Detection of 3-hydroxykynurenine in a plant pathogenic fungus

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A redox-active compound has been purified from the barley powdery mildew fungus *Blumeria (Erysiphe) graminis* f. sp. *hordei*. A combination of spectrophotometry, MS and NMR has identified it as 3-hydroxykynurenine (3OHKyn). This compound, never previously detected in any fungus or pathogen, is best known for its role in vertebrate cataracts. It is found abundantly in developing and germinating spores and also in runner hyphae.

Two roles for 3OHKyn are discussed: first, the presence of active oxygen species would enable 3OHKyn to cross-link the spore chemically with the plant. Secondly, it may be acting as an UV protectant and an antioxidant.

Key words: *Blumeria*, ferric reduction, hydroxykynurenine.

INTRODUCTION

Considerable effort has been spent on the elucidation of the early events in pathogen–plant interactions. The premise is that an understanding of these events will allow strategies to be developed that could help in controlling the disease by interfering with and manipulating these events. All successful pathogens must firmly attach themselves to the wall of the host, must counter the active and passive defence systems of the plants and must be capable of growing in full sunlight.

Blumeria (Erysiphe) graminis f. sp. *hordei* (BGH), the barley powdery mildew fungus, is an obligate biotroph and is dependent on the host plant to complete its life cycle. Shortly after settling on the leaf, the conidia produce a primary germ tube (PGT), which extends down to the surface of the leaf epidermis. The plant responds by producing a cross-linked patch under the PGT, known as a papilla. The role of the PGT appears to be to sense the surface characteristics, which, if favourable, stimulate the emergence of the second, appressorial germ tube (AGT). The AGT then extends, hooks and forms the appressorium, which attempts to penetrate the host epidermal cell. Initially, the fungus penetrates only one epidermal cell of the plant. Further development takes place on the leaf surface as runner hyphae and in aerial structures, where the conidiophores are produced which mature into conidia.

Attachment of conidia to the waxy epidermis has been attributed previously to a proteinaceous extracellular matrix [1], although details of the molecules involved are not known. In other fungal systems, attachment has been attributed to glycoproteins [2], to hydrophobins, a fungal-specific family of small Cys-rich amphipathic proteins [3], and to melanin which has also been suggested to play a role in redox protection, wall integrity and host–cell penetration [4,5]. Melanin has not been found in mildew.

Barley, like many plants, has been shown to produce active oxygen species (AOS), particularly hydrogen peroxide during the infection process. AOS are believed to act both as fungicides and also to strengthen the cell wall by cross-linking polymers and macromolecules [6–11]. AOS can be detected within 6 h of a spore landing on a leaf [12–15]. In cell suspension cultures, peroxide can be detected within 5–15 min of elicitor addi-

tion [16], indicating a rapid recognition and response to pathogen-produced elicitors. It is probable that successful pathogens have developed sophisticated methods to combat plant-derived AOS [17,18], although these processes are not well understood.

Recently, an expressed sequence tag (EST) project on BGH [19] revealed 1702 unique genes and has given new insights into the development and differentiation of this pathogen. Genes for several proteins possibly involved in host-surface degradation were discovered. These proteins could correspond to the hydrolases that have been suggested to degrade the epidermis and thereby prepare the ‘infection court’ [20].

Our original interest was in the elucidation of iron acquisition/utilization in BGH. Iron for the development of the fungus has to come from the plant and its stores. Data mining of the EST project suggested that the mechanism for iron uptake was similar to the high-affinity system present in yeast [21]. Ferric reductase activity of BGH can be demonstrated, but most of the reducing activity is chemical in nature and has been identified as 3-hydroxykynurenine (3OHKyn), a compound that has not been previously detected in fungi. We suggest that 3OHKyn may have a role in attachment and/or radical protection and thus may be an attractive target for fungicides.

EXPERIMENTAL

Fungal material

BGH race C15 [22] was used in all experiments. Conidia were propagated on barley cultivar Golden Promise as described previously [23]. Between 3 and 4 ml of spores were collected from plants that had been infected for 10–14 days.

Spore washing

Spores were washed with 10 ml of 100 mM citrate/phosphate buffer (38.85 ml of 0.1 M citric acid/63.15 ml of 0.2 M Na₂HPO₄; pH 6). The spores were gently suspended in this buffer and placed on ice for 20 min with occasional inversion. They were

Abbreviations used: AGT, appressorial germ tube; AOS, active oxygen species; BCS, bathocuprone disulphonic acid; BGH, *Blumeria (Erysiphe) graminis* f. sp. *hordei*; BPS, bathophenanthroline disulphonic acid; ESI, electrospray ionization; EST, expressed sequence tag; HMBC, heteronuclear multiple-bond correlation; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; 3OHKyn, 3-hydroxykynurenine; PGT, primary germ tube.

