be shown that Fe(III) and Cu(II) but not Fe(II) ions cause an absorbance change in the protein (see Fig. 2A, B, and C) suggesting that either Fe(III) and Cu(II) cause an oxidation of the chromophore or Fe(III) and Cu(II) cause a conformational change in the protein leading to a change in the absorbance of melanin.

The tight binding of Fe(II) to the protein can also be demonstrated by measuring the photosynthetic O_2 evolution with isolated thylakoid membranes from peas (see Table III). Fe(II) ions cause an about 50% inhibition of the O_2 evolution. When Fe(II) is preincubated with the melanopro-

Table I. ESI Q-ToF mass spectrometry of tryptic peptide fragments and identification of the derived peptide sequences by homology search.

Fragment	Mass [Da]	Derived amino acid sequence	Sequence similarity position sequence position	Possible function	Organism	Accession number
No. 1	2015.22	(K/Q)EFSTA(K/Q)NLVGTSGFNSAR	3 EFSTAKQNLVGTSGFNSAR 21 EF TAK NL TSGFNSAR 44 EFRTAK-NLETTSGFNSAR 61	Hypothetical protein	*Aspergillus nidulans*	AACD01000135
No. 2	1485.64	SWTSVGCNQLFDK	2 WTSVGCNQLFD 12 WTSVGC LFD 44 WTSVGC-RLFD 53	Hypothetical protein	*Rhizobium loti*	Q98IG7
No. 3	2401.65	YP(K/Q)SDPGPGANPDVLANYLGQVK	11 ANPDVLANYLG 21 ANPDV A YLG 252 ANPDVIAAYLG 262	Branched-chain amino acid ABC transporter	*Pseudomonas putida*	Q88JA1
No. 4	1295.42	VAVSVGSEDLYR	2 AVSVGSEDLYR 12 A+SVGSEDLYR 120 AISVGSEDLYR 130	Glucosidase	*Fusarium sporotrichioides*	Q876Y0
No. 5	1411.58	TPVA(K/Q)AVASPENAR	5 KQAVASPENA 14 K+AV SPENA 74 KEAVESPENA 83	Hypothetical protein	*Ustilago maydis*	AACP01000210
No. 6	1752.85	PYDPALDATAAQFEDK	4 PALDATAAQFEDK 16 PALDA AA F DK 277 PALDAIAA-FKDK 288	ABC transporter sugar-binding protein	*Bradyrhizobium japonicum*	Q89QC8
No. 7	885.98	LTDFFSR	1 LTDFFSR 7 LTDFFSR 24 LTDFFSR 30	Hypothetical protein	*Neurospora crassa*	Q7SCF9
No. 8	790.84	AALAGTSAE	1 AALAGTSAE 9 AALAGTS E 82 AALAGTSTE 90	1-Deoxy-D-xylulose 5-phosphate reductoisomerase	*Bradyrhizobium japonicum*	Q89KP9
No. 9	1313.35	NYGSTDASGVVKD	5 TDASGVVK 12 TDASGVVK 134 TDASGVVK 141	Dissimilatory sulfite reductase beta subunit	*Gamma-proteobacterium*	Q847X6
No. 10	757.85	YLGYLE	–	No homology	-	
No. 11	819.88	VLN(Q/K)TTSG	2 LNQKTTS 8 LNQKTTS 46 LNQKTTS 52	Ferredoxin NADP oxidoreductase,	*Arabidopsis thaliana*	Q9S9P8

Table II. Determination of the metal binding capacity of the purified melanoprotein from *V. inaequalis.*

Treatment of protein	Dialysis against H_2O [g atom/mol protein]	Dialysis against chelator [g atom/mol protein]
No preincubation	0	0
Preincubation with 160 μM $FeSO_4$	1.3	0.3
Preincubation with 160 μM $FeCl_3$	1.7	0.7
Preincubation with 160 μM $CuCl_2$	2.1	0

For this experiment the isolated melanoprotein (1.60 mg/ml) was preincubated with metal salts as indicated for 30 min and then dialysed against bidest. H_2O, 10 mM Na-EDTA, pH 7.0, or 1 mM *o*-phenanthroline, pH 7.0, for 48 h (with three changes of the dialysis solution). After the same treatment of the protein with $NiCl_2$ or $MnCl_2$ neither Ni nor Mn remained bound to the protein. The isolated protein before preincubation with metal contained neither iron nor copper or any other metal. The calculations of the metal:protein ratios were performed on the basis of the Lowry protein determination.

