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Characterisation of the saponin hydrolysing enzyme avenacoside-α-L-rhamnosidase from the fungal pathogen of cereals, *Stagonospora avenae*

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Abstract

The fungal pathogen *Stagonospora avenae* f. sp. *avenaria* infects oat leaves, which contain the saponins avenacoside A and B. The avenacosides are glycosylated steroidal saponins that occur within oat leaves in a non-fungitoxic form and are converted upon damage or pathogen invasion to their antifungal form by a plant enzyme. It has previously been shown that oat-attacking isolates of *S. avenae* are able to hydrolyse the sugar chain at the C3 position of the avenacosides. This carbohydrate moiety is a branched chain that consists of one α-L-rhamnose and two or three β-D-glucose residues in avenacosides A and B, respectively. Removal of the α-L-rhamnose residue is sufficient to detoxify the avenacosides. This work describes the purification of the avenacoside-degrading α-L-rhamnosidase–and determination of peptide sequence from the protein which represents the first α-L-rhamnosidase thus characterised in a fungal plant pathogen.

Abbreviations: 26-DGA – 26-desglucoavenacoside; DR-26-DGA – de-rhamnosyl-26-desglucoavenacoside.

Introduction

The ability of fungal pathogens to invade plant tissues and cause disease is dependent on the capacity of these organisms to evade or overcome plant defences. Plant resistance against disease involves a wide range of induced defence mechanisms that include the hypersensitive response, production of phytoalexins and papilla formation (Hammerschmidt, 1999; Heath, 2000; Thordal-Christensen et al., 1997). Plants also contain many different types of antimicrobial compounds that are produced constitutively and therefore act as preformed defence mechanisms against pathogens (Osbourn, 1996). Saponins represent a class of preformed antimicrobial secondary metabolites that are present in a broad range of plant species and that have been implicated in plant defence. The cultivated oat *Avena sativa* produces two classes of saponin, the avenacins and the avenacosides, which are present exclusively in the roots or in the leaves, respectively (Osbourn, 1996).

The avenacins, the best characterised of the oat saponins, are representatives of the glycosylated triterpenoid saponins and include four structurally-related molecules that are found in oat roots (Crombie et al., 1986; Crombie and Crombie, 1986). The avenacins are

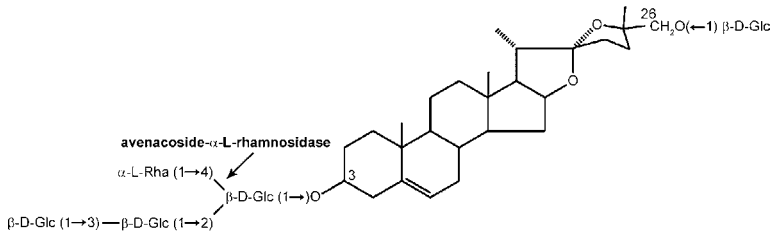

Figure 1. Structure of the oat leaf saponin avenacoside B. The site of avenacoside-α-L-rhamnosidase hydrolysis is indicated.

involved in defence against a variety of pathogens. The soilborne pathogen *Gaeumannomyces graminis* var. *avenae* relies on the enzymatic detoxification of these compounds in order to infect oat roots. A *G. graminis* strain that had undergone targeted disruption of the gene encoding the saponin-detoxifying enzyme avenacinase was unable to successfully infect oat roots, but retained full pathogenicity on wheat, which does not produce avenacins (Bowyer et al., 1995). Conversely, disease resistance is compromised in oat lines that are deficient in the production of avenacins. Avenacin-deficient mutants of diploid oat have enhanced susceptibility to fungal pathogens (Papadopoulou et al., 1999; Haralampidis et al., 2001).