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then spun at 3600 *g* for 10 min at 4 °C and the supernatant (wash) collected. The pellets were kept on ice until further processed. The wash was re-centrifuged and the supernatant size fractionated using a PD10 desalting column (Amersham Biosciences, Little Chalfont, Bucks., U.K.). The low-molecular-mass fractions were concentrated using a Vivaspin 5000 Da cut-off concentrator (Vivascience, Lincoln, U.K.).

LH20 column chromatography

The low-molecular-mass eluate from the Vivaspin column was loaded on to an LH20 column (30 cm × 1 cm; Amersham Biosciences). Elution was with water at a flow rate of 0.25 ml/min and 0.5 ml fractions were collected.

Metal-reducing activity

Ferric reductase activity was measured by the interaction of bathophenanthroline disulphonic acid (BPS) with generated Fe^{2+} . Each fraction (50 μl) from the LH20 column was incubated with the assay media (1 ml), containing a final concentration of 1 mM BPS and 100 μM Fe^{3+} (supplied as ferric chloride) in citrate buffer. The colour change to pink was observed and the absorbance measured at 535 nm. The cupric reductase activity was assayed using bathocuprone disulphonic acid (BCS) and cupric sulphate. A colour change from blue to yellow was observed and the absorbance measured at 484 nm. All stock solutions were prepared in MilliQ water. The most active fractions were pooled and dried, resuspended in 500 μl of water and re-chromatographed on the LH20 column.

UV and light absorbance profiling

UV and visible light absorbance were initially scanned from 210 to 800 nm. Fluorescence spectroscopy was performed using a Luminescence Spectrometer LH50B (PerkinElmer, Applied Biosystems, Warrington, Cheshire, U.K.). Excitation was at 226 nm and emission was followed from 300 to 800 nm.

Electrospray ionization (ESI)-MS

The ESI-MS were acquired in the positive mode (or negative mode) on a VG Quattro triple quadrupole mass spectrometer (Micromass Ltd., Wythenshaw, Manchester, U.K.) with aq. 50 % (v/v) CH_3CN , containing 1 % (v/v) formic acid as mobile phase. The samples were dissolved in water at a concentration of approx. 100 μM and injected into a 10 μl Rheodyne valve. They were introduced into the mass spectrometer at a flow rate of 8 $\mu\text{l}/\text{min}$ with a Syringe pump (Harvard Apparatus, MA, U.S.A.).

NMR

Spectra were recorded in 5 mm tubes at 600.13 MHz for ^1H and at 150.92 MHz for ^{13}C at 310 K, using a 10 mg solution of $^2\text{H}_2\text{O}$ in a Bruker DRX 600. The chemical shifts are given relative to acetone (2.225 p.p.m.) for ^1H and (28.05 p.p.m.) for ^{13}C . The double-quantum filtered phase-sensitive COSY experiment was performed using the Bruker standard program COSYDFPRT, with 0.409 s acquisition time and 4000 data points. F1 was zero-filled to give a matrix of 4000 × 1000 points and was resolution-enhanced in both dimensions by a shifted sinebell. The heteronuclear single-quantum coherence spectroscopy was performed using sensitivity improvement and echo/anti-echo

gradient selection. The heteronuclear multiple-bond correlation (HMBC) spectra were recorded using gradient-selection Bruker standard pulse program inv4gslplrnd with evolution delay of 60 ms. Authentic 3OHKyn was obtained from Sigma.

Matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) MS

A small fraction (0.1 ml) of the analyte solution (10 mg/ml) was added to the stainless-steel target and then dried at room temperature. The matrix α -cyano-4-hydroxycinnamic acid [10 mg in 1 ml of 70 % (v/v) acetonitrile] (0.1 μl) was added on the same spot and the sample dried at 40 °C. The spectrum was recorded in the positive-ion mode, on a Bruker ReflexTM III MALDI-TOF mass spectrometer. Spectra were obtained (1–100 pulses) using the lowest power required to facilitate desorption and ionization. Ions were accelerated towards the discrete dynode multiplier detector with an acceleration voltage of 20 kV. Bradykinin (1060.2 m-units), renin (1759.0 m-units) and mellitin (2846.5 m-units) were used as the standards for internal calibration of the mass.