Table III. Measurement of photosynthetic oxygen evolution of isolated thylakoid membranes from pea leaves in the presence of phenyl-*p*-benzoquinone as electron acceptor.

Additions	Photosynthetic O_2-evolution [μmol O_2/mg Chl $\times$ h]
None	70.0
50 μg melanoprotein	71.5
200 μM $FeCl_3$	34.0
200 μM $FeCl_3$ + 50 μg melanoprotein	70.0
200 μM $FeSO_4$	13.0
200 μM $FeSO_4$ + 50 μg melanoprotein	71.5
20 μM $CuSO_4$	2.0
20 μM $CuSO_4$ + 50 μg melanoprotein	71.5

The reaction mixture contained 50 mM Na-HEPES, pH 6.5, 1 mM phenyl-*p*-benzoquinone, thylakoid membranes corresponding to 25 μg chlorophyll and $FeSO_4$, $FeCl_3$ and $CuSO_4$ as indicated. The thylakoids membranes were preincubated in the presence of metal salts and/or melanoprotein in the Clark electrode for 5 min.

tein and this mixture is added to the reaction mixture, no inhibition of the O_2 evolution occurs. This suggests that Fe(II) is tightly bound to the melanoprotein.

Production of an antiserum against the melanoprotein and investigation of the localization of the protein in the infected M. domestica cv. Elstar

An antiserum against the purified melanoprotein was raised in a goat, and the antiserum was used to study the localization in *M. domestica* infected by *V. inaequalis*. In Western blot analyses of total leave extracts, intercellular washing fluid, and extracts of the infiltrated leaves from which the intercellular washing fluid was obtained, were investigated. The results show that the melanoprotein was only detectable in the leaves of the infected plant, and that it was mainly located in the intercellular washing fluid of the apoplast (see Fig. 3).
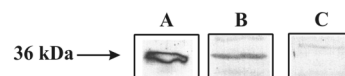

Fig. 3. Immunological detection of the melanoprotein in the apoplastic fluid of apple leaves. A: The purified melanoprotein from *V. inaequalis,* B: The apoplastic fluid from *V. inaequalis* infected leaves from *M. domestica* cv. Elstar; C: The apoplastic fluid from non-infected leaves. Samples were subjected to SDS-PAGE and subsequently immunostained by the anti-melanoprotein antiserum (dilution 1:500) followed by an alkaline phosphatase-coupled anti goat IgG. Each lane was loaded with 25 μg protein.

Conclusion

Previous results have indicated that the melanoprotein produced by *V. inaequalis* facilitates the progress of the apple scab disease (Hignett *et al.*, 1984). As our results show, the fungus *V. inaequalis* clone No. 36 isolated from *M. domestica* cv. Gloster also excretes a melanoprotein in relatively high amount. This might be of significant importance for optimisation of the infection of *M. domestica* by this fungus, since *V. inaequalis* does not form haustoria. This protein having a molecular mass of the native protein of 36 kDa was mainly detected in the apoplast of the infected *M. domestica*. Besides its function in increasing the number of deletions in the host plant, the protein might have an important function in recruiting iron for the pathogen. This should especially been seen under the aspect that iron is frequently a limiting factor due to its reduced bioavailability. Therefore, this melanoprotein might help the fungus in the competition of the host plant with the pathogen for this cofactor of many essential iron proteins/enzymes. It is interesting that this protein also binds copper

in addition to iron. Copper is a cofactor *e.g.* Cu/Zn superoxide dismutase. Besides its function in iron and/or copper homeostasis and transport the melanoprotein might also have a function in the detoxification of iron and copper leading to a reduction in oxidative stress in the apoplast. This would also protect the fungus, since the fungus is in part located in the apoplast. Although we are able to sequence almost 40% of the protein no significant sequence homologies to other proteins in databases were found. This makes a complete sequencing of the protein rather desirable to allow a more extended sequence analysis, especially with other siderophore type proteins or other iron (and copper) binding proteins to eventually elucidate the exact function which this melanoprotein has in the process of the infection of *M. domestica* plants by the fungus *V. inaequalis.*

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