Avenacosides A and B are steroidal saponins that are produced in oat leaves in a non-fungitoxic form with a single glucose residue at the C-26 position of the steroid molecule, and a more extensive sugar chain at the C-3 position which consists of a single α-L-rhamnose residue and two (avenacoside A) or three (avenacoside B) β-D-glucose residues (Figure 1). Hydrolysis of the C-26 glucose by a plant enzyme upon mechanical damage or pathogen invasion yields the antifungal 26-desglucoavenacosides A and B (26-DGAs). The antifungal activity of the 26-DGAs has been attributed to their membrane-permeabilising properties (Lüning and Schlösser, 1975, 1976).

Stagonospora avenae f. sp. *avenaria* (syn. *Septoria avenae* f. sp. *avenae*) is the causal agent of *Stagonospora* leaf blotch of oat (*A. sativa*), and produces purple-brown spots on leaves that later expand to form elliptical lesions (Cunfer, 2000). *Stagonospora avenae* f. sp. *avenaria* detoxifies the 26-DGAs by sequential hydrolysis of the sugar chain at the C-3 position of the steroid backbone (Wubben et al., 1996; Morrissey et al., 2000). An enzyme was previously purified from culture filtrates of *S. avenae* WAC1293 that hydrolysed both the α-L-rhamnose and β-D-glucose residues of the C-3 oligosaccharide. This protein, 110 kDa, was purified, N-terminal sequenced and identified as a β-glucosidase (Wubben et al., 1996, Morrissey et al., 2000). This was used to isolate and disrupt the savBGL1 gene encoding a *S. avenae* β-glucosidase. However, mutation of this gene did not have any obvious effect on tolerance to DGAs or pathogenicity (Morrissey et al., 2000). Further characterisation of the avenacoside-hydrolytic activity of *S. avenae* culture filtrate established that three enzymes are responsible for the degradation of the C-3 sugar chain; two β-glucosidases and one α-rhamnosidase. Furthermore, it was established that hydrolysis of the α-L-rhamnose residue represents the first step in the degradative process and is probably sufficient for the detoxification of the 26-DGA, converting them to de-rhamnosyl-26-desglucoavenacosides (DR-26-DGAs) (Morrissey et al., 2000; Figure 1).

This paper describes further characterisation of the avenacoside-hydrolytic activity of *S. avenae* culture filtrate, using the β-glucosidase (savBGL1) disrupted strain *S. avenae* SL362 and the β-glucosidase inhibitor deoxynojirimycin, to specifically target the avenacoside-α-L-rhamnosidase activity in assays. The avenacoside-α-L-rhamnosidase was purified from culture filtrate and identified as a 110 kDa protein that was subsequently characterised by Q-ToF mass spectrometry to obtain peptide sequence data.

Materials and methods

Fungal culture

Stagonospora avenae isolates WAC1293 and SL362 (Wubben et al., 1996; Morrissey et al., 2000) were maintained as sporulating cultures on Czapek Dox V8 agar containing a complete nutrient supplement (CzV8CS) under a mixture of continuous white and near-UV light at 17 °C (Newton and Caten, 1988). Conidia were harvested at 7 days by flooding the plates with water and mycelial debris removed by passing through three layers of sterile surgical gauze.

For isolation of α-rhamnosidase activity from culture filtrate, conidia of *S. avenae* SL362 ($\sim 10^5$) were inoculated into flasks containing 1 l of Jermyn's medium (Jermyn, 1959), modified by reducing the starch content to 1 g l^{-1}. Cultures were incubated for 5 days at 22 °C with orbital shaking at 200 rpm.