Whole leaf assay and microscopic localization

Barley leaves were attached to a Petri dish using double-sided sticky tape. To a cooled solution of 0.5 % agarose in citrate buffer, Fe^{3+} (final concentration 1 mM) and BPS (1 mM) were added, and the solution was poured over the leaves and allowed to set. A similar assay was performed using Cu^{2+} and BCS. Control overlays were without the addition of the metal ions. The plates were placed on an inverted microscope to view the localization of the reaction product. The reductase assay was also performed in solution with leaf material in a tube allowing the solution to come into contact with the entire surface of colony and leaf.

Ferritin reduction assay

Freshly collected spores were mixed in 10 ml of citrate/phosphate buffer (pH 6.0) with 100 mM BPS in a 15 ml Falcon tube and allowed to settle. Horse spleen ferritin (100 μl of 100 mg/ml; Sigma) in 5000 Da cut-off dialysis tubing was introduced into the Falcon tube and the colour change to pink was observed.

RESULTS

The *Blumeria* EST project [19] has given new insights into the development and differentiation of this pathogen. For example, the project revealed the presence of genes similar to the ferric reductase system found in *Saccharomyces cerevisiae* [21]. This study was originally conceived to investigate this process. We could readily show that BGH spores produced an activity that reduced Fe^{3+} to Fe^{2+} . This was detected using the ferrous-specific chelator, BPS, as a red product. Similarly, cupric reductase activity could be assayed using BCS.

Spores, collected directly from heavily infected barley leaves, were washed in buffer, pelleted by centrifugation and resuspended in citrate buffer. Ferric chloride and BPS were added to an aliquot. The rapid development of a pink pigment indicated the presence of ferrous iron. The spores were again pelleted and the supernatant tested for ferric reductase activity. Abundant ferric reductase activity could be detected in the wash supernatant. (A similar assay was performed for cupric reductase activity but using cupric sulphate and BCS chelator.) The supernatant was

Figure 1 The profile of reductase activity eluting from the LH20 column

Cupric (■) and ferric (◆) reductase activities are shown. Fractions (0.5 ml) were collected and 50 μ l portions assayed for cupric activity at 484 nm with chelator BCS and ferric activity at 535 nm with chelator BPS.

Table 1 Assignment of NMR data

	H (p.p.m.)	J (Hz)	C (p.p.m.)
1			121.2
2	7.39	8	125.8
3	6.64	8	119.2
4	6.98	8	122.2
5			147.3
6			143.6
C=O			203.2
7	3.67	4, 18	42.5
	3.65	7, 18	
8	4.10	4, 7	54.0
C=O			177.0

Figure 2 ESI-MS and MALDI-TOF analysis of the reactive fractions from LH20 column

(A) The ESI-MS trace of the most reactive fraction from the LH20 column. Several peaks are seen, with a major peak at m/z 224.76 and a minor one at m/z 226.24. (B) MALDI-TOF analysis of the same fraction gave a major peak at m/z 440.85 and a minor peak at m/z 442.85.

then fractionated using a size-exclusion column (PD10) and most of the reducing activity was found, not in the protein eluate but in the low-molecular-mass fractions. This supernatant was further fractionated by spin dialysis and the activity was found in the < 5000 Da fraction. The activity was unaffected by heat (100 °C for 20 min). The activity was retained over the pH range 2–14.

The fractions were further purified by chromatography using a Sephadex LH-20 column with water as the solvent. They eluted over several fractions and displayed a broad shoulder (Figure 1). The highest activity fractions were pooled, freeze-dried, dissolved in water and rechromatographed. The most active fractions were subjected to ESI-MS and MALDI-TOF. ESI-MS gave a mass of 224.76 Da, whereas MALDI-TOF analysis suggested the compound had a mass of 440.85 Da (Figure 2).

The compound was then analysed using NMR. Proton and COSY spectra identified two spin systems, one aromatic with three adjacent protons (7.39, 6.64 and 6.98 p.p.m.) and one aliphatic connecting a CH_2 (3.67/3.65 p.p.m.) and a CH (4.10 p.p.m.). Heteronuclear single-quantum coherence spectra assigned the carbon atoms to the two spin systems. HMBC spectra assigned the aromatic carbon atoms not bearing a proton to a three-bond proton/carbon coupling from H2 to C6 and from H3

to C1 and C5. The HMBC spectra also showed a long-range correlation from the aromatic proton H2 to a carbonyl (203.2 p.p.m.) and to the same carbonyl, a long-range correlation from H7 and H8. These correlations allowed the connection of the aromatic and aliphatic spin systems via a carbonyl. Similarly, it was possible to assign a carbonyl (177.0 p.p.m.) (acid) by a long-range correlation to H7 and H8 (see Table 1).