Avenacoside-α-L-rhamnosidase activity assay

α-L-rhamnosidase activity was assayed using purified 26-DGA-B as a substrate (Wubben et al., 1996) and deoxynojirimycin to inhibit further hydrolysis by glucosidase activity. Briefly, 1 µl samples of protein were incubated in 100 µl of 25 µM 26-DGA-B, 1.5 mM deoxynojirimycin in 20 mM sodium phosphate buffer pH 6.2 for 15 min before the reaction was stopped by plunging into liquid nitrogen (Morrissey et al., 2000). Samples were lyophilised and extracted with methanol (100 µl) before analysis by thin layer chromatography (TLC). Samples (10 µl) were spotted onto glass backed silica TLC plates (silica G-60, 0.25 mm; Merck AG, Darmstadt, Germany) and developed with a solvent mix of 70 : 35 : 5.5 chloroform : methanol : water. Chromatograms were stained by spraying with *p*-anisaldehyde (1 : 1 : 48 *p*-anisaldehyde : sulphuric acid : acetic acid) and baking at 170 °C. DR-26-DGA-B was visualised as a compound with higher mobility on TLC plates than 26-DGA-B, as previously confirmed by FAB-MS (Wubben et al., 1996). Assays were quantified by estimating the percentage conversion of 26-DGA-B to DR-26-DGA-B in partial rhamnosidase digests.

Purification of α-rhamnosidase activity from culture filtrate

Culture filtrate was prepared from 4 l of *S. avenae* SL362 by chilling to 4 °C and filtering through Miracloth (Merck A.G., Darmstadt, Germany). Total protein was precipitated by adding ammonium sulphate to 80% saturation (580 g l^{-1}). Precipitated protein was resuspended in distilled water containing protease inhibitor cocktail (Complete Protease Inhibitor, Roche Diagnostics, Lewes, UK) and incubated for 30 min before dialysis against 20 mM diethanolamine pH 8.5. Samples were then concentrated to ~500 µl using Centriplus YM-30 spin concentrators (Millipore, Billerica, MA). Concentrated samples (250 µl) were loaded onto a Poros HQ 20 anion exchange column (Perseptive Biosystems, Framingham, MA) equilibrated with 20 mM diethanolamine pH 8.5. Bound proteins were eluted with a gradient of 0–0.6 M NaCl in 20 mM diethanolamine over 30 column volumes of 1.662 ml at a flow rate of 7 ml min^{-1} on a Bio-Cad Sprint HPLC system (Perseptive Biosystems, Framingham, MA). Fractions (1 ml) were collected and analysed by 7.5% SDS-PAGE and silver staining (PlusOne Silver stain, Pharmacia, Uppsala, Sweden) and avenacoside-α-L-rhamnosidase activity assayed. Fractions selected for further purification were pooled and concentrated using Centricon C-30 spin concentrators (Millipore, Billerica, MA), exchanging the buffer to 20 mM sodium phosphate pH 7 containing 200 mM NaCl, to give a final volume of ~150 µl. The sample (120 µl) was loaded onto a TSK G3000 gel filtration column (TosoHAAS, Stuttgart, Germany) and was eluted isocratically with 20 mM sodium phosphate pH 7, 200 mM NaCl. Fractions (250 µl) were collected and analysed by SDS-PAGE and avenacoside-α-L-rhamnosidase activity assay. Protein recovery was assayed by the ESL Protein Assay (Roche Diagnostics).

Q-ToF mass spectrometry sequencing

Concentrated protein containing α-L-rhamnosidase activity pooled from HQ anion exchange fractions was loaded (100 µl, ~100 µg) to a 7.5% SDS-PAGE gel and separated. Protein was stained by colloidal Coomassie Blue G-250 (Neuhoff et al., 1988) and the α-rhamnosidase band excised, reduced, alkylated and digested with trypsin (Henzel et al., 1993). Peptides were separated by MALDI-ToF (Bruker REFLEX III, Bruker Daltonics Inc., MA) and MS/MS sequenced by Q-ToF mass spectrometry (Micromass Q-ToF II, Micromass (UK) Ltd, Manchester, UK) and analysed with ProteinLynx software (Micromass (UK) Ltd., Manchester, UK) by the IFR/ JIC Joint Proteomics Facility.

Results

Purification and characterisation of α-rhamnosidase from S. avenae *culture filtrate*

Fractions from anion exchange chromatography were assayed for avenacoside-α-L-rhamnosidase activity

and active fractions analysed by silver stained SDS-PAGE. Activity was detected as conversion of 26-DGA-B to DR-26-DGA-B, resulting in detection of a more mobile product on TLC plates. α-Rhamnosidase activity eluted from the column from fraction 22 onwards, but the peak of the activity was isolated between fractions 23 and 27 (Figure 2A). Comparison with SDS-PAGE gels showed that activity was correlated with two proteins at 110 kDa and 76 kDa (Figure 2B). Active fractions containing these proteins were pooled and exchanged into buffer for gel filtration chromatography.

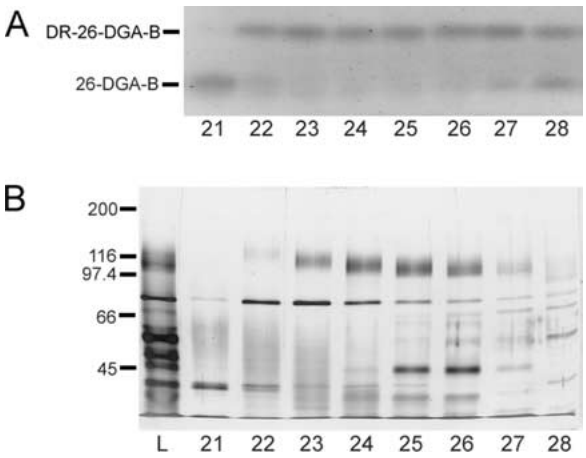

Figure 2. Anion exchange chromatography of *S. avenae* SL362 culture filtrate proteins. Proteins were fractionated on a Poros HQ 20 column with a 0–0.6 M NaCl gradient and 1 ml fractions collected. (A) Assay of fractions 21–28 for avenacoside-α-L-rhamnosidase activity, indicated by conversion of 26-DGA-B to DR-26-DGA-B. (B) Silver stained SDS-PAGE of proteins resolved in fractions 21–28. Lane L represents a sample of unfractionated proteins. The peak of the enzyme activity is correlated with the presence of a protein of ~110 kDa.

Proteins pooled from anion exchange chromatography were separated by gel filtration chromatography and fractions eluted were assayed for α-rhamnosidase activity and protein content. α-Rhamnosidase activity eluted from fraction 19 onwards, with activity declining from fraction 23/24 onwards (Figure 3A). Comparison with SDS-PAGE gels revealed that the activity correlated with the presence of the 110 kDa protein, rather than the 76 kDa protein (Figure 3B). The 110 kDa protein showed some heterogeneity in size between the different fractions, probably as a result of glycosylation which is a common feature of fungal extracellular proteins (Ruiz-Herrera, 1992). Fractions containing the electrophoretically pure α-rhamnosidase (fractions 19–22) were pooled, dialysed against 20 mM sodium phosphate buffer (pH 7) and concentrated to 100 µl in a Centricon C-30 ultrafilter. The purification process is summarised in Table 1. Final protein yield was 17 µg as measured by the Roche ESL protein assay (Roche Diagnostics).

Kinetic analysis by avenacoside-α-L-rhamnosidase assay revealed the α-rhamnosidase activity to have