The exact structure could not be unequivocally determined but this analysis suggested a simple structure, which favoured the 224.76 Da mass. Investigation of the databases of a natural compound with the 224 Da mass suggested 3OHKyn as a candidate compound (Figure 3). Authentic 3OHKyn was obtained and analysed. Its mass is 224.2 Da and it has an NMR spectrum that is close to our isolated compound (Figure 4). The UV, visible and fluorescence spectra of the compound for BGH and authentic 3OHKyn were also very similar. Figure 5 shows the UV spectrum from 210 to 400 nm. Chemical tests showed that authentic 3OHKyn reduced Fe^{3+} with similar kinetics and concentration dependence as the BGH reductase (results not shown).

Thus chemical activity and chemical structure as determined by NMR, MS and UV-visible spectroscopy and fluorescence all point to the identity of the BGH reductase activity to be

Figure 3 The structure of 3OHKyn

Figure 4 The NMR spectra of authentic 3OHKyn and the reductase isolated from *Blumeria* spore washings

Figure 5 The UV and visible spectra scan

The most informative region from 210 to 400 nm is shown. The upper trace is the reductase from *Blumeria* and the lower trace is authentic 3OHKyn.

3OHKyn. The anomalous MALDI-TOF mass is interpreted as an intermediate in the autooxidation of 3OHKyn to xanthommatin, which can be attributed to the UV laser.

The reductase activity, identified as 3OHKyn, had been obtained from the washings from unreleased spores. To investigate the function of 3OHKyn, spores were inoculated on to the leaf surface and allowed to germinate. After 24 h, the leaf was immersed in a solution containing BPS and Fe^{3+} to localize the 3OHKyn (and also enzymic ferric reductase activity). Figure 6(A) shows a germinated spore with both the primary germ and an AGT. Reductase activity is predominantly located on/in the body of the spore and in the developing appressoria. There is also activity at the site of haustorial initiation.

After 8 days, the colonies were well developed and reductase activity could be easily demonstrated over the whole colony. Also, by this time, conidiophores had produced abundant conidia (Figures 6B and 6C). Treatment of mature colonies with BPS and Fe^{3+} shows that reductase activity again concentrated on the spores still attached to the conidiophores. On prolonged incubation with the substrates, activity was also seen on the runner hyphae (Figure 6D). A reaction mixture of the buffer,

Figure 6 Germinated and developing spore stained for reductase activity

(A) The PGT can be seen. The developing appressoria and haustorial initiation structure are clearly evident. Scale bar, 5 μm . (B) Colonies after 8 days of development. The top leaf was immersed in a solution containing BCS, Cu^{2+} and the lower one in BPS and Fe^{3+} . The yellow reaction product is reduced copper and the red reaction product is reduced iron. Activity is visible only on the colonies and not on the uninfected leaf surface. Scale bar, 0.7 mm. (C) Reductase activity is mostly associated with the developing conidiophores. Scale bar, 15 μm . (D) Composite picture across the runner hyphae also displaying reductase activity. This is seen on prolonged exposure to the reagents (> 24 h). Scale bar=50 μm .

metal, chelator and melted agarose is poured on to colonies and allowed to set. This gave a strong reaction for the reduced metal that diffuses freely away from the colonies. This suggests that the activity is not enzymic but a diffusible chemical. Uninfected leaves do not have this capacity and neither free iron nor copper can be demonstrated on the leaf surface.

The ability of 3OHKyn to release iron from ferritin was tested. Horse spleen ferritin was taken in a dialysis bag and placed in a

solution of BPS, buffer and 3OHKyn. Within minutes, the solution had turned pink, indicating the presence of ferrous ions (results not shown).

DISCUSSION

EST analysis of BGH has shown that this organism contains homologues of the high-affinity iron (reduction–oxidation) uptake system in yeast [involving at least three genes *fet3* (dbEST AW790080), *fre1* (AW789227) and *zrt1* (AW92413)]. BGH does have ferric reductase activity (results not shown) but most of the reducing capacity is chemical in nature, and has been identified as 3OHKyn.