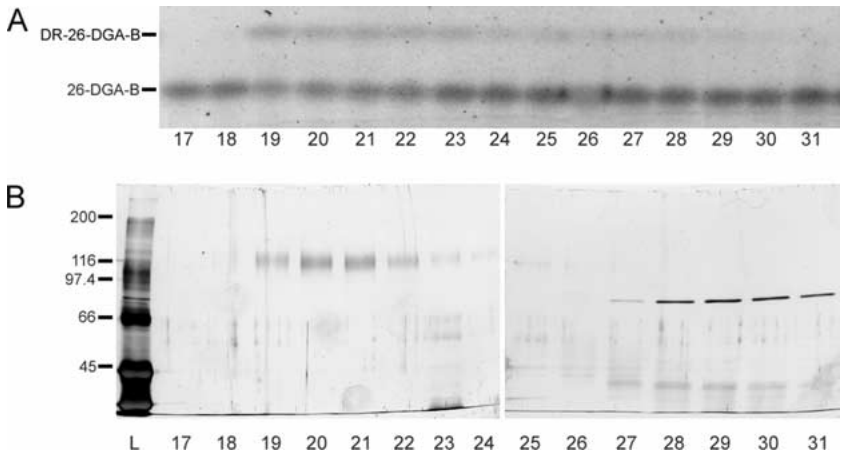

Figure 3. Gel filtration of *S. avenae* SL362 proteins. Proteins were eluted isocratically with 20 mM sodium phosphate pH 7, 200 mM NaCl and 250 µl fractions collected. (A) Assay of fractions 17–31 for avenacoside-α-L-rhamnosidase activity, indicated by conversion of 26-DGA-B to DR-26-DGA-B. (B) Silver stained SDS-PAGE of proteins resolved in fractions 17–31. Lane L represents a sample of unfractionated proteins. Rhamnosidase activity first elutes at fraction 19 and begins to decline at fraction 24, correlating with the presence of an electrophoretically pure 110 kDa protein between fractions 19 and 22.

Table 1. Purification of avenacoside-α-L-rhamnosidase

Purification stage	Protein (μg)	Specific activity[1]	Total units	Purification factor	% recovery
Ammonium sulphate precipitate	750	0.208	0.156	—	100.0
HQ pooled fractions	437	0.280	0.122	1.3	78.4
TSK-gel pooled fractions	17	1.103	0.019	5.3	12.2

[1]Specific activity = μmol substrate mg protein^{-1} min^{-1}.

Table 2. Amino acid sequences derived from avenacoside-α-L-rhamnosidase tryptic peptides by Q-ToF mass spectrometry

Peptide	Molecular weight	Sequence	Length
Rha1	1600.9	(L/I)[1](L/I)ADGG(L/I)DTVF(L/I)(L/I)VR	15
Rha2	1691.8	AA(L/I)EADDGETTMD(L/I)(L/I)K	16
Rha3	1204.6	PVTPGYTEWR	10

[1]Leucine and isoleucine are isomers and cannot be distinguished by Q-ToF

a K_M of 91 μM and a v_{max} of 2.94 μM min^{-1}. Experiments to assay α-rhamnosidase activity of *S. avenae* avenacoside-α-L-rhamnosidase using *p*-nitrophenyl-α-L-rhamnopyranoside and 4-O-methyl-umbelliferyl-L-rhamnopyranoside demonstrated that the *S. avenae* enzyme is unable to hydrolyse either of these substrates.

Peptide sequence of α-rhamnosidase

N-terminal sequencing of purified protein and of peptides derived from it by cyanogen bromide digestion was unsuccessful. As the α-rhamnosidase was isolated from proteins of similar molecular weight by anion exchange chromatography (Figure 2) and to avoid losses following gel filtration chromatography (Table 1), it was decided to isolate further protein for sequence characterisation by anion exchange chromatography followed by SDS-PAGE. Therefore, more total protein was prepared from culture filtrate and separated by anion exchange chromatography as before. Fractions containing α-rhamnosidase activity were pooled and concentrated to ~100 μl before loading onto a single well 7.5% SDS-PAGE minigel. Proteins were visualised by colloidal Coomassie Blue staining and the 110 kDa band excised from the gel and submitted for Q-ToF sequencing. Three peptides were successfully sequenced by Q-ToF (Table 2). None of the peptides were homologous to any known sequences in searches of GenBank by tblastn.