3OHKyn is a breakdown product of tryptophan and is in the pathway that leads to nicotinic acid synthesis and has been ascribed a protective role due to its ability to absorb UV irradiation [24]. The eye of the tropical fish, the three-spot gourami (*Trichogaster trichopterus*), has hydroxykynurenine, which acts as an UV filter [25]. 3OHKyn and its glucoside and glutathione adducts have all been suggested to play a role in UV protection in the eye [24,26,27]. It would appear that BGH, a fungus which lives on the leaf surface, has a compound capable of acting as an UV filter offering valuable protection to the developing colony. The free aromatic hydroxy group on 3OHKyn allows it to undergo complex oxidation processes, especially with compounds that contain a free amine. Hence, it could be involved in cross-linking proteins [28]. Cataract can be attributed to proteins, particularly α -crystallins, which have been cross-linked by 3OHKyn [29].

The cross-linking capacity of the compound is enhanced by (but not dependent on) the reduction of metal ions [28]. This reduction is coupled to the oxidation of 3OHKyn with a very reactive quinonimine intermediate, which can react with amine side chains on amino acids, converting the protein into a redox-reactive substrate. 3OHKyn is capable of generating hydrogen peroxide by reacting with copper and, to a lesser although still significant extent, with iron; this can also lead to the cross-linking of proteins [30]. Genes and enzymes putatively associated with 3OHKyn can be found in both the *Saccharomyces* and *Neurospora* genome databases (genome-www.stanford.edu/Saccharomyces and www-genome.wi.mit.edu). Yeast has potential homologues of the mammalian kynurenine 3-mono-oxygenase and a 3OHKyn-oxoglutarate transaminase (YBL098W). However, the role of neither the gene nor 3OHKyn has been detected in these or any other fungi. 3-Hydroxyanthranilic acid (3HAA) has been detected as a secretion product of *Saccharomyces* [31] and also of *Cryptococcus neoformans*, where it has been shown to be capable of reducing both iron and copper [32].

The very early events involved in mildew interaction with the host surface have attracted much attention [1,33]. We have shown that 3OHKyn is primarily located on spores that are newly deposited on the leaf surface and also on developing conidiophores (Figure 6). Less reductase activity was apparent on runner hyphae.

We have considered two potential roles for 3OHKyn: the first is as a passive defence against radicals produced from AOS and UV; and the second is an active role in cross-linking the fungus to the plant surface.

It is clear from other studies [12–14] that barley produces AOS in an attempt to prevent penetration. Whether the exact role of the AOS is direct antifungal killing, plant cell-wall strengthening and/or plant cell signalling has not been precisely determined. Nonetheless, it is clear that AOS are present and the role of 3OHKyn could be to counter them. Foliar plant pathogens are subject to the sun's rays and therefore must have effective means

for protection against UV. Melanin has been ascribed this role in *Alternaria alternata* [34]. In the opportunistic human fungal pathogen *C. neoformans*, it offers protection against the host immune system [35]. Melanin has not been detected in mildew. Ergothioneine, a constituent of the honeydew produced by the rye-infecting fungus *Claviceps purpurea*, has been shown to be a UV protectant and an antioxidant [36–39]. It also protects germinating conidia against hydrogen peroxide in an association with catalase [40]. The roles of UV and oxidant protection cannot be easily separated. 3OHKyn may have this passive role for BGH.

The possibility that 3OHKyn may have an active role in the cross-linking of the pathogen to the plant is intriguing. It is clear that adhesion to plants is rapid and powerful and is a crucial facet of pathogenicity. Mildew spores adhere to the highly hydrophobic barley epidermis within a few minutes after settling [41]. 3OHKyn is present on the surface of the spore and is solubilized in the water present on the leaf surface. The PGT emerges from the spore within 2 h but does not penetrate the epidermis. Instead, a papilla forms in the epidermal cell as a result of peroxide-mediated cross-linking. As mentioned above, hydrogen peroxide is present in abundance at the infection site. Such a mixture of 3OHKyn and peroxide provides the ingredients for the cross-linking of fungal and plant macromolecules and the adhesion of the spore to the leaf. The ability of 3OHKyn to liberate iron from ferritin would further enhance the cross-linking potential. Ferritins, and phytoferritin in plants, are the major storage protein complexes of iron. It effectively sequesters iron as Fe^{3+} hydride and thereby controls the amount of free iron (Fe^{2+}) in the cell. After fungal penetration of the epidermis, the release of iron in the presence of 3OHKyn and peroxide could help in anchoring the developing haustoria to plant cell membranes by cross-linking proteins.

To determine whether 3OHKyn has a role in radical protection or cross-linking requires further experimentation, which is beyond the scope of the present study. If such roles are confirmed, the synthesis and action of 3OHKyn could emerge as an attractive target for fungicides.

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