Discussion

Purification of the *S. avenae* avenacoside-α-L-rhamnosidase activity identified by Morrissey et al. (2000) was achieved using a three-stage process that involved ammonium sulphate precipitation, anion exchange chromatography and gel filtration. This resulted in the identification of a protein of 110 kDa that purified with the avenacoside-α-L-rhamnosidase activity in the *S. avenae* SL362 strain, deficient in the avenacosidase (β-D-glucosidase) purified by Wubben et al., (1996). This confirmed that the avenacoside-α-L-rhamnosidase activity identified by Wubben et al. (1996), and attributed to the 110 kDa enzyme purified in that study, was a co-purification of this separate 110 kDa α-L-rhamnosidase. Identification of the α-L-rhamnosidase allowed the isolation of further protein from polyacrylamide gel in sufficient quantity for tryptic digestion, MALDI-ToF MS and Q-ToF MS sequencing. Q-ToF MS sequencing provided sequence data for three peptides, two of which are of sufficient length to allow unambiguous primer design for PCR based cloning of the gene encoding the enzyme.

The lack of homology of the peptide sequences identified in this study to any known sequences is not surprising given the small number of α-L-rhamnosidases that have so far been purified and characterised, with the enzyme in the current study representing the first example of α-L-rhamnosidase sequence from a fungal pathogen of plants. The first full α-L-rhamnosidase gene sequence identified was the RamA of *Clostridium stercorarium*, which shares homology with putative α-L-rhamnosidase genes in *Streptomyces coelicolor* (Zverlov et al., 2000), along with other more recent putative bacterial (and one archaeal) homologues identified by BLAST searching. Recently, the sequences of two closely related α-L-rhamnosidases of *Aspergillus*

aculeatus have become available (Manzanares et al., 2001). No regions of either of the two proteins showed similarity to the peptides of the *S. avenae* α-L-rhamnosidase when alignment was attempted.

A recently reported saponin-hydrolysing α-L-rhamnosidase, the ginsenoside-α-L-rhamnosidase, may bear the greatest similarity to the avenacoside-α-L-rhamnosidase. Ginsenoside-α-L-rhamnosidase was purified from an *Absidia* species colonising koji (a fermented product of rice used in the production of sake) and hydrolyses the saponin ginsenoside Rg2 to ginsenoside Rh1 by hydrolysis of the α-1,2 linked rhamnose (Yu et al., 2002). Avenacoside-α-L-rhamnosidase, in common with the purified ginsenoside-α-L-rhamnosidase, was also unable to hydrolyse the standard synthetic substrate *p*-nitrophenyl-α-L-rhamnopyranoside. A notable feature of the avenacoside-α-L-rhamnosidase is that it hydrolyses α-1,4 linked L-rhamnose, whereas the *A. aculeatus* and *Absidia* enzymes act against α-1,2 and α-1,2/α-1,6 linked L-rhamnose residues, respectively. It would be of considerable interest to characterise the substrate specificity of the enzyme for other α-1,4 linked rhamnosyl substrates, for example the recently reported *Veronica multifida* saponin multifidoside and the aculeatisides produced by *Solanum aculeatissimum* (Ozipek et al., 2002; Saijo et al., 1983).

Further investigation of the *S. avenae*–oat interaction will be valuable to determine the role of the avenacoside-α-L-rhamnosidase in infection. Cloning and disruption of the gene encoding the enzyme will determine the importance of detoxification of the avenacosides to the ability of the pathogen to cause disease on oat. More detailed study of the infection process may allow the characterisation of post-penetration events. *In situ* hybridisation techniques using probes derived from a cloned gene sequence could be used in concert with these studies to determine whether expression of the avenacoside-α-L-rhamnosidase was correlated with post-penetration stages of the infection.

The ability of a fungal pathogen to detoxify saponins has also been demonstrated in the case of *Septoria lycopersici*, where replacement of the gene encoding tomatinase results in increased susceptibility to the steroidal glycoalkaloid saponin α-tomatine (Martin-Hernandez et al., 2000). More recently, the activity of tomatinase has been shown to suppress induced defence responses as a result of the product of hydrolysis, β_2-tomatine, interfering with the signalling processes leading to induced disease resistance (Bouarab et al., 2002). With the possibility open that similar processes could also occur in monocots, further characterisation of the role of the *S. avenae* avenacoside-degrading enzymes may lead to further insights into the potential for interaction between the passive and induced disease resistance mechanisms of plants.

Acknowledgements

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